# TGF-β Receptors on Human Chondrocytes: hetero-oligomerization and function

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy, Department of Surgical Research, McGill University, Montreal, Quebec, Canada

**by**

**Wendy Lynne Parker**

This thesis is dedicated to MM
for encouraging me to realize my
potential
and for reminding me
that to do more, to challenge
oneself, and to succeed
is one of the greatest of
accomplishments.

Thank you.

There in the sunshine
are my highest aspirations...
I can look up and see their beauty,
believe in them,
and try to follow where they lead.

- Louisa May Alcott

# TABLE OF CONTENTS

**Research Compliance Certificates**

Ethics Approval for Human Cartilage Harvest

Radioisotope Permit

# ABSTRACT

Human cartilage does not have the capacity for parent-like regeneration; instead, following injury, there is a programmed attempt at regeneration that ultimately results in fibrocartilage formation. This lack of intrinsic repair has been attributed to the avascular state of the tissue and chondrocyte dedifferentiation towards a cell incapable of type II collagen production and with altered responsiveness to the structural and regulatory mediators within its microenvironment. For several decades, debate has existed regarding the role Transforming Growth Factor-Beta (TGF-β) plays in modulating articular cartilage. Blocking of TGF-β signaling in mice by a dominant negative type II TGF-β receptor and transgenic knockouts of Smad 3, a central mediator of TGF-β signaling, result in osteoarthritic-like phenotypes, whereas local up-regulation of TGF-β promotes cartilage healing in degenerative joint disease models.

Despite TGF-β being implicated as a key player in the regulation of chondrocyte phenotype and extracellular matrix (ECM), little is known about TGF-β action in human chondrocytes. These investigations have established a critical link between variation in TGF-β receptor expression at the cell surface and TGF-β action in cartilage. In addition to the TGF-β signaling receptors, the presence of betaglycan and RIIB (a spliced variant of the type II signaling receptor) was confirmed on human chondrocytes. Moreover, the expression of three novel TGF-β receptors was identified, namely Sol RI (a soluble form of the type I receptor), Alk-1, and endoglin. These receptors formed a variety of heteromeric complexes and regulated TGF-β signaling. More importantly, RIIB and endoglin were demonstrated to regulate type II collagen levels and evidence was provided

that they likely represent chondrocyte phenotypic markers. A critical link between endoglin expression, cell phenotype, TGF-β responsiveness, and ECM was established. These results demonstrate the formation of TGF-β receptor heteromeric complexes of various subtype composition on chondrocytes suggesting that such complexes may regulate TGF-β signaling pathways in those cells. Cell surface TGF-β binding proteins, acting as phenotypic markers in human cartilage, are potential modulators of complex interactions between the cell and its microenvironment. Therefore, novel TGF-β receptors may provide an avenue to regulate the effects of TGF-β locally and establish new insights into cartilage regeneration or repair.

# RÉSUMÉ

Le cartilage humain n'a pas de pouvoir de régénération parentale; suite à une blessure, une tentative programmée de régénération résulte finalement en une formation de fibrocartilage. Cette absence de réparation intrinsèque est attribuée à l'état avasculaire du tissu, la dédifférenciation du chondrocyte en une cellule incapable de produire le collagène de type II, et la sensibilité modifiée aux médiateurs structuraux et régulateurs à l'intérieur de son micro-environnement. Pendant plusieurs dizaines d'années, il y a eu débat concernant le rôle joué par le Facteur de Croissance Transformant Bêta (TGF-β) dans la régulation du cartilage articulaire. Chez la souris, le blocage de la signalisation du TGF-β par l'empressions daim récepteur de type II à dominante négative et l'invalidation transgénique de Smad 3 - un médiateur central de signalisation du TGF-β - produisent des phénotypes analogues à ceux de l'ostéite, alors qu'une augmentation locale régulée du TGF-β favorise la guérison du cartilage dans les cas d'arthrose.

Bien que le TGF-β soit un acteur clé dans la régulation des phénotypes chondrocytaués et de la matrice extra-cellulaire (MEC), son action sur les chondrocytes humains est peu connue. Ces recherches ont permis d'établir un lien critique entre la variation de l'expression des récepteurs du TGF-β à la surface des cellules et l'action du TGF-β sur le cartilage. En plus des récepteurs de signalisation du TGF-β, la présence de béta-glycans et de RIIB (produit d'épis sage alternatif du gène du récepteur de signalisation de type II) a été confirmée dans les chondrocytes humains. De plus, l'expression de trois récepteurs atypiques du TGF-β a été identifiée, à savoir le Sol RI (une forme soluble du récepteur de type I), l'Alk-1, et l'endogline. Ces récepteurs constituent un assortiment de complexes

hétéromériques et régulent la signalisation du TGF-β. Mieux encore, il a été démontré que le RIIB et l'endogline pouvaient réguler les niveaux de collagène de type II ; il a aussi été prouvé qu'ils sont susceptibles de servir d'indicateurs des phénotypes chondrocytaués. Un lien critique entre l'expression de l'endogline, le phénotype des cellules, la sensibilité au TGF-β, et la MEC a été établi.

Ces résultats démontrent la formation de sous-compositions diverses de complexes hétéromériques des récepteurs du TGF-β sur les chondrocytes, évoquant la possibilité que de tels complexes puissent réguler les voies de signalisation du TGF-β dans ces cellules. Les protéines de liaison du TGF-β à la surface des cellules, agissant comme un indicateur de phénotype dans le cartilage humain, constituent potentiellement des modulateurs d'interactions complexes des cellules avec leur micro-environnement. C'est pourquoi les récepteurs atypiques de TGF-β pourraient ouvrir la voie à la régulation locale des effets du TGF-β et ainsi établir de nouvelles perspectives dans le domaine de la régénération et de la réparation du cartilage.

# CONTRIBUTION TO ORIGINAL KNOWLEDGE

1. I have characterized the TGF-β receptor profile on immortalized nonarticular and articular and primary articular human chondrocytes using affinity cross-link labeling with $^{125}$I-TGF-β1 and $^{125}$I-TGF-β2. I have confirmed the presence of the TGF-β signaling receptors, RIIB, a spliced variant of the type II signaling receptor, and betaglycan on human chondrocytes. More importantly, I have demonstrated the presence of three novel receptors not previously recognized on chondrocytes. These include the accessory receptor endoglin, the orphan receptor Activin Receptor-like kinase-1 (Alk-1), and a soluble form of the type I signaling receptor (Sol RI). The presence of these receptors was confirmed by immunoprecipitation studies and Western blot analysis.

2. I have demonstrated that endoglin is expressed on chondrocytes at levels comparable to human microvascular endothelial cells (HMEC-1) where it plays a role in vasculogenesis and neoangiogenesis. Endoglin's role in cartilage, an avascular tissue, is intriguing.

3. I have demonstrated that endoglin and betaglycan form a heteromeric complex on the chondrocyte surface in the presence of ligand and in a ligand independent manner and type II receptor (RII) independent manner.

4. I have also demonstrated the heteromeric complex formation between Alk-1 and betaglycan as well as Sol RI and betaglycan both at endogenous receptor concentrations

and ratios. Similarly, I showed that Alk-1 and Sol RI themselves associate on the chondrocyte surface.

5. I have presented data that overexpression of Alk-1 and Sol RI results in enhancement of TGF-β signaling in human chondrocytes.

6. I have demonstrated that endoglin is also expressed on DR26, 293, and L6 myoblast cells but at levels less than seen for human microvascular endothelial cells (HMEC-1) or chondrocytes.

7. I have confirmed the RII independent nature of the endoglin-betaglycan association and in addition demonstrated that this association occurs in the absence of the cytoplasmic domain of betaglycan or the glycosaminoglycan (GAG) side chains of its extracellular domain.

8. I have presented data that endoglin is able to bind TGF-β1 in the absence of RII which refutes the currently accepted TGF-β signaling paradigm.

9. I have also demonstrated that TGF-β binding by endoglin is enhanced by the presence of betaglycan. Moreover, I have shown that, in the absence of RII, endoglin requires betaglycan to be present to bind ligand.

10. I have shown using overexpression studies that endoglin inhibits whereas betaglycan enhances TGF-β1, -β2, and -β3 responses in human chondrocytes and have provided evidence that suggests the inhibitory effect by endoglin on -β2 signaling is likely mediated through its heteromeric complex formation with betaglycan.

11. I have identified the heteromeric receptor complex formation between RIIB and both the TGF-β signaling receptors (RI and RII) as well other TGF-β receptors (betaglycan, endoglin, Alk-1, and Sol RI) establishing the concept of a hetero-oligomeric receptor complex on human chondrocytes.

12. I have demonstrated that exogenous overexpression of RIIB in human chondrocytes enhances TGF-β signaling and type II collagen levels; however, I have shown that elevated endogenous RIIB levels correlate with a decreased TGF-β responsiveness. In addition, I have illustrated that RIIB expression on osteoarthritic (OA) chondrocytes is markedly enhanced as compared to normal primary articular cells suggesting RIIB may be a phenotypic marker in these cells.

13. I have utilized Morpholinos antisense oligo technology to block endoglin expression on human chondrocytes and demonstrate that this negates the inhibitory action on TGF-β signaling by endoglin in these cells.

14. Using serial photomicroscopy, I have extensively characterized the cell morphology of immortalized and primary human chondrocytes at various stages of differentiation in monolayer, 3-D alginate beads, and explant cultures.

15. I have presented evidence that endoglin is expressed in osteoarthritic (OA) chondrocytes at markedly higher levels than in normal primary articular cells.

16. I have demonstrated that endoglin expression correlates with type II collagen levels in human chondrocytes. More importantly, I have established a critical link between endoglin expression, the dedifferentiated state of the human chondrocyte, and the cells' responsiveness to TGF-β strongly suggesting that endoglin can be used as a phenotypic marker in these cells.

# ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. Anie Philip. She has contributed to my understanding of the scientific process yet encouraged me to think and work independently. She has enabled me to realize that research is a challenging process that can provide me a unique avenue to pursue my clinical questions in a basic science manner. She has helped me to acquire a love of investigation that I know will follow me throughout my surgical career.

My gratitude goes out to Dr. J.J. Lebrun for his support and direction and to the members of his lab for their friendship and training. In addition, I thank Dr. L. Rosenberg for his wisdom, encouragement, and advice and Dr. R. Chiu for his continued support. I would like to also thank the members of my committee: Dr. P. Roughley, Dr. J.J. Lebrun, and Dr. M. Zafarulla for their availability and support.

I sincerely appreciate the support of the McGill Department of Plastic Surgery which allowed me to take a three year leave of absence to pursue my research interests and which has supported my endeavours to present my work at various meetings. Also, I thank the McGill Surgeon Scientist Program for my salary support throughout this training.

The generous contribution of TGF-β by Genzyme (Framingham, MA) has been greatly appreciated. My sincere appreciation goes out to all of those people who have contributed reagents and tissue for my experiments all of whom have been acknowledged throughout the thesis. I would like to thank the members of the Montreal General Hospital Radiology department who allowed me such unlimited access to their equipment.

My appreciation for those students with which I have worked can not be overstated. I would like to thank Betty for training me and the members of Dr. Bernard's lab for sharing their space and time with me. In particular, I thank Galit and George for their understanding, encouragement, and honest friendship. I would like to thank the members of my own lab for their friendship, specifically Ranuka and Annie for their patience regarding my obsessive/compulsive behaviour and David for his help in the lab.

Most importantly, I would like to thank my family and friends. Not only have they provided me an endless amount of support over this time but they have continued to do so despite my absence from their lives. They have been understanding and generous with very limited returns. I am truly grateful. In particular, although he is too young to appreciate my sincerity, I thank my nephew, Nolan, for making me realize that there must be room in life for laughter.

# LIST OF FIGURES

Figures in this thesis include those contained within the five sequential manuscripts as listed and described in the figure legends of each manuscript.

Figures demonstrating additional results not present in the manuscripts because of limitation of space or focus have been included. These figures are presented as addendums following the manuscript to which the findings are most related. Explanations of these results are included in the bridging documents preceding the respective manuscripts. The figures have been labeled in a sequential manner as follows below with accompanying figure legends (**Results**). Also, two figures have been included with the introduction to provide an overview of the TGF-β signaling pathway and potential levels of regulation. Included with the discussion are two additional figures, a proposed theory of TGF-β receptor interactions forming hetero-oligomeric complexes at the chondrocyte surface and a schematic theorizing the complex multilevel influential factors regulating the chondrocyte and its microenvironment (**Schematics**).

**Results**

**Fig. A:** TGF-β responses in human chondrocytes

**i:** Stimulation of Smad 2 phosphorylation by TGF-β1.

**ii:** Triphasic growth response demonstrated by $^{3}$H-Thymidine incorporation.

**iii:** Effect on Col2a-promoter activity by TGF-β1.

**iv:** Stimulation of Smad 2 phosphorylation by TGF-β1, -β2, and -β3.

**Fig. B:** Human chondrocytes produce TGF-β.

# ABBREVIATIONS

| | |
|---|---|
| **β-gal** | -β-galactosidase |
| **2-D** | -two-dimensional |
| **3-D** | -three-dimensional |
| **ActRII** | -activin receptor type II |
| **Alk-1** | -Activin Receptor-like kinase-1 |
| **Alk-5** | -Activin Receptor-like kinase-5 |
| **AVM** | -arteriovenous malformation |
| **BG** | -betaglycan |
| **BMP** | -bone morphogenetic proteins |
| **BS3** | -Bis-sulfocsuccinimidyl suberate |
| **BSA** | -bovine serum albumin |
| **C** | -morpholinos control oligos |
| **CAM** | -cell associated matrix |
| **CD** | -cytoplasmic domain |
| **Co-IP** | -co-immunoprecipitation |
| **Col II** | -type II collagen |
| **Co-Smad** | -common mediator Smad |
| **CPM** | -continual passive motion |
| **DBM** | -demineralized bone matrix |
| **DEPC** | -diethyl pyrocarbonate |
| **DNA** | -deoxyribonucleic acid |

**DNRII** -dominant negative RII

**dPBS** -Dulbecco's PBS

**E** -early

**ECD** -extracellular domain

**ECM** -extracellular matrix

**eds** -editors

**Eg** -endoglin

**EGF** -epidermal growth factor

**ERK** -extracellular signal regulated kinase

**EV** -empty vector

**FBS** -fetal bovine serum

**FGF** -fibroblast growth factor

**Fig** -figure

**GAG** -glycosaminoglycan

**GF** -growth factor

**GPI** -glycosyl phosphatidylinositol

**HA** -hyaluronic acid

**HGFP** -high green fluorescent protein plasmid

**HHT** -hereditary hemorrhagic telangiectasia

**HMEC-1** -human microvascular endothelial cells

**IGF-1** -Insulin-like Growth Factor-1

**IgG** -immunoglobulin

**IL-1** -Interleukin-1

**IL-6** -Interleukin-6

| | |
|---|---|
| **IP** | -immunoprecipitation |
| **IPG** | -inositolphosphate glycan |
| **I-Smad** | -Inhibitory-Smads |
| **kDa** | -kilo Dalton |
| **L** | -late |
| **LAP** | -latency associated protein |
| **LBD** | -ligand binding domain |
| **LTBP** | -latent TGF-β binding protein |
| **MAP** | -mitogen activated protein |
| **MAPK** | -mitogen activated protein kinase |
| **MH** | -Mad homology |
| **MIS** | -Mullerian inhibitory substance |
| **MMP** | -matrix metalloproteinase |
| **MSC** | -mesenchymal stem cell |
| **N** | -normal |
| **NIP** | -nonimmunoprecipitated |
| **NLS** | -nuclear location signal |
| **nM** | -nanomolar |
| **NO** | -nitric oxide |
| **NOS** | -nitric oxide synthase |
| **NR** | -nonreducing |
| **NRS** | -normal rabbit serum |
| **NSAID** | -nonsteroidal anti-inflammatory |

| | |
|---|---|
| **OA** | -osteoarthritis/osteoarthritic |
| **p** | -plasmid |
| **PAI** | -plasminogen activator inhibitor |
| **PAS** | -protein A sepharose |
| **PBS** | -phosphate-buffered saline |
| **PCR** | -polymerase chain reaction |
| **PDGF** | -platelet derived growth factor |
| **PG** | -proteoglycan |
| **PGA** | -protein G agarose |
| **PIPLC** | -phosphatidylinositol phospholipase C |
| **PTH** | -parathyroid hormone |
| **R** | -reducing |
| **R/Rec** | -recovered |
| **RA** | -rheumatoid arthritis |
| **RI** | -type I TGF-β signaling receptor |
| **RII** | -type II TGF-β signaling receptor |
| **RIIB** | -type IIB TGF-β signaling receptor |
| **RNA** | -ribonucleic acid |
| **R-Smad** | -receptor-regulated Smad |
| **RT-PCR** | -Reverse Transcriptase Polymerase Chain Reaction |
| **SARA** | -Smad anchor for receptor activation |
| **SBE 4** | -Smad binding element 4 |
| **SDS-PAGE** | -sodium dodecyl sulphate-polyacrylamide gel electrophoresis |

**SFM** -serum free medium

**Smad 2P** -Smad 2 phosphorylation

**Sol RI** -soluble type I TGF-β receptor

**Stat** -signal transducers and activators of transcription

**TGF-β** -transforming growth factor-β

**TIMP** -tissue inhibitor of matrix metalloproteinase

**TM** -transmembrane

**TNF-α** -Tumour Necrosis Factor-α

# Section 1

## INTRODUCTION AND BACKGROUND KNOWLEDGE

# GENERAL THOUGHTS

**"from Hippocrates to the present age it is universally allowed that ulcerated cartilage is a troublesome thing and that, once destroyed, it is not repaired"**

**- Hunter (1743)**

Although cartilage damage and degenerative joint disease seem to be the endpoint, it is the failure of innate repair, as noted by Hippocrates, which defines the start line in the race towards cartilage regeneration and repair. Before beginning this race, it is necessary to understand: 1. the normal structure and physiology of cartilage; 2. the normal aging process of this specialized connective tissue; 3. what happens when this normal aging process goes awry and leads to the to pathogenic states of osteoarthritis (OA) and Rheumatoid arthritis (RA); and 4. the history of attempts at cartilage repair and their lack of success.

Armed with this background and prepared to enter the race, the discovery of growth factors and cytokines in the chondrocyte microenvironment and how they affect human cartilage becomes the starter's gun. Improved understanding of the complex microenvironment provides incentive to continue the race. Animal models provide evidence that implicate Transforming Growth Factor-Beta (TGF-β) in degenerative joint disease and cartilage healing, as researchers delve deeper into the action of TGF-β in chondrocytes. Using knowledge gained from the investigations of TGF-β receptors in other systems, one recognizes that accessory receptors may provide a novel avenue to modulate TGF-β responses in chondrocytes providing new found energy at mid-race.

Data is collected and analyzed as the race continues. Ultimately, to reach the finish line, one must have a vision of how to incorporate the action of TGF-β in human chondrocytes, specifically an understanding of the role its heteromeric receptor complexes hold in clinical applications to promote cartilage regeneration and repair.

## CARTILAGE

### INTRODUCTION

The rapid growth of our aging population has generated increased attention towards diseases which particularly impact this group. A preface to this must be an understanding of tissue aging (Clark 1999). Previously, much of this focus has been on cardiovascular and neuronal tissues with little research into cartilage aging. However, degenerative joint disease is the disease most related to increasing patient age (Buckwalter and Lappin 2000); it presents the greatest challenge against mobility, and few diseases impose a larger burden on health care (Praemer et al 1999). The cost of joint disease to the United States in the year 2000 was estimated at 95 billion dollars (Elders 2000).

### DEVELOPMENT/PHYSIOLOGY

Hyaline cartilage is distinct from elastic and fibrocartilage in its architecture and extracellular matrix (ECM) components. During skeletal development, cartilage serves as a template for long bone formation (Erlebacher et al 1995). This process of endochondral ossification begins by the condensation and differentiation of mesenchymal stem cells (MSC) into cartilage. Chondrocytes then undergo proliferation, differentiation, and hypertrophy; their ECM becomes calcified and apoptotic cell death follows. Articular cartilage, unlike that confined within the epiphyseal growth plate of these long bones, is

arrested before terminal hypertrophic differentiation and resides as a frictionless gliding surface for diarthrodial joints.

Cartilage is a very specialized connective tissue which affords joint surfaces both gliding and load resistance properties. It is composed of 95% highly hydrated ECM with a unique architecture and 5% of chondrocytes, its only resident cell type (Lieberman et al 2002). As a tissue, cartilage is aneural, alymphatic, and avascular. Chondromodulin-1, a cartilage specific growth modulating factor, has been implicated in angiogenesis inhibition in avascular zones of growth plate cartilage (Hiraki et al 1997) although this has not been investigated for articular cartilage. The ECM of articular cartilage, produced by resident chondrocytes, is composed primarily of proteoglycans (PG) and type II collagen. The PGs are highly negatively charged resulting in water retention and account for the load bearing ability of the tissue (van den Berg 1999). The most abundant PG is the large aggregating PG or aggrecan (Poole 2001). This molecule is found at increased concentration within the depths of the cartilage and has a leucine rich protein core with glycosaminoglycan (GAG) side chains of chondroitin sulphate and keratin sulphate. These carboxylated and sulphated groups are responsible for aggrecan's negative charge and water binding. Other leucine rich PGs found at lower concentration within the ECM are decorin, fibromodulin, and biglycan (Poole 2001). Decorin and biglycan are more concentrated at the articular surface where collagen fibrils tend to run parallel to the surface and where cartilage demonstrates its greatest tensile strength (Poole et al 1996).

Type II collagen, the other principal ECM component, forms fibrils which provide a cartilage endoskeleton. Other forms of collagen have also been identified in normal human hyaline cartilage including type IX, which resides primarily at the articular surface, and type XI, which is found both at the surface and within its substance. Within

the intrafibrillar spaces of the collagen backbone are resident chondrocytes and PGs linked to these fibrils via hyaluronic acid (HA). In addition to HA, many other proteins referred to as link proteins contribute to the organization within the ECM (Roughley 2001). Abnormalities of these link proteins have provided a great deal of insight into a genetic basis for various cartilage disorders.

Cartilage is not an inert tissue. Chondrocytes are thought to sense changes such as alteration in ECM composition, deformation, and load variation in their microenvironment. These cells tightly regulate their own environment primarily through growth factors (GF), cytokines, and enzymes in an autocrine and paracrine fashion to achieve a normal steady state of turnover. Interleukin -1 (IL-1), Insulin-like Growth Factor-1 (IGF-1), Tumour Necrosis Factor-α (TNF-α), Transforming Growth Factor-β (TGF-β), matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) are all considered important players in this regulation (Fukui et al 2001). However, in pathophysiologic states chondrocytes and ECM will also be affected by synovial factors.

It is clear from the structural organization of hyaline cartilage that it is not a homogeneous tissue (Aydelotte et al 1988, Aydelotte and Kuettner 1988). Similar to growth plates, articular cartilage has been divided into four zones, a superficial, transitional, least mature, and hypertrophic zone (Fukumura et al 1998) or more simply in to superficial, intermediate and deep layers (Moldovan et al 1997). It has been suggested that chondrocytes within superficial zones exhibit increased responsiveness to IL-1 and increased resistance to IL-1 antagonists (Hauselmann et al 1996). This suggests that these

cells may represent a distinct chondrocyte phenotype and play a specific role in ECM regulation.

NORMAL AGING

Normal aging of human articular cartilage involves changes in structure, matrix composition, and mechanical properties distinct from those seen in disease states (Roth and Mow 1980, Verzijl et al 2000, Buckwalter and Lane 1997, Buckwalter et al 2000). A change in chondrocyte function and its inability to maintain the tissue are implicated in this aging process. The ECM, specifically type II collagen turnover, is focused in a pericellular region for the first 30 to 35 years of life (Wu et al 2002, Nelson et al 1998). This corresponds to the peak in tensile strength noted at approximately 30 years of life (Kempson 1982). With time, however, this strength progressively declines and there is a shift to where ECM turnover is focused at the articular surface. At early stages, a zone of demarcation between superficial aging layers and a normal deeper zone is evident but cyclic damage and resynthesis of ECM proteins eventually progress from superficial to deep cartilage layers (Nelson et al 1998, Hollander et al 1995). Hyaline surface fibrillation is a universal age-related process and has not been associated with joint pain or dysfunction (Koepp et al 1999, Buckwalter et al 2000). With increasing age chondrocytes themselves show a decrease in mitotic rate (Bolton et al 1999, Martin and Buckwalter 2001). In addition, they demonstrate a decreased responsiveness to anabolic GFs (IGF-1) in their microenvironment (Martin and Buckwalter 2000) and a reduced capacity for protein synthesis (Campisi 1999). All human cells destined toward a differentiated maturity will eventually reach growth arrest; however, chondrocytes may achieve this early. One hypothesis as to why these cells become senescent is that of telomere erosion (Blackburn 1991, Campisi 1999) possibly from a history of repeated

vigorous mitotic activity in response to microtrauma. Oxidative damage may also be responsible for degeneration of chondrocyte mitochondria and multiple downstream effects leading to a lack of cell division (Dumont et al 2000, Toussaint et al 2000).

Regardless of mechanism, age-related changes in chondrocytes result in ECM disruption. Increased collagen crosslinking and decreased water content have been described (DeGroot et al 1999, Verzijl et al 2000). PGs are seen to decrease in size secondary to a decrease in individual lengths of protein cores, decrease in length of GAG side chains and decreased numbers of aggregating proteins per group. This is suggested to be a result of age-related changes in chondrocyte PG synthesis, an increased degradation, or a combination of both (Buckwalter et al 1985, Thonar et al 1986). Overall, an accumulation of collagen fibrils is seen and nonenzymatic glycation of end products leads to increased cartilage stiffness (Verzijl et al 2002).

These changes seen with normal cartilage aging were once thought to be the precursor of degenerative joint disease. It is now believed that advanced age simply increases the risk for OA and that OA results from a compromised ability of chondrocytes to restore the tissue after an insult (Buckwalter and Mankin 1997, Buckwalter et al 2000). What tips this delicate connective tissue balance over to a negative state of degeneration is uncertain. Genetic susceptibility may account for this shift as may anchorage dependence. This phenomenon presupposes that the initial perturbation in the ECM disturbs the chondrocytes' microenvironment and that cells relying on a cell-cell or cell-ECM interaction for survival will be unable to withstand these changes. The severely altered pericellular matrix seen in OA provides support for this theory (Poole et al 1991, Hambach et al 1998). Whether the fundamental problem lies within the chondrocytes or

their ECM, the pathophysiologic changes (when normal cartilage aging becomes degenerative joint disease) have a profound impact on functional outcome.

PATHOPHYSIOLOGY

**Degenerative Joint Disease**

The pathophysiologic state of hyaline articular cartilage is an identifying feature of both noninflammatory and inflammatory forms of arthritis. Despite the acceptance that OA does, in fact, have an inflammatory component it remains an entity distinct from RA and other related diseases. Each will be discussed separately.

Osteoarthritis

Incidence of OA either radiographically or clinically has been reported as 85% of the population by the age of 75 (Sack 1995) or radiographically in 80% over the age of 65 (Oddis 1996). The overall incidence in the adult population is thought to be upwards of 9% (Lawrence et al 1998) to 15% (Poole and Webb 2000) and with each decade over 40 there is a dramatic increase in the numbers affected (Praemer et al 1999, Buckwalter and Lappin 2000, Buckwalter et al 2000). Various risk factors have been cited including advanced age, female gender, obesity, occupation, sport activity, and genetics (Creamer and Hochberg 1997). Genetics have been implicated in the pathophysiology of OA since the description of Heberden's nodes in 1941 (Stecher 1941) and research has continued to support this theory with Col 2A1 as an example of a candidate gene target (Ala-Kokko et al 1990, Ritvaniemi et al 1995). Genetic background also appears to contribute to disease prevalence in 38-65% of all cases, with mutations in collagen genes most commonly affected (Spector et al 1996, Olsen 1995, Prockop and Kivirikko 1997). Occupation and sport and other mechanical factors as risk factors coincide with the theory of mechanical wear resulting in OA (Buckwalter 1995, Buckwalter and Mankin 1997, Gorman 1996).

However, now more commonly accepted is the idea of normal age-related changes resulting in a hyaline surface which is more susceptible to mechanical forces and altered joint loading leading to focal erosions (Neuhold et al 2001, Stoop et al 1999 and 2000). Therefore, age itself is likely not a risk factor but normal cartilage aging does predispose to further insult.

OA is not a single disease entity but instead represents a group of diseases with different underlying pathophysiologies, the development of which may take 20 to 30 years to reach clinical manifestations (Poole and Webb 2000). Regardless of etiology, OA generally presents with a degeneration of articular cartilage, new bone formation (osteophytes) at joint margins, a loss of PG and surface fibrillation and eventually cleft formation and erosion. Cartilage becomes soft; eburnation or sclerosis of underlying subchondral bone and cyst formation occur (Hamerman 1989). This results in joint space narrowing (Kellgren and Lawrence 1957) as well as pain and decreased range of motion for the patient.

Typical ultrastructural changes seen in OA include lacunar emptying with an accumulation of debris (Mitrovic et al 1983, Bullough 1997, Stockwell 1971) representative of cell death. Whether the loss of chondrocytes is from necrosis (Vignon et al 1976, Bullough 1997, Meachim et al 1965) or is a programmed apoptotic response (Blanco et al 1998, Hashimoto et al 1998a and 1998b) is controversial. Only a minor increase in empty lacunae in OA lesions has been clearly documented (Aigner et al 2001) and this may have been caused by technical artefact (Aigner and Kim 2002). Possible apoptotic initiators in OA include nitric oxide synthase (NOS) (Blanco et al 1995). Increased NOS levels have been measured in the synovial fluid of OA patients (Sakurai et al 1995). However, other well known mediators of apoptosis were not shown to modulate

chondrocyte death (Blanco et al 1995, Kuhn and Lotz 2001, Kim et al 2001a). Alternatively, a loss of type II collagen has been demonstrated to increase apoptosis in cartilage (Yang et al 1997, Cao et al 1999) and in addition, chondrocytes isolation protocols and ECM removal have also been linked to accelerated apoptosis (Kim et al 2001a, Blanco et al 1998). Whether ECM alterations directly increase cell death or are a secondary effect of chondrocyte loss is debateable. Mechanical stress at low levels has also been demonstrated to accentuate apoptosis (Loening et al 2000). Chondrocytes have a high capacity to recover from stress. The problem arises if cell death ensues as chondrocytes are the sole ECM regenerators in articular cartilage and undoubtedly tissue failure will follow.

It is likely that if apoptosis is a prominent feature of OA it occurs later in the disease. In fact, early stages of OA demonstrate clonal growth, a transient proliferation of chondrocytes forming clusters or chondrons of multicellular clones (van der Kraan and van den Berg 2000, reviewed in Mankin 1982). Accompanying this clonal growth is not only an increased rate of catabolic cytokine and enzyme synthesis but also increased production of anabolic factors, PG and type II collagen (reviewed in Poole et al 2002) and with this heightened ECM turnover. However, a loss of PG and type II collagen occurs at the joint surface along with an increase in water content and a decrease in strength (reviewed in Goldring 2000a). Decreased decorin, biglycan, and aggrecan are seen at the surface but increase in deeper levels and an increase in denatured type II collagen combined with abnormally produced collagen and PG (reviewed in Poole et al 2002) result in a severely altered chondrocyte microenvironment. This likely then contributes to cell death. In addition, it is believed that damaged fibrils block cell proliferation as the avascular state of the tissue precludes scavengers from clearing debris (Poole et al 2002).

A loss in cell number results in progressive changes in ECM. Late changes in OA include a decrease in cell proliferation and a shift in chondrocyte responsiveness (Goldring 2000a). OA is not considered an inflammatory arthritis but, in late stages, enhanced ECM breakdown and release of wear particles cause secondary inflammation of synovial tissues which then contributes to the proinflammatory state of the joint.

A change in chondrocyte phenotype in OA is evident. Van den Berg attributed the deranged phenotype to prolonged exposure to increased levels of destructive mediators and noted, interestingly, a disturbed receptor expression on these cells (van den Berg 1999). A change in gene expression pattern is seen for ECM synthesis (Goldring 2000a) with up-regulation of type III, VI, and X collagen which is normally a marker for hypertrophy in growth plate chondrocytes. An up-regulation of Matrilin-3, which effects ECM assembly, has also been documented (Pullig et al 2002). A characteristic feature of this phenotypically altered chondrocyte is an altered responsiveness to regulators within its microenvironment. Therefore, in addition to the increased synthesis of catabolic factors and inhibitory cytokines produced, the chondrocytes themselves have been shown to be more responsive to IL-1 and to have more IL-1 receptors (Martel-Pelletier et al 1992). A re-expression of type IIA procollagen, a chondroprogenitor spliced variant of type II procollagen, is also noted (Aigner et al 1999) with an increase in Sox 9 expression (Salminen et al 2001).

What incites the actual change in cell phenotype remains unclear but is most certainly related to changes in the ECM. This has generated a vicious cyclical argument regarding the etiology of OA. One possibility is that age-related changes present in cartilage result in chondrocytes less able to combat ECM insults. An insult, traumatic, genetic, or of unknown etiology then results in more aggressive changes to the ECM which by way of

local mediators or a shift in microarchitecture leads to a variation in chondrocyte phenotype. This results in a cell with less anabolic capabilities and continued ECM degeneration.

Rheumatoid Arthritis

Most reports suggest a 1% world wide incidence of RA (Alarcon 1995). There is a clear gender bias with incidence in women 2.5 fold higher than men (Lawrence et al 1998). In addition, RA encompasses many subtypes of disease with variation in clinical symptoms, age of onset, course, and outcome. Inflamed synovial tissue is pathonmuemonic for the articular component of this disease. Damage to the hyaline joint surface occurs secondary to an invading pannus and increased exposure to destructive cytokines and enzymes resulting in early loss of ECM and a decreased synthetic response by chondrocytes (van den Berg 1999).

RA can be considered in three stages (Szekanecz and Koch 2001). The first, initiation, is likely caused by a genetic susceptibility combined with an environmental trigger (Jirholt et al 2001). This is followed by a central stage with activated synovial cells which release GFs and cytokines to perpetuate the synovitis and result in pannus formation. The final stage is evidenced by cartilage and bone destruction.

The genetic susceptibility and autoimmune phenomenon associated with RA find a common link in the synovial tissue. Autoantigens found in this tissue, although as yet unidentified, are thought to be the target of a directed immune response involving monocytes, macrophages, lymphocytes, endothelial cells and fibroblasts. These cells contribute to enhanced cytokine expression which results in increased angiogenesis, chemotaxis, and inflammation eventually leading to aggressive growth and proliferation of synovial fibroblasts and surrounding joint destruction. As mentioned, cartilage and

underlying bony destruction are secondary to invasion by this advancing synovial tissue as well as an increased production of proteases (MMPs), plasminogen activators, toxic oxygen radicals, and arachadonic acid metabolites by the synovium (Konttinen et al 1999). Moreover, TIMPs and plasminogen activator inhibitor antagonists are decreased in RA synovial endothelium (Jackson et al 1998).

The first genetic association with RA was described in 1978 as HLA-D (Stastny 1978) but these associations have grown to include a variety of proposed triggers. Monozygotic and dizygotic twin studies have confirmed this genetic bias (Silman 1997, Seldin et al 1999, Wiles et al 1999). Despite this, these influences are thought to be polygenetic and are exceedingly complex. Regardless, continued focused studies of these conditions and potential genetic markers may provide avenues for therapy and further insight into the mechanisms of cartilage destruction.

LACK OF INTRINSIC REPAIR

Tissue repair falls under two categories, intrinsic and extrinsic. Intrinsic cartilage repair involves the replication of cells adjacent to a defect and an increase in ECM production to fill it. Extrinsic repair instead is the metaplasia of cells from other connective tissue stem cell sources such as synovium or subchondral bone marrow to chondrocytes (Sokoloff 1974 and 1978). Most cartilaginous lesions do not penetrate subchondral bone and the focal erosion that results leads to progressive OA changes and a painful debilitated joint (Lieberman et al 2002). Injury that does penetrate subchondral bone provides a source of progenitor cells. A fibrin clot initially fills the defect; vascular ingrowth follows. A few hyaline like chondrocytes may initially form but ultimately the defect is filled with fibrocartilage (reviewed in Mankin 1982).

There may be some symptomatic relief afforded by fibrocartilage reconstitution of the defect (Lieberman et al 2002). Even for those who claim to have generated a hyaline-like repair, within 12 months the tissue is fibrous and fibrillated (reviewed in Mankin 1982). There has been some evidence that continual passive motion (CPM) may lead to a more normal hyaline cartilage repair (Salter et al 1980) but this also has been inconsistent. Connective tissue such as bone, tendon, and synovium demonstrate healing with a parent-like tissue (Mankin 1982). However, articular cartilage, like many other human tissues lacks this intrinsic regenerative potential. Instead, it forms a fibro-cartilaginous scar resulting in an irregular joint surface which is unstable and eventually undergoes further degeneration.

This inability for self-renewal has been attributed to the following features: 1. a lack of chondrocyte proliferation; 2. the avascular state of the tissue that precludes clearing of debris and provision of new cellular elements; 3. a change in cell phenotype which shows altered sensitivity to catabolic and anabolic regulators in the microenvironment and subsequently a decreased synthetic capacity to retain or regenerate ECM. Despite not reaching an end result of repair, when cartilage injury occurs, a battle does ensue and has been described in three steps (Fukui et al 2001). Step 1 is the initial cartilage assault; in Step 2 the chondrocytes generate an attempt at repair with an enhanced rate of ECM turnover. Step 3 is the unsuccessful outcome of cartilage degradation. Why chondrocytes fail in Step 2 at repairing cartilage is not clear.

Chondrocytes from immature cartilage show evidence of mitosis (reviewed in Mankin 1982) and in this second phase of attempted healing there is a documented hypertrophic chondrocyte response and cell proliferation (Goldring 2000a). With simultaneous high matrix turnover, chondrocytes are subject to microenvironmental changes that likely

contribute to their phenotypic shift. Not only does the responsiveness profile of the cell change, its ECM production decreases and rather than generating type II collagen necessary for hyaline cartilage resiliency and visco-elastic properties, type I and III collagen predominate. These represent the main constituents of fibrocartilage, explain the presence of this tissue in the healing response, and are characteristic of a dedifferentiated chondrocyte (reviewed in Mankin 1982). At long term follow-up, dysfunctional fibrocartilage formed during the hypertrophic phase demonstrates significant substance loss and consistent with Step 3, failure of repair is noted.

THE HISTORY OF ATTMEPTED CARTILAGE REPAIR –

DE/REDIFFERENTIATION

***In vivo***

Research continues its aggressive efforts toward cartilage regeneration and repair because historically little success has been achieved. The race to restore a stable joint began early and on a large scale. In 1925, Lexer attempted transplants of half and whole joints from amputated limbs and cadavers to restore damaged joints (Lexer 1925). His failure was attributed to poor fixation and rejection by the host. Despite similar findings in many animal models which showed this approach to be impractical (reviewed in Bentley and Greer 1971), Volkov repeated these studies in 1970 (Volkov 1970) and faced the same problems. Prior to his work, however, Chesterman and Smith began a very dissimilar approach on a much smaller scale using isolated adult articular chondrocytes in suspension to fill the cartilage defect (Chesterman and Smith 1968). Their enthusiasm for the technique stemmed from the immune privileged nature of the tissue, its avascularity and the inhibitory action of dense ECM on invading antibodies (Chesterman and Smith 1968, reviewed in Bentley and Greer 1971). These attempts resulted in fibrocartilage

production. Two explanations were offered: 1. the cells were not secured in the defect and as a result the defect had healed by extrinsic repair from subchondral bone; 2. the moving joint created an "unfavourable" environment for healing. Bentley and Greer considered these results and those previously described and concluded that cell transplantation using immature cells capable of division would provide an advantage. They repeated this work with articular chondrocytes of immature rabbits; however, this again resulted in fibrocartilage formation and poor incorporation of the tissue into the defect (Bentley and Greer 1971). Holding to the theory that extrinsic repair from subchondral bone MSCs could result in hyaline cartilage repair, the previously established Pridie technique of subchondral drilling (Insall 1974), resurfaced (Mitchell and Shepard 1976). Rabbit femurs subjected to subchondral drilling showed resurfacing of defects but by 8 months fibrocartilage predominated and was poorly incorporated at the joint surface.

Throughout the 1970's, reports were published on the potential of perichondrium to generate cartilaginous tissue. Initially reports of the use of rib perichondrium to fill defects in rabbit femoral condyles were encouraging. Evidence of hyaline cartilage formation was reported although incorporation was not optimal and follow-up was only for short periods (Engkvist and Wilander 1979). The lack of success with extrinsic repair and the somewhat encouraging results of cell transplantation resulted in extensive literature in the 1980's and early 1990's. These provided an exhausting variation on connective tissue replacement techniques for cartilage defects. Use of perichondrium, periosteum, growth plate chondrocytes, isolated articular chondrocytes, cartilage grafts or explants, stem cell transfers including osteoblasts, chondroblasts, and MSCs were described as well as a return to innate repair through cartilage shaving (Wakitani et al 1994, Odenbring et al 1992, Goransson et al 1995). Some success was elucidated for

chondral shaving via arthroscopy although most investigators believe that it is the extensive joint washout with arthroscopy which simply alleviates patient symptoms (Mankin 1982). In one study, no healing was evident at 12 weeks with chondral shaving although variable results were evident with the addition of CPM (Kim et al 1991).

Similarly, connective tissue replacements were met with a lack of success. Cultured growth plate chondrocytes and articular and growth plate allograft inserted into papain induced and surgically created defects in rabbit knees resulted in a lack of graft incorporation, inadequate defect filling and eventually a fibrous repair (Aston and Bentley 1986). Attempts followed to secure grafts. Grande et al showed evidence of repair with autograft explants sutured in rabbit knees although only at 6 weeks of follow-up (Grande et al 1989). Homminga et al also showed encouraging results with costal perichondrium secured with fibrin glue into partial thickness defects of rabbit knees at 4 months (Homminga et al 1989). Kawabe and Yoshinao attached growth plate cartilage into full thickness defects with fibrin clot and demonstrated that all implants died by 12 weeks and were replaced by fibrous scar (Kawabe and Yoshinao 1991). In addition to securing the graft, CPM was compared to cast immobilization with periosteal transplants in rabbit knees. Increased chondrogenic potential with CPM was seen at 3 weeks (O'Driscoll et al 1986) and persisted at one year (O'Driscoll et al 1988). A similar study was reported using perichondrial autograft and CPM in rabbit knees but is difficult to interpret as failure of the graft to generate sufficient cartilage to fill the defect was considered an exclusion criterion (Woo et al 1987).

Although cartilage is considered an immune privileged tissue, Stevenson et al tested antigen matched and non-matched osteochondral allograft against autografts in dog knees. Non-matched tissues resulted in inflammation, synovial fibrosis and no healing

whereas matched grafts and autografts demonstrated repair but showed thinning and fibrillation at 11 months (Stevenson et al 1989).

The need to consider more than defect filling and graft incorporation when determining successful repair was established. Wakitani et al claimed success with allograft chondrocytes in a collagen gel suspension in full thickness defects at 24 weeks because he achieved an 80% type II collagen production (Wakitani et al 1989). One year later, Robinson et al reviewed the failure of the preceding decades-worth of research to attain a successful model of cartilage repair (Robinson et al 1990). He attributed the failure to a lack of adherence of transplanted tissues (Bentley and Greer 1971), resultant fibrocartilage production from dedifferentiated cells or extrinsic repair (Bentley and Greer 1971, Bentley et al 1978, Aston and Bentley 1986), and degeneration with time of any hyaline repair achieved. With this review, Robinson et al brought to the forefront the importance of the chondrocyte microenvironment. Cells were being seen as having a distinct phenotype which could be influenced by their architectural and regulatory surroundings.

Pursuing these ideas, transplanted cells were instead delivered on scaffolds. A past argument has been that grafts were inadequately secured (van Susante et al 1995); therefore, demineralised bone matrix (DBM) was trialed as a scaffold and seeded with articular chondrocytes. Tissue generated within the defects neither filled them nor was consistently hyaline in nature (Billings et al 1990, Dahlberg and Kreicbergs 1991). Scaffolds seeded with human articular and costal chondrocytes *in vitro* did, however, develop tissue resembling cartilage with type II collagen (Freed et al 1993). A combination of HA as a delivery substance with embryonal chondrocytes with theoretically improved proliferative capacity was described as successful in reconstituting

partial and full thickness defects in chickens although there was no conformation of collagen content or type noted (Robinson et al 1990). Perichondrial cells were successfully proliferated at high density on biodegradable carriers but at one year none appeared normal; all showed decreased GAG content and inadequate filling (Chu et al 1997).

The inconsistency of cartilage repair with chondrocyte transplantation seen in animal models warranted the slow progression of its use in humans. Early reports showed satisfactory resolution of symptoms up to 9 years post-treatment (Peterson et al 2000) but limited information exists on tissue composition at repair sites (Richardson et al 1999) and that which does exist demonstrates inconsistent healing and frequent fibrocartilage formation (Peterson et al 2000, Richardson et al 1999, Roberts et al 2001).

Approaches to regenerating articular cartilage have been extensive and diverse. Cell source has ranged from MSC, perichondrium, and periosteum to autogenous chondrocytes. Grafts have been secured with sutures, fibrin clot, glues, and DBM. Intrinsic and extrinsic healing has been pursued with shaving, drilling, and osteotomies. Transplanted cells have been delivered in collagen gels and HA or on non/biodegradable scaffolds. The common link between failures of these techniques is the formation of fibrocartilage production by a phenotypically altered chondrocyte.

***In vitro***

Paralleling the aforementioned *in vivo* research on cartilage repair, a tremendous body of literature was emerging based on phenotypic changes of chondrocytes *in vitro*. Benya and Shaffer introduced the term "dedifferentiated" chondrocyte in 1982 (Benya and Shaffer 1982) based on previous work (reviewed in Benya and Shaffer 1982, Thonar et al 1986). This dedifferentiated state of the chondrocyte was deemed responsible for the generation

of the type I and III collagen of fibrocartilage seen with both intrinsic and extrinsic cartilage repair noted in ongoing *in vivo* work. Benya and Shaffer theorized that monolayer culturing resulted in the dedifferentiated chondrocyte phenotype and that restoring a three-dimensional (3-D) architecture to the cells' microenvironment could redifferentiate them. Cells were cultured in monolayers, allowed to undergo dedifferentiation and then were suspended in 0.5% agarose. This was demonstrated to restore spherical morphology and ECM production typical of hyaline cartilage (Benya and Shaffer 1982). Many reports utilizing 3-D tissue culture techniques followed (Thompson et al 1985, Guo et al 1989, Hauselmann et al 1992 and 1994, Redini et al 1997).

Combining a 3-D tissue culture system with evidence that accumulation of hyaline matrix stabilizes phenotype (Searls and Janners 1969), rat muscle mesenchymal cells were cultured in agarose in combination with DBM extract. Chondrogenesis ensued with production of PG and type II collagen (Thompson et al 1985). Guo et al demonstrated that a 3-D matrix maintained chondrocyte morphology, caused cells to aggregate, and resulted in a pericellular matrix deposition (Guo et al 1989). Alginate had been introduced as a negatively charged unsulfated copolymer of L-guluronon and D-mannuronon which polymerizes in the presence of divalent cations ($Ca^{++}$) to form a gel (Martinsen et al 1989). Hauselmann et al saw the advantage of its use as a 3-D medium for chondrocytes because it could be readily depolymerised with $Ca^{++}$ chelation (NaCitrate) allowing the study of both cell and matrix. The original article using alginate for chondrocyte culture described ECM production as 1/3 cell associated matrix (CAM) and 2/3 scattered intercellularly (Hauselmann et al 1992). They later demonstrated that these cells could be maintained for up to 8 months in 3-D alginate matrix and that two cell populations

emerged, one of flattened morphology at the bead surface and one of rounded interior cells with CAM (Hauselmann et al 1994). The redifferentiation process of dedifferentiated chondrocytes within alginate beads has been confirmed using polymerase chain reaction (PCR) analysis for mRNA of type II collagen (Bonaventure et al 1994) and was also seen with suspension cultures and pellets (reviewed in Solursh 1991) but not with collagen matrix (van Susante et al 1995).

Pluripotent cells from periosteum in monolayer culture differentiated toward an osteoblastic phenotype but in agarose gel suspension differentiated toward a chondrogenic phenotype (Bahrami et al 2000). Dedifferentiated chondrocytes cultured in 3-D alginate beads at 5% and 21% oxygen showed that cells redifferentiated and type II collagen was produced at 5% but not at 21% (reviewed in Domm et al 2002).

*In vivo* and parallel *in vitro* studies have demonstrated that differentiated chondrocytes produce a predictable and tightly regulated ECM composed primarily of type II collagen, and minor amounts of type IX, XI, and X (Petit et al 1992, Mayne and Brewton 1993, Cancedda et al 1995). The OA chondrocyte is now considered a model of a phenotypically altered or dedifferentiated cell producing types I, III, and X collagen and an architecturally disturbed ECM (Ronziere et al 1990, Hoyland et al 1991, von der Mark et al 1992). The type of collagen produced appears to be affected by culture conditions and regulators within the cell's microenvironment (reviewed in Ronziere et al 1997). Recently, using new DNA array technology, several genes have been identified to be involved in the redifferentiation process including IL-6, gp130 (component of the IL-6 receptor) and various TGF-β related genes (Haudenschild et al 2001). In addition, Col 2A1 promoter-reporter activity was inhibited in monolayer cultures but rescued in 3-D

culture systems and appeared to be linked to Sox 9 modulation, a marker of OA chondrocyte phenotype (Stokes et al 2001).

CURRENT THERAPY DISSATISFACTION

Despite these advances in the understanding of chondrocyte phenotype and ECM, an effective cartilage regeneration or repair remains elusive. However, from this literature has grown the notion that GFs and cytokines within the chondrocyte microenvironment contribute equally with architecture in controlling phenotype. Therefore, efforts are being made to combine these strategies. Sanchez et al have used alginate bead cultures in conjunction with nonsteroidal anti-inflammatories (NSAIDS) to block $PGE_2$, to negatively regulate IL-6, and to enhance aggrecan synthesis (Sanchez et al 2002). Rabbit perichondrial cells cultured in alginate beads were seen to initiate type II collagen production with the addition of IGF-1 and TGF-β to the culture conditions (van Osch et al 2000).

These strategies to redifferentiate chondrocytes, maintain their phenotype, and generate hyaline cartilage are currently focused *in vitro*. Ultimately, intervention of joint disease would have to be at very early stages *in vivo*. For RA this means targeting the initiation phase and autoantigens. However, autoantigens have yet to be conclusively identified and current treatment strategies are based around the central phase of inflammation. Present strategies target inflammatory mediators through non-specific suppression of lymphocyte and macrophage activation. This inhibits inflammatory changes, synovial proliferation, and reduces patient symptoms but does not completely prevent progression toward cartilage destruction.

Similarly, medical management of OA disease targets patient symptoms but fails to prevent disease progression. Joint replacement is an invasive end stage option but has limited application in the hand and wrist and is even more limited in the spine. If the concepts of chondrocyte differentiation, the microenvironment (both architectural and regulatory agents such as growth factors within), and techniques of *in vivo* delivery could be incorporated into a unifying treatment strategy, disease progression could potentially be halted at early stages.

## TGF-β

FUNCTION

### The Superfamily

TGF-β belongs to a large group of multifunctional cytokines which regulate development and tissue homeostasis in invertebrates and vertebrates (reviewed in Massague et al 2000). They demonstrate a high degree of evolutional conservation and widespread expression. Over 30 members of structurally related proteins have now been identified as belonging to this TGF-β superfamily of polypeptides and are generally grouped into four major families (Massague 1992): (i) the Mullerian inhibitory substance family (MIS); (ii) the inhibin/activin family; (iii) the Vg-related family containing a variety of members including the bone morphogenetic proteins (BMPs); and (iv) the TGF-β family. This family is thought to be derived from a common ancestral gene (Massague 1990a) and each, with the exception of MIS, is encoded as a larger precursor which is then processed to a C-terminal monomeric unit of 100-134 amino acids (Cate et al 1986).

Some of these family members are expressed only in a few cell types or for limited time periods during development; however, others are ubiquitous and play diverse roles in

embryogenesis and in adult tissues (Roberts and Sporn 1990, Kingsley 1994, Goumans and Mummery 2000). Members of the TGF-β superfamily influence a variety of normal cellular processes including ECM secretion, cell adhesion through integrin expression, cell proliferation and apoptosis, and immune response. Moreover, alteration in superfamily signaling has been linked to various developmental abnormalities, cancer progression, inappropriate wound healing, and several fibrotic and other disease states (Arkwright et al 2000, Yamada et al 2000, Yokota et al 2000). BMPs are potent inducers of bone and cartilage formation and play important developmental roles in the induction of ventral mesoderm, differentiation of neural tissue, and organogenesis (reviewed in Kingsley 1994 and Hogan 1996). Activins, named so because of their activation of follicle stimulating hormone secretion from the pituitary, also promote erythropoeisis, mediate dorsal mesoderm induction, and contribute to survival of nerve cells (reviewed in Mathews 1994). MIS is a protein which regulates Mullerian duct regression in male embryos (Cate et al 1986).

**Characterization of TGF-β**

The TGF-β group of dimeric hormonally active polypeptides has been under much investigation. It is suggested that TGF-β was initially characterized as a growth-stimulating peptide capable of inducing anchorage independent growth in normal fibroblast cell lines (de Larco and Todaro 1978). However, Cartilage-inducing Factor-A, was previously isolated from DBM when it was shown to induce cartilage and bone formation in subcutaneous and intramuscular tissue (Urist 1965, Reddi and Huggins 1972, Hanamura et al 1980, Seyedin et al 1983) and was later confirmed to share the identity of TGF-β (Seyedin et al 1985). TGF-β was purified from human platelets and

placenta (Assoian et al 1983 et al, Frolik et al 1983) and characterized as a disulphide-linked homodimeric peptide with a molecular mass of 25 kDa. Five distinct genes encoding TGF-β have been identified in vertebrates but only three isoforms (with 60-80% homology) are present in mammals (TGF-β1, -β2, -β3) (Massague 1990a and 1990b). The TGF-β1 gene maps to chromosome 19q13.1-13.3, TGF-β2 to 1q41, and TGF-β3 to 14q23-24 (reviewed in Grande 1997).

The TGF-β isoforms are generally homodimers although heterodimers of -β2/3 and -β1/3 have been isolated from bone (Ogawa et al 1992). These isoforms are differentially expressed in tissues (reviewed in Bonewald 1999) suggesting isoform specific functions. TGF-β gene knockout studies in mice have also supported distinct functional roles for TGF-β isoforms. Interestingly, their *in vitro* biologic activities are similar whereas *in vivo* functions differ. The loss of an isoform is not compensated by the presence of others which may be explained by the tissue-specific and spatio-temporal expression differences in developing tissues. TGF-β1-deficient mice, if born alive, undergo early postnatal death from excessive infiltration of inflammatory lymphocytes and macrophages into several organs likely related to immune dysfunction (Diebold et al 1995, Shull et al 1992, Kulkarni and Karlsson 1993). Approximately 50% die in utero with severe defects in vasculogenesis and haematopoiesis (Dickson et al 1995a). TGF-β2-deficient mice exhibit multiple developmental malformations of cranial, cardiac, lung and genitor-urinary tissues resulting in perinatal death (Sanford et al 1997) and TGF-β3 null mice die shortly after birth secondary to abnormal pulmonary and palate development (Kaartinen et al 1995, Proetzel et al 1995).

**TGF-β Effects**

Unlike classical hormones, members of the TGF-β family produce different effects that depend on the type and state of the cell. Although TGF-β has been widely viewed as a growth-stimulatory factor for mesenchymal cells and a growth inhibitory factor for epithelial cells, TGF-β is pluripotent. TGF-β1 has the ability to suppress proliferation of normal and oncogenically transformed cells of epithelial, hematopoietic, and endothelial origin; to promote the development of bone and cartilage; to inhibit the differentiation of muscle, adipose, and hematopoietic progenitor cells; and to influence the expression of differentiated functions in cells of the immune and endocrine systems (reviewed in Massague 1985). Focal administration of TGF-β1 to chorioallantoic membrane *in vivo* has resulted in gross angiogenesis and increased cell density of fibroblasts, epithelial and endothelial cells. TGF-β inhibits cell proliferation by activating at least two distinct inhibitors of cyclin-dependent kinases that phosphorylate the retinoblastoma gene (Rb) (Reynisdottir et al 1995, Polyak 1996). In addition to this cell cycle regulation, TGF-β is also capable of triggering apoptosis in several tumour cell lines (Yamamoto et al 1996, Havrilesky et al 1995, Perry et al 1995). TGF-β's promotion of ECM deposition is a result of increased production of matrix macro molecules, decreased synthesis of proteases, and increased synthesis of protease inhibitors (Rosen et al 1988, Varga et al 1987, Madri et al 1988, Laiho et al 1986, Overall et al 1989). Taken together, with the evidence that TGF-β1 is a potent chemoattractant for monocytes (Wahl et al 1987) and fibroblasts (Postlethwaite et al 1987), these findings implicate TGF-β as a key player in wound healing.

Chemotactic effects of TGF-β1 for dermal fibroblasts occur at levels much lower than those required for growth inhibition or ECM synthesis (Postlethwaite et al 1987)

indicating that distinct functions may be determined simply by the specific isoform or its concentration. TGF-β1 and -β2 are indistinguishable in most assays *in vitro*. However, TGF-β1 has been reported as more potent than -β2 at inhibiting hematopoietic progenitor and aortic endothelial proliferation or at deactivating macrophages. TGF-β2 appears to be stronger at inducing mesodermal differentiation markers (all reviewed in Massague 1990a). TGF-β isoforms have both autocrine and paracrine effects on tumour cells and have been implicated in tumour suppression as well as progression and invasion (reviewed in Derynck et al 2001). Loss of TGF-β responsiveness appears to provide a distinct advantage for developing tumours. Abrogation of TGF-β signaling leads to a loss of growth inhibition and early tumour onset, paradoxically protecting against tumour progression. However, in later stages, alterations in the TGF-β signaling pathway contribute to increased tumour progression, invasion, and metastasis through TGF-β effects on cell proliferation and the microenvironment (reviewed in Derynck et al 2001). These findings exemplify the diversity and significance of TGF-β action in developmental and adult tissues which cannot be simply explained by variation in expression level of these highly homologous isoforms.

THE SIGNALING CASCADE

For all the diversity and physiologic importance of the responses that TGF-β can elicit, a seemingly disarmingly simple system lies at the core of its signaling pathway. All members of the TGF-β superfamily generate a response via two serine/threonine protein kinase receptors (a type I and type II receptor) and a family of central mediators of signaling known as Smads which act as substrates for the activated type I receptor. The Smads translocate to the nucleus as an assembly to regulate transcription through

interactions with other DNA binding factors known as co-factors, co-activators, and co-repressors. Multiple sites of the TGF-β signaling cascade have been identified as potential regulatable targets to modulate transcriptional response including ligand activation and receptor binding, Smads, and alternate DNA binding elements. In addition, evidence of crosstalk with other signaling cascades provides alternate avenues to regulate TGF-β action. A simple schematic for TGF-β signaling has been included (Fig. a).

**The Ligands**

As noted, all three TGF-β isoforms are structurally similar proteins with distinct functions *in vivo*. Each isoform is controlled by a differentially regulated promoter (Roberts et al 1991) which likely provides the first level of regulation of TGF-β action. Posttranscriptional control of TGF-β isoforms provides an additional level of modulation (Kim et al 1992). All of these members are synthesized as large precursor molecules that are proteolytically processed in the Golgi by endoproteases such as Furin. The precursor is cleaved into the mature C-terminal TGF-β fragment and the N-terminal remnant known as the latency-associated protein (LAP) (Munger et al 1997, Cui et al 1998). LAP remains noncovalently linked to TGF-β in the highly stable small latent complex. Prior to leaving the Golgi, LAP covalently associates with latent TGF-β binding proteins (LTBPs) to form a large latent complex (Taipale and Keski-Oja 1997). This large complex provides stability to TGF-β, ensures correct folding of the final ligand, and promotes secretion to the ECM for storage and activation (Munger et al 1997, Taipale and Keski-Oja 1997). LTBPs contain RGD sequences that allow these large latent complexes to bind integrins ($\alpha_v\beta_1$) at the cell surface (Munger et al 1998) which may allow TGF-β to activate integrin related signaling pathways or may promote TGF-β activation through changes in cell

shape and cell-cell or cell-ECM interactions. Diversity in the TGF-β signaling cascade can be explained by variability in LTBPs (types 1-4) and through potential interaction with the integrin signaling pathways.

TGF-β must be activated from its latent complex before it can bind its receptors on the cell surface. This requires TGF-β release from LAP through several potential mechanisms including: protease cleavage (plasmin, cathepsin), LAP binding to mannose-6-phosphate receptors (Munger et al 1997, Taipale and Keski-Oja 1997), its deglycosylation (Miyazono and Heldin 1989), exposure to reactive oxygen species (Barcellos-Hoff and Dix 1996), an acidic environment (Jullien et al 1989), or thrombospondin-1 which causes a conformational change in LAP releasing TGF-β (Crawford et al 1998). Biologically active TGF-β forms homo or heterodimers (Cheifetz et al 1987, Ogawa et al 1992, Massague 1998) which are then able to bind TGF-β signaling receptors on the cell surface. The active ligand can be modulated by several extracellular proteins which have been shown to bind and modify its activity. ECM proteins, decorin and biglycan, α2-macroglobulin, fibronectin, and type IV collagen have all been implicated in this regulation (Yamaguchi et al 1990, LaMarre et al 1991, Mather 1996, Paralkar et al 1991).

**The Signaling Receptors**

TGF-β signals through a heteromeric transmembrane (TM) complex consisting of two type I (RI) and two type II (RII) receptors. Upon binding ligand, RII, a constitutively active kinase, is autophosphorylated on its cytoplasmic tail and recruits RI into the complex. RII phosphorylates RI in the GS domain of its juxtamembrane region causing activation (reviewed in Piek et al 1999). RI is thought not to bind ligand in the absence of its corresponding RII. The signaling diversity provided at the level of these TM receptors

is multifold. Differential kinetics in receptor biosynthesis, receptor internalization, and down-regulation of both signaling receptors may contribute to signaling modification and downstream effects (Koli and Arteaga 1997, Wells et al 1997, Anders et al 1997 and 1998). It is also possible that competition by different ligands at the level of receptors contributes to this variability. Moreover, a number of cytoplasmic proteins (TRIP-1, STRAP, Bα, FKBP12, FTα) are known to interact with the kinase domains of both signaling receptors and modulate their activity (reviewed in Roberts and Dernyck 2001).

Because of the nature of the thesis and its focus on TGF-β receptors in human chondrocytes, TGF-β receptors, their interactions and regulation of TGF-β signaling is discussed in more detail below.

RI/RII

An increasing number of distinct high affinity cell surface binding proteins for TGF-β have been identified but the type I (RI) and type II (RII) remain as the key signaling receptors and are both required to transduce the signal (Attisano et al 1994, Massague 1990a, Laiho et al 1990 and 1991, Wrana et al 1992, Franzen et al 1993, Bassing et al 1994, ten Dijke et al 1994). RI (65 kDa) and RII (85-110 kDa) are TM serine/threonine kinases (Massague 1992). They share the following structure: an N-terminal signal sequence, a short cysteine-rich extracellular region, a single hydrophobic membrane-spanning domain and a cytoplasmic region containing the kinase domain (reviewed in Visser and Themmen 1998).

As noted, ligand activation of RII, its autophosphorylation, recruitment of RI, subsequent phosphorylation and activation of its kinase domain result in transduction of the signal to the nucleus via Smads. The heteromeric receptor complex between RI and RII has been

confirmed by co-immunoprecipitation studies (Wrana et al 1992, Franzen et al 1993) which concluded that RII can bind ligand in the absence of RI but RI cannot bind ligand independently of RII and must be recruited into the complex (Wrana et al 1994). Both RI and RII possess binding constants in the low nanomolar (nM) range (Massague 1992). In addition, the RI/II complex binds TGF-β1 and -β3 with higher affinity than -β2.

Evidence that RII exists in a homodimeric complex independent of ligand suggests that RI and RII form a heterotetrameric complex (Chen and Derynck 1994, Vivien et al 1995). Although it has been difficult to demonstrate homodimers of RI, kinase-deficient and activation-defective RI are complementary indicating that two RII are likely required for signaling (Weis-Garcia and Massague 1996). Therefore, RII appears essential in ligand binding, whereas RI is essential to propagate the downstream signal.

The ligand binding domain (LBD) of RII was found through determination of its crystal structure to be a 3-finger toxin fold in the ectodomain but within a region distinct from that seen for the type II activin receptor (ActRII) (Boesen et al 2002). Site directed mutagenesis was also used to map the LBD of RII to the extracellular domain (ECD) and showed it to reside within the finger 1 region (Guimond et al 2002a).

Murine knockouts of RII die in utero with defects in hematopoietic and vascular development (Oshima et al 1996) similar to Smad 2,4, and 5 knockouts. Mutations in the RII gene correlate with loss of sensitivity to TGF-β and have been associated with various cancers and pre-cancerous states (Gobbi et al 2000, Inagaki et al 1993, Ionov et al 1993, Markowitz et al 1995). These mutations result in a loss of RII surface expression and a decrease in mRNA transcripts. In addition, truncated versions of RII have been implicated

in tumour development (Park et al 1994) as have dominant negative inhibitory mutants of RII (Knaus et al 1996).

RI, also termed Activin Receptor-like kinase-5 (Alk-5), is one of now 8 Alk receptors identified (reviewed in de Caestecker et al 2002). Mutation analysis of ECD residues of RI indicated a region on the convex face of this structure that affected signaling and ligand internalization suggesting it to be a functional site of RI and RII interaction (Guimond et al 2002b). The process by which RI transmits the TGF-β signal is becoming increasingly complex. Caveolin-1, found within the cholesterol-rich membrane microdomains of caveolae, has been shown to interact through its scaffolding domain with RI to suppress Smad 2 phosphorylation and downstream signaling (Razani et al 2001). The complexity of and necessity for RI in signaling is evident. Mice lacking RI die at midgestation with severe defects in vascular development of the yolk sac and placenta and abnormal haematopoiesis (Oh et al 2000). RI was shown to be crucial for vascular development but the abnormal haematopoiesis resulting from its absence could be compensated for by Activin receptor-like kinase-1 Alk-1 (Larsson et al 2001).

Alk-1

In most cell types, TGF-β signals via RI (Alk-5) but, as suggested by its role in haematopoiesis rescue (Larson et al 2001), endothelial cells also express (Alk-1) which binds TGF-β1 (Oh et al 2000). However, Alk-5 signals through Smad 2 and 3 whereas Alk-1 has been shown to modulate phosphorylation of Smad 1 and 5 (Chen and Massague 1999, Oh et al 2000). The link between these receptors and their vital role in angiogenesis has been identified through gene ablation studies in mice (Goumans and Mummery 2000). A human vascular disorder, hereditary haemorrhagic telangiectasia (HHT) II, has

been attributed to mutations in Alk-1. The co-existence of Alk-1 and Alk-5 on endothelial cells and their opposing effects on cell migration and proliferation suggest that TGF-β regulated the activation states of these cells via a fine balance of Alk-1 and Alk-5 signaling (Goumans et al 2002).

Alk-1 shares a high degree of similarity with the other type I receptors (Attisano et al 1993, Hanks et al 1988, ten Dijke et al 1993). In its constitutively active state, it phosphorylates and activates Smad 1 and 5 (Macias-Silva et al 1998) but the true ligand and corresponding type II receptor for Alk-1 are unknown. In the presence of RII, Alk-1 binds TGF-β1 and in the presence of ActRII, it binds activin A (Attisano and Wrana 1998, ten Dijke et al 1994, De Winter et al 1996). However, neither of these complexes was shown to elicit a downstream signal; thus, Alk-1 has been considered an orphan receptor. In addition to binding TGF-β1 and activin A, Alk-1 binds TGF-β3 and an unknown ligand present in the serum (Lux et al 1999). It therefore is reasonable to group Alk-1 with the TGF-β signaling receptors.

**Other Novel TGF-β Receptors**

RI and RII Variants

It was observed that a truncated mutant of RII identified in gastric carcinoma (Park et al 1994) was able to suppress TGF-β signaling and thus its inhibition of cell proliferation. This suggests that variants of the TGF-β signaling receptors themselves may function as local regulators of the signaling cascade and TGF-β action. The presence of another variant of RII, RIIB, was previously described but has recently resurfaced as a potential regulator of TGF-β action.

The original description of RIIB appears to have been by Glansbeek et al in 1993 (Glansbeek et al 1993). Bovine chondrocytes freshly isolated had more of the higher molecular weight type II receptor ($RII_2$) as compared to the monolayers cultured cells by affinity labeling. This suggested that there was differential expression of the variant forms RII with chondrocyte phenotype (Glansbeek et al 1997).

Hirai and Fujita then referenced 4 articles that depicted cell types, including human vascular endothelial cells, in which TGF-β1 and -β2 act differently. They then isolated a variant of the type II receptor (RIIb) in these cells (Hirai and Fujita 1996) suggesting that this differential response was attributable to receptor heterogeneity. Using affinity labeling, both the RII and RIIb receptors were shown to be responsive to TGF-β1 competition but not to -β2, and RIIb was shown to be functional in cells absent of RII.

Recently, Rotzer et al have further characterized this type II receptor variant, RIIB, claiming that the expression of RIIB is restricted to cells originating from tissues such as bone where the TGF-β2 isoform plays a predominant role and thus must be important in isoform specific signaling. Response to the TGF-β2 isoform was restored through transfection with RIIB in the absence of betaglycan suggesting that RIIB transmits signals directly in response to TGF-β2 via RI. Co-immunoprecipitation of RIIB with RI, RII, and betaglycan were also shown (Rotzer et al 2001).

A soluble form of RI has also now been identified and is a potential regulator of TGF-β at the receptor level. A unique cDNA clone encoding a soluble form of Alk-5 (TGF-β type I receptor, RI) was identified from a neonatal rat kidney cDNA library. This soluble receptor (Sol RI) was demonstrated to occur naturally and bind TGF-β1 in the presence of

RII. Sol RI has been shown to be a functional receptor that acts either as an agonist to TGF-β action or possibly as a ligand chaperone (Choi 1999).

Various extra and intracellular proteins that have also been shown to physically interact with the TGF-β signaling receptors include FKBP12, BAMBI, TRIP-1, STRAP, Bα, FTα among others (reviewed in Piek et al 1999 and Roberts and Dernyck 2001). There is also evidence that regulation of these receptors can occur at the level of transcription and translation and may be regulated by crosstalk with alternate signaling pathways. RI and RII mRNA levels were induced in response to PDGF isoforms but only RII protein levels were noted to increase (Czuwara-Ladykowska et al 2001). Several other compounds are currently being investigated as potential regulators of receptor levels to locally modulate TGF-β action (Callahan et al 2002). Whether the regulation of alternate forms of signaling receptors directly influences TGF-β action or indirectly influences its action through heteromeric complexes with the classic signaling receptors (RI and RII) and/or additional accessory receptors is unclear.

Betaglycan

Betaglycan is an accessory TGF-β receptor which increases the complexity of the TGF-β signaling cascade and provides a novel avenue to locally modulate TGF-β action. It is the most commonly occurring accessory receptor (originally described as the type III TGF-β receptor) (Massague 1985, Fanger et al 1986) with a molecular mass of 200-350 kDa. Through biochemical interactions, betaglycan is able to form heteromeric complexes with the TGF-β signaling receptors. It has high affinity for TGF-β1 and -β2 isoforms (Cheifetz et al 1987 and 1988) and has been shown to enhance signaling through ligand donation to RII (Cheifetz et al 1987 and 1988, Ohta et al 1987, Ignotz and Massague 1987, Bassols

and Massague 1988, Sankar et al 1995). Betaglycan is found on the surface of cells with the same frequency as the signaling receptors (Cheifetz et al 1988). It contains a large mass of carbohydrate contributed by heparan and chondroitin sulphate glycosaminoglycan (GAG) chains. Digestion of these GAG chains did not prevent TGF-β binding to the receptor core polypeptide and did not affect its anchorage in the cell membrane. The binding site for the ligand appeared to reside in the 100-120 kDa core (Cheifetz et al 1988).

Increased details of the structure of betaglycan continue to be determined. The receptor has been shown to be cleaved into 2 fragments (95 and 58 kDa) held together by disulphide bonds. In addition, a soluble form of the ECD could be shed with temperature and ligand regulated protease cleavage near the TM domain (Philip et al 1999). Deletion mutagenesis revealed 2 regions of the betaglycan ECD capable of binding TGF-β, the N-terminal portion (Lopez-Casillas et al 1994) and the C-terminal portion (Pepin et al 1994). These regions were further characterized (Esparza-Lopez et al 2001). Both were found to have higher affinity for TGF-β2 than -β1 but only the N-terminal region could increase -β2 labeling of RII yet, both could modulate Smad 2 phosphorylation. In addition, the N-terminal region was seen to bind inhibin A with greater affinity than TGF-β1 but less affinity than -β2. These properties alluded to the potential regulatory mechanisms of betaglycan in TGF-β signaling.

Elucidation of the functional roles of the cytoplasmic domain (CD) of betaglycan was also investigated (Blobe et al 2001). Betaglycan has a short CD; the interaction of betaglycan with RII was previously concluded to be an ECD association as deletion of the CD of betaglycan did not alter the interaction (Lopez-Casillas et al 1994). However, it

was demonstrated that the CD of betaglycan and RII interact specifically in a manner dependent on the kinase activity of RII and its ability to autophosphorylate (Blobe et al 2001). It has become clear that betaglycan has an important, albeit uncertain, role in regulation of TGF-β signaling. This was confirmed in cell lines lacking endogenous betaglycan (Eickelberg et al 2002). Interestingly, in L6 myoblasts betaglycan expression was seen to enhance TGF-β signaling whereas in LLC-$PK_1$ cells, betaglycan expression prevented the association of RI/RII in a GAG dependent manner and resulted in an inhibition of response (Eickelberg et al 2002). Evidence has been presented that the soluble ectodomain of betaglycan could enhance and inhibit signaling dependent upon TGF-β concentration (Fukushima et al 1993), although in most cases soluble betaglycan was believed to bind and sequester ligand acting as a receptor antagonist (Lopez-Casillas et al 1991, 1993 and 1994, Philip et al 1999).

Full-length forms of betaglycan have previously been reported to both enhance and inhibit TGF-β signaling. This inhibitory role, however, was attributed to the competitive binding of inhibin by betaglycan which results in inhibition of downstream TGF-β responses as opposed to activin binding which enhances responses (Lewis et al 2000). Based on the previous findings that the soluble form of betaglycan could inhibit TGF-β signaling, it was ascertained that recombinant soluble betaglycan could inhibit angiogenesis and tumour growth of breast cancer *in vivo* (Bandyopadhyay et al 2002). Similarly, overexpression of full-length betaglycan in breast cancer cells restored their TGF-β responsiveness and decreased clonogenic tendencies (Chen et al 1997a).

GPI-Anchored Proteins

Affinity labeling allowed for detection of many novel TGF-β binding proteins of different molecular masses in various cells (reviewed in Cheifetz and Massague 1991). Although most of the integral membrane proteins identified are inserted into the membrane via a polypeptide anchor, several proteins have been described which use a glycosyl-phosphotidylinositol anchor (reviewed in Low 1989). Sensitivity to a phosphatidylinositol-specific phospholipase C (PIPLC) enzymatic cleavage suggests the presence of such an anchor. Novel TGF-β binding proteins with these characteristics have been identified including a 180 kDa protein that preferentially binds TGF-β1 and 60 and 140 kDa proteins that preferentially bind TGF-β2 (Cheifetz and Massague 1991). Recently, a 180 kDa PIPLC sensitive TGF-β binding protein was identified on human skin fibroblasts and shown to form a heteromeric complex with the signaling receptors (Tam et al 1998). Another GPI-anchored TGF-β binding protein (r150) was subsequently identified on human keratinocytes (Tam et al 2001). It was demonstrated that the GPI anchor was contained within the 150 kDa protein and that upon enzymatic release the soluble form of r150 could bind TGF-β1.

Endoglin

Endoglin is a homodimeric integral membrane protein of two 95 kDa subunits (Quackenbush et al 1986, Gougos and Letarte 1988) joined by a disulphide bond ($Cys^{330}$-$Cys^{412}$; Raab et al 1999) in the ECD (Bellon et al 1993). It was first identified with a monoclonal antibody against the HOON pre B leukemic cell line (Quackenbush and Letarte 1985) and termed CD105 (Lastres et al 1994). Endoglin was subsequently cloned and its structure determined to consist of an ECD, TM region, and a short 47 residue CD. It has 4 potential N-linked glycosylation sites in the N-terminal domain and a probable O-

glycan domain rich in serine/threonine residues proximal to the TM region. An RGD tripeptide sequence has also been identified which is a key recognition structure in cell adhesion and infers a potential role for endoglin in integrin recognition (Gougos and Letarte 1990). Two forms of endoglin have been identified differing in their cytoplasmic tails. The L or full length form predominates (Bellon et al 1993) and has a 47 amino acid tail (Gougos and Letarte 1990). It contains 3 sites of mainly serine residues which are phosphorylated 8 times the magnitude of the single region on the S form (Lastres et al 1994). This S or short form is a spliced variant with a cytoplasmic tail of 14 amino acids (Bellon et al 1993, Gougos and Letarte 1990). Interestingly, administration of TGF-β1 has been shown to decrease endoglin phosphorylation (Lastres et al 1994), whereas endoglin was previously thought to exist in a constitutively phosphorylated state unaltered by TGF-β1 stimulation (Miyazono et al 1993).

Endoglin is expressed at increased levels on human endothelial cells of capillaries, arterioles, and venules (Quackenbush and Letarte 1985, Gougos and Letarte 1988); however, it is now known to be widely expressed but at variable levels. This glycoprotein has been documented at low levels on acute lymphoblastic and myelocytic leukemia cells and in a small percentage of normal bone marrow cells (Quackenbush and Letarte 1985, Hanks et al 1988). It has been shown on macrophages (Lastres et al 1992, O'Connell et al 1992), erythroid precursors (Buhring et al 1991), placental syncytiotrophoblasts (Gougos et al 1992), fibroblasts (Gougos and Letarte 1988), as well as ovarian tissue, uterus, cardiac tissue, skeletal muscle and spleen. Its distribution has been compared and found to be quite similar to TGF-β1 (St-Jacques et al 1994) and Alk-1 expression (Jonker and

Arthur 2002). Endoglin's expression on both normal and malignant tissues has been reviewed (Fonsatii et al 2001).

Endoglin is recognized as an accessory TGF-β receptor. It shares 71% sequence homology with betaglycan in the TM region and cytoplasmic tail (Cheifetz et al 1992, Gougos and Letarte 1990, Bellon et al 1993). Of this cytoplasmic tail, 40% is composed of serine/threonine residues (Gougos and Letarte 1990, Gougos et al 1992, Lopez-Casillas et al 1991) which suggest a possible site of phosphorylation. Endoglin binds TGF-β1 and -β3 with high affinity (Wrana et al 1992) but does not demonstrate affinity for TGF-β2 (Cheifetz et al 1992, Miyazono et al 1993). Approximately 1% of endoglin on endothelial cells is thought to bind ligand (Cheifetz et al 1992); moreover, RII is presumed to be required to enable this binding (Barbara et al 1999, Letamendia et al 1998a). Endoglin has also been demonstrated to bind BMP 7 and activin A in the presence of ActRII and ActRIIB respectively and BMP 2 in the presence of Alk-3 and Alk-6 (Barbara et al 1999). This receptor has been shown to form complexes with RI and RII (Cheifetz et al 1992, Zhang et al 1996, Lastres et al 1996, Miyazono et al 1993) as well as betaglycan (Wong et al 2000) and Alk-1 (Lux et al 1999).

Full length endoglin has been shown to interact with both RI and RII, independently of their kinase activation state or the presence of exogenous TGF-β1. Truncated endoglin constructs demonstrated that the association with the signaling receptors occurred through both extracellular and cytoplasmic domains, although distinct sites for the endoglin/RI and endoglin/RII interactions were noted. RI and RII phosphorylated endoglin's CD and conversely endoglin expression resulted in an altered phosphorylation state of the

signaling receptors, downstream Smads, as well as modulation of TGF-β signaling. (Guerrero-Esteo et al 2002).

Insight into the function of endoglin has been gained through studies of HHT I or Rendu-Osler-Weber syndrome. This is an autosomal dominant vascular disorder affecting 1-2:100,000 individuals with age-related penetrance (Guttmacher et al 1995, Plauchu et al 1989) and a variable phenotype. Gene mutations on chromosome 9q33-34 linked endoglin to this vascular disorder (McDonald et al 1994, Shovlin et al 1994). Forty-one mutations associated with HHT I have been described (reviewed in Paquet et al 2001). According to gene mutation studies in humans, there is a termination mutation in the ECD and if translated should result in soluble proteins. However, analysis of mutant proteins showed them to be unstable and to undergo intracellular degradation leading to a 50% decrease in endoglin expression at the surface of endothelial cells (Pece et al 1997). This haploinsufficiency model has been supported by several other studies (Shovlin et al 1997, reviewed in Paquet et al 2001). A single study demonstrated a dominant negative mutant which formed heterodimers on the cell surface (McAllister et al 1995).

Phenotypic characteristics associated with HHT I have been reviewed (Haitjema et al 1996) and classified into diagnostic criteria (Shovlin et al 2000). Typically, abnormal vasculogenesis is noted in the nasal mucosa, alimentary tract, pulmonary and cerebral tissue, and hepatic vasculature beds with telangiectasias as a classic feature and pulmonary and cerebral arteriovenous malformations (AVM) present in 15-20% and 10-15% of patients, respectively. Sophisticated murine models of endoglin heterozygote knockouts have been developed. Null mutants die at day 10-10.5 in utero as a result of defects in vasculogenesis and cardiac malformations (Bourdeau et al 1999) accounted for

by poor vascular smooth muscle development and arrested endothelial remodelling (Li et al 1999). Phenotype heterogeneity seen in human disease is also represented in these models (Bourdeau et al 2001).

As with other accessory TGF-β receptors, endoglin has been shown to modulate TGF-β signaling (Letamendia et al 1998a, Lastres et al 1996, Li et al 2000a). Overexpression of endoglin in monocytes has led to an inhibition of TGF-β responses (Lastres et al 1996) and myoblasts (Letamendia et al 1998a). Endoglin has been demonstrated to increase ligand binding to RI and RII (Letamendia et al 1998a) and endoglin itself appears to be regulated by TGF-β1 at mRNA and protein levels (Lastres et al 1996, Rius et al 1998, Botella et al 2001). TGF-β1 increased endoglin, -β1, RII and ECM (type I and IV collagen, fibronectin) in human mesangial cells while endoglin overexpression decreased collagen levels (Diez-Marques et al 2002).

Endoglin appears to have 2 distinct but likely interacting roles in angiogenesis and ECM regulation. Administration of TGF-β1 resulted in increased type I collagen, -β1, and endoglin levels consistent with its increased expression in renal glomerular sclerosis (Rodriguez-Pena et al 2001). Increased endoglin expression has also been documented in fibroblasts of scleroderma patients but whether it functions to increase ECM production or its overexpression is part of a negative feedback loop to decrease TGF-β response is unclear (Leask et al 2002). Mouse fibroblasts demonstrated decreased migration with endoglin overexpression possibly related to integrin (α5β1) expression (Guerrero-Esteo et al 1999).

Developmentally, endoglin plays a clear role in cardiogenesis (Arthur et al 2000, Qu et al 1998) as well as in vasculogenesis and angiogenesis (RayChaudhury and D'Amore 1991,

Folkman and D'Amore 1996). In fact, endoglin is now considered as a cell marker for both endothelial damage and repair and for angiogenesis, specifically in malignant tissues (reviewed in Li et al 2000a,b,c). Increased endoglin expression has been documented on endothelial cells of the central nervous system and breast carcinoma and malignant melanoma (Bodey et al 1998a, b, c). This increased expression of endoglin in tumours has been correlated with markers of cell proliferation (cyclin A and Ki-67) (Miller et al 1999). Interestingly, a soluble form of endoglin has been observed in human serum and is thought to result from a normal physiologic shedding process (Wang et al 1994) and increased levels of soluble endoglin in cancer patients have been suggested as a potential marker of metastatic risk (Li et al 2000a and b).

**The Smads**

Smads are the only known direct substrates of TGF-β (reviewed in Roberts and Dernyck 2001). R-Smads or receptor regulated Smads for TGF-β in mammalian cells are Smad 2 and 3. Their Co-Smad or co-operative Smad is Smad 4, whereas Smad 7 represents the I-Smad or inhibitory Smad. Once active, RI is thought to undergo a conformational change. The protein SARA contains a FEV1 zinc finger domain which anchors it to the intracellular surface of the cell membrane. Sara associates with RI and is thought to target Smads to the cell surface. Activation of RI causes a corresponding conformational change in SARA resulting in presentation of the R-Smads to RI for phosphorylation on C-terminus, activation, and release from SARA. These activated R-Smads form a complex, via the L3 loop, with Smad 4 (Co-Smad) in the cytoplasm and translocate to the nucleus. The nuclear localization mechanism of Smads appears highly complex but seems dependent on the C-terminus of Smad 4. However, it is now better appreciated that Smads

are also likely sequestered along cytoplasmic microdomains possibly organized by intracellular structural proteins.

**Gene Transcription**

Once in the nucleus, these Smads function as transcription factors to control target gene expression. Smad 3, once activated by RI, undergoes a conformational change which results in a dissociation of its terminal MH domains. The MH1 domain is then available for DNA binding. Smad 4 is also able to bind DNA and in its complex with Smad 3 does so at specific sequences known as Smad binding elements (SBEs). Smad 2 has not been shown to bind DNA directly. The Smad complex, however, requires the presence of cofactors or other transcription factors to efficiently bind DNA. FAST-1 is an example of a cofactor which binds not only DNA but also Smad 4 and 2 and provides further DNA interactions. AP-1 is another well known transcription factor which interacts at the level of DNA with Smads and provides an avenue for crosstalk with the mitogen-activated protein kinase (MAPK) pathway. In addition, various coactivators (p300, CBP) and corepressors bind DNA as part of the Smad and cofactor complex and modify the transcriptional activity. Smad 7 does not bind DNA but instead inhibits the TGF-β signaling cascade through negatively regulating the R and Co-Smad complex formation or by blocking activation of the R-Smads at the level of RI. Therefore, although the process of activation and trafficking of Smads to the nucleus seems quite structured, many avenues are present for regulation of signaling. Proteins that interact with RI or SARA and prevent Smad activation will interfere with signaling. Similarly, many proteins are now known to bind either of the MH domains of Smads and prevent their activation, complex formation, nuclear localization, or DNA binding. Cofactors,

coactivators, and corepressors are all capable of altering Smad function at the level of gene transcription (above reviewed in Piek et al 1999, Massague 2000, and Roberts et al 2001). All of the above modulators of TGF-β receptors and their central mediators, Smads, demonstrate the actual complexity of the TGF-β signaling and suggest potential avenues of regulation of TGF-β action in cells.

CROSSTALK

The possible involvement of alternate signaling pathways modulating TGF-β action was recognized when classic effects of TGF-β were found to occur independently of Smad 4. Pathways which have been implicated in mediating TGF-β effects include: RhoA, MAPK, PI3K and its downstream target Akt/PKN, Wnt, TNF-α and IFN-γ mediated cascades, and others. The direct links of these pathways to TGF-β receptors are unknown apart from the recently described role of the adaptor protein DAXX in mediating activation of JNK by TGF-β (Perlman et al 2001). However, several members of these pathways have been shown to directly interact with Smads and or have been activated themselves through TGF-β (reviewed in Roberts 2002). A schematic representation has been included to illustrate the level of complexity of TGF-β signaling and potential interactions among various signaling pathways (Fig. b). The TGF-β signaling pathway receives regulatory inputs pre and post receptor phase. The TGF-β pathway shows some evidence of internal regulation through inhibition of Smad complexes and Smad-RI interactions by I-Smads (Smad 7) and through ligand induced receptor internalization. More importantly, novel TGF-β receptors, through interactions with RI and RII appear to provide another avenue to locally influence TGF-β action. In addition, alternate signaling

pathways indirectly modulate the TGF-β cascade via interactions with TGF-β receptors, Smads, and other transcriptional regulatory proteins (reviewed in Massague et al 2000).

Fig. a

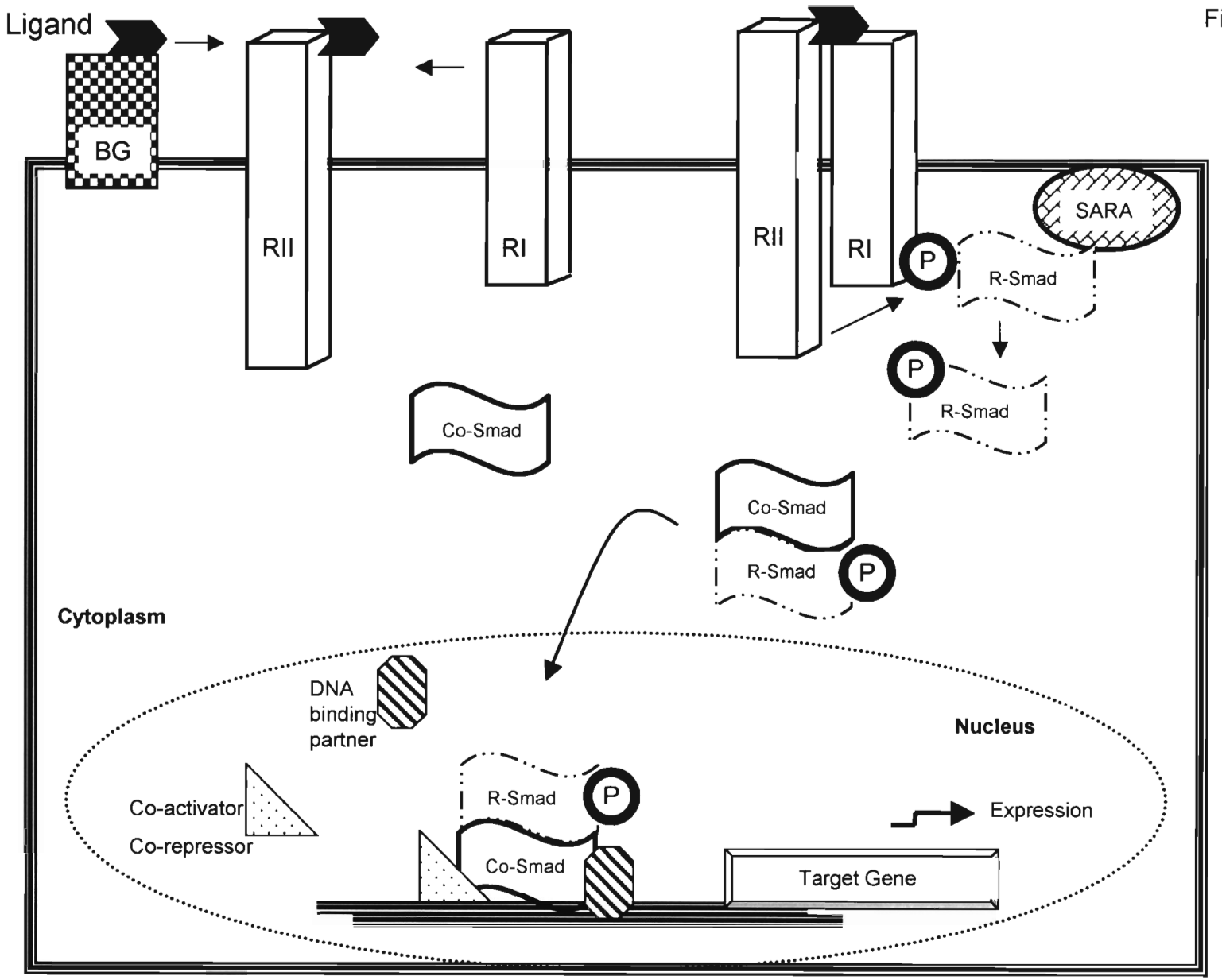

Ligand
BG
RII
RI
RII
RI
SARA
P
R-Smad
P
R-Smad
Co-Smad
Co-Smad
R-Smad
P
Cytoplasm
DNA
binding
partner
Nucleus
Co-activator
Co-repressor
R-Smad
P
Co-Smad
Expression
Target Gene

**Fig. a: Schematic representation of the TGF-β signaling cascade.** The basic TGF-β signaling motif consists of two serine/threonine protein kinase receptors (receptors type I (RI) and type II (RII)) and a family of central mediators of signaling known as Smads. Ligand binds RII (possibly with the aid of betaglycan (BG), autophosphorylates and recruits RI into a complex. RI is phosphorylated by RII and its kinase domain is activated. R-Smads (Smad 2 and 3) act as substrates for the activated RI and are sequestered at the membrane by SARA. The R-Smads associate with the Co-Smad (Smad 4) and all are localized to the nucleus as an assembly to regulate transcription. Smad 3 and 4 are able to bind DNA but with much higher affinity in the presence of transcriptional cofactors or DNA binding partners. In addition, co-activators and co-repressors modulate transcription at the level of DNA binding.

Fig. b

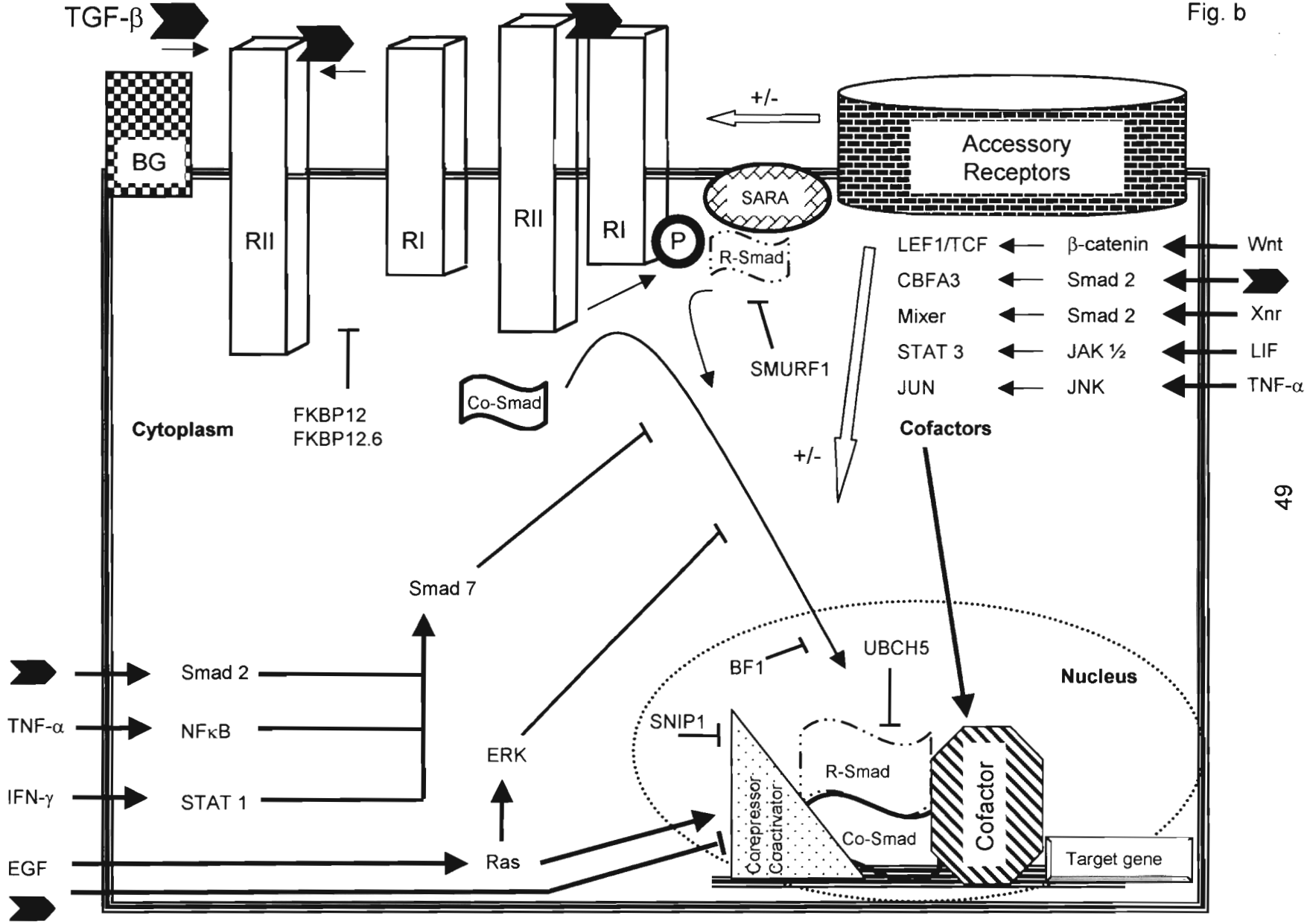

TGF-β
BG
RII
RI
RII
RI
P
SARA
+/-
Accessory Receptors
R-Smad
SMURF1
FKBP12
FKBP12.6
Co-Smad
Cytoplasm
LEF1/TCF
β-catenin
Wnt
CBFA3
Smad 2
Mixer
Smad 2
Xnr
STAT 3
JAK ½
LIF
JUN
JNK
TNF-α
Cofactors
+/-
Smad 7
Smad 2
TNF-α
NFκB
IFN-γ
STAT 1
EGF
ERK
Ras
BF1
UBCH5
Nucleus
SNIP1
Corepressor
Coactivator
R-Smad
Co-Smad
Cofactor
Target gene

**Fig. b: Potential levels of regulation of the TGF-β cascade and of crosstalk with other signaling pathways.** Several avenues of potential TGF-β regulation have been demonstrated including: transcriptional regulation of the TGF-β genes, their translation to protein products and storage as a latent complex, ligand activation, receptor expression and ligand binding, Smad regulation, and gene transcription through modulation by co-factors, co-activators, and co-repressors. Moreover, interaction with signaling pathways of alternate regulators of cell function provides yet another level of complexity to this system. TGF-β accessory receptors are now accepted as modulators of TGF-β signaling at the level of receptor interactions. Regulation of ligand storage and activation has been omitted for clarity.

## TGF-β AND CARTILAGE

### TGF-β PRODUCTION AND CHONDROGENESIS

TGF-β has been documented in abundance in cartilage (Ellingsworth et al 1986, Frazer et al 1991, Morales and Roberts 1988). mRNA of TGF-β1, -β2, and -β3 is evident in cultured human chondrocytes as well as produced in the culture medium (Villiger et al 1993). Moreover, human cartilage explants liberate TGF-β (Lafeber et al 1993). However, the ratio of isoform production has yet to be investigated.

Chondrogenesis, the process of differentiation from an immature precursor cell to one with a chondrogenic phenotype, is regulated by TGF-β. Cartilage-inducing Factor-A, later identified as TGF-β (Seyedin et al 1985), was isolated from DBM and shown to induce cartilage and bone formation in subcutaneous and intramuscular tissue (Urist 1965, Reddi and Huggins 1972, Hanamura et al 1980, Seyedin et al 1983). Some controversy exists as to whether TGF-β promotes this differentiation process and stabilizes the chondrocyte phenotype or promotes dedifferentiation into a fibroblastic-like cell.

Various studies support the role of TGF-β in chondrocyte differentiation. TGF-β3 in conjunction with Dexamethasone was shown to differentiate human MSCs toward a chondrocyte phenotype (Mackay et al 1998). TGF-β induced rat muscle MSCs toward a chondrocyte phenotype evidenced by increased type II collagen and PG production (Seyedin et al 1985 and 1986, Sampath et al 1987). Interestingly, human MSCs were noted to express endoglin (Majumdar et al 2000). These cells, when cultured in 3-D alginate beads with TGF-β3, up-regulated type II collagen mRNA and protein levels as compared to 3-D cultures in serum free medium (SFM). TGF-β1 has also been shown to

promote chondrogenesis in periosteum *in vitro* (O'Driscoll et al 1994, Miura et al 1994) and *in vivo* (O'Driscoll and Salter 1984). Moreover, synovial rabbit explants have undergone chondrogenesis when cultured with TGF-β1 (Nishimura et al 1999). TGF-β1 in bone marrow MSCs showed an increased chondrocyte differentiation in a dose dependent manner (Worster et al 2000).

In contrast, rabbit articular chondrocytes grown in TGF-β with or without fibroblast growth factor (FGF) became fibroblastic (Inoue et al 1989) and could not induce colony formation on agar (Iwamoto et al 1989). TGF-β1 has been shown to increase adult human articular chondrocyte proliferation in monolayer culture but it also contributed to dedifferentiation and a decrease in type II collagen synthesis (Jakob et al 2001). Similarly, rat chondroblasts *in vitro* could not be differentiated toward chondrocytes with TGF-β1 (Rosen et al 1988). No type II collagen was produced by these cells, decreased levels of PG were noted, and a change in cell morphology was evident. This suggested that this differentiation process or chondrogenesis also relied on a cell-ECM interaction.

GROWTH PLATE CHONDROCYTES

Many of the early studies on the role of TGF-β in cartilage were done in growth plate chondrocytes. TGF-β was demonstrated to have a dose related biphasic effect on PG synthesis in chick growth plate chondrocytes (O'Keefe et al 1988). TGF-β was also demonstrated to have a biphasic effect on cell growth but resulted in a decrease in type II collagen synthesis in these cells (Rosier et al 1989). This appeared to be related to the particular zone of cartilage and the proportion of "high affinity" versus "low affinity" TGF-β receptors on the cells. Unloading of rat joints was noted to cause a decrease in

cartilage height within the growth plate but TGF-β2 administration responded by increasing cell number in the proliferative zone (Zerath et al 1997).

Theoretical problems are inherent with these growth plate models. The response to TGF-β of chondrocytes, as for other cells, is proportional to their state of differentiation (Rosier et al 1989, Centrella et al 1995). In addition, TGF-β1 has been shown to have different effects on old versus young cells (Guerne et al 1995, van Beuningen et al 1994, Rosenthal et al 1994, Recklies et al 1989) or in normal versus diseased cells (Recklies et al 1989, Rayan and Hardingham 1994, van Beuningen et al 1994). Chondrocytes of different origins also respond differently to TGF-β (Lee et al 1997). Therefore, to investigate the role of TGF-β action on growth plate chondrocytes and attempt to extrapolate findings to articular cells is problematic.

ARTICULAR CHONDROCYTES

**TGF-β Regulation *In Vitro***

Because of the inherent difficulties posed by studies in growth plates, *in vitro* investigations using articular chondrocytes emerged. However, inconsistency in TGF-β action is also apparent in these studies. *In vitro* evidence has suggested that TGF-β could have both a mitogenic effect on chondrocytes and a role in ECM production. Early studies of cells in monolayer cultures showed increased DNA synthesis, cell growth (Skantze et al 1985), increased GAG synthesis (Anastassiades et al 1998) with TGF-β stimulation and synergy with platelet derived growth factor (PDGF) or IGF-1 (Chopra and Anastassiades 1998). This up-regulation of PG synthesis was subsequently demonstrated in many cell systems. In rabbit articular chondrocytes, TGF-β increased PG synthesis (Redini et al 1991) independent of cell proliferation (Redini et al 1988); in

calves it decreased PG losses (Morales and Roberts 1988) and in canine articular cells it increased PG synthesis (Venn et al 1990). In equine articular cells, TGF-β1 increased PG synthesis but this -β1 responsiveness was seen to decline with advancing age (Iqbal et al 2000). TGF-β2 has been described as a more potent stimulator of chondrogenesis, PG synthesis and cell proliferation of rabbit articular chondrocytes *in vitro* as compared to other TGF-β isoforms (Okazaki et al 1996).

In addition to PG synthesis, fibronectin was shown to be up-regulated in canine articular explants by TGF-β1 (Burton-Wurster and Lust 1990) and TGF-β increased type II and XI collagen and PG in rabbit articular chondrocytes and was able to counteract the destructive effects of IL-1 (Pujol et al 1991). TGF-β1 and -β2 administration with IGF-1 or insulin resulted in an increase in type II collagen and aggrecan in dedifferentiated adult cells (Yaeger et al 1997). Taken together, these investigations make a convincing argument for the regenerative potential of TGF-β in cartilage. However, notably few studies have been performed with human articular chondrocytes.

Despite this evidence of TGF-β promoting the chondrocyte phenotype and matrix synthesis, data also exist that suggests TGF-β negatively regulates these parameters. In rabbit articular cells, TGF-β inhibited type II collagen and PG synthesis (Rosen et al 1988). In bovine articular chondrocytes, TGF-β2 initially stimulated proliferation in monolayer cultures but could not retain the cell phenotype and within several passages type II collagen expression was lost (de Haart et al 1999).

**Alternate Regulation**

Indirect effects of TGF-β on chondrocytes and ECM have also been described. TGF-β can exert its effects on cartilage indirectly through MMPs and TIMPs or other ECM

players. Decorin and biglycan (1-2% of PG) bind TGF-β and could potentially mediate its effects on cell proliferation and ECM synthesis (Yamaguchi et al 1990, Hildebrand et al 1994, Iozzo and Murdoch 1996). It is quite well established that TGF-β regulates MMP and TIMP production (Edwards et al 1987). Human articular chondrocytes have been shown to produce increased collagenase 3 (MMP-13) with the addition of TGF-β in OA explant cultures (Moldovan et al 1997) which correlated with the cell's physiologic state (Moldovan et al 2000) and appeared to be mediated by increased furin levels (Tardif et al 1999). In addition, TGF-β1 has been demonstrated to increase aggrecan synthesis in CAM, intracellular MMP-13 and TIMP-1 and TIMP -3 all corresponding to RII levels in human articular chondrocytes cultured in agarose gel (Wong et al 2000).

Furthermore, other members of the TGF-β superfamily have been implicated in cartilage regulation. BMP-2 was seen to promote healing in full-thickness defects in rabbit articular cartilage but with evidence of poor integration of regenerated tissue (Frenkel et al 2000). BMP-2 was also noted to increase TIMP-1 mRNA and protein expression in bovine articular chondrocytes (Frenkel et al 2000).

TGF-β has also been combined with various 3-D culture systems to promote repair while maintaining cell phenotype. In bovine articular chondrocytes, increase of exogenous type II collagen in the culture conditions augmented the TGF-β1 up-regulation of aggrecan, DNA synthesis, and mRNA for procollagen αI(II) (Qi and Scully 1998), implicating ECM as a regulator of TGF-β action in cartilage. TGF-β caused an increase in dense pericellular matrix in bovine articular chondrocytes cultured in alginate beads (van Susante et al 2000) and up-regulation of PG synthesis by TGF-β1 was greater in alginate beads than monolayer cultures (Demoor-Fossard et al 1998). The increase in PG

observed in monolayer was found only in the medium but in bead cultures the increase was noted intracellularly and in the CAM (Demoor-Fossard et al 1999). Redifferentiation of human articular chondrocytes in pellets with the addition of TGF-β1 resulted in the largest increase in type II collagen mRNA, aggrecan, and tissue accumulation as compared to other factors tested (Jakob et al 2001).

**TGF-β Regulation *In Vivo***

It has been argued that the inconsistency with TGF-β administration to *in vitro* systems is a result of the invariable dedifferentiation of chondrocytes in monolayer cultures. An alternative to *in vitro* TGF-β administration to assess its regulation of articular chondrocytes is local delivery to the cell or joint surface. Adenoviral transfers have been shown to be a feasible option for gene delivery in chondrocytes (Smith et al 2000). Subsequently, transfer of the TGF-β1 gene in rabbit articular cells was demonstrated to result in increased PG and type II collagen synthesis in a dose dependent manner (Shuler et al 2000). Attempts have been made to activate TGF-β locally through photodynamic therapy (Sullivan et al 2002). This caused an increase in both latent and active TGF-β, and a decrease in IL-$1_\beta$ stimulated loss of PG.

Repeated injection of TGF-β1 into murine knee joints has been shown to prevent IL-1 induced destruction (van Beuningen et al 1993). Similarly, surgically created full-thickness defects in rabbit knees with implanted periosteal cells on fibrin gel underwent chondrocyte differentiation and increased PG synthesis with the addition of TGF-β1 (Perka et al 2000).

Despite this apparent regenerative potential of TGF-β *in vivo*, in other studies TGF-β has demonstrated adverse effects leading to joint inflammation, osteophytes and cartilage

destruction (Elford et al 1992, van den Berg et al 1993). Moreover, TGF-β1 administered to unloaded joints in growing rats caused age-related changes throughout the articular zones consistent with degenerative and inflammatory joint disease (Itayem et al 1999).

**Theoretical Problems with these Models**

Articular cartilage can be morphologically divided into zones thought to reflect different phenotypes and responsiveness of cells (Iwamoto et al 1989, Moldovan et al 2000). Growth effects of TGF-β are proportional to proliferative state of the cell population, that is the distribution of cells within each phase of the cell cycle (Sigel et al 1996). $G_0/G_1$ rabbit articular chondrocytes were growth inhibited by TGF-β1 whereas actively dividing S phase cells were shown to be growth stimulated (Vivien et al 1990 and 1992). There is also evidence that S phase cells demonstrate lower TGF-β1 binding (Vivien et al 1993a). In addition, TGF-β in rabbit articular chondrocytes causes recruitment of chondrocytes from the non-cycling SQ or $G_2Q$ phases and results in stimulation of cell proliferation which is clearly cell cycle dependent (Vivien et al 1990).

O'Connor et al have reviewed this TGF-β controversy in cartilage (O'Connor et al 2000). TGF-β has been reported to both increase and decrease PG synthesis, type II collagen synthesis, cell proliferation and differentiation, TIMPs and MMPs. One possible explanation offered for this inconsistency is the effect on TGF-β or cells directly from other cytokines or serum factors (reviewed in O'Connor et al 2000). In addition, other modulators may affect chondrocyte responsiveness to TGF-β. These include the state of cell differentiation or proliferation (cell cycle), culture conditions (monolayer, 3-D, serum, cytokines), as well as cell origin, species, and age, all of which may vary between *in vivo* and *in vitro* conditions and are likely to contribute to the TGF-β controversy.

Therefore, the therapeutic potential of TGF-β in cartilage repair will remain unrealized until its mechanism of action in this specialized connective tissue is further elucidated. A clearer understanding of the mechanism of action of TGF-β in chondrocytes may be gained through knowledge of TGF-β receptor expression on these cells.

TGF-β RECEPTORS IN CHONDROCYTES

Resting, proliferative, and mature zones of growth plate chondrocytes of growing rats express RI. RII is co-expressed in these zones but was only detected up to 6 weeks of development (Matsunaga et al 1999). RI, RII, betaglycan, and RV (likely a higher order complex) have been described in rabbit articular chondrocytes (O'Grady et al 1991) and shown to form complexes on the cell surface (Hall et al 1996). RI and RII have been localized to distinct zones of rat articular cartilage including the superficial, transitional, and least mature zones (Fukumura et al 1998). Addition of TGF-β1 in rabbit articular chondrocytes resulted in increased inositolphosphate glycan (IPG) levels which were inhibited with anti-TGF-β1 antibody (Vivien et al 1993b). The same increase in IPG was seen with PIPLC treatment suggesting the presence of a GPI anchored protein in rabbit articular chondrocytes. No evidence was presented that this represented a TGF-β binding protein but the increased IPG levels corresponded to an up-regulation of DNA synthesis and were thought to mimic TGF-β action in these cells.

Rapid turnover of TGF-β receptors on human bone has been described for growth plate chondrocytes (Centrella et al 1996). RI and RII have been demonstrated in hypertrophic and mineralizing zones of human growth plate chondrocytes (Horner et al 1998, Moldovan et al 1997). The limited information available on TGF-β receptors in human cartilage contributes minimally to our understanding of TGF-β action in cartilage.

REVELANCE OF TGF-β ACTION

Some interesting concepts implicating TGF-β in cartilage regeneration and repair have arisen out of the aforementioned *in vitro* and *in vivo* studies and ongoing investigations of diseased and normal cartilage.

Spontaneous regeneration of partial-thickness articular cartilage defects with hyaline cartilage have been documented in lambs in utero (Namba et al 1998). However, beyond in utero development cartilage does not undergo intrinsic repair with type II collagen. Cartilage injury can be regarded as a wound healing process in which growth factors, particularly TGF-β, play an integral role in many tissues (reviewed in Bos et al 2001). Increased growth factor expression including TGF-β1 and -β3 have been demonstrated after cartilage injury for up to 2 weeks which appears to correspond to the hypertrophic phase of attempted regeneration (Bos et al 2001). Although there has been controversy regarding the articular cartilage healing potential of TGF-β1 *in vivo*, it has been implicated in joint disease in 2 murine models. Transgenic mice, expressing a cytoplasmically truncated functionally inactive RII (dominant negative), develop joints which histologically resemble human OA (Serra et al 1997). Similarly, degenerative joint disease results in null mice with deletions in exon 8 of Smad 3 (Yang et al 2001). These models implicate TGF-β dysregulation in the development of OA phenotypes.

That TGF-β receptor levels correlate with cell phenotype and may reflect cell responsiveness has been demonstrated in normal articular chondrocytes. RI expression was found to be several fold higher in G0/G1 cells and overexpression of RI in the S phase suppressed TGF-β's mitogenic effect. This suggested that the ratio of RI/RII regulated cell proliferation as a function of cell cycle (Boumediene et al 1998b). A

change of TGF-β receptor expression has also been implicated in the development of an OA chondrocyte phenotype. OA cells have been described as having decreased RII expression (Boumediene et al 1998a). In addition, TGF-β1 effects were demonstrated to differ in OA versus normal cells causing an increase in PG synthesis in OA cells. Chondrocytes in upper layers of explants, more dedifferentiated, exhibited increased sensitivity to TGF-β1 (Lafeber et al 1997). However, TGF-β was found to up-regulate aggrecanase-1 gene expression in human synovial cells. This gene is also expressed in OA and RA chondrocytes but was not inducible by TGF-β indicating these cells may not be responsive to TGF-β (Yamanishi et al 2002). Despite this, increased TGF-β1 expression in synovial fluid of OA and RA patients has been described (reviewed in Bakker et al 2001).

It appears that dysregulation of TGF-β signaling may be correlated with an altered chondrocyte phenotype, TGF-β receptor expression profile, and TGF-β responsiveness. Over-administration of TGF-β by repeated *in vivo* injections of active TGF-β1 resulted in hyperplasia of synovium and osteophytes (van Beuningen et al 1994, Lafyatis et al 1989, Chu et al 1992). In contrast, appropriate local modulation of TGF-β appears to positively regulate cartilage repair. Adenoviral mediated TGF-β1 delivery into a synovial depleted joint did not demonstrate degenerative changes (Bakker et al 2001). Similarly, TGF-β increased α3/α5 integrin expression in bovine articular chondrocytes leads to an increase in cell adhesion to fibronectin and type II collagen (Loeser 1997). These findings underscore that multiple factors in the chondrocyte microenvironment are potentially regulating cell phenotype directly or indirectly by modulating TGF-β responsiveness.

Therefore, it is not surprising that TGF-β genes have recently been described as candidate genes for degenerative joint disease (Yamada et al 2000).

## RATIONALE

Degenerative joint disease inflicts over 60% of the aging population (Ferlic 1985, Praemer et al 1999, Oddis 1996). The articular cartilage of these diarthrodial joints is physiologically unique in that it does not possess the intrinsic healing capabilities seen in other connective tissues. This had been attributed to the avascular state of the tissue, a lack of proliferation by mature chondrocytes, and their dedifferentiation into a fibroblastic cell which produces type I and III rather than type II collagen and which displays altered sensitivity to anabolic and catabolic cytokines. This loss of visco-elastic cartilage results in a non-gliding, painful, and often unstable joint and ultimately a negative global impact on functioning. Despite extensive investigative efforts to achieve cartilage regeneration, hyaline repair has been elusive. Therefore, current medical treatment of degenerative joint disease focuses on alleviating patient symptoms and surgical treatment involves invasive soft tissue rebalancing or joint replacement and is focussed on end stage disease.

Research aimed at restoring hyaline cartilage has been fuelled by the success of tissue engineering in other areas. Although investigators have demonstrated the ability to steer mesenchymal stem cells and other precursors toward a chondrocyte phenotype (Johnstone et al 1998), this approach has its own limitations. The foundations of this work are *in vitro* studies where individual components of a cells microenvironment can be independently modulated. However, *in vivo* applications remain limited because of the complex milieu of regulatory factors influencing the tissue.

An important avenue of the tissue engineering field has been the investigation of wound healing and it is here that TGF-β has grown in stature. It has become readily apparent that TGF-β is intimately related to all phases of the wound healing process, namely cell growth and differentiation, ECM regulation, angiogenesis, and immune response. Applying the analogy of wound healing to cartilage injury led to the discovery of TGF-β's role as a key mediator in cartilage repair. Despite conflicting reports on the action of TGF-β on ECM *in vitro*, *in vivo* animal studies suggested that it promotes healing of full and partial thickness articular defects. In addition, homozygote Smad 3 (exon 8) knockout mice (Yang et al 2001) and transgenic mice expressing a dominant negative form of RII (Serra et al 1997) develop degenerative joint disease. In mice with a papain induced OA, an inhibition of endogenous TGF-β by addition of a scavenger soluble form of the type II signaling receptor, resulted in an enhanced proteoglycan loss and impaired cartilage repair (Scharstuhl et al 2002). These models provide a convincing link between dysregulation of the TGF-β signaling cascade and articular cartilage degeneration.

Although clearly implicated in the pathophysiology of cartilage degeneration, little is known about TGF-β action in human chondrocytes. Even less has been elucidated regarding TGF-β receptors on these cells. A single study by Horner et al (Horner et al 1998) describes the expression of RI and RII in growth plate chondrocytes. Immunolocalization of RI, RII, and betaglycan in distinct zones of OA cartilage explants has been documented (Moldovan et al 2000). In an insightful paper, Boumedeine et al demonstrate that OA chondrocytes are less sensitive than normal cells to TGF-β and exhibit decreased RII mRNA levels (Boumediene et al 1998). This change in functional

activity of OA cells and their altered ECM was attributed to the variation in receptor expression.

Unlike may other complex cell signaling pathways, TGF-β signals through a highly structured cascade, with Smads as direct substrates, to influence multiple target genes. Therefore, to elicit the appropriate downstream response, diversity in TGF-β signaling must arise at another level, potentially the TGF-β receptors at the cell surface. Increased information of TGF-β action in chondrocytes must be obtained before its application in cartilage repair and regeneration is realized. More clearly defining the expression profiles of TGF-β receptors and biochemical interactions between them, leading to the formation of hetero-oligomeric complexes in human chondrocytes, is critical to this understanding of TGF-β and its role in degenerative joint disease.

## HYPOTHESES

(1) that novel cell surface TGF-β binding proteins are expressed on human chondrocytes

(2) that these novel receptors are able to form hetero-oligomeric complexes with the TGF-β signaling receptors (RI and RII) and with other TGF-β receptors

(3) that these novel TGF-β receptors and complexes modulate TGF-β signaling and thus action in human chondrocytes

(4) that TGF-β receptors are potential markers of human chondrocyte phenotype and reflect cell responsiveness to TGF-β

(5) that TGF-β receptor expression modulates chondrocyte ECM production

## OBJECTIVES

(i) to define the expression profile of TGF-β receptors on human chondrocytes

(ii) to determine the structural features of TGF-β receptor interactions on the cell surface

(iii) to determine the functional role of TGF-β receptors and complexes in regulating TGF-β signaling in human chondrocytes

(iv) to investigate phenotypic differences in TGF-β receptor expression among chondrocytes at different stages of differentiation and determine if this correlates with cells responsiveness to TGF-β and ultimately ECM regulation

# Section 2

## MANUSCRIPTS AND ADDITIONAL RESULTS

# CHAPTER 1

# BRIDGE

Characterization of immortalized and primary human chondrocytes. Preliminary identification of TGF-β receptors on these cells with specific emphasis on endoglin and its interaction with betaglycan.

## CHAPTER 1

BRIDGING DOCUMENT

### Introduction and Rationale

The dramatic consequences of articular cartilage injury leading to degenerative joint disease arise from the lack of inherent repair seen in this specialized connective tissue. Efforts made to facilitate cartilage repair and regeneration have had limited success. TGF-β has emerged as a potential regulator of chondrocyte growth, differentiation, and ECM turnover but its action is poorly understood. Accessory TGF-β receptors in other cell types have been shown to regulate TGF-β signaling; however, the expression profile in chondrocytes has been poorly defined.

### Hypotheses

(1) that human chondrocytes are responsive to TGF-β1, -β2, and -β3 isoforms

(2) that novel cell surface TGF-β binding proteins are expressed on these cells

(3) that these novel receptors form heteromeric complexes on the chondrocyte surface

### Objectives

(i) to demonstrate the responsiveness of human chondrocytes to TGF-β1, -β2, and -β3 isoforms

(ii) to define TGF-β receptor expression profiles on human chondrocytes

(iii) to identify biochemical interactions between receptors leading to the formation of oligomeric complexes on the cell surface

### Summary of Manuscript Findings

In the first manuscript, the expression profiles of TGF-β receptors on human articular and nonarticular chondrocytes are demonstrated. Endoglin is shown to be expressed on

chondrocytes at levels comparable to human microvascular endothelial cells and its formation of a heteromeric complex with the type I (RI) and type II (RII) TGF-β receptors is confirmed. Moreover, endoglin's ability to form a heteromeric complex with betaglycan at endogenous receptor concentrations and ratios is demonstrated and shown to occur in the presence of ligand and ligand-independent manner and independently of RII.

**Additional Data**

In addition to the relevant findings noted within the first manuscript, other important data requires presentation. The role of TGF-β in chondrocytes *in vitro* has been controversial. This has been attributed to the diverse systems within which TGF-β has been studied (O'Connor et al 2000). Therefore, the immortalized and primary human chondrocytes obtained for these investigations were first confirmed to be representative of human chondrocytes (manuscript 1). The immediate aims were then to confirm that these cells were responsive to all three TGF-β isoforms and to optimize investigative techniques which were to be subsequently utilized.

The following section contains this pertinent information, describes the experimental procedures used, the relevant findings with accompanying figures and legends, and the conclusions drawn from these results in conjunction with those contained within the first manuscript.

Acknowledgements

We thank Dr. M. O'Connor McCourt (Montreal, Quebec) for the gift of anti-betaglycan (272, 277) antibody and the Bolton Hunter prepared $^{125}$I-TGF-β1; Dr. L. Rosenberg

(Montreal, Quebec) for the IL-1β; Dr. Mary Goldring (Boston, MA) for the plasmid encoding Col 2a; and Dr. D. Rifkin (New York, NY) MLEC-clone 32 cells.

Experimental Procedures

**Western Blot Analysis**

Immortalized human chondrocytes were obtained from Dr. M. Goldring (Boston, MA). Human juvenile costal cartilage was used to isolate the C-28/I2 cell line by retroviral infection with SV-40 large T antigen. Similarly, tsT/AC62 cells were isolated from adult human articular cartilage with retrovirus expressing a temperature-sensitive mutant of SV-40 large T antigen functional at 32° C (Robbins et al 2000). TGF-β1 and -β2 were obtained in standardized concentrations from Genzyme Inc. (Framingham, MA) and TGF-β3 was purchased (standardized concentration) from Austral Biochemical (San Ramon, CA).

To determine the regulation of the phosphorylated form of Smad 2 by TGF-β1 and to optimize conditions for future chondrocyte stimulation, cells were grown in 60mm dishes, serum starved for 24 hours, then treated with increasing concentrations (0, 1, 10, 50, 100, 250, 500 pM) of TGF-β1 for 60 minutes to generate a dose response curve. Cell lysates were prepared and samples were standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad; Mississauga, Ont.). The extracts were then fractionated by 7.5% SDS-PAGE and transferred by electrophoresis to nitrocellulose membranes (Scheicher and Schuell; Keene, NH.). The membrane was blocked for 3 hours in a milk protein/Tween blocking solution (1M Tris, pH 7.5; 5M NaCl; Tween 20,Sigma;Carnation Skim milk powder) and incubated with a rabbit polyclonal anti-phosphoSmad 2 antibody (a gift from Dr. S. Souchelnytskyi, Uppsala, Sweden; Souchelnytskyi et al 2001, Nakao et

al 1997) at 4°C overnight. The membrane was washed and incubated for 1 hour with goat anti-rabbit HRP conjugated secondary antibody (Santa Cruz Biotechnology Inc.; Santa Cruz, CA) at room temperature and protein was detected using the ECL system (Amersham Pharmacia Biotech Inc.; Baie d'Urfe, Que.). The membrane was reprobed with anti-Smad 2 (non-phosphorylated form) or anti-STAT 3 antibody (a member of the JAK/STAT pathway not inducible by TGF-β stimulation) (both from Santa Cruz Biotechnology Inc.; Santa Cruz, CA) to confirm equal protein loading.

**Thymidine Incorporation**

Regulation of DNA synthesis was determined using the Thymidine incorporation assay with modifications as described previously by our lab (Wong et al 2000). Briefly, cells were seeded at a density of $7.0x10^5$ cells/24 well plate and cultured for 24 hours. The cells were washed with PBS then serum starved for 4 hours. Wells were then incubated increasing concentrations of TGF-β1 under serum free conditions overnight. [$^3$H]-Thymidine ($1\mu Ci \cdot ml^{-1}$) (Amersham Pharmacia Biotech Inc.) was added per well for the final 4 hours of TGF-β1 treatment. The cells were washed 3 times with PBS and once with 5% trichloroacetic acid (A&C American Chemicals Ltd.; Montreal, Quebec). They were then solubilized in 1% SDS and incorporated radioactivity was determined by liquid scintillation counting.

**Luciferase Reporter Assay**

The Col 2a, an IL-$1_\beta$ and TGF-β-inducible promoter-reporter construct (Robbins et al 2000), containing the luciferase gene under the control of a portion of the type II collagen promoter region, was used to determine cellular responsiveness to TGF-β. Chondrocytes were grown to 90% confluency in a 6-well plate and transiently transfected with 1μg of

pCol 2a and 1μg of pβ-galactosidase (β-gal) per well using Lipofectamine Reagent (Life Technologies) in serum-free medium according to the manufacturer's specifications. Cells were allowed to recover overnight, serum starved the following day for 4 hours, then treated with 100pM of TGF-β1, 2ng/ml of IL-$1_\beta$, or a combination of both overnight. The cells were lysed and assayed for luciferase activity (BD Pharmingen; Mississauga, Ont.) using the EG&G Berthold Microplate Luminometer (Berthold Technologies USA; Oak Ridge, TN). Light emission by the TGF-β1 treated cells was expressed as a percentage of the emission by the control cells and adjusted for transfection efficiency obtained using the βgal assay.

**PAI Assay**

C-28/I2 and tsT/AC-62 cells were plated in 24 well tissue culture plates each at a density of 2.5x $10^5$ cells for 2 wells. The following day cells were washed with PBS and 500μl of serum free medium was added per well and incubated for 24 hours. At the same time that serum free medium was added, MLEC cells (stable transfectants of mink lung epithelial cells with the PAI-1-luciferase reporter; Mazzieri et al 2000) were plated into a 96 well plate incorporating blanks, triplicate wells for TGF-β standards (0pm, 5, 10, 20, and 40), and triplicate wells for the treatment media at 100μl per well from a stock of cells of 1.65x $10^5$ cells/ml. At 24 hours, media was collected from the C-28/I2 and tsT/AC-62 cells and samples, kept at 37°C (active) or heated to 70°C (total) to activate TGF-β, were then mixed (50μl:50μl) with .1%BSA-DMEM. Standard concentrations of TGF-β1 were also prepared in .1%BSA-DMEM. 100μl of the TGF-β standard solutions or treatment media were added to individual wells of MLEC. The following day cells were lysed and luciferase activity determined. Luciferase activity from TGF-β standard wells were used

to generate a standard curve and TGF-β concentration of the total and active treatment samples was determined from the standard curve and depicted as pM concentration for cell density.

**Chloramine T TGF-β Iodination**

Iodination of TGF-β1 or TGF-β2 (gifts from Genzyme Inc.; Framingham, MA) using the Chloramine T method was performed at the Biomedical Research Institute (BRI; Montreal, Quebec) as previously described (Philip and O'Connor 1991). A regulatory leaded and ventilated hood was used and appropriate safety precautions maintained throughout the procedure.

Briefly, a PD-10 Column (Amersham Pharmacia Biotech.) was equilibrated with column buffer (0.2 g BSA, 0.88 g NaCl, 200 ml $dH_2O$) and capped until ready for use. 5μl of 1M NaPO4 (pH 7.4) was added to 2μg of TGF-β (carrier free; prepared in 4nM HCl to dilute, pH 7.4) then 10μl of NA-$^{125}$ I (1mCi; Amersham Pharmacia Biotech.) was added and iodination was achieved with three sequential additions of 5μl of Chloramine T (10mg/ml in 1M $NaPO_4$) at 0, 2 and 3.5 minute intervals. The reaction was terminated at 4.5 minutes with 20μl of L-Tyrosine (9mg/ml in 50mM $NaPO_4$, pH 7.4), 200μl KI (10mg/ml in 50mM $NaPO_4$, pH 7.4) and 200μl urea (1g/ml in 1M HCl) to stop reaction. The final volume was chromatographed on the pre-equilibrated column and twelve 0.8ml fractions were collected into siliconized eppendorfs containing storage buffer (0.4 g BSA, 40 ml 4mM HCl).

5μl aliquots of above collected fractions were transferred to scintillation vials and radioactivity determined. The two hottest fractions were pooled into the final product. Labeling efficiency (product specific activity) and concentration were determined (1μCi

of NA-$^{125}$ I equal to $2.22x10^6$ cpm and a final volume of 1600µl). The final product was stored in lead pigs at -80°C. The specific activity of $^{125}$I-TGF-β1 ranged from 2-4 mCi/pmole and biologic activity was evaluated using a receptor binding assay on C-28/I2 cells.

**Affinity Labeling**

Affinity labeling was performed as described previously with modifications (Wong et al 2000). Briefly, monolayers of cells were washed with ice-cold binding buffer [Dulbecco's PBS (dPBS) with $Ca^{++}$ and $Mg^{++}$, pH 7.4 containing 0.1% bovine serum albumin (BSA)] and were incubated with 100 pM of $^{125}$I-TGF-β1 or $^{125}$I-TGF-β2 prepared using the Chloramine T (described above) or Bolton Hunter method (gift from Dr. O'Connor-McCourt; Montreal, Quebec) in the absence or presence of increasing concentrations of unlabeled TGF-β1, -β2 or -β3 (Genzyme Inc. or purchased from Austral Biochemical; San Ramon, CA). The receptor ligand complexes were cross-linked with Bis-sulfosuccinimidyl suberate (BS3; Pierce; Rockford, Il.). The reaction was stopped by the addition of glycine and the cell membrane extracts were prepared and separated by SDS-PAGE (3-11% gradient) under reducing (with β-Mercaptoethanol, Sigma) conditions and analyzed by autoradiography.

**Immunoprecipitation of TGF-ß Receptors**

The anti-RI and anti-RII TGF-β receptor antibodies were obtained from Santa Cruz Biotechnology Inc (Santa Cruz Biotechnology Inc.). The anti-betaglycan antibody (Get 1; corresponding to the intracellular domain of betaglycan raised against the peptide sequence GETARRQQVPTSPPASENSS) was a gift from Dr. S. Souchelnytskyi (Uppsala, Sweden; Piek et al 1997). This peptide sequence is not present in human

endoglin (Accession Number J05481 GenBank) and the Get 1 antibody exhibits no crossreactivity to endoglin (personal communication Dr. S. Souchelnytskyi; Uppsala, Sweden). The anti-pig endoglin antibody (EG(591-609)); corresponding to the intracellular domain of endoglin raised against the peptide sequence KREPVVAVAAPASSESSST) was a gift from Dr. K. Miyazono (Japan; Yamashita et al 1994). This peptide sequence is not present in human betaglycan (Accession Number XM_001924 GenBank. The anti-betaglycan antibodies, 277 and 272, were a gift from Dr. M. O'Connor-McCourt (BRI; Montreal, Quebec; Philip et al 1999) or purchased from Santa Cruz Biotechnology Inc.

Immunoprecipitation studies were performed as described previously (Wong et al 2000, Dumont et al 1995) with modifications. Cells were affinity labeled with 200 pM $^{125}$I-TGF-β1, and the membrane extracts were incubated with 3μg/ml of the various antibodies or respective IgG controls. Immune complexes were then incubated with protein G-Agarose (PGA) slurry (Roche Diagnostics; Laval, Que.) or protein A-sepharose (PAS) (Amersham Pharmacia Biotech Inc.) and the beads were pelleted by centrifugation. The immune complexes were analyzed by SDS-PAGE under non-reducing or reducing conditions followed by autoradiography.

Results

These results demonstrate the optimization of investigative techniques and present data which are relevant to and support that which is presented in the manuscript to follow (manuscript 1).

**TGF-β stimulates Smad 2 phosphorylation, DNA synthesis, and regulates Col 2a promoter activity in human chondrocytes**

To illustrate the sensitivity of human chondrocytes to TGF-β, various endpoints were chosen. TGF-β treatment was to be used extensively throughout these studies; thus, in illustrating the cellular response to TGF-β, a time curve and dose response to determine optimal treatment conditions were generated. In addition, because there is a consensus that cells may respond differently to the three TGF-β isoforms, the chondrocyte response to TGF-β1, -β2, and -β3 isoforms was compared.

To demonstrate the TGF-β sensitivity of chondrocytes and to illustrate signaling through Smad 2, a central mediator of TGF-β action, TGF-β induced Smad 2 phosphorylation was examined. Fig. Ai (panel A) demonstrates that incubation of C-28/I2 chondrocytes with increasing concentrations of TGF-β1 for 60 minutes resulted in a graded increase in Smad 2 phosphorylation and detected stimulation with as little as 1-10 pm of treatment. Of note, a corresponding decrease is seen in Smad 2 levels. However, this likely represents somewhat unequal loading of protein rather than a true finding as it was not repeated. Despite, the decrease protein loaded the increase in Smad 2 phosphorylation is convincing. A time-dependent increase in Smad 2 phosphorylation with 100 pM of TGF-β1 treatment is evident in panel B with maximum stimulation seen at 60 minutes but as early as 5 minutes after treatment. Fig. Aiv demonstrates that chondrocytes were responsive to all three TGF-β isoforms. Treatment with 10 or 250 pM of TGF-β1, -β2, or -β3 for 15 minutes demonstrated an up-regulation of Smad 2 phosphorylation. Interestingly, the cells appear to be more responsive to the TGF-β2 isoform both at low and high concentrations which is consistent with the view that this isoform is particularly important in bone related tissues (Rotzer et al 2001).

Growth response (DNA synthesis) to TGF-β was demonstrated using a [$^3$H]-Thymidine incorporation assay (Fig. Aii). Regulation of DNA synthesis was determined and used to express TGF-β responsiveness in tsT/AC-62 cells with increasing concentrations of TGF-β1 treatment. The results demonstrate a triphasic response to TGF-β1 stimulation with growth stimulation as compared to untreated cells at low dose range (1 pM, p= 0.04; 10 pm, p= 0.09), marked growth inhibition at mid dose range (50 pM; p =0.006), no stimulation at 100 pM (p=0.45) and again stimulation at high dose range (200 pM; p =0.05). The data shown is representative of three experiments done in quadruplicate. Similar results were seen for C-28/I2 cells (data not shown).

Cellular signaling has been demonstrated by a Col 2a-driven luciferase reporter assay as a marker for TGF-β1 responsiveness (Robbins et al 2000). Chondrocytes were transiently transfected with pCol 2a and the induction of luciferase activity by exogenous TGF-β1, IL-$1_\beta$, or a combination of both was measured. As illustrated in Fig. Aiii, luciferase activity of cells treated with 100 pM of TGF-β1 was inhibited approximately 40%, with 2ng/ml of IL-$1_\beta$ or a combination 60%, as compared to untreated cells (control) for the tsT/AC-62 cell line (upper panel). Luciferase activity of cells treated with 100 pM of TGF-β1 was inhibited approximately 20%, with 2ng/ml of IL-$1_\beta$ or a combination 40-50%, for C-28/I2 chondrocytes (lower panel). The results were normalized by co-transfection of the β-gal plasmid. Transfection of the empty vector and treatment with TGF-β in control experiments did not result in any alteration of luciferase activity (data not shown).

**Chondrocytes produce TGF-β**

Fig. A demonstrated chondrocytes sensitivity to all three TGF-β isoforms and illustrated an appropriate dose range and time points for subsequent exogenous TGF-β treatment. The question remained as to whether these cells produce endogenous TGF-β which could interfere with exogenous TGF-β stimulation. To demonstrate TGF-β production by human chondrocytes, a PAI-luciferase assay was performed using MLEC cells (stable transfectants of mink lung epithelial cells with the PAI-1-luciferase reporter; Mazzieri et al 2000). Fig. B illustrates TGF-β production at 24 hours by both tsT/AC-62 and C-28/I2 cells in the range of 40-70 pM of total and 5-20 pM for active TGF-β from confluent wells of a 24 well plate ($1.25x10^5$ cells). To eliminate this endogenously produced TGF-β, cells are serum starved or washed extensively with a mild acid (0.1% glacial acetic acid; Glick et al 1990) prior to any exogenous TGF-β stimulation.

**TGF-β receptor profiles on human chondrocytes**

Affinity labeling with $^{125}$I-TGF-β1 is a standard technique used to determine expression profiles of TGF-β receptors on various cells. To optimize this technique for chondrocyte, cells were affinity labeled with $^{125}$I-TGF-β1 prepared by the Chloramine T method (as described in Experimental Procedures) or Bolton Hunter (gift from Dr. M. O'Connor-McCourt; Montreal, Quebec) and analyzed by SDS-PAGE (3-11% gradient). The receptor profiles representative of human chondrocytes are illustrated in Fig. C. The competition using 2.0 nM of unlabeled TGF-β1 and -β2 demonstrated that both forms of $^{125}$I-TGF-β1 labeled the cells equally well and the relative binding affinity of the receptors for the TGF-β1 and -β2 isoforms appeared similar using both iodination techniques. The type I TGF-β receptor (RI) was observed at 65 kDa, the type II TGF-β receptor (RII) at 85 kDa, betaglycan at 250-300 kDa, and an additional protein with TGF-

β affinity was noted at 180 kDa. The remainder of the affinity labeling experiments were performed using Chloramine T prepared $^{125}$I-TGF-β1.

Competition profiles using increasing concentrations of unlabeled TGF-β1, TGF-β2, and TGF-β3 isoforms depict the relative binding affinity of the receptors for the TGF-β isoforms (manuscript 1). To demonstrate that very low concentrations of unlabeled ligand had been accurately prepared, affinity labeling was performed simultaneously with two sources of TGF-β1 (Genzyme Inc. and Austral Biochemical; Fig. D). The competition using 2.0 nM of both unlabeled TGF-β1 sources demonstrated equally well displacement of labeled ligand from the TGF-β receptors. The remainder of the affinity labeling experiments were performed using the TGF-β1 donated by Genzyme Inc.

TGF-β receptor-ligand affinity can be determined by affinity labeling with $^{125}$I labeled ligand and competition with an unlabeled ligand. Increasing concentration of unlabeled TGF-β isoforms will displace receptor-bound labeled ligand proportionate to the receptor's affinity for the particular ligands. A standard approach to determine TGF-β2 affinity involves labeling the cells with $^{125}$I-TGF-β1 and competing with the -β2 isoform. An alternative is to label receptors directly with $^{125}$I-TGF-β2 (Fig. E.) The competition using increasing concentrations of unlabeled TGF-β1, TGF-β2, and TGF-β3 isoforms demonstrated the relative binding affinity of the receptors for the TGF-β isoforms. $^{125}$I-TGF-β2 labeling in the absence of unlabeled ligand (lanes designated as '0') revealed three major binding complexes of relative molecular weights of 65, 85, and 250-300 kDa under reducing conditions. The migration patterns and isoform specificities of these complexes were characteristic of the cloned type I receptor (RI), type II receptor (RII), and type III (betaglycan) receptor, respectively (Wong et al 2000, Tam and Philip 1998).

This was confirmed later by immunoprecipitation studies using specific anti-receptor antibodies (see below). All receptors demonstrated affinity for all three TGF-β isoforms.

**TGF-β signaling receptors form heteromeric complexes**

To confirm the identity of the TGF-β signaling receptors expressed on human chondrocytes and to study potential associations between them, cells were affinity labeled with $^{125}$I-TGF-β1 and immunoprecipitated using specific anti-receptor antibodies directed against RI and RII. In these studies, whereas immunoprecipitation with a specific anti-receptor antibody confirmed the identity of its cognate TGF-β receptor, co-immunoprecipitation of another receptor which is not recognized by this antibody was indicative of heteromeric complex formation between those receptors. In addition, both PAS and PGA were used to incubate the immune complexes to determine any difference in their efficacy.

As shown in Fig. F (C-28/I2 left panel and tsT/AC-62 right panel), the nonimmunoprecipitated membrane lysate (NIP) of 50μg and 100μg of protein demonstrated the presence of TGF-β binding proteins consistent in mobility and size with RI, RII, and betaglycan (Lane 1,2,7, 8). The anti-RI antibody with PAS (α-RI-PAS, Lane 4) immunoprecipitated RI and co-immunoprecipitated trace amounts of RII and betaglycan. Similarly, with PGA, the anti-RI antibody (α-RI-PGA, Lane 10) precipitated RI and co-immunoprecipitated RII and betaglycan. The anti-RII antibody with PAS (α-RII-PAS, Lane 5) immunoprecipitated RII and co-immunoprecipitated RI and betaglycan. However, with PGA, the anti-RII antibody (α-RII-PGA, Lanes 6 and 11) precipitated RII and co-immunoprecipitated RI and betaglycan with greater efficacy suggesting that PGA may be more effective at sequestering the antibody-receptor complexes. The co-

immunoprecipitation of RI and RII with betaglycan in other cells is well documented (Franzen et al 1993) and has been confirmed on human chondrocytes (Horner et al 1998). No receptors were immunoprecipitated with the control (rabbit) immunoglobulin (IgG, Lane 3, 6).

Multiple antibodies against TGF-β receptors are commercially available or have been independently generated by individual laboratories. Antibody sensitivities may vary and thus affect detection of co-immunoprecipitation of associated receptors. Immunoprecipitation with anti-betaglycan antibody demonstrated betaglycan but also suggested co-immunoprecipitation of endoglin (manuscript 1). To determine which available anti-betaglycan antibody would be most efficient in detecting endoglin co-immunoprecipitation, studies were performed with various anti-betaglycan antibodies under nonreducing (Fig. Gi) and reducing (Fig. Gii) conditions. In Fig. Gi, the anti-endoglin antibody (α-Eg, Lane 6) immunoprecipitated the endoglin dimer which is also evident in the non-immunoprecipitated lanes (NIP, Lane 1, 2). The anti-betaglycan antibody which both demonstrated the highest level of betaglycan immunoprecipitation and traces of endoglin co-immunoprecipitation was Get 1 (Lane 3). This was also evident under reducing conditions (Fig. Gii, Lane 2). No receptors were immunoprecipitated with the control (rabbit) immunoglobulin (Fig. Gii; IgG, Lane 1).

**Conclusions**

This additional data confirmed the responsiveness of human chondrocytes to all three TGF-β isoforms and suggested optimal conditions for exogenous TGF-β treatment (Fig. A) used in subsequent studies (manuscripts 1-5). Fig. B demonstrated the production of TGF-β by human chondrocytes and suggested that the active form comprises

approximately 15-25% of the total TGF-β produced. This illustrates the need to serum starve cells prior to exogenous TGF-β treatment to remove endogenous ligand which, if unequally produced by cells, will confound results. This endogenous TGF-β must be removed with mild acid wash (0.1% glacial acetic acid; Glick et al 1990) to conclude ligand independence when determining receptor interactions. These modifications were thus made in subsequent investigations (manuscripts 1-5).

Optimization of affinity labeling and immunoprecipitation techniques has been demonstrated. Fig. C indicated that the efficiency of affinity labeling with Chloramine T labeled $^{125}$TGF-β1 was equal to that of commercially available Bolton Hunter labeled $^{125}$TGF-β1 and thus the Chloramine T form was subsequently used for all affinity labeling studies (manuscripts 1-5). Similarly, data indicated that the unlabeled TGF-β1 (a gift from Genzyme) was accurately diluted and prepared as compared to that purchased from Austral (Fig. D) and could be used for further competition profiles with confidence. The effectiveness of TGF-β anti-receptor antibodies was also determined. Anti-RI and anti-RII antibodies, combined with PAS or PGA, were investigated (Fig. F). It was apparent that the immune complex was more efficiently detected using PGA and therefore PGA was used for betaglycan-endoglin co-immunoprecipitation studies (manuscript 1). The presence of the TGF-β signaling receptors and betaglycan on human chondrocytes and the heteromeric receptor complexes between them (Horner et al 1998) was confirmed for the chondrocyte cell lines (Fig. F). The efficacy of the anti-betaglycan antibodies required for endoglin co-immunoprecipitation studies (manuscript 1) were investigated (Fig. G) and indicated that the anti-endoglin antibody obtained from Dr. K. Miyazono (Tokyo, Japan; Yamashita et al 1994) was effective at detecting this receptor. In addition, Get 1

(Piek et al 1997) was the most efficient antibody for betaglycan detection as well as for endoglin co-immunoprecipitation. All immunoprecipitations for betaglycan were subsequently performed using Get 1 antibody (manuscript 1-5).

Results demonstrated in Fig. E confirmed the receptor affinities for TGF-β isoforms using labeled $^{125}$I-TGF-β2. The TGF-β signaling receptors (RI and RII) and betaglycan are shown to bind TGF-β2 and demonstrate affinity for all three unlabeled isoforms.

Therefore, the results presented above have demonstrated the optimization of techniques used throughout the thesis (manuscript 1-5). Together with the data contained in manuscript 1, the responsiveness of human chondrocytes to all three TGF-β isoforms has been confirmed. TGF-β production by human chondrocytes (15-25% active form) is documented. The TGF-β receptor expression profile of chondrocytes is well characterized and the heteromerization of the signaling receptors with betaglycan are demonstrated. For the first time, endoglin expression on human chondrocytes at high concentrations is demonstrated. It is shown to form higher order complexes with RI and RII and more importantly, a heteromeric complex with betaglycan on these cells. Also, that this endoglin-betaglycan complex formation occurs at physiological receptor concentrations and ratios, and in the presence of ligand and ligand-independent manner has been illustrated. Furthermore, this heteromeric complex can occur independently of RII. Elucidating the role of endoglin in the regulation of TGF-β signaling and understanding the significance of the endoglin-betaglycan association in human chondrocytes are critical to unravelling the molecular mechanisms governing TGF-β action in these cells and thus the role of TGF-β in mediating cartilage repair.

## CONTRIBUTION OF AUTHORS

# MANUSCRIPT ONE

Endoglin is expressed on human chondrocytes and forms a heteromeric complex with betaglycan in a ligand and type II TGF-β receptor independent manner

**WL Parker:** experimental designs, procedures, and analysis, preparation of figures and text

**MB Goldring:** contribution of C-28/I2 and tsT/AC-62 cell lines, manuscript editorial assistance

**A Philip:** assistance with experimental design and analysis and manuscript editorial assistance

# Endoglin is expressed on human chondrocytes and forms a heteromeric complex with betaglycan in a ligand and type II TGF-β receptor independent manner

Wendy L. Parker[1], Mary B. Goldring[2], and Anie Philip[1]*.

[1]Division of Plastic Surgery, Department of Surgery, McGill University, Montreal, Quebec, Canada

[2]Beth Israel Deaconness Medical Center, Harvard Institutes of Medicine, Boston, Massachusetts, USA

*Correspondence to: Anie Philip, Division of Plastic Surgery, Montreal General Hospital, 1650 Cedar Avenue, Room C9-177, Montreal, Quebec, Canada H3G 1A4.

Telephone: (514) 937 6011 ext 44533; Fax: (514) 934 8289

E-mail: anie.philip@mcgill.ca

Running Title: Endoglin expression on chondrocytes

Acknowledgements

We thank Genzyme Inc. (Framingham, MA) for the gift of TGFβ1 and TGFβ2; Dr. M. O'Connor McCourt (Montreal, Quebec) for the gift of plasmids encoding the Type II TGFβ receptor and betaglycan; Dr. S. Souchelnytskyi (Uppsala, Sweden) for the anti-betaglycan (Get1) and anti-phospho Smad 2 antibodies; Dr. K. Miyazono (Tokyo, Japan) for the anti-endoglin antibody; Dr. S. St Jacques (Quebec City, Quebec) for the 44G4 anti-endoglin antibody; Dr. C. Bernabeu (Madrid, Spain) for the L-endoglin plasmid; Dr. F.W. Ades and Dr. T.J. Lawley (Atlanta, GA) for the human microvascular endothelial cells (HMEC-1), Dr. M. Tanzer for human articular cartilage specimens and Dr. JJ Lebrun (Montreal, Quebec) for his expert advice on transient transfections and RTPCR studies.

Summary

Previous work has implicated transforming growth factor β (TGFβ) as an essential mediator of cartilage repair, and TGFβ signaling as a requirement for the maintenance of articular cartilage in vivo. However, the mechanisms regulating TGFβ action in chondrocytes are poorly understood. Endoglin, an accessory receptor of the TGFβ receptor superfamily, is highly expressed on endothelial cells and has been shown to potently modulate TGFβ responses. It is not known whether chondrocytes express endoglin or whether it modulates TGFβ signaling in these cells. In the present study, we demonstrate that endoglin is expressed on human chondrocytes at comparable levels to endothelial cells and that it forms higher order complexes with the types I and II TGFβ receptors. More importantly, we show that endoglin forms a heteromeric complex with betaglycan on these cells at endogenous receptor concentrations and ratios. Endoglin complexes with betaglycan in a ligand independent and dependent manner as indicated by co-immunoprecipitation in the absence of TGFβ and after affinity labeling with radiolabeled TGFβ, respectively. Also, the endoglin-betaglycan association can occur independently of the type II TGFβ receptor. These findings, taken together with the available evidence that endoglin and betaglycan are potent modulators of TGFβ signal transduction, imply that the complex formation between endoglin and betaglycan may be of critical significance in the regulation of TGFβ signaling in chondrocytes.

**Key Words:** chondrocytes, TGF-β receptors, endoglin, betaglycan, cartilage

## INTRODUCTION

Cartilage, one of the body's five major connective tissues, displays poor intrinsic healing which is the key element in a variety of common joint diseases. Although the potential for restoring diseased or injured cartilage has generated much interest, a successful model of cartilage regeneration or repair has been elusive. This has been attributed to the lack of regenerative or proliferative ability of mature chondrocytes, their dedifferentiation into fibrocartilage (type I and type III collagen producing cells) (1) and the avascular state of the tissue (2). Increased attention has focused on the chondrocyte environment as a critical determinant of phenotypic matrix synthesis and reparative capacity in which growth factors play important roles. Transforming growth factor β (TGFβ)[1] has emerged as a potential regulator of chondrocyte growth and differentiation. For example, a positive relationship between cellular maturity and proliferative response to TGFβ has been demonstrated in growth plate chondrocytes (3). Furthermore, chondrocytes derived from the different layers of articular cartilage are differentially stimulated by TGFβ (4).

TGFβ is a member of a large family of multifunctional proteins intricately involved in growth, differentiation, and development (5) and was described initially as "cartilage inducing factor" (6). Three distinct isoforms of TGFβ (TGFβ 1, 2, and 3) which are encoded by distinct genes have been described in mammals (5). TGFβ is secreted in a latent form that requires activation before it can bind to its receptors. The TGFβ signal is transduced by a pair of transmembrane serine/threonine kinases, known as the types I and

[1] The abbreviations used are: TGFβ, transforming growth factor beta; PAI – 1, plasminogen activator inhibitor-1; RI, TGFβ type I receptor; RII, TGFβ type II receptor; HHT, hereditary hemorrhagic ; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum; DMEM, Dulbecco's minimal essential medium; dPBS, Dulbecco's phosphate buffered saline; BSA, bovine serum albumin; βgal, beta-galactosidase.

II receptors which are present on almost all cell types (7, 8, 9, 10). The type I receptor does not bind TGFβ in the absence of the type II receptor. The binding of TGFβ to the type II receptor, a constitutively active kinase, results in the recruitment, phosphorylation and concomitant activation of the type I receptor. The activated type I receptor in turn transmits the signal via downstream mediators such as Smads, resulting in the regulation of target gene expression. Other cell surface TGFβ binding proteins include the accessory receptors, betaglycan (type III TGFβ receptor) and endoglin (CD105) which have a limited tissue distribution. Betaglycan, a membrane proteoglycan, binds all three TGFβ isoforms with high affinity and is believed to facilitate TGFβ binding to the type II TGFβ receptor (11, 12, 13).

Endoglin binds TGFβ1 and TGFβ3 with high affinity through its association with the type II receptor (reviewed in 14). Furthermore, endoglin appears to interact not only with TGFβ but also with activin and bone morphogenic protein in the presence of their respective ligand binding receptor (15). Endoglin was originally identified with the monoclonal antibody 44G4 generated against the HOON pre-B leukemic cell line (16) but was subsequently found to be highly expressed on endothelial cells. Other cell types that express endoglin include monocytes, lymphocytes and placental and uterine cells (17,18,19). Endoglin, a homodimeric transmembrane protein of ~180 kDa, composed of disulfide-linked protein subunits of ~95 kDa, has limited species-specificity and shows 70% homology to betaglycan. Two different isoforms, L and S-endoglin, have been described and both are constitutively phosphorylated. The functional role of endoglin in TGFβ signaling is poorly understood. It has been shown to facilitate ligand binding to the types I and II receptors (20). However, over-expression of endoglin decreases TGFβ

responses in monocytes and myoblasts (19,20,21). In contrast, overexpression of betaglycan enhanced TGFβ responses in these cells (20).

The gene encoding endoglin is located on chromosome 9q34 (22). Hereditary hemorrhagic telangiectasia (HHT) is attributable to mutations in the endoglin gene with each mutation providing a slightly modified disease phenotype (23). Heterozygotes, both human and animal models, display the classic phenotype of epistaxis, telangiectasia, and visceral vascular malformations (24). Null alleles are embryonically lethal secondary to abnormal yolk sac vasculogenesis and abnormal cardiac development (25). The specific function of endoglin responsible for HHT is likely related to alterations in TGFβ action (20,26).

TGFβ receptor profiles on human chondrocytes are poorly defined and it is not known whether chondrocytes express novel TGFβ receptors. Expression of TGFβ accessory receptors such as endoglin and glycosyl phosphatidylinositol-anchored TGFβ binding proteins have been shown to regulate TGFβ signaling and TGFβ responses in other cell types (20,27,28). Defining the expression profiles of the TGFβ receptor types and the biochemical interactions between these receptors leading to the formation of oligomeric complexes on chondrocytes is critical to understanding the mechanism of TGFβ action in these cells.

In the present study, we report the expression profiles of TGFβ receptors on human articular and nonarticular chondrocytes and demonstrate for the first time that endoglin is expressed on chondrocytes. More importantly, we show that in addition to the well documented formation of a heteromeric complex with the type I/type II TGFβ receptors, endoglin forms a heteromeric complex with betaglycan on chondrocytes at endogenous

receptor concentrations and ratios. Also, we present data to illustrate that the heteromerization of endoglin with betaglycan occurs in a ligand-induced and ligand-independent manner as shown by their co-immunoprecipitation after affinity cross-link labeling with radiolabeled TGFβ and their association in the absence of TGFβ, respectively. Furthermore, we demonstrate that the complex formation between endoglin and betaglycan can occur independently of the type II TGFβ receptor.

## EXPERIMENTAL PROCEDURES

### Cell Culture

Primary human articular chondrocytes were obtained from cartilage specimens obtained at total knee replacement surgery. Digestion of the specimens was performed according to standard techniques (29). Briefly, cartilage was washed with phosphate-buffered saline (PBS), incubated with 0.25% trypsin (10ml/g of tissue) (Life Technologies; Burlington, ON.) for 30 to 45 minutes, minced with a scalpel, and incubated with hyaluronidase (10ml/g of tissue) and collagenase (1mg/ml; 10ml/g of tissue) (both from Sigma; Oakville, ON.) overnight. The suspension was pelleted, washed, and plated at $1x10^6$ cells/ml. The immortalized human chondrocyte cell lines (C-28/I2 and tsT/AC62) have been described previously (29,30). The C-28/I2 cell line was developed using chondrocytes isolated from juvenile human costal cartilage by retroviral infection with SV-40 large T antigen. The tsT/AC62 cells were developed using chondrocytes isolated from adult human articular cartilage by immortalization with a retrovirus expressing a temperature-sensitive mutant of SV-40 large T antigen. This mutant is functional when the cells are cultured at 32°C but not at 37°C (29). The 293 cells (CRL-1573) were obtained from the American Type Culture Collection (Rockville, MD) and the human

microvascular endothelial cells (HMEC-1) were a gift from Dr. F.W. Ades and Dr. T.J. Lawley (National Center for Infectious Disease; Atlanta, GA). All cells with the exception of HMEC-1 were grown in DMEM/Ham's F12 (1:1, v:v) containing 10% fetal bovine serum (FBS) and 100U/ml penicillin, 100μg/ml streptomycin and 0.25μg/ml amphoteracin (all from Life Technologies). HMEC-1 were grown in MCDB 131 (Life Technologies) containing 10% fetal bovine serum (FBS) and 100U/ml penicillin, 100μg/ml streptomycin and 0.25μg/ml amphoteracin (all from Life Technologies). Cells were cultured at 37°C in an atmosphere of 5% CO2/air with the exception of the tsT/AC62 line which was maintained at 32°C.

**Analysis of RNA by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

To verify chondrocyte phenotype of the immortalized cell lines and primary chondrocytes, aggrecan and type II collagen mRNAs were analyzed by RT-PCR as described previously (29) with modifications. Total RNA was extracted using Trizol Reagent (Life Technologies) and chloroform with final resuspension of the RNA in DEPC-treated water (Sigma). The concentration of RNA in the samples was determined using spectrophotometry and RNA stability was determined on a 1% agarose-ethidium bromide gel. RTPCR was performed with 5μg samples using a Superscript First Strand Kit and custom primers (Life Technologies) according to the manufacturer's instructions. Final DNA preparations were visualized on a 1.5% agarose-ethidium bromide gel and photographed.

**Luciferase Reporter Assay**

The p3TP-Lux, a TGFβ-inducible promoter-reporter construct (31), containing the luciferase gene under the control of a portion of the plasminogen activator inhibitor-1

promoter region was used to determine cellular responsiveness to TGFβ. Chondrocytes were grown to 90% confluency in a 6-well plate and transiently transfected with 1μg of p3TP-Lux and 1μg of pβ-galactosidase (β-gal) per well using Lipofectamine Reagent (Life Technologies) in serum-free medium according to the manufacturer's specifications. Cells were allowed to recover overnight in regular medium, serum starved the following day for 4 hours, then treated with 100pM of TGFβ1 overnight. The cells were lysed and assayed for luciferase activity (BD Pharmingen; Mississauga, Ont.) using the EG&G Berthold Microplate Luminometer (Berthold Technologies USA; Oak Ridge, TN).Light emission by the TGFβ1 treated cells was expressed as a percentage of the emission by the control cells and adjusted for transfection efficiency obtained using the βgal assay. Optimal transfection conditions were determined using pHGFP (high green fluorescent protein plasmid; Q-biogene; Carlsbad, CA.).

**Western Blot Analysis**

To determine the regulation of the phosphorylated form of Smad 2 by TGFβ1, chondrocytes were grown in T25 flasks and serum starved for 24 hours. Cells were then washed with PBS and treated with TGFβ1 as indicated. Cell lysates were prepared and samples were standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad; Mississauga, Ont.). The extracts were then fractionated by 7.5% SDS-PAGE and transferred by electrophoresis to nitrocellulose membranes (Scheicher and Schuell; Keene, NH.). The membrane was blocked for 3 hours in a milk protein/Tween blocking solution (1M Tris, pH 7.5; 5M NaCl; Tween 20,Sigma;Carnation Skim milk powder) and incubated with a rabbit polyclonal anti-phosphoSmad2 antibody (a gift from Dr. S. Souchelnytskyi, Uppsala, Sweden) at 4°C overnight. The membrane was washed and

incubated for 1 hour with goat anti-rabbit HRP conjugated secondary antibody (Santa Cruz Biotechnology Inc.; Santa Cruz, CA) at room temperature and detected using the ECL system (Amersham Pharmacia Biotech Inc.; Baie d'Urfe, Que.). The membrane was reprobed with anti-Smad 2 (non-phosphorylated form) antibody (Santa Cruz Biotechnology Inc.) to confirm equal protein loading. To determine the expression of endoglin in chondrocytes and HMEC-1, cells were grown in 60mm dishes until confluent. Cell lysates were prepared, and analyzed by Western blot using a rabbit polyclonal anti-endoglin antibody (SN6h; Dako Inc.; Carpinteria, CA). The addition of secondary antibody and detection were performed as described above. The membrane was reprobed with anti-Smad 2/3 (non-phosphorylated form) antibody (Santa Cruz Biotechnology Inc.) to confirm equal protein loading.

To verify the presence of endoglin in cartilage, a plasma membrane fraction from fresh human articular cartilage was prepared as described by Gruppuso et al (32). Briefly, tissue was rinsed with dPBS, weighed, minced, and homogenized using a hand homogenizer in 4 volumes of homogenization buffer (Sucrose .25M ,EDTA 1mM, Hepes 50mM pH 7.5) containing a mixture of protease inhibitors (1mM phenylmethanesulfonyl fluoride, 20 $\mu g \cdot ml^{-1}$ aprotinin, 20 $\mu g \cdot ml^{-1}$ leupeptin, 20 $\mu g \cdot ml^{-1}$ soybean trypsin inhibitor, and 25 mM benzamidine; all from Sigma). The membrane fraction was centrifuged at 100,000 x g for 2 hours at 20°C. The supernatant was removed and the pellet resuspended in 1x sample buffer and boiled for 5 minutes. The samples were then analyzed by 7.5% SDS-PAGE under nonreducing or reducing (with β-Mercaptoethanol, Sigma) conditions and immunoblotted as noted above for endoglin.

**Affinity Labeling of Cells**

Affinity labeling was performed as described previously with modifications (33) Briefly, monolayers of cells were washed with ice-cold binding buffer [Dulbecco's PBS (dPBS) with $Ca^{++}$ and $Mg^{++}$, pH 7.4 containing 0.1% bovine serum albumin (BSA)] and were incubated with 100 pM of $^{125}$I-TGFβ1 in the absence or presence of varying concentrations of non-radioactive TGFβ1, 2, or 3. The receptor ligand complexes were cross-linked with Bis-sulfocsuccinimidyl suberate (BS3; Pierce; Rockford, Il.). The reaction was stopped by the addition of glycine and the cell membrane extracts were prepared. The solubilized samples were separated by SDS-PAGE on a 3-11% polyacrylamide gradient gels under non-reducing or reducing (with β-Mercaptoethanol, Sigma) conditions and analyzed by autoradiography.

**Immunoprecipitation of TGF-ß Receptors**

The anti-type I TGFβ receptor antibody and anti-type II TGFβ receptor antibody were from Santa Cruz Biotechnology Inc (Santa Cruz Biotechnology Inc.). The anti-betaglycan antibody (Get 1; corresponding to the intracellular domain of betaglycan raised against the peptide sequence GETARRQQVPTSPPASENSS) was a gift from Dr. S. Souchelnytskyi (Uppsala, Sweden; 34). This peptide sequence is not present in human endoglin (Accession Number J05481 GenBank) and the Get 1 antibody exhibits no crossreactivity to endoglin (personal communication Dr. S. Souchelnytskyi; Uppsala, Sweden). The monoclonal anti-human endoglin antibody (44G4; 35) was a gift from Dr. S. St. Jacques (Université Laval, Canada), the anti-pig endoglin antibody (EG(591-609)); corresponding to the intracellular domain of endoglin raised against the peptide sequence KREPVVAVAAPASSESSST) was a gift from Dr. K. Miyazono (Japan; 36). This peptide

sequence is not present in human betaglycan (Accession Number XM_001924 GenBank). The SN6h anti-human endoglin antibody was from Dako Inc.

Immunoprecipitation studies were performed as described previously (33,37) with modifications. Cells were affinity labeled with 200pM $^{125}$I-TGFβ1, and the membrane extracts were incubated with 3μg/ml of the various antibodies and with respective IgG controls. Immune complexes were then incubated with protein G-Agarose (Roche Diagnostics; Laval, Que.) slurry and the beads were pelleted by centrifugation. The immune complexes were analyzed by SDS-PAGE under non-reducing or reducing conditions followed by autoradiography.

**Overexpression of endoglin and betaglycan**

cDNAs encoding human RII and rat betaglycan subcloned into pcDNA3 were obtained from Dr. M. O'Connor-McCourt, Montreal (38), and the pcEXV-Endo-L expression vector encoding the human L-endoglin isoform was from Dr. C. Bernabeu, Spain (39). The 293 cells grown in 6-well plates were transiently transfected with 1μg each of pEndo-L, pbetaglycan and pβgal per well. In some experiments cells were also transfected with pRII. Parallel transfections with pcDNA3 empty vector and pβgal were performed as mock controls. The transfections were carried out using Superfect Reagent (Life Technologies) according to the manufacturer's specifications. Cells were affinity labeled with 200 pM $^{125}$I-TGFβ1 and membrane extracts were left nonimmunoprecipitated or immunoprecipitated with the indicated anti-receptor antibodies, fractionated by 3-11% gradient gels, and analyzed by autoradiography. In parallel, transfected cells were not affinity labeled, but immunoprecipitated as indicated, fractionated on 3-11% gradient gels and electrophoresed onto nitrocellulose prior to

Western blotting with anti-endoglin antibody (SN6h; Dako) using the ECL detection system (Amersham Pharmacia Biotech Inc.).

**Immunoprecipitation/Western Blot Analysis**

Chondrocytes and 293 cells overexpressing endoglin and betaglycan were washed three times with 0.1% BSA-dPBS, twice with dPBS, and membrane extracts of cells were prepared, and immunoprecipitated with anti-betaglycan antibody (Get 1) or anti-human endoglin antibody (SN6h; Dako), or not immunoprecipitated. In some experiments, cells were washed with a mild acid (0.1% glacial acetic acid) to ensure complete removal of endogenous TGFβ (40, 41) before membrane extraction and subsequent immunoprecipitation. In some other experiments, cells were pretreated with 100 pM TGFβ1 at 37°C for 2 hrs before being washed twice with dPBS followed by membrane extraction and immunoprecipitation. The extracts or immune complexes were fractionated by SDS-PAGE on 3-11% gradient gels and Western blot analysis was performed as described above using anti-human endoglin antibody (SN6h; Dako) and the ECL system for detection (Amersham Pharmacia Biotech Inc.).

**Two-Dimensional Gel Electrophoresis**

Two-dimensional gel electrophoresis was performed as described by MacKay et al (42) except that 3-mercaptopropionic acid was omitted in the second dimension (40). Solubilized membrane extracts of cells were affinity labeled with $^{125}$I-TGFβ1 were first fractionated on a 1.0 mm-thick 3-11% gradient gel under non-reducing conditions in the first dimension and on a 1.5 mm-thick 3-11% gradient gel under reducing conditions in the second dimension. The gel was then subjected to autoradiography.

**RESULTS**

**Expression of Aggrecan and Type II Collagen by Chondrocytes**

The tsT/AC62 and C-28/I2 cell lines provide a reproducible model that mimics human articular and costal phenotypes respectively (43,44,45,46). To confirm the chondrocyte phenotype of the immortalized cell lines and the human primary articular chondrocytes in culture, we determined the production of aggrecan and type II collagen by these cells using RTPCR. As displayed in Fig. 1A, aggrecan and type II collagen mRNAs were expressed by both chondrocyte cell lines and the primary chondrocytes. In addition, light microscopic examination of the cells in culture at early passage showed that they displayed the typical chondrocyte morphology as detected by a spherical to polygonal shape with a cell-associated matrix when suspended in alginate bead cultures (data not shown).

**TGFβ1 Stimulates PAI promoter Activity and Smad2 Phosphorylation in Chondrocytes**

To illustrate the sensitivity of the chondrocyte cell lines and primary chondrocytes to TGFβ, cellular signaling was determined by a PAI-driven luciferase reporter assay using p3TP-Lux which has been used extensively as a marker for TGFβ responsiveness (47,11). The chondrocytes were transiently transfected with p3TP-Lux and the induction of luciferase activity by exogenous TGFβ1 was measured. As illustrated in Fig. 1B, luciferase activity of cells treated with 100pM of TGFβ1 was stimulated approximately a 10-fold compared to untreated cells for the C28/I2 line, 6-fold for TST/AC62, and 5-fold for primary chondrocytes. The results were normalized by co-transfection of the β-gal plasmid and are representative of three different experiments each done in triplicate.

Transfection of the empty vector and treatment with TGFβ in control experiments did not result in any alteration of luciferase activity (data not shown).

To demonstrate further the TGFβ sensitivity of these chondrocytes and to illustrate the cellular signaling through Smad 2, a central mediator of TGFβ action, we examined TGFβ induced Smad 2 phosphorylation. Cells were treated with 100 pM of TGFβ1 for 15 or 30 minutes or were left untreated and the phosphorylated form of Smad 2 was determined using Western blot analysis. As shown in Fig. 1C that is representative of three different experiments, incubation of C-28/I2 chondrocytes with 100 pM of TGFβ1 for 15 minutes resulted in a marked increase in Smad 2 phosphorylation (upper panel). There was a time-dependent increase in Smad 2 phosphorylation as detected by 30 minutes of TGFβ1 treatment (data not shown). Immunoblotting of total Smad2 was also performed to demonstrate equal protein loading (lower panel). Similar results were obtained using tsT/AC62 cells and primary chondrocytes (data not shown).

**TGFβ receptor profiles on human chondrocytes**

To analyze TGFβ receptor profiles on human chondrocytes, cells were affinity labeled with $^{125}$I-TGFβ1 and the labeled receptors were analyzed by SDS-PAGE. The receptor profiles representative of human articular and nonarticular chondrocytes are illustrated in Fig. 2 and 3 respectively. The competition using increasing concentrations of unlabeled TGFβ1, TGFβ2, and TGFβ3 isoforms demonstrated the relative binding affinity of the receptors for the TGFβ isoforms. $^{125}$I-TGFβ1 labeling in the absence of unlabeled ligand (lanes designated as '0') reveals five major binding complexes of relative molecular weights of 65, 85, 100, 180 and 200-300 kDa under reducing conditions (Fig. 2). The migration patterns and isoform specificities of the 65, 85, 100, and 200-300 kDa

complexes were characteristic of the cloned type I receptor (RI), type II receptor (RII), endoglin monomer and type III (betaglycan) receptor respectively (48,49). This was confirmed later by immunoprecipitation studies using specific anti-receptor antibodies (see below). The band at 180 kDa may represent the endoglin homodimer which was cross-linked inadvertently during the affinity labeling procedure and thus became reductant insensitive (Fig. 2). All receptor complexes showed high affinity for TGFβ1 and an intermediate affinity for TGFβ3. With the exception of betaglycan, these receptors showed virtually no affinity for TGFβ2.

TGFβ receptor profiles of $^{125}$I-TGFβ1 labeled chondrocytes analyzed under non-reducing conditions are shown in Fig. 3. In addition to the RI (65 kDa), RII (85 kDa), endoglin dimer (180 kDa) and betaglycan (200-300 kDa), two binding complexes of relative molecular weights of 115 kDa and 145 kDa were detected in the absence of unlabeled ligands (middle lane designated as '0'). These 115 and 145 kDa complexes were confirmed later as the RI homodimer and RI and RII heterodimer respectively by two-dimensional gel electrophoresis (see below), consistent with our previous observations in other cell types (33,40). Also evident were two higher molecular weight bands at 240 kDa and 320 kDa which were unmasked in the presence of the unlabeled β2 isoform which has very low affinity for these bands but competes out betaglycan effectively. The 240 kDa and 320 kDa bands were later identified as oligomeric complexes containing endoglin, RII and/or RI (see below). Once again, all receptor complexes, with the exception of betaglycan, show high affinity for TGFβ1 and virtually no affinity for TGFβ2.

As expected of endoglin, the 100 kDa band (monomer under reducing conditions, Fig. 2) and the 180 kDa band (dimer under non-reducing conditions, Fig. 3) displayed high affinity for TGFβ1, intermediate affinity for TGFβ3 and no affinity for TGFβ2. However, the almost complete lack of affinity of the types I and II receptors for the TGFβ2 isoform in these cells which express ample amounts of betaglycan (which exhibits high affinity for TGFβ2) is intriguing.

**Endoglin is present in human articular cartilage and is expressed on chondrocytes at a level comparable to that on endothelial cells**

The results shown in Figure 2 and 3 demonstrate binding patterns consistent with the presence of endoglin. Endoglin had not been documented previously on chondrocytes. To confirm the presence of endoglin on these cells, its expression on primary human articular chondrocytes and chondrocyte cell lines (nonarticular and articular) was compared to that of human microvascular endothelial cells using Western blot and affinity labeling. In addition to confirming the expression of endoglin on human chondrocytes at levels comparable to those on HMEC-1, results shown in figure 4A and 4B under conditions of equal protein loading suggest that articular chondrocytes express higher levels of endoglin than nonarticular chondrocytes. Also, the primary articular cells display higher amounts than the articular cell line (tsT/AC62). Figure 4C demonstrates the presence of endoglin in human cartilage tissue by Western blot confirming that its expression is not an artefact of chondrocytes cultured in monolayers.

**Endoglin forms a heteromeric complex with betaglycan in a ligand-induced manner**

To confirm the identity of the TGFβ receptors expressed on human chondrocytes and to study potential associations between endoglin and other TGFβ receptors, chondrocytes

were affinity labeled with $^{125}$I-TGFβ1 and immunoprecipitated using specific anti-receptor antibodies directed against RI and RII, betaglycan and endoglin. In these studies, whereas immunoprecipitation with a specific anti-receptor antibody confirmed the identity of its cognate TGFβ receptor, co-immunoprecipitation of another type of receptor which is not recognized by this antibody was indicative of heteromeric complex formation between those receptors.

As shown in Fig. 5A, SDS-PAGE under reducing conditions of the nonimmunoprecipitated membrane lysate (NIP) demonstrated the presence of TGFβ binding proteins consistent in mobility and size with RI, RII, the endoglin monomer and betaglycan (lane 1). The anti-betaglycan antibody (α-BG) immunoprecipitated betaglycan and co-immunoprecipitated not only RI and RII but also trace amounts of the endoglin monomer (Lane 2). Similarly, the anti-endoglin antibody (α-Eg; 44G4) precipitated the endoglin monomer, thus confirming the presence of endoglin on human chondrocytes (Lane 3). Interestingly, α-Eg co-immunoprecipitated betaglycan in addition to RI and RII. The co-immunoprecipitation of RI and RII with α-BG (8,36) or with α-Eg (50) is well documented. However, the co-immunoprecipitation of betaglycan with α-Eg and that of endoglin with α-BG has not been reported previously with the exception of our recent demonstration on microvascular endothelial cells (33). The findings of co-immunoprecipitation of endoglin with α-BG and that of betaglycan with α-Eg suggest that endoglin and betaglycan form a heteromeric complex on the cell surface of chondrocytes. The anti-RI antibody (α-RI) immunoprecipitated RI and co-immunoprecipitated RII and betaglycan (lane 4). Similarly, the anti-RII antibody (α-RII) precipitated RII, RI, and betaglycan (Lane 5). The patterns of co-immunoprecipitation

observed with the anti-RI and RII antibodies have previously been documented and confirm the association of these receptors on the cell surface. No receptors were immunoprecipitated with the control (rabbit) immunoglobulin (IgG, Lane 6).

Under nonreducing conditions (Fig. 5B), the α-BG immunoprecipitated betaglycan (Lane 2), and the α-Eg precipitated the endoglin dimer (Lane 3) as expected. Again, α-BG co-immunoprecipitated the endoglin dimer along with the RI, and RII, as well as the 115 kDa and 145 kDa bands that were later identified as the RI homodimer and RI-RII heterodimer, respectively (Lane 2). Significantly, α-Eg co-immunoprecipitated trace amounts of betaglycan as well as RI and RII (Lane 3). The 240 kDa band which was later identified as an endoglin-containing heteromeric complex was also precipitated. No receptor was immunoprecipitated using control (rabbit) immunoglobulin (IgG, Lane 1). The immunoprecipitation results with α-BG and α-Eg under reducing (Fig. 5A) and nonreducing (Fig. 5B) conditions on chondrocytes affinity labeled with $^{125}$I-TGFβ1 strongly suggest that endoglin associates with betaglycan in a ligand-dependent manner.

**Endoglin associates with betaglycan in a type II TGFβ receptor independent manner**

Since RII has been shown to form a complex with betaglycan and with endoglin, it can be argued that the association between endoglin and betaglycan observed on chondrocytes is a result of the complex formation of RII with both betaglycan and endoglin simultaneously. Therefore, we tested whether the endoglin-betaglycan association occurs in the absence of the type II receptor using a cell type exhibiting virtually no type II receptors, the 293 cells (38). The 293 cells were transiently transfected with cDNAs encoding L-endoglin and betaglycan with and without the cDNA encoding RII. Cells were then affinity labeled with $^{125}$I-TGFβ1, immunoprecipitated with α-Eg or α-BG, and

analyzed by SDS-PAGE under reducing conditions, as shown in Fig. 5C. On 293 cells transfected with the empty vector, pcDNA3, it was not possible to detect any TGFβ binding proteins except trace amounts of betaglycan (Lanes 1-5). In contrast, the 293 cells transfected with vectors encoding endoglin and betaglycan, displayed those receptors on the cell surface visualized as $^{125}$I-TGFβ1 labeled complexes in non-immunoprecipitated lysates (NIP, Lane 6). The migration patterns of betaglycan and endoglin in transfected 293 cells, however, were different from those of their endogenous counterparts in chondrocytes, with betaglycan migrating at 180-250 kDa and endoglin at 130 kDa. This may be due to differential glycosylation in 293 cells compared with chondrocytes. Importantly, immunoprecipitation with α-BG resulted in the immunoprecipitation of both betaglycan and endoglin (Lane 8). Similarly, immunoprecipitation with α-Eg resulted in the precipitation of both endoglin and betaglycan , where the co-immunoprecipitation of betaglycan was more abundant than the precipitation of endoglin (Lane 9). Immunoprecipitation with a rabbit control IgG showed no receptor complexes (Lane 7). Finally, 293 cells co-transfected with RII in addition to endoglin and betaglycan, when affinity labeled but not immunoprecipitated, showed the expression of all three transfected receptors and specified the migration position of all three receptors. Results shown in lanes 1 and 5 confirm that the 293 cells express virtually no endogenous RII. Taken together, these findings indicate that endoglin forms a complex with betaglycan independent of RII.

**The association of endoglin and betaglycan can occur in a ligand-independent manner**

Thus far, our immunoprecipitation studies of affinity labeled chondrocytes and 293 cells indicated that endoglin forms a heteromeric complex with betaglycan in a ligand-induced manner, indicating ligand dependence. It was important to confirm this association and to determine whether such complex formation also could occur in the absence of ligand. Membrane extracts of chondrocytes, extensively washed with dPBS, were prepared and immunoprecipitated with α-BG, α-Eg, or with control IgG, or not immunoprecipitated. They were then fractionated by SDS-PAGE under reducing conditions and analyzed by Western blotting using α-Eg (SN6h), as shown in Fig.6A. No endoglin was detected in the control experiment in which the immunoprecipitation was done with rabbit control IgG (IgG) before Western blotting (Lane 1). Western blotting of membrane extracts immunoprecipitated with α-Eg (Lane 4) confirmed the presence of endoglin monomer at 100 kDa. Importantly, Western blotting of extracts immunoprecipitated with α-BG (Lane 2) revealed the endoglin monomer at 100 kDa, thus demonstrating that endoglin was co-immunoprecipitated with betaglycan in the absence of TGFβ. In addition, when chondrocytes were pretreated with 100 pM TGFβ1, there was no increase in the amount of endoglin monomer detected (Lane 3, α-BG/β1). Western blotting of nonimmunoprecipitated membrane extracts (NIP, Lane 5) also confirmed the presence of endoglin in chondrocytes. Furthermore, parallel experiments in which cells were washed with mild acid to ensure complete removal of any endogenous TGFβ (40,41) before membrane extraction and immunoprecipitation with α-BG revealed similar levels of endoglin upon Western blot using α-Eg (data not shown). These results taken together confirm that endoglin forms a complex with betaglycan in a ligand independent manner. This is consistent with our observation in microvascular endothelial cells (33).

To further confirm that endoglin associates with betaglycan in the absence of ligand and in the absence of RII, 293 cells were transiently transfected with endoglin and betaglycan and the immunoprecipitation/Western blot analysis was done as above, and the data are shown in Fig.6B. Western blotting of nonimmunoprecipitated membrane extracts of 293 cells (Lane 1, NIP) and those immunoprecipitated with α-Eg (Lane 4) demonstrated the endoglin monomer at 100 kDa that is expressed in the transfected 293 cells. Once again, Western blot of extracts immunoprecipitated with α-BG (Lane 3) revealed the endoglin monomer at 100 kDa, indicating endoglin-betaglycan association while extracts immunoprecipitated with control IgG revealed no detectable bands (Lane 2). These results obtained in the absence of TGFβ in 293 cells which express no type II receptors but overexpression of endoglin and betaglycan confirm that endoglin associates with betaglycan in a ligand- and RII- independent manner.

**Endoglin forms higher order complexes with TGFβ signaling receptors on human chondrocytes**

When chondrocytes labeled with $^{125}$I-TGFβ1 were analyzed under nonreducing conditions, the results represented in Fig. 3 showed binding complexes at 115 kDa, 145 kDa, 270 kDa and 320 kDa, in addition to RI, RII, endoglin and betaglycan. To further characterize the nature of these binding complexes and to test whether they represent oligomeric complexes of TGFβ receptors, two-dimensional gel electrophoresis was performed. Chondrocytes were affinity labeled with $^{125}$I-TGFβ1 and the membrane extracts were not immunoprecipitated (Fig. 7A) or precipitated with α-Eg (SN6h)(Fig. 7B) and two-dimensional gel electrophoresis was performed under nonreducing conditions in the first dimension and reducing conditions in the second dimension. The

results shown in Fig 7A revealed a spot of identical mobility as the endoglin monomer that fell from the 180 kDa position. Traces of spot with identical mobility as RI fell from the 115 kDa position. Similarly, two spots with mobilities identical to those of RI and RII fell from the 145 kDa complex. These results suggest that the 180 kDa complex represents endoglin while the 115 kDa complex corresponds to the RI homodimer and the 145 kDa complex to the RI/RII heterodimer. These data are consistent with our observations in microvascular endothelial cells (33). The 240 kDa and the 320 kDa complexes were reductant sensitive and gave rise to spots of mobilities identical to those of the endoglin monomer and detectable amounts of RII and RI indicating that endoglin forms higher order complexes with the TGFβ signaling receptors on chondrocytes. The 180 kDa that fell from the 240 and 320 kDa complexes may correspond to the endoglin dimer that is either reductant insensitive or to endoglin monomers that were cross-linked inadvertently during the affinity labeling procedure.

Membrane extracts immunoprecipitated with α-Eg (SN6h) upon two dimensional electrophoresis revealed a 100 kDa spot of identical mobility as endoglin monomer that fell from 180 kDa, 240 kDa and 320 kDa positions (Fig. 7B). These results provide further evidence that the 180 kDa complex represents endoglin and demonstrate that the 240 and 320 kDa complexes contain endoglin.

**DISCUSSION**

Although TGFβ has been implicated as a key mediator of cartilage repair and TGFβ signaling has been demonstrated to be required for the maintenance of articular cartilage (50), the mechanisms regulating TGFβ action in chondrocytes are poorly understood. The question of whether chondrocytes express novel or accessory TGFβ receptors has not

been addressed in previous studies. Localization of the types I, II and III TGFβ receptors (RI, RII and betaglycan) have been demonstrated in the growth plate and articular cartilage of growing rats and rabbits using immunohistochemistry, in situ hybridization or RT-PCR (49,51,52). However, information on the expression of TGFβ receptors in the human cartilage or chondrocytes is limited to a single study by Horner et al (53) who described the expression of RI and RII in growing human bone. On the other hand, several recent studies report that TGFβ rapidly induces several signaling pathways such as the Smad and mitogen activated protein kinases (MAPK) pathways such as the extracellular signal regulated kinase (ERK), and p38 pathways in chondrocytes (48,54,55). The relative contribution of these pathways in determining the chondrocyte response to TGFβ is unknown. The expression profiles of accessory TGFβ receptors and their interactions with the signaling TGFβ receptors at the membrane level leading to the formation of heteromeric receptor complexes may be critical in specifying the relative contributions of the various signaling pathways, and thus may play a central role in regulating the diverse actions of TGFβ in chondrocytes. Interaction of novel and accessory receptors with TGFβ signaling receptors have been reported previously to be important regulators of TGFβ signaling in other cell types (10,20,28).

In the present study we demonstrate for the first time that endoglin is expressed on human articular and nonarticular chondrocytes at high concentrations and that it forms higher order complexes with RI and RII on the cell surface. More importantly, endoglin forms a heteromeric complex with betaglycan on human chondrocytes. This complex formation occurs at normal physiological receptor concentrations since the chondrocytes used were not transfected with any receptor cDNAs. Significantly, we found that endoglin

heteromerizes with betaglycan in a ligand-induced manner as indicated by their co-immunoprecipitation after affinity cross-link labeling with $^{125}$I-TGFβ1. Interestingly, endoglin complexes with betaglycan also in the absence of TGFβ, demonstrating ligand-independent association. Furthermore, endoglin-betaglycan association is observed in the absence of RII, indicating that the association can occur independently of RII.

The phenotype of the human chondrocytes used in the present study has been characterized extensively (43,44). The primary human articular chondrocytes and immortalized chondrocytes expressed both aggrecan and type II collagen mRNAs, and their responsiveness to TGFβ was shown by stimulation of PAI promoter activity and Smad 2 phosphorylation. Prior studies analyzing the interactions between TGFβ receptors and the stoichiometry of the signaling complex employed primarily mutant cell lines or cells overexpressing the wild-type or chimeric receptors (56,57). In the present study, we used normal TGFβ responsive human chondrocytes to demonstrate the association of endoglin with betaglycan, and the formation of higher order complexes containing endoglin and TGFβ signaling receptors. Thus, our results illustrate that these associations occur at endogenous receptor concentrations and ratios.

The presence of endoglin on chondrocytes has not been documented previously. Endoglin is primarily expressed on endothelial cells, and its expression at comparable levels on chondrocytes which resides in an avascular tissue, cartilage, is intriguing. While it is possible that the variable expression of endoglin observed in the three cell types studied (primary articular versus articular cell line versus nonarticular cell line) is related to the phenotype of the cell, further studies are needed to make more definitive conclusions. However, it is interesting to note that the stimulation of TGFβ–induced PAI

promoter activity was inversely related to the level of endoglin observed in these cells (Figure 1). In fact, our preliminary data suggest that overexpression of endoglin in chondrocytes leads to a diminution in TGF-β responses (Parker and Philip, unpublished observation).

In addition to providing confirmation of the identity of endoglin, the findings of immunoprecipitation studies using cells affinity labeled with $^{125}$I-TGFß1, indicated that endoglin forms a ligand-induced heteromeric complex with betaglycan. This was demonstrated by co-immunoprecipitation of endoglin with α-BG and that of betaglycan with α-Eg when analyzed under either reducing or non-reducing conditions. Furthermore, the endoglin-betaglycan association was detectable not only on chondrocytes expressing endogenous receptors, but also on 293 cells transiently transfected with cDNAs encoding betaglycan and endoglin. Although immunoprecipitation results provided evidence for the occurrence of TGFβ induced complex formation between endoglin and betaglycan, it was not possible to detect an endoglin-betaglycan heteromeric complex on SDS-PAGE analysis. The very large molecular weight of such a complex and the highly heterogeneous nature of betaglycan will preclude the detection of that complex.

That endoglin exists in a heteromeric complex with betaglycan on chondrocyte cell surface was confirmed using membrane extracts that were immunoprecipitated with α-BG and then subjected to Western blotting with α-Eg. In addition, since the latter study was done in the absence of TGFβ, it suggested that the endoglin-betaglycan complex formation could occur in a ligand-independent manner. Also, the occurrence of this complex was observed even after a mild acid wash (which ensure complete removal of

endogenous TGFβ without affecting receptor integrity, (41) of chondrocytes before immunoprecipitation and Western analysis, thus providing further evidence for ligand independence of endoglin-betaglycan interaction. Furthermore, similar results were obtained when the experiment was repeated with 293 cells transiently transfected to express endoglin and betaglycan.

The results presented argue against the possibility that the association between betaglycan and endoglin observed in our study is a result of RII interacting with betaglycan and also with endoglin. For example, 293 cells expressing virtually no RII, exhibit endoglin-betaglycan complex formation endogenously and upon transfection with cDNAs encoding endoglin and betaglycan. In addition, the observation that betaglycan does not associate with RII in the absence of ligand (58), but complexes with endoglin in the absence TGFβ, argues against the involvement of RII in the endoglin-betaglycan association.

The finding that the betaglycan-endoglin association occurs on chondrocytes is consistent with our recent demonstration of such an association on endothelial cells (33). Although numerous studies have shown that both endoglin and betaglycan interact with RI and RII, the observation that endoglin forms a complex with its homologue, betaglycan is novel. That this association occurs in a ligand-induced manner and in a ligand independent fashion is intriguing. Betaglycan has been shown to facilitate TGFβ binding to the TGFβ signaling receptors, specifically the binding of the TGFβ2 isoform to RII, which occurs at low affinity in the absence of betaglycan (59,60). However, the property of ligand presentation of betaglycan does not account for the strict requirement of betaglycan for the epithelial-mesenchymal transition involved in the heart valve formation (61). Thus, it

has been postulated that betaglycan may play a more direct, albeit unknown, role in TGFβ signaling. In addition, a functional role for the cytoplasmic tail of betaglycan in regulating TGFβ signaling has recently been described where autophosphorylated RII phosphorylates the betaglycan cytoplasmic tail, thereby enhancing TGFβ2 signaling (62). Although there is increasing evidence to show that endoglin and betaglycan potently modulate TGF- signaling, the mechanisms by which they exert their effects are poorly defined. The vascular disorder HHT1 has been attributed to mutation in the endoglin gene and thus alteration in TGFβ action is believed to be responsible for HHT1. In addition, TGFβ treatment upregulated endoglin expression on human and rat mesangial cells (63). Interestingly, it has recently been reported that both the types I and II TGF-β receptors phosphorylate endoglin cytoplasmic tail and that endoglin expression is associated with increased phosphorylation of the type I TGFβ receptor and Smad 2, and enhanced signaling (64). This is in contrast to the earlier studies which have demonstrated that endoglin expression results in an inhibition of TGFβ signaling (20). This discrepancy may reflect the differences in the activation by endoglin of the different gene promoters examined in the above studies (64). Our preliminary results on the overexpression of endoglin in chondrocytes indicate that endoglin inhibits transcriptional activity and Smad 2 phosphorylation in these cells (Parker and Philip, unpublished observation). Regardless of the precise mechanisms involved, it is clear that endoglin and betaglycan are strong modulators or direct participants in TGFβ signal transduction. This in turn implies that the complex formation between endoglin and betaglycan may be of critical significance in the regulation of TGFβ signaling.

The two dimensional gel electrophoresis provided information on the nature of the higher molecular weight receptor complexes, in addition to demonstrating the occurrence of RI/RII heterodimers and RI homodimers on chondrocytes. This is consistent with our previous observation on endothelial cells and the mechanism explaining their occurrence has been detailed (33). The true molecular weight of the 240 kDa and 320 kDa complexes containing endoglin, type II and or type I TGFβ receptors are likely to be different since high molecular weight glycoprotein complexes are known to migrate anomalously on SDS-PAGE. It is possible that they are derived from higher order complexes. Current evidence indicates that the TGFβ signaling complex is a heterotetramer consisting of one molecule each of the type I and type II receptor associated with each monomer of a TGFβ dimer molecule (65). While it is difficult to estimate the precise stoichiometry of the endoglin-containing complexes, it is possible that endoglin associates with the heterotetrameric TGFβ signaling complex. Moreover, multiple high molecular weight receptor complexes containing endoglin, RI and RII with or without betaglycan may be formed depending on the efficiency of cross-linking of the individual receptor components to the $^{125}$I-TGFβ1 subunits. Alternatively, TGFβ receptor complexes of different subtypes and ratios may exist in parallel. Oligomerization of TGFβ receptors to form complexes consisting of differing subtype composition and ratio may represent modes of regulating distinct TGFβ responses.

Based on the results in the present study, we propose a model to illustrate the significance of the endoglin-betaglycan complex formation in the modulation of TGF-β signaling in chondrocytes (Figure 8). Endoglin associates with betaglycan on the cell surface and this heteromeric complex may interact directly with the signaling receptors.

However, the relative expression level of the individual receptor components may determine the outcome on TGFβ signaling. Thus, as mentioned above, overexpression of endoglin can inhibit (20) or enhance (64) TGFβ signaling. However, as shown in Figure 8, complex formation between endoglin and betaglycan will allow the regulation of each other's function at the level of ligand binding (A) or downstream signaling (B). Thus, endoglin-betaglycan association may be critical for achieving a fine balance between the positive and the negative regulation of TGF-β signaling. It is interesting to mention in this regard that complex formation between endoglin and betaglycan association may also modulate signaling by other members of the TGFβ receptor superfamily in the light of recent observations which show that endoglin is an accessory receptor not only for TGFβ, but also for the bone morphogenic proteins and activin (15), and that betaglycan, in addition to binding the TGFβ isoforms, binds inhibin, thereby facilitating inhibin antagonism of activin signaling (66).

In summary, the present results demonstrate for the first time that endoglin is expressed on human chondrocytes at high concentrations and that it forms higher order complexes with RI and RII on the cell surface. More importantly, we demonstrate that endoglin forms a heteromeric complex with betaglycan on these cells. Also, our results illustrate that this complex formation occurs at physiological receptor concentrations and ratios. Significantly, we found that the endoglin complexes with betaglycan in a ligand-induced and ligand-independent manner. Furthermore, this complex formation can occur independently of RII. Elucidating the role of endoglin in the regulation of TGFβ signaling in chondrocytes and in the avascular cartilage and understanding the significance of endoglin-betaglycan association in these cells are critical to unraveling the

molecular mechanisms governing TGFβ action in chondrocytes, and thus the role of TGFβ in mediating cartilage formation and repair.

**FIGURE LEGENDS**

**Fig. 1. Expression of cartilage-specific matrix gene expression and TGFβ responses in primary chondrocytes and nonarticular (C28/I2) and articular (tsT/AC62) chondrocyte cell lines.** **A:** Expression of aggrecan and type II collagen mRNAs: Chondrocytes were cultured to confluence, total RNA was extracted, 5μg of total RNA was reverse transcribed and PCR was performed using specific primers. Products were analyzed on a 1.5% agarose-ethidium bromide gel. **B:** Stimulation of plasminogen activator inhibitor-1 (PAI-1) promoter activity by TGFβ. The chondrocytes were transiently transfected with the PAI-1 promoter-luciferase reporter construct, p3TP-Lux and pβgal. 24 hours after transfection, cells were treated with 100pM of TGFß1 for 24 hours or were left untreated. Luciferase activity was determined, normalized using the βgal assay and expressed as a percent control of untreated cells. The data shown are representative of three different experiments each done in triplicates. **C:** Stimulation of Smad 2 phosphorylation by TGFβ1. Solubilized extracts of C-28/I2 cells treated with 100pM of TGFß1 for 15 minutes or left untreated were analyzed by SDS-PAGE (3-11% gradient gels) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against the phosphorylated form of Smad2 (upper panel). Immunoblotting using an antibody directed against the nonphosphorylated form of Smad 2 was performed to illustrate equal protein loading (lower panel). The ECL system was used for chemiluminescence detection. Similar

results were obtained using the tsT/AC62 cell line and primary chondrocytes (data not shown).

**Fig. 2. Affinity labeling of human chondrocytes with $^{125}$I-TGFβ1 and analysis under reducing conditions.** Confluent monolayers of tsT/AC62 cells were affinity labeled with 100pM of $^{125}$I-TGFβ1 in the absence or presence of the indicated concentrations of unlabeled TGFβ1, β2, or β3. Solubilized cell extracts were analyzed by SDS-PAGE on 3-11% polyacrylamide gradient gels under reducing conditions followed by autoradiography. Similar receptor profiles were observed using primary chondrocytes and the C-28/I2 cell line (data not shown).

**Fig. 3. Affinity labeling of human chondrocytes with $^{125}$I-TGFß1 and analysis under nonreducing conditions.** Confluent monolayers of tsT/AC62 cells were affinity labeled with 100pM of $^{125}$I-TGFβ1 in the absence or presence of the indicated concentrations of unlabeled TGFβ1, or β2 or β3. Solubilized cell extracts were analyzed by SDS-PAGE (3-11% gradient gels) under nonreducing conditions followed by autoradiography. Similar receptor profiles were observed using primary chondrocytes and the C-28/I2 cell line (data not shown).

**Fig. 4. Endoglin is expressed in human articular cartilage and its expression in** primary **chondrocytes and chondrocyte cell lines is comparable to that in endothelial cells (HMEC-1). A:** Solubilized extracts of primary chondrocytes, C-28/I2, tsT/AC62, and HMEC-1 cells were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against endoglin (SN6h, Dako) (upper panel). Immunoblotting using an antibody directed against the nonphosphorylated form of Smad

2/3 was performed to illustrate equal protein loading (lower panel). The ECL system was used for chemiluminescence detection. **B:** C-28/I2, tsT/AC62, and HMEC-1 cells were affinity labeled with 100 pM of $^{125}$I-TGFβ1 in the absence or presence of 2.5 nM of unlabeled TGFβ1. Solubilized cell extracts were analyzed by SDS-PAGE (3-11% gradient gels) under nonreducing conditions followed by autoradiography. **C:** Plasma membrane fraction of human articular cartilage was prepared as described in Methods and were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions or nonreducing and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against endoglin (SN6h, Dako). The ECL system was used for chemiluminescence detection.

**Fig. 5. Immunoprecipitation of TGFβ binding proteins on human chondrocytes and transiently transfected 293 cells.** Cells were affinity labeled with 200 pM of $^{125}$I-TGFβ1 and solubilized cell extracts were immunoprecipitated with 3μg/ml each of anti-receptor antibodies or control IgG. Complexes were fractionated on SDS-PAGE (3-11% gradient gels) under reducing (A and C) or nonreducing (B) conditions and visualized by autoradiography. **A:** Non-immunoprecipitated (NIP) cell extract of C28/I2 is shown in Lane 1. Immunoprecipitations were performed using anti-betaglycan (Lane 2, α-BG), anti-endoglin (Lane 3, α-Eg), anti-RI (Lane 4, α-RI), or anti-RII (Lane 5, α-RII) or control IgG (Lane 6). **B:** tsT/AC62 cell extracts were immunoprecipitated with control IgG (Lane 1), anti-betaglycan (Lane 2, α-BG), or anti-endoglin (Lane 3, α-Eg). **C:** 293 cells were transiently transfected with empty vector (pcDNA3, Lanes 1-5), or L-endoglin (Eg) and betaglycan (BG) (Lanes 6-9) or Eg , BG and RII (Lane 10). After affinity labeling, non-immunoprecipitated cell extracts (NIP) were analyzed by SDS-PAGE

(Lanes 1, 6 & 10) or immunoprecipitations were performed using control IgG (Lanes 2 & 7) or specific anti-receptor antibodies as indicated (Lanes 3-5, 8-9) before analysis by SDS-PAGE.

**Fig. 6. Western blot analysis demonstrating complex formation between endoglin and betaglycan on chondrocytes. A:** Solubilized extracts of human chondrocytes (C-28/I2) were not immunoprecipitated (NIP, Lane 5), or immunoprecipitated with control IgG (Lane 1), or anti-betaglycan antibody (α-BG, Lane 2) or anti-endoglin antibody (α-Eg, Lane 4). In Lane 3, chondrocytes were pretreated with 100 pM of TGFβ1 at 37°C for 2 hrs before membrane extraction and immunoprecipitation. Complexes were fractionated by SDS-PAGE (7.5% acrylamide) under reducing conditions and Western blotting was performed, as described in Methods using anti-endoglin antibody (SN6h). **B:** Solubilized extracts of 293 cells transiently transfected with L-endoglin (Eg) and betaglycan (BG) were not immunoprecipitated (NIP, Lane 1), or immunoprecipitated with control IgG (Lane 2), or anti-betaglycan (α-BG, Lane 3), or anti-endoglin (α-Eg, Lane 4) antibody. Complexes were then fractionated and immunoblotted as in **A** above.

**Fig. 7. Two-dimensional gel electrophoresis of TGFβ receptor complexes on human chondrocytes.** tsT/AC62 cells were affinity labeled with 100 pM of $^{125}$I-TGFβ1 and solubilized extracts were not immunoprecipitated **(A)** or immunoprecipitated with anti-Endoglin antibody (SN6h) **(B)** and analyzed by SDS-PAGE (3-11% gradient gel) under nonreducing conditions in the first dimension. The individual lane was then cut out, laid horizontally on a second 3-11% gradient SDS-PAGE gel and analyzed under reducing conditions in the second dimension.

**Figure 8. Schematic representation of a possible model illustrating the significance of endoglin-betaglycan complex formation in modulating TGFβ signaling.** Endoglin associates with betaglycan on the cell surface and this heteromeric complex may interact directly with the signaling receptors. Overexpression of endoglin may inhibit or enhance TGFβ signaling as has been reported using different promoter-reporter constructs (20,64). Also. overexpression of betaglycan is believed to enhance signaling by facilitating ligand binding to the signaling receptors and by an alternate mechanism involving the phosphorylation of its cytoplasmic tail (62,65). However, formation of a heteromeric complex between endoglin and betaglycan will allow the regulation of each other's function at the level of ligand binding (A) and/or downstream signaling (B). Thus, the interaction between endoglin and betaglycan may be of critical significance for achieving a fine balance between the positive and the negative regulation of the TGFβ signaling pathways. Complex formation between endoglin and the signaling receptors has been omitted for clarity.

## REFERENCES


1. Robinson D, Halperin N, Nevo Z 1990 Regenerating hyaline cartilage in articular defects of old chickens using implants of embryonal chick chondrocytes embedded in a new natural delivery substance. Calc Tissue Int 46:246-253.
2. Aston JE, Bentley G 1986 Repair of articular surfaces by allografts of articular and growth-plate cartilage. J Bone Joint Surg 68-B:29-35.
3. Rosier RN, O'Keefe RJ, Crabb ID, Puzas JE 1989 Transforming growth factor beta: An autocrine regulator of chondrocytes. Connect Tissue Res 20:295-301.

4. Howard S, Anastasiades T 1993 Differential effects of bone associated factors on newly synthesized anionic glycoconjugates by articular chondrocyte cultures from adult and immature bovines. J Rheumatol 20:2083-2094.

5. Roberts AB, Sporn MB 1990 The transforming growth factor beta's. In: Sporn MB and Roberts AB (eds) Peptide Growth Factors and Their Receptors, vol 95. Spriner-Verlag, New York, pp. 419-472.

6. Seyedin S, Thompson A, Bentz H, Rosen DM, McPherson JM, Conti A, Siegel NR, Galluppi GR, and Piez KA 1986 Cartilage-inducing Factor-A. J Biol Chem 261:5693-5695.

7. Lin HV, Wang X-F, Ng-Eaton E, Weinberg RA, Lodish H F 1992 Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. Cell 68:775-785.

8. Franzen P, ten Dijke P, Ichijo H, Yamashita H, Schultz P, Heldin C H, Miyazono K 1993 Cloning of TGF-β type I receptor that forms a Heterodimeric complex with the type II TGF-β receptor. Cell 75: 681-692.

9. Piek E, Heldin C-H, ten Dijke P 1999 Specificity, diversity, and regulation in TGF-β superfamily FASEB 13:2105-2124.

10. Massague J, Chen Y-G 2000 Controlling TGF-beta signaling. Genes Dev 14:627-644.

11. Wang X F, Lin H F, Ng-Eaton E, Downward J, Lodish H F, Weinberg R A 1991 Expression cloning and characterization of the TGF-β type III receptor. Cell 67:797-805.

12. Lopez-Casillas F, Wrana JL, Massague J 1993 Betaglycan presents ligand to the TGF-β signaling receptor. Cell 73:1435-1444.

13. Massague J 2000 How cells read TGF-ß signals. Nature (reviews) 1:169-178.

14. Fonsatii E, del Vechchio L, Altomonte M, Sigalotti L, Nicotra MR, Coral S, Natali PG, Maio M 2001 Endoglin: An accessory component of the TGF-β-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. J Cell Phys. 188:1-7.

15. Barbara NP, Wrana JL, Letarte M 1999 Endoglin is an accessory protein that interacts with the signalling receptor complex of multiple members of the transforming growth factor-β super family. J Biol Chem 274(2):584-594.

16. Quackenbush EJ, Letarte M 1985 Identification of several cell surface proteins of non-T, non-B acute lymphoblastic leukemia by using monoclonal antibodies. J Immunol 134:1276-1285.

17. Arthur HM, Ure J, Smith AJH, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG 2000 Endoglin, an ancillary TGF-ß receptor, is required for extra embryonic angiogenesis and plays a key role in heart development. Dev Biol 217:42-53.

18. Li C, Harpson L, Kumar P, Bernabeu C, Kumar S 2000 CD105 antagonizes the inhibitory signaling of transforming growth factor ß1 on human vascular endothelial cells. FASEB J 14:55-64.

19. Guerrero-Esteo M, Lastres P, Letamendia A, Perez-Alvarez MJ, Langa, C, Lopez LA, Fabra A, Garcia-Pardo A, Vera S, Letarte M, Bernabeu C 1999 Endoglin

overexpression modulates cellular morphology, migration, and adhesion of mouse fibroblasts. E J Cell Biol 78:614-623.

20. Letamendia A, Lastres P, Botella LM, Raab U, Langa C, Velasco B, Attisano L, Bernabeu C 1998 Role of endoglin in cellular responses to transforming growth factor-β. A comparative study with betaglycan. J Biol Chem 273(49):33011-33019.

21. Lastres P, Letamendia A, Zhang H, Rius C, Almendro N, Raab U, Loepez LA, Langa C, Fabra A, Letarte M, Bernabeu C 1996 Endoglin modulates cellular responses to TGF-βI. J Cell Biol 133:1109-1121.

22. Ferandez-Riuz E, St-Jacques S, Bellon T, Letarte M, Bernabeu C 1993 Assignment of the human endoglin gene (END) to 9q34-->qter. Cytogenet Cell Genet 64:204-207.

23. McAllister KA, Baldwin MA, Thukkani AK, Gallione CJ, Berg JN, Porteous ME, Guttmacher AE, Marchuk DA 1995 Six novel mutations in the endoglin gene in hereditary hemorrhagic telangiectasia type 1 suggest a dominant-negative effect of receptor function. Hum Mol Genet 4:1983-1985.

24. Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJJ, Kjeldsen AD, Plauchu H 2000 Diagnostic criteria for Hereditary Hemorrhagic Telangiectasia (Rendu-Osler-Weber Syndrome) Am J Med Gen 91:66-67.

25. Bourdeau A, Dumont DJ, Letarte M 1999 A murine model of hereditary hemorrhagic telangiectasia. J Clin Invest 104:1343-1351.

26. Pece N, Vera S, Cymerman U, White RJ, Wrana JL, Letarte M 1997 Mutant endoglin in Hereditary Hemorrhagic Telangiectasia type 1 is transiently expressed intracellularly and is not a dominant negative. J Clin Invest 100:2568-2579.

27. Tam B, Germain L, Philip A 1998 A 150kDa GPI-anchored TGF-β1 binding protein forms heteromeric complex with the types I and II TGF-β signalling receptors. J Cell Biochem 70:573-586.

28. Tam B, Larouche D, Germain L, Hooper NM, Philip A 2001 Characterization of a 150 kDa accessory receptor for TGFb1 on keratinocytes: Direct evidence for a GPI-anchor, and ligand binding of the released form. J Cell Biochem 83:494-507.

29. Robbins JR, Goldring MB 1997 Preparation of immortalized human chondrocyte cell lines. In: Morgan JR, Yarmush ML (eds) Methods in Molecular Medicine, vol 18. Humana Press Inc., Totowa, N.J. pp. 173-192.

30. Goldring MB, Birkhead JR, Suen L-F, Yamin R, Mizuno S, Glowacki J, Apperley JF 1994 Successful immortalization of human chondrocytes exhibiting stable expression of cartilage-specific type II collagen. J Clin Invest 94:2307-2316.

31. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J 1994 Mechanism of activation of the TGF-β receptor. Nature 370:341-347.

32. Gruppuso PA, Mead JE, Fausto N 1990 Transforming growth factor receptors in liver regeneration following partial hepatectomy in the rat. Cancer Res 50:1464-1469.

33. Wong SH, Hamel L, Chevalier S, Philip A 2000 Endoglin expression on human microvascular endothelial cells: demonstration of higher order complexes with TGFβ signaling receptors and heteromerization with betaglycan. Eur J Biochem 267:5550-5560.

34. Piek E, Franzen P, Heldin CH, ten Dijke P 1997 Characterization of a 60-kDa cell surface-associated transforming growth factor-beta binding protein that can

interfere with transforming growth factor-beta receptor binding. J Cell Physiol 173(3):447-59.

35. Gougos A, Letarte M 1988 Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line. J Immunol 141:1925-1933.

36. Yamashita H, Ichijo H, Grimsby S, Moron A, ten Dijke P, Miyazono K 1994 Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor beta. J Biol Chem 269:1995-2001.

37. Dumont N, O'Connor-McCourt M, Philip A 1995 Transforming growth factor-β receptors on human endometrial cells: Identification of the type I, II and III receptors, and glycosylphosphatidylinositol anchored TGF-β binding proteins. Mol Cell Endocrinol 111:57-66.

38. Zwaagstra JC, Kassam Z, O'Connor-McCourt MD 1999 Down-regulation of transforming growth factor-beta receptors: cooperativity between the types I, II, and III receptors and modulation at the cell surface. Exp Cell Res 252:352-36.

39. Bellon T, Corbi A, Lastres P, Cales C, Cebrain M, Vera S, Chefeitz M, Massague J, Letarte M, Bernabeu C 1993 Identification and expression of two forms of the human transforming growth factor-β-binding protein endoglin with distinct cytoplasmic regions. Eur J Immunol 23 : 2340-2345.

40. Tam B, Philip A 1998 Transforming growth factor-β receptor expression on human skin fibroblasts; dimeric complex formation of type I and type II receptors and identification of glycosyl phosphatidylinositol-anchored transforming growth factor-β binding proteins. J Cell Physiol 176:553-564.

41. Glick AB, Danielpour D, Morgan D, Sporn MB, Yuspa SH 1990 Induction and autocrine receptor binding of TGF-β2 during terminal differentiation of primary mouse keratinocytes. Mol Endocrinol 4:46-52.

42. MacKay K, Robbins AR, Bruce M.D, Danielpour D 1990 Identification of disulfide-linked transforming growth factor-beta 1-specific binding proteins in rat glomeruli. J Biol Chem 265:9351-9356.

43. Robbins JR, Thomas B, Tan L, Choy B, Arbiser JL, Berenbaum F, Goldring MB 2000 Immortalized human adult articular chondrocytes maintain cartilage-specific phenotype and responses to interleukin-1beta. Arthritis Rheum 43:2189-201.

44. Goldring MB, Berenbaum F 1999 Human chondrocyte culture models for studying cyclooxygenase expression and prostaglandin regulation of collagen gene expression. Osteoarthritis Cartilage 7:386-88.

45. Kokenyesi R, Tan L, Robbins JR, Goldring MB 2000 Proteoglycan production by immortalized human chondrocyte cell lines cultured under conditions that promote expression of the differentiated phenotype. Arch Biochem Biophys 383:79-90.

46. Loeser R, Sadiev S, Tan L, Goldring MB 2000 Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for α1β1 and α2β1 integrins in mediating chondrocyte adhesion to types II and VI collagen. Osteoarthritis Cartilage 8:96-105.

47. Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J 1992 TGF-β signals through a heteromeric protein kinase receptor complex. Cell 71:1003-1014.

48. Yang X, Chen L, Xu X, Li C, Huang C, Deng C-X 2001 TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol 153:35-46.

49. Fukumura K, Matsunaga S, Yamamoto T, Nagamine T, Ishidou Y, Sakou T 1998 Immunolocalization of transforming growth factor-beta s and type I and type II receptors in rat articular cartilage. Anticancer Res 18:4189-4194.

50. Lux A, Attisano L, Marchuk DA 1999 Assignment of transforming growth factor β1 and β3 and a third new ligand to the type I receptor ALK-1 J Biol Chem 274:9984-9992.

51. Kabasawa Y, Ejiri S, Matsuki Y, Hara K, Ozawa H 1998 Immunoreactive localization of transforming growth factor-beta type II receptor-positive cells in rat tibiae. Bone 22:93-98.

52. Matsunaga S, Yamamoto T, Fukumura K 1999 Temporal and spatial expressions of transforming growth factor-betas and their receptors in epiphyseal growth plate. Int J of Onc 14:1063-1067.

53. Horner A, Kemp P, Summers C, Bord S, Bishop NJ, Kelsall AW, Coleman N, Compston JE 1998 Expression and distribution of transforming growth factor-beta isoforms and their signaling receptors in growing human bone. Bone 23:95-102.

54. Miyazaki Y, Tsukazaki T, Hirota Y, Yonekura A, Osaki M, Shindo H, Yamashita S 2000 Dexamethasone inhibition of TGF beta-induced cell growth and type II collagen mRNA expression through ERK-integrated AP-1 activity in cultured rat articular chondrocytes. Osteoarthritis Cartilage 8:378-385.

55. Watanabe H, de Caestecker MP, Yamada Y 2001 Transcriptional cross-talk between Smad, ERK1/2 and p38 mitogen activated protein kinase pathways regulates transforming growth factor-beta-induced aggrecan gene expression in chondrogenic ATDC5 cells. J Biol Chem. 276:14466-14473.

56. Laiho M, Decaprio J, Ludlow JW, Livingston DM, Massague. J 1990 Growth inhibition by TGF-β linked to suppression of retinoblastoma protein phosphorylation. Cell 62:175-185.

57. Wells RG, Gilboa L, Sun Y, Liu X, Henis YI, Lodish HF 1999 Transforming growth factor-beta induces formation of a dithiothreitol-resistant type I/Type II receptor complex in live cells. J Biol Chem 274:5716-5722.

58. Matsuzaki K, Kan M, McKeehan WL 1996 Reconstitution of a pentameric complex of dimeric transforming growth factor beta ligand and a type I, II, III receptor in baculoviral-infected insect cells. In Vitro Cell Dev Biol Animal 32:345-360.

59. Chen X, Weisberg V, Fridmacher M, Watanabe G, Naco G, Whitman M 1997 Smad4 and FAST-1 in the assembly of activin-responsive factor. Nature 389:85-89.

60. Sun L, Chen C 1997 Expression of transforming growth factor beta type III receptor suppresses tumorigenicity of human breast cancer MDA-MB-231 cells. J Biol Chem 272:25367-25372.

61. Brown CB, Boyer AS, Runyan RB, Barnett JV 1999 Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. Science 283:2080-2082.

62. Blobe GC, Schiemann WP, Pepin M-C, Beauchemin M, Moustakas A, Lodish HF, O'Connor-McCourt MD 2001 Functional roles for the cytoplasmic domain of the

type III transforming growth factor beta receptor in regulating transforming growth factor beta signaling. J Biol Chem 276:24627-24637.

63. Rodriguez-Barbero A, Obreo J, Eleno N, Rodriguez-Pena A, Duwel A, Jeric M, Sanchez-Rodriguez A, Bernabeu C, Lopez-Novoa JM 2001 Endoglin expression in human and rat mesangial cells and its upregulation by TGF-β1. Biochem Biophys Res Com 282:142-147.

64. Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C 2002 Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-β receptors I and II. J Biol Chem In Press.

65. Massague J 1998 TGF-beta signal transduction. Ann Rev Biochem 67:753-791.

66. Lewis KA, Gray PC, Blount AL, Macconell LA, Waiter E, Bilezikjian LM, Vale W 2000 Betaglycan binds inhibin and can mediate functional antagonism of activin signaling. Nature 404:411-414.

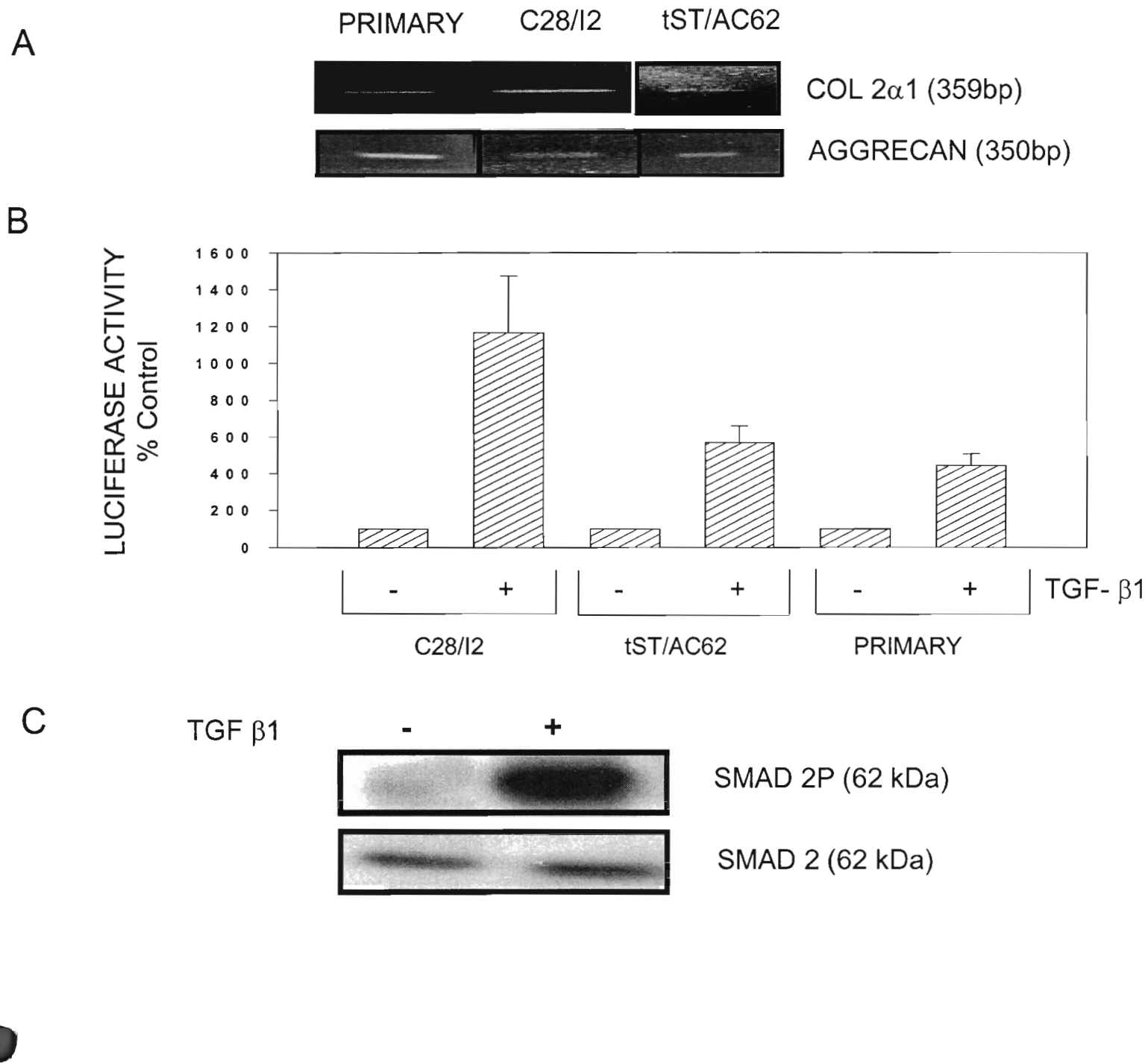
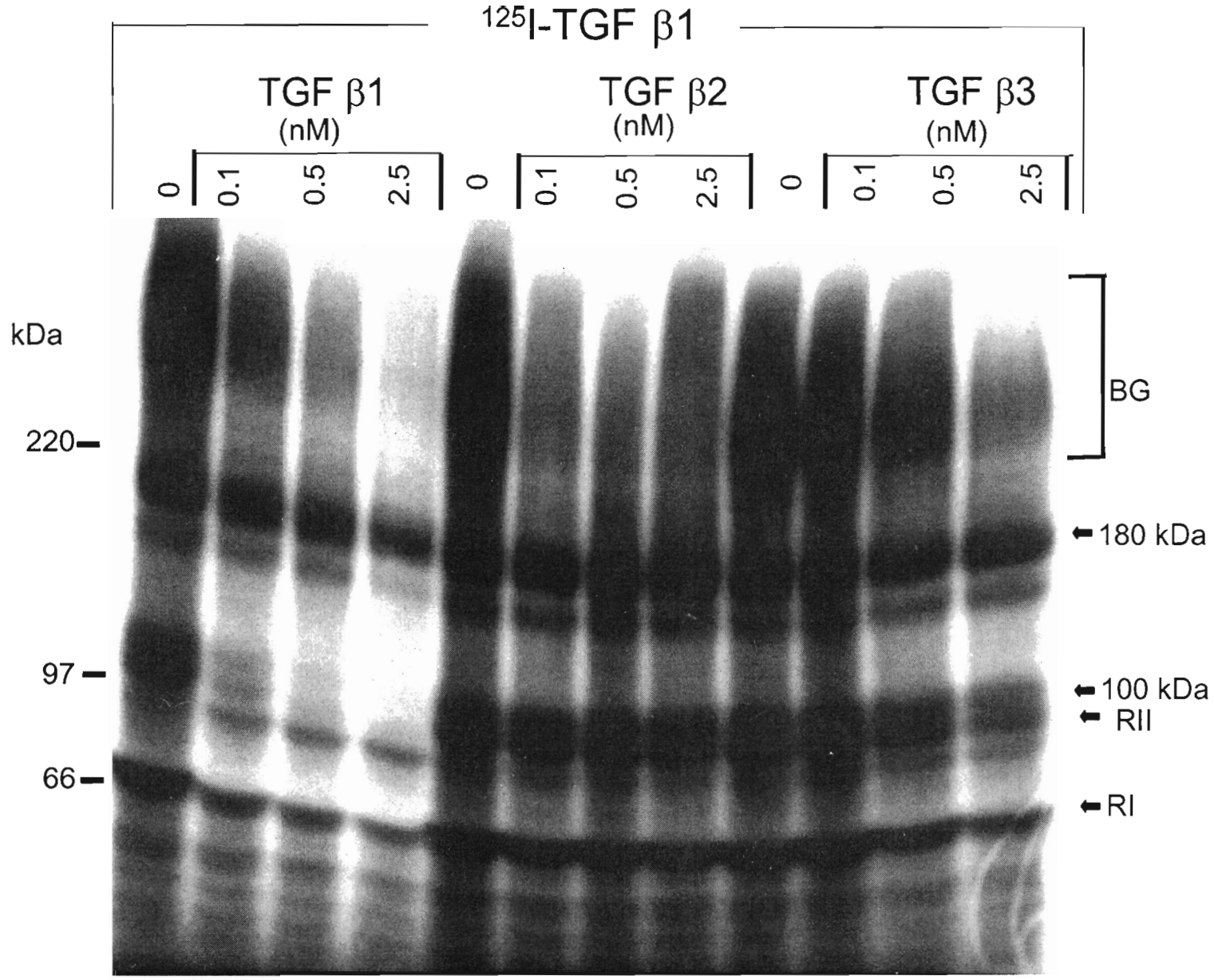
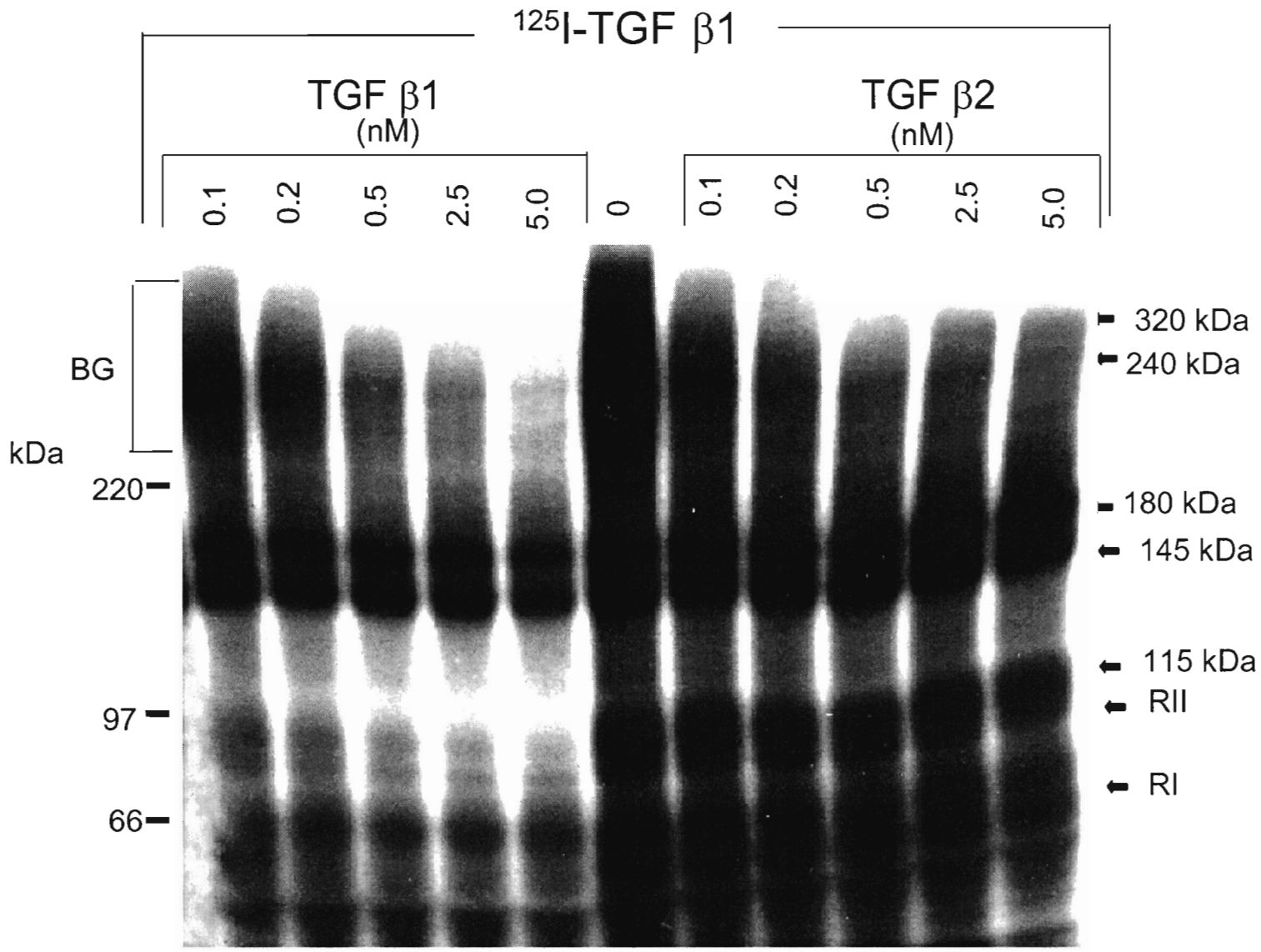

A
PRIMARY
C28/I2
tST/AC62
COL 2α1 (359bp)
AGGRECAN (350bp)
B
LUCIFERASE ACTIVITY
% Control
1600
1400
1200
1000
800
600
400
200
0
-
+
-
+
-
+
TGF- β1
C28/I2
tST/AC62
PRIMARY
C
TGF β1
-
+
SMAD 2P (62 kDa)
SMAD 2 (62 kDa)

FIGURE 2


125I-TGF β1
TGF β1
(nM)
0
0.1
0.5
2.5
TGF β2
(nM)
0
0.1
0.5
2.5
TGF β3
(nM)
0
0.1
0.5
2.5
kDa
220
97
66
BG
180 kDa
100 kDa
RII
RI

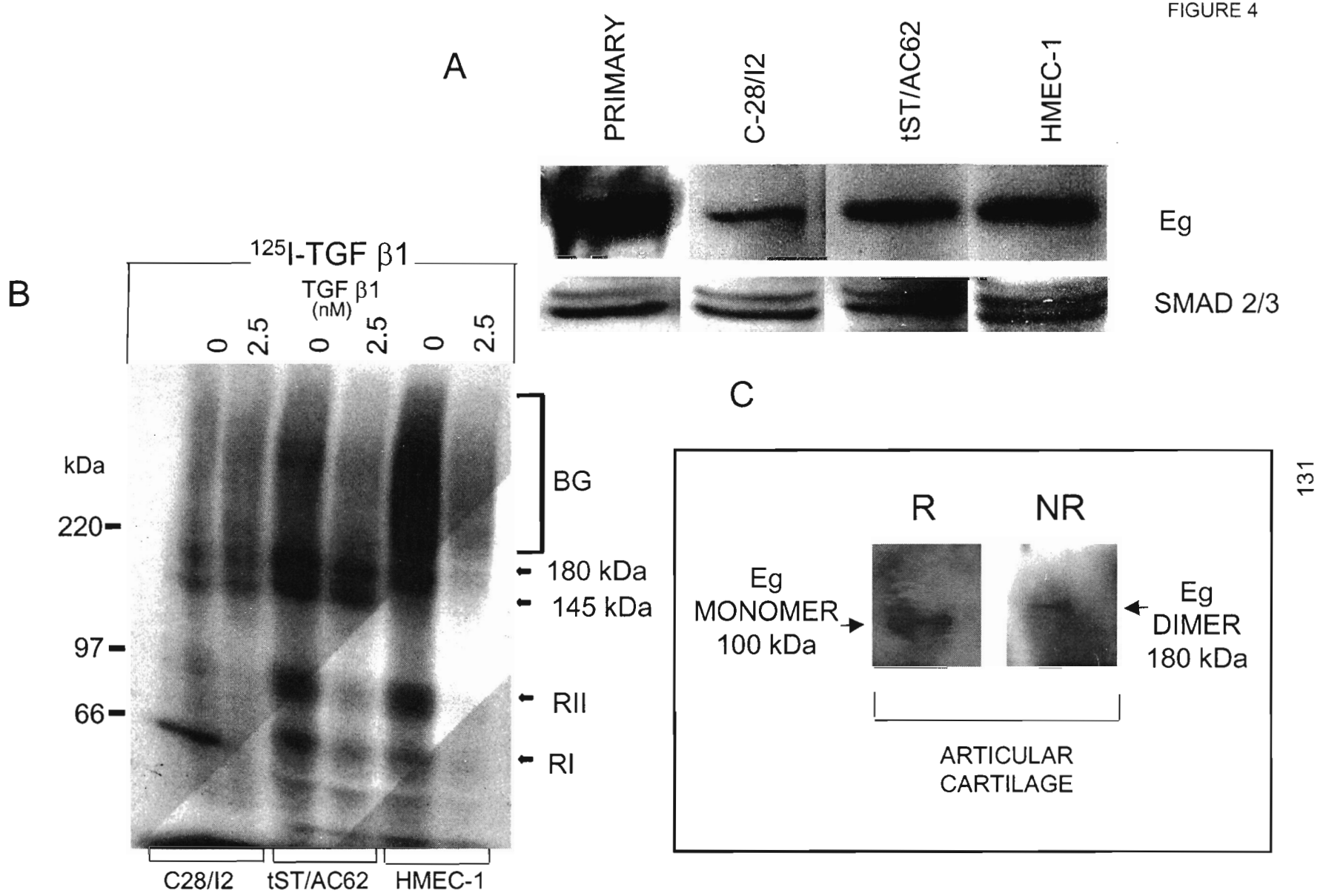
129

FIGURE 3


125I-TGF β1
TGF β1
(nM)
0.1
0.2
0.5
2.5
5.0
0
TGF β2
(nM)
0.1
0.2
0.5
2.5
5.0
BG
kDa
220
97
66
320 kDa
240 kDa
180 kDa
145 kDa
115 kDa
RII
RI

130

FIGURE 4


A
PRIMARY
C-28/I2
tST/AC62
HMEC-1
Eg
SMAD 2/3
B
125I-TGF β1
TGF β1
(nM)
0
2.5
0
2.5
0
2.5
kDa
220
97
66
BG
180 kDa
145 kDa
RII
RI
C28/I2
tST/AC62
HMEC-1
C
R
NR
Eg
MONOMER
100 kDa
Eg
DIMER
180 kDa
ARTICULAR
CARTILAGE

131

FIGURE 5A

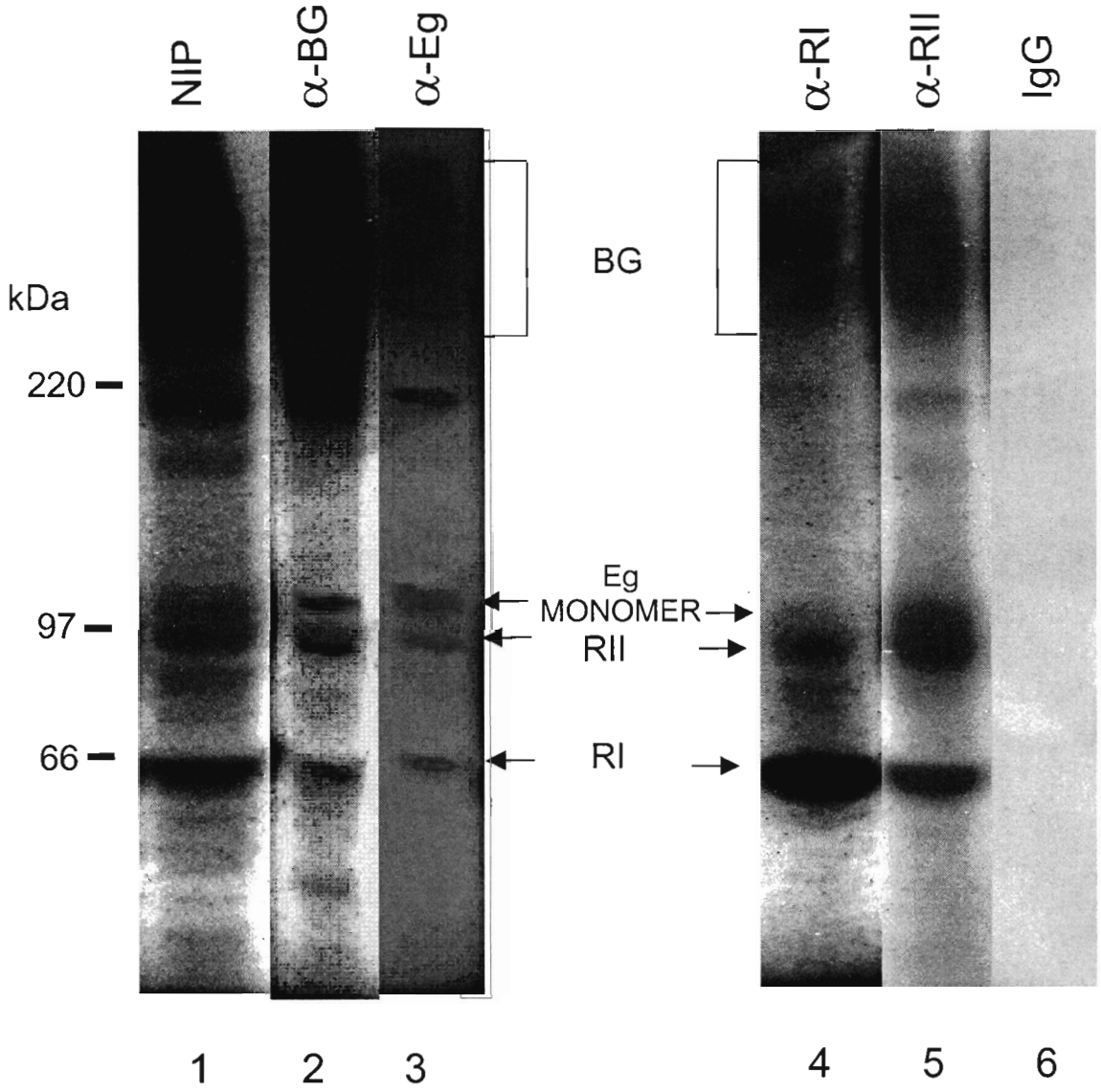

NIP
α-BG
α-Eg
α-RI
α-RII
IgG
kDa
220
97
66
BG
Eg
MONOMER
RII
RI
1
2
3
4
5
6

FIGURE 5B

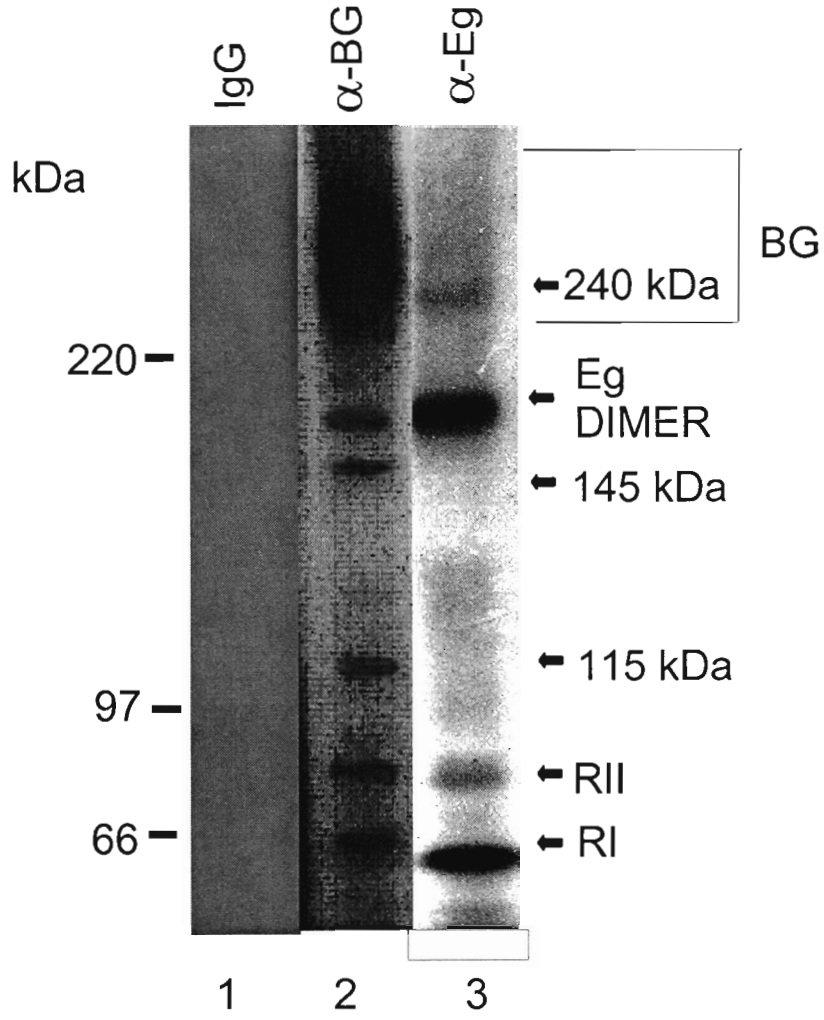
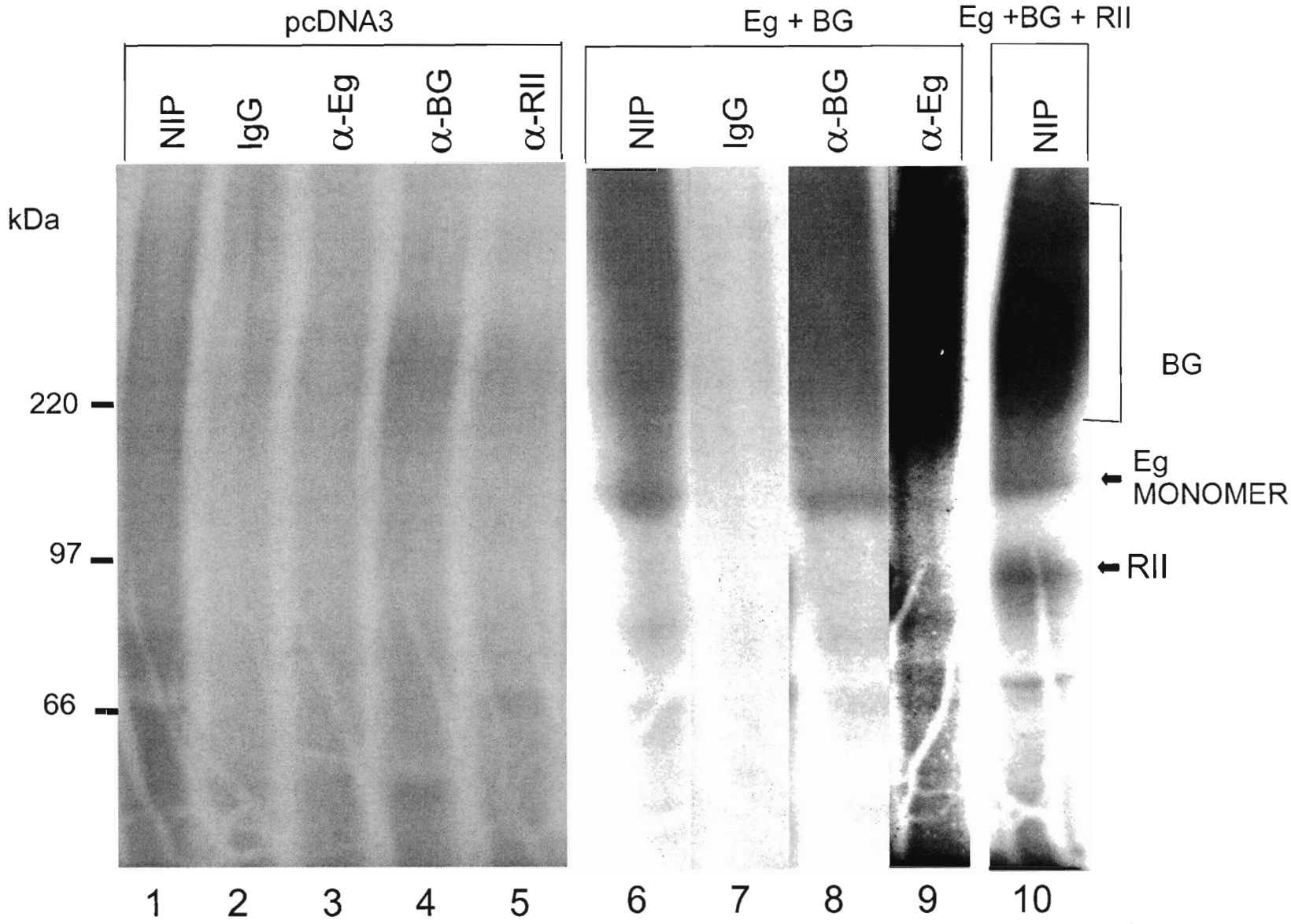
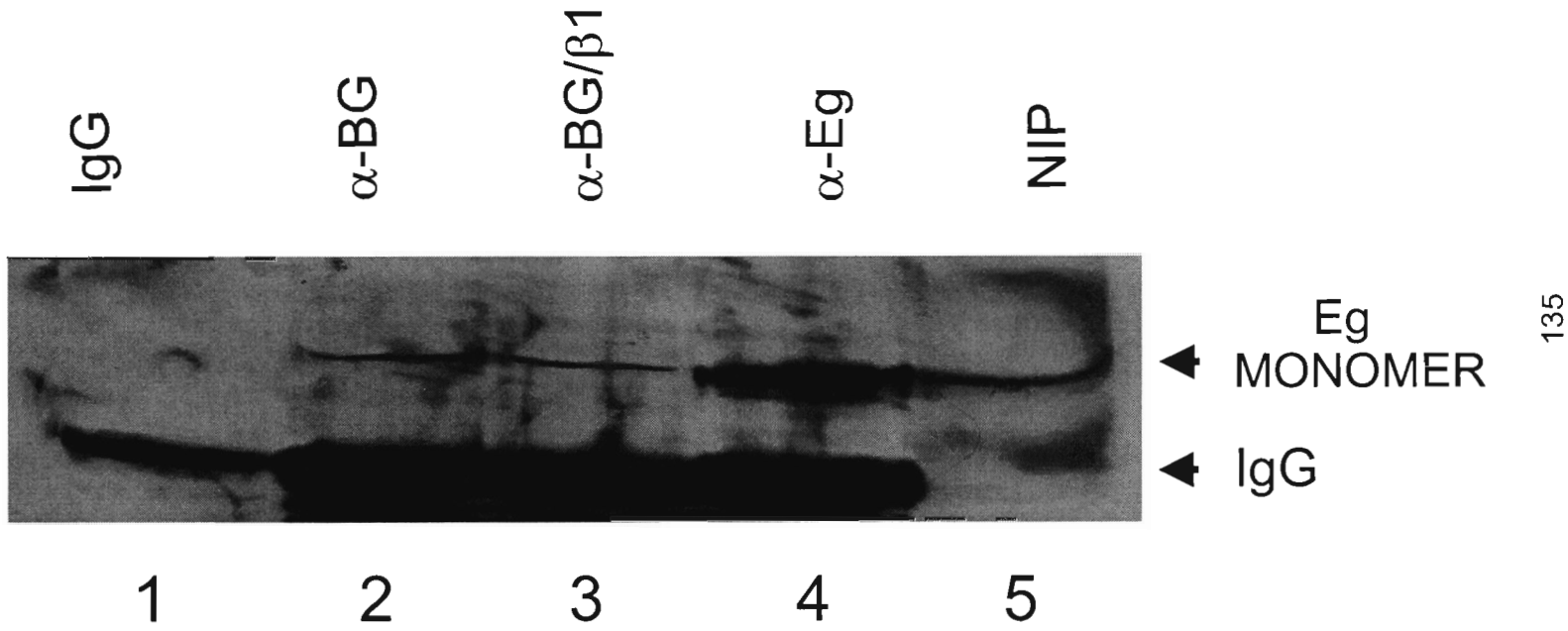

IgG
α-BG
α-Eg
kDa
BG
240 kDa
220
Eg
DIMER
145 kDa
115 kDa
97
RII
66
RI
1
2
3

FIGURE 5C



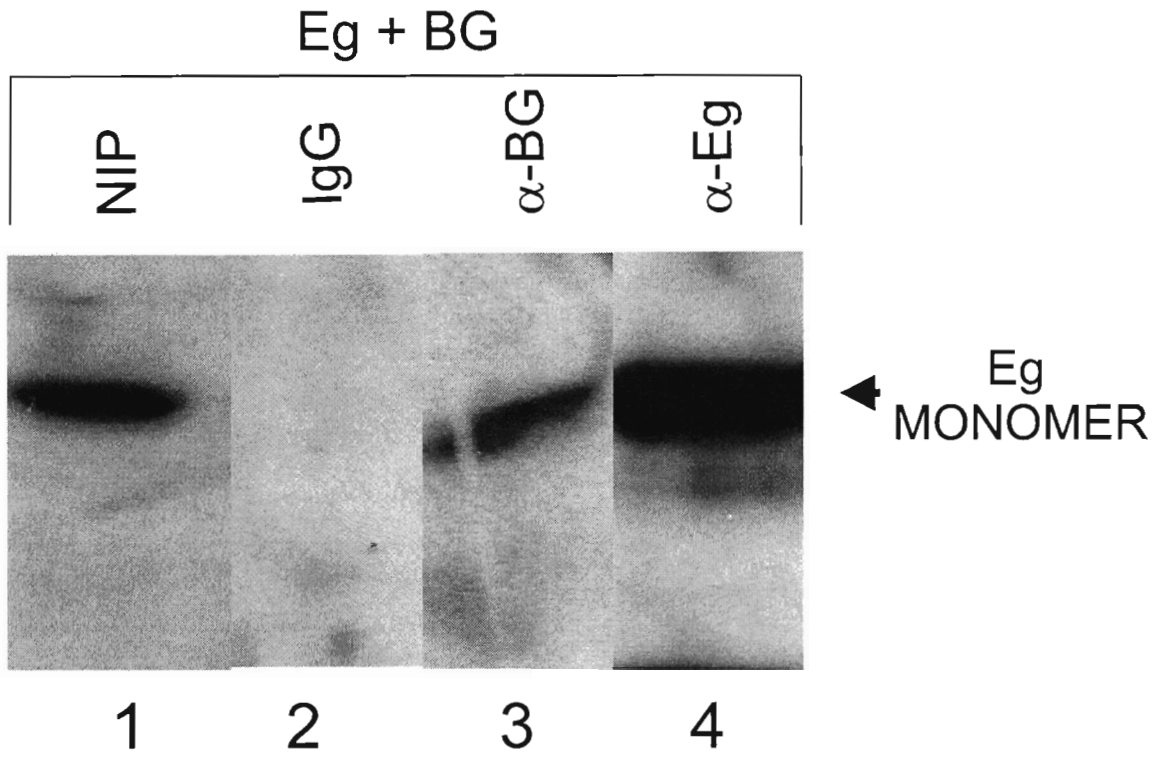

134

FIGURE 6A




135

FIGURE 6B




136

FIGURE 7A

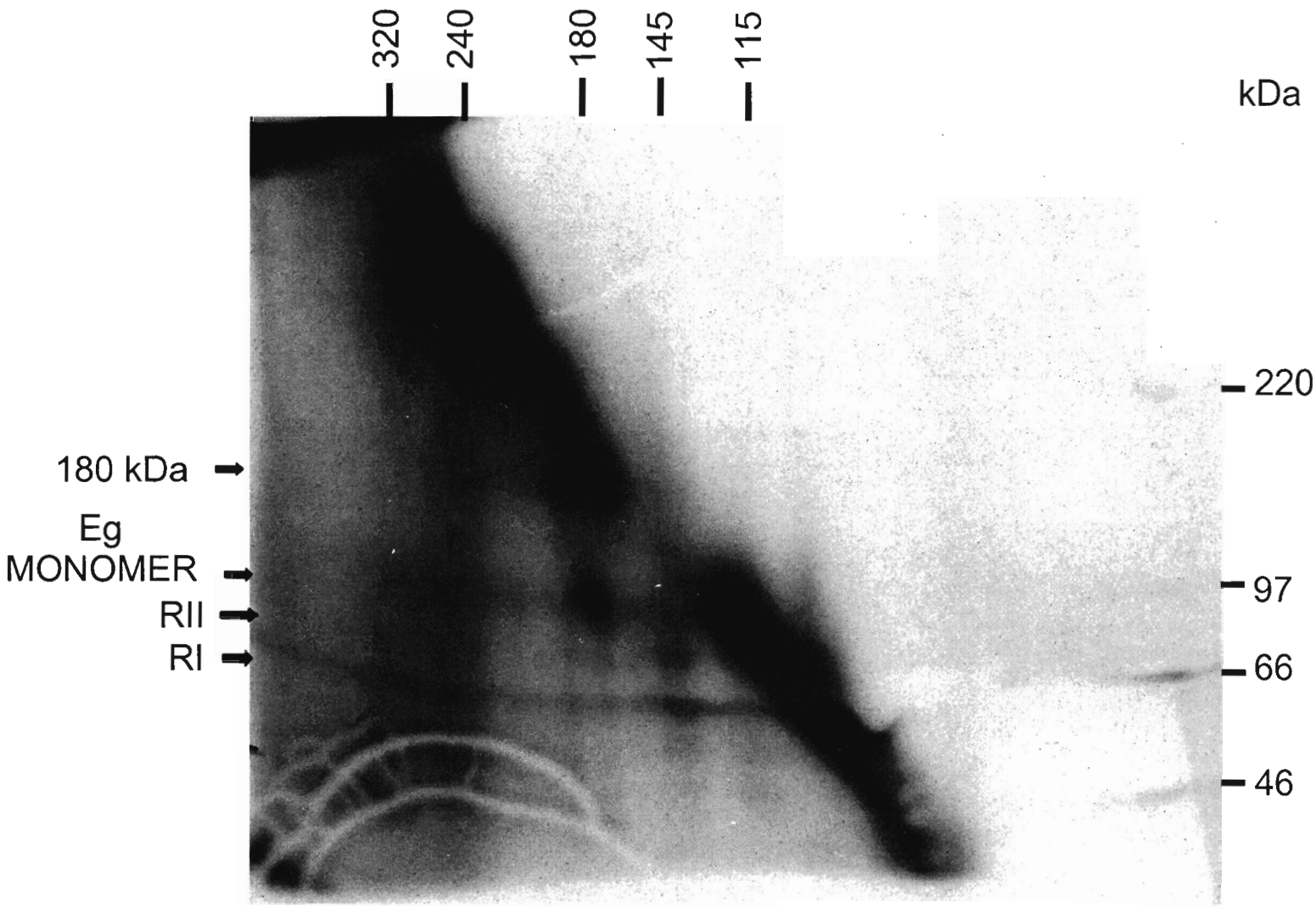


FIGURE 7B

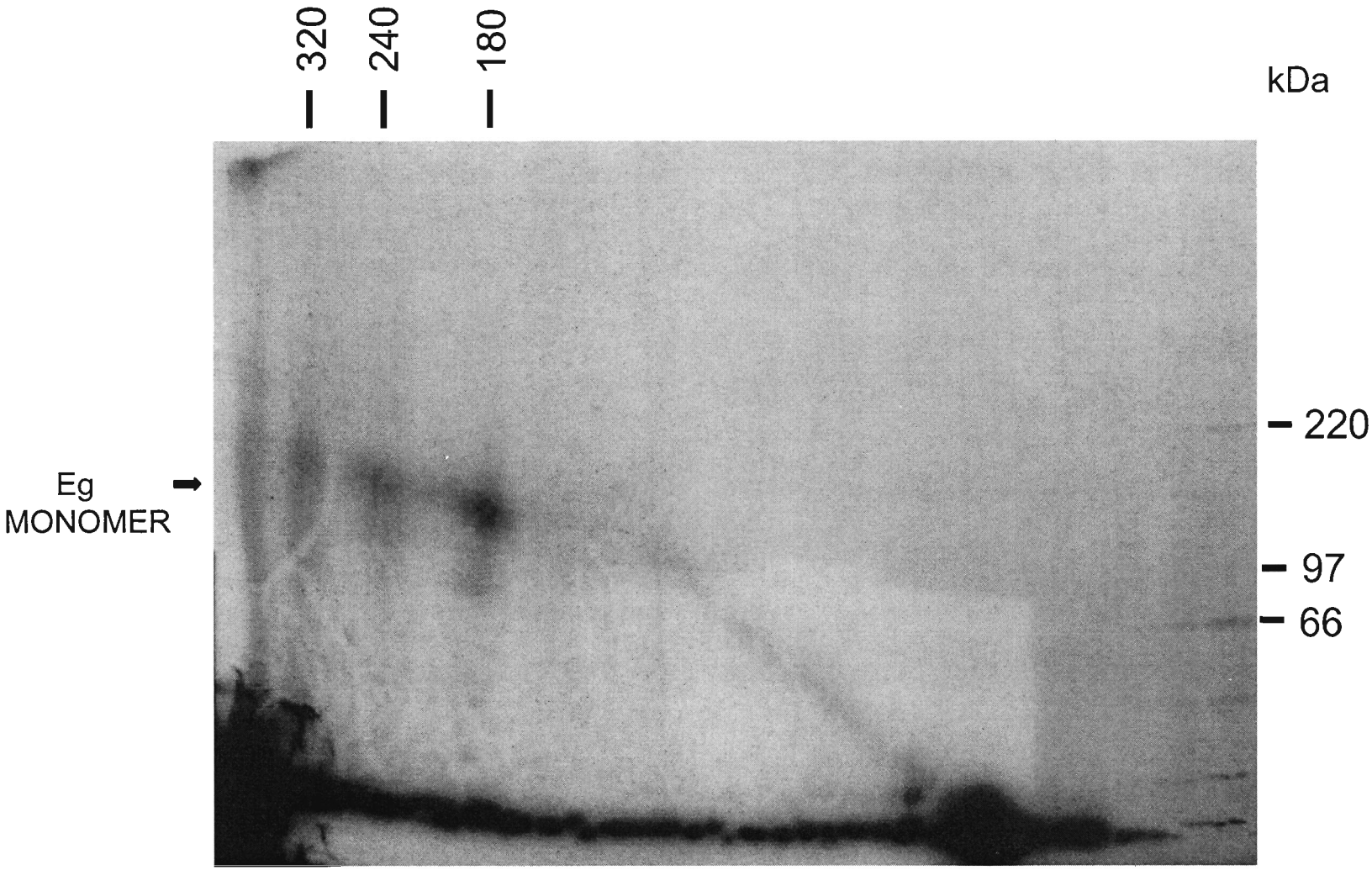
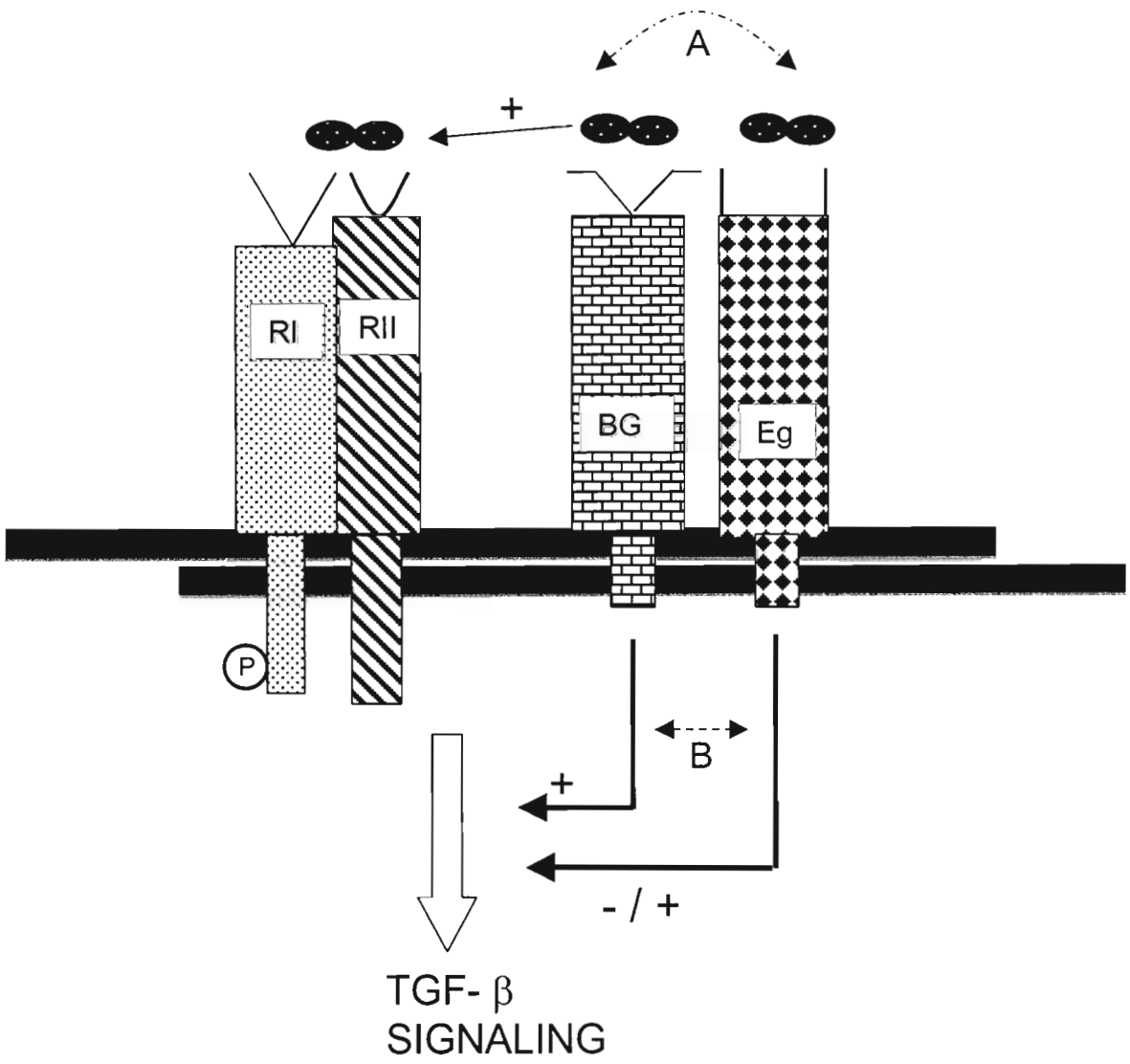
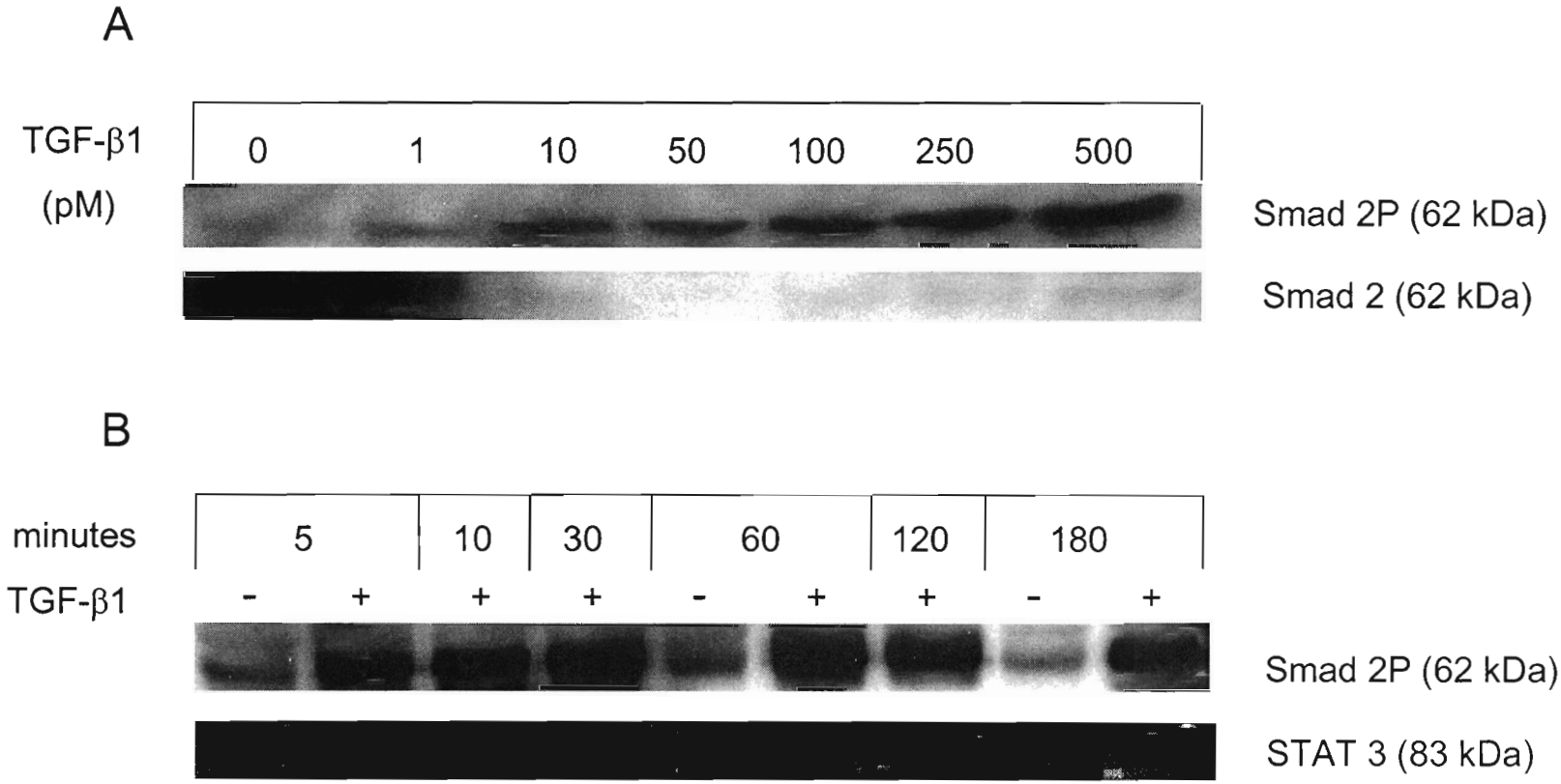


FIGURE 8



ADDITIONAL FIGURES AND LEGENDS

**CHAPTER 1**


A
TGF-β1
(pM)
0
1
10
50
100
250
500
Smad 2P (62 kDa)
Smad 2 (62 kDa)
B
minutes
5
10
30
60
120
180
TGF-β1
- + + + - + + - +
Smad 2P (62 kDa)
STAT 3 (83 kDa)

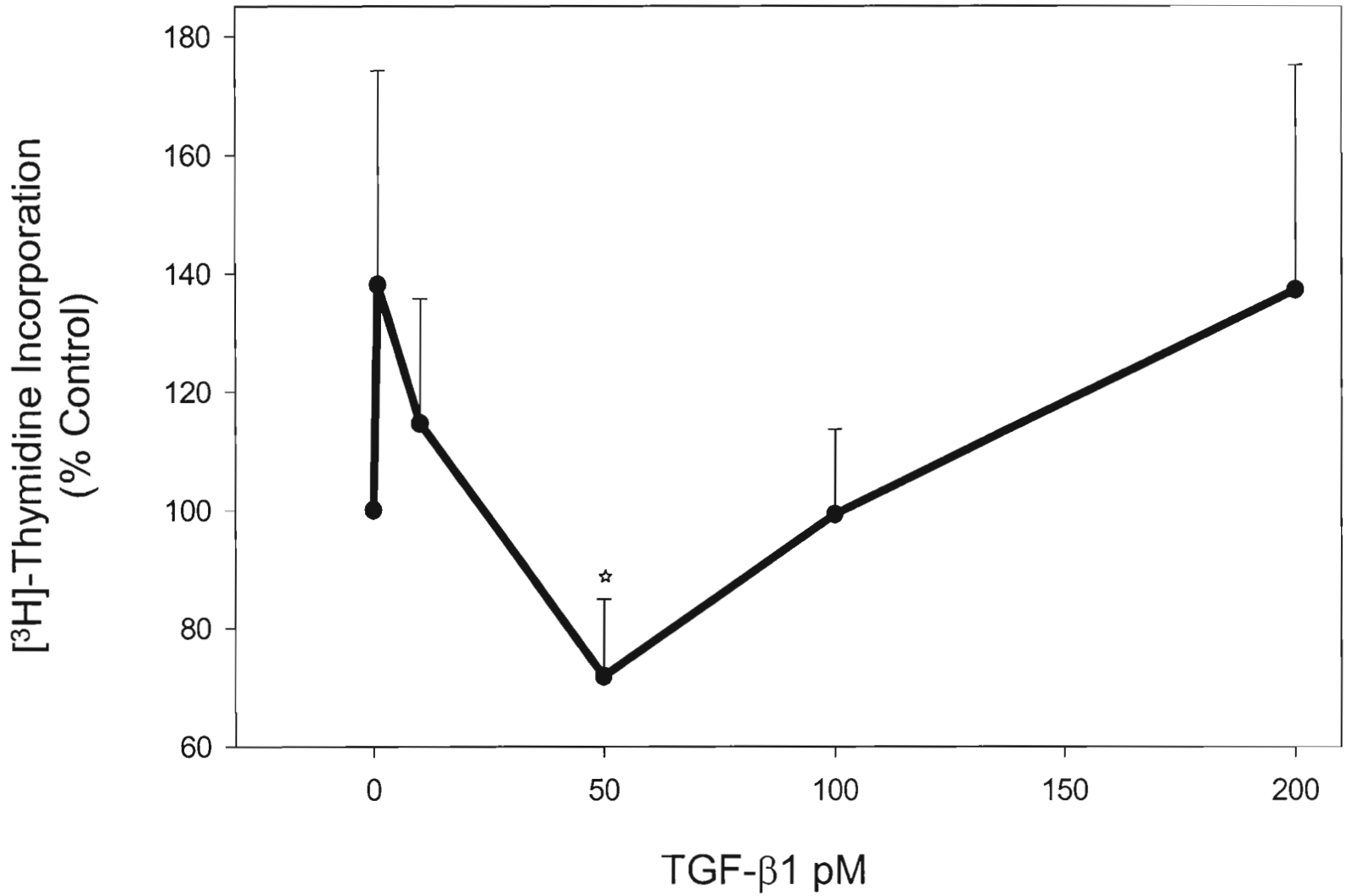

[3H]-Thymidine Incorporation
(% Control)
180
160
140
120
100
80
60
0
50
100
150
200
TGF-β1 pM

Fig. Aiii

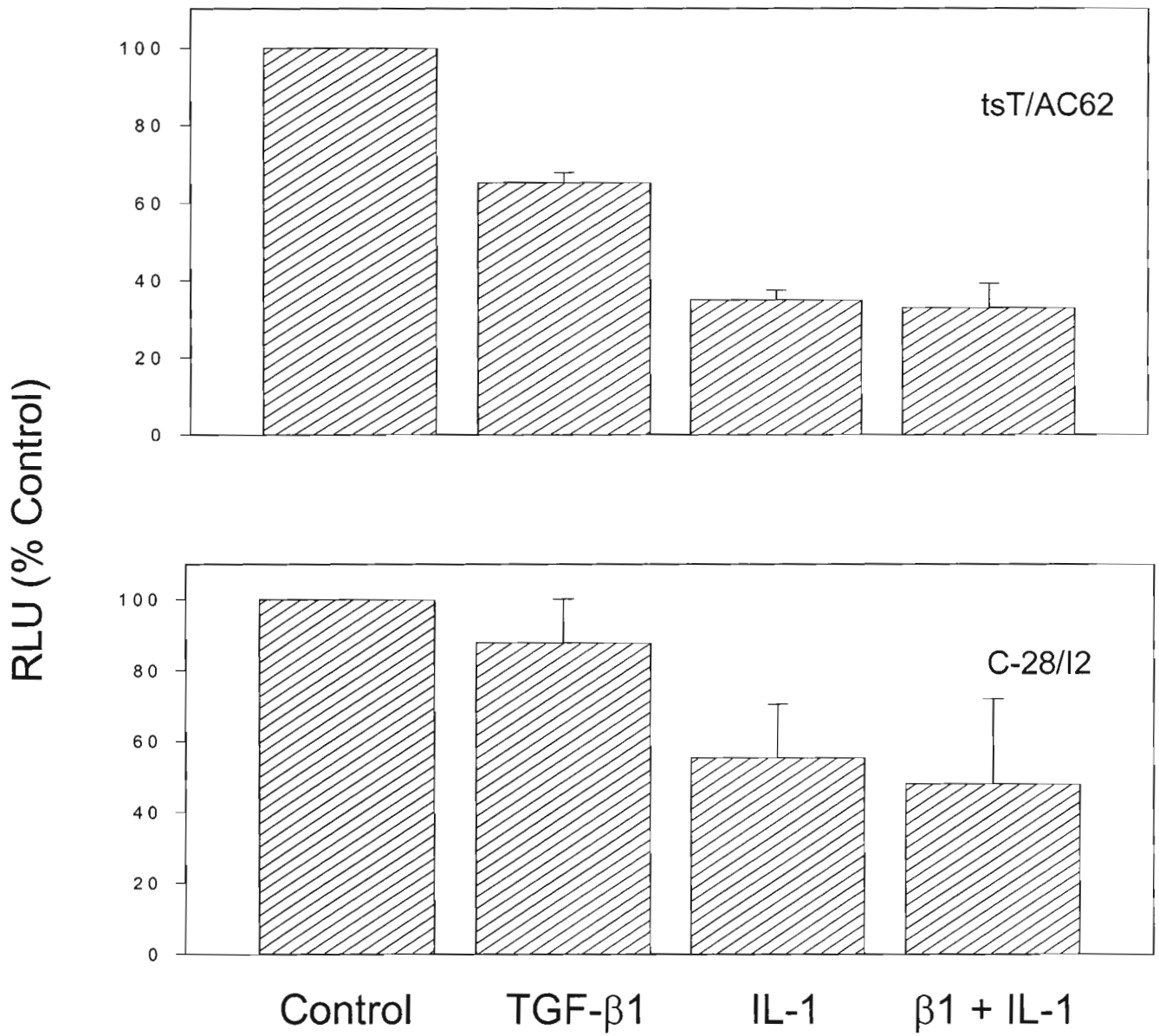

RLU (% Control)
100
80
60
40
20
0
tsT/AC62
C-28/I2
Control
TGF-β1
IL-1
β1 + IL-1

Fig. Aiv

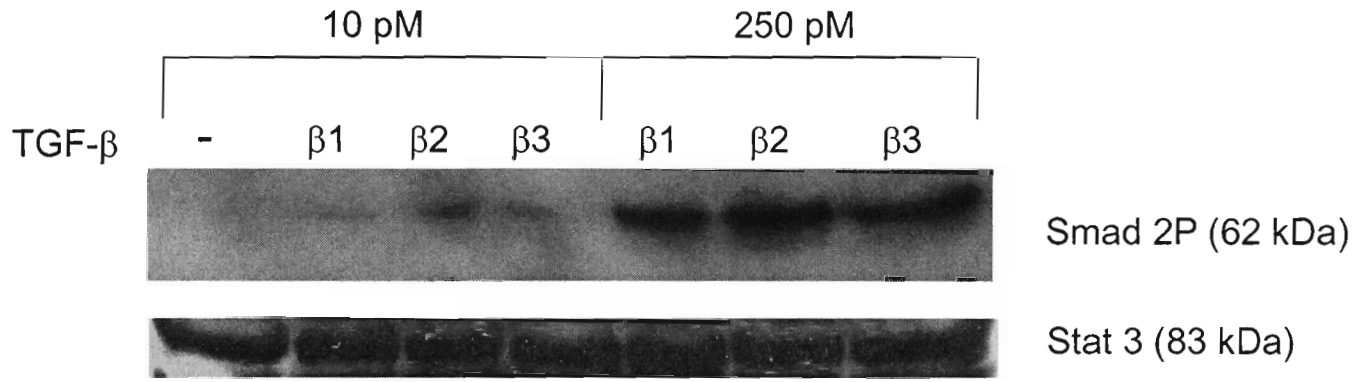


**Fig. A. TGF-β Responses in Human Chondrocytes. i):** Stimulation of Smad 2 phosphorylation by TGF-β1. Solubilized extracts of C-28/I2 cells treated with increasing concentrations of TGF-ß1 (0, 1, 10, 50, 250, 500 pM) for 60 minutes (panel A) or treated with 100 pM of TGF-β1 or left untreated for increasing time increments (5, 10, 30, 60, 120, 180 minutes) (panel B) were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and Western blotting performed with an antibody against the phosphorylated form of Smad 2 (upper panel). The membranes were reprobed with anti-Smad 2 or anti-STAT 3 to illustrate equal protein loading. The ECL system was used for chemiluminescence detection. **ii):** Triphasic growth response demonstrated by [$^3$H]-Thymidine incorporation. Confluent monolayers of tsT/AC-62 cells were seeded at a density of $7.0x10^5$ cells/24 well plate and cultured for 24 hours. The cells were serum starved for 4 hours and incubated with increasing concentrations of TGF-β1 under serum free conditions overnight. [$^3$H]-Thymidine ($1\mu Ci \cdot ml^{-1}$) was added per well for the final 4 hours of TGF-β1 treatment. The cells were solubilized in 1% SDS, and incorporated radioactivity was determined by liquid scintillation counting. The data shown are representative of a minimum of three different experiments each done in triplicates and statistically significant changes from control untreated cells are noted by an asterisk ($p<0.01$). This triphasic response was also seen for C-28/I2 cells (data not shown). **iii):** Effect on Col 2a-promoter activity by TGF-β1. Chondrocytes (tsT/AC-62, upper panel; C-28/I2, lower panel) were transiently transfected with the 1μg of pCol2a and pβ-gal. After 24 hours, cells were serum starved for 4 hours then treated with 100 pM of TGF-ß1, 2ng/ml of IL-$1_\beta$ or a combination of both overnight or were left untreated (control). Luciferase activity was determined, normalized using the β-gal assay and expressed as a

percent control of untreated cells. The data shown are representative of three different experiments each done in triplicates. **iv):** Stimulation of Smad 2 phosphorylation by TGF-β1, -β2, and -β3. Solubilized extracts of C-28/I2 cells treated with 10 and 250 pM of TGF-ß1 for 15 minutes or left untreated (-) were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against the phosphorylated form of Smad 2 (upper panel) and reprobed with anti-STAT 3 antibody to illustrate equal protein loading. The ECL system was used for chemiluminescence detection.

Fig. B

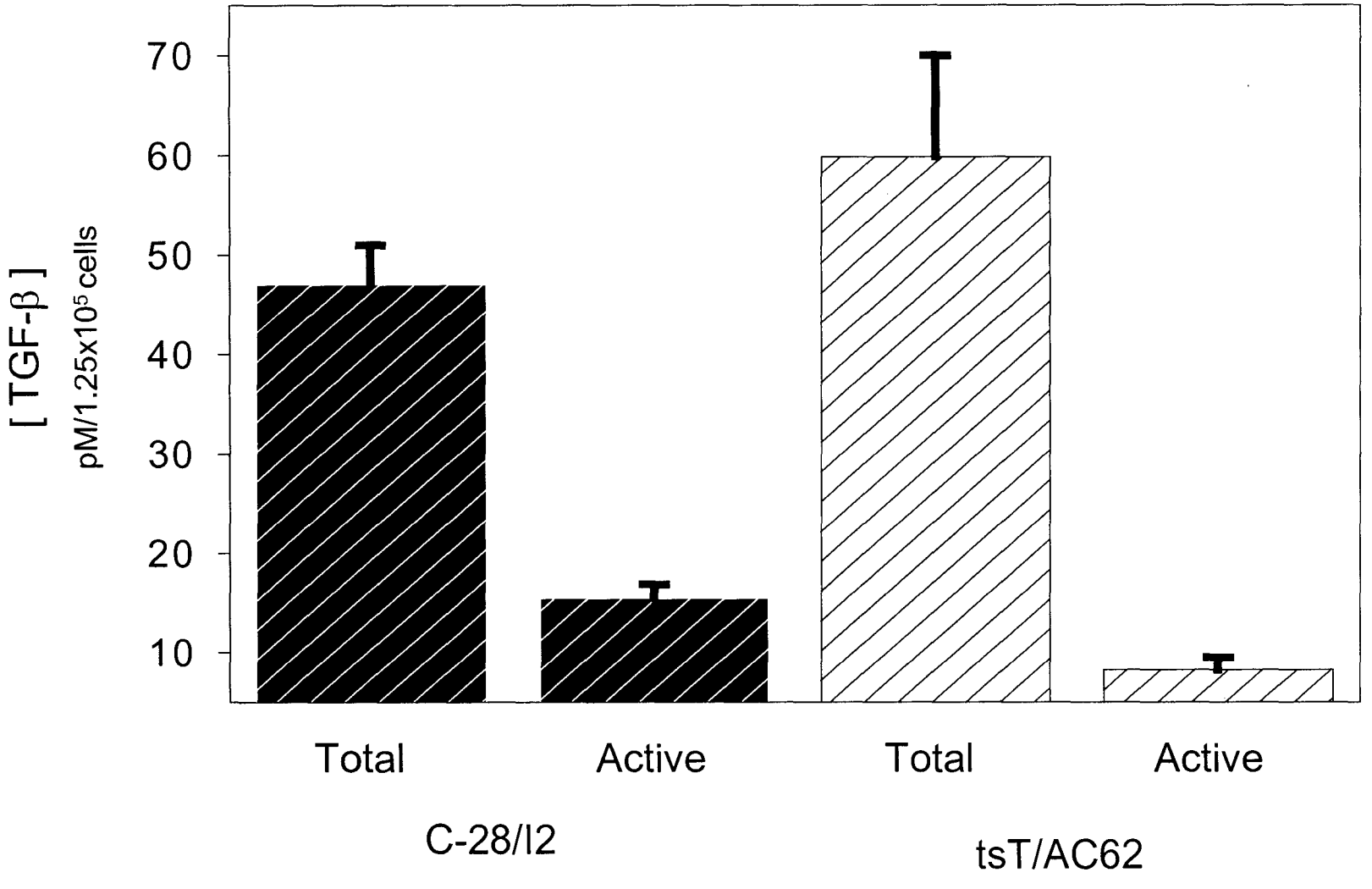

[ TGF-β ]
pM/1.25x10^5 cells
70
60
50
40
30
20
10
Total
Active
Total
Active
C-28/I2
tsT/AC62

**Fig. B. Human chondrocytes produce TGF-β.** C-28/I2 and tsT/AC-62 cells were plated in 24 well tissue culture plates each at a density of 2.5x $10^5$ cells for 2 wells. Cells were serum starved; medium was collected after 24 hours and kept at 37°C (active) or heated to 70°C (total) to activate the TGF-β. Samples and standard concentrations of TGF-β1 were prepared in .1%BSA-DMEM and applied to TMLC cells, luciferase activity measured and TGF-β concentration determined from the standard curve and depicted as pM concentration as described in Experimental Procedures.

Fig. C

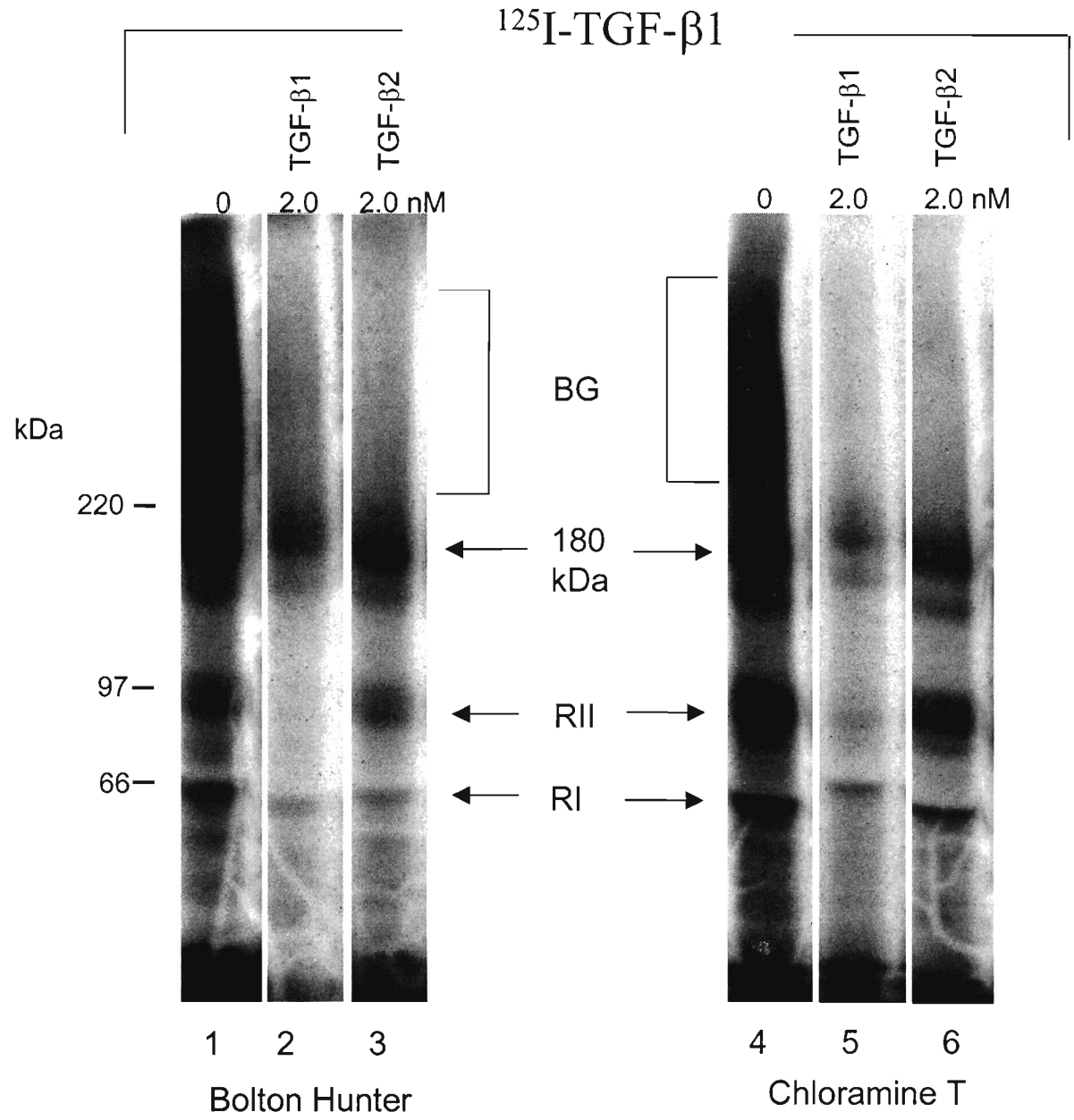
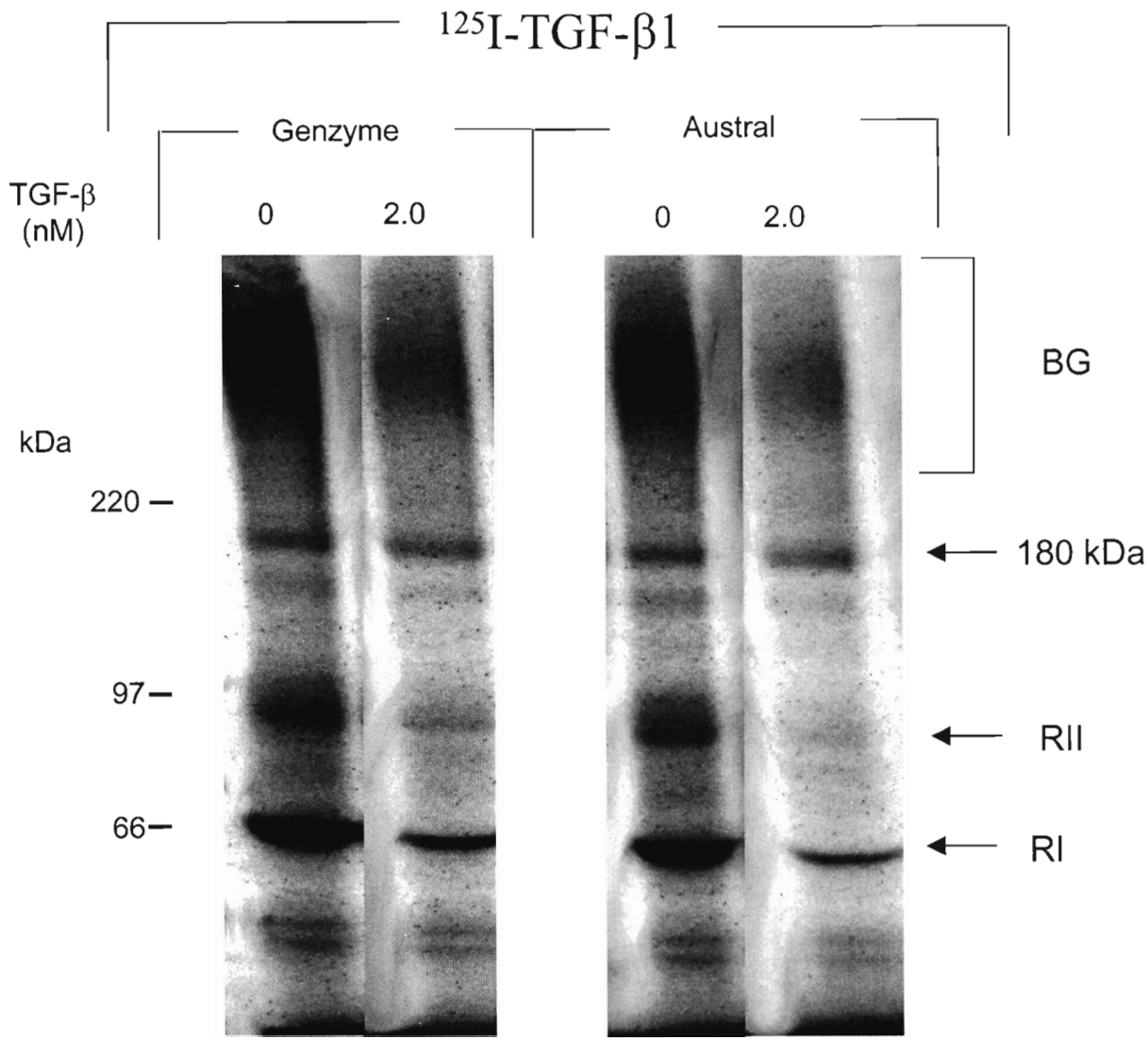

125I-TGF-β1
TGF-β1
TGF-β2
0
2.0
2.0 nM
TGF-β1
TGF-β2
0
2.0
2.0 nM
kDa
220
97
66
BG
180
kDa
RII
RI
1
2
3
4
5
6
Bolton Hunter
Chloramine T

**Fig. C. Affinity labeling of human chondrocytes with $^{125}$I-TGF-β1 prepared using the Chloramine T versus Bolton Hunter method of iodination analyzed.** Confluent monolayers of cells were affinity labeled with 100 pM of $^{125}$I-TGF-β1 prepared by the Bolton Hunter (Lanes 1-3) or Chloramine T (Lanes 4-6) method in the absence or presence of 2.0 nM of unlabeled TGF-β1 or -β2. Solubilized cell extracts were analyzed by SDS-PAGE (3-11% gradient) under reducing conditions followed by autoradiography.

Fig. D


125I-TGF-β1
Genzyme
Austral
TGF-β (nM)
0
2.0
0
2.0
kDa
220
97
66
BG
180 kDa
RII
RI

151

**Fig. D. Competition profile of $^{125}$I-TGF-β1 affinity labeled chondrocytes with unlabeled TGF-β1 (Genzyme versus Austral).** Confluent monolayers of tsT/AC-62 cells were affinity labeled with 100 pM of $^{125}$I-TGF-β1 in the absence or presence of the indicated concentrations of unlabeled Genzyme and Austral TGF-β1. Solubilized cell extracts were analyzed by SDS-PAGE (3-11% gradient) under reducing conditions followed by autoradiography.

Fig. E

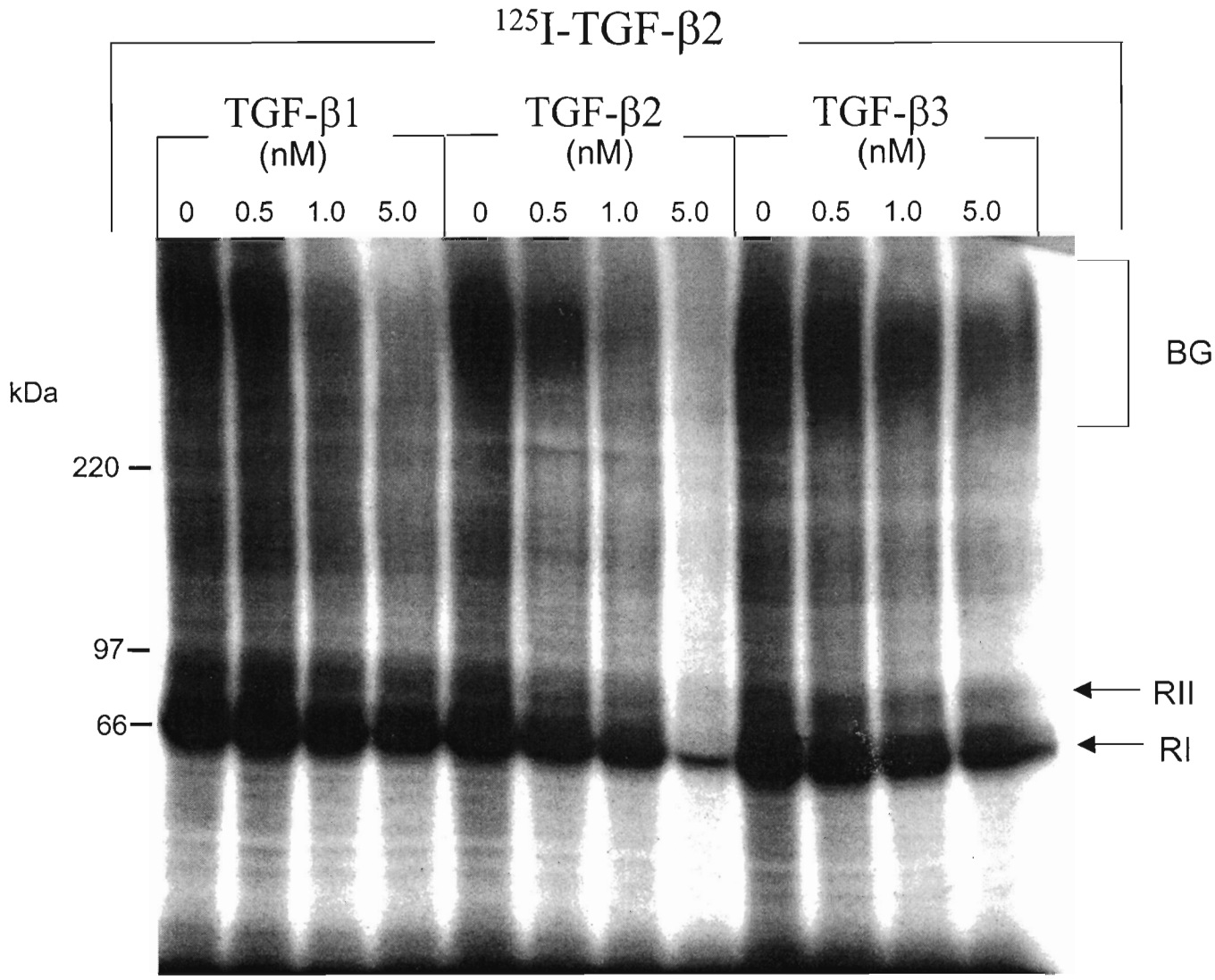

125I-TGF-β2
TGF-β1 (nM)
TGF-β2 (nM)
TGF-β3 (nM)
0 0.5 1.0 5.0 0 0.5 1.0 5.0 0 0.5 1.0 5.0
kDa
220
97
66
BG
RII
RI

**Fig. E. Affinity labeling with $^{125}$I-TGF-β2.** Confluent monolayers of tsT/AC-62 cells were affinity labeled with 100 pM of $^{125}$I-TGF-β1 in the absence or presence of the indicated concentrations of unlabeled TGF-β1, -β2, or -β3. Solubilized cell extracts were analyzed by SDS-PAGE (3-11% gradient) under reducing conditions followed by autoradiography. Similar receptor profiles were observed using primary chondrocytes and the C-28/I2 cell line (data not shown).

Fig. F

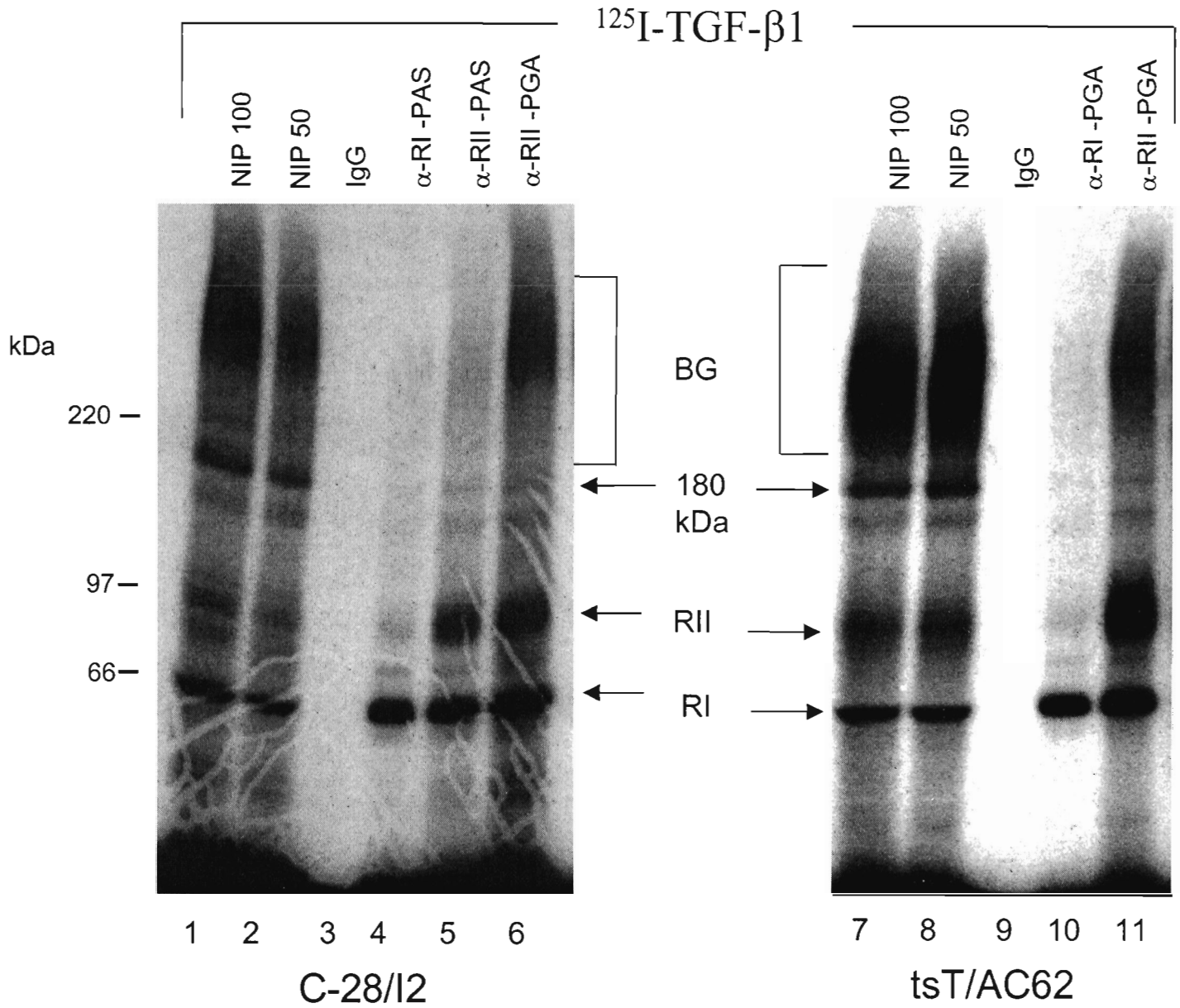

125I-TGF-β1
NIP 100
NIP 50
IgG
α-RI -PAS
α-RII -PAS
α-RII -PGA
NIP 100
NIP 50
IgG
α-RI -PGA
α-RII -PGA
kDa
220
97
66
BG
180
kDa
RII
RI
1 2 3 4 5 6
7 8 9 10 11
C-28/I2
tsT/AC62

**Fig. F. Immunoprecipitation of TGF-β signaling receptors on human chondrocytes.** Cells were affinity labeled with 200 pM of $^{125}$I-TGF-β1 and solubilized cell extracts were immunoprecipitated with 3μg/ml each of anti-receptor antibodies or control IgG. Complexes were fractionated on SDS-PAGE (3-11% gradient) under reducing conditions and visualized by autoradiography. 50μg and 100μg of protein of non-immunoprecipitated (NIP) cell extract of C28/I2 and tsT/AC-62 cells is shown in Lanes 1, 2 and Lanes 7, 8 respectively. Immunoprecipitations were performed using anti-RI with PAS (Lane 4, α-RI-PAS), anti-RI with PGA (Lane 10, α-RI-PGA), anti- RII with PAS (Lane 5, α-RII-PAS), or anti-RII with PGA (Lanes 6 and 11, α-RII-PGA) antibody or control IgG (Lane 3, 9).

Fig. Gi

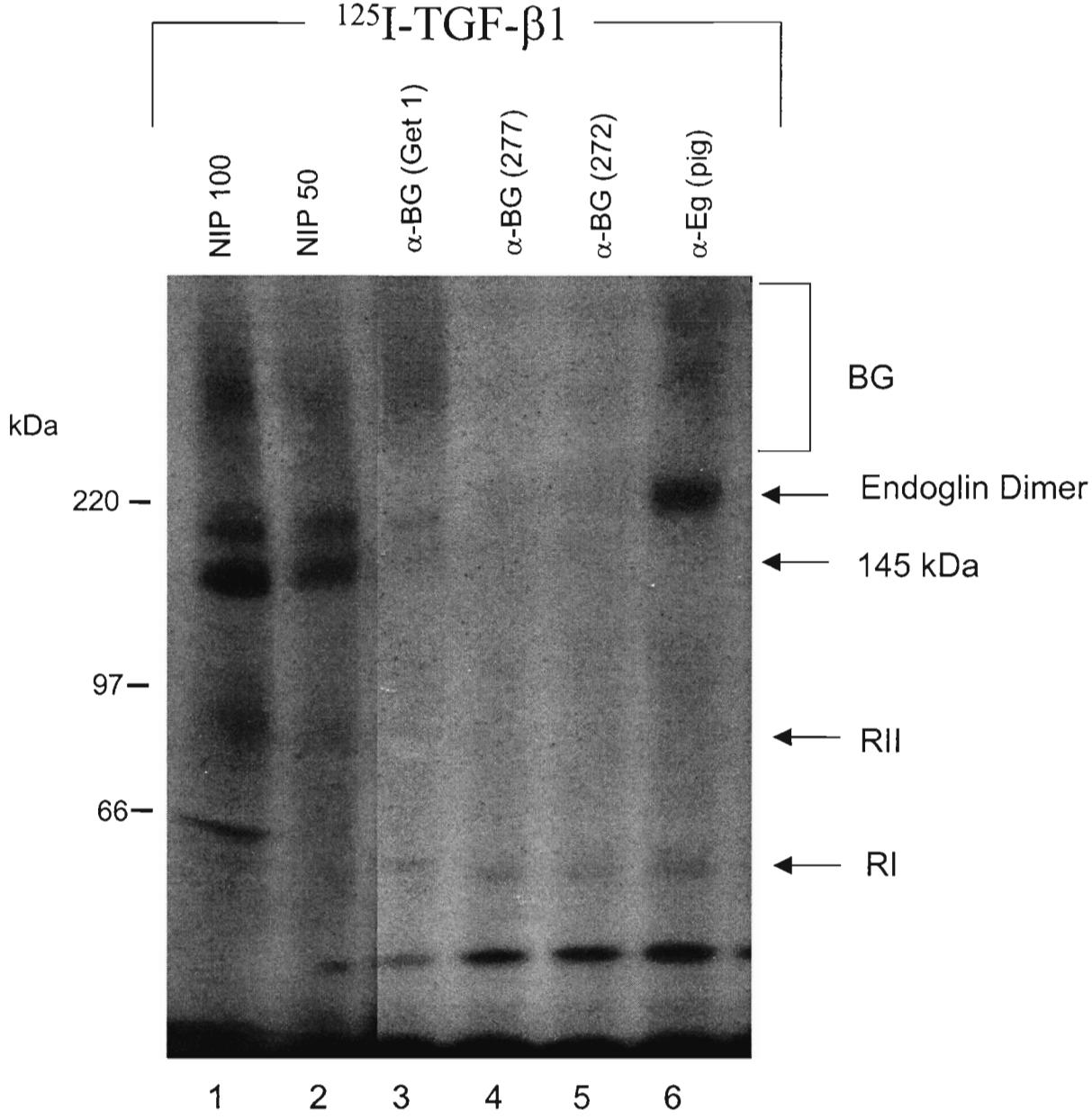

125I-TGF-β1
NIP 100
NIP 50
α-BG (Get 1)
α-BG (277)
α-BG (272)
α-Eg (pig)
kDa
220
97
66
BG
Endoglin Dimer
145 kDa
RII
RI
1
2
3
4
5
6

Fig. Gii

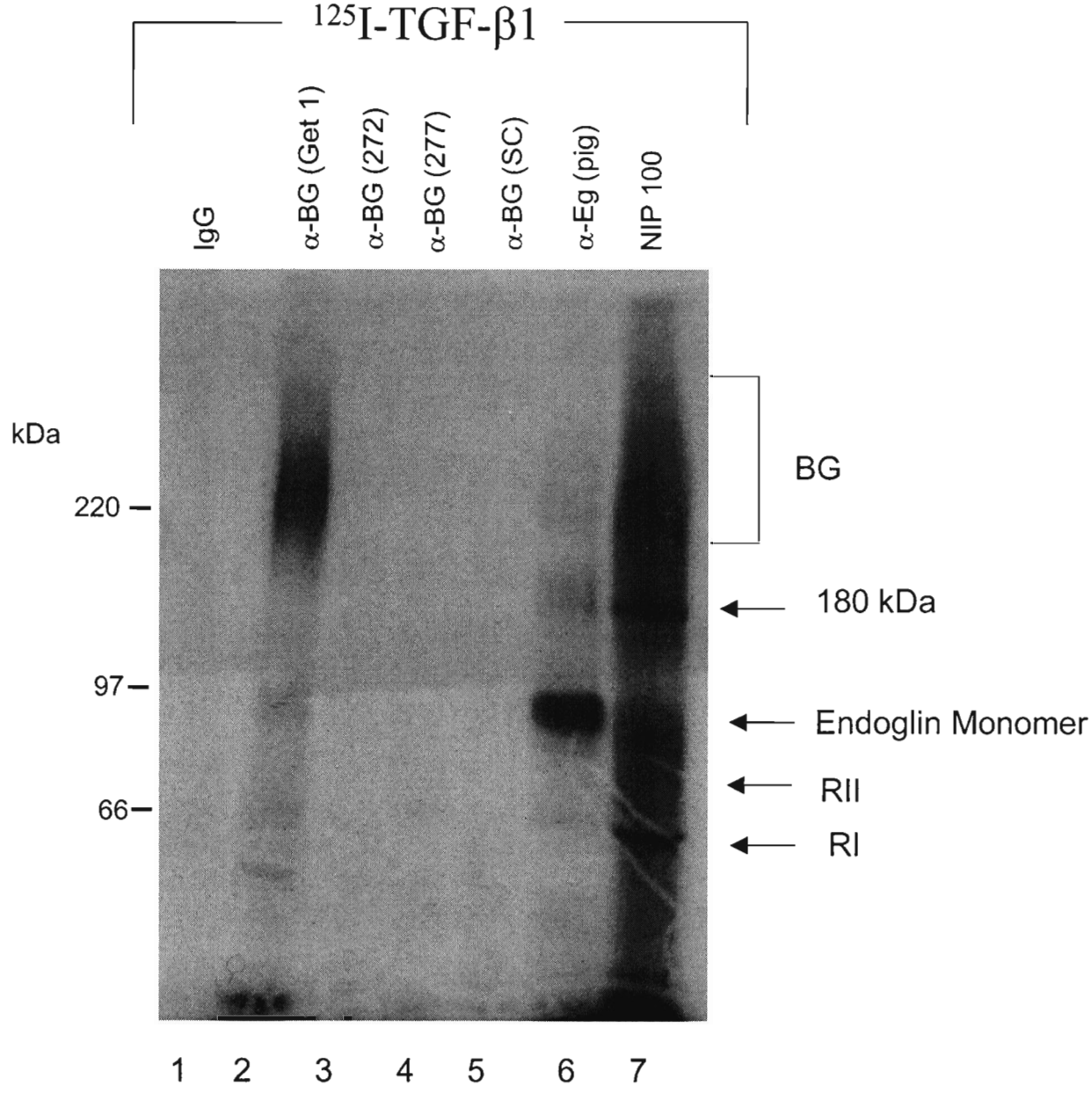

$^{125}$I-TGF-β1
IgG
α-BG (Get 1)
α-BG (272)
α-BG (277)
α-BG (SC)
α-Eg (pig)
NIP 100
kDa
220
97
66
BG
180 kDa
Endoglin Monomer
RII
RI
1 2 3 4 5 6 7

**Fig. G. Immunoprecipitation of betaglycan: antibody efficacy for endoglin co-immunoprecipitation.** Cells were affinity labeled with 200 pM of $^{125}$I-TGF-β1 and solubilized cell extracts were immunoprecipitated with 3μg/ml each of anti-receptor antibodies or control IgG. Complexes were fractionated on SDS-PAGE (3-11% gradient gels) under nonreducing **(i)** or reducing **(ii)** conditions and visualized by autoradiography. **i):** Non-immunoprecipitated (NIP) cell extract of T/AC-62 is shown in Lanes 1 and 2. Immunoprecipitations were performed using anti-betaglycan antibodies (Get 1, Lane 3; 277, Lane 4; 272, Lane 5) or anti-endoglin antibody (α-Eg, pig, Lane 5). **ii):** Non-immunoprecipitated (NIP) cell extract of C-28/I2 is shown in Lane 7. Immunoprecipitations were performed using anti-betaglycan antibodies (Get 1, Lane 2; 272, Lane 3; 277, Lane 4; Santa Cruz, Lane 5), anti-endoglin antibody (α-Eg, pig, Lane 6) or control IgG (Lane 1).

# CHAPTER 2

# BRIDGE

Identification of Activin Receptor-like kinase-1 (Alk-1) and Soluble type I receptor (Sol RI) on human chondrocytes. The novel interaction of these receptors with the TGF-β signaling receptors and additional accessory receptors and their modulation of TGF-β response.

## CHAPTER 2

BRIDGING DOCUMENT

**Introduction and Rationale**

Identification of endoglin and its biochemical interaction with betaglycan and the TGF-β signaling receptors confirmed that novel accessory receptors exist on human chondrocytes and form heteromeric complexes on the cell surface. In other cells, a soluble variant of RI (Sol RI) has been identified and shown to regulate TGF-β signaling (Choi 1999). Previous investigations identified an additional band at 47 kDa by affinity labeling techniques which showed affinity for TGF-β1 and -β3 isoforms (manuscript 1, Fig. D) but less affinity for TGF-β2. The novel Sol RI cloned was found to have a molecular weight of 47 kDa; thus, it was hypothesized that the novel 47 kDa band may indicate that a soluble form of RI (Sol RI) also exists on chondrocytes.

Alk-1, an orphan receptor, one of the eight cloned mammalian type I TGF-β receptors, has been previously described. This receptor has been noted to co-exist with Alk-5 (RI) on the surface of endothelial cells (Goumans et al 2002) and has been shown to form heteromeric complexes with RI and RII (Attisano et al 1993). Alk-1 mutations in humans are implicated in HHT II and homozygote embryos die at midgestation with severe vascular abnormalities. Endoglin gene mutations in humans result in HHT I with a remarkably similar phenotype. This, taken together with the finding of endoglin on human chondrocytes, raised the question of the presence of Alk-1 on these cells.

In addition, it is becoming increasingly accepted that accessory TGF-β receptors play an important regulatory role in TGF-β signaling and action in many cells. Whether novel TGF-β

receptors identified on human chondrocytes could act as regulators of TGF-β signaling was also considered.

**Hypotheses**

(1) that novel cell surface TGF-β binding proteins, namely Sol RI and Alk-1, are expressed on human chondrocytes

(2) that these novel receptors form heteromeric complexes on the chondrocyte surface

(3) that Sol RI and Alk-1 modulate TGF-β signaling in these cells

**Objectives**

(i) to identify Sol RI and Alk-1 and their heteromeric complex formations with TGF-β signaling and other accessory receptors on human chondrocytes

(ii) to determine the role of Sol RI and Alk-1 in modulating TGF-β signaling in these cells

**Summary of Manuscript Findings**

In the second manuscript, the novel expression of Sol RI and Alk-1 on human chondrocytes was reported. For the first time, in addition to the documented formation of heteromeric complexes with the type I and II TGF-β signaling receptors (Docagne et al 2001, Choi 1999, Lux et al 1999, Oh et al 2000), the association of both Alk-1 and Sol RI with betaglycan at the cell surface, at endogenous receptor concentrations and ratios, is demonstrated. Also, evidence is provided that Alk-1 and Sol RI form a heteromeric complex and when overexpressed both enhance TGF-β signaling. This supports the theory that expression of novel TGF-β receptors and their formation of heteromeric receptor complexes at the cell surface may be critical in specifying the relative contributions of the various signaling

pathways, and thus may play a central role in regulating the diverse action of TGF-β in chondrocytes.

**Conclusions**

Therefore, with the results demonstrated above (manuscript 2) the expression profile of TGF-β receptors on human chondrocytes has been expanded to include Alk-1 and Sol RI. Evidence has been provided that, in addition to endoglin and betaglycan, both Alk-1 and Sol RI form heteromeric complexes with the signaling receptors (RI and RII) and with betaglycan on the chondrocyte surface and that for Sol RI this occurs independently of betaglycan's GAG chains. By demonstrating that Sol RI and Alk-1 overexpression results in an enhancement of TGF-β signaling, the importance of accessory receptors and their complex formations in these cells is illustrated. With these findings, a theory of a variable hetero-oligomeric TGF-β receptor complex in human chondrocytes in which the relative expression level of the individual receptor components may determine the outcome of TGF-β signaling is being constructed. Thus, receptor associations and levels of expression may be critical for achieving a fine balance between the positive and the negative regulation of TGF-β signaling.

**CONTRIBUTION OF AUTHORS**

# MANUSCRIPT TWO

Identification of Activin Receptor-like Kinase-1 and Soluble type I TGF-β receptor on human chondrocytes: hetero-oligomerization and regulation of TGF-β signaling

**WL Parker:** experimental designs, procedures, and analysis, preparation of figures and text

**ME Choi:** contribution of anti-soluble type I antibody and the expression plasmid for Sol RI

**A Philip:** assistance with experimental design and analysis and manuscript editorial assistance

# Identification of Activin Receptor-like Kinase-1 and Soluble type I TGF-β receptor on human chondrocytes: hetero-oligomerization and regulation of TGF-β signaling

Wendy L. Parker[1], Mary E. Choi[2] and Anie Philip[1]*.

[1]Division of Plastic Surgery, Department of Surgery, McGill University, Montreal, Quebec, Canada

[2] Section of Nephrology and Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut, USA

*Correspondence to: Anie Philip, Division of Plastic Surgery, Montreal General Hospital, 1650 Cedar Avenue, Room C9-177, Montreal, Quebec, Canada H3G 1A4.

Telephone: (514) 937 1934 ext 44533; Fax: (514) 934 8289

E-mail: anie.philip@mcgill.ca

Running Title: Alk-1 and Sol RI enhance TGF-β signaling

Acknowledgements

We thank Genzyme Inc. (Framingham, MA) for the gift of TGF-β1 and TGF-β2; Dr. M. O'Connor McCourt (Montreal, Quebec) for the gift of plasmid encoding the type I TGF-β receptor; Dr. L. Attisano (Toronto, Ontario) for the Alk-1-HA tagged plasmid; Dr. Bert Vogelstein (Baltimore, ML) for the SBE 4 expression vector; Dr. S. Souchelnytskyi (Uppsala, Sweden) for the anti-betaglycan (Get 1) and anti-phospho Smad 2 antibodies; Dr. M. Goldring (Boston, MA) for the immortalized human chondrocyte lines (C-28/I2 and tsT/AC62); and Dr. J. Massague (New York, NY ) for the R1B-L17 cells.

Summary

Degenerative joint disease is evident in two murine models of TGF-β dysregulation. Despite this and the implication of TGF-β as a key mediator of cartilage repair, its action in human chondrocytes is poorly understood. The TGF-β signal is transduced by a pair of transmembrane serine/threonine kinases, the type I receptor (RI) and the type II receptor (RII). It is now acknowledged that accessory TGF-β binding proteins are able to modulate this signaling through complex formations with RI and RII. We have recently described two such accessory receptors, endoglin and betaglycan, in human chondrocytes and their heteromeric complex formation in these cells. In the present study we document the novel expression of the soluble type I receptor (Sol RI) and Activin Receptor-like kinase-1 receptor (Alk-1) on human chondrocytes and their heteromeric complex formation with both the TGF-β signaling receptors and betaglycan. We demonstrate that Alk-1 and Sol RI overexpression leads to enhancement of TGF-β responses in these cells. The expression of these receptors and their interactions at the cell surface support the theory of a hetero-oligomeric receptor complex in which modulation of individual components may specify alternate TGF-β signaling pathways and action in chondrocytes.

**Key Words**: chondrocytes, TGF-β receptors, TGF-β signaling, Alk-1, Soluble type I TGF-β receptor, cartilage

## INTRODUCTION

Injury to articular cartilage resulting in degenerative joint disease affects the majority of the aging population. Yet the potential for restoring diseased or injured cartilage, despite generating much research interest, has remained elusive. Previous work has implicated transforming growth factor-β (TGF-β)[1] as an essential mediator of cartilage repair, and TGF-β signaling as a requirement for the maintenance of articular cartilage (1-6). However, the mechanisms regulating TGF-β action in chondrocytes are poorly understood. In fact, knowledge of the expression of TGF-β receptors in human cartilage or chondrocytes has been very limited (7-9).

TGF-β is a member of a large family of multifunctional proteins intricately involved in growth, differentiation, and development (10-13) and was described initially as "cartilage inducing factor" (14). Three distinct isoforms of TGF-β (TGF-β 1, 2, and 3) which are encoded by distinct genes have been described in mammals (10,15,16). TGF-β is secreted in a latent form that requires activation before it can bind to its receptors. The TGF-β signal is transduced by a pair of transmembrane serine/threonine kinases, known as the types I (RI) and II (RII) receptors which are present on almost all cell types (17-20). The type I receptor does not bind TGF-β in the absence of the type II receptor. The binding of TGF-β to the type II receptor, a constitutively active kinase, results in the

---

[1] The abbreviations used are: TGF-β, transforming growth factor beta; PAI – 1, plasminogen activator inhibitor-1; RI, TGF-β type I receptor; RII, TGF-β type II receptor; Alk-1, Activin Receptor-like Kinase-1; Sol RI, soluble type I TGF-β receptor; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum; DMEM, Dulbecco's minimal essential medium; dPBS, Dulbecco's phosphate buffered saline; BSA, bovine serum albumin; β-gal, beta-galactosidase; GPI, glycosyl phosphatidylinositol; SBE, Smad binding element; HHT, hereditary hemorrhagic telangiectasia.

recruitment, phosphorylation and concomitant activation of the type I receptor. The activated type I receptor in turn transmits the signal via downstream mediators such as Smads, resulting in the regulation of target gene expression. Accessory TGF-β receptors are being increasingly recognized and are thought to play an important role in local regulation of TGF-β action by forming heteromeric complexes with the signaling receptors (21-23). These receptors include glycosyl phosphatidylinositol (GPI)-anchored proteins (24-27), the type III TGF-β receptor (betaglycan) which binds all three TGF-β isoforms (28,29) and endoglin which binds the β1 and β3 but not β2 isoforms (22,30-31). We have recently identified both endoglin and betaglycan on human chondrocytes. In addition, we have shown that these novel receptors form a complex in the presence of ligand and ligand independent manner and a type II receptor (RII) independent manner (9).

It is also apparent that recombinant forms of TGF-β receptors overexpressed at the cell surface may function to modulate TGF-β responsiveness (32-34). We identified a unique cDNA clone encoding a soluble form (Sol RI) of Alk-5 (TGF-β type I receptor, RI) from a neonatal rat kidney cDNA library. This soluble receptor (Sol RI) was demonstrated to occur naturally and bind TGF-β1 in the presence of RII. We have shown this to be a functional receptor which appears to act either as an agonist to TGF-β action or as a ligand chaperone (35). Others constructed a recombinant form of this receptor from the extracellular domain of RI. This recombinant Sol RI required the presence of both RI and RII signaling receptors to bind TGF-β1 and to generate a signal. In the absence of TGF-β this receptor mimicked TGF-β induced transcriptional and growth responses (34).

Currently, eight mammalian type I TGF-β receptors have been cloned and for the majority the ligand and corresponding type II receptor identified (reviewed in 36). Alk-1 however remains an orphan receptor. Its true ligand and corresponding type II receptor are unknown. Yet, it is known that Alk-1 binds TGF-β1 in the presence of RII and Activin A in the presence of ActR-II (37-39). In addition, Alk-1 has been demonstrated to bind TGF-β3 and an unknown ligand present in human serum (36). Alk-1 gene mutations in humans are implicated in HHT II and homozygote embryos die at midgestation with severe vascular abnormalities (40). Importantly, during angiogenesis, Alk-1 has been shown to modulate TGF-β signaling (41) and during this process signals through Smads with an end point which differs with respect to Alk-5 in mice (42). The apparent potential of Alk-1 and Sol RI to regulate TGF-β action and the recent identification of endoglin an accessory receptor on human chondrocytes led us to investigate whether these TGF-β receptors were present on human cartilage and to determine if they could modulate TGF-β response in these cells.

In the present study we report for the first time the expression of Sol RI and Alk-1 on human chondrocytes. More importantly, we demonstrate that in addition to the documented formation of heteromeric complexes with the type I and II TGF-β signaling receptors (34-36,41) both Alk-1 and Sol RI associate with betaglycan at the cell surface at endogenous receptor concentrations and ratios. In addition, we have provided evidence that Alk-1 associates with Sol RI and that when overexpressed, both Alk-1 and Sol RI enhance TGF-β signaling.

## EXPERIMENTAL PROCEDURES

### Cell Culture

The immortalized human chondrocyte cell lines (C-28/I2 and tsT/AC62), a gift from Dr. M. Goldring (Beth Israel Deaconness Medical Center, Harvard Institutes of Medicine, Boston, MA) have been described previously (43,44). The C-28/I2 cell line was developed using chondrocytes isolated from juvenile human costal cartilage by retroviral infection with SV-40 large T antigen. The tsT/AC62 cells were developed using chondrocytes isolated from adult human articular cartilage by immortalization with a retrovirus expressing a temperature-sensitive mutant of SV-40 large T antigen. This mutant is functional when the cells are cultured at 32°C but not at 37°C (43). The 293 cells (CRL-1573) were obtained from the American Type Culture Collection (Rockville, MD) and the R1B-L17 cells were a gift from Dr. J. Massague (Howard Hughes Medical Institute; New York, NY; 45). Chondrocytes and 293 cells were grown in DMEM/Ham's F12 (1:1, v:v) containing 10% fetal bovine serum (FBS) and 100U/ml penicillin, 100μg/ml streptomycin and 0.25μg/ml amphoteracin (all from Invitrogen Life Technologies, Burlington, ON) and maintained at 37°C (C-28/I2, 293) or 32°C (tsT/AC62) in an atmosphere of 5% CO2/air. R1B-L17 cells were cultured in MEM, non-essential amino acids, 10% FBS (dialyzed), Histidinol .5mM, 1x glutamine, and 100U/ml penicillin, 100μg/ml streptomycin and 0.25μg/ml amphoteracin (all from Invitrogen Life Technologies) and maintained at 37°C.

**Affinity Labeling of Cells**

Affinity labeling was performed as described previously with modifications (46). Briefly, monolayers of cells were washed with ice-cold binding buffer [Dulbecco's PBS (dPBS) with $Ca^{++}$ and $Mg^{++}$, pH 7.4 containing 0.1% bovine serum albumin (BSA)] and were incubated with 100 pM of $^{125}$I-TGF-β1 in the absence or presence of varying

concentrations of non-radioactive TGF-β1, -β2, or -β3. The receptor ligand complexes were cross-linked with Bis-sulfocsuccinimidyl suberate (BS3; Pierce; Rockford, Il.). The reaction was stopped by the addition of glycine and the cell membrane extracts were prepared. The solubilized samples were separated by SDS-PAGE on a 3-11% polyacrylamide gradient gel under reducing (with β-Mercaptoethanol; Sigma Aldrich, Oakville, ON) conditions and analyzed by autoradiography.

**Immunoprecipitation of TGF-ß Receptors**

The anti-betaglycan antibody (Get 1) was a gift from Dr. S. Souchelnytskyi (Uppsala, Sweden; 47). The anti-Alk-1 antibody, anti-RI, and IgG control were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The Sol RI antibody was raised against the divergent COOH-terminal region of the extracellular domain of Sol RI (Sma I) (35) to specifically detect Sol RI. The Immunoprecipitation studies were performed as described previously (25,46) with modifications. Cells were affinity labeled with 200 pM $^{125}$I-TGF-β1 and the membrane extracts were incubated with 3μg/ml of the various antibodies and with respective normal rabbit serum controls or IgG. Immune complexes were then incubated with protein A-sepharose slurry (Amersham Pharmacia Biotech Inc.; Baie d'Urfe, Que.) and the beads were pelleted by centrifugation. The immune complexes were analyzed by SDS-PAGE under non-reducing or reducing conditions followed by autoradiography. Where Sol RI was overexpressed to determine antibody specificity, RIB-L17 were transiently transfected with 1μg of either empty vector (EV, pcMV5), pSol RI, pRI for 48 hours per well using Lipofectamine Reagent (Invitrogen Life Technologies) in serum-free medium according to the manufacturer's specifications. In addition, prior to affinity labeling and immunoprecipitation, some of the cell lysates were

pretreated with or without enzymes to cleave the glycosaminoglycan side chains (GAGs) from betaglycan. The procedure was performed as previously described (48) with some modifications. Briefly, confluent monolayers of cells were washed with dPBS and incubated with chondroitinase-ABC and heparinase (0.5U/ml and 4U/ml respectively, both from Sigma Aldrich) for 3 hours at room temperature. Thereafter, the cells were washed with 0.1% BSA in dPBS and affinity labeled with 200 pM of $^{125}$I-TGF-β1 as described above. Extracts were fractionated by SDS-PAGE on 3-11% polyacrylamide gels and analyzed by autoradiography. The membrane extracts were incubated with 3μg/ml of the various antibodies and with respective normal rabbit serum controls or IgG as described above.

**Western Blot of TGF-ß Receptors**

Western blotting of TGF-β receptors was performed as previously described (9). Briefly, chondrocytes were grown to 90% confluency in 6 well plates and not transfected, transfected with empty vector (EV, pcMV5), or receptor (pAlk-1 or pSol RI) for 48 hours. Cells were then washed with PBS and lysed. Cell lysates were prepared and samples were standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad; Mississauga, Ont.). Samples were then fractionated on SDS-PAGE (7.5% acrylamide) and transferred by electrophoresis to nitrocellulose membrane (Scheicher and Schuell; Keene, NH.). The membrane was blocked for 3 hours in a milk protein/Tween blocking solution (1M Tris, pH 7.5; 5M NaCl; Tween 20,Sigma Aldrich; Carnation Skim milk powder) and incubated with anti-Alk-1 antibody, control IgG (both from Santa Cruz Biotechnology Inc.), or anti-Sol RI antibody at room temperature for 1-2 hours followed by incubation for 1 hour with goat anti-rabbit HRP conjugated secondary antibody (Santa

Cruz Biotechnology Inc.) at room temperature and detected using the ECL system (Amersham Pharmacia Biotech Inc.).

**Immunoprecipitation/Western Blot Analysis**

Immunoprecipitation followed by Western blotting to detect TGF-β receptors was performed as previously described (9, 46). Briefly, chondrocytes were washed three times with 0.1% BSA-dPBS, twice with dPBS, and membrane extracts of cells were prepared and immunoprecipitated with anti-betaglycan antibody (Get 1), anti-Alk-1 antibody, anti-Sol RI antibody, control IgG, or not immunoprecipitated. In some experiments, cells were washed with a mild acid (0.1% glacial acetic acid; American Chemicals Ltd., Montreal, Quebec) to ensure complete removal of endogenous TGF-β (26,49) before membrane extraction and subsequent immunoprecipitation. In other experiments, cells were pretreated with 100 pM TGF-β1 at 37°C for 2 hrs before being washed twice with dPBS followed by membrane extraction and immunoprecipitation. The extracts or immune complexes were fractionated by SDS-PAGE on 3-11% gradient gels or 7.5% SDS-PAGE and Western blot analysis was performed using anti-Sol RI antibody, anti-Alk-1 antibody or anti-HA antibody and the ECL system was used for detection (Amersham Pharmacia Biotech Inc.) as discussed above.

**Two-Dimensional Gel Electrophoresis**

Two-dimensional gel electrophoresis was performed as described by MacKay et al (50) except that 3-mercaptopropionic acid was omitted in the second dimension (9,26). Solubilized membrane extracts of cells affinity labeled with $^{125}$I-TGF-β1 were immunoprecipitated with anti-betaglycan antibody (Get 1), anti-Sol RI, anti-Alk-1 antibody, or not immunoprecipitated and then fractionated on a 1.0 mm-thick 3-11%

gradient gel under non-reducing conditions in the first dimension and on a 1.5 mm-thick 3-11% gradient gel under reducing conditions in the second dimension. The gel was then subjected to autoradiography or Western blotted as described above with anti-Alk-1 antibody (Santa Cruz Biotechnology Inc.).

**Receptor Overexpression and Western Blot Analysis**

To determine the regulation of the phosphorylated form of Smad 2 by TGF-β1 treatment in chondrocytes overexpressing Alk-1 or Sol RI, cells were grown in 6 well plates until 90% confluency. Cells were then transiently transfected with 1μg per well of pAlk-1, pSol RI, pRI, or empty vectors (EV, pcMV5 and pcDNA3) using Lipofectamine Reagent (Invitrogen Life Technologies) in serum-free medium according to the manufacturer's specifications. Cells were allowed to recover for 48 hours in regular medium, serum starved the following day for 4 hours, then treated with 50 pM of TGF-β1 for 15 minutes. Cell lysates were prepared and samples were standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad). The extracts were then fractionated by SDS-PAGE (7.5% acrylamide) and transferred by electrophoresis to nitrocellulose membrane (Scheicher and Schuell). The membrane was blocked for 3 hours in a milk protein/Tween blocking solution (1M Tris, pH 7.5; 5M NaCl; Tween 20,Sigma Aldrich; Carnation Skim milk powder) and incubated with a rabbit polyclonal anti-phosphoSmad 2 antibody (a gift from Dr. S. Souchelnytskyi, Uppsala, Sweden; 51,52) at 4°C overnight. The membrane was washed and incubated for 1 hour with goat anti-rabbit HRP conjugated secondary antibody (Santa Cruz Biotechnology Inc.) at room temperature and detected using the ECL system (Amersham Pharmacia Biotech Inc.). The membrane was reprobed with anti-STAT 3 antibody (Santa Cruz Biotechnology Inc.) to confirm equal protein loading.

### Receptor Overexpression and Luciferase Reporter Assay

The p3TP-Lux, a TGF-β-inducible promoter-reporter construct (53), containing the luciferase gene under the control of a portion of the plasminogen activator inhibitor-1 promoter region was used to determine cellular responsiveness to TGF-β. pSBE 4-luc is also a TGF-β sensitive reporter plasmid which has been used extensively to determine cell response to TGF-β (34,54-56). Chondrocytes were grown to 90% confluency in a 6-well plate and transiently transfected with 1μg of p3TP-Lux or pSBE 4 and 1μg of pβ-galactosidase (β-gal) per well using Lipofectamine Reagent (Invitrogen Life Technologies) in serum-free medium according to the manufacturer's specifications. In addition the reporter cells were either transfected with the empty vector (EV, pcMV5) or one of pRI, pAlk-1, or pSol RI. Cells were allowed to recover for 48 hours in regular medium, serum starved the following day for 4 hours, then treated with 100 pM of TGF-β1 overnight. The cells were lysed and assayed for luciferase activity (BD Pharmingen; Mississauga, Ont.) using the EG&G Berthold Microplate Luminometer (Berthold Technologies USA; Oak Ridge, TN). Light emission by the TGF-β1 treated cells was expressed as a percentage of the emission by the control cells and adjusted for transfection efficiency obtained using the β-gal assay.

## RESULTS

### TGF-β receptor profiles on human chondrocytes

The tsT/AC62 and C-28/I2 cell lines provide a reproducible model that mimics human articular and costal phenotypes respectively (57-60). The chondrocyte phenotype of the immortalized cell lines and the human primary articular chondrocytes in culture, have previously been confirmed by determination of the production of aggrecan and type II

collagen (9) and by ascertaining typical chondrocyte morphology as detected by a spherical to polygonal shape with a cell-associated matrix when suspended in alginate bead cultures (data not shown).

To determine whether human chondrocytes express novel TGF-β receptors, the cells were affinity labeled with $^{125}$I-TGF-β1 and the labeled receptors were analyzed by SDS-PAGE. A receptor profile representative of human articular and nonarticular chondrocytes is illustrated in Fig. 1. The competition using increasing concentrations of unlabeled TGF-β1, TGF-β2, and TGF-β3 isoforms demonstrated the relative binding affinity of the receptors for the TGF-β isoforms. We have previously reported on four major binding complexes of relative molecular weights of 65, 85, 100, and 200-300 kDa in human chondrocytes under reducing conditions. These bands represented the cloned type I receptor (RI/Alk-5), type II receptor (RII), endoglin monomer, and type III (betaglycan) receptor respectively (62,63). This was confirmed later by immunoprecipitation studies using specific anti-receptor antibodies (9).

In addition to the aforementioned receptors, a novel band at 47 kDa was detectable in the absence of unlabeled ligands (Fig. 1, lanes designated as '0'). The migratory pattern (47 kDa) and isoform specificity of this band is characteristic of our recently identified soluble type I TGF-β receptor (35) which shows affinity for all three TGF-β isoforms with β1>β3>β2. That this band represents Sol RI was later confirmed by immunoprecipitation studies using a specific anti-Sol RI receptor antibody (see below). The presence of Alk-1 was also confirmed on these cells by Western blot and immunoprecipitation (see below) and thus has been labeled at the 65 kDa position (Fig. 1)

in addition to RI. Alk-1 is partially responsible for the binding of $^{125}$I-TGF-β and the intensity of the 65 kDa band noted.

**Sol RI and Alk-1 are expressed on human cartilage**

The results of Western blotting shown in Fig. 2 demonstrated the presence of Alk-1 and Sol RI in human articular (tsT/AC62) and nonarticular (C-28/I2) chondrocytes. Receptor detection in untransfected cells (Ø) confirms that Alk-1 and Sol RI are present endogenously. This endogenous presence was also noted with transfection of empty vector (EV). When Alk-1 and Sol RI were overexpressed (Alk-1, Sol RI) the receptors were observed to co-migrate with the endogenous receptors thus confirming their identity and molecular weights.

**Alk-1 and Sol RI form heteromeric complexes with betaglycan in the presence of ligand**

To confirm the identity of Alk-1 and Sol RI on human chondrocytes and to study potential associations between these and other TGF-β receptors, chondrocytes were affinity labeled with $^{125}$I-TGF-β1 and immunoprecipitated using specific anti-receptor antibodies directed against Alk-1, Sol RI, and betaglycan. In these studies, whereas immunoprecipitation with a specific anti-receptor antibody confirmed the identity of its cognate TGF-β receptor, co-immunoprecipitation of another type of receptor which is not recognized by this antibody was indicative of heteromeric complex formation between those receptors.

As shown in Fig. 3A, SDS-PAGE of the nonimmunoprecipitated membrane lysate demonstrated the presence of TGF-β binding proteins consistent in mobility and size with RI/Alk-1, RII, Sol RI, betaglycan, and the endoglin monomer (Lane 3; NIP). The anti-

betaglycan antibody (Lane5; α-BG) immunoprecipitated betaglycan and co-immunoprecipitated RI/Alk-1, RII, and Sol RI. Similarly, the anti-Sol RI antibody (Lane 6;α-Sol RI) precipitated Sol RI, thus confirming the presence of Sol RI on human chondrocytes. Interestingly, anti-Sol RI antibody co-immunoprecipitated betaglycan in addition to RI/Alk-1 and RII. The co-immunoprecipitation of RI and RII with α-BG (18,30) is well documented. However, the co-immunoprecipitation of betaglycan with α-Sol RI and that of Sol RI with α-BG has not been previously reported and suggests that Sol RI and betaglycan form a heteromeric complex on the cell surface of chondrocytes. The anti-RI and anti-RII antibodies were used as controls (Lane 1; α-RI and Lane 2; α-RII respectively). No receptors were immunoprecipitated with the normal rabbit serum control (Lane 4; NRS).

Fig. 3B demonstrated that this novel association between Sol RI and betaglycan does not require the GAGs of betaglycan to be present. Prior to affinity labeling cells were either left untreated (-) or were treated (+) with a combination of chondroitinase ABC and heparinase enzymes (C/H) which are known to cleave the GAG chains from betaglycan's extracellular domain (48). This results in the full length betaglycan being reduced to its core molecule of 120 kDa (Lane 2,3; NIP). Again immunoprecipitation with anti-betaglycan antibody (Lane 4,5; α-BG) revealed betaglycan and co-immunoprecipitated Sol RI. In addition, immunoprecipitation with anti-Sol RI antibody (Lane 6,7; α-Sol RI) revealed Sol RI and co-immunoprecipitated betaglycan. These co-immunoprecipitations were apparent in the absence or presence of enzyme treatment confirming that this association occurs independently of betaglycan's carbohydrate side chains.

As shown in Fig. 3C, SDS-PAGE under reducing conditions of the nonimmunoprecipitated membrane lysate demonstrated the presence of TGF-β binding proteins consistent in mobility and size with RI, RII, and betaglycan (Lane 1; NIP). The anti-Alk-1 antibody precipitated a band at 65 kDa, thus confirming the presence of Alk-1 on human chondrocytes (Lane 3; α-Alk-1). However, this band could be partially comprised of RI also 65 kDa and know to co-immunoprecipitate with Alk-1. Interestingly, α-Alk-1 co-immunoprecipitated betaglycan in addition to RII. This novel finding suggests that Alk-1 and betaglycan form a heteromeric complex on the cell surface of chondrocytes. No receptors were immunoprecipitated with the normal rabbit serum control (Lane 2; NRS).

**Confirmation of the expression of Sol RI on chondrocytes and characterization of anti-Sol RI antibody**

The Sol RI antibody was raised against the divergent COOH-terminal region of the extracellular domain of Sol RI (35) to specifically detect Sol RI. This antibody specificity is demonstrated in Fig. 4A using R1B-L17 cells which are stably mutated Mink lung epithelial cells exhibiting no type I TGF-β receptor (45). R1B-L17 cells were transfected with empty vector (EV, Lane 2), Sol RI (Lane 3,4), RI (Lane 6) or left untransfected (Lane 1, 5,7). As anticipated the transfection efficiency of these cells is extremely low, therefore, it is difficult to observe an increased expression of the transfected receptors. Cell lysates were then immunoprecipitated using the anti-Sol RI antibody (α-Sol RI, Lane 4-6), control rabbit IgG (Lane 7), or not immunoprecipitated (Lane 1-3). The absence of type I receptor is apparent in the R1B-L17 cells (Lane 1-7) as compared to the 65 kDa band representing RI evident by immunoprecipitation with anti-RI antibody of C28-I2

cell lysates (Lane 8) known to express RI. The α-Sol RI antibody was able to detect both endogenous levels of Sol RI (Lane 5, 6) as well as overexpressed Sol RI (Lane 4). It was previously noted that these cells did not express RI, both from affinity labeling of non-immunoprecipitated lysates and an inability to immunoprecipitate RI (data not shown). Of note, when RI is overexpressed in RIB-L17 cells α-Sol RI antibody is unable to detect RI (Lane 6) indicating the Sol RI antibody does not cross-react with RI. Western blot using the same α-Sol RI antibody confirms the presence of Sol RI on chondrocytes (Fig. 4B). Human chondrocyte lysates immunoblotted for Sol RI (right panel) demonstrate the presence of the receptor at 47 kDa. However, Immunoblotting with control IgG (left panel) failed to demonstrate a signal at 47 kDa.

**The associations of Alk-1 and Sol RI with betaglycan can occur in the presence of ligand and ligand-independent manner**

Using immunoprecipitation studies of affinity labeled chondrocytes, we have thus far demonstrated that Alk-1 and Sol RI form heteromeric complexes with betaglycan in the presence of ligand. For confirmation and to determine whether such complex formation also could occur in the absence of ligand, immunoprecipitation studies in tandem with Western blot were performed. Membrane extracts of chondrocytes, extensively washed with dPBS, were prepared and immunoprecipitated with α-BG, α-Alk-1, α-Sol RI antibodies or with control IgG, or not immunoprecipitated. They were then fractionated by SDS-PAGE under reducing conditions and analyzed by Western blot using α-Alk-1 antibody, as shown in Fig.5A or α-Sol RI antibody as shown in Fig.5B.

Alk-1 and Sol RI were not detected in the control experiments where immunoprecipitation was preformed with rabbit control IgG (IgG) before Western blotting (Lane 1; Fig. 5A and B). Western blotting of membrane extracts immunoprecipitated with α-Alk-1 antibody (Fig. 5A; Lane 6) and with α-Sol R1 antibody (Fig. 5B; Lane 6) confirmed the presence of Alk-1 at 65 kDa and Sol RI at 46 kDa respectively. Importantly, Western blotting of extracts immunoprecipitated with α-BG antibody (Lane 3) revealed Alk-1 at 65 kDa (Fig. 5A) and Sol RI (Fig. 5B), thus demonstrating that betaglycan co-immunoprecipitated Alk-1 and also Sol RI in the absence of exogenous TGF-β. Furthermore addition of mild acid wash (0.1% glacial acetic acid) to remove any endogenous TGF-β followed by immunoprecipitation with α-BG antibody also demonstrated these associations (Lane 4, BG/acid; Fig. 5A and B). When chondrocytes were pretreated with 100 pM TGF-β1, there was no apparent increase in either the amount of Alk-1 or Sol RI detected (Lane 5, BG/TGF-β1; Fig. 5A and B). Western blotting of nonimmunoprecipitated membrane extracts (NIP, Lane 2) also confirmed the presence of Alk-1 (Fig. 5A) and Sol RI (Fig. 5B) in chondrocytes. Interestingly, immunoprecipitation with α-Sol RI antibody co-immunoprecipitated Alk-1 (Fig. 5A; Lane 7) suggesting that these two receptors are also capable of forming a heteromeric receptor complex on the chondrocyte surface.

To further confirm the novel finding that Alk-1 associates with betaglycan, 293 cells were transfected with HA-tagged Alk-1 pcDNA (Fig. 5C, Lane 3,4), empty vector (EV, Lane 2) or were left untransfected (Lane 1). Cell lysates were not immunoprecipitated (Lane 1-3) or immunoprecipitated with α-BG antibody (Lane 4; α-BG) and analyzed by SDS-PAGE under reducing conditions. Western blotting with α-HA antibody demonstrated the

presence of Alk-1 in Lane 3 (overexpressed, not immunoprecipitated) and 4 (overexpressed, immunoprecipitated with α-BG antibody) confirming the betaglycan-Alk-1 association.

**Sol RI and Alk-1 form higher order complexes with TGF-β signaling receptors on human chondrocytes**

To further characterize the nature of these various TGF-β binding receptors and to test whether they represent oligomeric complexes two-dimensional gel electrophoresis was performed. Chondrocytes were affinity labeled with $^{125}$I-TGF-β1 and the membrane extracts were immunoprecipitated with α-BG (Fig. 6A and D), α-Sol RI (Fig. 6B), or α-Alk-1 (Fig. 6C) antibody and two-dimensional gel electrophoresis was performed under nonreducing conditions in the first dimension and reducing conditions in the second dimension. Fig 6A revealed spots at 250-300 kDa (betaglycan) and 85 kDa (RII) as well as 2 spots (spot 1 and 2) at 47 kDa which represent Sol RI. It is apparent that these spots have fallen from complexes of approximately 135 and 350 kDa off the diagonal. This suggests that Sol RI forms a higher order complex with betaglycan (spot 1) and likely RII (spot 2). Membrane extracts, immunoprecipitated with α-Sol RI antibody (Fig. 6B) again revealed 2 spots of 47 kDa demonstrating the presence of Sol RI. Betaglycan is also detected near the diagonal at 250-300 kDa and appears to form a higher order complex with Sol RI (spot 1). Similarly, membrane extracts immunoprecipitated with α-Alk-1 antibody (Fig. 6C) revealed 2 spots at 47kDa representing Sol RI (spot 1 and 3) and 2 spots at 65 kDa representing Alk-1 (spot 1 and 2). As noted for the Sol RI immunoprecipitate, betaglycan is observed at 250-300 kDa and is forming a higher order complex with Alk-1 and Sol RI (spot 1). In Fig. 6D cells were immunoprecipitated with

anti-betaglycan antibody, two dimensional gel electrophoresis was performed and followed by Western blotting with α-Alk-1 antibody. Two spots of 65 kDa are observed (spot 1 and 2) representative of Alk-1. The presence of spot 2 (the co-immunoprecipitation of Alk-1) and the presence of spot 1 (Alk-1 descending from a higher order complex) confirm the association of betaglycan with Alk-1 and their formation of higher order complexes on chondrocytes.

**Overexpression of Sol RI and Alk-1 enhances TGF-β response in human chondrocytes**

Chondrocytes were transfected with pcDNA of RI, Sol RI, Alk-1, or empty vector (Fig. 7). Cells were then left untreated (Lane 1,3,5,7) or treated with 100 pM of TGF-β1 for 15 minutes (Lane 2,4,6,8) and Western blotting was performed to detect the phosphorylated form of Smad 2 (Fig. 7A, upper panel). The overexpression of RI demonstrated an upregulation of Smad 2 phosphorylation (Lane 3,4) consistent with its ability to enhance TGF-β signaling (36,61). Interestingly, both Alk-1 (Lane 7,8) and Sol RI (Lane 5,6) overexpression demonstrated an upregulation of Smad 2 phosphorylation, in untreated and TGF-β1 treated lysates. Thus, it appears that at endogenous TGF-β levels and with TGF-β treatment, overexpression of these receptors can enhance signaling. The membrane was reprobed for STAT3 (lower panel) to confirm equal protein loading in all lanes.

The enhanced TGF-β signaling with Sol RI and Alk-1 overexpression was also demonstrated as stimulation of transcriptional activity. In Fig. 7B and C chondrocytes were transfected with pcDNA of RI, Sol RI, or Alk-1 in addition to either 3TP-Lux (Fig. 7B) or SBE 4 (Fig. 7C) and pβ-gal. After 48 hours cells were serum starved for 4 hours

then left untreated (-) or were treated with 100 pM of TGF-β1 (+) overnight and luciferase activity was measured and standardized with a β-gal assay. Data obtained with the 3TP-Lux reporter construct demonstrated that overexpression of Sol RI ($p=0.04$) and Alk-1 ($p=0.001$) caused a marked enhancement of TGF-β response as compared to control untransfected cells. Overexpression of RI was used as a comparison as it has previously been reported to up-regulate TGF-β response (36,61) but here did not show a significant upregulation ($p=0.19$). Data obtained with the SBE-4 reporter construct demonstrated that overexpression of Sol RI ($p=0.12$) and Alk-1 ($p=0.13$) caused an enhancement of TGF-β response as compared to control untransfected cells, although not at statistically significant levels.

**DISCUSSION**

Evidence indicates that cartilage destruction in osteoarthritis (OA) is linked to dysregulation of cytokine expression. Transgenic mice with a dominant negative form of RII (64) and a Smad 3 heterozygote murine knock out (62) have been established. These two murine models develop early degenerative joint disease suggesting that dysregulation of the TGF-β signaling cascade may contribute to inadequate cartilage regeneration. Chondrogenesis, the process of differentiation from an immature precursor cell to one with a chondrogenic phenotype, is regulated by TGF-β. In addition, *in vitro* evidence suggests that TGF-β can have both a mitogenic effect on chondrocytes and a role in ECM production. Despite TGF-β's implication as a key mediator of cartilage repair (1-6,36,65-67) the mechanisms regulating TGF-β action in chondrocytes are poorly understood. One potential avenue of local modulation of TGF-β action is at the level of its membrane bound receptors. Recently we characterized the TGF-β receptor expression profile on

human chondrocytes (9) and demonstrated that novel accessory receptors do form heteromeric complexes in these cells. Specifically we identified the presence of endoglin but more importantly documented its association with betaglycan and demonstrated that they form a heteromeric complex in the presence of ligand and independent manner and a type II receptor independent manner. The expression profiles of novel TGF-β binding proteins and their interactions with the TGF-β signaling receptors at the membrane level leading to the formation of heteromeric receptor complexes may play a central role in regulating the diverse actions of TGF-β in chondrocytes.

In the present study we demonstrated for the first time that Sol RI and Alk-1 are expressed on human chondrocytes. Not only do these receptors form higher order complexes with RI and RII on the cell surface but also associate with each other and more importantly with betaglycan. These novel interactions of Alk-1 and Sol RI with betaglycan are shown to occur in the presence of ligand and ligand independent manner. The complex formation between Sol RI and betaglycan occurs independent of the carbohydrate side chains of betaglycan. Furthermore our results demonstrate that the above receptor interactions occur at physiological receptor concentrations and ratios at the cell surface and were not an artefact of overexpression. Importantly we demonstrate that overexpression of Sol RI and Alk-1 enhances TGF-β responses in human chondrocytes.

The phenotype of the human chondrocytes used in the present study has been characterized extensively (57-60). In addition, we have shown that both primary human articular chondrocytes and immortalized cells express aggrecan and type II collagen mRNAs and are responsive to TGF-β (9). Prior studies analyzing the interactions between

TGF-β receptors and the stoichiometry of the signaling complex employed primarily mutant cell lines or cells overexpressing the wild-type or chimeric receptors (68,69). In the present study, we used TGF-β responsive human chondrocytes to demonstrate the association of Alk-1 and Sol RI with betaglycan, and the formation of higher order complexes with TGF-β signaling receptors. Thus, our results illustrated that these associations occur at endogenous receptor concentrations and ratios.

In addition to providing confirmation of the identity of Sol RI and Alk-1 (Fig. 1 and 2), the findings of immunoprecipitation studies using cells affinity labeled with $^{125}$I-TGF-ß1, indicated that they form heteromeric complexes with betaglycan in the presence of ligand. This was demonstrated by co-immunoprecipitation of Alk-1 and Sol RI with α-BG antibody and that of betaglycan with α-Sol RI and α-Alk-1 antibody when analyzed with SDS-PAGE (Fig. 3A,3C). Although immunoprecipitation results provided evidence for TGF-β induced complex formation between Alk-1 and betaglycan, and Sol RI and betaglycan, it was not possible to detect these heteromeric complexes on SDS-PAGE analysis. The large molecular weight of such complexes and the highly heterogeneous nature of betaglycan will preclude their detection.

That Sol RI and Alk-1 exists in heteromeric complexes with betaglycan on the chondrocyte surface was confirmed using membrane extracts that were immunoprecipitated with α-betaglycan antibody and then subjected to Western blotting with either α-Sol RI or α-Alk-1 antibody (Fig. 5A,5B). In addition, cells were either left untreated, treated with TGF-β1 or washed with a mild acid to ensure complete removal of endogenous TGF-β without affecting receptor integrity (49) demonstrating that the above heteromeric complexes occur in the presence of ligand and ligand independent fashion.

Furthermore, the Alk-1-betaglycan association is not unique to chondrocytes since this interaction was detectable also on 293 cells transiently transfected with cDNA encoding HA-tagged Alk-1 (Fig. 5C). In addition, the association between Sol RI and betaglycan was seen to occur independent of betaglycan's carbohydrate side chains (Fig. 3B) suggesting that this may be core protein interaction.

Two dimensional gel electrophoresis provided information on the nature of the higher molecular weight receptor complexes. Fig. 6A – D demonstrated that both Alk-1 and Sol RI were able to form higher order complexes with betaglycan as well as the type II TGF-β signaling receptor. Sol RI could be seen to fall from a higher order complex involving betaglycan and RII (350 kDa; Fig. 6A, 6B, 6C and 135 kDa; Fig. 6A respectively). Alk-1 could be seen to fall from a higher order complex involving betaglycan (365 kDa; Fig. 6D). It was also apparent that Sol RI and Alk-1 together could associate with betaglycan in a higher order complex (415 kDa; Fig. 6C). In addition, it is possible that all of these receptors associate with the heterotetrameric TGF-β signaling complex, although not specifically investigated here. Moreover, multiple high molecular weight receptor complexes containing the signaling receptors with or without betaglycan, Alk-1 and Sol RI may be formed during the cross-linking procedure.

Numerous studies have shown that accessory receptor such as endoglin and betaglycan interact with RI and RII (30,55,69). Evidence has recently been presented that betaglycan may play a more direct role in TGF-β signaling. A functional role for the cytoplasmic tail of betaglycan in regulating TGF-β signaling has been described where autophosphorylated RII phosphorylates the betaglycan cytoplasmic tail, thereby enhancing TGF-β2 signaling (55). Therefore, additional TGF-β binding proteins which

form heteromeric complexes with betaglycan will undoubtedly modify its actions and subsequent interactions with the signaling receptors.

Others have alluded to the regulatory potential of accessory TGF-β receptors. It has been well established that betaglycan facilitates TGF-β binding to the TGF-β signaling receptors, specifically the binding of the TGF-β2 isoform to RII, which occurs at low affinity in the absence of betaglycan (70,71). However, the property of ligand presentation of betaglycan does not account for the strict requirement of betaglycan for the epithelial-mesenchymal transition involved in the heart valve formation (72). In addition, its extracellular domain can be shed into the medium where it may act as a TGF-β antagonist (29,73). As previously mentioned, we recently identified endoglin on human chondrocytes and demonstrate a complex formation with betaglycan (9).

We have now added Alk-1 and Sol RI to the human chondrocyte TGF-β receptor expression profile. The ligand and corresponding type II receptor for Alk-1 are unknown. In the presence of RII it binds TGF-β1 and in the presence of ActRII it binds activin A (37-39). However, neither of the complexes were shown to elicit a signal and Alk-1 was considered an orphan receptor. More recently, however, Alk-1 has been shown to modulate phosphorylation of Smad 1 and 5 (74, 41) suggesting it may play a role in signaling of the TGF-β superfamily. Sol RI occurs naturally (35) and has been demonstrated by us and others to associate with RII (34,35) although its role in modulating TGF-β signaling is not certain A recombinant form of this receptor was constructed from the extracellular domain of RI. This recombinant Sol RI required the presence of both RI and RII signaling receptors to bind TGF-β1 and to generate a signal. In the absence of TGF-β this receptor mimicked TGF-β induced transcriptional and

growth responses (34). Whether or not the enhancement of TGF-β signaling observed here with Sol RI overexpression is related to Sol RI mimicking ligand, providing an additional signaling pathway through RII, or modulating signaling through its interaction with betaglycan remains to be determined. Importantly, we have provided evidence for a functional role for Alk-1 and Sol RI since overexpression of both of these receptors enhanced TGF-β response in human chondrocytes (Fig. 7). Whether this is a result of a direct interaction with signaling receptors or indirect through their associations with betaglycan remains to be determined.

Current evidence indicates that the TGF-β signaling complex is a heterotetramer consisting of one molecule each of the type I and type II receptor associated with each monomer of a TGF-β dimer molecule (75). While it is difficult to estimate the precise stoichiometry of the Sol RI and Alk-1 containing complexes, it is possible that these receptors associate with the heterotetrameric TGF-β signaling complex. However, multiple high molecular weight receptor complexes containing Alk-1 and Sol RI, RI and RII with or without betaglycan may be formed as an artefact of the cross-linking procedure which would not be present in endogenous states. Oligomerization of TGF-β receptors to form complexes consisting of differing subtype composition and ratio may represent modes of regulating distinct TGF-β responses.

Based on the results in the present study, we propose a model to illustrate the significance of the Alk-1-betaglycan and Sol RI-betaglycan complex formation in the modulation of TGF-β signaling in chondrocytes (Fig. 8). Alk-1 and Sol RI associate with betaglycan on the cell surface and this heteromeric complex may interact directly with the signaling receptors. However, the relative expression level of the individual receptor components

may determine the outcome of TGF-β signaling. We demonstrated that overexpression of Alk-1 and Sol RI enhances TGF-β signaling. However, as shown in Figure 8, this complex interaction between Alk-1, betaglycan and Sol RI may (A) regulate each others function at the level of ligand binding or regulate the function of signaling receptors by facilitating or decreasing ligand binding by RI and RII (B). Alternatively, these receptors or their complex may regulate signaling receptors by interacting with them at the extracellular domain level (C) or (D) at the downstream signaling level. Thus, these associations may be critical for achieving a fine balance of TGF-β signaling and thus for specifying a particular TGF-β response. It is interesting to mention in this regard that the novel complex formation between Alk-1 and betaglycan may also modulate signaling by other members of the TGF-β receptor superfamily in the light of recent observations which show that Alk-1 is able to bind Activin A in the presence of ActRII (39) and that betaglycan binds inhibin (76).

In summary, our results demonstrated for the first time that Alk-1 and Sol RI are expressed on human chondrocytes and that they form higher order complexes with RI and RII on the cell surface. More importantly, we demonstrated that they each form a heteromeric complex with betaglycan and that these complex formations occur at physiological receptor concentrations and ratios. Significantly, we found that Alk-1 and Sol RI complex with betaglycan in the presence of ligand and ligand-independent manner and that the complex of Sol RI and betaglycan occurs independent of betaglycan's carbohydrate side chains. Furthermore we have shown that overexpression of both Sol RI and Alk-1 enhance TGF-β signaling in human chondrocytes.

It is becoming increasingly evident that accessory TGF-β receptors likely have functional cytoplasmic domains and that their interactions with signaling receptors play a more predominant role in regulation of TGF-β action than previously thought. TGF-β plays a diverse role in the maintenance of cartilage from regulating cell proliferation and differentiation to governing ECM turnover. Despite this the TGF-β and Smad mediated signaling cascade appears quite rigid. The relative contributions of novel TGF-β receptors and their formation of heteromeric receptor complexes at the cell surface may be critical in specifying these various TGF-β signaling pathways and in regulating TGF-β action towards cartilage regeneration or repair.

**FIGURE LEGENDS**

**Fig. 1. Affinity labeling of human chondrocytes with $^{125}$I-TGF-β1 and analysis under reducing conditions.** Confluent monolayers of tsT/AC62 cells were affinity labeled with 100 pM of $^{125}$I-TGF-β1 in the absence or presence of the indicated concentrations of unlabeled TGF-β1, β2, or β3. Solubilized cell extracts were analyzed by SDS-PAGE on 3-11% polyacrylamide gradient gels under reducing conditions followed by autoradiography. Similar receptor profiles were observed using primary chondrocytes and the C-28/I2 cell line (data not shown).

**Fig. 2. Sol RI and Alk-1 are expressed on human articular cartilage.** Solubilized extracts of cell lines (C-28/I2 and tsT/AC62) transfected with pAlk-1 (Alk-1), pSol RI (Sol RI), pcMV5 (EV) or left untransfected (Ø), were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions. They were then transferred onto nitrocellulose membrane and the membranes were immunoblotted with anti-Alk-1 (upper panel) or anti-

Sol RI (lower panel) antibody. The ECL system was used for chemiluminescence detection.

**Fig. 3. Immunoprecipitation of TGF-β binding proteins on human chondrocytes.** Cells were affinity labeled with 200 pM of $^{125}$I-TGF-β1 and solubilized cell extracts were immunoprecipitated with 3μg/ml each of anti-receptor antibodies or control normal rabbit serum. Complexes were fractionated on SDS-PAGE (3-11% gradient gels) under reducing conditions and visualized by autoradiography. **A:** Non-immunoprecipitated (NIP) cell extract of tsT/AC62 is shown in Lane 3. Immunoprecipitations were performed using anti-betaglycan (Lane 5, α-BG), anti-Sol RI (Lane 6, α-Sol RI) or anti-RI (Lane 1,α-RI), anti-RII (Lane 2, α-RII) or control normal rabbit serum (Lane 4, NRS). **B:** Cells were left untreated (-) or treated (+) with chondroitinase ABC and heparinase enzymes (C/H) prior to affinity labeling. Non-immunoprecipitated (NIP) cell extract of C28/I2 is shown in Lane 2 and 3. Immunoprecipitations were performed using anti-betaglycan (Lane 4 and 5, α-BG), anti-Sol RI (Lane 6 and 7, α-Sol RI) or control rabbit IgG (Lane 1). **C:** Non-immunoprecipitated (NIP) cell extract of tsT/AC62 is shown in Lane 1. Immunoprecipitations were performed using anti-Alk-1 (Lane 3, α-Alk-1) or control normal rabbit serum (Lane 2).

**Fig. 4. Confirmation of the expression of Sol RI on chondrocytes and characterization of anti-Sol RI antibody. A:** RIB-L17 cells were cultured to 90% confluency in 6 well plates and transiently transfected with pcMV5 (EV, Lane 2), pRI (RI, Lane 6) or Sol RI (Sol RI, Lane 3,4) or not transfected (Lane 1,5,7). C28/I2 cells were not transfected (Lane 8). All cells were affinity labeled with 200 pM of $^{125}$I-TGF-β1 and solubilized cell extracts were immunoprecipitated with anti-Sol RI antibody (Lane 4-

6), anti-RI antibody (Lane 8), or control IgG (Lane 7) or were not immunoprecipitated (Lane 1-3). Complexes were fractionated on SDS-PAGE (3-11% gradient gels) under reducing conditions and visualized by autoradiography. **B:** Solubilized extracts of immortalized chondrocytes, C-28/I2 and tsT/AC62, were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membranes were immunoblotted with anti-IgG (left panel, α-IgG) or anti-Sol RI antibody (right panel, α-Sol RI). The ECL system was used for chemiluminescence detection.

**Fig. 5. Western blot analysis demonstrating complex formations between betaglycan and Alk-1 and betaglycan and Sol RI on chondrocytes. A/B:** Chondrocytes were left untreated (Lane 1-3, 6 and 7), were treated with 100 pM of TGF-β for 2 hours (Lane 5), or were subject to a mild acid wash (Lane 4). **A:** Solubilized extracts of human chondrocytes were not immunoprecipitated (NIP, Lane 2), or immunoprecipitated with control IgG (Lane 1), anti-betaglycan antibody (α-BG, Lane 3; α-BG/acid, Lane 4; α-BG/TGF-β1, Lane 5), anti-Alk-1 antibody (α-Alk-I, Lane 6), or anti-Sol RI antibody (α-Sol RI, Lane 7). Complexes were fractionated on 3-11% gradient gels under reducing conditions and Western blotting was performed, as described in Experimental Procedures using anti-Alk-1 primary antibody. **B:** Solubilized extracts of human chondrocytes were not immunoprecipitated (NIP, Lane 2), or immunoprecipitated with control IgG (Lane 1), anti-betaglycan antibody (α-BG, Lane 3; α-BG/acid, Lane 4; α-BG/TGF-β1, Lane 5), or anti-Sol RI antibody (α-Sol RI, Lane 6). Complexes were fractionated on 3-11% gradient gels under reducing conditions and Western blotting was performed, as described in Experimental Procedures using anti-Sol RI primary antibody. **C:** 293 cells were cultured to 90% confluency in 6 well plates and transiently transfected with pcMV5 (EV, Lane 2)

or HA-tagged Alk-1 (Alk-1·HA, Lane 3 and 4) or not transfected (Lane 1). After 48 hours, solubilized extracts were not immunoprecipitated (Lane 1-3) or immunoprecipitated with anti-betaglycan antibody (α-BG, Lane 4) and complexes were fractionated by SDS-PAGE (7.5% acrylamide) under reducing conditions and Western blotting was performed, as described in Experimental Procedures using anti-HA antibody.

**Fig. 6. Two-dimensional gel electrophoresis of TGF-β receptor complexes on human chondrocytes.** Chondrocytes were affinity labeled with 100 pM of $^{125}$I-TGF-β1 (**A-C**) or not affinity labeled (**D**) and solubilized extracts were immunoprecipitated with anti-BG antibody **(A and D),** anti-Sol RI antibody **(B)**, or anti-Alk-1 antibody **(C)** and analyzed by SDS-PAGE (3-11% gradient gel) under nonreducing conditions in the first dimension. The individual lane was then cut out, laid horizontally on a second gel (3-11% gradient) and analyzed under reducing conditions in the second dimension and subjected to autoradiography. In **D** the gel was transferred to nitrocellulose membrane and Western blotted with anti-Alk-1 antibody, as described above.

**Fig. 7. TGF-β responses in chondrocyte cell lines overexpressing the Alk-1 and Sol RI receptors. A:** Stimulation of Smad 2 phosphorylation by TGF-β1. Chondrocytes were transiently transfected with pAlk-1 (Lane 7,8; Alk-1), pSol RI (Lane 5,6; Sol RI), or pRI (Lane 3,4; RI), or empty vector (Lane 1,2; EV). After 48 hours cells were treated with 100 pM of TGF-ß1 (+) for 15 minutes or left untreated (-). Solubilized extracts were analyzed by SDS-PAGE (3-11% gradient gels) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against the phosphorylated form of Smad 2 (upper panel). Immunoblotting using an antibody directed against STAT 3 was performed to illustrate equal protein loading

(lower panel). The ECL system was used for chemiluminescence detection. **B/C:** Stimulation of plasminogen activator inhibitor-1 (PAI-1) promoter activity and Smad binding element 4 (SBE) promoter activity by TGF-β. The chondrocytes were transiently transfected with the PAI-1 promoter-luciferase reporter construct, p3TP-Lux **(B)**, SBE 4 **(C)**, pβ-gal, in addition to either pSol RI or pAlk-1. 48 hours after transfection, cells were treated with 100 pM of TGF-ß1 (+) overnight or were left untreated (-). Luciferase activity was determined, normalized using the β-gal assay and expressed as a fold increase of untreated cells. The data shown are representative of three different experiments each done in triplicates with statistically significant upregulation noted by an asterisk.

**Fig. 8. Schematic representation of a possible model illustrating the significance of the Alk-1, Sol RI, and betaglycan complex formation in modulating TGF-β signaling.** Alk-1 and Sol RI associate with betaglycan and each other on the cell surface and this heteromeric complex may interact directly with the signaling receptors, although the possibility that these receptors may interact individually with RI and RII cannot be ruled out. Overexpression of Alk-1 and Sol RI appear to enhance TGF-β signaling. In addition, overexpression of betaglycan is believed to enhance signaling by facilitating ligand binding to the signaling receptors and by an alternate mechanism involving the phosphorylation of its cytoplasmic tail (55,75). This complex interaction between Alk-1, betaglycan and Sol RI may (A) regulate each others function at the level of ligand binding or regulate the function of signaling receptors by facilitating or decreasing ligand binding by RI and RII (B). Alternatively, these receptors or their complex may regulate signaling receptors by interacting with them at the extracellular domain level (C) or (D) at the

downstream signaling level. Thus, the interaction amongst these novel receptors may be of critical significance as the relative expression level of the individual receptor components may determine the outcome on TGF-β signaling. Complex formation of Alk-1, Sol RI, betaglycan, with the signaling receptors has been omitted for clarity.

**REFERENCES**


1. Howard, S. and Anastasiades, T. (1993) *J. Rheumatol.* **20**, 2083-2094
2. Demoor-Fossard, M., Redini, F., Boittin, M., and Pujol, J.P. (1998) *Biochem. Biophys. Acta.* **1398,** 179-91
3. Redini, F., Galera, P., Mauviel, A., Loyau, G., and Pujol, J.P. (1988) *FEBS Lett* **234,** 172-6
4. Morales, T.I. and Roberts, A.B. (1988) *J. Biol. Chem.* **263,** 12828-31
5. Pujol, J.P., Galera, P., Redini, F., Mauviel, A., and Loyau, G. (1991) *J. Rheumatol. Suppl.* **27,** 76-9
6. Tsukazaki, T., Matsumoto, T., Enomoto, H., Usa, T., Ohtsuru, A., Namba, H., Iwasaki, K., and Yamashita, S. (1994) *Osteoarthritis Cartilage* **2**:259-67
7. Horner, A., Kemp, P., Summers, C., Bord, S., Bishop, N.J., Kelsall, A.W., Coleman, N., and Compston, J.E. (1998) *Bone* **23**:95-102
8. Moldovan, F., Pelletier, J.P., Mineau, F., Dupuis, M., Cloutier, J.M., and Martel-Pelletier, J. (2000) *Arthritis Rheum.* **43**, 2100-9
9. Parker, W.L., Goldring, M.B., and Philip, A. (2003) *J Bone Miner. Res.* **18**:289-302
10. Roberts, A.B. and Sporn, M.B. (1990) The transforming growth factor beta's. In: Sporn MB and Roberts AB (eds) Peptide Growth Factors and Their Receptors, vol 95. Spriner-Verlag, New York, pp. 419-472

11. Massague, J. (1987) *Cell* **49**:437-8

12. Sporn, M.B., Roberts, A.B., Wakefield, L.M., and de Crombrugghe, B. (1987) *J. Cell Biol.* **105**:1039-45

13. Roberts, A.B. and Sporn, M.B. (1987) *Int. J. Rad. Appl. Instrum. B.* **14**:435-9

14. Seyedin, S., Thompson, A., Bentz, H., Rosen, D.M., McPherson, J.M., Conti, A., Siegel, N.R., Galluppi, G.R., and Piez, K.A. (1986) *J. Biol. Chem.* **261**:5693-5695

15. Proetzel, G., Pawlowski, S.A., Wiles, M.V., Yin, M., Boivin, G.P., Howles, P.N., Ding, J., Ferguson, M.W., and Doetschman, T. (1995) *Nature Genet.* **11**:409-419

16. Letterio, J.J. and Roberts, A.B. (1996) *J. Leukoc. Biol.* **59**:769-74

17. Lin, H.V., Wang, X-F., Ng-Eaton, E., Weinberg, R.A., and Lodish, H.F. (1992) *Cell* **68**:775-785

18. Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schultz, P., Heldin, C.H., and Miyazono, K. (1993) *Cell* **75**: 681-692

19. Piek, E., Heldin, C-H., and ten Dijke, P. (1999) *FASEB J.* **13**:2105-2124

20. Massague, J. and Chen, Y-G. (2000) *Genes Dev.* **14**:627-644

21. Lastres, P., Letamendia, A., Zhang, H., Rius, C., Almendro, N., Raab, U., Loepez, L.A., Langa, C., Fabra, A., Letarte, M., and Bernabeu, C. (1996) *J. Cell Biol.* **133:**1109-1121

22. Letamendia, A., Lastres, P., Botella, L.M., Raab, U., Langa, C., Velasco, B., Attisano, L., and Bernabeu, C. (1998) *J. Biol. Chem.* **273,** 33011-33019

23. Li, C., Harpson, L., Kumar, P., Bernabeu, C., and Kumar, S. (2000) *FASEB J.* **14**:55-64

24. Cheifetz, S. and Massague, J. (1991) *J. Biol. Chem.* **266**, 20767-20772

25. Dumont, N., O'Connor-McCourt, M., and Philip, A. (1995) *Mol. Cell Endocrinol.* **111**:57-66

26. Tam, B. and Philip, A. (1998) *J. Cell Physiol.* **176**:553-564

27. Tam, B., Germain, L., and Philip, A. (1998) *J. Cell Biochem.* **70**:573-586

28. Lopez-Casillas, F., Wrana, J.L., and Massague, J. (1993) *Cell* **73**:1435-1444

29. Philip, A., Hannah, R., and O'Connor-McCourt, M. (1999) *Europ. J. Biochem.* **261**:618-628

30. Yamashita, H., Ichijo, H., Grimsby, S., Moron, A., ten Dijke, P., and Miyazono, K. (1994) *J. Biol. Chem.* **269**:1995-2001

31. Guerrero-Esteo, M., Sanchez-Elsner, T., Letamendia, A., and Bernabeu, C. (2002) *J. Biol. Chem.* **277**, 29197-209

32. Komesli, S., Vivien, D., and Dutartre, P. (1998) *Eur. J. Biochem.* **254**:505-13

33. Rotzer, D., Roth, M., Lutz, M., Lindemann, D., Sebald, W., and Knaus, P. (2001) *EMBO J.* **20**:480-490

34. Docagne, F., Colloc'h, N., Bougueret, V., Page, M., Paput, J., Tripier, M., Dutartre, P., MacKenizie, E.T., Buisson, A., Komesli, S., and Vivien, D. (2001) *J. Biol. Chem.* **276**: 46243-46250

35. Choi, M.E. (1999) *Am. J. Physiol.* **276**: (Renal Physiol. 45):F88-F95

36. Lux, A., Attisano, L., and Marchuk, D.A. (1999) *J. Biol. Chem.* **274**:9984-9992

37. Attisano, L., Carcamo, J., Ventura, F., Weis, F.M., Massague, J., and Wrana, J.L. (1993) *Cell* **75**: 671

38. ten Dijke, P., Yamashita, H., Ichijo, H., Franzen, P., Laiho, M., Miyazono, K., Heldin, C.H. (1994) *Science* **264**:101-4

39. De Winter, J.P., De Vries, C.J., Van Achterberg, T.A., Ameerun, R.F., Feijen, A., Sugino, H., De Waele, P., Huylebroeck, D., Verschueren, K., and Van Den Eijden-Van Raaij, A.J. (1996) *Exp. Cell Res.* **224**:323-34

40. Johnson, D.W., Berg, J.N., Baldwin, M.A., Gallione, C.J., Marondel, I., Yoon, S.J., Stenzel, T.T., Speer, M., Pericak-Vance, M.A., Diamond, A., Guttmacher, A.E., Jackson, C.E., Attisano, L., Kucherlapati, R., Porteous, M.E., and Marchuk, D.A. (1996) *Nat. Genet.* **13**:189-95

41. Oh, S.P., Seki, T., Goss, K.A., Imamura, T., Yi, Y., Donahoe, P.K., Li, L., Miyazono, K., ten Dijke, P., Kim, S., and Li, E. (2000) *Proc. Natl. Acad. Sci. USA* **97**:2626-31

42. Goumans, M.J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P., and ten Dijke, P. (2002) *EMBO J.* **21**:1743-53

43. Robbins, J.R. and Goldring, M.B. (1997) Preparation of immortalized human chondrocyte cell lines. In: Morgan JR, Yarmush ML (eds) Methods in Molecular Medicine, vol 18. Humana Press Inc., Totowa, N.J. pp. 173-192

44. Goldring, M.B., Birkhead, J.R., Suen, L-F., Yamin, R., Mizuno, S., Glowacki, J., and Apperley, J.F. (1994) *J. Clin. Invest.* **94**:2307-2316

45. Boyd, F.T. and Massague, J. (1989) *J. Biol. Chem.* **264**:2272-8

46. Wong, S.H., Hamel, L., Chevalier, S., and Philip, A. (2000) *Eur. J. Biochem.* **267**:5550-5560

47. Piek, E., Franzen, P., Heldin, C.H., and ten Dijke, P. (1997) *J. Cell Physiol.* **173**:447-59

48. Cheifetz, S., Bassols, A., Stanley, K., Ohta, M., Greenberger, J., and Massague, J. (1988) *J. Biol. Chem.* **263**:10783-10789

49. Glick, A.B., Danielpour, D., Morgan, D., Sporn, M.B., and Yuspa, S.H. (1990) *Mol. Endocrinol.* **4**:46-52

50. MacKay, K., Robbins, A.R., Bruce, M.D., and Danielpour, D. (1990) *J. Biol. Chem.* **265**:9351-9356

51. Souchelnytskyi, S., Ronnstrand, L., Heldin, C.H., and ten Dijke, P. (2001) *Methods Mol. Biol.* **124**:107-20

52. Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C.H., Miyazono, K., and ten Dijke, P. (1997) *EMBO J.* **16**:5353-62

53. Wrana, J.L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) *Nature* **370**:341-347

54. Eickelberg, O., Centrella, M., Reiss, M., Kashgarian, M., and Wells, R.G. (2002) *J. Biol. Chem.* **277**:823-9

55. Blobe, G.C., Schiemann, W.P., Pepin, M-C., Beauchemin, M., Moustakas, A., Lodish, H.F., and O'Connor-McCourt, M.D. (2001) *J. Biol. Chem.* **276**:24627-24637

56. Zawel, L., Dai, J.L., Buckhaults, P., Zhou, S., Kinzler, K.W., Vogelstein, B., and Kern, S.E. (1998) *Mol. Cell* **1**:611-617

57. Robbins, J.R., Thomas, B., Tan, L., Choy, B., Arbiser, J.L., Berenbaum, F., and Goldring, M.B. (2000) *Arthritis Rheum.* **43**:2189-201

58. Goldring, M.B. and Berenbaum, F. (1999) *Osteoarthritis Cartilage* **7**:386-88

59. Kokenyesi, R., Tan, L., Robbins, J.R., and Goldring, M.B. (2000) *Arch. Biochem. Biophys.* **383**:79-90

60. Loeser, R., Sadiev, S., Tan, L., Goldring, M.B. (2000) *Osteoarthritis Cartilage* **8**:96-105

61. Chen, F. and Weinberg, R.A. (1995) *Proc. Natl. Acad. Sci. USA* **92**:1565-9

62. Yang, X., Chen, L., Xu, X., Li, C., Huang, C., and Deng, C-X. (2001) *J. Cell Biol.* **153**:35-46

63. Fukumura K, Matsunaga S, Yamamoto T, Nagamine T, Ishidou Y, Sakou T 1998 Immunolocalization of transforming growth factor-betas and type I and type II receptors in rat articular cartilage. Anticancer Res 18:4189-4194.

64. Serra, R., Johnson, M., Filvaroff, E.H., LaBorde, J., Sheehan, D.M., Derynck, R., Moses, H.L. (1997) *J. Cell Biol.* **139**:541-52

65. Fahlgren, A., Andersson, B., and Messner, K. (2001) *Osteoarthritis Cartilage* **9**:195-202

66. Fawthrop, F.W., Frazer, A., Russell, R.G., and Bunning, R.A. (1997) *Br. J. Rheumatol.* **36**:729-34

67. Iqbal, J., Dudhia, J., Bird, J.L., and Bayliss, M.T. (2000) *Biochem. Biophys. Res. Commun.* **274**:467-71

68. Laiho, M., Decaprio, J., Ludlow, J.W., Livingston, D.M., and Massague, J. (1990) *Cell* **62**:175-185

69. Wells, R.G., Gilboa, L., Sun, Y., Liu, X., Henis, Y.I., and Lodish, H.F. (1999) *J. Biol. Chem.* **274**:5716-5722

70. Chen, X., Weisberg, V., Fridmacher, M., Watanabe, G., Naco, G., and Whitman, M. (1997) *Nature* **389**:85-89

71. Sun, L. and Chen, C. (1997) *J. Biol. Chem.* **272**:25367-25372

72. Brown, C.B., Boyer, A.S., Runyan, R.B., and Barnett, J.V. (1999) *Science* **283,** 2080-2082

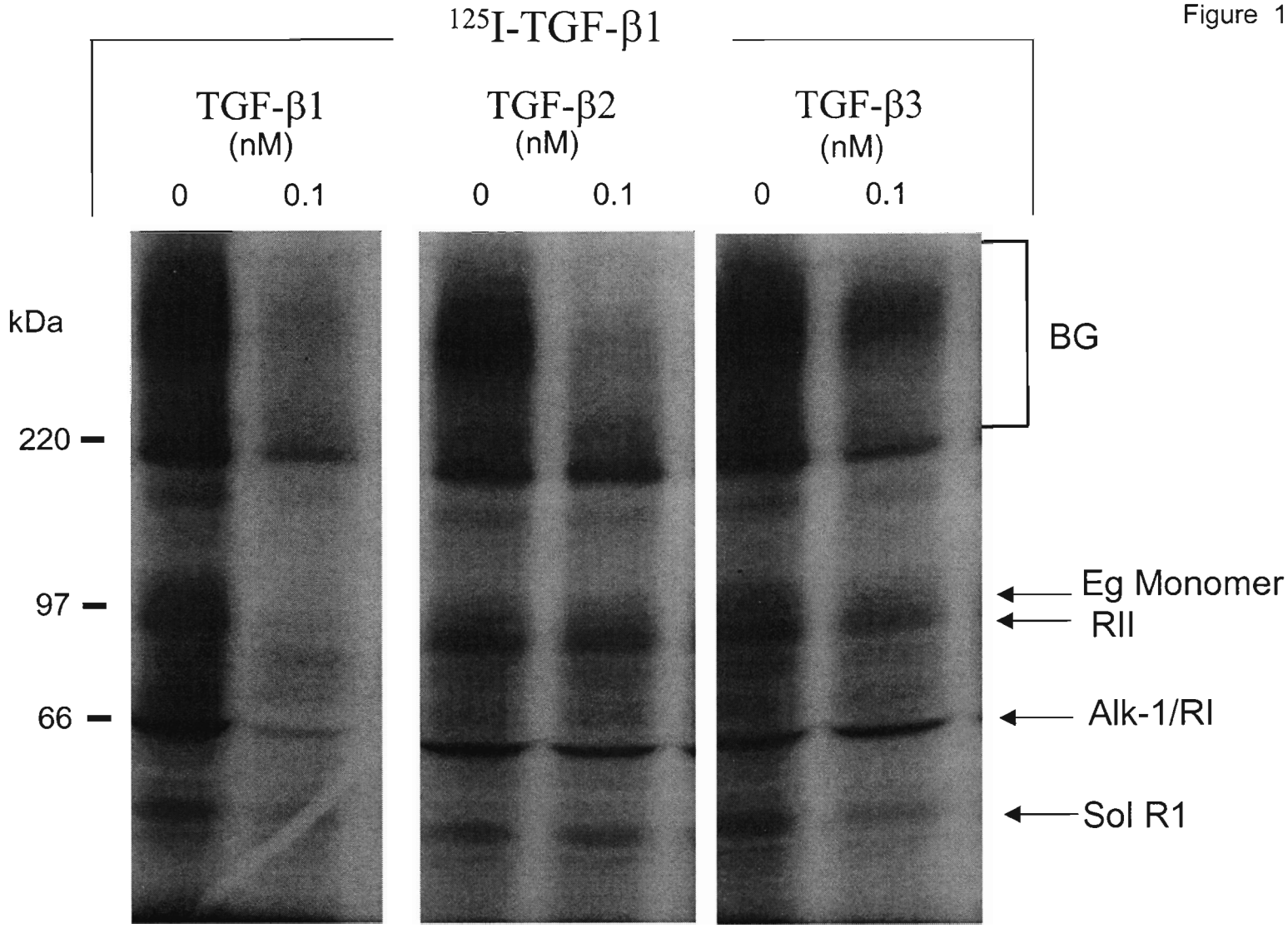
73. Lopez-Casillas, F., Payne, H.M., Andres, J.L., and Massague, J. (1994) *J. Cell Biol.* **124**, 557-568

74. Chen, Y.G. and Massague, J. (1999) *J. Biol. Chem.* **274**:3672-7

75. Massague, J. (1998) *Ann Rev Biochem* **67**:753-791

76. Lewis, K.A., Gray, P.C., Blount, A.L., Macconell, L.A., Waiter, E., Bilezikjian, L.M., and Vale, W. (2000) *Nature* **404,** 411-414

Figure 1


125I-TGF-β1
TGF-β1
(nM)
0
0.1
TGF-β2
(nM)
0
0.1
TGF-β3
(nM)
0
0.1
kDa
220
97
66
BG
Eg Monomer
RII
Alk-1/RI
Sol R1

204

Figure 2

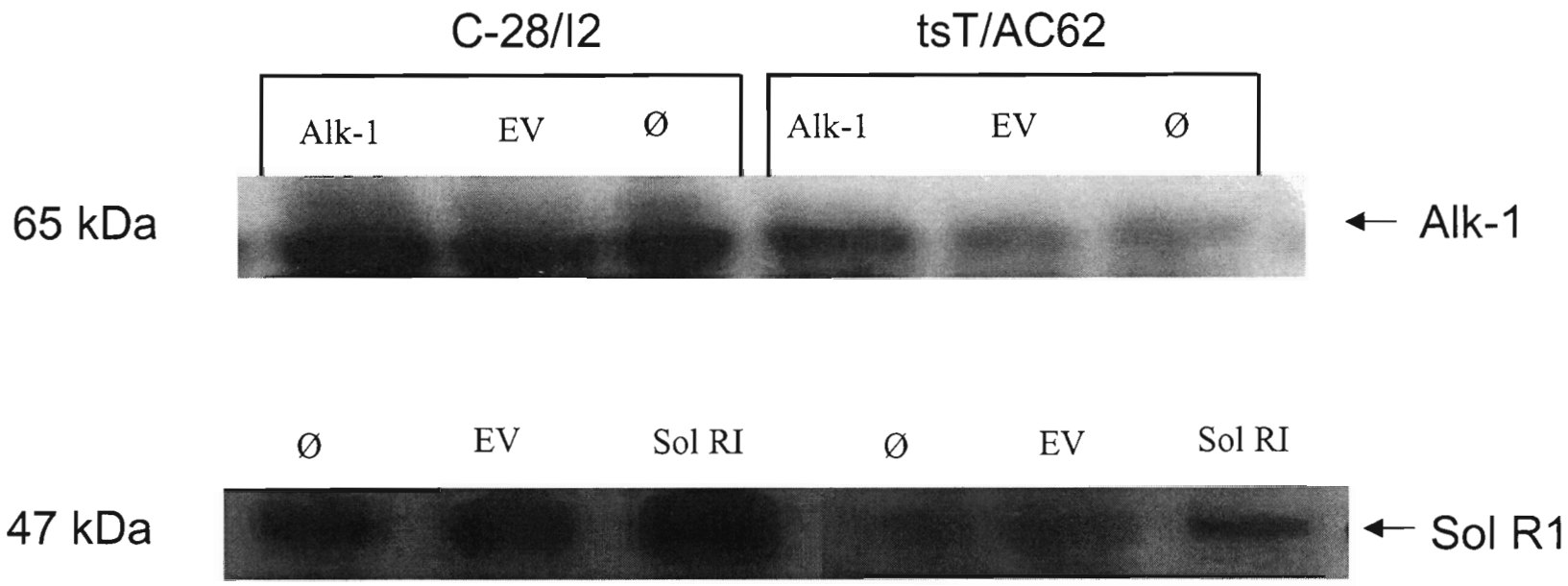
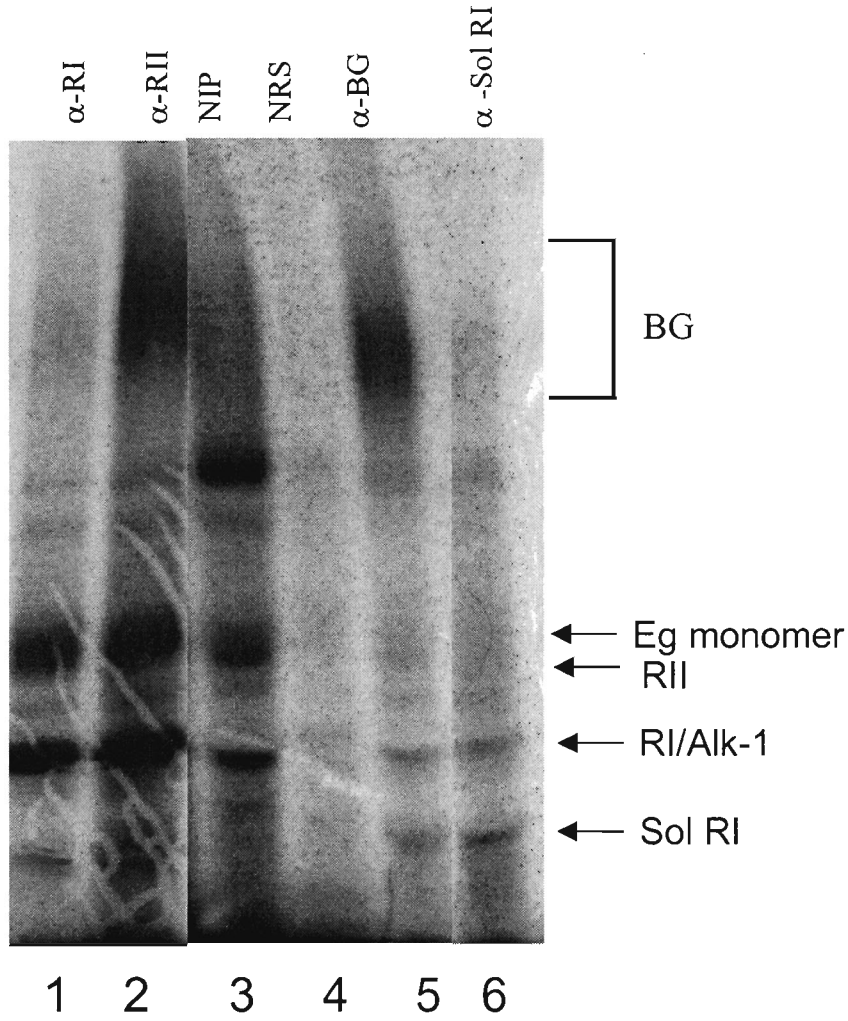

C-28/I2
tsT/AC62
Alk-1
EV
Ø
Alk-1
EV
Ø
65 kDa
Alk-1
Ø
EV
Sol RI
Ø
EV
Sol RI
47 kDa
Sol R1

Figure 3A




206

Figure 3B

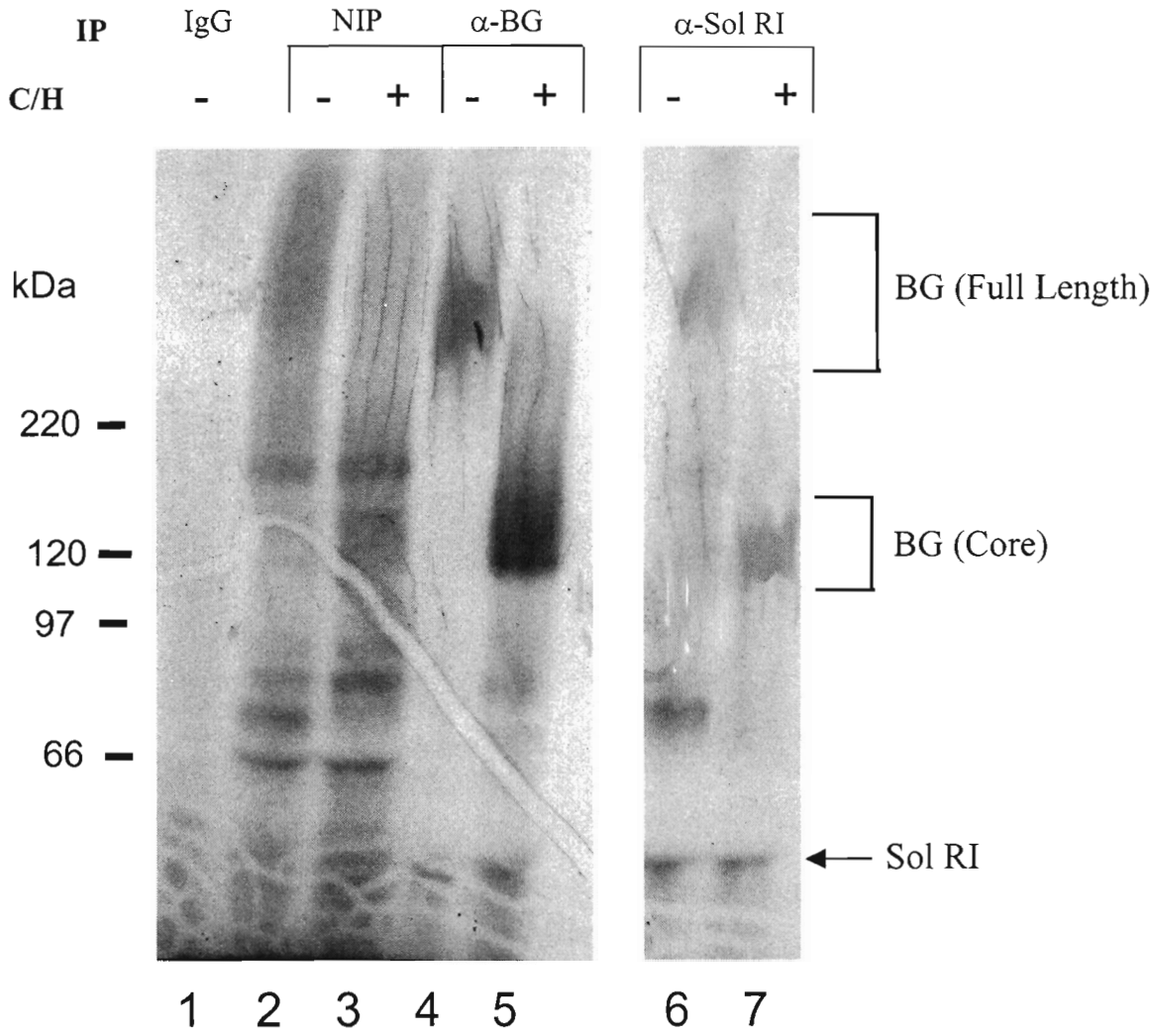

IP
IgG
NIP
α-BG
α-Sol RI
C/H
-
-
+
-
+
-
+
kDa
220
120
97
66
BG (Full Length)
BG (Core)
Sol RI
1
2
3
4
5
6
7

Figure 3C

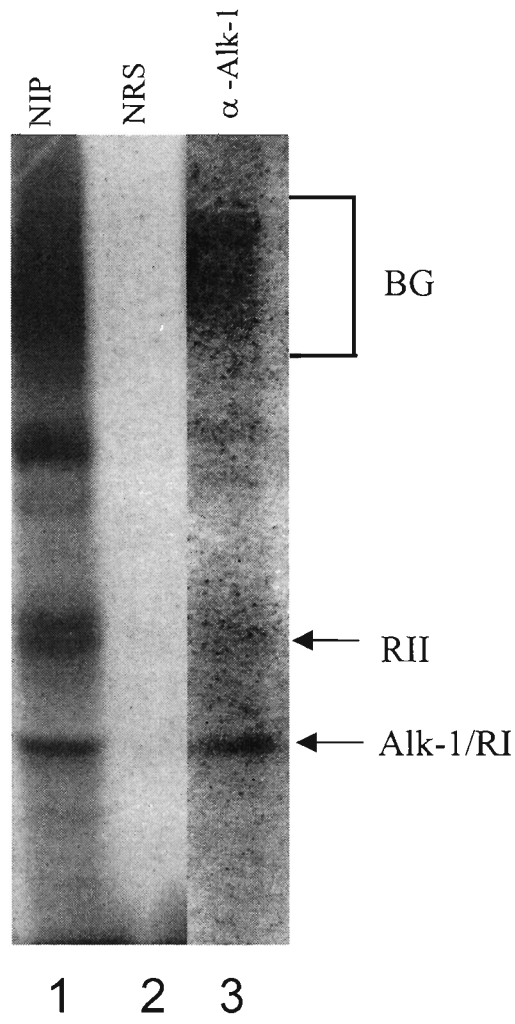


Figure 4A

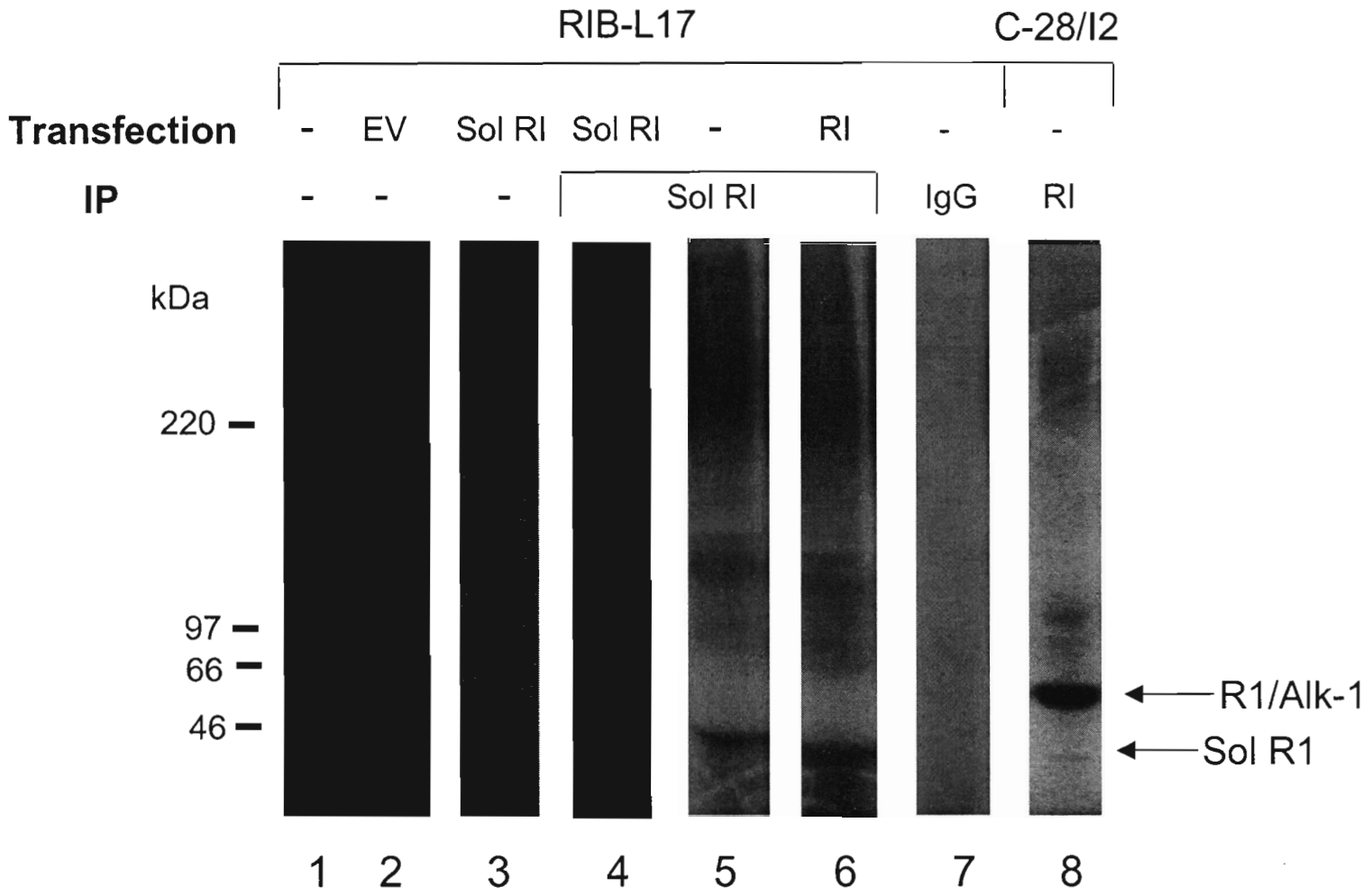
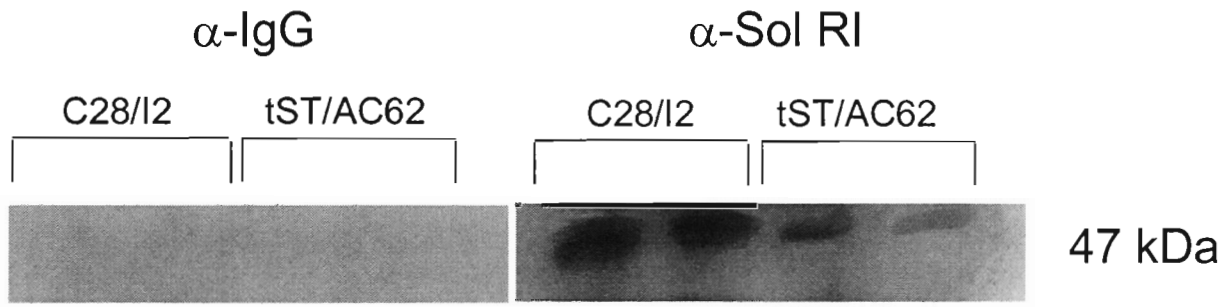

RIB-L17
C-28/I2
Transfection
-
EV
Sol RI
Sol RI
-
RI
-
-
IP
-
-
-
Sol RI
IgG
RI
kDa
220
97
66
46
R1/Alk-1
Sol R1
1
2
3
4
5
6
7
8

Figure 4B




210

Figure 5A

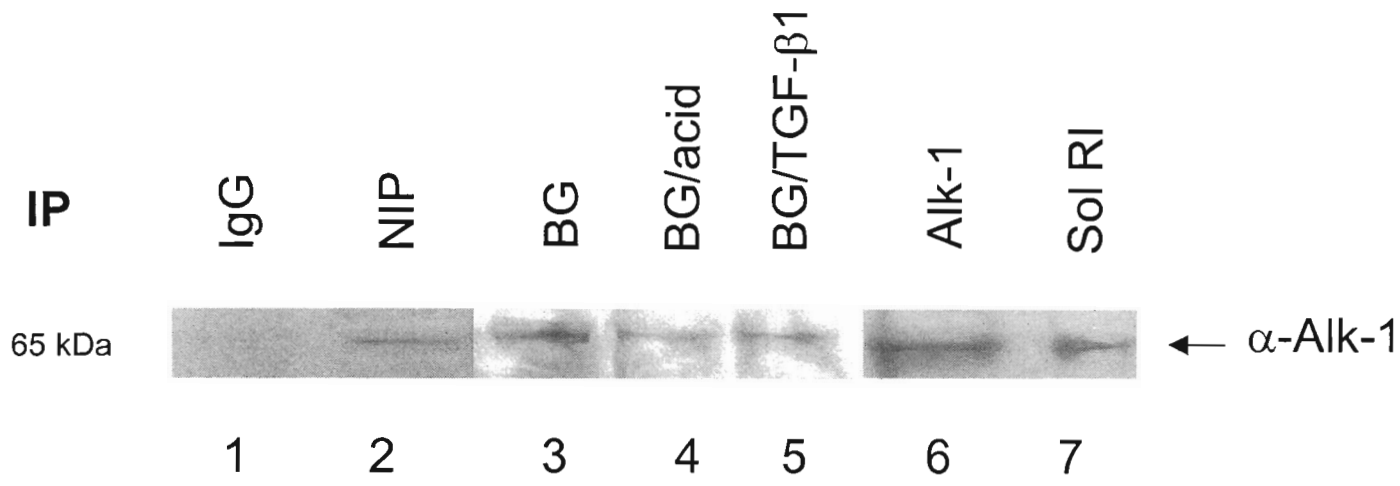


Figure 5B

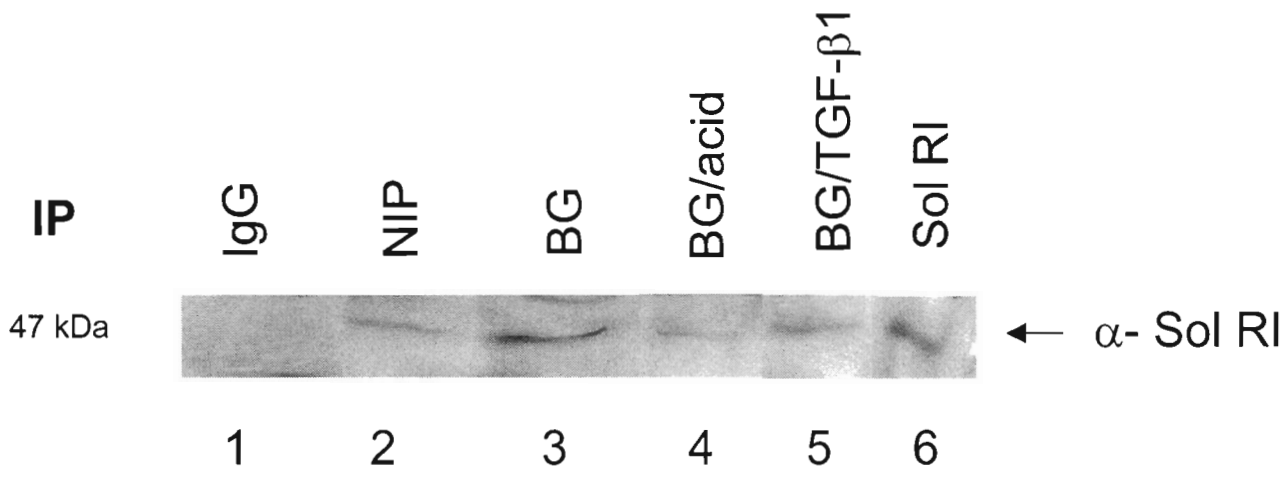


Figure 5C

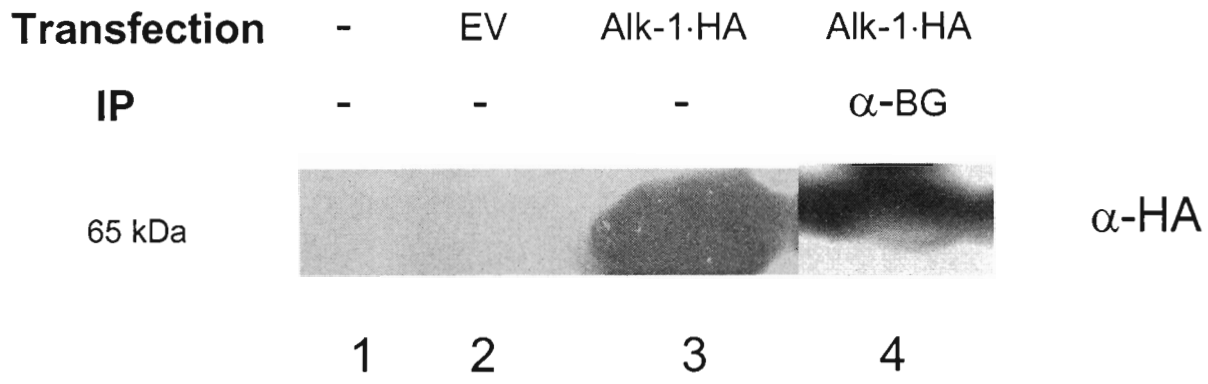


Figure 6A

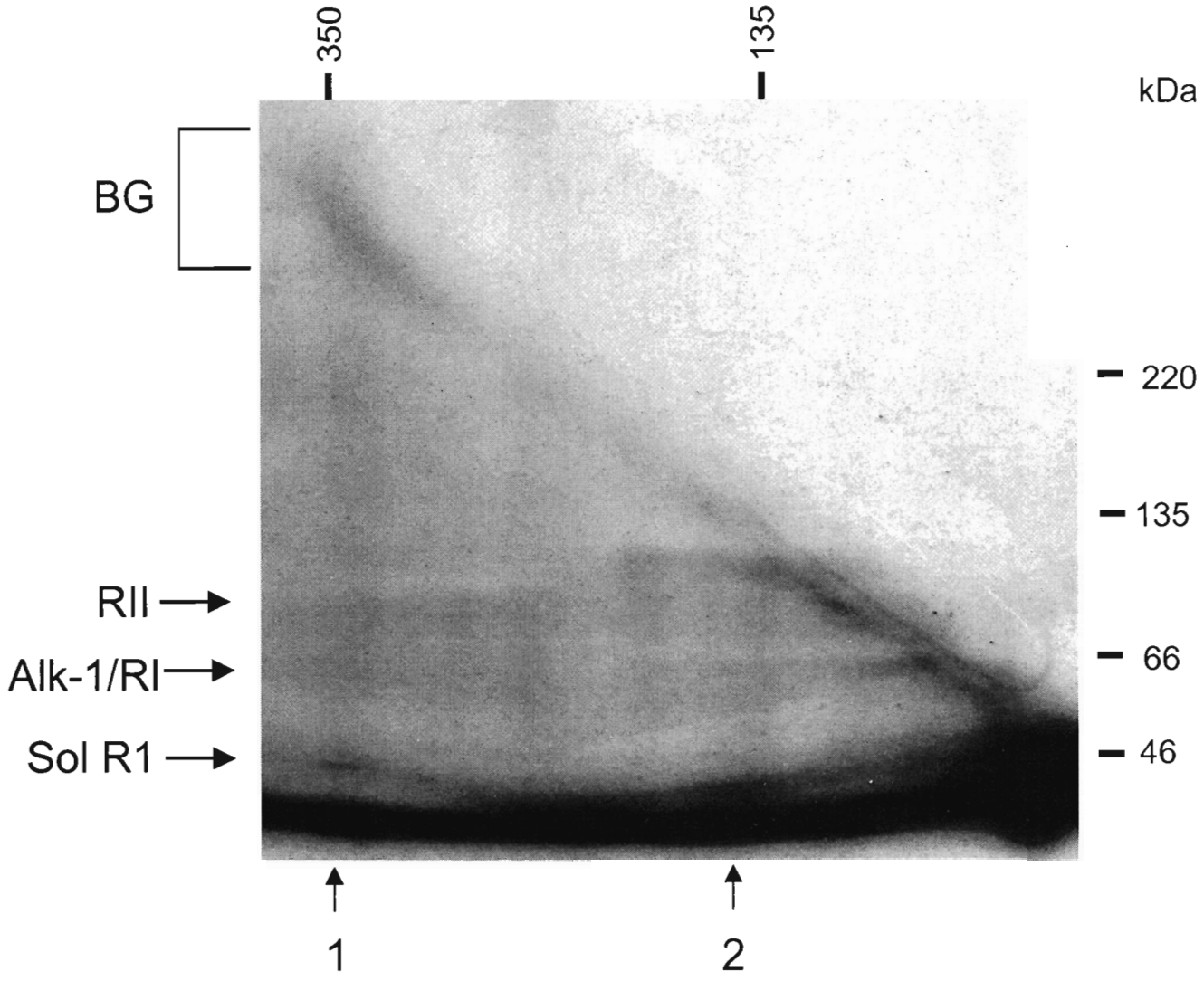


Figure 6B

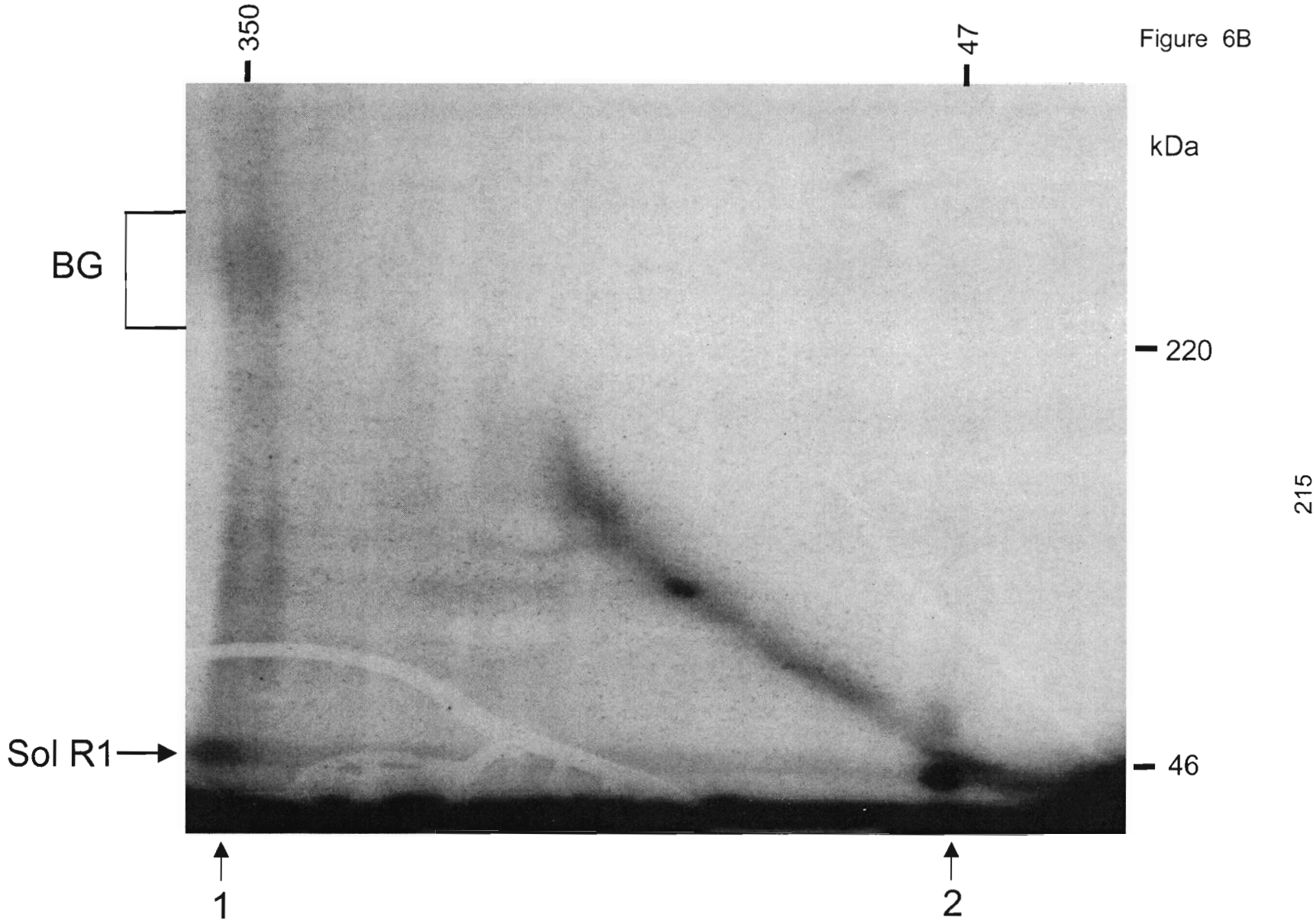


Figure 6C

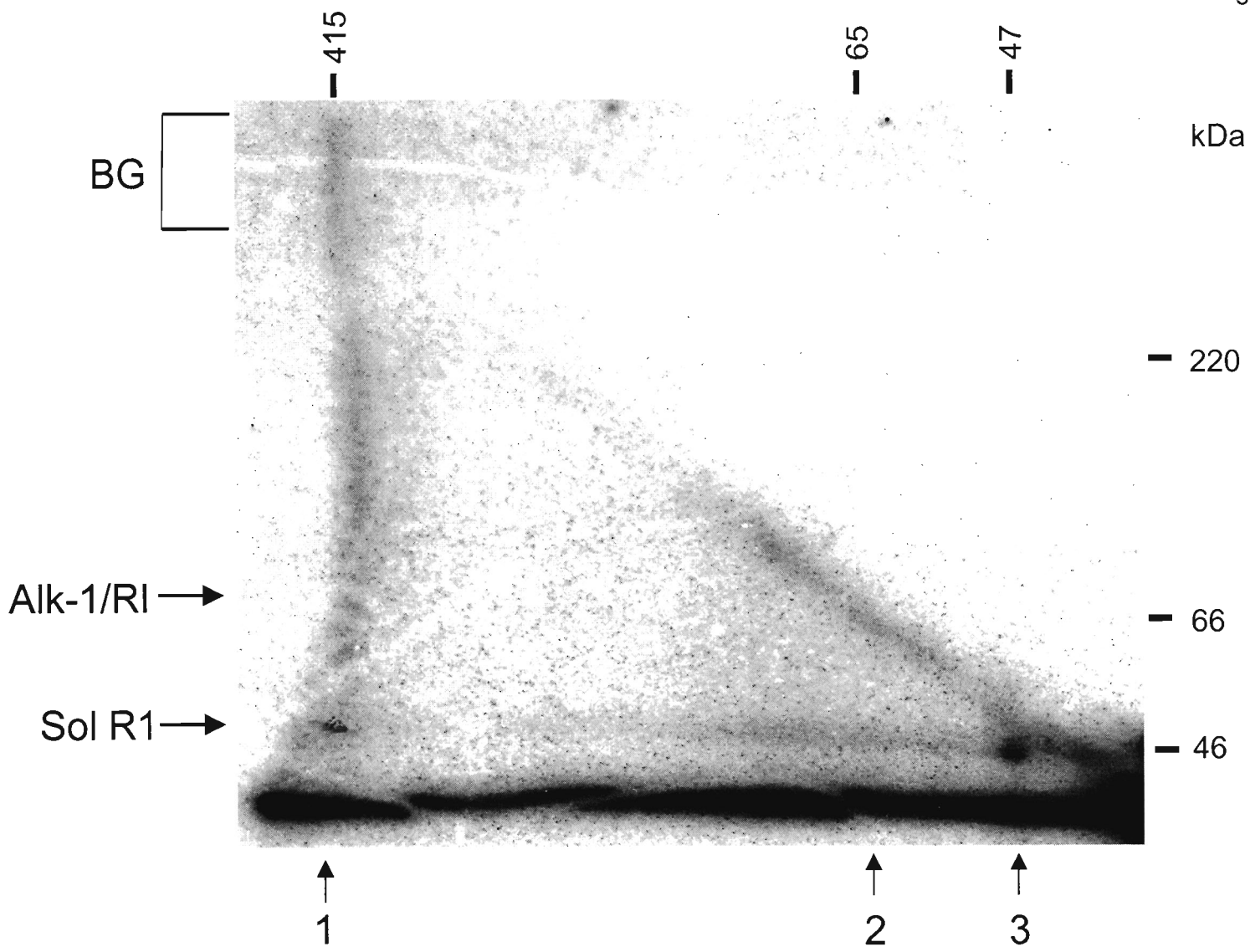


Figure 6D

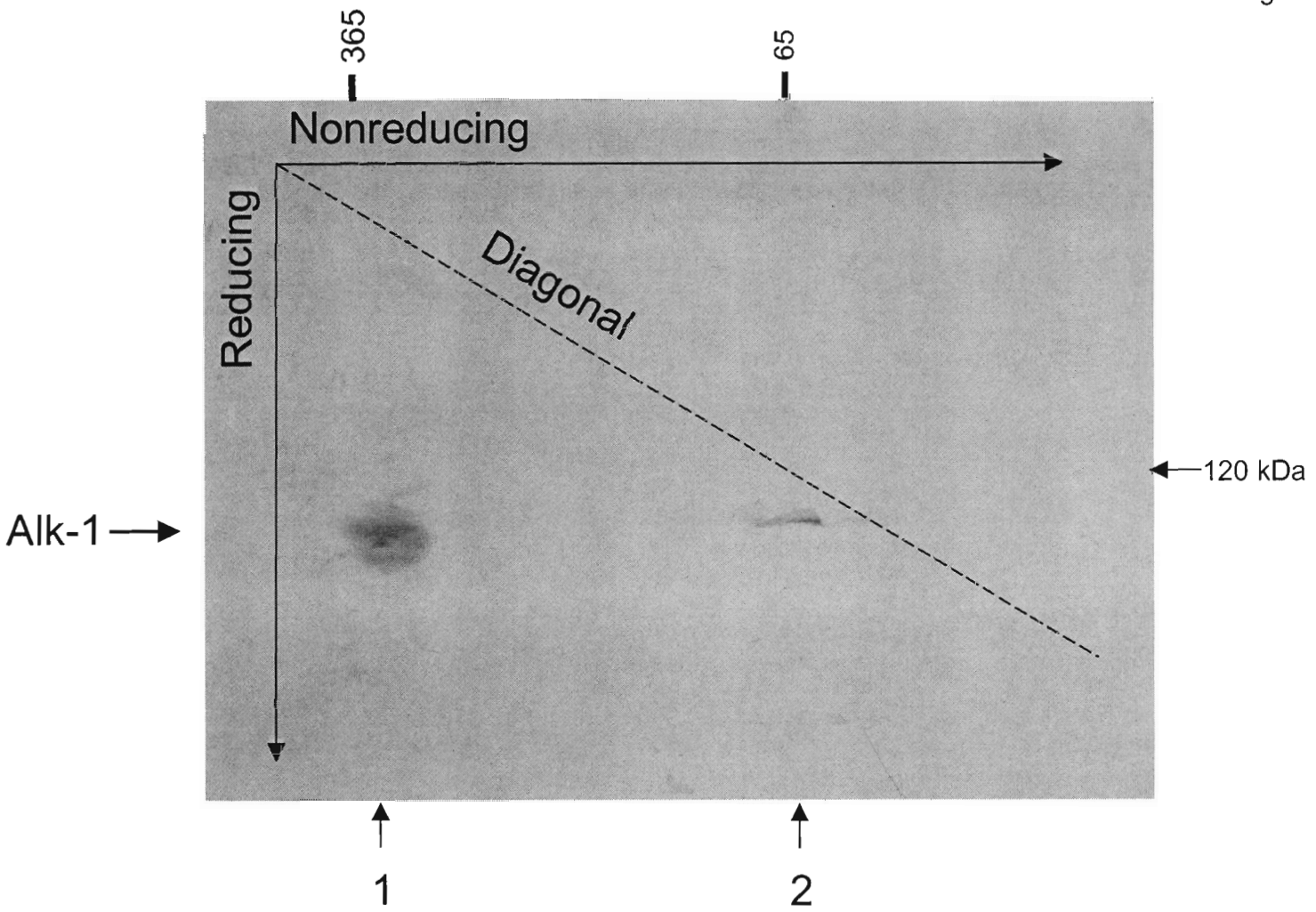
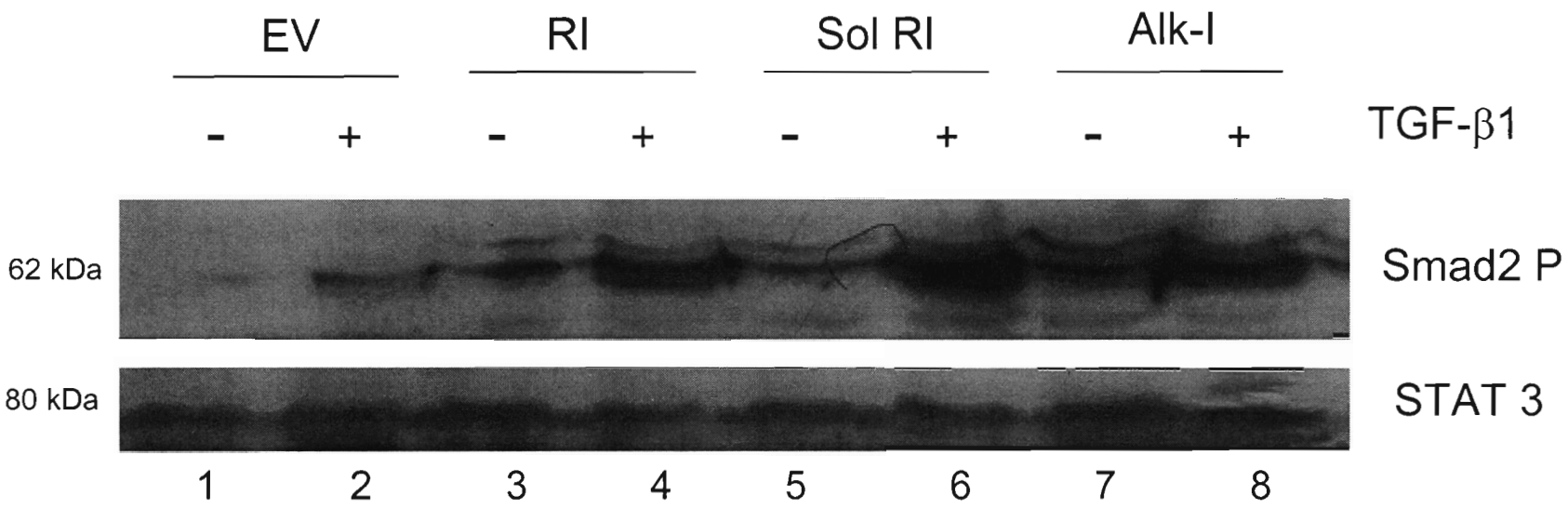


Figure 7A




218

Figure 7B

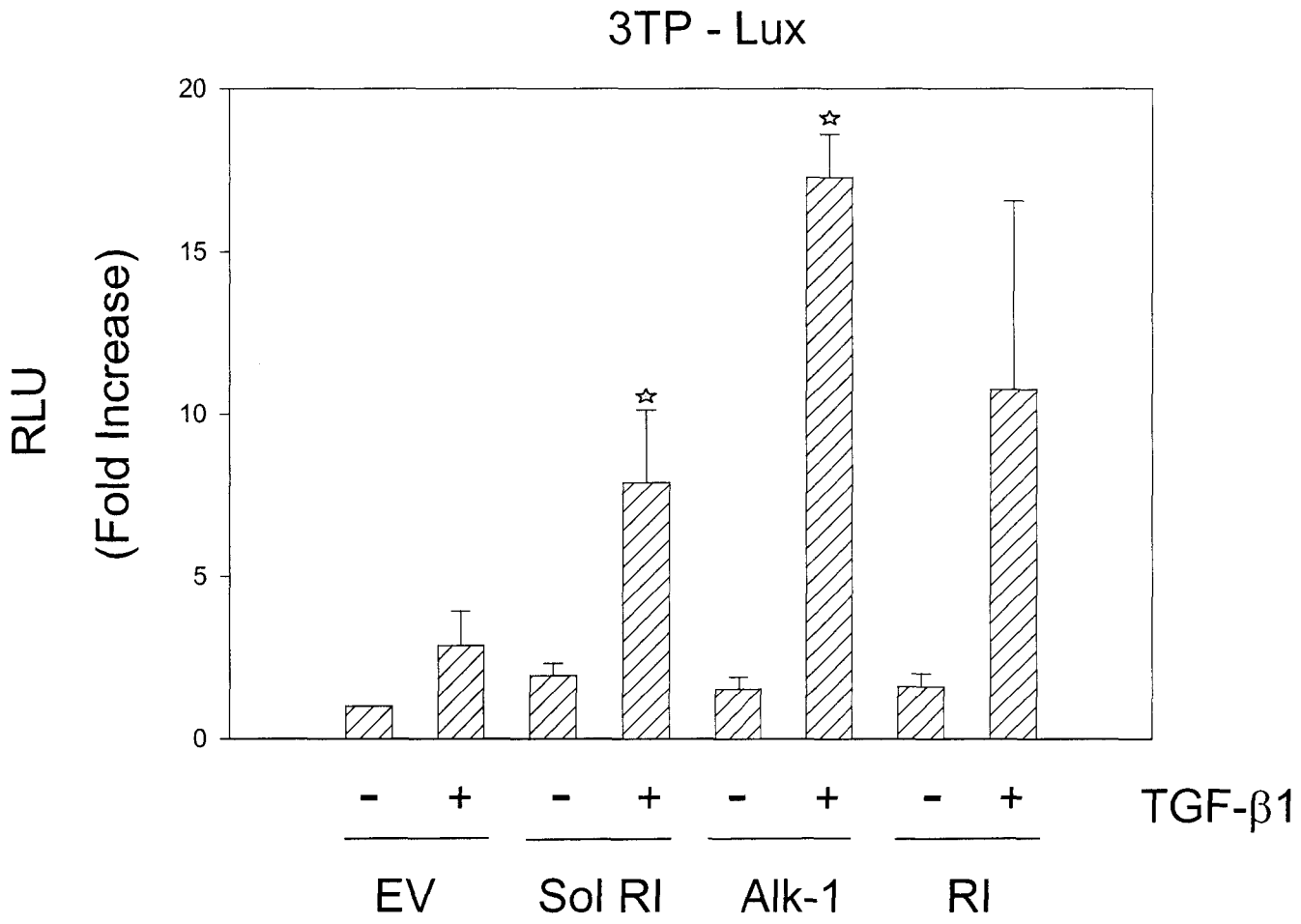
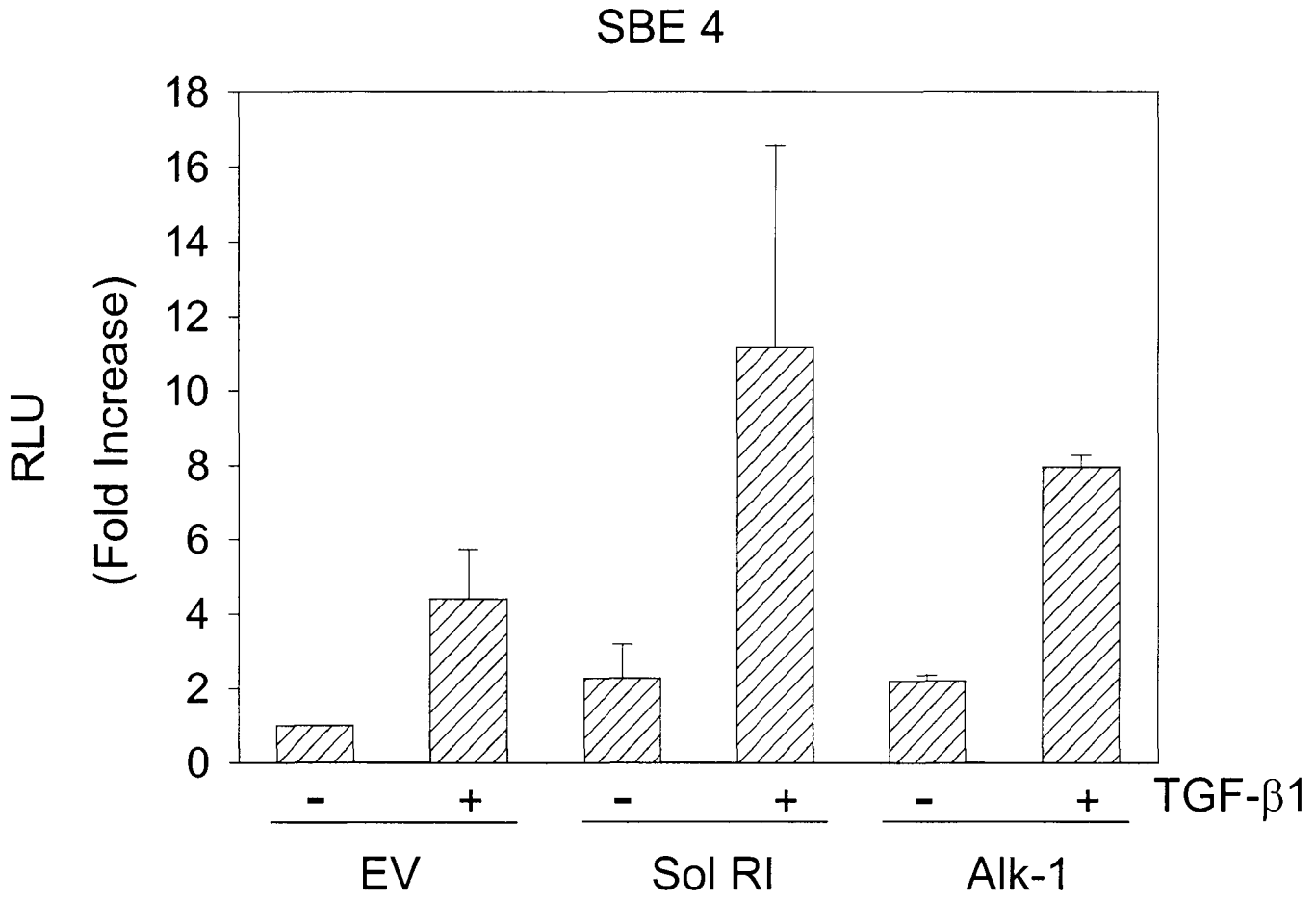
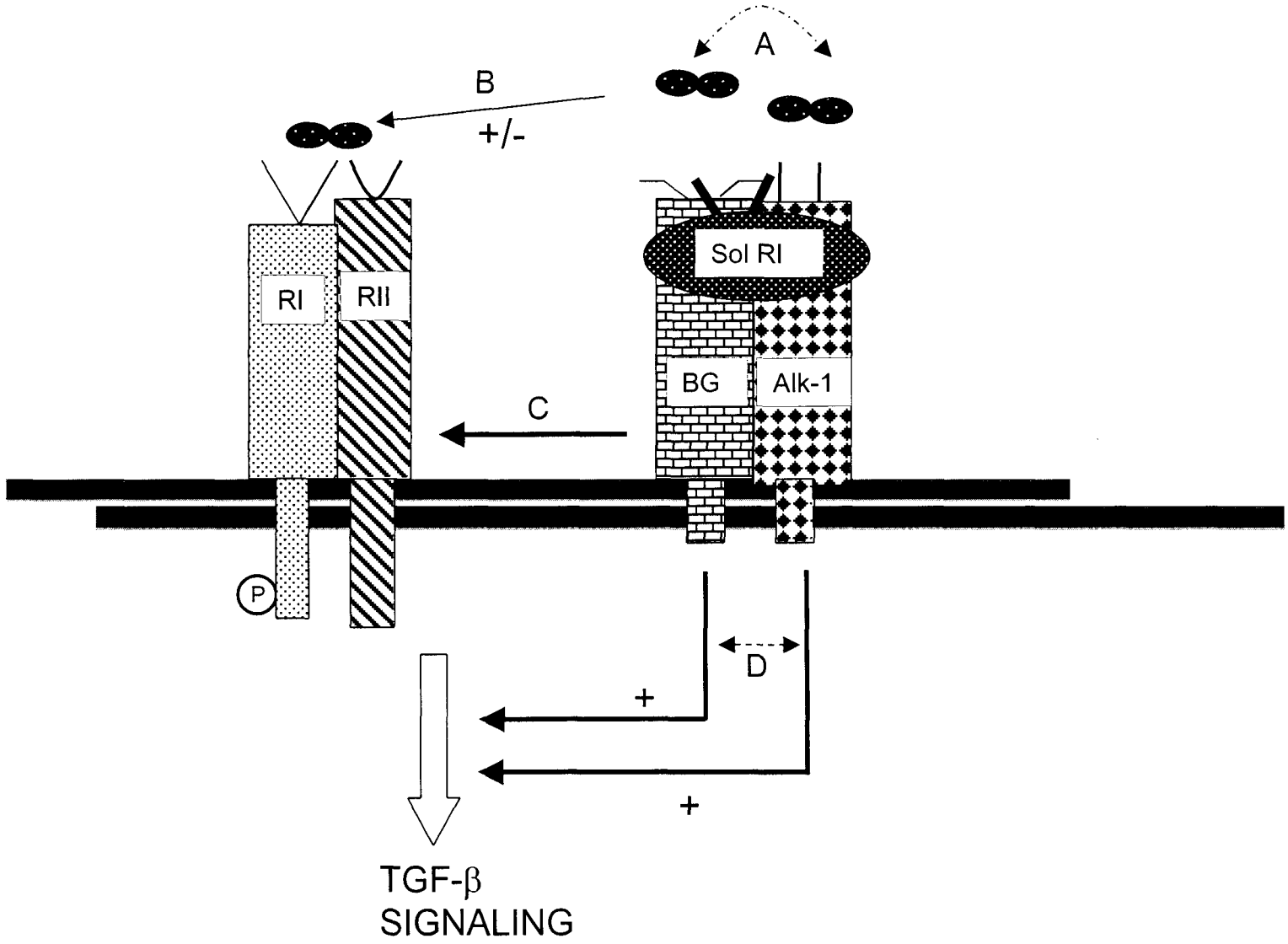


Figure 7C




220

Figure 8




221

# CHAPTER 3

# BRIDGE

Confirmation of the type II signaling receptor (RII) independent nature of the endoglin-betaglycan association and the identification of further structural determinants of this interaction. The novel discovery of endoglin's ability to bind the TGF-β1 isoform independently of RII but requiring betaglycan and endoglin's inhibition of the TGF-β signaling cascade.

## CHAPTER 3

BRIDGING DOCUMENT

### Introduction and Rationale

TGF-β receptor heteromeric complex formations on the surface of cells are often complicated biochemical interactions with specific structural determinants. Previously, endoglin and betaglycan were shown to form a heteromeric complex on the surface of human chondrocytes in the presence of ligand and ligand independent manner. Studies using 293 cells (lacking RII) suggested that this heteromeric complex forms in the absence of RII (manuscript 1). Therefore, it was important to confirm the RII independent nature but also to elucidate the structural determinants governing the betaglycan-endoglin association. It was previously considered that the short cytoplasmic tails of betaglycan and endoglin were devoid of catalytic activity (Cheifetz et al 1992, Lopez-Casillas et al 1993) but recent evidence suggests that endoglin is able to modulate the phosphorylation of the TGF-β signaling receptors (Geurerro-Esteo et al 2002). However, regulation of TGF-β signaling by endoglin and betaglycan varies among different cells with both receptors seen to inhibit and enhance responses (Lastres et al 1996, Li 1999 and 2000a, Letamendia et al 1998a, Blobe et al 2001). The current TGF-β signaling paradigm dictates betaglycan facilitating ligand binding by the signaling receptors, playing an important role for the -β2 ligand as RII has low affinity for TGF-β2. Endoglin, however, is thought not to bind ligand in the absence of RII and its role in signaling remains unclear. The specifics of their association and the role of these two receptors in human chondrocytes remained to be determined.

### Hypotheses

(1) that the endoglin–betaglycan heteromeric complex on the chondrocyte surface can occur independently of RII and may be a core protein interaction

(2) that endoglin and betaglycan have distinct roles in regulating TGF-β signaling in human chondrocytes

**Objectives**

(i) to confirm that the complex formation between endoglin and betaglycan on human chondrocytes occurs in the absence of RII

(ii) to determine the structural requirements of the endoglin-betaglycan heteromeric complex on these cells

(iii) to elucidate the role of endoglin and betaglycan in the modulation of TGF-β signaling in chondrocytes

**Summary of Manuscript Findings**

In the third manuscript, endoglin is shown to be expressed on DR26, L6, and 293 cells but at endogenous levels less than seen for human microvascular endothelial cells or chondrocytes. Most importantly, for the first time, endoglin is demonstrated to bind TGF-β1 in the absence of RII, and that not only is this binding facilitated by but requires betaglycan. The endoglin-betaglycan heteromeric complex is shown to occur independently of RII and, in addition, independently of betaglycan's cytoplasmic domain or carbohydrate side chains. Lastly, the overexpression of endoglin is seen to inhibit whereas betaglycan enhances TGF-β1, β2, and β3 responses in human chondrocytes.

**Conclusions**

Prior to the investigations seen in the following manuscript (manuscript 3) several novel TGF-β receptors on human chondrocytes were identified and confirmed to form associations

at the cells surface. Of these heteromeric receptor complexes, the Sol RI and betaglycan complex was shown to occur in the absence of betaglycan's GAG chains and the endoglin-betaglycan complex in the presence or absence of ligand. That this endoglin-betaglycan association forms independently of RII has been confirmed and that neither the GAG chains nor betaglycan's cytoplasmic domain are necessary for this interaction has been demonstrated. Investigations which illustrated the presence of endoglin on DR26, 293, and L6 myoblasts led to more important findings. For the first time, endoglin's ability to bind TGF-β1 in the absence of RII and that this binding is not only enhanced by betaglycan but also requires its presence is shown. In addition, evidence that endoglin inhibits whereas betaglycan enhances TGF-β1, -β2, and -β3 signaling in human chondrocytes is provided. Endoglin is not known to bind TGF-β2; despite this, inhibition of TGF-β2 signaling by endoglin was evident. This suggests that endoglin may be mediating this inhibition of TGF-β signaling through direct interactions with signaling receptors or alternatively, through its heteromeric complex formation with betaglycan. Taken together, this information provides new insights into the TGF-β signaling cascade and the passive manner in which accessory receptors were once thought to participate in signaling is no longer an acceptable theory.

## CONTRIBUTION OF AUTHORS

# MANUSCRIPT THREE

Characterization of endoglin-betaglycan heteromerization: TGF-β binding by endoglin requires betaglycan but not the type II receptor

**WL Parker:** experimental designs, procedures, and analysis, preparation of figures and text

**GC Blobe:** will contribute manuscript editorial assistance, donated L6 myoblast cell lines including parent cells and stable transfectants as well as a full length betaglycan construct

**A Philip:** assistance with experimental design and analysis and manuscript editorial assistance

# Characterization of endoglin-betaglycan heteromerization: TGF-β binding by endoglin requires betaglycan but not the type II TGF-β receptor

Wendy L. Parker[1], Gerald C. Blobe[2], and Anie Philip[1]*

[1]Division of Plastic Surgery, Department of Surgery, McGill University, Montreal, Quebec, Canada

[2]Department of Medicine, Duke University Medical Center, Durham, North Carolina, USA

*Correspondence to: Anie Philip, Division of Plastic Surgery, Montreal General Hospital, 1650 Cedar Avenue, Room C9-177, Montreal, Quebec, Canada H3G 1A4.

Telephone: (514) 937 1934 ext 44533; Fax: (514) 934 8289

E-mail: anie.philip@mcgill.ca

Running Title: Endoglin binds TGF-β1 without RII

Acknowledgements

We thank Genzyme Inc. (Framingham, MA) for the gift of TGF-β1 and TGF-β2; Dr. M. O'Connor McCourt (Montreal, Quebec) for the gift of plasmids encoding the type II and type I TGF-β receptors and betaglycan; Dr. K. Miyazono (Tokyo, Japan) for the anti-endoglin antibody; Dr. Bert Vogelstein (Baltimore, ML) for the SBE 4 expression vector; Dr. S. Souchelnytskyi (Uppsala, Sweden) for the anti-betaglycan (Get 1) and anti-phospho Smad 2 antibodies; Dr. C. Bernabeu (Madrid, Spain) for the L-endoglin plasmid; Dr. J. Massague (New York, NY) for DR26 cells; Dr. F.W. Ades and Dr. T.J. Lawley (Atlanta, GA) for the human microvascular endothelial cells (HMEC-1); Dr. Rik Derynk (San Francisco, CA) for the plasmid encoding the dominant negative form of the type II TGF-β receptor (XF120,DNRII); Dr. Rebecca Wells (New Haven, Connecticut) for the plasmids encoding cytoplasmically and GAG deficient betaglycan; and Dr. Mary Goldring (Boston, MA) for the C-28/I2 immortalized human chondrocytes.

Summary

TGF-β's ability to regulate almost all aspects of the tissue repair process has incited interest in its therapeutic use to promote cartilage regeneration. Despite this, the mechanism of action of TGF-β in human chondrocytes and in particular its receptor expressions and interactions have been poorly defined. Endoglin and betaglycan are accessory TGF-β receptors and the current TGF-β signaling paradigm indicates that endoglin is unable to bind ligand in the absence of the type II signaling receptor (RII). Endoglin's role in modulating TGF-β response is uncertain. Previously, we have shown that endoglin forms a heteromeric complex with betaglycan. In the present study we confirm the RII independent nature of this interaction and demonstrate it occurs in the absence of betaglycan's glycosaminoglycan (GAG) chains and cytoplasmic domain. More importantly, we show for the first time that endoglin is able to bind ligand in the absence of RII but requires the presence of betaglycan and paradoxically that endoglin inhibits whereas betaglycan enhances TGF-β signaling in human chondrocytes. Taken together these novel findings further implicate accessory TGF-β receptors as key modulators of TGF-β action. The endoglin-betaglycan complex may redefine the delicate balance of TGF-β signaling critical in regulating cartilage repair and regeneration.

**Key Words**: chondrocytes, TGF-β receptors, TGF-β signaling, endoglin, betaglycan

## INTRODUCTION

Tissue repair is a complex programmed biologic process that involves cell proliferation and differentiation, neovascularization and extracellular matrix deposition. TGF-β[1] has the potential to regulate almost all aspects of the tissue repair process (1) and of the myriad of growth factors that have been studied in the context of tissue healing it has the broadest spectrum of effects (2-4).

TGF-β is a member of a large family of multifunctional proteins intricately involved in growth, differentiation, and development (5-9) and was described initially as "cartilage inducing factor" (4). Three distinct isoforms of TGF-β (TGF-β 1, 2, and 3) which are encoded by distinct genes have been described in mammals (6). TGF-β is secreted in a latent form that requires activation before it can bind to its receptors. The current model of TGF-β signaling invokes a pair of transmembrane serine/threonine kinases, known as the type I (RI) and type II (RII) receptors which are present in almost all cell types analyzed (10). According to this model RI does not bind TGF-β in the absence of RII. The binding of TGF-β to RII, a constitutively active kinase, results in the recruitment and phosphorylation of RI in its 'GS sequence' upstream from the kinase domain, resulting in its concomitant activation. Recent evidence indicates that accessory receptors are important regulators of TGF-β action. Expression of TGF-β accessory receptors such as betaglycan (the type III receptor), endoglin and glycosyl phosphatidylinositol (GPI)-

---

[1] The abbreviations used are: TGF-β, transforming growth factor beta; PAI – 1, plasminogen activator inhibitor-1; RI, TGF-β type I receptor; RII, TGF-β type II receptor; Alk-1, Activin Receptor-like kinase-1; Sol RI, soluble type I TGF-β receptor; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum; DMEM, Dulbecco's minimal essential medium; dPBS, Dulbecco's phosphate buffered saline; BSA, bovine serum albumin; β-gal, beta-galactosidase; GPI, glycosyl phosphatidylinositol.

anchored TGF-β binding proteins have been shown to regulate TGF-β signaling and responses in various cell types (11-14). Betaglycan binds all three TGF-β isoforms (15,16) while endoglin has been shown to bind TGF-β1 and TGF-β3, but not TGF-β2 (11,17). Endoglin and betaglycan are approximately 70% homologous. Their short cytoplasmic tails were previously thought to be devoid of catalytic activity (15,18) but recently endoglin has been shown to be intricately involved in phosphorylation of and by the TGF-β signaling receptors (19). Betaglycan is a proteoglycan which facilitates ligand binding by the signaling receptors (15,18). We have demonstrated that RII and betaglycan interact at their cytoplasmic domains in a manner dependent on the RII kinase activity and its ability to autophosphorylate resulting in phosphorylation of betaglycan's cytoplasmic tail (20). Although endoglin has also been shown to form a complex with RII (17), its role in TGF-β signaling is poorly understood. When overexpressed in myoblasts, endoglin inhibits while betaglycan enhances TGF-β responses (11,21). Endoglin has also been reported to antagonize TGF-β action in human microvascular endothelial cells (22,23). The current signaling paradigm is that endoglin cannot bind ligand in the absence of the respective type II ligand binding receptor (11,24). Recently we identified the presence of endoglin on human chondrocytes and its complex formation with betaglycan occurring in the presence of ligand and ligand independent manner. In addition, utilizing 293 cells (severely deficient in RII) we provided evidence that this association may occur independently of RII.

In the present report we further characterize the nature and significance of the endoglin-betaglycan association and the influence of this association on ligand binding using cell lines of varying receptor status. We demonstrate that endoglin is expressed endogenously

on DR26, L6 myoblast, and 293 cells at levels comparable with that of human microvascular endothelial (HMEC-1) cells and chondrocytes (C-28/I2). We show that TGF-β1 binding by endoglin requires the presence of betaglycan and can be enhanced by its overexpression. More importantly, we refute the current TGF-β signaling paradigm and demonstrate for the first time that endoglin binds TGF-β1 independently of RII. We present the association of endoglin and betaglycan in DR26 cells (lacking RII) and in chondrocytes overexpressing the dominant negative form of RII, which abolishes TGF-β signaling, confirming that this accessory receptor association does occur in a RII independent manner and that endoglin binds TGF-β in the absence of RII. Furthermore we provide evidence that TGF-β1 binding by endoglin requires the presence of betaglycan. We also show that the endoglin-betaglycan heteromeric complex occurs independently of betaglycan's cytoplasmic domain or carbohydrate side chains. Lastly, we demonstrate that overexpression of endoglin inhibits whereas betaglycan enhances TGF-β1, β2, and β3 responses in human chondrocytes. Taken together, these findings suggest that endoglin alone, or as part of the endoglin-betaglycan heteromeric receptor complex, likely plays an intricate role in regulating the fine balance of TGF-β signaling. These studies may provide important information to determine endoglin's role in modulating TGF-β action in human chondrocytes.

## EXPERIMENTAL PROCEDURES

### Cell Culture

The immortalized human chondrocyte cell line (C-28/I2; obtained from Dr. Mary Goldring, Beth Israel Deaconness Medical Center, Harvard Institutes of Medicine, Boston, MA) has been described previously (25,26). The C-28/I2 cell line was developed

using chondrocytes isolated from juvenile human costal cartilage by retroviral infection with SV-40 large T antigen. The 293 cells (CRL-1573) were obtained from the American Type Culture Collection (Rockville, MD) and the human microvascular endothelial cells (HMEC-1) were a gift from Dr. F.W. Ades and Dr. T.J. Lawley (National Center for Infectious Disease; Atlanta, GA). DR26 cells, stable mutants devoid of the type II TGF-β signaling receptor generated from mink lung epithelial cells, were a gift from Dr. J. Massague (Howard Hughes Medical Institute; New York, NY; 27). L6 myoblast have been extensively characterized (20,28-30). The L6 parent cells lack betaglycan, whereas stable transfectants have been developed to express either full length betaglycan or a form deficient in its cytoplasmic domain (lacking residues 812-853) (20,28-30). The C-28/I2, 293, L6 parent myoblasts and DR26 cells were grown in DMEM/Ham's F12 (1:1, v:v) containing 10% fetal bovine serum (FBS) and 100U/ml penicillin, 100μg/ml streptomycin and 0.25μg/ml amphoteracin (all from Invitrogen Life Technologies; Burlington ON). HMEC-1 were grown in MCDB 131 (Invitrogen Life Technologies) containing 10% fetal bovine serum (FBS) and 100U/ml penicillin, 100μg/ml streptomycin and 0.25μg/ml amphoteracin (all from Invitrogen Life Technologies). L6 cytoplasmic deficient and full length betaglycan stable mutants were cultured in DMEM (1x glutamine), 10% FBS , 100U/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphoteracin, and .3mg/ml G418 (Geneticin, Invitrogen Life Technologies). Cells were cultured at 37°C in an atmosphere of 5% CO2/air.

**Western and Immunoprecipitation-Western Blot of Endoglin**

Western and Immunoprecipitation-Western blot procedures were performed as previously described (31,32). Briefly, 293, DR26, L6 myoblasts, and HMEC-1 cells were grown to

90% confluency in 6 well plates. Cells were then washed with PBS and lysed. Cell lysates were prepared and samples were standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad; Mississauga, Ont.). Samples were then fractionated on SDS-PAGE (7.5% acrylamide) under reducing or nonreducing conditions (with β-Mercaptoethanol, Sigma Aldrich, Oakville, ON.) and transferred by electrophoresis to nitrocellulose membrane (Scheicher and Schuell; Keene, NH.). The membrane was blocked for 3 hours in a milk protein/Tween blocking solution (1M Tris, pH 7.5; 5M NaCl; Tween 20,Sigma Aldrich; Carnation Skim milk powder) and incubated with anti-endoglin antibody (SN6h; Dako Inc.; Carpinteria, CA) overnight at 4°C followed by incubation for 2 hours with rabbit anti-mouse HRP conjugated secondary antibody (Santa Cruz Biotechnology Inc. Santa Cruz, CA) at room temperature and detected using the ECL system (Amersham Pharmacia Biotech Inc.; Baie d'Urfe, Que.). Alternatively, cells were grown to confluency in T75 flasks and washed three times with 0.1% BSA-dPBS, twice with dPBS, and membrane extracts of cells were prepared and immunoprecipitated with anti-endoglin antibody (SN6h) (1:100 dilution) or not immunoprecipitated. The extracts or immune complexes were fractionated by SDS-PAGE (3-11% gradient or 7.5% acrylamide) and Western blot analysis was performed using anti-endoglin antibody, and the ECL system for detection (Amersham Pharmacia Biotech Inc.) as discussed above.

**Affinity Labeling With or Without TGF-β Receptor Overexpression**

cDNAs encoding human RII and RI and rat betaglycan subcloned into pcDNA3 were obtained from Dr. M. O'Connor-McCourt (Montreal, Quebec; 33), and the pcEXV-Endo-L expression vector encoding the human L-endoglin isoform was from Dr. C. Bernabeu (Madrid, Spain; 34). The 293 cells grown in 6-well plates were transiently transfected

with 1μg each of pEndo-L, pbetaglycan, pRII, pRI, or combinations of these plasmids and pβ-galactosidase (pβ-gal). Parallel transfections with pcDNA3 (empty vector) and pβ-gal were performed as mock controls. The transfections were carried out using Superfect Reagent (Invitrogen Life Technologies) according to the manufacturer's specifications. Similarly C-28/I2, DR26, and L6 myoblast cells were grown in 12 well plates to 90% confluency were transiently transfected with 1μg each of pcDNA3 (EV) or plasmids encoding full length, cytoplasmically mutated (lacking residues 812-853) or GAG mutated (serine to alanine mutations at positions 535 and 546) betaglycan (30), or were not transfected.

Affinity labeling of all cells were then performed as described previously (35) with modifications (31,32). Briefly, the 293 monolayers were washed with ice-cold binding buffer [Dulbecco's PBS (dPBS) with $Ca^{++}$ and $Mg^{++}$, pH 7.4 containing 0.1% bovine serum albumin (BSA)] and were incubated with 100 pM of $^{125}$I-TGF-β1 in the absence or presence of 2.0 nM of unlabeled TGF-β1. The receptor ligand complexes were cross-linked with Bis-sulfocsuccinimidyl suberate (BS3; Pierce; Rockford, IL). The reaction was stopped by the addition of glycine and cell membrane extracts were prepared. The solubilized samples were separated by SDS-PAGE (3-11% gradient) under reducing (with β-Mercaptoethanol, Sigma Aldrich) or nonreducing conditions and analyzed by autoradiography.

**Immunoprecipitation of TGF-β Receptors**

The anti-type II TGF-β receptor antibody was obtained from Santa Cruz Biotechnology Inc. The anti-betaglycan antibody (Get 1; corresponding to the intracellular domain of betaglycan raised against the peptide sequence GETARRQQVPTSPPASENSS) was a gift

from Dr. S. Souchelnytskyi (Uppsala, Sweden; 36). This peptide sequence is not present in human endoglin (Accession Number J05481 GenBank) and the Get 1 antibody exhibits no crossreactivity to endoglin (personal communication Dr. S. Souchelnytskyi; Uppsala, Sweden). The anti-pig endoglin antibody (EG(591-609)), corresponding to the intracellular domain of endoglin raised against the peptide sequence KREPVVAVAAPASSESSST) was a gift from Dr. K. Miyazono (Japan; 17). This peptide sequence is not present in human betaglycan (Accession Number XM_001924 GenBank). The SN6h anti-human endoglin antibody was from Dako Inc.

Immunoprecipitation studies were performed as described previously (31,32,35,37) with modifications. The various cells were transiently transfected with the pEXV (EV), pendoglin (Eg), pDNRII (XF120, DNRII; 38-41) for 48 hours or left untransfected and then affinity labeled with 200 pM $^{125}$I-TGF-β1. The membrane extracts were incubated with 3μg/ml of the various antibodies and with respective IgG controls. Immune complexes were then incubated with protein G-Agarose (Roche Diagnostics; Laval, Que.) slurry and the beads were pelleted by centrifugation. The immune complexes were analyzed by SDS-PAGE (3-11% gradient) under non-reducing or reducing conditions followed by autoradiography.

**Immunoprecipitation/Western Blot Analysis**

DR26 cells were washed three times with 0.1% BSA-dPBS, twice with dPBS, and membrane extracts of cells were prepared, and immunoprecipitated with anti-betaglycan (Get 1) or anti-endoglin (SN6h; Dako) antibody, or not immunoprecipitated. The extracts or immune complexes were fractionated by SDS-PAGE (3-11% gradient) and Western

blot analysis was performed as described above using anti-endoglin antibody (SN6h; Dako) and the ECL system for detection (Amersham Pharmacia Biotech Inc.).

**Luciferase Reporter Assay**

The p3TP-Lux, a TGF-β-inducible promoter-reporter construct (42), containing the luciferase gene under the control of a portion of the plasminogen activator inhibitor-1 promoter region was used to determine the effect of the DNRII plasmid (XF120; 38-41) on cellular responsiveness to TGF-β. 293 cells were grown to 90% confluency in a 6-well plate and transiently transfected with 1μg/per well of p3TP-Lux with pDNRII or pcMV5 (EV) and 1μg of pβ-galactosidase (β-gal) using Lipofectamine Reagent (Invitrogen Life Technologies) in serum-free medium according to the manufacturer's specifications. Cells were allowed to recover for 48 hours, serum starved the following day for 4 hours, then treated with 100 pM of TGF-β1 overnight. The cells were lysed and assayed for luciferase activity using the EG&G Berthold Microplate Luminometer (Berthold Technologies USA; Oak Ridge, TN). Light emission by the TGF-β1 treated cells was expressed as a fold change of the emission by the untreated cells and adjusted for transfection efficiency using the β-gal assay.

**Chondroitinase/Heparinase Enzyme Treatment and Immunoprecipitation**

Prior to affinity labeling and immunoprecipitation of lysates, C-28/I2 cells were pretreated with enzymes to cleave the glycosaminoglycan side chains (GAG) from betaglycan or were left untreated. The procedure was performed as previously described (43) with some modifications (32). Briefly, confluent monolayers of cells were washed with dPBS and incubated with chondroitinase-ABC and heparinase (0.5U/ml and 4U/ml respectively, both from Sigma Aldrich) for 3 hours at room temperature. Thereafter, the

cells were washed with 0.1% BSA in dPBS and affinity labeled with 200 pM of $^{125}$I-TGF-β1 and not immunoprecipitated or immunoprecipitated with anti-betaglycan antibody as described above. Extracts were fractionated by SDS-PAGE (3-11% gradient) and analyzed by autoradiography.

**Receptor Overexpression and TGF-β Responses**

Receptor overexpression was performed as previously described (32). Briefly, to determine the regulation of the phosphorylated form of Smad 2 by TGF-β1 treatment in chondrocytes (C-28/I2) overexpressing endoglin, betaglycan, or both receptors combined, cells were grown in 6 well plates until 90% confluency. Cells were then transiently transfected with 1μg/per well of plasmids encoding endoglin, betaglycan (full length) alone or in combination, or empty vectors (pEXV and pcDNA3) using Lipofectamine Reagent (Invitrogen Life Technologies) in serum-free medium according to the manufacturer's specifications. Cells were allowed to recover for 48 hours in regular medium, serum starved the following day for 4 hours, then treated with 50 pM of TGF-β1, β2, or β3 for 15 minutes. Cell lysates were prepared and samples were standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad). The extracts were then fractionated by SDS-PAGE (7.5% acrylamide) and transferred by electrophoresis to nitrocellulose membrane (Scheicher and Schuell). The membrane was blocked for 3 hours in a milk protein/Tween blocking solution (1M Tris, pH 7.5; 5M NaCl; Tween 20,Sigma Aldrich; Carnation Skim milk powder) and incubated with a rabbit polyclonal anti-phosphoSmad 2 antibody (a gift from Dr. S. Souchelnytskyi, Uppsala, Sweden; 44,45) at 4°C overnight. The membrane was washed and incubated for 1 hour with goat anti-rabbit HRP conjugated secondary antibody (Santa Cruz Biotechnology Inc.) at room

temperature and detected using the ECL system (Amersham Pharmacia Biotech Inc.). The membrane was reprobed with anti-STAT 3 antibody (Santa Cruz Biotechnology Inc.) to confirm equal protein loading.

The p3TP-Lux (42) was also used to determine cellular responsiveness to TGF-β with receptor overexpression. Chondrocytes were grown to 90% confluency in a 6-well plate and transiently transfected with 1μg/per well of p3TP-Lux, pβ-gal, empty vectors or plasmids encoding endoglin or betaglycan alone or in combination. Cells were allowed to recover for 48 hours, serum starved the following day for 4 hours, then treated with 20 pM of TGF-β1, β2, or β3 overnight. The cells were lysed and assayed for luciferase activity as described above.

## RESULTS

### Endoglin is expressed on 293, DR26, and L6 myoblast cells

We recently identified endoglin on human chondrocytes (31) and have shown that it forms a heteromeric receptor complex in the presence of ligand and ligand independent manner. We also provided preliminary evidence that this association was occurring independently of RII (31). To further characterize the nature of the endoglin-betaglycan association and to determine its influence on ligand binding, we used cell lines lacking or deficient in TGF-β receptors, namely 293 (deficient in RII; 35), DR26 (lacking RII; 27), and L6 myoblasts (lacking betaglycan; 20,28-30). We demonstrate the expression of endoglin on these cell lines using Western blot and immunoprecipitation-Western blot under nonreducing and reducing conditions (Fig. 1) and show that the levels of expression on these cells are comparable to that of HMEC-1. In panel A cell extracts were analyzed by SDS-PAGE (7.5% acrylamide) under nonreducing (NR) and reducing (R) conditions,

transferred to nitrocellulose membrane, and Western blotted with anti-endoglin antibody. Endoglin is detectable on all cell lines (Lane 1-4). In Panel B extracts of cells were immunoprecipitated with anti-endoglin antibody (upper panel, Lane 2,4,6; lower panel, Lane 1-4) or not immunoprecipitated (upper panel, Lane 1,3,5) and Western blotting for endoglin was performed as above under nonreducing (NR) and reducing (R) conditions. Again, endoglin is seen to be expressed in all cell lines tested and its level of expression is notably higher in immunoprecipitated extracts (upper panel, Lane 2,4,6).

**TGF-β1 binding by endoglin is enhanced by overexpression of betaglycan but not the signaling receptors**

Affinity labeling of 293 cells (Fig. 2) illustrates that in cells transfected with the empty vectors (Lane 1; EV), plasmids encoding endoglin (Lane 2; Eg), endoglin and betaglycan (Lane 3; Eg/BG), betaglycan alone (Lane 4; BG), RI (Lane 7; RI), or endoglin and RI (Lane 8; Eg/RI) no RII is detectable confirming the low expression levels of RII in these cells. Overexpression of RII (Lane 5,6; RII and Eg/RII) results in a band present at 85 kDa consistent with RII. The presence of the endoglin monomer at 100 kDa is not detected in those cells transfected with the empty vectors (Lane 1). Overexpression of endoglin results in a slight increase in the intensity of the 100 kDa band (Lane 2) as anticipated. Of note, overexpression of betaglycan alone (Lane 4) or in combination with endoglin (Lane 3) results in a marked increase of intensity of the band representing the endoglin monomer suggesting that the presence of betaglycan is facilitating endoglin's ability to bind ligand. This effect of enhancing ligand binding by endoglin is not evident with overexpression of the TGF-β signaling receptors alone or in combination with endoglin overexpression (Lane 5-9) as no appreciable increase in the endoglin monomer

is detectable. Thus betaglycan appears to facilitate TGF-β1 binding by endoglin which may be mediated through the complex formation of these receptors independently of the signaling receptors.

**Endoglin binds TGF-β1 independently of RII**

Results shown in Fig. 1 demonstrate the presence of endoglin in DR26 cells, a mutant mink lung epithelial cell that does not express RII (27). TGF-β receptor profiles on DR26 cells were analyzed by affinity labeling with $^{125}$I-TGF-β1 and SDS-PAGE. The receptor profiles under reducing (R) conditions are illustrated in Fig. 3A (left panel). The competition using 2.0 nM of unlabeled TGF-β1 demonstrate the relative binding affinity of the receptors for the TGF-β1 isoform. $^{125}$I-TGF-β1 labeling in the absence of unlabeled ligand (Lanes designated as '0') reveals binding complexes of molecular weights of 65, 85, 100, 180 and 200-300 kDa under reducing conditions in C-28/I2 cells and 100, 180 and 200-300 kDa in DR26 cells. The migration patterns and isoform specificities of the 65, 85, 100, and 200-300 kDa complexes are characteristic of the cloned type I receptor (RI), type II receptor (RII), endoglin monomer and type III (betaglycan) receptor respectively (31). This was confirmed later by immunoprecipitation studies using specific anti-receptor antibodies (see below). All receptors showed high affinity for the TGF-β1 isoform. TGF-β receptor profiles of $^{125}$I-TGF-β1 labeled chondrocytes analyzed under non-reducing (NR) conditions are also shown in Fig. 3A (right panel). RI (65 kDa), RII (85 kDa), the endoglin dimer (180 kDa) and betaglycan (200-300 kDa) are evident in C-28/I2 cells and the endoglin dimer (180 kDa) and betaglycan (200-300 kDa) in DR26 cells. Once again, these receptors show high affinity for TGF-β1. Since detection by autoradiography of a receptor required that it

binds $^{125}$I-TGF-β1 at the cell surface, the appearance of both the endoglin dimer and monomer in DR26 cells indicates that endoglin in fact is able to bind ligand independently of RII.

**Endoglin and betaglycan form a heteromeric complex in the absence of RII**

Previously we demonstrated that endoglin and betaglycan could form a heteromeric receptor complex, using 293 cells, indicating that this association could occur in the absence of RII (31). Results shown in Fig. 3B confirm this association occurs independently of RII, using DR26 cells which are completely devoid of RII. Moreover, these results confirm endoglin's ability to bind ligand in the absence of RII. C28-I2 and DR26 cells affinity labeling with $^{125}$I-TGF-β1 and analyzed by SDS-PAGE under (3-11% gradient) nonreducing (left panel, NR) and reducing (right panel, R) conditions are shown. The non-immunoprecipitated extracts reveal the presence of betaglycan (200-300 kDa) and the endoglin dimer (180 kDa) under nonreducing conditions (Lane 3, NIP) or faint levels of the endoglin monomer (100 kDa) under reducing conditions (Lane 6, NIP). This suggests that endoglin is able to bind ligand independently of RII. Immunoprecipitation of labeled DR26 extracts with anti-RII antibody (Lane 4, α-RII) demonstrates the absence of RII in these cells. This can be compared with the immunoprecipitation of RII in C-28/I2 cells (Lane 1, α-RII) which demonstrates a band at 85 kDa representative of RII. Control IgG failed to immunoprecipitate any receptors (Lane 2, IgG).

In these immunoprecipitation studies, whereas immunoprecipitation with a specific anti-receptor antibody confirms the identity of its cognate TGF-β receptor, co-immunoprecipitation of another type of receptor which is not recognized by this antibody

is indicative of heteromeric complex formation between those receptors. In DR26 cells anti-betaglycan antibody (Lane 7; α-BG) immunoprecipitated betaglycan and co-immunoprecipitated the endoglin monomer. Similarly, the anti-endoglin antibody (Lane 5; α-Eg) immunoprecipitated the endoglin monomer, thus confirming the presence of endoglin in DR26 cells but also co-immunoprecipitated betaglycan. This co-immunoprecipitation of endoglin and betaglycan in DR26 cells confirms that their heteromeric complex occurs in the absence of RII.

The co-immunoprecipitation of betaglycan with α-Eg and that of endoglin with α-BG has not been reported previously with the exception of our recent demonstration on microvascular endothelial cells (35) and human chondrocytes (31). The findings of co-immunoprecipitation of endoglin with α-BG and that of betaglycan with α-Eg confirm the association of endoglin and betaglycan in the absence of RII but more importantly, demonstrate that endoglin is present on DR26 cells and binds TGF-β1 independently of RII. In addition, these results illustrate that endoglin associates with betaglycan independently of RII at endogenous receptor concentrations and ratios and endogenous ligand concentrations under reducing and nonreducing conditions (Fig. 3B). This heteromeric complex formation of endoglin and betaglycan occurring independently of RII was confirmed using immunoprecipitation-Western blot (Fig. 3C). Solubilized extracts of unlabeled DR26 cells were immunoprecipitated with anti-betaglycan (Lane 3; BG), anti-endoglin (Lane 4; Eg) antibody, control IgG (Lane 1; IgG), or were not immunoprecipitated (Lane 2; NIP), analyzed by SDS-PAGE (3-11% gradient), and transferred to nitrocellulose membrane. Western blotting performed with anti-endoglin antibody revealed the presence of endoglin in the nonimmunoprecipitated (Lane 2) and

anti-endoglin immunoprecipitated (Lane 4) DR26 extracts confirming its expression on these cells. However, endoglin was also detectable in anti-betaglycan immunoprecipitates (Lane 3) demonstrating the heteromeric association of endoglin and betaglycan in DR26 cells and thus in the absence of RII. Western blotting was not performed using the anti-RII antibody for RII as a control for this experiment because RII is known not to be expressed in DR26 cells. However, affinity labeling of these cells did not reveal the presence of this receptor and neither did affinity labeling followed by immunoprecipitation for RII (Fig. 3B) confirming the absence of expression on these cells.

**The dominant negative form of RII abolishes TGF-β signaling in 293 cells**

It could be argued that although RII levels are undetectable in 293 cells there are enough RII molecules to permit TGF-β signaling. Fig. 4A demonstrated that 293 cells are in fact TGF-β responsive. Cellular signaling was determined by a PAI-driven luciferase reporter assay (p3TP-Lux) which has been used extensively as a marker for TGF-β responsiveness (42). 293 cells were transiently transfected with p3TP-Lux and the induction of luciferase activity by exogenous TGF-β1 was measured. As illustrated in Fig. 4A, luciferase activity of cells treated with 100 pM of TGF-β1 was stimulated approximately a 6-fold compared to untreated cells. However, in 293 cells overexpressing the dominant negative form of RII (DNRII; 38-41) TGF-β-induced signaling was abolished ($p=0.01$). The decrease in response was also detectable at endogenous ligand levels in the absence of exogenous administration of TGF-β1 ($p=0.005$). The results were normalized by co-transfection of the β-gal plasmid and are representative of three different experiments each done in triplicate. These results demonstrate that DNRII can

be used to generate cells devoid of RII to study receptor interactions in the absence of RII.

To explore whether endoglin associates with betaglycan on chondrocytes in the absence of RII, we used C28-I2 cells transfected with DNRII (Fig. 4B). C-28/I2 cells were either not transfected (Lane 7-10; Ø) or transfected with empty vector (Lane 11; EV), DNRII (Lane 1,2; DNRII), endoglin and betaglycan (Lane 3,4; Eg/BG) or endoglin and betaglycan in combination with DNRII (Lane 5,6; DNRII/Eg/BG). Chondrocytes were affinity labeled with $^{125}$I-TGF-β1 and cells extracts were immunoprecipitated using specific anti-receptor antibodies directed against betaglycan (Lane 2,4,6,10; α-BG), endoglin (Lane 1,3,5,9; α-Eg), RII (Lane 8), or not immunoprecipitated (Lane 7,11). The co-immunoprecipitations of betaglycan with anti-endoglin antibody and endoglin with anti-betaglycan antibody are evident at endogenous receptor levels in untransfected cells (Lane 9,10) and with receptor overexpression (Lane 3,4). Overexpression of DNRII (Lane 1,2,5,6) results in the absence of RII as compared to cells not overexpressing DNRII (Lane 3,4,7-11). Despite the absence of RII with DNRII overexpression (Lane 1,2,5,6) the association between endoglin and betaglycan is demonstrated confirming the RII independent nature of the complex formation. Furthermore, the detection of endoglin in the absence of RII again suggests its ability to bind ligand independently of RII.

**TGF-β1 binding by endoglin requires betaglycan but not the GAG chains of betaglycan**

We have provided evidence that endoglin binds TGF-β1 independently of RII (Fig. 3A,3B; Fig. 4B). Moreover, we have shown that this binding is enhanced by betaglycan overexpression (Fig. 2). To determine if endoglin binding of TGF-β1 is dependent upon

the presence of betaglycan, L6 myoblasts which lack betaglycan (20,28-30) were used to investigate ligand binding by endoglin (Fig. 5A). Results shown in Fig. 1 demonstrated the presence of endoglin in L6 myoblasts. To analyze TGF-β receptor profiles on these cells they were affinity labeled with $^{125}$I-TGF-β1 and the labeled receptors were analyzed by SDS-PAGE (3-11% gradient). The receptor profile of L6 myoblasts transfected with empty vector under nonreducing conditions is illustrated in Fig. 5A (Lane 1; EV). Of note, only RI and trace amounts of RII are evident, whereas no betaglycan is detectable. However in cells transfected with full length betaglycan, under both reducing (Lane 4,5; BG) and nonreducing (Lane 2,3; BG) conditions, RI and RII receptors are readily identified at 65 and 85 kDa respectively. The band at 120 kDa represents the core of betaglycan. The fact that the affinity labeling studies were performed 24 hours after betaglycan transfection likely accounts for the observed core protein rather than a glycosylated full length form as the cells may not have had adequate time to add these complex carbohydrate chains to the betaglycan core. It is not uncommon to see the presence of additional bands during affinity labeling procedures which are representative of non-specific protein binding rather than TGF-β binding proteins with clear ligand affinity. Various competition studies have been performed prior to these final studies to identify the presence of such proteins on various cell types. Thus, they are not noted here. That betaglycan enhances ligand binding to RI and RII is well established (43,46-49). More importantly, we show the expression of betaglycan resulted in ligand binding by endoglin as demonstrated by the appearance of endoglin dimer (180 kDa) and endoglin monomer (100 kDa) under nonreducing (Lane 2,3) and reducing (Lane 4,5) conditions respectively (Fig. 5A). Although endoglin could be demonstrated in these cells by

Western blot (Fig. 1) endoglin is not identified in the empty vector transfected L6 myoblasts (Lane 1), but became apparent with the expression of betaglycan (Fig. 5A; Lane 2,4). The competition using 2.0 nM of unlabeled TGF-β1 demonstrated the relative binding affinity of the receptors for the TGF-β1 isoform (Lane 3,5). This suggested that betaglycan not only enhances but is in fact required for endoglin to bind TGF-β1. Also, that the betaglycan transfection at 24 hours resulted in only the core protein being expressed suggested that perhaps the GAG chains of betaglycan are not required to allow TGF-β1 binding by endoglin.

We therefore tested the significance of GAG chains of betaglycan in enhancing endoglin binding of TGF-β using L6 myoblasts transfected with empty vectors or GAG deficient mutant and full length forms of betaglycan. The cells were then allowed to recover for 48 hours (rather than 24 hours) and analyzed under nonreducing conditions (Fig. 5B). Again endoglin is not detected in cells transfected with empty vector (EV) but with either full length (BG-FL) or GAG (BG-GAG) transfected betaglycan the endoglin dimer became apparent. The appearance of $^{125}$I-TGF-β1 bound endoglin in cells transfected with full length betaglycan but not in cells transfected with empty vector confirms the requirement of betaglycan for endoglin binding. Of note, after 48 hours full length betaglycan is detectable illustrating that GAG chains are being synthesized onto betaglycan. Importantly, however, ligand binding by endoglin even in the absence of GAG chains of betaglycan demonstrates that GAG chains are not required for ligand binding by endoglin.

**The endoglin-betaglycan heteromerization occurs independently of betaglycan's cytoplasmic domain or GAG chains**

To determine the significance of the cytoplasmic domain of betaglycan and its extracellular GAG chains on the endoglin-betaglycan association, affinity labeling studies and immunoprecipitations were carried out in stable mutants of L6 myoblasts expressing betaglycan deficient in its cytoplasmic domain or parent cells transiently transfected with betaglycan deficient in carbohydrate side chains (GAG) (20,28-30). Specifically we investigated whether the endoglin-betaglycan association could occur independently of betaglycan's GAG chains or cytoplasmic domain. In Fig. 6A, L6 stable mutants expressing cytoplasmic deficient betaglycan were transfected with plasmid encoding endoglin (Lane 5-8; Eg) or its empty vector (pEXV; Lane 1-4; EV) and affinity labeled with $^{125}$I-TGF-β1 and analyzed by SDS-PAGE (3-11% gradient). The receptor profile under nonreducing conditions is illustrated. Cells lysates were either not immunoprecipitated (Lane 1,2,5,6) or immunoprecipitated with anti-endoglin (Lane 3,7) or anti-betaglycan (Lane 4,8) antibody. Despite the absence of betaglycan's cytoplasmic domain in these stable transfectants the endoglin-betaglycan heteromeric complex was detectable. This was seen both at endogenous receptor concentrations and ratios (Lane 3,4) and with receptor overexpression (Lane 7,8).

To confirm that the betaglycan-endoglin heteromerization could occur in L6 cells with betaglycan in its full length form L6 myoblasts expressing full length betaglycan were used (Fig. 6B). Cells were transiently transfected with endoglin (Lane 4-6,9,10) or pEXV (Lane 1-3, 7,8) then affinity labeled and immunoprecipitated as noted above for Fig. 6A under both reducing (Lane 1-6) and nonreducing conditions (Lane 7-10). The co-immunoprecipitation of endoglin by anti-betaglycan antibody (Lane 3, 8) and betaglycan

by anti-endoglin antibody (Lane 2,7) at endogenous receptor concentrations was not enhanced by overexpression of endoglin (Lane 5,6 and 9,10 respectively).

It was determined that the presence of the GAG chains of betaglycan were not a requirement for endoglin to bind ligand. To determine their significance in the endoglin-betaglycan association L6 myoblasts were transfected with plasmid encoding the GAG deficient form of betaglycan. Cells were affinity labeled and immunoprecipitated with anti-endoglin and anti-betaglycan antibodies as noted above under both reducing and nonreducing conditions and the endoglin-betaglycan co-immunoprecipitation was detected (data not shown) confirming the GAG independent nature of the association. To confirm the cytoplasmic independent nature of the endoglin-betaglycan complex seen in Fig. 6A, L6 myoblasts were transfected with plasmid encoding the cytoplasmic deficient form of betaglycan, affinity labeled and immunoprecipitated as described above (data not shown). The presence of the endoglin-betaglycan co-immunoprecipitation independently of betaglycan's cytoplasmic domain was observed.

To determine if the GAG independent nature of the endoglin-betaglycan heteromerization was also applicable to human chondrocytes, enzymatic removal of these side chains of betaglycan was carried out (Fig. 6C). C-28/I2 cells were affinity labeled then either left untreated (Lane 1,3,5; -) or were treated (Lane 2,4,6; +) with a combination of chondroitinase ABC and heparinase enzymes which are known to cleave the GAG chains from betaglycan's extracellular domain (43). This results in the full length betaglycan being reduced to its core protein of 120 kDa. Cell extracts were then not immunoprecipitated (Lane 3,4; NIP) or immunoprecipitated with anti-betaglycan antibody (Lane 5,6; α-BG) or control IgG (Lane 1,2; IgG) and visualized by

autoradiography. The heteromerization between endoglin monomer (100 kDa) and betaglycan (200-300 kDa) is detected in the presence (Lane 6) and absence (Lane 5) of enzyme treatment confirming that the presence of betaglycan's GAGs are not required for the association.

**Overexpression of endoglin inhibits whereas betaglycan enhances TGF-β responses in human chondrocytes**

Chondrocytes (C-28/I2) were transfected (Fig. 7A) with plasmid encoding endoglin (panel A, Lane 5-8; Eg), betaglycan (panel B, Lane 1-4; BG), or both receptors in combination (panel B, Lane 5-8; BG+Eg) or with respective empty vectors (panel A, Lane 1-4; EV). Cells were then left untreated (-) or treated with 50 pM of TGF-β1, β2, or β3 for 15 minutes and Western blotting was performed to detect the phosphorylated form of Smad 2 (panel A and B, upper panels). Interestingly, endoglin overexpression inhibited TGF-β isoform-induced Smad 2 phosphorylation, in the presence of all three isoforms (panel A; Lane 6-8). Betaglycan had an opposite effect enhancing Smad 2 phosphorylation with all three TGF-β isoforms (panel B; Lane 2-4). Overexpression of both endoglin and betaglycan in these cells resulted in up-regulation of TGF-β-induced Smad 2 phosphorylation (panel B; Lane 6-8) but to a lesser extent than with betaglycan alone (panel B; Lane 2-4). This suggests that endoglin's inhibition of TGF-β signaling is able to partially override betaglycan's enhancement. The membranes were reprobed for STAT 3 (lower panels) to confirm equal protein loading. The data shown are representative of three different experiments each done in triplicates.

The regulation of TGF-β-induced transcriptional activity by endoglin and betaglycan is shown in Fig. 7B. Chondrocytes again were transfected with plasmid encoding endoglin

(panel B, Eg), betaglycan (panel A, BG), both receptors in combination (panel C, Eg+BG) or with respective empty vectors (panel A, B, C; EV) in addition to 3TP-Lux (42) and pβ-gal. Cells were allowed to recover for 48 hours and then were treated with 20 pM of TGF-β1 overnight or left untreated (-) and luciferase activity was measured and standardized with a β-gal assay. Corresponding to the results obtained with Smad 2 phosphorylation, endoglin was noted to inhibit responses with all three TGF-β isoforms whereas betaglycan enhanced luciferase activity. For endoglin this inhibition was significant at endogenous ligand levels ($p=0.01$) and with addition of exogenous TGF-β1 ($p=0.05$), TGF-β2 ($p=0.02$), and TGF-β3 ($p=0.01$). The enhanced activity with betaglycan was also significant at both endogenous levels ($p=0.03$) and with all three isoforms, TGF-β1, -β2, and -β3 ($p=0.03$, $p=0.03$, $p=0.01$ respectively). When both endoglin and betaglycan were simultaneously overexpressed enhanced luciferase activity was noted in response to TGF-β2 ($p=0.07$) and -β3 ($p=0.03$) isoform treatment but not with TGF-β1 ($p=0.19$), although only TGF-β3 and endogenous ligand ($p=0.03$) levels were significant.

**DISCUSSION**

Dysregulation of the TGF-β signaling cascade has been shown to result in degenerative joint disease in two murine models clearly depicting the significance of TGF-β signaling in chondrocytes (50,51). TGF-β has the potential to regulate almost all aspects of the tissue repair process, from cell proliferation and differentiation, to neovascularization and ECM deposition and turnover (1-4,52,55).

There has been inconsistency and controversy as to TGF-β's mechanism of action in cartilage. This may be attributable to the diverse models in which TGF-β and cartilage

have been studied, that chondrocyte phenotype may change in culture, and a multitude of regulatory agents present in the in vivo environment which may oppose or augment TGF-β actions (56). The inconsistencies observed with exogenous administration of TGF-β in cartilage *in vivo* and *in vitro* demand an alternative means to regulate TGF-β-induced cell proliferation, maintenance of phenotype and ECM production. Modulation of the action of endogenous TGF-β at the level of its receptors provides such an avenue. Interaction of novel and accessory receptors with TGF-β signaling receptors have been reported previously to be important regulators of TGF-β signaling in other cell types (11,57,58). However, information on the expression of TGF-β receptors in cartilage or chondrocytes is limited (59,60-63). We have recently identified several novel TGF-β receptors on human chondrocytes (Alk-1, Sol RI, endoglin) and demonstrated various heteromeric complex formations between them, the TGF-β signaling receptors and also betaglycan (31,32). Further elucidation of the expression profiles of novel TGF-β receptors and their interactions leading to the formation of heteromeric receptor complexes may be critical in defining TGF-β signaling pathways and thus action in chondrocytes.

In the present study we demonstrated for the first time that endoglin binds TGF-β1 independently of RII but requires the presence of betaglycan. We show that endoglin is expressed on DR26 and 293 cells, and L6 myoblasts and using these cell lines show that TGF-β1 binding by endoglin is enhanced by betaglycan but not the signaling receptors. Moreover, we confirm that the heteromeric complex formation of endoglin and betaglycan occurs in an RII independent manner and in the absence of betaglycan's cytoplasmic domain or carbohydrate side chains. Lastly, overexpression of endoglin is

demonstrated to inhibit whereas betaglycan enhances TGF-β1, -β2, and -β3 responses in human chondrocytes.

The phenotype of the human chondrocytes used in the present study has been characterized extensively (64-67). The primary human articular chondrocytes and immortalized chondrocytes expressed both aggrecan and type II collagen mRNAs, and their responsiveness to TGF-β was shown by stimulation of PAI promoter activity and Smad 2 phosphorylation (31). The DR26 cells, developed from a mink lung epithelial cells line, have been shown not to express RII (27) and the L6 myoblasts are known not to express betaglycan (20,28-30). We have previously characterized the TGF-β receptor profiles on human articular and nonarticular chondrocytes. We have identified, in addition to RI, RII, and betaglycan, Alk-1 and Sol RI (32), and more importantly, endoglin (31). We have previously demonstrated that endoglin on human chondrocytes forms a heteromeric complex with betaglycan and that this complex occurs in the presence of ligand and ligand independent manner. Betaglycan is found on most mammalian cells (68). Endoglin was originally identified with the monoclonal antibody 44G4 generated against the HOON pre-B leukemic cell line (69) but was subsequently found to be highly expressed on endothelial cells. Other cell types known to express endoglin include monocytes, lymphocytes and placental and uterine cells (23,70,71). Endoglin, a homodimeric transmembrane protein of ~180 kDa composed of disulfide-linked protein subunits of ~95 kDa, has limited species-specificity and shows 70% homology to betaglycan. The functional role of endoglin in TGF-β signaling remains poorly understood.

In the present study we have identified endoglin on DR26, 293 and L6 myoblast cells (Fig. 1). Western blot for endoglin (panel A) demonstrated the expression of endoglin at levels similar to human microvascular endothelial cells (HMEC-1). Immunoprecipitation studies using anti-endoglin antibody followed by Western blot of the immunoprecipitates with anti-endoglin antibody revealed the receptor (panel B). The expression profile of endoglin is likely much more diverse than previously thought. This accessory TGF-β receptor may not been identified more frequently on other tissues because of low ligand binding affinity in detection techniques relying on ligand binding, or simply because it has not been considered.

We had previously noted that betaglycan and endoglin formed a heteromeric complex in 293 cells (31) suggesting that this association could occur in the absence of RII. In addition, this suggested that endoglin may bind ligand in the absence of RII as the results relied on affinity labeling and $^{125}$I-TGF-β1 binding by receptors. To further define the ligand binding properties of endoglin, overexpression of various combinations of TGF-β receptors in 293 cells were performed followed by affinity labeling (Fig. 2). We demonstrated that endoglin binding of TGF-β1 is enhanced by the presence of betaglycan but not by the presence of the signaling receptors (Fig. 2). Betaglycan overexpression resulted in a marked increase in intensity of the band representing the endoglin monomer indicating enhancement of ligand binding by endoglin which was not apparent with RI and RII overexpression.

Betaglycan is known to bind all three TGF-β isoforms and it is considered to facilitate ligand binding by the signaling receptors (15,28,57) while it is generally accepted that endoglin binds TGF-β1 and TGF-β3, but not TGF-β2 (11,24). Despite this, the roles of

betaglycan and endoglin in signaling are poorly defined. Endoglin has been shown to associate with Alk-1 and RI in a ligand independent manner (72) and in fact it is thought that only 1% of endoglin at the cell surface actually binds ligand (18). The mechanism by which betaglycan is enhancing TGF-β1 binding by endoglin is unclear. It is possible that the heteromeric complex between these receptors induces a conformational change in the ligand binding domain of endoglin facilitating TGF-β1 binding.

The current signaling paradigm is that endoglin cannot bind ligand in the absence of the respective type II ligand binding receptor (11,24). The results presented in Fig. 3A and B argue against this theory. These results demonstrate that endoglin binds TGF-β in the absence of RII. We had previously demonstrated the presence of endoglin on DR26 cells (Fig. 1A). Analysis of TGF-β receptor profiles on these cells (devoid of type II receptor) revealed the endoglin dimer and monomer (Fig. 3A), later confirmed by immunoprecipitation (Fig.3B), indicating that endoglin in fact is able to bind ligand independently of RII.

In addition, co-immunoprecipitation studies in DR26 cells demonstrated that the heteromeric association of endoglin and betaglycan formed in the absence of RII (Fig. 3B). Confirmation of this was achieved using immunoprecipitation-Western blot in these cells (Fig. 3C).

Evidence that the betaglycan-endoglin association was also occurring independently of RII in human chondrocytes has been presented (Fig. 4B). The complex formation between endoglin and betaglycan was detectable by co-immunoprecipitation in chondrocytes overexpressing the dominant negative form of RII (DNRII). Moreover, the

detection of endoglin in chondrocytes overexpressing DNRII (Fig. 4B) (no functional RII present) supports our theory of endoglin's ability to bind TGF-β independently of RII.

Thus far we have provided evidence that endoglin binds TGF-β1 in the absence of RII and that this receptor-ligand affinity is enhanced by betaglycan. Therefore, we investigated whether TGF-β1 binding by endoglin required the presence of betaglycan. L6 myoblasts, devoid of betaglycan (20,28-30), were affinity labeled with $^{125}$I-TGF-β1 (Fig. 5A). This suggested that betaglycan not only enhances but in fact is required for endoglin to bind TGF-β1. We therefore repeated this experiment with L6 myoblasts transfected with empty vector or transfected with full length and GAG deficient mutant forms of betaglycan (Fig. 5B). Again endoglin was not detected in empty vector transfected cells but with expression of either full length or GAG deficient betaglycan the endoglin dimer became apparent confirming that endoglin required the presence of betaglycan to bind ligand.

If betaglycan is required for endoglin to bind TGF-β1 it is important to further elucidate the nature of their heteromeric complex formation. We have thus far confirmed that this association occurs independently of RII. To investigate the role of betaglycan's cytoplasmic domain and glycosaminoglycan side chains (GAG) in this association we utilized stable transfectants of L6 myoblasts expressing betaglycan deficient in these domains and transiently transfected parent cells with betaglycan expression plasmids also lacking these regions. The findings presented in Fig. 6A, B, and C indicate that neither the GAG chains nor cytoplasmic domain of betaglycan are essential in its complex formation with endoglin. This suggests a core-core protein interaction of their extracellular domains.

Although their short cytoplasmic tails were previously thought to be devoid of catalytic activity (15,18), there is increasing evidence to show that endoglin and betaglycan potently modulate TGF-β signaling. Endoglin has been shown to be intricately involved in phosphorylation of and by the TGF-β signaling receptors (19) and betaglycan' role in regulating TGF-β signaling is becoming increasingly complex (19,20). These receptors have been shown to demonstrate opposing actions in cells. When overexpressed in myoblasts, endoglin inhibits, whereas betaglycan enhances TGF-β responses (11,21). Endoglin has also been reported to antagonize TGF-β action in human microvascular endothelial cells (22,23). Moreover, endoglin has been shown to increase fibronectin production in response to TGF-β but inhibit cell adhesion in the same system (21) demonstrating its diversity in modulating TGF-β response.

TGF-β responsiveness of human chondrocytes overexpressing endoglin and betaglycan were used to determine their role in modulating TGF-β signaling in these cells. Western blot for Smad 2 phosphorylation (Fig. 7A) and TGF-β induced luciferase activity (Fig. 7B) were used as indicators of TGF-β responsiveness. Endoglin overexpression was observed to inhibit whereas betaglycan overexpression enhanced TGF-β signaling in chondrocytes for all three TGF-β isoforms at significant levels. Combined receptor overexpression resulted in an up-regulation of TGF-β signaling but to a lesser extent than with betaglycan alone and only significant for endogenous ligand levels and for TGF-β3 stimulation. The results presented here are representative of the experiment performed in triplicate all with similar findings.

Endoglin's influence on TGF-β1 and β3 responses may be directly mediated through interactions with the signaling receptors (17, reviewed in 73). It is well accepted that

endoglin does not bind the β2 ligand (24). Despite this, endoglin overexpression in chondrocytes inhibits the β2-induced TGF-β response. We hypothesize that this inhibition is mediated through the endoglin-betaglycan heteromeric complex perhaps by preventing betaglycan's donation of the β2 isoform to the signaling receptors. In our previous characterization of the TGF-β receptor profile in human chondrocytes we noted that RI and RII showed relatively poor affinity for the TGF-β2 isoform despite the presence of betaglycan in these cells. This may be explained by the abundance of endoglin and its heteromeric complex with betaglycan in human chondrocytes. Two regions of betaglycan's ECD bind ligand, one region at the amino end known as the endoglin related region (74) and one region at the C terminal end or uromodulin related domain important for inhibin A binding (75). Both of these domains bind TGF-β2 with increased affinity than -β1, however, only the endoglin related region increases -β2 labeling of RII (76). Despite this, both domains cause a TGF-β2 dependent increase in Smad 2 phosphorylation. These structural details provide insight into the potential role of the endoglin-betaglycan heteromeric complex in its regulation of TGF-β2 signaling. If in fact endoglin, during its association with betaglycan, prevents TGF-β2 donation to the signaling receptors this may explain their apparent low affinity for this ligand.

In summary these results have demonstrated the presence of endoglin on 293, DR26 and L6 myoblast cells. Most importantly, we showed for the first time that endoglin binds TGF-β1 in the absence of RII, and that not only is this binding facilitated by but also appears to require betaglycan. In addition, we confirmed that the betaglycan-endoglin heteromeric complex formation occurs independently of RII. Significantly, we demonstrated that this association does not require betaglycan's cytoplasmic domain or

GAG chains. Furthermore, in human chondrocytes we demonstrated that endoglin inhibits whereas betaglycan enhances TGF-β1, β2, and β3 isoform responses and propose that endoglin's inhibition of the β2-induced response is mediated through its complex formation with betaglycan.

Taken together this information provides new insights into the TGF-β signaling cascade and argues against the concept of endoglin requiring the presence of RII to bind ligand. We now see the TGF-β signaling paradigm begin to shift. The passive manner in which accessory receptors were once thought to participate in signaling is no longer acceptable. The potential impact of accessory receptors in modulating TGF-β action has far reaching implications in disease states where TGF-β dysregulation is prominent (77-80, reviewed in 81). From angiogenesis and chronic fibrotic disease states to malignancy and degenerative joint disease, these accessory receptors may provide novel avenues of regulation of TGF-β action towards tissue repair.

**FIGURE LEGENDS**

**Fig. 1. Endoglin is expressed in 293, DR26, and L6 myoblast cells.** Solubilized extracts of 293, DR26, L6 myoblasts and HMEC-1 cells were not immunoprecipitated (panel A; panel B-NR, Lane 1,3,5) or immunoprecipitated with anti-endoglin antibody (panel B-NR, Lane 2,4,6; panel B-R, Lane 1-4) then analyzed by SDS-PAGE (7.5% acrylamide) under nonreducing (NR) or reducing (R) conditions and transferred onto nitrocellulose membrane. Membranes were immunoblotted with anti-endoglin antibody and the ECL system was used for chemiluminescence detection.

**Fig. 2. TGF-β receptor overexpression and affinity labeling with $^{125}$I-TGF-ß1 in 293 cells.** Confluent monolayers of 293 cells were transfected with empty vectors (Lane 1;

EV), plasmids encoding RI (Lane 7), RII (Lane 5), endoglin (Lane 2), betaglycan (Lane 4), endoglin/betaglycan (Lane 3), endoglin/RII (Lane 6), endoglin/RI (Lane 8), or endoglin/RII/RI (Lane 9) for 48 hours and then affinity labeled with 100 pM of $^{125}$I-TGF-β1. Solubilized cell extracts were analyzed by SDS-PAGE (3-11% gradient) under reducing conditions followed by autoradiography.

**Fig. 3. Endoglin forms a heteromeric complex with betaglycan and binds TGF-β1 in the absence of RII.** Affinity labeling (**A**), Co-immunoprecipitation (**B**), and Immunoprecipitation-Western blot (**C**) in DR26 cells lacking RII indicate that endoglin binds TGF-β1 in the absence of RII and confirm the RII independent nature of the endoglin-betaglycan complex. **A:** Confluent monolayers of DR26 and C-28/I2 cells were affinity labeled with 100 pM of $^{125}$I-TGF-β1 in the absence or presence of 2.0 nM of unlabeled TGF-β1. Solubilized cell extracts were analyzed by SDS-PAGE (3-11% gradient) under reducing (R) and nonreducing (NR) conditions followed by autoradiography. **B:** DR26 and C-28/I2 cells were affinity labeled with 200 pM of $^{125}$I-TGF-β1 and solubilized cell extracts were immunoprecipitated with 3μg/ml each of anti-receptor antibodies or control IgG. Complexes were fractionated on SDS-PAGE (3-11% gradient) under nonreducing (NR) and reducing (R) conditions and visualized by autoradiography. Non-immunoprecipitated cell extract (Lane 3,6; NIP) and immunoprecipitates of anti-betaglycan (Lane 7; α-BG), anti-endoglin (Lane 5; α-Eg), anti-RII (Lane 1,4; α-RII) antibodies or control IgG (Lane 5; IgG) are shown. **C:** Solubilized extracts of DR26 cells were not immunoprecipitated (Lane 2; NIP), or immunoprecipitated with control IgG (Lane 2; IgG), anti-betaglycan (Lane 3; α-BG) or anti-endoglin (Lane 4; α-Eg) antibody. Complexes were fractionated by SDS-PAGE (3-

11% gradient) under nonreducing conditions and Western blotting was performed, as described in Experimental Procedures using anti-endoglin antibody. The ECL system was used for chemiluminescence detection.

**Fig. 4. The dominant negative form of RII abolishes signaling in 293 cells but fails to prevent the endoglin-betaglycan complex formation in human chondrocytes. A:** 293 cells were transiently transfected with the PAI-1 promoter-luciferase reporter construct, (3TP-Lux), pβ-gal and the plasmid encoding the dominant negative RII (DNRII) or the empty vector (EV). 48 hours after transfection, cells were treated with 100 pM of TGF-ß1 overnight or were left untreated. Luciferase activity was determined, normalized using the β-gal assay and expressed in relative light units (RLU) as a fold change of untreated cells. Statistically significant changes are noted by an asterisk. **B:** C-28/I2 cells were not transfected (Lane 7-10; NIP), transfected with pcMV5 (Lane 11; EV), or transfected with plasmids encoding DNRII (Lane 1,2; DNRII) endoglin and betaglycan alone (Lane 3,4; Eg/BG) or in combination with DNRII (Lane 5,6; DNRII/Eg/BG). Cells were then affinity labeled with 200 pM of $^{125}$I-TGF-β1 and solubilized cell extracts were immunoprecipitated with 3μg/ml each of anti-receptor antibodies. Complexes were fractionated on SDS-PAGE (3-11% gradient) under nonreducing conditions and visualized by autoradiography. Non-immunoprecipitated cell extract (Lane 7,11; NIP) and immunoprecipitates of anti-betaglycan (Lane 2,4,6,10; α-BG), anti-endoglin (Lane 1,3,5, 9; α-Eg), or anti-RII (Lane 8; α-RII) are shown.

**Fig. 5. TGF-β1 binding by endoglin is enhanced by betaglycan. A:** Confluent monolayers of L6 myoblasts were transiently transfected with plasmids encoding betaglycan (Lane 2-5; BG) or pcDNA3 (Lane 1; EV). Cells were allowed to recover for

24 hours and affinity labeled with 100 pM of $^{125}$I-TGF-β1 in the absence or presence of 2.0 nM of unlabeled TGF-β1. Solubilized cell extracts were analyzed by SDS-PAGE (3-11% gradient) under nonreducing (NR) or reducing (R) conditions followed by autoradiography. Of note at 24 hours post-transfection only the betaglycan core can be detected by autoradiography as it likely has not yet undergone addition of carbohydrate chains. **B:** Confluent monolayers of L6 myoblasts were transiently transfected with plasmid encoding full length betaglycan (BG-FL), the GAG mutant of betaglycan (BG-GAG), or empty vector (EV) for 48 hours then affinity labeled with 100 pM of $^{125}$I-TGF-β1. Solubilized cell extracts were analyzed by SDS-PAGE (3-11% gradient) under nonreducing conditions followed by autoradiography. Of note at 48 hours post-transfection the betaglycan core and full length forms of betaglycan can be visualized by autoradiography.

**Fig. 6. The endoglin-betaglycan association does not require the cytoplasmic domain or GAG chains of betaglycan.** L6, cytoplasmic deficient or full length betaglycan, stable mutants were transiently transfected with pEXV (EV) or plasmid encoding endoglin (Eg) for 48 hours. Cells were affinity labeled with 200 pM of $^{125}$I-TGF-β1 in the absence or presence of 2.0 nM of unlabeled TGF-β1 and solubilized cell extracts were immunoprecipitated with 3μg/ml each of anti-receptor antibodies or not immunoprecipitated. Complexes were fractionated on SDS-PAGE (3-11% gradient) under reducing (panel A,B,C) or nonreducing (panel B) conditions and visualized by autoradiography. **A:** Non-immunoprecipitated extracts of L6 cytoplasmic deficient betaglycan mutants (Lane 1,2,5,6; NIP) and immunoprecipitates of anti-betaglycan (Lane 4,8; α-BG) or anti-endoglin (Lane 3,7; α-Eg) antibody are shown. **B:** Non-

immunoprecipitated extracts of L6 full length betaglycan stable mutants (Lane 1,4; NIP) and immunoprecipitates of anti-betaglycan (Lane 3,6,8,10; α-BG) or anti-endoglin (Lane 2,5,7,9; α-Eg) antibody are shown. **C:** C-28/I2 cells were left untreated (Lane 1,3,5; -) or treated (Lane 2,4,6; +) with chondroitinase ABC and heparinase enzymes (C/H) prior to affinity labeling. Non-immunoprecipitated extract of C28/I2 cells (Lane 3,4; NIP) and immunoprecipitates of anti-betaglycan (Lane 5,6; α-BG) or control rabbit IgG (Lane 1,2; α-IgG) are shown.

**Fig. 7. Endoglin and betaglycan overexpression regulates TGF-β responses in human chondrocytes.** **A:** Regulation of TGF-β isoform-induced Smad 2 phosphorylation by endoglin and betaglycan. Chondrocytes were transiently transfected with plasmids encoding endoglin (panel A, Lane 5-8; Eg), betaglycan (panel B, Lane 1-4; BG), both in combination (panel B, Lane 5-8; BG + Eg) or empty vectors (panel A, Lane 1-4; EV). After 48 hours solubilized extracts of cells treated with 50 pM of TGF-ß1 (Lane 2,6) -β2 (Lane 3,7), or -β3 (Lane 4,8) for 15 minutes or left untreated (Lane 1,5; -) were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membranes were immunoblotted with an antibody against the phosphorylated form of Smad 2 (upper panels) and reprobed with anti-STAT 3 antibody to illustrate equal protein loading (lower panels). The ECL system was used for chemiluminescence detection. **B:** Regulation of TGF-β isoform-induced plasminogen activator inhibitor-1 (PAI-1) promoter activity by endoglin and betaglycan. The chondrocytes were transiently transfected with plasmids encoding the PAI-1 promoter-luciferase reporter construct (3TP-Lux), β-gal, in addition to either endoglin (panel B, Eg), betaglycan (panel A, BG), both in combination (panel C, Eg + BG), or their

respective empty vectors (panel A, B, C; EV). 48 hours after transfection, cells were treated with 20 pM of TGF-ß1, β2, or β3 overnight or were left untreated (-). Luciferase activity was determined, normalized using the β-gal assay, and expressed as a fold increase of untreated cells. The data shown are representative of three different experiments each done in triplicates and statistically significant values are indicated by an asterisk.

**REFERENCES**


1. Roberts AB, Sporn MB 1996 Transforming growth factor beta. In: Clark RAF (ed) The molecular and cellular biology of wound repair (2nd edition), Plenum Press, New York, NY, pp.275-308.
2. Rosier RN, O'Keefe RJ, Crabb ID, Puzas JE 1989 Transforming growth factor beta: An autocrine regulator of chondrocytes. Connect Tissue Res 20:295-301.
3. Howard S, Anastasiades T 1993 Differential effects of bone associated factors on newly synthesized anionic glycoconjugates by articular chondrocyte cultures from adult and immature bovines. J Rheumatol 20:2083-2094.
4. Seyedin S, Thompson A, Bentz H, Rosen DM, McPherson JM, Conti A, Siegel NR, Galluppi GR, and Piez KA 1986 Cartilage-inducing Factor-A. J Biol Chem 261:5693-5695.
5. Roberts AB 1998 Molecular and cell biology of TGF-ß. Miner Electrolyte Metab 24:111-119.
6. Roberts AB, Sporn MB 1990 The transforming growth factor beta's. In: Sporn MB and Roberts AB (eds) Peptide Growth Factors and Their Receptors, vol 95. Spriner-Verlag, New York, pp. 419-472.

7. Sporn MB, Roberts AB, Wakefield LM, de Crombrugghe B 1987 Some recent advances in the chemistry and biology of transforming growth factor-beta. J Cell Biol 105:1039-45.

8. Massague J 1987 The TGF-beta family of growth and differentiation factors. Cell 49:437-8.

9. Bonewald LF 1999 Regulation and regulatory activities of transforming growth factor beta. Crit Rev Eukaryot Gene Expr 9:33-44.

10. Massague J 1998 TGF-beta signal transduction. Ann Rev Biochem 67:753-791.

11. Letamendia A, Lastres P, Botella LM, Raab U, Langa C, Velasco B, Attisano L, Bernabeu C 1998 Role of endoglin in cellular responses to transforming growth factor-β. A comparative study with betaglycan. J Biol Chem 273(49):33011-33019.

12. Tam B, Philip A 1998 Transforming growth factor-β receptor expression on human skin fibroblasts; dimeric complex formation of type I and type II receptors and identification of glycosyl phosphatidylinositol-anchored transforming growth factor-β binding proteins. J Cell Physiol 176:553-564.

13. Tam B, Germain L, Philip A 1998 A 150kDa GPI-anchored TGF-β1 binding protein forms heteromeric complex with the types I and II TGF-β signalling receptors. J Cell Biochem 70:573-586.

14. Cheifetz S, Massague J 1991 Isoform-specific transforming growth factor-beta binding proteins with membrane attachments sensitive to phosphatidylinositol-specific phospholipase C. J Biol Chem 266, 20767-20772.

15. Lopez-Casillas F, Wrana JL, Massague J 1993 Betaglycan presents ligand to the TGF-β signaling receptor. Cell 73:1435-1444.

16. Philip A, Hannah R, O'Connor-McCourt M 1999 Ectodomain cleavage and shedding of the type III TGF-β receptor in the rat lung membranes: Effect of temperature, ligand binding and membrane solubilization. Europ J Biochem 261:618-628.

17. Yamashita H, Ichijo H, Grimsby S, Moron A, ten Dijke P, Miyazono K 1994 Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor beta. J Biol Chem 269:1995-2001.

18. Cheifetz S, Bellon T, Cales C, Vera S, Bernabeu C, Massague J, Letarte M 1992 Endoglin is a component of the transforming growth factor-β receptor system in human endothelial cells. J Biol Chem 267:19027-19030.

19. Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C 2002 Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-β receptors I and II. J Biol Chem 277: 29197-209.

20. Blobe GC, Schiemann WP, Pepin M-C, Beauchemin M, Moustakas A, Lodish HF, O'Connor-McCourt MD 2001 Functional roles for the cytoplasmic domain of the type III transforming growth factor beta receptor in regulating transforming growth factor beta signaling. J Biol Chem 276:24627-24637.

21. Lastres P, Letamendia A, Zhang H, Rius C, Almendro N, Raab U, Loepez LA, Langa C, Fabra A, Letarte M, Bernabeu C 1996 Endoglin modulates cellular responses to TGF-βI. J Cell Biol 133:1109-1121.

22. Li DY, Sorensen LK, Brook BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP 1999 Defective angiogenesis in mice lacking Endoglin. Science 284:1534-1537.

23. Li C, Harpson L, Kumar P, Bernabeu C, Kumar S 2000 CD105 antagonizes the inhibitory signaling of transforming growth factor β1 on human vascular endothelial cells. FASEB J 14:55-64.

24. Barbara NP, Wrana JL, Letarte M 1999 Endoglin is an accessory protein that interacts with the signalling receptor complex of multiple members of the transforming growth factor-β super family. J Biol Chem 274(2):584-594.

25. Robbins JR, Goldring MB 1997 Preparation of immortalized human chondrocyte cell lines. In: Morgan JR, Yarmush ML (eds) Methods in Molecular Medicine, vol 18. Humana Press Inc., Totowa, N.J. pp. 173-192.

26. Goldring MB, Birkhead JR, Suen L-F, Yamin R, Mizuno S, Glowacki J, Apperley JF 1994 Successful immortalization of human chondrocytes exhibiting stable expression of cartilage-specific type II collagen. J Clin Invest 94:2307-2316.

27. Laiho M, Weis FMB, Massague J 1990 Concomitant loss of Transforming Growth Factor (TGF)-β receptor types I and II in TGF-β-resistant cell mutants implicates both receptor types in signal transduction. J Biol Chem 265: 18518-18524.

28. Wang XF, Lin HF, Ng-Eaton E, Downward J, Lodish HF, Weinberg RA 1991 Expression cloning and characterization of the TGF-β type III receptor. Cell 67:797-805.

29. Lopez-Casillas F, Cheifetz S, Doody J, Andres JL, Lane WS, Massague J 1991 Structure and expression of the membrane protein betaglycan, a component of the TGF-β receptor system. Cell 15:785-95.

30. Eickelberg O, Centrella M, Reiss M, Kashgarian M, Wells RG 2002 Betaglycan inhibits TGF-beta signaling by preventing type I-type II receptor complex

formation. Glycosaminoglycan modifications alter betaglycan function. J Biol Chem 277:823-9.

31. Parker WL, Goldring MB, Philip A 2003 Endoglin is expressed on human chondrocytes and forms a heteromeric complex with betaglycan in a ligand and type II TGF-β receptor independent manner. J Bone Miner Res 18:289-302.

32. Parker WL, Choi ME, Philip A Identification of Activin Receptor-like Kinase-1 and Soluble type I TGF-β receptor on human chondrocytes: hetero-oligomerization and regulation of TGF-β signaling. Submitted.

33. Zwaagstra JC, Kassam Z, O'Connor-McCourt MD 1999 Down-regulation of transforming growth factor-beta receptors: cooperativity between the types I, II, and III receptors and modulation at the cell surface. Exp Cell Res 252:352-36.

34. Bellon T, Corbi A, Lastres P, Cales C, Cebrain M, Vera S, Chefeitz M, Massague J, Letarte M, Bernabeu C 1993 Identification and expression of two forms of the human transforming growth factor-β-binding protein endoglin with distinct cytoplasmic regions. Eur J Immunol 23: 2340-2345.

35. Wong SH, Hamel L, Chevalier S, Philip A 2000 Endoglin expression on human microvascular endothelial cells: demonstration of higher order complexes with TGF-β signaling receptors and heteromerization with betaglycan. Eur J Biochem 267:5550-5560.

36. Piek E, Heldin C-H, ten Dijke P 1999 Specificity, diversity, and regulation in TGF-β superfamily FASEB 13:2105-2124.

37. Dumont N, O'Connor-McCourt M, Philip A 1995 Transforming growth factor-β receptors on human endometrial cells: Identification of the type I, II and III

receptors, and glycosylphosphatidylinositol anchored TGF-β binding proteins. Mol Cell Endocrinol 111:57-66.

38. Lucas PJ, Kim SJ, Melby SJ, Gress RE 2000 Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. J Exp Med 191:1187-96.

39. Kim TK, Mo EK, Yoo CG, Lee CT, Han SK, Shim YS, Kim YW 2001 Alteration of cell growth and morphology by overexpression of transforming growth factor beta type II receptor in human lung adenocarcinoma cells. Lung Cancer 31:181-91.

40. Hahm KB, Im YH, Parks TW, Park SH, Markowitz S, Jung HY, Green J, Kim SJ 2001 Loss of transforming growth factor beta signalling in the intestine contributes to tissue injury in inflammatory bowel disease. Gut 49:190-8.

41. Hahm KB, Lee KM, Kim YB, Hong WS, Lee WH, Han SU, Kim MW, Ahn BO, Oh TY, Lee MH, Green J, Kim SJ 2002 Conditional loss of TGF-beta signalling leads to increased susceptibility to gastrointestinal carcinogenesis in mice. Aliment Pharmacol Ther16 Suppl 2:115-27.

42. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J 1994 Mechanism of activation of the TGF-β receptor. Nature 370:341-347.

43. Cheifetz S, Bassols A, Stanley K, Ohta M, Greenberger J, Massague J 1988 J Biol Chem 263:10783-10789.

44. Souchelnytskyi S, Ronnstrand L, Heldin CH, ten Dijke P 2001 Phosphorylation of Smad signaling proteins by receptor serine/threonine kinases. Methods Mol Biol 124:107-20.

45. Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P 1997 TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. EMBO J 16:5353-62.

46. Ohta M, Greenberger JS, Anklesaria P, Bassols A, Massague J 1987 Two forms of transforming growth factor-beta distinguished by multipotential haematopoietic progenitor cells. Nature 329:539-41.

47. Ignotz RA, Massague J 1987 Cell adhesion protein receptors as targets for transforming growth factor-beta action. Cell 51:189-97.

48. Bassols A, Massague J 1988 Transforming growth factor beta regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. J Biol Chem 263:3039-45.

49. Sankar S, Mahooti-Brooks N, Centrella M, McCarthy TL, Madri JA 1995 Expression of transforming growth factor type III receptor in vascular endothelial cells increases their responsiveness to transforming growth factor beta 2. J Biol Chem 270:13567-72.

50. Yang X, Chen L, Xu X, Li C, Huang C, Deng C-X 2001 TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol 153:35-46.

51. Serra R, Johnson M, Filvaroff EH, LaBorde J, Sheehan DM, Derynck R, Moses HL 1997 Expression of a truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. J Cell Biol139:541-52.

52. Demoor-Fossard M, Redini F, Boittin M, Pujol JP 1998 Expression of decorin and biglycan by rabbit articular chondrocytes. Effects of cytokines and phenotypic modulation. Biochem Biophys Acta 1398:179-91.

53. Howard S, Anastasiades T 1993 Differential effects of bone associated factors on newly synthesized anionic glycoconjugates by articular chondrocyte cultures from adult and immature bovines. J Rheumatol 20:2083-2094.

54. Redini F, Galera P, Mauviel A, Loyau G, Pujol JP 1988 Transforming growth factor beta stimulates collagen and glycosaminoglycan biosynthesis in cultured rabbit articular chondrocytes. FEBS Lett 234:172-6.

55. Perka C, Schultz O, Spitzer RS, Lindenhayn K 2000 The influence of transforming growth factor beta1 on mesenchymal cell repair of full-thickness cartilage defects. J Biomed Mater Res 52:543-52.

56. O'Connor WJ, Botti T, Khan SN, Lane JM 2000 The use of growth factors in cartilage repair. Orthop Clin North Am 31:399-410.

57. Massague J, Chen Y-G 2000 Controlling TGF-beta signaling. Genes Dev 14:627-644.

58. Tam B, Larouche D, Germain L, Hooper NM, Philip A 2001 Characterization of a 150 kDa accessory receptor for TGF-β1 on keratinocytes: Direct evidence for a GPI-anchor, and ligand binding of the released form. J Cell Biochem 83:494-507.

59. Fukumura K, Matsunaga S, Yamamoto T, Nagamine T, Ishidou Y, Sakou T 1998 Immunolocalization of transforming growth factor-betas and type I and type II receptors in rat articular cartilage. Anticancer Res 18:4189-4194.

60. Hall FL, Benya PD, Padilla SR, Carbonaro-Hall D, Williams R, Buckley S, Warburton D 1996 Transforming growth factor-beta type-II receptor signalling: intrinsic/associated casein kinase activity, receptor interactions and functional effects of blocking antibodies. Biochem J 316( Pt 1):303-10.

61. Matsunaga S, Yamamoto T, Fukumura K 1999 Temporal and spatial expressions of transforming growth factor-betas and their receptors in epiphyseal growth plate. Int J of Onc 14:1063-1067.

62. Boumediene K, Conrozier T, Mathieu P, Richard M, Marcelli C, Vignon E, Pujol J-P 1998 Decrease of cartilage transforming growth factor-β receptor II expression in the rabbit experimental osteoarthritis-potential role in cartilage breakdown. Osteoarth Cart 6:146-149.

63. Horner A, Kemp P, Summers C, Bord S, Bishop NJ, Kelsall AW, Coleman N, Compston JE 1998 Expression and distribution of transforming growth factor-beta isoforms and their signaling receptors in growing human bone. Bone 23:95-102.

64. Robbins JR, Thomas B, Tan L, Choy B, Arbiser JL, Berenbaum F, Goldring MB 2000 Immortalized human adult articular chondrocytes maintain cartilage-specific phenotype and responses to interleukin-1beta. Arthritis Rheum 43:2189-201.

65. Goldring MB, Berenbaum F 1999 Human chondrocyte culture models for studying cyclooxygenase expression and prostaglandin regulation of collagen gene expression. Osteoarthritis Cartilage 7:386-88.

66. Kokenyesi R, Tan L, Robbins JR, Goldring MB 2000 Proteoglycan production by immortalized human chondrocyte cell lines cultured under conditions that promote expression of the differentiated phenotype. Arch Biochem Biophys 383:79-90.

67. Loeser R, Sadiev S, Tan L, Goldring MB 2000 Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for α1β1 and α2β1 integrins in mediating chondrocyte adhesion to types II and VI collagen. Osteoarthritis Cartilage 8:96-105.

68. Massague J, Cheifetz S, Ignotz RA, Boyd FT 1987 Multiple type-beta transforming growth factors and their receptors. J Cell Physiol Suppl Suppl 5:43-7.

69. Quackenbush EJ, Letarte M 1985 Identification of several cell surface proteins of non-T, non-B acute lymphoblastic leukemia by using monoclonal antibodies. J Immunol 134:1276-1285.

70. Arthur HM, Ure J, Smith AJH, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG 2000 Endoglin, an ancillary TGF-ß receptor, is required for extra embryonic angiogenesis and plays a key role in heart development. Dev Biol 217:42-53.

71. Guerrero-Esteo M, Lastres P, Letamendia A, Perez-Alvarez MJ, Langa, C, Lopez LA, Fabra A, Garcia-Pardo A, Vera S, Letarte M, Bernabeu C 1999 Endoglin overexpression modulates cellular morphology, migration, and adhesion of mouse fibroblasts. E J Cell Biol 78:614-623.

72. Lux A, Attisano L, Marchuk DA 1999 Assignment of transforming growth factor β1 and β3 and a third new ligand to the type I receptor ALK-1 J Biol Chem 274:9984-9992.

73. Fonsattii E, del Vechchio L, Altomonte M, Sigalotti L, Nicotra MR, Coral S, Natali PG, Maio M 2001 Endoglin: An accessory component of the TGF-β-binding

receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. J Cell Phys 188:1-7.

74. Lopez-Casillas F, Payne HM, Andres JL, Massague J 1994 Betaglycan can act as a dual modulator of TGF-β access to signalling receptors: mapping of ligand binding and GAG attachment sites. J Cell Biol 124:557-568.

75. Pepin MC, Beauchemin M, Plamondon J, O'Connor-McCourt MD 1994 Mapping of the ligand binding domain of the transforming growth factor beta receptor type III by deletion mutagenesis. Proc Natl Acad Sci USA 91:6997-7001.

76. Esparza-Lopez J, Montiel JL, Vilchis-Landeros MM, Okadome T, Miyazono K, Lopez-Casillas F 2001 Ligand binding and functional properties of betaglycan, a co-receptor of the transforming growth factor-beta superfamily. Specialized binding regions for transforming growth factor-beta and inhibin A. J Biol Chem 276:14588-96.

77. Dickson K, Philip A, Warshawsky H, O'Connor-McCourt M Bergeron JJM 1995 Specific binding of endocrine TGFβ1 to vascular endothelium. J Clin Invest 95: 2539-2554.

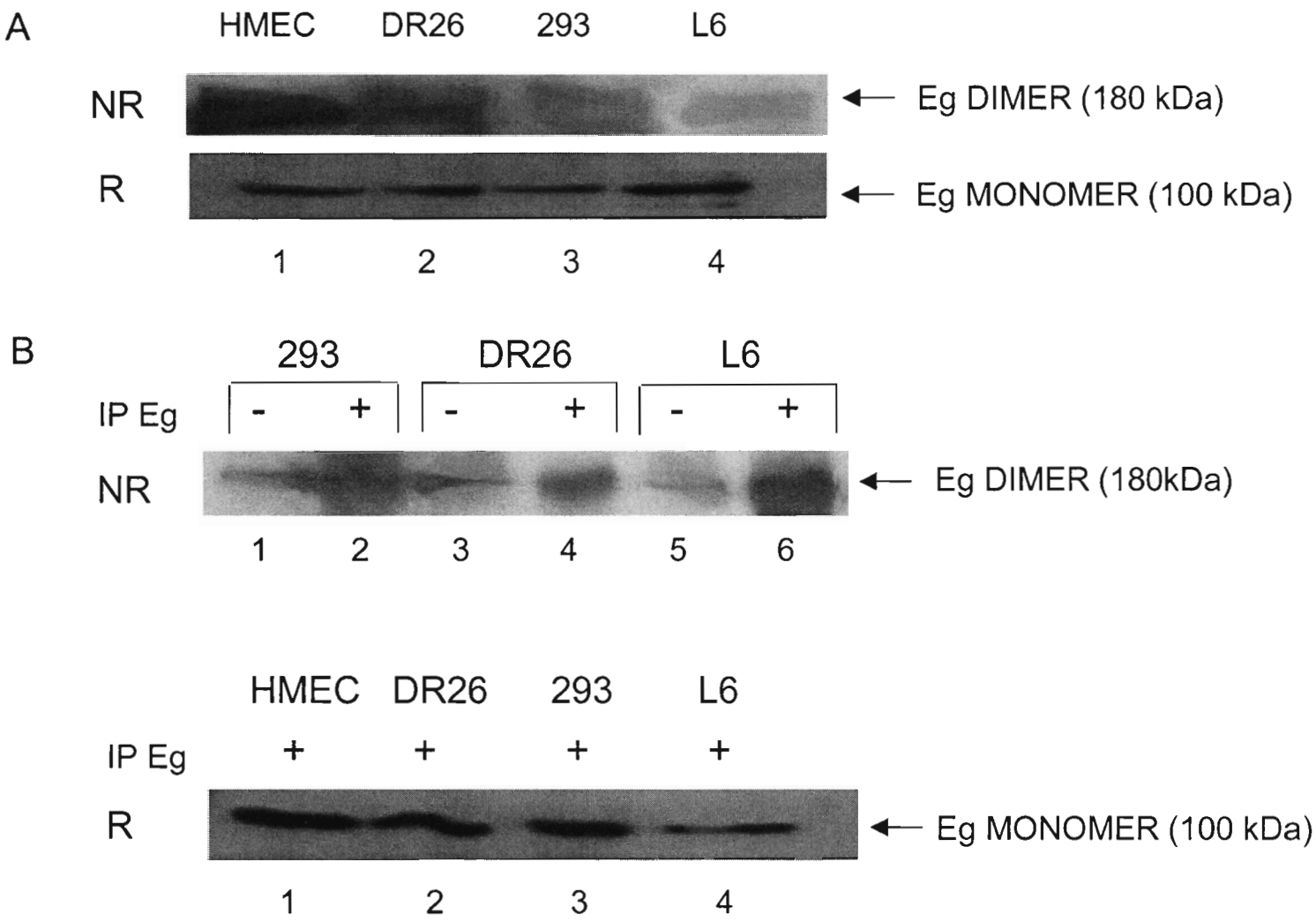
78. Khalil N, Parekh TV, O'Connor R, Antman N, Kepron W, Yehaulaeshet T, Xu YD, Gold LI 2001 Regulation of the effects of TGF-beta 1 by activation of latent TGF-beta 1 and differential expression of TGF-beta receptors (T beta R-I and T beta R-II) in idiopathic pulmonary fibrosis. Thorax 56:907-15.

79. Weber AJ, De Bandt M 2000 Angiogenesis: general mechanisms and implications for rheumatoid arthritis. Joint Bone Spine 67:366-83.

80. Chin GS, Liu W, Peled Z, Lee TY, Steinbrech DS, Hsu M, Longaker MT 2001 Differential expression of transforming growth factor-beta receptors I and II and activation of Smad 3 in keloid fibroblasts. Plast Reconstr Surg 108:423-9.

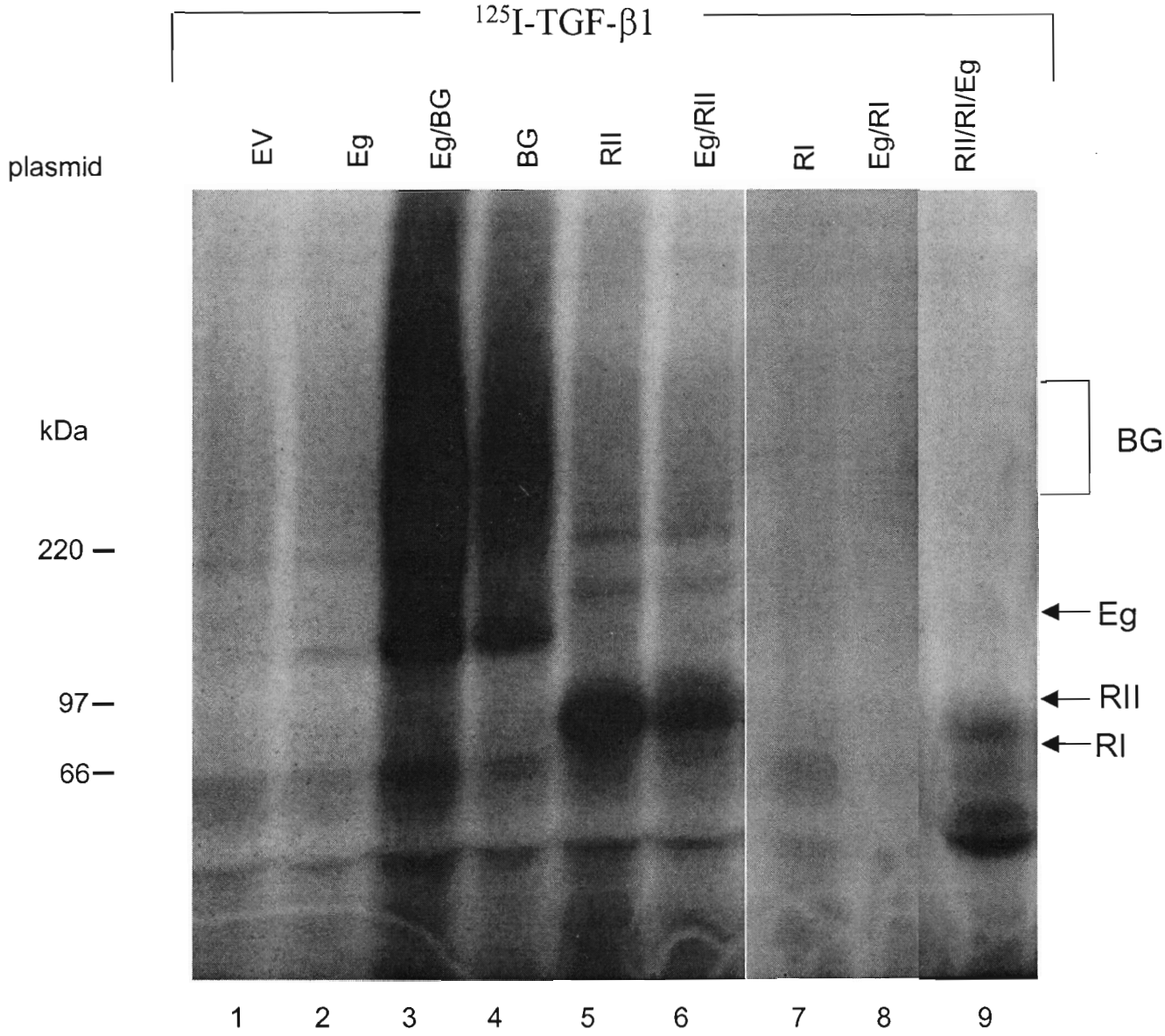
81. Kelly DL, Rizzino A 1999 Growth regulatory factors and carcinogenesis: the roles played by transforming growth factor beta, its receptors and signaling pathways. Anticancer Res 19:4791-807.

Fig. 1


A
HMEC
DR26
293
L6
NR
Eg DIMER (180 kDa)
R
Eg MONOMER (100 kDa)
1
2
3
4
B
293
DR26
L6
IP Eg
-
+
-
+
-
+
NR
Eg DIMER (180kDa)
1
2
3
4
5
6
HMEC
DR26
293
L6
IP Eg
+
+
+
+
R
Eg MONOMER (100 kDa)
1
2
3
4

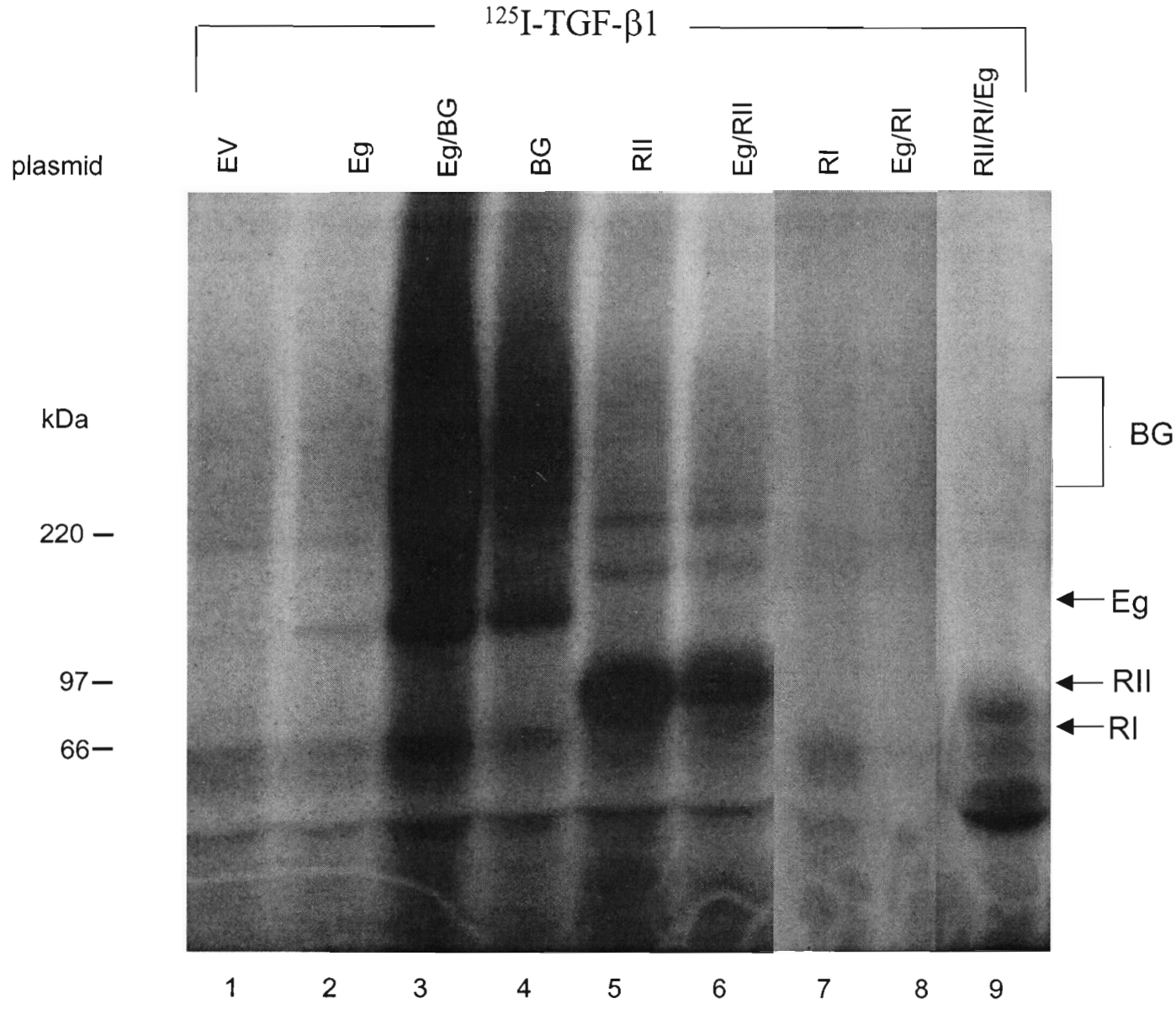
276

Fig. 2


125I-TGF-β1
plasmid
EV
Eg
Eg/BG
BG
RII
Eg/RII
RI
Eg/RI
RII/RI/Eg
kDa
220
97
66
BG
Eg
RII
RI
1
2
3
4
5
6
7
8
9

277

Fig. 2


125I-TGF-β1
plasmid
EV
Eg
Eg/BG
BG
RII
Eg/RII
RI
Eg/RI
RII/RI/Eg
kDa
220
97
66
BG
Eg
RII
RI
1
2
3
4
5
6
7
8
9

278

Fig. 3A

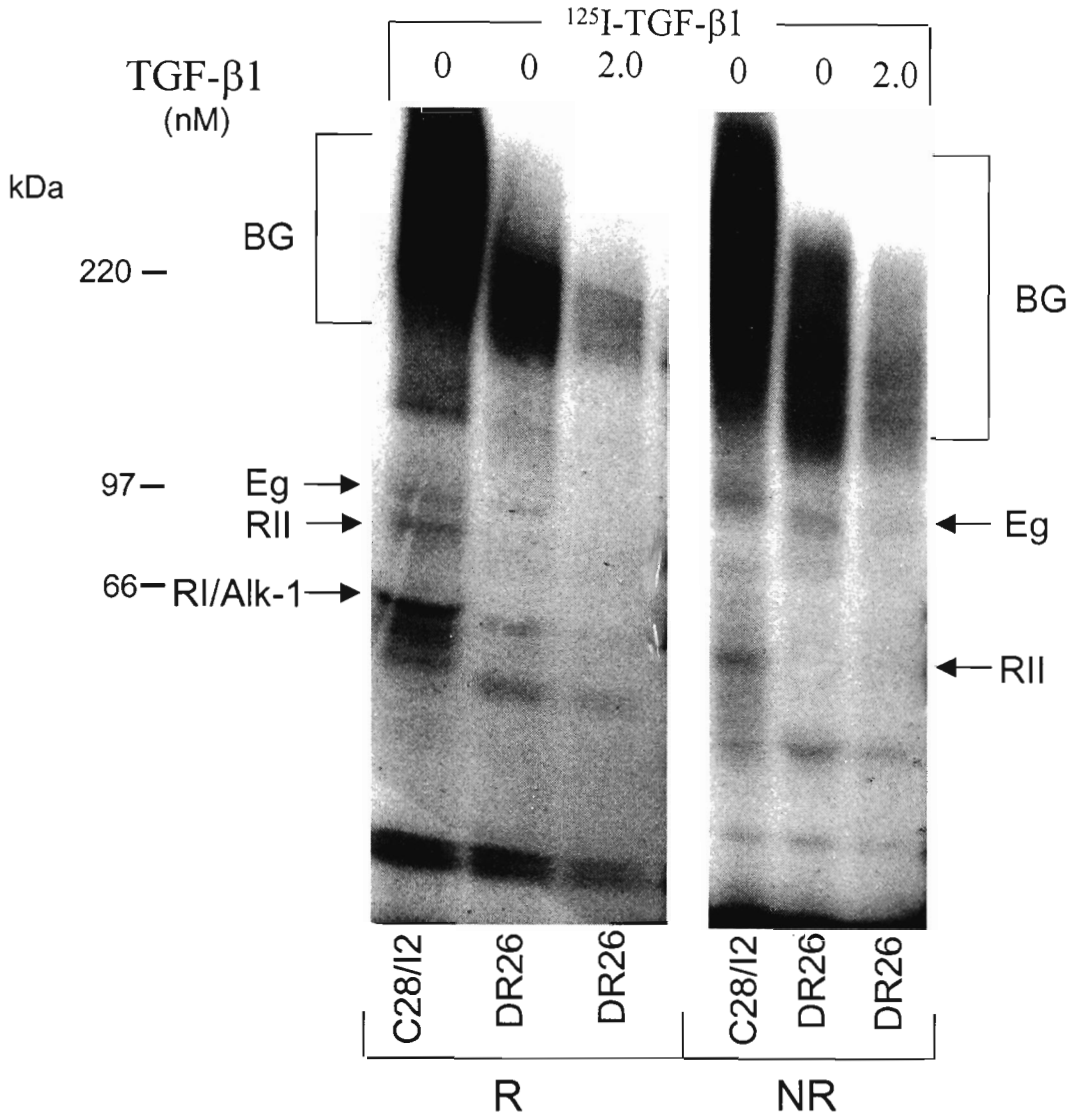

125I-TGF-β1
TGF-β1
(nM)
0 0 2.0 0 0 2.0
kDa
220
BG
97
Eg
RII
66
RI/Alk-1
BG
Eg
RII
C28/I2
DR26
DR26
C28/I2
DR26
DR26
R
NR

Fig. 3B

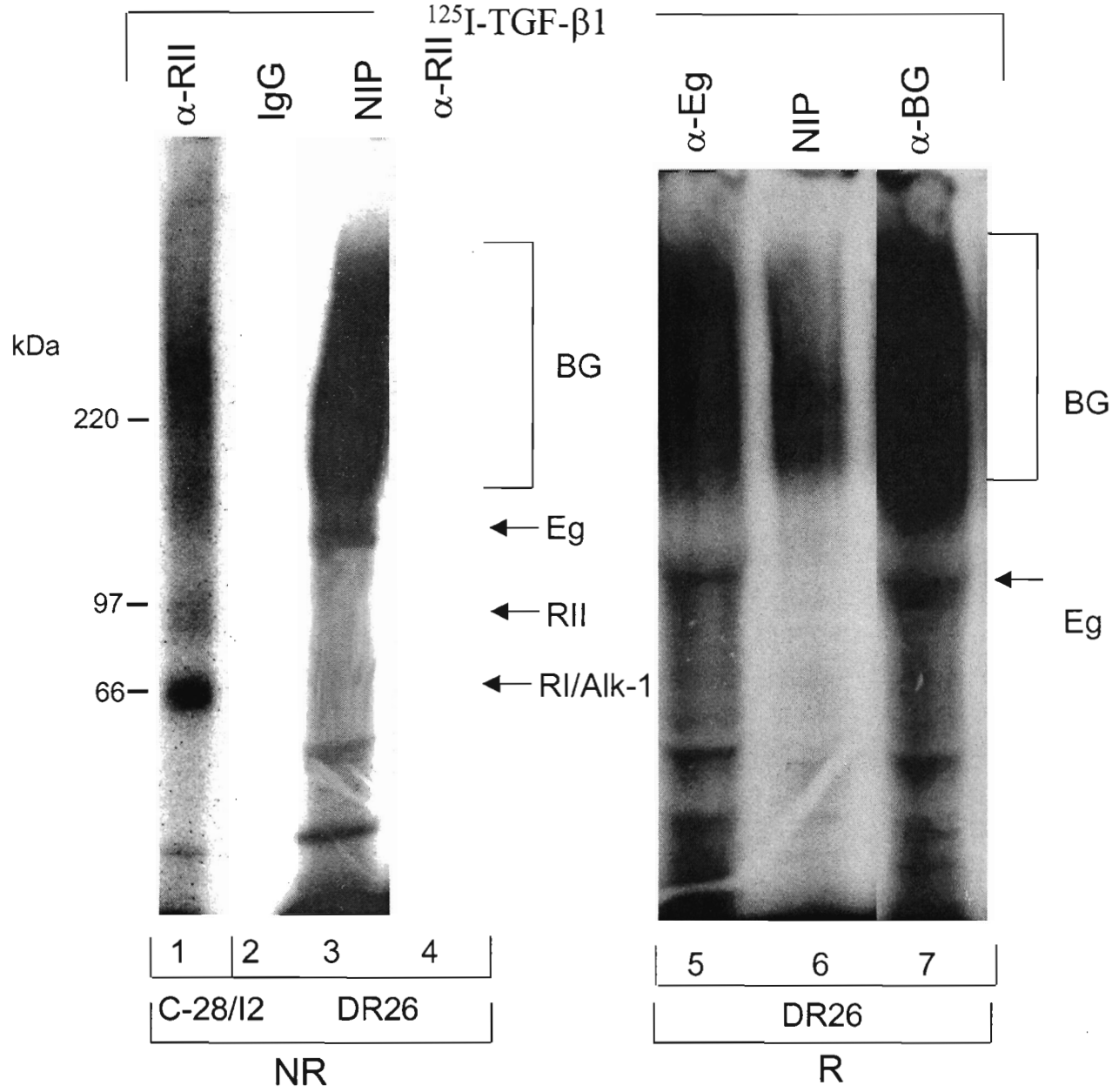
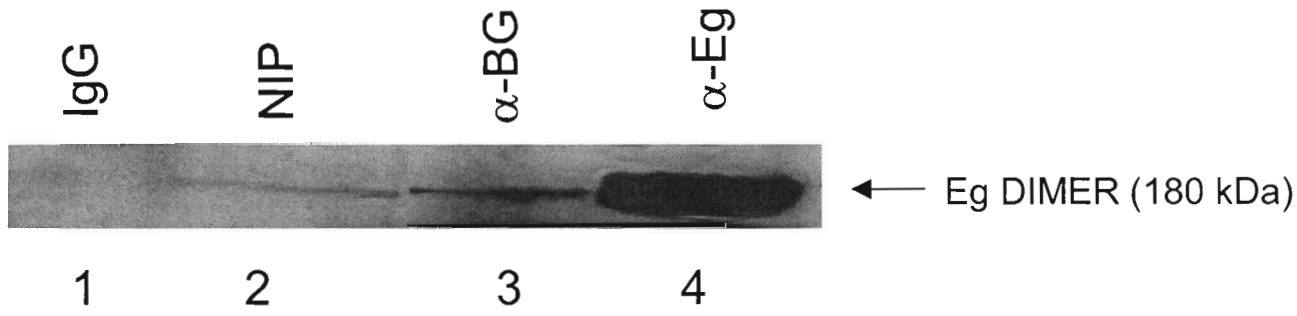

125I-TGF-β1
α-RII
IgG
NIP
α-RII
α-Eg
NIP
α-BG
kDa
220
97
66
BG
Eg
RII
RI/Alk-1
BG
Eg
1
2
3
4
5
6
7
C-28/I2
DR26
DR26
NR
R

Fig. 3C




281

Fig. 4A

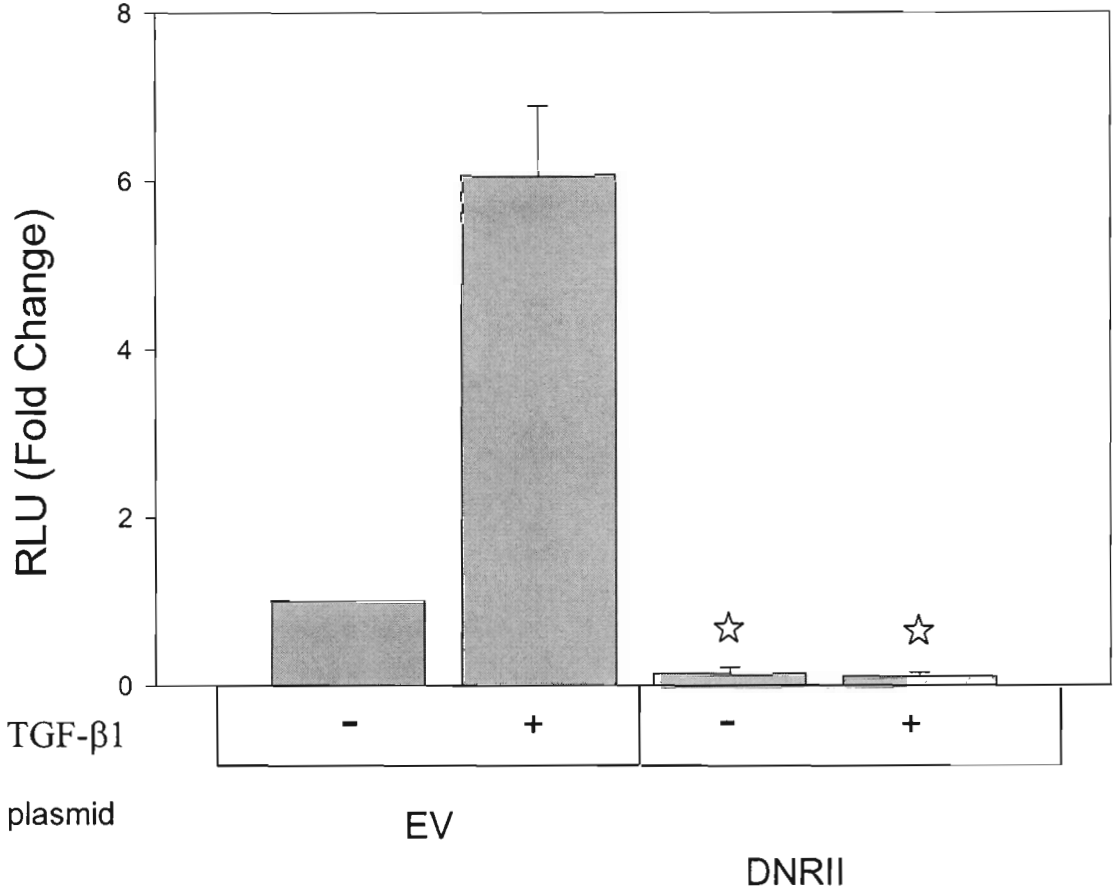


Fig. 4B

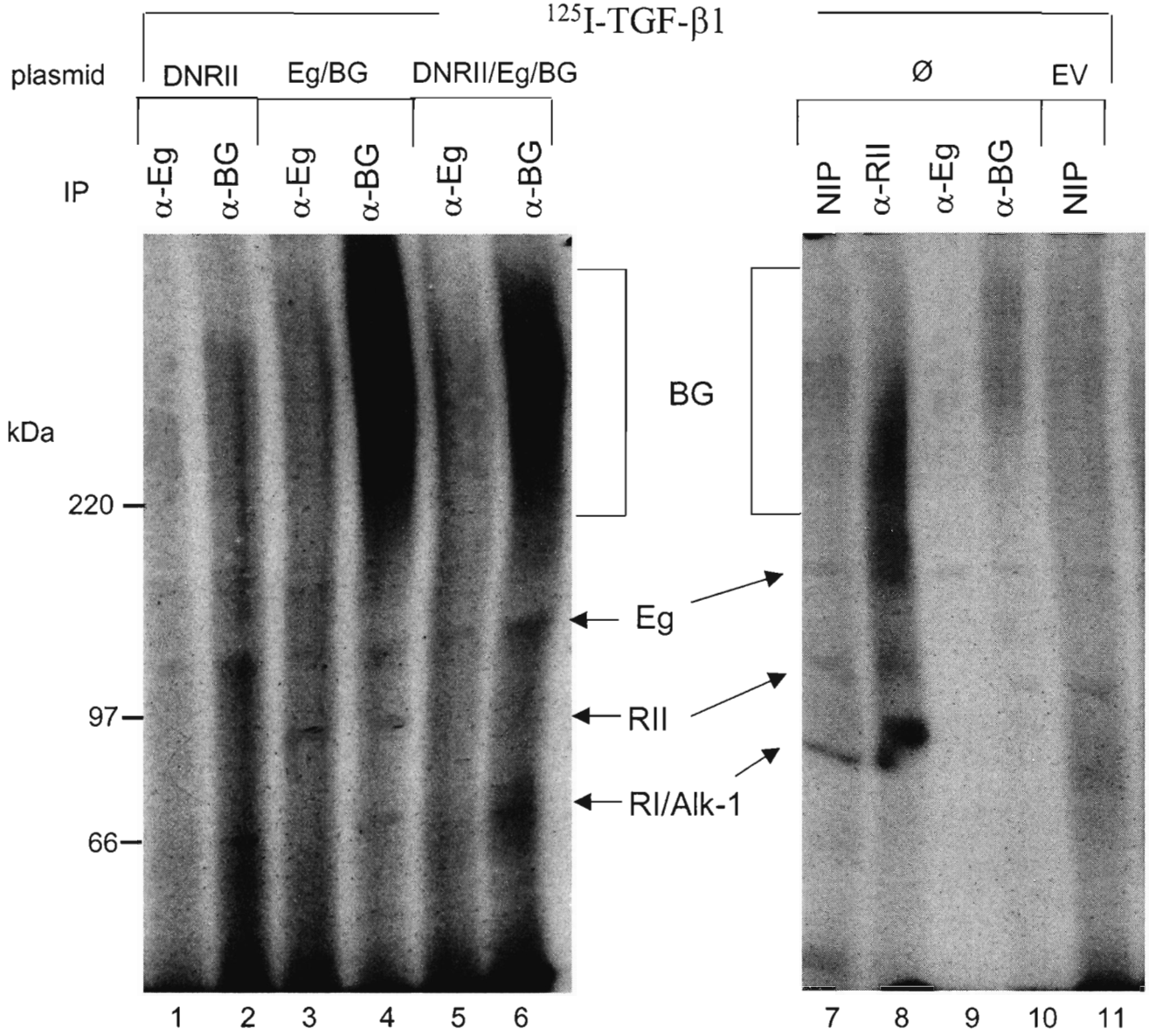

125I-TGF-β1
plasmid
DNRII
Eg/BG
DNRII/Eg/BG
Ø
EV
IP
α-Eg
α-BG
α-Eg
α-BG
α-Eg
α-BG
NIP
α-RII
α-Eg
α-BG
NIP
kDa
220
97
66
BG
Eg
RII
RI/Alk-1
1
2
3
4
5
6
7
8
9
10
11

Fig. 5A

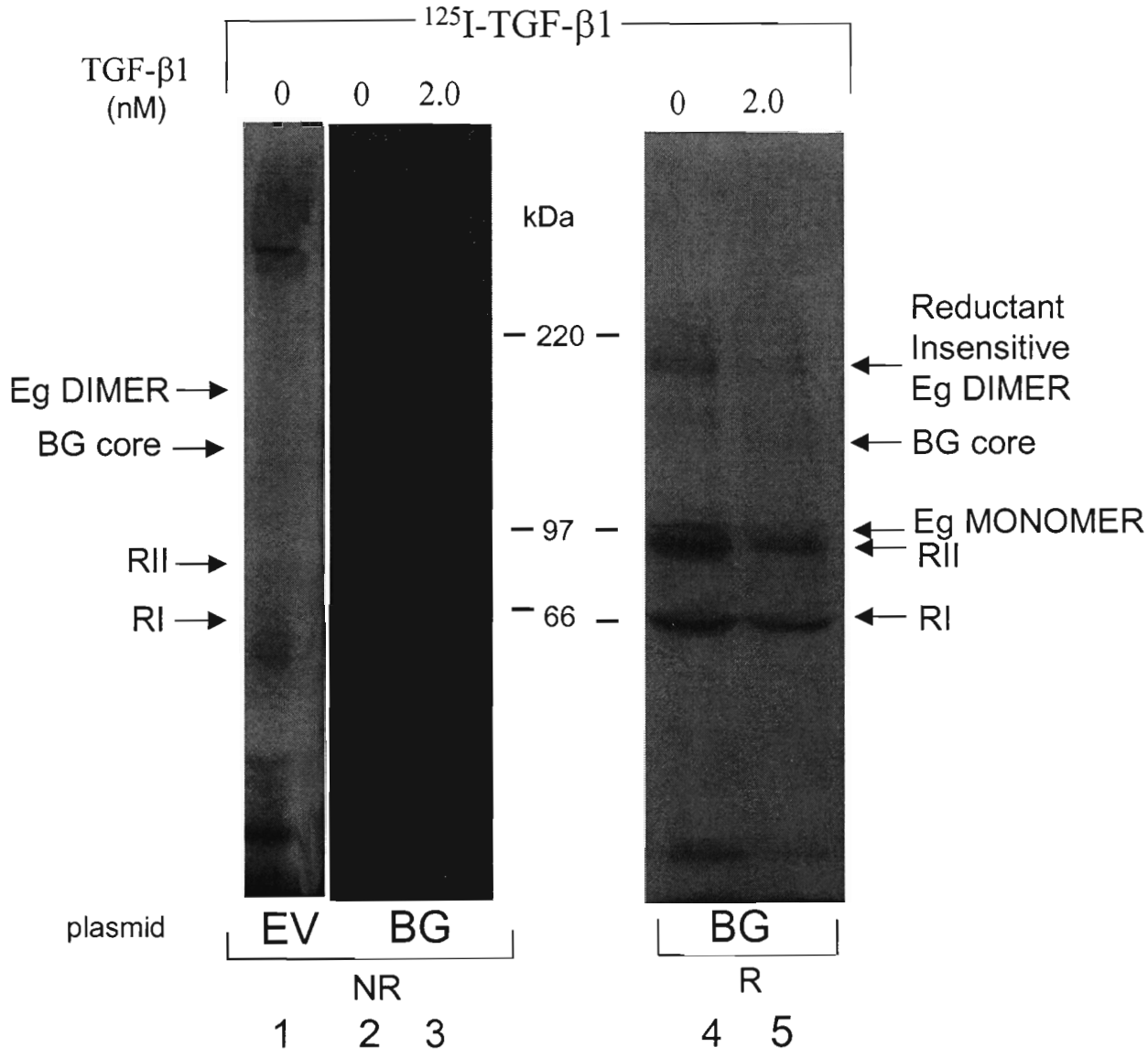

125I-TGF-β1
TGF-β1
(nM)
0
0
2.0
0
2.0
kDa
220
97
66
Eg DIMER
BG core
RII
RI
Reductant
Insensitive
Eg DIMER
BG core
Eg MONOMER
RII
RI
plasmid
EV
BG
BG
NR
R
1
2
3
4
5

Fig. 5B

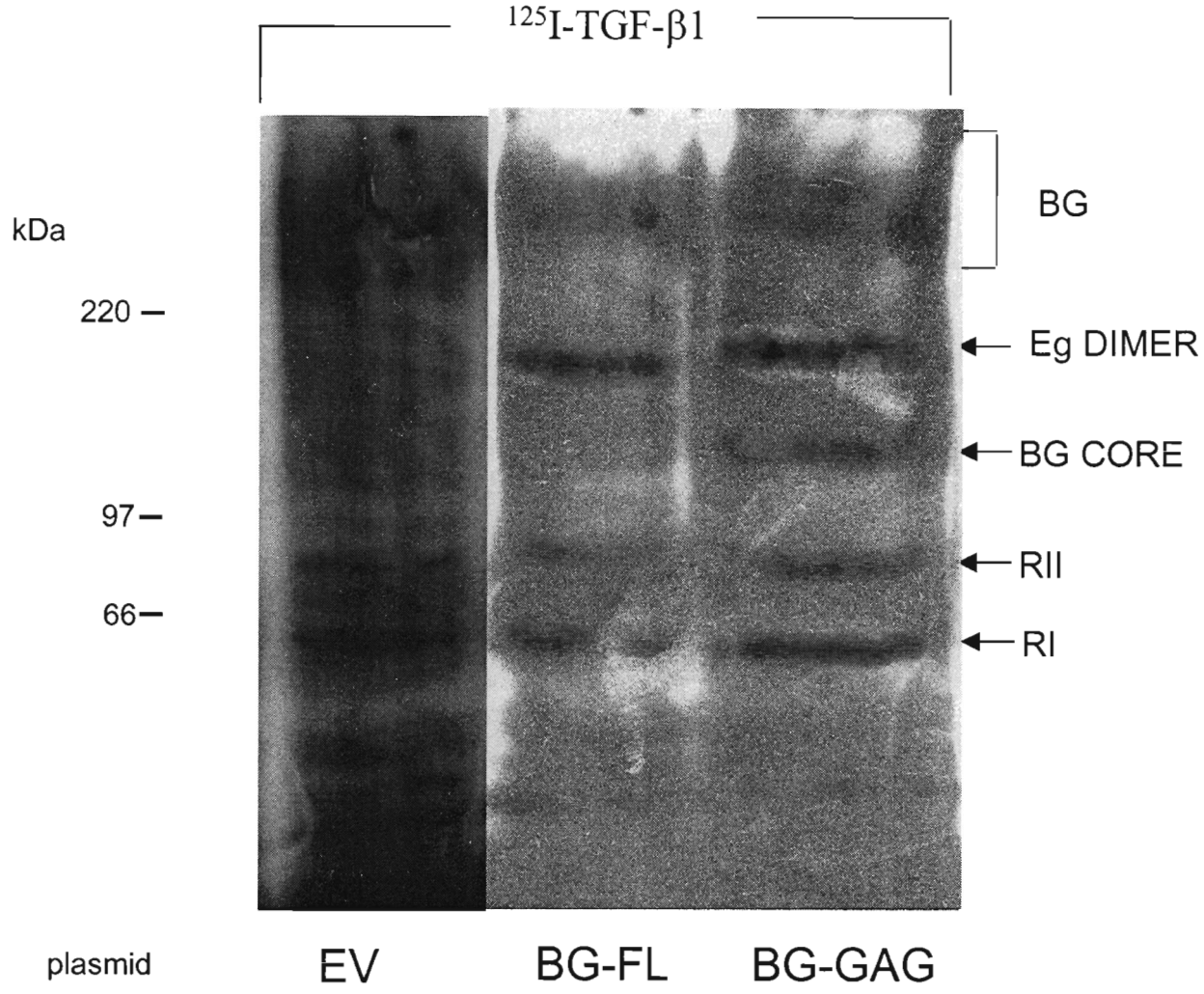

125I-TGF-β1
kDa
220
97
66
BG
Eg DIMER
BG CORE
RII
RI
plasmid
EV
BG-FL
BG-GAG

Fig. 6A

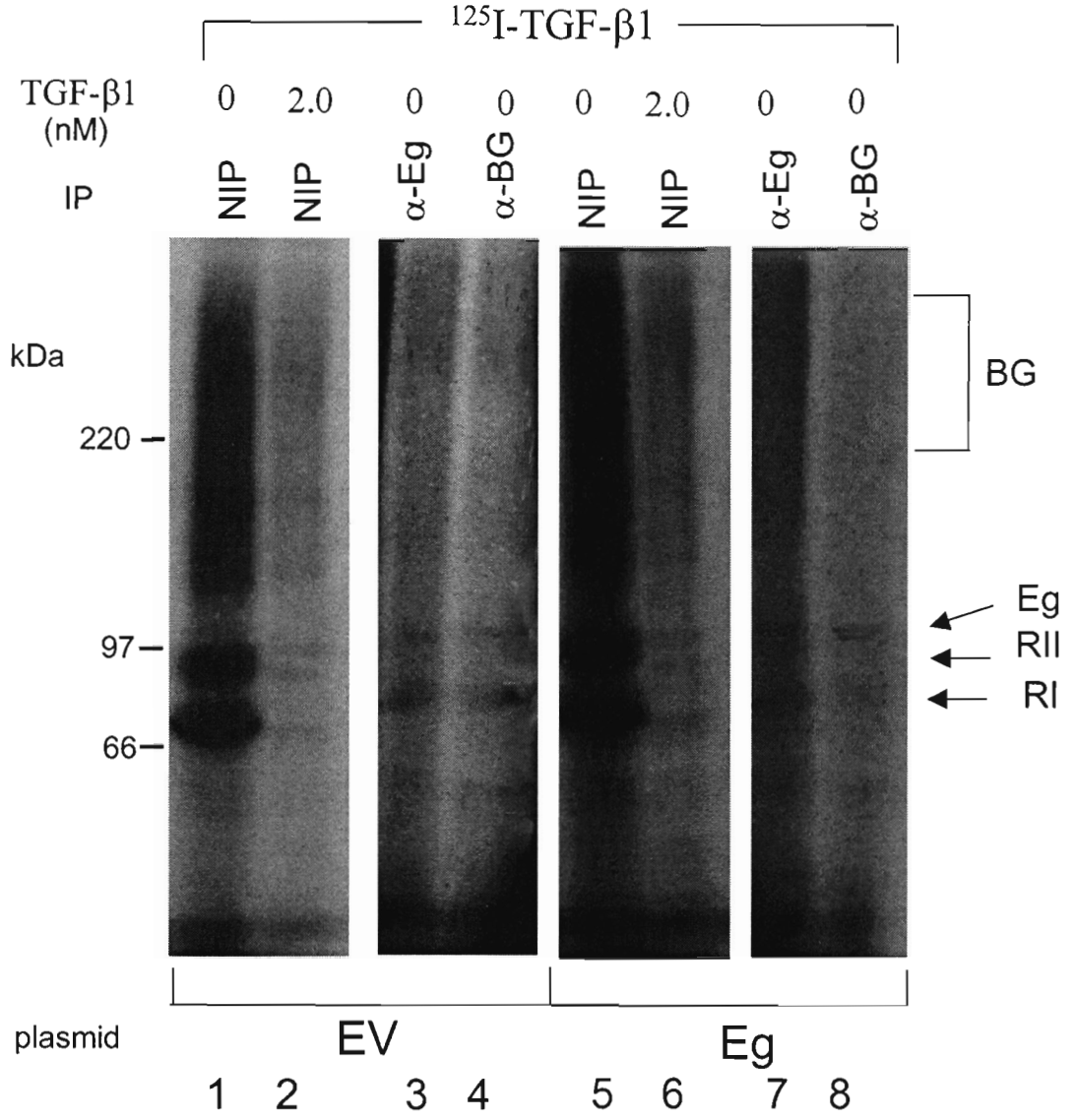
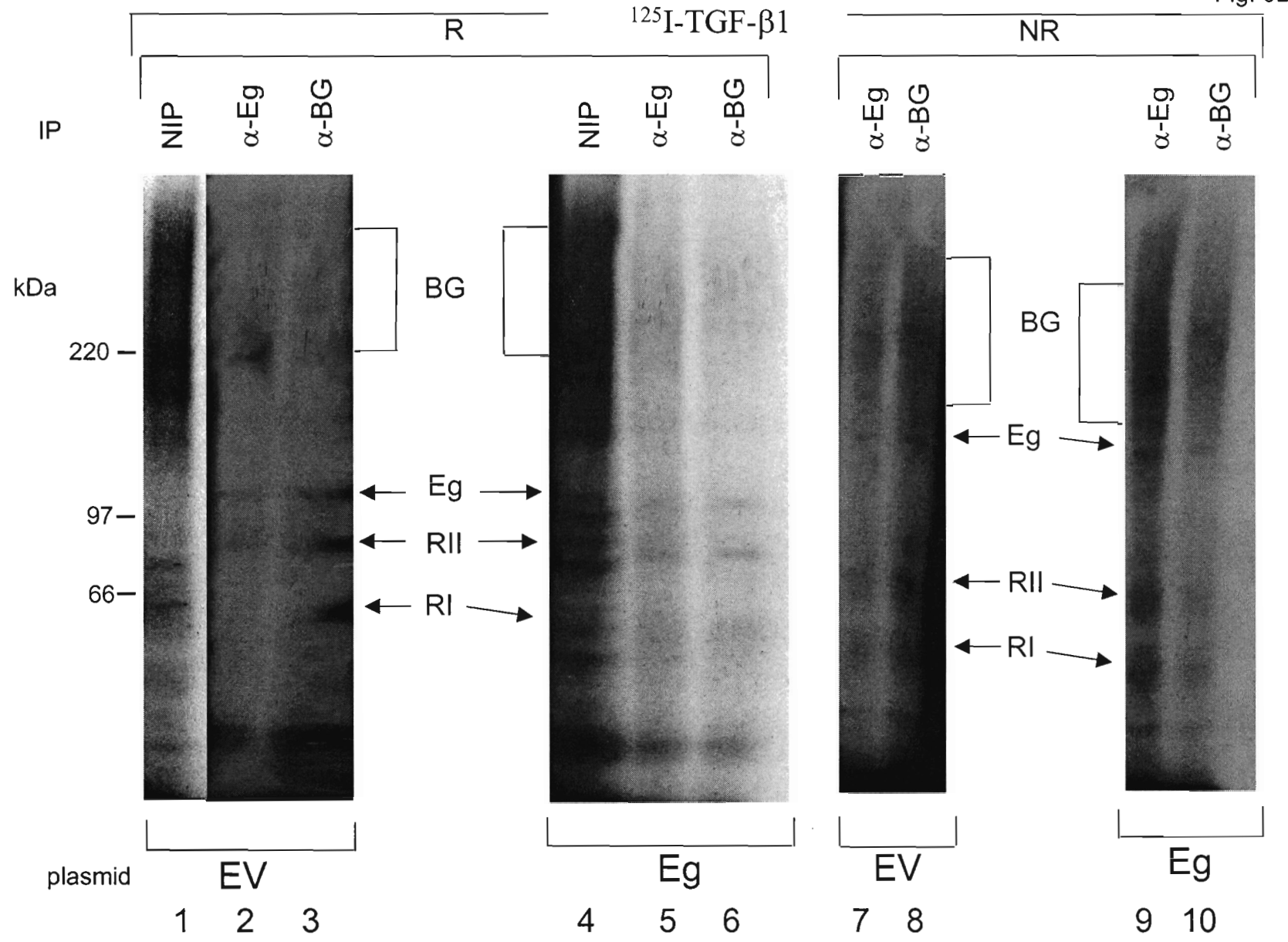
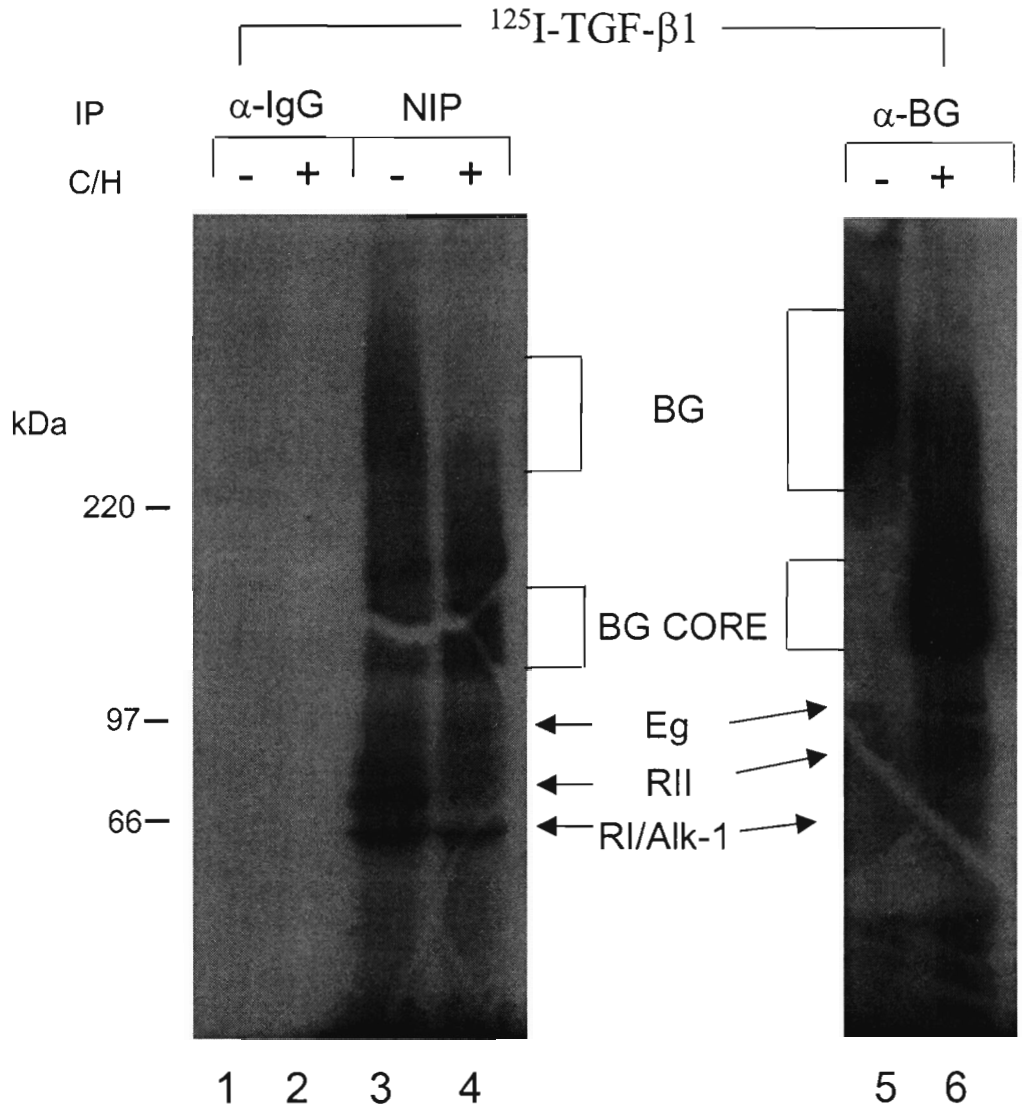

125I-TGF-β1
TGF-β1 (nM)
0
2.0
0
0
0
2.0
0
0
IP
NIP
NIP
α-Eg
α-BG
NIP
NIP
α-Eg
α-BG
kDa
220
97
66
BG
Eg
RII
RI
plasmid
EV
Eg
1 2 3 4 5 6 7 8

Fig. 6B


R
125I-TGF-β1
NR
IP
NIP
α-Eg
α-BG
NIP
α-Eg
α-BG
α-Eg
α-BG
α-Eg
α-BG
kDa
220
97
66
BG
Eg
RII
RI
BG
Eg
RII
RI
plasmid
EV
Eg
EV
Eg
1 2 3
4 5 6
7 8
9 10

287

Fig. 6C


125I-TGF-β1
IP
α-IgG
NIP
α-BG
C/H
- + - +
- +
kDa
220
97
66
BG
BG CORE
Eg
RII
RI/Alk-1
1 2 3 4
5 6

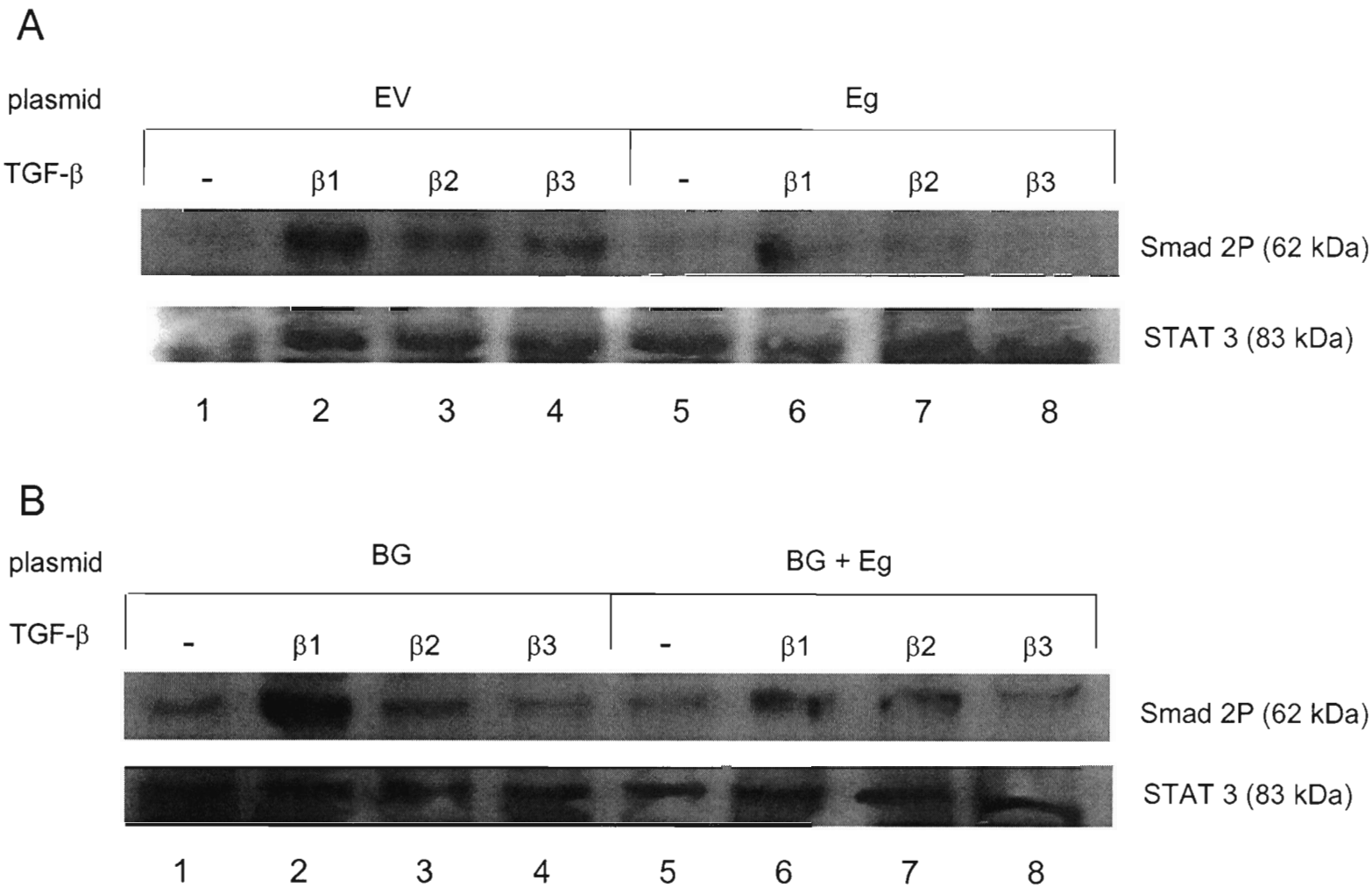
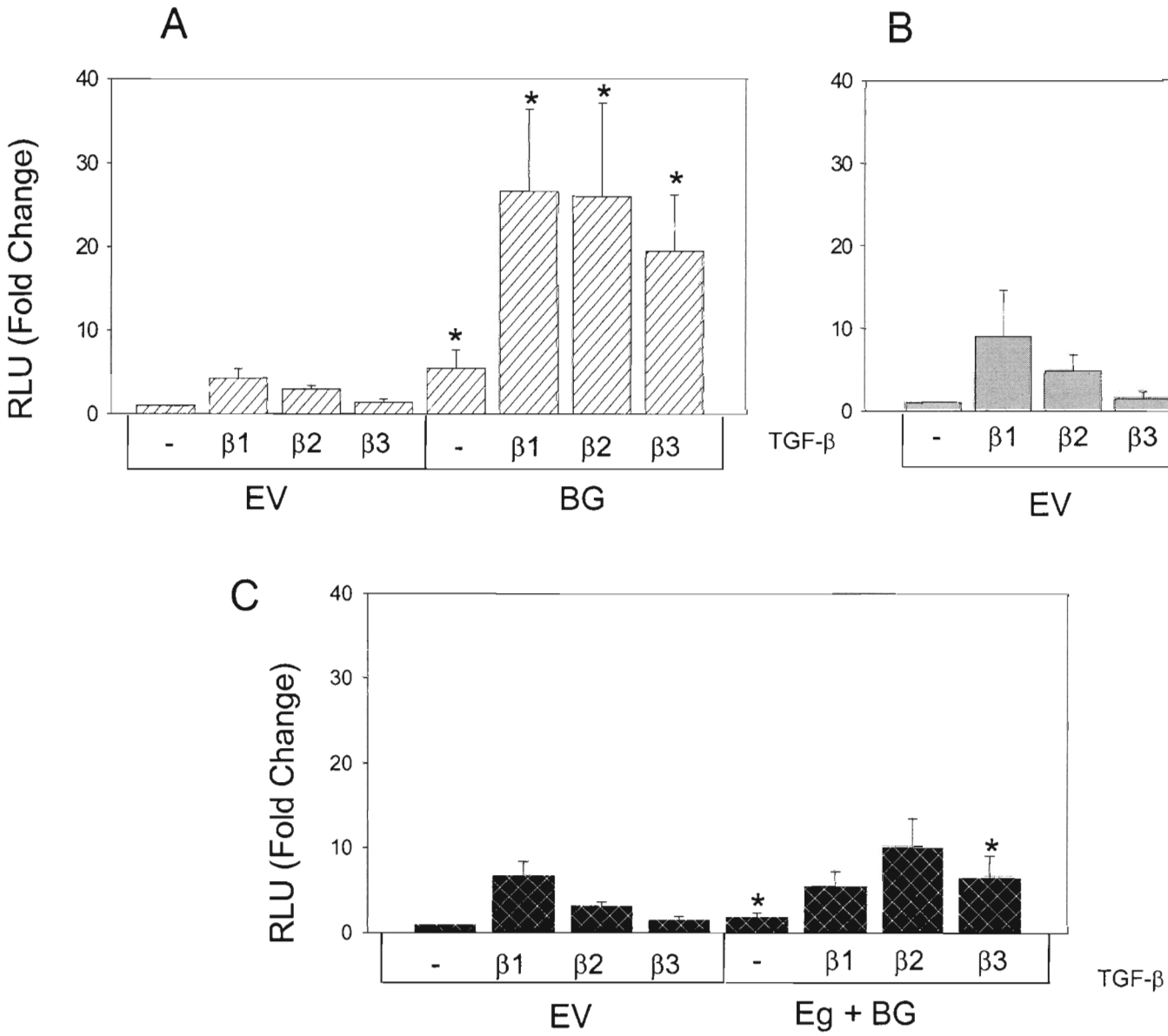

A
plasmid
EV
Eg
TGF-β
-
β1
β2
β3
-
β1
β2
β3
Smad 2P (62 kDa)
STAT 3 (83 kDa)
1
2
3
4
5
6
7
8
B
plasmid
BG
BG + Eg
TGF-β
-
β1
β2
β3
-
β1
β2
β3
Smad 2P (62 kDa)
STAT 3 (83 kDa)
1
2
3
4
5
6
7
8

Fig. 7B




290

# CHAPTER 4

# BRIDGE

Identification of a spliced variant of the TGF-β type II signaling receptor (RIIB) on human chondrocytes and its receptor associations. The effects on TGF-β responsiveness of RIIB overexpression as compared to endogenous RIIB levels and the correlation of expression with cell phenotype.

## CHAPTER 4

BRIDGING DOCUMENT

### Introduction and Rationale

In 1997, Glansbeek et al identified an additional TGF-β binding protein on human chondrocytes (Glansbeek et al 1997) which was believed to be a variant of RII. However, this finding was only based on the presence of a band noted with affinity labeling. In addition, the authors mistakenly identified RII as the variant and the variant as RII. This receptor was later cloned and shown to have a predominant role in TGF-β2 signaling (Rotzer et al 2001). It was suggested that this spliced variant, RIIB, is restricted to cells originating from tissues such as bone; however, it has previously been identified and cloned on human vascular endothelial cells (Hirai and Fujita 1996). Affinity labeling profiles of human chondrocytes (manuscript 1) had demonstrated a potential TGF-β binding protein with a molecular weight of approximately 12 kDa higher than RII which was thought to potentially represent RIIB.

Gene targets and cellular responses of TGF-β are numerous; yet, TGF-β signaling appears to follow a relatively simplistic cascade involving Smads, which until recently were believed to be the only direct substrate of TGF-β. Thus, there must exist a means of distinguishing the signal to initiate the intended downstream response. One possibility is that variations in signaling and accessory receptors, in forming complexes with different receptor subtypes and concentrations provide this needed diversity. Evidence for this has been observed in growth plate chondrocytes. The biphasic growth response seen in growth plate chondrocytes has been attributed to a differential expression of "high affinity" receptors (Rosier et al 1989). In addition, the ratio of RI to RII as also been shown to modulate TGF-β responsiveness with respect to cell cycle and proliferation (Vivien et al 1993a). An alternative means of

distinguishing the TGF-β signal may be afforded by ligand concentration; TGF-β is known to result in differential responses at low and high dose ranges (Pepper et al 1993).

**Hypotheses**

(1) that a variant of RII (RIIB) is present on human chondrocytes and similar to RII is able to form complexes with accessory receptors

(2) that the expression of this novel signaling receptor will vary between chondrocytes of distinct phenotypes

(3) that overexpression of RIIB will regulate TGF-β signaling and ECM in these cells

**Objectives**

(i) to confirm the presence of RIIB and its heteromeric complexes with other TGF-β receptors on human chondrocytes

(ii) to investigate phenotypic differences in RIIB expression between differentiated and dedifferentiated chondrocytes

(iii) to determine the functional role of RIIB in modulating TGF-β signaling and ECM in these cells

**Summary of Manuscript Findings**

In the fourth manuscript, the presence of RIIB on human chondrocytes and its complex formation with the signaling receptors and novel TGF-β receptors including endoglin, betaglycan, Alk-1, and Sol RI is documented. RIIB's affinity for all three TGF-β isoforms (β1>β3>β2) is demonstrated. Evidence is provided that exogenous or overexpressed RIIB enhances TGF-β responses whereas endogenous RIIB levels correlate with a decreased TGF-β responsiveness and that this corresponds to chondrocyte phenotype. The novel finding of marked increased RIIB expression in OA versus normal human chondrocytes is presented

and increased RIIB expression in these dedifferentiated primary cells suggests that RIIB may, in addition to its important role in TGF-β signaling, be a marker of chondrocyte phenotype and thus play a role in governing TGF-β responsiveness in these cells.

## Conclusions

The results demonstrated in the following manuscript show that the expression profile of TGF-β receptors on human chondrocytes has been expanded to include RIIB. Evidence suggests that, in addition to the signaling receptors (RI and RII), RIIB forms heteromeric complexes with endoglin, betaglycan, Sol RI and Alk-1. These findings add merit to the theory of a fluid hetero-oligomeric TGF-β receptor complex on human chondrocytes in which the relative expression level of the individual receptor components may determine the outcome of TGF-β signaling. More importantly, RIIB and the state of chondrocyte differentiation are critically linked suggesting that RIIB may be a phenotypic marker in these cells. The high levels of RIIB expression in dedifferentiated OA chondrocytes and the type II collagen regulation evident with its overexpression demonstrate that RIIB may play a role in governing TGF-β responsiveness in these cells. Thus, receptor associations and levels of expression may be critical for achieving the fine balance of TGF-β signaling and ECM regulation.

**CONTRIBUTION OF AUTHORS**

# MANUSCRIPT FOUR

## The type IIB TGF-β receptor on human chondrocytes: phenotypic variability of expression and differential effects of endogenous versus exogenous expression

**WL Parker:** experimental designs, procedures, and analysis, preparation of figures and text

**P Knaus:** contribution of anti-RIIB antibody and the expression plasmid for RIIB

**A Philip:** assistance with experimental design and analysis and manuscript editorial assistance

# The type IIB TGF-β receptor on human chondrocytes: phenotypic variability of expression and differential effects of endogenous versus exogenous expression

Wendy L. Parker[1], Petra Knaus[2], and Anie Philip[1]*

[1]Division of Plastic Surgery, Department of Surgery, McGill University, Montreal, Quebec, Canada

[2]Department of Physiological Chemistry, Biocenter and Department of Virology, University of Wurzburg, Germany

*Correspondence to: Anie Philip, Division of Plastic Surgery, Montreal General Hospital, 1650 Cedar Avenue, Room C9-177, Montreal, Quebec, Canada H3G 1A4.

Telephone: (514) 937 1934 ext 44533; Fax: (514) 934 8289

E-mail: anie.philip@mcgill.ca

Running Title: Opposing actions of RIIB

Acknowledgements

We thank Dr. S. Souchelnytskyi (Uppsala, Sweden) for the anti-betaglycan (Getl) and anti-phospho Smad 2 antibodies; Dr. M. O'Connor McCourt (Montreal, Quebec) for the gift of plasmid encoding the type II TGF-β receptor; Dr. M. Choi (New Haven, Connecticut) for the anti-Sol RI antibody; Dr. R. Poole (Montreal, Quebec) for the anti-type II collagen antibody; Dr. Mary Goldring (Boston, MA) for the immortalized human chondrocyte lines (C-28/I2 and tsT/AC62); Dr. Eric Lenzcner (Montreal, Quebec) for the human articular cartilage; and Genzyme Inc. (Framingham, MA) for the gift of TGF-β1 and TGF-β2.

Summary

The poor innate reparative ability of human cartilage has been attributed to the lack of mature chondrocyte proliferation, their differentiation into a fibroblastic-like cell and the avascular state of the tissue. TGF-β, a key player in the chondrocyte microenvironment, has been implicated in cartilage healing, in modulating the phenotypic state of the cell and ultimately the turnover of the extracellular matrix (ECM). The mechanisms by which TGF-β and its receptors modulate these diverse actions in cartilage are unclear. In the current study, we document the presence of RIIB, a spliced variant of the type II receptor (RII) in human chondrocytes and demonstrate its heteromeric complex formation with novel TGF-β receptors (endoglin, betaglycan, Sol RI, Alk-1) in addition to signaling receptors on these cells. Moreover, we show that RIIB varies with cell phenotype; it is markedly up-regulated in osteoarthritic (OA) cells as compared to normal chondrocytes while the level of RII in these cells remains unchanged. We provide evidence that overexpression of RIIB enhances TGF-β signaling in human chondrocytes whereas high endogenous expression of RIIB correlates with inhibition of response. Thus RIIB appears to be a marker of cell differentiation and reflects chondrocyte responsiveness to TGF-β. This taken together with the potential regulation of TGF-β action by novel receptors suggests that variation in RIIB expression may contribute to TGF-β's role in cartilage healing.

**Key Words**: chondrocytes, TGF-β receptors, TGF-β signaling, RIIB, phenotype, cartilage, osteoarthritis

## INTRODUCTION

Articular cartilage injury, whether post-traumatic, of unknown etiology, or associated with systemic chronic autoimmune diseases, results in progressive unrelenting joint degradation and ultimately dysfunction. Medical management of hyaline cartilage injury is limited apart from controlling synovitis and symptomatology in arthritis patients. Despite advances in surgical techniques for rebalancing soft tissues at early stages and vascularized or implant based joint replacements, joint dysfunction is never completely regained.

With advances in tissue engineering, interest in the potential to regenerate or repair diseased or injured cartilage is growing as an alternative to conventional surgical reconstruction. However, a successful model of cartilage restoration has been elusive. This has been attributed to the lack of regenerative or proliferative ability of mature chondrocytes, their dedifferentiation into fibrocartilage (type I and type III collagen producing cells) (1) and the avascular state of the tissue (2). It is hypothesized that chondrocytes express distinct phenotypes and this phenotypic "fingerprint" determines the cells capacity to not only produce extracellular matrix (ECM[1]) but also modulate the

[1] The abbreviations used are: ECM, extracellular matrix; TGF-β, transforming growth factor beta; PAI – 1, plasminogen activator inhibitor-1; RI, TGF-β type I receptor; RII, TGF-β type II receptor; Alk-1, Activin Receptor-like Kinase-1; Sol RI, soluble type I TGF-β receptor; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum; DMEM, Dulbecco's minimal essential medium; dPBS, Dulbecco's phosphate buffered saline; BSA, bovine serum albumin; β-gal, beta-galactosidase; GPI, glycosyl

constituent collagen. As chondrocytes dedifferentiate, type II collagen production diminishes with a corresponding increase in type I and III collagen (3-6) and ultimately fibrocartilage is formed. This results in a scarred joint surface rather than the uniform gliding viscoelastic surface seen in a normal joint.

What controls this process of chondrocytes dedifferentiation has generated much research interest as it poses a potential avenue of regulation of ECM synthesis and thus cartilage repair. Equally important is identifying phenotypic markers of chondrocytes as these markers may also provide clues as to triggers of dedifferentiation and a means of preventing of this phenomenon. The concept being that if chondrocytes within an injured articular joint surface could be encouraged to proliferate and maintain their distinct phenotype of type II collagen secreting cells, cartilage repair could be encouraged as in situ phenomena – regeneration, as is seen with various other connective tissues such as ligament and bone. TGF-β has emerged as a key player within the chondrocyte microenvironment. It likely plays a role in governing the dedifferentiation/redifferentiation process of chondrocytes and in addition TGF-β receptors may in fact serve as phenotypic cell markers.

TGF-β is a member of a large family of multifunctional proteins intricately involved in growth, differentiation, and development (7) and was described initially as "cartilage inducing factor" (8). Three distinct isoforms of TGF-β (TGF-β 1, 2, and 3) which are encoded by distinct genes have been described in mammals (7). TGF-β is secreted in a latent form that requires activation before it can bind to its receptors. The TGF-β signal is

---

phosphatidylinositol; SBE, Smad binding element; Col II, type II collagen; OA, osteoarthritis; N, normal.

transduced by a pair of transmembrane serine/threonine kinases, known as the types I and II receptors which are present on almost all cell types (9-12). The type I receptor does not bind TGF-β in the absence of the type II receptor. The binding of TGF-β to the type II receptor, a constitutively active kinase, results in the recruitment, phosphorylation and concomitant activation of the type I receptor. The activated type I receptor in turn transmits the signal via downstream mediators such as Smads, resulting in the regulation of target gene expression. Accessory receptors which form heteromeric complexes with the type I (RI) and type II (RII) signaling receptors have been identified and may play a role in modulating TGF-β signaling and response. These include glycosyl phosphatidylinositol (GPI)-anchored proteins (13-16), the type III TGF-β receptor (betaglycan) which binds all three TGF-β isoforms (17,18) and endoglin which binds the β1 and β3 but not β2 isoforms (19-21). We have now expanded the TGF-β receptor profile on chondrocytes to include endoglin, a soluble variant of RI (Sol RI), and the orphan receptor Activin Receptor-like kinase I (Alk-1) in addition to RI, RII, and betaglycan (22,23). We have demonstrated that these novel receptors form hetero-oligomeric complexes with both the signaling receptors and with other receptors and in doing so influence TGF-β response in human chondrocytes.

In addition to Sol RI a variant of RI (24), a variant of RII known as RIIB has been observed and we believed it to be restricted to tissues in which the TGF-β2 isoform plays a predominant role (25). We have shown that RIIB is a functional receptor and can restore TGF-β2 response in cells deficient in betaglycan (25). The original description of this RII variant was in chondrocytes (26); however, the variant was mistakenly identified as the lower molecular weight receptor of the two type II signaling receptors (27). The presence

of RIIB was later noted in human vascular endothelial cells and its signaling capacity confirmed in DR26 cells lacking RII (28).

The gene targets and cellular responses to TGF-β are numerous yet in isolation the TGF-β signaling appears to follow a relatively simplistic cascade involving Smads as central mediators. Thus, there must exist a means of distinguishing the signal to provide variable and correct downstream responses. One possible level of regulation is at the cell surface receptors and their complex formations. These receptors may provide means of modulating TGF-β response and ultimately cell growth, differentiation, and ECM production. Therefore, further defining TGF-β receptors in human chondrocytes and identifying them as potential markers of cell phenotype are critical to understanding the role of TGF-β in cartilage repair.

In the current study we established the presence of RIIB on human chondrocytes and its complex formation with the TGF-β signaling receptors as well as several other receptors on these cells (endoglin, Sol R1, Alk-1, and betaglycan). We demonstrated that RIIB has affinity for all three TGF-β isoforms (β1>β3>β2) on chondrocytes and that its affinity for TGF-β2 is higher than that seen for RII. Moreover, we have shown that the expression of RIIB is enhanced in OA cells. Importantly, we provide evidence that high endogenous RIIB levels correlate with a decreased TGF-β responsiveness whereas exogenous overexpression of RIIB enhances TGF-β response in chondrocytes. This suggests that RIIB may, in addition to its important role in TGF-β signaling, be a marker of chondrocyte phenotype and play a role in governing TGF-β responsiveness.

## EXPERIMENTAL PROCEDURES

### Cell Culture

The immortalized human chondrocyte cell lines (C-28/I2 and tsT/AC62), a gift from Dr. Mary Goldring (Beth Israel Deaconness Medical Center, Harvard Institutes of Medicine, Boston, MA), have been described previously (29,30). The C-28/I2 cell line was developed using chondrocytes isolated from juvenile human costal cartilage by retroviral infection with SV-40 large T antigen. The tsT/AC62 cells were developed using chondrocytes isolated from adult human articular cartilage by immortalization with a retrovirus expressing a temperature-sensitive mutant of SV-40 large T antigen. This mutant is functional when the cells are cultured at 32°C but not at 37°C (29).

Human articular cartilage was obtained intraoperatively from adult total knee arthroplasty specimens (osteoarthritic cartilage, OA) or from adults with traumatic open joint injuries with no history of degenerative joint disease and normal appearing cartilage (normal cartilage, N). For experiments involving fresh cartilage specimens OA samples were obtained from at least 3 different individuals and for normal tissue from at least 2 different individuals. Therefore experiments comparing diseased versus normal cartilage were performed in triplicate. Specimens were either digested to obtain monolayer cultures or protein extracts were obtained by two methods of solubilization.

Digestion of the specimens was performed according to standard techniques (29). Briefly, cartilage was washed with phosphate-buffered saline (PBS), incubated with 0.25% trypsin (10ml/g of tissue) (Invitrogen Life Technologies; Burlington, ON) for 30 to 45 minutes, minced with a scalpel, and incubated with hyaluronidase (10ml/g of tissue) and collagenase (1mg/ml; 10ml/g of tissue) (both from Sigma Aldrich; Oakville, ON) overnight. The suspension was pelleted, washed, and plated at $1x10^6$ cells/ml. All chondrocytes were grown in DMEM/Ham's F12 (1:1, v:v) containing 10% fetal bovine

serum (FBS) and 100U/ml penicillin, 100μg/ml streptomycin and 0.25μg/ml amphoteracin (all from Invitrogen Life Technologies) and maintained at 37°C (C-28/I2, 293) or 32°C (tsT/AC62) in an atmosphere of 5% CO2/air.

Protein extracts from fresh human articular cartilage were prepared as described by Gruppuso et al (31) or as in Current Protocols (32). Briefly, for Sucrose Preparation tissue was rinsed with dPBS, weighed, minced, and homogenized using a hand homogenizer in 4 volumes of homogenization buffer (Sucrose .25M, EDTA 1mM, HEPES 50mM pH 7.5) containing a mixture of protease inhibitors (1mM phenylmethanesulfonyl fluoride, 20 $\mu g \cdot ml^{-1}$ aprotinin, 20 $\mu g \cdot ml^{-1}$ leupeptin, 20 $\mu g \cdot ml^{-1}$ soybean trypsin inhibitor, and 25 mM benzamidine; all from Sigma Aldrich). This fraction was obtained by centrifuging at 100,000 x g for 2 hours at 20°C. The supernatant was removed and the pellet resuspended in HEPES buffer. For SDS Preparation of protein extracts, tissue was homogenized in a similar fashion but 2ml of Sodium Dodecyl Sulfate (SDS; Bioshop, Burlington, ON)/solubilization buffer (for 10 ml final volume: 0.1g 2-(N-cyclohexylamino) ethanesulfonic acid (CHES), 0.2g SDS, 0.1g DTT, 1.0ml glycerol, distilled water to 10 mls) per 100mg of tissue and boiled for 5 minutes prior to centrifugation.

All experiments were done at early passage (Passages 1-5) unless stated otherwise. Late passage experiments were performed with cells at Passage 10 or above and recovered passage experiments were performed with chondrocytes immediately recovered from 3-dimensional (3-D) alginate bead cultures and allowed to settle onto tissue culture plates. The 3-D alginate culture system has been described previously (33-36) and is used to redifferentiate chondrocytes to their type II collagen producing phenotype. Briefly, several 10cm plates of chondrocyte monolayers are trypsinized and washed with PBS.

Pelleted cells are resuspended in a 1.2% alginate (Keltone LVCR, NF; ISP Alginates Inc., San Diego, CA.) solution at 4 x $10^6$ cells/ml and expressed through a 10ml syringe with a 25 gauge needle into a polypropylene tube containing 102 mM $CaCl_2$. Cells are allowed to polymerize for 10 minutes then are washed with sterile saline. Beads are decanted into T75 flasks and maintained in regular medium as noted above. After 3 weeks in 3-D culture cells are recovered. Medium is aspirated and beads are washed with PBS. Alginate is depolymerised with 55mM Na Citrate (Sigma Aldrich) at 37° for 10 min, cells are dislodged, pelleted, washed with PBS then plated as required for analysis.

**Western Blot of TGF-ß Receptors**

Western blotting was performed as previously described (22). Briefly, chondrocytes were grown to 90% confluency in 6 well plates and not transfected, transfected with pcDNA1 (empty vector, EV), receptor (pRIIB or pRII) for 48 hours. Cells were then washed with PBS and lysed. Cell lysates were prepared and samples were standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad; Mississauga, Ont.). Alternatively, protein extracts of human cartilage were prepared as described above and standardized for protein content. Samples were then fractionated on SDS-PAGE (7.5% acrylamide) and transferred by electrophoresis to nitrocellulose membrane (Scheicher and Schuell; Keene, NH.). The membrane was blocked for 3 hours in a milk protein/Tween blocking solution (1M Tris, pH 7.5; 5M NaCl; Tween 20, Sigma Aldrich; Carnation Skim milk powder) and incubated with primary antibody. For RIIB detection, anti-RIIB antibody and for RII detection, anti-RII antibody (Santa Cruz Biotechnology Inc.) or a preincubated mixture of anti-RII antibody-blocking peptide at a ratio of 1:10. All antibodies were applied at room temperature for 2 hours and were followed by incubation for 1 hour with goat anti-rabbit

HRP conjugated secondary antibody (Santa Cruz Biotechnology Inc. Santa Cruz, CA) at room temperature. For type II collagen detection Immunoblotting was performed with anti-type II collagen antibody (a gift from Dr. AR Poole; Montreal, Quebec; 37) followed by goat anti-mouse HRP conjugated secondary antibody (Santa Cruz Biotechnology Inc. Santa Cruz, CA) at room temperature. The ECL system (Amersham Pharmacia Biotech Inc.; Baie d'Urfe, Que.) was used for detection. Equal protein loading was verified by reprobing membranes with Smad 2, Smad 2/3, or STAT 3 antibody (all from Santa Cruz Biotechnology Inc.).

**Affinity Labeling of Cells**

Affinity labeling was performed as described previously (38) with modifications. Briefly, monolayers of cells were washed with ice-cold binding buffer [Dulbecco's PBS (dPBS) with $Ca^{++}$ and $Mg^{++}$, pH 7.4 containing 0.1% bovine serum albumin (BSA)] and were incubated with 100 pM of $^{125}$I-TGF-β1 or $^{125}$I-TGF-β2 in the absence or presence of varying concentrations of non-radioactive TGF-β1 or -β2. The receptor ligand complexes were cross-linked with Bis-sulfocsuccinimidyl suberate (BS3; Pierce; Rockford, IL). The reaction was stopped by the addition of glycine and the cell membrane extracts were prepared. The solubilized samples were separated by SDS-PAGE (3-11% gradient) under reducing (with β-Mercaptoethanol, Sigma Aldrich) or nonreducing conditions and analyzed by autoradiography. In one experiment cells were not transfected, transfected with empty vector, or transfected with RIIB for 48 hours prior to affinity labeling in the presence or absence of 0.5 nM unlabeled TGF-β1 to determine the relative molecular weight of the exogenous RIIB.

**Immunoprecipitation of TGF-ß Receptors**

The anti-betaglycan antibody (Get 1) was a gift from Dr. S. Souchelnytskyi (Uppsala, Sweden; 39) and the anti-RII was purchased from Santa Cruz Biotechnology Inc. Immunoprecipitation studies were performed as described previously (14,38) with modifications. Cells were affinity labeled with 200 pM $^{125}$I-TGF-β1, and the membrane extracts were incubated with 3μg/ml of the various antibodies and with respective normal rabbit serum controls or IgG. Immune complexes were then incubated with protein A-sepharose slurry (Amersham Pharmacia Biotech Inc.) and pelleted by centrifugation. The immune complexes were analyzed by SDS-PAGE under non-reducing or reducing conditions followed by autoradiography.

**Immunoprecipitation/Western Blot Analysis**

The anti-betaglycan antibody (Get 1) was a gift from Dr. S. Souchelnytskyi (Uppsala, Sweden; 39) and the anti-Sol RI antibody was a gift from Dr. M. Choi, (New Haven, Connecticut; 23,24). The anti-Alk-1 antibody and control IgG were purchased from Santa Cruz Biotechnology Inc. and the anti-endoglin antibody (SN6h) was purchased from Dako Inc. (Carpinteria, CA). Immunoprecipitation was performed as previously described (22). Briefly, chondrocytes were washed three times with 0.1% BSA-dPBS, twice with dPBS, and membrane extracts of cells were prepared, and immunoprecipitated with anti-betaglycan, anti-Alk-1, anti-Sol RI, anti-endoglin antibody or control IgG, or not immunoprecipitated. The extracts or immune complexes were fractionated by SDS-PAGE (3-11% gradient or 7.5% acrylamide) and Western blot analysis was performed using anti-RIIB antibody or anti-endoglin antibody and the ECL system was used for detection (Amersham Pharmacia Biotech Inc.) as discussed above.

**Two-Dimensional Gel Electrophoresis-Western Blot**

Two-dimensional gel electrophoresis was performed as described by MacKay et al (40) except that 3-mercaptopropionic acid was omitted in the second dimension (16). Solubilized membrane extracts of cells immunoprecipitated with anti-RII antibody (Santa Cruz Biotechnology Inc.) were affinity labeled with $^{125}$I-TGF-β1 were first fractionated on a 1.0 mm-thick 3-11% gradient gel under non-reducing conditions in the first dimension and on a 1.5 mm-thick 3-11% gradient gel under reducing conditions in the second dimension. The protein was then transferred to nitrocellulose membrane and immunoblotted as described above with anti-RIIB antibody.

**Receptor Overexpression and Western Blot Analysis**

To determine the regulation of the phosphorylated form of Smad 2 by TGF-β1 treatment in chondrocytes overexpressing RIIB, chondrocytes were grown in 6 well plates until 90% confluency. Cells were then transiently transfected with 1μg of pRIIB, or empty vector (EV, pcDNA1) per well using Lipofectamine Reagent (Invitrogen Life Technologies) in serum-free medium according to the manufacturer's specifications. Cells were allowed to recover for 48 hours in regular medium, serum starved the following day for 4 hours, then treated with 50 pM of TGF-β1 for 15 minutes. Cell lysates were prepared and samples were standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad.). Immunoblotting was performed as described above using primary rabbit polyclonal anti-phosphoSmad 2 antibody (a gift from Dr. S. Souchelnytskyi, Uppsala, Sweden; 41,42) or anti-RIIB antibody (to confirm receptor overexpression) at 4°C overnight followed by goat anti-rabbit HRP conjugated secondary antibody (Santa Cruz Biotechnology Inc.) for 1 hour at room temperature. In other experiments cells of differing phenotypes (C-28/I2, tsT/AC62, primary articular chondrocytes) or stages of

differentiation (early, late, recovered) were plated and treated with 50 pM of β1 for 15 or 30 minutes, or not treated, and Smad 2 phosphorylation determined as above.

**Receptor Overexpression and Luciferase Reporter Assay**

The p3TP-Lux, a TGF-β-inducible promoter-reporter construct (43), containing the luciferase gene under the control of a portion of the plasminogen activator inhibitor-1 promoter region was used to determine cellular responsiveness to TGF-β. Chondrocytes were grown to 90% confluency in a 12-well plate and transiently transfected with 1μg of p3TP-Lux and 1μg of pβ-galactosidase (β-gal) per well using Lipofectamine Reagent (Invitrogen Life Technologies) in serum-free medium according to the manufacturer's specifications. In addition to the promoter-reporter construct, cells were either transfected with the empty vector (EV, pcDNA1), pRIIB or pRII or were not transfected. Cells were allowed to recover for 48 hours in regular medium, serum starved the following day for 4 hours, then treated with 100 pM of TGF-β1 overnight. The cells were lysed and assayed for luciferase activity using the EG&G Berthold Microplate Luminometer (Berthold Technologies USA; Oak Ridge, TN). Light emission by the TGF-β1 treated cells was expressed as a percentage of the emission by the control cells and adjusted for transfection efficiency using the β-gal assay.

**[$^3$H]-Thymidine Incorporation**

Regulation of DNA synthesis was determined using the Thymidine incorporation assay with modifications as described previously by our lab (38). Briefly, cells at various stages of differentiation were seeded at a density of $7.0x10^5$ cells/24 well plate and cultured for 24 hours. The cells were washed with PBS then serum starved for 4 hours. Wells were then incubated with 50 or 200 pM of TGF-β1 under serum free conditions overnight. [$^3$H]-

Thymidine ($1\mu Ci \cdot ml^{-1}$) (Amersham Pharmacia Biotech Inc.) was added per well for the final 4 hours of TGF-β1 treatment. The cells were washed 3 times with PBS and once with 5% trichloroacetic acid (A&C American Chemicals Ltd.; Montreal, Quebec), then were solubilized in 1% SDS and incorporated radioactivity was determined by liquid scintillation counting.

## RESULTS

### RIIB is expressed on human chondrocytes as detected with a specific anti-RIIB antibody

The tsT/AC62 and C-28/I2 cell lines provide a reproducible model that mimics human articular and costal phenotypes respectively (44-47). We have previously confirmed the chondrocyte phenotype of these immortalized cell lines and the human primary articular chondrocytes in culture by demonstrating the production of aggrecan and type II collagen using RTPCR (22) and by documenting their typical chondrocyte morphology as detected by a spherical to polygonal shape with a cell-associated matrix when suspended in alginate bead cultures (data not shown).

The results of Western blotting shown in Fig. 1A demonstrate endogenous expression of RIIB (lanes represented by Ø) in human articular (tsT/AC62) and nonarticular (C-28/I2) chondrocytes as compared to overexpression (lanes represented by RIIB) and empty vector controls (lanes represented by EV). The relative molecular weight of endogenous RIIB is confirmed by the co-migration of overexpressed RIIB on SDS-PAGE (upper panel). The membrane was reprobed with anti-STAT 3 antibody to confirm equal protein loading (lower panel).

Human immortalized chondrocytes were analyzed for levels of RII with Western blot (Fig. 1B). The antibody used to detect RII (Santa Cruz biotechnology Inc.) produced two bands, one representative of RII (85 kDa) and one of RIIB (97 kDa) (upper panel). Of note, increased levels of both receptors were evident in articular (tsT/AC62) as compared to nonarticular (C-28/I2) cells. To determine the specificity of the anti-RII and anti-RIIB antibodies both were used for Immunoblotting and the resulting bands compared. OA and normal primary human chondrocytes were used (primary cells digested and plated as monolayer cultures had previously been observed to exhibit both type II receptors by affinity labeling – data not shown). Blotting with anti-RII antibody (lower left panel; α-RII) resulted in three bands; however, when preincubated with blocking peptide the upper two bands are no longer apparent leaving only the lower non-specific band (lower center panel; α-RII+BP). The anti-RIIB antibody was also used for Immunoblotting a parallel membrane. This demonstrated a single band corresponding to the upper band of the anti-RII antibody treated membrane and represents RIIB (lower right panel; α-RIIB). The anti-RIIB antibody did not detect RII. Interestingly, a comparison of the relative intensity of the RIIB bands suggest that OA cells express greater amounts of RIIB than normal chondrocytes whereas the relative abundance of RII did not differ between these phenotypically distinct cells. (This difference in RIIB expression was later confirmed – see below).

**Overexpression of exogenous RIIB can be demonstrated by affinity labeling and demonstrates affinity for TGF-β1, β2, and β3 isoforms**

To analyze the expression profile of RIIB and its affinity for the three TGF-β isoforms, human chondrocytes were affinity labeled with $^{125}$I-TGF-β1 and the cell lysates were

analyzed by SDS-PAGE. Initially C-28/I2 cells were either transfected with pcDNA1 (EV), pRIIB (RIIB), or not transfected (Ø) (Fig. 2A) prior to affinity labeling in the absence or presence of unlabeled TGF-β1. An increased intensity at 97 kDa in the cells transfected with pRIIB confirmed its size and position as the same as that of the endogenous RIIB. Alternate lanes demonstrate the TGF-β receptor expression profile of cells labeled in the presence of 0.5 nM of unlabeled TGF-β1. In these lanes bands which decrease in intensity from those affinity labeled in the absence of unlabeled ligand (0 nM) demonstrate affinity for TGF-β1 by displacing $^{125}$I-TGF-β1. This demonstrates binding proteins with specificity for TGF-β at predictable molecular weights including previously identified receptors RI (65 kDa), RII (85 kDa), betaglycan (250-300 kDa), the endoglin dimer (180 kDa), and a 145 kDa heteromeric complex of RI/RII, RI/endoglin, or RII/endoglin (22).

Competition profiles were then created with TGF-β1 and β3 (Fig. 2B) to more clearly define the relative ligand binding affinity of overexpressed RIIB. It is apparent that RIIB has affinity for the β1 isoform as shown by others evidenced by the graded displacement of $^{125}$I-TGF-β1 with increasing concentrations of unlabeled -β1 ligand. Some RIIB affinity is seen for the TGF-β3 isoform but to a much lower extent and only at higher concentrations of unlabeled ligand. Although no apparent affinity could be appreciated with $^{125}$I-TGF-β1 affinity labeling and cold competition with TGF-β2 for RIIB (data not shown), evidence that RIIB does bind the -β2 isoform is evident in Fig. 2C. As anticipated, RII also shows affinity for the TGF-β1 and -β3 isoforms and to a similar extent as RIIB. This is also demonstrated in Fig. 2C in which affinity labeling was performed with $^{125}$I-TGF-β2. Both RII and RIIB are apparent thus confirming their ability

to bind the TGF-β2 isoform. Competition with unlabeled TGF-β1 and -β2 shows that RIIB's affinity for both of these ligands exceeds that of RII. This finding of RIIB's affinity for TGF-β2 is consistent with our previously reported observations that in cells deficient in betaglycan the cells' response to the -β2 ligand could be restored through overexpression of RIIB (25). However, others have been unable to document RIIB as having affinity for the β2 isoform in the presence of betaglycan (28).

**RIIB forms a higher order complex with RII on human chondrocytes**

We have previously shown that RIIB is able to form complexes with the TGF-β signaling receptors using Cos-7 cells (25). To confirm that this association occurs on human chondrocytes, two-dimensional gel electrophoresis was performed. In these studies, whereas immunoprecipitation with a specific anti-receptor antibody confirmed the identity of its cognate TGF-β receptor, co-immunoprecipitation of another type of receptor which is not recognized by this antibody was indicative of heteromeric complex formation between those receptors.

Extracts of tsT/AC62 cells were immunoprecipitated with α-RII antibody. Two-dimensional gel electrophoresis was performed under nonreducing conditions in the first dimension and reducing conditions in the second dimension. Protein was transferred to nitrocellulose membrane and immunoblotted with anti-RIIB antibody and the ECL kit was used for detection. The anti-RIIB antibody detected three spots of 95 kDa, the molecular weight of and representative of RIIB (Fig 3A; spots 1-3). Spot 3 falls on the diagonal at 95 kDa and consists of uncomplexed RIIB. Spot 1 and 2 have fallen from higher molecular weights of 180 and 165 kDa respectively and represent RIIB which has descended from higher order receptor complexes. Because this follows

immunoprecipitation of RII, it is likely that the higher order complex observed at 180 kDa is that of RII and RIIB and at 160 kDa is that of RI and RIIB as we have previously demonstrated the RI/RII association on chondrocytes (22).

**RIIB forms heteromeric complexes with accessory receptors in the presence of ligand**

To confirm the identity of RIIB expressed on human chondrocytes and to study potential associations between it and other TGF-β receptors, chondrocytes were affinity labeled with $^{125}$I-TGF-β1 and immunoprecipitated using anti-RIIB antibody or not immunoprecipitated. As shown in Fig. 3B, SDS-PAGE under nonreducing conditions of the nonimmunoprecipitated membrane lysate (Lane 1, NIP) demonstrated the presence of TGF-β binding proteins consistent in mobility and size with Sol RI, RI/Alk-1, RII, RIIB, the endoglin dimer, the 145 kDa RI-RII heterodimer, and betaglycan (22). The anti-RIIB antibody (Lane 2, α-RIIB) immunoprecipitated RIIB and co-immunoprecipitated the aforementioned receptors. As mentioned we have previously demonstrated the co-immunoprecipitation of RI, RII, and betaglycan with α-RIIB in Cos-7 cells (25). However, the co-immunoprecipitation of RIIB and endoglin or of RIIB and Sol RI has not been reported.

To confirm the RIIB-accessory receptor interactions IP-Western blot analysis was performed. Membrane extracts prepared from extensively washed (dPBS) chondrocytes, were immunoprecipitated with α-betaglycan, α-Alk-1, α-Sol RI, α-endoglin, α-RIIB antibodies or with control IgG, or not immunoprecipitated. Immunoprecipitates were then fractionated by SDS-PAGE (3-11% gradient) under reducing conditions and

analyzed by Western blotting using α-RIIB (Fig. 3C, panel A and C) or α-endoglin antibody (Fig. 3C, panel B).

Fig. 3C demonstrates that no RIIB was detected in the control experiment in which the immunoprecipitation was done with rabbit control IgG before Western blotting (panel A and C, Lane 1; IgG). Western blotting of nonimmunoprecipitated membrane extracts (panel A and C, Lane 2; NIP) confirmed the presence of RIIB in chondrocytes. Western blotting of extracts immunoprecipitated with α-endoglin antibody (panel A, Lane 3; α-Eg) revealed RIIB at 97 kDa, thus demonstrating that RIIB was co-immunoprecipitated with endoglin in the absence of exogenous TGF-β. Western blot of extracts immunoprecipitated with α-RIIB antibody revealed endoglin at 100 kDa further demonstrating this co-immunoprecipitation of RIIB and endoglin (panel B, Lane 2; α-RIIB). A control lane of immunoprecipitated endoglin confirms its presence on the chondrocytes (panel B, Lane 1; α-Eg). Western blotting of extracts immunoprecipitated with α-betaglycan (panel C, Lane 3; α-BG), α-Alk-1 (panel C, Lane 4; α-Alk-1), and α-Sol RI antibody (panel C, Lane 5; α-Sol RI) antibody revealed RIIB at 97 kDa, thus demonstrating that RIIB was co-immunoprecipitated with these accessory receptors in the absence of exogenous TGF-β.

**Chondrocytes of distinct phenotypes express variable amounts of endogenous RIIB**

A preliminary Western blot used to demonstrate the presence of RIIB on human immortalized chondrocytes (Fig. 1B) alluded to the fact that tsT/AC62 cells expressed higher levels of not only RII but also RIIB than C-28/I2 cells. This differential RIIB expression is also evident after immunoprecipitation with anti-RIIB and anti-RII antibodies (Fig. 4A). SDS-PAGE under nonreducing conditions of the

nonimmunoprecipitated membrane lysate demonstrated the presence of TGF-β binding proteins consistent in mobility and size with RI, RII, Sol RI, the endoglin dimer, betaglycan, and RIIB (Lane 1; NIP) (22,23). The anti-RII antibody (Lane 2; α-RII) co-immunoprecipitated the aforementioned receptors as did the anti-RIIB antibody (Lane 3; α-RIIB). It is difficult to address whether the co-immunoprecipitation of RIIB with α-RII seen here is a true association as we have shown that this antibody cross-reacts with the RIIB receptor. However, we have shown above by immunoprecipitation-2D-Western blot that this association is in fact occurring (Fig. 3A). Fig. 4A also confirms the other RIIB-receptor associations with betaglycan, RI, Sol RI, and endoglin. Importantly, this demonstrates the increased expression of RIIB as well as RII on articular as compared to nonarticular cells.

Not only did we observe a difference in RIIB expression between articular and nonarticular chondrocytes but also we noted that its expression varied according to state of cell passage and thus phenotype. As seen in Fig. 4B, for both C-28/I2 and tsT/AC62 cells, early and recovered passages of cells express more RIIB by affinity labeling than late passage whereas the levels of RII remain relatively unchanged. The autorads chosen are representative of multiple repeats of affinity labeling (n>10). Early passage monolayers (E) and chondrocytes harvested directly from 3-D alginate bead cultures (R) are in a more differentiated state than late passage cultures (L) and have been shown to produce higher amounts of type II collagen (6,33,36,48). That cells begin to dedifferentiate with successive passages in monolayer culture and can be redifferentiated to their original phenotype through various 3-D matrix culturing systems has been well established (6,33-36,48,49). This variability of RIIB expression was confirmed using

Western blot. Fig. 4C (upper panel) demonstrates RIIB expression at increased levels in early (Lane 1,4,7) and recovered (Lane 3,6,9) chondrocytes for both articular and nonarticular cell lines as well as for primary articular cells as compared to late passage cells (Lane 2,5,8). The membrane was reprobed with anti-Smad 2/3 antibody to demonstrate equal protein loading (lower panel). This is representative of three separate trials.

The results presented above suggest that the phenotype of human chondrocytes correlates with RIIB expression. We thus considered whether OA and normal human articular chondrocytes which display distinct phenotypes would exhibit varied RIIB expression. We tested this hypothesis by determining RIIB expression on normal and osteoarthritic cartilage using Western blot (Fig. 4D, panel A,B,C; upper panels). Increased RIIB expression in OA cells is evident for protein extracts of SDS (panel A, Lane 1,2) and Sucrose (panel B, Lane 1,2) preparations. Monolayers of OA and normal cartilage prepared by enzymatic digestion or generated from explant cultures also demonstrate increased RIIB levels in OA cells (panel C, Lane 5,6). The membranes were reprobed with anti-STAT 3 antibody to demonstrate equal protein loading (lower panels). This may signify the importance of the RIIB receptor in modulating TGF-β response in phenotypically distinct chondrocytes.

**Increased expression of endogenous RIIB correlates with inhibition of TGF-β response**

We have shown that late passage dedifferentiated chondrocytes express decreased levels of RIIB as compared to early or recovered passages and that more RIIB is expressed in

tsT/AC62 than C-28/I2 (Fig. 4A-C). Thus, it was of interest to test whether the endogenous expression levels of RIIB correlated to the magnitude of TGF-β response.

To illustrate the sensitivity of the chondrocyte cell lines and primary chondrocytes to TGF-β, cellular signaling was determined by a Plasminogen Activator Inhibitor (PAI)-driven luciferase reporter assay (p3TP-Lux) which has been used extensively as a marker for TGF-β responsiveness (43). The chondrocytes were transiently transfected with p3TP-Lux and the induction of luciferase activity by exogenous TGF-β1 was measured. As illustrated in Fig. 5A, luciferase activity of cells treated with 100 pM of TGF-β1 is expressed as a percent control of untreated cells and it is evident that the C-28/I2 line are almost twice as responsive to ligand as tsT/AC62 cells (panel A; $p=0.02$). Enhanced gene transcriptional activation for late passage tsT/AC62 cells as compared to recovered passages is also noted but not statistically significant (panel B; $p=0.08$). The results were normalized by co-transfection of the β-gal plasmid and are representative of three different experiments each done in triplicates.

To demonstrate further that this variable response to TGF-β may be related to cell phenotype we examined TGF-β induced Smad 2 phosphorylation. Enhanced responsiveness to TGF-β was seen for C-28/I2 and primary articular chondrocytes which express lower levels of endogenous RIIB (Fig. 5B). Cells were left untreated (Lane 1,2,3; upper panel) or were treated with 50 pM of TGF-β1 for 15 minutes (Lane 4,5,6; upper panel). Up-regulation of phosphorylated Smad 2 is apparent for C-28/I2 (Lane 4) and primary chondrocytes (Lane 6) whereas minimal increase with TGF-β1 stimulation is noted for tsT/AC62 cells (Lane 5). The membranes were reprobed with anti-STAT 3 antibody to demonstrate equal protein loading (lower panel).

In addition, data shown in Fig. 5C illustrates that late passage cells which exhibit decreased levels of endogenous RIIB, in the presence of exogenous TGF-β, demonstrate higher levels of phosphorylated Smad 2 than early and recovered passages. Early (E), late (L) and recovered (R) passages of C-28/I2 (Lane 1,2,3), tsT/AC62 (lane 4,5,6), and primary articular cells (Lane 7,8,9) were treated for 15 minutes with TGF-β1. Levels of phosphorylated Smad 2 are enhanced for late passage cells (Lane 2,5,8) as compared to early (Lane 1,4,7) or recovered (Lane 3,6,9) passages.

Late passage cells of C-28/I2 demonstrate this enhanced response with treatment of TGF-β1 at 15 and 30 minutes (Fig. 5D). Early, late, and recovered passages of C-28/I2 cells were left untreated (Lane 1,3,5 respectively) or were treated with 50 pM of TGF-β1 for 15 (upper panel) or 30 minutes (lower panel) (Lane 2,4,6 respectively). Responsiveness to TGF-β1 was determined by immunoblotting with anti-phosphoSmad 2 antibody (upper panels). Late passage chondrocytes (Lane 4) exhibit up-regulation of phosphoSmad 2 as compared to early (Lane 2) or recovered (Lane 6) cells. The membranes were reprobed with anti-STAT 3 antibody to demonstrate equal protein loading (lower panels). The results demonstrated in the above Western blots are representative of duplicate or triplicate trials.

Finally, differential response related to cell phenotype was demonstrated with [$^3$H]-Thymidine incorporation (Fig. 5E). C-28/I2 cells were seeded at a density of $7.0x10^5$ cells/24 well plate and cultured for 24 hours. The cells were serum starved for 4 hours and incubated with 50 or 200 pM of TGF-β1 under serum free conditions overnight. [$^3$H]-Thymidine ($1\mu Ci \cdot ml^{-1}$) was added per well for the final 4 hours of TGF-β1 treatment incorporated radioactivity was determined by liquid scintillation counting. [$^3$H]-Thymidine incorporation expressed as a ratio of late (Late) to

recovered (Rec) cells demonstrates an almost two-fold higher sensitivity to TGF-β1 of late passage chondrocytes than recovered cells, although statistically significant values were seen only with the 200 pm ($p=0.05$) and not the 50 pm treatment ($p=0.10$).

Thus the data presented in Fig. 5 illustrates that lower levels of RIIB expression as seen in C28-I2 and late passage chondrocytes correlates with an enhanced responsiveness to TGF-β and suggests that RIIB may act as a marker of cell phenotype.

**Exogenous /Overexpressed RIIB enhances TGF-β1, β2, and β3 response in human chondrocytes**

The effect of exogenous overexpression of RIIB on human chondrocyte TGF-β responsiveness was also investigated. Chondrocytes were transfected with plasmid encoding RIIB (RIIB) or pcDNA1 (EV) (Fig. 6A). Cells were then left untreated (Lane 1,3; -) or treated with 100 pM of TGF-β1 (Lane 2,4; +) for 15 minutes and Western blotting was performed to detect the phosphorylated form of Smad 2 (panel A; upper panel). The overexpression of RIIB demonstrated an upregulation of Smad 2 phosphorylation (Lane 4). The membrane was reprobed for STAT 3 (panel A; lower panel) to confirm equal protein loading. On a parallel gel, extracts of cells transfected with plasmid encoding RIIB (panel B, Lane 2; RIIB) or pcDNA1 (panel B, Lane 1; EV) were analyzed by Western blot for RIIB to confirm receptor overexpression.

Fig. 6B illustrates that overexpression of RIIB results in enhanced gene transcriptional activity. Chondrocytes were transfected with plasmids encoding RIIB (RIIB), RII (RII), or pcDNA1 (EV) in addition to 3TP-Lux (a PAI-driven luciferase reporter construct; 43) and pβ-gal. Cells were allowed to recover for 48 hours and were then left untreated (-) or were treated with 100 pM of TGF-β1, β2, or β3 overnight and luciferase activity was

measured and standardized with a β-gal assay. Overexpression of RIIB caused an enhancement of TGF-β response as compared to control untransfected cells; however, this was only significant for the TGF-β3 isoform (endogenous TGF-β, p=0.07; -β1, p=0.07; -β2, p=0.08; -β3, p=0.02). Overexpression of RII was used as a comparison as it is well known to enhance TGF-β response (24,50). This increase in responsiveness with RII overexpression was evident with all isoforms but was not significant for TGF-β1 (endogenous TGF-β, p=0.004; -β1, p=0.22; -β2, p=0.007; -β3, p=0.01).

To demonstrate that the intended receptor was being overexpressed, residual lysates used in the 3TP-Lux assay above (Fig. 6B) were separated by SDS-PAGE (7.5% acrylamide) and Western blotting was performed to detect RII and RIIB expression (Fig. 6C; upper panel). Enhanced detection of RII (Lane 2) and RIIB (Lane 6) was evident where receptors have been overexpressed in these cells. The membranes were reprobed with the non-phosphorylated form of anti-Smad 2 antibody to demonstrate equal protein loading (lower panel).

The effect of RIIB overexpression on type II collagen production is seen in Fig. 6D (upper panel). Cells were transfected with pcDNA1 (Lane 3,4; EV) or pRIIB (Lane 5,6; RIIB) for 48 hours or not transfected (Lane 1,2; Ø). Western blotting was performed using anti-type II collagen antibody (37) to detect type II collagen which was seen to increase with RIIB overexpression (Lane 5 and 6). The membrane was reprobed with anti-STAT 3 antibody to demonstrate equal protein loading (lower panel). This is representative of duplicate experiments and multiple membrane blots.

**DISCUSSION**

The inability of chondrocytes to maintain their type II collagen producing phenotype has been implicated in the lack of regenerative capacity of mature articular cartilage. TGF-β administration has been linked to increased ECM production, enhanced cell proliferation, and cartilage healing (51-60) whereas dysregulation of the TGF-β signaling cascade has been shown to result in degenerative joint disease in two murine models (61,62). TGF-β is known to influence cell phenotype through an intricate involvement in growth, differentiation, and development (7,8). TGF-β receptors may provide a means of regulating the TGF-β signaling pathway. It has been suggested that various receptor complexes or levels of receptor expression may impart specificity in TGF-β signaling (63,64) or infer variable responsiveness to different TGF-β isoforms (28). In addition, attempts have been made to identify receptors as potential markers of cell differentiation (65-67).

RIIB, an alternatively spliced variant of RII, interacts with the TGF-β signaling receptors at overexpressed levels and is implicated in TGF-β2 isoform responses (25). That RIIB expression has been demonstrated on human chondrocytes (27) and that the TGF-β2 isoform is uniquely expressed in bone-related tissue (25) both suggest RIIB may play a predominant role in human chondrocytes. Identifying and further defining RIIB, its heteromeric complex formations with other TGF-β receptors, and its effect on TGF-β signaling in human chondrocytes is critical. This may provide insight into its function as a phenotypic marker or gatekeeper in determining specificity and sensitivity in TGF-β signaling and thus variable response to TGF-β isoforms in specialized tissues.

In the present study we confirmed the presence of RIIB on human chondrocytes and its complex formation with RI and RII in addition to other novel receptors namely

betaglycan, endoglin, Alk-1, and Sol RI. We demonstrated that RIIB has affinity for all three TGF-β isoforms (β1>β3>β2) and that its affinity for TGF-β2 exceeds that of RII for β2. Moreover, we present evidence that RIIB may function as a phenotypic marker in chondrocytes. RIIB expression varies between chondrocytes of distinct phenotypes while RII expression remains relatively unchanged. In keeping with this, we present the novel finding of markedly increased RIIB expression in OA as compared to normal primary articular chondrocytes. We provide important evidence that exogenous or overexpressed RIIB enhances TGF-β response whereas increased endogenous RIIB levels correlate with a diminished response.

The phenotype of the human chondrocytes used in the present study has been characterized extensively (44-47). The primary human articular chondrocytes and immortalized chondrocytes expressed both aggrecan and type II collagen mRNAs, and their responsiveness to TGF-β was shown by stimulation of PAI promoter activity and Smad 2 phosphorylation (22). We demonstrated that RIIB is expressed in human chondrocytes and that this expression is significantly higher in tsT/AC62 than C-28/I2 cells (Fig. 1A, 1B). In addition, RIIB expression is more abundant in OA than normal human cartilage (Fig. 1B).

The possibility of a type II TGF-β receptor variant was first suggested in bovine chondrocytes (26). The authors suggested that that freshly harvested bovine chondrocytes expressed more of the higher molecular weight type II receptor than cells progressively cultured in monolayer, or dedifferentiated cells. Glansbeek et al later demonstrated that RIIB was expressed in both murine and human articular chondrocytes and that the level of expression varied between species and with passages in monolayer cultures (27).

Human macrovascular endothelial cells have also been shown to express RIIB (28). RIIB has been cloned by us and others (25,28) and we have suggested it to be a predominant receptor in bone related tissues (25) where the TGF-β2 isoform is abundant.

Using L6 myoblasts, lacking betaglycan, RIIB overexpression was shown to restore cell responsiveness to the TGF-β2 isoform and suggests that RIIB may be able to transmit its signal directly in response to TGF-β2 via RI (25). Our results of affinity labeling of human chondrocytes depict RIIB at approximately a 12 kDa higher molecular weight than RII as anticipated from its cloned sequence (25). Moreover, transfection with plasmid encoding RIIB confirmed the presence of endogenous RIIB in these cells and the nature of the plasmid (Fig.2A). Fig. 2B demonstrates that RIIB has high affinity for the TGF-β1 isoform and lesser affinity for the β3 isoforms, although others have been unable to demonstrate this (28). In addition, RIIB displays a moderate affinity for the -β2 isoform and this affinity supersedes that of RII at exogenous and endogenous levels (Fig. 2C and data not shown). However, the true affinity of RII and RIIB for the TGF-β isoforms is difficult to interpret in the presence of betaglycan which is expressed on these cells.

Prior studies analyzing the interactions between RIIB and TGF-β receptors and the stoichiometry of these heteromeric complexes have employed primarily mutant cell lines or cells overexpressing the wild-type or chimeric receptors (25,28). In the present study, we used normal TGF-β responsive human chondrocytes to demonstrate the association of RIIB with other TFG-β receptors. Thus, our results illustrate that these associations occur at endogenous receptor concentrations and ratios. Fig. 3A, an immunoprecipitation with anti-RII antibody, 2-D SDS-PAGE, followed by Western blotting with anti-RIIB antibody depicts complex formations between RII and RIIB as well as RI and RIIB. In addition to

providing confirmation of the identity of RIIB, the findings of immunoprecipitation studies of cells affinity labeled with $^{125}$I-TGF-ß1, indicated that RIIB forms a heteromeric complex with the signaling receptors, betaglycan, and Sol RI in the presence of ligand, as detected by co-immunoprecipitation of these receptors with anti-RIIB antibody (Fig. 3B). That RIIB exists in a heteromeric complex with other TGF-β receptors (endoglin, betaglycan, Alk-1, and Sol RI) on the chondrocyte surface was confirmed using membrane extracts that were immunoprecipitated with combinations of anti-receptor antibodies (α-BG, α-Eg, α-Alk-1, α-Sol RI or α-RIIB) and then subjected to Western blotting with α-RIIB or α-Eg (Fig. 3C).

We have previously demonstrated that endoglin and betaglycan form a heteromeric receptor complex on the chondrocyte surface in the presence of ligand and ligand independent manner and RII independent manner (22). Also, we have shown that Alk-1 and Sol RI also form similar heteromeric associations with betaglycan (23). These findings and that of RIIB forming various TGF-β receptor interactions suggest a fluid TGF-β receptor complex at the chondrocyte cell surface. That TGF-β receptors may all interact at the cell surface and that their spatial or temporal expression within this complex may regulate TGF-β signaling pathways to specify which of the diverse TGF-β responses will result. Evidence in support of this theory is already available. Alk-1 and its downstream effector, Smad 5, are unable to rescue cells deficient in Alk-5 (RI) from severe defects in vascular development despite its complex formation with other TGF-β receptors (63). In endothelial cells it has been demonstrated that TGF-β can activate two distinct type I receptor/Smad signaling pathways with Alk-1/Smad1/5 inhibiting and Alk-5/Smad 2 enhancing cell migration and proliferation (64). These opposing results

could be attributed to the presence or absence of additional TGF-β receptors in the signaling complex.

That novel TGF-β receptors and specifically RIIB may provide an avenue of TGF-β regulation is supported by the data presented in Fig. 6. Overexpression of RIIB in human chondrocytes resulted in enhanced response to TGF-β as determined by Smad 2 phosphorylation (Fig. 6A) and luciferase activity of a TGF-β sensitive promoter (Fig. 6B). We also demonstrated that overexpression of RIIB results in an upregulation of type II collagen detected by Western blot (Fig. 6D). Conversely, endoglin overexpression in human mesangial cells has been shown to conversely decrease type I and IV collagen production (68). This implies that TGF-β modulation of ECM turnover in tissues may be regulated thorough its receptor expression. TGF-β itself has been shown to regulate the level of its own receptors (68,69) possibly through internalization and turnover at the cell surface (reviewed in 70).

Loss of chondrocyte type II collagen producing phenotype and subsequent dedifferentiation into a type I and III collagen producing fibroblast-like cell plays a role in the lack of regenerative potential of injured or diseased cartilage. Our preliminary data (Fig. 1B) suggests that RIIB levels are higher in dedifferentiated cells which fail to produce type II collagen (6,33-36,48,49). However, this is not in keeping with RIIB overexpression resulting in increased type II collagen production (Fig. 6D). We hypothesized that this observed inconsistency may be explained by endogenous RIIB levels and thus further investigated endogenous RIIB expression on phenotypically distinct cells.

Affinity labeling and immunoprecipitation studies revealed that articular chondrocytes (tsT/AC62) express significantly higher levels of RIIB as compared to costal chondrocytes (C-28/I2) (Fig. 4A, 4B). RIIB expression was reduced in early and recovered passage chondrocytes (Fig. 4B, 4C). Interestingly, the level of RIIB expression was found to correspond to TGF-β responsiveness in these cells. Luciferase activity, Smad 2 phosphorylation, and [$^3$H]-Thymidine incorporation (Fig. 5) depict differences in TGF-β sensitivity of chondrocytes of distinct phenotypes. An increased endogenous expression of RIIB appears to correspond to a decreased TGF-β responsiveness in human chondrocytes. Hirai and Fujita proposed that variation in RIIB expression could account for differential response to TGF-β by phenotypically distinct cells but was unable to draw specific conclusions from his investigations (28). It thus seems contradictory and controversial that exogenous overexpression of RIIB was seen to enhance TGF-β responsiveness (Fig. 6A, 6B) whereas endogenous levels correspond to decreased responsiveness. What could account for this apparent discrepancy is intriguing. It does seem evident that RIIB forms a complex with the other TGF-β signaling receptors. Perhaps RIIB at endogenous levels competes with RII for ligand; therefore in cells with high expression of RIIB less ligand is available for the signaling complex and TGF-β response is diminished. Alternatively, exogenous overexpression of RIIB may be causing the enhanced signaling through alternate means. It has been hypothesized that when proteins are transfected they may have cellular effects within the cytosol before their expression is manifested at the cell surface or in addition to this. It can be speculated that RIIB within the cell may alter the TGF-β signaling pathway intermediates which could potentially enhance responsiveness.

OA cells represent a distinct chondrocyte phenotype that exhibit a reduced capacity for type II collagen production and ultimately a poor innate reparative capacity (71-75). We have presented the novel finding of enhanced RIIB expression in OA versus normal human articular chondrocytes (Fig. 4D). This was confirmed both with cells obtained from monolayer cultures of digested cartilage and explant cultures as well as from whole cartilage protein extracts (SDS and Sucrose Preparations). Perhaps it is the abundant expression of endogenous RIIB and its inhibitory regulatory potential on TGF-β signaling in OA chondrocytes which impairs their capacity to aid in cartilage repair or regeneration. In summary, the present results have shown that RIIB is differentially expressed on human chondrocytes of distinct phenotypes and that it forms higher order complexes with RI and RII on the cell surface. More importantly, we illustrated that RIIB forms a hetero-oligomeric complex with betaglycan, endoglin, Alk-1, and Sol RI on these cells. Significantly, we demonstrated that exogenous/overexpressed RIIB enhances TGF-β response whereas high endogenous levels correspond to diminished TGF-β signaling. Moreover we have shown that OA human articular chondrocytes express high levels of RIIB as compared to normal non-diseased cells while RII expression remains relatively unchanged. Enhanced RIIB expression on OA cells may contribute to the inherent poor reparative capacity of OA cartilage. Further elucidation of the molecular mechanisms governing RIIB action in chondrocytes may lead to novel therapeutic avenues towards cartilage regeneration and repair.

**FIGURE LEGENDS**

**Fig. 1. RIIB is expressed on human articular cartilage and chondrocytes. A:** Solubilized extracts of immortalized chondrocytes, C-28/I2 and tsT/AC62 transfected

with pRIIB (RIIB), pcDNA1 (EV) or not transfected (Ø), were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with anti-RIIB (upper panel) and reprobed with anti-STAT 3 antibody (lower panel). The ECL system was used for chemiluminescence detection. **B:** Solubilized extracts of C-28/I2 and tsT/AC62 were analyzed by Immunoblotting with anti-RII antibody (upper panel, α-RII) which detects both RII and RIIB. Immunoblotting with α-RII and α-RIIB antibodies are shown in the lower panels using OA and normal (N) human primary chondrocytes. The left panel represents blotting with anti-RII antibody (α-RII), the right panel with anti-RIIB antibody (α-RIIB), and the center with anti-RII antibody preincubated with blocking peptide (α-RII+BP).

**Fig. 2. Affinity labeling of human chondrocytes with $^{125}$I-TGF-β1 and $^{125}$I-TGF-β2.** **A:** Confluent monolayers of C-28/I2 cells were not transfected (Ø) or were transfected with pcDNA1 (EV) or pRIIB (RIIB) for 48 hours and then affinity labeled with 100 pM of $^{125}$I-TGF-β1 in the absence or presence of 0.5 nM of unlabeled TGF-β1. Solubilized cell extracts were analyzed by SDS-PAGE (3-11% gradient) under nonreducing conditions followed by autoradiography. **B:** Confluent monolayers of C-28/I2 cells were transfected with pRIIB for 48 hours and affinity labeled with 100 pM of $^{125}$I-TGF-β1 in the absence or presence of indicated concentrations of unlabeled TGF-β1 or -β3. Solubilized cell extracts were analyzed by SDS-PAGE (3-11% gradient) under nonreducing conditions followed by autoradiography. **C:** Confluent monolayers of tsT/AC62 cells were affinity labeled with 100 pM of $^{125}$I-TGF-β2 in the absence or presence of 1.0 nM of unlabeled TGF-β1 or -β2. Solubilized cell extracts were analyzed

by SDS-PAGE (3-11% gradient) under nonreducing conditions followed by autoradiography.

**Fig. 3. Identification of TGF-β receptor complexes on human chondrocytes. A:** Solubilized extracts of chondrocytes were immunoprecipitated with anti-RII antibody and analyzed by SDS-PAGE (3-11% gradient gel) under nonreducing conditions in the first dimension. The individual lane was then cut out, laid horizontally on a second gel (3-11% gradient) and analyzed under reducing conditions in the second dimension. The gel was transferred to nitrocellulose membrane and Western blotted with anti-RIIB antibody. The ECL system was used for chemiluminescence detection. Cells were affinity labeled (**B**) with 100 pM of $^{125}$I-TGF-β1 or not affinity labeled (**C**) and solubilized cell extracts were immunoprecipitated with 3μg/ml each of anti-receptor antibodies, IgG, or not immunoprecipitated. Complexes were fractionated on SDS-PAGE (3-11% gradient gels) under nonreducing (**B**) or reducing (**C**) conditions and visualized by autoradiography (**B**) or the gel was transferred to nitrocellulose membrane and Western blotted (**C**) with anti-RIIB antibody (panel A and C) or anti-endoglin antibody (panel B). **B:** Non-immunoprecipitated (NIP) cell extract of tsT/AC62 is shown in Lane 1. Immunoprecipitation was performed using anti-RIIB antibody (Lane 2, α-RIIB). **C:** Non-immunoprecipitated (NIP) cell extract of C-28/I2 is shown in Lane 2 of panel A and C. Immunoprecipitations were performed using anti-endoglin (panel A, Lane 3; panel B, Lane 1; α-Eg), anti-RIIB antibody (panel B, Lane 2; α-RIIB), anti-betaglycan antibody (panel C, Lane 3; α-BG), anti-Alk-1 antibody (panel C, Lane 4; α-Alk-1), anti-Sol RI antibody (panel C, Lane 5; α-Sol R1) or control rabbit IgG (Lane 1, panel A and C; IgG).

**Fig. 4. Endogenous RIIB expression varies with chondrocyte phenotype. A:** Chondrocytes were affinity labeled with 100 pM of $^{125}$I-TGF-β1 and solubilized extracts were immunoprecipitated with anti-RII (Lane 2, α-RII) or anti-RIIB (Lane 3, α-RIIB) antibodies, or not immunoprecipitated (Lane 1, NIP) and analyzed by SDS-PAGE (3-11% gradient gel) under nonreducing conditions and visualized by autoradiography. **B:** Confluent monolayers of early (E), late (L), and recovered (R) passages of chondrocytes were affinity labeled with 100 pM of $^{125}$I-TGF-β1 and solubilized extracts were analyzed by SDS-PAGE on a 3-11% polyacrylamide gradient gel under reducing conditions followed by autoradiography. **C:** Confluent monolayers of early (E), late (L), and recovered (R) passages of chondrocytes were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with anti-RIIB antibody (upper panel) and reprobed with anti-Smad 2/3 antibody (lower panel) to confirm equal protein loading. The ECL system was used for chemiluminescence detection. **D:** OA and normal cartilage was harvested and either digested with enzymes or cultured as explants to obtain confluent monolayers (panel C). Alternatively, SDS (panel A) and Sucrose (panel B) Preparations of protein extracts were obtained. Solubilized extracts were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with anti-RIIB antibody (upper panels of A, B, and C) and reprobed with anti-STAT 3 antibody (lower panels of A, B, and C) to confirm equal protein loading. The ECL system was used for chemiluminescence detection.

**Fig. 5. TGF-β responses in phenotypically distinct chondrocytes correlate with endogenous RIIB expression. A:** Stimulation of plasminogen activator inhibitor-1 (PAI-1)

promoter activity by TGF-β. Chondrocytes, C-28/I2 and tsT/AC62 (Late passage; panel A) and tsT/AC62 (Late and Recovered (Rec) passages; panel B) were transiently transfected with the PAI-1 promoter-luciferase reporter construct (p3TP-Lux) and pβ-gal. 24 hours after transfection, cells were treated with 100 pM of TGF-ß1 overnight or were left untreated. Luciferase activity was determined, normalized using the β-gal assay and expressed as a percent control of untreated cells. The data shown are representative of three different experiments each done in triplicates and statistically significant values are indicated by an asterisk. **B-D:** Stimulation of Smad 2 phosphorylation by TGF-β1. Solubilized extracts of C-28/I2, tsT/AC62, and primary chondrocytes or early (E), late (L), and recovered (R) passages of these cells were treated (+) with 50 pM of TGF-ß1 for 15 minutes, 30 minutes, or left untreated (-) were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against the phosphorylated form of Smad 2 (upper panels) and reprobed with anti-STAT 3 antibody to illustrate equal protein loading (lower panels). The ECL system was used for chemiluminescence detection. **E:** Confluent monolayers of late (Late) and recovered (Rec) passages of C-28/I2 were seeded at a density of $7.0x10^5$ cells/24 well plate and cultured for 24 hours. The cells were serum starved for 4 hours and incubated with 50 or 200 pM of TGF-β1 under serum free conditions overnight. [$^3$H]-Thymidine ($1\mu Ci \cdot ml^{-1}$) was added per well for the final 4 hours of TGF-β1 treatment. The cells were washed 3 times with PBS and once with 5% trichloroacetic acid, solubilized in 1% SDS, and incorporated radioactivity was determined by liquid scintillation counting. [$^3$H]-Thymidine incorporation by chondrocytes is expressed as a ratio of Late to Recovered

cells. The data shown are representative of three different experiments each done in quadruplicates and statistically significant values are indicated by an asterisk.

**Fig. 6. TGF-β responses in chondrocyte cell lines overexpressing the RIIB. A:** Stimulation of Smad 2 phosphorylation by TGF-β1. Chondrocytes were transiently transfected with pRIIB (RIIB) or pcDNA1 (EV). After 48 hours cells were treated with 50 pM of TGF-ß1 for 15 minutes (+) or left untreated (-) were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against the phosphorylated form of Smad 2 (panel A, upper panel) and reprobed with anti-STAT 3 antibody to illustrate equal protein loading (panel A, lower panel). Immunoblotting with the anti-RIIB antibody (panel B) was used to confirm receptor overexpression. The ECL system was used for chemiluminescence detection. **B:** Stimulation of PAI-1 promoter (3TP-Lux) activity by TGF-β. Chondrocytes were transiently transfected with p3TP-Lux, pcDNA1 and pβ-gal (EV) or p3TP-Lux, pβ-gal and pRII (RII) or pRIIB (RIIB). 48 hours after transfection, cells were treated with 100 pM of TGF-ß1, β2, or β3 for 24 hours or were left untreated (-). Luciferase activity was determined, normalized using the β-gal assay and expressed as a fold increase of untreated cells. The data shown are representative of three different experiments each done in triplicates and statistically significant values are indicated by an asterisk. **C:** Confirmation of receptor overexpression is shown. Extracts from **B** were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membranes were immunoblotted with anti-RII (upper panel, Lane 1,2,3; α-RII) or anti-RIIB antibody (upper panel, Lane 4,5,6; α-RIIB) and reprobed with anti-Smad 2 antibody

(lower panel, Lane 1-6) to confirm equal protein loading. The ECL system was used for chemiluminescence detection. **D:** Chondrocytes were transiently transfected with pRIIB (RIIB), pcDNA1 (EV), or not transfected (Ø). After 48 hours solubilized extracts were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against type II collagen (upper panel) and reprobed with anti-STAT 3 antibody (lower panel) to confirm equal protein loading. The ECL system was used for chemiluminescence detection.

**REFERENCES**


1. Robinson D, Halperin N, Nevo Z 1990 Regenerating hyaline cartilage in articular defects of old chickens using implants of embryonal chick chondrocytes embedded in a new natural delivery substance. Calc Tissue Int 46:246-253.
2. Aston JE, Bentley G 1986 Repair of articular surfaces by allografts of articular and growth-plate cartilage. J Bone Joint Surg 68-B:29-35.
3. Petit B, Freyria A-M, van der Rest M, Herbage D 1992 Cartilage collagens. In: Adolphe M (ed) Biological Regulation of the Chondrocytes. CRC Press, Boca Raton, FL, pp.33-84.
4. Mayne R, Brewton RG 1993 Extracellular matrix of cartilage: collagen. In: Woessner JF Jr, Howell DS (eds) Joint Cartilage Degradation. Basic and Clinical Aspects. Marcel Dekker, New York, NY, pp. 81-108.
5. Cancedda R, Descalzi Cancedda F, Castagnola P 1995 Chondrocyte differentiation. Int Rev Cytol 159:265-358.
6. Benya PD, Shaffer JD 1982 Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215-224.

7. Roberts AB, Sporn MB 1990 The transforming growth factor beta's. In: Sporn MB and Roberts AB (eds) Peptide Growth Factors and Their Receptors, vol 95. Spriner-Verlag, New York, NY, pp. 419-472.

8. Seyedin S, Thompson A, Bentz H, Rosen DM, McPherson JM, Conti A, Siegel NR, Galluppi GR, and Piez KA 1986 Cartilage-inducing Factor-A. J Biol Chem 261:5693-5695.

9. Lin HV, Wang X-F, Ng-Eaton E, Weinberg RA, Lodish H F 1992 Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. Cell 68:775-785.

10. Franzen P, ten Dijke P, Ichijo H, Yamashita H, Schultz P, Heldin C H, Miyazono K 1993 Cloning of TGF-β type I receptor that forms a heterodimeric complex with the type II TGF-β receptor. Cell 75: 681-692.

11. Piek E, Heldin C-H, ten Dijke P 1999 Specificity, diversity, and regulation in TGF-β superfamily FASEB 13:2105-2124.

12. Massague J, Chen Y-G 2000 Controlling TGF-beta signaling. Genes Dev 14:627-644.

13. Cheifetz S, Massague J 1991 Isoform-specific transforming growth factor-beta binding proteins with membrane attachments sensitive to phosphatidylinositol-specific phospholipase C. J Biol Chem 266, 20767-20772.

14. Dumont N, O'Connor-McCourt M, Philip A 1995 Transforming growth factor-β receptors on human endometrial cells: Identification of the type I, II and III receptors, and glycosylphosphatidylinositol anchored TGF-β binding proteins. Mol Cell Endocrinol 111:57-66.

15. Tam B, Germain L, Philip A 1998 A 150kDa GPI-anchored TGF-β1 binding protein forms heteromeric complex with the types I and II TGF-β signalling receptors. J Cell Biochem 70:573-586.

16. Tam B, Philip A 1998 Transforming growth factor-β receptor expression on human skin fibroblasts; dimeric complex formation of type I and type II receptors and identification of glycosyl phosphatidylinositol-anchored transforming growth factor-β binding proteins. J Cell Physiol 176:553-564.

17. Lopez-Casillas F, Wrana JL, Massague J 1993 Betaglycan presents ligand to the TGF-β signaling receptor. Cell 73:1435-1444.

18. Philip A, Hannah R, O'Connor-McCourt M 1999 Ectodomain cleavage and shedding of the type III TGF-β receptor in the rat lung membranes: Effect of temperature, ligand binding and membrane solubilization. Europ J Biochem 261:618-628.

19. Yamashita H, Ichijo H, Grimsby S, Moron A, ten Dijke P, Miyazono K 1994 Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor beta. J Biol Chem 269:1995-2001.

20. Letamendia A, Lastres P, Botella LM, Raab U, Langa C, Velasco B, Attisano L, Bernabeu C 1998 Role of endoglin in cellular responses to transforming growth factor-β. A comparative study with betaglycan. J Biol Chem 273(49):33011-33019.

21. Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C 2002 Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-β receptors I and II. J Biol Chem 277: 29197-209.

22. Parker WL, Goldring MB, Philip A 2003 Endoglin is expressed on human chondrocytes and forms a heteromeric complex with betaglycan in a ligand and type II TGF-β receptor independent manner. J Bone Miner Res 18:289-302.

23. Parker WL, Choi ME, Philip A Identification of Activin Receptor-like Kinase-1 and Soluble type I TGF-β receptor on human chondrocytes: hetero-oligomerization and regulation of TGF-β signaling. Submitted.

24. Choi ME 1999 Cloning and characterization of a naturally occurring soluble form of TGF-β type I receptor. Am J Physiol 276: (Renal Physiol. 45):F88-F95.

25. Rotzer D, Roth M, Lutz M, Lindemann D, Sebald W, Knaus P 2001 Type III TGF-β receptor-independent signalling of TGF-β2 via TβRII-B, an alternatively spliced TGF-β type II receptor. EMBO 20:480-490.

26. Glansbeek HL, van der Kraan PM, Vitters EL, van den Berg WB 1993 Correlation of the size of type II transforming growth factor β (TGF-β) receptor with TGF-β responses of isolated bovine articular chondrocytes. Ann Rheum Dis 52:812-816.

27. Glansbeek HL, van der Kraan PM, Lafeber FPJG Vitters EL, van den Berg WB 1997 Species-specific expression of type II TGF-β receptor isoforms by articular chondrocytes: affect on proteoglycan depletion and aging. Cytokine 9:347-351.

28. Hirai R, Fujita T 1996 A human transforming growth factor-β type II receptor that contains an insertion in the extracellular domain. Exp Cell Res 223:135-141.

29. Robbins JR, Goldring MB 1997 Preparation of immortalized human chondrocyte cell lines. In: Morgan JR, Yarmush ML (eds) Methods in Molecular Medicine, vol 18. Humana Press Inc., Totowa, N.J. pp. 173-192.

30. Goldring MB, Birkhead JR, Suen L-F, Yamin R, Mizuno S, Glowacki J, Apperley JF 1994 Successful immortalization of human chondrocytes exhibiting stable expression of cartilage-specific type II collagen. J Clin Invest 94:2307-2316.

31. Gruppuso PA, Mead JE, Fausto N 1990 Transforming growth factor receptors in liver regeneration following partial hepatectomy in the rat. Cancer Res 50:1464-1469.

32. Chanda VB 1995 Support Protocol: Sample Solubilization. In: Chanda VB (ed) Current Protocols in Molecular Biology, Supplement, John Wiley & Sons, Canada, pp. 10.3.8-10.

33. Hauselmann HJ, Aydelotte MB, Schumacher BL, Kuettner KE, Gitelis SH, Thonar J-MA 1992 Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. Matrix 12:116-129.

34. Hauselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block JA, Aydelotte MB, Kuettner KE, Thonar EJ 1994 Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J Cell Sci 107:17-27.

35. Guo J, Jourdian GW, MacCallum DK 1989 Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Conn Tissue Res 19:277-297.

36. Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Friesinger P 1994 Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp Cell Res 212:97-104.

37. Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, Poole AR 1994 Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. J Clin Invest 93:1722-32.

38. Wong SH, Hamel L, Chevalier S, Philip A 2000 Endoglin expression on human microvascular endothelial cells: demonstration of higher order complexes with TGF-β signaling receptors and heteromerization with betaglycan. Eur J Biochem 267:5550-5560.

39. Piek E, Franzen P, Heldin CH, ten Dijke P 1997 Characterization of a 60-kDa cell surface-associated transforming growth factor-beta binding protein that can interfere with transforming growth factor-beta receptor binding. J Cell Physiol 173(3):447-59.

40. MacKay K, Robbins AR, Bruce M.D, Danielpour D 1990 Identification of disulfide-linked transforming growth factor-beta 1-specific binding proteins in rat glomeruli. J Biol Chem 265:9351-9356.

41. Souchelnytskyi S, Ronnstrand L, Heldin CH, ten Dijke P 2001 Phosphorylation of Smad signaling proteins by receptor serine/threonine kinases. Methods Mol Biol 124:107-20.

42. Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P 1997 TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. EMBO J 16:5353-62.

43. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J 1994 Mechanism of activation of the TGF-β receptor. Nature 370:341-347.

44. Robbins JR, Thomas B, Tan L, Choy B, Arbiser JL, Berenbaum F, Goldring MB 2000 Immortalized human adult articular chondrocytes maintain cartilage-specific phenotype and responses to interleukin-1beta. Arthritis Rheum 43:2189-201.

45. Goldring MB, Berenbaum F 1999 Human chondrocyte culture models for studying cyclooxygenase expression and prostaglandin regulation of collagen gene expression. Osteoarthritis Cartilage 7:386-88.

46. Kokenyesi R, Tan L, Robbins JR, Goldring MB 2000 Proteoglycan production by immortalized human chondrocyte cell lines cultured under conditions that promote expression of the differentiated phenotype. Arch Biochem Biophys 383:79-90.

47. Loeser R, Sadiev S, Tan L, Goldring MB 2000 Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins in mediating chondrocyte adhesion to types II and VI collagen. Osteoarthritis Cartilage 8:96-105.

48. Domm C, Schunke M, Christesen K, Kurz B 2002 Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. Osteoarthritis Cartilage 10:13-22.

49. Thompson AY, Piez KA, Seyedin SM 1985 Chondrogenesis in agarose culture. Exp Cell Res 157:483-494.

50. Lux A, Attisano L, Marchuk DA 1999 Assignment of transforming growth factor $\beta 1$ and $\beta 3$ and a third new ligand to the type I receptor ALK-1 J Biol Chem 274:9984-9992.

51. Howard S, Anastasiades T 1993 Differential effects of bone associated factors on newly synthesized anionic glycoconjugates by articular chondrocyte cultures from adult and immature bovines. J Rheumatol 20:2083-2094.

52. Demoor-Fossard M, Redini F, Boittin M, Pujol JP 1998 Expression of decorin and biglycan by rabbit articular chondrocytes. Effects of cytokines and phenotypic modulation. Biochem Biophys Acta 1398:179-91.

53. Redini F, Galera P, Mauviel A, Loyau G, Pujol JP 1988 Transforming growth factor beta stimulates collagen and glycosaminoglycan biosynthesis in cultured rabbit articular chondrocytes. FEBS Lett 234:172-6.

54. Perka C, Schultz O, Spitzer RS, Lindenhayn K 2000 The influence of transforming growth factor beta1 on mesenchymal cell repair of full-thickness cartilage defects. J Biomed Mater Res 52:543-52.

55. Fahlgren A, Andersson B, Messner K 2001 TGF-beta1 as a prognostic factor in the process of early osteoarthrosis in the rabbit knee. Osteoarthritis Cartilage 9:195-202.

56. Fawthrop FW, Frazer A, Russell RG, Bunning RA 1997 Effects of transforming growth factor beta on the production of prostaglandin E and caseinase activity of unstimulated and interleukin 1-stimulated human articular chondrocytes in culture. Br J Rheumatol 36:729-34.

57. Iqbal J, Dudhia J, Bird JL, Bayliss MT 2000 Age-related effects of TGF-beta on proteoglycan synthesis in equine articular cartilage. Biochem Biophys Res Commun 274:467-71.

58. Morales TI, Roberts AB 1988 Transforming growth factor beta regulates the metabolism of proteoglycans in bovine cartilage organ cultures. J Biol Chem. 263:12828-31.

59. Tsukazaki T, Matsumoto T, Enomoto H, Usa T, Ohtsuru A, Namba H, Iwasaki K, Yamashita S 1994 Growth hormone directly and indirectly stimulates articular chondrocyte cell growth. Osteoarthritis Cartilage 2:259-67.

60. Pujol JP, Galera P, Redini F, Mauviel A, Loyau G 1991 Role of cytokines in osteoarthritis: comparative effects of interleukin 1 and transforming growth factor-beta on cultured rabbit articular chondrocytes. J Rheumatol Suppl 27:76-9.

61. Serra R, Johnson M, Filvaroff EH, LaBorde J, Sheehan DM, Derynck R, Moses HL 1997 Expression of a truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. J Cell Biol139:541-52.

62. Yang X, Chen L, Xu X, Li C, Huang C, Deng C-X 2001 TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol 153:35-46.

63. Laarsons J, Goumans M-J, Sjostrand LJ, van Rooijen MA, Ward D, Leveen, P, Xu X, ten Dijke P, Mummery C, Karlsson S 2001 Abnormal angiogenesis but intact hematopoietic potential in TGF- type I receptor deficient mice . EMBO 20:1663-1673.

64. Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P 2002 Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J 21:1743-53.

65. Letamendia A, Lastres P, Almendro N, Raab U, Buhring H-J, Kumar S, Bernabeu C 1998 Endoglin, a component of the TGF-β receptor system, is a differentiation marker of human choriocarcinoma cells. Int J Cancer 76:541-546.

66. Arthur HM, Ure J, Smith AJH, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG 2000 Endoglin, an ancillary TGF-ß receptor, is required for extra embryonic angiogenesis and plays a key role in heart development. Dev Biol 217:42-53.

67. Szekanecz Z, Haines GK, Harlow LA, Shah MR, Fong TW, Fu R, Lin J-W, Rayan G, Koch AE 1995 Increased synovial expression of transforming growth factor (TGF)-β receptor endoglin and TGF-β1 in rheumatoid arthritis: possible interactions in the pathogenesis of the disease. Clin Immunol Immunopath 76:187-194.

68. Diez-Marques L, Ortega-Velazquez R, Langa C, Rodriguez-Barbero A, Lopez-Novoa JM, Lamas S, Bernabeu C 2002 Expression of endoglin in human mesangial cells: modulation of extracellular matrix synthesis. Biochem Biophys Acta 1587:36-44.

69. Rodriguez-Barbero A, Obreo J, Yuste L, Montero A, Rodriguez-Pena A, Pandiella A, Bernabeu C, Lopez-Novoa JM 2001 Transforming growth factor-β1 induces collagen synthesis and accumulation via p38 mitogen-activated protein kinase (MAPK) pathway in cultured $L_6E_9$ myoblasts. FEBS Letters 513:282-288.

70. Zwaagstra JC, El-Alfy M, O'Connor-McCourt MD 2001 Transforming growth factor (TGF)-β1 internalization: modulation by ligand interaction with TGF-β receptors types I and II and a mechanism that is distinct from clathrin-mediated endocytosis. J Biol Chem 276:27237-27245.

71. Fukui N, Purple CR, Sandell LJ 2001 Cell biology of osteoarthritis: the chondrocyte's response to injury. Curr Rheum Reports 3:496-505.

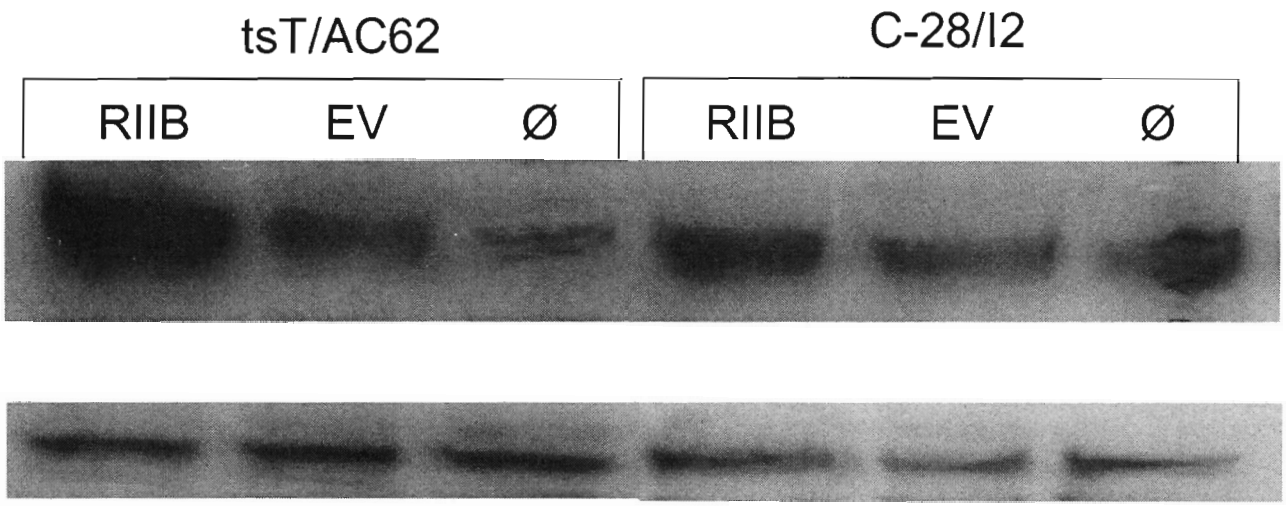
72. Roughley PJ 2001 Articular cartilage and changes in arthritis: Noncollagenous proteins and proteoglycans in the extracellular matrix of cartilage. Arthritis Res 3:342-347.

73. Goldring MB 2000 Osteoarthritis and cartilage: the role of cytokines. Curr Rheumatol Rep 2:459-65.

74. Boumediene K, Conrozier T, Mathieu P, Richard M, Marcelli C, Vignon E, Pujol J-P 1998 Decrease of cartilage transforming growth factor-β receptor II expression in the rabbit experimental osteoarthritis-potential role in cartilage breakdown. Osteoarth Cart 6:146-149.

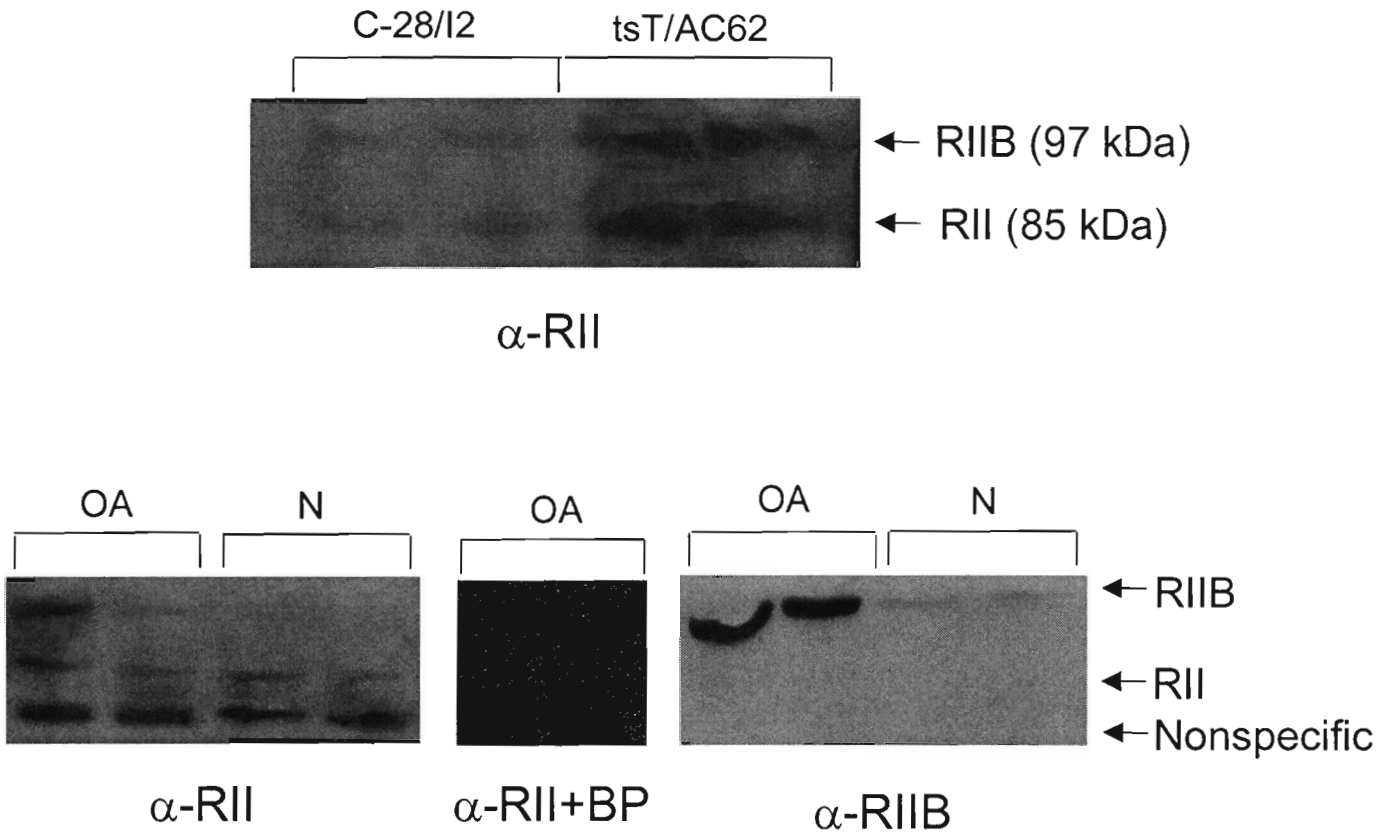
75. van den Berg WB 1999 From Cartilage metabolism to clinics: The role of cytokines and growth factors in cartilage destruction in osteoarthritis and rheumatoid arthritis. J Rheumatol 58:136-141.

Fig. 1A




345

Fig. 1B


C-28/I2
tsT/AC62
RIIB (97 kDa)
RII (85 kDa)
α-RII
OA
N
OA
OA
N
RIIB
RII
Nonspecific
α-RII
α-RII+BP
α-RIIB

346

Fig. 2A

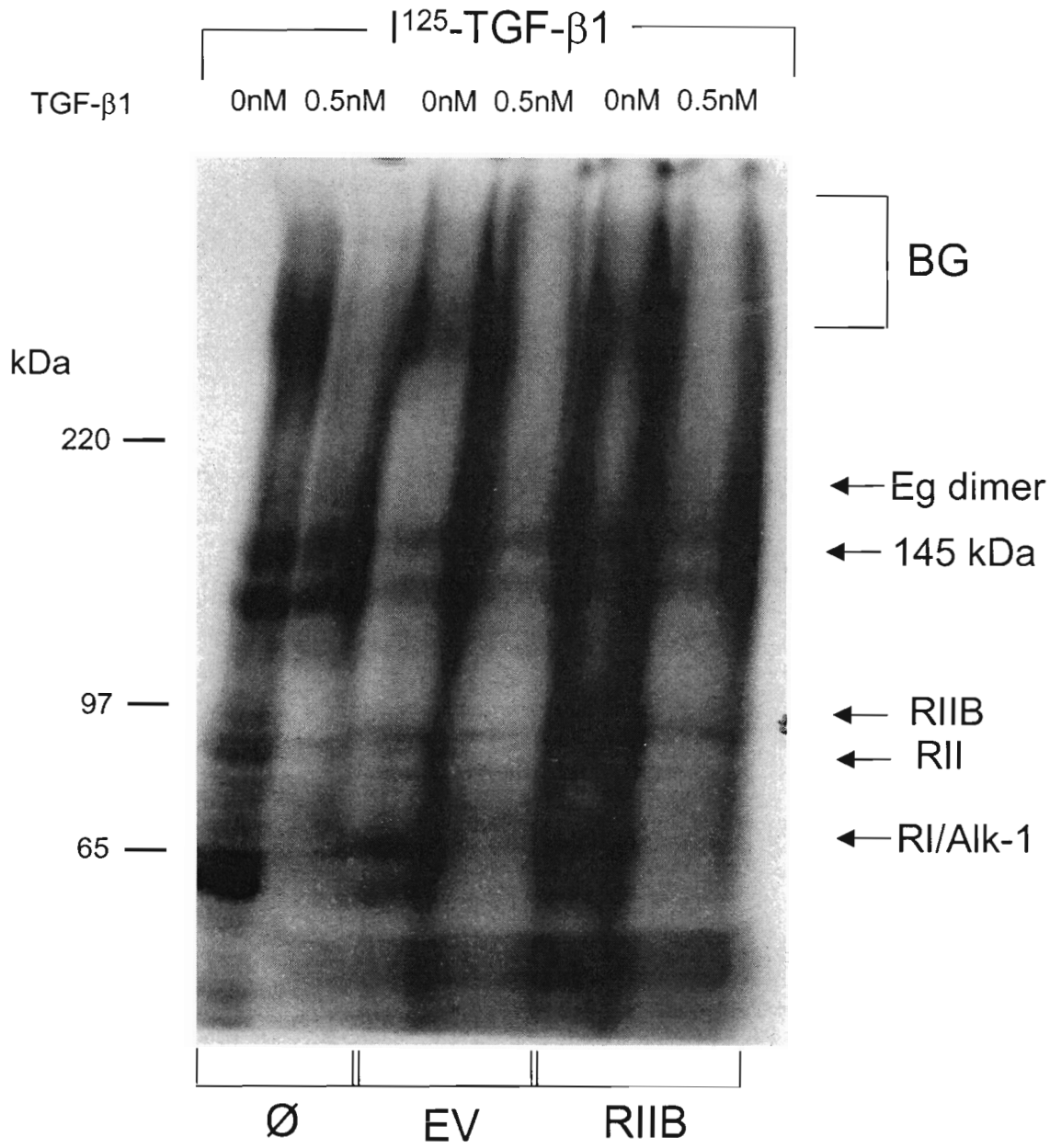
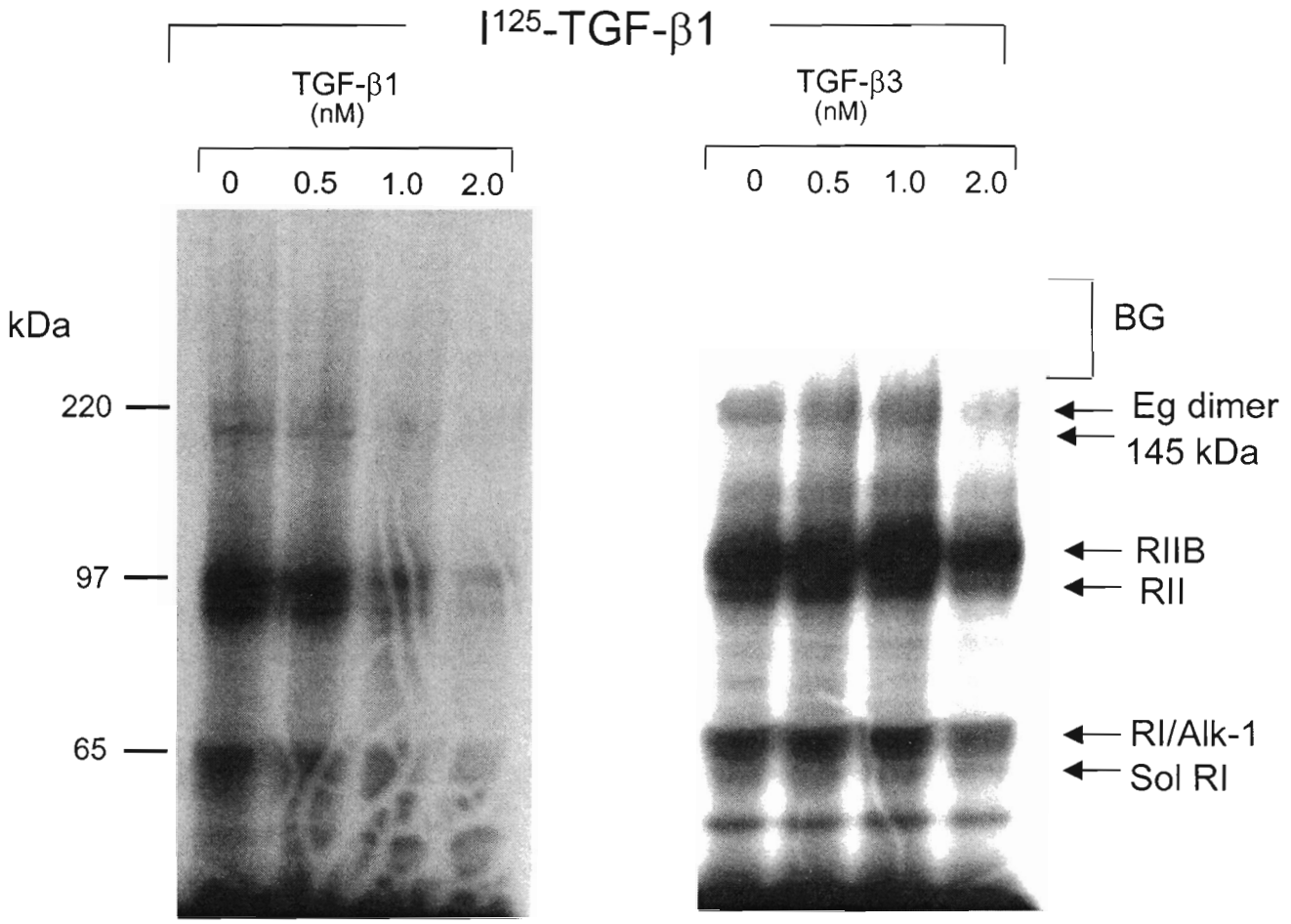

I125-TGF-β1
TGF-β1
0nM 0.5nM 0nM 0.5nM 0nM 0.5nM
kDa
220
97
65
BG
Eg dimer
145 kDa
RIIB
RII
RI/Alk-1
Ø
EV
RIIB

Fig. 2B


I125-TGF-β1
TGF-β1
(nM)
0
0.5
1.0
2.0
TGF-β3
(nM)
0
0.5
1.0
2.0
kDa
220
97
65
BG
Eg dimer
145 kDa
RIIB
RII
RI/Alk-1
Sol RI

348

Fig. 2C

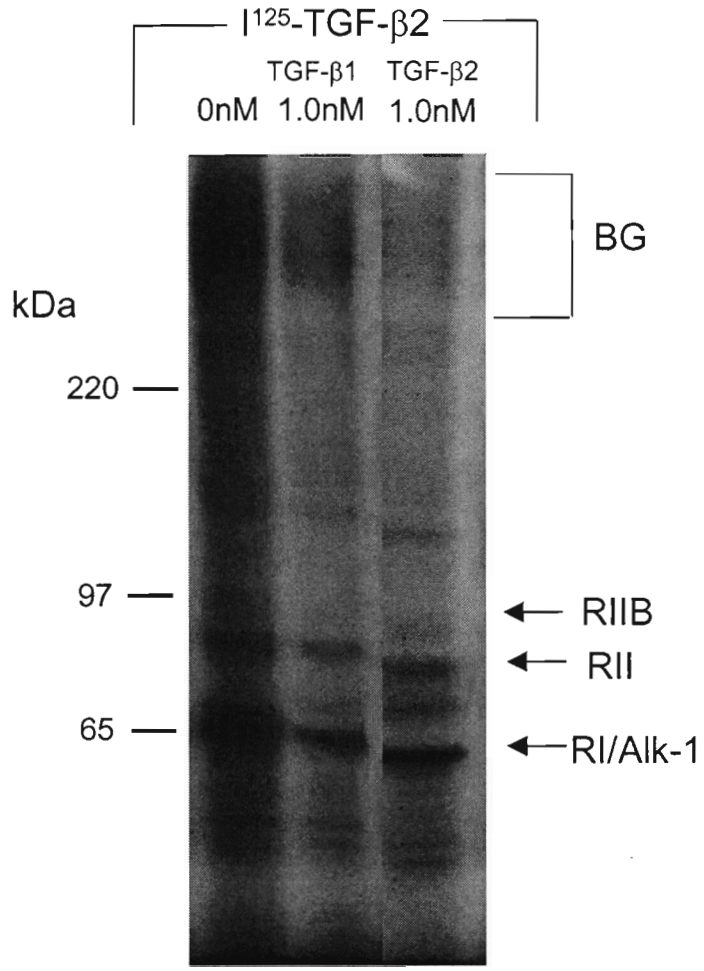

I125-TGF-β2
TGF-β1
TGF-β2
0nM
1.0nM
1.0nM
kDa
220
97
65
BG
RIIB
RII
RI/Alk-1

Fig. 3A

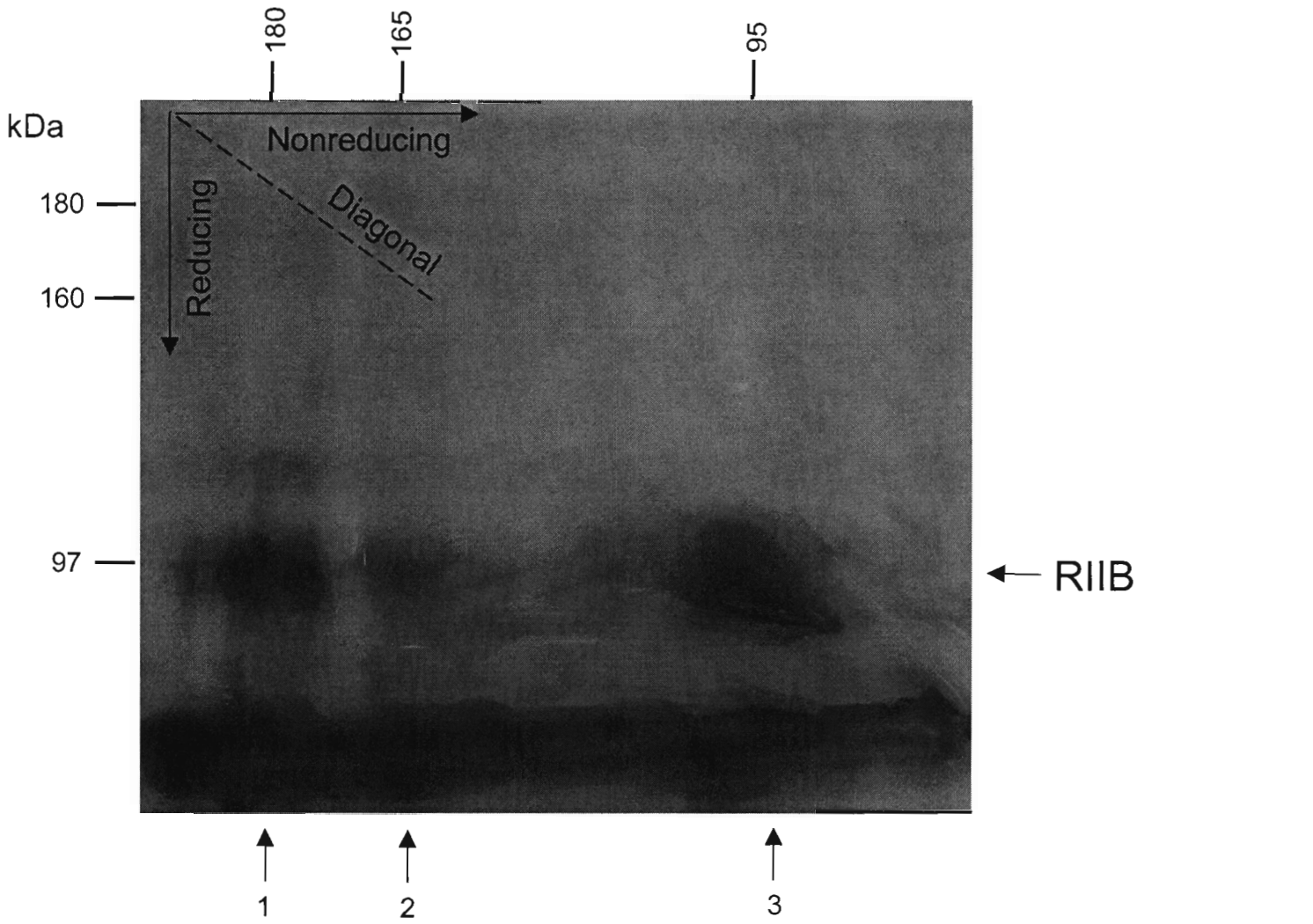
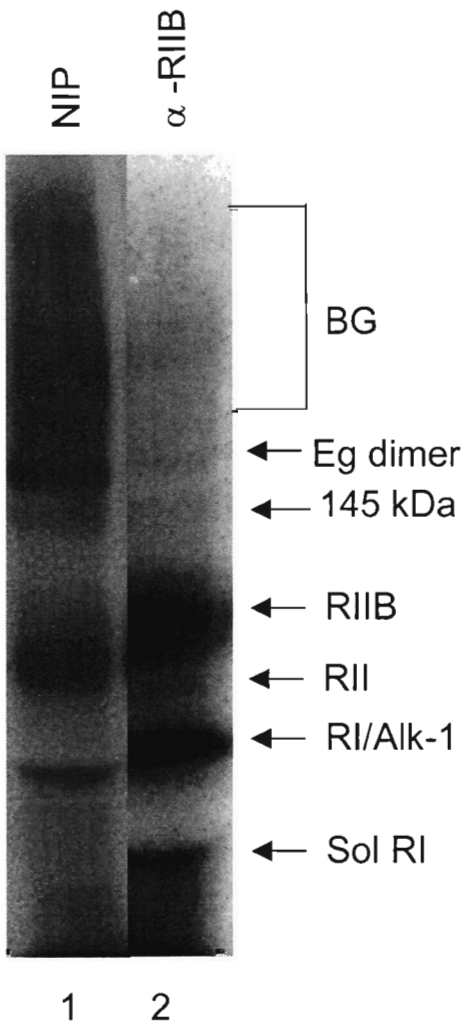
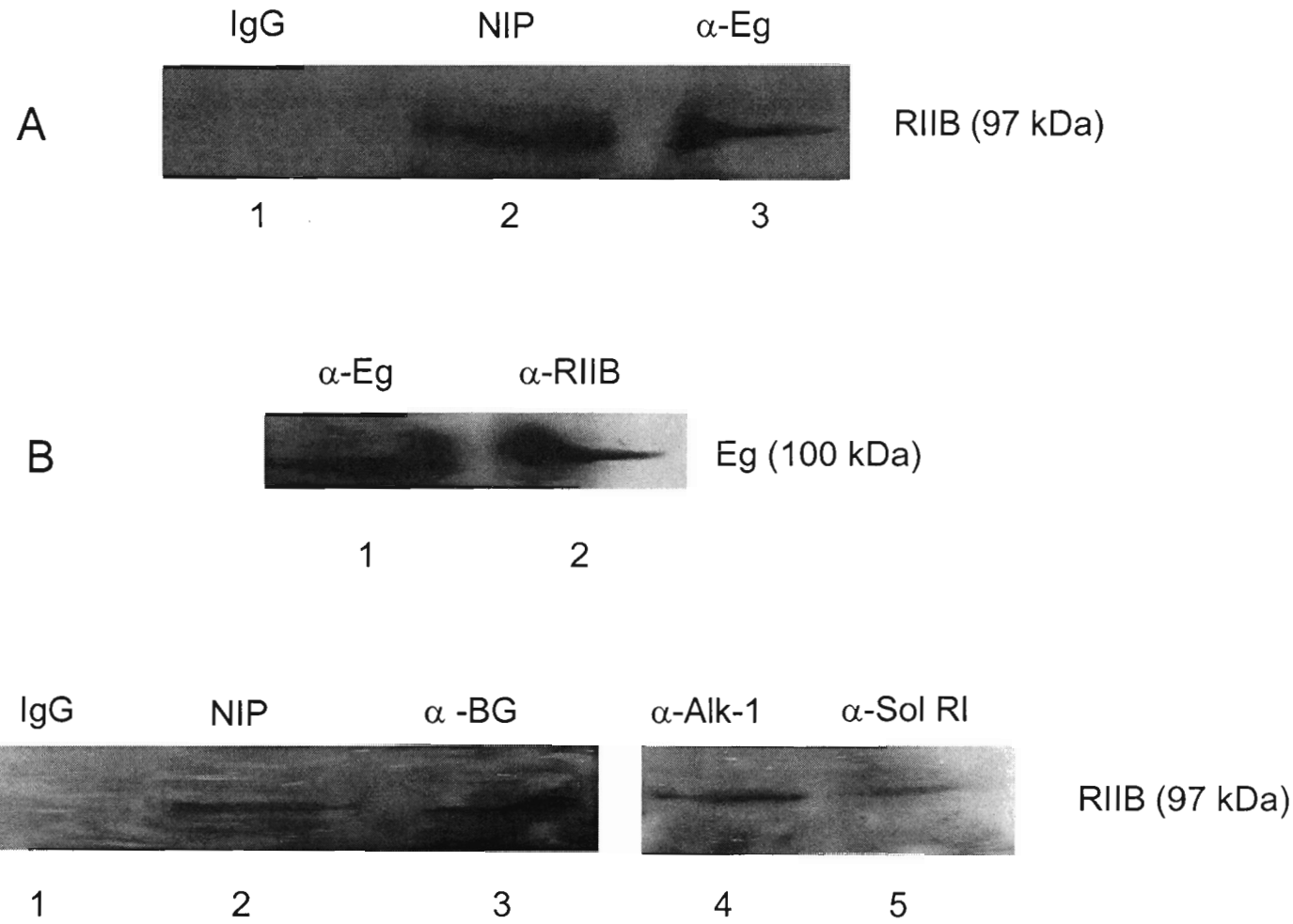

180
165
95
kDa
Nonreducing
Reducing
Diagonal
180
160
97
RIIB
350
1
2
3

Fig. 3B




351

Fig. 3C


IgG
NIP
α-Eg
A
RIIB (97 kDa)
1
2
3
α-Eg
α-RIIB
B
Eg (100 kDa)
1
2
IgG
NIP
α -BG
α-Alk-1
α-Sol RI
C
RIIB (97 kDa)
1
2
3
4
5

352

Fig. 4A

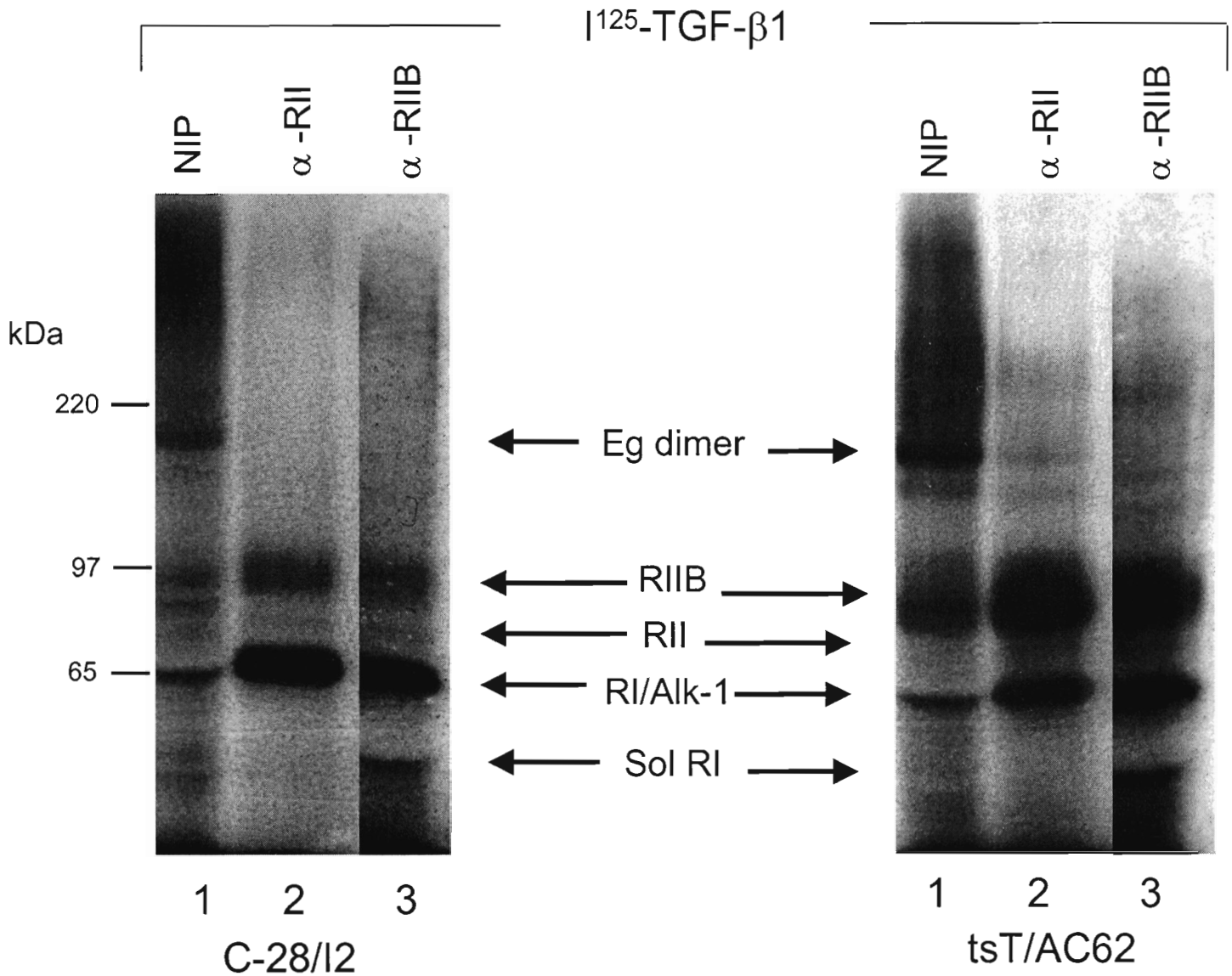
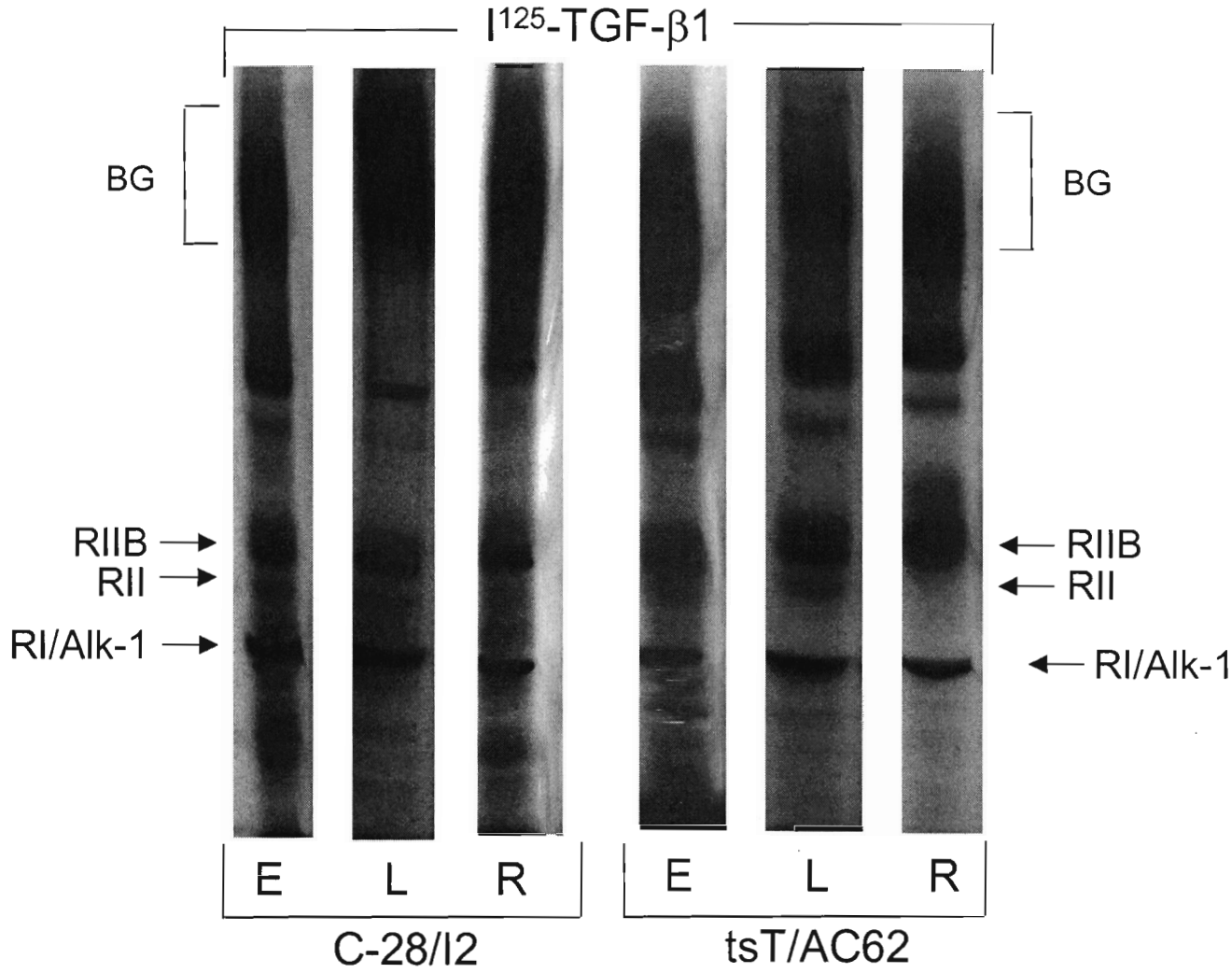
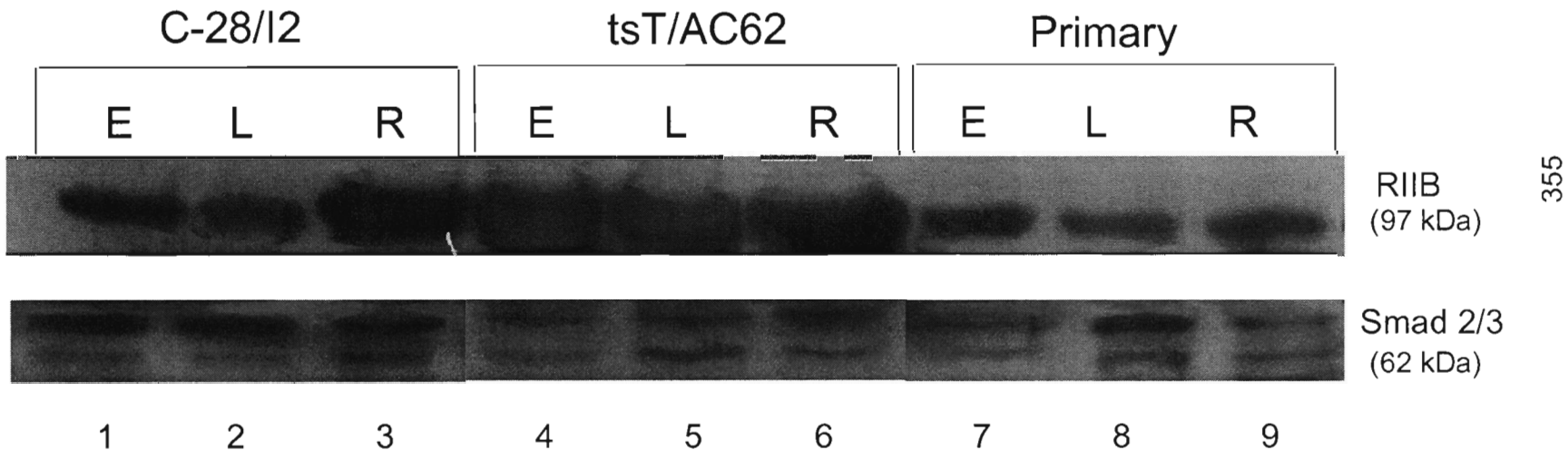

I125-TGF-β1
NIP
α -RII
α -RIIB
NIP
α -RII
α -RIIB
kDa
220
97
65
Eg dimer
RIIB
RII
RI/Alk-1
Sol RI
1 2 3
C-28/I2
1 2 3
tsT/AC62

Fig. 4B


I125-TGF-β1
BG
BG
RIIB
RII
RI/Alk-1
RIIB
RII
RI/Alk-1
E
L
R
E
L
R
C-28/I2
tsT/AC62

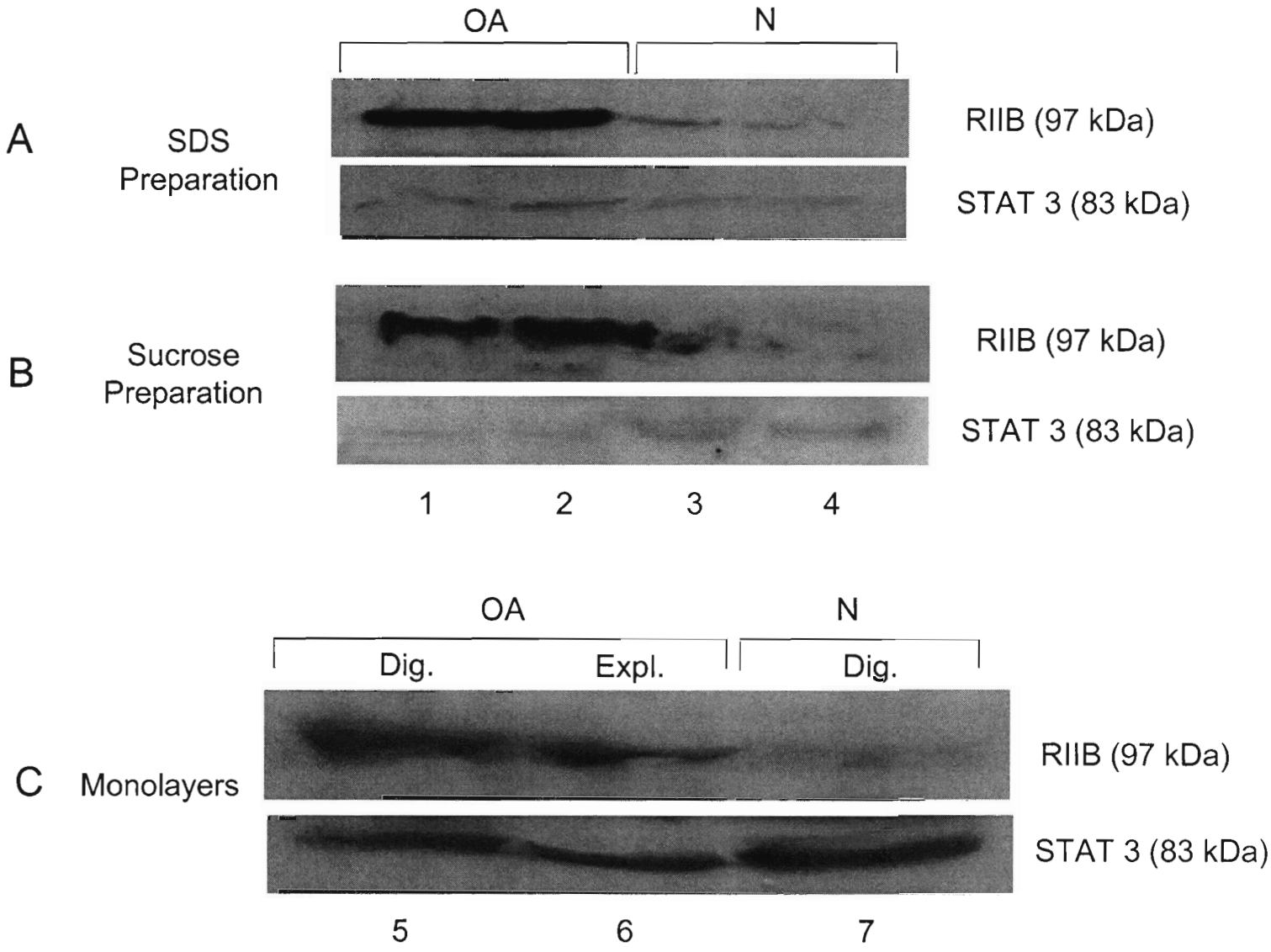
354

Fig. 4C


C-28/I2
tsT/AC62
Primary
E
L
R
E
L
R
E
L
R
RIIB
(97 kDa)
Smad 2/3
(62 kDa)
1
2
3
4
5
6
7
8
9

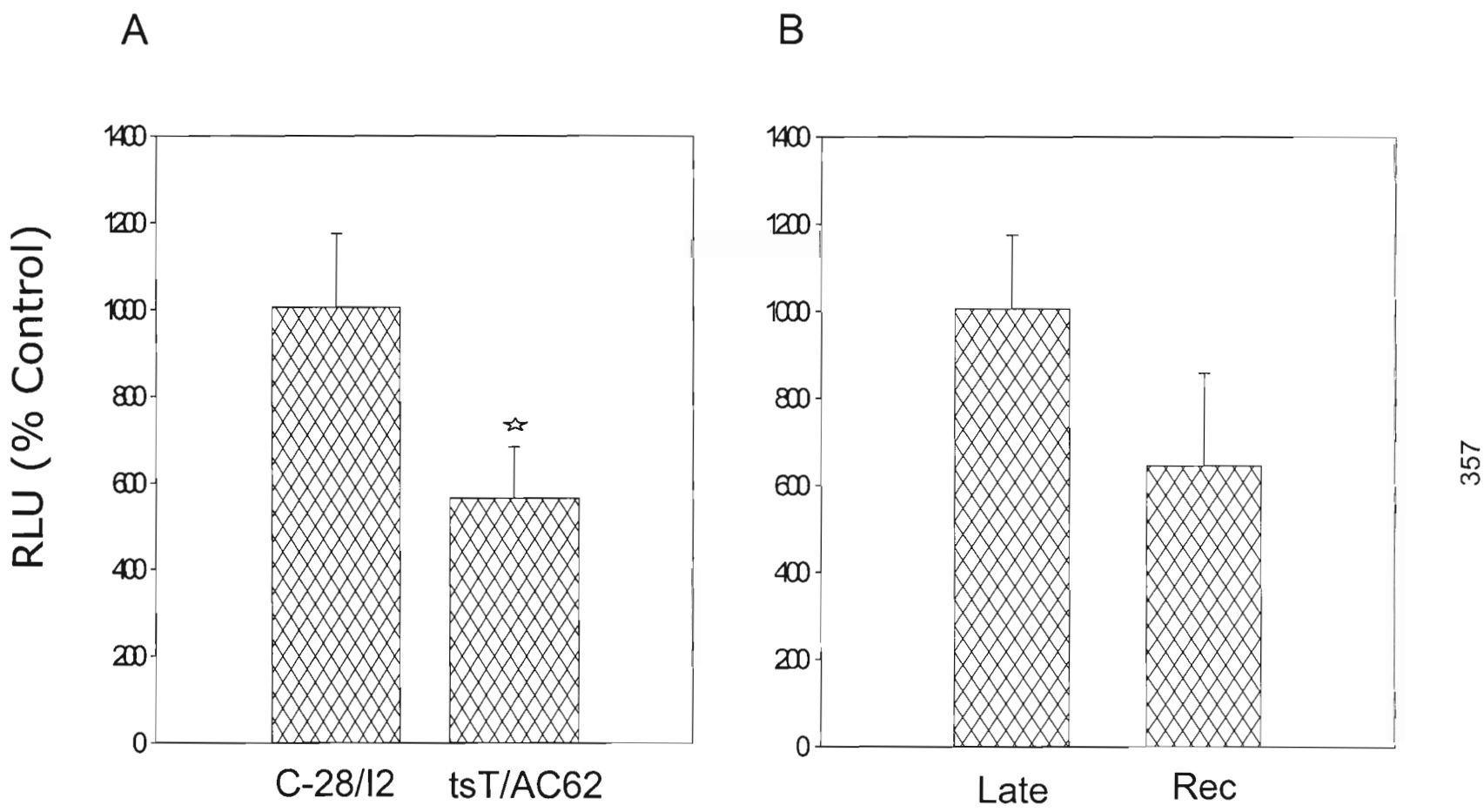
355

Fig. 4D


OA
N
A
SDS
Preparation
RIIB (97 kDa)
STAT 3 (83 kDa)
B
Sucrose
Preparation
RIIB (97 kDa)
STAT 3 (83 kDa)
1
2
3
4
OA
N
Dig.
Expl.
Dig.
C
Monolayers
RIIB (97 kDa)
STAT 3 (83 kDa)
5
6
7

356

Fig. 5A


A
B
RLU (% Control)
1400
1200
1000
800
600
400
200
0
C-28/I2
tsT/AC62
Late
Rec

357

Fig. 5B

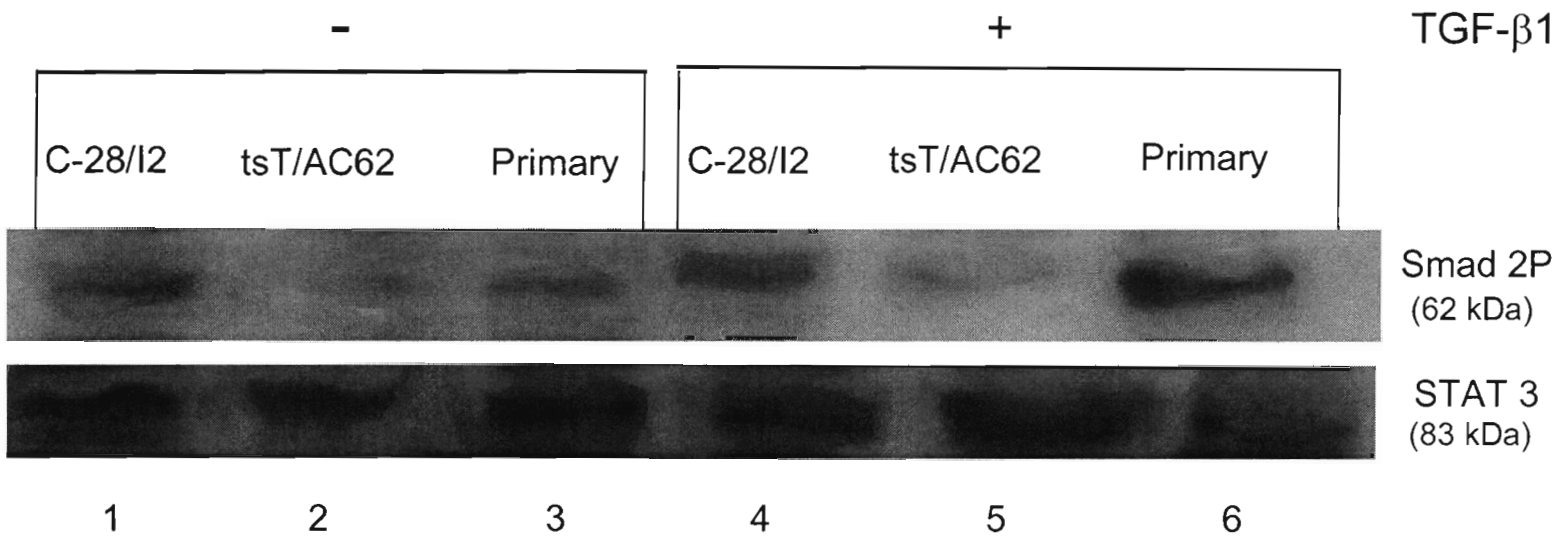
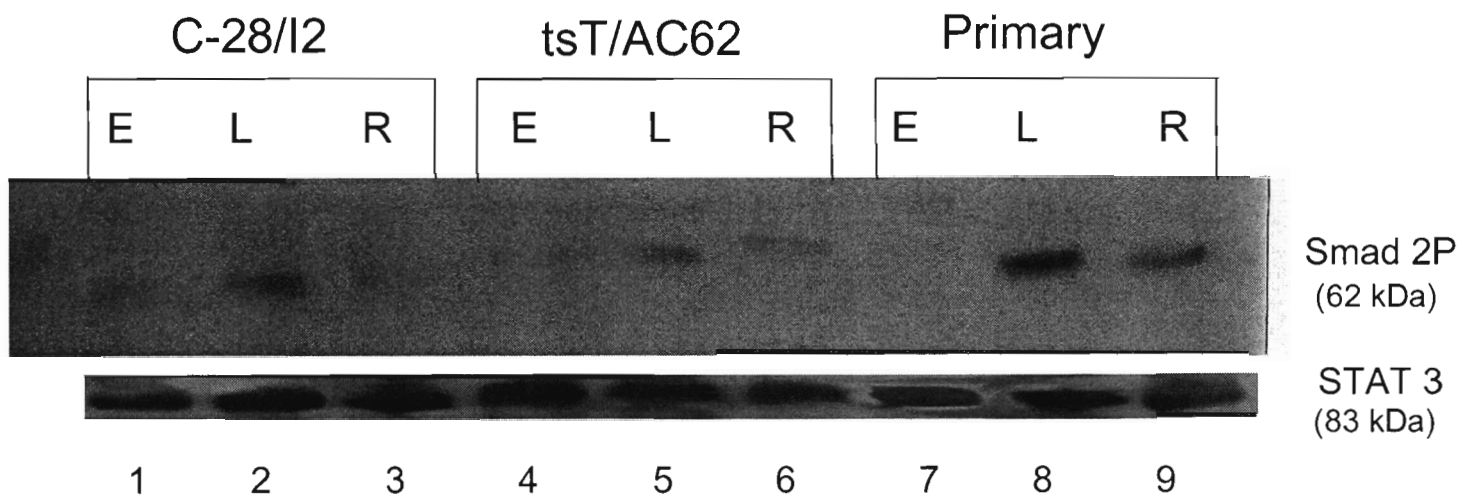
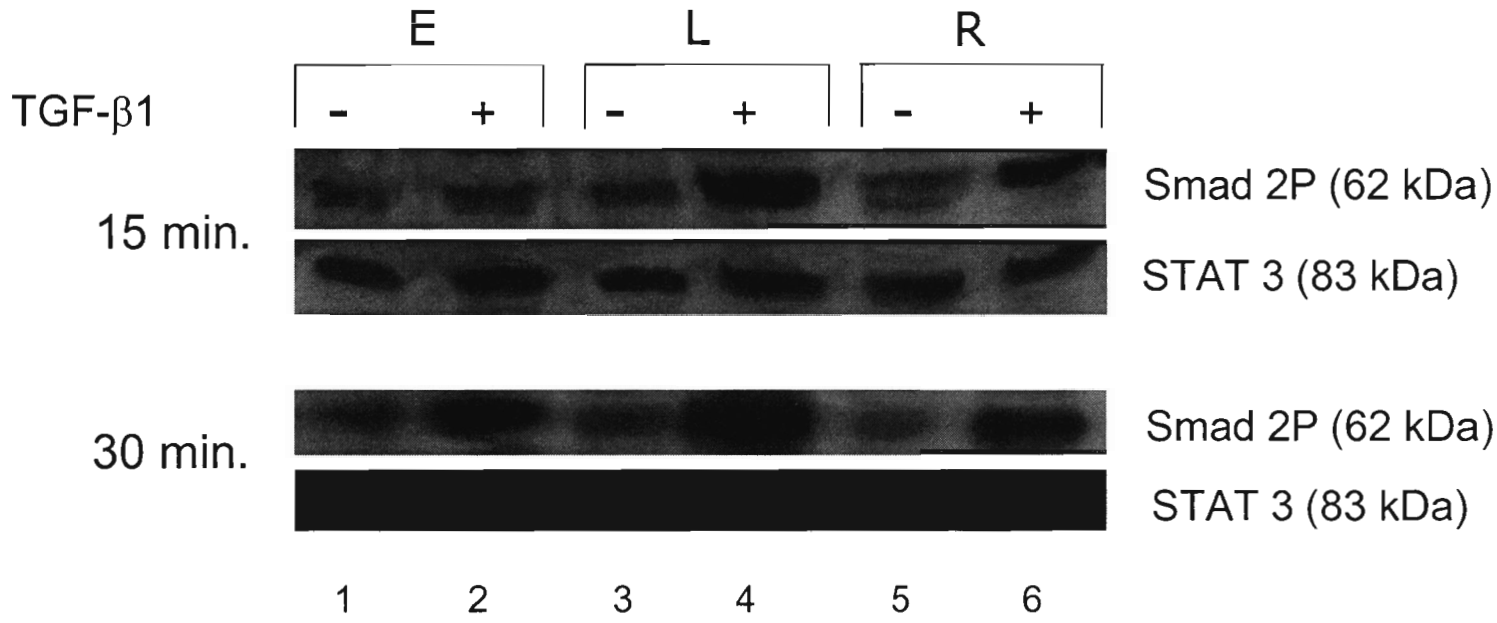

-
+
TGF-β1
C-28/I2
tsT/AC62
Primary
C-28/I2
tsT/AC62
Primary
Smad 2P
(62 kDa)
STAT 3
(83 kDa)
1
2
3
4
5
6

Fig. 5C


C-28/I2
tsT/AC62
Primary
E
L
R
E
L
R
E
L
R
Smad 2P
(62 kDa)
STAT 3
(83 kDa)
1
2
3
4
5
6
7
8
9

359

Fig. 5D


E
L
R
TGF-β1
-
+
-
+
-
+
15 min.
Smad 2P (62 kDa)
STAT 3 (83 kDa)
30 min.
Smad 2P (62 kDa)
STAT 3 (83 kDa)
1
2
3
4
5
6

360

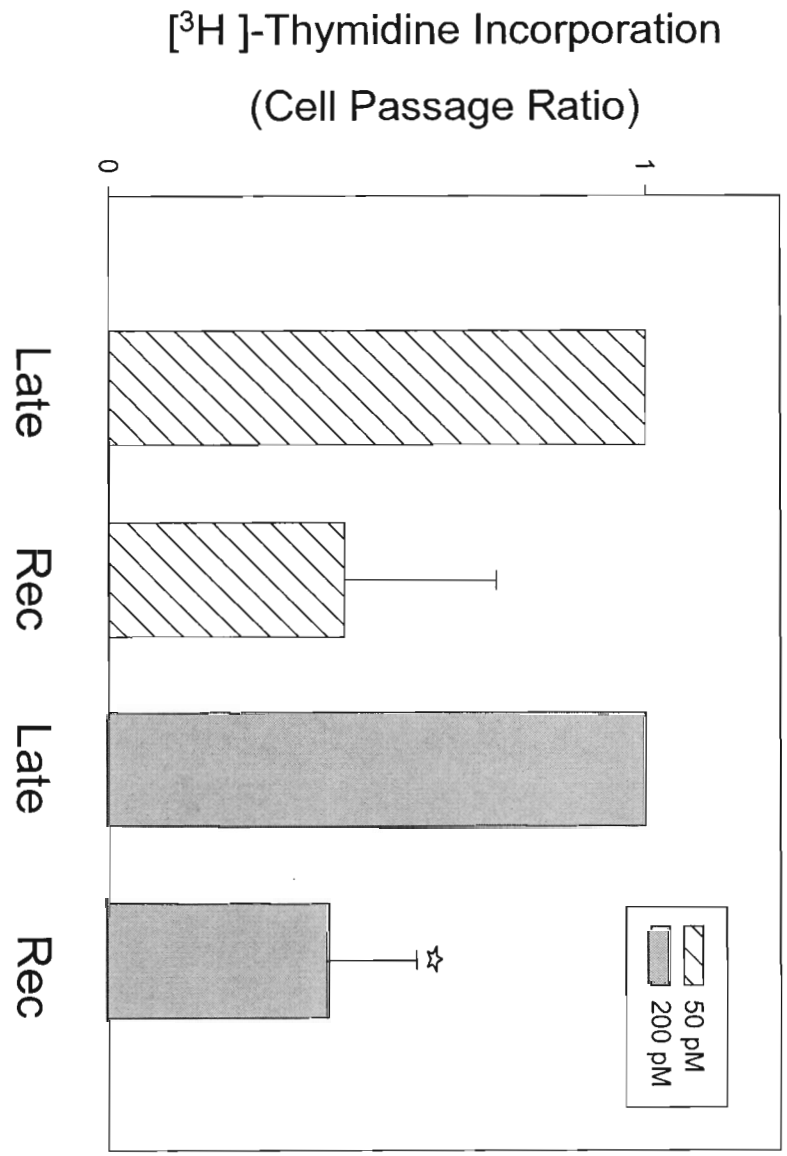
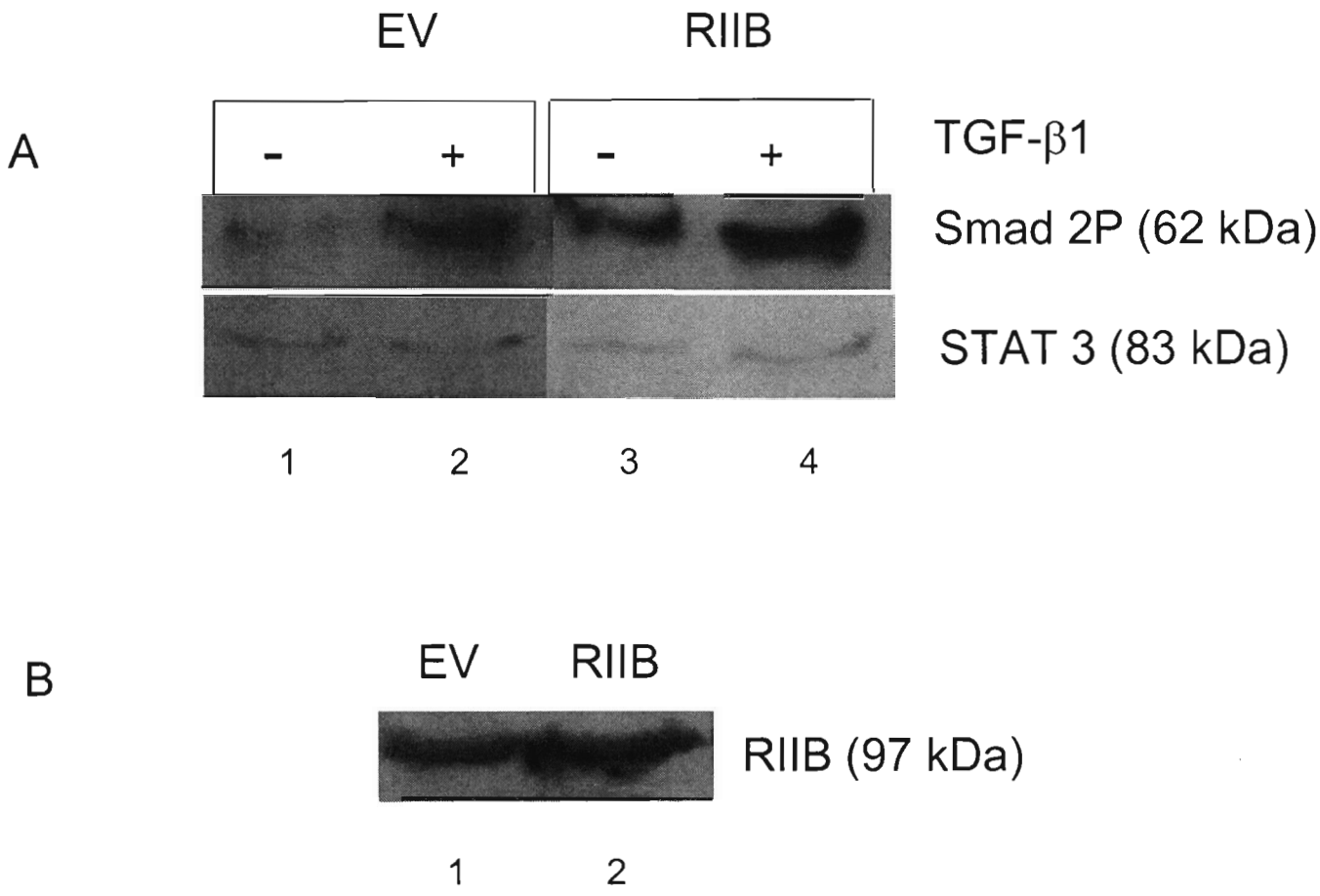

[3H ]-Thymidine Incorporation
(Cell Passage Ratio)
0
1
Late
Rec
Late
Rec
50 pM
200 pM

Fig. 5E

Fig. 6A


EV
RIIB
A
-
+
-
+
TGF-β1
Smad 2P (62 kDa)
STAT 3 (83 kDa)
1
2
3
4
B
EV
RIIB
RIIB (97 kDa)
1
2

362

Fig. 6B

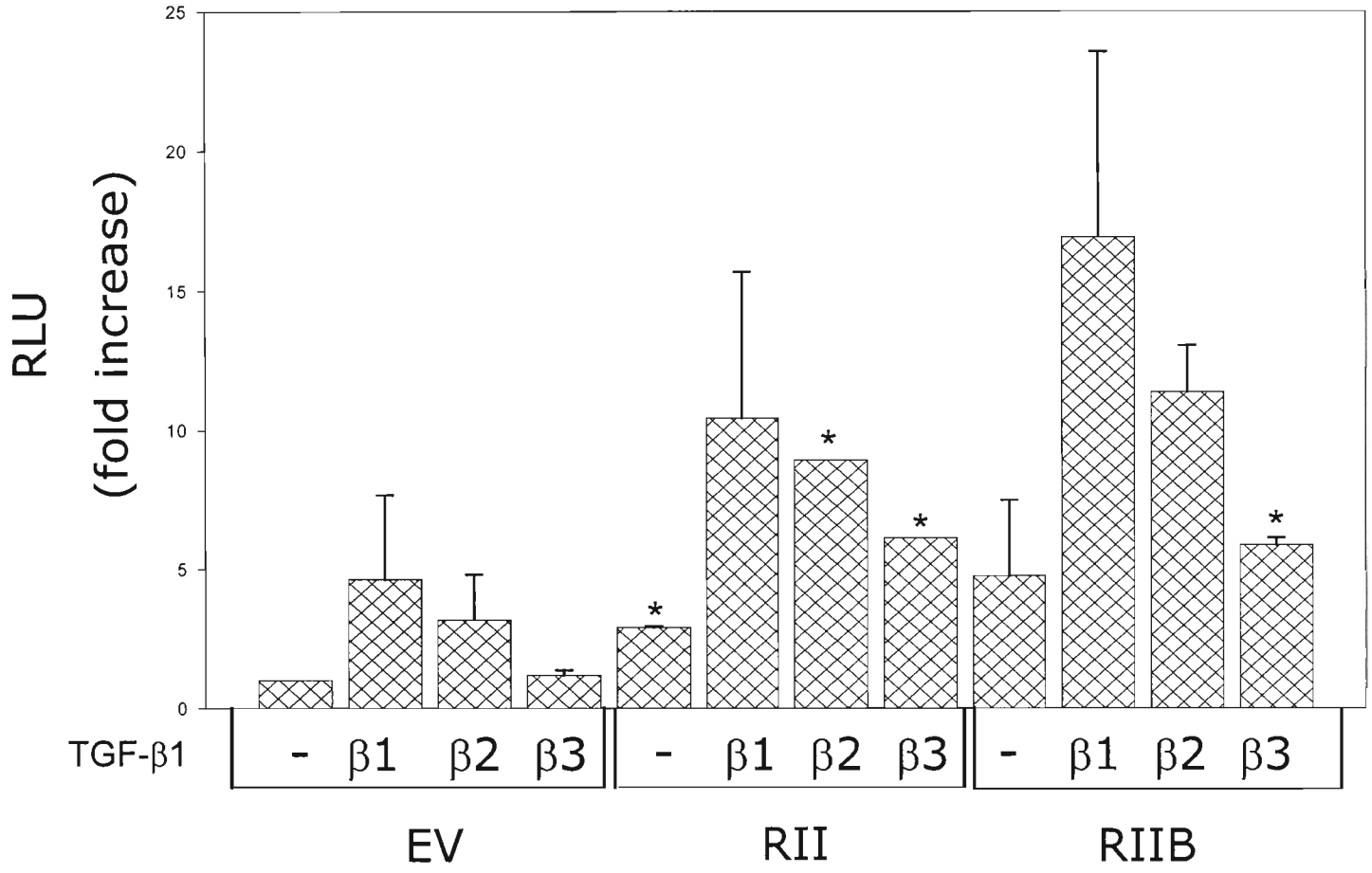

RLU
(fold increase)
25
20
15
10
5
0
TGF-β1
-
β1
β2
β3
-
β1
β2
β3
-
β1
β2
β3
EV
RII
RIIB
*
*
*
*
*

Fig. 6C

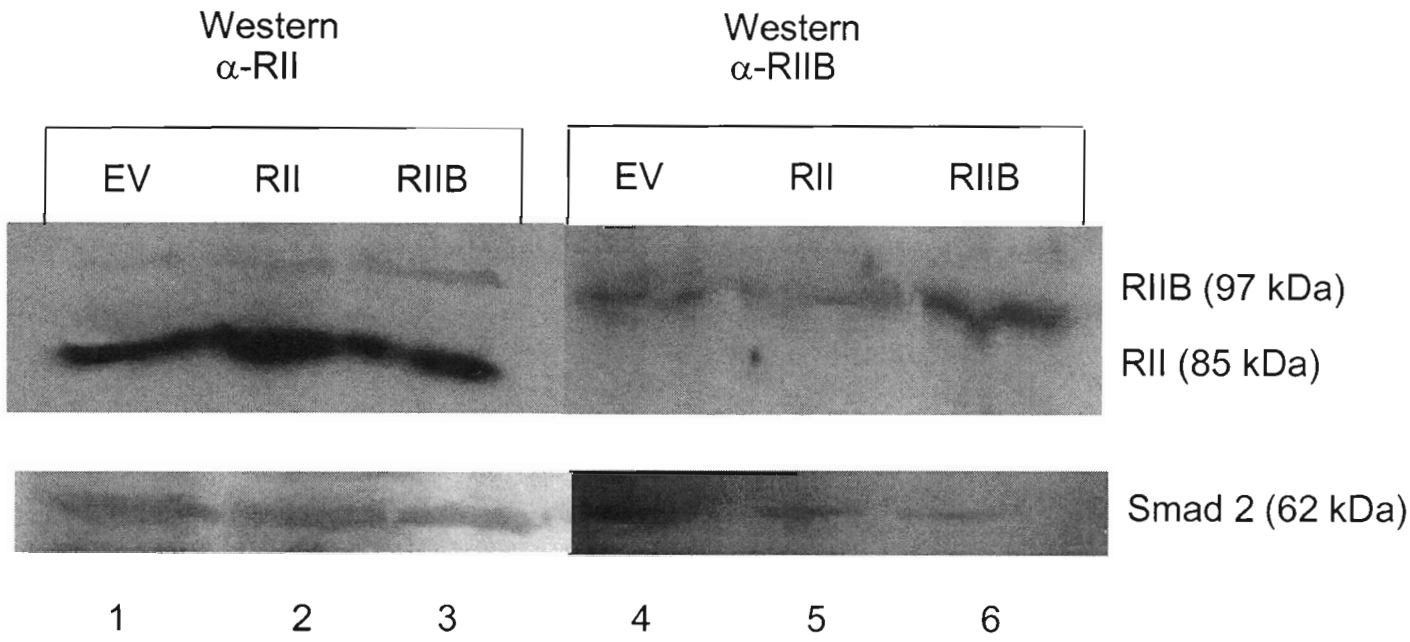
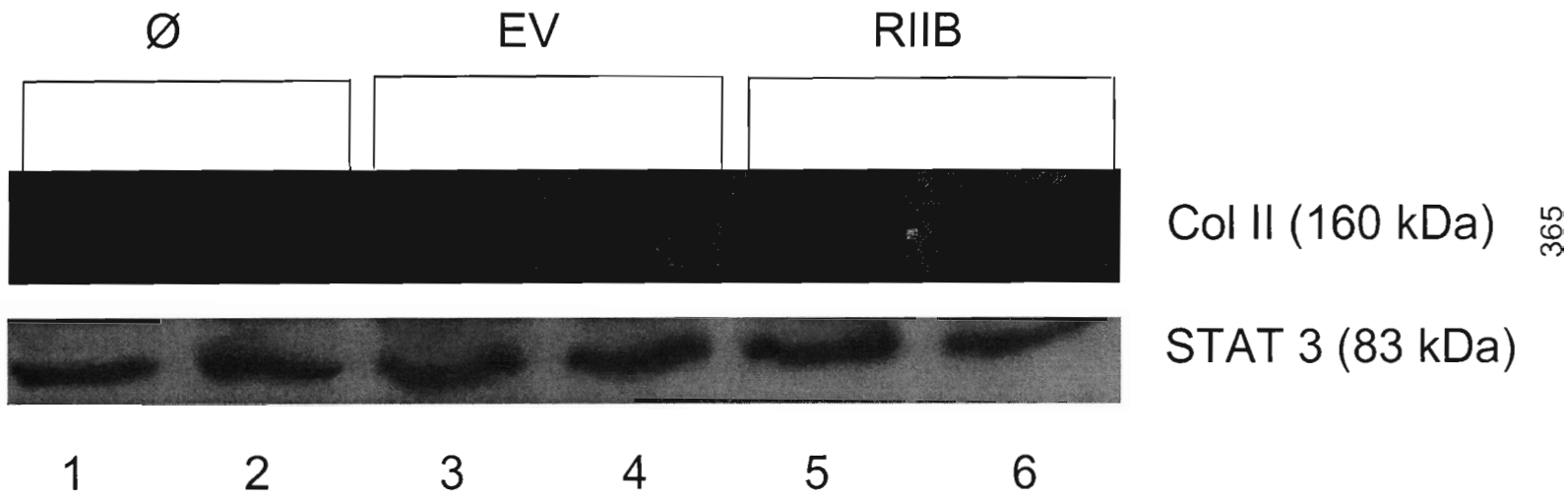

Western
α-RII
Western
α-RIIB
EV
RII
RIIB
EV
RII
RIIB
RIIB (97 kDa)
RII (85 kDa)
Smad 2 (62 kDa)
1
2
3
4
5
6

Fig. 6D




365

# CHAPTER 5

# BRIDGE

The concept of endoglin as a phenotypic marker of human chondrocytes and the critical link between endoglin expression, cell differentiation, and TGF-β responsiveness.

## CHAPTER 5

BRIDGING DOCUMENT

### Introduction and Rationale

Lack of innate healing by articular cartilage has been attributed to a decrease proliferation of mature chondrocytes, the avascular state of the tissue, and a dedifferentiation of chondrocytes into a fibroblast-like cell. This change in phenotype results in a cell with decreased responsiveness to anabolic cytokines (van den Berg 1999), increased responsiveness to catabolic cytokines (Martel-Pelletier et al 1992) and a shift in collagen production from type II to type I and III. This altered phenotype is a well established characteristic of OA chondrocytes. It has been postulated that a disturbed receptor expression profile accounts for this dedifferentiation in OA cells (van den Berg 1999). Alternately, TGF-β receptors may act as a marker of cell phenotype, reflecting the cells' responsiveness to TGF-β. The variable expression of RIIB on human chondrocytes of distinct phenotypes, its modulation of ECM, and association with TGF-β responsiveness suggests that RIIB is a phenotypic marker in these cells. Endoglin has been described as a phenotypic marker in choriocarcinoma cells (Letamendia et al 1998b, Arthur et al 2000) and up-regulation of its expression in synovial tissues of OA and RA patients (Szekanecz et al 1995) has been demonstrated. Whether endoglin represents a phenotypic marker in human chondrocytes was uncertain.

### Hypotheses

(1) that the expression of endoglin on human chondrocytes varies between undifferentiated and differentiated cells and thus endoglin acts as a marker of cell phenotype

(2) that chondrocytes of distinct phenotypes show varied responsiveness to TGF-β

(3) that levels of endoglin expression, cell phenotype, and TGF-β responsiveness correlate with ECM production

**Objectives**

(i) to investigate the phenotypic differences in endoglin expression between differentiated and dedifferentiated human chondrocytes

(ii) to identify whether the state of chondrocyte differentiation is related to TGF-β responsiveness

(iii) to determine if there is a link between endoglin expression, TGF-β responsiveness, and ECM in these cells

**Summary of Manuscript Findings**

In the fifth manuscript, the morphology of immortalized and primary human chondrocytes which depict a progressive dedifferentiation in monolayer culture is extensively characterized. The use of morpholino antisense oligos to block endoglin expression is shown to negate endoglin's inhibitory effect on TGF-β signaling in chondrocytes. Significantly, evidence is provided that endoglin expression modulates type II collagen levels. More importantly, it is demonstrated that endoglin expression correlates with the dedifferentiated phenotype of human chondrocytes, decreased levels of type II collagen, and a decreased TGF-β responsiveness.

**Additional Data**

Acknowledgements

No additional materials than those noted in the following manuscript were required to complete this additional investigation.

Experimental Procedures

**Western Blot Analysis**

To determine the regulation of the phosphorylated form of Smad 2 by TGF-β1, normal primary articular chondrocytes (late passage monolayer cultures from cartilage digestion and early passage monolayers from explants) were treated with 100 pM of TGF-β1 for 15 and 30 minutes and Western blotting was performed as previously described (Chapter 1, Bridging Document; Experimental Procedures).

Results

**Normal primary human chondrocytes of distinct phenotypes demonstrate variable TGF-β responsiveness**

To compare the TGF-β responsiveness of late monolayer, dedifferentiated primary chondrocytes with those obtained from early passage explant differentiated cultures, cells were treated with 100 pM of TGF-β1 for 15 and 30 minutes or were left untreated and the phosphorylated form of Smad 2 was determined using Western blot analysis. Fig. H illustrated that incubation of late passage dedifferentiated chondrocytes with TGF-β1 resulted in an increase in Smad 2 phosphorylation (Lane 2,4); however, a greater enhancement of Smad 2 phosphorylation was apparent for TGF-β1 treated early passage differentiated cells (Lane 6,8).

**Conclusions**

These final results offer a cohesive picture of TGF-β receptors, their heteromeric complex formations, and roles on human chondrocytes. In addition to the TGF-β signaling receptors, several other TGF-β receptors have been demonstrated on these cells. Betaglycan has been previously documented on chondrocytes (Horner et al 1998) and although confused with

RII, RIIB also has been noted on these cells (Glansbeek et al 1997). The presence of both RIIB and betaglycan were confirmed on primary and immortalized chondrocytes. For the first time, the expression of endoglin, Alk-1, and Sol RI on human chondrocytes is presented and multiple heteromeric complex formations between the signaling, accessory, and other novel TGF-β receptors in various combinations are presented. Furthermore, some of the structural determinants of these interactions have been elucidated. The relative expression of individual receptors within an oligo-heteromeric TGF-β receptor complex may determine the outcome of TGF-β signaling and provide diversity to the stringent downstream intracellular Smad-mediated TGF-β pathway.

By demonstrating that endoglin is able to bind TGF-β in the absence of RII, a discrepancy in the TGF-β signaling paradigm becomes apparent. That this ligand binding is not only enhanced by betaglycan but also requires its presence is novel and intriguing.

More importantly, these novel TGF-β receptors on human chondrocytes appear to play a distinct role in modulating TGF-β signaling. Overexpression of betaglycan, Alk-1, Sol RI, and RIIB enhanced signaling while overexpression of endoglin inhibited signaling and interestingly, endogenous RIIB levels corresponded with decreased TGF-β responsiveness. In addition to regulating TGF-β signaling, endoglin and RIIB were demonstrated to be markers of chondrocyte phenotype with increased expression of both apparent in OA cells. Endoglin expression was shown to correlate with the dedifferentiated phenotype of human chondrocytes, decreased levels of type II collagen and a decreased TGF-β responsiveness establishing a critical link among these factors.

Although clearly implicated in the pathophysiology of cartilage injury, little is known about TGF-β action in human chondrocytes. Even less has been elucidated regarding TGF-β

receptors on these cells. With this data, a complex interplay of TGF-β receptors on the surface of human chondrocytes, which play distinct roles in TGF-β signaling, has been established. These receptors may, in fact, confer signaling specificity, define the phenotype of these cells, infer or alter their TGF-β responsiveness, and ultimately affect their surrounding ECM. Although these studies are now beginning to define the roles of TGF-β receptors and their hetero-oligomeric complexes in human chondrocytes, they are but a part of the highly complex microenvironment of the cell. Further elucidation of their regulatory actions in the chondrocyte microenvironment must precede the successful application of TGF-β in cartilage repair and regeneration.

## CONTRIBUTION OF AUTHORS

# MANUSCRIPT FIVE

Endoglin Expression Links Chondrocyte Phenotype, TGF-β Responsiveness, and Type II Collagen Production: Relevance to Degenerative Joint Disease

**WL Parker:** experimental designs, procedures, and analysis, preparation of figures and text

**MB Goldring:** provision of the chondrocyte cell lines (C-28/I2 and tsT/AC62)

**A Philip:** assistance with experimental design and analysis and manuscript editorial assistance

# Endoglin Expression Links Chondrocyte Phenotype, TGF-β Responsiveness, and Type II Collagen Production: Relevance to Degenerative Joint Disease

Wendy L. Parker[1], Mary B. Goldring[2] and Anie Philip[1]*.

[1]Division of Plastic Surgery, Department of Surgery, McGill University, Montreal, Quebec, Canada

[2]Beth Israel Deaconness Medical Center, Harvard Institutes of Medicine, Boston, Massachusetts, USA

*Correspondence to: Anie Philip, Division of Plastic Surgery, Montreal General Hospital, 1650 Cedar Avenue, Room C9-177, Montreal, Quebec, Canada H3G 1A4.

Telephone: (514) 937 1934 ext 44533; Fax: (514) 934 8289

E-mail: anie.philip@mcgill.ca

Running Title: Endoglin's role cartilage degeneration

Acknowledgements

We thank Genzyme Inc. (Framingham, MA) for the gift of TGF-β1 and TGF-β2; Dr. Bert Vogelstein (Baltimore, ML) for the SBE 4 expression vector; Dr. S. Souchelnytskyi (Uppsala, Sweden) for the anti-phospho Smad 2 antibody; Dr. C. Bernabeu (Madrid, Spain) for the L-endoglin plasmid; and Dr. Eric Lenczner and Dr. Harvey C. Brown (Montreal, Quebec) for the human cartilage.

Summary

Degenerative joint disease constitutes a major medical problem in the aging population and the innate reparative ability of other connective tissues is not shared by articular cartilage. There is a flaw in the process of regeneration following cartilage injury which results in a dedifferentiation of type II collagen producing chondrocytes into fibroblastic-like type I and III collagen producing cells. TGF-β has emerged as a key player in the human chondrocyte proliferation and dedifferentiation with contributions to cartilage healing. Dysregulation of its signaling cascade has been shown to result in degenerative joint disease in animal models. Despite this, the available information on TGF-β receptors and action in human chondrocytes is limited. Endoglin an accessory TGF-β receptor binds TGF-β1 and -β3 with high affinity in the presence of RII but does not bind TGF-β2. This receptor has been shown to form complexes with RI and RII, as well as betaglycan and Alk-1, and to modulate TGF-β signaling. We have previously reported the presence of Eg on human chondrocytes and its complex formation with BG occurring in the presence of ligand and ligand independent manner. We have also shown that endoglin inhibits TGF-β signaling in chondrocytes.

In the present study, using morpholino antisense oligos of endoglin we show the suppression of endoglin expression on chondrocytes and ablation of its inhibitory effect on TGF-β signaling in these cells. More importantly, we establish a critical link between endoglin expression, the phenotypic state of the human chondrocyte, its TGF-β responsiveness, and type II collagen expression. Our results demonstrating endoglin as a potential marker of chondrocyte phenotype, and establishing the association between its expression, TGF-β signaling, and extracellular matrix (ECM) suggest that this accessory

receptor plays a crucial role in TGF-β signaling in chondrocytes. Regulation of endoglin expression and action may lead to novel therapeutic approaches towards enhancing cartilage regeneration and repair to combat degenerative joint disease.

**Key Words**: chondrocytes, osteoarthritis, TGF-β receptors, TGF-β signaling, endoglin, phenotype, cartilage, morpholinos

## INTRODUCTION

Joint damage and subsequent dysfunction from articular cartilage injury is an expanding health problem faced by our aging population. Osteoarthritis (OA[1]), a slowly progressive primarily monoarticular disorder is the most prevalent of all joint diseases and is now thought to approach an incidence of 60% in those people over the age of 60 years (1,2). It is characterized clinically by pain, deformity, and a limited range of motion all a result of underlying cartilage destruction and accompanying joint changes. Articular injury and hyaline cartilage destruction are also prominent features of rheumatoid arthritis (RA). Although the prevalence of this chronic systemic inflammatory condition is only 0.3-1.5% of the population, it affects large subsets of younger patients and being polyarticular in nature has a profound impact on joint function and activities of daily living (3,4).

Medical treatment of joint diseases has aimed at alleviating patient symptoms but more recently has targeted the underlying pathophysiology of arthritic changes (reviewed in 5). Surgical treatment is currently limited to invasive soft tissue rebalancing procedures or end stage joint replacement. Although the potential for restoring diseased or injured cartilage has generated much interest, a successful model of cartilage regeneration or repair has been elusive. This has been attributed to the lack of regenerative or proliferative ability of mature chondrocytes, their dedifferentiation into fibrocartilage (type I and type III collagen producing cells) (6) and the avascular state of the tissue (7).

---

[1] The abbreviations used are: OA, osteoarthritis; RA, rheumatoid arthritis; N, normal; ECM, extracellular matrix; TGF-β, transforming growth factor beta; PAI – 1, plasminogen activator inhibitor-1; RI, TGF-β type I receptor; RII, TGF-β type II receptor; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum; DMEM, Dulbecco's minimal essential medium; dPBS, Dulbecco's phosphate buffered saline; BSA, bovine serum albumin; β-gal, beta-galactosidase; GPI, glycosyl phosphatidylinositol; SBE, Smad binding element; Col II, type II collagen;

OA has been viewed as three stages. The first is that of injury. The second is of attempted repair which rather than regenerating a smooth viscoelastic gliding joint surface leads to stage 3 highlighted by catabolism and further injury (8). What triggers this inherent flaw in the process of regeneration, where there is a change from attempt to regenerate matrix to not only a lack of repair but also to disease progression, is unknown but many theories exist (8). One is an upregulation of cytokines which are destructive in nature rather than anabolic. These lead to increased inflammation and enhanced cell death. Another is a change in chondrocyte phenotype which demonstrates decreased responsiveness to anabolic factors in the microenvironment of the cell (9) and increased responsiveness to catabolic factors (10,11). It seems then that it would be of interest to not only be capable of recognizing chondrocyte phenotype but also to be able to manipulate it. Reliable markers of phenotype may provide clues as to problems within the cell as well as its microenvironment and thus avenues to alter this environment to potentiate cartilage regeneration or repair.

Transforming growth factor β (TGF-β) has emerged as a potential regulator of chondrocyte growth and differentiation (12,13). It is a member of a large family of multifunctional proteins intricately involved in growth, differentiation, and development (14) and was described initially as "cartilage inducing factor" (15). Three distinct isoforms of TGF-β (TGF-β 1, 2, and 3) which are encoded by distinct genes have been described in mammals (14). TGF-β is secreted in a latent form that requires activation before it can bind to its receptors. The TGF-β signal is transduced by a pair of transmembrane serine/threonine kinases, known as the types I (RI) and II (RII) receptors which are present on almost all cell types (16-19). RI does not bind TGF-β in the absence

of RII. The binding of TGF-β to RII, a constitutively active kinase, results in the recruitment, phosphorylation and concomitant activation of RI. Activated RI in turn transmits the signal via downstream mediators such as Smads, resulting in the regulation of target gene expression.

The diminished sensitivity to TGF-β seen by OA chondrocytes has been attributed to decreased levels of RII (20). Recently we described no difference in RII expression between normal and OA human primary articular cells but a significant increase in RIIB expression, a spliced functional variant of RII. Moreover high endogenous levels of RIIB correlated with decreased TGF-β responsiveness in phenotypically distinct chondrocytes (21). Endoglin an accessory TGF-β receptor, has been implicated as a phenotypic marker in choriocarcinoma cells (22,23) and shows enhanced expression in RA and OA synovium (24). Classic indicators of chondrocyte phenotype include cell morphology and ECM production. Cells have been shown to undergo a change in morphology from a rounded differentiated type II collagen producing phenotype to an elongated, spindle-shaped fibroblastic looking cell with multiple long processes. In this dedifferentiated state the cell's capacity for type II collagen production is lost and replaced by enhanced type I and III collagen synthesis. It is now well established that altering chondrocyte culture conditions to a three-dimensional (3-D) system maintains the hyaline cartilage phenotype and also results in redifferentiation of dedifferentiated cells toward the type II collagen producing phenotype (25-31). In addition, increasing levels of type II collagen in these 3-D culture systems have also been shown to maintain chondrocyte phenotype and may restore cell response to anabolic factors in the microenvironment (32). The established link between RIIB and chondrocyte phenotype, identification of endoglin as a cell marker

in disease states, and their association with cell responsiveness and ECM regulation suggest that cell surface proteins such as TGF-β receptors can be added to the list of accepted markers of chondrocyte differentiation.

With the change in chondrocyte phenotype, there is an accompanying decrease in cell proliferation, a decrease in cell responsiveness to local factors, and an abnormal matrix production which all may aid in maintaining this dedifferentiated phenotype and provide resistance to innate repair. The problem becomes circular in nature. Inability to control cell phenotype leads to an abnormal microenvironment which then contributes to this phenotype. Alternatively a change in this microenvironment through injury or altered levels of regulatory anabolic or catabolic factors may trigger the cell dedifferentiation to initiate this process.

TGF-β has been manipulated in the chondrocyte microenvironment to promote increased cell proliferation, maintenance of phenotype and ECM production to mediate cartilage repair (13,33-41). Despite this, local exogenous administration has been plagued with problems (42,43). An alternative is to modulate the action of endogenous TGF-β at the level of its receptors. There is increasing evidence that accessory receptors play an important role in regulating TGF-β action locally (44-46). Therefore if TGF-β receptor expression could be correlated to chondrocyte phenotype and TGF-β responsiveness then these receptors could potentially be regulated locally to restore dedifferentiated cells, enhance TGF-β response - cell proliferation and ECM synthesis, and promote cartilage repair.

In the present study, we begin characterizing the cell morphology of immortalized nonarticular and articular chondrocytes and primary diseased versus normal articular

chondrocytes and illustrate that morphological changes depict a progressive dedifferentiation in monolayer culture. We illustrate how cells derived from primary OA, RA, and normal primary articular explant cultures follow a predictable cell morphology related to phenotype. We block endoglin expression and demonstrate that this negates endoglin's inhibitory effect on TGF-β signaling in chondrocytes. Significantly, we have provided evidence that endoglin expression influences type II collagen production. More importantly, we show that endoglin expression correlates with the dedifferentiated phenotype of human chondrocytes, decreased levels of type II collagen and a decreased TGF-β responsiveness. This critical link between endoglin and the dedifferentiated chondrocyte, poorly responsive to TGF-β, provides insight into the inadequate inherent reparative ability of human chondrocytes and suggests that accessory TGF-β receptors may provide a novel avenue to locally modulate TGF-β action to facilitate cartilage regeneration.

## EXPERIMENTAL PROCEDURES

### Cell Culture

The immortalized human chondrocyte cell lines (C-28/I2 and tsT/AC62) have been described previously (47,48). The C-28/I2 cell line was developed using chondrocytes isolated from juvenile human costal cartilage by retroviral infection with SV-40 large T antigen. The tsT/AC62 cells were developed using chondrocytes isolated from adult human articular cartilage by immortalization with a retrovirus expressing a temperature-sensitive mutant of SV-40 large T antigen. This mutant is functional when the cells are cultured at 32°C but not at 37°C (47).

Human articular cartilage was obtained intraoperatively from adult total knee arthroplasty specimens (osteoarthritic cartilage, OA) or from adults with traumatic open joint injuries with no history of degenerative joint disease and normal appearing cartilage (normal cartilage, N). For experiments involving fresh cartilage specimens OA samples were obtained from at least 3 different individuals and for normal tissue from at least 2 different individuals. Therefore experiments comparing diseased versus normal cartilage were performed in triplicate. Specimens were either digested to obtain monolayer cultures or protein was directly extracted by solubilization (see below). Digestion of the specimens was performed according to standard techniques (47). Briefly, cartilage was washed with phosphate-buffered saline (PBS), incubated with 0.25% trypsin (10ml/g of tissue) (Invitrogen Life Technologies; Burlington, ON.) for 30 to 45 minutes, minced with a scalpel, and incubated with hyaluronidase (10ml/g of tissue) and collagenase (1mg/ml; 10ml/g of tissue) (both from Sigma Aldrich; Oakville, ON.) overnight. The suspension was pelleted, washed, and plated at $1x10^6$ cells/ml. All chondrocytes were grown in DMEM/Ham's F12 (1:1, v:v) containing 10% fetal bovine serum (FBS) and 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphoteracin (all from Invitrogen Life Technologies) and maintained at 37°C (C-28/I2, 293) or 32°C (tsT/AC62) in an atmosphere of 5% CO2/air.

All experiments were done at early passage (Passage 1-5; P1-5) unless stated otherwise. Late passage experiments were performed with cells at Passage 10 (P10) or above and recovered passage experiments were performed with chondrocytes immediately recovered from 3-dimensional (3-D) alginate bead cultures and allowed to settle onto tissue culture plates. The 3-D alginate culture system has been described previously (27) and is used to

redifferentiate chondrocytes to their type II collagen producing phenotype. Briefly, several 10cm plates of chondrocyte monolayers are trypsinized and washed with PBS. Pelleted cells are resuspended in a 1.2% alginate (Keltone LVCR, NF; ISP Alginates Inc., San Diego, CA.) solution at 4 x $10^6$ cells/ml and expressed through a 10ml syringe with a 25 gauge needle into a polypropylene tube containing 102 mM $CaCl_2$. The suspension was allowed to polymerize for 10 minutes then are washed with sterile saline. Beads are decanted into T75 flasks and maintained in regular medium as noted above. After 3 weeks in 3-D culture cells are recovered. Medium is aspirated and beads are washed with PBS. Alginate is depolymerised with 55 mM Na Citrate (Sigma Aldrich) at 37° for 10 min, cells are dislodged, pelleted, washed with PBS then plated as required for analysis.

Explant cultures were plated from whole cartilage specimens obtained intraoperatively as described above. Briefly, cartilage was washed repeatedly with sterile PBS and minced into 2x2mm slices with a scalpel. The bottom of 10cm tissue culture plates were scratched with a glass pipette to create a grid. Specimens were transferred to the plates and left exposed to air for a few minutes to promote adherence to the plate and then covered with medium. After 3-5 weeks, chondrocytes could be seen growing in monolayers surrounding the explants. Once confluent the cells were trypsinized and passaged as monolayers.

**Phase Contrast Microscopy and H&E Staining**

Monolayer cultures of immortalized and primary (OA and N) human chondrocytes were followed through sequential passages by phase contrast microscopy (Olympus B202; Carson Group Inc., Markham, ON) and photographed (DC-330; Dage-MTI Inc., Michigan City, IN) to document changes in cell morphology. Similarly, explant cultures

were observed and chondrocytes derived from these explants were followed in monolayer cultures. Cells suspended in 3-D alginate beads were also visualised and photographed in this fashion. Both the 3-D matrix as well as the cells within the matrix is identifiable. Some of these beads were also subjected to cryopreservation and H&E staining prior to being imaged as described previously (49) with modifications. Briefly, beads were rinsed with PBS and placed in 15ml tubes with 4% paraformaldehyde solution to cover (Sigma Aldrich) and left at 4°C for 24 hours. Following this, they were rinsed with PBS and placed in 20% sucrose solution (Sigma Aldrich) again at 4°C for 24 hrs. The solution was aspirated beads were covered with tissue embedding media (Histo-Prep; Fisher Scientific, Nepean, ON) and placed in liquid nitrogen. The beads were stored at -80°C until sectioning. Samples were cut in 5 μm sections on a microtome and stained in the standard H&E fashion and left at 50°C overnight to dry.

**Blocking Endoglin Expression**

The endoglin gene expression was blocked using Morpholino antisense oligos (sequence CGCGGTCCATGCTGTCCACGT; Gene Tools LLC; Philomath, OR) to bind and inactivate endoglin mRNA sequences. Morpholinos have the advantage of better specificity and stability compared to the more commonly used antisense oligos Phosphorothioates (50). The morpholino oligos were delivered to the chondrocytes as per manufacturer's instructions (Gene Tools Inc.;51) Briefly, chondrocytes were grown to 90% confluency and the EPEI delivery agent was mixed with either the control oligos (sequence CCTCTTACCTCAgTTACAATTTATA; Gene Tools LLC) or anti-endoglin oligos and left at room temperature for 20 minutes. This mixture was then applied to the cells in serum free medium for 3 hours at 37°C. The reagent was then replaced with

regular medium and cells were allowed to recover for 24 hours or 48 hours for the initial experiments. Remaining experiments were then conducted all at 48 hours after delivery of the oligos as this was found to be the optimal time to reduce endoglin expression.

**Affinity Labeling of Cells**

Affinity labeling was performed as described previously with modifications (52) Briefly, monolayers of cells were washed with ice-cold binding buffer [Dulbecco's PBS (dPBS) with $Ca^{++}$ and $Mg^{++}$, pH 7.4 containing 0.1% bovine serum albumin (BSA)] and were incubated with 100 pM of $^{125}$I-TGF-β1 in the absence or presence of varying concentrations of unlabeled TGF-β1, -β2, or -β3. The receptor ligand complexes were cross-linked with Bis-sulfocsuccinimidyl suberate (BS3; Pierce; Rockford, Il.). The reaction was stopped by the addition of glycine and the cell membrane extracts were prepared. The solubilized samples were separated by SDS-PAGE (3-11% gradient) under non-reducing or reducing (with β-Mercaptoethanol, Sigma Aldrich) conditions and analyzed by autoradiography. In some experiments chondrocytes were pretreated with the morpholinos antisense oligos for endoglin or control oligos and a 48 hour recovery time was permitted prior to affinity labeling.

**Immunoprecipitation of Endoglin**

The SN6h anti-human endoglin antibody used for immunoprecipitation was from Dako Inc (Carpinteria, CA.) Immunoprecipitation studies were performed as described previously (52,53) with modifications. Cells were affinity labeled with 100 pM $^{125}$I-TGF-β1, and the membrane extracts were incubated with 3μg/ml of the various antibodies and with respective IgG controls. Immune complexes were then incubated with protein G-Agarose (Roche Diagnostics; Laval, Que.) slurry and the beads were pelleted by

centrifugation. The immune complexes were analyzed by SDS-PAGE (3-11% gradient) under reducing conditions followed by autoradiography.

**Western Blot Analysis**

To determine the regulation of the phosphorylated form of Smad 2 by TGF-β1, chondrocytes were grown to confluency in 6 well plates and serum starved overnight. In some experiments, prior to Western blotting, cells underwent treatment with morpholino endoglin or control antisense oligos (Gene Tools) for 48 hours. Alternatively, endoglin was overexpressed as previously described by our lab (54). Cells were transiently transfected with 1μg of endoglin expression plasmid or empty vector (EV, pEXV) per well using Lipofectamine Reagent (Invitrogen Life Technologies) in serum-free medium according to the manufacturer's specifications. Cells were allowed to recover for 48 hours in regular medium and serum starved the following day for 4 hours. Cells were then washed with PBS and treated with 100 pM of TGF-β1 or -β2 as indicated or left untreated. Cell lysates were prepared and samples were standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad; Mississauga, Ont.). The extracts were then fractionated by SDS-PAGE (7.5% acrylamide) and transferred by electrophoresis to nitrocellulose membranes (Scheicher and Schuell; Keene, NH.). The membrane was blocked for 3 hours in a milk protein/Tween blocking solution (1M Tris, pH 7.5; 5M NaCl; Tween 20,Sigma Aldrich; Carnation Skim milk powder) and incubated with a rabbit polyclonal anti-phosphoSmad2 antibody (a gift from Dr. S. Souchelnytskyi, Uppsala, Sweden; 55,56) or rabbit polyclonal anti-endoglin antibody (SN6h; Dako Inc.) at 4°C overnight. The membrane was washed and incubated for 1 hour with goat anti-rabbit or rabbit anti-mouse HRP conjugated secondary antibody (Santa Cruz

Biotechnology Inc.; Santa Cruz, CA) at room temperature and detected using the ECL system (Amersham Pharmacia Biotech Inc.; Baie d'Urfe, Que.). The membrane was reprobed with anti-Smad 2 (non-phosphorylated form), anti-Smad2/3, or anti-STAT 3 antibody (all from Santa Cruz Biotechnology Inc.) to confirm equal protein loading.

**Luciferase Reporter Assays**

The p3TP-Lux (containing the luciferase gene under the control of a portion of the plasminogen activator inhibitor-1 promoter region; 57) and SBE 4 (containing the luciferase gene under the control of the Smad Binding Element; 58-61) both TGF-β-inducible promoter-reporter constructs were used to determine cellular responsiveness to TGF-β. OA and normal primary human articular chondrocytes were grown to 90% confluency in 6-well plates and transiently transfected with 1μg p3TP-Lux of or pSBE 4 and 1μg of pβ-galactosidase (β-gal) per well using Lipofectamine Reagent (Invitrogen Life Technologies) in serum-free medium according to the manufacturer's specifications. Cells were allowed to recover for 48 hours in regular medium, serum starved the following day for 4 hours, then treated with 100 pM of TGF-β1 overnight. The cells were lysed and assayed for luciferase activity using the EG&G Berthold Microplate Luminometer (Berthold Technologies USA; Oak Ridge, TN). Light emission by the TGF-β1 treated cells was expressed as a percentage of the emission by the control untreated cells and adjusted for transfection efficiency obtained using the β-gal assay.

**Type II Collagen Detection**

Western blot analysis was used to detect levels of type II collagen (Col II). The anti-type II collagen antibody was purchased from Developmental Studies Hybridoma Band (DSHB Iowa University, Iowa City, Iowa) and used in the manner previously described

(25) with modifications. Briefly, chondrocytes were grown to confluency in T25 flasks. Cells were then washed with PBS, lysates were prepared and samples were standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad). The extracts were then fractionated by SDS-PAGE (7.5% acrylamide) and transferred by electrophoresis to nitrocellulose membranes (Scheicher and Schuell). The membrane was blocked overnight at 4°C in a milk protein/Tween blocking solution (1M Tris, pH 7.5; 5M NaCl; Tween 20, Sigma Aldrich; Carnation Skim milk powder) and incubated with the rabbit serum anti-type II collagen antibody at room temperature for 2 hours. The membrane was washed and incubated for 2 hours with rabbit anti-mouse HRP conjugated secondary antibody (Santa Cruz Biotechnology Inc.) at room temperature and detected using the ECL system (Amersham Pharmacia Biotech Inc.). The membrane was reprobed with anti-Smad 2/3 or anti-STAT 3 antibody (both from Sigma Aldrich) to confirm equal protein loading. In experiments where chondrocytes were to be treated with morpholino endoglin antisense oligos (Gene Tools) or transiently transfected with endoglin expression plasmid (Lipofectamine; Invitrogen Life Technologies) prior to collagen detection the cells were grown in 6 well plates.

The protocol for extracting protein directly from 3-D alginate chondrocyte beads was followed as previously described (25). Approximately 5 beads were obtained per sample required and dissolved in the standard fashion with NaCitrate as noted above with a final volume of 400µl of buffer per sample. 100µl of .25M acetic acid and 100µl of pepsin (Sigma Aldrich) (1mg/ml in 50mM acetic acid; Sigma Aldrich) were added and the sample left at 4°C for 24 hours. The following day samples were incubated at 37°C with the addition of 100µl of 10X TSB (1M Tris, 2M NaCl, 50mM $CaCl_2$, pH 8) and 100µl of

pancreatic elastase (Sigma Aldrich) (1mg/ml in 1x TSB) for 30 minutes. They were then centrifuged for 10 minutes at 9000 x g and standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad; Mississauga, Ont.). The extracts were then fractionated by SDS-PAGE (7.5% acrylamide), transferred by electrophoresis to nitrocellulose membranes (Scheicher and Schuell; Keene, NH.), and blotted for type II collagen as described above.

**Endoglin Detection on Fresh Human Cartilage**

To compare endoglin expression by OA and N human cartilage samples, protein extracts were prepared as described by Gruppuso et al (62) and as in Current Protocols (63). Briefly, for Sucrose Preparation, tissue was rinsed with dPBS, weighed, minced, and homogenized using a hand homogenizer in 4 volumes of homogenization buffer (Sucrose .25M, EDTA 1mM, HEPES 50mM pH 7.5) containing a mixture of protease inhibitors (1mM phenylmethanesulfonyl fluoride, 20 $\mu g \cdot ml^{-1}$ aprotinin, 20 $\mu g \cdot ml^{-1}$ leupeptin, 20 $\mu g \cdot ml^{-1}$ soybean trypsin inhibitor, and 25 mM benzamidine; all from Sigma Aldrich). The protein fraction was centrifuged at 100,000 x g for 2 hours at 20°C. The supernatant was removed and the pellet resuspended in HEPES buffer. For SDS Preparation, tissue was homogenized in a similar fashion but 2ml of Sodium Dodecyl Sulfate (SDS; Bioshop, Burlington, ON)/solubilization buffer per 100mg of tissue and boiled for 5 minutes prior to centrifugation. Protein content of samples was determined using the Bradford Protein Assay Kit (Bio-Rad), prepared in 1X sample buffer, and boiled for 5 minutes. The samples were then analyzed by 7.5% SDS-PAGE under nonreducing or reducing (with β-Mercaptoethanol, Sigma Aldrich) conditions and immunoblotted as noted above. Equal

protein loading was verified by reprobing the membranes with anti-STAT 3 antibody (Sigma Aldrich).

## RESULTS

### Growth characteristics and morphology of human chondrocyte lines in monolayer and alginate beads.

The tsT/AC62 and C-28/I2 cell lines provide a reproducible model that mimics human articular and costal phenotypes respectively (64-67). We have previously confirmed the chondrocyte phenotype of the immortalized cell lines and the human primary articular chondrocytes in culture, as shown by the production of aggrecan and type II collagen by these cells using RTPCR (68).

Here we document the morphology of these cells through serial passages in monolayer cultures and in 3-D alginate beads. Phase contrast microscopy of early passage C-28/I2 cells in monolayer cultures (Fig. 1A) can be seen at low (10X; a) and high (20x; b) optical magnification and late passage monolayers at low (10X; c) and high (20X; d) optical magnification. C-28/I2 cell morphology remains unchanged through multiple passages in monolayer culture. These cells display the typical rounded to polygonal shape on 2-D tissue culture plates. They are large vacuolated cells which proliferate rapidly in culture and their growth is not impaired by contact inhibition. Representative chondrocytes have been outlined to demonstrate this morphology in 2-D. Costal chondrocytes (C-28/I2) were trypsinized and suspended in 3-D alginate beads and maintained in culture for 3 weeks. The ability of cells to maintain this rounded morphology is evident (Fig. 1B) at low (10X; a,b) and high (20X; c-e) optical magnification.

Consistent with the accepted concept of dedifferentiation of articular chondrocytes in monolayer cultures (27-29,31,69-71), tsT/AC62 cells when observed through serial

passages were noted to change morphology (Fig. 2A). Cells in early passage are seen under high (20X; a,b) optical magnification to have a polygonal shape; however, they progressively acquire spindle shape morphology with long processes similar to a fibroblast-like cell as demonstrated by low (10X; c) and high (20X; d) optical magnification. Dedifferentiated articular cells (tsT/AC62) of fibroblastic-like morphology were trypsinized and suspended in 3-D alginate beads and maintained in culture for 3 weeks (Fig. 2B). These cells are evenly distributed throughout the alginate matrix and maintain the appearance of a rounded differentiated cell as seen under low (10X; a,b) and high (20X; c-e) optical magnification. The capacity to redifferentiate chondrocytes to their original type II collagen producing phenotype has been described (25,27-29,31). Cryopreservation and H&E staining of some of these alginate beads was performed as previously described (49) to further demonstrate the nature of the articular cells within the 3-D matrix (Fig. 2C). Eosinophilic stained cytoplasm of chondrocytes is evident under low (10X; a) optical magnification. To further delineate the haematoxylin stained nuclear material within the cells (arrows) two cells have been enlarged (b,c). Despite artefact created within the 3-D matrix caused by cryopreservation and sectioning of the beads, the polygonal shape of the redifferentiated articular chondrocytes is appreciated.

**Growth characteristics and morphology of primary human chondrocytes in monolayer and alginate beads.**

Human primary articular chondrocytes were obtained intraoperatively from adult total knee arthroplasty specimens (osteoarthritic cartilage, OA) or from adults with traumatic open joint injuries with no history of degenerative joint disease and normal appearing cartilage (normal cartilage, N). Specimens were either enzymatically digested to obtain monolayer cultures or cultured as explants. These cells were also observed using phase

contrast microscopy and morphology was documented through serial monolayer passages with photography. Cell derived from normal healthy adult articular cartilage specimens are seen at first passage in Fig. 3A. As noted for early passage tsT/AC62 cells, these cells demonstrate a rounded to polygonal shape on 2-D tissue culture plates throughout early passages (Passage 1-5; Fig. 3A, a,b) at high (20X) optical magnification. Representative cells have been outlined to illustrate morphology in 2-D. However, these cells did undergo morphologic changes consistent with dedifferentiation such as development of a spindle shape and long processes with late passages (Passage 10 and greater, data not shown).

Phase contrast microscopy of human articular cartilage explants after 3 weeks in culture are shown in Fig. 3B at low (10X; a,b) and high (20X; c,d) optical magnification. In (a) arrows indicate the regularity of the explant surface. In (c) the arrow denotes a cluster of chondrocytes and in (d) the arrow denotes the pallisading nature of the cells toward the cartilage surface. Monolayers of chondrocytes originating from the explants are noted after 3 weeks in culture as seen at high (20X; e,f) optical magnification. The large roughened surface seen in the upper right corner of the image in (e) is an area of the tissue which has been scraped to allow for explant adhesion. Representative cells have been outlined (e,f) to illustrate morphology in 2-D.

Cell derived from osteoarthritic (OA) adult articular cartilage specimens are seen in Fig. 4A at low (10X; a,b) optical magnification. As noted for late passage tsT/AC62 cells (Fig. 2A; c,d), these cells demonstrate a spindle shape on 2-D tissue culture plates at first passage (Fig. 4A; a,b) which persists throughout late passage monolayers (data not shown). Arrows denote chondrons surrounded by cells of dedifferentiated spindle-shaped fibroblastic morphology.

Phase contrast microscopy of human OA cartilage explants after 3 weeks in culture are depicted in Fig. 4B at low (10X; a,b) and high (20X; e,f) optical magnification. In (b) arrows indicate the irregularity of the fibrillated explant surface. Monolayers of chondrocytes originating from the explants are evident after 3 weeks in culture as seen by low (10X; c,e) and high (20X; d,f) optical magnification. In (e,f) arrows illustrate the dedifferentiated phenotype of the cells as they emerge from the explants prior to monolayer passaging. A representative cell has been outlined (d) to illustrate the fibroblastic spindle-like morphology in 2-D.

RA represents another disease state of articular cartilage with poor innate reparative capacity and insufficient type II collagen production (9). Phase contrast microscopy of human RA cartilage explants after 3 weeks in culture are seen at low (10X; a,b) and high (20X; c,d) optical magnification (Fig. 4C). In (b) arrows indicate the regularity of the explant surface. However, in (c,d) arrows illustrate the fibroblastic morphology consistent with a dedifferentiated phenotype of the cells as they emerge from the explants. Monolayers of chondrocytes originating from the explants are evident after 3 weeks in culture at low (10X; e) and high (20X; f) optical magnification. In (e) the arrow illustrates the dedifferentiated phenotype of the cells as seen with monolayers derived from OA cartilage (Fig. 4A and B).

**Blocking endoglin expression with morpholino antisense oligos.**

Traditional approaches to blocking protein expression by cells have involved Phosphorothioates (50) but morpholinos have recently become available and have the advantage of better specificity and stability (51). The results shown in Fig. 5 demonstrate its effectiveness in blocking endoglin expression in human chondrocytes. OA and normal (N) human primary cells were treated with either the anti-endoglin antisense oligos (Eg)

or control oligos (C) and allowed to recover for 24 or 48 hours (Fig. 5A). We have previously reported the presence of endoglin in human chondrocytes and extensively characterized it's interaction with other TGF-β receptors by affinity labeling, immunoprecipitation (IP), Western blot, and combined IP-Western (21,54,68). By 24 and 48 hours following antisense treatment endoglin is not apparent in normal primary cells (Lane 2,5 and 6). Similarly in OA cells endoglin levels are reduced at 24 hours (Lane 8) and nearly absent at 48 hours (Lane 10). Possible explanations for the residual small band remaining at the 180 kDa position of the endoglin dimer include that OA primary human chondrocytes are seen to express very high levels of endoglin (see below) and although morpholino antisense is preventing new protein production residual endoglin may be expressed for longer periods of time on the cell surface. In addition, morpholino antisense is generally not 100% effective; there is some protein production usually of 10-20% (51). To confirm that the morpholinos was in fact blocking new endoglin expression, Western blot analysis was performed for endoglin (Fig. 5B). It is evident that the level of endoglin detectable in OA cells treated with antisense oligos (Lane 3,4; Eg) is reduced as compared to control oligos (Lane 1,2; C).

**Blocking endoglin expression alters TGF-β responsiveness.**

To determine the effect blocking endoglin expression would have on TGF-β response, OA chondrocytes were pretreated with control (Lane 2,5; C) or endoglin antisense (Lane 3,6; Eg) oligos or not treated (Lane 1,4; -). Cells were allowed to recover for 48 hours then serum starved and treated with 100 pM of TGF-β1 (+) and Smad 2 phosphorylation (Smad 2P) was determined by Western blotting with anti-Smad 2P antibody (Fig. 6A). It has been suggested that endoglin inhibits TGF-β signaling (22) in other cells and we have

previously reported this in immortalized chondrocyte cell lines where betaglycan enhances signaling (54). In cells not treated with morpholino antisense (-) or treated with control oligos (C) TGF-β caused a slight up-regulation of Smad 2P (upper panel; Lane 4,5 respectively) as compared to untreated cells (upper panel; Lane 1,2 respectively). However, cells treated with antisense oligos (Eg) when stimulated with TGF-β1 resulted in a marked upregulation of Smad 2P (upper panel; Lane 6) as compared to no TGF-β1 stimulation (upper panel; Lane 3). Equal protein loading is demonstrated by STAT 3 Western blotting (lower panel).

The effect of blocking endoglin expression by morpholinos in C-28/I2 cells as compared to endoglin overexpression on Smad 2P was also investigated (Fig. 6B). In panel A cells treated with control oligos (Lane 1,3; C) or endoglin antisense (Lane 2,4; Eg) were allowed to recover for 48 hours, then were serum starved for 4 hours and were left untreated (-) or treated (+) with 100 pM of TGF-β for 15 minutes. A marked up-regulation of Smad 2P with TGF-β1 stimulation is seen for endoglin antisense treated chondrocytes (Lane 4) as compared to control oligo treated cells (Lane 3). In panel B cells were transfected with plasmid encoding endoglin (Lane 2,4; Eg) or empty vector (Lane 1,3; EV) for 48 hours. Cells were then serum starved overnight and left untreated (-) or treated (+) with 100 pM of TGF-β1. Endoglin overexpression is noted to inhibit Smad 2P both at endogenous (Lane 2) and exogenous (Lane 4) TGF-β 1 levels as compared to empty vector overexpression (Lane1,3). Equal protein loading is demonstrated by STAT 3 Western blotting (panel A and B; lower panels).

**Endoglin expression influences type II collagen production.**

Accessory TGF-β receptors are emerging as potential modulators of TGF-β action (22,45,53,72-75) and in turn may regulate downstream target genes and products. We were interested in investigating how endoglin expression would affect type II collagen levels. In Fig. 6C C-28/I2 cells were transfected with plasmid encoding endoglin (panel A, Lane 3,4; Eg) or empty vector (panel A, Lane1,2; EV) or were treated with endoglin antisense (panel B, Lane 3; Eg), control oligos (panel B, Lane 2; C) or were left untreated (panel B, Lane 1; -). Cells were allowed to recover for 48 hours then solubilized extracts were analyzed for type II collagen by Western blot. Endoglin overexpression (panel A, Lane 3,4) appeared to result in a downregulation of type II collagen levels whereas blocking endoglin expression (panel B, Lane 3) caused an increase in type II collagen levels. Equal protein loading is demonstrated by STAT 3 Western blotting (panel A and B, lower panels).

**Endoglin expression varies with phenotype of immortalized human chondrocytes.**

To analyze endoglin expression on human chondrocytes, cells were affinity labeled with $^{125}$I-TGF-β1 and the labeled receptors were analyzed by SDS-PAGE. The receptor profile representative of human articular chondrocytes is illustrated in Fig.7A. We have previously demonstrated seven major binding complexes of relative molecular weights of 47, 65, 85, 97, 145,180 and 200-300 kDa under nonreducing conditions in human chondrocytes (68). In addition to Sol RI (47 kDa), RI/Alk-1 (65 kDa), RII (85 kDa), RIIB (97 kDa), the endoglin dimer (180 kDa) and betaglycan (200-300 kDa), a complex of molecular weight of 145 kDa was detected. This 145 kDa complex likely represents heterodimers of RI/RII, RI/endoglin, or RII/endoglin consistent with our previous observations (21,68). Also evident is a molecular weight band at 240 kDa representative

of a higher order complex involving endoglin (68). Results presented here suggest levels of endoglin expression vary between cells of distinct phenotypes. Late and early passage tsT/AC62 cells affinity labeled with 100 pM of $^{125}$I-TGF-β1 under nonreducing conditions (Fig. 7A) reveal that the level of endoglin expression appears higher in late passage (Lane 1; Late) than in recovered passage (Lane 2; Rec) cells.

This differential endoglin expression is also evident by immunoprecipitation (Fig. 7B). Early and late passage tsT/AC62 cells were affinity labeled with $^{125}$I-TGF-β1 and immunoprecipitated using anti-endoglin antibody (Lane 2,4; α-Eg) or were not immunoprecipitated (Lane 1,3; NIP). SDS-PAGE under reducing conditions of the nonimmunoprecipitated membrane lysate (Lane 1,3; NIP) demonstrated the presence of TGF-β binding proteins consistent in mobility and size with RI, RII, RIIB, the endoglin monomer, and betaglycan. The anti-endoglin antibody (α-Eg) immunoprecipitated endoglin in both early (Lane 4) and late passage (Lane 2) chondrocytes confirming its expression on these cells yet, more importantly, demonstrated that it is found at slightly higher levels on late passage chondrocytes. Of note, anti-endoglin antibody co-immunoprecipitated the TGF-β signaling receptors, RIIB, and betaglycan as previously described by our lab (68,76).

Early, late, and recovered passage tsT/AC62 chondrocytes were also analyzed for levels of endoglin with Western blot (Fig. 7C; upper panel). Increased endoglin expression appears to be observed in late passage cells (Lane 2, L) whereas both early (Lane 1, E) and recovered (Lane 3, R) cells expressed lower levels. The membrane was reprobed with anti-STAT 3 antibody to demonstrate equal protein loading (lower panel). These blots are representative of the experiments performed in triplicates.

**Cell phenotype and endoglin expression correlate with type II collagen levels.**

As mentioned chondrocytes are thought to maintain or regain their inherent phenotype of a type II collagen producing cell when cultured in 3-D systems (25-31). To confirm that tsT/AC62 cells in 3-D alginate beads (Fig. 2B) were in fact retaining this phenotype we assessed their type II collagen production by Western blot. Protein extracts were obtained as previously described (25). Fig. 7D (panel A) shows detectable levels of type II collagen from ECM of redifferentiated tsT/AC62 cells cultured in 3-D alginate beads for 3 weeks (Lane 2,3). Standard type II bovine collagen was used as an internal control (Lane 1).

Similarly we would anticipate that increased levels of type II collagen would be detected from solubilized extracts of early and recovered tsT/AC62 cells which express a more differentiated morphology than late passage cells (Fig. 2A). Western blot for type II collagen (Fig. 7D, panel B) supports this claim. Early (Lane 1, E), late (Lane 2, L) and recovered (Lane 3, R) passage chondrocytes were cultured in monolayer and solubilized extracts were analyzed for type II collagen levels. Late passage cells (Lane 2) seem to exhibit reduced type II collagen levels than early or recovered chondrocytes.

Moreover, OA cells were noted to display a morphology similar to late passage dedifferentiated tsT/AC62 cells (Fig. 4A and B) suggesting a distinct phenotype from that of normal human primary chondrocytes which appear to maintain their morphology at early passages and with explant cultures (Fig. 3A and B). Results of Western blot in Fig. 7D (panel C) suggest that a dedifferentiated morphology of OA chondrocytes also corresponds to decreased levels of type II collagen. Monolayers of OA and normal articular chondrocytes were obtained from explant cultures and solubilized extracts were

analyzed for levels of type II collagen. It appeared that higher levels of type II collagen were detectable from normal (Lane 2; N) than from OA cell cultures (Lane 1; OA). Interestingly solubilized extracts of tsT/AC62 early and recovered passage cells (Fig. 7D, panel B) and protein extracted from beads (Fig. 7D, panel A) apparently had the highest detectable levels of type II collagen which correlates with diminished endoglin expression (Fig. 7A, B, and C) on these cells.

**Endoglin expression on OA and normal primary human chondrocytes corresponds to phenotype and TGF-β responsiveness.**

Thus far evidence is provided that levels of endoglin expression correlate with phenotype of immortalized chondrocytes and levels of type II collagen. Levels of type II collagen in OA primary cells appeared lower that that of normal cells (Fig. 7D, panel C) which also appeared to correspond to a dedifferentiated cell morphology (Fig. 3A and B and Fig. 4A and B). To determine if these findings could also be related to levels of endoglin found in diseased OA versus normal human articular chondrocytes Western blot (Fig. 8A) to detect endoglin on cartilage protein extracts was performed. Sucrose (panel A) and SDS (panel B) Preparations of primary OA and normal cartilage were obtained as described in Experimental Procedures and Western blotting was performed to detect endoglin expression. Endoglin expression appeared higher in protein extracts (Lane 1, panel A and B) for OA cartilage. Equal protein loading is demonstrated by STAT 3 Western blotting (panel A and B; lower panels).

To illustrate the sensitivity of the OA versus normal primary chondrocytes to TGF-β, cellular signaling was determined by both a PAI-driven (57) and SBE 4-driven luciferase reporter assays (58-61). The chondrocytes were transiently transfected with p3TP-Lux or

pSBE 4 and allowed to recover overnight, then serum starved for 4 hours and were treated with 100 pM of TGF-β1 overnight or were left untreated. As illustrated in Fig. 8B, luciferase activity of OA cells treated with 100 pM of TGF-β1 (+) was only mildly stimulated as compared to untreated cells (-) whereas luciferase activity of normal treated cells (+) was stimulated to a significantly higher level (3TP Lux p=0.03; SBE-4 p=0.05). The results were normalized by co-transfection of the β-gal plasmid.

To demonstrate further the TGF-β responsiveness of phenotypically distinct chondrocytes we examined TGF-β induced Smad 2P. Cells were treated with 100 pM of TGF-β1 or -β2 for 15 minutes or were left untreated and the phosphorylated form of Smad 2 was determined using Western blot analysis. In Fig. 8C (panel A) stimulation of C-28/I2 cells with TGF-β1 resulted in an up-regulation Smad 2P (Lane 3) as compared to untreated cells (Lane 1). Up-regulation of Smad 2P was also noted for C-28/I2 cells stimulated with TGF-β2 (Lane 5) but to a lesser extent than that seen with -β1 treatment. OA chondrocytes treated with TGF-β1 (Lane 4) show a slight up-regulation of Smad 2P as compared to untreated cells (Lane 2). However, treatment of OA chondrocytes with TGF-β2 did not result in an increase in Smad 2P levels (Lane 6). The membrane was reprobed with anti-STAT 3 antibody to demonstrate equal protein loading (lower panel). Responsiveness to TGF-β1 was also compared for OA and normal primary articular chondrocytes using Smad 2P as a marker of responsiveness (Fig. 8C, panel B). Up-regulation of Smad 2P is evident for normal chondrocytes treated with TGF-β1 (Lane 4) as compared to untreated cells (Lane 2). Again, stimulation of OA cells with TGF-β1 (Lane 3) results in a mild increase in Smad 2P levels as compared to untreated cells (Lane 1). This data is representative of duplicate experiments. C-28/I2 cells were shown to have

lower levels of endoglin expression by affinity labeling and Western blot (data not shown) as compared to articular cells (tsT/AC62). OA chondrocytes were demonstrated to have high levels of endoglin expression (Fig. 8A) as compared to normal chondrocytes. This suggests that endoglin expression is increased on dedifferentiated cells, which corresponds to a significant decrease in TGF-β responsiveness seen for OA chondrocytes (Fig. 8B and C).

**DISCUSSION**

Injury to a joint, whether traumatic, associated with autoimmune phenomena or of unknown etiology, results in disruption of the regular viscoelastic hyaline cartilage and ultimately joint dysfunction manifested by pain and decreased range of motion. Connective tissue such as ligament, synovium and bone have the capacity for innate repair following injury, however, articular cartilage is not afforded this regenerative ability. This has been attributed to the lack of regenerative or proliferative ability of mature chondrocytes, their dedifferentiation into fibrocartilage (type I and type III collagen producing cells) (6) and the avascular state of the tissue (7). This dedifferentiation into fibrocartilage cells and shift in collagen production away from type II highlights the importance of cell phenotype in governing tissue repair. In turn, the influence of a 3-D culture system on regulating chondrocyte phenotype is well established (25-31).

Not only do structural aspects of the cells' microenvironment modulate their state of differentiation but growth factors and cytokines have also been implicated in this control. TGF-β has emerged as a potential regulator of chondrocyte growth and differentiation (12,13). A positive relationship between cellular maturity and proliferative response to

TGF-β has been demonstrated in growth plate chondrocytes (12). Furthermore, chondrocytes derived from the different layers of articular cartilage are differentially stimulated by TGF-β (13). TGF-β administration has been linked to increased ECM production, enhanced cell proliferation, and cartilage healing (33-41,77) whereas dysregulation of the TGF-β signaling cascade has been shown to result in degenerative joint disease in two murine models (78,79). Therefore it is imperative to understand how TGF-β relates to cell phenotype and conversely how cell phenotype correlates with TGF-β responsiveness and to elucidate whether TGF-β receptors are markers of human chondrocyte phenotype. Determining how phenotype, TGF-β receptors and responsiveness all correspond to ECM turnover is critical to understanding cartilage regeneration and repair.

In the present study we characterized the cell morphology of human chondrocytes and demonstrated a progressive dedifferentiation of articular cells in monolayer culture in agreement with the findings of others. We illustrated that cells derived from primary OA and RA articular explant cultures exhibit dedifferentiated cell morphology unlike those derived from normal articular explants. We have blocked endoglin expression with the use of morpholino antisense oligo nucleotide technology to demonstrate that this negates the inhibitory effect of endoglin on TGF-β signaling in chondrocytes. We provided evidence that endoglin overexpression appears to lower type II collagen levels and that blocking endoglin expression appears to raise this level. More importantly, we have shown that endoglin expression correlates with the dedifferentiated phenotype of human chondrocytes, decreased levels of type II collagen and a decreased TGF-β responsiveness establishing a critical link amongst these factors.

The tsT/AC62 and C-28/I2 cell lines provide a reproducible model that mimics human articular and costal phenotypes respectively (64-67). Our results presented in Fig. 1 and 2 substantiate the reported behaviour of articular and nonarticular chondrocytes in culture. The polygonal morphology of C-28/I2 cells remained unchanged through multiple passages in monolayer culture (Fig. 1A) and when suspended in 3-D alginate beads the chondrocytes maintain this rounded morphology beyond 3 weeks in culture (Fig. 1B).

The phenomenon of articular chondrocyte dedifferentiation when proliferated in monolayer culture is well accepted (25-31,80-82). Specifically, ECM of differentiated articular chondrocytes consists primarily of type II collagen and cartilage-specific proteoglycan. During serial monolayer culture this phenotype is replaced by that of a type I and III collagen and low level of proteoglycan producing cell. Various 3-D culture systems including alginate bead suspensions are seen to redifferentiate these cells toward a spherical morphology and distinct type II collagen containing ECM which can be separated into a cell associated or interterritorial matrix. The loss of normal 3-D architecture of hyaline cartilage likely contributes to this loss of phenotype by altering cell-cell and cell-matrix interactions. In addition it may alter the delivery of other local regulatory factors to the chondrocyte indirectly affecting their responsiveness to them. tsT/AC62 cells observed through serial passages were noted to change morphology. At early passage cells can be seen to have a polygonal shape, however, they progressively acquire spindle shape morphology with long processes similar to a fibroblast-like cell (Fig. 2A). The capacity to redifferentiate chondrocytes to their original type II collagen producing phenotype has been described (25-31) and an actual cell associated ECM can be appreciated as distinct from more distant generalized ECM within these systems (27,34,38). We show that articular cells of fibroblastic-like morphology suspended in 3-D

alginate beads acquire the appearance of a rounded differentiated cell which is maintained beyond 3 weeks in culture (Fig. 2B) and thus is not an artefact of trypsinization from monolayer.

This morphologic change represents one aspect of the articular chondrocytes' phenotypic expression. Cells also exhibit an increased expression of proteolytic enzymes and suppression of their inhibitors, increased expression of pro-inflammatory cytokines (IL-1, IL-6, and TNF-$\alpha$), increased cell death through apoptosis, and increased levels of nitric oxide (83). Sox9 a critical enhancer of transcription of type II collagen is upregulated in mice OA chondrocytes likely as attempt to regenerate injured ECM (84), however, type III and X collagen are also upregulated (85) suggesting a misdirected attempt at repair.

The morphologic change toward a fibroblastic-like cell is a well recognized phenomenon of accompanying dedifferentiation of articular chondrocytes in monolayer cultures. However, results presented in Figs. 3 and 4 demonstrate that this dedifferentiation is not simply a manifestation of cells grown in monolayer culture but is also likely evident *in vivo*. Cells derived from digestion of normal healthy adult articular cartilage specimens (Fig. 3A; a,b) demonstrated a rounded to polygonal shape on 2-D tissue culture plates throughout early passages and dedifferentiated into fibroblastic-like cells after serial monolayer passages (data not shown). Similarly, cells derived from normal cartilage explants at early passages (Fig. 3B; e,f) appeared to have a differentiated morphology. Interestingly, cells derived from OA articular cartilage specimens (Fig. 4A; a,b) at first passage already exhibit a spindle shaped dedifferentiated morphology. Moreover, chondrocytes budding from OA explants display a spindle shape with long process consistent with a dedifferentiated phenotype (Fig. 4B; c-f). This appearance of OA

articular cells thus is not simply a manifestation of progressive monolayer culture and suggests that these cells probably exhibit this deranged phenotype *in vivo*. RA represents another disease state of articular cartilage with poor innate reparative capacity and insufficient type II collagen production to overcome injury (reviewed in 9). Cells immediately derived from RA explants (Fig. 4C; c,e,f) displayed a similar dedifferentiated morphology as those cells derived from OA explants which suggests that these cells also exhibit a deranged phenotype *in vivo* and not simply as a result of monolayer culturing.

Endoglin, a homodimeric transmembrane protein of ~180 kDa, composed of disulfide-linked protein subunits of ~95 kDa, has limited species-specificity and shows 70% homology to betaglycan and was thought to have a limited tissue distribution (23,54,86-88). Two different isoforms, L and S-endoglin, have been described with their encoding gene on chromosome 9q34 (89). Hereditary hemorrhagic telangiectasia (HHT) is attributable to mutations in the endoglin gene with each mutation providing a slightly modified disease phenotype (90). Heterozygotes, both human and animal models, display the classic phenotype of epistaxis, telangiectasia, and visceral vascular malformations (91). Null alleles are embryonically lethal secondary to abnormal yolk sac vasculogenesis and abnormal cardiac development (92). The specific function of endoglin responsible for HHT is likely related to alterations in TGF-β action (22,93).

Endoglin is able to form a complex with RII (94) and to facilitate ligand binding to the types I and II receptors (22). However, over-expression of endoglin decreases TGF-β responses in monocytes and myoblasts (22,72,87). The short cytoplasmic tail of endoglin was previously thought to be devoid of catalytic activity (95,96) but recently has been

shown to be intricately involved in phosphorylation of and by the TGF-β signaling receptors (75). Despite this, its role in TGF-β signaling is poorly understood. When overexpressed in myoblasts, endoglin inhibits, while betaglycan enhances TGF-β responses (22,72). Endoglin has also been reported to antagonize TGF-β action in human microvascular endothelial cells (88). The current signaling paradigm is that endoglin cannot bind ligand in the absence of the respective type II ligand binding receptor (22,97). Recently we identified the presence of Eg on human chondrocytes and its complex formation with BG occurring in the presence of ligand and ligand independent manner (68) and in the absence of betaglycan's cytoplasmic domain or carbohydrate side chains (54). We have also shown that TGF-β1 binding by endoglin is enhanced by betaglycan but not the signaling receptors. Also, we have demonstrated that endoglin binds TGF-β1 independently of RII but requires the presence of betaglycan. Overexpression of endoglin was seen to inhibit while betaglycan enhanced TGF-β1, β2, and β3 responses in human chondrocytes (54).

Here we show the use of morpholino antisense oligo nucleotide technology to block endoglin expression and that this negates the inhibitory effect of endoglin on TGF-β signaling in chondrocytes. Fig. 5A and B demonstrate, by affinity labeling and Western blot of endoglin, the ability of morpholino antisense oligos to block endoglin expression in OA and normal primary human articular chondrocytes and chondrocyte cell lines (data not shown). The effect of blocking endoglin expression by antisense on TGF-β responsiveness was determined (Fig. 6A and B). TGF-β1 treatment of chondrocytes overexpressing endoglin was demonstrated to inhibit Smad 2P whereas blocking of endoglin expression in cells caused an up-regulation of Smad 2P with TGF-β1 treatment.

This confirms that endoglin overexpression inhibits TGF-β1 signaling in chondrocytes and blocking endoglin expression negates this inhibition.

Fig. 6C demonstrated that endoglin expression influences type II collagen levels in human chondrocytes. Endoglin overexpression resulted in a decreased levels of type II collagen and blocking its expression negated this effect. TGF-β has been extensively implicated in the regulation of ECM (98-103, reviewed in 104). In addition, endoglin itself has been implicated in the regulation of ECM; its overexpression in human mesangial cells has been shown to conversely decrease type I and IV collagen production (105). Recently, we have shown that RIIB overexpression in chondrocytes leads to an increased expression of type II collagen (21).

TGF-β receptor expression in chondrocytes likely contributes to the TGF-β responsiveness of the cell and also its regulation of ECM. Decreased levels of RII in OA chondrocytes has been implicated in their diminished sensitivity to TGF-β (20). We were unable to detect a difference in RII expression between normal and OA human primary articular cells but noted a significant increase in RIIB (a spliced functional variant of RII) expression in OA chondrocytes as compared to normal cells (76). Moreover, we found that the endogenous levels of RIIB correlated with decreased TGF-β responsiveness in phenotypically distinct chondrocytes (21). Here we show that endoglin expression modulates TGF-β responses in human chondrocytes and regulates type II collagen levels.

Endoglin has been implicated as a phenotypic marker in choriocarcinoma cells (22) and shows enhanced expression in RA and OA synovium (24). To determine whether endoglin also acts as a phenotypic marker in human chondrocytes we analyzed the expression of endoglin in cells of distinct phenotype. Fig. 7A, B, and C demonstrated that

endoglin levels appeared higher in late passage dedifferentiated cells than in early passage cells or those immediately recovered from alginate beads suggesting that it is differentially expressed by cells of distinct phenotypes. We show that, interestingly, this endoglin expression not only correlates with the dedifferentiated phenotype but also with type II collagen levels. Levels of type II collagen seemed to be higher in cultures of differentiated chondrocytes was compared to cells of dedifferentiated phenotype (Fig. 7D). This finding was consistent for chondrocyte lines as well as primary cells. OA cells are considered to represent a dedifferentiated phenotype (Fig. 4A, B) (reviewed in 8,106). As anticipated we found these cells to express large amounts of endoglin (Fig. 8A) and corresponding decreased type II collagen levels (Fig. 7D) as compared to normal primary articular cells.

Thus, we determined that endoglin expression corresponds to chondrocyte phenotype and type II collagen levels. As mentioned, the diminished TGF-β sensitivity of OA chondrocytes has been attributed to decreased levels of RII (20). In OA human explants where upper layers of chondrocytes were shown to have an altered phenotype they in fact appeared to be more TGF-β sensitive (107). Our new data of increased endoglin expression on dedifferentiated cells, taken together with its negative regulation of TGF-β signaling, suggests that OA cells should show a decreased TGF-β responsiveness. Luciferase reporter assays (Fig. 8B, statistically significant) and Western blot for Smad 2P (Fig. 8C) confirmed that phenotypically deranged OA chondrocytes exhibit markedly reduced TGF-β responsiveness as compared to normal primary cells.

Mice expressing a dominant negative form of RII (78) have joints histologically resembling human osteoarthritis and similarly homozygote Smad 3 knockout mice

develop degenerative joint disease (79). Both of these models suggest that a dysregulation of the TGF-β signaling pathway results in articular cartilage degeneration and joint changes. A third model of TGF-β dysregulation in mice, however, does not result in any recognizable joint changes. Mice which are homozygote knockouts for endoglin die in utero from abnormal yolk sac development and disorganized vasculogenesis. Heterozygotes survive and display a phenotype remarkably similar to human patients with Hereditary Hemorrhagic Telangiectasia (HHT I). The abnormality in their TGF-β signaling cascade in this case is a downregulation of endoglin expression. We have shown that endoglin inhibits TGF-β signaling in chondrocytes and is related to a decrease in type II collagen levels. Therefore, reduction in endoglin expression may provide joint protection through up-regulation of TGF-β signaling and higher levels of type II collagen. HHT mice do not show evidence of degenerative joint disease (91,92,108-110) and a recent review of 78 human patients with HHT which did not reveal any trend towards joint disease in these patients regardless of advancing age (personal communication from Dr. S. Shapshay; Boston, MA ; 111,112). This supports the theory of diminished endoglin expression protecting against degenerative joint changes since the development of degenerative joint disease is a prominent feature of several mice lineages at advanced age as well as having high incidence in the aging human population.

In summary we have characterized the cell morphology of immortalized nonarticular and articular chondrocytes and primary diseased versus normal articular chondrocytes all of which, apart from costal chondrocytes, display a progressive dedifferentiation in serial monolayer cultures. We demonstrated that cells derived immediately from normal articular cartilage display morphologic characteristics of differentiated cells whereas cells

derived immediately from OA or RA cartilage present morphologic characteristics consistent with a dedifferentiated phenotype. This suggests that the deranged phenotype of diseased chondrocytes is an *in vivo* phenomena and not a manifestation of monolayer cultures. In addition, we showed the ability to block endoglin expression through the use of morpholino antisense oligo nucleotide technology and that this negated the inhibitory effect of endoglin on TGF-β signaling in chondrocytes. Significantly, we provided evidence that endoglin overexpression inhibits type II collagen levels and blocking of its expression results in an upregulation of type II collagen. More importantly, we have demonstrated that endoglin expression correlates with the dedifferentiated phenotype of human chondrocytes, decreased levels of type II collagen, and a decreased TGF-β responsiveness. We have established a novel association between endoglin expression, dedifferentiated chondrocytes and the inherent decrease in TGF-β responsiveness of OA chondrocytes. This identification of endoglin as a potential marker of chondrocyte phenotype and its critical link with ECM, specifically type II collagen, may provide an avenue to locally modulate TGF-β action towards cartilage regeneration and repair.

**FIGURE LEGENDS**

**Fig. 1. Growth characteristics and morphology of human costal chondrocytes in monolayer and alginate beads. A:** Phase contrast microscopy of C-28/I2 cells in monolayer cultures. Early passage monolayers. Magnification 10X (a) and 20X (b) optical zoom. Late passage monolayers. Magnification 10X (c) and 20X (d) optical zoom. Representative cells have been outlined to illustrate morphology in 2-D. **B:** Phase contrast microscopy of C-28/I2 cells suspended in 3-D alginate beads and maintained for 3 weeks in culture. Magnification 10X (a,b) and 20X (c-e) optical zoom.

**Fig. 2. Growth characteristics and morphology of human articular chondrocytes in monolayer and alginate beads. A:** Phase contrast microscopy of tsT/AC62 cells in monolayer cultures. Early passage monolayers. Magnification 10X (a) and 20X (b) optical zoom. Late passage monolayers. Magnification 10X (c) and 20X (d) optical zoom. **B:** Phase contrast microscopy of tsT/AC62 suspended in 3-D alginate beads and maintained for 3 weeks in culture. Magnification 10X (a,b) and 20X (c-e) optical zoom. **C:** Histology of tsT/AC62 cells in 3-D alginate beads. Magnification 10X (a) optical zoom. Inserts of cells have been enlarged (b,c). Note that the cryopreservation has caused some distortion of the cells and surrounding architecture.

**Fig. 3. Growth characteristics and morphology of primary human articular explants and chondrocytes in monolayer. A:** Phase contrast microscopy of primary human articular chondrocytes obtained through enzymatic digestion of cartilage as described in Experimental Procedures in monolayer cultures. First passage monolayers. Magnification 20X (a,b) optical zoom. Representative cells have been outlined to illustrate morphology in 2-D. **B:** Phase contrast microscopy of human articular cartilage explants after 3 weeks in culture. Magnification 10X (a,b) and 20X (c,d) optical zoom. In (a) arrows indicate the regularity of the explant surface. In (c) the arrow denotes a cluster of chondrocytes and in (d) the arrow denotes the pallisading nature of the cells toward the cartilage surface. Monolayers of chondrocytes originating from the explants are noted after 3 weeks in culture. Magnification 20X (e,f) optical zoom. The large roughened surface seen in the upper right corner of the image in (e) is an area of the tissue culture plate which has been scraped to allow for explant adhesion. Representative cells have been outlined (e,f) to illustrate morphology in 2-D.

**Fig. 4. Growth characteristics and morphology of primary human OA and RA explants and chondrocytes in monolayer. A:** Phase contrast microscopy of primary human OA chondrocytes obtained through enzymatic digestion of cartilage as described in Experimental Procedures in monolayer cultures. First passage monolayers. Magnification 10X (a,b) optical zoom. Arrows denote chondrons surrounded by cells of dedifferentiated spindle-shaped fibroblastic morphology. **B:** Phase contrast microscopy of human OA cartilage explants after 3 weeks in culture. Magnification 10X (a,b) optical zoom. In (b) arrows indicate the irregularity of the explant surface. Monolayers of chondrocytes originating from the explants are noted after 3 weeks in culture. Magnification 10X (c,e) and 20X (d,f) optical zoom. In (e,f) arrows illustrate the dedifferentiated phenotype of the cells as they emerge from the explants. A representative cell has been outlined (d) to illustrate morphology in 2-D. **C:** Phase contrast microscopy of human RA cartilage explants after 3 weeks in culture. Magnification 10X (a,b) and 20X (c,d) optical zoom. In (b) arrows indicate the regularity of the explant surface. In (c,d) arrows illustrate the dedifferentiated phenotype of the cells as they emerge from the explants. Monolayers of chondrocytes originating from the explants are noted after 3 weeks in culture. Magnification 10X (e) and 20X (f) optical zoom. In (e) the arrow illustrates the dedifferentiated phenotype of the cells as is seen with monolayers derived from OA cartilage (Fig. 4A and B).

**Fig. 5. Blocking endoglin expression with morpholino antisense oligos.** Affinity labeling and Western blot demonstrate inhibition of endoglin expression by antisense. **A:** Normal and OA primary human chondrocytes were grown in monolayers and then were treated with morpholino endoglin antisense (Eg; Lane 2,5,6,8,10) or control oligos (C; Lane 1,3,4,7,9). At 24 (Lane 1,2,7,8) or 48 (Lane 3-6,9,10) hours cells were affinity

labeled with 100 pM of $^{125}$I-TGF-β1 and solubilized extracts analyzed by SDS-PAGE (3-11% gradient gels) under nonreducing conditions followed by autoradiography. **B:** OA monolayer cultures were treated with morpholino endoglin antisense (Lane 3,4; Eg) or control oligos (Lane 1,2; C). At 48 hours solubilized cell extracts were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with anti-endoglin antibody (upper panel) and the ECL system was used for chemiluminescence detection. The membrane was reprobed with anti-STAT 3 antibody to verify equal protein loading.

**Fig. 6. The effect of endoglin overexpression or antisense on TGF-β response and type II collagen in human chondrocytes.** Western blot of anti-phosphoSmad 2 (**A/B**) and type II collagen (**C**). OA (Fig. 6A) or C-28/I2 (Fig. 6B) cells were grown in monolayers and then were treated with morpholino endoglin antisense (Fig. 6A, Lane 3,6; Fig. 6B, panel A-Lane 2,4; Fig. 6C, panel B-Lane 3; Eg), control oligos (Fig. 6A, Lane 2,5; Fig. 6B, panel A-Lane 1,3; Fig. 6C, panel B-Lane 2; C) or were left untreated (Fig. 6A, Lane 1,4; Fig. 6C, panel B-Lane 1; -). Alternatively, cells were transiently transfected with pEXV (Fig. 6B, panel B-Lane 1,3; Fig. 6C, panel A-Lane 1,2; EV) or pEg (Fig. 6B, panel B-Lane 2,4; Fig. 6C, panel A-Lane 3,4; Eg). At 48 hours solubilized cell extracts pretreated with 100 pM of TGF-ß1 for 15 minutes (Fig. 6A, Lane 4-6; Fig. 6B, panel A and B, Lane 3,4; +) or left untreated (Fig. 6A, Lane 1-3; Fig. 6B, panel A and B, Lane 1,2; -) were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against the phosphorylated form of Smad 2 (**A/B**, upper panels) or anti-type II collagen antibody (**C**, upper panels). The ECL system was used for chemiluminescence

detection. Immunoblotting using an antibody directed against the STAT 3 was performed to illustrate equal protein loading (lower panels).

**Fig. 7. Endoglin expression correlates with phenotype of human chondrocytes. A/B:** Recovered and early passage differentiated human articular chondrocytes express lower levels of endoglin than late passage cells. tsT/AC62 cells grown in early or late passage monolayers or recovered from beads (allowed to settle onto tissue culture plates for 24 hours prior to treatment) were affinity labeled with 100 pM of $^{125}$I-TGF-β1. Solubilized cell extracts immunoprecipitated with anti-endoglin antibody (Fig. 7B, Lane 2,4; α-Eg) or not immunoprecipitated (Fig. 7A, Lane 1,2; Fig. 7B, Lane 1,3; NIP) were analyzed by SDS-PAGE (3-11% gradient gels) under nonreducing (**A**) or reducing (**B**) conditions followed by autoradiography. **C:** Endoglin expression as detected by Western blot is higher in late passage articular cells as compared to early and recovered passages. Early (Lane 1, E), late (Lane 2, L) and recovered (Lane 3, R) passages of tsT/AC62 cells were grown to confluency in T25 flasks and solubilized cell were analyzed by SDS-PAGE (7.5% acrylamide) under nonreducing conditions and transferred onto nitrocellulose membrane. Western blotting was performed using antibody directed against endoglin (upper panel) and the ECL system was used for chemiluminescence detection. The membrane was reprobed with antibody directed against STAT 3 (lower panel) to verify equal protein loading. **D:** Cell phenotype and endoglin expression correlate with type II collagen levels. tsT/AC62 cells were resuspended in alginate beads and maintained in culture for three weeks. Protein was extracted directly from 3-D alginate beads (described in Experimental Procedures; panel A, Lane 2,3). Early (Lane 1, E), late (Lane 2, L), and recovered (Lane 3, R) passages of tsT/AC62 cells (panel B) or OA (Lane 1, OA) and

normal (Lane 2, N) primary articular chondrocytes (panel C) were grown to confluency in T25 flasks. Western blotting for type II collagen was performed by described in **C** with anti-type II collagen antibody. In panel A bovine collagen (Lane 1) was used as a standard control. Membranes were reprobed with antibody directed against Smad 2/3 (panel B) or STAT 3 (panel C) to verify equal protein loading.

**Fig. 8. Endoglin expression in OA and normal human articular cartilage corresponds to phenotype and TGF-β responsiveness. A:** Western blot shows variable endoglin expression with distinct chondrocyte phenotypes. Sucrose (panel A) and SDS (panel B) Preparations of protein extracts of human articular OA (Lane 1, OA) and normal (Lane 2, N) cartilage were prepared as described in Experimental Procedures and were analyzed by SDS-PAGE (7.5% acrylamide) under reducing (panel A) and nonreducing (panel B) conditions respectively and transferred onto nitrocellulose membrane. Western blotting was performed with anti-endoglin antibody (upper panels) and the ECL system was used for chemiluminescence detection. Membranes were reprobed with antibody directed against STAT 3 (lower panels) to verify equal protein loading. **B/C:** TGF-β1 responsiveness of costal and primary articular OA and normal chondrocytes. **B:** Normal and OA cells obtained by enzymatic digestion of cartilage were grown to confluency in 6 well plates and then transiently transfected with p3TP-Lux or pSBE 4 and pβ-gal. 24 hours after transfection, cells were treated with 100 pM of TGF-ß1 (+) overnight or left untreated (-). Luciferase activity was determined, normalized using the β-gal assay and expressed in relative light units (RLU) as a percent control of untreated cells. Values of statistical significance are indicated by an asterisk. **C:** Normal (panel B, Lane 2,4) and OA (panel A, Lane 2,4,6; panel B, Lane 1,3) primary articular

chondrocytes and C-28/I2 (panel A, Lane 1,3,5) cells were grown to confluency in monolayers. Solubilized extracts pretreated with 100 pM of TGF-ß1 (panel A, Lane 3,4; panel B, Lane 3,4) or -β2 (panel A, Lane 5,6) for 15 minutes or left untreated (panel A, Lane 1,2; panel B, Lane 1,2) were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membranes were immunoblotted with an antibody against the phosphorylated form of Smad 2 (upper panels) and reprobed with antibody directed against non-phosphorylated Smad 2 or STAT 3 to illustrate equal protein loading.

**REFERENCES**


1. Ferlic DC 1985 Osteoarthritis of the hand. Orthop Surg Update Series, L36 3:1-8.
2. Isani A 1997 Upper limb arthritis. In: Aston SJ, Beasley RW and Thorn CHM (eds) Grabb and Smith's Plastic Surgery (5th edition). Lippincott-Raven, Philadelphia, NY, pp.999-1008.
3. Rodnam GP, Schumacher HR 1983 The arthritis Foundation: Primer on the Rheumatic Diseases. Osteoarthritis 36:104.
4. Lawrence RC, Helmick CG, Arnett FC, Deyo RA, Felson DT, Giannini EH, Heyse SP, Hirsch R, Hochberg MC, Hunder GG, Liang MH, Pillemer SR, Steen VD, Wolfe F 1998 Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. Arthritis Rheum 41:778-99.
5. Lieberman JR, Daluiski A, Einhorn TA 2002 The role of growth factors in the repair of bone. Biology and clinical applications. J Bone Joint Surg Am 84-A:1032-44.

6. Robinson D, Halperin N, Nevo Z 1990 Regenerating hyaline cartilage in articular defects of old chickens using implants of embryonal chick chondrocytes embedded in a new natural delivery substance. Calc Tissue Int 46:246-253.

7. Aston JE, Bentley G 1986 Repair of articular surfaces by allografts of articular and growth-plate cartilage. J Bone Joint Surg 68-B:29-35.

8. Fukui N, Purple CR, Sandell LJ 2001 Cell biology of osteoarthritis: the chondrocyte's response to injury. Curr Rheum Reports 3:496-505.

9. van den Berg WB 1999 From Cartilage metabolism to clinics: The role of cytokines and growth factors in cartilage destruction in osteoarthritis and rheumatoid arthritis. J Rheumatol 58:136-141.

10. Moldovan F, Pelletier JP, Mineau F, Dupuis M, Cloutier JM, Martel-Pelletier 2000 Modulation of collagenase 3 in human osteoarthritic cartilage by activation of extracellular transforming growth factor beta: role of furin convertase. Arthritis Rheum 43:2100-9.

11. Tardif G, Pelletier JP, Dupuis M, Geng C, Cloutier JM, Martel-Pelletier 1999 Collagenase 3 production by human osteoarthritic chondrocytes in response to growth factors and cytokines is a function of the physiologic state of the cells. Arthritis Rheum 42:1147-58.

12. Rosier RN, O'Keefe RJ, Crabb ID, Puzas JE 1989 Transforming growth factor beta: An autocrine regulator of chondrocytes. Connect Tissue Res 20:295-301.

13. Howard S, Anastasiades T 1993 Differential effects of bone associated factors on newly synthesized anionic glycoconjugates by articular chondrocyte cultures from adult and immature bovines. J Rheumatol 20:2083-2094.

14. Roberts AB, Sporn MB 1990 The transforming growth factor beta's. In: Sporn MB and Roberts AB (eds) Peptide Growth Factors and Their Receptors, vol 95. Spriner-Verlag, New York, NY, pp. 419-472.

15. Seyedin S, Thompson A, Bentz H, Rosen DM, McPherson JM, Conti A, Siegel NR, Galluppi GR, and Piez KA 1986 Cartilage-inducing Factor-A. J Biol Chem 261:5693-5695.

16. Lin HV, Wang X-F, Ng-Eaton E, Weinberg RA, Lodish H F 1992 Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. Cell 68:775-785.

17. Franzen P, ten Dijke P, Ichijo H, Yamashita H, Schultz P, Heldin C H, Miyazono K 1993 Cloning of TGF-β type I receptor that forms a heterodimeric complex with the type II TGF-β receptor. Cell 75: 681-692.

18. Piek E, Heldin C-H, ten Dijke P 1999 Specificity, diversity, and regulation in TGF-β superfamily FASEB 13:2105-2124.

19. Massague J, Chen Y-G 2000 Controlling TGF-beta signaling. Genes Dev 14:627-644.

20. Boumediene K, Conrozier T, Mathieu P, Richard M, Marcelli C, Vignon E, Pujol J-P 1998 Decrease of cartilage transforming growth factor-β receptor II expression in the rabbit experimental osteoarthritis-potential role in cartilage breakdown. Osteoarth Cart 6:146-149.

21. WL, Knaus P, Philip A The type IIB TGF-β receptor on human chondrocytes: phenotypic variability of expression and differential effects of endogenous versus exogenous expression. Submitted.

22. Letamendia A, Lastres P, Botella LM, Raab U, Langa C, Velasco B, Attisano L, Bernabeu C 1998 Role of endoglin in cellular responses to transforming growth factor-β. A comparative study with betaglycan. J Biol Chem 273(49):33011-33019.

23. Arthur HM, Ure J, Smith AJH, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG 2000 Endoglin, an ancillary TGF-ß receptor, is required for extra embryonic angiogenesis and plays a key role in heart development. Dev Biol 217:42-53.

24. Szekanecz Z, Haines GK, Harlow LA, Shah MR, Fong TW, Fu R, Lin J-W, Rayan G, Koch AE 1995 Increased synovial expression of transforming growth factor (TGF)-β receptor endoglin and TGF-β1 in rheumatoid arthritis: possible interactions in the pathogenesis of the disease. Clin Immunol Immunopath 76:187-194.

25. Domm C, Schunke M, Christesen K, Kurz B 2002 Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. Osteoarthritis Cartilage 10:13-22.

26. Thompson AY, Piez KA, Seyedin SM 1985 Chondrogenesis in agarose culture. Exp Cell Res 157:483-494.

27. Hauselmann HJ, Aydelotte MB, Schumacher BL, Kuettner KE, Gitelis SH, Thonar J-MA 1992 Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. Matrix 12:116-129.

28. Hauselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block JA, Aydelotte MB, Kuettner KE, Thonar EJ 1994 Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J Cell Sci 107:17-27.

29. Guo J, Jourdian GW, MacCallum DK 1989 Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Conn Tissue Res 19:277-297.

30. Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Friesinger P 1994 Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp Cell Res 212:97-104.

31. Benya PD, Shaffer JD 1982 Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215-224.

32. Qi WN, Scully SP 1998 Effect of type II collagen in chondrocyte response to TGF-beta 1 regulation. Exp Cell Res 241:142-50.

33. Lux A, Attisano L, Marchuk DA 1999 Assignment of transforming growth factor β1 and β3 and a third new ligand to the type I receptor ALK-1 J Biol Chem 274:9984-9992.

34. Demoor-Fossard M, Redini F, Boittin M, Pujol JP 1998 Expression of decorin and biglycan by rabbit articular chondrocytes. Effects of cytokines and phenotypic modulation. Biochem Biophys Acta 1398:179-91.

35. Redini F, Galera P, Mauviel A, Loyau G, Pujol JP 1988 Transforming growth factor beta stimulates collagen and glycosaminoglycan biosynthesis in cultured rabbit articular chondrocytes. FEBS Lett 234:172-6.

36. Morales TI, Roberts AB 1988 Transforming growth factor beta regulates the metabolism of proteoglycans in bovine cartilage organ cultures. J Biol Chem. 263:12828-31.

37. Tsukazaki T, Matsumoto T, Enomoto H, Usa T, Ohtsuru A, Namba H, Iwasaki K, Yamashita S 1994 Growth hormone directly and indirectly stimulates articular chondrocyte cell growth. Osteoarthritis Cartilage 2:259-67.

38. Pujol JP, Galera P, Redini F, Mauviel A, Loyau G 1991 Role of cytokines in osteoarthritis: comparative effects of interleukin 1 and transforming growth factor-beta on cultured rabbit articular chondrocytes. J Rheumatol Suppl 27:76-9.

39. Fahlgren A, Andersson B, Messner K 2001 TGF-beta1 as a prognostic factor in the process of early osteoarthrosis in the rabbit knee. Osteoarthritis Cartilage 9:195-202.

40. Fawthrop FW, Frazer A, Russell RG, Bunning RA 1997 Effects of transforming growth factor beta on the production of prostaglandin E and caseinase activity of unstimulated and interleukin 1-stimulated human articular chondrocytes in culture. Br J Rheumatol 36:729-34.

41. Iqbal J, Dudhia J, Bird JL, Bayliss MT 2000 Age-related effects of TGF-beta on proteoglycan synthesis in equine articular cartilage. Biochem Biophys Res Commun 274:467-71.

42. Itayem R, Mengarelli-Widholm S, Reinholt FP 1999 The long-term effect of a short course of transforming growth factor-beta1 on rat articular cartilage. APMIS 107:183-92.

43. Bakker AC, Van de Loo FA, Joosten LA, Bennink MB, Arntz OJ, Dmitriev IP, Kashentsera EA, Curiel DT, van den Berg WB 2001 A tropism-modified adenoviral vector increased the effectiveness of gene therapy for arthritis. Gene Ther 8:1785-93.

44. Letamendia A, Lastres P, Almendro N, Raab U, Buhring H-J, Kumar S, Bernabeu C 1998 Endoglin, a component of the TGF-β receptor system, is a differentiation marker of human choriocarcinoma cells. Int J Cancer 76:541-546.

45. Tam B, Germain L, Philip A 1998 A 150kDa GPI-anchored TGF-β1 binding protein forms heteromeric complex with the types I and II TGF-β signalling receptors. J Cell Biochem 70:573-586.

46. Tam B, Larouche D, Germain L, Hooper NM, Philip A 2001 Characterization of a 150 kDa accessory receptor for TGF-β1 on keratinocytes: Direct evidence for a GPI-anchor, and ligand binding of the released form. J Cell Biochem 83:494-507.

47. Robbins JR, Goldring MB 1997 Preparation of immortalized human chondrocyte cell lines. In: Morgan JR, Yarmush ML (eds) Methods in Molecular Medicine, vol 18. Humana Press Inc., Totowa, N.J. pp. 173-192.

48. Goldring MB, Birkhead JR, Suen L-F, Yamin R, Mizuno S, Glowacki J, Apperley JF 1994 Successful immortalization of human chondrocytes exhibiting stable expression of cartilage-specific type II collagen. J Clin Invest 94:2307-2316.

49. van Susante JL, Buma P, van Osch GJ, Versleyen D, van der Kraan PM, van der Berg WB, Homminga GN 1995 Culture of chondrocytes in alginate and collagen carrier gels. Acta Orthop Scand 66:549-56.

50. Taylor MF, Paulauskis JD, Weller DD, Kobzik L 1996 In Vitro Efficacy of Morpholino-modified Antisense Oligomers Directed against Tumor Necrosis Factor-α mRNA. J Biol Chem 271:17445-17452.

51. Morcos PA 2001 Achieving efficient delivery of morpholino oligos in cultured cells. Genesis 30:94-102.

52. Wong SH, Hamel L, Chevalier S, Philip A 2000 Endoglin expression on human microvascular endothelial cells: demonstration of higher order complexes with TGF-β signaling receptors and heteromerization with betaglycan. Eur J Biochem 267:5550-5560.

53. Dumont N, O'Connor-McCourt M, Philip A 1995 Transforming growth factor-β receptors on human endometrial cells: Identification of the type I, II and III receptors, and glycosylphosphatidylinositol anchored TGF-β binding proteins. Mol Cell Endocrinol 111:57-66.

54. Parker WL, Blobe GC, Philip A Characterization of endoglin-betaglycan heteromerization: TGF-β binding by endoglin requires betaglycan but not the type II receptor. Submitted.

55. Souchelnytskyi S, Ronnstrand L, Heldin CH, ten Dijke P 2001 Phosphorylation of Smad signaling proteins by receptor serine/threonine kinases. Methods Mol Biol 124:107-20.

56. Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P 1997 TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. EMBO J 16:5353-62.

57. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J 1994 Mechanism of activation of the TGF-β receptor. Nature 370:341-347.

58. Blobe GC, Schiemann WP, Pepin M-C, Beauchemin M, Moustakas A, Lodish HF, O'Connor-McCourt MD 2001 Functional roles for the cytoplasmic domain of the type III transforming growth factor beta receptor in regulating transforming growth factor beta signaling. J Biol Chem 276:24627-24637.

59. Eickelberg O, Centrella M, Reiss M, Kashgarian M, Wells RG 2002 Betaglycan inhibits TGF-beta signaling by preventing type I-type II receptor complex formation. Glycosaminoglycan modifications alter betaglycan function. J Biol Chem 277:823-9.

60. Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B, Kern SE 1998 Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cell 1:611-617.

61. Docagne F, Colloc'h N, Bougueret V, Page M, Paput J, Tripier M, Dutartre P, MacKenizie ET, Buisson A, Komesli S, Vivien D 2001 A soluble TGF-β type-1 receptor mimics TGF-β responses. J Biol Chem 276: 46243-46250.

62. Gruppuso PA, Mead JE, Fausto N 1990 Transforming growth factor receptors in liver regeneration following partial hepatectomy in the rat. Cancer Res 50:1464-1469.

63. Chanda VB 1995 Support Protocol: Sample Solubilization. In: Chanda VB (ed) Current Protocols in Molecular Biology, Supplement, John Wiley & Sons, Canada, pp. 10.3.8-10.

64. Robbins JR, Thomas B, Tan L, Choy B, Arbiser JL, Berenbaum F, Goldring MB 2000 Immortalized human adult articular chondrocytes maintain cartilage-specific phenotype and responses to interleukin-1beta. Arthritis Rheum 43:2189-201.

65. Goldring MB, Berenbaum F 1999 Human chondrocyte culture models for studying cyclooxygenase expression and prostaglandin regulation of collagen gene expression. Osteoarthritis Cartilage 7:386-88.

66. Kokenyesi R, Tan L, Robbins JR, Goldring MB 2000 Proteoglycan production by immortalized human chondrocyte cell lines cultured under conditions that promote expression of the differentiated phenotype. Arch Biochem Biophys 383:79-90.

67. Loeser R, Sadiev S, Tan L, Goldring MB 2000 Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins in mediating chondrocyte adhesion to types II and VI collagen. Osteoarthritis Cartilage 8:96-105.

68. Parker WL, Goldring MB, Philip A 2003 Endoglin is expressed on human chondrocytes and forms a heteromeric complex with betaglycan in a ligand and type II TGF-$\beta$ receptor independent manner. J Bone Miner Res 18:289-302.

69. Petit B, Freyria A-M, van der Rest M, Herbage D 1992 Cartilage collagens. In: Adolphe M (ed) Biological Regulation of the Chondrocytes. CRC Press, Boca Raton, FL, pp.33-84.

70. Mayne R, Brewton RG 1993 Extracellular matrix of cartilage: collagen. In: Woessner JF Jr, Howell DS (eds) Joint Cartilage Degradation. Basic and Clinical Aspects. Marcel Dekker, New York, NY, pp. 81-108.

71. Cancedda R, Descalzi Cancedda F, Castagnola P 1995 Chondrocyte differentiation. Int Rev Cytol 159:265-358.

72. Lastres P, Letamendia A, Zhang H, Rius C, Almendro N, Raab U, Loepez LA, Langa C, Fabra A, Letarte M, Bernabeu C 1996 Endoglin modulates cellular responses to TGF-$\beta$I. J Cell Biol 133:1109-1121.

73. Cheifetz S, Massague J 1991 Isoform-specific transforming growth factor-beta binding proteins with membrane attachments sensitive to phosphatidylinositol-specific phospholipase C. J Biol Chem 266, 20767-20772.

74. Tam B, Philip A 1998 Transforming growth factor-β receptor expression on human skin fibroblasts; dimeric complex formation of type I and type II receptors and identification of glycosyl phosphatidylinositol-anchored transforming growth factor-β binding proteins. J Cell Physiol 176:553-564.

75. Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C 2002 Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-β receptors I and II. J Biol Chem 277:29197-209.

76. Parker W, Knaus P, Philip A The type IIB TGF-β receptor on human chondrocytes: phenotypic variability of expression and differential effects of endogenous versus exogenous expression. Submitted.

77. Glansbeek, H.L., van Beuningen HM., van Vitters, B.H., van der Kraan, E.L, and van den Berg WB. (1998). Stimulation of articular cartilage repair in established arthritis by local administration of transforming growth factor-$\beta$ into murine knee joints. Lab Invest. 78:133-142.

78. Serra R, Johnson M, Filvaroff EH, LaBorde J, Sheehan DM, Derynck R, Moses HL 1997 Expression of a truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. J Cell Biol139:541-52.

79. Yang X, Chen L, Xu X, Li C, Huang C, Deng C-X 2001 TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol 153:35-46.

80. von der Mark K, Gauss V, von der Mark H, Muller P 1977 Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature 267: 531

81. Kuettner KE, Memoli VA, Pauli BU, Wrobel NC, Thonar EJ-MA, Daniel JC 1982 Synthesis of cartilage matrix by mammalian chondrocytes in vitro. II. Maintenance of collagen and proteoglycan phenotype. J Cell Biol 93:751-7.

82. Bounelis P, Daniel JC 1983 The effect of ascorbate on embryonic chick sternal chondrocytes cultured in agarose. Tissue Cell 15:683.

83. Aigner T, Zhu Y, Chansky HH, Matsen FA III, Maloney WJ, Sandell LJ 1999 Reexpression of type IIA procollagen by adult articular chondrocytes in osteoarthritic cartilage. Arthritis Rheum 42:1443-1450.

84. Salminen H, Vuorio E, Saamanen Am 2001 Expression of Sox9 and type IIA procollagen during attempted repair of articular cartilage damage in a transgenic mouse model of osteoarthritis. Arthritis Rheum 44:947-955.

85. Boos N, Nerlich AG, Wiest I, von der Mark K, Ganz R, Aebi M 1999 Immunohistochemical analysis of type-X-collagen expression in osteoarthritis of the hip joint. J Orthop Res 17:495-502.

86. Quackenbush EJ, Letarte M 1985 Identification of several cell surface proteins of non-T, non-B acute lymphoblastic leukemia by using monoclonal antibodies. J Immunol 134:1276-1285.

87. Guerrero-Esteo M, Lastres P, Letamendia A, Perez-Alvarez MJ, Langa, C, Lopez LA, Fabra A, Garcia-Pardo A, Vera S, Letarte M, Bernabeu C 1999 Endoglin overexpression modulates cellular morphology, migration, and adhesion of mouse fibroblasts. E J Cell Biol 78:614-623.

88. Li C, Harpson L, Kumar P, Bernabeu C, Kumar S 2000 CD105 antagonizes the inhibitory signaling of transforming growth factor β1 on human vascular endothelial cells. FASEB J 14:55-64.

89. Ferandez-Riuz E, St-Jacques S, Bellon T, Letarte M, Bernabeu C 1993 Assignment of the human endoglin gene (END) to 9q34-->qter. Cytogenet Cell Genet 64:204-207.

90. McAllister KA, Baldwin MA, Thukkani AK, Gallione CJ, Berg JN, Porteous ME, Guttmacher AE, Marchuk DA 1995 Six novel mutations in the endoglin gene in hereditary hemorrhagic telangiectasia type 1 suggest a dominant-negative effect of receptor function. Hum Mol Genet 4:1983-1985.

91. Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJJ, Kjeldsen AD, Plauchu H 2000 Diagnostic criteria for Hereditary Hemorrhagic Telangiectasia (Rendu-Osler-Weber Syndrome) Am J Med Gen 91:66-67.

92. Bourdeau A, Dumont DJ, Letarte M 1999 A murine model of hereditary hemorrhagic telangiectasia. J Clin Invest 104:1343-1351.

93. Pece-Barabara N, Wrana JL, Letarte M 1999 Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-β superfamily. JBC 274:584-594.

94. Yamashita H, Ichijo H, Grimsby S, Moron A, ten Dijke P, Miyazono K 1994 Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor beta. J Biol Chem 269:1995-2001.

95. Cheifetz S, Bellon T, Cales C, Vera S, Bernabeu C, Massague J, Letarte M 1992 Endoglin is a component of the transforming growth factor-β receptor system in human endothelial cells. J Biol Chem 267:19027-19030.

96. Lopez-Casillas F, Wrana JL, Massague J 1993 Betaglycan presents ligand to the TGF-β signaling receptor. Cell 73:1435-1444.

97. Barbara NP, Wrana JL, Letarte M 1999 Endoglin is an accessory protein that interacts with the signalling receptor complex of multiple members of the transforming growth factor-β super family. J Biol Chem 274(2):584-594.

98. Mustoe TA, Pierce GF, Thomason A, Gramates P, Sporn MB, Deuel TF 1987 Accelerated healing of incisional wounds in rats induced by transforming growth factor-ß. Science 237:1333-1336.

99. Quaglino D, Nanney LB, Ditesheim JA, Davidson JM 1991 Transforming growth factor-ß stimulates wound healing and modulates extracellular matrix gene expression in pig skin: incisional wound model. J Invest Dermatol 97: 34-42.

100. Dickson K, Philip A, Warshawsky H, O'Connor-McCourt M Bergeron JJM 1995 Specific binding of endocrine TGFβ1 to vascular endothelium. J Clin Invest 95: 2539-2554.

101. Khalil N, Parekh TV, O'Connor R, Antman N, Kepron W, Yehaulaeshet T, Xu YD, Gold LI 2001 Regulation of the effects of TGF-beta 1 by activation of latent TGF-

beta 1 and differential expression of TGF-beta receptors (T beta R-I and T beta R-II) in idiopathic pulmonary fibrosis. Thorax 56:907-15.

102. Weber AJ, De Bandt M 2000 Angiogenesis: general mechanisms and implications for rheumatoid arthritis. Joint Bone Spine 67:366-83.

103. Chin GS, Liu W, Peled Z, Lee TY, Steinbrech DS, Hsu M, Longaker MT 2001 Differential expression of transforming growth factor-beta receptors I and II and activation of Smad 3 in keloid fibroblasts. Plast Reconstr Surg 108:423-9.

104. Kelly DL, Rizzino A 1999 Growth regulatory factors and carcinogenesis: the roles played by transforming growth factor beta, its receptors and signaling pathways. Anticancer Res 19:4791-807.

105. Diez-Marques L, Ortega-Velazquez R, Langa C, Rodriguez-Barbero A, Lopez-Novoa JM, Lamas S, Bernabeu C 2002 Expression of endoglin in human mesangial cells: modulation of extracellular matrix synthesis. Biochem Biophys Acta 1587:36-44.

106. Goldring MB 2000 Osteoarthritis and cartilage: the role of cytokines. Curr Rheumatol Rep 2:459-65.

107. Lafeber FPJG, van der Kraan PM, Huber-Bruning O, van den Berg WB, Bijlsma JWJ 1993 Osteoarthritic human cartilage is more sensitive to transforming growth factor β than is normal cartilage. Br J Rheumatol 32:281-286.

108. Shovlin CL 1999 Supermodels and disease: insights from the HHT mice. J Clin Invest 104:1335-6.

109. Haitjema T, Westermann CJ, Overtoom TT, Timmer R, Disch F, Mauser H, Lammers JW 1996 Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu

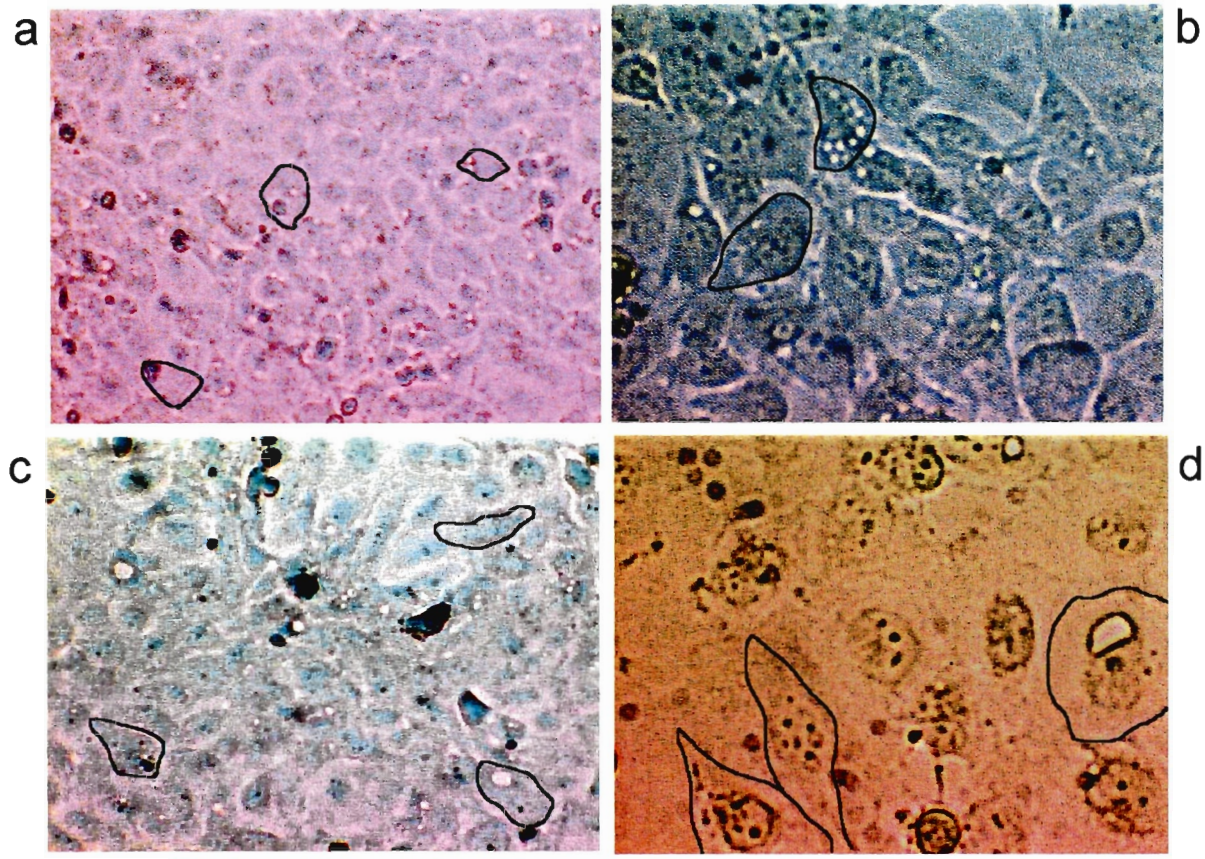
disease): new insights in pathogenesis, complications, and treatment. Arch Intern Med 156:714-9.

110. Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP 1999 Defective angiogenesis in mice lacking endoglin. Science 284:1534-7.

111. Shah RK, Dhingra JK, Shapshay SM 2002 Hereditary hemorrhagic telangiectasia: a review of 76 cases. Laryngoscope 112:767-73.

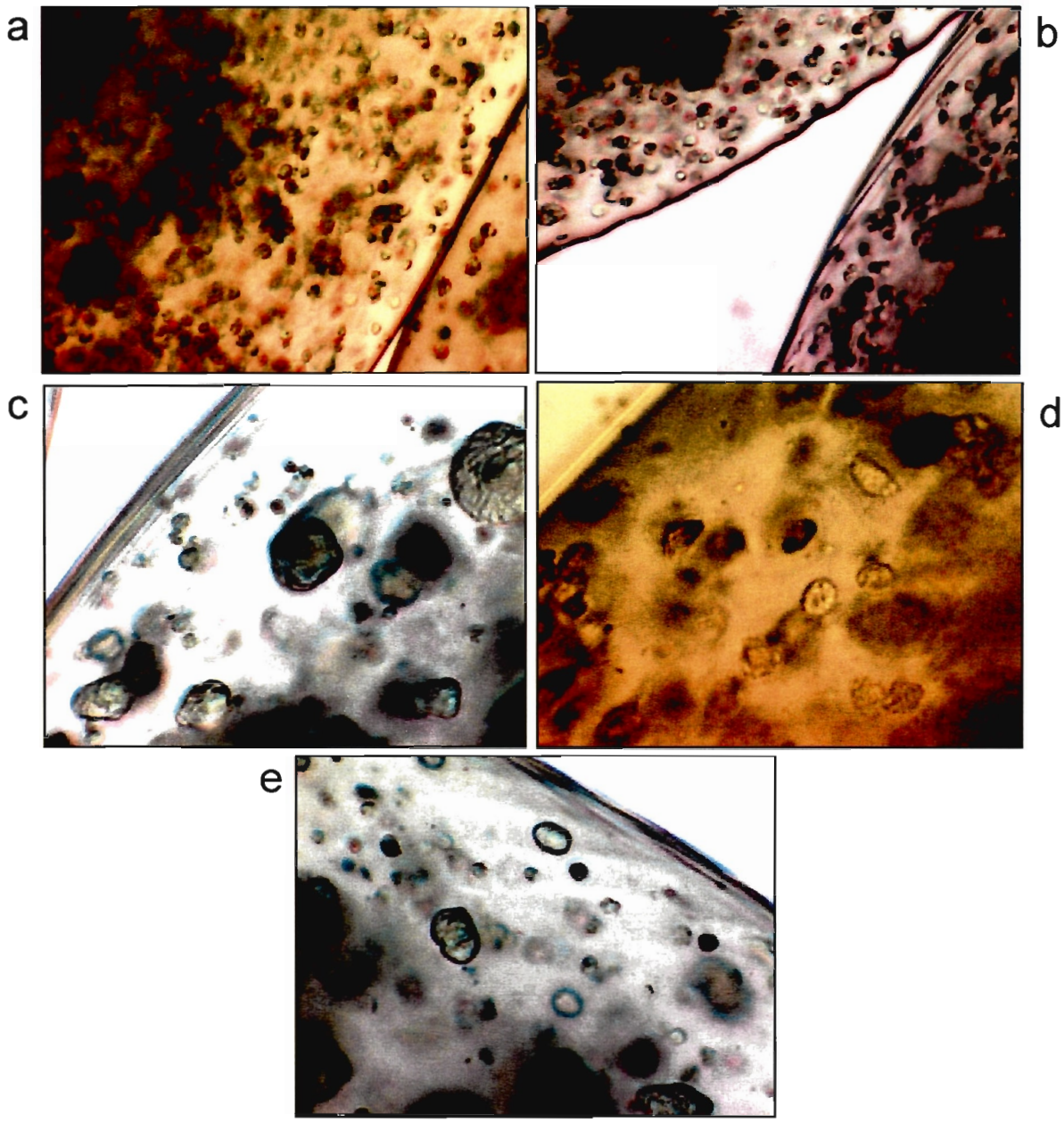
112. Abdalla SA, Pece-Barbara N, Vera S, Tapia E, Paez E, Bernabeu C, Letarte M 2000 Analysis of ALK-1 and endoglin in newborns from families with hereditary hemorrhagic telangiectasia type 2. Hum Mol Genet 9:1227-37.

Fig. 1A



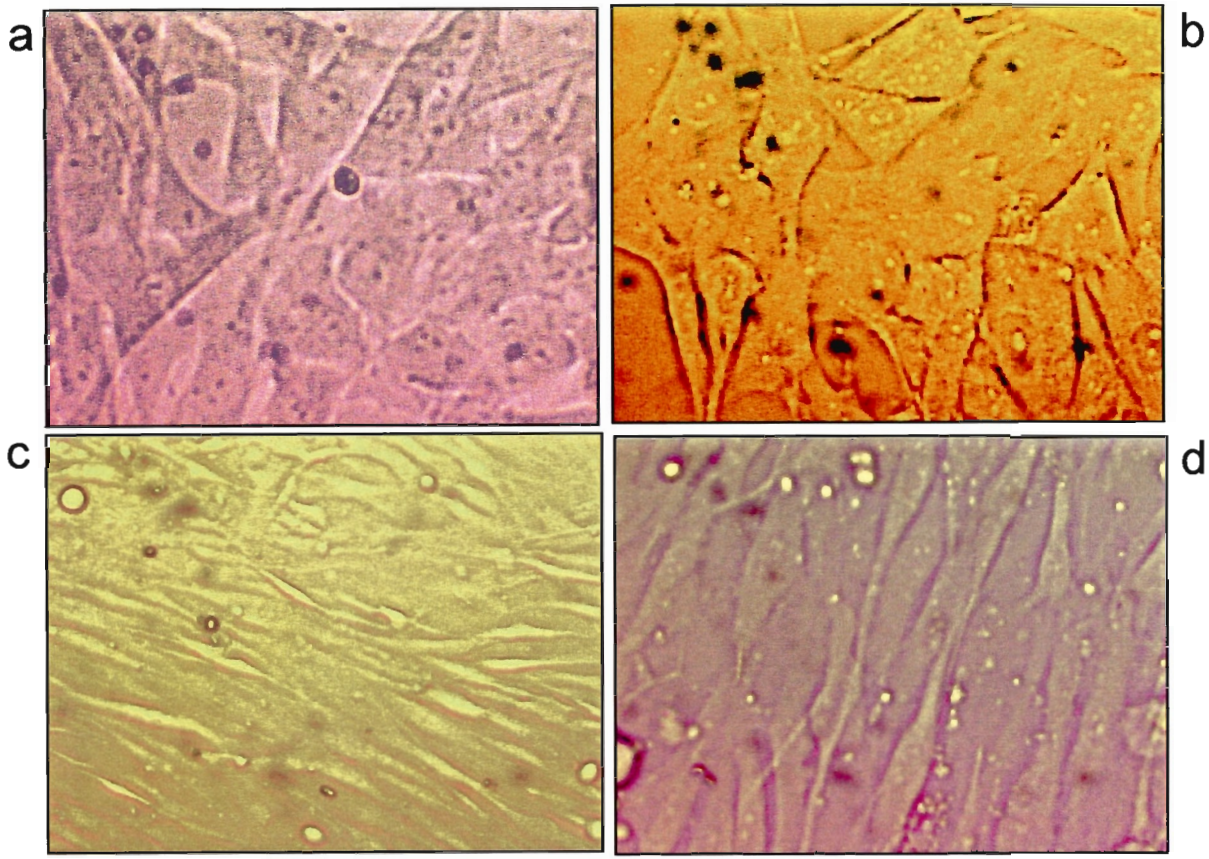

432

Fig. 1B



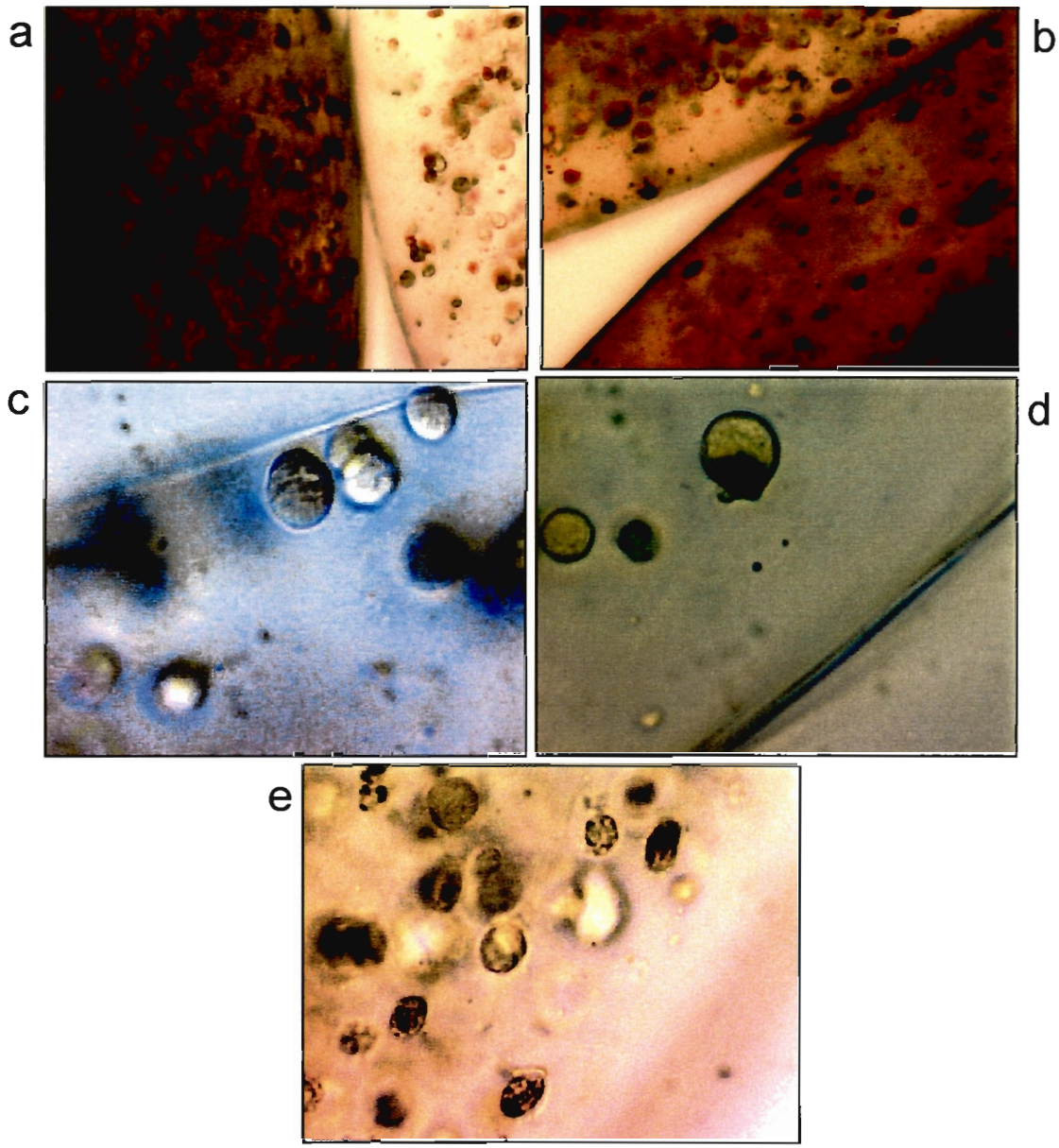

433

Fig. 2A



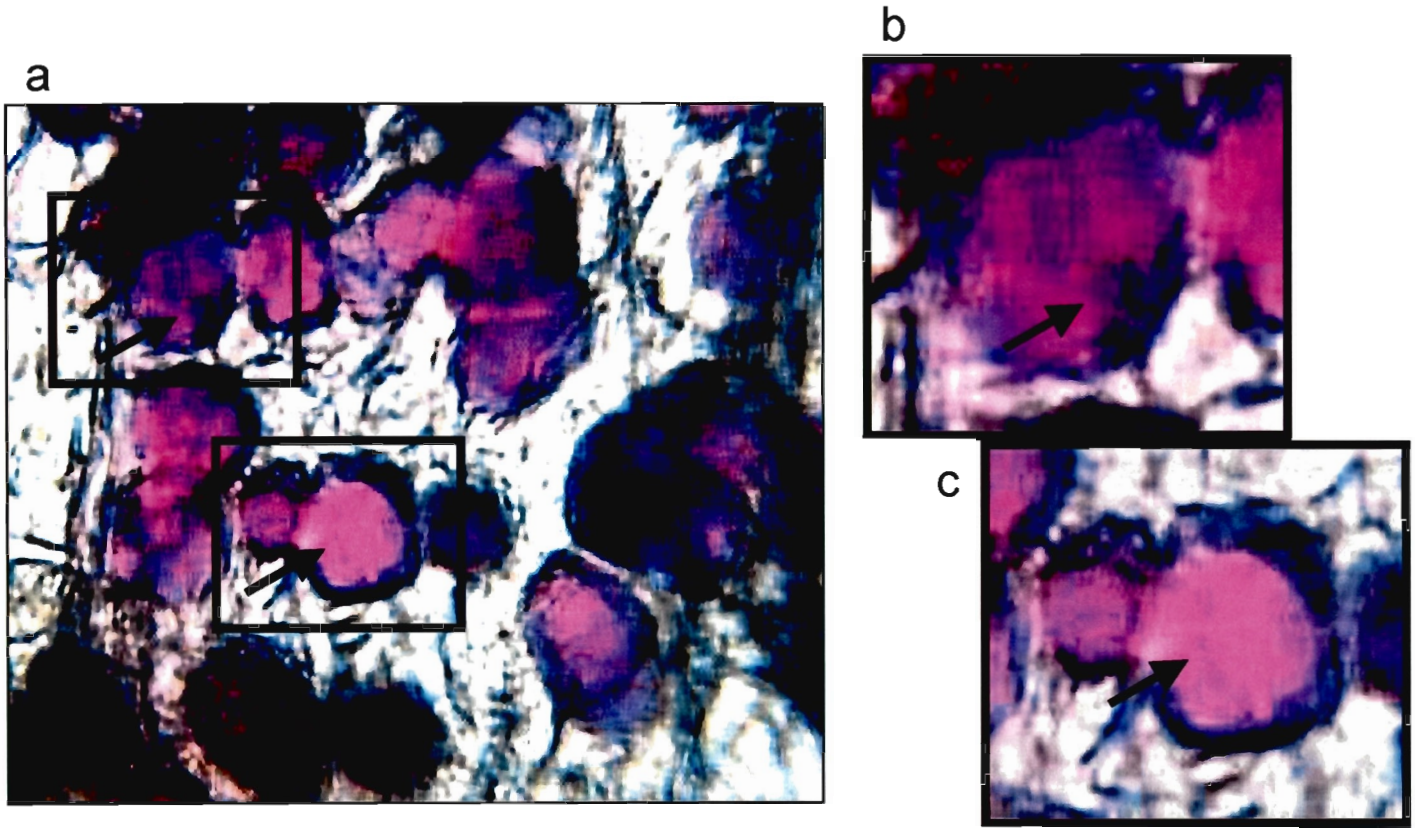

434

Fig. 2B



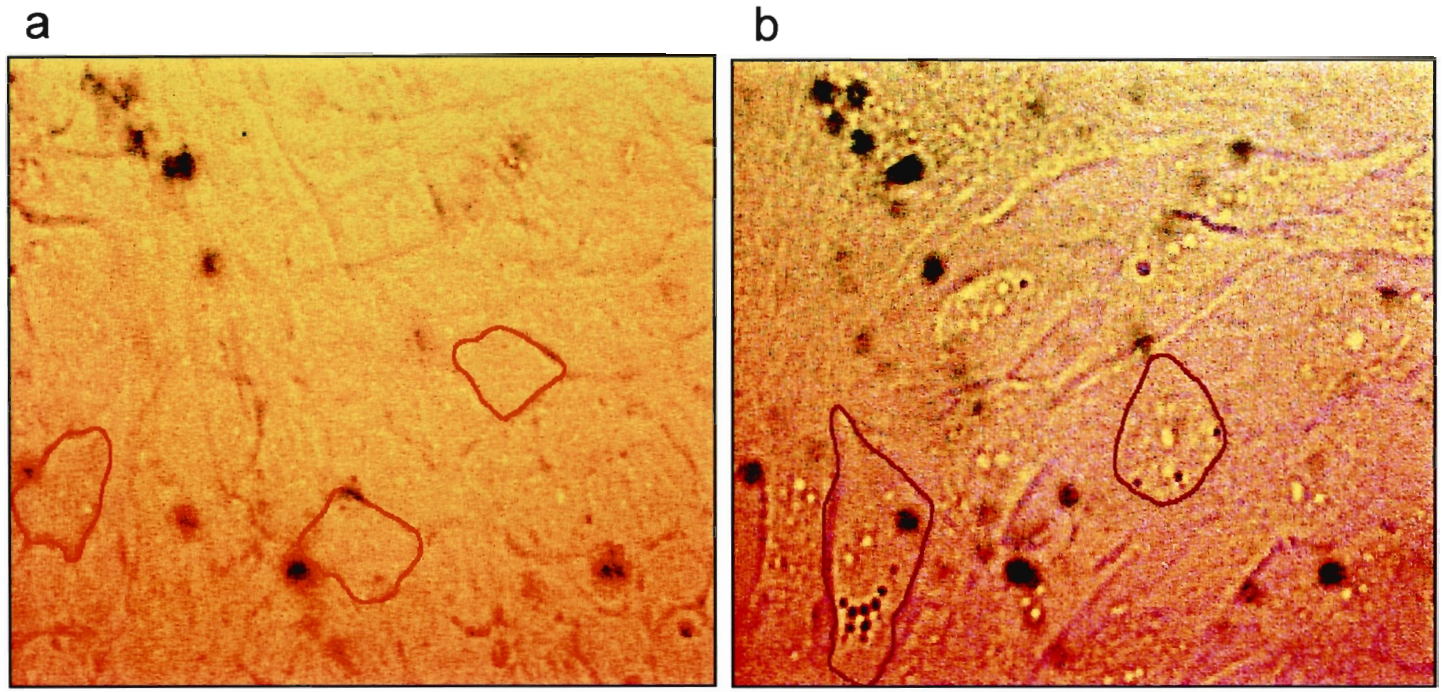

435

Fig. 2C




436

Fig. 3A




437

Fig. 3B

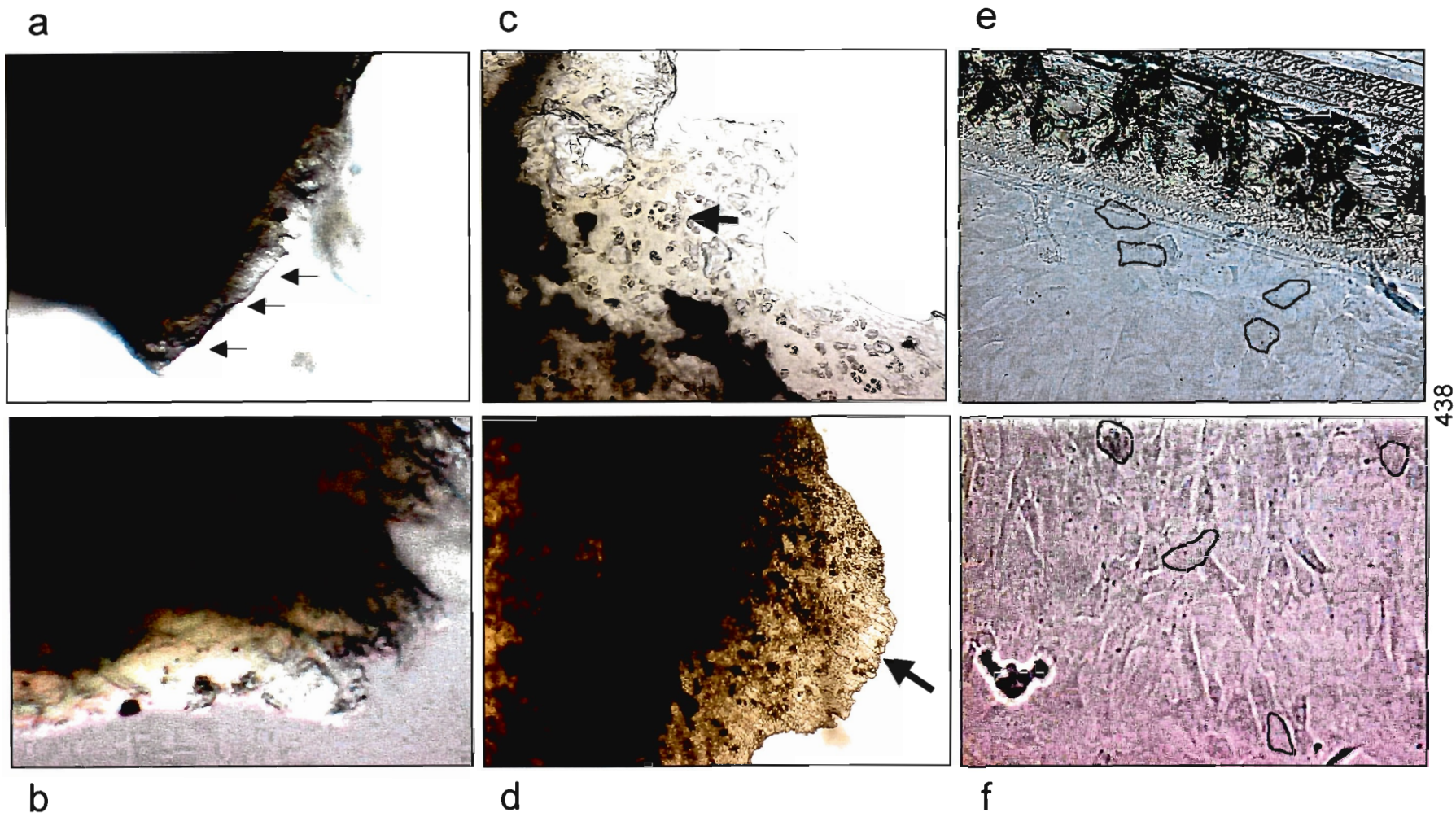
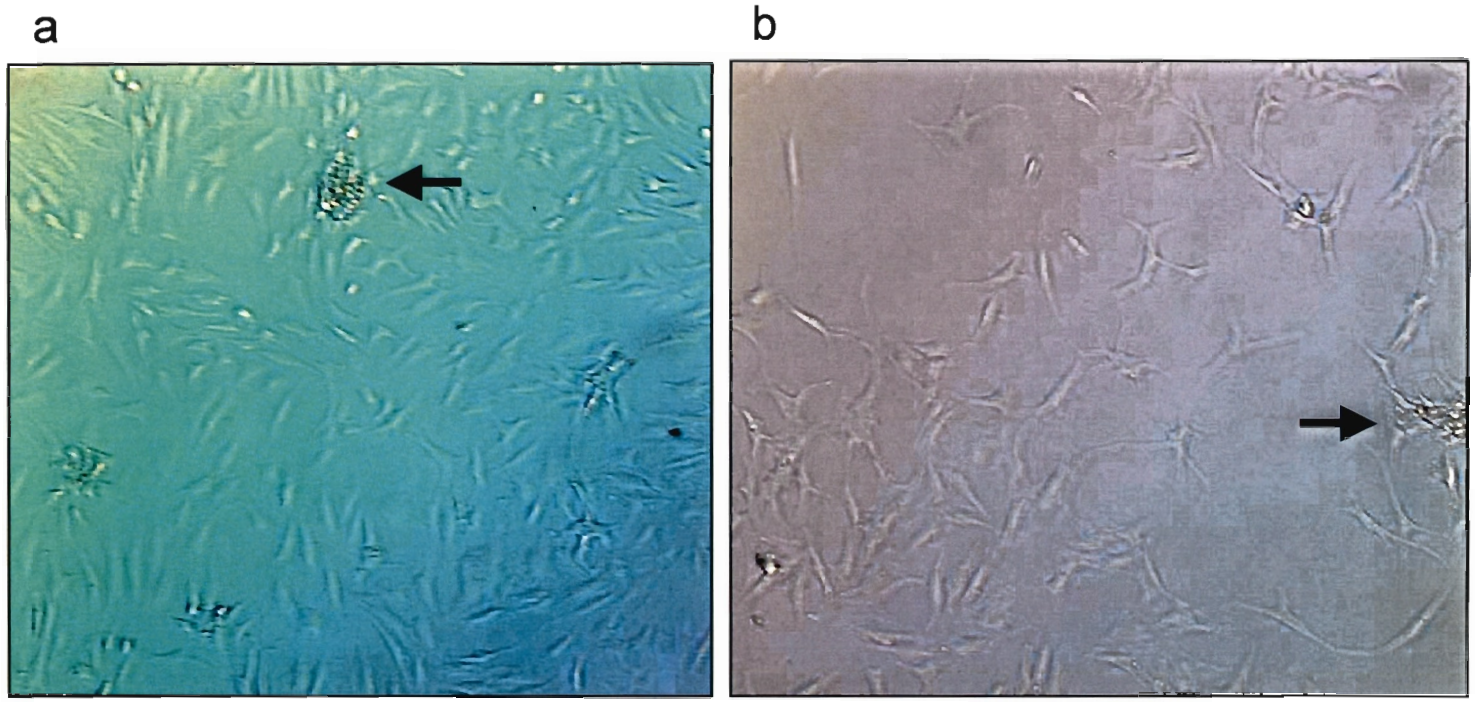


Fig. 4A




439

Fig. 4B

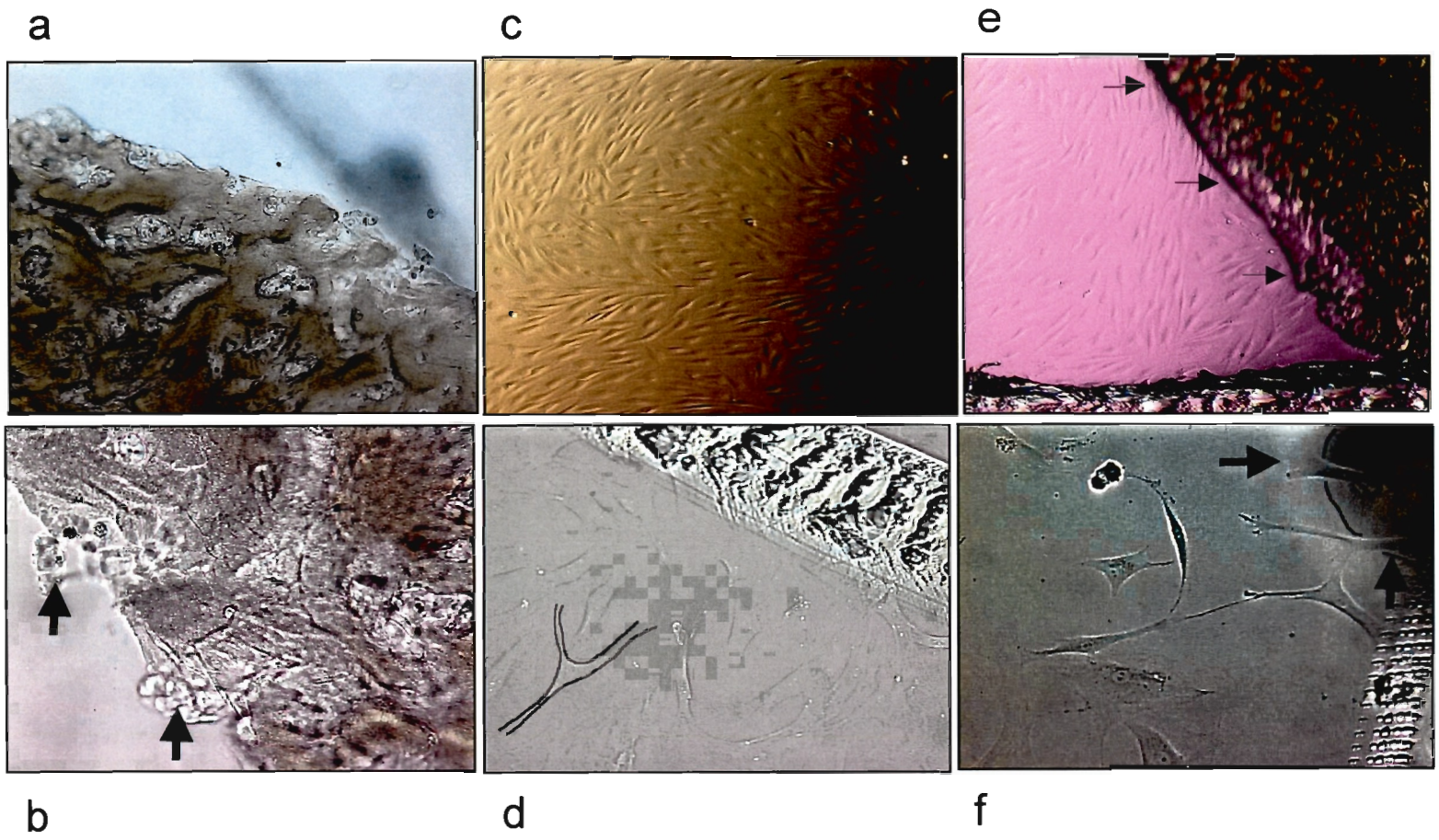
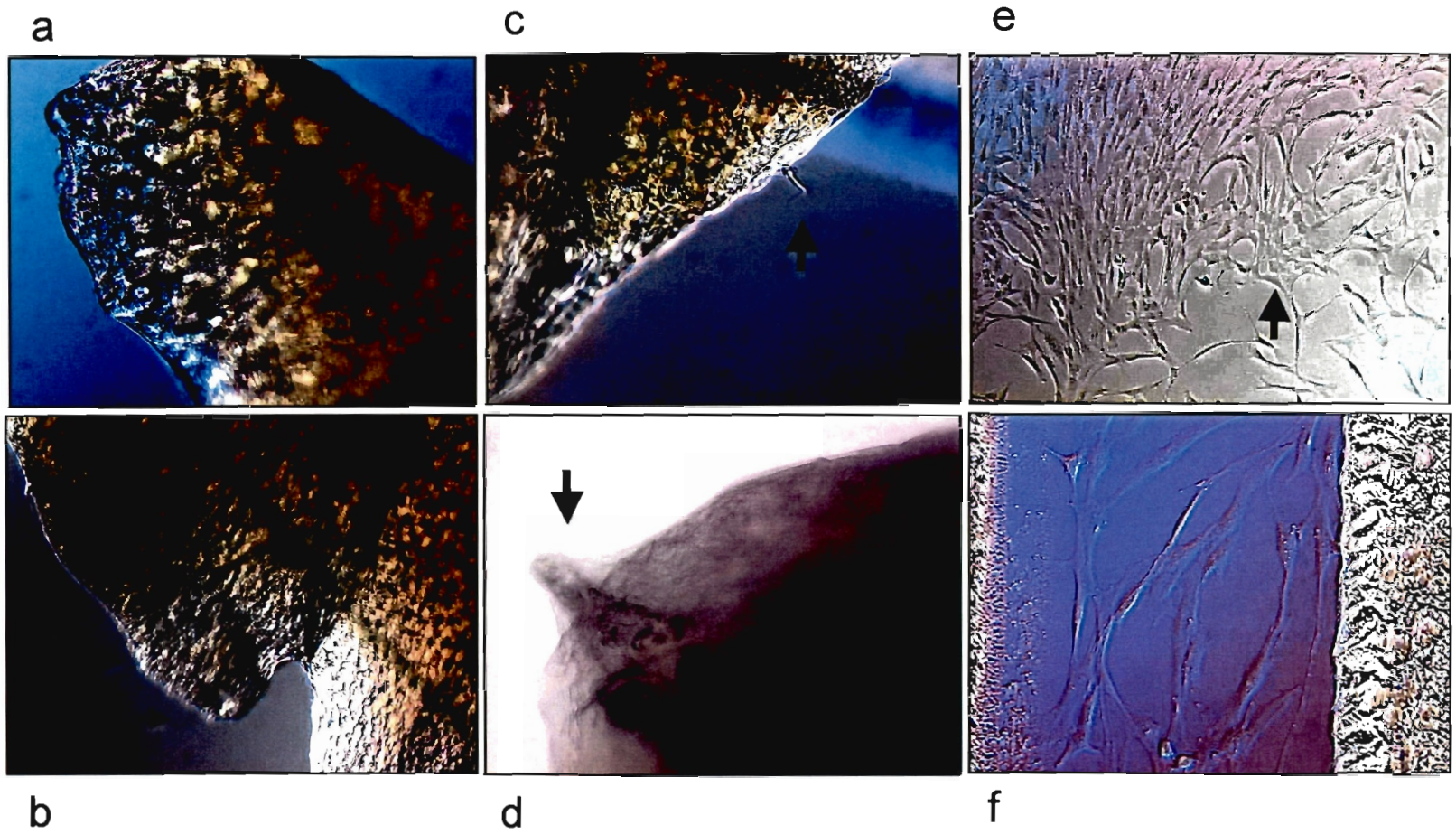
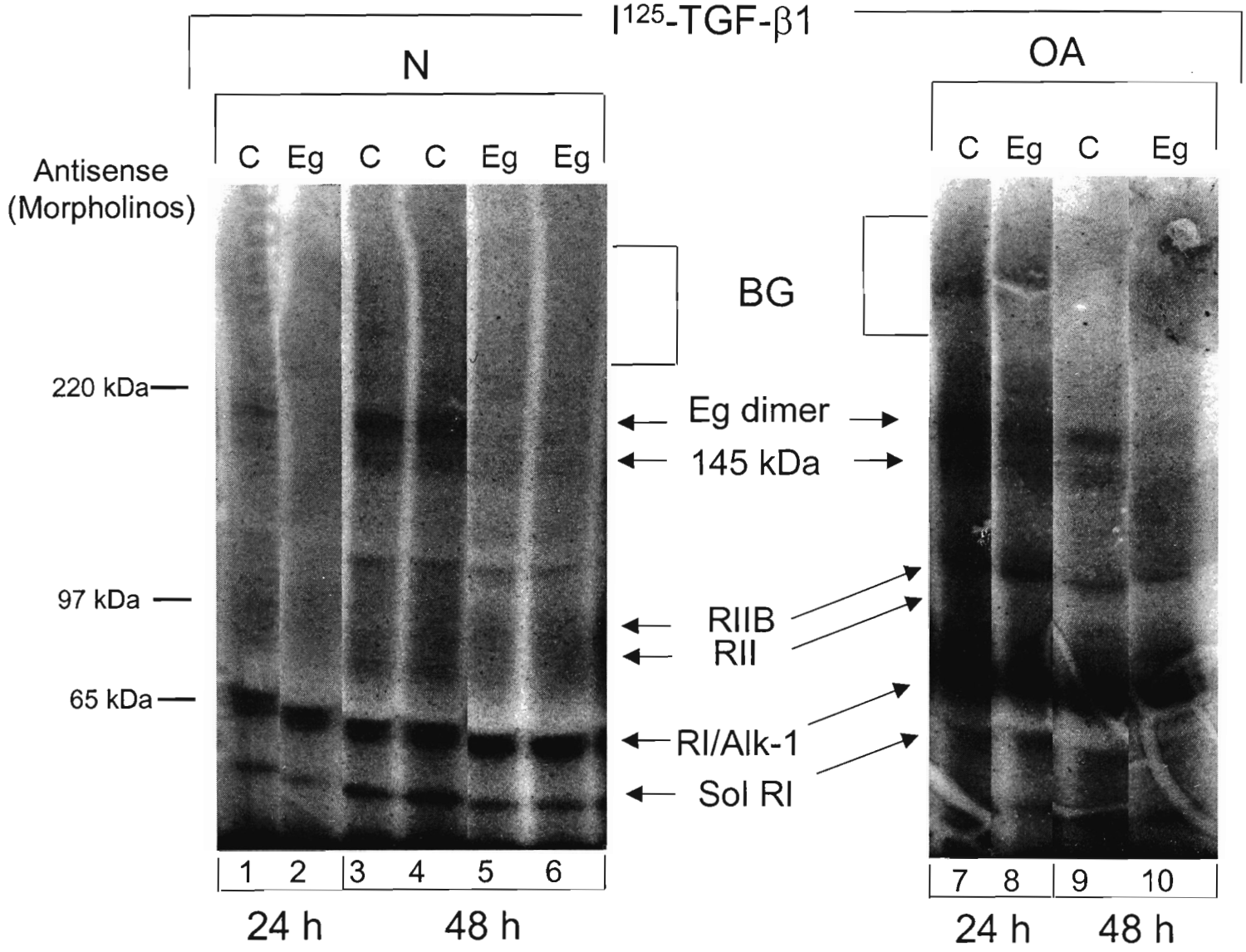


Fig. 4C




441

Fig.5A


I125-TGF-β1
N
OA
C Eg C C Eg Eg
C Eg C Eg
Antisense
(Morpholinos)
BG
220 kDa
Eg dimer
145 kDa
97 kDa
RIIB
RII
65 kDa
RI/Alk-1
Sol RI
1 2 3 4 5 6
7 8 9 10
24 h 48 h
24 h 48 h

442

Fig.5B

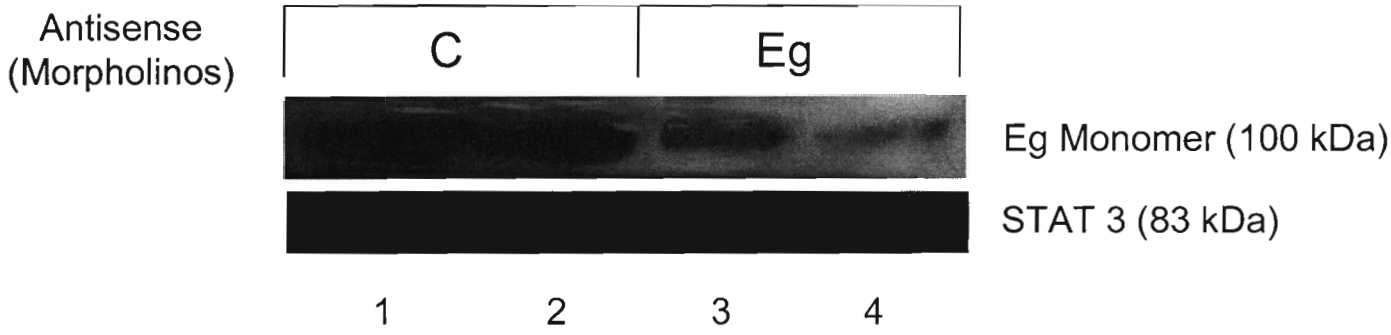

Antisense
(Morpholinos)
C
Eg
Eg Monomer (100 kDa)
STAT 3 (83 kDa)
1
2
3
4

Fig. 6A

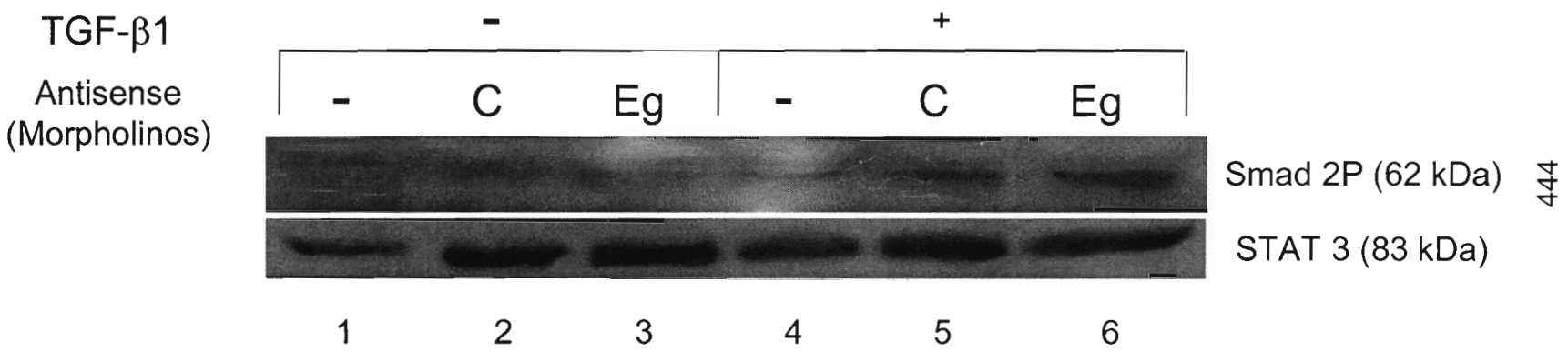

TGF-β1
-
+
Antisense
(Morpholinos)
-
C
Eg
-
C
Eg
Smad 2P (62 kDa)
STAT 3 (83 kDa)
1
2
3
4
5
6

Fig.6B

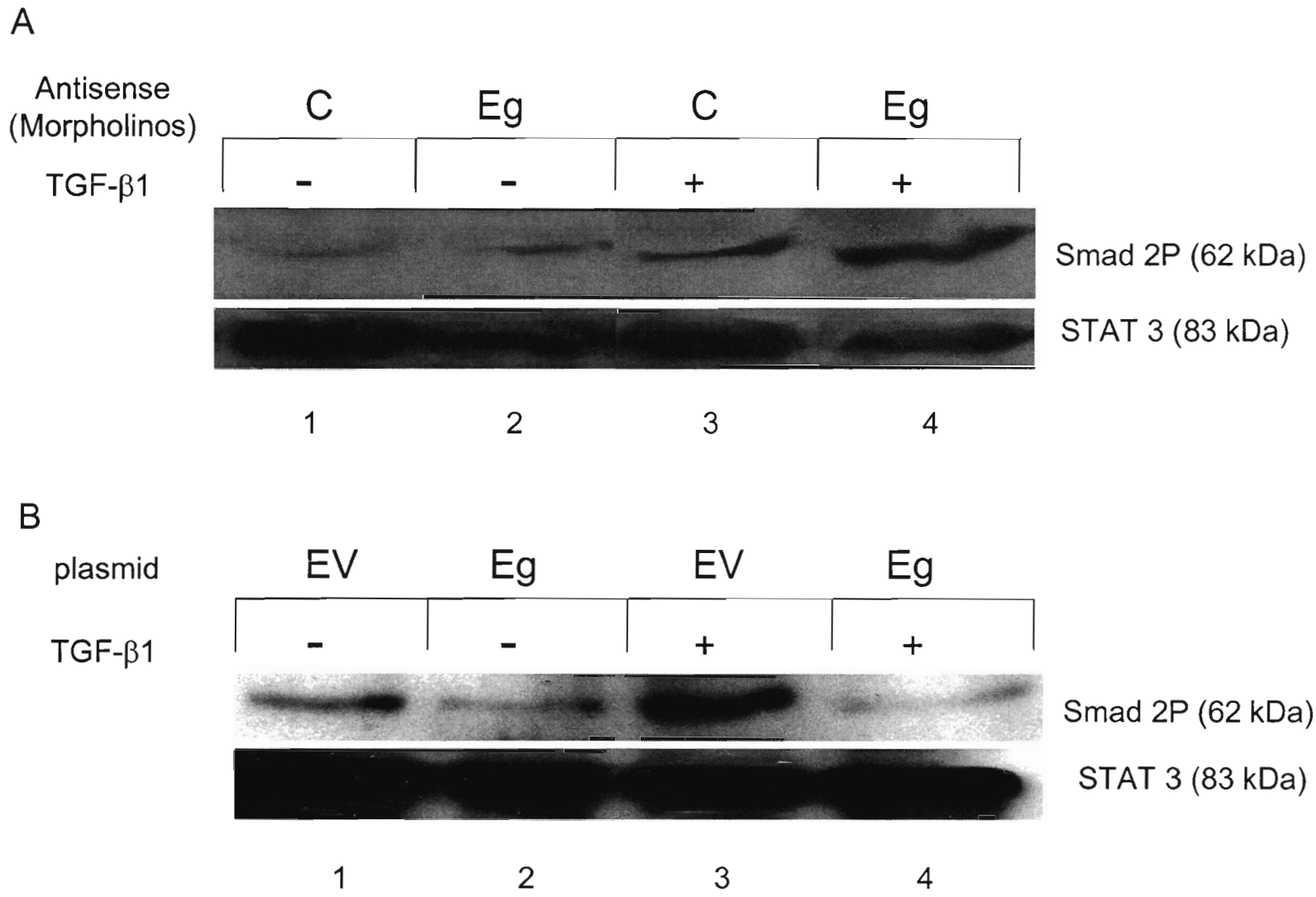

A
Antisense
(Morpholinos)
C
Eg
C
Eg
TGF-β1
-
-
+
+
Smad 2P (62 kDa)
STAT 3 (83 kDa)
1
2
3
4
B
plasmid
EV
Eg
EV
Eg
TGF-β1
-
-
+
+
Smad 2P (62 kDa)
STAT 3 (83 kDa)
1
2
3
4

Fig.6C

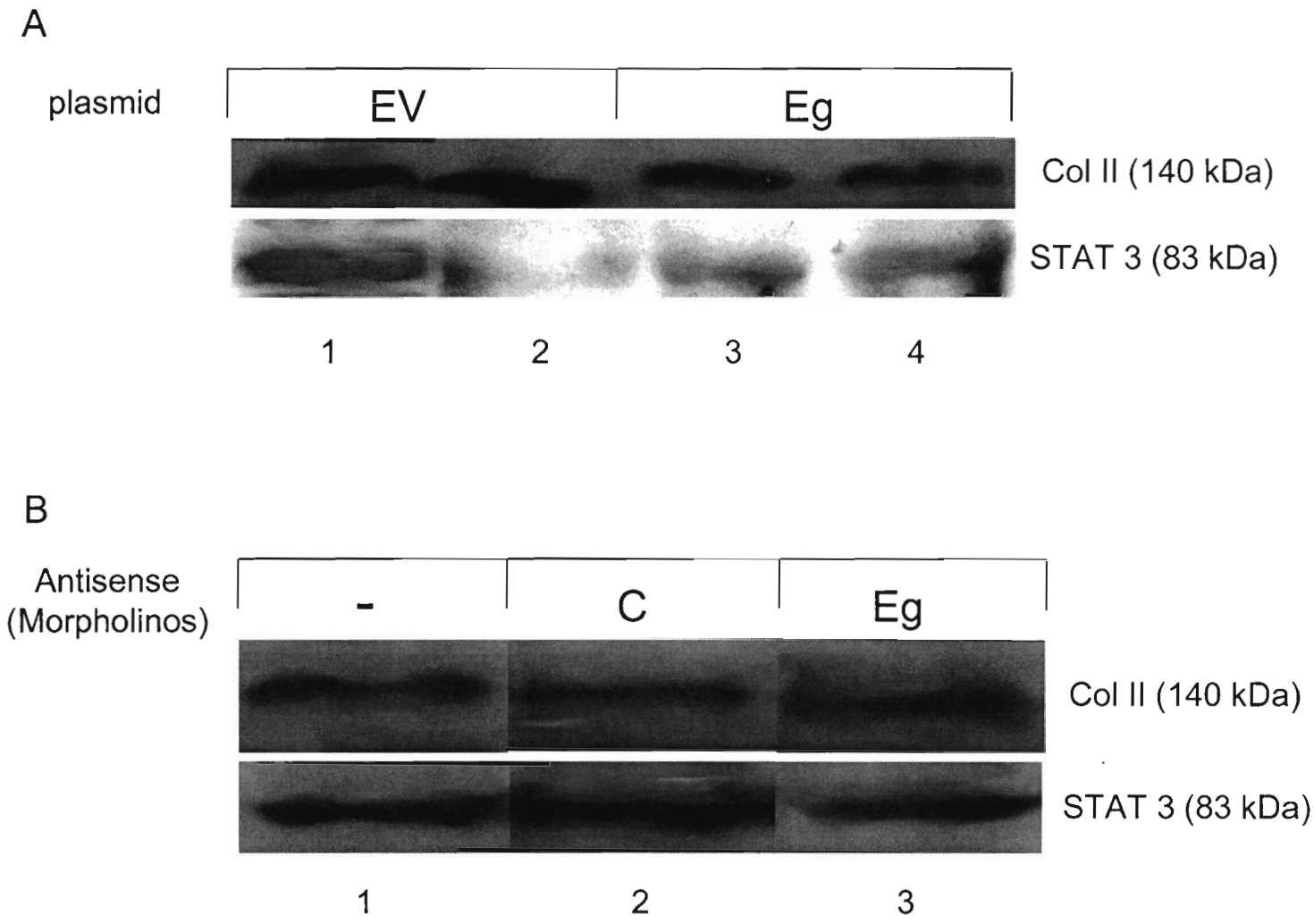

A
plasmid
EV
Eg
Col II (140 kDa)
STAT 3 (83 kDa)
1
2
3
4
B
Antisense
(Morpholinos)
-
C
Eg
Col II (140 kDa)
STAT 3 (83 kDa)
1
2
3

Fig. 7A

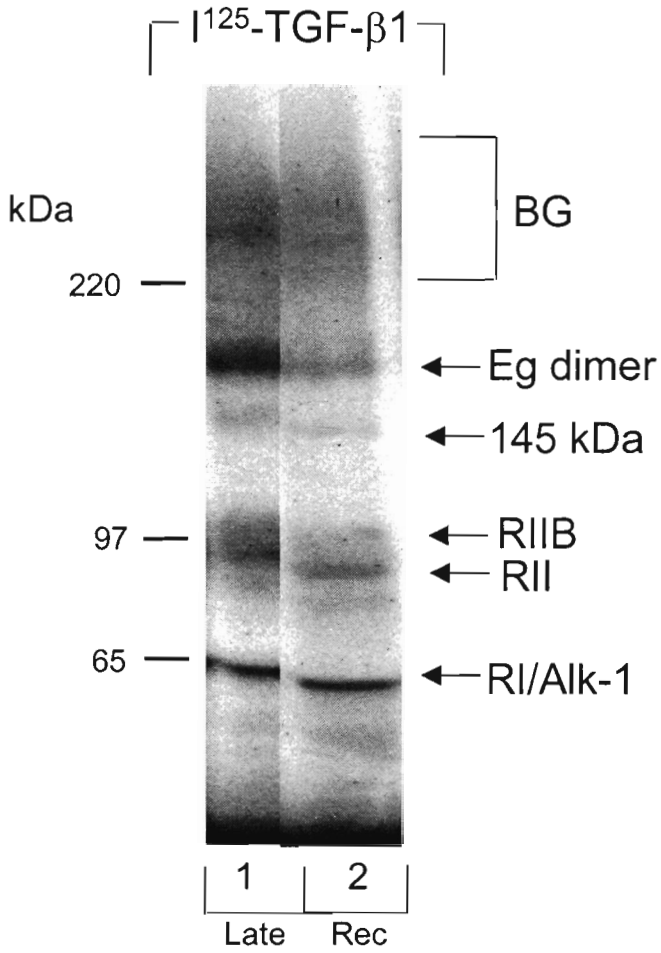
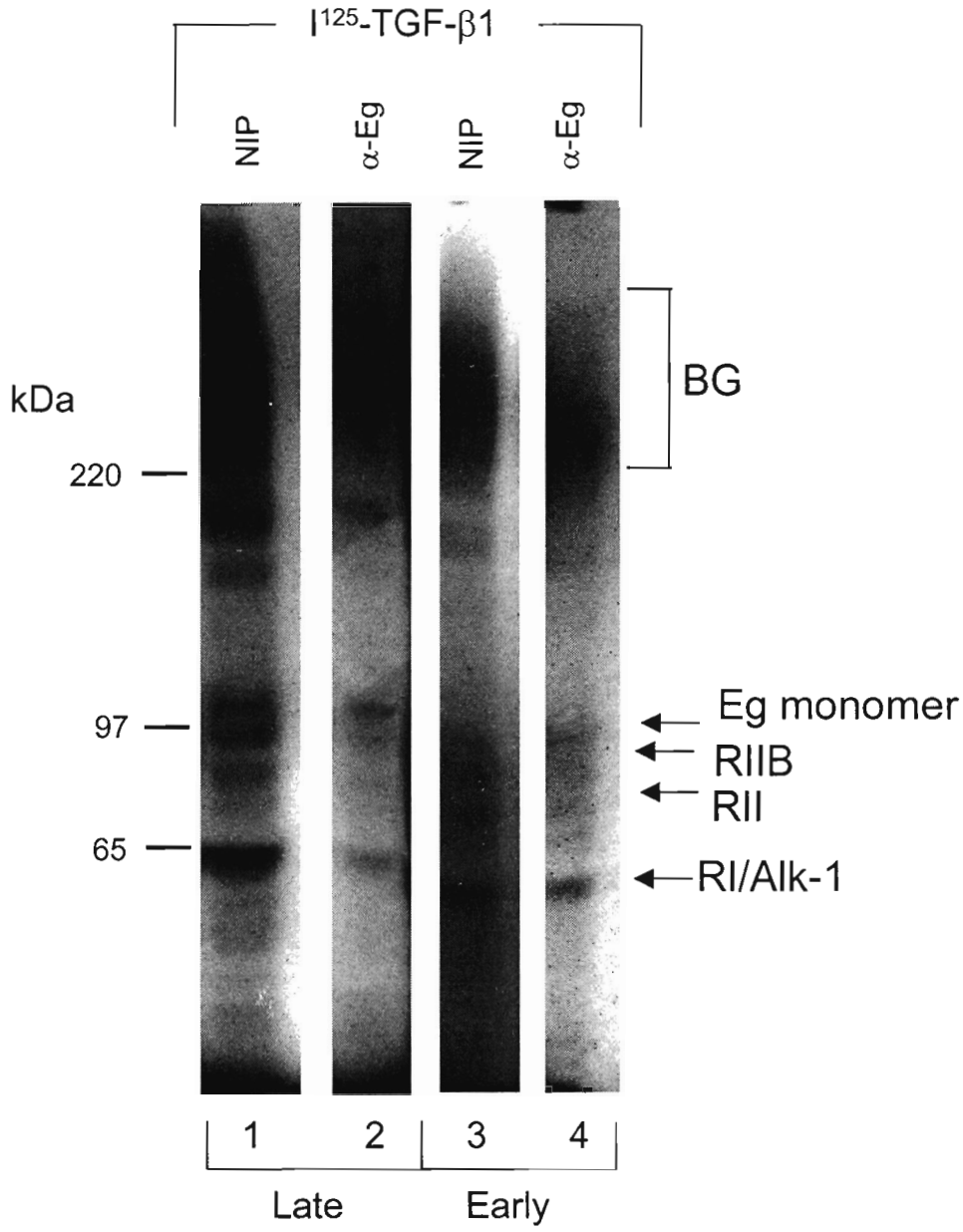

I125-TGF-β1
kDa
220
97
65
BG
Eg dimer
145 kDa
RIIB
RII
RI/Alk-1
1
2
Late
Rec

Fig. 7B


I125-TGF-β1
NIP
α-Eg
NIP
α-Eg
kDa
220
97
65
BG
Eg monomer
RIIB
RII
RI/Alk-1
1
2
3
4
Late
Early

448

Fig. 7C

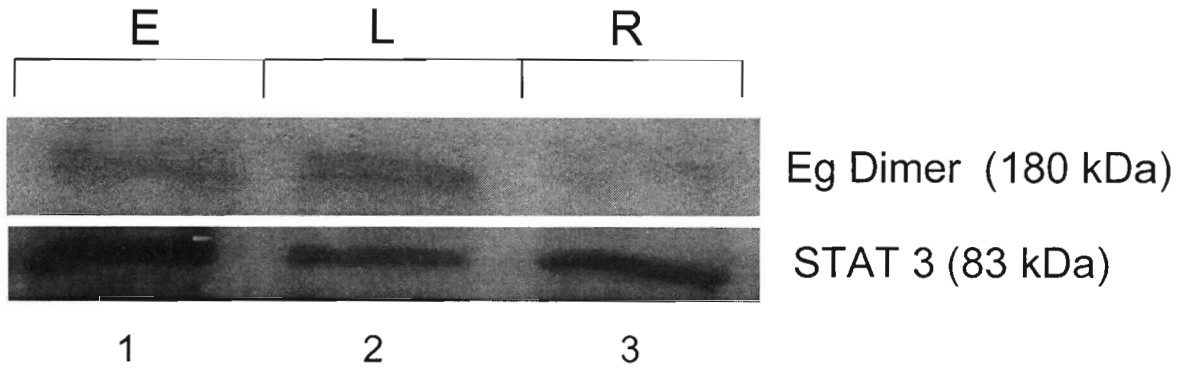
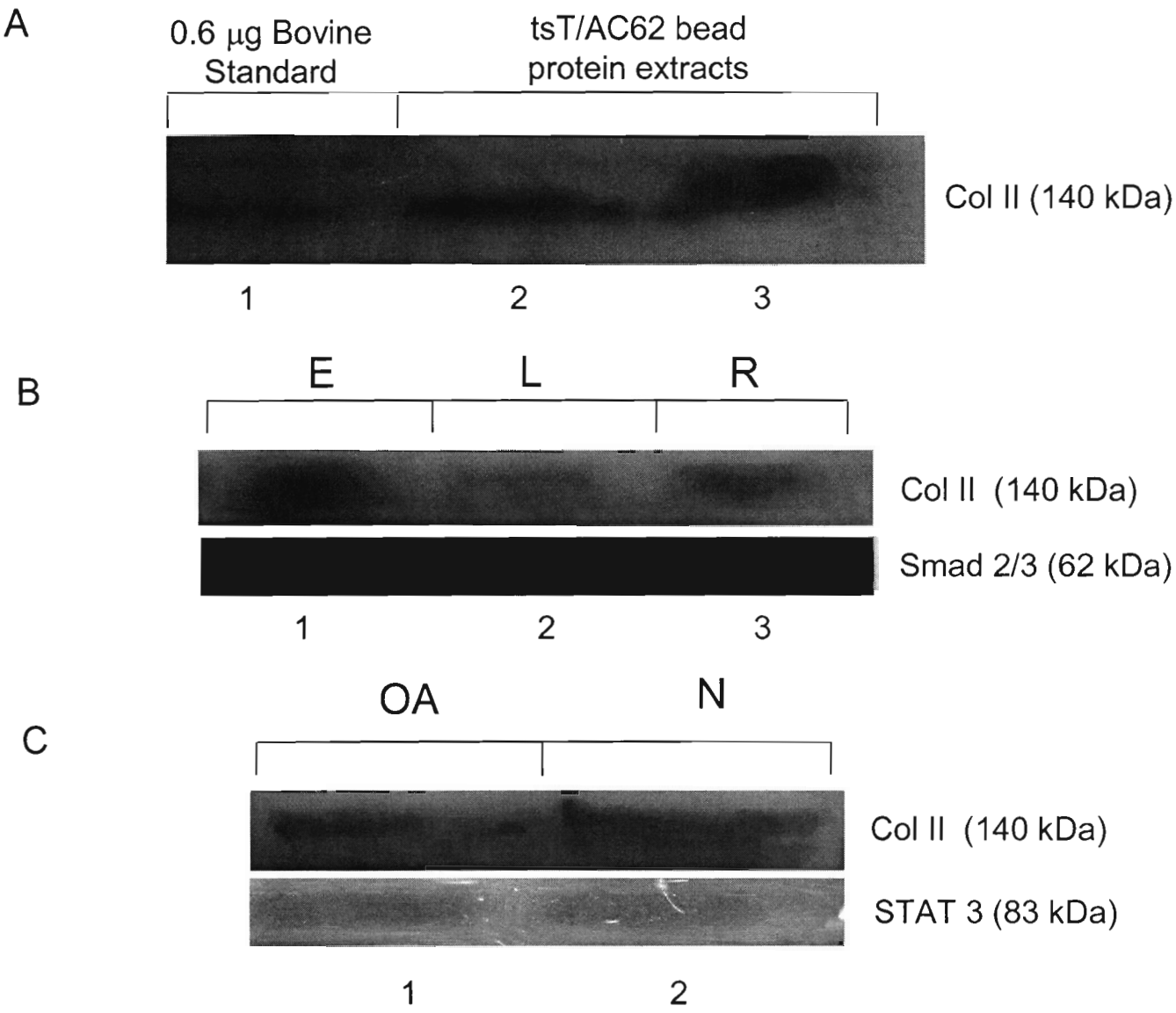


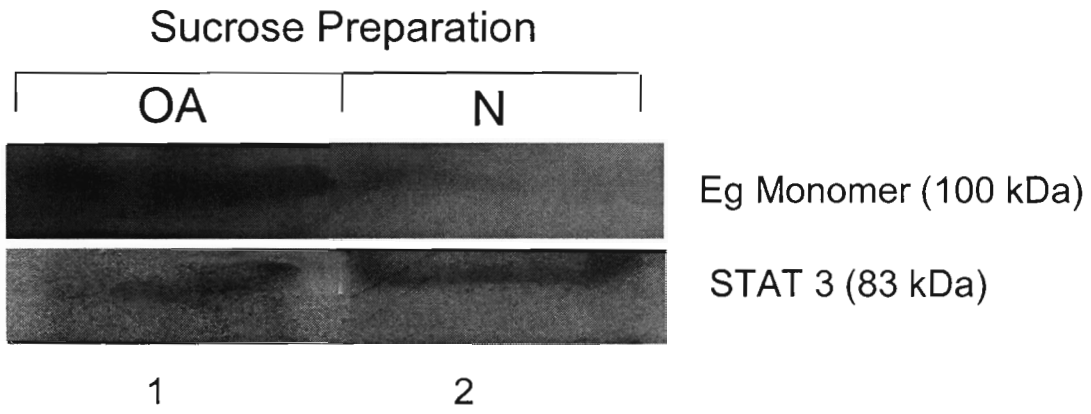
Fig.7D


A
0.6 µg Bovine Standard
tsT/AC62 bead protein extracts
Col II (140 kDa)
1
2
3
B
E
L
R
Col II (140 kDa)
Smad 2/3 (62 kDa)
1
2
3
C
OA
N
Col II (140 kDa)
STAT 3 (83 kDa)
1
2

450

Fig. 8A

A


Sucrose Preparation
OA
N
Eg Monomer (100 kDa)
STAT 3 (83 kDa)
1
2

451

B

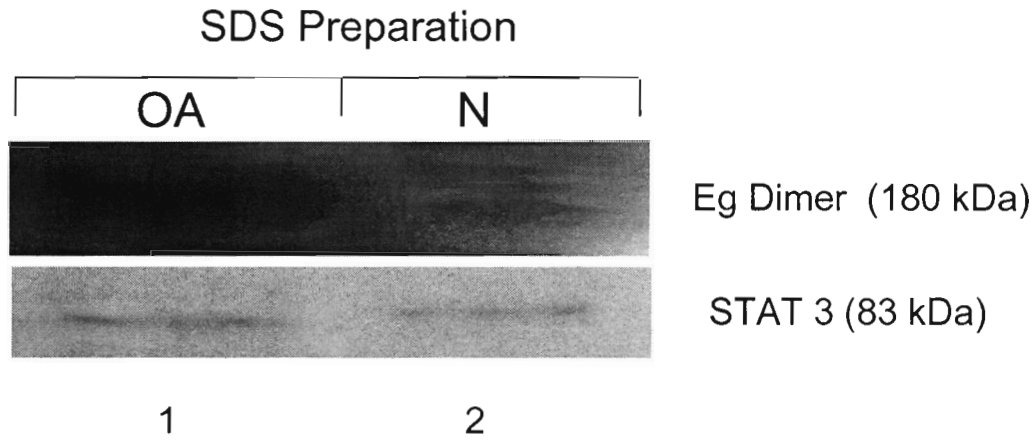
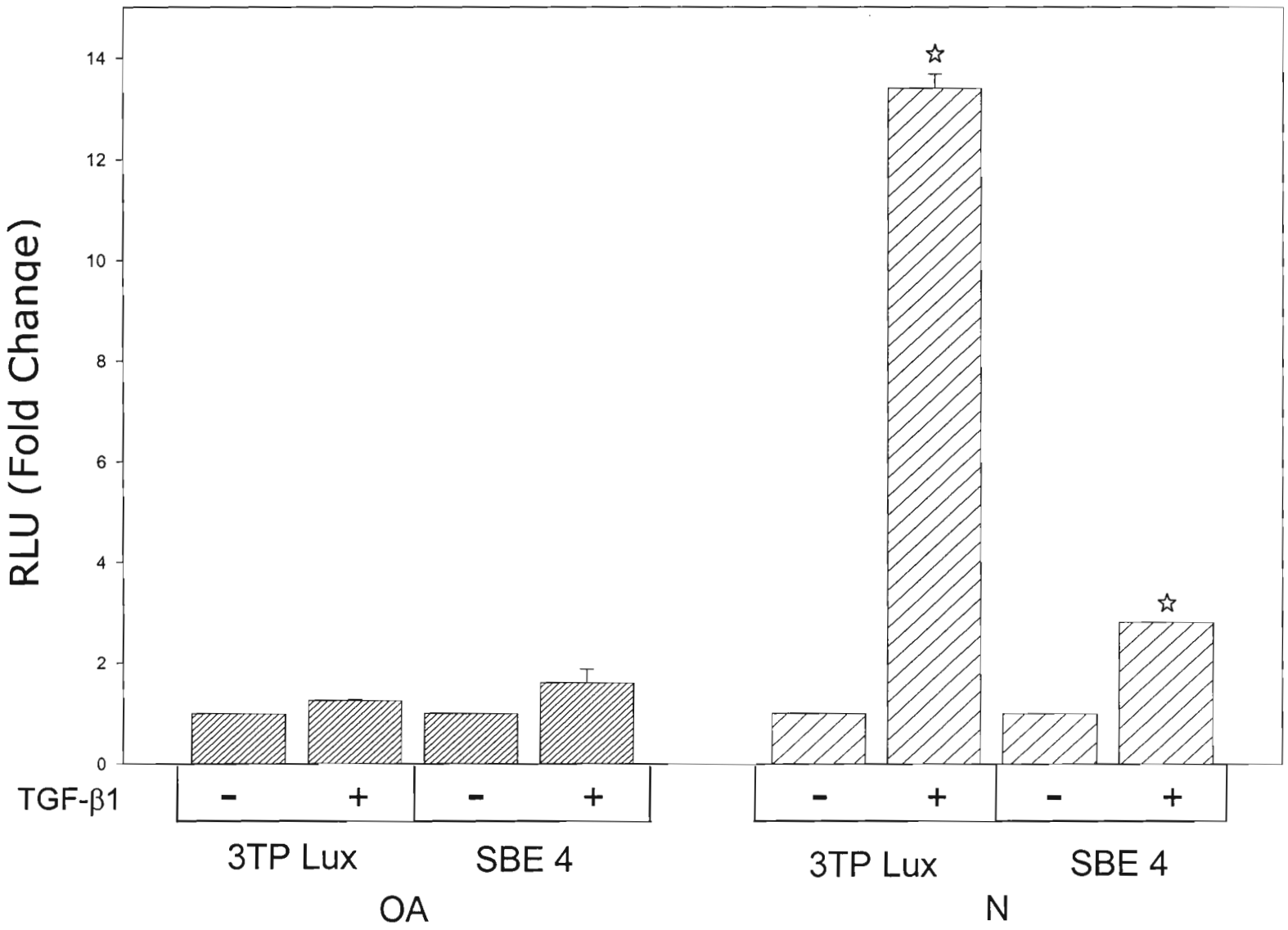
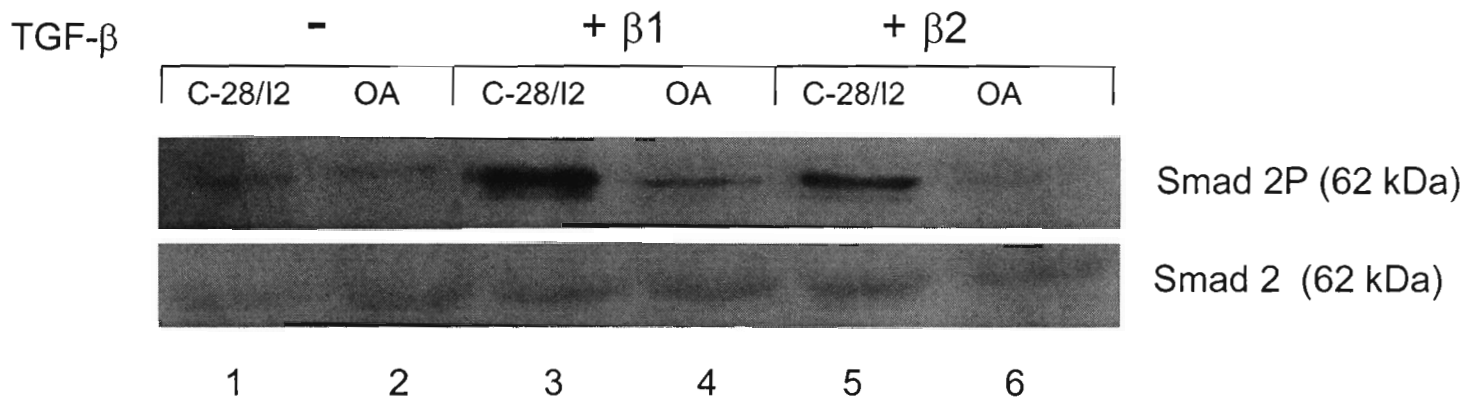

SDS Preparation
OA
N
Eg Dimer (180 kDa)
STAT 3 (83 kDa)
1
2

Fig. 8B

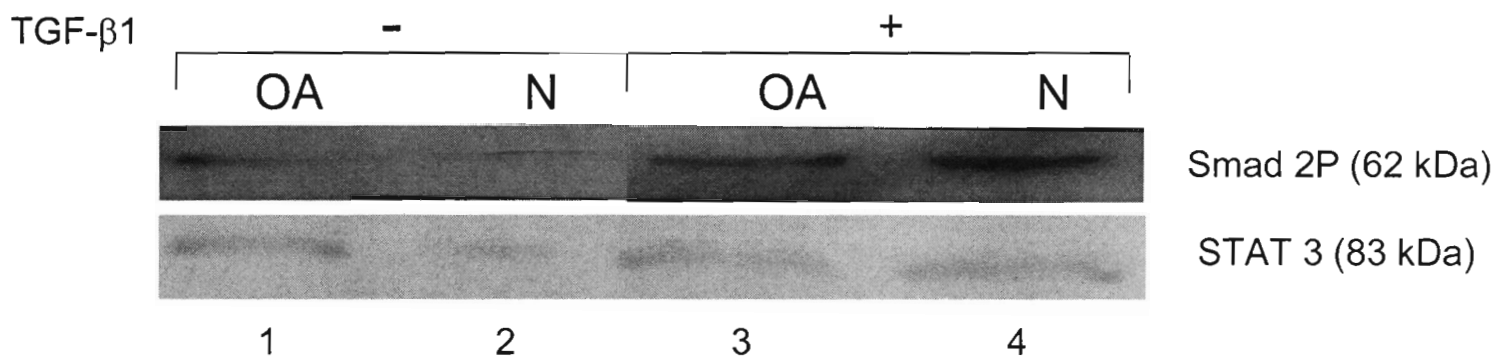

RLU (Fold Change)
14
12
10
8
6
4
2
0
TGF-β1
-
+
-
+
-
+
-
+
3TP Lux
SBE 4
3TP Lux
SBE 4
OA
N

452

A


TGF-β
-
+ β1
+ β2
C-28/I2
OA
C-28/I2
OA
C-28/I2
OA
Smad 2P (62 kDa)
Smad 2 (62 kDa)
1
2
3
4
5
6

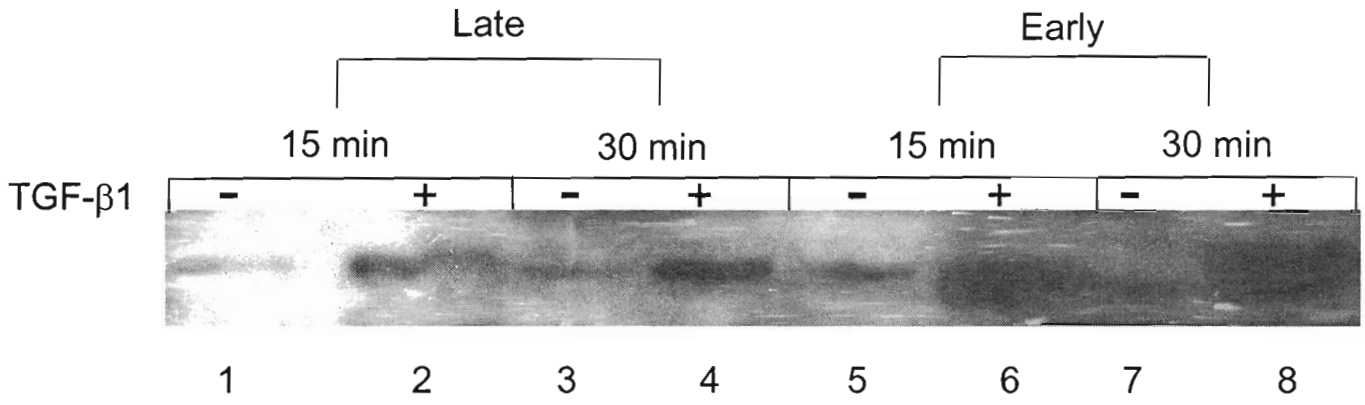
B


TGF-β1
-
+
OA
N
OA
N
Smad 2P (62 kDa)
STAT 3 (83 kDa)
1
2
3
4

ADDITIONAL FIGURE AND LEGEND

**CHAPTER 5**

Fig. H




Smad 2P (62 kDa)

**Fig. H. Dedifferentiated primary articular chondrocytes are less TGF-β responsive than dedifferentiated cells.** Stimulation of Smad 2 phosphorylation by TGF-β1. Normal articular cartilage was either enzymatically digested and plated through serial monolayer passages (Late) representing dedifferentiated cells or early passage monolayer cultures were obtained from articular cartilage explants (Early) representing differentiated cells. Chondrocytes were treated with 100pM of TGF-ß1 for 15 or 30 minutes (Lane 2,4,6,8; +) minutes or were left untreated (Lane 1,3,5,7; -), analyzed by SDS-PAGE (3-11% gradient) under reducing conditions. Western blotting was performed with an antibody against the phosphorylated form of Smad 2 and the ECL system was used for chemiluminescence detection.

# Section 3

## DISCUSSION

# DISCUSSION

## BACKGROUND AND OVERVIEW OF FINDINGS

The human articular cartilage, which covers the ends of diarthrodial joints, is an extremely complex tissue. The intricate association between chondrocytes, its sole host cell, and their surrounding specialized ECM provide cartilage with its unique visco-elastic properties. It is this character of hyaline cartilage which allows for a joint surface that not only resists force and absorbs shock but also permits for a gliding, painless range of motion.

Because of its prevalence in the elderly population (Sack 1995, Oddis 1996, Praemer et al 1999, Buckwalter and Lappin 2000, Buckwalter et al 2000), degenerative joint disease has been considered to result from tissue aging. However, it has now been ascertained that the processes which accompany the normal aging of articular cartilage are distinct from true pathologic states (Martin and Buckwalter 2002). Normal aging results in an increased risk of pathologic joint changes by compromising the ability of the chondrocyte to maintain or restore tissue. In the aging state, hyaline cartilage is vulnerable to secondary insults - morphologic, biochemical, or biomechanical in nature. Whether this insult is in the form of direct joint injury, inflammation, or an imbalance in regulatory factors in the microenvironment, it disrupts the tightly regulated ECM-chondrocyte milieu and leads to cartilage degeneration.

However, human articular cartilage degeneration does not result in healing with parent-like tissue as is seen with other connective tissues (Mankin 1982). Instead, following a partial thickness injury, inappropriate intrinsic repair with fibrocartilage is inevitable (reviewed in Mankin 1982). Extrinsic repair of full thickness defects, impinging on

subchondral bone, involves healing through fibrin clot formation and MSC precursors. Although this form of tissue regeneration may initially stimulate hyaline cartilage production, similar to intrinsic repair, it ultimately results in a fibrocartilage filled defect and a scarred unstable joint surface (Lieberman et al 2002, reviewed in Mankin 1982).

Throughout the preceding four decades, the approaches aimed at cartilage repair have been numerous and diverse. Attempts to promote extrinsic healing by converting partial thickness lesions to full thickness defects were made before it was fully appreciated that the nature of the tissue filling the defect was fibrocartilage and that this did not constitute a physiologically and biomechanically acceptable repair (Insall 1974, Mitchell and Shepard 1976). Attempts to transplant mature or precursor cells into the defect, in hopes that they would regenerate the deficient hyaline ECM, were equally unsuccessful. In addition, whole tissue explants of hyaline cartilage, periosteum, and perichondrium were attempted; however, these did not consistently incorporate into joint defects, and at best resulted in a mixed picture of hyaline and fibrocartilage and a non-uniform articular surface (Engkvist and Wilander 1979, Wakitani et al 1994, Odenbring et al 1992, Aston and Bentley 1986, Kawabe and Yoshinao 1991).

The lack of appropriate intrinsic hyaline repair by articular cartilage has been attributed to the avascular state of the tissue, the poor proliferative capacity of mature chondrocytes, and their phenotypic shift to a fibroblast-like cell.

The avascular state of cartilage impairs the normal wound healing process seen in other tissues; with injury, a fibrin clot does not form, inflammatory cells and mediators are not present, and importantly, no precursor cells are available for chondrogenesis or to reconstitute the ECM.

Controversy has surrounded the concept of chondrocyte proliferation. Mature chondrocytes, albeit in a relatively quiescent state within their ECM, are able to proliferate. In the hypertrophic phase of injury response, they replicate and form clusters or chondrons within the matrix representative of clonal colonies (van der Kraan and van den Berg 2000, reviewed in Mankin 1982).

Therefore, the shift in chondrocyte phenotype rather than a deficiency in cell population may be responsible for the inadequate repair. This phenotypic shift can similarly affect mature cells of explanted tissue introduced into the defect iatrogenically or bone marrow precursors from full thickness defects invading subchondral bone. This dedifferentiation of chondrocytes results in a fibroblastic cell that, as opposed to producing type II collagen typical of hyaline cartilage, synthesizes increased amounts of type I and III collagen (Benya and Shaffer 1982). The distinct rounded to polygonal morphology typical of articular chondrocytes, instead, becomes that of an elongated spindle-shaped cell with multiple long processes. Both of these features, the change in ECM components and change in cell morphology, are fundamental to the production of fibrocartilage. To date, the limited success in regenerating or repairing articular cartilage has encouraged researchers to focus on this phenomena of chondrocyte dedifferentiation and to elucidate which events promote or maintain the differentiated state.

Acknowledging advances and applying principles from the field of tissue engineering, several key players have been recognized as modulators of chondrocyte phenotype and thus, the tightly regulated cell–ECM microenvironment. These players include the 3-D matrix itself (the architectural component of the microenvironment), cytokines, growth factors and enzymes (the regulators within the microenvironment), and lastly, external forces which act to modulate these. Direct injury, inflammatory processes, and other

stresses such as change in pH, oxygen tension, and hydrostatic pressures are examples of such external forces acting on the chondrocyte microenvironment. Alternatively, external forces may have a more direct effect on chondrocytes by way of various intracellular signaling pathways mediated through cell surface receptors or cytoskeletal changes.

Paralleling this increasing understanding of chondrocyte dedifferentiation, attempts at cartilage repair have been made utilizing techniques that promote the type II collagen producing chondrocyte phenotype. Awareness that proliferation of cells in monolayer cultures results in progressive dedifferentiation has resulted in the use of 3-D tissue culture techniques (Thompson et al 1985, Guo et al 1989, Hauselmann 1992 and 1994, Redini et al 1997). Agarose, alginate, pellet, and suspension cultures have all been shown to promote chondrogenesis and to redifferentiate cells to their native phenotype (Hauselmann 1994, Bonaventure et al 1994, reviewed in Solursh 1991). Unfortunately, there have been relatively few *in vivo* approaches using 3-D culture systems to maintain cell phenotype. Instead, various non-biodegradable scaffolds have been seeded with mature and precursor cells and transplanted into defects; however, chondrocytes proliferate along these matrices in a manner similar to that seen in 2-D monolayer cultures and, ultimately, form fibrocartilage.

Cytokines, growth factors, and enzymes have long been regarded as key mediators in wound healing and tissue repair processes. In cartilage, they provide a means for chondrocytes to regulate their ECM in both an autocrine and paracrine manner (Ellingsworth et al 1986, Frazer et al 1991, Morales and Roberts 1988, Villiger et al 1993). These regulators are directly and intricately linked to matrix turnover in articular cartilage (Anastassiades et al 1998, Burton-Wurster and Lust 1990, Redini et al 1991, Morales and Roberts 1988, Venn et al 1990, Iqbal et al 2000, Pujol et al 1991, Okazaki et

al 1996, Yaeger 1997, Rosen et al 1988, de Haart et al 1999) and influence cell phenotype.

Despite conflicting reports of the role TGF-β plays in the regulation of chondrocyte phenotype and ECM turnover, the potential of this growth factor to modulate cartilage repair is explicit. Confounding factors of culture conditions, chondrocyte source and cell cycle state, the presence of other regulatory factors, as well as the delivery, concentration, and timing of TGF-β administration, seen in *in vitro* and *in vivo* studies, may be avoided through the use of OA murine models. A transgenic mouse expressing a DNRII (Serra et al 1997) and a Smad $3^{exon8}$ knockout (Yang et al 2001) both exhibit degenerative joint disease, implying, that dysregulation of the TGF-β signaling cascade results in a pathologic process of articular cartilage consistent with the development of OA. Similarly, administration of TGF-β was able to block IL-1 induced OA in murine joints (van Beuningen et al 1994 and 1993).

A convincing link has been established between TGF-β, articular cartilage injury, and attempted repair. TGF-β has been implicated in chondrocyte growth, differentiation, and ECM regulation; however, limited information is available as to TGF-β action in human chondrocytes. The diversity required by the TGF-β signaling cascade to mediate the various TGF-β governed responses in cartilage may be provided by heterogeneity in its receptor expression and their complex formations. Thus, defining the TGF-β receptor expression profile, novel receptor interactions, and their role in modulating TGF-β signaling will provide valuable information to better define the varied actions of TGF-β in human chondrocytes. Moreover, it has been suggested that diseased or injured chondrocytes, representative of a dedifferentiated phenotype, display a deranged receptor

profile (van den Berg 1999, Boumediene et al 1998a) that may be responsible for observed differences in their sensitivity to cytokines (Martel-Pelletier et al 1992, reviewed in Goldring 2000a). In addition, chondrocytes are thought to sense the altered composition and conformation of ECM seen in OA and RA. Whether these ECM alterations trigger the phenotypic change in the chondrocyte or alternatively, are a manifestation of dedifferentiated cells with abnormal synthetic and replicative properties has not been elucidated.

Throughout the course of these investigations, a TGF-β receptor expression profile on human chondrocytes has been established. The presence of the TGF-β signaling receptors, betaglycan, and RIIB was confirmed on these cells and, for the first time, endoglin, Alk-1, and Sol RI were identified. All receptors were demonstrated to form heteromeric complexes with betaglycan and the TGF-β signaling receptors. Endoglin overexpression was shown to inhibit TGF-β signaling with all three isoforms whereas betaglycan overexpression enhanced these responses. The use of morpholinos antisense oligos to inhibit endoglin expression resulted in up-regulation of TGF-β signaling. Overexpression of Alk-1, Sol RI, and RIIB was also seen to enhance TGF-β signaling. Interestingly, endogenous RIIB levels correlated with a decreased TGF-β responsiveness in phenotypically distinct cells. Moreover, evidence has been provided implicating RIIB and endoglin as markers of chondrocyte phenotype and modulators of ECM synthesis.

Pursuing the relevance of chondrocyte phenotype, cells were followed morphologically through serial passage monolayer cultures and their dedifferentiation documented. Redifferentiation and regained capacity for type II collagen production were demonstrated with 3-D alginate bead cultures. Chondrocytes derived from OA and RA

cartilage explants immediately (P0) displayed a phenotype consistent with dedifferentiated cells and, more importantly, OA cells demonstrated a decreased TGF-β responsiveness. Furthermore, the OA chondrocytes displayed high levels of RIIB and endoglin expression and a decrease type II collagen production establishing a critical link between novel receptors, phenotype, TGF-β responsiveness and ECM.

Identification of novel TGF-β receptors, their heteromeric complex formations and function, provide insight into TGF-β action in human chondrocytes. However, these detailed and specific findings increase in value when incorporated into the concept of the chondrocyte microenvironment. They provide a bridge between cell and matrix and when viewed from a wound healing or tissue engineering angle, become more applicable to cartilage regeneration.

RELEVANCE OF FINDINGS

**Cell Characterization and TGF-β Responsiveness**

Cells of all tissues do not exist in isolation. The appreciation of the microenvironment of cells has greatly increased the understanding of cellular function and the complexity of associations between the cell and its ECM. In no tissue is this more evident than in human cartilage. TGF-β has emerged as a key player within the chondrocyte microenvironment; it is a potent regulator of cell proliferation, phenotype, and ECM turnover. However, to modulate such diverse gene targets, TGF-β must transmit signals to the chondrocyte and the cells must in turn be responsive to these signals.

Therefore, these initial investigations involved characterization of human chondrocytes and demonstration of their responsiveness to TGF-β. Characterization of cells was based on the premise that differentiated articular chondrocytes produce two structural

macromolecules that have been used to define their specific phenotype, PG (aggrecan) and type II collagen (reviewed in Benya and Shaffer 1982). When removed from their ECM and grown in serial passage monolayers, these cells eventually stop producing type II collagen and assume a dedifferentiated state with a shift towards type I and III collagen production.

Although the phenotype of the immortalized human chondrocytes (C-28/I2 and tsT/AC62) used throughout these studies has been extensively characterized (Robbins et al 2000, Goldring and Berenbaum 1999), the expression of aggrecan and type II collagen mRNA was confirmed for these cell lines using RTPCR (manuscript 1, Fig. 1). In addition, primary human chondrocytes harvested through digestion of OA and normal articular cartilage were also shown to express aggrecan and type II collagen mRNA (manuscript 1, Fig. 1). The production of type II collagen by both cell lines and primary chondrocytes was also later illustrated at the protein level by Western blot (see below). Articular chondrocyte (tsT/AC-62 and normal primary cells) in early passage monolayer culture demonstrated a rounded to polygonal morphology and, as anticipated, became fibroblastic with serial passages. Cells suspended in 3-D alginate beads regained a rounded morphology and the capacity for type II collagen production (see below). These features, characteristic of human chondrocytes, allowed for the establishment of a cell culture system to investigate phenotypically distinct cells. Experiments performed on early passage chondrocytes in monolayer culture or those redifferentiated and recovered from 3-D alginate beads were considered to represent a differentiated phenotype whereas late passage cells were deemed to be in a dedifferentiated state.

The TGF-β responsiveness of these human chondrocytes was subsequently established. Smad proteins are the central mediators of TGF-β signaling and undergo phosphorylation by RI after it is activated by the ligand-RII complex. Thus, detection of intracellular phosphorylated Smad 2 is an indicator of cellular responsiveness to TGF-β. Human chondrocytes exhibited a dose and time dependent increase in Smad 2 phosphorylation with TGF-β1, -β2, and -β3 isoforms confirming their TGF-β sensitivity (manuscript 1, Fig. 1; Fig. Ai, Aiv). Interestingly, these cells appeared to be particularly responsive to the TGF-β2 isoform. It has been suggested that the TGF-β2 isoform plays a distinct role in bone related tissues (Rotzer et al 2001). Moreover, in tissues where TGF-β2 signaling is prominent, increased expression of betaglycan is noted. This is in keeping with the pronounced betaglycan expression which was later observed for human chondrocytes (see below).

As previously mentioned, TGF-β regulates diverse responses by chondrocytes including proliferation and ECM synthesis. Therefore, it was investigated whether TGF-β would regulate these parameters in the human chondrocyte lines and primary cells.

The growth response of human chondrocytes to TGF-β was studied. A [$^3$H]-Thymidine incorporation assay, used to determine DNA synthesis, demonstrated a triphasic response to TGF-β1 (Fig. Aii). Early passage differentiated chondrocytes were growth stimulated at low dose ranges (1-10 pM), inhibited at mid dose ranges (50 pM), and stimulated again at high doses (200 pM) of TGF-β illustrating a variable response with differing concentrations of TGF-β administered. TGF-β is known to result in differential responses at low and high dose ranges (Pepper et al 1993). Therefore, the triphasic nature of the

growth response demonstrated at varying concentrations of TGF-β (Fig. Aii) is not unusual.

In addition, cells may respond differently to cytokines and growth factors in a manner proportional to their state of differentiation or cell cycle. As growth plate chondrocytes mature, the mitogenic TGF-β response increases (Rosier et al 1989). It has also been shown that growth responsiveness depends on the proliferative state of the cell and therefore, the distribution of cells within each phase of the cell cycle (Sigel et al 1996). $G_0/G_1$ rabbit articular chondrocytes were growth inhibited by TGF-β whereas cells in S phase were stimulated (Vivien et al 1990 and 1992). Mitogenic affects of TGF-β also appear to differ with culture conditions. Rapidly proliferating undifferentiated rat microvascular endothelial cells in 2-D monolayers were growth inhibited by TGF-β1; however, 3-D cultures of these cells were refractory to TGF-β1 growth inhibition (Sankar et al 1996). Therefore, chondrocyte proliferation is likely influenced by the phenotype of the cells, their stage of cell cycle, and the concentration of TGF-β administered.

The ability of TGF-β to modulate the ECM of human chondrocytes was also investigated. Stimulation of PAI-promoter-reporter (Wrana et al 1994) activity by TGF-β was confirmed in these cells (manuscript 1, Fig. 1). Of note, the promoter up-regulation differed among cell types with costal (C-28/I2) exceeding that of articular cells (tsT/AC62 and primary). As alluded to previously, chondrocyte phenotype is thought to be factor in determining cellular responsiveness to TGF-β.

TGF-β was also seen to regulate activity of the Col 2a-lucifersase reporter (Fig. Aiii). A down-regulation of Col 2a gene transcription with TGF-β1 administration was observed. Similarly, IL-1 treatment, alone or in combination with TGF-β1, inhibited luciferase

activity. The inhibition of Col 2a gene transcription by TGF-β, although unanticipated, can be explained. TGF-β regulation of type II collagen has been plagued with controversy (Rosier et al 1989, Pujol et al 1991, Yaeger 1997, Rosen et al 1988, de Haart et al 1999). The inconsistency of type II collagen production by TGF-β (stimulation or inhibition) has been attributed to variability in culture conditions, cell source, age, phenotype and presence of additional regulatory agents (O'Connor et al 2000). The down-regulation of Col 2a activity observed here (Fig. Aiii) might be explained by an inappropriate concentration of TGF-β treatment. The concentration of TGF-β used to stimulate the cells was the same as was used to measure PAI-induced luciferase activity (manuscript 1, Fig. 1) but may not adequately stimulate the Col 2a gene. As noted, despite extensive *in vitro* investigations, the role of TGF-β in modulating type II collagen is controversial and inconsistent (O'Connor et al 2000). Therefore, stimulation of type II collagen by TGF-β was not investigated; however, if used, higher concentrations of TGF-β may have enhanced type II collagen levels and Col 2a gene transcription. Despite this, the modulation of Col 2a gene transcription by TGF-β confirmed the responsiveness of human chondrocytes and suggests that TGF-β is a regulator of their ECM.

Human chondrocytes are not only responsive to TGF-β but also have been shown to produce TGF-β which then functions in an autocrine or paracrine manner. Human cartilage explants have also been shown to produce TGF-β (Lafeber et al 1993). In addition, mRNA for all three TGF-β isoforms has been detected in human chondrocytes grown in monolayer and protein has been found in chondrocyte culture medium; both were regulated by IL-1 and IL-6 (Villiger 1993). To confirm the production of TGF-β by the human chondrocyte cell lines (C-28/I2 and tsT/AC62), stably transfected mink lung

epithelial cells containing a PAI-1-luciferase reporter construct were treated with culture medium obtained from the chondrocytes after 24 hours in culture (Fig. B). Interestingly, the articular cells (tsT/AC62) produced slightly more TGF-β than the costal cells (C-28/I2) but a decreased percentage of the total produced was in the active form. It is important to consider endogenous TGF-β production when interpreting results of experiments. Variation in endogenous TGF-β production by different cell types or cells at different states of differentiation may interfere with responses of cells to exogenous TGF-β administration. Therefore, serum starvation of cells prior to treatment with exogenous ligand, repeated washing of cells, and application of acid washes (Glick et al 1990) to remove endogenous ligand were utilized in the subsequent investigations.

Thus, the human chondrocyte cell lines and primary cells to be used for the remaining investigations were characterized and their responsiveness to TGF-β, proliferation and ECM regulation, was demonstrated.

**TGF-β Receptors and Signaling**

The effects of TGF-β on human chondrocytes are diverse. Other than ECM turnover and cell proliferation, cell differentiation (chondrogenesis) is also under the influence of TGF-β (Urist 1965, Reddi and Huggins 1972, Hanamura et al 1980, Seyedin et al 1983, 1985, and 1986, Sampath et al 1987, MacKay 1998, Nishimura et al 1999, Worster et al 2000). With respect to ECM regulation, type II collagen levels have been shown to be modulated by TGF-β (Rosier et al 1989, Pujol et al 1991, Yeager 1997, Rosen et al 1988, de Haart et al 1999); in addition, fibronectin, PG and other noncollagenous proteins are TGF-β regulated (O'Keefe et al 1988, Howard and Anastasiades 1993, Burton-Wurster and Lust 1990, Redini et al 1991 and 1988, Venn et al 1990). Moreover, TGF-β administration has

been shown to alter synthesis of other ECM regulating cytokines in the chondrocyte microenvironment (reviewed in Goldring 2000a and 2000b).

Despite this observed multifunctional role in cartilage, TGF-β signals through a tightly regulated and highly structured pathway with Smads as the central mediators. The accepted TGF-β signaling paradigm dictates that two serine/threonine protein kinase receptors (RI and RII) bind ligand and that a family of central mediators of signaling, known as Smads, act as substrates for activated RI. The Smads are translocated to the nucleus as an assembly to regulate transcription (Fig. a). There is a need, however, to account for the multitude of downstream targets of TGF-β and how a seemingly simplistic cascade can achieve such diverse responses. Novel TGF-β receptors on the surface of human chondrocytes may provide such an avenue to locally modulate TGF-β signaling and specify a definitive downstream response. Despite this, information regarding the expression of TGF-β receptors in human chondrocytes is limited.

RI, RII, and betaglycan expression have been described in several animal growth plate models (Matsunaga et al 1999, O'Grady et al 1991, Hall et al 1996) and similarly on articular cells (Fukumura et al 1998). With respect to human chondrocytes, rapid turnover of TGF-β receptors has been described for growth plate chondrocytes (Centrella et al 1996). RI and RII have been demonstrated in hypertrophic and mineralizing zones of human growth plate chondrocytes (Horner et al 1998, Moldovan et al 1997) constituting the only studies describing TGF-β receptors in human articular cells. Therefore, studies were designed to define the expression profile of TGF-β receptors on human chondrocytes, to identify and determine the requirements of TGF-β receptor interactions

on the cell surface, and to determine the functional role of TGF-β receptors and complexes in regulating TGF-β signaling in human chondrocytes.

Using affinity labeling techniques, in combination with immunoprecipitation and Western blot, the TGF-β receptor profile on human chondrocytes was characterized. The presence of the types I and II signaling receptors (RI and RII) and betaglycan, was confirmed on human chondrocytes (manuscript 1, Fig. 2, 3, 5A; Fig. C, D, E, F, G). RIIB, the spliced variant of RII previously described on human chondrocytes (Glansbeek et al 1997), was confirmed on these human chondrocyte lines and primary cells (manuscript 4, Fig 1, 2A, 2C, 3, 4). In addition, for the first time, Sol RI, Alk-1 (manuscript 2, Fig. 1, 2, 3), and endoglin (manuscript 1, Fig. 4, 5A, 5B, 7; Fig. Gi, Gii), were found to be expressed on human chondrocytes.

Several variants of the signaling receptors as well as accessory TGF-β binding proteins have been identified on the surface of many cell types. Furthermore, the role these binding proteins play in modulating signaling is becoming increasingly complex. RII initiates signaling by binding ligand. The presence of a spliced variant of this receptor, RIIB, confirmed here in human chondrocytes, has been described in other cells (Hirai and Fujita 1996, Rotzer et al 2001). It was also observed in human chondrocytes but mistaken for RII (Glansbeek et al 1993 and 1997). It is thought to be important in transmission of TGF-β2 signals and has been demonstrated to form heteromeric complexes with RI and RII (Hirai and Fujita 1996, Rotzer et al 2001). The soluble variant of the type I receptor (Sol RI) identified here was cloned from a neonatal rat kidney cDNA library (Choi 1999). Alk-1 is a member of the 8 identified type I signaling receptors of the TGF-β superfamily (reviewed in de Caestecker et al 2002). Although the ligand and corresponding type II

receptor for Alk-1 are unknown, Alk-1 is able to form complexes with the TGF-β signaling receptors (Attisano and Wrana 1998, ten Dijke et al 1994, De Winter et al 1996). Betaglycan and endoglin, identified here on human chondrocytes, are accessory TGF-β receptors. Accessory receptors are TGF-β binding proteins which do not have an intracellular kinase domain but are able to bind ligand at the cell surface. These receptors have been identified in isolation and in complexes with the signaling receptors. Betaglycan is the most prevalent of these accessory receptors (originally described as the type III TGF-β receptor) (Massague 1985, Fanger et al 1986). It has high affinity for TGF-β and -β2 isoforms (Cheifetz et al 1987 and 1988), and is found on the surface of cells with the same frequency as the signaling receptors (Cheifetz et al 1988). Endoglin shares 71% sequence homology with betaglycan (Cheifetz et al 1992, Gougos and Letarte 1990, Bellon et al 1993) and binds TGF-β1 and -β3 with high affinity (Wrana et al 1992) but does not demonstrate affinity for TGF-β2 (Cheifetz et al 1992, Miyazono et al 1993). Therefore, through the investigations presented here, the TGF-β receptor expression profile of human chondrocytes has taken shape. Moreover, various heteromeric receptor interactions were identified. RIIB, Sol RI, Alk-1, and endoglin were observed in complexes with the signaling receptors, RI and RII. The three novel receptors, Alk-1 and Sol RI (manuscript 2, Fig. 3, 5, 6), and endoglin (manuscript 1, Fig. 5A, 5B, 6, 7) were also seen to associate with betaglycan on the chondrocyte surface. Notably, these associations occurred in the presence of ligand and ligand independent manner. In addition, heteromeric complexes were noted between RIIB and endoglin (manuscript 4, Fig. 3B, 3C, 4A), Alk-1 and Sol RI (manuscript 2, Fig. 5A, 5B), RIIB and Alk-1, as well

as RIIB and Sol RI (both in manuscript 4, Fig. 3B, 3C, 4A). All of these complexes were observed at physiologic receptor concentrations and ratios.

It was also demonstrated that the Sol RI-betaglycan complex formed in the absence of the GAG chains of betaglycan (manuscript 2, Fig. 3B). Similarly the endoglin-betaglycan association was depicted in the absence of these carbohydrate side chains (manuscript 3, Fig. 5B, 6). More importantly, it was shown that the endoglin-betaglycan heteromeric complex formed independently of RII (manuscript 1, Fig. 5C; manuscript 3, Fig. 3). This confirmed that this receptor interaction was not a consequence of endoglin and betaglycan's independent associations with the signaling receptors.

The existence of such an extensive group of TGF-β binding proteins on human chondrocytes and their multiple complex formations is consistent with the notion that receptors expressed on the cell surface may modulate TGF-β signaling.

The co-existence of Alk-1 and Alk-5 on endothelial cells and their opposing effects on cell migration and proliferation suggests that TGF-β regulates the activation states of these cells via a fine balance of Alk-1 and Alk-5 signaling (Goumans et al 2002). RIIB is able to mediate TGF-β2 signaling via the Smad pathway in the absence of betaglycan (Rotzer et al 2001). Sol RI is a functional receptor which appears to act either as an agonist to TGF-β action or a ligand chaperone (Choi 1999). A recombinant form of this receptor was constructed from the extracellular domain of RI. In the absence of TGF-β this receptor variant mimicked TGF-β induced transcriptional and growth responses (Docagne et al 2001). Betaglycan and endoglin also regulate TGF-β signaling through modifying ligand donation to the signaling receptors (Cheifetz et al 1987 and 1988, Ohta et al 1987, Ignotz and Massague 1987, Bassols and Massague 1988, Sankar et al 1995,

Cheifetz et al 1992, Zhang et al 1996, Lastres et al 1996, Miyazono et al 1993), through biochemical interactions with these receptors (Eickelberg et al 2002), and through interactions with their CDs altering levels of phosphorylation and subsequent activation (Guerrero-Esteo et al 2002).

It is also possible that ratios of receptors and thus their relative contributions in heteromeric complexes may define the intended downstream target of TGF-β signaling. Evidence for this theory can be found within of the TGF-β superfamily.

It has been suggested for BMPs that signaling specificity may be determined in part by the particular type I receptor complexed with the type II receptor and the ligand associated. In developing bone and precursor cells, the type Ib signaling receptor regulated apoptosis whereas the type Ia receptor maintained cell differentiation (Zou and Niswander 1996, Zou et al 1997, Chen et al 1998). Studies have shown that the preformed complex preferentially signals through the Smad pathway whereas the BMP 2-ligand-induced complex signals through the p38/MAPK pathway to regulate alkaline phosphatase activity (Nohe et al 2002, Knaus and Sebald 2001). Rabbit periosteal grafts (*in vitro*), while undergoing chondrogenesis, differentially express the type I and II BMP receptor combinations. The type Ib receptor expression increases in response to TGF-β1 treatment and precedes increases in type II collagen expression (Sanyal et al 2002).

Thus, it is evident that novel TGF-β binding proteins exist on cells and form heteromeric complexes with the signaling receptors; these receptors and their complexes increase the diversity of the signaling cascade and provide a means of modulating TGF-β activity. Therefore, the next objective was to determine the role of these novel receptors, identified on human chondrocytes, in TGF-β signaling regulation.

Overexpression of Alk-1 and Sol RI in human chondrocytes resulted in an enhancement of TGF-β signaling (manuscript 2, Fig. 7). This is consistent with previous investigations in other cells. Sol RI has been shown to bind TGF-β1 in the presence of RII and regulate signaling (Choi 1999). In the presence of RII, Alk-1 binds TGF-β1 and in the presence of ActRII binds activin A (Attisano and Wrana 1998, ten Dijke et al 1994, De Winter et al 1996). Alk-1 shares a high degree of similarity with the other type I receptors (Attisano et al 1993, Hanks et al 1988, ten Dijke et al 1993) and in its constitutively active state it phosphorylates Smad 1 and 5 (Macias-Silva et al 1998). In addition to binding TGF-β1 and activin A, Alk-1 has been shown to bind TGF-β3 and an unknown ligand present in the serum (Lux et al 1999).

Similarly, RIIB overexpression resulted in an enhancement of TGF-β signaling (manuscript 4, Fig. 6A, 6B). Interestingly, increased endogenous levels of RIIB in chondrocytes correlated with a decrease in TGF-β responsiveness (manuscript 4, Fig. 5) suggesting that RIIB expression may play a more diverse role in these cells. RIIB has been shown here to bind all three TGF-β isoforms and modulate signaling. In addition, in the absence of betaglycan, RIIB overexpression has been shown to restore TGF-β signaling (Rotzer et al 2001). It is possible that RIIB may compete for ligand with RII at the cell surface. Alternatively, through its formation of various heteromeric receptor complexes, RIIB may prevent the RII/RI association as has been seen with betaglycan (Eickelberg et al 2002) or modulate other interactions between novel TGF-β receptors and the signaling receptors.

Endoglin was shown to be expressed on human chondrocytes at levels comparable to human microvascular endothelial cells (HMEC-1) (manuscript 1, Fig. 4). Considering the

avascular nature of cartilage and the high levels of endoglin expression in this tissue, the role of endoglin was intriguing. Thus, an important objective was to determine the functional significance of this novel receptor on human chondrocytes. Overexpression of endoglin was found to inhibit TGF-β signaling whereas betaglycan enhanced signaling. This was evident for all 3 TGF-β isoforms (manuscript 3, Fig. 7). Similarly, Morpholinos antisense oligos were used to bind and inactivate endoglin mRNA sequences. As anticipated from overexpression studies, blocking endoglin expression with antisense resulted in an up-regulation of Smad 2 phosphorylation with a corresponding decrease in endoglin expression (manuscript 5, Fig. 5A, 5B, 6A, 6B).

The ability of betaglycan, and more importantly endoglin, to modulate signaling of all three TGF-β isoforms is interesting. It has previously been shown that a soluble form of betaglycan prevents binding of ligand to the signaling receptors and therefore blocks TGF-β actions (Lopez-Casillas et al 1994). This was most dramatic for TGF-β2 activity (Segarini et al 1987, Andres 1991). Two regions of betaglycan's ECD bind ligand, one region at the amino terminal end, known as the endoglin related region (Lopez-Casillas et al 1994) and one region at the C terminal end, or uromodulin related domain, are important for inhibin A binding (Pepin et al 1994). Both of these domains bind TGF-β2 with more affinity than -β1; however, only the endoglin related region increases -β2 labeling of RII (Esparza-Lopez et al 2001). Despite this, both domains cause a TGF-β2 dependent increase in Smad 2 phosphorylation. These structural details provide insight into the endoglin-betaglycan heteromeric complex and its regulation of TGF-β2 signaling.

For example, endoglin has not been shown to bind TGF-β2 (Cheifetz et al 1992, Miyazono et al 1993). However, in these studies, when human chondrocytes were stimulated with TGF-β2, endoglin overexpression still resulted in inhibition of the TGF-β2 response and was able to down-regulate the betaglycan stimulation (manuscript 3, Fig. 7). Endoglin may be mediating its effects on TGF-β signaling through direct interactions with RI and RII. *In vitro* phosphorylation assays have demonstrated the phosphorylation of endoglin's CD by RI and RII and the regulation of RI and RII phosphorylation by endoglin (Guerrero-Esteo et al 2002). On the other hand, it is possible that with the endoglin-betaglycan heteromeric complex formation a structural change occurs which prevents the donation of ligand to RII. If this structural change occurred in the N terminal (endoglin associated) domain of betaglycan, preventing TGF-β2 donation, this could potentially explain endoglin's ability to inhibit signaling despite not binding TGF-β2.

The rigid paradigm of the TGF-β signaling cascade is gradually becoming more flexible. Evidence to support this change is apparent from the following endoglin-ligand binding studies. Initial investigations of the determinants of the endoglin-betaglycan heteromeric complex suggested that this was a RII independent association. These studies were performed with overexpression of endoglin and betaglycan in 293 cells, severely deficient in RII (Wong et al 2000) (manuscript 1, Fig. 5C). It was noted that in control experiments, where receptors were not overexpressed, a band corresponding to endoglin was apparent; however, endoglin had not previously been documented on 293 cells. Therefore, the expression of endoglin on several other cell types (293, DR26, and L6 myoblasts) was investigated and endoglin was found to be present on these cells, but at levels lower than observed for HMEC-1 or chondrocytes (manuscript 3, Fig. 1). As

mentioned, 293 cells are severely deficient in RII (Wong et al 2000). DR26 cells are mutants of mink lung epithelial cells which do not express RII (Laiho et al 1990) and L6 myoblasts which do not expressing betaglycan (Wang et al 1991, Lopez-Casillas et al 1991, Blobe et al 2001). Utilizing the above properties of these cell lines and the knowledge of their endoglin expression permitted investigations as to the requirements of ligand binding by endoglin.

The initial aim was to confirm the RII independent nature of the endoglin-betaglycan association. The co-immunoprecipitation of endoglin and betaglycan in DR26 cells (not expressing RII) provided this confirmation (manuscript 3, Fig. 3B, 3C). To illustrate that the endoglin-betaglycan complex could form in the absence of a functional RII in human chondrocytes, cells were transfected with the dominant negative form of RII (DNRII). This construct contains a mutant form of RII which binds to RII either intracellularly or at the cell surface and prevents a TGF-β signal from being transduced (manuscript 3, Fig. 4A). Despite the inactivation of RII, the heteromeric endoglin-betaglycan complex was still observed (manuscript 3, Fig. 4B).

Affinity labeling, followed by immunoprecipitation, was used to demonstrate the co-immunoprecipitation of endoglin and betaglycan. This technique relies on the binding of $^{125}$I-TGF-β1 by endoglin and betaglycan to visualize the receptor complex. Therefore, endoglin was observed not only to form a complex with betaglycan independently of RII but also was able to bind ligand independently of RII. This is the first time that endoglin has been demonstrated to bind ligand in the absence of the TGF-β signaling receptors.

It has been previously argued that endoglin is unable to bind ligand in the absence of RII (Barbara et al 1999, Letamendia et al 1998a). These previous studies both involved

transient transfections with endoglin and RII in affinity labeled COS cells and were unable to detect the presence of endoglin without RII overexpression. Interestingly, RII was immunoprecipitated in cells overexpressing endoglin; however, the presence of this endogenous RII did not allow detection of endoglin (Barbara et al 1999) which contradicts their argument. The discrepancy between these authors findings and those seen here, using 293, DR26 cells, and human chondrocytes (manuscript 3, Fig. 2,3,4B), could be explained in several ways. The investigations performed by the previous authors were done in COS cells which exhibit a different receptor profile and binding affinities than the cells used here. Poor separation between the endoglin monomer and RII is noted in these COS cells and the band observed may not represent endoglin but instead be the overexpressed RII. (Letamendia et al 1998a). It is difficult to conclude in their study that endoglin required RII to bind ligand as only portions of the autorads are included and the quality is not optimal both of which may impair endoglin detection (Letamendia et al 1998a). Similarly, in the study by Barbara et al, there appears to be unequal protein loading in the non-immunoprecipitated lanes of transfected COS cells (Barbara et al 1999). This may also be obscuring endoglin detection.

The requirement of betaglycan for the endoglin-ligand interaction was subsequently investigated. L6 myoblasts (not expressing betaglycan) stably transfected with full-length betaglycan and betaglycan absent in its CD, and transiently transfected parent cells expressing betaglycan deficient in its CD or GAG chains were used. Results demonstrated that betaglycan overexpression enhanced ligand binding by endoglin (manuscript 3, Fig. 2) and, in fact, betaglycan expression was a requirement of this receptor-ligand interaction (manuscript 3, Fig. 5). In addition, the co-immunoprecipitation

of betaglycan and endoglin was confirmed to occur independently of betaglycan's CD and GAG chains (manuscript 3, Fig. 6A, 6C).

Thus, it is clearly evident that multiple TGF-β binding proteins are present on human chondrocytes and that these receptors, through biochemical interactions, form heteromeric complexes in various combinations. Evidence suggests that those associations involving betaglycan tend to be of the ectodomain core independent of its GAG chains or CD. It has been demonstrated that overexpression or blocking of various receptor expression modulates TGF-β signaling in these cells. Taken together with the previous findings of receptor regulation in other systems, the potential of receptor expression to regulate TGF-β action in human chondrocytes is convincing. Moreover, the novel finding that endoglin binds ligand in the absence of RII refutes the TGF-β signaling paradigm. Endoglin, if able to bind ligand independently of the signaling receptor complex, may mediate its effects on the TGF-β cascade indirectly through interactions with other novel receptors or by regulating ligand bioavailability. This provides an additional level of diversity to its seemingly rigid scheme.

The TGF-β binding protein complexes on the surface of human chondrocytes are likely fluid in nature (Fig. c). This heterogeneity in endogenous ratios or concentrations of individual receptors may be critical in specifying the relative contributions of various TGF-β signaling pathways. In addition, endoglin and other novel receptors may act independently of TGF-β in conjunction with other signaling pathways. Therefore, modification of receptor expression through overexpression or morpholino antisense provides an avenue to locally regulate TGF-β action in these cells.

**TGF-β Receptors, Chondrocyte Phenotype and their Microenvironment**

Although controversial, TGF-β's role in regulating ECM turnover is evident (O'Connor et al 2000). To determine whether changes in TGF-β receptor expression could result in changes in ECM, effects on type II collagen were investigated. Overexpression of RIIB was noted to increase type II collagen protein levels (manuscript 4, Fig. 6D). Endoglin, however, when overexpressed decreased type II collagen levels and when expression was blocked, by morpholino antisense, increased type II collagen levels (manuscript 5, Fig. 6C).

The ability of TGF-β receptor levels to modulate ECM synthesis provides evidence of the intricate association between the cell, its phenotype and the microenvironment. The concept of cell phenotype is one of the most important advancements in the field of tissue engineering and repair. The process of chondrocyte dedifferentiation has been recognized in zones of attempted cartilage repair, both intrinsic and extrinsic, and was implicated in the fibrocartilage healing response (Sandell and Aigner 2001, Aigner and Dudhia 1997). In addition, chondrocytes of OA and RA cartilage are now recognized as having a distinct phenotype which changes as the disease progresses (Fukui et al 2001). OA cells re-express type IIa procollagen, demonstrate enhanced Sox9 levels and, similar to hypertrophic cells, increased production of type III and X collagen (Fukui et al 2001).

To further investigate the association of cell phenotype, ECM, and the TGF-β signaling cascade, the phenotype of the human chondrocytes were documented in varied culture conditions. Cell lines (C-28/I2 and tsT/AC62) were serially passaged in monolayer cultures and morphology was documented through photomicroscopy. Costal chondrocytes (C-28/I2) maintained a rounded to polygonal morphology throughout sequential passages and after three weeks in 3-D alginate bead cultures (manuscript 5,

Fig. 1A). Articular cells (tsT/AC62), however, became fibroblastic after several passages in monolayer cultures (manuscript 5, Fig. 2A). The same dedifferentiation was noted for primary articular chondrocytes after serial monolayer culturing (manuscript 5, Fig. 3A and data not shown). When resuspended in 3-D alginate beads, the morphology of these cells returned to that of a differentiated cell with a rounded appearance and was maintained at three weeks in culture (manuscript 5, Fig. 1B, 2B, 2C). In addition, these redifferentiated chondrocytes were shown to re-express type II collagen (manuscript 5, Fig. 7D).

OA, RA, and normal cartilage explants were also cultured. Within three weeks, chondrocytes originating from the explants were observed to proliferate in monolayer (manuscript 5, Fig. 3B, 4B, 4C). Interestingly, cells originating from the OA and RA explants were morphologically similar to the dedifferentiated articular chondrocytes seen after repeated passages in monolayer cultures; they originated from the explants (P0) with a fibroblast-like appearance (manuscript 5, Fig. 4A, 4B, 4C). This suggests that OA and RA chondrocytes are representative of dedifferentiated cells, that this phenotypic shift is not necessarily a product of non-physiologic monolayer cultures, and that other factors in the chondrocyte microenvironment must be influencing cell phenotype.

The current concept is that the chondrocyte, as a result of various cues from its microenvironment, shifts ECM production and morphology which reflect the cell phenotype. It is also recognized that the cell may also alter receptor and other protein expression on its surface as an indicator of its state of differentiation or in response to this change.

Thus, the expression levels of RIIB and endoglin in human chondrocytes and the correlation with cell phenotype were studied to determine the validity of this hypothesis.

Endogenous RIIB expression was found to be increased in early and recovered passage chondrocytes (manuscript 4, Fig. 4B, 4C). However, RIIB was expressed in OA cells to a greater extent than normal primary articular chondrocytes (manuscript 4, Fig. 4D). This seems contradictory in that early and recovered cells represent a more differentiated phenotype whereas OA cells are thought to be dedifferentiated.

It is unlikely that the expression of only one receptor governs or reflects the phenotypic state of a single cell. Therefore, the relationship between chondrocyte phenotype and TGF-β receptor expression was further investigated and endoglin expression also correlated with cell phenotype. Enhanced expression was observed in dedifferentiated cells, both late passage monolayer cultures of chondrocyte lines and in primary OA cells (manuscript 5, Fig. 4B, 4C, 4D). This suggested that, in addition to RIIB, endoglin may function as a marker of the state of cell differentiation for human chondrocytes.

That cell surface receptor expression may reflect phenotype has been previously suggested in various cell types. Rat microvascular endothelial cells, in 2-D, behave as undifferentiated cells but in 3-D cultures undergo a phenotypic shift of decreased cell growth (Sankar et al 1996). These cells, in 3-D, have been found to express lower levels of RII than cells cultured in 2-D inferring that RII expression is associated with the undifferentiated phenotype. Furthermore, Parathyroid hormone (PTH) receptors in rabbit costal chondrocytes can be regulated by retinoic acid and are expressed at higher levels in differentiated cells suggesting they may be a marker of cell phenotype (Takigawa et al 1991). RI and RII are expressed in normal lung cells; however, decreased RI levels have been noted in areas of injury in idiopathic pulmonary fibrosis (Khalil et al 2001). Increased levels of RI and RII expression are observed in keloid fibroblasts along with

increased Smad 3 phosphorylation (Chin et al 2001). *In vitro*, chondrocytes with dedifferentiated fibroblastic phenotype express FGFR-3 (Robinson et al 1999) which is lost when cells redifferentiate (Robinson et al 1998). IL-1 receptors are overexpressed in OA chondrocytes whereas mRNA levels of RII are significantly decreased in early stages of the disease process (Boumediene et al 1998a). A change in TGF-β RIIB expression with phenotype in human articular cells has also been described (Glansbeek et al 1997). This link between chondrocyte phenotype and receptor expression has been hypothesized by others (Vivien et al 1990, 1991, and 1993a) but remains unclear.

The concept of endoglin as a marker of cell phenotype has been previously described in other systems (O'Connell et al 1992). In addition, endoglin expression in synovial cells of RA and OA patients is higher than in patients without disease (Szekanecz et al 1995). In choriocarcinoma tissue, increased endoglin expression was noted on syncytiotrophoblast-like cells whereas no expression could be detected on cytotrophoblastic cells (Letamendia et al 1998b). Increased endoglin expression has been observed with various inflammatory skin conditions (Westphal et al 1993) such as in psoriatic skin (Rulo 1995).

Other cell surface proteins, notably integrins, are also differentially expressed in correlation with cell phenotype. Cartilage-derived retinoic acid-sensitive protein (CD-RAP) levels are increased in human synovial cells of diseased joints and have been suggested as a marker to monitor disease progression (Schmidt-Rohlfing et al 2002).

Integrins are heterodimeric transmembrane glycoproteins of α and β subunits with ECDs, which bind ECM proteins, and CD, which interact with cytoskeletal proteins. These glycoproteins mediate changes in cell shape and are involved in signal transduction (Albelda and Buck 1990, Hynes 1992, Ruoslahti 1991). Adult articular chondrocytes

express several integrins including $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_5$ (Loeser 1997). There is evidence that $\alpha_1\beta_1$ mediates adhesion to type II collagen and $\alpha_5\beta_1$ to fibronectin. Various components of the chondrocyte microenvironment, which reflect cell phenotype, also correspond to levels of integrin expression on these cells (reviewed in Loeser 1997 and Lee et al 2002).

There is an intriguing link between shift of endoglin and that of integrin expression with state of chondrocytes differentiation. Endoglin expression on endothelial cells is hypothesized to be important in ingress of polymorphonuclear cells in RA synovium (Szekanecz et al 1995). Endoglin contains an RGD motif in its ECD that implicates it as a potential adhesion molecule (Lastres et al 1992, Gougos and Letarte 1990). The potential of endoglin to bind ECM components and mediate signaling between the cell and the architectural component of the microenvironment could implicate endoglin in modulation of cell-matrix interactions.

Therefore, a link has been established between endoglin, phenotype, and possibly ECM; and, it is becoming increasingly difficult to separate these components as the phenotype itself infers properties of ECM production as well as morphology. Moreover, all of these factors play key roles in defining and disease states.

In OA cartilage, it is apparent that the ECM is abnormal and the phenotype of the cell is dedifferentiated. The observation that endoglin is markedly overexpressed on dedifferentiated (manuscript 5, Fig. 4B, 4C) and degenerative cells (manuscript 5, Fig. 4D) provides a critical link between ECM dysregulation and cell phenotype. It will be important to further characterize cellular changes, expression profiles of receptors, and

variation in gene expression to better understand this association of cell phenotype and microenvironmental influences.

In addition to being a potential indicator of chondrocyte phenotype, differences in TGF-β receptor expression may also be responsible for the variable sensitivity to TGF-β stimulation observed for cells in alternate states of differentiation which again may be reflected and likely contribute to variations seen in abnormal tissue states. Although it was observed that RIIB overexpression resulted in an enhancement of TGF-β response (manuscript 4, Fig. 6A,6B), endogenous RIIB expression appeared to correlate with the TGF-β responsiveness of human chondrocytes. Cell expressing high levels of RIIB were less responsive to TGF-β than cells expressing less RIIB. This was observed for Smad 2 phosphorylation (manuscript 4, Fig. 5B, 5C, 5D), [$^3$H]-Thymidine incorporation (manuscript 4, Fig. 5E), and PAI-1-promoter activity (manuscript 4, Fig. 5A). Hirai and Fujita suggested that the differential responsiveness of cells to TGF-β isoforms was a consequence of variable RIIB/RII expression but were unable to demonstrate this (Hirai and Fujita 1996).

Why overexpression of RIIB leads to an enhanced TGF-β response whereas endogenous levels correspond to a decreased cell responsiveness is not clear. However, this suggests that several factors are contributing to the regulation of chondrocyte responsiveness which is likely intricately linked to cell phenotype, TGF-β receptor expression, and the ECM. Corresponding to this hypothesis, cells expressing increased levels of endoglin (OA cells (manuscript 5, Fig. 8A) and articular cells (manuscript 5, Fig. 7A, 7B, 7C)) were noted to be less responsive to TGF-β (manuscript 5, Fig. 8B, 8C).

The concept of receptor expression determining cell responsiveness to growth factors and cytokines has been previously described. In normal (non-OA) chondrocytes, TGF-β receptor ratios appear to contribute to cell responsiveness. The RII/RI ratio, in rabbit articular chondrocytes, appears to govern the stimulatory or inhibitory effects of TGF-β on cell proliferation (Boumediene et al 1998b) corresponding to a decreased level of RI and a down-regulation of binding of -β1 in S phase cells (Vivien et al 1993a). Similarly, chick growth plate chondrocytes obtained from distinct zones express different levels of "low and high affinity" TGF-β receptors. The differential effects of TGF-β related to cell phenotype were related to this receptor expression (Rosier et al 1989).

Similarly and as was seen in the investigations presented here with human chondrocytes, the expression of receptors on OA chondrocytes has been shown to correspond to the cell's responsiveness to cytokines and growth factors.

IL-1 plays a central role in OA. These chondrocytes are more sensitive to IL-1 and have higher levels of IL-1 receptor expression (Boumediene et al 1998a). Thus, the ratio of TGF-β receptor subtypes could also be a mechanism by which TGF-β responsiveness is modulated. The discrepancy presented, with regards to the increased TGF-β responsiveness of OA cells previously reported (Moldovan et al 1997) and the decreased sensitivity observed in manuscript 5, Fig. 8B and 8C or the diminished sensitivity to TGF-β seen by OA chondrocytes attributed to decreased levels of RII (Boumediene et al 1998a), requires explanation. The results presented by Moldovan et al demonstrate that OA cells were more responsive to TGF-β than normal chondrocytes; however, these studies were performed using cells found in the upper layers of OA human explants (Moldovan et al 1997). However, articular cartilage is not a homogenous tissue and has

been described as having a zonal phenomenon similar to growth plate cells (Fukumura et al 1998). This may account for the demonstrated differences in TGF-β responsiveness seen in OA chondrocytes. Moreover, increased endoglin expression on dedifferentiated cells and endoglin's role in inhibiting TGF-β signaling suggests that OA cells, representative of this dedifferentiated state, should show a decreased TGF-β responsiveness as was noted (manuscript 5, Fig. 8B, 8C).

Progressing one step farther, attempts were previously made to correlate cell responsiveness and ECM. Early loss of PG in OA may depend on TGF-β receptor expression (Glansbeek et al 1993) and on the relative state of cell differentiation (Vivien et al 1990, 1991, and 1993a). Furthermore, TGF-β induced increases in type II collagen synthesis were preferentially noted in these dedifferentiated cells (Galera et al 1992). The decreased RII expression in 3-D cultures of rat microvascular endothelial cells was thought to be responsible for their refractory nature to TGF-β1 inhibition of cell proliferation whereas RI expression appeared to mediate ECM regulation (Sankar et al 1996). Therefore, ECM composition and architecture as well as phenotypic changes of cells may be regulated by changes in receptor expression and thus chondrocyte sensitivity to TGF-β. This produces a complex picture in which the lines between the chondrocyte, its phenotype, and correspondingly, the ECM and the cell's TGF-β responsiveness become blurred. However, this sets the stage for microenvironmental cues to result in diverse chondrocyte responses proportional to the cell's phenotypic characteristics.

**Regulators of the Microenvironment**

The manner in which a cell responds to signals from the microenvironment is likely related to the chondrocyte phenotype and furthermore may be reflected in expression of

various cell surface proteins. *In vivo*, chondrocytes reside in a complex 3-D microenvironment which is subject to various extrinsic forces such as injury, inflammation, and stress (Fig. d). These factors may result in changes to the 3-D architecture through degradation of structural proteins, modulation of growth factors, cytokines, and enzymes within the ECM or they may directly cause cell damage. Chondrocytes are believed to detect alterations in their microenvironment and, through an intricate circular system of regulation, in turn, alter the surrounding ECM. The cells may detect architectural variation through integrin and CAM related cell-cell and cell-ECM interactions. In addition, signaling pathways mediated through cell surface receptors transmit information from the microenvironment to the cell. Chondrocyte responses may involve growth, changes in synthesis of ECM structural components as well as autocrine and paracrine regulatory factors, or a shift in cell phenotype. This change in phenotype, or state of cell differentiation, may be manifested as a change in cell morphology and expression of cell surface molecules. Therefore, receptors and integrins at the cell surface could be considered as phenotypic markers, may provide clues as to changes occurring in the microenvironment, and may alter the cell's responsiveness to further changes. TGF-β receptors likely play a vital role in mediating interactions between the chondrocyte and both its architectural and regulatory microenvironment.

Thus far, the results presented suggest that human chondrocytes exhibit specific phenotypic characteristics which vary with culture conditions. Moreover, chondrocytes obtained directly from diseased cartilage (P0) appeared dedifferentiated, irrespective of culture conditions, suggesting this phenotype is reflective of the *in vivo* state of the cell. In addition, expression of RIIB and endoglin were linked to the dedifferentiated state of

human chondrocytes and to their ECM production. Furthermore, expression of these receptors was demonstrated to correspond to chondrocyte TGF-β responsiveness. Taken together, these results suggest a critical link between chondrocyte phenotype, ECM, TGF-β receptors and responsiveness. However, because of the complexity of these associations, it becomes increasingly difficult to distinguish cause and effect.

The other issue which arises from these investigations is that of unravelling the complexity of the microenvironment within which the chondrocytes reside. Not only do interactions between the chondrocyte and TGF-β need to be better defined, but also the multitude of additional regulatory agents within the microenvironment which directly or indirectly affect the cell or TGF-β need to be addressed.

Many of these regulators within cartilage and their effects on chondrocytes have been extensively investigated and can be considered within the realm of normal, aging, and diseased cartilage.

In normal human cartilage, cell-matrix interactions are tightly regulated. Cells sense matrix deformation as well as changes in matrix composition, notably regulatory factors within the microenvironment. Three classes of regulatory factors are prominent players in the ECM and contribute to the cell-microenvironment association. These include destructive cytokines (IL-1, IL-17, TNF-α, leukemia inhibitory factor (LIF)), regulatory cytokines (IL-4, Il-10, IL-13, IL-6, and enzyme inhibitors), and anabolic cytokines or growth factors (IGF-1, TGF-β, BMPs, and chondrocyte derived morphogenic proteins (CDMPs) (reviewed in van den Berg 1999). The destructive cytokines suppress PG and collagen synthesis and stimulate chondrocytes to produce destructive proteases. Regulatory cytokines, directly or through intermediates, decrease production of their

destructive counterparts, increase inhibitors of these destructive factors, and increase TIMPs. Anabolic cytokines and growth factors enhance the general synthetic activity of chondrocytes and directly counteract destructive cytokines.

An interesting association between these regulatory agents and chondrocytes has been established through studies of integrins. Growth factors have been shown to modulate integrin expression (Heino et al 1989, Heino and Massague 1989, Santala and Heino 1991, Zambruno et al 1995, Zhang et al 1993). Specifically, TGF-β1 increases $\alpha_5\beta_1$ levels (Loeser et al 1995) and IGF-1, together with TGF-β1, increases $\alpha_3\alpha_5$ expression and enhances articular chondrocyte adhesion to fibronectin and type II collagen (Loeser 1997). Furthermore, these integrins may play a central role in cartilage repair via promoting the assembly of ECM components and mediating changes in the production of MMPs by these cells (Werb et al 1989). Therefore, integrins appear to provide an important link between regulatory agents within the microenvironment and the cell.

Similar to integrins, cell surface receptors also provide a means to detect alterations in the ECM; moreover, their expression may also be adjusted in response to these changes. Retinoic acid, epidermal growth factor (EGR) and fibroblast growth factor (FGF) have been shown to increase PTH receptors on costal chondrocytes whereas IGF-1 and TGF-β decreased receptor levels (Takigawa et al 1991). Thus, both integrins and cellular receptors provide an avenue for the chondrocyte to detect and respond to their microenvironment.

ECM-chondrocyte interactions and extrinsic factors which regulate them contribute to the understanding of changes seen in aging cartilage. Age-related decreases in NO production by articular cells are thought to be associated with a decrease in cellular responsiveness to

IL-1 (Hauselmann et al 1998). There is also an age-related down-regulation in anabolic response to IGF-1 and a proportionate up-regulation of IGF-1 binding proteins (Martin and Buckwalter 2002). Noncollagenous ECM proteins number over 40 and differ in structure, distribution, and function (Roughley 2001). These proteins may also vary with age and, because of their structural and regulatory role, may directly influence chondrocytes or, via their degradation products, alter the cell phenotype and responsiveness.

Not only are changes in chondrocyte responsiveness thought to be a prominent factor in cartilage aging, but also the contribution of cell death is significant and again reflects the importance of the cells' microenvironment. In addition, cell death associated with aging cartilage, the emptying of lacunae, and the resultant structural ECM changes may affect the responsiveness of chondrocytes and has been suggested to be induced by changes in cell-ECM interactions (Fukui et al 2001).

Furthermore, biomechanical features of the microenvironment have been shown to affect aging cartilage. High magnitude cyclic tensile loads have been demonstrated to increase destructive cytokines or alter regulatory cytokine levels to promote destructive changes, which then may trigger the shift from normal aging to a disease state (Fukui et al 2001). Alternatively, smaller tensile forces change fluid flow through the porous cartilage matrix and have been demonstrated to exhibit anabolic effects on chondrocytes by delivering positive growth factors to them (Bonassar et al 2001).

The importance of this complex relationship between the chondrocyte and its microenvironment, both structural and regulatory, in the context of diseased cartilage is paramount. Morphologic, biochemical, molecular and biomechanical changes contribute to the altered state of cartilage seen in degenerative joint disease.

Early disruption or alteration of structure and composition of ECM is seen in OA and may constitute the triggering mechanism from aging to disease state (Martin and Buckwalter 2002). These ECM changes stimulate cell anabolism and the proliferative responses seen in stage 2, the hypertrophic phase, of attempted repair (Fukui et al 2001). This phase, however, progresses to stage 3, where characteristic thinning and decreased stiffness of the joint surface further accelerate ECM losses (Martin and Buckwalter 2002). These studies have demonstrated that chondrocytes obtained from diseased cartilage, in keeping with the tissue changes observed, exhibit a dedifferentiated phenotype (manuscript 5, Fig. 4B). This altered phenotype is likely a manifestation of the cell's response to stressful cues from its microenvironment coupled with normal aging. Thus, despite a programmed response to injury and an attempt to repair the tissue, degeneration ensues.

Growth factors and cytokines have been demonstrated to provide such microenvironmental cues and directly or indirectly regulate chondrocyte phenotype and ECM turnover (reviewed in Fukui et al 2001 and Goldring 2000a). Increased expression and activation of growth factors are observed in OA cartilage and in synovium of both OA and RA patients despite the distinct pathogenesis of these diseases (van den Berg 1999) suggesting that regulatory agents may be a trigger to initiate the disease process in cartilage and may also be modulated in response to it.

The role of regulatory agents within the chondrocyte microenvironment, in normal and aging tissue, implicate these mediators in the pathophysiology of degenerative joint diseases. The net effect of these mediators depends on their abundance, their inhibitors (for example soluble receptors) and the balance between them (van der Kraan and van den Berg 2000). Altered receptor types, concentrations, and ratios may have profound

consequences on the functional activity of cells and properties of the cartilage matrix. They may also play a role in cell proliferation, all of which are essential to tissue healing. IL-1, as a mediator of cells and ECM in joint disease, is a likely target; it has been repeatedly implicated in the pathogenesis of RA (van den Berg 1999) but its role in OA disease is less clear. OA cells have been shown to be more sensitive to IL-1 and to express higher levels of the IL-1 receptor than normal cartilage (Moldovan et al 1997).

The failure of attempted repair in degenerative joint disease is likely related to interplay of IL-1 and many other factors in the microenvironment including TGF-β. A serious complicating factor in interpreting the roles of these cytokines and specifically TGF-β is the deranged phenotype of chondrocytes and their disturbed receptor expression (van den Berg 1999, Boumediene et al 1998a). The confusion lies in whether the phenotypic change of the cell resulting in ECM disruption or degenerative joint disease may actually be a result of the cells' prolonged exposure to mediators or that this pathology results from normal levels of regulatory agents but a disturbed cellular responsiveness to them, which in a circular argument, is linked to aberrant receptor expression (van den Berg 1999). There is clearly a need to further characterize these regulatory factors and determine their perspective roles in degenerative joint disease in hopes of attaining an appropriate balance.

**The Relevance of TGF-β and its Receptors in Repair**

Regulatory agents residing in the microenvironment have been defined as key mediators of chondrocyte-ECM interactions. They appear to influence cell phenotype, reflected by cell proliferation and morphology, ECM turnover, and responsiveness to the growth

factors themselves. TGF-β is one such growth factor that has been clearly implicated in the regulation of both normal and diseased cartilage.

Protective and pathologic roles of TGF-β have been studied extensively (Shuler et al 2000, van Beuningen et al 1993, Elford et al 1992, van den Berg et al 1993, Itayem et al 1999, Perka et al 2000). The conflicting nature of its actions is possibly related to the changing phenotype of cells and their altered TGF-β receptor expression profile and responsiveness (van der Kraan and van den Berg 2000). New perspectives to combat degenerative joint changes will have to address all of these parameters and their complex interactions to target cartilage repair; however, limited information is available on TGF-β receptors in human chondrocytes and yet their role in the cell-microenvironment associations appears to be extensive.

The potential of TGF-β receptors to: allow the chondrocyte to detect and respond to changes in the microenvironment, act as markers of cell phenotype, contribute to cell responsiveness to TGF-β and other regulatory factors, function as detectors of structural changes in the ECM to mediate cell-ECM interactions, alter the ability of the cell to maintain its ECM 3-D structure and composition make them ideal targets for new therapeutic regimens aimed at treating degenerative joint disease. Thus, the regulation of chondrocyte function in physiologic and pathologic states may depend on manipulation of TGF-β receptor expression in a temporally and spatially dynamic fashion.

The link established throughout these investigations between TGF-β receptor expression, cell phenotype, cell responsiveness and the ECM, is critical to understanding the role of TGF-β action in joint disease and potential cartilage repair. However, the cyclic interactions between chondrocyte and microenvironment result in identification of

influential factors but without a specific start or finish line. Ironically, these investigations of human chondrocytes, TGF-β receptors and TGF-β action in these cells do not result in crossing any finish line in the race towards cartilage regeneration. Instead, the race has become a relay in which an expanding complex picture of TGF-β, the chondrocyte, and its microenvironment predominates. Despite this, new strategies have been acquired to pursue the ultimate goal of cartilage repair.

Treatment strategies aimed at redifferentiating chondrocytes, maintaining their phenotype, and generating hyaline cartilage are currently investigated primarily *in vitro*. Ultimately, intervention of joint disease needs to occur at very early stages; presently, it targets patient symptoms and fails to prevent disease onset or progression. To accomplish early disease intervention, the concepts of cell aging and phenotype must be combined. The decrease in chondrocyte numbers seen in normal aging cartilage must be replaced with cells of a stable hyaline cartilage producing phenotype that respond appropriately to alterations in the microenvironment and can maintain their surrounding ECM. Alternatively, this cell senescence must be delayed. Mechanisms employed to promote and maintain the differentiated phenotype will likely involve 3-D culture systems and implant devices in addition to modulation of growth factors locally, specifically TGF-β, potentially through regulation of its receptor expression.

There is evidence that treatment strategies are already moving in this direction. Controlling cytokine and growth factor expression locally has generated a large focus of therapeutic effort. However, the usefulness of growth factors to improve tissue repair has been limited by delivery problems and the inability to sustain anabolic factors at an appropriate level (reviewed in Lieberman et al 2002). The feasibility of delivering genes

to chondrocytes has been demonstrated *in vitro* (Arai et al 1997) and *in vivo* (Baragi et al 1997, Kang et al 1997). The concept, to deliver genes encoding for therapeutic cytokines which thus could be synthesized locally at sustainable concentrations, followed. Ideally, these genes could be regulated so that local cytokine production would correspond to appropriate cell phenotypic changes and would also combat increasing levels of destructive cytokines. The delivery of the TGF-β1 gene by adenoviral transfer has been demonstrated to result in a dose dependent up-regulation of PG, collagenous and noncollagenous protein synthesis in monolayer cultures of rabbit articular chondrocytes (Shuler et al 2000).

Alternate strategies to locally regulate TGF-β action have been suggested. *In vivo* delivery of a soluble form of RII (TGF-β scavenger molecule) was shown to penetrate cartilage (van Lent et al 1987) and increase PG loss and a decreased thickness of articular cartilage in a papain induced chicken OA model (Scharstuhl et al 2002). The potential replication of a competent virus or insertional mutagenesis, the generation of an immune response with repeat transfer, and the restricted size of transferable DNA pose disadvantages with adenoviral transfers; however, lipofection transfer is a realistic alternative (Stove et al 2002). Alternatively, liposomes and microspheres have been shown to deliver TGF-β1 to full thickness defects in articular cartilage (Hunziker 2001 and Hunziker et al 2001). *In vitro* transfer of regulatory agents through dense cartilage matrix can be difficult but has been accomplished with liposomes and other vectors (reviewed in Lieberman et al 2002). A related approach would be to genetically modify autogenous cells *ex vivo* and optimize their expansion prior to transplantation. IGF-1, BMPs, and TGF-β all promote phenotypic stability in chondrocytes (reviewed in

Lieberman et al 2002); however, cells must be proliferated and transferred in a physiologic-like 3-D matrix to preserve the differentiated state. An elegant *in vivo* approach to tissue engineering using non-invasive injectables has recently been described (Elisseeff et al 2001). Bovine articular chondrocytes were encapsulated with polyethylene oxide dimethacrylate (PEODM) microspheres of IGF-1, with or without TGF-β, in an injectable hydrogel. The hydrogel was photopolymerized through the skin resulting in a slow controlled release of the growth factors and an increased GAG production (Elisseeff et al 2001).

On a molecular level, experimental treatments for OA now target production of catabolic cytokines using anti-cytokine therapy, MMP inhibitors, or alternatively by targeting protein kinases involved in their signaling pathways including SAPKs/JNKS, p38MAPK, and $NF_{\kappa}B$. However, to affect these targets, there is a need to genetically engineer cells prior to transplantation to promote cartilage-specific matrix synthesis and counteract inflammation and destructive cytokines. This is a daunting task when considering the complex regulation of these factors by binding proteins, receptors, and various signaling pathways. A successful approach will likely require coordinative therapies to exploit the chondrogenic potential of the cells.

Epidemiologic studies have identified genetic influences as risk factors for the development of degenerative joint disease. In RA, most treatments target the central (activation and progression of synovitis) or the late (cartilage and bone destruction) phase of disease. There is a need, rather, to target the initiation phase where a genetic susceptibility and a triggering event begin the cascade of inflammation and destruction. Similarly, in OA, identifying the genes involved in critical pathways leading to disease

initiation or progression may be useful in developing new therapeutic strategies. DNA microarrays and other readily advancing genetic tools will allow analysis of gene expression profiles in these joint disease states to identify candidate genes as potential targets for intervention. Despite these aims, current pharmacologic treatment is for symptom alleviation and not disease prevention. Therefore, the need for early detection of joint changes is critical if new strategies will be successful.

Utilizing the principle that gadolinium is normally excluded from the ECM by aggrecan, magnetic resonance imaging with gadolinium can detect early aggrecan loss (reviewed in Poole et al 2002). Assays have been developed to detect denatured type II collagen (Hollander et al 1994). Alternatively, synthesis of type II procollagen can be ascertained through measurement of released propeptides when procollagen is incorporated into fibrils (Poole et al 2002). Detection of these degradative and synthetic markers has already been used to predict joint space narrowing. Therefore, these biomarkers may be used to detect joint damage at early stages, follow injury progression, to study the effects of new treatment strategies, and possibly provide a screening tool to establish genetic links to OA and RA.

It is unethical to take human cartilage biopsies. Advances in the fields of cytokines and growth factors, 3-D matrices, and phenotypic modulation of cells and the combination of these concepts thus require sound animal models of joint injury to determine their impact on healing. In addition, as new detection strategies are employed and new therapeutic targets are discovered, a model to test novel treatments will be essential. Ideally, this animal model is "a homogeneous set which have inherited, naturally acquired, or experimentally induced a biological process amenable to scientific investigation that in one or more aspects resembles the disease in humans" (Pritzker 1994). It has been

difficult to establish such a model of human joint conditions. Humans have a larger body size than most models, stature is varied, and load distribution to joints is unique. Cartilage of animals has different properties and therefore, results cannot be directly translated to human conditions (Helminen et al 2002).

Various murine models of OA, genetically developed through mutations in collagen, fibromodulin, biglycan, MMP-13, $\beta_1$ integrins, and growth hormones have been used extensively (reviewed in Helminen et al 2002). STR/ORT and BCBC/4 mice show spontaneous OA development. Tiptoe walking and progressive ankylosis murine models have defects in the pathways controlling mineral deposition which results in calcium crystal deposits in synovial joints and degenerative joint disease (reviewed in Newman and Wallis 2002). Two significant problems present in all of these models are that chondrocyte density is much greater in murine cartilage than human and OA is of early onset rather than the progressive late onset seen in humans.

Despite their shortcomings, murine models provide an ethical alternative to study degenerative joint disease and novel treatment strategies. In addition, studying the development of disease and genetic links in these models may identify new therapeutic targets. Fifty to seventy percent of Smad $3^{exon8}$ homozygote murine knockouts die within 3 months of life from uncontrolled infectious processes; however, the surviving mice develop early skeletal abnormalities that progressively worsen with age (Yang et al 2001). These mice demonstrate abnormal hypertrophic differentiation of articular chondrocytes. By 4 months, there is obvious unusual differentiation of cells with surface fibrillation, increased type I collagen, osteocalcin, cartilage loss and osteophyte formation (Yang et al 2001). Similarly, transgenic mice expressing a cytoplasmically truncated form

of RII have been developed (Serra et al 1997). This mutant RII acts in a dominant negative fashion to inhibit TGF-β signaling; these develop skeletal defects by 3 months with progressively stiffening joints. With time, decreased levels of PG and increased type X collagen are observed and formation of chondrons can be seen in deeper cartilage layers. Cartilage becomes disorganized, calcified, hypertrophic cells are noted, and fibrillation follows with osteophyte formation (Serra et al 1997). Taken together, these two murine models implicate TGF-β dysregulation in the development of degenerative joint disease and suggest that further investigation into the role of TGF-β and cartilage is warranted. Progressive understanding of the chondrocyte, its microenvironment and the overwhelming complexity of their association may provide clues as to the nature of the degenerative changes in diseased cartilage and may provide novel avenues to modulate this association toward regeneration or repair.

## CONCLUSIONS

Because of the complexity of degenerative joint disease, treatment strategies, often aimed at late stage disease, are limited. Problems faced in attempts at repair of damaged cartilage include the avascular state of the tissue and a dedifferentiation of chondrocytes resulting in a cell with a varied response profile to cytokines and growth factors, a decreased anabolic capacity and an inability to maintain its surrounding matrix.

TGF-β promotes the differentiated phenotype of chondrocytes, enhances cartilage healing, and dysregulation of its signaling cascade results in degenerative joint disease. TGF-β has been implicated in cell proliferation and differentiation and ECM regulation in cartilage. The investigations presented here have established a critical link whereby the action of TGF-β in cartilage may be explained through variation in receptor expression at

the cell surface. In addition to the TGF-β signaling receptors on chondrocytes, the presence of betaglycan and RIIB was confirmed and the novel expression of Sol RI, Alk-1, and endoglin was identified. These receptors were observed to form a variety of heteromeric complexes and to regulate TGF-β signaling. More importantly, RIIB and endoglin were demonstrated to regulate type II collagen levels and evidence was provided that they likely represent chondrocyte phenotypic markers. Furthermore, a critical association between endoglin expression, cell phenotype, cell responsiveness, and ECM was established. These results suggest that TGF-β binding proteins in human cartilage are potential modulators of complex interactions between the cell and its microenvironment and may provide a novel avenue to regulate the effects of TGF-β locally.

Fig. c

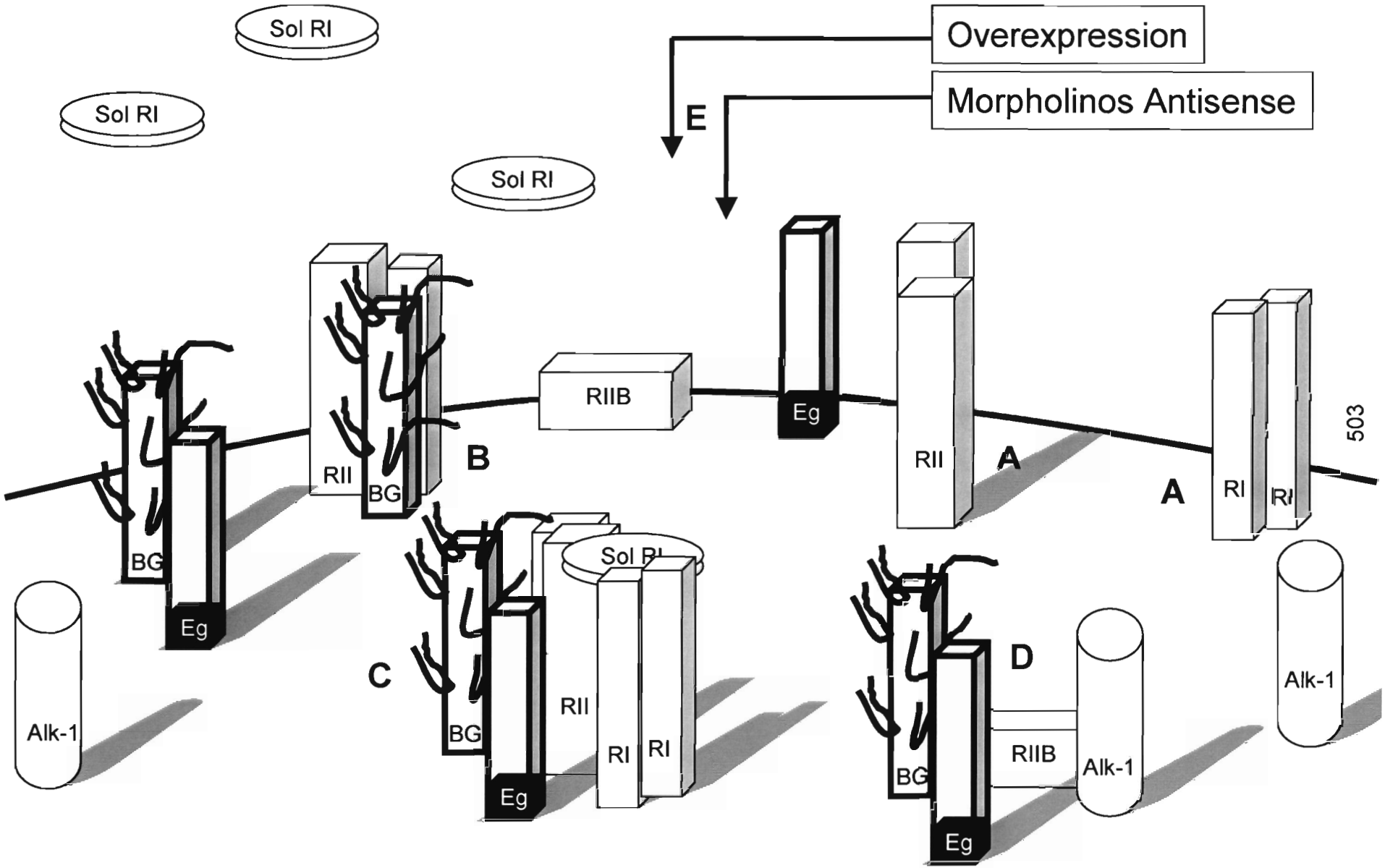
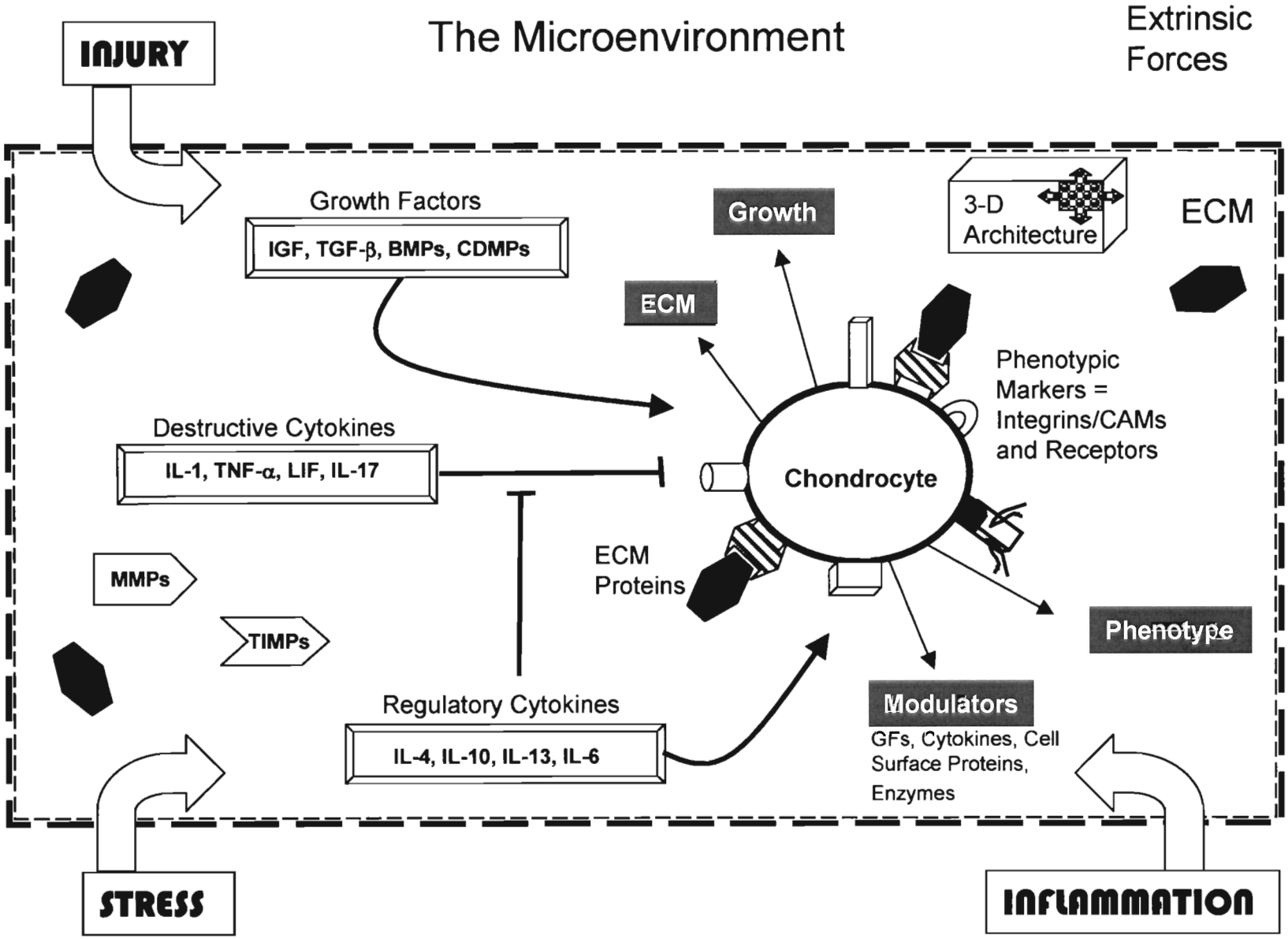

Sol RI
Sol RI
Sol RI
Overexpression
Morpholinos Antisense
E
RIIB
Eg
RII
A
A
RI
RI
503
B
RII
BG
BG
Eg
Alk-1
C
Sol RI
RII
BG
RI
RI
Eg
D
BG
RIIB
Alk-1
Eg
Alk-1

**Fig. c: The hypothesized fluid hetero-oligomeric TGF-β receptor complex on human chondrocytes.** Identification of TGF-β receptors in human chondrocytes has revealed the presence of RI and RII signaling receptors and variants of these namely Sol RI and RIIB. In addition, Alk-1 has been demonstrated on the cell surface as have the accessory receptors endoglin and betaglycan and a GPI-anchored 180 kDa TGF-β binding protein. The signaling receptors form homodimeric (A) and heterodimeric complexes which may also involve accessory receptors such as betaglycan (B). The signaling receptors are thought to form a heterotetramer when transducing the TGF-β response (C). Novel receptors likely provide a tight regulation of TGF-β signaling through heteromeric complex formations with each other as well as RI and RII (C). These complexes, however, are potentially heterogeneous in nature (D). The ratios and concentration of individual components may be critical in specifying the relative contributions of the various signaling pathways, and thus, may play a central role in regulating the diverse actions of TGF-β in chondrocytes. As evidence, overexpression of various receptors or inhibition of their expression (E) has been shown to modulate TGF-β signaling in these cells.

Fig. d


The Microenvironment
Extrinsic Forces
INJURY
Growth Factors
IGF, TGF-β, BMPs, CDMPs
Growth
3-D Architecture
ECM
ECM
Phenotypic Markers = Integrins/CAMs and Receptors
Destructive Cytokines
IL-1, TNF-α, LIF, IL-17
Chondrocyte
ECM Proteins
MMPs
TIMPs
Phenotype
Regulatory Cytokines
IL-4, IL-10, IL-13, IL-6
Modulators
GFs, Cytokines, Cell Surface Proteins, Enzymes
STRESS
INFLAMMATION

505

**Fig. d: The human chondrocyte, its microenvironment and the overwhelming complexity of their association.** *In vivo*, chondrocytes reside in a complex 3-D microenvironment which is subject to various extrinsic forces such as injury, inflammation, and stress. These factors may result in changes to the 3-D architecture through degradation of structural proteins, modulation of growth factors, cytokines, and enzymes within the ECM or may directly cause cell damage. Chondrocytes are believed to detect changes in their microenvironment and, in turn, alter the surrounding ECM. Chondrocyte response to these signals may involve growth, a change in synthesis of ECM structural components as well as autocrine and paracrine regulatory factors, or a change in cell phenotype manifested as a change in cell morphology or expression of cell surface molecules. Therefore, receptors and integrins at the cell surface could be considered as phenotypic markers, may provide clues as to changes occurring in the microenvironment, and may alter the cell's responsiveness to further changes. TGF-β receptors likely play a vital role in mediating interactions between the chondrocyte and both its architectural and regulatory microenvironment.

FUTURE DIRECTIONS

The failure to establish a reliable bioavailability model for the administration of TGF-β to the joint surface lends support to the concept of local regulation of endogenous levels. In addition, *in vivo* modulation of endogenous TGF-β allows for chondrocytes to remain in their physiologic 3-D microenvironment. Alternatively, and to address the problem of chondrocyte senescence, autogenous cells could be manipulated *in vitro* and delivered to joint defects in 3-D matrices which mimic the microenvironment. TGF-β receptor modulation, because of their association with cell phenotype, responsiveness and ECM, is a promising route to locally regulate TGF-β. A variety of TGF-β regulatory agents have been identified, some of which directly effect receptor expression.

Anti-inflammatory medications are often used in symptomatic treatment of degenerative and inflammatory joint disease. Administration of misoprostil to bovine articular chondrocytes has been shown to stimulate GAG synthesis by a TGF-β1 dependent mechanism (Anastassiades et al 1998).

Many other potential TGF-β regulatory agents have been proposed. For example, NO has been shown to act as an important regulator of TGF-β (reviewed in Studer et al 1996). In OA chondrocytes, Doxycycline administration depressed MMP-1 and MMP-13 levels, enhanced TGF-β3 levels and up-regulated both TGF-β signaling receptors (Shlopov et al 1999 and 2001). In addition, Retinoic acid (vitamin A) increases TGF-β2 synthesis and its effects are antagonized by -β2 administration (Morales and Roberts 1992). Retinoic acid has been demonstrated to inhibit PG synthesis (Caputo et al 1987, Campbell and Handley 1987) and there is some evidence that nuclear receptor signaling of RA and the TGF-β2 signaling cascade may be linked (Glick et al 1989 and 1991). Methotrexate can

up-regulate endoglin expression (Letamendia et al 1998b) and work with anti-endoglin antibodies and cyclophosphamides in blood vessels of human tumours implicate endoglin as a potential target of cell modulation.

Steroids, another frequently employed agent used in treating joint disease, have well known associations with TGF-β (Roberts and Sporn 1992). Steroids increase expression of TGF-β isoforms and can induce expression of TGF-β receptors (Glick et al 1989 and 1990, Ruscetti et al 1991). Glucocorticoids have been demonstrated to inhibit cartilage growth (Datuin et al 2001) as well as increase osteopenia and enhance apoptosis in bone and cartilage (Silvestrini et al 2000). Glucocorticoids are known to promote chondrocyte differentiation but by unknown mechanisms (reviewed in Sekiya et al 2001).

The effects of glucocorticoids on chondrocyte differentiation may be mediated directly; Dexamethasone increases Sox9 mRNA expression which in turn increases mRNA of Col 2A1 (Sekiya et al 2001). On the other hand, indirect actions of glucocorticoids include up-regulation of betaglycan mRNA (Nakayama et al 1994), increased TGF-β1 binding to betaglycan (Centrella et al 1991, Nakayama et al 1994) but not RII (Nakayama et al 1994), and decreased binding to RI (Centrella et al 1991). Glucocorticoid receptors inhibit TGF-β induced PAI-1 and SBE 4 luciferase activity via an interaction that inactivates Smad 3 (Song et al 1999).

The link between endogenous steroids and cartilage is also evident. Women on long term estrogen replacement therapy exhibit increased cartilage thickness (Wluka et al 2001). Moreover, epidemiologic studies indicate female gender as a risk factor for the development of both inflammatory and degenerative joint disease. Taken together, with the observation of functional estrogen receptors ($ER_\alpha$ and $ER_\beta$) in articular cartilage, this

information identifies estrogen ($E_2$) as a potential regulator of cartilage. More importantly, responses in growth plate chondrocytes to $E_2$ (17-β estradiol) are regulated by TGF-β1 (Nasatzky et al 1999). $E_2$ has been shown to inhibit chondrocyte proliferation, stimulate protein synthesis and promote cell differentiation (reviewed in Nasatzky et al 1999).

The involvement of these agents in regulating chondrocytes, their microenvironment, the interactions between them, and the potential regulation of them by TGF-β makes steroids and other factors likely candidates to address local modulation of TGF-β action in cartilage.

Further understanding of the action of TGF-β in human cartilage and better elucidation of the role of TGF-β receptors in this process is critical. Specifically, the aims of future investigations in human chondrocytes should be:

1. to further elucidate the structural determinants of TGF-β receptor interactions and complex formations;
2. to better define the role of receptors and receptor complexes in modulating TGF-β signaling;
3. to determine if levels of TGF-β receptor expression at the cell surface or their contributions to heterogeneous complexes result in unique signaling pathways to specify a particular TGF-β induced response among the diverse downstream targets

To accomplish these aims, the following objectives should be met:

(i). modify TGF-β receptor expression to locally regulate TGF-β action in cartilage;

(ii). identify regulatory agents of TGF-β receptors as a means of indirectly modulating TGF-β signaling;

(iii). determine the role of endoglin in cartilage as a regulator of ECM turnover and cell phenotype in modulating cell-ECM interactions;

(iv). determine if endoglin plays a role in promoting the avascular state of the tissue.

The use of available murine models (Smad $3^{exon8}$, DNRII, and HHT I) may allow for these objectives to be pursued *in vivo* and will likely aid in identifying the role of TGF-β and its receptors in joint disease.

FINAL THOUGHTS

The results presented and the literature reviewed agree that chondrocyte phenotype, cell responsiveness to cytokines and growth factors, and the avascular state of the tissue contribute to the lack of appropriate repair by injured cartilage. There is a need to engineer replacement tissue with appropriate biochemical and biomechanical properties or tissue failure will ensue. The production of a stable type II collagen containing hyaline matrix may be realized if the microarchitecture of articular cartilage can be recreated. However, the mechanical demands on this specialized tissue are a harsh discriminator of inadequate repair. There is an increasing acceptance that degenerative joint disease is not the equivalent of normal aging, rather, that normal age-related changes in cartilage make the tissue more vulnerable to triggering events.

Independently, both *in vitro* and *in vivo* investigations have provided much insight into cartilage engineering through genetics, studies of growth factors and cytokines, 3-D matrices and local delivery of regulatory agents. What remains is to integrate these concepts into a unifying direction. The appropriate timing of interventions and

appropriate models for their application must be in place to provide novel therapeutic regimens that may include TGF-β and its receptors.

**"there are ... no instances in which a lost portion of cartilage has been restored with new and well formed permanent cartilage in the human subject"**

**-Paget 1853**

The move towards cartilage regeneration or repair appears to have become a marathon since the time Paget withdrew from the simple race. It is with the hope that a concerted effort and a combination of knowledge and strategies will provide the answers to restore this specialized connective tissue that the race is continued.

# Section 4

## LITERATURE CITED

Aigner T, Dudhia J 1997 Phenotypic modulation of chondrocyte as a potential therapeutic target in osteoarthritis: a hypothesis. Ann Rheum Dis 56:287-291.

Aigner T, Hemmel M, Neureiter D, Gebhard PM, Zeiler G, Kirchner T, McKenna L 2001 Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritis human articular knee cartilage: a study of proliferation, programmed cell death (apoptosis), and viability of chondrocytes in normal and osteoarthritic human knee cartilage. Arthritis Rheum 44:1304-12.

Aigner T, Kim HA 2002 Apoptosis and cellular vitality: issues in osteoarthritic cartilage degeneration. Arthritis Rheum 46:1986-96.

Aigner T, Zhu Y, Chansky HH, Matsen FA III, Maloney WJ, Sandell LJ 1999 Reexpression of type IIA procollagen by adult articular chondrocytes in osteoarthritic cartilage. Arthritis Rheum 42:1443-1450.

Ala-Kokko L, Baldwin CT, Moskowitz RW, Prockop DJ 1990 Single base mutation in the type II procollagen gene (COL2A1) as a cause of primary osteoarthritis associated with a mild chondrodysplasia. Proc Natl Acad Sci U S A 87:6565-8.

Alarcon GS 1995 Epidemiology of rheumatoid arthritis. Rheum Dis Clin North Am 21:589-604.

Albelda SM, Buck CA 1990 Integrins and other cell adhesion molecules. FASEB J 4:2868-80.

Anastassiades T, Chopra R, Law C, Wong E 1998 In vitro suppression of transforming growth factor-beta induced stimulation of glycosaminoglycan synthesis by acetylsalicylic acid and its reversal by misoprostol. J Rheumatol 25:1962-7.

Anders RA, Arline SL, Dore JJ, Leof EB 1997 Distinct endocytic responses of heteromeric and homomeric transforming growth factor beta receptors. Mol Biol Cell 8:2133-43.

Anders RA, Dore JJ Jr, Arline SL, Garamszegi N, Leof EB 1998 Differential requirement for type I and type II transforming growth factor beta receptor kinase activity in ligand-mediated receptor endocytosis. J Biol Chem 273:23118-25.

Andres JL, Ronnstrand L, Cheifetz S, Massague J 1991 Purification of the transforming growth factor-beta (TGF-beta) binding proteoglycan betaglycan. J Biol Chem 266:23282-7.

Arai Y, Kubo T, Kobayashi K, Takeshita K, Takahashi K, Ikeda T, Imanishi J, Takigawa M, Hirasawa Y 1997 Adenovirus vector-mediated gene transduction to chondrocytes: in vitro evaluation of therapeutic efficacy of transforming growth factor-beta 1 and heat shock protein 70 gene transduction. J Rheumatol 24:1787-95.

Arkwright PD, Laurie S, Super M, Pravica V, Schwarz MJ, Webb AK, Hutchinson IV 2000 TGF-beta(1) genotype and accelerated decline in lung function of patients with cystic fibrosis. Thorax 55:459-62.

Arthur HM, Ure J, Smith AJH, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG 2000 Endoglin, an ancillary TGF-ß receptor, is required for extra embryonic angiogenesis and plays a key role in heart development. Dev Biol 217:42-53.

Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB 1983 Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. J Biol Chem 258:7155-60.

Aston JE, Bentley G 1986 Repair of articular surfaces by allografts of articular and growth-plate cartilage. J Bone Joint Surg 68-B:29-35.

Attisano L, Carcamo J, Ventura F, Weis FM, Massague J, Wrana JL 1993 Identification of Human Activin and TGF Type Receptors That Form Heteromeric Kinase Complexes with Type II Receptors. Cell 75: 671.

Attisano L, Wrana JL 1998 Mads and Smads in TGF beta signalling. Curr Opin Cell Biol 10:188-94.

Attisano L, Wrana JL, Lopez-Casillas F, Massague J 1994 TGF-beta receptors and actions. Biochem Biophys Acta 1222:71-80.

Aydelotte MB, Greenhill RR, Kuettner KE 1988 Differences between sub-populations of cultured bovine articular chondrocytes. II. Proteoglycan metabolism. Connect Tissue Res. 18:223-34.

Aydelotte MB, Kuettner KE 1988 Differences between sub-populations of cultured bovine articular chondrocytes. I. Morphology and cartilage matrix production. Connect Tissue Res 18:205-22.

Bahrami S, Stratmann U, Wiesmann HP, Mokrys K, Bruckner P, Szuwart T 2000 Periosteally derived osteoblast-like cells differentiate into chondrocytes in suspension culture in agarose. Anat Rec 259:124-30.

Bakker AC, Van de Loo FA, Joosten LA, Bennink MB, Arntz OJ, Dmitriev IP, Kashentsera EA, Curiel DT, van den Berg WB 2001 A tropism-modified

adenoviral vector increased the effectiveness of gene therapy for arthritis. Gene Ther 8:1785-93.

Bandyopadhyay A, Zhu Y, Malik SN, Kreisberg J, Brattain MG, Sprague EA, Luo J, Lopez-Casillas F, Sun LZ 2002 Extracellular domain of TGFbeta type III receptor inhibits angiogenesis and tumor growth in human cancer cells. Oncogene 21:3541-51.

Baragi VM, Renkiewicz RR, Qiu L, Brammer D, Riley JM, Sigler RE, Frenkel SR, Amin A, Abramson SB, Roessler BJ 1997 Transplantation of adenovirally transduced allogeneic chondrocytes into articular cartilage defects in vivo. Osteoarthritis Cartilage 5:275-82.

Barbara NP, Wrana JL, Letarte M 1999 Endoglin is an accessory protein that interacts with the signalling receptor complex of multiple members of the transforming growth factor-β super family. J Biol Chem 274:584-594.

Barcellos-Hoff MH, Dix TA 1996 Redox-mediated activation of latent transforming growth factor-beta 1. Mol Endocrinol 10:1077-83.

Bassing CH, Yingling JM, Howe DJ, Wang T, He WW, Gustafson ML, Shah P, Donahoe PK, Wang XF 1994 Transforming growth factor-β type-I receptor that signals to activate gene expression. Science 263:87-89.

Bassols A, Massague J 1988 Transforming growth factor beta regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. J Biol Chem 263:3039-45.

Bellon T, Corbi A, Lastres P, Cales C, Cebrain M, Vera S, Chefeitz M, Massague J, Letarte M, Bernabeu C 1993 Identification and expression of two forms of the human

transforming growth factor-β-binding protein endoglin with distinct cytoplasmic regions. Eur J Immunol 23: 2340-2345.

Bentley G, Greer RB 3rd 1971 Homotransplantation of isolated epiphyseal and articular cartilage chondrocytes into joint surfaces of rabbits. Nature 230:385-8.

Bentley G, Smith AU, Mukerjhee R 1978 Isolated epiphyseal chondrocyte allografts into joint surfaces. An experimental study in rabbits. Ann Rheum Dis 37:449-58.

Benya PD, Shaffer JD 1982 Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215-224.

Billings E Jr, von Schroeder HP, Mai MT, Aratow M, Amiel D, Woo SL, Coutts RD 1990 Cartilage resurfacing of the rabbit knee. The use of an allogeneic demineralized bone matrix-autogeneic perichondrium composite implant. Acta Orthop Scand 61:201-6.

Blackburn EH 1991 Structure and function of telomeres. Nature 350:569-73.

Blanco FJ, Guitian R, Vazquez-Martul E, de Toro FJ, Galdo F 1998 Osteoarthritis chondrocytes die by apoptosis. A possible pathway for osteoarthritis pathology. Arthritis Rheum 41:284-9.

Blanco FJ, Ochs RL, Schwarz H, Lotz 1995 Chondrocyte apoptosis induced by nitric oxide. Am J Pathol 146:75-85.

Blobe GC, Schiemann WP, Pepin M-C, Beauchemin M, Moustakas A, Lodish HF, O'Connor-McCourt MD 2001 Functional roles for the cytoplasmic domain of the type III transforming growth factor beta receptor in regulating transforming growth factor beta signaling. J Biol Chem 276:24627-24637.

Bodey B, Bodey B Jr, Siegel SE, Kaiser HE 1998a Upregulation of endoglin (CD105) expression during childhood brain tumor-related angiogenesis. Anti-angiogenic therapy. Anticancer Res 18:1485-500.

Bodey B, Bodey B Jr, Siegel SE, Kaiser HE 1998b Over-expression of endoglin (CD105): a marker of breast carcinoma-induced neo-vascularization. Anticancer Res 18:3621-8.

Bodey B, Bodey B Jr, Siegel SE, Kaiser HE 1998c Immunocytochemical detection of endoglin is indicative of angiogenesis in malignant melanoma. Anticancer Res 18:2701-10.

Boesen CC, Motyka SA, Patamawenu A, Sun PD 2002 Crystallization and preliminary crystallographic studies of human TGF-beta type II receptor ligand-binding domain. Acta Crystallogr D Biol Crystallogr 58(Pt 7):1214-6.

Bolton MC, Dudhia J, Bayliss MT 1999 Age-related changes in the synthesis of link protein and aggrecan in human articular cartilage: implications for aggregate stability. Biochem J 337:77-82.

Bonassar LJ, Grodzinsky AJ, Frank EH, Davila SG, Bhaktav NR, Trippel SB 2001 The effect of dynamic compression on the response of articular cartilage to insulin-like growth factor-I. J Orthop Res 19:11-7.

Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Friesinger P 1994 Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp Cell Res 212:97-104.

Bonewald LF 1999 Regulation and regulatory activities of transforming growth factor beta. Crit Rev Eukaryot Gene Expr 9:33-44.

Bos PK, van Osch GJ, Frenz DA, Verhaar JA, Verwoerd-Verhoef HL 2001 Growth factor expression in cartilage wound healing: temporal and spatial immunolocalization in a rabbit auricular cartilage wound model. Osteoarthritis Cartilage 9:382-9.

Botella LM, Sanchez-Elsner T, Rius C, Corbi A, Bernabeu 2001 Identification of a critical Sp1 site within the endoglin promoter and its involvement in the transforming growth factor-beta stimulation. J Biol Chem 276:34486-94.

Boumediene K, Conrozier T, Mathieu P, Richard M, Marcelli C, Vignon E, Pujol J-P 1998a Decrease of cartilage transforming growth factor-β receptor II expression in the rabbit experimental osteoarthritis-potential role in cartilage breakdown. Osteoarth Cart 6:146-149.

Boumediene K, Felisaz N, Pujol JP 1998b Cell-cycle-dependent expression of transforming growth factor beta type I receptor correlates with differential proliferative effects of TGFbeta1 in articular chondrocytes. Exp Cell Res 243:173-84.

Bourdeau A, Dumont DJ, Letarte M 1999 A murine model of hereditary hemorrhagic telangiectasia. J Clin Invest 104:1343-1351.

Bourdeau A, Faughnan ME, McDonald ML, Paterson AD, Wanless IR, Letarte M 2001 Potential role of modifier genes influencing transforming growth factor-beta1 levels in the development of vascular defects in endoglin heterozygous mice with hereditary hemorrhagic telangiectasia. Am J Pathol 158:2011-20.

Buckwalter JA 1995 Osteoarthritis and articular cartilage use, disuse, and abuse: experimental studies. J Rheumatol Suppl 43:13-5.

Buckwalter JA Mankin HJ 1997 Articular Cartilage II. Degeneration and osteoarthrosis, repair, regeneration, and transplantation. J Bone Joint Surg 79A:612-632.

Buckwalter JA, Kuettner KE, Thonar EJ 1985 Age-related changes in articular cartilage proteoglycans: electron microscopic studies. J Orthop Res 3:251-7.

Buckwalter JA, Lane NE 1997 Athletics and osteoarthritis. Am J Sports Med 25:873-81.

Buckwalter JA, Lappin DR 2000 The disproportionate impact of chronic arthralgia and arthritis among women. Clin Orthop 372:159-68.

Buckwalter JA, Martin J, Mankin HJ 2000 Synovial joint degeneration and the syndrome of osteoarthritis. Instr Course Lect. 49:481-9.

Buhring HJ, Muller CA, Letarte M, Gougos A, Saalmuller A, van Agthoven AJ, Busch FW 1991 Endoglin is expressed on a subpopulation of immature erythroid cells of normal human bone marrow. Leukemia 5:841-7.

Bullough PG 1997 The role of bone biopsy in evaluating bone disease. Rev Rheum Engl Ed. 64:37S-43S.

Burton-Wurster N, Lust G 1990 Fibronectin and proteoglycan synthesis in long term cultures of cartilage explants in Ham's F12 supplemented with insulin and calcium: effects of the addition of TGF-beta. Arch Biochem Biophys 283:27-33.

Callahan JF, Burgess JL, Fornwald JA, Gaster LM, Harling JD, Harrington FP, Heer J, Kwon C, Lehr R, Mathur A, Olson BA, Weinstock J, Laping NJ 2002 Identification of novel inhibitors of the transforming growth factor beta1 (TGF-beta1) type 1 receptor (ALK5). J Med Chem 45:999-1001.

Campbell MA, Handley CJ 1987 The effect of retinoic acid on proteoglycan turnover in bovine articular cartilage cultures. Arch Biochem Biophys 258:143-55.

Campisi J 1999 Replicative senescence and immortalization. In: Stein GS (ed) The Molecular Basis of Cell Cycle and Growth Control, Wiley-Liss, New York, NY, pp. 348-373.

Cancedda R, Descalzi Cancedda F, Castagnola P 1995 Chondrocyte differentiation. Int Rev Cytol 159:265-358.

Cao L, Lee V, Adams ME, Kiani C, Zhang Y, Hu W, Yang BB 1999 beta-Integrin-collagen interaction reduces chondrocyte apoptosis. Matrix Biol 18:343-55.

Caputo CB, Sygowski LA, Wolanin DJ, Patton SP, Caccese RG, Shaw A, Roberts RA, DiPasquale G 1987 Effect of synthetic metalloprotease inhibitors on cartilage autolysis in vitro. J Pharmacol Exp Ther 240:460-5.

Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, Ninfa EG, Frey AZ, Gash DJ, Chow EP, et al 1986 Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells. Cell 45(5):685-98.

Centrella M, Casinghino S, Kim J, Pham T, Rosen V, Wozney J, McCarthy TL 1995 Independent changes in type I and type II receptors for transforming growth factor beta induced by bone morphogenetic protein 2 parallel expression of the osteoblast phenotype. Mol Cell Biol 15:3273-81.

Centrella M, Ji C, Casinghino S, McCarthy TL 1996 Rapid flux in transforming growth factor-beta receptors on bone cells. J Biol Chem 271:18616-22.

Centrella M, McCarthy TL, Canalis E 1991 Glucocorticoid regulation of transforming growth factor beta 1 activity and binding in osteoblast-enriched cultures from fetal rat bone. Mol Cell Biol 11:4490-6.

Cheifetz S, Bassols A, Stanley K, Ohta M, Greenberger J, Massague J 1988 Heterodimeric transforming growth factor beta. Biological properties and interaction with three types of cell surface receptors. J Biol Chem 263:10783-10789.

Cheifetz S, Bellon T, Cales C, Vera S, Bernabeu C, Massague J, Letarte M 1992 Endoglin is a component of the transforming growth factor-β receptor system in human endothelial cells. J Biol Chem 267: 19027-19030.

Cheifetz S, Massague J 1991 Isoform-specific transforming growth factor-beta binding proteins with membrane attachments sensitive to phosphatidylinositol-specific phospholipase C.J Biol Chem 266, 20767-20772.

Cheifetz S, Weatherbee JA, Tsang MLS, Anderson JK, Mole JE, Lucas R, Massague J 1987 The transforming growth factor-beta system, a complex pattern of cross-reactive ligands and receptors. Cell 48: 409-415.

Chen C, Wang X-F, Sun L 1997a Expression of Transforming Growth Factor β (TGFβ) Type III receptor restored autocrine TGF-β1 activity in human breast cancer MCF-7 Cells. J Biol Chem 272:12862-12867.

Chen D, Ji X, Harris MA, Feng JQ, Karsenty G, Celeste AJ, Rosen V, Mundy GR, Harris SE 1998 Differential roles for bone morphogenetic protein (BMP) receptor type IB and IA in differentiation and specification of mesenchymal precursor cells to osteoblast and adipocyte lineages. J Cell Biol. 142:295-305.

Chen RH, Derynck R 1994 Homomeric interactions between type II transforming growth factor-beta receptors. J Biol Chem 269:22868-74.

Chen YG, Massague J 1999 Smad 1 recognition and activation by the ALK1 group of transforming growth factor-beta family receptors. J Biol Chem 274:3672-7.

Chesterman PJ, Smith AU 1968 Homotransplantation of articular cartilage and isolated chondrocytes. An experimental study in rabbits. J Bone Joint Surg Br. 50:184-97.

Chin GS, Liu W, Peled Z, Lee TY, Steinbrech DS, Hsu M, Longaker MT 2001 Differential expression of transforming growth factor-beta receptors I and II and activation of Smad 3 in keloid fibroblasts. Plast Reconstr Surg 108:423-9.

Choi ME 1999 Cloning and characterization of a naturally occurring soluble form of TGF-β type I receptor. Am J Physiol 276: (Renal Physiol. 45):F88-F95.

Chopra R, Anastassiades T 1998 Specificity and synergism of polypeptide growth factors in stimulating the synthesis of proteoglycans and a novel high molecular weight anionic glycoprotein by articular chondrocyte cultures. J Rheumatol 25:1578-84.

Chu CQ, Field M, Allard S, Abney E, Feldmann M, Maini RN 1992 Detection of cytokines at the cartilage/pannus junction in patients with rheumatoid arthritis: implications for the role of cytokines in cartilage destruction and repair. Br J Rheumatol 31:653-61.

Chu CR, Dounchis JS, Yoshioka M, Sah RL, Coutts RD, Amiel D 1997 Osteochondral repair using perichondrial cells. A 1-year study in rabbits. Clin Orthop 340:220-9.

Clark WR 1999 A Means to an End: the Biological Basis of Aging and Death. Oxford University Press, New York, NY.

Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SMF, Lawler J, Hynes RO, Boivin GP Bouck N 1998 Thrombospondin-1 is a major activator of TGF-β1 *in vivo*. Cell 93:1159-1170.

Creamer P, Hochberg MC 1997 Osteoarthritis. Lancet 350:503-8.

Cui Y, Jean F, Thomas G, Christian JL 1998 BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryonic development. EMBO J 17:4735-43.

Czuwara-Ladykowska J, Gore EA, Shegogue DA, Smith EA, Trojanowska M 2001 Differential regulation of transforming growth factor-beta receptors type I and II by platelet-derived growth factor in human dermal fibroblasts. Br J Dermatol 145:569-75.

Dahlberg L, Kreicbergs A 1991 Demineralized allogeneic bone matrix for cartilage repair. J Orthop Res 9:11-9.

Datuin JP, Ng KP, Hayes TB, Bern HA 2001 Effects of glucocorticoids on cartilage growth and response to IGF-I in the tilapia (Oreochromis mossambicus). Gen Comp Endocrinol 121:289-94.

de Caestecker MP, Bottomley M, Bhattacharyya S, Payne TL, Roberts AB, Yelick PC 2002 The novel type I serine-threonine kinase receptor ALK8 binds TGF-β in the presence of TGF-βRII. Biochem Biophys Res Com 293:1556-1565.

de Haart M, Marijnissen WJ, van Osch GJ, Verhaar JA 1999 Optimization of chondrocyte expansion in culture. Effect of TGF beta-2, bFGF and L-ascorbic acid on bovine articular chondrocytes. Acta Orthop Scand 70:55-61.

de Larco JE, Todaro GJ 1978 Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci U S A 75:4001-5.

De Winter JP, De Vries CJ, Van Achterberg TA, Ameerun RF, Feijen A, Sugino H, De Waele P, Huylebroeck D, Verschueren K, Van Den Eijden-Van Raaij AJ 1996 Truncated activin type II receptors inhibit bioactivity by the formation of heteromeric complexes with activin type I receptors. Exp Cell Res 224:323-34.

DeGroot J, Verzijl N, Bank RA, Lafeber FP, Bijlsma JW, TeKoppele JM 1999 Age-related decrease in proteoglycan synthesis of human articular chondrocytes: the role of nonenzymatic glycation. Arthritis Rheum. 42:1003-9.

Demoor-Fossard M, Boittin M, Redini F, Pujol JP 1999 Differential effects of interleukin-1 and transforming growth factor beta on the synthesis of small proteoglycans by rabbit articular chondrocytes cultured in alginate beads as compared to monolayers. Mol Cell Biochem 199:69-80.

Demoor-Fossard M, Redini F, Boittin M, Pujol JP 1998 Expression of decorin and biglycan by rabbit articular chondrocytes. Effects of cytokines and phenotypic modulation. Biochem Biophys Acta 1398:179-91.

Derynck R, Akhurst RJ, Balmain A 2001 TGF-beta signaling in tumor suppression and cancer progression. Nat Genet 29:117-29.

Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ 1995a Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development 121:1845-54.

Diebold RJ, Eis MJ, Yin M, Ormsby I, Boivin GP, Darrow BJ, Saffitz JE, Doetschman T 1995 Early-onset multifocal inflammation in the transforming growth factor beta 1-null mouse is lymphocyte mediated. Proc Natl Acad Sci USA 92:12215-9.

Diez-Marques L, Ortega-Velazquez R, Langa C, Rodriguez-Barbero A, Lopez-Novoa JM, Lamas S, Bernabeu C 2002 Expression of endoglin in human mesangial cells: modulation of extracellular matrix synthesis. Biochem Biophys Acta 1587:36-44.

Docagne F, Colloc'h N, Bougueret V, Page M, Paput J, Tripier M, Dutartre P, MacKenzie ET, Buisson A, Komesli S, Vivien D 2001 A soluble TGF-β type-1 receptor mimics TGF-β responses. J Biol Chem 276: 46243-46250.

Domm C, Schunke M, Christesen K, Kurz B 2002 Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. Osteoarthritis Cartilage 10:13-22.

Dumont N, O'Connor-McCourt M, Philip A 1995 Transforming growth factor-β receptors on human endometrial cells: Identification of the type I, II and III receptors, and glycosylphosphatidylinositol anchored TGF-β binding proteins. Mol Cell Endocrinol 111:57-66.

Dumont P, Burton M, Chen QM, Gonos ES, Frippiat C, Mazarati JB, Eliaers F, Remacle J, Toussaint O 2000 Induction of replicative senescence biomarkers by sublethal oxidative stresses in normal human fibroblast. Free Radic Biol Med 28:361-73.

Edwards DR, Murphy G, Reynolds JJ, Whitham SE, Docherty AJ, Angel P, Heath JK 1987 Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J 6:1899-904.

Eickelberg O, Centrella M, Reiss M, Kashgarian M, Wells RG 2002 Betaglycan inhibits TGF-beta signaling by preventing type I-type II receptor complex formation. Glycosaminoglycan modifications alter betaglycan function. J Biol Chem 277:823-9.

Elders MJ 2000 The increasing impact of arthritis on public health. J Rheumatol Suppl 60:6-8.

Elford PR, Graeber M, Ohtsu H, Aeberhard M, Legendre B, Wishart WL, MacKenzie AR 1992 Induction of swelling, synovial hyperplasia and cartilage proteoglycan loss upon intra-articular injection of transforming growth factor beta-2 in the rabbit. Cytokine 4:232-8.

Elisseeff J, McIntosh W, Fu K, Blunk BT, Langer R 2001 Controlled-release of IGF-I and TGF-beta1 in a photopolymerizing hydrogel for cartilage tissue engineering. J Orthop Res 19:1098-104.

Ellingsworth LR, Brennan JE, Fok K, Rosen DM, Bentz H, Piez KA, Seyedin SM 1986 Antibodies to the N-terminal portion of cartilage-inducing factor A and transforming growth factor beta. Immunohistochemical localization and association with differentiating cells. J Biol Chem 261:12362-7.

Engkvist O, Wilander E 1979 Formation of cartilage from rib perichondrium grafted to an articular defect in the femur condyle of the rabbit. Scand J Plast Reconstr Surg. 13:371-76.

Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R 1995 Towards a molecular understanding of skeletal development. Cell 80:371-378.

Esparza-Lopez J, Montiel JL, Vilchis-Landeros MM, Okadome T, Miyazono K, Lopez-Casillas F 2001 Ligand binding and functional properties of betaglycan, a co-receptor of the transforming growth factor-beta superfamily. Specialized binding regions for transforming growth factor-beta and inhibin A. J Biol Chem 276:14588-96.

Fanger BO, Wakefield LM, Sporn MB 1986 Structure and properties of the cellular receptor for transforming growth factor type beta. Biochemistry 25:3083-91.

Ferlic DC 1985 Osteoarthritis of the hand. Orthop Surg Update Series, L36 3:1-8.

Folkman J, D'Amore PA 1996 Blood vessel formation: what is its molecular basis? Cell 87:1153-5.

Fonsatii E, del Vechchio L, Altomonte M, Sigalotti L, Nicotra MR, Coral S, Natali PG, Maio M 2001 Endoglin: An accessory component of the TGF-β-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. J Cell Phys 188:1-7.

Franzen P, ten Dijke P, Ichijo H, Yamashita H, Schultz P, Heldin C H, Miyazono K 1993 Cloning of TGF-β type I receptor that forms a heterodimeric complex with the type II TGF-β receptor. Cell 75: 681-692.

Frazer A, Seid JM, Hart KA, Bentley H, Bunning RA, Russell RG 1991 Detection of mRNA for the transforming growth factor beta family in human articular chondrocytes by the polymerase chain reaction. Biochem Biophys Res Commun 180:602-8.

Freed LE, Marquis JC, Nohria A, Emmanual J, Mikos AG, Langer R 1993 Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers. J Biomed Mater Res 27:11-23.

Frenkel SR, Saadeh PB, Mehrara BJ, Chin GS, Steinbrech DS, Brent B, Gittes GK, Longaker M 2000 Transforming growth factor beta superfamily members: role in cartilage modeling. Plast Reconstr Surg 105:980-90.

Frolik CA, Dart LL, Meyers CA, Smith DM, Sporn MB 1983 Purification and initial characterization of a type beta transforming growth factor from human placenta. Proc Natl Acad Sci USA 80:3676-80.

Fukui N, Purple CR, Sandell LJ 2001 Cell biology of osteoarthritis: the chondrocyte's response to injury. Curr Rheum Reports 3:496-505.

Fukumura K, Matsunaga S, Yamamoto T, Nagamine T, Ishidou Y, Sakou T 1998 Immunolocalization of transforming growth factor-beta s and type I and type II receptors in rat articular cartilage. Anticancer Res 18:4189-4194.

Fukushima D, Butzow R, Hildebrand A, Ruoslahti E 1993 Localization of transforming growth factor beta binding site in betaglycan. Comparison with small extracellular matrix proteoglycans. J Biol Chem 268:22710-5.

Galera P, Redini F, Vivien D, Bonaventure J, Penfornis H, Loyau G, Pujol JP 1992 Effect of transforming growth factor-beta 1 (TGF-beta 1) on matrix synthesis by monolayer cultures of rabbit articular chondrocytes during the dedifferentiation process. Exp Cell Res 200:379-92.

Glansbeek HL, van der Kraan PM, Lafeber FPJG Vitters EL, van den Berg WB 1997 Species-specific expression of type II TGF-β receptor isoforms by articular chondrocytes: affect on proteoglycan depletion and aging. Cytokine 9:347-351.

Glansbeek HL, van der Kraan PM, Vitters EL, van den Berg WB 1993 Correlation of the size of type II transforming growth factor β (TGF-β) receptor with TGF-β responses of isolated bovine articular chondrocytes. Ann Rheum Dis 52:812-816.

Glick AB, Danielpour D, Morgan D, Sporn MB, Yuspa SH 1990 Induction and autocrine receptor binding of TGF-β2 during terminal differentiation of primary mouse keratinocytes. Mol Endocrinol 4:46-52.

Glick AB, Flanders KC, Danielpour D, Yuspa SH, Sporn MB 1989 Retinoic acid induces transforming growth factor-beta 2 in cultured keratinocytes and mouse epidermis. Cell Regul 1:87-97.

Glick AB, McCune BK, Abdulkarem N, Flanders KC, Lumadue JA, Smith JM, Sporn MB 1991 Complex regulation of TGF beta expression by retinoic acid in the vitamin A-deficient rat. Development 111:1081-6.

Gobbi H, Arteaga CL, Jensen RA, Simpson JF, Dupont WD, Olson SJ, Schuyler PA, Plummer WD Jr, Page DL 2000 Loss of expression of transforming growth factor beta type II receptor correlates with high tumour grade in human breast in-situ and invasive carcinomas. Histopathology 36:168-77.

Goldring MB 2000a Osteoarthritis and Cartilage: The role of Cytokines. Curr Rheum Reports 2:459-465.

Goldring MB 2000b The role of the chondrocyte in osteoarthritis. Arthritis and Rheumatism 43:1916-26.

Goldring MB, Berenbaum F 1999 Human chondrocyte culture models for studying cyclooxygenase expression and prostaglandin regulation of collagen gene expression. Osteoarthritis Cartilage 7:386-88.

Goransson H, Lehtosalo J, Vuola J, Patiala H, Rokkanen P 1995 Regeneration of defects in articular cartilage with callus and cortical bone grafts. An experimental study. Scand J Plast Reconstr Surg Hand Surg 29:281-7.

Gorman C 1996 Relief for swollen joints. TIME: 86.

Gougos A, Letarte M 1988 Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line. J Immunol 141:1925-1933.

Gougos A, Letarte M 1990 Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. J Biol Chem 265:8361-4.

Gougos A, St Jacques S, Greaves A, O'Connell PJ, d'Apice AJ, Buhring HJ, Bernabeu C, van Mourik JA, Letarte M 1992 Identification of distinct epitopes of endoglin, an RGD-containing glycoprotein of endothelial cells, leukemic cells, and syncytiotrophoblasts. Int Immunol 4:83-92.

Goumans MJ, Mummery C 2000 Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. Int J Dev Biol 44:253-65.

Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P 2002 Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J 21:1743-53.

Grande DA, Pitman MI, Peterson L, Menche D, Klein M 1989 The repair of experimentally produced defects in rabbit articular cartilage by autologous chondrocyte transplantation. J Orthop Res 7:208-18.

Grande JP 1997 Role of transforming growth factor-beta in tissue injury and repair. Proc Soc Exp Biol Med 214:27-40.

Guerne PA, Blanco F, Kaelin A, Desgeorges A, Lotz M 1995 Growth factor responsiveness of human articular chondrocytes in aging and development. Arthritis Rheum 38:960-8.

Guerrero-Esteo M, Lastres P, Letamendia A, Perez-Alvarez MJ, Langa, C, Lopez LA, Fabra A, Garcia-Pardo A, Vera S, Letarte M, Bernabeu C 1999 Endoglin overexpression modulates cellular morphology, migration, and adhesion of mouse fibroblasts. E J Cell Biol 78:614-623.

Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C 2002 Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-β receptors I and II. J Biol Chem In Press.

Guimond A, Sulea T, Pen A, Ear P, O'Connor-McCourt MD 2002a Site-directed mutagenesis of the type II TGF-beta receptor indicates a ligand-binding site distinct from that of the type II activin receptor. FEBS Lett 515:13-9.

Guimond A, Sulea T, Zwaagstra JC, Ekiel I, O'Connor-McCourt MD 2002b Identification of a functional site on the type I TGF-beta receptor by mutational analysis of its ectodomain. FEBS Lett 513:147-52.

Guo J, Jourdian GW, MacCallum DK 1989 Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Conn Tissue Res 19:277-297.

Guttmacher AE, Marchuk DA, White RI Jr 1995 Hereditary hemorrhagic telangiectasia. N Engl J Med 333:918-24.

Haitjema T, Westermann CJ, Overtoom TT, Timmer R, Disch F, Mauser H, Lammers JW 1996 Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu disease): new insights in pathogenesis, complications, and treatment. Arch Intern Med 156:714-9.

Hall FL, Benya PD, Padilla SR, Carbonaro-Hall D, Williams R, Buckley S, Warburton D 1996 Transforming growth factor-beta type-II receptor signalling: intrinsic/associated casein kinase activity, receptor interactions and functional effects of blocking antibodies. Biochem J 316(Pt 1):303-10.

Hambach L, Neureiter D, Zeiler G, Kirchner T, Aigner T 1998 Severe disturbance of the distribution and expression of type VI collagen chains in osteoarthritic articular cartilage. Arthritis Rheum 41:986-96.

Hamerman D 1989 The biology of osteoarthritis. N Engl J Med 320:1322-30.

Hanamura H, Higuchi Y, Nakagawa M, Iwata H, Nogami H, Urist MR 1980 Solubilized bone morphogenetic protein (BMP) from mouse osteosarcoma and rat demineralized bone matrix. Clin Orthop 148:281-90.

Hanks SK, Quinn AM, Hunter T 1988 The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241:42-52.

Hashimoto S, Ochs RL, Komiya S, Lotz M 1998a Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. Arthritis Rheum 41:1632-8.

Hashimoto S, Takahashi K, Amiel D, Coutts RD, Lotz M 1998b Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis. Arthritis Rheum 41:1266-74.

Haudenschild DR, McPherson JM, Tubo R, Binette F 2001 Differential expression of multiple genes during articular chondrocyte redifferentiation. Anat Rec 263:91-8.

Hauselmann HJ, Aydelotte MB, Schumacher BL, Kuettner KE, Gitelis SH, Thonar J-MA 1992 Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. Matrix 12:116-129.

Hauselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block JA, Aydelotte MB, Kuettner KE, Thonar EJ 1994 Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J Cell Sci 107:17-27.

Hauselmann HJ, Flechtenmacher J, Michal L, Thonar EJ, Shinmei M, Kuettner KE, Aydelotte MB 1996 The superficial layer of human articular cartilage is more

susceptible to interleukin-1-induced damage than the deeper layers. Arthritis Rheum 39:478-88.

Hauselmann HJ, Stefanovic-Racic M, Michel BA, Evans CH 1998 Differences in nitric oxide production by superficial and deep human articular chondrocytes: implications for proteoglycan turnover in inflammatory joint diseases. J Immunol 160:1444-8.

Havrilesky LJ, Hurteau JA, Whitaker RS, Elbendary A, Wu S, Rodriguez GC, Bast RC Jr, Berchuck 1995 Regulation of apoptosis in normal and malignant ovarian epithelial cells by transforming growth factor beta. Cancer Res 55:944-8.

Heino J, Ignotz RA, Hemler ME, Crouse C, Massague J 1989 Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. J Biol Chem 264:380-8.

Heino J, Massague J 1989 Transforming growth factor-beta switches the pattern of integrins expressed in MG-63 human osteosarcoma cells and causes a selective loss of cell adhesion to laminin. J Biol Chem 264:21806-11.

Helminen HJ, Saamanen AM, Salminen H, Hyttinen MM 2002 Transgenic mouse models for studying the role of cartilage macromolecules in osteoarthritis. Rheumatology 41:848-56.

Hildebrand A, Romaris M, Rasmussen LM, Heinegard D, Twardzik DR, Border WA, Ruoslahti E 1994 Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. Biochem J 302 (Pt 2):527-34.

Hirai R, Fujita T 1996 A human transforming growth factor-β type II receptor that contains an insertion in the extracellular domain. Exp Cell Res 223:135-141.

Hiraki Y, Inoue H, Iyama K, Kamizono A, Ochiai M, Shukunami C, Iijima S, Suzuki F, Kondo J 1997 Identification of chondromodulin I as a novel endothelial cell growth inhibitor. Purification and its localization in the avascular zone of epiphyseal cartilage. J Biol Chem 272:32419-26.

Hogan BL 1996 Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev 10:1580-94.

Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck, Poole AR 1994 Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. J Clin Invest 93:1722-1732.

Hollander AP, Pidoux I, Reiner A, Rorabeck C, Bourne R, Poole AR 1995 Damage to type II collagen in aging and osteoarthritis starts at the articular surface, originates around chondrocytes, and extends into the cartilage with progressive degeneration. J Clin Invest 96:2859-69.

Homminga GN, van der Linden TJ, Terwindt-Rouwenhorst EA, Drukker J 1989 Repair of articular defects by perichondrial grafts. Experiments in the rabbit. Acta Orthop Scand 60:326-9.

Horner A, Kemp P, Summers C, Bord S, Bishop NJ, Kelsall AW, Coleman N, Compston JE 1998 Expression and distribution of transforming growth factor-beta isoforms and their signaling receptors in growing human bone. Bone 23:95-102.

Howard S, Anastasiades T 1993 Differential effects of bone associated factors on newly synthesized anionic glycoconjugates by articular chondrocyte cultures from adult and immature bovines. J Rheumatol 20:2083-2094.

Hoyland JA, Thomas JT, Donn R, Marriott A, Ayad S, Boot-Handford RP, Grant ME, Freemont AJ 1991 Distribution of type X collagen mRNA in normal and osteoarthritic human cartilage. Bone Miner 15:151-63.

Hunziker EB 2001 Growth-factor-induced healing of partial-thickness defects in adult articular cartilage. Osteoarthritis Cartilage 9:22-32.

Hunziker EB, Driesang IM, Morris EA 2001 Chondrogenesis in cartilage repair is induced by members of the transforming growth factor-beta superfamily. Clin Orthop 391 Suppl: S171-81.

Hynes RO 1992 Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11-25.

Ignotz RA, Massague J 1987 Cell adhesion protein receptors as targets for transforming growth factor-beta action. Cell 51:189-97.

Inagaki M, Moustakas A, Lin HY, Lodish HF, Carr BI 1993 Growth inhibition by transforming growth factor beta (TGF-beta) type I is restored in TGF-beta-resistant hepatoma cells after expression of TGF-beta receptor type II cDNA. Proc Natl Acad Sci U S A 90:5359-63.

Inoue H, Kato Y, Iwamoto M, Hiraki Y, Sakuda M, Suzuki F 1989 Stimulation of cartilage-matrix proteoglycan synthesis by morphologically transformed chondrocytes grown in the presence of fibroblast growth factor and transforming growth factor-beta. J Cell Physiol 138:329-37.

Insall J 1974 The Pridie debridement operation for osteoarthritis of the knee. Clin Orthop 101:61-7.

Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M 1993 Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 363:558-61.

Iozzo RV, Murdoch AD 1996 Proteoglycans of the extracellular environment: clues from the gene and protein side offer novel perspectives in molecular diversity and function. FASEB J 10:598-614.

Iqbal J, Dudhia J, Bird JL, Bayliss MT 2000 Age-related effects of TGF-beta on proteoglycan synthesis in equine articular cartilage. Biochem Biophys Res Commun 274:467-71.

Itayem R, Mengarelli-Widholm S, Reinholt FP 1999 The long-term effect of a short course of transforming growth factor-beta1 on rat articular cartilage. APMIS 107:183-92.

Iwamoto M, Sato K, Nakashima K, Fuchihata H, Suzuki F, Kato Y 1989 Regulation of colony formation of differentiated chondrocytes in soft agar by transforming growth factor-beta. Biochem Biophys Res Commun 159:1006-11.

Jackson CJ, Arkell J, Nguyen M 1998 Rheumatoid synovial endothelial cells secrete decreased levels of tissue inhibitor of MMP (TIMP1). Ann Rheum Dis 57:158-61.

Jakob M, Demarteau O, Schafer D, Hintermann B, Dick W, Heberer M, Martin I 2001 Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro. J Cell Biochem 81:368-77.

Jirholt J, Lindqvist A-KB, Holmdahl R 2001 The genetics of rheumatoid arthritis and the need for animal models to find and understand the underlying genes. Arthritis Res 3:87-97.

Johnstone B Hering TM, Caplan AI, Goldberg VM, Yoo JU 1998 In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238:265-72.

Jonker L, Arthur HM 2002 Endoglin expression in early development is associated with vasculogenesis and angiogenesis. Mech Dev 110:193-6.

Jullien P, Berg TM, Lawrence DA 1989 Acidic cellular environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF. Int J Cancer 43:886-91.

Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J 1995 Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. Nat Genet 11:415-21.

Kang R, Marui T, Ghivizzani SC, Nita IM, Georgescu HI, Suh JK, Robbins PD, Evans CH 1997 Ex vivo gene transfer to chondrocytes in full-thickness articular cartilage defects: a feasibility study. Osteoarthritis Cartilage 5:139-43.

Kawabe N, Yoshinao M 1991 The repair of full-thickness articular cartilage defects. Immune responses to reparative tissue formed by allogeneic growth plate chondrocyte implants. Clin Orthop 268:279-93.

Kellgren J, Lawrence J 1957 Radiological assessment of osteoarthritis. Ann Rheum Dis 16:494-502.

Kempson GE 1982 Relationship between the tensile properties of articular cartilage from the human knee and age. Ann Rheum Dis 41:508-11.

Khalil N, Parekh TV, O'Connor R, Antman N, Kepron W, Yehaulaeshet T, Xu YD, Gold LI 2001 Regulation of the effects of TGF-beta 1 by activation of latent TGF-beta 1 and differential expression of TGF-beta receptors (T beta R-I and T beta R-II) in idiopathic pulmonary fibrosis. Thorax 56:907-15.

Kim HA, Suh DI, Song YW 2001a Relationship between chondrocyte apoptosis and matrix depletion in human articular cartilage. J Rheumatol 28:2038-45.

Kim HK, Moran ME, Salter RB 1991 The potential for regeneration of articular cartilage in defects created by chondral shaving and subchondral abrasion. An experimental investigation in rabbits. J Bone Joint Surg Am 73:1301-15.

Kim S J, Park K, Koeller D, Kim KY, Wakefield LM, Sporn MB, Roberts AB 1992 Post-transcriptional regulation of the human transforming growth factor-β1 gene. J Biol Chem 267:13702-13707.

Kingsley DM 1994 The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev 8:133-46.

Knaus P, Sebald W 2001 Cooperativity of binding epitopes and receptor chains in the BMP/TGFbeta superfamily. Biol Chem 382:1189-95.

Knaus PI, Lindemann D, DeCoteau JF, Perlman R, Yankelev H, Hille M, Kadin ME, Lodish HF 1996 A dominant inhibitory mutant of the type II transforming growth factor beta receptor in the malignant progression of a cutaneous T-cell lymphoma. Mol Cell Biol 16:3480-9.

Koepp H, Eger W, Muehleman C, Valdellon A, Buckwalter JA, Kuettner KE, Cole AA. 1999 Prevalence of articular cartilage degeneration in the ankle and knee joints of human organ donors. J Orthop Sci 4:407-12.

Koli KM, Arteaga CL 1997 Processing of the transforming growth factor beta type I and II receptors. Biosynthesis and ligand-induced regulation. J Biol Chem 272:6423-7.

Konttinen YT, Ainola M, Valleala H, Ma J, Ida H, Mandelin J, Kinne RW, Santavirta S, Sorsa T, Lopez-Otin C, Takagi M 1999 Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. Ann Rheum Dis 58:691-7.

Kuhn K, Lotz M 2001 Regulation of CD95 (Fas/APO-1)-induced apoptosis in human chondrocytes. Arthritis Rheum 44:1644-53.

Kulkarni AB, Karlsson S 1993 Transforming growth factor-beta 1 knockout mice. A mutation in one cytokine gene causes a dramatic inflammatory disease. Am J Pathol 143:3-9.

Lafeber FPJG, van der Kraan PM, Huber-Bruning O, van den Berg WB, Bijlsma JWJ 1993 Osteoarthritic human cartilage is more sensitive to transforming growth factor $\beta$ than is normal cartilage. Br J Rheumatol 32:281-286.

Lafeber FPJG, van Roy HLAM, van der Kraan PM, Berg WB, Bijlsma JWJ 1997 Transforming growth factor-$\beta$ predominantly stimulates phenotypically changed chondrocytes in osteoarthritis human cartilage. J Rheumatol 24:536-542.

Lafyatis R, Thompson NL, Remmers EF, Flanders KC, Roche NS, Kim SJ, Case JP, Sporn MB, Roberts AB, Wilder RL 1989 Transforming growth factor-beta production by synovial tissues from rheumatoid patients and streptococcal cell wall arthritic rats. Studies on secretion by synovial fibroblast-like cells and immunohistologic localization. J Immunol 143:1142-8.

Laiho M, Saksela O, Andreasen PA, Keski-Oja J 1986 Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-beta. J Cell Biol 103:2403-10.

Laiho M, Weis FM, Boyd FT, Ignotz RA, Massague J 1991 Responsiveness to transforming growth factor-beta (TGF-beta) restored by genetic complementation between cells defective in TGF-beta receptors I and II. J Biol Chem 266:9108-12.

Laiho M, Weis FMB, Massague J 1990 Concomitant loss of Transforming Growth Factor (TGF)-β receptor types I and II in TGF-β-resistant cell mutants implicates both receptor types in signal transduction. J Biol Chem 265: 18518-18524.

LaMarre J, Hayes MA, Wollenberg GK, Hussaini I, Hall SW, Gonias SL 1991 An alpha 2-macroglobulin receptor-dependent mechanism for the plasma clearance of transforming growth factor-beta 1 in mice. J Clin Invest 87:39-44.

Larsson J, Goumans M-J, Sjostrand LJ, van Rooijen MA, Ward D, Leveen P, Xu X, ten Dijke P, Mummery CL, Karlsson S 2001 Abnormal angiogenesis but intact hematopoietic potential in TGF-β type I receptor-deficient mice. EMBO J 20:1663-1673.

Lastres P, Bellon T, Cabanas C, Sanchez-Madrid F, Acevedo A, Gougos A, Letarte M, Bernabeu C 1992 Regulated expression on human macrophages of endoglin, an Arg-Gly-Asp-containing surface antigen. Eur J Immunol 22:393-7.

Lastres P, Letamendia A, Zhang H, Rius C, Almendro N, Raab U, Loepez LA, Langa C, Fabra A, Letarte M, Bernabeu C 1996 Endoglin modulates cellular responses to TGF-βI. J Cell Biol 133:1109-1121.

Lastres P, Martin-Perez J, Langa C, Bernabeu C 1994 Phosphorylation of the human-transforming-growth-factor-beta-binding protein endoglin. Biochem J 301(Pt 3):765-8.

Lawrence RC, Helmick CG, Arnett FC, Deyo RA, Felson DT, Giannini EH, Heyse SP, Hirsch R, Hochberg MC, Hunder GG, Liang MH, Pillemer SR, Steen VD, Wolfe F 1998 Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. Arthritis Rheum 41:778-99.

Leask A, Abraham DJ, Finlay DR, Holmes A, Pennington D, Shi-Wen X, Chen Y, Venstrom K, Dou X, Ponticos M, Black C, Bernabeu C, Jackman JK, Findell PR, Connolly MK 2002 Dysregulation of transforming growth factor beta signaling in scleroderma: overexpression of endoglin in cutaneous scleroderma fibroblasts. Arthritis Rheum 46:1857-65.

Lee JD, Hwang O, Kim SW, Han S 1997 Primary cultured chondrocytes of different origins respond differently to bFGF and TGF-beta. Life Sci 61:293-9.

Lee JW, Qi WN, Scully SP 2002 The involvement of beta1 integrin in the modulation by collagen of chondrocyte-response to transforming growth factor-beta1. J Orthop Res 20:66-75.

Letamendia A, Lastres P, Almendro N, Raab U, Buhring H-J, Kumar S, Bernabeu C 1998b Endoglin, a component of the TGF-β receptor system, is a differentiation marker of human choriocarcinoma cells. Int J Cancer 76:541-546.

Letamendia A, Lastres P, Botella LM, Raab U, Langa C, Velasco B, Attisano L, Bernabeu C 1998a Role of endoglin in cellular responses to transforming growth factor-β. A comparative study with betaglycan. J Biol Chem 273:33011-33019.

Lewis KA, Gray PC, Blount AL, Macconell LA, Waiter E, Bilezikjian LM, Vale W 2000 Betaglycan binds inhibin and can mediate functional antagonism of activin signaling. Nature 404:411-414.

Lexer E 1925 Surg Gynec Obstet 40:782.

Li C, Guo B, Wilson PB, Stewart A, Byrne G, Bundred N, Kumar S 2000b Plasma levels of soluble CD105 correlate with metastasis in patients with breast cancer. Int J Cancer 89:122-6.

Li C, Harpson L, Kumar P, Bernabeu C, Kumar S 2000a CD105 antagonizes the inhibitory signaling of transforming growth factor β1 on human vascular endothelial cells. FASEB J 14:55-64.

Li CG, Bethell H, Wilson PB, Bhatnagar D, Walker MG, Kumar S 2000c The significance of CD105, TGFbeta and CD105/TGFbeta complexes in coronary artery disease. Atherosclerosis 152:249-56.

Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP 1999 Defective angiogenesis in mice lacking endoglin. Science 284:1534-7.

Lieberman JR, Daluiski A, Einhorn TA 2002 The role of growth factors in the repair of bone. Biology and clinical applications. J Bone Joint Surg Am 84-A:1032-44.

Loening AM, James IE, Levenston ME, Badger AM, Frank EH, Kurz B, Nuttall ME, Hung HH, Blake SM, Grodzinsky AJ, Lark MW 2000 Injurious mechanical compression of bovine articular cartilage induces chondrocyte apoptosis. Arch Biochem Biophys 381:205-12.

Loeser R 1997 Growth factor regulation of chondrocyte integrins. Differential effects of insulin-like growth factor 1 and transforming growth factor beta on alpha 1 beta 1

integrin expression and chondrocyte adhesion to type VI collagen. Arthritis Rheum 40:270-6.

Loeser RF, Carlson CS, McGee MP 1995 Expression of beta 1 integrins by cultured articular chondrocytes and in osteoarthritic cartilage. Exp Cell Res 217:248-57.

Lopez-Casillas F, Cheifetz S, Doody J, Andres JL, Lane WS, Massague J 1991 Structure and expression of the membrane protein betaglycan, a component of the TGF-β receptor system. Cell 15:785-95.

Lopez-Casillas F, Payne HM, Andres JL, Massague J 1994 Betaglycan can act as a dual modulator of TGF-β access to signalling receptors: mapping of ligand binding and GAG attachment sites. J Cell Biol 124:557-568.

Lopez-Casillas F, Wrana JL, Massague J 1993 Betaglycan presents ligand to the TGF-β signaling receptor. Cell 73:1435-1444.

Low MG 1989 Glycosyl-phosphatidylinositol: a versatile anchor for cell surface proteins. FASEB J 3:1600-8.

Lux A, Attisano L, Marchuk DA 1999 Assignment of transforming growth factor β1 and β3 and a third new ligand to the type I receptor ALK-1 J Biol Chem 274:9984-9992.

Macias-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL 1998 Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. J Biol Chem 273:25628-36.

Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF 1998 Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 4:415-28.

Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF 1998 Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 4:415-28.

Madri JA, Pratt BM, Tucker AM 1988 Phenotypic modulation of endothelial cells by transforming growth factor-beta depends upon the composition and organization of the extracellular matrix. J Cell Biol 106:1375-84.

Majumdar MK, Banks V, Peluso DP, Morris EA 2000 Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells. J Cell Physiol 185:98-106.

Mankin HJ 1982 The response of articular cartilage to mechanical injury. J Bone Joint Surg Am 64:460-6.

Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, Fan RS, Zborowska E, Kinzler KW, Vogelstein B, et al 1995 Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 268:1336-8.

Martel-Pelletier J, McCollum R, DiBattista J, Faure MP, Chin JA, Fournier S, Sarfati M, Pelletier JP 1992 The interleukin-1 receptor in normal and osteoarthritic human articular chondrocytes. Identification as the type I receptor and analysis of binding kinetics and biologic function. Arthritis Rheum 35:530-40.

Martin JA, Buckwalter JA 2000 The role of chondrocyte-matrix interactions in maintaining and repairing articular cartilage. Biorheology 37:129-40.

Martin JA, Buckwalter JA 2001 Roles of articular cartilage aging and chondrocyte senescence in the pathogenesis of osteoarthritis. Iowa Orthop J 21:1-7.

Martin JA, Buckwalter JA 2002 Aging, articular cartilage chondrocyte senescence and osteoarthritis. Biogerontology 3:257-64.

Martinsen A, Skjak-Braek G, Smidsrod O 1989 Alginate as immobilization material. 1. Correlation between chemical and physical properties of alginate gel beads. Biotechnol Bioeng 26:53-58.

Massague J 1985 Transforming growth factors. Isolation, characterization, and interaction with cellular receptors. Prog Med Virol. 1985;32:142-58.

Massague J 1990a The transforming growth factor-beta family. Annu Rev Cell Biol 6:597-641.

Massague J 1990b The TGF-beta family of growth and differentiation factors. Cell 49:437-8.

Massague J 1992 Receptors for the TGF-beta family. Cell 69:1067-70.

Massague J 1998 TGF-beta signal transduction. Ann Rev Biochem 67:753-791.

Massague J 2000 How cells read TGF-ß signals. Nature (reviews) 1:169-178.

Massague J, Blain SW, Lo RS 2000 TGFbeta signaling in growth control, cancer, and heritable disorders. Cell 103:295-309.

Mather JP 1996 Follistatins and alpha 2-macroglobulin are soluble binding proteins for inhibin and activin. Horm Res 45:207-10.

Mathews LS 1994 Activin receptors and cellular signaling by the receptor serine kinase family. Endocr Rev 15:310-25.

Matsunaga S, Yamamoto T, Fukumura K 1999 Temporal and spatial expressions of transforming growth factor-betas and their receptors in epiphyseal growth plate. Int J of Onc 14:1063-1067.

Mayne R, Brewton RG 1993 Extracellular matrix of cartilage: collagen. In: Woessner JF Jr, Howell DS (eds) Joint Cartilage Degradation. Basic and Clinical Aspects. Marcel Dekker, New York, NY, pp. 81-108.

Mazzieri R, Munger JS, Rifkin DB 2000 Measurement of active TGF-beta generated by cultured cells. Methods Mol Biol 142:13-27.

McAllister KA, Baldwin MA, Thukkani AK, Gallione CJ, Berg JN, Porteous ME, Guttmacher AE, Marchuk DA 1995 Six novel mutations in the endoglin gene in hereditary hemorrhagic telangiectasia type 1 suggest a dominant-negative effect of receptor function. Hum Mol Genet 4:1983-1985.

McDonald MT, Papenberg KA, Ghosh S, Glatfelter AA, Biesecker BB, Helmbold EA, Markel DS, Zolotor A, McKinnon WC, Vanderstoep JL, et al 1994 A disease locus for hereditary haemorrhagic telangiectasia maps to chromosome 9q33-34. Nat Genet 6:197-204.

Meachim G, Ghadially FN, Collins DH 1965 Regressive changes in the superficial layer of human articular cartilage. Ann Rheum Dis 24:23-30.

Miller DW, Graulich W, Karges B, Stahl S, Ernst M, Ramaswamy A, Sedlacek AH, Muller R, Adamkiewicz J 1999 Elevated expression of Endoglin, a component of the TGF-β-receptor complex, correlates with proliferation of tumor endothelial cells. Int J Cancer 81:568-572.

Mitchell N, Shepard N 1976 The resurfacing of adult rabbit articular cartilage by multiple perforations through the subchondral bone. J Bone Joint Surg Am 58:230-3.

Mitrovic D, Quintero M, Stankovic A, Ryckewaert A 1983 Cell density of adult human femoral condylar articular cartilage. Joints with normal and fibrillated surfaces. Lab Invest 49:309-16.

Miura Y, Fitzsimmons JS, Commisso CN, Gallay SH, O'Driscoll SW 1994 Enhancement of periosteal chondrogenesis in vitro. Dose-response for transforming growth factor-beta 1 (TGF-beta 1). Clin Orthop 301:271-80.

Miyazono K, Heldin CH 1989 Role for carbohydrate structures in TGF-β. Nature 338:158-160.

Miyazono K, Ichijo H, Heldin CH 1993 Transforming growth factor-beta: latent forms, binding proteins and receptors. Growth Factors 8:11-22.

Moldovan F, Pelletier JP, Hambor J, Cloutier JM, Martel-Pelletier J 1997 Collagenase-3 (matrix metalloprotease 13) is preferentially localized in the deep layer of human arthritic cartilage in situ: in vitro mimicking effect by transforming growth factor beta. Arthritis Rheum 40:1653-61.

Moldovan F, Pelletier JP, Mineau F, Dupuis M, Cloutier JM, Martel-Pelletier 2000 Modulation of collagenase 3 in human osteoarthritic cartilage by activation of extracellular transforming growth factor beta: role of furin convertase. Arthritis Rheum 43:2100-9.

Morales TI, Roberts AB 1988 Transforming growth factor beta regulates the metabolism of proteoglycans in bovine cartilage organ cultures. J Biol Chem. 263:12828-31.

Morales TI, Roberts AB 1992 The interaction between retinoic acid and the transforming growth factors-beta in calf articular cartilage organ cultures. Arch Biochem Biophys 293:79-84.

Munger JS, Harpel JG, Giancotti FG, Rifkin DB 1998 Interactions between growth factors and integrins: latent forms of transforming growth factor-beta are ligands for the integrin alphavbeta1. Mol Biol Cell 9:2627-38.

Munger JS, Harpel JG, Gleizes P-M, Mazzieri R, Nunes I, Rifkin DB 1997 Latent transforming growth factor-β structural features and mechanism of activation. Kidney Int51:1376-1382.

Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P 1997 TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. EMBO J 16:5353-62.

Nakayama H, Ichikawa F, Anders JL, Massague J, Noda M 1994 Dexamethasone enhancement of betaglycan (TGF-β type III receptor) gene expression in osteoclast-like cells. Exp Cell Res 211:301-306.

Namba RS, Meuli M, Sullivan KM, Le AX, Adzick NS 1998 Spontaneous repair of superficial defects in articular cartilage in a fetal lamb model. J Bone Joint Surg Am 80:4-10.

Nasatzky E, Grinfeld D, Boyan BD, Dean DD, Ornoy A, Schwartz Z 1999 Transforming growth factor-beta1 modulates chondrocyte responsiveness to 17beta-estradiol. Endocrine 11:241-9.

Nelson F, Dahlberg L, Laverty S, Reiner A, Pidoux I, Ionescu M, Fraser GL, Brooks E, Tanzer M, Rosenberg LC, Dieppe P, Robin Poole A 1998 Evidence for altered synthesis of type II collagen in patients with osteoarthritis. J Clin Invest 102:2115-25.

Neuhold LA, Killar L, Zhao W, Sung ML, Warner L, Kulik J, Turner J, Wu W, Billinghurst C, Meijers T, Poole AR, Babij P, DeGennaro LJ 2001 Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. J Clin Invest 107:35-44.

Newman B, Wallis GA 2002 Is osteoarthritis a genetic disease? Clin Invest Med 25:139-49.

Nishimura K, Solchaga LA, Caplan AI, Yoo JU, Goldberg VM, Johnstone B 1999 Chondroprogenitor cells of synovial tissue. Arthritis Rheum 42:2631-7.

Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P 2002 The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. J Biol Chem 277:5330-8.

O'Driscoll SW, Keeley FW, Salter RB 1988 Durability of regenerated articular cartilage produced by free autogenous periosteal grafts in major full-thickness defects in joint surfaces under the influence of continuous passive motion. A follow-up report at one year. J Bone Joint Surg Am 70:595-606.

O'Driscoll SW, Salter RB 1984 The induction of neochondrogenesis in free intra-articular periosteal autografts under the influence of continuous passive motion. An experimental investigation in the rabbit. J Bone Joint Surg Am 66:1248-57.

O'Keefe RJ, Puzas JE, Brand JS, Rosier RN 1988 Effects of transforming growth factor-β on matrix synthesis by chick growth plate chondrocytes. Calcif Tissue Int 43:352-8.

O'Connell PJ, McKenzie A, Fisicaro N, Rockman SP, Pearse MJ, d'Apice AJ 1992 Endoglin: a 180-kD endothelial cell and macrophage restricted differentiation molecule. Clin Exp Immunol 90:154-9.

O'Connor WJ, Botti T, Khan SN, Lane JM 2000 The use of growth factors in cartilage repair. Orthop Clin North Am 31:399-410.

Oddis CV 1996 New perspectives on osteoarthritis. Am J Med 100:10S-15S.

Odenbring S, Egund N, Lindstrand A, Lohmander LS, Willen H 1992 Cartilage regeneration after proximal tibial osteotomy for medial gonarthrosis. An arthroscopic, roentgenographic, and histologic study. Clin Orthop 277:210-6.

O'Driscoll SW, Keeley FW, Salter RB 1986 The chondrogenic potential of free autogenous periosteal grafts for biological resurfacing of major full-thickness defects in joint surfaces under the influence of continuous passive motion. An experimental investigation in the rabbit. J Bone Joint Surg Am 68:1017-35.

O'Driscoll SW, Recklies AD, Poole AR 1994 Chondrogenesis in periosteal explants. An organ culture model for in vitro study. J Bone Joint Surg Am 76:1042-51.

Ogawa Y, Schmidt DK, Dasch JR, Chang RJ, Glaser CB 1992 Purification and characterization of transforming growth factor-beta 2.3 and -beta 1.2 heterodimers from bovine bone. J Biol Chem 267:2325-8.

O'Grady P, Huang SS, Huang JS 1991 Expression of a new type high molecular weight receptor (type V receptor) of transforming growth factor beta in normal and transformed cells. Biochem Biophys Res Commun 179:378-85.

Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, Li E 2000 Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. Proc Natl Acad Sci USA 97:2626-31.

Ohta M, Greenberger JS, Anklesaria P, Bassols A, Massague J 1987 Two forms of transforming growth factor-beta distinguished by multipotential haematopoietic progenitor cells. Nature 329:539-41.

Okazaki R, Sakai A, Nakamura T, Kunugita N, Norimura T, Suzuki K 1996 Effects of transforming growth factor beta s and basic fibroblast growth factor on articular chondrocytes obtained from immobilised rabbit knees. Ann Rheum Dis 55:181-6.

Olsen BR 1995 Mutations in collagen genes resulting in metaphyseal and epiphyseal dysplasias. Bone 17:45S-49S.

Oshima M, Oshima H, Taketo MM 1996 TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. Dev Biol 179:297-302.

Overall CM, Wrana JL, Sodek J 1989 Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor-beta. J Biol Chem 264:1860-9.

Paquet ME, Pece-Barbara N, Vera S, Cymerman U, Karabegovic A, Shovlin C, Letarte M 2001 Analysis of several endoglin mutants reveals no endogenous mature or secreted protein capable of interfering with normal endoglin function. Hum Mol Genet 10:1347-57.

Paralkar VM, Vukicevic S, Reddi AH 1991 Transforming growth factor beta type 1 binds to collagen IV of basement membrane matrix: implications for development. Dev Biol 143:303-8.

Park K, Kim SJ, Bang YJ, Park JG, Kim NK, Roberts AB, Sporn MB 1994 Genetic changes in the transforming growth factor beta (TGF-beta) type II receptor gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF-beta. Proc Natl Acad Sci U S A 91:8772-6.

Parker WL, Blobe GC, Philip A Characterization of endoglin-betaglycan heteromerization: TGF-β binding by endoglin requires betaglycan but not the type II receptor. Submitted.

Parker WL, Choi ME, Philip A Identification of Activin Receptor-like Kinase-1 and Soluble type I TGF-β receptor on human chondrocytes: hetero-oligomerization and regulation of TGF-β signaling. Submitted.

Parker WL, Goldring MB, Philip A Endoglin is expressed on human chondrocytes and forms a heteromeric complex with betaglycan in a ligand and type II TGF-β receptor independent manner. J Bone Miner Res 18:289-302.

Parker WL, Goldring, MB, Philip A Endoglin Expression Links Chondrocyte Phenotype, TGF-β Responsiveness, and Type II Collagen Production: Relevance to Degenerative Joint Disease. Submitted.

Parker WL, Knaus P, Philip A The type IIB TGF-β receptor on human chondrocytes: phenotypic variability of expression and differential effects of endogenous versus exogenous expression. Submitted.

Pece N, Vera S, Cymerman U, White RJ, Wrana JL, Letarte M 1997 Mutant endoglin in Hereditary Hemorrhagic Telangiectasia type 1 is transiently expressed intracellularly and is not a dominant negative. J Clin Invest 100:2568-2579.

Pepin MC, Beauchemin M, Plamondon J, O'Connor-McCourt MD 1994 Mapping of the ligand binding domain of the transforming growth factor beta receptor type III by deletion mutagenesis. Proc Natl Acad Sci USA 91:6997-7001.

Pepper MS, Vassalli JD, Orci L, Montesano R 1993 Biphasic effect of transforming growth factor-beta 1 on in vitro angiogenesis. Exp Cell Res 204:356-63.

Perka C, Schultz O, Spitzer RS, Lindenhayn K 2000 The influence of transforming growth factor beta1 on mesenchymal cell repair of full-thickness cartilage defects. J Biomed Mater Res 52:543-52.

Perlman R, Schiemann WP, Brooks MW, Lodish HF, Weinberg RA 2001 TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. Nat Cell Biol 3:708-14.

Perry RR, Kang Y, Greaves BR 1995 Relationship between tamoxifen-induced transforming growth factor beta 1 expression, cytostasis and apoptosis in human breast cancer cells. Br J Cancer 72:1441-6.

Peterson L, Minas T, Brittberg M, Nilsson A, Sjogren-Jansson E, Lindahl A 2000 Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. Clin Orthop 374:212-34.

Petit B, Freyria A-M, van der Rest M, Herbage D 1992 Cartilage collagens. In: Adolphe M (ed) Biological Regulation of the Chondrocytes. CRC Press, Boca Raton, FL, pp.33-84.

Philip A, Hannah R, O'Connor-McCourt M 1999 Ectodomain cleavage and shedding of the type III TGF-β receptor in the rat lung membranes: Effect of temperature, ligand binding and membrane solubilization. Europ J Biochem 261:618-628.

Philip A, O'Connor-McCourt M 1991 Interaction of transforming growth factor-β with alpha-2–macroglobulin; Role in of transforming growth factor-β clearance. J Biol Chem 266:22290-22296.

Piek E, Franzen P, Heldin CH, ten Dijke P 1997 Characterization of a 60-kDa cell surface-associated transforming growth factor-beta binding protein that can

interfere with transforming growth factor-beta receptor binding. J Cell Physiol 173:447-59.

Piek E, Heldin C-H, ten Dijke P 1999 Specificity, diversity, and regulation in TGF-β superfamily FASEB 13:2105-2124.

Plauchu H, de Chadarevian JP, Bideau A, Robert JM 1989 Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. Am J Med Genet 32:291-7.

Polyak K 1996 Negative regulation of cell growth by TGF beta. Biochem Biophys Acta 1242:185-99.

Poole AR 2001 Cartilage in health and disease. In: Koopman W (ed) Arthritis and allied conditions. A textbook of rheumatology, 14th Edition. Lippincott, Williams, and Wilkins, Philadelphia PA pp.226-84.

Poole AR Webb G 2000 Osteoarthritis. In: Tsoukas GC (ed) Current Molecular Medicine: principles of molecular rheumatology. Humana Press, Totawa NJ pp.401-411.

Poole AR, Kobayashi M, Yasuda T, Laverty S, Mwale F, Kojima T, Sakai T, Wahl C, El-Maadawy S, Webb G, Tchetina E, Wu W 2002 Type II collagen degradation and its regulation in articular cartilage in osteoarthritis. Ann Rheum Dis 61 Suppl 2:ii78-81.

Poole AR, Rosenberg LC, Reiner A, Ionescu M, Bogoch E, Roughley PJ 1996 Contents and distributions of the proteoglycans decorin and biglycan in normal and osteoarthritic human articular cartilage. J Orthop Res 14:681-9.

Poole CA, Matsuoka A, Schofield JR 1991 Chondrons from articular cartilage. III. Morphologic changes in the cellular microenvironment of chondrons isolated from osteoarthritic cartilage. Arthritis Rheum 34:22-35.

Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH 1987 Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. J Exp Med 165:251-6.

Praemer A, Furner S, Rice DP 1999 Musculoskeletal conditions in the United States. American Academy of Orthopaedic Surgeons, Rosemont IL

Pritzker KP 1994 Animal models for osteoarthritis: processes, problems and prospects. Ann Rheum Dis 53:406-20.

Prockop DJ, Kivirikko KI 1995 Collagens: molecular biology, diseases, and potentials for therapy.

Annu Rev Biochem 64:403-34.

Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN, Ding J, Ferguson MW, Doetschman T 1995 Transforming growth factor-β-3 is required for secondary palate fusion. Nature Genet 11:409-419.

Pujol JP, Galera P, Redini F, Mauviel A, Loyau G 1991 Role of cytokines in osteoarthritis: comparative effects of interleukin 1 and transforming growth factor-beta on cultured rabbit articular chondrocytes. J Rheumatol Suppl 27:76-9.

Pullig O, Weseloh G, Klatt AR, Wagener R, Swoboda 2002 Matrilin-3 in human articular cartilage: increased expression in osteoarthritis. Osteoarthritis Cartilage 10:253-63.

Qi WN, Scully SP 1998 Effect of type II collagen in chondrocyte response to TGF-beta 1 regulation. Exp Cell Res 241:142-50.

Qu R, Silver MM, Letarte M 1998 Distribution of endoglin in early human development reveals high levels on endocardial cushion tissue mesenchyme during valve formation. Cell Tissue Res 292:333-43.

Quackenbush EJ, Gougos A, Baumal R, Letarte M 1986 Differential localization within human kidney of five membrane proteins expressed on acute lymphoblastic leukemia cells. J Immunol 136:118-24.

Quackenbush EJ, Letarte M 1985 Identification of several cell surface proteins of non-T, non-B acute lymphoblastic leukemia by using monoclonal antibodies. J Immunol 134:1276-1285.

Raab U, Velasco B, Lastres P, Letamendia A, Cales C, Langa C, Tapia E, Lopez-Bote JP, Paez E, Bernabeu C 1999 Expression of normal and truncated forms of human endoglin. Biochem J 339 (Pt 3):579-88.

Rayan V, Hardingham T 1994 The recovery of articular cartilage in explant culture from interleukin-1 alpha: effects on proteoglycan synthesis and degradation. Matrix Biol 14:263-71.

RayChaudhury A, D'Amore PA 1991 Endothelial cell regulation by transforming growth factor-beta. J Cell Biochem 47:224-9.

Razani B, Zhang XL, Bitzer M, von Gersdorff G, Bottinger EP, Lisanti MP 2001 Caveolin-1 regulates transforming growth factor (TGF)-beta/SMAD signaling through an interaction with the TGF-beta type I receptor. J Biol Chem 276:6727-38.

Recklies AD, Roughley PJ, Bilimora KM 1989 Differential response of young and old human articular chondrocytes to IGF-1 and TGF-β. Tran Orthop Re soc 14:281.

Reddi AH, Huggins C 1972 Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. Proc Natl Acad Sci U S A 69:1601-5.

Redini F, Daireaux M, Mauviel A, Galera P, Loyau G, Pujol JP 1991 Characterization of proteoglycans synthesized by rabbit articular chondrocytes in response to transforming growth factor-beta (TGF-beta). Biochem Biophys Acta 1093:196-206.

Redini F, Galera P, Mauviel A, Loyau G, Pujol JP 1988 Transforming growth factor beta stimulates collagen and glycosaminoglycan biosynthesis in cultured rabbit articular chondrocytes. FEBS Lett 234:172-6.

Redini F, Min W, Demoor-Fossard M, Boittin M, Pujol JP 1997 Differential expression of membrane-anchored proteoglycans in rabbit articular chondrocytes cultured in monolayers and in alginate beads. Effect of transforming growth factor-beta 1. Biochem Biophys Acta 1355:20-32.

Reynisdottir I, Polyak K, Iavarone A, Massague J 1995 Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes Dev 9:1831-45.

Richardson JB, Caterson B, Evans EH, Ashton BA, Roberts S 1999 Repair of human articular cartilage after implantation of autologous chondrocytes. J Bone Joint Surg Br 81:1064-8.

Ritvaniemi P, Korkko J, Bonaventure J, Vikkula M, Hyland J, Paassilta P, Kaitila I, Kaariainen H, Sokolov BP, Hakala M, et al 1995 Identification of COL2A1 gene

mutations in patients with chondrodysplasias and familial osteoarthritis. Arthritis Rheum 38:999-1004.

Rius C, Smith JD, Almendro N, Langa C, Botella LM, Marchuk DA, Vary CP, Bernabeu C 1998 Cloning of the promoter region of human endoglin, the target gene for hereditary hemorrhagic telangiectasia type 1. Blood 92:4677-90.

Robbins JR, Thomas B, Tan L, Choy B, Arbiser JL, Berenbaum F, Goldring MB 2000 Immortalized human adult articular chondrocytes maintain cartilage-specific phenotype and responses to interleukin-1beta. Arthritis Rheum 43:2189-201.

Roberts AB 2002 The ever-increasing complexity of TGF-beta signaling. Cytokine Growth Factor Rev 13:3-5.

Roberts AB, Dernyck R 2001 Meeting report: signaling schemes for TGF-beta. Sci STKE. 18:PE43.

Roberts AB, Kim SJ, Noma Y, Glick AB, Lafyatis R, Lechleider R, Jakowlew SB, Geiser A, O'Reilly MA, Danielpour D, et al. 1991 Multiple forms of TGF-β: distinct promoters and differential expression. Ciba Found Symp 157:7-28.

Roberts AB, Sporn MB 1990 The transforming growth factor beta's. In: Sporn MB and Roberts AB (eds) Peptide Growth Factors and Their Receptors, vol 95. Spriner-Verlag, New York, pp. 419-472.

Roberts AB, Sporn MB 1992 Mechanistic interrelationship between superfamilies: the steroid/retinoid receptors and transforming growth factor-β. Canc Surveys 14:205-220.

Roberts S, Hollander AP, Caterson B, Menage J, Richardson JB 2001 Matrix turnover in human cartilage repair tissue in autologous chondrocyte implantation. Arthritis Rheum 44:2586-98.

Robinson D, Halperin N, Nevo Z 1990 Regenerating hyaline cartilage in articular defects of old chickens using implants of embryonal chick chondrocytes embedded in a new natural delivery substance. Calc Tissue Int 46:246-253.

Robinson D, Hasharoni A, Cohen N, Yayon A, Moskowitz RM, Nevo Z 1999 Fibroblast growth factor receptor-3 as a marker for precartilaginous stem cells. Clin Orthop 367 Suppl:S163-75.

Robinson D, Nevo Z, Halperin N 1998 Assaf Harofe Experience in isolated chondrocytes transplantation. Orthop Bull 18:63.

Rodriguez-Pena A, Prieto M, Duwel A, Rivas JV, Eleno N, Perez-Barriocanal F, Arevalo M, Smith JD, Vary CP, Bernabeu C, Lopez-Novoa JM 2001 Up-regulation of endoglin, a TGF-beta-binding protein, in rats with experimental renal fibrosis induced by renal mass reduction. Nephrol Dial Transplant 16 Suppl 1:34-9.

Ronziere MC, Farjanel J, Freyria AM, Hartmann DJ, Herbage D 1997 Analysis of types I, II, III, IX and XI collagens synthesized by fetal bovine chondrocytes in high-density culture. Osteoarthritis Cartilage 5:205-14.

Ronziere MC, Ricard-Blum S, Tiollier J, Hartmann DJ, Garrone R, Herbage D 1990 Comparative analysis of collagens solubilized from human foetal, and normal and osteoarthritic adult articular cartilage, with emphasis on type VI collagen. Biochem Biophys Acta 1038:222-30.

Rosen DM, Stempien SA, Thompson AY, Seyedin SM 1988 Transforming growth factor-beta modulates the expression of osteoblast and chondroblast phenotypes in vitro. J Cell Physiol 134:337-346.

Rosenthal AK, Ryan LM 1994 Ageing increases growth factor-induced inorganic pyrophosphate elaboration by articular cartilage. Mech Ageing Dev 75:35-44.

Rosier RN, O'Keefe RJ, Crabb ID, Puzas JE 1989 Transforming growth factor beta: An autocrine regulator of chondrocytes. Connect Tissue Res 20:295-301.

Roth V, Mow VC 1980 The intrinsic tensile behavior of the matrix of bovine articular cartilage and its variation with age. J Bone Joint Surg Am 62:1102-17.

Rotzer D, Roth M, Lutz M, Lindemann D, Sebald W, Knaus P 2001 Type III TGF-β receptor-independent signalling of TGF-β2 via TβRII-B, an alternatively spliced TGF-β type II receptor. EMBO 20:480-490.

Roughley PJ 2001 Articular cartilage and changes in arthritis: Noncollagenous proteins and proteoglycans in the extracellular matrix of cartilage. Arthritis Res 3:342-347.

Rulo HF, Westphal JR, van de Kerkhof PC, de Waal RM, van Vlijmen IM Ruiter DR 1995 Expression of endoglin in psoriatic involved and uninvolved skin. J Dermatol Sc 10:103-109.

Ruoslahti E 1991 Integrins. J Clin Invest 87:1-5.

Ruscetti FW, Dubois C, Falk LA, Jacobsen SE, Sing G, Longo DL, Wiltrout RH, Keller JR 1991 In vivo and in vitro effects of TGF-beta 1 on normal and neoplastic haemopoiesis. Ciba Found Symp 157:212-27.

Sack KE 1995 Osteoarthritis. A continuing challenge. West J Med 163:579-86.

Sakurai H, Kohsaka H, Liu MF, Higashiyama H, Hirata Y, Kanno K, Saito I, Miyasaka N 1995 Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J Clin Invest 96:2357-63.

Salminen H, Vuorio E, Saamanen AM 2001 Expression of Sox9 and type IIA procollagen during attempted repair of articular cartilage damage in a transgenic mouse model of osteoarthritis. Arthritis Rheum 44:947-955.

Salter RB, Simmonds DF, Malcolm BW, Rumble EJ, MacMichael D, Clements ND 1980 The biological effect of continuous passive motion on the healing of full-thickness defects in articular cartilage. An experimental investigation in the rabbit. J Bone Joint Surg Am 62:1232-51.

Sampath TK, Muthukumaran N, Reddi AH 1987 Isolation of osteogenin, an extracellular matrix-associated, bone-inductive protein, by heparin affinity chromatography. Proc Natl Acad Sci USA 84:7109-13.

Sanchez C, Mateus MM, Defresne MP, Crielaard JM, Reginster JY, Henrotin YE 2002 Metabolism of human articular chondrocytes cultured in alginate beads. Longterm effects of interleukin 1beta and nonsteroidal antiinflammatory drugs. J Rheumatol 29:772-82.

Sandell LJ, Aigner T 2001 Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. Arthritis Res 3:107-13.

Sanford LP, Ormsby I, Gittenberger-deGroot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T 1997 TGF-β2 knock out mice have multiple developmental defects that are nonoverlapping with other TGF-β knockout phenotypes. Development 124:2659-2670.

Sankar S, Mahooti-Brooks N, Bensen L, McCarthy TL, Centrella M, Madri JA 1996 Modulation of transforming growth factor beta receptor levels on microvascular endothelial cells during in vitro angiogenesis. J Clin Invest 97:1436-46.

Sankar S, Mahooti-Brooks N, Centrella M, McCarthy TL, Madri JA 1995 Expression of transforming growth factor type III receptor in vascular endothelial cells increases their responsiveness to transforming growth factor beta 2. J Biol Chem 270:13567-72.

Santala P, Heino J 1991 Regulation of integrin-type cell adhesion receptors by cytokines. J Biol Chem 266:23505-9.

Sanyal A, Oursler MJ, Clemens VR, Fukumoto T, Fitzsimmons JS, O'Driscoll SW 2002 Temporal expression patterns of BMP receptors and collagen II (B) during periosteal chondrogenesis. J Orthop Res 20:58-65.

Scharstuhl A, Glansbeek HL, van Beuningen HM, Vitters EL, van der Kraan PM, van den Berg WB 2002 Inhibition of endogenous TGF-beta during experimental osteoarthritis prevents osteophyte formation and impairs cartilage repair. J Immunol 169:507-14.

Schmidt-Rohlfing B, Schneider U, Thomsen M, Bosserhoff AK 2002 Correlation of a novel matrix protein with the degree of cartilage degradation. Rheumatol Int 22:165-9.

Searls RL, Janners MY 1969 The stabilization of cartilage properties in the cartilage-forming mesenchyme of the embryonic chick limb. J Exp Zool 170:365-75.

Segarini PR, Roberts AB, Rosen DM, Seyedin SM 1987 Membrane binding characteristics of two forms of transforming growth factor-beta. J Biol Chem 262:14655-62.

Sekiya I, Koopman P, Tsuji K, Mertin S, Harley V, Yamada Y, Shinomiya K, Nifuji A, Noda M 2001 Dexamethasone enhances SOX9 expression in chondrocytes. J Endocrin 169:573-579.

Seldin MF, Amos CI, Ward R, Gregersen PK 1999 The genetics revolution and the assault on rheumatoid arthritis. Arthritis Rheum 42:1071-9.

Serra R, Johnson M, Filvaroff EH, LaBorde J, Sheehan DM, Derynck R, Moses HL 1997 Expression of a truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. J Cell Biol139:541-52.

Seyedin S, Thompson A, Bentz H, Rosen DM, McPherson JM, Conti A, Siegel NR, Galluppi GR, and Piez KA 1986 Cartilage-inducing Factor-A. J Biol Chem 261:5693-5695.

Seyedin SM, Thomas TC, Thompson AY, Rosen DM, Piez KA 1985 Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. Proc Natl Acad Sci USA 82:2267-71.

Seyedin SM, Thompson AY, Rosen DM, Piez KA 1983 In vitro induction of cartilage-specific macromolecules by a bone extract. J Cell Biol 97:1950-3.

Shlopov BV, Smith GN Jr, Cole AA, Hasty KA 1999 Differential patterns of response to doxycycline and transforming growth factor beta1 in the down-regulation of collagenases in osteoarthritic and normal human chondrocytes. Arthritis Rheum 42:719-27.

Shlopov BV, Stuart JM, Gumanovskaya ML, Hasty KA 2001 Regulation of cartilage collagenase by doxycycline. J Rheumatol 28:835-42.

Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJJ, Kjeldsen AD, Plauchu H 2000 Diagnostic criteria for Hereditary Hemorrhagic Telangiectasia (Rendu-Osler-Weber Syndrome) Am J Med Gen 91:66-67.

Shovlin CL, Hughes JM, Scott J, Seidman CE, Seidman JG 1997 Characterization of endoglin and identification of novel mutations in hereditary hemorrhagic telangiectasia. Am J Hum Genet 61:68-79.

Shovlin CL, Hughes JM, Tuddenham EG, Temperley I, Perembelon YF, Scott J, Seidman CE, Seidman JG 1994 A gene for hereditary haemorrhagic telangiectasia maps to chromosome 9q3. Nat Genet 6:205-9.

Shuler FD, Georgescu HI, Niyibizi C, Studer RK, Mi Z, Johnstone B, Robbins RD, Evans CH. 2000 Increased matrix synthesis following adenoviral transfer of a transforming growth factor beta1 gene into articular chondrocytes. J Orthop Res18:585-92.

Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, et al 1992 Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature 359:693-9.

Sigel AV, Centrella M, Eghbali-Webb M 1996 Regulation of proliferative response of cardiac fibroblasts by transforming growth factor-beta 1. J Mol Cell Cardiol 28:1921-9.

Silman AJ 1997 Problems complicating the genetic epidemiology of rheumatoid arthritis. J Rheumatol 24:194-6.

Silvestrini G, Ballanti P, Patacchioli FR, Mocetti P, Di Grezia R, Wedard BM, Angelucci L, Bonucci E 2000 Evaluation of apoptosis and the glucocorticoid receptor in the

cartilage growth plate and metaphyseal bone cells of rats after high-dose treatment with corticosterone. Bone 26:33-42.

Skantze KA, Brinckerhoff CE, Collier JP 1985 Use of agarose culture to measure the effect of transforming growth factor beta and epidermal growth factor on rabbit articular chondrocytes. Cancer Res 45:4416-21.

Smith P, Shuler FD, Georgescu HI, Ghivizzani SC, Johnstone B, Niyibizi C, Robbins PD, Evans CH 2000 Genetic enhancement of matrix synthesis by articular chondrocytes: comparison of different growth factor genes in the presence and absence of interleukin-1. Arthritis Rheum 43:1156-64.

Sokoloff L 1974 Cell biology and the repair of articular cartilage. J Rheumatol 1:9-16.

Sokoloff L 1978 Invitro cultures of skeletal tissues. In: Sokoloff L (ed) The knee joints and synovial fluid. vol II, Academic Press, New York NJ, pp.1-27.

Solursh M 1991 Formation of cartilage tissue in vitro. J Cell Biochem 45:258-60.

Song CZ, Tian X, Gelehrter TD 1999 Glucocorticoid receptor inhibits transforming growth factor-beta signaling by directly targeting the transcriptional activation function of Smad3. Proc Natl Acad Sci U S A 96:11776-81.

Souchelnytskyi S, Ronnstrand L, Heldin CH, ten Dijke P 2001 Phosphorylation of Smad signaling proteins by receptor serine/threonine kinases. Methods Mol Biol 124:107-20.

Spector TD, Cicuttini F, Baker J, Loughlin J, Hart D 1996 Genetic influences on osteoarthritis in women: a twin study. BMJ 312:940-3.

Stastny P 1978 HLA-D and Ia antigens in rheumatoid arthritis and systemic lupus erythematosus.

Arthritis Rheum 21(5 Suppl):S139-43.

Stecher RM 1941 Heberden's nodes: heredity in hypertrophic arthritis of the finger joints. Am J Med Sci 201:801.

Stevenson S, Dannucci GA, Sharkey NA, Pool RR 1989 The fate of articular cartilage after transplantation of fresh and cryopreserved tissue-antigen-matched and mismatched osteochondral allografts in dogs. J Bone Joint Surg Am 71:1297-307.

St-Jacques S, Cymerman U, Pece N, Letarte M 1994 Molecular characterization and in situ localization of murine endoglin reveal that it is a transforming growth factor-beta binding protein of endothelial and stromal cells. Endocrinology 134:2645-57.

Stockwell RA 1971 The interrelationship of cell density and cartilage thickness in mammalian articular cartilage. J Anat 109:411-21.

Stokes DG, Liu G, Dharmavaram R, Hawkins D, Piera-Velazquez S, Jimenez SA 2001 Regulation of type-II collagen gene expression during human chondrocyte de-differentiation and recovery of chondrocyte-specific phenotype in culture involves Sry-type high-mobility-group box (SOX) transcription factors. Biochem J 360(Pt 2):461-70.

Stoop R, Buma P, van der Kraan PM, Hollander AP, Clark Billinghurst R, Robin Poole A, van den Berg WB 2000 Differences in type II collagen degradation between peripheral and central cartilage of rat stifle joints after cranial cruciate ligament transection. Arthritis Rheum 43:2121-31.

Stoop R, van der Kraan PM, Buma P, Hollander AP, Billinghurst RC, Poole AR, van den Berg WB 1999 Type II collagen degradation in spontaneous osteoarthritis in C57Bl/6 and BALB/c mice. Arthritis Rheum 42:2381-9.

Stove J, Fiedler J, Huch K, Gunther KP, Puhl W, Brenner R 2002 Lipofection of rabbit chondrocytes and long lasting expression of a lacZ reporter system in alginate beads. Osteoarthritis Cartilage 10:212-7.

Studer RK, DeRubertis FR, Craven PA 1996 Nitric oxide suppresses increases in mesangial cell protein kinase C, transforming growth factor beta, and fibronectin synthesis induced by thromboxane. J Am Soc Nephrol 7:999-1005.

Sullivan LG, Hasan T, Wright M, Mankin HJ, Towle CA 2002 Photodynamic treatment has chondroprotective effects on articular cartilage. J Orthop Res 20:241-8.

Szekanecz Z, Haines GK, Harlow LA, Shah MR, Fong TW, Fu R, Lin J-W, Rayan G, Koch AE 1995 Increased synovial expression of transforming growth factor (TGF)-β receptor endoglin and TGF-β1 in rheumatoid arthritis: possible interactions in the pathogenesis of the disease. Clin Immunol Immunopath 76:187-194.

Szekanecz Z, Koch AE 2001 Update on synovitis. Curr Rheumatol Rep 3:53-63.

Taipale J, Keski-Oja J 1997 Growth factors in the extracellular matrix. FASEB J 11:51-59.

Takigawa M, Kinoshita A, Enomoto M, Asada A, Suzuki F 1991 Effects of various growth and differentiation factors on expression of parathyroid hormone receptors on rabbit costal chondrocytes in culture. Endocrinology 129:868-76.

Tam B, Philip A 1998 Transforming growth factor-β receptor expression on human skin fibroblasts; dimeric complex formation of type I and type II receptors and identification of glycosyl phosphatidylinositol-anchored transforming growth factor-β binding proteins. J Cell Physiol 176:553-564.

Tam B, Germain L, Philip A 1998 A 150kDa GPI-anchored TGF-β1 binding protein forms heteromeric complex with the types I and II TGF-β signalling receptors. J Cell Biochem 70:573-586.

Tam B, Larouche D, Germain L, Hooper NM, Philip A 2001 Characterization of a 150 kDa accessory receptor for TGF-β1 on keratinocytes: Direct evidence for a GPI-anchor, and ligand binding of the released form. J Cell Biochem 83:494-507.

Tardif G, Pelletier JP, Dupuis M, Geng C, Cloutier JM, Martel-Pelletier 1999 Collagenase 3 production by human osteoarthritic chondrocytes in response to growth factors and cytokines is a function of the physiologic state of the cells. Arthritis Rheum 42:1147-58.

ten Dijke P, Ichijo H, Franzen P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K 1993 Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene 8:2879-87.

ten Dijke P, Yamashita H, Ichijo H, Franzen P, Laiho M, Miyazono K, Heldin CH 1994 Characterization of type I receptors for transforming growth factor-beta and activin. Science 264:101-4.

Thompson AY, Piez KA, Seyedin SM 1985 Chondrogenesis in agarose culture. Exp Cell Res 157:483-494.

Thonar EJ, Buckwalter JA, Kuettner KE 1986 Maturation-related differences in the structure and composition of proteoglycans synthesized by chondrocytes from bovine articular cartilage. J Biol Chem 261:2467-74.

Toussaint O, Medrano EE, von Zglinicki T 2000 Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. Exp Gerontol 35:927-45.

Urist MR 1965 Bone: formation by autoinduction. Science 150:893-9.

van Beuningen HM, van der Kraan PM, Arntz OJ, van den Berg WB 1993 Protection from interleukin 1 induced destruction of articular cartilage by transforming growth factor beta: studies in anatomically intact cartilage in vitro and in vivo. Ann Rheum Dis 52:185-91.

van Beuningen HM, van der Kraan PM, Arntz OJ, van den Berg WB 1994 In vivo protection against interleukin-1-induced articular cartilage damage by transforming growth factor-beta 1: age-related differences. Ann Rheum Dis 53:593-600.

van den Berg WB 1999 From Cartilage metabolism to clinics: The role of cytokines and growth factors in cartilage destruction in osteoarthritis and rheumatoid arthritis. J Rheumatol 58:136-141.

van den Berg WB, van Osch GJ, van der Kraan PM, van Beuningen HM 1993 Cartilage destruction and osteophytes in instability-induced murine osteoarthritis: role of TGF beta in osteophyte formation? Agents Actions 40:215-9.

van der Kraan PM, van den Berg WB 2000 Anabolic and destructive mediators in osteoarthritis. Curr Opin Clin Nutr Metab Care 3:205-11.

van Lent PL, van den Berg WB, Schalkwijk J, van de Putte LB, van den Bersselaar L 1987 The impact of protein size and charge on its retention in articular cartilage. J Rheumatol 14:798-805.

van Osch GJ, van der Veen SW, Burger EH, Verwoerd-Verhoef HL 2000 Chondrogenic potential of in vitro multiplied rabbit perichondrium cells cultured in alginate beads in defined medium. Tissue Eng 6:321-30.

van Susante JL, Buma P, van Beuningen HM, van den Berg WB, Veth RP 2000 Responsiveness of bovine chondrocytes to growth factors in medium with different serum concentrations. J Orthop Res 18:68-77.

van Susante JL, Buma P, van Osch GJ, Versleyen D, van der Kraan PM, van der Berg WB, Homminga GN 1995 Culture of chondrocytes in alginate and collagen carrier gels. Acta Orthop Scand 66:549-56.

Varga J, Rosenbloom J, Jimenez SA 1987 Transforming growth factor beta (TGF beta) causes a persistent increase in steady-state amounts of type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts. Biochem J 247:597-604.

Venn G, Lauder RM, Hardingham TE, Muir H 1990 Effects of catabolic and anabolic cytokines on proteoglycan biosynthesis in young, old and osteoarthritic canine cartilage. Biochem Soc Trans 18:973-4.

Verzijl N, DeGroot J, Ben ZC, Brau-Benjamin O, Maroudas A, Bank RA, Mizrahi J, Schalkwijk CG, Thorpe SR, Baynes JW, Bijlsma JW, Lafeber FP, TeKoppele JM 2002 Crosslinking by advanced glycation end products increases the stiffness of the collagen network in human articular cartilage: a possible mechanism through which age is a risk factor for osteoarthritis. Arthritis Rheum 46:114-23.

Verzijl N, DeGroot J, Oldehinkel E, Bank RA, Thorpe SR, Baynes JW, Bayliss MT, Bijlsma JW, Lafeber FP, Tekoppele JM 2000 Age-related accumulation of

Maillard reaction products in human articular cartilage collagen. Biochem J 350:381-7.

Vignon E, Arlot M, Patricot LM, Vignon G 1976 The cell density of human femoral head cartilage. Clin Orthop 121:303-8.

Villiger PM, Kusari AB, ten Dijke P, Lotz M 1993 IL-1 beta and IL-6 selectively induce transforming growth factor-beta isoforms in human articular chondrocytes. J Immunol 151:3337-44.

Visser JA, Themmen APN 1998 Downstream factors in transforming growth factor-beta family signaling. Mol Cell Endocrinol 146:7-17.

Vivien D, Attisano L, Wrana JL, Massague J 1995 Signaling activity of homologous and heterologous transforming growth factor-beta receptor kinase complexes. J Biol Chem 270:7134-41.

Vivien D, Galera P, Lebrun E, Daireaux M, Loyau G, Pujol JP 1992 TGF-beta-induced G2/M delay in proliferating rabbit articular chondrocytes is associated with an enhancement of replication rate and a cAMP decrease: possible involvement of pertussis toxin-sensitive pathway. J Cell Physiol 150:291-8.

Vivien D, Galera P, Lebrun E, Loyau G, Pujol J-P 1990 Differential effects of Transforming Growth Factor-β and Epidermal Growth Factor on the cell cycle of cultured rabbit articular chondrocytes. J Cell Phys 143:534-545.

Vivien D, Galera P, Loyau G, Pujol JP 1991 Differential response of cultured rabbit articular chondrocytes (RAC) to transforming growth factor beta (TGF-beta)-evidence for a role of serum factors. Eur J Cell Biol 54:217-23.

Vivien D, Petitfrere E, Martiny L, Sartelet H, Galera P, Haye B, Pujol JP 1993b IPG (inositolphosphate glycan) as a cellular signal for TGF-beta 1 modulation of chondrocyte cell cycle. J Cell Physiol 155:437-44.

Vivien D, Redini F, Galera P, Lebrun E, Loyau G, Pujol JP. 1993a Rabbit articular chondrocytes (RAC) express distinct transforming growth factor-beta receptor phenotypes as a function of cell cycle phases. Exp Cell Res 205:165-70.

Volkov M 1970 Allotransplantation of joints. J Bone Joint Surg Br 52:49-53.

von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Gluckert K, Stoss H 1992 Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. Arthritis Rheum 35:806-11.

Wahl SM, Hunt DA, Wakefield L 1987 Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci 84:5788-92.

Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI, Goldberg VM 1994 Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Joint Surg Am 76:579-92.

Wakitani S, Kimura T, Hirooka A, Ochi T, Yoneda M, Yasui N, Owaki H, Ono K 1989 Repair of rabbit articular surfaces with allograft chondrocytes embedded in collagen gel. J Bone Joint Surg Br 71:74-80.

Wang JM, Wilson PB, Kumar S, Pye D, Hunter RD 1994 Quantitation of endothelial cell specific protein E-9 employing a single monoclonal antibody in an indirect sandwich ELISA. J Immunol Methods 171:55-64.

Wang XF, Lin HF, Ng-Eaton E, Downward J, Lodish HF, Weinberg RA 1991 Expression cloning and characterization of the TGF-β type III receptor. Cell 67:797-805.

Weis-Garcia F, Massague J 1996 Complementation between kinase-defective and activation-defective TGF-beta receptors reveals a novel form of receptor cooperativity essential for signaling. EMBO J 15:276-89.

Wells RG, Yankelev H, Lin HY, Lodish HF 1997 Biosynthesis of the type I and type II TGF-beta receptors. Implications for complex formation. J Biol Chem 272:11444-51.

Werb Z, Tremble PM, Behrendtsen O, Crowley E, Damsky CH 1989 Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J Cell Biol 109:877-89.

Westphal JR, Willems HW, Schalkwijk CJ, Ruiter DJ, de Waal RM 1993 A new 180-kDa dermal endothelial cell activation antigen: in vitro and in situ characteristics. J Invest Dermatol 100:27-34.

Wiles N, Symmons DP, Harrison B, Barrett E, Barrett JH, Scott DG, Silman AJ 1999 Estimating the incidence of rheumatoid arthritis: trying to hit a moving target? Arthritis Rheum 42:1339-46.

Wluka AE, Davis SR, Bailey M, Stuckey SL, Cicuttini FM 2001 Users of oestrogen replacement therapy have more knee cartilage than non-users. Ann Rheum Dis 60: 332-336.

Wong SH, Hamel L, Chevalier S, Philip A 2000 Endoglin expression on human microvascular endothelial cells: demonstration of higher order complexes with TGF-β signaling receptors and heteromerization with betaglycan. Eur J Biochem 267:5550-5560.

Woo SL, Kwan MK, Lee TQ, Field FP, Kleiner JB, Coutts RD 1987 Perichondrial autograft for articular cartilage. Shear modulus of neocartilage studied in rabbits. Acta Orthop Scand 58:510-5.

Worster AA, Nixon AJ, Brower-Toland BD, Williams J 2000 Effect of transforming growth factor beta1 on chondrogenic differentiation of cultured equine mesenchymal stem cells. Am J Vet Res 61:1003-10.

Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J 1992 TGF-β signals through a heteromeric protein kinase receptor complex. Cell 71:1003-1014.

Wrana JL, Attisano L, Wieser R, Ventura F, Massague J 1994 Mechanism of activation of the TGF-β receptor. Nature 370:341-347.

Wu W, Billinghurst RC, Pidoux I, Antoniou J, Zukor D, Tanzer M, Poole AR 2002 Sites of collagenase cleavage and denaturation of type II collagen in aging and osteoarthritic articular cartilage and their relationship to the distribution of matrix metalloproteinase 1 and matrix metalloproteinase 13. Arthritis Rheum 46:2087-94.

Yaeger PC, Masi TL, de Ortiz JL, Binette F, Tubo R, McPherson JM 1997 Synergistic action of transforming growth factor-beta and insulin-like growth factor-I induces expression of type II collagen and aggrecan genes in adult human articular chondrocytes. Exp Cell Res 237:318-25.

Yamada Y, Okuizumi H, Miyauchi A, Takagi Y, Ikeda K, Harada A. 2000 Association of transforming growth factor beta1 genotype with spinal osteophytosis in Japanese women. Arthritis Rheum 43:452-60.

Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K 1995 Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science 270:2008-2011.

Yamaguchi Y, Mann DM, Ruoslahti E 1990 Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Natureb346:281-4.

Yamamoto M, Maehara Y, Sakaguchi Y, Kusumoto T, Ichiyoshi Y, Sugimachi K 1996 Transforming growth factor-beta 1 induces apoptosis in gastric cancer cells through a p53-independent pathway. Cancer 77(Suppl):1628-33.

Yamanishi Y, Boyle DL, Clark M, Maki RA, Tortorella MD, Arner EC, Firestein GS 2002 Expression and regulation of aggrecanase in arthritis: the role of TGF-beta. J Immunol 168:1405-12.

Yamashita H, Ichijo H, Grimsby S, Moron A, ten Dijke P, Miyazono K 1994 Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor beta. J Biol Chem 269:1995-2001.

Yang C, Li SW, Helminen HJ, Khillan JS, Bao Y, Prockop DJ 1997 Apoptosis of chondrocytes in transgenic mice lacking collagen II. Exp Cell Res 235:370-3.

Yang X, Chen L, Xu X, Li C, Huang C, Deng C-X 2001 TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol 153:35-46.

Yokota M, Ichihara S, Lin TL, Nakashima N, Yamada Y 2000 Association of a T29-->C polymorphism of the transforming growth factor-beta1 gene with genetic susceptibility to myocardial infarction in Japanese. Circulation 101:2783-7.

Zambruno G, Marchisio PC, Marconi A, Vaschieri C, Melchiori A, Giannetti A, De Luca M 1995 Transforming growth factor-beta 1 modulates beta 1 and beta 5 integrin

receptors and induces the de novo expression of the alpha v beta 6 heterodimer in normal human keratinocytes: implications for wound healing. J Cell Biol 129:853-65.

Zerath E, Holy X, Mouillon JM, Farbos B, Machwate M, Andre C, Renault S, Marie PJ 1997 TGF-beta2 prevents the impaired chondrocyte proliferation induced by unloading in growth plates of young rats. Life Sci 61:2397-406.

Zhang H, Shaw AR, Mak A, Letarte M 1996 Endoglin is a component of the transforming growth factor (TGF)-beta receptor complex of human pre-B leukemic cells. J Immunol 156:564-73.

Zhang Z, Tarone G, Turner DC 1993 Expression of integrin alpha 1 beta 1 is regulated by nerve growth factor and dexamethasone in PC12 cells. Functional consequences for adhesion and neurite outgrowth. J Biol Chem 268:5557-65.

Zou H, Niswander L 1996 Requirement for BMP signaling in interdigital apoptosis and scale formation. Science 272:738-41.

Zou H, Wieser R, Massague J, Niswander L 1997 Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. Genes Dev 11:2191-203.

# Section 5

## APPENDICES

JOURNAL OF BONE AND MINERAL RESEARCH
Volume 18, Number 2, 2003


# Endoglin Is Expressed on Human Chondrocytes and Forms a Heteromeric Complex With Betaglycan in a Ligand and Type II TGFβ Receptor Independent Manner

WENDY L PARKER,[1] MARY B GOLDRING,[2] and ANIE PHILIP[1]

## ABSTRACT

**Previous work has implicated transforming growth factor β (TGFβ) as an essential mediator of cartilage repair and TGFβ signaling as a requirement for the maintenance of articular cartilage in vivo. However, the mechanisms regulating TGFβ action in chondrocytes are poorly understood. Endoglin, an accessory receptor of the TGFβ receptor superfamily, is highly expressed on endothelial cells and has been shown to potently modulate TGFβ responses. It is not known whether chondrocytes express endoglin or whether it modulates TGFβ signaling in these cells. In this study, we show that endoglin is expressed on human chondrocytes at levels comparable with endothelial cells and that it forms higher order complexes with the types I and II TGFβ receptors. More importantly, we show that endoglin forms a heteromeric complex with betaglycan on these cells at endogenous receptor concentrations and ratios. Endoglin complexes with betaglycan in a ligand-independent and -dependent manner as indicated by co-immunoprecipitation in the absence of TGFβ and after affinity labeling with radiolabeled TGFβ, respectively. Also, the endoglin-betaglycan association can occur independently of the type II TGFβ receptor. These findings, taken together with the available evidence that endoglin and betaglycan are potent modulators of TGFβ signal transduction, imply that the complex formation between endoglin and betaglycan may be of critical significance in the regulation of TGFβ signaling in chondrocytes. (J Bone Miner Res 2003;18:289–302)**

**Key words: chondrocytes, transforming growth factor β receptors, endoglin, betaglycan, cartilage**

## INTRODUCTION

CARTILAGE, ONE OF the body's five major connective tissues, displays poor intrinsic healing, which is the key element in a variety of common joint diseases. Although the potential for restoring diseased or injured cartilage has generated much interest, a successful model of cartilage regeneration or repair has been elusive. This has been attributed to the lack of regenerative or proliferative ability of mature chondrocytes, their dedifferentiation into fibrocartilage (type I and type III collagen producing cells),[1] and the avascular state of the tissue.[2] Increased attention has focused on the chondrocyte environment as a critical determinant of phenotypic matrix synthesis and reparative capacity in which growth factors play important roles. Transforming growth factor β (TGFβ) has emerged as a potential regulator of chondrocyte growth and differentiation. For example, a positive relationship between cellular maturity and proliferative response to TGFβ has been demonstrated in growth plate chondrocytes.[3] Furthermore, chondrocytes derived from the different layers of articular cartilage are differentially stimulated by TGFβ.[4]

TGFβ is a member of a large family of multifunctional proteins intricately involved in growth, differentiation, and development[5] and was described initially as "cartilage inducing factor."[6] Three distinct isoforms of TGFβ (TGFβ 1, 2, and 3), which are encoded by distinct genes, have been described in mammals.[5] TGFβ is secreted in a latent form that requires activation before it can bind to its receptors. The TGFβ signal is transduced by a pair of transmembrane serine/threonine kinases, known as the types I and II receptors, which are present on almost all cell types.[7–10] The type I receptor does not bind TGFβ in the absence of the type II receptor. The binding of TGFβ to the type II receptor, a constitutively active kinase, results in the recruitment, phosphorylation, and concomitant activation of the type I

The authors have no conflict of interest.

[1]Division of Plastic Surgery, Department of Surgery, McGill University, Montreal, Quebec, Canada.
[2]Beth Israel Deaconness Medical Center, Harvard Institutes of Medicine, Boston, Massachusetts, USA.

receptor. The activated type I receptor in turn transmits the signal through downstream mediators such as Smads, resulting in the regulation of target gene expression. Other cell surface TGFβ binding proteins include the accessory receptors, betaglycan (type III TGFβ receptor) and endoglin (CD105), which have a limited tissue distribution. Betaglycan, a membrane proteoglycan, binds all three TGFβ isoforms with high affinity and is believed to facilitate TGFβ binding to the type II TGFβ receptor.[11–13]

Endoglin binds TGFβ1 and TGFβ3 with high affinity through its association with the type II receptor.[14] Furthermore, endoglin appears to interact not only with TGFβ but also with activin and bone morphogenic protein in the presence of their respective ligand binding receptor.[15] Endoglin was originally identified with the monoclonal antibody 44G4 generated against the HOON pre-B leukemic cell line[16] but was subsequently found to be highly expressed on endothelial cells. Other cell types that express endoglin include monocytes, lymphocytes, and placental and uterine cells.[17–19] Endoglin, a homodimeric transmembrane protein of ~180 kDa, composed of disulfide-linked protein subunits of ~90 kDa, has limited species-specificity and shows 70% homology to betaglycan. Two different isoforms, L- and S-endoglin, have been described, and both are phosphorylated. The functional role of endoglin in TGFβ signaling is poorly understood. It has been shown to facilitate ligand binding to the types I and II receptors.[20] However, overexpression of endoglin decreases TGFβ responses in monocytes and myoblasts.[19–21] In contrast, overexpression of betaglycan enhanced TGFβ responses in these cells.[20]

The gene encoding endoglin is located on chromosome 9q34.[22] Hereditary hemorrhagic telangiectasia (HHT) is attributable to mutations in the endoglin gene, with each mutation providing a slightly modified disease phenotype.[23] Heterozygotes, both human and animal models, display the classic phenotype of epistaxis, telangectasia, and visceral vascular malformations.[24] Null alleles are embryonically lethal secondary to abnormal yolk sac vasculogenesis and abnormal cardiac development.[25] The specific function of endoglin responsible for HHT is likely related to alterations in TGFβ action.[20,26]

TGFβ receptor profiles on human chondrocytes are poorly defined, and it is not known whether chondrocytes express novel TGFβ receptors. Expression of TGFβ accessory receptors such as endoglin and glycosyl phosphatidylinositol-anchored TGFβ binding proteins have been shown to regulate TGFβ signaling and TGFβ responses in other cell types.[20,27,28] Defining the expression profiles of the TGFβ receptor types and the biochemical interactions between these receptors leading to the formation of oligomeric complexes on chondrocytes is critical to understanding the mechanism of TGFβ action in these cells.

In this study, we report the expression profiles of TGFβ receptors on human articular and nonarticular chondrocytes and show for the first time that endoglin is expressed on chondrocytes. More importantly, we show that in addition to the well documented formation of a heteromeric complex with the type I/type II TGFβ receptors, endoglin forms a heteromeric complex with betaglycan on chondrocytes at endogenous receptor concentrations and ratios. Also, we present data to illustrate that the heteromerization of endoglin with betaglycan occurs in a ligand-induced and ligand-independent manner as shown by their co-immunoprecipitation after affinity cross-link labeling with radiolabeled TGFβ and their association in the absence of TGFβ, respectively. Furthermore, we show that the complex formation between endoglin and betaglycan can occur independently of the type II TGFβ receptor.

## MATERIALS AND METHODS

### *Cell culture*

Primary human articular chondrocytes were obtained from cartilage specimens obtained at total knee replacement surgery. Digestion of the specimens was performed according to standard techniques.[29] Briefly, cartilage was washed with phosphate-buffered saline (PBS), incubated with 0.25% trypsin (10 ml/g of tissue; Life Technologies, Burlington, Ontario, Canada) for 30–45 minutes, minced with a scalpel, and incubated with hyaluronidase (10 ml/g of tissue) and collagenase (1 mg/ml; 10 ml/g of tissue; both from Sigma, Oakville, Ontario, Canada) overnight. The suspension was pelleted, washed, and plated at $1 \times 10^6$ cells/ml. The immortalized human chondrocyte cell lines (C-28/I2 and tsT/AC62) have been described previously.[29,30] The C-28/I2 cell line was developed using chondrocytes isolated from juvenile human costal cartilage by retroviral infection with SV-40 large T antigen. The tsT/AC62 cells were developed using chondrocytes isolated from adult human articular cartilage by immortalization with a retrovirus expressing a temperature-sensitive mutant of SV-40 large T antigen. This mutant is functional when the cells are cultured at 32°C but not at 37°C.[29] The 293 cells (CRL-1573) were obtained from the American Type Culture Collection (Rockville, MD, USA), and the human microvascular endothelial cells (HMEC-1) were a gift from Dr FW Ades and Dr TJ Lawley (National Center for Infectious Disease, Atlanta, GA, USA). All cells with the exception of HMEC-1 were grown in DMEM/Ham's F12 (1:1, v:v) containing 10% FBS and 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphoteracin (all from Life Technologies). HMEC-1 were grown in MCDB 131 (Life Technologies) containing 10% FBS, 100U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphoteracin (all from Life Technologies). Cells were cultured at 37°C in an atmosphere of 5% $CO_2$/air, with the exception of the tsT/AC62 line, which was maintained at 32°C.

### *Analysis of RNA by reverse transcriptase polymerase chain reaction*

To verify chondrocyte phenotype of the immortalized cell lines and primary chondrocytes, aggrecan and type II collagen mRNAs were analyzed by a modified reverse transcriptase polymerase chain reaction (RT-PCR) as described previously.[29] Total RNA was extracted using Trizol Reagent (Life Technologies) and chloroform with final resuspension of the RNA in DEPC-treated water (Sigma). The concentration of RNA in the samples was determined using spectrophotometry, and RNA stability was determined on a

1% agarose-ethidium bromide gel. RT-PCR was performed with 5-$\mu$g samples using a Superscript First Strand Kit and custom primers (Life Technologies) according to the manufacturer's instructions. Final DNA preparations were visualized on a 1.5% agarose-ethidium bromide gel and photographed.

*Luciferase reporter assay*

The p3TP-Lux, a TGF$\beta$-inducible promoter-reporter construct,[31] containing the luciferase gene under the control of a portion of the plasminogen activator inhibitor-1 promoter region was used to determine cellular responsiveness to TGF$\beta$. Chondrocytes were grown to 90% confluency in a 6-well plate and transiently transfected with 1 $\mu$g of p3TP-Lux and 1 $\mu$g of p$\beta$-galactosidase ($\beta$-gal) per well using Lipofectamine Reagent (Life Technologies) in serum-free medium according to the manufacturer's specifications. Cells were allowed to recover overnight in regular medium, serum starved the following day for 4 h, and then treated with 100 pM of TGF$\beta$1 overnight. The cells were lysed and assayed for luciferase activity (Pharmingen, Mississauga, Ontario, Canada) using the EG&G Berthold Microplate Luminometer (Berthold Detection Systems, Pforzheim, Germany). Light emission by the TGF$\beta$1 treated cells was expressed as a percentage of the emission by the control cells and adjusted for transfection efficiency obtained using the $\beta$gal assay. Optimal transfection conditions were determined using pHGFP (high green fluorescent protein plasmid; Q-Biogene, Carlsbad, CA, USA).

*Western blot analysis*

To determine the regulation of the phosphorylated form of Smad 2 by TGF$\beta$1, chondrocytes were grown in T25 flasks and serum starved for 24 h. Cells were then washed with PBS and treated with TGF$\beta$1 as indicated. Cell lysates were prepared, and samples were standardized for protein content using the Bradford Protein Assay Kit (Bio-Rad, Mississauga, Ontario, Canada). The extracts were then fractionated by 7.5% SDS-PAGE and transferred by electrophoresis to nitrocellulose membranes (Scheicher and Schuell, Keene, NH, USA). The membrane was blocked for 3 h in a milk protein/Tween blocking solution (1M Tris, pH 7.5; 5M NaCl; Tween 20;Sigma; Carnation Skim milk powder) and incubated with a rabbit polyclonal anti-phosphoSmad2 antibody (a gift from Dr S Souchelnytskyi, Uppsala, Sweden) at 4°C overnight. The membrane was washed and incubated for 1 h with goat anti-rabbit HRP conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at room temperature and detected using the ECL system (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). The membrane was reprobed with anti-Smad 2 (non-phosphorylated form) antibody (Santa Cruz Biotechnology Inc.) to confirm equal protein loading. To determine the expression of endoglin in chondrocytes and HMEC-1, cells were grown in 60-mm dishes until confluent. Cell lysates were prepared and analyzed by Western blot using a rabbit polyclonal anti-endoglin antibody (SN6h; Dako, Carpinteria, CA, USA). The addition of secondary antibody and detection were performed as described above. The membrane was reprobed with anti-Smad 2/3 (non-phosphorylated form) antibody (Santa Cruz Biotechnology Inc.) to confirm equal protein loading.

To verify the presence of endoglin in cartilage, a plasma membrane fraction from fresh human articular cartilage was prepared as described by Gruppuso et al.[32] Briefly, tissue was rinsed with Dulbecco's PBS (dPBS), weighed, minced, and homogenized using a hand homogenizer in four volumes of homogenization buffer (sucrose 25 M, EDTA 1 mM, HEPES 50 mM, pH 7.5) containing a mixture of protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 20 $\mu$g/ml aprotinin, 20 $\mu$g/ml leupeptin, 20 $\mu$g/ml soybean trypsin inhibitor, and 25 mM benzamidine; all from Sigma). The membrane fraction was obtained by centrifugation at 100,000$g$ for 2 h at 20°C. The supernatant was removed, and the pellet resuspended in 1× sample buffer and boiled for 5 minutes. The samples were then analyzed by 7.5% SDS-PAGE under nonreducing or reducing (with $\beta$-mercaptoethanol; Sigma) conditions and immunoblotted as noted above for endoglin.

*Affinity labeling of cells*

Affinity labeling was performed as described previously with modifications.[33] Briefly, monolayers of cells were washed with ice-cold binding buffer (dPBS with $Ca^{2+}$ and $Mg^{2+}$, pH 7.4, containing 0.1% bovine serum albumin [BSA]) and were incubated with 100 pM of $^{125}$I-TGF$\beta$1 in the absence or presence of varying concentrations of non-radioactive TGF$\beta$1, 2, or 3. The receptor ligand complexes were cross-linked with bis-sulfocsuccinimidyl suberate (BS3; Pierce, Rockford, IL, USA). The reaction was stopped by the addition of glycine and the cell membrane extracts were prepared. The solubilized samples were separated by SDS-PAGE on a 3–11% polyacrylamide gradient gels under nonreducing or reducing (with $\beta$-bercaptoethanol; Sigma) conditions and analyzed by autoradiography.

*Immunoprecipitation of TGF$\beta$ receptors*

The anti-type I TGF$\beta$ receptor antibody and anti-type II TGF$\beta$ receptor antibody were from Santa Cruz Biotechnology Inc.. The anti-betaglycan antibody (Get 1; corresponding to the intracellular domain of betaglycan raised against the peptide sequence GETARRQQVPTSPPASENSS) was a gift from Dr S Souchelnytskyi (Uppsala, Sweden).[34] This peptide sequence is not present in human endoglin (GenBank accession no. J05481), and the Get 1 antibody exhibits no cross-reactivity to endoglin (S Souchelnytskyi, personal communication, 2001). The monoclonal anti-human endoglin antibody (44G4)[35] was a gift from Dr S St Jacques (Université Laval, Canada), the anti-pig endoglin antibody [EG(591–609), corresponding to the intracellular domain of endoglin raised against the peptide sequence KREPVVAVAAPASSESSST] was a gift from Dr K Miyzono (Tokyo, Japan).[36] This peptide sequence is not present in human betaglycan (GenBank accession no. XM_001924). The SN6h anti-human endoglin antibody was from Dako.

Immunoprecipitation studies were performed as described previously[33,37] with modifications. Cells were affinity labeled with 200 pM $^{125}$I-TGF$\beta$1, and the membrane extracts were incubated with 3 $\mu$g/ml of the various anti-

bodies and with respective IgG controls. Immune complexes were then incubated with protein G-agarose (Roche, Laval, Quebec, Canada) slurry, and the beads were pelleted by centrifugation. The immune complexes were analyzed by SDS-PAGE under nonreducing or reducing conditions followed by autoradiography.

*Overexpression of endoglin and betaglycan*

cDNAs encoding human RII and rat betaglycan subcloned into pcDNA3 were obtained from Dr M O'Connor-McCourt (Montreal, Canada),[38] and the pcEXV-Endo-L expression vector encoding the human L-endoglin isoform was from Dr C Bernabeu, (Madrid, Spain).[39] The 293 cells grown in 6-well plates were transiently transfected with 1 $\mu$g each of pEndo-L, p$\beta$glycan, and p$\beta$gal per well. In some experiments, cells were also transfected with pRII. Parallel transfections with pcDNA3 empty vector, and p$\beta$gal were performed as mock controls. The transfections were carried out using Superfect Reagent (Life Technologies) according to the manufacturer's specifications. Cells were affinity labeled with 200 pM $^{125}$I-TGF$\beta$1 and membrane extracts were left non-immunoprecipitated or immunoprecipitated with the indicated antireceptor antibodies, fractionated on 3–11% gradient gels, and analyzed by autoradiography. In parallel, transfected cells were not affinity labeled, but immunoprecipitated as indicated, fractionated on 3–11% gradient gels, and electrophoresed onto nitrocellulose before Western blotting with anti-endoglin antibody (SN6h; Dako) using the ECL detection system (Amersham Pharmacia Biotech).

*Immunoprecipitation/Western blot analysis*

Chondrocytes and 293 cells overexpressing endoglin and betaglycan were washed three times with 0.1% BSA-dPBS, twice with dPBS, and membrane extracts of cells were prepared and immunoprecipitated with anti-betaglycan antibody (Get 1) or anti-human endoglin antibody (SN6h; Dako) or not immunoprecipitated. In some experiments, cells were washed with a mild acid (0.1% glacial acetic acid) to ensure complete removal of endogenous TGF$\beta$[40,41] before membrane extraction and subsequent immunoprecipitation. In other experiments, cells were pretreated with 100 pM TGF$\beta$1 at 37°C for 2 h before being washed twice with dPBS and followed by membrane extraction and immunoprecipitation. The extracts or immune complexes were fractionated by SDS-PAGE on 3–11% gradient gels, and Western blot analysis was performed as described above using anti-human endoglin antibody (SN6h; Dako) and the ECL system for detection (Amersham Pharmacia Biotech).

*Two-dimensional gel electrophoresis*

Two-dimensional gel electrophoresis was performed as described by MacKay et al.,[42] except that 3-mercaptopropionic acid was omitted in the second dimension.[40] Solubilized membrane extracts of cells affinity labeled with $^{125}$I-TGF$\beta$1 were first fractionated on a 1.0-mm-thick 3–11% gradient gel under nonreducing conditions in the first dimension and then on a 1.5-mm-thick 3–11% gradient gel under reducing conditions in the second dimension. The gel was then subjected to autoradiography.

## RESULTS

*Expression of aggrecan and type II collagen by chondrocytes*

The tsT/AC62 and C-28/I2 cell lines provide a reproducible model that mimics human articular and costal phenotypes, respectively.[43–46] To confirm the chondrocyte phenotype of the immortalized cell lines and the human primary articular chondrocytes in culture, we determined the production of aggrecan and type II collagen by these cells using RT-PCR. As displayed in Fig. 1A, aggrecan and type II collagen mRNAs were expressed by both chondrocyte cell lines and the primary chondrocytes. In addition, light microscopic examination of the cells in culture at early passage showed that they displayed the typical chondrocyte morphology as detected by a spherical to polygonal shape with a cell-associated matrix when suspended in alginate bead cultures (data not shown).

*TGF$\beta$1 stimulates PAI promoter activity and Smad2 phosphorylation in chondrocytes*

To illustrate the sensitivity of the chondrocyte cell lines and primary chondrocytes to TGF$\beta$, cellular signaling was determined by a PAI-driven luciferase reporter assay using p3TP-Lux, which has been used extensively as a marker for TGF$\beta$ responsiveness.[11,47] The chondrocytes were transiently transfected with p3TP-Lux and the induction of luciferase activity by exogenous TGF$\beta$1 was measured. As illustrated in Fig. 1B, luciferase activity of cells treated with 100 pM of TGF$\beta$1 was stimulated approximately a 10-fold compared with untreated cells for the C28/I2 line, 6-fold for TST/AC62, and 5-fold for primary chondrocytes. The results were normalized by co-transfection of the $\beta$-gal plasmid and are representative of three different experiments each done in triplicate. Transfection of the empty vector and treatment with TGF$\beta$ in control experiments did not result in any alteration of luciferase activity (data not shown).

To show further the TGF$\beta$ sensitivity of these chondrocytes and to illustrate the cellular signaling through Smad 2, a central mediator of TGF$\beta$ action, we examined TGF$\beta$ induced Smad 2 phosphorylation. Cells were treated with 100 pM of TGF$\beta$1 for 15 or 30 minutes or were left untreated, and the phosphorylated form of Smad 2 was determined using Western blot analysis. As shown in Fig. 1C, which is representative of three different experiments, incubation of C-28/I2 chondrocytes with 100 pM of TGF$\beta$1 for 15 minutes resulted in a marked increase in Smad 2 phosphorylation (top panel). There was a time-dependent increase in Smad 2 phosphorylation as detected by 30 minutes of TGF$\beta$1 treatment (data not shown). Immunoblotting of total Smad2/3 was also performed to show equal protein loading (bottom panel). Similar results were obtained using tsT/AC62 cells and primary chondrocytes (data not shown).

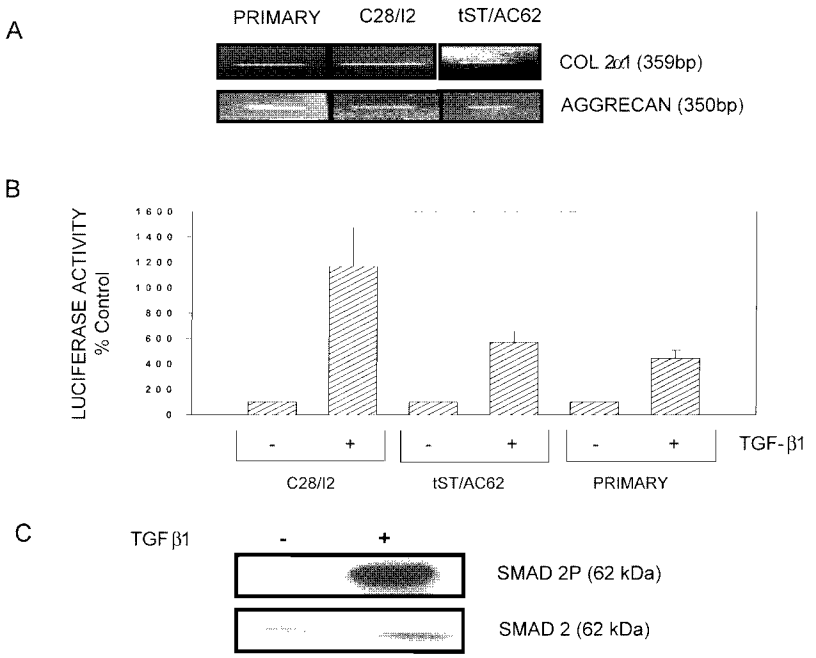



**FIG. 1.** Expression of cartilage-specific matrix gene expression and TGFβ responses in primary chondrocytes and nonarticular (C28/I2) and articular (tsT/AC62) chondrocyte cell lines. (A) Expression of aggrecan and type II collagen mRNAs: chondrocytes were cultured to confluence, total RNA was extracted, 5 μg of total RNA was reverse transcribed, and PCR was performed using specific primers. Products were analyzed on a 1.5% agarose-ethidium bromide gel. (B) Stimulation of plasminogen activator inhibitor-1 (PAI-1) promoter activity by TGFβ. The chondrocytes were transiently transfected with the PAI-1 promoter-luciferase reporter construct, p3TP-Lux, and pβgal. Twenty-four hours after transfection, cells were treated with 100 pM of TGFβ1 for 24 h or were left untreated. Luciferase activity was determined, normalized using the βgal assay, and expressed as a percent control of untreated cells. The data shown are representative of three different experiments each done in triplicates. (C) Stimulation of Smad 2 phosphorylation by TGFβ1. Solubilized extracts of C-28/I2 cells treated with 100 pM of TGFβ1 for 15 minutes or left untreated were analyzed by SDS-PAGE (3–11% gradient gels) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against the phosphorylated form of Smad2 (top panel). Immunoblotting using an antibody directed against the non-phosphorylated form of Smad 2 was performed to illustrate equal protein loading (bottom panel). The ECL system was used for chemiluminescence detection. Similar results were obtained using the tsT/AC62 cell line and primary chondrocytes (data not shown).

*TGFβ receptor profiles on human chondrocytes*

To analyze TGFβ receptor profiles on human chondrocytes, cells were affinity labeled with $^{125}$I-TGFβ1 and the labeled receptors were analyzed by SDS-PAGE. The receptor profiles representative of human articular and nonarticular chondrocytes are illustrated in Figs. 2 and 3, respectively. The competition using increasing concentrations of unlabeled TGFβ1, TGFβ2, and TGFβ3 isoforms demonstrated the relative binding affinity of the receptors for the TGFβ isoforms. $^{125}$I-TGFβ1 labeling in the absence of unlabeled ligand (lanes designated as "0") reveals five major binding complexes of relative molecular weights of 65, 85, 100, 180, and 200–300 kDa under reducing conditions (Fig. 2). The migration patterns and isoform specificities of the 65, 85, 100, and 200–300 kDa complexes were characteristic of the cloned type I receptor (RI), type II receptor (RII), endoglin monomer, and type III (betaglycan) receptor, respectively.[48,49] This was confirmed later by immunoprecipitation studies using specific antireceptor antibodies (see below). The band at 180 kDa may represent the endoglin homodimer that was cross-linked inadvertently during the affinity labeling procedure and thus became reductant insensitive (Fig. 2). All receptor complexes showed high affinity for TGFβ1 and an intermediate affinity for TGFβ3. With the exception of betaglycan, these receptors showed virtually no affinity for TGFβ2.

TGFβ receptor profiles of $^{125}$I-TGFβ1-labeled chondrocytes analyzed under nonreducing conditions are shown in Fig. 3. In addition to the RI (65 kDa), RII (85 kDa), endoglin dimer (180 kDa), and betaglycan (200–300 kDa), two binding complexes of relative molecular weights of 115 and 145 kDa were detected in the absence of unlabeled ligands (middle lane designated as "0"). These 115- and 145-kDa complexes were confirmed later as the RI homodimer and RI and RII heterodimer, respectively, by two-dimensional gel electrophoresis (see below), consistent with our previous observations in other cell types.[33,40] Also evident were two higher molecular weight bands at 240 and 320 kDa that were unmasked in the presence of the unlabeled β2 isoform, which has very low affinity for these bands but competes with betaglycan effectively. The 240- and 320-kDa bands were later identified as oligomeric complexes containing endoglin, RII, and/or RI (see below). Once again, all receptor complexes, with the exception of betaglycan, show high affinity for TGFβ1 and virtually no affinity for TGFβ2.

As expected of endoglin, the 100-kDa band (monomer under reducing conditions, Fig. 2) and the 180-kDa band (dimer

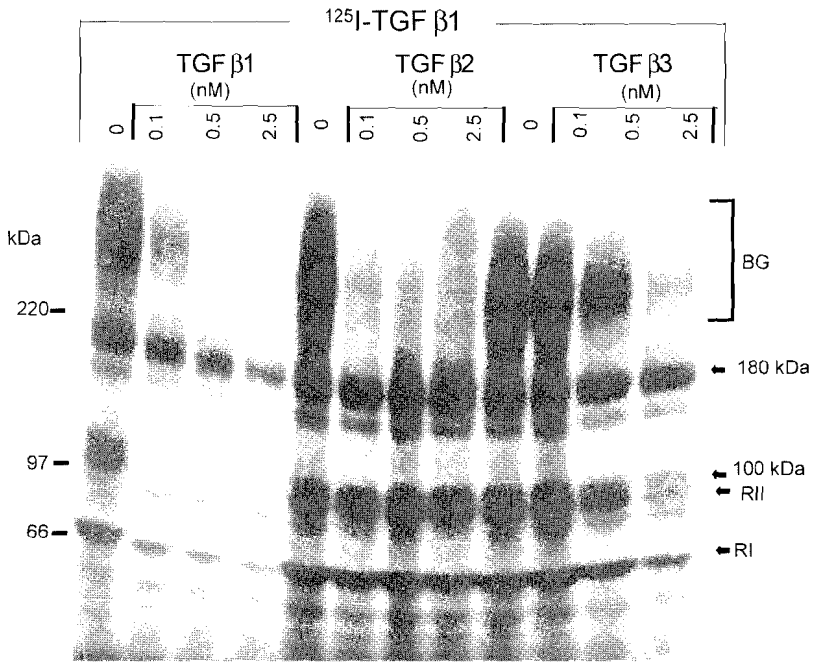



**FIG. 2.** Affinity labeling of human chondrocytes with $^{125}$I-TGF$\beta$1 and analysis under reducing conditions. Confluent monolayers of tsT/AC62 cells were affinity labeled with 100 pM of $^{125}$I-TGF$\beta$1 in the absence or presence of the indicated concentrations of unlabeled TGF$\beta$1, $\beta$2, or $\beta$3. Solubilized cell extracts were analyzed by SDS-PAGE on 3–11% polyacrylamide gradient gels under reducing conditions followed by autoradiography. Similar receptor profiles were observed using primary chondrocytes and the C-28/I2 cell line (data not shown).

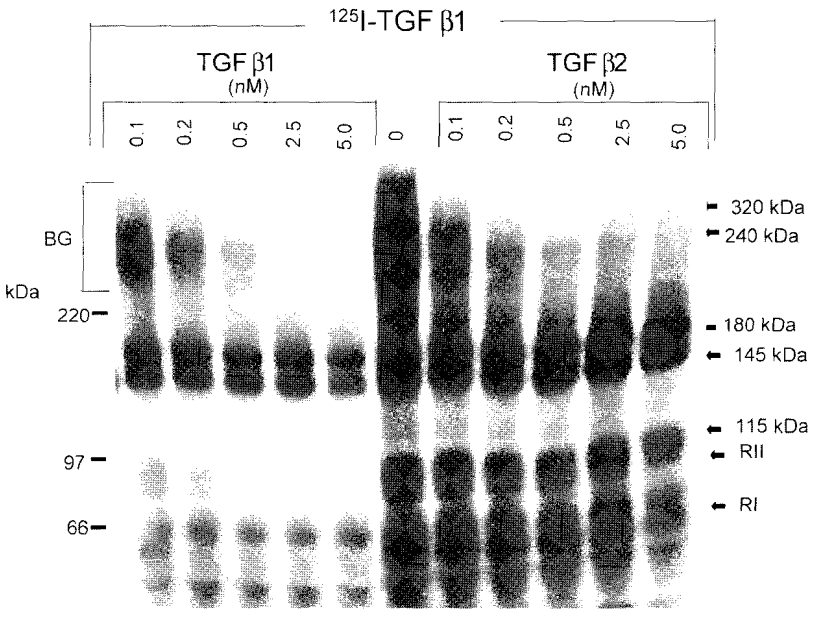



**FIG. 3.** Affinity labeling of human chondrocytes with $^{125}$I-TGF$\beta$1 and analysis under nonreducing conditions. Confluent monolayers of tsT/AC62 cells were affinity labeled with 100 pM of $^{125}$I-TGF$\beta$1 in the absence or presence of the indicated concentrations of unlabeled TGF$\beta$1, $\beta$2, or $\beta$3. Solubilized cell extracts were analyzed by SDS-PAGE (3–11% gradient gels) under nonreducing conditions followed by autoradiography. Similar receptor profiles were observed using primary chondrocytes and the C-28/I2 cell line (data not shown).

under nonreducing conditions, Fig. 3) displayed high affinity for TGF$\beta$1, intermediate affinity for TGF$\beta$3, and no affinity for TGF$\beta$2. However, the almost complete lack of affinity of the types I and II receptors for the TGF$\beta$2 isoform in these cells that express ample amounts of betaglycan (which exhibits high affinity for TGF$\beta$2) is intriguing.

*Endoglin is present in human articular cartilage and is expressed on chondrocytes at a level comparable with that on endothelial cells*

The results shown in Figs. 2 and 3 show binding patterns consistent with the presence of endoglin. Endoglin had not been documented previously on chondrocytes. To confirm the presence of endoglin on these cells, its expression on primary human articular chondrocytes and chondrocyte cell lines (nonarticular and articular) was compared with that of human microvascular endothelial cells using Western blot and affinity labeling. In addition to confirming the expression of endoglin on human chondrocytes at levels comparable with those on HMEC-1, results shown in Figs. 4A and 4B under conditions of equal protein loading suggest that articular chondrocytes express higher levels of endoglin than nonarticular chondrocytes. Also, the primary articular cells display higher amounts than the articular cell line

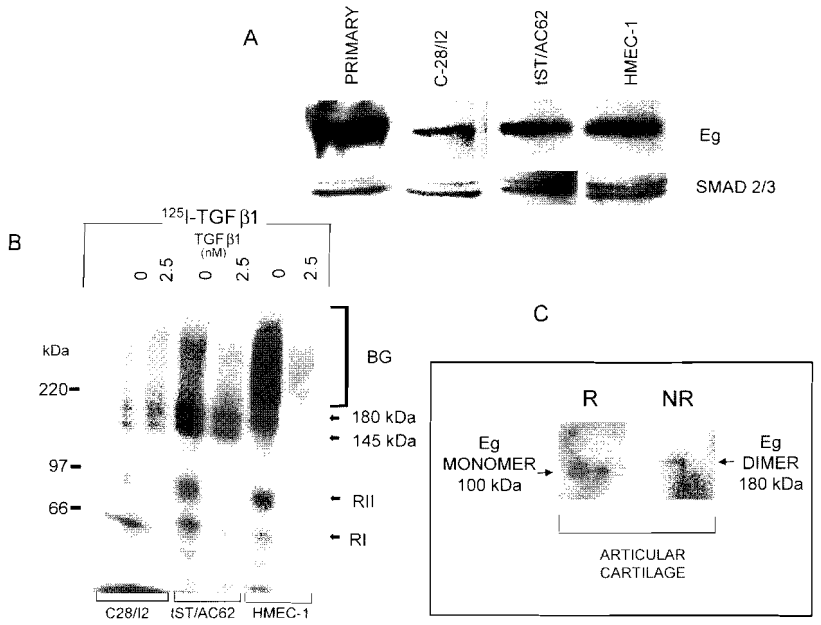



**FIG. 4.** Endoglin is expressed in human articular cartilage and its expression in primary chondrocytes and chondrocyte cell lines is comparable with to that in endothelial cells (HMEC-1). (A) Solubilized extracts of primary chondrocytes, C-28/I2, tsT/AC62, and HMEC-1 cells were analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against endoglin (SN6h, Dako) (top panel). Immunoblotting using an antibody directed against the non-phosphorylated form of Smad 2/3 was performed to illustrate equal protein loading (bottom panel). The ECL system was used for chemiluminescence detection. (B) C-28/I2, tsT/AC62, and HMEC-1 cells were affinity labeled with 100 pM of $^{125}$I-TGF$\beta$1 in the absence or presence of 2.5 nM of unlabeled TGF$\beta$1. Solubilized cell extracts were analyzed by SDS-PAGE (3–11% gradient gels) under nonreducing conditions followed by autoradiography. (C) Plasma membrane fraction of human articular cartilage was prepared as described in the Materials and Methods section and analyzed by SDS-PAGE (7.5% acrylamide) under reducing conditions or nonreducing and transferred onto nitrocellulose membrane. The membrane was immunoblotted with an antibody against endoglin (SN6h, Dako). The ECL system was used for chemiluminescence detection.

(tsT/AC62). Figure 4C shows the presence of endoglin in human cartilage tissue by Western blot, confirming that its expression is not an artifact of chondrocytes cultured in monolayers.

### *Endoglin forms a heteromeric complex with betaglycan in a ligand-induced manner*

To confirm the identity of the TGF$\beta$ receptors expressed on human chondrocytes and to study potential associations between endoglin and other TGF$\beta$ receptors, chondrocytes were affinity labeled with $^{125}$I-TGF$\beta$1 and immunoprecipitated using specific antireceptor antibodies directed against RI and RII, betaglycan, and endoglin. In these studies, whereas immunoprecipitation with a specific antireceptor antibody confirmed the identity of its cognate TGF$\beta$ receptor, co-immunoprecipitation of another type of receptor that is not recognized by this antibody was indicative of heteromeric complex formation between those receptors.

As shown in Fig. 5A, SDS-PAGE under reducing conditions of the nonimmunoprecipitated membrane lysate (NIP) demonstrated the presence of TGF$\beta$ binding proteins consistent in mobility and size with RI, RII, the endoglin monomer, and betaglycan (lane 1). The anti-betaglycan antibody ($\alpha$-BG) immunoprecipitated betaglycan and co-immunoprecipitated not only RI and RII, but also trace amounts of the endoglin monomer (lane 2). Similarly, the anti-endoglin antibody ($\alpha$-Eg; 44G4) precipitated the endoglin monomer, thus confirming the presence of endoglin on human chondrocytes (lane 3). Interestingly, $\alpha$-Eg co-immunoprecipitated betaglycan in addition to RI and RII. The co-immunoprecipitation of RI and RII with $\alpha$-BG[8,36] or with $\alpha$-Eg[50] is well documented. However, the co-immunoprecipitation of betaglycan with $\alpha$-Eg and that of endoglin with $\alpha$-BG has not been reported previously with the exception of our recent demonstration on microvascular endothelial cells.[33] The findings of co-immunoprecipitation of endoglin with $\alpha$-BG and that of betaglycan with $\alpha$-Eg suggest that endoglin and betaglycan form a heteromeric complex on the cell surface of chondrocytes. The anti-RI antibody ($\alpha$-RI) immunoprecipitated RI and co-immunoprecipitated RII and betaglycan (lane 4). Similarly, the anti-RII antibody ($\alpha$-RII) precipitated RII, RI, and betaglycan (lane 5). The patterns of co-immunoprecipitation observed with the anti-RI and RII antibodies have previously been documented and confirm the association of these receptors on the cell surface. No receptors were immunoprecipitated with the control (rabbit) immunoglobulin (IgG, lane 6).

Under nonreducing conditions (Fig. 5B), the $\alpha$-BG immunoprecipitated betaglycan (lane 2) and the $\alpha$-Eg precipitated the endoglin dimer (Lane 3) as expected. Again, $\alpha$-BG co-immunoprecipitated the endoglin dimer along with the RI and RII, as well as the 115- and 145-kDa bands that were later identified as the RI homodimer and RI-RII heterodimer, respectively (lane 2). Significantly, $\alpha$-Eg co-

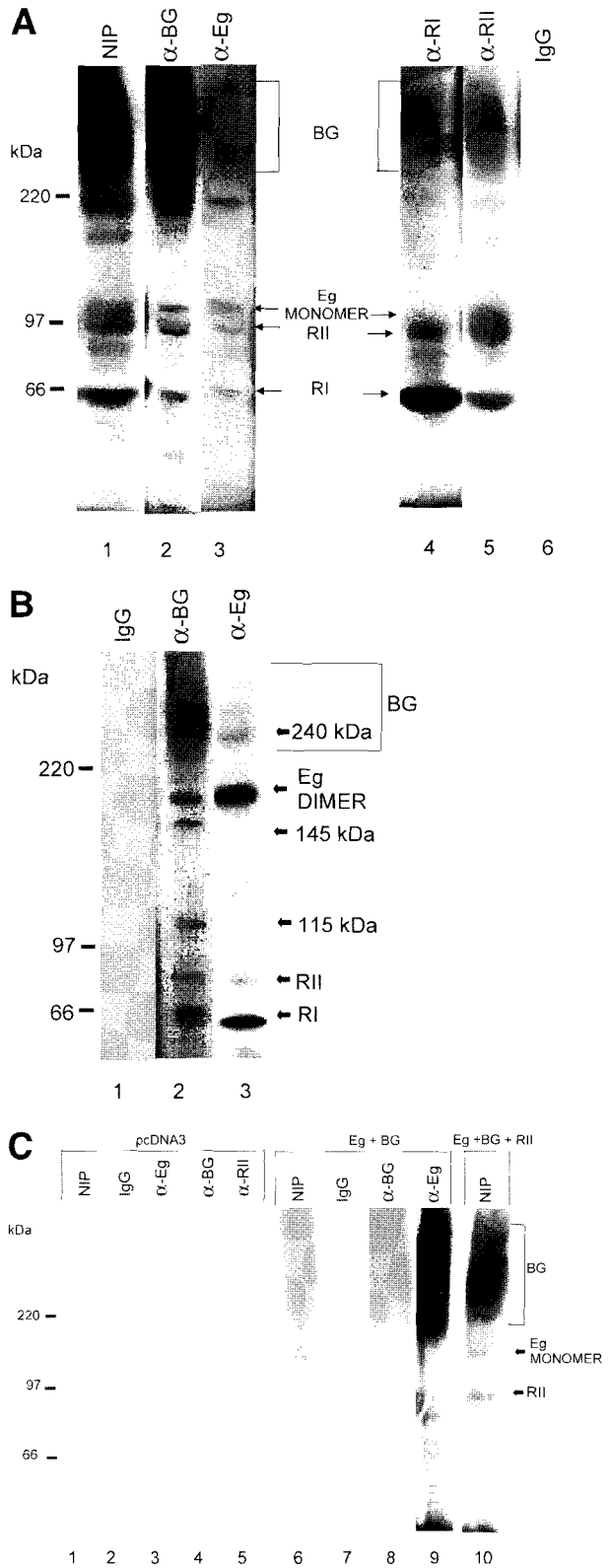



**FIG. 5.** Immunoprecipitation of TGFβ binding proteins on human chondrocytes and transiently transfected 293 cells. Cells were affinity labeled with 200 pM of $^{125}$I-TGFβ1 and solubilized cell extracts were immunoprecipitated with 3 μg/ml each of antireceptor antibodies or control IgG. Complexes were fractionated on SDS-PAGE (3–11% gradient gels) under (A and C) reducing or (B) nonreducing conditions and visualized by autoradiography. (A) Non-immunoprecipitated (NIP) cell extract of C28/I2 is shown in lane 1. Immunoprecipitations were performed using anti-betaglycan (lane 2, α-BG), anti-endoglin (lane 3, α-Eg), anti-RI (lane 4, α-RI), anti-RII (lane 5, α-RII), or control IgG (lane 6). (B) tsT/AC62 cell extracts were immunoprecipitated with control IgG (lane 1), anti-betaglycan (lane 2, α-BG), or anti-endoglin (lane 3, α-Eg). (C) 293 cells were transiently transfected with empty vector (pcDNA3, lanes 1–5), or L-endoglin (Eg) and betaglycan (BG; lanes 6–9) or Eg, BG, and RII (lane 10). After affinity labeling, non-immunoprecipitated cell extracts (NIP) were analyzed by SDS-PAGE (lanes 1, 6, and 10) or immunoprecipitations were performed using control IgG (lanes 2 and 7) or specific antireceptor antibodies as indicated (lanes 3–5 and 8–9) before analysis by SDS-PAGE.

immunoprecipitated trace amounts of betaglycan as well as RI and RII (lane 3). The 240-kDa band, which was later identified as an endoglin-containing heteromeric complex, was also precipitated. No receptor was immunoprecipitated using control (rabbit) immunoglobulin (IgG, lane 1). The immunoprecipitation results with α-BG and α-Eg under reducing (Fig. 5A) and nonreducing (Fig. 5B) conditions on chondrocytes affinity labeled with $^{125}$I-TGFβ1 strongly suggest that endoglin associates with betaglycan in a ligand-dependent manner.

### *Endoglin associates with betaglycan in a type II TGFβ receptor independent manner*

Because RII has been shown to form a complex with betaglycan and with endoglin, it can be argued that the association between endoglin and betaglycan observed on chondrocytes is a result of the complex formation of RII with both betaglycan and endoglin simultaneously. Therefore, we tested whether the endoglin-betaglycan association occurs in the absence of the type II receptor using a cell type exhibiting virtually no type II receptors, the 293 cells.[38] The 293 cells were transiently transfected with cDNAs encoding L-endoglin and betaglycan with and without the cDNA encoding RII. Cells were then affinity labeled with $^{125}$I-TGFβ1, immunoprecipitated with α-Eg or α-BG, and analyzed by SDS-PAGE under reducing conditions, as shown in Fig. 5C. On 293 cells transfected with the empty vector, pcDNA3, it was not possible to detect any TGFβ binding proteins except trace amounts of betaglycan (lanes 1–5). In contrast, the 293 cells transfected with vectors encoding endoglin and betaglycan displayed those receptors on the cell surface visualized as $^{125}$I-TGFβ1 labeled complexes in non-immunoprecipitated lysates (NIP, lane 6). The migration patterns of betaglycan and endoglin in transfected 293 cells, however, were different from those of their endogenous counterparts in chondrocytes, with betaglycan migrating at 180–250 kDa and endoglin at 130 kDa. This may be caused by differential glycosylation in 293 cells compared with chondrocytes. Importantly, immunoprecipitation with α-BG resulted in the immunoprecipitation of both betaglycan and endoglin (lane 8). Similarly, immunoprecipitation with α-Eg resulted in the precipitation of both endoglin and betaglycan, where the co-immunoprecipitation of betaglycan was more abundant than the precipitation of endoglin (lane 9). Immunoprecipitation with a rabbit control IgG showed no receptor complexes (lane 7). Finally, 293 cells

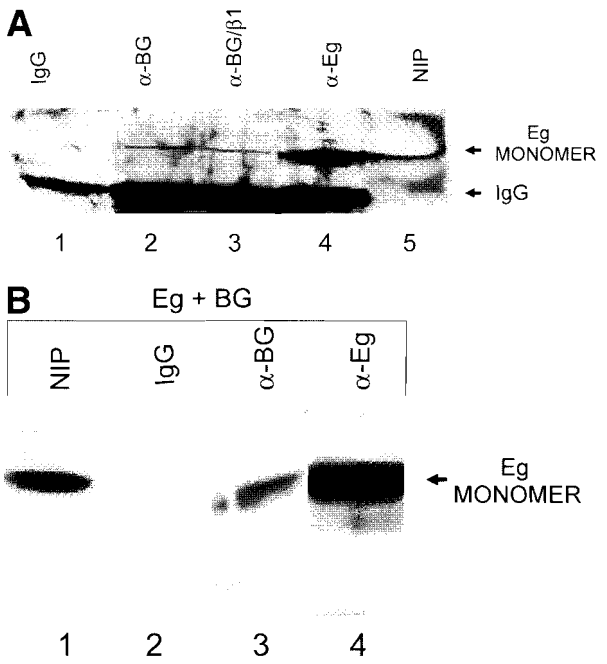



**FIG. 6.** Western blot analysis showing complex formation between endoglin and betaglycan on chondrocytes. (A) Solubilized extracts of human chondrocytes (C-28/I2) were not immunoprecipitated (NIP, Lane 5), or immunoprecipitated with control IgG (lane 1), anti-betaglycan antibody (α-BG, lane 2), or anti-endoglin antibody (α-Eg, lane 4). In lane 3, chondrocytes were pretreated with 100 pM of TGFβ1 at 37°C for 2 h before membrane extraction and immunoprecipitation. Complexes were fractionated by SDS-PAGE (7.5% acrylamide) under reducing conditions, and Western blotting was performed as described in the Materials and Methods section using anti-endoglin antibody (SN6h). (B) Solubilized extracts of 293 cells transiently transfected with L-endoglin (Eg) and betaglycan (BG) were not immunoprecipitated (NIP, lane 1) or immunoprecipitated with control IgG (lane 2), anti-betaglycan (α-BG, lane 3), or anti-endoglin (α-Eg, lane 4) antibody. Complexes were then fractionated and immunoblotted as in A.

co-transfected with RII in addition to endoglin and betaglycan, when affinity labeled but not immunoprecipitated, showed the expression of all three transfected receptors and specified the migration position of all three receptors. Results shown in lanes 1 and 5 confirm that the 293 cells express virtually no endogenous RII. Taken together, these findings indicate that endoglin forms a complex with betaglycan independently of RII.

### *Association of endoglin and betaglycan can occur in a ligand-independent manner*

Thus far, our immunoprecipitation studies of affinity labeled chondrocytes and 293 cells indicated that endoglin forms a heteromeric complex with betaglycan in a ligand-induced manner, indicating ligand dependence. It was important to confirm this association and to determine whether such complex formation also could occur in the absence of ligand. Membrane extracts of chondrocytes, extensively washed with dPBS, were prepared and immunoprecipitated with α-BG, α-Eg, or control IgG, or not immunoprecipitated. They were then fractionated by SDS-PAGE under reducing conditions and analyzed by Western blotting using α-Eg (SN6h), as shown in Fig. 6A. No endoglin was detected in the control experiment in which the immunoprecipitation was done with rabbit control IgG before Western blotting (lane 1). Western blotting of membrane extracts immunoprecipitated with α-Eg (lane 4) confirmed the presence of endoglin monomer at 100 kDa. Importantly, Western blotting of extracts immunoprecipitated with α-BG (lane 2) revealed the endoglin monomer at 100 kDa, thus showing that endoglin was co-immunoprecipitated with betaglycan in the absence of TGFβ. In addition, when chondrocytes were pretreated with 100 pM TGFβ1, there was no increase in the amount of endoglin monomer detected (lane 3, α-BG/β1). Western blotting of nonimmunoprecipitated membrane extracts (NIP, lane 5) also confirmed the presence of endoglin in chondrocytes. Furthermore, parallel experiments in which cells were washed with mild acid to ensure complete removal of any endogenous TGFβ[40,41] before membrane extraction and immunoprecipitation with α-BG revealed similar levels of endoglin on Western blot using α-Eg (data not shown). These results taken together confirm that endoglin forms a complex with betaglycan in a ligand-independent manner. This is consistent with our observation in microvascular endothelial cells.[33]

To further confirm that endoglin associates with betaglycan in the absence of ligand and in the absence of RII, 293 cells were transiently transfected with endoglin and betaglycan, the immunoprecipitation/Western blot analysis was done as above, and the data are shown in Fig. 6B. Western blotting of nonimmunoprecipitated membrane extracts of 293 cells (lane 1, NIP) and those immunoprecipitated with α-Eg (lane 4) demonstrated the endoglin monomer at 100 kDa that is expressed in the transfected 293 cells. Once again, Western blot of extracts immunoprecipitated with α-BG (lane 3) revealed the endoglin monomer at 100 kDa, indicating endoglin-betaglycan association while extracts immunoprecipitated with control IgG revealed no detectable bands (lane 2). These results were obtained in the absence of TGFβ in 293 cells that express no type II receptors, but overexpression of endoglin and betaglycan confirm that endoglin associates with betaglycan in a ligand- and RII-independent manner.

### *Endoglin forms higher order complexes with TGFβ signaling receptors on human chondrocytes*

When chondrocytes labeled with $^{125}$I-TGFβ1 were analyzed under nonreducing conditions, the results represented in Fig. 3 showed binding complexes at 115, 145, 270, and 320 kDa, in addition to RI, RII, endoglin, and betaglycan. To further characterize the nature of these binding complexes and to test whether they represent oligomeric complexes of TGFβ receptors, two-dimensional gel electrophoresis was performed. Chondrocytes were affinity labeled with $^{125}$I-TGFβ1 and the membrane extracts were not immunoprecipitated (Fig. 7A) or precipitated with α-Eg (SN6h; Fig. 7B) and two-dimensional gel electrophoresis was performed under nonreducing conditions in the first dimension and reducing conditions in the second dimension. The results shown in Fig. 7A revealed a spot of identical mobility as the endoglin monomer that fell from

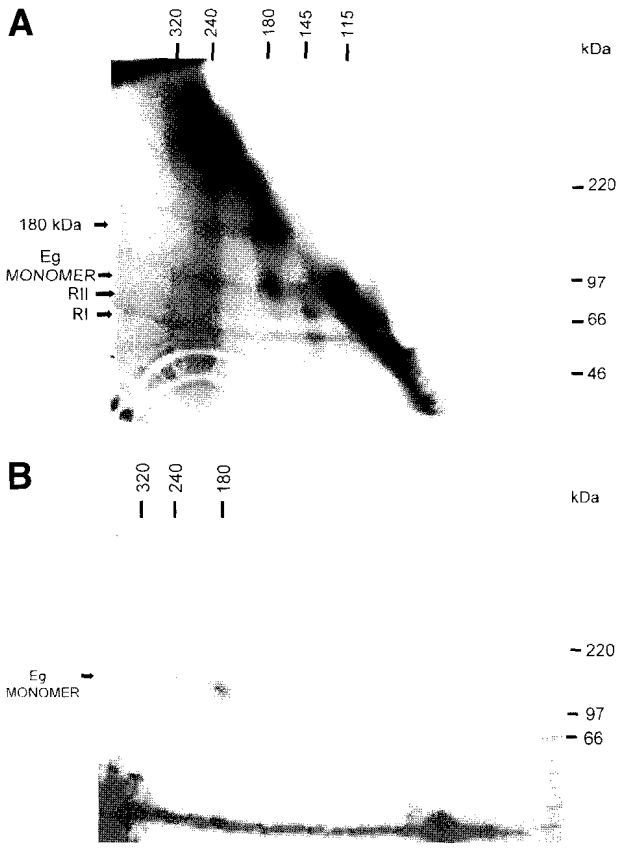



**FIG. 7.** Two-dimensional gel electrophoresis of TGFβ receptor complexes on human chondrocytes. tsT/AC62 cells were affinity labeled with 100 pM of $^{125}$I-TGFβ1 and solubilized extracts were (A) not immunoprecipitated or (B) immunoprecipitated with anti-Endoglin antibody (SN6h) and analyzed by SDS-PAGE (3–11% gradient gel) under nonreducing conditions in the first dimension. The individual lane was then cut out, laid horizontally on a second 3–11% gradient SDS-PAGE gel, and analyzed under reducing conditions in the second dimension.

the 180-kDa position. Traces of spot with identical mobility as RI fell from the 115-kDa position. Similarly, two spots with mobilities identical to those of RI and RII fell from the 145-kDa complex. These results suggest that the 180-kDa complex represents endoglin, whereas the 115-kDa complex corresponds to the RI homodimer and the 145-kDa complex to the RI/RII heterodimer. These data are consistent with our observations in microvascular endothelial cells.[33] The 240- and 320-kDa complexes were reductant sensitive and gave rise to spots with mobilities identical to those of the endoglin monomer and of RII and RI signaling receptors (Fig. 7A), indicating that endoglin forms higher order complexes with the TGFβ signaling receptors on chondrocytes. The 180-kDa complex that fell from the 240- and 320-kDa complexes may correspond to the endoglin dimer that is either reductant insensitive or to endoglin monomers that were cross-linked inadvertently during the affinity labeling procedure.

Membrane extracts immunoprecipitated with α-Eg (SN6h) when analyzed on two-dimensional electrophoresis revealed a 100-kDa spot of identical mobility as a endoglin monomer that fell from 180-, 240-, and 320-kDa positions (Fig. 7B). These results provide further evidence that the 180-kDa complex represents endoglin and show that the 240- and 320-kDa complexes contain endoglin.

## DISCUSSION

Although TGFβ has been implicated as a key mediator of cartilage repair and TGFβ signaling has been demonstrated to be required for the maintenance of articular cartilage,[50] the mechanisms regulating TGFβ action in chondrocytes are poorly understood. The question of whether chondrocytes express novel or accessory TGFβ receptors has not been addressed in previous studies. Localization of the types I, II, and III TGFβ receptors (RI, RII, and betaglycan) have been demonstrated in the growth plate and articular cartilage of growing rats and rabbits using immunohistochemistry, in situ hybridization, or RT-PCR.[49,51,52] However, information on the expression of TGFβ receptors in the human cartilage or chondrocytes is limited to a single study by Horner et al.,[53] who described the expression of RI and RII in growing human bone. On the other hand, several recent studies report that TGFβ rapidly induces several signaling pathways such as the Smad and mitogen-activated protein kinases (MAPK) pathways such as the extracellular signal regulated kinase (ERK), and p38 pathways in chondrocytes.[48,54,55] The relative contribution of these pathways in determining the chondrocyte response to TGFβ is unknown. The expression profiles of accessory TGFβ receptors and their interactions with the signaling TGFβ receptors at the membrane level leading to the formation of heteromeric receptor complexes may be critical in specifying the relative contributions of the various signaling pathways and thus may play a central role in regulating the diverse actions of TGFβ in chondrocytes. Interaction of novel and accessory receptors with TGFβ signaling receptors have been reported previously to be important regulators of TGFβ signaling in other cell types.[10,20,28]

In this study, we show for the first time that endoglin is expressed on human articular and nonarticular chondrocytes at high concentrations and that it forms higher order complexes with RI and RII on the cell surface. More importantly, endoglin forms a heteromeric complex with betaglycan on human chondrocytes. This complex formation occurs at normal physiological receptor concentrations because the chondrocytes used were not transfected with any receptor cDNAs. Significantly, we found that endoglin heteromerizes with betaglycan in a ligand-induced manner as indicated by their co-immunoprecipitation after affinity cross-link labeling with $^{125}$I-TGFβ1. Interestingly, endoglin also complexes with betaglycan in the absence of TGFβ, showing ligand-independent association. Furthermore, the endoglin-betaglycan association is observed in the absence of RII, indicating that the association can occur independently of RII.

The phenotype of the human chondrocytes used in this study has been characterized extensively.[43,44] The primary human articular chondrocytes and immortalized chondrocytes expressed both aggrecan and type II collagen mRNAs, and their responsiveness to TGFβ was shown by stimulation

of PAI promoter activity and Smad 2 phosphorylation. Prior studies analyzing the interactions between TGFβ receptors and the stoichiometry of the signaling complex used primarily mutant cell lines or cells overexpressing the wild-type or chimeric receptors.[56,57] In this study, we used normal TGFβ responsive human chondrocytes to show the association of endoglin with betaglycan, and the formation of higher order complexes containing endoglin and TGFβ signaling receptors. Thus, our results illustrate that these associations occur at endogenous receptor concentrations and ratios.

The presence of endoglin on chondrocytes has not been documented previously. Endoglin is primarily expressed on endothelial cells, and its expression at comparable levels on chondrocytes that resides in an avascular tissue, cartilage, is intriguing. While it is possible that the variable expression of endoglin observed in the three cell types studied (primary articular vs. articular cell line vs. nonarticular cell line) is related to the phenotype of the cell, further studies are needed to make more definitive conclusions. However, it is interesting to note that the stimulation of TGFβ-induced PAI promoter activity was inversely related to the level of endoglin observed in these cells (Fig. 1). In fact, our preliminary data suggest that overexpression of endoglin in chondrocytes leads to a diminution in TGF-β responses (W Parker and A Philip, unpublished observations, 2002).

In addition to providing confirmation of the identity of endoglin, the findings of immunoprecipitation studies using cells affinity labeled with $^{125}$I-TGFβ1 indicated that endoglin forms a ligand-induced heteromeric complex with betaglycan. This was demonstrated by co-immunoprecipitation of endoglin with α-BG and that of betaglycan with α-Eg when analyzed under either reducing or nonreducing conditions. Furthermore, the endoglin-betaglycan association was detectable not only on chondrocytes expressing endogenous receptors, but also on 293 cells transiently transfected with cDNAs encoding betaglycan and endoglin. Although immunoprecipitation results provided evidence for the occurrence of TGFβ-induced complex formation between endoglin and betaglycan, it was not possible to detect an endoglin-betaglycan heteromeric complex on SDS-PAGE analysis. The very large molecular weight of such a complex and the highly heterogeneous nature of betaglycan will preclude the detection of that complex.

That endoglin exists in a heteromeric complex with betaglycan on chondrocyte cell surface was confirmed using membrane extracts that were immunoprecipitated with α-BG and then subjected to Western blotting with α-Eg. In addition, because the latter study was done in the absence of TGFβ, it suggested that the endoglin-betaglycan complex formation could occur in a ligand-independent manner. Also, the occurrence of this complex was observed even after a mild acid wash (which ensure complete removal of endogenous TGFβ without affecting receptor integrity)[41] of chondrocytes before immunoprecipitation and Western analysis, thus providing further evidence for ligand independence of endoglin-betaglycan interaction. Furthermore, similar results were obtained when the experiment was repeated with 293 cells transiently transfected to express endoglin and betaglycan.

The results presented argue against the possibility that the association between betaglycan and endoglin observed in our study is a result of RII interacting with betaglycan and endoglin. For example, 293 cells expressing virtually no RII exhibit endoglin-betaglycan complex formation endogenously and on transfection with cDNAs encoding endoglin and betaglycan. In addition, the observation that betaglycan does not associate with RII in the absence of ligand,[58] but complexes with endoglin in the absence TGFβ, argues against the involvement of RII in the endoglin-betaglycan association.

The finding that the betaglycan-endoglin association occurs on chondrocytes is consistent with our recent demonstration of such an association on endothelial cells.[33] Although numerous studies have shown that both endoglin and betaglycan interact with RI and RII, the observation that endoglin forms a complex with its homolog, betaglycan, is novel. That this association occurs in a ligand-induced manner and in a ligand independent fashion is intriguing. Betaglycan has been shown to facilitate TGFβ binding to the TGFβ signaling receptors, specifically the binding of the TGFβ2 isoform to RII, which occurs at low affinity in the absence of betaglycan.[59,60] However, the property of ligand presentation of betaglycan does not account for the strict requirement of betaglycan for the epithelial-mesenchymal transition involved in the heart valve formation.[61] Thus, it has been postulated that betaglycan may play a more direct, albeit unknown, role in TGFβ signaling. In addition, a functional role for the cytoplasmic tail of betaglycan in regulating TGFβ signaling has recently been described where autophosphorylated RII phosphorylates the betaglycan cytoplasmic tail, thereby enhancing TGFβ2 signaling.[62]

Although there is increasing evidence to show that endoglin and betaglycan potently modulate TGF-signaling, the mechanisms by which they exert their effects are poorly defined. The vascular disorder HHT1 has been attributed to mutation in the endoglin gene and thus alteration in TGFβ action is believed to be responsible for HHT1. In addition, TGFβ treatment upregulated endoglin expression on human and rat mesangial cells.[63] Interestingly, it has recently been reported that both the types I and II TGF-β receptors phosphorylate endoglin cytoplasmic tail and that endoglin expression is associated with increased phosphorylation of the type I TGFβ receptor and Smad 2 and enhanced signaling.[64] This is in contrast to the earlier studies that have demonstrated that endoglin expression results in an inhibition of TGFβ signaling.[20] This discrepancy may reflect the differences in the activation by endoglin of the different gene promoters examined in the above studies.[64] Our preliminary results on the overexpression of endoglin in chondrocytes indicate that endoglin inhibits transcriptional activity and Smad 2 phosphorylation in these cells (W Parker and A Philip, unpublished observations, 2002). Regardless of the precise mechanisms involved, it is clear that endoglin and betaglycan are strong modulators or direct participants in TGFβ signal transduction. This in turn implies that the complex formation between endoglin and betaglycan may be of critical significance in the regulation of TGFβ signaling.

The two-dimensional gel electrophoresis provided information on the nature of the higher molecular weight receptor complexes, in addition to showing the occurrence of RI/RII heterodimers and RI homodimers on chondrocytes. This is consistent with our previous observation on endothelial cells and the mechanism explaining their occurrence has been detailed.[33] The true molecular weight of the 240- and 320-kDa complexes containing endoglin, type II, and or type I TGFβ receptors are likely to be different because high molecular weight glycoprotein complexes are known to migrate anomalously on SDS-PAGE. It is possible that they are derived from higher order complexes. Current evidence indicates that the TGFβ signaling complex is a heterotetramer consisting of one molecule each of the type I and type II receptor associated with each monomer of a TGFβ dimer molecule.[65] Whereas it is difficult to estimate the precise stoichiometry of the endoglin-containing complexes, it is possible that endoglin associates with the heterotetrameric TGFβ signaling complex. Moreover, multiple high molecular weight receptor complexes containing endoglin, RI, and RII with or without betaglycan may be formed depending on the efficiency of cross-linking of the individual receptor components to the $^{125}$I-TGFβ1 subunits. Alternatively, TGFβ receptor complexes of different subtypes and ratios may exist in parallel. Oligomerization of TGFβ receptors to form complexes consisting of differing subtype composition and ratio may represent modes of regulating distinct TGFβ responses.

Based on the results in this study, we propose a model to illustrate the significance of the endoglin-betaglycan complex formation in the modulation of TGF-β signaling in chondrocytes (Fig. 8). Endoglin associates with betaglycan on the cell surface, and this heteromeric complex may interact directly with the signaling receptors. However, the relative expression level of the individual receptor components may determine the outcome on TGFβ signaling. Thus, as mentioned above, overexpression of endoglin can inhibit[20] or enhance[64] TGFβ signaling. However, as shown in Fig. 8, complex formation between endoglin and betaglycan will allow the regulation of each other's function at the level of ligand binding (Fig. 8A) or downstream signaling (Fig. 8B). Thus, endoglin-betaglycan association may be critical for achieving a fine balance between the positive and the negative regulation of TGF-β signaling. It is interesting to mention in this regard that complex formation between endoglin and betaglycan association may also modulate signaling by other members of the TGFβ receptor superfamily in the light of recent observations that show that endoglin is an accessory receptor not only for TGFβ, but also for the bone morphogenic proteins and activin,[15] and that betaglycan, in addition to binding the TGFβ isoforms, binds inhibin, thereby facilitating inhibin antagonism of activin signaling.[66]

In summary, the present results show for the first time that endoglin is expressed on human chondrocytes at high concentrations and that it forms higher order complexes with RI and RII on the cell surface. More importantly, we show that endoglin forms a heteromeric complex with betaglycan on these cells. Also, our results illustrate that this complex formation occurs at physiological receptor concentrations

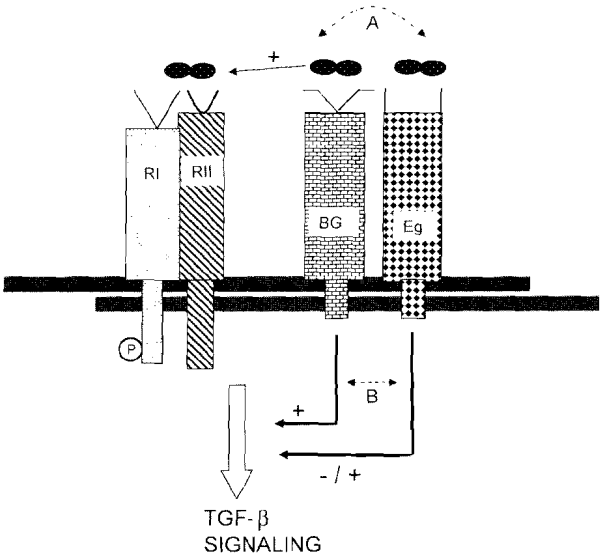



**FIG. 8.** Schematic representation of a possible model illustrating the significance of endoglin-betaglycan complex formation in modulating TGFβ signaling. Endoglin associates with betaglycan on the cell surface and this heteromeric complex may interact directly with the signaling receptors. Overexpression of endoglin may inhibit or enhance TGFβ signaling as has been reported using different promoter-reporter constructs.[20,64] Also, overexpression of betaglycan is believed to enhance signaling by facilitating ligand binding to the signaling receptors and by an alternate mechanism involving the phosphorylation of its cytoplasmic tail.[62,65] However, formation of a heteromeric complex between endoglin and betaglycan will allow the regulation of each other's function at the level of (A) ligand binding and/or (B) downstream signaling. Thus, the interaction between endoglin and betaglycan may be of critical significance for achieving a fine balance between the positive and the negative regulation of the TGFβ signaling pathways. Complex formation between endoglin and the signaling receptors has been omitted for clarity.

and ratios. Significantly, we found that the endoglin complexes with betaglycan in a ligand-induced and ligand-independent manner. Furthermore, this complex formation can occur independently of RII. Elucidating the role of endoglin in the regulation of TGFβ signaling in chondrocytes and in the avascular cartilage and understanding the significance of endoglin-betaglycan association in these cells are critical to unraveling the molecular mechanisms governing TGFβ action in chondrocytes and thus the role of TGFβ in mediating cartilage formation and repair.

## ACKNOWLEDGMENTS

We thank Genzyme Inc. (Framingham, MA, USA) for the gift of TGFβ1 and TGFβ2; Dr M O'Connor McCourt (Montreal, Quebec, Canada) for the gift of plasmids encoding the type II TGFβ receptor and betaglycan; Dr S Souchelnytskyi (Uppsala, Sweden) for the anti-betaglycan (Getl) and anti-phospho Smad 2 antibodies; Dr K Miyazono (Tokyo, Japan) for the anti-endoglin antibody; Dr S St Jacques (Quebec City, Quebec, Canada) for the 44G4 anti-endoglin antibody; Dr C Bernabeu (Madrid, Spain) for the L-endoglin plasmid; Drs FW Ades and TJ Lawley (Atlanta, GA, USA) for the human microvascular endothelial cells (HMEC-1),

Dr M Tanzer for human articular cartilage specimens; and Dr JJ Lebrun (Montreal, Quebec, Canada) for his expert advice on transient transfections and RT-PCR studies.

## REFERENCES


1. Robinson D, Halperin N, Nevo Z 1990 Regenerating hyaline cartilage in articular defects of old chickens using implants of embryonal chick chondrocytes embedded in a new natural delivery substance. Calcif Tissue Int **46:**246–253.
2. Aston JE, Bentley G 1986 Repair of articular surfaces by allografts of articular and growth-plate cartilage. J Bone Joint Surg Br **68:**29–35.
3. Rosier RN, O'Keefe RJ, Crabb ID, Puzas JE 1989 Transforming growth factor beta: An autocrine regulator of chondrocytes. Connect Tissue Res **20:**295–301.
4. Howard S, Anastasiades T 1993 Differential effects of bone associated factors on newly synthesized anionic glycoconjugates by articular chondrocyte cultures from adult and immature bovines. J Rheumatol **20:**2083–2094.
5. Roberts AB, Sporn MB 1990 The transforming growth factor betas. In: Sporn MB and Roberts AB (eds.) Peptide Growth Factors and Their Receptors, vol 95. Springer-Verlag, New York, NY, USA, pp. 419–472.
6. Seyedin S, Thompson A, Bentz H, Rosen DM, McPherson JM, Conti A, Siegel NR, Galluppi GR, and Piez KA 1986 Cartilage-inducing factor-A. J Biol Chem **261:**5693–5695.
7. Lin HV, Wang X-F, Ng-Eaton E, Weinberg RA, Lodish HF 1992 Expression cloning of the TGF-$\beta$ type II receptor, a functional transmembrane serine/threonine kinase. Cell **68:**775–785.
8. Franzen P, ten Dijke P, Ichijo H, Yamashita H, Schultz P, Heldin C H, Miyazono K 1993 Cloning of TGF-$\beta$ type I receptor that forms a Heterodimeric complex with the type II TGF-$\beta$ receptor. Cell **75:**681–692.
9. Piek E, Heldin C-H, ten Dijke P 1999 Specificity, diversity, and regulation in TGF-$\beta$ superfamily. FASEB **13:**2105–2124.
10. Massague J, Chen Y-G 2000 Controlling TGF-beta signaling. Genes Dev **14:**627–644.
11. Wang XF, Lin HF, Ng-Eaton E, Downward J, Lodish HF, Weinberg RA 1991 Expression cloning and characterization of the TGF-$\beta$ type III receptor. Cell **67:**797–805.
12. Lopez-Casillas F, Wrana JL, Massague J 1993 Betaglycan presents ligand to the TGF-$\beta$ signaling receptor. Cell **73:**1435–1444.
13. Massague J 2000 How cells read TGF-$\beta$ signals. Nature **1:**169–178.
14. Fonsatii E, del Vechchio L, Altomonte M, Sigalotti L, Nicotra MR, Coral S, Natali PG, Maio M 2001 Endoglin: An accessory component of the TGF-$\beta$-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. J Cell Physiol **188:**1–7.
15. Barbara NP, Wrana JL, Letarte M 1999 Endoglin is an accessory protein that interacts with the signalling receptor complex of multiple members of the transforming growth factor-$\beta$ super family. J Biol Chem **274:**584–594.
16. Quackenbush EJ, Letarte M 1985 Identification of several cell surface proteins of non-T, non-B acute lymphoblastic leukemia by using monoclonal antibodies. J Immunol **134:**1276–1285.
17. Arthur HM, Ure J, Smith AJH, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG 2000 Endoglin, an ancillary TGF-$\beta$ receptor, is required for extra embryonic angiogenesis and plays a key role in heart development. Dev Biol **217:**42–53.
18. Li C, Harpson L, Kumar P, Bernabeu C, Kumar S 2000 CD105 antagonizes the inhibitory signaling of transforming growth factor $\beta$1 on human vascular endothelial cells. FASEB J **14:**55–64.
19. Guerrero-Esteo M, Lastres P, Letamendia A, Perez-Alvarez MJ, Langa, C, Lopez LA, Fabra A, Garcia-Pardo A, Vera S, Letarte M, Bernabeu C 1999 Endoglin overexpression modulates cellular morphology, migration, and adhesion of mouse fibroblasts. Eur J Cell Biol **78:**614–623.
20. Letamendia A, Lastres P, Botella LM, Raab U, Langa C, Velasco B, Attisano L, Bernabeu C 1998 Role of endoglin in cellular responses to transforming growth factor-$\beta$. A comparative study with betaglycan. J Biol Chem **273:**33011–33019.
21. Lastres P, Letamendia A, Zhang H, Rius C, Almendro N, Raab U, Loepez LA, Langa C, Fabra A, Letarte M, Bernabeu C 1996 Endoglin modulates cellular responses to TGF-$\beta$1. J Cell Biol **133:**1109–1121.
22. Ferandez-Riuz E, St-Jacques S, Bellon T, Letarte M, Bernabeu C 1993 Assignment of the human endoglin gene (END) to 9q34→qter. Cytogenet Cell Genet **64:**204–207.
23. McAllister KA, Baldwin MA, Thukkani AK, Gallione CJ, Berg JN, Porteous ME, Guttmacher AE, Marchuk DA 1995 Six novel mutations in the endoglin gene in hereditary hemorrhagic telangiectasia type 1 suggest a dominant-negative effect of receptor function. Hum Mol Genet **4:**1983–1985.
24. Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJJ, Kjeldsen AD, Plauchu H 2000 Diagnostic criteria for Hereditary Hemorrhagic Telangiectasia (Rendu-Osler-Weber Syndrome). Am J Med Gen **91:**66–67.
25. Bourdeau A, Dumont DJ, Letarte M 1999 A murine model of hereditary hemorrhagic telangectasia. J Clin Invest **104:**1343–1351.
26. Pece N, Vera S, Cymerman U, White RJ, Wrana JL, Letarte M 1997 Mutant endoglin in Hereditary Hemorrhagic Telangiectasia type 1 is transiently expressed intracellularly and is not a dominant negative. J Clin Invest **100:**2568–2579.
27. Tam B, Germain L, Philip A 1998 A 150kDa GPI-anchored TGF-$\beta$1 binding protein forms heteromeric complex with the types I and II TGF-$\beta$ signalling receptors. J Cell Biochem **70:**573–586.
28. Tam B, Larouche D, Germain L, Hooper NM, Philip A 2001 Characterization of a 150 kDa accessory receptor for TGFb1 on keratinocytes: Direct evidence for a GPI-anchor, and ligand binding of the released form. J Cell Biochem **83:**494–507.
29. Robbins JR, Goldring MB 1997 Preparation of immortalized human chondrocyte cell lines. In: Morgan JR, Yarmush ML (eds.) Methods in Molecular Medicine, vol 18. Humana Press Inc., Totowa, NJ, USA, pp. 173–192.
30. Goldring MB, Birkhead JR, Suen L-F, Yamin R, Mizuno S, Glowacki J, Apperley JF 1994 Successful immortalization of human chondrocytes exhibiting stable expression of cartilage-specific type II collagen. J Clin Invest **94:**2307–2316.
31. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J 1994 Mechanism of activation of the TGF-$\beta$ receptor. Nature **370:**341–347.
32. Gruppuso PA, Mead JE, Fausto N 1990 Transforming growth factor receptors in liver regeneration following partial hepatectomy in the rat. Cancer Res **50:**1464–1469.
33. Wong SH, Hamel L, Chevalier S, Philip A 2000 Endoglin expression on human microvascular endothelial cells: Demonstration of higher order complexes with TGF$\beta$ signaling receptors and heteromerization with betaglycan. Eur J Biochem **267:**5550–5560.
34. Piek E, Franzen P, Heldin CH, ten Dijke P 1997 Characterization of a 60-kDa cell surface-associated transforming growth factor-beta binding protein that can interfere with transforming growth factor-beta receptor binding. J Cell Physiol **173:**447–459.
35. Gougos A, Letarte M 1988 Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line. J Immunol **141:**1925–1933.
36. Yamashita H, Ichijo H, Grimsby S, Moron A, ten Dijke P, Miyazono K 1994 Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor beta. J Biol Chem **269:**1995–2001.
37. Dumont N, O'Connor-McCourt M, Philip A 1995 Transforming growth factor-$\beta$ receptors on human endometrial cells: Identification of the type I, II and III receptors, and glycosylphosphatidylinositol anchored TGF-$\beta$ binding proteins. Mol Cell Endocrinol **111:**57–66.
38. Zwaagstra JC, Kassam Z, O'Connor-McCourt MD 1999 Down-regulation of transforming growth factor-beta receptors: Cooperativity between the types I, II, and III receptors and modulation at the cell surface. Exp Cell Res **252:**352–362.
39. Bellon T, Corbi A, Lastres P, Cales C, Cebrain M, Vera S, Chefeitz M, Massague J, Letarte M, Bernabeu C 1993 Identification and expression of two forms of the human transforming growth factor-$\beta$-binding protein endoglin with distinct cytoplasmic regions. Eur J Immunol **23:**2340–2345.
40. Tam B, Philip A 1998 Transforming growth factor-$\beta$ receptor expression on human skin fibroblasts; dimeric complex formation of type I and type II receptors and identification of glycosyl phosphatidylinositol-anchored transforming growth factor-$\beta$ binding proteins. J Cell Physiol **176:**553–564.

41. Glick AB, Danielpour D, Morgan D, Sporn MB, Yuspa SH 1990 Induction and autocrine receptor binding of TGF-β2 during terminal differentiation of primary mouse keratinocytes. Mol Endocrinol **4:**46–52.
42. MacKay K, Robbins AR, Bruce MD, Danielpour D 1990 Identification of disulfide-linked transforming growth factor-beta 1-specific binding proteins in rat glomeruli. J Biol Chem **265:** 9351–9356.
43. Robbins JR, Thomas B, Tan L, Choy B, Arbiser JL, Berenbaum F, Goldring MB 2000 Immortalized human adult articular chondrocytes maintain cartilage-specific phenotype and responses to interleukin-1beta. Arthritis Rheum **43:**2189–2201.
44. Goldring MB, Berenbaum F 1999 Human chondrocyte culture models for studying cyclooxygenase expression and prostaglandin regulation of collagen gene expression. Osteoarthritis Cartilage **7:**386–388.
45. Kokenyesi R, Tan L, Robbins JR, Goldring MB 2000 Proteoglycan production by immortalized human chondrocyte cell lines cultured under conditions that promote expression of the differentiated phenotype. Arch Biochem Biophys **383:**79–90.
46. Loeser R, Sadiev S, Tan L, Goldring MB 2000 Integrin expression by primary and immortalized human chondrocytes: Evidence of a differential role for α1β1 and α2β1 integrins in mediating chondrocyte adhesion to types II and VI collagen. Osteoarthritis Cartilage **8:**96–105.
47. Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J 1992 TGF-β signals through a heteromeric protein kinase receptor complex. Cell **71:**1003–1014.
48. Yang X, Chen L, Xu X, Li C, Huang C, Deng C-X 2001 TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol **153:**35–46.
49. Fukumura K, Matsunaga S, Yamamoto T, Nagamine T, Ishidou Y, Sakou T 1998 Immunolocalization of transforming growth factor-betas and type I and type II receptors in rat articular cartilage. Anticancer Res **18:**4189–4194.
50. Lux A, Attisano L, Marchuk DA 1999 Assignment of transforming growth factor β1 and β3 and a third new ligand to the type I receptor ALK-1. J Biol Chem **274:**9984–9992.
51. Kabasawa Y, Ejiri S, Matsuki Y, Hara K, Ozawa H 1998 Immunoreactive localization of transforming growth factor-beta type II receptor-positive cells in rat tibiae. Bone **22:**93–98.
52. Matsunaga S, Yamamoto T, Fukumura K 1999 Temporal and spatial expressions of transforming growth factor-betas and their receptors in epiphyseal growth plate. Int J Oncol **14:**1063–1067.
53. Horner A, Kemp P, Summers C, Bord S, Bishop NJ, Kelsall AW, Coleman N, Compston JE 1998 Expression and distribution of transforming growth factor-beta isoforms and their signaling receptors in growing human bone. Bone **23:**95–102.
54. Miyazaki Y, Tsukazaki T, Hirota Y, Yonekura A, Osaki M, Shindo H, Yamashita S 2000 Dexamethasone inhibition of TGF beta-induced cell growth and type II collagen mRNA expression through ERK-integrated AP-1 activity in cultured rat articular chondrocytes. Osteoarthritis Cartilage **8:**378–385.
55. Watanabe H, de Caestecker MP, Yamada Y 2001 Transcriptional cross-talk between Smad, ERK1/2 and p38 mitogen activated protein kinase pathways regulates transforming growth factor-beta-induced aggrecan gene expression in chondrogenic ATDC5 cells. J Biol Chem **276:**14466–14473.
56. Laiho M, Decaprio J, Ludlow JW, Livingston DM, Massague J 1990 Growth inhibition by TGF-β linked to suppression of retinoblastoma protein phosphorylation. Cell **62:**175–185.
57. Wells RG, Gilboa L, Sun Y, Liu X, Henis YI, Lodish HF 1999 Transforming growth factor-beta induces formation of a dithiothreitol-resistant type I/type II receptor complex in live cells. J Biol Chem **274:**5716–5722.
58. Matsuzaki K, Kan M, McKeehan WL 1996 Reconstitution of a pentameric complex of dimeric transforming growth factor beta ligand and a type I, II, III receptor in baculoviral-infected insect cells. In Vitro Cell Dev Biol Anim **32:**345–360.
59. Chen X, Weisberg V, Fridmacher M, Watanabe G, Naco G, Whitman M 1997 Smad4 and FAST-1 in the assembly of activin-responsive factor. Nature **389:**85–89.
60. Sun L, Chen C 1997 Expression of transforming growth factor beta type III receptor suppresses tumorigenicity of human breast cancer MDA-MB-231 cells. J Biol Chem **272:**25367–25372.
61. Brown CB, Boyer AS, Runyan RB, Barnett JV 1999 Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. Science **283:**2080–2082.
62. Blobe GC, Schiemann WP, Pepin M-C, Beauchemin M, Moustakas A, Lodish HF, O'Connor-McCourt MD 2001 Functional roles for the cytoplasmic domain of the type III transforming growth factor beta receptor in regulating transforming growth factor beta signaling. J Biol Chem **276:**24627–24637.
63. Rodriguez-Barbero A, Obreo J, Eleno N, Rodriguez-Pena A, Duwel A, Jeric M, Sanchez-Rodriguez A, Bernabeu C, Lopez-Novoa JM 2001 Endoglin expression in human and rat mesangial cells and its upregulation by TGF-β1. Biochem Biophys Res Commun **282:** 142–147.
64. Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C 2002 Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-β receptors I and II. J Biol Chem **277:**29197–29207.
65. Massague J 1998 TGF-beta signal transduction. Ann Rev Biochem **67:**753–791.
66. Lewis KA, Gray PC, Blount AL, Macconell LA, Walter E, Bilezikjian LM, Vale W 2000 Betaglycan binds inhibin and can mediate functional antagonism of activin signaling. Nature **404:**411–414.

Address reprint requests to:
*Anie Philip, PhD*
*Division of Plastic Surgery*
*Montreal General Hospital*
*1650 Cedar Avenue, Room C9–177*
*Montreal, Quebec H3G 1A4, Canada*
*E-mail: anie.philip@mcgill.ca*

Received in original form April 8, 2002; in revised form August 6, 2002; accepted August 21, 2002.

JBMR
JOURNAL OF BONE AND MINERAL RESEARCH



3209 Guess Road, Suite 201, Durham, NC 27705
ph : (919) 620-0681 fax: (919) 620-8465
email: journal@jbmr.org
http://www.jbmronline.org

March 12, 2003



Dr. Wendy L. Parker
3475 de la Montagne #816
Montreal, Quebec
Canada H3G 2A4

Dear Dr. Parker:

Permission is granted to reproduce the *Journal of Bone and Mineral Research* article referenced below with your Ph.D. thesis. The article should be reproduced without modification.

Please contact me if you need additional information.

Sincerely,

Adrienne Lea

Adrienne Lea
Director of Publications

*J Bone Miner Res* 2003; 18:289-302 reproduced with permission of the American Society for Bone and Mineral Research

**Centre universitaire de santé McGill**
**McGill University Health Centre**

May 30, 2002



Dr. Anie Philip
Department of Surgery
Division of Plastic Surgery
Montreal General Hospital

**RE: REC#01-054 entitled "Characterization of Transforming Growth Factor ß (TGFß) Action in Human Cartilage Cells (Participants with Arthritis)."**

Dear Dr. Philip:

We are writing in response to your request for review by the Montreal General Hospital Research Ethics Committee of the research proposal referenced above.

We are pleased to inform you that the study was found to be within ethical guidelines for conduct at the McGill University Health Centre. Approval for the study protocol, and the revised English and French Consent Forms (dated May 9, 2002), was provided via expedited review of the Chair on May 30, 2002.

The Research Ethics Boards (REBs) of the McGill University Health Centre are registered REBs working under the published guidelines of the Tri-Council Policy Statement, in compliance with the "Plan d'action ministériel en éthique de la recherche et en intégrité scientifique" (MSSS, Qc) and the Food and Drugs Act (17 June, 2001); and acting in conformity with standards set forth in the (US) Code of Federal Regulations governing human subjects research, functions in a manner consistent with internationally accepted principles of good clinical practice.

We wish to advise you that this document completely satisfies the requirement for REB Attestation as stipulated by Health Canada.

We ask you to note that all research requires review at a regular interval and approval for the study will remain in effect until April 2003. It is the responsibility of the principal investigator to submit an application for Continuing Review before the expiration date of the study approval.

The project was assigned MUHC study number REC#01-054 and should any revision to the research or other unanticipated development occur prior to the next required review, please advise the REB promptly and prior to initiating a proposed revision.

We trust this will prove entirely satisfactory to you.

Sincerely,

Cournoyer

Denis Cournoyer, M. D.
Chairman
Research Ethics Committee
MUHC-Montreal General Hospital

Cc: REC#01-054

Centre universitaire de santé McGill
McGill University Health Centre

# Internal Permit #: 6-1007-04

**Permit Holder:** Anie Philip Hani Shennib **Office:** C9.185

**Department:** Surgery **Telephone:** 44533/4809

**(A) Location**

| Room | Classification |
|---|---|
| B7.103 | Basic |
| C10.140 | Basic |
| C10.153 | Basic |
| C10.181 | Radioactive Materials Area |
| C9.112 Hall | Radioactive Materials Area |
| C9.145.1 | Basic |
| C9.177 | Basic |

**(B) Authorized Activity**

| Isotope | Max Manipulated (MBq) | Max Purchase (MBq) |
|---|---|---|
| I125 | 0.1 | 9.25 |
| H3 | 3.7 | 185 |
| P32 | 3.7 | 9.25 |
| C14 | 0.185 | 0.185 |

**(C) Authorised Users**

| Last Name | First Name | P32 | P33 | S35 | H3 | C14 | Fe59 | I125 | Ca45 | Co57 | Cr51 | Na22 | Rb86 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Benhomeid | Osama | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Endo | Monamoto | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Haghighat | Roya | ☑ | ☐ | ☐ | ☐ | ☑ | ☐ | ☑ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Horvat | Ann Mary | ☐ | ☐ | ☐ | ☑ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kosa | Jamal | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Mok | David | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Negi | Ranuka | ☐ | ☐ | ☐ | ☑ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Parker | Wendy | ☑ | ☐ | ☐ | ☑ | ☑ | ☐ | ☑ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Wang | Jintang | ☑ | ☐ | ☐ | ☑ | ☑ | ☐ | ☑ | ☐ | ☐ | ☐ | ☐ | ☐ |

**(D) Conditions**

| Conditions |
|---|
| I125 vials transported from BRI must not have more than 2.5 uCi/vial |

**The internal permit holder and the persons listed in section (C) are authorised to use the designated radioisotopes. The radioisotopes and their respective activities listed in section (B) can only be used in the laboratories listed in section (A) in accordance with the conditions listed in section (D). Importation, storage, manipulation and disposition of radioactive material must be performed in conformity with our CNSC licence, with Federal regulations and with the MUHC Radiation Safety Policies and Procedures. A copy of our CNSC licence is posted at RVH Research secretariat office and is also available at Radiation Protection Service (room S4.79, local 36133).**

**Approved by:** Daniel Alie for Manon Rouleau
**Radiation Safety Manager (36133)**

**Date issued: 01 November, 2002**

**Expiration date: June 01, 2004**

# Permis interne: 6-1007-04

**Détenteur de permis:** Anie Philip Hani Shennib **Bureau:** C9.185

**Département:** Surgery **Téléphone:** 44533/4809

**(A) Localisation**

| Room | Classification |
|---|---|
| B7.103 | Élémentaire |
| C10.140 | Élémentaire |
| C10.153 | Élémentaire |
| C10.181 | Zone de matériaux radioactifs |
| C9.112 Hall | Zone de matériaux radioactifs |
| C9.145.1 | Élémentaire |
| C9.177 | Élémentaire |

**(B) Activité authorisée**

| Isotope | Max Manipulated (MBq) | Max Purchase (MBq) |
|---|---|---|
| I125 | 0.1 | 9.25 |
| H3 | 3.7 | 185 |
| P32 | 3.7 | 9.25 |
| C14 | 0.185 | 0.185 |

**(C) Personnel authorisé**

| Last Name | First Name | P32 | P33 | S35 | H3 | C14 | Fe59 | I125 | Ca45 | Co57 | Cr51 | Na22 | Rb86 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Benhomeid | Osama | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Endo | Monamoto | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Haghighat | Roya | ☑ | ☐ | ☐ | ☐ | ☑ | ☐ | ☑ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Horvat | Ann Mary | ☐ | ☐ | ☐ | ☑ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kosa | Jamal | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Mok | David | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Negi | Ranuka | ☐ | ☐ | ☐ | ☑ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Parker | Wendy | ☑ | ☐ | ☐ | ☑ | ☑ | ☐ | ☑ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Wang | Jintang | ☑ | ☐ | ☐ | ☑ | ☑ | ☐ | ☑ | ☐ | ☐ | ☐ | ☐ | ☐ |

**(D) Conditions**

| Condition |
|---|
| Les fioles transported de BRI ne peuvent pas avoire plus que 2.5 uCi/fiole |

Le détenteur de permis interne et les personnes inscrites dans la section (C) sont autorisés à utiliser les radioisotopes désignés. Les radioisotopes et leur activité respective listé dans la section (B) peuvent être utilisé dans les laboratoires listés dans la section (A), en conformité avec les conditions inscrites dans la section (D). L'importation, le storage, la manipulation ainsi que la disposition du matériel radioactif doit être effectué en conformité avec la license de la CCSN, les règles fédérales ainsi que les politiques et procédures de la radioprotection. La copie de la license de la CCSN est disponible au secrétariat de la recherche (HRV) et au bureau de la radioprotection (HRV salle S4.79, ext. 36133)

**Approuvée par:** _____
Chef du Service de radioprotection (36133)

**Date d'entrée en vigueur:** 01 November, 2002

**Date d'expiration:** 01 Juin, 2004