TGF-β Action in Skin Cells: Regulation by Oxygen Tension and Steroids

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy,
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Your file Votre référence
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Our file Notre référence
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Many centuries ago, a very powerful Iranian king who had an interest in
Philosophy gave a distinctive command to his special consoler
to gather the detailed information about the life of all the
people who have lived on earth so far. After many
challenging reduction of numerous books
to one, the king looked at the book
and again he demanded a daring
request for his consoler:
summarize it in
one phrase.

After a moment of deep thinking, his wise counselor responded as:

They came, suffered, and gone.

This thesis is dedicated to children and all those
who still have not been given the
opportunity to feel the
beauty of life.

ABSTRACT

In this era of modern medicine, impaired wound healing is still a major medical problem with limited treatment options, especially for the elderly and for diabetic patients. One of the molecules that are recognized to be critical in wound healing is transforming growth factor (TGF-β). Exogenous direct application of TGF-β to the wound promotes healing in animal models. Since this property of TGF-β is cell context-dependent, the present study investigated the role of ischemia/reperfusion using a pig-skin flap model (*in vivo*). In addition, we studied the effects of: i) hypoxia/reperfusion, ii) steroids, and iii) the combined effect of both hypoxia and steroids on the TGF-β signaling pathway (TGF-β, its receptors and its downstream mediators, Smad2 and Smad3) *in vitro*; all of these were studied using human primary skin fibroblasts and a human keratinocyte cell line (HaCat cells).

Our *in vivo* results showed that the TGF-β receptors (RI, RII, and RIII) and TGF-β1 are markedly increased under acute ischemic conditions in dermal blood vessels and fibroblasts. Our *in vitro* experiments showed that short-term (2 hours) and long-term (24 hours) exposure to hypoxia differentially regulates TGF-β components, namely, active TGF-β, RII mRNA, phosphorylated (P) and total Smad2 and Smad3. Our studies have also shown that steroids are able to strongly modulate TGF-β signaling and that these effects are critically dependent on the type of steroid used and on treatment duration. Analysis of the combined effect of hypoxia and steroids revealed that steroid effects on the TGF-β signaling machinery is potentially modulated by oxygen tension, with hypoxia acting synergistically or antagonistically in a steroid-specific manner.

In summary, we have identified oxygen tension and steroids as regulators of the action of TGF-β in skin cells. We demonstrated that their effects are critically dependent on the duration of treatment and that they may act synergistically or antagonistically on the components of the TGF-β pathway, emphasizing the complexity of TGF-β signaling. Identification of these agents as modulators of TGF-β signaling provides a basis for the development of strategies for the manipulation of TGF-β action in the skin. This may contribute towards the development of agents that promote wound healing.

RÉSUMÉ

Dans cette époque de la médecine moderne, la guérison altérée de plaies demeure toujours un problème majeur, offrant des options de traitement limitées chez les personnes âgées et les diabétiques. Une des molécules reconnues comme critique (vital) pour la guérison de plaies est le facteur de croissance transformateur (TGF-β). L'application exogène directe du TGF-β à la plaie favorise la guérison chez les modèles animaux. Puisque cette propriété du TGF-β dépend du contexte de la cellule, la présente étude a examiné le rôle de l'ischémie et de la réperfusion sur un modèle rabat de peau porcine (*en vivo).* De plus, nous avons étudié les effets de : i) l'hypoxie/la réperfusion, ii) les stéroïdes, et iii) l'effet combiné, et de l'hypoxie et des stéroïdes, sur les voies de signalisation du TGF-β (sur le TGF-β, ses récepteurs et ses médiateurs en aval, Smad2 et Smad3) e*n vitro*; ceux-ci ont été étudiés utilisant des fibroblastes humains primaires provenant de la peau, ainsi qu'une lignée cellulaire humaine de kératinocytes (cellules HaCat).

Nos résultats *in vivo* démontrent que le nombre de récepteurs de TGF-β (RI, RII et RIII) et de TGF-β1 est nettement augmenté dans les vaisseaux sanguins du derme et dans les fibroblastes sous des conditions ischémiques aiguës. Nos expériences *in vitro* ont démontré que des expositions à court terme (2 heures) et à long terme (24 heures) à l'hypoxie régularisent les composantes de TGF-β différentiellement, entre autres, le TGF-β actif, l'ARN messager RII, Smad2 et Smad3 phophorylés et totaux. Nos études ont également prouvées que les stéroïdes sont capables de moduler fortement la signalisation du TGF-β, et que ces effets sont critiquement dépendants sur le type de stéroïde utilisé et de la durée du traitement. L'analyse de l'effet combiné de l'hypoxie

et des stéroïdes sur les lignées cellulaires mentionnées ci-haut révèlent que l'effet des stéroïdes sur la machinerie de signalisation du TGF-β est potentiellement modulée par la tension de l'oxygène, avec l'hypoxie agissant synergiquement ou antagoniquement d'une manière spécifique au stéroïde utilisé.

En résumé, nous avons identifié la tension de l'oxygène et les stéroïdes comme régulateurs de l'action du TGF-β dans les cellules de la peau. Nous avons démontré que leurs effets sont critiquement dépendants sur la durée du traitement et qu'ils peuvent agir synergiquement ou antagoniquement sur les composantes de la voie du TGF-β, soulignant la complexité de la signalisation du TGF-β. L'identification de ces agents agissant comme modulateurs pour la signalisation du TGF-β fournit une base pour le développement de stratégies pour la manipulation de l'action du TGF-β sur la peau. Ceci pourrait contribuer au développement d'agents qui pourrait promouvoir la guérison de plaies.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

1) I have demonstrated for the first time that ischemia and reperfusion leads to rapid and dynamic regulation of TGF-β receptors (type I, II, and III) and TGF-β1 in the skin at the protein level in a cell type specific manner *in vivo* using pig skin flap model.

2) I have demonstrated for the first time that active TGF-β and its receptors are differentially regulated by short (2 hours) and longer (24 hours) term hypoxia in human skin cells *in vitro*. This is consistent with the beneficial effect of initial hypoxia on wound healing and the deleterious effect of sustained hypoxia on wound healing.

3) I have shown for the first time the differential modulation of phosphorylated forms of Smad2 and Smad3 by hypoxia at 24 hours in human skin cells *in vitro*. In addition, I demonstrated opposing effects of hypoxia on total Smad2 and total Smad3 at 1 hour and 24 hours.

4) I have demonstrated that long term exposure (24 hours) to steroids (dexamethasone, RU486, estrogen, tamoxifen) decreased the active levels of TGF-β while short term exposure (2 hours) to glucocorticoids (dexamethasone and RU486) increased active and total TGF-β in human skin cells *in vitro*.

5) I have demonstrated the effect of steroids on TGF-β receptors (RI, RII, and RIII) in skin cells *in vitro* which indicated that short term (2 hours) and longer term (24 hours) exposure of steroids (dexamethasone, RU486, estrogen, or tamoxifen) upregulated and testosterone (24 hours) down regulated the expression of RII mRNA. Dexamethasone or RU486 treatment (24 hours) increased RI mRNA expression, as well as RI, RII, and RIII proteins.

6) I have shown for the first time the differential regulation of Smad proteins by dexamethasone and RU486 at short term (1 hour) exposure in skin cells *in vitro*. Dexamethasone had no effect and RU486 had upregulating effect on Smad2P and Smad3P. In contrast, RU486 had negligible effect on total Smad2 and Smad3 while dexamethasone upregulated them.

7) I have demonstrated for the first time that hypoxia (24 hours) counteracted the downregulatory effects of estrogen and tamoxifen on active levels of TGF-β in skin cells *in vitro*. On the other hand, hypoxia displayed an additive effect in down regulating the active levels of TGF-β in dexamethasone and RU486 treated cells.

8) I have demonstrated that hypoxia (24 hours) decreased the upregulatory effect of dexamethasone and RU486 on expression of both RI and RII in skin cells, and that of estrogen, or tamoxifen on expression of RII with testosterone having no effect.

9) I have shown for the first time that hypoxia (24 hours) reversed the dexamethasone and RU486 induced downregulation of total Smad2 and total Smad3 in skin cells *in vitro*.

PREFACE

This thesis is assembled in accordance with the regulations as detailed by the Faculty of Graduate Studies and Research, McGill University. It consists of an Abstract, Resume, and five chapters entitled Introduction (Chapter 1); Materials and Methods (Chapter 2); the effect of ischemia-reperfusion injury on TGF-β receptors in skin cells (Chapter 3); Modulation of TGF-β signaling pathway by oxygen deprivation in skin cells (Chapter 4.A); modulation of TGF-B signaling pathways by steroids in skin cells (Chapter 4.B); the impact of hypoxia, and steroids on TGF-B signaling pathway in skin cells (Chapter 4.C), and the final chapter provides conclusion consisting of: i) summary of results, general Conclusion and Future Perspectives (Chapter 5). A bibliography containing all the literature cited is placed at the end of this thesis.

Chapter 3 in the thesis was reported in the following article:

Mortazavi-Haghighat R, Taghipour-Khiabani K, David S, Kerrigan CL, Philip A. (2002). Rapid and dynamic regulation of TGF-β receptors on blood vessels and fibroblasts during ischemia-reperfusion injury. *Am J Physiol Cell Physiol.* 282(5):C1161-9.

Nimeh T, **Mortazavi-Haghighat R**, Khiabani K, Noura M, Watters K, Philip A. Ischemia regulates endoglin expression in a pig skin flap model. *MJM*, 6(1):2001.

All the data presented in this thesis are the work of the author with the following exceptions:

1) Hybridization of membrane that was used for RNase protection assay was performed by Jin Tang Wang, a research assistant in Dr. Philip's laboratory.

2) The preparation of the early passage of primary human fibroblast cells from healthy volunteers at breast reduction surgery was accomplished by Betty Yuet Ye Tam, a graduate student in Dr. Philip's laboratory.

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My most earnest gratitude goes to the chairman of Experimental Surgery, Dr Lawrence Rosenberg, for his wisdom and his kindness in helping me out whenever I needed it. I wish to express many thanks to him for letting me use his state-of-the-art microscope facility.

Special thanks go to Dr. Michael Tanzer for permitting me to stay in his lab while our lab was under renovation. I wish to thank Dorota Karabasz in Dr. Tanzer's lab for her friendship, kindness and her well-mannered way, which made for a pleasant atmosphere in their lab.

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Very special thanks must be given to Debby and Maria in Dr. Rosenberg's lab for letting me use their instruments. They were quite helpful to me. My thanks also go to Dr. Chiu and Minh for letting me use their cell culture hood. I would also like to thank Claude, Paul and Anne-Marie for their friendly attitude.

I would especially like to thank Mrs. Janet Laganière for welcoming me to her lab to use the cryostat. Her expertise, kindness and warm character made the boring task of cutting the hard, frozen pig skin into a pleasing task, so much so that I would forget the time, and I would cut more!

I was pleased everyday to see Ms. Allanna Melanson; I thank her for her assistance and her precious sense of humor. You are definitely the sunshine of C9!

Many thanks go to Dr. Osama Benhameid, and Marie-Linda Boughdady for their unbelievably special, nice character. I greatly appreciate the acceptance of Ms. Boughdady to do the French translation of my abstract.

My children Farinaz and Arvin soon recognized the existence of TGF-β and the fact that they have to compete very hard with it to gain my attention. I really thank them for their patience, for their perseverance and for their attempt. Their appreciation for the field of TGF-β and for research is cherished! I hope that one day they will fully understand why I went that far and will forgive me for not having been there for them as much as they wanted.

I would also like to thank my husband Fari for his support and for his patience throughout this project.

Last but not least, I would like to express my profound gratitude and affection toward my parents for teaching me that nothing in life sustain but *knowledge*. Thank you.

Though, regrettably, a recent sudden incident prevented my father from witnessing his final influential impact on me.

Oh wheel of the Spheres, destruction comes of thy spite
Justice has ever since been thy profession
And thou, oh Earth, if they were to Cleave thy bosom
Many a precious jewel would they find within

(By Iranian poet: Omar Khayam. Rendered into English Verse by Edward Fitzgerald)

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ABBREVIATIONS

ACTH - adrenocorticotropic hormone

ActRI - activin receptor type I

Alk-1 - activin receptor- like kinase1

Alk-4 - activin receptor- like kinase4

Alk-5 - activin receptor- like kinase5

AP-1 - activator-protein-1

AR - androgen receptor

ARNT - aryl hydrocarbon receptor nuclear translocator

ATP - adenosine triphosphate

BAMBI - BMP and activin membrane-bound inhibitor

b-HLH - basic-helix-loop-helix

BMP - bone morphogenetic proteins

BSA - bovine serum albumin

CBFa1 - core binding factor a1

Cdk - cyclin-dependent kinases

CD44 - hyaluronan receptor

cDNA - chromosomal DNA

Co-Smad - common mediator Smad

CBP - creb binding protein

Dex - dexamethasone

DHEA - dehydroepiandrosterone

DNA - deoxyribonucleic acid

PBS - Dulbecco's PBS

ECM - extracellular matrix

EGF - epidermal growth factor

EGFR - epidermal growth factor receptor

Estrogen - 17 β estradiol

ER - estrogen receptor

ERK - extracellular signal regulated kinase

FAST-1 - forkhead activin signal transducer-1

FBS - fetal bovine serum

FGF - fibroblast growth factor

FYVE - Fab-1, YGL023, Vps27, and EEA1 a small, cysteine-rich Zn^{2+} binding domain of 200 amino acids

GC - glucocorticoids

GDF-1 - growth differentiation factor 1

GDNF - glial derived neurotropic growth factor

GPI - glycosyl phosphatidylinositol

GR - glucocorticoids receptor

GRE - glucocorticoids response element

HIF-1 - hypoxic-inducible factor-1

HNPCC - hereditory non-polyposis colorectal cancer

Hsps - heat shock protein

HUVEC - human umbilicalvein endothelial cells

IGF-I - Insulin-like Growth Factor-I

IGF-II - Insulin-like Growth Factor-II

IgG - immunoglobulin

IL-1 - Interleukin-1

JNKs - c-Jun N-terminal kinase

kDa - kilo Dalton

LAP - latency associated protein

LTBP - latent TGF-β binding protein

M6P - mannose-6-phosphate

MAP - mitogen activated protein

MAPK - mitogen activated protein kinase

MEK - MAPK/ERK kinase

MEKK - MEK kinase

MH - Mad homology

MH1 - mad homologous region 1

MH2 - mad homologous region 2

MIC-1 - macrophage inhibitory cytokine

MIS -Mullerian inhibitory substance

MKK - MAPK kinase

MMP - matrix metalloproteinase

mRNA - messenger ribonucleic acid

NF-κB - nuclear factor kappa B

nM - nanomolar

NF-YA - Nuclear factor YA

NRK - rat kidney fibroblasts

p15 - a protein belonging to INK4 family that causes cell cycle arrest

p21 - a protein belonging to CIP/KIP family that causes cell cycle arrest

p38 - a MAPK signaling pathway poorly activated by mitogen

PAI - plasminogen activator inhibitor

PAI-I - type I plasminogen activator inhibitor

PBS - phosphate-buffered saline

PDGF - platelet derived growth factor

PKB - protein kinase B

PG - progesterone

PR - Progesterone receptor

RI - type I TGF-β signaling receptor

RII - type II TGF-β signaling receptor

RIIB - type IIB TGF-β signaling receptor

RIII - type III TGF-β receptor, betaglycan

RNA - ribonucleic acid

Smad - a class of intracellular signaling protein for the TGF-β superfamily

R-Smad - receptor-regulated Smad

SAPKs - stress-activated protein kinases

SARA - Smad anchor for receptor activation

SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SMURF - Smad ubiquitination regulatory factor

STAT - signal transducers and activators of transcription

TAM - tamoxifen

TGF-α - transforming growth factor-α

TGF-β - transforming growth factor-β

TIMP - tissue inhibitor of matrix metalloproteinase

TNF-α - tumour necrosis factor-α

TSP-1 - thrombospondin-1

uPA - urokinase-type plasminogen activator

VDR - vitamin D receptor

VEGF - vascular endothelial growth factor

Chapter 1: Introduction

1.1. Introduction

Technological advances during the last century have considerably increased our understanding of human disease states and have allowed us to identify and measure the changes in concentration of molecules and chemicals in our body with a very high precision. Despite this, our understanding of the many interactions and effects that exist at the molecular level is primitive. This lack of knowledge is a barrier to design effective medications without side effect for the various human disorders. In this regard, one of the identified cytokines that is involved in many aspects of cellular events such as: proliferation, differentiation, adhesion and migration, is transforming growth factor-β (TGF-β). The disruption of its signaling pathway is implicated in impaired wound healing, inflammation and cancer. Other factors believed to play a role in these processes include oxygen tension and steroid hormones. Oxygen starvation of tissues is called hypoxia and a more complex condition that includes inadequate supply of oxygen and nutrients is defined as ischemia. During ischemia, there is an additional decreased removal of metabolic by-products that lead to profound pathological alterations and damage to cells, tissues, and organs. Many biological activities such as metabolic, reproductive, immune, and neuroendocrine responses are controlled by steroid hormones. The effect of hypoxia and steroids on TGF-β signaling pathway is not fully understood. Our objective was to study the effect of hypoxia and steroids on TGF-β

signaling pathway. This chapter present a review of literature on TGF-β, hypoxia and steroids with on emphasize on their role in wound healing.

1.2. TGF-β and TGF-β signaling in normal cells

Transforming growth factor-β superfamily

TGF-β was first isolated from conditioned media obtained from murine sarcoma virus-transformed mouse fibroblasts, that enabled phenotypically normal rat kidney fibroblasts (NRK cells) to grow as colonies in semisolid agar in the presence of epidermal growth factor (EGF), a feature of malignant cells (Roberts et al, 1981). Subsequently, two distinct types of transforming growth factors were isolated from the above conditioned medium, and were called TGF-α and TGF-β (de Larco and Todaro, 1978; Anzano et al, 1983). The ability of TGF-α (5 kDa) and TGF-β (25 kDa) in combination, to promote the growth of colonies in semisolid agar opened a new era in the study of cancer cell biology. It is now known that TGF-β super family members are critically involved in cell-cycle control, regulation of early development and differentiation, extracellular matrix (ECM) formation, hematopoiesis, angiogenesis, chemotaxis, as well as immune function (Figure 1) (Roberts et al, 1990a; Kingsley, 1994; Kim and Letterio, 2003; Luethviksson and Gunnlaugsdottir, 2003)

Since the initial cloning of TGF-β, more than 40 members of a large superfamily of secreted factors that are structurally related to TGF-β have been discovered

Figure 1. *TGF-β is a multifunctional regulator.*

For example, TGF-β inhibits cell growth and promotes apoptosis, stimulates extracellular matrix production, regulates the proliferation and migration of endothelial and vascular smooth muscle cells, and regulates the proliferation, differentiation, and activation of immune cells. Deregulated TGF-β signaling has been implicated in various human diseases, including cancer, fibrosis, vascular disorders, and autoimmune diseases.

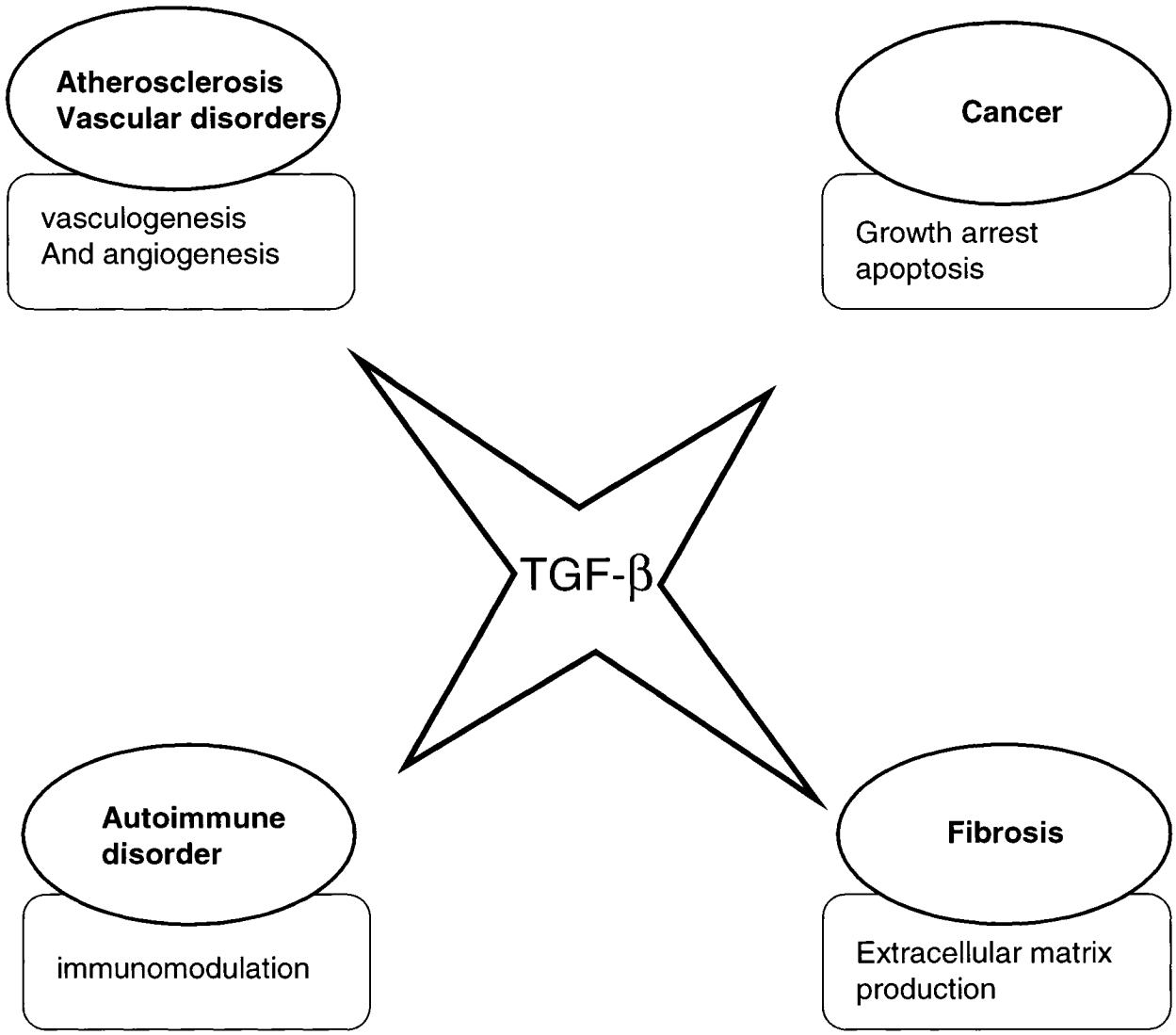

Atherosclerosis
Vascular disorders
vasculogenesis
And angiogenesis
Cancer
Growth arrest
apoptosis
TGF-β
Autoimmune
disorder
immunomodulation
Fibrosis
Extracellular matrix
production

Figure. 1

(Kingsley, 1994; Dube et al, 1998; Hogan, 1996; Roberts et al, 2003). The TGF-β superfamily includes the activins, inhibins, bone morphogenetic proteins (BMPs), growth and differentiation factor 1 (GDF-1), Mullerian inhibiting substance (MIS), glial derived neurotropic growth factor (GDNF), Nodal, and lefty (Kingsley, 1994; Dube et al, 1998; Hogan, 1996). Recently, macrophage inhibitory cytokine (MIC-1) was identified as a divergent member of the TGF-β superfamily (Bootcov et al, 1997). A common structural feature of these molecules is the cysteine knot motif (McDonald and Hendrickson, 1993) whose function is to allow TGF-β superfamily members to form biologically active homo- or hetero-dimers through intermolecular disulphide bonding (Cheifetz et al, 1988; Schlunegger and Grutter, 1994). Several members of the family (i.e., GDF-9, BMP-15, GDF-3, lefty-1, and lefty-2) have a substitution of a serine for the cysteine in the knot: while dimers of most family members are held together covalently, the proteins with this substitution would be expected to be noncovalently associated resulting in less stable dimers (Chang et al, 2002).

The TGF-β superfamily members, "activin" and "inhibin" were initially named based on their antagonistic effects in several biological systems. Activins stimulate synthesis of follicle-stimulating hormone (FSH) in pituitary gonadotropes and play an important role in erythroid differentiation (Vale et al, 1986; Eto et al, 1987), while inhibins block FSH production (Ling et al, 1985). Inhibin is a glycoprotein secreted by the gonads. It exists as a dimer of two subunits (α and βA or βB) that form inhibin A (αβA) and B (αβB). Activin, in contrast to inhibin, is produced by many non

reproductive tissues including liver, bone and blood vessels. Activins are built by homodimerization of two inhibin beta subunits. After menopause with the depletion of functional follicles, circulating alpha subunit, inhibin A and B decrease to nondetectable levels (Burger et al, 1998) although serum activin levels remain elevated (Muttukrishna et al, 1996).

The TGF-β superfamily also includes the pleiotropic proteins "BMP", a group of morphogens that were identified originally by virtue of their osteoinductive capacity in cartilage (Wozney et al, 1988). BMPs are also involved in sculpting the body plan and regulating development of many organs, including nervous system, lung, kidney, gonads, and skin (Hogan, 1996).

GDNF was first isolated from a glial cell line (B49) conditioned medium as a survival factor for cultured midbrain dopaminergic neurons (Lin et al, 1993). Recently, the GDNF family has expanded to include neurturin, persephin, and artemin/neublastin (Baloh et al, 1998; Milbrandt et al, 1998; Rosenblad et al, 2000). These proteins represent the most divergent subgroup within the TGF-β superfamily, and their carboxy-terminal domains share only about 20% sequence similarity with other members. Moreover, they signal through a receptor complex that is distinct from TGF-β superfamily receptors and consists of the tyrosine kinase c-Ret and a glycosylphosphatidylinositol (GPI)-anchored α-receptor (Treanor et al, 1996; Worby et al, 1996).

TGF-β ligand

In mammals, TGF-β exists in three different isoforms, TGF-β1, TGF-β2, and TGF-β3. Each isoform has a molecular weight of approximately 25 KDa and is secreted as a latent form that is activated into a mature form. Mature TGF-β represent the carboxy-terminal of the precursor as a peptide of 112 amino acids. TGF-β isoforms show a high degree of conservation: TGF-β2 is 70-74% similar to -β1 while TGF-β3 is 78-80% similar to -β1 and 80-82% to -β2 (Cheifetz et al, 1988; Kingsley, 1994; Massague et al, 1992b). In contrast , comparison of the three precursor sequences shows less similarity, with TGF-β1 having a G-C-rich sequence, -β2 an A-T-rich sequence and -β3 a mixture of G-C and A-T (Roberts and Sporn, 1990b).

Each TGF-β isoform is encoded by distinct genes located on different chromosomes. The TGF-β1 gene has been located at 19q13 in the human, and chromosome 7 in the mouse (Roberts and Sporn, 1990b). The gene for TGF-β2 is at chromosome 1q41 in the human, and at chromosome 1 in the mouse. The TGF-β3 gene, the last one to be cloned, is at 14q24 in the human and at 2 in the mouse.

TGF-β isoforms are differentially expressed during embryogenesis (Wall and Hogan, 1994; Jaskoll and Melnick, 1999; Akhurst et al, 1990; Heine et al, 1987), carcinogenesis (Gorsch et al, 1992; Thompson et al, 1992; Friess et al, 1993; Jennings et al, 1994; Friedman et al, 1995; Kloen et al, 1997; Naef et al, 1997), inflammation and

tissue repair (Kulkarni et al, 1993; Levine et al, 1993; Shihab et al, 1995; Horvath et al, 1996; Riesle et al, 1997; Yamamoto et al, 1999), suggesting that the isoforms may have divergent functional roles.

The functional roles of TGF-β isoforms have been studied in knock out (KO) mice. The role of TGF-β1 as a central regulator of immune homeostasis was clearly seen in the TGF-β1 KO mouse. TGF-β1 null mice died within four weeks of birth due to massive infiltration of lymphocytes and macrophages in multiple organs including the heart, lung, liver, pancreas, stomach and intestine (Shull et al, 1992; Kulkarni et al, 1993). Ablation of TGF-β2 resulted in death shortly after birth due to heart, limb, craniofacial, ear, eye, and urogenital defects (Sanford et al, 1997). Finally, the TGF-β3 KO mice display cleft palate and immature lung development (Kaartinen et al, 1995; Proetzel et al, 1995).

TGF-β can induce apoptosis in both normal and cancer cells and is considered to be a tumor suppressor (Alevizopoulos et al, 1997; Chiarugi et al, 1997). For example, increased levels of TGF-β following androgen ablation therapy directly induced apoptosis of prostate cancer cells in rat (Kyprianou and Isaacs, 1989; Hsing et al, 1996). Overexpression of TGF-β receptor type II in a human prostate cancer cell line, LNCaP, treated with TGF-β1, caused suppression of tumorigenic growth (Guo and Kyprianou, 1999).

TGF-βs are potent chemoattractants for immune cells, fibroblasts, smooth muscle cells, and certain tumor cells, including a human prostate cancer cell line, PC-3

(Koyama et al, 1990; Postlethwaite et al, 1987; Festuccia et al, 1999). Due to its immunosuppressive properties, TGF-β1 can prevent allograft rejection in animal models (Letterio and Roberts, 1998; Josien et al, 1998). On the other hand, induction of TGF-β1 by cyclosporin (an immunosuppressant) directly promoted tumor growth and metastasis (Hojo et al, 1999; Shin et al, 1998; Khanna et al, 1999). TGF-β1 is also a potent inducer of extracellular matrix proteins, including collagens, fibronectin, proteoglycans, laminin, and tenascin (Ignotz et al, 1987; Laiho et al, 1987). TGF-β is one of the most powerful growth inhibitors of epithelial cells but it is a strong stimulator of DNA synthesis in cells of mesenchymal origin (Leof et al, 1986; Shipley et al, 1986).

TGF-β activation

TGF-β effects are mediated through intracellular mediatory molecules such as Smad proteins resulting in a signaling cascade resulting in the induction of specific target genes. The process of gene regulation by TGF-β ligand can be separated into five steps: i) activation of TGF-β ligand, ii) binding of TGF-β ligand to its receptors, iii) activation of Smad proteins, iv) translocation of Smad proteins to the nucleus, and v) binding of Smad proteins to DNA and initiation of gene transcription (Shi and Massague, 2003). It is essential to understand the structure and functional properties of

each of these elements before viewing them in a big picture of TGF-β signaling pathway.

TGF-β is secreted as a latent complex (LTGF-β) and is found *in vivo* primarily in the latent form which makes it distinct from other cytokines (Miyazono et al, 1993). Mature TGF-β must be released from the latent complex to have a biological effect through binding to receptors. Latent TGF-β exists in two different forms (Olofsson et al, 1992). The small latent complex contain the 25 kDa TGF-β homodimer noncovalently associated with a dimer of its 75 kDa N-terminal precursor protein (latency associated peptide, LAP). The large latent complex contains a second gene product, the latent TGF-β binding protein (LTBP) which is disulfide-linked to LAP (Miyazono et al, 1993). In both complexes, LAP confers latency to the complex and prevents receptor binding of the mature TGF-β (Gleizes et al, 1997; Zhu et al, 2001).

Four different LTBP isoforms (120-210 kDa) have been identified (Kanzaki et al, 1990; Moren et al 1994; Yin et al, 1995; Giltay et al, 1997; Lack et al, 2003). A functional role for LTBP in regulating the local activity of TGF-β has emerged from several studies using antibodies to LTBP-1. For example, the endocardial cushion mesenchymal cells of the mouse embryonic heart is surrounded by LTBP-1 and anti-LTBP-1 antiserum inhibits the endothelial-mesenchymal transformation in artrio-ventricular endocardial cells. Application of TGF-β is able to reverse the effects, suggesting that LTBP-1 can act as a regulator of TGF-β availability during embryonic development (Nakajima et al, 1997). In osteoblast-like cells, modulation of the LTBP-1

Figure 2. *TGF-β secretion, deposition, and activation.*

TGF-β is synthesized as a biological inactive precursor protein, a dimer containing the mature TGF-β and pro-domain (propeptide) called LAP (TGF-β latency-associated protein). Transcription of the TGF-β precursor is coregulated with LTBP (latent TGF-β binding protein). LTBP possesses a central role in the processing and secretion of TGF-β. After secretion, LTBP targets TGF-β for deposition in the extracellular matrix. The release and activation of TGF-β is controlled tightly at many levels including cellular microenvironments and various proteolytic enzymes.

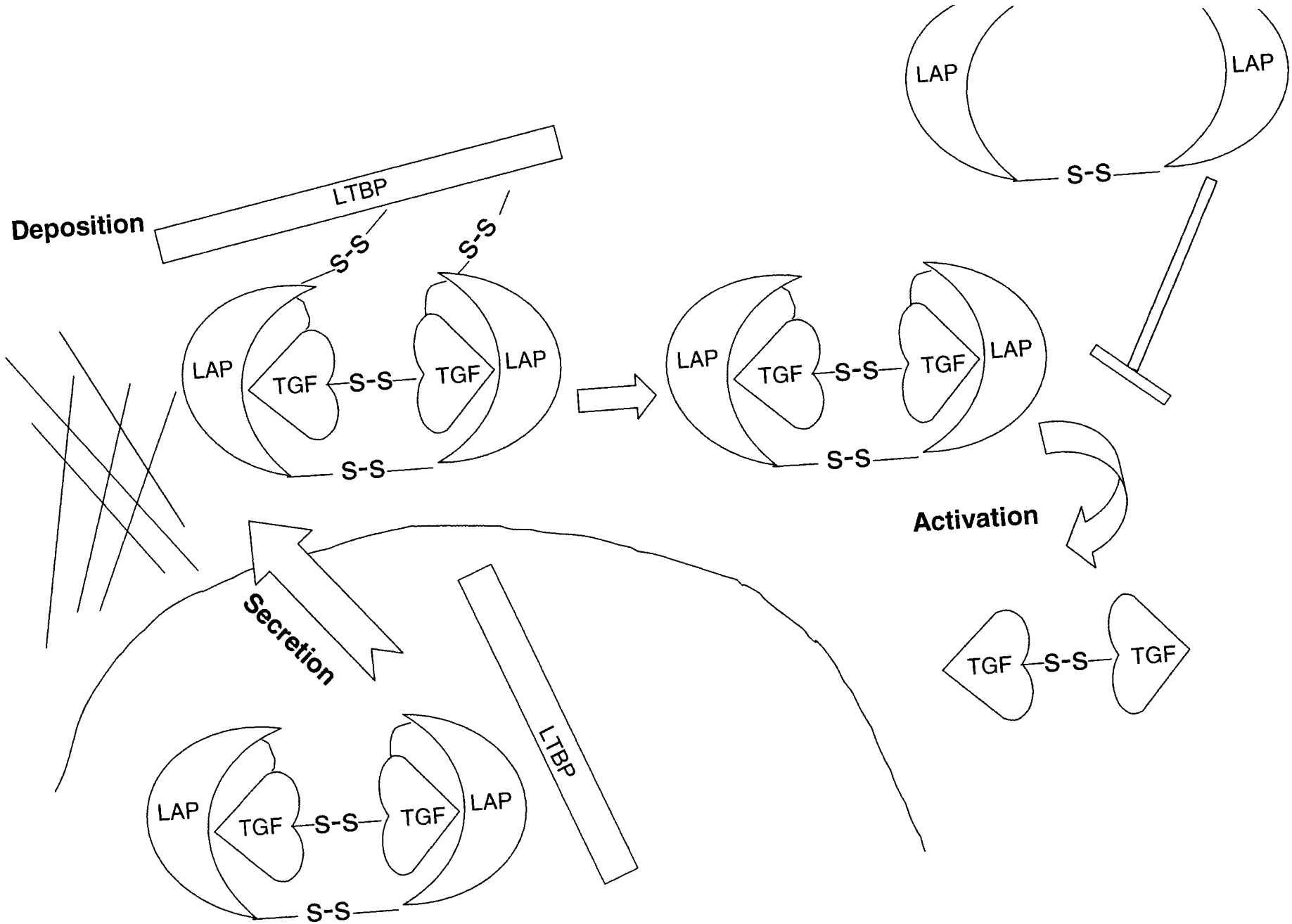

LAP
LAP
S-S
Deposition
LTBP
S-S
S-S
LAP
TGF
S-S
TGF
LAP
S-S
LAP
TGF
S-S
TGF
LAP
S-S
Activation
TGF
S-S
TGF
Secretion
LTBP
LAP
TGF
S-S
TGF
LAP
S-S

Figure. 2

content of latent TGF-β complexes may act to regulate their function (Dallas et al, 1995). The LTBP-2 null mice die at the implantation stage (Shipley et al, 2000). This phenotype differs markedly from that of TGF-β1-null mice (Shull et al, 1992), indicating that LTBP-2 has an unidentified activity in early development. LTBP-3 null mice display bone phenotypes including osteoarthritis and osteoporosis (Dabovic et al, 2002) which also occur in mice that have defective TGF-β signaling pathways resulting from either mutations in Smad3 (osteoarthritis) (Yang et al, 2001b) or the expression of a dominant negative type II TGF-β receptor in osteoblasts (osteopetrosis) (Filvaroff et al, 1999). LTBP-4 null mice develop pulmonary emphysema, cardiac myopathy and colorectal cancer (Sterner-Kock et al, 2002). Interestingly, the defects in LTBP-4 null animals are consistent with both increased and decreased TGF-β activity: i) emphysema has been associated with both increased and decreased TGF-β activity (Kaartinen et al, 1995; Zhou et al, 1996); ii) cardiac myopathy is associated with increased TGF-β activity (Schultz Jel et al, 2002); and iii) colorectal cancer is associated with a lack of TGF-β activity (Gold, 1999). These observations indicate that an isolated deficit in TGF-β activation could not account for the observed phenotypes and emphasizes the importance of other regulatory role of LTBPs that have not been recognized yet.

LTBPs are glycoproteins that are structurally regarded as a subfamily of the extracellular microfibrillar protein fibrillin 1 and 2. Both LTBPs and fibrillin contain an arginine-glycine-aspartic acid (RGD) sequence, 16 EGF-like repeats, a β2-laminin binding sequence, and 8 cysteine repeats (Kanzaki et al, 1990; Corson et al, 1993).

LTBP does not confer latency to the complex, and is known to facilitate assembly and secretion of latent TGF-β (Miyazono et al, 1991); to participate in the proteolytical activation of TGF-β (Taipale et al, 1992; Taipale et al, 1995); and to target the large complex to the extracellular matrix (Taipale et al, 1994; Nunes et al, 1997) where it is involved in the formation of fibrillar structures (Dallas et al, 1995). Latent TGF-β is stored in extracellular matrix by the transglutaminase-mediated cross-linking of the N-terminal portion of LTBP to the ECM (Dallas et al, 1995; Nunes et al, 1997).

TGF-β isoforms have a knot motif composed of six cysteine residues joined together by three intrachain disulfide bonds that stabilize β-sheet bands. One free cysteine forms an interchain disulfide bond with an identical monomeric chain to generate the mature TGF-β dimer. Activation of TGF-β is a complex process involving conformational changes of LTGF-β complex. This results from the dissociation of the LAP-TGF-β complex by cleavage of LAP by proteases such as plasmin, and thrombin (Schultz-Cherry et al, 1994b), by plasma transglutaminase or endoglycosylases, as well as by conformational rearrangement of the LAP by other proteins such as thrombospondin-1 (TSP-1) (Schultz-Cherry et al, 1994a; Schultz-cherry et al, 1994b; Murphy-Ullrich et al, 2000), CD44 (Yu and Stamenkovic, 2000), or integrin α_v β_6 (Munger et al, 1999). Plasmin- mediated proteolysis of LAP is under the positive and negative control of urokinase-type plasminogen activator (uPA) and type I plasminogen activator inhibitor (PAI-I), respectively (Vassali et al, 1991), which act by regulating the

serine protease, plasmin. The interaction of mannose-6-phosphate (M6P) domains in the LAP with M6P/insulin-like growth factor II (M6P/IGF-II) receptors on the cell, localize latent TGF-β on the cell surface which facilitate the cleavage of LAP by plasmin (Sato et al, 1990; Nunes et al, 1995; Khalil et al, 1996).

TSP-1 is a homotrimeric protein that activates TGF-β by exposing the receptor binding sites in TGF-β through conformational changes in LAP (Schultz-Cherry et al, 1994b; Schultz-Cherry et al, 1995). This is mediated by the interaction of "activation sequence", KRFK, in TSP-1 with a highly conserved LSKL segment in the N-terminal portion of LAP (Ribeiro et al, 1999).

In addition, CD44, a receptor for hyaluronan, can activate TGF-β by dissociating LAP from the mature TGF-β through localization of matrix metalloproteinase MMP-2 and MMP-9 on the cell surface (Yu and Stamenkovic, 2000). TGF-β can also be activated *in vivo* by integrin α_v β_6 via RGD sequences in LAP (Munger et al, 1999). The important role of integrin in bioactivation of TGF-β was demonstrated by generation of β_6 null mice which display inflammation in the lung and skin similar to the TGF-β1 knockout mice (Huang et al, 1996). Finally, *in vitro* activation of latent TGF-β can also be achieved by using dissociating agents such as acid, chaotropic agents, or heat (Brown et al, 1990). Activation of TGF-β is likely to be a critical determinant of TGF-β bioavailability and action.

TGF-β receptors

Most TGF-β superfamily members exert their effects on cells by binding to cell-surface proteins termed type I (RI; 55 KDa), and type II (RII; 75 KDa) receptors which are members of the serine/threonine protein kinases family. These receptors can be distinguished from each other based on their structural and functional properties. While the receptors I and II are responsible for signal transduction, the type III receptor (betaglycan, 200-300 KDa) might modulate ligand access to the signaling receptors (Lin, 1992; Massague, 1992a; Massague, 1998; Kingsley, 1994). In mammals, five type II receptors and seven types I receptors have been identified (Itoh et al, 2000; Moustakas et al, 2001). Combinatorial interactions in the tetrameric receptor complex permit differential ligand binding or differential signaling in response to the same ligand (Derynck and Feng, 1997) (Table 1). For example, the type II receptors ActRII and ActRIIB can combine with the type I receptor ActRIB/ALK4 and mediate activin signaling, whereas their interactions with BMP-RIA or BMP-RIB allow BMP binding and signaling instead. The BMP type II receptor BMP-RII can combine with three type I receptors, BMP-RIA, BMP-RIB and ActRI/ALK2 to bind several BMPs and mediate BMP signaling.

Act-RI, also known as ALK-2 can bind to various ligands, including activins, BMP2/4, and BMP7 (Yamashita et al, 1995; Ten Dijke et al, 1994a; Ten Dijke et al, 1994b). The TGF-β type II receptor (RII) interacts with RI/ALK5. Another type I

receptor that can bind to both TGF-β and activins, is ALK1 which is expressed in blood vessels during embryogenesis (Roelen et al, 1997) and adult stages (Panchenko et al, 1996). The widely expressed ALK5 induces the phosphorylation of Smad2 and Smad3, while ALK1, which is predominantly expressed in endothelial cells (ECs) and at specific sites of epithelial-mesenchymal interactions (Roelen et al, 1997), stimulates Smad1 and Smad5 phosphorylation (Chen and Massagué, 1999; Oh et al, 2000). The TGFβ/ALK5 and TGFβ/ALK1 pathways have opposite effects on ECs behavior; ALK5 inhibits EC migration and proliferation while ALK1 stimulates both processes. Moreover, the balance between the ALK1 and ALK5 signaling pathways in endothelial cells plays a crucial role in angiogenesis (Oh et al, 2000). Furthermore, ALK1 is involved in arterialization and remodeling of arteries (Seki et al, 2003) and mutations of the ALK1 gene have been linked to the type II hereditary hemorrhagic telangiectasia (Johnson et al, 1996).

TGF-β receptors were initially identified by affinity cross-linking experiments (Cheifetz and Massague, 1989; Massague, 1992a). Type I and type II receptors each consists of a short extracellular domain, a single transmembrane region, and a kinase domain (Franzen et al, 1993; Lin et al, 1992). The extracellular regions of these receptors contain about 150 amino acids with 10 or more cysteines that determine the folding of this region. The type I TGF-β receptor distinguishes itself from the type II TGF-β receptor by several features. First the type II TGF-β receptor is a constitutively active kinase.

Table 1. *Mammalian TGF-β superfamily receptors and their alternative names.*

BMPRII = bone morphogenetic protein receptor II, ActRII = activin receptor type II, RI = TGF-β type I receptor, RII = TGF-β type II receptor, Alk-2 = activin receptor-like kinase 2, Alk-3 = activin receptor-like kinase 3, Alk-4 = activin receptor-like kinase 4, Alk-5 = activin receptor-like kinase 5, Alk-6 = activin receptor-like kinase 6, Alk-7 = activin receptor-like kinase 7.

TYPE II RECEPTORS	TYPE I RECEPTORS
BMPRII	Alk-2 (ActRI) Alk-3 (BMP-RIA) ALK-6 (BMP-RIB)
ActRII, ActRIIB	ALK-4 (ActRIB)
ActRIIB	ALK-7
RII	ALK-5 (RI) ALK-1 ALK-2

Second, the type I TGF-β receptor has a 30 amino acid region rich in glycine and serine residues (GS domain) in the juxtamembrane region immediately preceding the receptor kinase domain (Huse et al, 1999). Ligand-induced phosphorylation of the serines and threonines in the GS domain is required for activation of signaling (Souchelnytskyi et al, 1996). Distal to the GS sequence, the type I TGF-β receptor has an LP motif. The immunophilin 12-kDa FK506 binding protein binds the LP sequence of the type I TGF-β receptor, inhibiting TGF-β signaling (Chen et al, 1997).

Accessory TGF-β receptors

Betaglycan and endoglin are considered as accessory TGF-β receptors that regulate TGF-β signaling (Wang et al, 1991; Gougos and Letarte, 1990). Endoglin is a cell surface protein expressed at high levels in endothelial cells and at lower levels in monocytes, erythroid precursors, and other cell types (Gougos et al, 1990; Lastres et al, 1996). A high degree of homology exists between endoglin and the cytoplasmic domain of the type III receptor, which may be an indication of a conserved role for the cytoplasmic domain of these receptors. Endoglin binds TGF-β1 and TGF-β3 but not TGF-β2. Mutations in the endoglin gene result in hereditary hemorrhagic telangiectasia type I (McAllister et al, 1994). This was the first description of a mutation in a TGF-β receptor resulting in a human disease.

Betaglycan functions predominantly in ligand presentation. In this process, it binds to TGF-β and then the type II receptor and presents TGF-β to the type II receptor (Cheifetz and Massague, 1991; Segarini et al, 1989). However, this simple view of functional property of the type III receptor has recently been challenged. Several studies have indicated that: i) hematopoietic and endothelial cells that do not express the type III receptor do not respond to TGF-β2 (Sankar et al, 1995); ii) the type III receptor is essential in mediating the effects of TGF-β (TGF-β1 or TGF-β2) on mesenchymal transformation in chick embryonic heart development (Brown et al, 1999a); iii) the loss of functional type III receptor expression on intestinal goblet cells is sufficient to mediate resistance to TGF-β (Deng et al, 1999); and iv) the type III receptor binds and modulates signaling by inhibin (Lewis et al, 2000).

Sequence analysis of type III receptor revealed that it is a transmembrane proteoglycan (Lopez-Casillas et al, 1991) with a short and highly conserved cytoplasmic domain. Type III receptor has no apparent signaling motif (Lopez-Casillas et al, 1991; Wang et al, 1991) and thus has no transducing signal activity (Brown et al, 1999a). However, its cytoplasmic domain can be phosphorylated by the type II receptor which is able to enhance TGF-β2 signaling (Blobe et al, 2001).

Soluble betaglycan is able to inhibit the binding of TGF-β to the RI-RII complex (corresponding to its extracellular domain) (Lopez-Casillas et al, 1994). Interestingly, soluble betaglycan was able to inhibit tumorigenesis and metastasis of breast cancer cell

line (MDA-MB-231) by reducing the activity of TGF-β1 and TGF-β2 secreted by these cells (Bandyopadhyay et al, 1999).

Smad proteins and TGF-β signaling

The Sma- and Mad- related proteins (Smads) mediate signaling by the TGF-β superfamily of cytokines, including TGF-β, activin, and BMP. At least 10 vertebrate Smad proteins have been identified to date (Massague and Chen, 2000b). Smads are ubiquitously expressed throughout development and in all adult tissues (Flanders et al, 2001; Luukko et al, 2001). Smad proteins have highly conserved domains at the N- and C terminus named MH1 (MAD homologous region 1) and MH2 (MAD homologous region 2), respectively. The linker region between the MH1 and MH2 domains is highly variable (Massague, 1998; Heldin et al, 1997; Wrana, 1998). Based on their structural and functional properties, Smad proteins are categorized in three distinct groups: i) receptor-regulated Smads or R-Smad (Smads1, 2, 3, 5, and 8), ii) common Smads or co-Smads (Smad4), and iii) inhibitory Smads (Smad6 and Smad7) (Figure 3).

Upon binding of ligand to RII, RI is recruited into the complex and phosphorylated by RII at its cytoplasmic GS domain (Figure 4). Interestingly, RI activation does not result from increased kinase activity but is rather from the creation of a binding site for Smad proteins which act as substrates for RI (Huse et al, 2001). The domain important for the RI-Smad interaction is located in the kinase domain of RI,

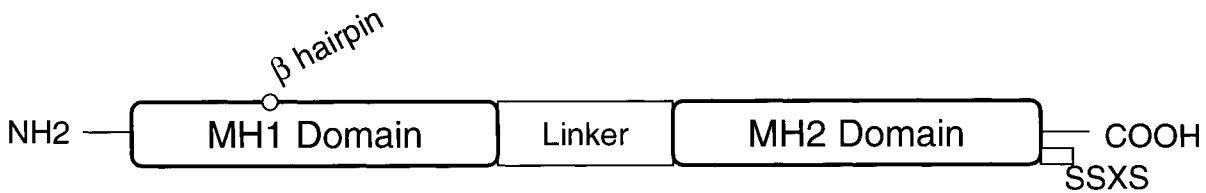
Figure 3. *Smad family.*

Diagrammatic representation of the three subfamilies of Smads. Smad family can be divided in three distinct subgroups: R-Smads, Co-Smads, and I-Smads. MH1 and MH2 domains are N- and C-terminal regions of high similarity, respectively. Inhibitory Smads lack a recognizable MH1 domain. The β-hairpin loop, which is important for interaction with DNA, is indicated. The serine residues in the C-terminal SSXS motif in R-Smads can be phosphorylated by activated type I receptors which result in R-Smad activation, heteromerization with Smad4, and subsequent translocation into the cell nucleus. The MH1 domain recognizes the DNA sequence CAGAC; the MH2 domain is involved in protein/protein interactions with co-Smad, transcriptional coactivators (p300, CBP), corepressors (TGIF, SnoN, c-Ski, or CtBP), and transcription factors (FAST, mixer, milk).

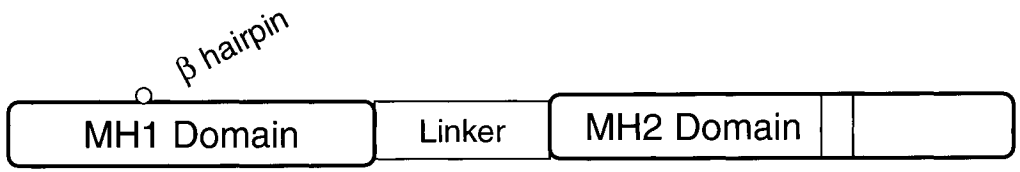
MH1 domain:
- DNA binding
- Interaction with transcription factor

MH2 domain:
- Receptor interaction (R-Smad, Smad6/7)
- Smad oligomerization
- Transcription activation
- Interaction with CBP/p300
- Interaction with transcription factors

R-Smad
(Smad1, Smad2, Smad3, Smad5, Smad8)



Co-Smad
(Smad4)



I-Smad
(Smad6/7)

Figure. 3

which is termed the L45-loop (Feng and Derynck, 1997). RI sends downstream signals for gene activation by directly phosphorylating Smad2 or Smad3 at serine residues.

Receptor-regulated Smads (Smads 1-3, 5 and 8) are phosphorylated by RI on two conserved serines at the COOH-terminus (Masssague, 1998; Heldin et al, 1997). Phosphorylation of R-Smads induces the release from the receptor complex as well as from the Smad anchor for receptor activation protein (SARA) that recruits Smads to the membrane. Phosphorylation also stimulates R-Smads to migrate and accumulate in the nucleus as heteromeric complexes with Smad4. Smad4 forms a complex with Smad1, Smad5 or Smad8 when BMP pathways are activated, and forms a complex with Smad2, or Smad3 when activin or TGF-β pathways are activated (Macias-Silva, 1996; Newfeld et al, 1999). Smad4 has a characteristic insertion in the MH2 domain and lack the C-terminal SSXS motif, the site of phosphorylation by type I receptors (Zhang et al, 1996). Because of this sequence alteration, Smad4 is not a substrate of type I receptors.

The complex of Smad4 and R-Smad migrate to the nucleus and regulate gene transcription either through direct DNA binding, through the MH1 domain of the Smad proteins, or through association with other specific transcription factors such as forkhead activin signal transducer-1 (FAST-1) or FAST-2 (Labbe et al, 1998; Liu et al, 1999; Nagarajan et al, 1999). The MH2 domain of Smad proteins contains a transactivating activity that is mediated by interaction with two closely related proteins, CREB-binding protein (CBP) and p300 which function as transcriptional co-activators and link Specific

Figure 4. *Schematic diagram of TGF-β signal transduction.*

TGF-β is secreted in a latent form, whereby TGF-βs association with LAP renders it biologically inactive. Activation of TGF-β resulting from the dissociation of LAP, permits TGF-β to bind the constitutively active type II receptor. The type I receptor is subsequently recruited into the complex and is phosphorylated in GS domain by the type II kinase. The activated type I kinase then phosphorylates Smad2/3 (R-Smads), which leads to heteromerization with Smad4 (Co-Smad). The hetero-oligomeric Smad complex then translocates into the nucleus and regulates the expression of target genes.

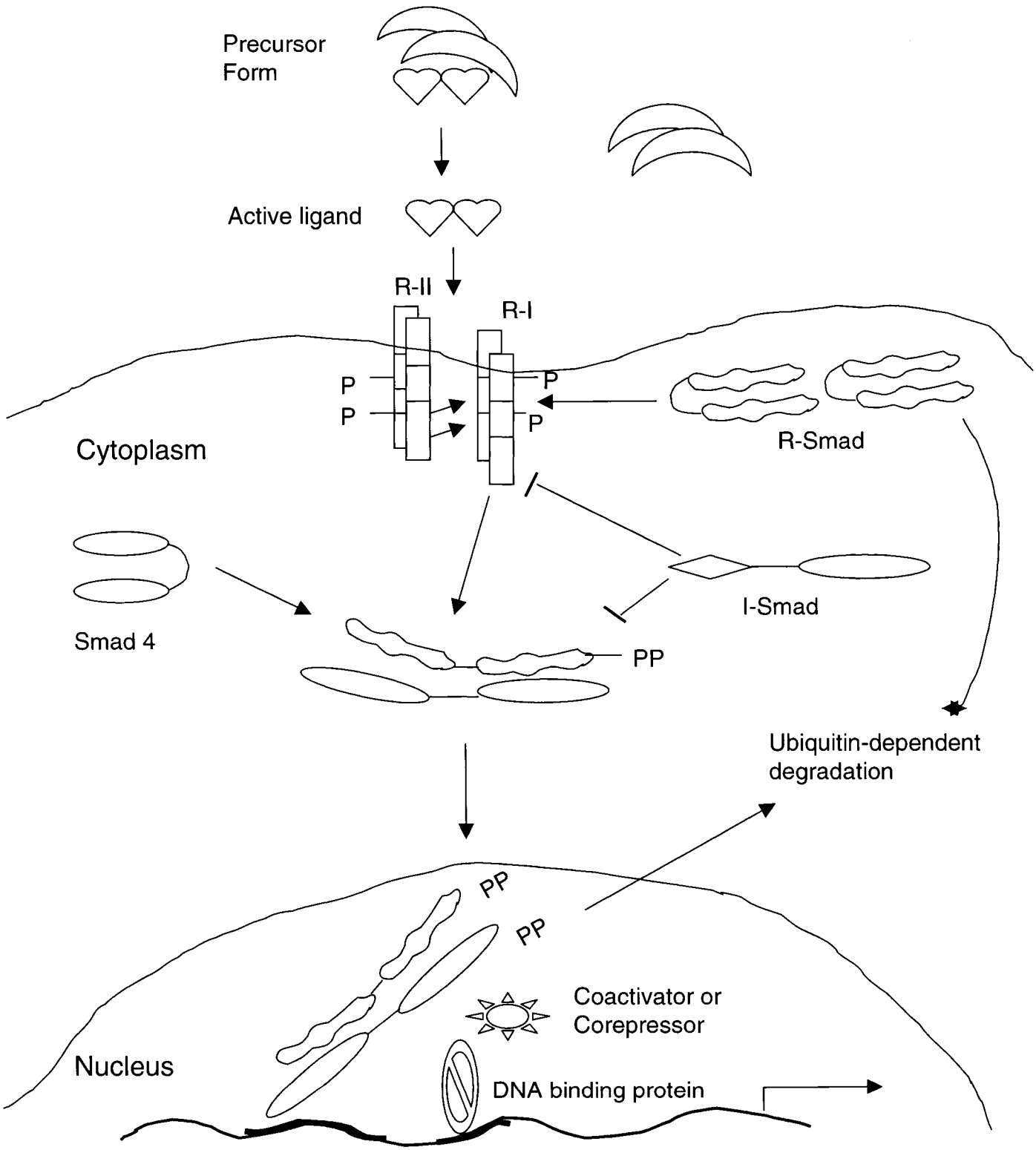

Precursor
Form
Active ligand
R-II
R-I
P
P
P
P
Cytoplasm
R-Smad
Smad 4
I-Smad
PP
Ubiquitin-dependent
degradation
PP
PP
Coactivator or
Corepressor
Nucleus
DNA binding protein

Figure. 4

transcription factors to the basal transcriptional machinery (Janknecht et al,1998; Feng et al, 1998; Pouponnot et al, 1998). The recruitment of co-repressors such as p107 (Chen et al, 2002) determine the repression of the target gene. Other co-repressors such as SKI, SNON, TGIF, EVI1 and SIP1 attenuate SMAD-mediated transactivation (Ten Dijke et al, 2002). The complex formation of Smad protein with transcriptional factors and/or co activator(s) and co repressor(s) at the DNA site determines the activation of the specific gene(s) in the nucleus (Massague and Wotton, 2000c). Besides its classical role in transcription, new observations hint at an additional role for Smads in controlling the protein turnover (Moustakas et al, 2001; Roberts and Drynck, 2001a; Attisano and Wrana, 2002; Strochein et al, 2001; Wan et al, 2001). For example, the human enhancer of filamentation 1 (HEF1) levels are regulated by proteasomal degradation induced by the Smad pathway (Liu et al, 2000). The paradigm of Smads acting as mediators of ubiquitination of cellular proteins may be extensive and may involve various mechanisms and molecular partners.

Regulation of TGF-β signaling

TGF-β signaling is controlled by positive and negative regulators. Positive regulators for TGF-β signaling are: i) betaglycan which binds to TGF-β and increases its affinity for the signaling receptors, and ii) SARA which binds Smad2 and Smad3 and facilitates their interaction with TGF-β receptors (Tsukazaki et al, 1998). In addition,

TGF-β signaling is also negatively regulated by Smad6 and Smad7 in invertebrate (Massague and Chen, 2000b). Smad7 inhibits phosphorylation of R-Smads by occupying the type I receptors for TGF-β, activin and BMP (Massague and Wotton, 2000c). Smad6 preferentially inhibits BMP signaling by competing with Smad4 for binding to receptor activated Smad1 and forms an inactive Smad1-Smad6 complex (Masssague, 1998; Heldin et al, 1997).

The immunophilin FKBP12 provides an additional regulatory control for type I receptor, by binding to the GS domain of the RI, and preventing it from ligand-independent receptor phosphorylation. Furthermore, type I receptors can also be controlled by BAMBI which acts as a pseudo-receptor and prevents signaling by forming inactive dimers with type I receptor. Interaction of Smads with coactivators or corepressors results in transcriptional activation or repression of several different Smad responsive promoters (Akiyoshi et al, 1999; Luo et al, 1999; Stroschein et al, 1999). Lastly, calmodulin, the primary mediator of calcium signaling, physically interacts with R-Smads and Co-Smads *in vitro*, and inhibits Smad mediated trans-activation of multiple TGF-β responsive promoters.

Other proteins are known to interact with Smads - suggesting that they may play an important regulatory role in TGF-β signaling. TGF-β receptors can associate with caveolin, a protein found in plasma membrane invaginations called caveaolae, and can also interact with sorting nexins resulting in modulation of TGF-β signaling. The abundance of Smad protein is also regulated by the ubiquitin proteasome pathway

through association of Smads with E3 ubiquitin ligases such as Jab1, Roc1, and Smurfs (Podos et al, 2001; Massague et al, 2000a).

The TGF-β signaling pathway is also under positive and negative regulation by the mitogen-activated protein kinase (MAPK) pathway (Rockey et al, 1992; Kretzschmar et al, 1997; Kretzschmar et al, 1999). The linker region of R-Smads is phosphorylated by the Erk kinases which itself is activated via the Ras pathway. This phosphorylation inhibits nuclear accumulation of the Smad complex. The cross-talk among different signaling pathways may also enhance or inhibit TGF-β responses, as IFN-gamma inhibits TGF-β signaling by transcriptional induction of Smad7 (Ulloa et al, 1999).

Steroids can also regulate the TGF-β signaling pathway in different tissues such as ovary and prostate (Evangelou et al, 2003; Blanchere et al, 2002). In prostatic cells, androgens negatively regulate TGF-β1 ligand (Kyprianou and Isaacs, 1989; Zatelli et al 2000) and receptor expression (Kyprianou, and Isaacs, 1988; Wikstrom et al, 1999), along with Smad expression and activation (Brodin et al, 1999). Recent reports show that androgen receptor (AR) associates with Smad3 and that this association may either enhance (Kang et al, 2001) or inhibit (Hayes et al, 2001) AR-mediated transcription. Vitamin D can also regulate the levels of bioactive TGF-β1 and appears to affect aspects of the TGF-β1 signaling system (Aschenbrenner et al, 2001). Vitamin D receptor (VDR) is a member of the nuclear receptor superfamily (Issa et al, 1998): physical interaction of liganded VDR, Smad3, and SRC-1/TIF2 can mediate cross-talk between vitamin D and

TGF-β signaling pathways (Yanagi, et al, 1999; Yanagisawa et al, 1999). Glucocorticoids (GCs) are hormones that are essential for normal growth and development of cells. Some of their effects have been shown to be mediated through modulation of the components of the TGF-β signaling pathway including TGF-β ligand, its receptors, and Smad proteins.

Hypoxia is another regulator of TGF-β signaling. Exposure of human umbilical vein endothelial cells (HUVECs) to hypoxia (1% O_2) selectively up-regulates transcription and expression of TGF-β2 by as much as 20-fold and induces Smad2, Smad3, and Smad4 to associate with DNA (Akman et al, 2001), possibly through direct interaction with the transcriptional activatory protein, hypoxia-inducible factor-1 (HIF-1) (Sanchez-Elsner et al, 2001; Semenza 2000b).

Smad-independent TGF-β signaling

Mad-independent TGF-β signaling pathways have also been identified (Figure 5) (Moustakas et al, 2001; Roberts and Drynck, 2001a; de Caestecker et al, 2000; Massague et al, 2000a; Attisano and wrana, 2002). TGF-β can activate the mitogen-activated protein kinases (MAPKs) (Hartsough and Mulder 1995; Frey and Mulder, 1997a; Frey and Mulder, 1997b; Hartsough and Mulder, 1997; Mulder, 2000). MAPKs are responsible for the conversion of a large number of extracellular stimuli into specific cellular responses that range from positive and negative roles on cell proliferation,

differentiation, and apoptosis to regulation of inflammatory and stress responses. MAPKs are serine/threonine kinases that are activated by phosphorylation on threonine and tyrosine residues in response to a wide array of extracellular stimuli. Three distinct groups of MAPKs have been identified in mammalian cells, including extracellular signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs), and p38 (Kyriakis et al, 1998). MAPK cascades convey a signal in the form of phosphorylation events. Each of the MAPKs is phosphorylated (activated) by specific MAP kinase kinases (MAPKKs). The complexity and diversity of the MAPK signaling pathways is further enhanced due to the activation of each MAPKK by more than one MAPKKK. MAPKKKs are regulated by multiple mechanisms (Cobb, 1999; Pearson et al, 2001). G proteins such as Ras (upstream effector of Raf activation), Rac and RhoA can activate the MAPKKKs (Kyriakis et al, 1998). TGF-β can activate Ras, TAK1, protein kinase B (PKB, also called Akt), and Rho family guanosine triphosphatases (GTPases). Overexpression of a dominant negative mutant of Ras (RasN17), or addition of the specific MEK1 (a MAPKK specific for ERK) inhibitor PD98059 blocks the ability of TGF-β to activate ERK1 (Hartsough et al, 1996; Yue et al, 1998). In osteoblast and 293T cells, the activation of TAK1 by TGF-β in turn activates MKK4, and p38 or JNKs (Yamaguchi et al 1995; Shibuya et al, 1996; Zhou et al, 1999). Interestingly, expression of a dominant-negative mutant of RII abrogates the ability of TGF-β to activate JNK in Hs578T cells, emphasizing the importance of TGF-β receptors for TGF-β activation of JNK1.

Figure 5. *MAPK signaling cascades activated by TGF-β.*

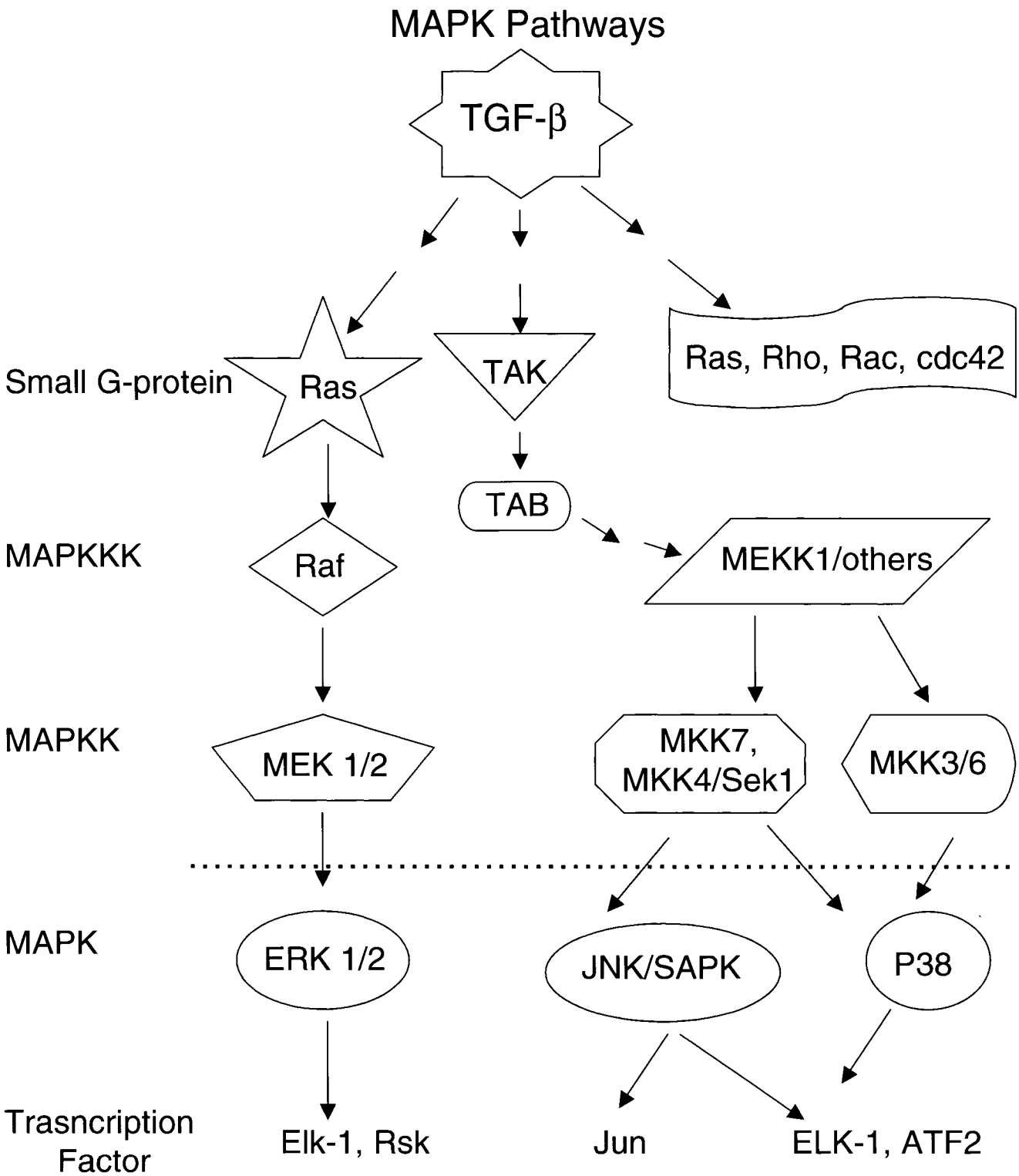


Figure. 5

(Yue and Mulder, 2001)

Other signaling pathways, including Hedgehog, Wnt, protein kinase A, G1 cyclin-cyclin dependent kinase complexes also intersect with TGF-β pathways (Vervoort, 2000; Yue and Mulder, 2001).

1.3. Dysregulation of TGF-β action in wound healing

Wound healing is a complex process that involves numerous cell types, multiple growth factors and the extracellular matrix. This process can be divided into three phases that overlap in time: inflammation, tissue formation, and tissue remodeling. Wound healing involves a reduction in wound size secondary to contraction and re-epithelialization accompanied by an increase in collagen deposition (Ashcroft et al, 1995). Impaired wound healing is an important issue in many different disease states such as reconstructive surgery in cancer patients, and diabetes mellitus.

TGF-β isoforms play a central role in all phases of wound healing process (Amento and Beck, 1991; McCune et al, 1993; O'Kane and Ferguson, 1997). Although TGF-β is released from degranulating platelets at the time of wounding, all of the different cell types involved in wound healing such as keratinocytes, fibroblasts, inflammatory cells, and endothelial cells can produce and respond to TGF-β during the course of the healing process (Schmid et al, 1998; Frank et al, 1996).

Due to the great impact of TGF-β on cells involved in wound healing, the deficiency or excesses of TGF-β may in fact lead to the disorders of wound healing (Beck et al, 1990; Bullard et al, 1997). Wound healing is severely impaired in diabetic patients. These patients show prolonged inflammation and impaired neovascularization at the site of the wound. In genetically diabetic mice the level of TGF-β1 is significantly reduced as compared to nondiabetics (Frank et al, 1996). Also a great improvement has been reported by the addition of recombinant TGF-β1 protein to the site of the wound in diabetic animals (Bitar and Labbad, 1996; Bitar et al, 1999; Bitar, 2000; Puolakkainen et al, 1995). In a report by Chesnoy et al (2003), TGF-β1 appears to have provided a higher cell proliferation rate in the granulation tissue and in the wound edges as compared to control wound.

All TGF-β isoforms, RI/II receptors and activated Smad3 proteins were found in high concentrations in the fibrotic scar tissue of various organs and in keloids. Keloids are defined as an overgrowth of scar tissue at the site of a skin injury (O'Kane and Ferguson, 1997; Chin et al, 2001). In patients with rhinophyma, RII is significantly increased in the proliferating scar tissue of the affected face compared to nonaffected skin areas (Pu et al, 2000). Moreover, studies of the scarless healing of fetal skin wounds in animal experiments revealed the lack of presence of RII mRNA. The healing process of similar wounds in adult animals that formed scar tissue, the expression of RII mRNA and the protein were both detected (Cowin et al, 2001; Hsu et al, 2001). The fibrosis-promoting properties of TGF-β include enhanced collagen deposition, stimulation of the

synthesis of matrix components by fibroblasts, inhibition of collagenase and plasminogen activators, and chemotaxis for fibroblasts, monocytes, and macrophages (Adzick and Lorenz, 1994; Noble et al, 1992). The significant role of Smad3 in wound healing has been explored recently. Acceleration of epithelialization of incisional wounds has been observed in Smad3 null mice as compared to wild-type (WT) littermates (Ashcroft et al, 1999b). Furthermore, wound made in skin of irradiated Smad3 null mice showed increased rate of epithelialization and reduced inflammatory cell infiltrate compared to WT littermate controls (Flanders et al, 2003).

Critical role of Fibroblasts in wound healing

Fibroblasts play essential roles in cutaneous wound repair and remodeling. They function as a synthetic cell, and also secrete the growth factors important for cell-cell communication during the repair process (Takehara, 2000; Mansbridge et al, 1999). The second phase of wound healing is associated with growth oriented cytokines and factors, capable of initiating extracellular matrix deposition and cellular proliferation. Fibroblasts have an important role in this process by proliferating to expand, migrate into the wound bed and express thick actin bundles as myofibroblasts (Singer and Clark, 1999). As a result of loss of contact inhibition, fibroblasts respond by secreting exctracellular components such as type I, and III collagen to form granulation or scar tissue (Clark, 1985), extracellular protease inhibitors (TIMP-2), and peptide growth

factors (TGF-β, PDGF, EGF, TGF-α, IGF, and VEGF) (Enholm, 1997; Steinbrech et al, 1999; Kunz-Schughart and Knuechel, 2002). TGF-β can considerably increase the contractile effect of human fibroblasts in the collagen matrix (Montesano and Orci, 1988), as well as the synthesis and maturation of collagen in early wounds (Cromack et al, 1993).

Dysregulation of apoptosis during the wound process is an important factor in formation of chronic wounds. Apoptosis occurs during the early phase to eliminate inflammatory cells and during the transition between granulation tissue and scar to eliminate both fibroblasts and endothelial cells. The wound is cleared from apoptotic cells by the phagocytotic action of infiltrated neutrophils and macrophages. In this process, fibroblasts produce macrophage colony-stimulating factor (M-CSF) and human granulocyte-macrophage colony-stimulating factor (GM-CSF). M-CSF is a direct stimulators of macrophages (Leibovich and Ross, 1975; Cohen et al, 1987; Danon et al, 1989) and an indirect stimulator of angiogenesis (Hamilton et al, 1993). GM-CSF facilitates wound contraction, causes local recruitment of inflammatory cells, and induces keratinocytes proliferation (Groves and Schmidt-Lucke, 2000).

Different diseases states have been recognized as a result of dysregulation of fibroblast function. For example, systematic sclerosis is tightly linked to the activation of fibroblasts. Skin fibroblasts cultured from patients with systemic sclerosis produce more collagen type I and IIII than fibroblasts from normal controls. Furthermore, in systemic sclerosis skin fibroblasts are resistant to certain forms of apoptosis (Santiago et

al, 2001; Jelaska and Korn, 2000) which might explain the persistence of clonally expanded fibroblasts with high production of matrix in systemic sclerosis.

In addition to having an important role in wound healing, fibroblasts are also linked to tumor progression. Interactions of tumor (often epithelial) cells with normal fibroblasts or their conditioned medium can enhance the invasive and metastatic phenotype of tumor cells *in vivo* (Picard et al, 1986; Tanaka et al, 1988; Camps et al, 1990; Saiki et al, 1994). Frequently, stromal fibroblastic hyperplasia was observed within and around the growing nodule (Clark, 1988). Fibrotic disorders of the breast, lung, and liver have been correlated with a higher incidence of cancer (Vorherr, 1986; Fraire and Greenberg, 1973).

TGF-β action in keratinocytes

The epidermis forms the outer layer of the skin. The majority of cells in the epidermis are keratinocytes, which are organized into four layers: the basal layer, the spinous layer, the granular layer and the cornified layer. Of these, only the basal layer contains mitotic cells. TGF-β exerts a wide range of biological effects on keratinocytes, such as growth inhibition, production of extracellular matrix, and synthesis of plasminogen activator and its inhibitor (Shipley et al, 1986; Hashiro et al, 1991; Keski-Oja and Koli, 1992). Although *in vitro* studies have documented that TGF-β acts as a potent growth inhibitor for keratinocytes (Pittelkow et al, 1988), *in vivo* studies utilizing

transgenic mouse models revealed contradicting roles for TGF-β in epidermal proliferation. When the TGF-β1 transgene was overexpressed in both the basal (proliferative) and suprabasal (differentiated) layers of the epidermis, essentially all basal cell proliferation ceased in the epidermis, resulting in neonatal lethality in these mice (Sellheyer et al, 1993). In contrast, transgenic mice that express TGF-β1 in the suprabasal keratinocyte compartment showed an increased epidermal mitotic rate with no histological changes. The transgene, however, acted in the expected fashion, as a negative regulator of cell growth, when hyperplasia was induced by treatment by 12-tetradecanoyl-phorbol-13-acetate (TPA) (Cui et al, 1995).

In keratinocytes, the c-myc oncogene is considered a major target of TGF-β-induced G1 arrest, because TGF-β1 treatment of keratinocytes results in a marked reduction of c-myc expression (Pietenpol et al, 1990a, Pietenpol et al, 1990b). Moreover, transgenic mice overexpressing c-myc in the epidermis exhibit a hyperplastic skin phenotype (Waikel et al, 1999). *In vitro* studies have demonstrated that growth inhibition by TGF-β is a consequence of promoting G1 arrest of the cell cycle via transcriptional up-regulation of inhibitors of cyclin-dependent kinases (Cdk), p21 (WAF1) that binds cdk2 and cdk4-cyclin complexes (Datto et al, 1995; Reynisdottir et al, 1995; Satterwhite and Moses, 1994) and p15 (INK4b) that binds cdk4 and cdk6 cyclin complexes (Hannon and Beach, 1994; Reynisdottir et al, 1995). p21 and p15 transcription was found to be elevated by TGF-β in cultured keratinocytes, and

correlated with TGF-β-induced growth arrest (Li et al, 1995; Reynisdottir et al, 1995). TGF-β also increases $p27^{kip1}$ protein levels (Polyak et al, 1994)

TGF-β and wound healing

Wound healing is a highly complex process comprising a number of well-defined temporally overlapping phases, all of which involve TGF-β. TGF-β signaling modulates wound healing through multiple pathways that influence cell infiltration, proliferation, angiogenesis, extracellular matrix synthesis and remodeling (Sieweke and Bissell, 1994; Cordeiro et al, 2000; Robert et al, 1986; Shah et al, 1995; Tyrone et al, 2000; Hosokawa et al, 2003). The role of TGF-β in accelerating wound healing has been demonstrated in healing-impaired models such as radiation-exposed animals or animals treated with antiproliferative-agents or glucocorticoids (Beck et al, 1993; Salomon et al, 1990; Shukla et al, 1999; Wicke et al, 2000).

TGF-β released from degranulating platelets chemotactically attracts inflammatory cells (Wahl et al, 1987) and fibroblasts (Postlethwaite et al, 1987) into the wound site. This effect may be mediated by TGF-β signaling through Smad3. In Smad3 deficient mice, neutrophils and monocytes are largely absent in the early wound, suggesting that Smad3 is also critical for TGF-β-mediated chemotaxis of inflammatory cells (Ashcroft and Roberts, 2000). However, deletion of Smad3 leads to enhanced re-

epithelialization and contracted wound areas indicating an inhibitory role for Smad3 in wound healing.

TGF-β extends or amplifies its effect on repair processes through autoinduction, which has been reported to occur in macrophages (Wahl et al, 1990) and fibroblasts (Van-Obberghen-Schilling et al, 1988). Stimulation of fibroblasts by TGF-β results in: i) increased production of a wide spectrum of matrix proteins such as collagen, proteoglycans, elastin, and fibronectin; ii) increased expression of protease inhibitors such as plasminogen activator inhibitor and tissue inhibitor of metalloproteinase (TIMP) (Bennett and Schultz, 1993); iii) reduced expression of many different degrading enzymes including collagenase, stromelysin, and plasminogen activator; and iv) increased numbers of integrin receptors. These effects result in increased matrix formation and modulation of cell-cell interactions at the wound site.

TGF-β1 and TGF-β2 are potent stimulators of dermal scarring (Gruschwitz et al, 1990; Roberts et al, 1986; Shah et al, 1995). Application of antibodies to TGF-β1 and TGF-β2 in adult cutaneous wounds markedly reduced scarring (Shah et al, 1995) by inhibiting transdifferentiation of fibroblasts to myofibroblasts (Fan et al, 1999). Furthermore, fetal wounds heal with minimal scarring most likely because of low levels of TGF-β1 in these wounds (Whitby and Ferguson, 1991). On the other hand, TGF-β3 reduces scar formation by changing the dynamic balance of type I collagen accumulation and degradation. It reduces type I collagen deposition by restricting myofibroblast differentiation and consequential type I collagen production by these cells

(Hosokawa et al, 2003). Thus, the ratio of TGF-β1/TGF-β3 appears to be critical determinant of dermal scarring.

The complex regulatory role of TGF-β on extracellular matrix production and cell communication implicate a requirement for an upregulation of TGF-β expression in normal wound repair (Frank et al, 1996); while, deregulation and sustained overproduction of TGF-β leads to the collapse of repair process and contributes to tissue fibrosis (Gruschwitz et al, 1990; Cotton et al, 1998; Yu et al, 2003).

TGF-β and inflammation

Elimination of TGF-β or disrupting its downstream signaling cascade leads to inflammatory disease. For example, expression of a dominant negative TGF-β type II receptor in a transgenic mice model leads to CD4+ T cell hyperactivity and enhances the immune response with increased production of autoantibodies which leads to autoimmunity (Gorelik and Flavell, 2000; Hahm et al, 2001). Similarly, the TGF-β1 knock-out mouse exhibits a profound self-targeting multifocal inflammatory response mediated by lymphocytes and is characterized by the overproduction of autoantibodies that kill the animal very early in life (Shull et al, 1992; Kulkarni et al, 1993; Diebold et al, 1995; Yaswen et al, 1996).

Dysregulation of the Smad pathway also plays an important role in formation of inflammatory diseases. Transgenic mice demonstrating the expression of Smad7 develop

glomerulonephritis due to the activity of antibodies directed against the glomerular basal membrane (Kanamaru et al, 2001) as well as respiratory tract inflammation (Nakao et al, 2000). Moreover, ablation of Smad3 results in early post-natal death due to leukocytosis and impaired mucosal immunity that leads to severe chronic infection (Datto et al, 1999; Yang et al, 1999).

TGF-β also has a role in formation and progression of cancer. If we acknowledge that carcinogenesis is a multistep process, and TGF-β is a multifunctional cytokine that is involved in proliferation, differentiation, migration and apoptosis, then it should not be surprising that TGF-β plays a significant role in tumor invasion and/or metastasis (Derynck and Akhurst, 2001; Akhurst and Derynck, 2001; Wakefield and Roberts, 2002; Janda et al, 2002; Kakonen et al, 2002; Oft et al, 2002). TGF-β regulates the growth of cancer cells in an autocrine or a paracrine fashion (Roberts et al, 1990a; Hartsough and Mulder 1997). TGF-β can stimulate tumor angiogenesis, alter the stromal environment, and cause local and systemic immunosuppressant. Loss of normal TGF-β function is involved in pathogenesis of cancer, atherosclerosis, and autoimmune and inflammatory diseases, while excessive TGF-β production has been implicated in fibroproliferative disorders, immunosuppression, successful parasite infection, and metastasis (Massague et al, 2000a; de Caestecker et al, 2000; Reiss, 1999; Topper, 2000; Chen and Wahl, 1999; Branton and Kopp, 1999; Reed, 1999).

Initially, TGF-β can function as a tumor suppressor; however, in late stage of human tumors there is an increase in expression of TGF-β isoforms that is associated

with increased metastasis and poor prognosis (Dumont and Arteaga, 2000). Transgenic mice with overexpression of active TGF-β1 in mammary epithelium are resistant to oncogene- or carcinogen-induced mammary cancers (Pierce et al, 1993; Jhappan et al,1993; Pierce et al, 1995). On the other hand transgenic mice with hemizygous or homozygous TGFβ1-null animals show an increased incidence of chemically or spontaneously induced tumors, respectively (Derynck and Akhurst, 2001; Akhurst and Derynck, 2001; Wakefield and Robert, 2002; Tang et al, 1998; Engle et al, 1999). Moreover, in a mouse skin model of chemical carcinogenesis, expression of TGF-β1 in keratinocytes suppresses the formation of benign skin tumors (Cui et al, 1996). However, once the tumor was developed, TGF-β1 actually enhanced tumor progression to a highly invasive spindle cell phenotype (Cui et al, 1996). Ha-Ras-induced mammary tumor cells secrete high levels of TGF-β and display highly invasive characteristic *in vitro* and *in vivo* (Oft et al, 1996).

The invasiveness of cancer cells can be controlled by decreasing of TGF-β expression. For example, a soluble chimeric protein composed of the extracellular domain of the RII and the Fc portion of the murine IgG1 heavy chain (Fc:RII) that targeted circulating TGF-β1 converted mammary tumor cells toward a more differentiated less motile/migratory phenotype, diminished the production of matrix metalloproteinase 2 (MMP2) and MMP9 (important in tumor invasion, migration, and intravasation), and increased apoptosis (Muraoka et al, 2002). Furthermore, prolonged

exposure to antagonists can significantly suppress metastasis in mice without any adverse side effects (Yang et al, 2002).

Mutations in RII were first identified in hereditary non-polyposis colorectal cancer (HNPCC) (Markowitz et al, 1995; Reiss, 1997), and later on were detected in gastric cancer, endometrial cancer, and head and neck cancers (Iacopetta et al, 1999; Nakashima et al, 1999; Garrigue-Antar et al, 1995). A progressive decrease in the expression of RII with less well-differentiated cells was observed in head and neck squamous cell carcinomas (Muro-Cacho et al, 1999). Mutation in RI has also been observed in LnCaP human prostate cancer cells in which TGF-β1 is no longer able to inhibit the proliferation of these cells. Transfection of RI back into LnCaP cells restores the ability of TGF-β1 to inhibit the proliferation of LnCap cells (Kim et al, 1996).

Analysis of Smad knock-out mice revealed that their functional inactivation may play a role in tumorigenesis (Goumans and Mummery, 2000). Smad3 null mice developed colon cancer (Zhu et al, 1998). Some colon cancers also have shown mutation in Smad2. Smad4 mutations have also been detected in many colorectal carcinomas, pancreatic carcinomas and to a lesser extent in ovarian, breast, head, neck, prostatic, esophageal, and gastric cancers (Schutte et al, 1996; Hahn et al, 1996). *In vivo* studies have shown that restoration of Smad4 to Smad4 deficient pancreatic carcinoma cells increased the steady state mRNA levels of thrombospondin-1 (TSP-1), and suppressed tumor formation by repressing tumor angiogenesis (Schwarte-Waldhoff et al, 2000).

1.4. Hypoxia

Since cells do not store oxygen, the reduction of oxygen supply by any means leads to cellular dysfunction and death. Having said that, it is absolutely impossible to avoid situations in which cells would not be exposed to low levels of oxygen. For example, starting from gestation, the fertilized egg grows in hypoxic conditions prior to making connection with the host through blood vessels, or the unavoidable accidental occurrence of wounding that happen throughout life time. Hence, the question is, whether hypoxia per se is bad or good. Is it an element that basically shuts down the normal haemostasis of cells because of inhibition of ATP production, or is it able to initiate the expression of other genes necessary for survival? Based on studies of embryogenesis, hypoxia actually initiates differentiation and angiogenesis. Interestingly, the same pattern is observed in acute wound and cancer.

Hypoxia and activation of genes

Over the years of evolution, nature has developed survival strategies to adapt to oxygen deficiency and increase the oxygen delivery to tissue. In mammalian systems, the adaptive response to hypoxia is accompanied by an increase in the expression of a variety of genes at the levels of transcription and mRNA stability. Hypoxia induces expression of a variety of genes such as the hematopoietic growth factor erythropoietin

(which stimulates red cell formation and consequently improves blood oxygen transport), vascular endothelial growth factor (VEGF) (which induces the formation of new capillaries), glycolytic enzymes (which increase the efficiency of nonoxidative energy generation), and inducible nitric oxide synthetase and heme oxygenase (Semenza and Wang, 1992; Forsythe et al, 1996; Semenza et al, 1994; Semenza, 2001; Melillo et al, 1995). These genes are transcriptionally activated by hypoxia and also by transition metals such as cobalt and by iron chelators such as desferrioxamine. Most of these genes are regulated by a common oxygen-sensing pathway involving the hypoxia-inducible factor-1 (HIF-1) protein complex (Scortegagna et al, 2003; Koshikawa et al, 2003; Minchenko et al, 2002; Yang and Zou, 2001; Wenger and Gassmann, 1997; Wang and Semenza, 1993).

The initial view was that activation of HIF-1α (one of the heterodimer of HIF-1) signaling pathway occurs only under hypoxic conditions. However growth-factors such as insulin and insulin-like growth factor-I (IGF-I) activate HIF-1 and this has been shown to occur through pathways separated from that employed by the classical hypoxic pathway (Zelzer et al, 1998). Factors that stimulate HIF-α includes many growth-factors, cytokines and circulatory factors such as PDGF, EGF, FGF-2, IGF-II, TGF-β1, HGF, TNFα, IL-1β, angiotensin-2 and thrombin (Zhong et al, 2000; Feldser et al, 1999 ; Hellwig-Burgel et al, 1999; Richard et al, 2000; Gorlach et al, 2001 ; Tacchini et al, 2001; Thornton et al, 2000). In addition, oncogenes (HER2 ^{NEU}, Ras, v-Src) (Laughner et al, 2001; Chen et al, 2001; Jiang et al, 1997), and mutations in the tumor suppressor

PTEN (Zundel et al, 2000), have also been shown to affect HIF-1α activity through these same signaling pathways. Other HIF-1α stimuli include signaling intermediates such as NO (Sandau et al, 2000; Sheta et al, 2001) and the *in vitro* phenomena of cell culture confluence (Sheta et al, 2001; Mukhopadhyay et al, 1998).

HIF-1 and its mechanism of action

The biochemical purification of the HIF-DNA binding complex revealed the presence of one subunit of about 120 kDa, HIF-1α, and a second subunit HIF-1β with polypeptides of 91, 93 and 94 kDa with a similar tryptic digestion composition. Both subunits belong to a subfamily of basic-helix-loop-helix (b-HLH) transcription factors containing a PAS domain. HIF-1β is an aryl hydrocarbon receptor nuclear translocator (ARNT) protein. HIF-1α has a oxygen dependent degradation domain (ODD) that can confer hypoxic stabilization to HIF-1α (Srinivas et al, 1999). It seems under normoxic conditions, this domain receives a signal that primes the HIF-1α protein for ubiquitination and subsequent degradation in proteasomes. Normoxic HIF-1α subunit degradation involves the von-Hipple-Lindau tumor suppressor protein (pVHL) which recognizes and links HIF-1α subunits to the ubiquitination machinery (Maxwell et al, 1999). However, during hypoxic conditions, the ODD of HIF-1α is stable and allows nuclear translocation and gene regulation.

The recognition of the HIF-1 subunit by pVHL is dependent upon hydroxylation of conserved proline residues within the ODD under normoxic conditions (Ivan et al, 2001; Jaakkola et al, 2001; Yu et al, 2001). Under hypoxic conditions, hydroxylation and subsequent pVHL-mediated ubiqitination of HIF-1α, does not take place, resulting in HIF-1 accumulation (Bruick, 2003).

The C-terminal portion of HIF-1α binds specifically to p300, a general transcriptional activator that participates in a number of biological functions such as induction of various tissue-specific enhancers, regulation of cell cycle and stimulation of differentiation pathways (Arany et al, 1996).

Hypoxia and wound healing

Disruption of the vasculature is a characteristic feature of wound sites. Regeneration of wounded tissue requires both inflammatory leukocytes such as neutrophils and macrophages, and energy which are restricted by hypoxic conditions that are often observed near wounds. Hypoxia occurs in early wounds due to decreased perfusion caused by damage to the vasculature and to the intense metabolic activity of cells infiltrating the wound. Hypoxia initially induces cellular proliferation as well as induction of gene expression of several growth factors such as platelet-derived growth factor (PDGF), TGF-β (Falanga et al, 1991), and VEGF. The augmented expression of these genes is important in promoting wound healing.

On the other hand, persistent hypoxia, which leads to tissue ischemia, delays wound healing as observed in chronic wounds (LaVan and Hunt, 1990; Xia et al, 2001). In fact tissue ischemia is known to be one of the most significant factors leading to chronic wounds (Van de Kerkhof et al, 1994). Chronic wounds that have been treated with hyperbaric oxygen show an increase in wound granulation tissue formation and accelerate wound contraction and closure (Boykin, 2000; Williams, 1997).

Hypoxia and cancer

Oxygen availability is known to play a key role in the growth-regulatory process underlying carcinogenesis (Brown and Giaccia, 1998). Initially, hypoxia inhibits cell division and induces cell death (Graeber et al, 1994; Schmaltz et al, 1998; Yao et al, 1995): during this process, groups of cells adapt to alterations in the microenvironment enabling them to survive (Czyzyk-Krzeska, 1997). The mechanisms leading to adaptation of this new *favourable* environment for tumor cells is still poorly understood (Zhong et al, 1999). These groups of cells are able to respond to hypoxia by inducing angiogenesis and the activation of anaerobic metabolism. HIF-1 has a direct impact on these responses since deletion of either HIF-1α or ARNT delays tumor growth (Ryan, and Lo, 1998; Maxwell et al, 1997) as a result of decreased vascularization and increased apoptosis.

Clinical studies have indicated that there is a direct association between the tumor oxygen level and the rate of metastasis in patients with carcinoma of the cervix (Hockel et al, 1996) or soft tissue sarcomas (Brizel et al, 1996). The existence of hypoxia in the closed area of tumor cells may in turn be a leading cause of mutation of p53 which subsequently destabilizes the cells and increases this malignant potential. In addition, HIF-1 may associate with p53 protein, thus increasing the stability of p53 (An et al, 1998). The above explanation is supported by the observation that there is a striking reduction in hypoxia-mediated apoptosis with the loss of wild-type p53 (Graeber et al, 1996). In addition, hypoxia increases the rate of mutation of other genes and acts as a strong stimulant for the expression of genes that promote the growth and survival of cancer cells. These include the angiogenic agents such as vascular endothelial growth factor, as well as glycolytic enzymes and signaling molecules (Blancher and Harris, 1998).

Low oxygen levels have been detected in a variety of human solid tumors, including those of the brain, head and neck, breast, cervix and soft tissue sarcomas (Knisely and Rockwell, 2002; Becker et al, 2002; Nordsmark et al, 2001; Birner et al, 2000). The median oxygen concentration in normal tissues is in the range of 40-60 mmHg, whereas half of all solid tumors have mean values less than 10 mmHg (Brown, 1999b). Two main therapies that are used for cancer patients are ionizing radiation and chemotherapy. Response of tumor cells to both radiotherapy and chemotherapy is

dependent on the degree of hypoxia within the tumor cells (Nordsmark et al, 1996; Brizel et al, 1999; Rofstad et al, 2000). This is due to the fact that oxygen molecules react rapidly with the free-radical damage produced by ionizing radiation in DNA which leads to cell death. The effectiveness of anticancer drugs depends on two main concepts: accessibility of tumor cells through the blood vessels, and proliferating stage of cells (Brown, 2000). These two criteria do not apply to the hypoxic cancer cells, as they are located away from functioning blood vessels and they divide at very slow rate which make them resistant to therapy.

1.5. Steroids and wound healing

Clinical studies have indicated that the rate of wound healing is different in men as compared to women, suggesting that sex steroids may play a role in regulating wound healing. The mechanism of action of steroids in the process of wound healing is not clear. Postmenopausal female skin undergoes profound changes, including a decrease in dermal collagen and reduced skin thickness (Chotnopparatpattara et al, 2001). Furthermore, acute wounds in post-menopausal females heal at slower rate as compared to those who take systemic hormone replacement therapy (Ashcroft et al, 1999a).

Estrogen

Estrogens are hormones with a multitude of biological effects on different tissues such as the reproductive tract, bone, blood vessels and skin. Estrogens act through two types of nuclear receptors, estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β) (Kuiper and Gustafsson, 1997). Estrogen receptors are ligand-dependent transcriptional activators that regulate gene transcription by binding directly to the estrogen-responsive element with a consensus sequence of 5'-GGTCAnnnTGACC-3' (Shang and Brown, 2002) or by interacting with other transcription factors such as Sp1 and AP1 (Shang and Brown, 2002). Regulation of gene transcription by estrogen is accompanied by recruiting coactivators such as CBP/p300 (Chrivia et al, 1993; Kamei et al, 1996; Smith et al, 1996), SRC-1 (Onate et al, 1995; Takeshita et al, 1996), TIF2 (Voegel, et al 1996), GRIP1 (Hong et al, 1997; Hong et al, 1996), TIF1 (Thenot et al, 1997; Cavailles et al, 1995), and RIP140 (Le Douarin et al, 1995; L'Horset et al, 1996), or corepressors like SMRT (Horlein et al, 1995; Kurokawa et al, 1995) and N-CoR (Jackson et al, 1997; Chen and Evans, 1995).

Estrogen receptors (ERs) are expressed in skin fibroblasts (Bentley et al, 1986), emphasizing the ability of estrogens to regulate connective tissue synthesis. Studies on reproductive hormone levels in wound healing, have suggested that estrogen causes accelerated wound healing in females via its induction of dermal fibroblast production of TGF-β1 (Ashcroft et al, 1997). Estrogens have profound effect on the dermis where they

are important regulators of connective tissue molecules such as collagen and hyaluronic acid (Brincat, 2000; Shah and Maibach, 2001). In postmenopausal women decreased skin collagen levels are associated with reduced blood estrogen levels (Affinito et al, 1999; Brincat et al, 1987). Experimental models have shown that the naturally occurring estrogens (17β-E2 or 17α-E2) cause skin thickening in mice. Increased hyaluronan (hyaluronic acid) content in the dermis (Bentley et al, 1986; Uzuka et al, 1980) leads to water accumulation and thus thickening of skin (Uzuka et al, 1980). Hyaluronan synthesis is regulated by a family of HAS enzymes (Weigel et al, 1997), whose activity is increased by 17β-E2 (Uzuka et al, 1980). This mechanism may explain the estrogen induced increases in skin thickness seen in women (Callens et al, 1996; Maheux et al, 1994) and may explain the effect of aging on skin in menopausal women.

Tamoxifen

Tamoxifen (TAM) is a triphenylethylene compound that was first synthesized more than 30 years ago. TAM is a highly lipophilic molecule that is able to partition within the lipid bilayers of biomembranes, including cell membranes and mitochondrial membranes. Tamoxifen may initiate signaling by changing: i) the membrane fluidity, ii) redox state, iii) conformation and/or release of membrane-associated proteins. Most of the studies have indicated that tamoxifen stabilizes the membrane by decreasing its fluidity (Custodio et al, 1994; Custodio et al, 1998) which might be due to its structural

similarity of cholesterol. TAM exhibits estrogen-like and antiestrogen actions based on the target tissue being studied. TAM is an ER antagonist in breast tissue (Jordan, 1992) but an ER agonist in bone (Love et al, 1992) and uterine tissue (Kedar et al, 1994). The discrepancy observed in the activity of TAM in different tissue is believed to be related to the unidentified cofactors that bind to the antiestrogen-modulated ER (Shang and Brown, 2002). TAM is well known for its use as a single agent of choice in the treatment of hormone responsive breast cancer; although at the same time it may increase the development of uterine cancer by more than 40% (Mandlekar and Kong, 2001).

Initially, it was postulated that TAM exerts its anti-proliferative actions by inhibiting the ER-α which is a transcription factor that has a role in the regulation of growth-promoting genes such as TGF-α and epidermal growth factor receptor (EGFR). TAM is a partial agonist of ER-α. The antiestrogenic effects of TAM mediated by ER-α involve cytostatic or a cytotoxic effect leading to tumor shrinkage and regression *in vivo* (Budtz, 1999). Recent studies have indicated that TAM acts through other pathways using ER-β and through TAM metabolites. TAM is a pure antagonist of ER-β. This newly identified receptor showed that the anti-proliferative action of TAM was not solely due to interaction between TAM and ER-α. Furthermore, TAM is extensively metabolized to active metabolites: 4-hydroxy (OHT) and N-desmethyl (DMT) which possesses ER-binding affinities higher than or equal to that of TAM, respectively (Fabian et al, 1981). Interestingly, both derivatives are also able to induce apoptotic cell

death in ER-negative MDA MB 231 and BT-20 breast caner cells at micro molar concentrations (Mandlekar et al, 2000).

TGF-β is secreted by both ER-negative and ER-positive breast cancer cell lines *in vitro* and *in vivo*. The role of TGF-β in TAM-mediated signaling is complex and the signaling pathway is not well understood. *In vitro* studies have shown that TAM treatment of human breast cancer cell line MCF-7, and glioblastoma cells result in stimulation and production of TGF-β mRNA and protein respectively (Chen et al, 1996; Puchner et al, 2002). Furthermore, TAM increases the biological activity of TGF-β secreted in the cell culture media (Koli et al, 1997; Perry et al, 1995; Colletta et al, 1990; Chen et al, 1996). The apoptotic activity of TAM was inhibited by addition of an anti TGF-β antibody. TGF-β1 inhibition of ER-α expression occurs within 3 hours in MCF-7 breast cancer cells (Stoica et al, 1997) which suggest the importance of the cross-talk that exists between estrogen receptors and TGF-β signaling in breast cancer cells. Recent results show that Smad4 functions as a transcription corepressor for human ER-α, and the interaction was induced by antiestrogen ligands such as tamoxifen (Wu et al, 2003). Tamoxifen has a promising role in controlling the excess formation of collagen in skin diseases. In keloid fibroblasts, tamoxifen is able to decrease the production of TGF-β which is a key cytokines in formation of fibrotic conditions. Moreover, tamoxifen neutralizes or downregulates TGF-β2 in fibroblasts (Kuhn et al, 2002). The observed interactions indicate the potential therapeutic significance of TAM in manipulating wound healing events.

Testosterone

Testosterone secreted by the testes is the main circulating androgenic hormone in mature male mammals. In adult males, 95% of circulating testosterone is derived from testicular secretion, with the remainder arising from metabolic conversion of precursors that are predominantly secreted by the adrenal cortex, such as DHEA, DHEA sulfate, and androstenedione (Ashcroft and Mills, 2002). Although, studies have shown that acute wounds heal more slowly in males than females, and that males have an altered inflammatory response (Taylor et al, 2002), little information is available on the role and mechanism of action of testosterone on wound healing. The role of gonadal androgens in the pathogenesis of impaired wound healing may involve an increase in the inflammatory response and upregulation of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), and a delay in collagen deposition (Ashcroft and Mills, 2002). AR is expressed by a variety of cell types not only within normal skin but also in acute wounds, including epithelial cells, hair follicles, fibroblasts, and macrophages. Castration or direct AR blockade with flutamide accelerates wound healing through a dampened inflammatory response, increased matrix deposition, and downregulation of TNF-α. These effects of androgens suggest that androgens modulate multiple pathways that involve the local inflammatory response and the wound healing process.

RU486

RU486 (mifepristone) was first synthesized in 1980 and it has two important properties. First, it is a potent anti-progesterone (that is used to terminate early pregnancy). Second, it also binds with high affinity to the glucocorticoid receptor (GR) and exerts an antiglucocorticoid activity. The mechanism of action of RU486 with progesterone receptor (PR) is not fully understood. Studies have suggested that i) RU486 has higher affinity to bind to PR as compared to progesterone (PG), and this binding occurs at the site different from ligand binding domain (Vegeto et al, 1992; Benhamou et al, 1992), ii) RU486 induce a stronger dimerization of PR and tighter association of PR with DNA than hormone agonists (Demarzo et al, 1992; Skafar, 1991), iii) RU486 alter conformation in the C-carboxyl terminal tail of PR that is distinct from hormone agonist (Tetel et al, 1999), and iv) RU486 interacts with other transcription factors (Bamberger et al, 1996).

The affinity of RU486 for the glucocorticoid receptor is approximately three-fold higher than that for dexamethasone (Philibert et al, 1981). In animals, RU486 consistently inhibits the various effects induced by dexamethasone in classical experimental animal models. In glucocorticoid antagonism, RU486 appear to have at least two mechanisms of action. It binds to cytosolic GR and block translocation of GR to the nucleus. It also can act at a post-DNA binding step after binding to the receptor (Beck et al, 1993), meaning that transcriptional activation is significantly diminished in

comparison to agonist-bound receptors. Thus, it seems that RU486 inhibits both the glucocorticoid receptor activation and gene transcription phenomenon.

Since GCs levels are often increased in diabetic patients, it is possible that these higher GCs concentrations are responsible for the impaired wound healing (a major diabetic complication) in these patients. Insulin treatment or counteracting the excessive actions of GCs by using RU486 enhanced wound healing in diabetic rats (Bitar et al, 1999). In fact, treatment with RU486 resulted in healing rates comparable to those of control animals. This illuminates the therapeutic potential of RU486 in promoting wound repair under hypercortisolemic conditions including diabetes and Cushing's syndrome.

Glucocorticoids

Glucocorticoids (GCs) are produced and secreted by adrenal cortex. The glucocorticoids get their name from their effect of raising the level of blood glucose. All somatic cells express glucocorticoid receptors and these hormones are necessary for normal growth and development, liver function, immune functions, and play a key role in mediating stress responses (Barnes, 1998; Sapolsky et al, 2000). GCs have potent anti-inflammatory and immunosuppressive properties through prevention of T cell activation and by a multitude of mechanisms, including increased expression of the immunosuppressive cytokine TGF-β, and decreased production of prostaglandins and

leukotrienes. Their anti-inflammatory properties are achieved by formation of a GR-ligand complex that binds to pro-inflammatory transcription factors such as nuclear factor kappa B (NF-κB) (Dumont et al, 1998; Wiegers and Reul, 1998; Wissink et al, 1998) or activator protein-1 (AP-1). Furthermore, ligands such as dexamethasone antagonize AP-1 by altering the functional domains of Fos and Jun (Jonat et al, 1990; Yang-Yen et al, 1990; Schule et al, 1990; Heck et al, 1994), preventing gene expression by these pro-inflammatory transcription factors (McKay and Cidlowski, 1999; Webster and Cidlowski, 1999; Finkenzeller et al 1995; Cabral et al, 2001; Mckay and Cidlowski, 1998). Thus, it is not surprising that GCs inhibits wound healing by suppressing the inflammatory phase.

GR are ligand-dependent transcription factors belonging to the superfamily of steroid/thyroid/retinoic acid receptors (Pratt and Toft, 1997), and contain a number of structural motifs including a DNA binding domain, ligand binding domain and two transactivation motifs AF1 and AF2 (Kumar et al, 1999). GR regulates a large number of genes in a hormone-or ligand-dependent manner. It is predominantly cytosolic and requires association with heat shock proteins for ligand binding (Morishima et al, 2000; Dittmar and Pratt, 1997). A favored model involves binding of hormone to a cytoplasmic GR which exist as a heteromeric complex containing heat shock proteins (hsps), such as hsp90 and hsp56 (Sanchez et al, 1985; Sanchez et al, 1990). Upon hormone binding, the GR dissociates from the hsps (Marchetti et al, 2000) and the hormone-bound GR translocates to the nucleus (Sanchez et al, 1987). In the nucleus, the

hormone-receptor complex binds to specific DNA sequences called glucocorticoid response elements (GREs) in the promoter region of target genes (Picard et al, 1990; Scherrer et al, 1993). Ligand-GR complexes are also capable of activating transcription by synergizing with other regulatory factors leading to chromatin remodeling and basal transcription machinery assembly and the initiation of transcription (Refojo et al, 2001). Depending on the nature of the glucocorticoid response element (GRE), GR binding can result in activation or repression of genes containing GR-binding sites.

GCs can also act through GRE-independent mechanisms such as protein-protein interactions of GR with other sequence-specific DNA binding factors or coactivators. This takes place at the promoter responsive elements of these factors (Refojo et al, 2001). Proteins such as CBP/p300, SRC-1, GR interacting protein-1 in mouse, transcriptional intermediary factor 2 in humans (GRIP1/TIF2), RAP-46 and signal transducer and activator of transcription-5 (STAT) have GR coactivatory activity (Dumont et al, 1998).

Ligands have no effect on the GR-DNA binding affinity and cooperativity; however, they have distinct and measurable allosteric effects on GR-DNA dissociation. Ligand binding may promote the entry of other DNA remodeling factors that prepare the site for transcription by RNA polymerase (Pandit et al, 2002). An antagonist like RU486, slows down the dissociation of GR from DNA, which may prevent the access of DNA remodeling factors to the site of transcription. It has been shown that other components present in the cell induce the effects on GR-DNA dissociation in a ligand-

dependent manner. For example, the molecular chaperone protein p23 forms a ternary complex with hormone-bound GR and chromatin DNA and enhances disassembly of the transcriptional complex *in vivo* in an ATP-dependent manner (Hutchison et al, 1995).

The sensitivity of cells to glucocorticoids has been shown to be dependent on the number of GRs found in the cell (Hoeck et al, 1989; Silva et al, 1994). GR expression is regulated at multiple levels including mRNA and protein. Upon ligand binding the GR undergoes a process of homologous down-regulation (Burnstein, 1990; Bellingham et al, 1992). Glucocorticoid treatment decreases GR mRNA levels by 50-80% in many different tissues (Burnstein et al, 1994; Dong et al, 1988) and leads to a subsequent decrease in the ability of the receptor to bind hormone.

TGF-β and glucocorticoid signaling pathways interact both positively and negatively in regulating a variety of physiological and pathological process. TGF-β and GCs can induce growth arrest in human monocytoid leukemia, prostate cancer and osteosarcoma cells (Kanatani et al, 1996; Reyes-Moreno et al, 1995; Boulanger et al, 1995). On the other hand, glucocorticoids and TGF-β antagonistically regulate bone formation (Chang et al, 1998) and tight junction activity (Woo et al, 1996). Furthermore, GCs inhibit the TGF-β induced expression of extracellular matrix proteins including fibronectin (Guller et al, 1995), collagen (Slavin et al, 1994), and proteinase inhibitors, including certain inhibitors of metalloproteinases (Su et al, 1996). Hence, GCs and TGF-β may be considered as important regulators in the homeostatic control of cell

growth, wound healing and fibrosis. The molecular mechanisms involved in the cross-talk between TGF-β and glucocorticoid signaling pathways remain to be established.

Dexamethasone enhances betaglycan gene expression and increases the abundance of betaglycan message at least in part via transcriptional events in osteoblast-like cells (Nakayama et al, 1994). Moreover, dexamethasone stimulation up-regulates betaglycan at both the transcriptional and translational level in hepatic stellate cells and at the transcriptional level in myofibroblasts in a time-and dose-dependent manner while no changes were detected in expression of RI and RII mRNA in these cell types (Wickert et al, 2004). On the other hand, reduction of RII level and a stimulation of the RI mRNA are reported in normal and wounded skin of mice treated with dexamethasone (Frank et al, 1996). Dexamethasone suppresses core binding factor a1 (CBFa1) expression and indirectly represses RI expression in matrix producing bone cells (Ji et al, 1997; Chang 1998). Dexamethasone is able to inhibit the increased expression of RI and RII mRNA levels by FSH in hamster ovarian cells (Roy and Kole, 1995) and the mechanism(s) may involve the reduction in the level of cellular nuclear factor Y (NF-YA) (Roy et al, 2003), a transcription factor that upregulates RI/RII promoter activity in many cell types (Park et al, 2002). These observations further indicate the contextual and cell dependent regulation of TGF-β signaling pathways.

One of the first cellular responses to TGF-β is the rapid transcriptional activation of a set of specific genes, some of which are genes encoding transcription factors such as AP-1 and NF-κB (Takeshita et al, 1998; Han et al, 1998). Besides having a role in

inflammation, AP-1 and NF-κB also play a critical role in the regulation of cell growth, and immunity (Yang-Yen et al, 1990; McKay and Cidlowski, 1998). The inhibition of GR-mediated gene expression by TGF-β is associated with increased c-Jun and c-Fos binding to AP-1 sites. TGF-β transactivates c-Fos gene expression through the serum responsive element (SRE) (Osaki et al, 1999). It has recently been demonstrated that Smad3 could transactivate AP-1 sites by direct binding to DNA, and that this transactivation is enhanced by the direct interaction of Smad3 with Smad4, c-Fos and c-Jun (Zhang et al, 1998). These findings suggest that dexamethasone exerts its negative effect by inhibiting ERK and/or Smad signaling cascades of TGF-β. Dexamethasone selectively inhibits TGF-β-induced MEK-ERK pathway but not Smad pathway in cultured rat articular chondrocytes (Miyazaki et al, 2000). Furthermore, GR inhibits Smad3 and Smad4 activation function in a ligand-dependent manner. GR both functionally and physically interacts with the C-terminal activated domain of Smad3. The repression of Smad4 might be due to the interactions that exist between Smad4 and Smad3. Thus, there is extensive cross-talk between TGF-β and GCs signaling pathways.

1.6. Rationale of experiments in the thesis

Wound healing is a complex programmed biological process that involves cell proliferation, differentiation and extracellular matrix deposition. The chronic non-healing skin wound that is observed in elderly, burn patients, diabetic ulcers, venous

ulcers and pressure sores is an important medical issue. Thus, there is a need to develop agents that promote wound healing. Factors that have profound effects on wound healing are TGF-β, hypoxia and steroids. TGF-β is involved in all phases of wound healing. Several animal models have demonstrated the efficacy of exogenous TGF-β in enhancing wound healing with TGF-β increasing ECM synthesis and the strength of the wound. However, overstimulation can cause scarring. Decreasing TGF-β1 activity with neutralizing antibodies, antisense oligonucleotides or Smad7 reduces scarring as does local administration of TGF-β3. Thus, inappropriate control of the expression and function of TGF-β isoforms locally may contribute to impaired wound healing and hypertrophic scarring. Tissue ischemia further impairs cutaneous wound healing. Initially, hypoxia increases angiogenesis and collagen synthesis by fibroblasts (Falanga et al, 1993; Malonne et al, 1999; Han et al, 2003). Hypoxia has been demonstrated to induce TGF-β expression and to increase TGF-β responsiveness in cultured skin fibroblasts. However, the specific mechanism of action of hypoxia on TGF-β signaling pathway and wound healing at the molecular level is not clearly understood. Another important factor that has an enormous impact on wound healing is steroids. For example, estrogen replacement therapy in menopausal women prevents loss of skin thickness and accelerates wound healing while glucocorticoids impair wound healing. The effects of steroids may be mediated by the modulation of TGF-β action. TGF-β and its receptors are differentially regulated by glucocorticoids during wound healing in mice.

The regulation of TGF-β action most likely occurs at the levels of its bioavailability, its receptors and components of its signaling pathway. Sex steroids and glucocorticoids have been shown to have a strong effect in the regulation of TGF-β isoforms, and its receptors. Considering the important role of hypoxia, steroids, and TGF-β in wound healing, our understanding of dynamic interactions that exist among TGF-β signaling pathway, oxygen tension and steroids at the molecular level is limited. We hypothesize that steroids and oxygen tension may regulate TGF-β bioavailability and TGF-β receptors and downstream TGF-β signaling pathway (Smad2 and Smad3) in skin cells. To explore this, we have compared elements of the TGF-β signaling pathway at normoxic condition treated with steroids such as (estrogen, tamoxifen, dexamethasone, RU486, and testosterone), or normoxic condition was compared to hypoxia, and reoxygenated condition with or without the treatment of steroids at different time.

Our objective is to identify the effects of oxygen tension and steroids on TGF-β ligand, receptor, and Smad2 and Smad3 in skin cells. To do so, we chose skin fibroblasts and keratinocytes as experimental models. Fibroblasts and keratinocytes have important functional roles in healing of wounds. Fibroblasts invade the wound in the first few days of healing, and have multiple functions important to wound repair, such as collagen synthesis, extracellular matrix reorganization, and wound contraction (Lerman et al, 2003). Furthermore, fibroblasts secret growth factors important for cell-cell communication during the repair process (Takehara, 2000; Mansbridge et al, 1999).

After skin wounding, keratinocytes migrate rapidly to the wound site in order to close the wound, and proliferate strongly during the first and second phase of epidermal reepithelialization. Thus, any impediments to fibroblast, or keratinocytes function could prevent normal wound healing and result in chronic, non-healing wounds.

Our research is aimed at exploring the effect of hypoxia and steroids on TGF-β signaling pathway at the molecular level using skin cells as experimental models. We hope that our findings will lead to the development of strategies for manipulating TGF-β action using regulatory factors to promote wound healing and reduce scarring.

Chapter 2: Materials and Methods

2.1. Pig skin flap

2.1.1. Surgical preparation and experimental design

Six female White Landrace pigs (10–14 week old) were housed in a temperature-controlled (20–22°C) animal holding room. All the pigs were offered the same commercial pig diet and tap water ad libitum. Food was withheld the evening before surgery. Animals were sedated with intramuscular injections of ketamine (20 mg/kg), xylazine (2 mg/kg), and atropine (1 mg). Animals were anesthetized with an intravenous injection of pentobarbital sodium (6 mg/kg). They were then intubated, and general anesthesia was maintained with spontaneous inhalation of oxygen (8 L/min) and halothane (0.5–1.0%). The protocol for use of pigs in this experiment was approved by the McGill University Animal Care Committee.

In each pig, bilateral buttock skin flaps (10 - 18 cm) based on the vascular pedicle of the superficial circumflex artery and its accompanying vena comitans as well as the lateral femoral cutaneous nerve were elevated and later returned and sutured to their beds with 3-0 nylon skin sutures (Figure 6). The procedure used was described previously by Kerrigan et al. (Kerrigan et al, 1986). Briefly, the flap on one side was randomly assigned to 1 hour of arterial occlusion with the flap on the contra lateral side acting as a nonischemic control. Arterial ischemia in these island flaps was created by clamp application on the artery, which mimics the clinical scenario of an ischemic free flap. Complete occlusion of the vascular pedicle was achieved by application of an Acland V2 microvascular clamp to the branch of the circumflex iliac artery supplying

Figure 6. *Experimental pig model.*

In each pig, bilateral buttock skin flaps (10 x 18 cm) based on the vascular pedicle of the superficial circumflex artery and its accompanying vena comitantes as well as the lateral femoral cutaneous nerve were elevated, and later returned and sutured to their beds using 3-0 nylon skin sutures. The flap on one side was randomly assigned to 1 hour of arterial occlusion with the flap on the contra lateral side acting as a non-ischemic control. Arterial ischemia was created by clamp application on the artery. Complete occlusion of the vascular pedicle was achieved by application of an Acland V2 micro vascular clamps to the branch of the circumflex iliac artery supplying the buttock flap, and was verified by application of 10% sodium fluorescein dye (15 mg/Kg). Absence of fluorescein in the skin 15 minutes after dye injection indicated complete occlusion of the vascular pedicle. After one hour of ischemia, the micro vascular clamps were removed to allow reflow. Skin biopsies (4 x 8 mm pieces from the central portion in the proximal third of the flap) were taken at 1 hour after the induction of ischemia and at 1 hour after reflow from the experimental flaps (ischemic), and at corresponding times from the control flaps (non-ischemic) and unoperated buttock skin.

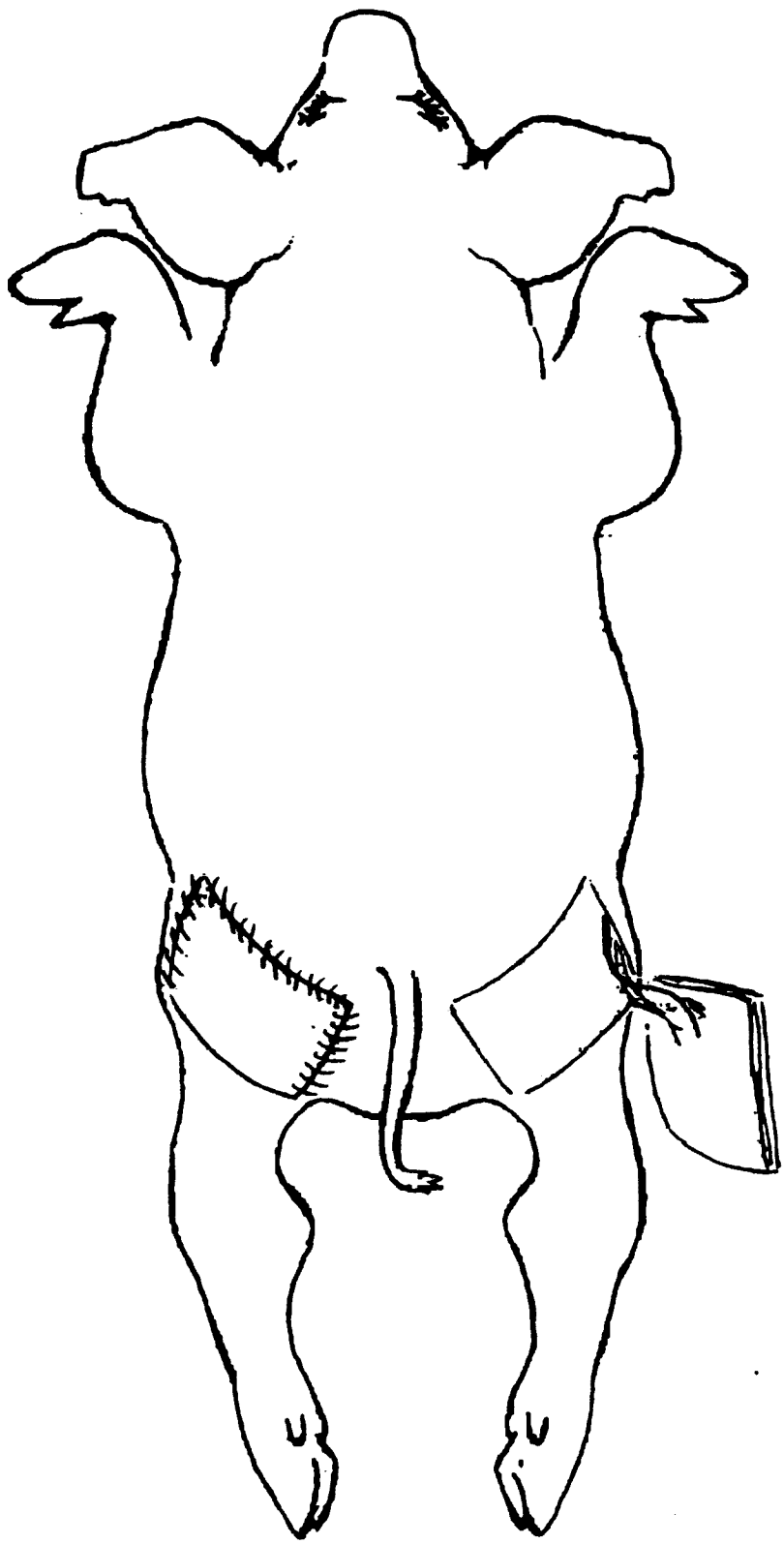


Figure. 6

the buttock flap and was verified by application of 10% sodium fluorescein dye (15 mg/kg). Absence of fluorescein in the skin 15 min after dye injection indicated complete occlusion of the vascular pedicle. After 1 hour of ischemia, the microvascular clamps were removed to allow reflow. From each pig, skin biopsies (4 – 8 mm pieces from the central portion in the proximal third of the flap) were taken at 1 hour after the induction of ischemia and at 1 hour after reflow from the experimental flap (ischemic) and at corresponding times from the control flap (nonischemic) and unoperated buttock skin. This experiment was repeated in all six pigs. Thus each group (unoperated skin, nonischemic control flap, ischemic flap, and reperfusion flap) represents 6 animals or 12 flaps. In a similar manner, an additional pair of bilateral skin flaps (thoracic area) was created on each of the six pigs (i.e., 12 additional flaps). Global arterial ischemia in these flaps was achieved by clamping the thoracodorsal artery. Biopsies were collected after 4 hours of ischemia and at 4 hours after reflow. No necrosis was observed in the flaps during the ischemia-reperfusion time period that we tested.

2.1.2. Preparation of pig skin tissue sections

Biopsies collected from the skin flaps and unoperated skins were fixed in 4% paraformaldehyde for 8 hours followed by immersion in 15% sucrose for 30 hours at 4°C. They were then embedded in Tissue Tek and frozen in liquid nitrogen. Serial sections of the frozen tissue were prepared with a cryostat. Each slide contained tissue sections (in duplicates or triplicates) from all four groups: 1) unoperated skin, 2)

nonischemic control flap, 3) ischemic flap, and 4) reperfusion flap. A minimum of 15 slides each were analyzed for type I, II, and III receptors and TGF-β1 from each group.

2.1.3. Antibodies used

Expression of type I, II, and III TGF-β receptors and TGF-β1 in tissue sections was detected by immunohistochemistry with their respective specific anti-peptide antibodies. The anti-type I and anti-type II receptor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were both rabbit polyclonal IgGs. The anti-type I antibody recognizes amino acids 158–179 of the precursor form of the type I TGF-β receptor (ALK5) of human origin, whereas the anti-type II antibody recognizes amino acids 550–565 of the precursor form of the human type II TGF-β receptor. The anti-type III antibody and the anti-TGF-β1 antibody were kind gifts from Dr. M. O'Connor-McCourt (Biotechnology Re-search Institute, Montreal, PQ, Canada). The peptide sequences used and the procedure employed for the preparation of the type III receptor antibody were exactly the same as described by Moustakas et al. (Moustakas et al, 1993). Briefly, polyclonal rabbit antisera were raised against a COOH-terminal epitope of human type III receptor, the immunoglobulins were prepared, and their ability to specifically immunoprecipitate the receptor was tested. In comparison studies, this antibody displayed the same specificity as the antibodies obtained from Dr. A. Moustakas (Whitehead Institute for Medical Research, Cambridge, MA). The procedure

for the preparation of anti-TGF-β1 antibody involved coupling of TGF-β1 to keyhole limpet hemocyanin and injection of rabbits and was described previously (Moulin et al, 1997). Normal rabbit IgG used as a negative control was obtained from Lipshaw Immunon (Pittsburgh, PA). The immunizing peptides or protein (same as used for the preparation of antibodies) that were used in control experiments to show the specificity of antibodies during the immunohistochemistry procedure were obtained from Santa Cruz Biotechnology (type I and II peptide), synthesized locally at Sheldon Biotechnology Institute (Montreal, PQ, Canada; type III peptide), or bought from R&D Systems (Minneapolis, MN; TGF-β1).

2.1.4. Immunohistochemistry

Immunohistochemical localization of type I, II, and III TGF-β receptors and TGF-β1 ligand was performed on 8-μm-thick cryostat sections of skin tissue placed onto gelatin-coated glass slides. The sections were washed and permeabilized three times for 15 min each with phosphate-buffered saline pH 7.5 (PBS) containing 0.1% Triton X-100. Endogenous peroxidase activity was then quenched by treating the sections with 1% H_2O_2 in 99% methanol for 45 min at room temperature. The sections were incubated in a humidified chamber for 3 hours with blocking solution (PBS containing 1% normal goat serum, 0.3% Triton X-100, and 0.5% BSA) to block excess proteins and prevent nonspecific antibody binding. The primary antibodies diluted in the blocking solution

were applied to the sections over-night at 4°C in a humidified chamber. The anti-type I and anti-type II antibodies were diluted 1:100 to a final concentration of 2 μg/ml, whereas the anti-type III antibody and anti-TGF-β1 antibody were diluted 1:400 and 1:250, respectively. The next day, slides were washed two times with PBS containing 0.1% Triton X-100 and once with PBS alone. The slides were incubated with biotinylated goat anti-rabbit secondary antibody (diluted to 0.5% in PBS containing 0.5% BSA and 1.5% normal goat serum) for 1 hour. The sections were washed two times with PBS containing 0.1% Triton X-100 and once with PBS alone. This was followed by incubation with avidin-biotin complex (ABC; Vector Laboratories) diluted in PBS for 1 hour. The slides were again washed two times with PBS containing 0.1% Triton X-100 and once with PBS alone. The brown color, indicating immunoreactivity, was developed with 0.05% of 3,3′-diaminobenzidine tetrahydro-chloride (Sigma, St. Louis, MO) in 0.1% H_2O_2 in PBS for 3 min. Sections were rinsed in distilled water. Finally, the slides were dehydrated and mounted with permount (Sigma).

A corresponding set of slides was counterstained with Gill's hematoxylin before mounting. All tissue sections that were compared were treated at the same time and for the same length of time. Normal rabbit IgG (Lipshaw Immunon) at the same concentration as the primary antibody was used as a negative control for the immunostaining protocol. Additional procedural control involved incubation without the primary antibody, with the rest of the protocol unchanged. Antibody specificity was proven by absorbing each antibody with a 50 X excess of immunizing peptide or protein

(see Antibodies used) to the antibody. Briefly, the peptide or protein was incubated with the primary antibody overnight at 4°C, centrifuged, and applied to the tissue samples instead of the unabsorbed antibody. Positive staining on tissue sections was not observed under any of the above circumstances.

2.1.5. Evaluation of staining and statistical analysis

The results of immunohistochemistry studies were assessed in a blinded fashion by three separate investigators. The evaluation of positively staining skin structures (cell types) was performed semiquantitatively on an arbitrary scale ranging from 0 to 4 for each structure: 0, negative reaction; 1, positive reaction in a few cells; 2, reaction in a moderate number of cells; 3, reaction in a large number of cells; 4, reaction in almost all cells. The Kruskal-Wallis test was used to analyze the differences between groups (unoperated buttock skin, nonischemic control flap, ischemic flap, and reperfusion flap) in the expression of type I, II, and III TGF-β receptors and TGF-β1. Differences with a P value of < 0.05 was considered significant (2-sided test). Analysis of the data using a second non-parametric test, Monte Carlo estimates for the exact test, gave results similar to those obtained by the Kruskal-Wallis test. SAS version 8.0 statistical software (SAS Institute, 1999) was used for computation and analysis.

2.2. Cell Culture

2.2.1. Human fibroblasts

Dermal fibroblasts were isolated from fresh biopsy specimens obtained from the skin tissue of healthy volunteers at breast reduction surgery after informed consent. A standard explant method was used to establish the primary fibroblast cultures from the operative specimens. This explant method was performed using sterile technique under a laminar flow hood. The specimens were collected and underwent antimicrobial treatment with Dulbecco's Minimal Essential Medium (D-MEM) with 5% penicillin/streptomycin/amphotericin for several times. The tissue was minced into small fragments measuring approximately 0.3 mm^3 thickness in a petri dish with a sterile scalpel blade. The explants were distributed into T-25cm^2 tissue culture flasks and cultured in D-MEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate and 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (Gibco, Burlington, Ontario, Canada). The cultures were maintained in a controlled humidified incubator containing a 5% CO2/air at 37°C.

The explant-containing flasks were examined daily under light microscopy for outgrowth of fibroblasts from the tissue fragments. Culture medium was changed every three days until light microscopy demonstrated such fibroblasts outgrowth. After three weeks, the fibroblast outgrowth approached confluence, and the tissue fragments were removed. Fibroblast cells were then trypsinized (0.05% Trypsin-EDTA, Gibco) and

subcultured were grown in T-75cm^2 flasks. Subsequent subculturing was performed every four days. Keratinocytes and other contaminating cells were removed in the first 2-3 subculturings. The purity and homogeneity of the early passage fibroblasts were determined by immunofluorescence microscopic studies using a mouse monoclonal antibody to vimentin (Sigma, St Louis, Missouri) and a rhodamine conjugated goat anti-mouse IgG (Jackson, Laboratories, Westgrove, PA.) was used as the secondary antibody. The immunofluorescence data indicated that more than 99% of the fibroblast population is positive for vimentin, a marker specific for stromal cells. Cells from the fourth to tenth passages were used for experiments.

2.2.2. Human keratinocytes

An immortalized human keratinocyte cell line, HaCat, was obtained from Dr. P. Boukamp (Hedielberg, Germany). HaCat is a spontaneously immortalized cell line which displays no major differences in differentiation as compared to normal keratinocytes. It possesses a transformed phenotype, with no tumourigenic activity (Boukamp et al, 1988). HaCat cells were cultured in D-MEM containing 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine and 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (all Gibco, Burlington, Ontario).

2.3. Treatment of Cells

2.3.1. Hypoxic conditions

Skin cells (fibroblasts or keratinocytes) grown as monolayers in 150 mm petri dishes (Starsted) to 70% confluence were exposed to hypoxia for the various time periods using a Gas Pak system (Figure 7) (BBL Gas Pak Plus system; Becton-Dickinson, Lincoln Park, NJ) at 37°C, as described by Detmar et al. (1997). Before exposure to hypoxia, skin cells (Fibroblasts or keratinocytes) were cultured for 24 hours in a medium that was supplemented with 10% charcoal treated FBS. The medium was changed again 3 hours before exposure to hypoxia. Briefly, the two basic components of the Gas Pak anaerobic system are the Gas Pak hydrogen and carbon dioxide generator envelope and a room temperature palladium catalyst. The Gas Pak envelopes and catalyst were placed within a durable polycarbonate jar designed with a special lid and clams, to assure adequate and safe gaseous exchange reactions within the jar. Hydrogen is generated from a sodium borohydride tablet following the addition of water, combines in the presence of the palladium catalyst with the oxygen in the jar to form water. In our experiments oxygen concentration was maintained at 0.2% during hypoxia. This was verified by using a colored paper indicator placed inside the jar. Control cultures consisted of monolayers of fibroblasts at 70% confluence maintained under normoxic conditions (5% CO_2-95% air) at 37°C. For control, cultures were incubated under normoxic conditions for the same length of time. reoxygenated condition (reexposure to

normoxia) was achieved by taking the petri dishes out of hypoxic chamber and incubating them at normoxia in the incubator for 2 hours. At different time points of experiment, the condition medium was collected, and cells were lysed for RNA or protein extraction and were stored at -80C.

2.3.2. Steroids stimulation

Before steroids treatment, skin cells (Fibroblasts or keratinocytes) were cultured for 24 hours in a medium that was supplemented with 10% charcoal treated FBS. The medium was changed again (no FBS) 3 hours before stimulation with steroids. Skin cells were then treated with final concentration of 100 nM of steroids in the medium (no FBS). A control with the corresponding vehicle (<1% ethanol) concentration was carried along. At different time points, the conditioned medium was collected, and cells were lysed for RNA or protein extraction and were stored at -80C.

2.4. Northern blot analysis

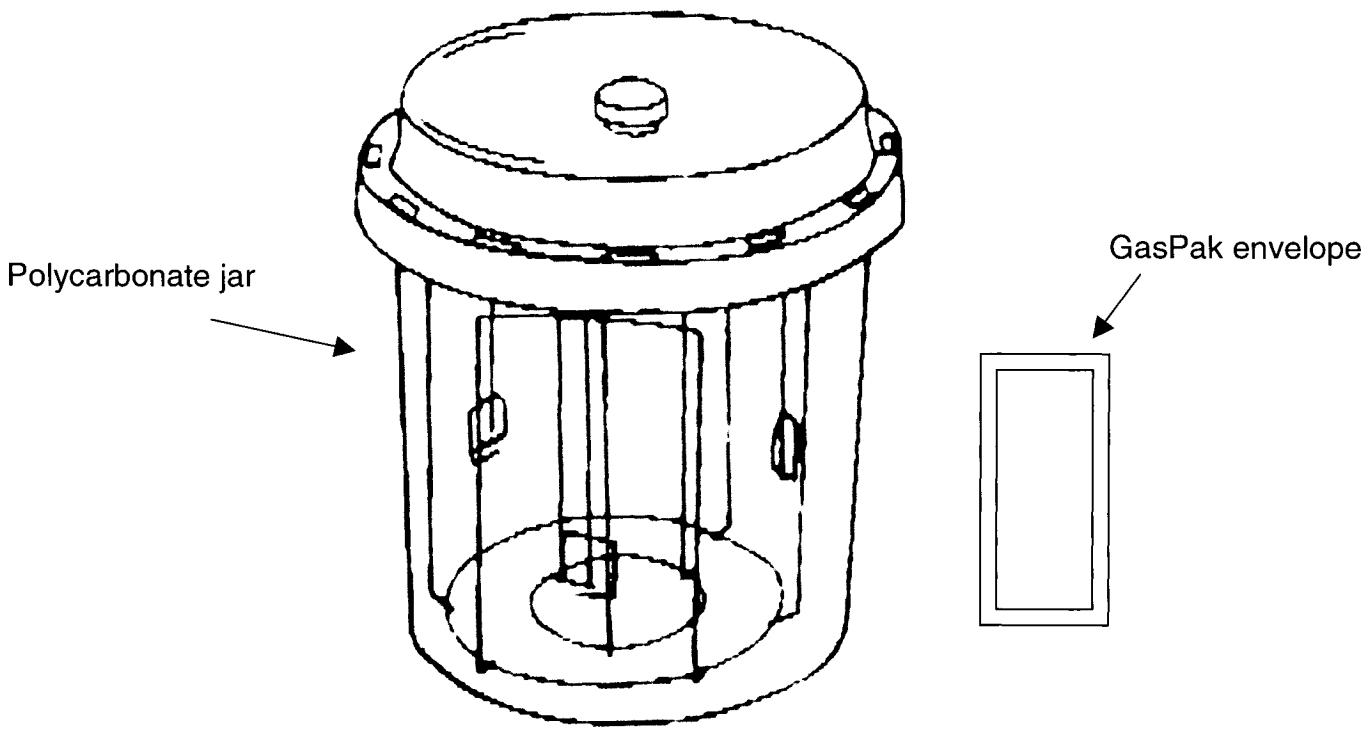
2.4.1. Isolation of RNA using GTC

Total RNA from skin fibroblasts cultured under normoxic and hypoxic conditions was isolated by homogenization in 4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5 M *N*-lauroylsarcosine and 0.1 M mercaptoethanol (Sigma) as described previously (Chomczynski and Sacchi, 1987).

Figure 7. *Anaerobic System.*

Anaerobic system was generated by using GasPak hydrogen and carbon dioxide generator envelope and a palladium catalyst. The GasPak envelopes and catalyst are placed within a large jar that includes a two piece lid for positive locking and sealing. The lid serves as a carrier for the catalyst reaction chambers and has a recessed crown to permit stacking of jars if desired. The polycarbonate lid consists of an outer lid and thumbscrew, and an inner lid with an O-ring gasket and three double-screened reaction chambers.

Anaerobic Systems



Inner Lid and O-ring Gasket

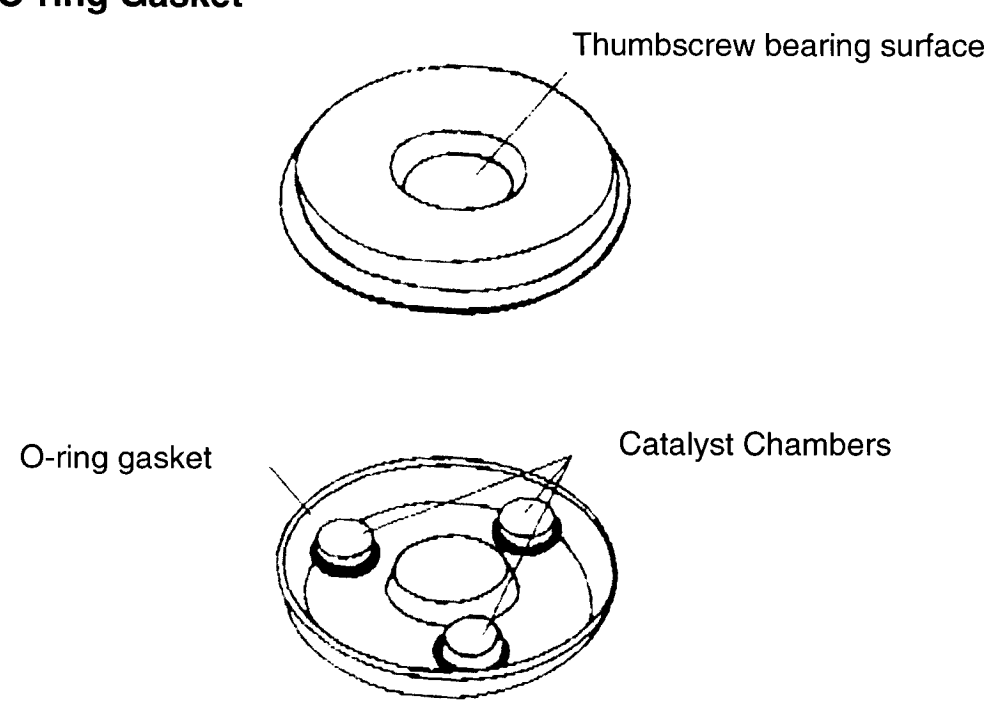


Figure. 7

2.4.2. Isolation of RNA using TRIZOL reagent

Trizol reagent is a mono-phasic solution of phenol and guanidine isothiocyanate, and is an improvement of the single-step RNA isolation method (Chomczynski and Sacchi, 1987). Briefly, cells were lysed directly in a culture dish by adding 2 ml of TRIZOL reagent to a 10 cm diameter dish, and passing the cells lysate several times through a pipette. The Homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.4 ml of chloroform was added to the TRIZOL reagent. Tubes were shaken for 20 seconds and incubated for 4 minutes at room temperature. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase containing RNA was transferred to a fresh tube, and RNA was precipitated using 1 ml of isopropyl alcohol. After 10 minutes of incubation, the samples were centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and RNA was washed with 75% ethanol, and centrifuged at 7,500 x g for 5 minutes at 4°C. The pellet was dried and dissolved with RNase-free water.

2.4.3. Preparation of membrane

RNA (20 μg/well) was electrophoresed on a 1% agarose formaldehyde gel, transferred to nylon membrane (Boehringer Mannheim), and ultraviolet cross-linked.

2.4.4. Preparing a DIG RNA probe

DIG RNA probes were made using DIG RNA Labeling Kit from Boehringer Mannheim. This kit generates DIG-labeled, single-stranded RNA probes of defined length by transcription using SP6, or T7 RNA polymerase. In a standard RNA labeling reaction approximately 10 µg newly synthesized DIG-RNA probe is transcribed from 1 µg DNA template. RNA probe yield was estimated using a spot test with a DIG-labeled control was used. Serial dilutions were made from starting concentration of 20 ng/µl to 0.1 pg/µl. 1 µl of the diluted controls was spotted on a piece of nylon membrane. In a second row, 1 µl of the corresponding dilutions of the experimental probe was spotted. The nucleic acids were fixed to the membrane by cross-linking with UV-light. The membrane was washed briefly in washing buffer. The membrane was incubated in blocking solution for 30 minutes at room temperature. Anti-DIG-alkaline phosphatase was diluted 1:5000 in blocking solution. The membrane was incubated in the diluted antibody solution for 30 minutes at room temperature. The membrane was washed twice, 15 minutes per wash in washing buffer at room temperature. The membrane was incubated in detection buffer for 2 minutes 45 µl of NBT and 35 µl of BCIP was mixed in 10 ml of detection buffer. The color substrate was added to the membrane. The reaction was stopped by washing the membrane with TE buffer for 5 minutes. The spot intensity of control was compared with experimental dilution to estimate the concentration of the experimental probe.

2.4.5. Hybridization using a DIG probe

After electrophoresis, the formaldehyde gel was equilibrated in 20 x SSC (DEPC-treated) for 2 x 15 min. The RNA was transferred to Boehringer Mannheim Nylon Membranes, from the gel using capillary transfer overnight at 4°C with 10 x SSC (DEPC). The next day, membrane was UV-cross linked. The blot was prehybridized with 15 ml of prehybridization solution (5 x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent from the 10% blocking reagent stock solution provided by Boehringer Mannheim) in a hybridization bag for 1 hour at 68°C. The DIG-labeled probe was heat-denatured using boiling water for 10 minutes, and diluted in prehybridization solution to a final concentration of 50-100 ng/ml. The membranes were hybridized overnight at 68°C. The next day, the membrane was washed twice for 15 minutes per wash in 2 x wash solution (2 x SSC, containing 0.1% SDS) at room temperature, followed by additional washes for 15 minutes in 0.5 x wash solution (0.5 x SSC, containing 0.1% SDS).

2.4.6. Detection

The membrane was equilibrated in washing buffer (dilution of 1/10 of: 100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20° C); 0.3% (v/v) Tween® 20) for 1 minute, and then incubated in blocking solution for 45 minutes. The membrane was incubated for 30 minutes in the antibody solution containing anti-Digoxigenin-AP diluted 1:10000 in

blocking solution. The membrane was washed twice for 15 minutes in washing buffer. The membrane was equilibrated in detection buffer for two minutes. Chemiluminescent alkaline phosphatase substrate (CSPD) was diluted 1:100 in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5 (+20° C)) and was added on top of the membrane for 5 minutes. The membrane was sealed in a plastic bag and exposed to X-ray film.

2.4.7. Hybridization using a ^{32}P labeled probe

Membranes were prehybridized overnight at 42°C and transferred to fresh hybridization solution (5 x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent from the 10% blocking reagent stock solution provided by Boehringer Mannheim) containing ^{32}P-labeled probe. The type II TGF-β receptor probe was a 474-bp (521–995) fragment labeled with [^{32}P] dCTP with nick translation (GIBCO-BRL). After hybridization overnight, the membrane was washed twice, and blot was exposed to Kodak X-ray film with an intensifying screen for about 96 hours at -80°C. Equivalent RNA loading and transfer were determined by subsequent studies using a probe for 18S rRNA that was radiolabeled as above. Scanning densitometry was performed to quantify relative mRNA abundance.

2.5. RNase protection assay

Total RNA was prepared from treated and control human primary skin fibroblasts using TRIzol (Gibco) and quantified using spectrophotometer. Ribonuclease protection analysis was performed using the RiboQuant Multi-Probe RNase protection assay system (PharMingen, San Diego, CA). In this assay, high-specific-activity RNA probes are synthesized from a mixture of DNA templates of distinct lengths that correspond to a sequence in a distinct messenger RNA (mRNA) species. Template sets hCR4 (PharMingen) were used: This set contains templates for RI, RII, and the ubiquitously expressed genes for ribosomal protein L32 and GAPDH. T7 polymerase-directed antisense RNA probe synthesis, hybridization to total RNA, RNase treatment, and gel resolution of protected probes were done as described by the manufacturer. Briefly, probe was synthesized by labeling the template with [$^{\alpha-32}$P] UTP (Amersham) using T7 RNA polymerase. A total of 6 x 10^{5} cpm of labeled probe in hybridization solutions was added to 1 μg total RNA, and the samples were overlaid with mineral oil before brief heating to 90°C; hybridization continued for 16 hours at 56°C. After hybridization, the samples were digested for 45 minutes at 30°C with a mixture of RNase A and RNase T1 (as recommended in the kit), treated with proteinase k, extracted with phenol/chloroform/isoamyl alcohol (50:50:50), precipitated wit ethanol and resolved on 5% denaturing polyacrylamide sequencing gels. Radioactive fragments were detected by autoradiography using Kodak X-ray film. To control for differences in

sample processing, hybridization signals in each sample were divided by the signal for L32 which does not change with exposure to hypoxia. The cytokine/L32 signal ratio from hypoxic skin fibroblast cells was divided by the cytokine/L32 ratio obtained from normoxic fibroblast cells to determine fold increases in mRNA levels in hypoxic human primary fibroblast cells compared with control.

2.6. Iodination of TGF-β

Iodination of TGF-β1 was performed by the chrolamine T method as previously described (Philip and O' Connor- McCourt, 1991). Briefly, 2 μg of carrier free TGF-β1, prepared in 4 mM HCl, and was diluted up to 20 μl with 1 M sodium phosphate (pH 7.4). The ligand was iodinated with 1 mCi ^{125}I-Na (Amersham-Pharmacia Biotech) by three additions of 10 μl chloramine T (10 mg/ml in 1 M sodium phosphate (pH 7.4)) at set time points of 0, 2, and 3.5 minutes. At 4.5 minutes, the reaction was halted by the addition of 20 μl tyrosine (9 mg/ml in 50 mM sodium phosphate (pH 7.4)), 200 μl of potassium iodide (10 mg/ml in 50 mM sodium phosphate, (pH 7.4)), and 200 μl of urea (1 g/ml in 1 M HCl). The radiolabeled ligand was chromatographed on a PD10 column (Amersham-Pharmacia-Biotech) and twelve 0.8 ml fractions were collected in microtubes supplemented with 0.5 ml of 4 mM HCl containing 0.1% bovine serum albumin (BSA). The radioactivity of a 5 μl aliquot from each fraction was determined using a gamma counter. The two fractions containing the highest radioactivity were

pooled. The specific activity of ^{125}I-TGF-β was 2-4 mCi/pmole: the binding activity was verified by performing receptor binding assays using cultured fibroblasts.

2.6.1. Affinity labeling of cells

Affinity cross-link labeling techniques were performed as detailed by Dumont et al (1995). Briefly, monolayers of cells were washed with ice cold binding buffer (PBS with Ca^{2+} and Mg^{2+}, pH 7.4 containing 0.1% BSA) three times over a thirty minute period. The cells were then incubated at 4°C for three hours with 200 pM of ^{125}I-TGF-β1, in the presence or absence of varying concentrations of non-radioactive TGF-β1, (Austral Biochemical). The receptor-ligand complexes were then cross-linked with 1mM Bis (Sulfocsuccinimidyl) suberate (BS3) (Pierce, Rockford Il). After 10 minutes, the reaction was stopped by the addition of 500 mM glycine and further incubated for 5 minutes. The cells were then washed twice with PBS and lysed with solubilization buffer (20 mM Tris-HCl, pH 7.4 containing 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 μM phenylmethylsulfonylfloride (PMSF), 200 μg/ml BSA, 1 μg/mL leupeptin, 10 μg/ml soyabean trypsin inhibitor, 10 μg/ml benzamide and 2 μg/ml pepstatin). The solubilized material was collected and 1/5 volume of 5X electrophoresis sample buffer (0.25 M Tris-HCl (pH 6.8), 5% SDS, 50% glycerol and trace bromophenol blue) was added. The samples were run on a 1.5 mm-thick 3%-11% gradient SDS-PAGE gel under reducing (5% β-mercaptoethanol) conditions. Results

were analyzed using autoradiography followed by quantitative densitometry. (14C) methylated molecular weight protein markers included myosin (H-chain) (200-220 kDa), phosphorylase-β (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa) (In vitrogene or Amersham-Pharmacia Biotech).

2.6.2. Regulation of TGF-β receptors by hypoxia or steroids (Dexamethasone or RU486) using affinity labeling

Cell monolayers were serum starved overnight. Cells were incubated for 24 hours at 37°C: i) at hypoxia (24 hours) and reoxygenated (2 hours) condition, or ii) with 100nM of dexamethasone or RU486. Following treatment, Confluent monolayers of cells were then subjected to a receptor binding assay using affinity cross-link labeling followed by SDS-PAGE under reducing conditions followed by autoradiography to evaluate the regulation of TGF-β receptor subtypes. Any alterations in cell number or total protein content in cultures due to treatment were monitored by counting cells using a hemocytometer or by determining protein concentrations using a Bio-Rad protein assay based on the Bradford method (Bio-Rad).

2.7. Plasminogen activator inhibitor-1/ Luciferase (PAI/L) Assay

PAI-L assay is a highly sensitive and specific, nonradioactive quantitative bioassay for TGF-β based on its ability to induce plasminogen activator inhibitor-I (PAI-I) expression. Mink lung epithelial cells (MLEC) were stably transfected with an 800 base pair fragment (799 → +71) of the 5' end of the human PAI-I gene fused to the firefly luciferase reporter gene in a p19LUC-based vector containing the neomycin-resistance gene from pMAMneo. These stably transfected MLEC cells (TMLC) were a kind gift from Dr. Rifkin (Abe et al, 1992). Addition of TGF-β (0 to > 40 pM) to the transfectants resulted in a dose-dependent increase in luciferase activity in the cell lysates. Conditioned medium was collected after treatment of skin cells with hypoxia or steroids at different time points in siliconized eppendorf tubes containing protease inhibitor cocktails (10ul phenylmethylsulfonyl fluoride (PMSF), 20 µl protease inhibitor (PI/ml of medium). In order to measure TGF-β in the medium, TGF-β1 standards, (0-40 pM) (R&D Biotechnology) were prepared in a DMEM medium with no FBS. Transfected Mink Lung Cells (TMLC) were plated at $1.6x10^5$ cells/ml in a 96 well plate in DMEM medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin (Gibco), and geniticin. Cells were allowed to attach for 3 hours at 37°C in a 5% CO_2/95% air atmosphere incubator. After 3 hours, wells were aspirated and TGF-β1 standards, or conditioned medium collected from cells that were treated with steroids or hypoxia were added 100 µl per well in triplicates. This

allowed the determination of active TGF-β in this conditioned medium. In order to analyze the total level of TGF-β (latent + active), the conditioned medium was heat treated at 70°C for 10 minutes to activate endogenous latent TGF-β. After cooling, the samples were diluted with DMEM prior to addition to TMLC. The plate was incubated overnight. The next day, wells were aspirated and washed once with cold PBS. After washing, 70 µl of 1X PharMingen Lysis Buffer (BD PharMingen) was added to each well. After 30-45 minutes incubation, the lysates (45 µl) of each well were transferred to luminometer plates along with 10 µl of ATP cocktail (ddH_2O: 4 µl; 0.1M ATP: 3 µl; 0.5M KH_2PO_4 (pH 7.8): 2 µl; 1M $MgCl_2$: 0.5 µl). Each well received 50 µl of 0.25 mM Luciferin (Roche Diagnostics) and the plate was read with a Berthold Luminometer (Tropics Inc.). Luciferase activity was reported as relative light units (RLU). The concentration of TGF-β1 in the samples read from the standard curve, taking into consideration the appropriate dilution factors.

2.7.1. Specificity of PAI-L Assay

A pan TGF-β neutralizing antibody (ID11, Genzyme), that recognizes all three TGF-β isoforms (TGF-β1, -β2, and -β3) was used to assess the specificity of the induction of luciferase activity by determining its efficacy in neutralizing the TGF-β isoforms in the conditioned medium. For cells treated with steroids, antibody was added to the cells prior the addition of steroids.

2.8. Western blotting

Fibroblast cells were grown in 60 mm dishes (Falcon) to 70-80% confluency and serum starved overnight. Cells were washed with PBS and were left untreated or treated with TGF-β1 (50 pM), dexamethasone (100 nM), or RU486 (100nM) for the indicated times at 37°C in normoxic or hypoxic conditions. The cells were solubilized with 500 µl of lysis buffer (50mM Tris (pH 7.5), 150mM NaCl, 50mM NaF, 50mM β-glycerophosphate, 1mM sodium orthovanadate, 1mM DTT, 5mM EDTA (pH 8.0), 1% NP-40, 10% glycerol, 10 µM PMSF, 200 µg/ml BSA, 1 µg/mL leupeptin, 10 µg/ml soya bean trypsin inhibitor, 10 µg/ml benzamide and 2 µg/ml pepstatin) for at least 30 minutes at 4°C with mild agitation. The protein concentration of the each sample was determined using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad). An equal amount of protein (25 µg), with 1/5 volume of 5X electrophoresis reducing sample buffer, and 5% mercaptoethanol were boiled for 5 minutes. Total cell extracts were then separated on a 7.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked for at least three hours to overnight in blocking buffer (TBS-T buffer: 30 mM Tris (pH 7.5), 150 mM NaCl, 0.05% w/v Tween 20, containing 5% w/v of skim milk powder). The blot was then incubated for 60 minutes at room temperature with the specific antibody that recognize phosphorylated Smad2 and Smad3 (Santa Cruz), or an antibody that recognize both phosphorylated and unphosphorylated (total) Smad2 and Smad3 (Santa Cruz), or Stat3 (Santa Cruz). The blot was then washed in washing buffer (30 mM Tris (pH 7.5), 150 mM NaCl, 0.05% w/v Tween 20) and

incubated for 45 minutes at room temperature with a secondary anti-rabbit, or anti-goat antibody conjugated to horseradish peroxidase (HRP) (1:5000) (Pierce). The signals in the membrane were then developed using the ECL chemiluminescence analysis (Amersham-Pharmacia-Biotech) and Kodak X-OMAT film (Kodak). In order to determine equal protein loading, membranes were immunoblotted with a rabbit polyclonal anti-Stat3 antibody (Santa Cruz).

Chapter 3: The Effect of Ischemia-Reperfusion Injury on TGF-β Receptors in Skin Cells

3.1. Rationale

Ischemic insult, an inevitable consequence of vascular injury, occlusion, or tissue transfer has detrimental effects on tissue viability. Paradoxically, reestablishment of normal vascular flow can incite continued and often intensified tissue injury (Kerrigan and Stotland MA, 1993). Numerous studies using a variety of animal models have shown that the deleterious effects of ischemic injury are mediated by oxygen-derived free radicals such as superoxide anions (Cotran et al, 1998; McCord, 1985; Semenza 2000a), and locally released cytokines such as interleukin-1 and tumor necrosis factor-α (Gilmont et al, 1996; Herskowitz et al, 1995; Sanchez-Elsner et al, 2001). However, not all cytokines have detrimental effects: and some cytokines have been shown to attenuate ischemic tissue damage. For example, external administration of TGF-β provides protection against ischemia-reperfusion injury in organs such as the heart and brain in animal models (Lefer et al, 1990; McNeill et al, 1994; Mehta et al, 1999).

TGF-β is a member of a large family of multi functional proteins important in growth, differentiation and development (Roberts et al, 1990a; Roberts, 1995; Roberts, 1998). Three distinct isoforms of TGF-β (TGF-β1, 2, and 3) have been described in mammals, which are encoded by distinct genes (Roberts, 1998). The most commonly expressed form is TGF-β1, which has been shown to have potent effects on extracellular matrix synthesis, immune modulation, endothelial adhesiveness and tissue repair (Diebold et al, 1995; O'Kane and Fergueson, 1997; Roberts et al, 1988). There are three cloned TGF-β receptors termed type I, type II and type III that are expressed on most

cell types. The TGF-β signal is transduced by the types I and II receptors which are transmembrane serine/threonine kinases (Franzen et al, 1993; Lin et al, 1992). The type III TGF-β receptor which is a membrane proteoglycan is believed to facilitate TGF-β binding to the signaling receptors (Lopez-Casillas et al, 1993; Tam and Philip, 1988).

Because TGF-β is implicated in the protection of tissue from ischemic damage, it is important to understand the molecular basis of TGF-β action under ischemic conditions. Hypoxia has been shown to upregulate the expression of TGF-β (Flalanga et al, 1994) and to down regulate TGF-β binding (Falanga et al, 1991) during *in vitro* studies using skin fibroblasts in culture. Upregulation of TGF-β following tissue ischemia has been demonstrated in central nervous tissues and kidney (Basile et al, 1996; Knuckey et al, 1996). Less information is available on the effect of tissue ischemia and reperfusion on the expression of TGF-β receptors. Recently, upregulation of TGF-β receptors following ischemia has been reported in the brain (Ata et al, 1999). However, in this study, the expression of receptors was analyzed one or more days after the induction of ischemia. In addition, the effect of reperfusion was not studied. Considering that TGF-β has potent tissue protective effects during ischemia-reperfusion injury, our objective was to determine whether the expression of TGF-β and its receptors is regulated by acute ischemia and reperfusion in different cell types of the skin using pig skin flap model.

3.2. RESULTS

3.2.1. Immunohistochemical localization patterns of types I, II and III TGF-β receptors and TGF-β1 ligand

To analyze the regulation of TGF-β receptors and TGF-β1 during ischemia-reperfusion injury, a pig skin flap model was used. Immunohistochemical localization of the types I, II, and III TGF-β receptors and TGF-β1 ligand was performed in tissue sections prepared from non-ischemic control flap (contralateral flap with no arterial occlusion), ischemic flap, reperfused flap and unoperated buttock skin. Comparative analysis of immunostaining of types I, II and III receptors revealed that the expression of these receptors were increased in the non-ischemic control flaps, ischemic flaps and reperfused flaps compared to the unoperated skin (Figures 1 and 2).

The skin structures that showed the most dramatic increases were the blood vessels (endothelial cells), fibroblasts, and the basal layer of the epidermis. The immunoreactivity of the three receptors was the highest in the ischemic flap with the non-ischemic control flap showing significantly lower immunostaining. The expression of TGF-β1 in the ischemic flap, on the other hand, was not significantly different from that of the non-ischemic control. Importantly, TGF-β1 expression in the non-ischemic control is markedly higher than in the unoperated skin (Figure 2). Semiquantitative evaluation of immunostaining and statistical analysis of the differences between groups

(unoperated, non-ischemic control, ischemic, and reperfused) of types I, II and III receptors and TGF-β1 are shown in Table 1.

The skin structures immunostained for the types I , II and III TGF-β receptors and TGF-β1 ligand in the skin flaps were in general similar in the unoperated skin (Figures 1 and 2). In the epidermis, no detectable immunostaining for types I, II, and III receptors and TGF-β1 was observed in the stratum corneum.

The stratum luciderm showed the strongest immunostaining while stratum granulosum and spinosum showed strong but less robust expression. However, these two layers displayed no dramatic alterations between groups. In the stratum basale, the immunostaining of the three receptors and TGF-β1 was moderate but showed marked increased in the expression of type I receptor in ischemic control flap as compared to non ischemic control flap, and TGF-β1 in reperfused condition as compared to ischemic condition.

There was no significant difference in immunostaining patterns of the type I, II and III receptors and TGF-β1 between flaps exposed to 1 hour and 4 hours of ischemia (data not shown). The data from flaps representing 1 hour of ischemia and 1 hour of reperfusion are shown in Table 1.

Table 1. *Semiquantitative analyses of immunoreactivity of the types I, II, and III TGF-β receptors and TGF-β1 in unoperated skin, non-ischemic control flap, ischemic flap, and reperfused flap.*

Data represent values from 6 pigs (12 flaps) analyzed by Kruskal-Wallis test on means ± SD of samples collected at 1 hour of ischemia and at 1 hour after reflow from experimental (ischemic or reperfused) flaps and from control (nonischemic) flaps (Cont) and unoperated skin (Unop).

TGF, transforming growth factor; R, receptor; Ischem, ischemic flap; Reper, reperfused flap. P values are indicated in parenthesis (NS, not significant).

	RI	**RII**	**RIII**	**TGF-β1**
Blood Vessels				
Cont vs Ischem	Ischem ↑ ($P < 0.0004$)	Ischem ↑ ($P < 0.01$)	Ischem ↑ ($P < 0.0009$)	NS
Ischem vs Reper	NS	NS	NS	NS
Unop vs Cont	Cont ↑ ($P < 0.04$)	Cont ↑ ($P < 0.02$)	Cont ↑ ($P < 0.002$)	Cont ↑ ($P < 0.0005$)
Fibroblast				
Cont vs Ischem	Ischem ↑ ($P < 0.004$)	Ischem ↑ ($P < 0.001$)	Ischem ↑ ($P < 0.002$)	NS
Ischem vs Reper	Ischem ↑ ($P < 0.01$)	Ischem ↑ ($P < 0.01$)	NS	NS
Unop vs Cont	Cont ↑ ($P < 0.005$)	Cont ↑ ($P < 0.03$)	Cont ↑ ($P < 0.0004$)	Cont ↑ ($P < 0.005$)
Stratum basale				
Cont vs Ischem	Ischem ↑ ($P < 0.003$)	NS	NS	NS
Ischem vs Reper	NS	NS	NS	Reper ↑ ($P < 0.05$)
Unop vs Cont	NS	NS	NS	NS

3.2.2. Types I and II TGF-β receptor expression

The immunoreactivity patterns of types I and II receptors are shown in Figure 1. The expression of both types I and II receptors was the highest in the ischemic flap (c_I, c_{II}). The immunostaining of types I and II receptors on blood vessels ($P< 0.0004$ and $P< 0.01$, respectively) and fibroblasts ($P<0.004$ and $P<0.001$, respectively) in the ischemic flap (c_I, c_{II}) was significantly higher as compared to the non-ischemic control flap (b_I, b_{II}) (Table 1). A higher magnification of the non-ischemic control flap (e_I, e_{II}, for types I and II respectively) and ischemic flap (f_I, f_{II}, for types I and II respectively) is shown to emphasize the effect of ischemia on receptor expression and to better depict the cell types involved.

Although the types I and II receptor staining was also increased in the stratum basale in the ischemic flap compared to the non-ischemic control flap, it was significant ($P<0.003$) only for the type I receptor. The immunoreactivity in the reperfused flap as compared to ischemic flap was significantly lower for the type I (d_I vs c_I, $P< 0.01$) and type II (d_{II} vs c_{II}, $P< 0.01$) receptors on fibroblasts with no significant difference in blood vessels and stratum basale.

The expression of types I and II receptors in the non-ischemic control flap (b_I, b_{II}) as compared to the unoperated skin (a_I, a_{II}) was significantly increased in blood vessels ($P< 0.04$ and $P< 0.02$, for types I and II respectively) and fibroblasts ($P< 0.005$ and $P< 0.03$, for types I and II respectively) but not in stratum basale (Table 1). No immunoreactivity was observed in control experiments when the type I (g) and type II

(h) antibodies preincubated with their respective immunizing peptides were used. The control slides (g and h) were counter stained using Gill's hematoxylin before mounting to show the histology.

3.2.3. Type III TGF-β receptor and TGF-β1 expression

The immunoreactivity patterns of types III receptor and TGF-β1 ligand are shown in Figure 2. As observed above for the types I and II receptors, the expression of the type III receptor was the highest in the ischemic flap (c_{III}) with the blood vessels ($P< 0.0009$) and fibroblasts ($P<0.002$) of this flap showing markedly higher immunoreactivity than those of the non-ischemic control flap (b_{III}) (Table 1).

In the stratum basale, the type III immunostaining was not significantly different between the non-ischemic control and ischemic flaps, as observed for type II but not type I in this cell layer. When the reperfused flap (d_{III}) was compared to ischemic flap (c_{III}), the type III receptor immunoreactivity was unchanged in blood vessels, fibroblasts and stratum basale. A higher magnification of the type III receptor immunostaining in the non-ischemic control flap (e_{III}) and ischemic flap (f_{III}) is shown to highlight the alterations in immunoreactivity and cell types involved. When the type III receptor immunoreactivity in the non-ischemic control flap (b_{III}) was compared to that of the unoperated skin (a_{III}), it was significantly increased in the control flap on blood vessels ($P<0.002$) and fibroblasts ($P<0.0004$), but not in the stratum basale.

Figure 1. *Immunohistochemical localization of types I and II TGF-β receptors in the pig skin.*

Immunostaining patterns of types I and II receptors in unoperated skin (a_I and a_{II}, respectively), non-ischemic control flap (b_I and b_{II}, respectively), ischemic flap (c_I and c_{II}, respectively), reperfused flap (d_I and d_{II}, respectively), non-ischemic control flap (e_I and e_{II} respectively, higher magnification, 40 x original) and ischemic flap (f_I and f_{II} respectively, higher magnification, 40 x original). The immunostaining patterns show dynamic regulation of types I and II receptors in a cell type specific manner. Significant alterations are observed in blood vessels (➔), fibroblasts (→), and stratum basale (⇨). Control experiments in which the primary antibodies - anti-type I (g) and anti-type II (h) receptor antibodies - were preincubated with their respective immunizing peptides do not show any immunoreactivity. Sections (g) and (h) are counter stained with hematoxylin before mounting.

The result shown is representative of samples collected at 1 hour after ischemia and at 1 hour after reflow from experimental flaps and at corresponding times from control flaps and unoperated skin.

Figure. 1

In contrast to what was observed for the types I, II and III receptors, the TGF-β1 immunoreactivity in the ischemic flap was not significantly different from that of non-ischemic control in blood vessels, fibroblasts or stratum basale. More importantly, however, TGF-β1 immunostaining in the non-ischemic control flap ($b_{\beta 1}$) was markedly higher than that of the unoperated skin ($a_{\beta 1}$) on blood vessels ($P<0.0005$) and fibroblasts ($P<0.005$). Stratum basale showed no significant difference. Interestingly, TGF-β1 immunostaining in the reperfused flap ($d_{\beta 1}$) as compared to ischemic flap ($c_{\beta 1}$) was significantly higher in stratum basale ($P<0.05$), with no significant difference in blood vessels and fibroblasts.

In control experiments when the anti-type III receptor (g) and anti-TGF-β1 (h) antibodies preincubated with their respective immunizing peptides were used, no detectable immunoreactivity was seen. The control slides (g and h) were counter stained using Gill's hematoxylin before mounting to show the histology.

3.2.4. Northern blot analysis of type II TGF-β receptor expression

Although, both type I and type II receptor components appear to be required for signal transduction of TGF-β's antiporliferative activity (Laiho et al, 1990), a close association between inactivation of type II receptor and escape from TGF-β-mediated growth inhibition has been demonstrated in several tumor cell lines (Geiser et al, 1992; Park et al, 1994). Moreover an expanding body of evidence has implicated a

dysfunctional TGF-β-mediated signal transduction pathway, due to loss of type II receptor function, in the development and progression of several human malignancies, including breast cancer (Sun et al, 1994), T-cell lymphomas (Kadin et al, 1994, Rooke et al, 1999), gastric carcinoma (El-Rifai and Powell, 2002), colon cancer cells (Markowitz et al, 1995, Nagayama et al, 2002, Furukawa et al, 2002), and head and neck squamous carcinoma (Garrigue-Antar et al, 1995). Furthermore, malignant conversion frequency and carcinoma incidence are both increased and carcinoma latency is decreased in the dominant negative type II TGF-β receptor in mouse skin (Amendt et al, 1998).

To determine whether hypoxia is able to regulate the expression of type II TGF-β receptors in skin fibroblasts *in vitro*, early passage of human skin fibroblasts were subjected to hypoxic condition for 2 hours while the control cells remained under normoxic conditions. Expression of the type II receptor was determined by Northern blot analysis. Results shown in Figure 3 demonstrate that exposure to hypoxic condition for 2 hours markedly increased the abundance of type II receptor mRNA in early passage of skin fibroblasts. This observation illustrates that acute exposure to hypoxia leads to an increase in the expression of type II receptor in early passage skin fibroblasts, and supports the *in vivo* immunohistochemistry results presented above.

3.3. Discussion

The pathophysiologic mechanisms involved in ischemia-reperfusion injury, a common denominator in clinical conditions such as myocardial infarction, cerebral ischemia,

tissue transplantation and free tissue transfer, are poorly understood. TGF-β has been shown to provide protection against ischemia-reperfusion injury in many organ systems, and is known to be a key regulator of the tissue repair process. However, little is known about the regulation of TGF-β receptors during ischemia and reperfusion. Our results demonstrate the occurrence of dynamic and cell type specific regulation of TGF-β receptors and TGF-β1 *in vivo* during ischemia-reperfusion, using a pig skin flap model. This model was designed to study the early dynamic changes (1 hour and 4 hours) during ischemia as well as 'recovery' from ischemia. This time frame was chosen as it represents the clinically significant period during which intervention with therapeutic agents might have a beneficial effect on the deleterious effects of injury. Thus, the model we have used does not allow us to evaluate the late effects of ischemia-reperfusion injury. The bilateral skin flap design with the contralateral flap acting as the control avoid animal to animal variation, allows us to tease out the effect of ischemia from that of wounding (creation of the flap) which is also associated with ischemia on the regulation of TGF-β/TGF-β receptor system *in vivo*. In addition, the cutaneous anatomy and vascular supply of the pig are similar to that of the man, making the model relevant to the human.

Figure 2. *Immunohistochemical localization of types III TGF-β receptor and TGF-β1 ligand in the pig skin.*

Immunostaining of types III receptors and TGF-β1 in unoperated skin (a_{III} and $a_{\beta 1}$, respectively), non-ischemic control flap (b_{III} and $b_{\beta 1}$, respectively), ischemic flap (c_{III} and $c_{\beta 1}$, respectively), and reperfused flap (d_{III} and $d_{\beta 1}$, respectively). The immunostaining patterns show dynamic regulation of type III receptors and TGF-β1 in a cell type specific manner. Marked alterations are observed in blood vessels (➔), fibroblasts (→), and stratum basale (⇨). Control experiments in which the primary antibodies - anti-type III receptor (g) and anti-TGF-β1 (h) antibodies - were preincubated with their respective immunizing peptides do not show any immunoreactivity. Sections (g) and (h) are counter stained with hematoxylin before mounting. The result shown is representative of samples collected at 1 hour after ischemia and at 1 hour after reflow from experimental flaps and at corresponding times from control flaps and unoperated skin.

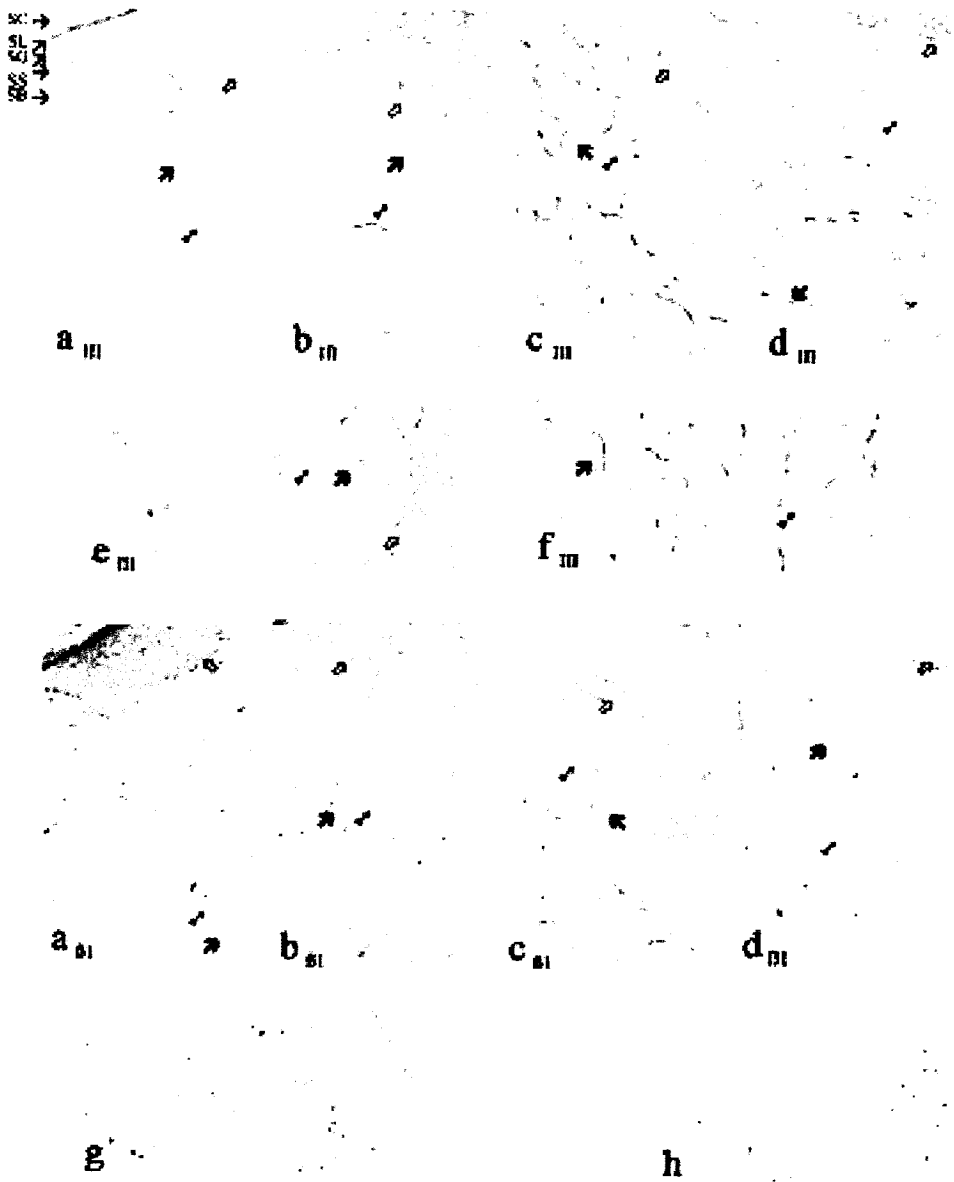


Figure. 2

Figure 3. *Hypoxia regulates expression of type II TGF-β receptor mRNA expression in skin fibroblasts in vitro.*

Early passage human skin fibroblasts were cultured and subjected to 2 hours of hypoxia as described in Materials and Methods. Total RNA was extracted and Northern blot was done using a cDNA probe for the type II TGF-β receptor. The type II TGF-β receptor mRNA expression under normoxia (N) (20% oxygen) and hypoxia (H) (< 0.2% oxygen) conditions are shown (upper panel). The 18 S ribosomal RNA is shown to demonstrate equal loading of RNA (bottom panel).

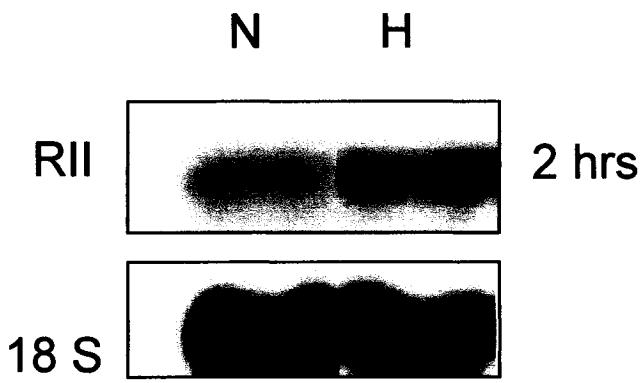


Figure. 3

The most important finding in the present study is that global ischemia resulted in rapid (within 1hour) upregulation of types I, II and III TGF-β receptors on blood vessels (endothelial cells) and fibroblasts. Immunostaining of TGF-β receptors in those cell types were markedly higher in the ischemic skin flap (subjected to global ischemia) than in the non-ischemic control flap. This increase was maintained at 4 hours of ischemia (data not shown). Importantly, creation of the flap (which involves wounding and partial ischemia) resulted in an increase in the expression of all three TGF-β receptors and TGF-β1 in endothelial cells and fibroblasts, as shown by significantly higher immunostaining in non-ischemic control flap than in the unoperated skin. The absence of a further increase in TGF-β1 expression in endothelial cells (blood vessels) and fibroblast after the induction of global ischemia is intriguing. It is possible that the expression of TGF-βl is already maximal due to the partial ischemia and wounding induced by the creation of the flap, and subsequent global ischemia may have no further effect.

While the expression of the TGF-β receptors was increased on endothelial cells and fibroblasts within 1 hour of the induction of ischemia, subsequent reperfusion led to a significant decrease in the types I and II receptors in fibroblasts, but this decrease was not significant in blood vessels. The rapid upregulation of TGF-β receptors and TGF-β1, and thus enhanced TGF-β signal transducing machinery, in endothelial cells and fibroblasts during ischemia-reperfusion provide an explanation at the molecular level for the potent effect of TGF-β under these conditions, namely the tissue protective effect

that TGF-β exerts against ischemia/reperfusion injury in several animal models (Lefer et al, 1990; McNeill et al, 1994; Mehta et al, 1999). The endothelial cell is the principal cell type involved in the development of ischemia reperfusion injury, and TGF-β has been shown to be a key regulator of several endothelial responses important in attenuating ischemia reperfusion injury. For example, TGF-β has been demonstrated to potently inhibit endothelial adhesiveness to polymorphonuclear leukocytes (Lefer et al 1990; Rhodes et al 1995) to inhibit free radical generation and preserve vasomotor tone (Lefer et al, 1990; Mehta et al, 1999).

Although the middle layers of the epidermis (granulosum, spinosum and luciderm) showed strong immunoreactivity for the types I, II and III receptors and TGF-β1, it was the stratum basale (basal keratinocytes) which exhibited significant differences in immunoreactivity, specifically that for type I receptor during ischemia and that for TGF-β1 after reflow. However, it is possible that the high expression of the receptors and the ligand in the middle layers precluded the detection of alterations in immunoreactivity. The significance of the upregulation of type I, but not types II and III receptors or TGF-β1 by global ischemia (non-ischemic control vs ischemia) and the enhanced expression of TGF-β1, but not types I, II and III receptors following reperfusion on basal keratinocytes is not known.

Although sustained ischemia results in the failure of wounds to heal, ischemia in the initial phase of the wound healing process stimulates fibroplasia and angiogenesis. As such, both occlusive dressings which promote hypoxia and hyperbaric oxygen have

been shown to enhance wound healing (Stadelmann et al, 1998). Interestingly, it has recently been demonstrated that TGF-β was capable of accelerating wound healing under both non-ischemic and ischemic conditions while fibroblast growth factor and platelet derived growth factors were ineffective under ischemic conditions (Wu and Mustoe, 1995). That TGF-β was effective under ischemic conditions would be predicted from our results which show that the expressions of TGF-β/TGF-β receptors system are enhanced during ischemia. Thus the impaired wound healing observed in ischemic tissue is not likely due to decreased TGF-β action, but may be accounted for by the reduced supply of nutrients and immune cells, or increased production of oxygen free radicals (Senel et al, 1997) or decreased action of other growth factors (Wu and Mustoe, 1995) or all of the above. Limited information is available on the regulation of TGF-β receptors in skin cells under ischemic conditions. In *in vitro* culture studies using skin fibroblasts, hypoxia has been reported to induce TGF-β1 expression (Falanga et al, 1991) and to decrease TGF-β receptor mRNA and binding (Falanga et al, 1994). The decreased expression of TGF-β receptors *in vitro* during hypoxia reported by this group (Falanga et al, 1994) is not consistent with our results *in vivo* (Table 1) and *in vitro* (Figure 3), or with those of Ata et al *in vivo* in the brain. Differences in experimental conditions may explain this discrepancy. Although our study does not allow us to determine the precise temporal relationship between the induction of ligand and receptors, the rapid upregulation of all three TGF-β1or TGF-β receptors by partial or global ischemia suggests that ischemia may have a direct effect on the expression of

these receptors and ligand. Our *in vitro* results demonstrating the upregulation of type II receptor mRNA at 2 hours of hypoxia in skin fibroblasts (Figure 3) support this conclusion. Although Ata et al (1999) has recently demonstrated that the types I and II TGF-β receptors are upregulated by ischemia in the cerebral tissue, the expression of receptors was analyzed at days 1 and 3 following ischemia in this study. Regulation of gene expression by low oxygen concentration is now a well documented phenomenon (Bunn and Poyton, 1996; Ratcliffe et al, 1998). Whether hypoxia-inducible factor-1 (HIF-1), a master regulator of oxygen homeostasis (Semenza, 2000a; Wu and Mustoe 1995), is involved in the induction of the TGF-β/TGF-β receptors system during ischemia remains to be determined. It is interesting to note in this regard that hypoxia and TGF-β have recently been reported to synergistically cooperate to induce vascular endothelial growth factor expression (Sanchez-Elsner et al, 2001), and that this cooperation may involve a physical association between HIF-1α and Smad3, a central mediator of TGF-β action.

The cellular distribution of the three types of receptors was similar to that of TGF-β1. The concomitant expression profiles and the co-localization of the TGF-β/TGF-β receptors system in the same cell types is consistent with the numerous studies showing the heteromeric complex formation of the three receptors and their high affinity for the TGF-β1 ligand. The co-localization and the synchrony in the regulation of the types I and II TGF-β receptors on endothelial cells and fibroblasts is not consistent with

the notion that activation of these receptors leads to distinct TGF-β signalling pathways performing independent functions (Chen et al, 1993; Feng and Derynck, 1996), but suggest that they cooperate to initiate the TGF-β signalling cascade.

In summary, our results demonstrate that the TGF-β/TGF-β receptor system is dynamically regulated during ischemia-reperfusion in a cell type specific manner in the skin. The data presented define an increase in the expression of TGF-β receptors and TGF-β1 as a rapid adaptive response to partial or global ischemia. Taken together, our results indicate that TGF-β action is enhanced under ischemic conditions, and provide an explanation at the molecular level for the potent effects of TGF-β under these conditions. The augmented TGF-β responsiveness may be an important determinant for the tissue protective effect of TGF-β against ischemia/reperfusion injury.

Bridging Document:

In the previous chapter, a pig skin flap model was used to study the possible regulatory role of ischemia and reperfusion on TGF-β receptors (RI, RII, and RIII), and TGF-β ligand. Since our *in vivo* experiments showed the susceptibility of TGF-β receptors and ligand to ischemia and reperfusion in fibroblast cells and the fact that fibroblast and keratinocyte cells play important role in the process of wound healing, these cells were selected to study the effect of steroids and oxygen tension on TGF-β signaling as a simple *in vitro* model. Wound healing is a complex process and is not an isolated event. It is affected by other regulatory molecules such as steroids and hypoxia. This prompted us to analyze the regulation of TGF-β action by oxygen tension and steroid using individual cell types to obtain fundamental information under controlled condition. Thus, in following chapter, I studied the effect of oxygen tension, steroids, and the combined effects of oxygen tension and steroids on the TGF-β signaling pathway (TGF-β ligand, receptors, and Smad2 and Smad3).

Chapter 4: Regulation of TGF-β Signaling by Hypoxia, and Steroids in Skin Cells

4.1. Rationale

Impaired wound healing is a major medical problem and costs the health services over $9 billion per year in the United States. TGF-β is a key cytokine that plays a role in all phases of wound healing (Yang et al, 2001), through its effects on angiogenesis, cell differentiation, and inflammation. In the inflammatory phase of wound healing, neutrophils, monocytes and T cells are attracted to the wound by TGF-β and clear the area of dead tissue and bacteria (Wahl et al, 1987; Moulin 1995; Slavin, 1996). TGF-β also attracts keratinocytes and fibroblasts, and has a great impact on tissue remodeling by stimulating the synthesis of collagen while downregulating ECM degrading proteases and upregulating synthesis of protease inhibitors (Ignotz and Massague, 1986; Overall et al, 1991). Hypoxia is an inevitable result of wounding and is recognized as a common denominator of chronic wounds. Interestingly, exogenous application of TGF-β enhances wound healing under both normal and ischemic conditions (Mustoe et al, 1987; Quaglino et al, 1991; Wu and Mustoe, 1995). In wound healing, hypoxia alters the overall cellular behavior as a consequence of, or in addition to activating specific genetic pathway. In wounds, initially low oxygen tension modulates the pace of healing and wound resolution in part by increasing collagen synthesis and accelerating wound reepithelialization (La Van and Hunt, 1990; Woodley, 1996). Hypoxia by itself is not the only determinant that affects the fate of a wound healing. Interactions between oxygen levels and other elements in the cellular environment dictate the outcome of healing. The validity of this reasoning has been shown by observation that fetal skin

wounds heals scarlessly in a physiologically hypoxic condition as compared to a hypoxic adult wounds.

Steroids have a profound effect on the cell homeostasis, proliferation and progression of wound healing. Animal studies have shown that estrogens play a crucial role in cutaneous wound healing and that wound repair is significantly delayed in their absence (Ashcroft et al, 1999a). Tamoxifen is a partial estrogen antagonist with antiangiogenic properties. It favorably modifies certain aspects of wound healing, most notably keloid formation and scarring (Mikulec et al, 2001; Chau et al. 1998).

Another class of steroids involved in wound healing are glucocorticoids (GCs). GCs are immunosuppressive and anti-inflammatory agents that also able to stimulate the expression of TGF-β. Glucocorticoids inhibit wound healing and may exert a major role in the pathogenesis of impaired wound healing in diabetes (Bitar, 2000). Interestingly, RU486 a glucocorticoid receptor blocker has been shown to ameliorate various phases of the diabetes-induced wound healing impairment (Bitar et al, 1999). In connection to hypoxia, *in vivo* studies have shown that pretreatment with synthetic glucocorticoid dexamethasone provides protection against hypoxic-ischemic brain damage (Buisson et al, 2003; Dhandapani and Brann, 2003).

Numerous *in vivo* studies have shown that TGF-β is implicated in the protection of tissue and attenuates ischemic tissue damage. Taking into the consideration that oxygen tension, steroids and TGF-β play critical role in the wound repair process, it is essential to explore their interactions at the molecular level. Our aim is to characterize

the regulation of TGF-β signaling pathway by oxygen tension, and steroids (dexamethasone, RU486, estrogen, tamoxifen, and testosterone) by using skin cells (fibroblast skin cells and keratinocytes).

In this chapter I have characterized the regulation by oxygen tension and steroids of: i) TGF-β ligand, ii) TGF-β receptors, iii) phosphorylation of Smad2, and Smad3, and iv) total Smad2 and Smad3.

4.2. Modulation of TGF-β Signaling Pathway by Oxygen Tension in Skin Cells

4.2.1. RESULTS

4.2.1.1. A representative of standard curve for TGF-β1 using PAI-L assay

A luciferase reporter assay (PAI/L) using plasminogen activator inhibitor promoter was used to detect the TGF-β expression. This assay is a very sensitive method which is used widely for detection of TGF-β in conditioned medium (Abe et al, 1994). Mink Lung Cells stably transfected with PAI-I promoter fused to firefly luciferase reporter gene (TMLC) were plated at $1.6x10^5$ cells/ml in a 96 well plate. In order to obtain a standard curve, known concentrations of TGF-β (0, 1, 3, 5, 10, 20 and 40 pM), were added to each well. After 20 hours incubation, the cells were solubilized and the intensity of light produced by the induction of luciferase activity (RLU) was determined as described in chapter 2 (Figure 4).

The PAI-L assay does not distinguish between the three TGF-β isoforms-all three (TGF-β1, β2, and β3) are measured in this procedure. The specificity of the PAI-L assay for the TGF-β isoforms was examined using a pan anti-TGF-β antibody which recognizes all three isoforms and completely neutralized the TGF-β activity in the conditioned medium. Since no residual activity could be detected after the anti-TGF-β antibody neutralization, it implies that values obtained with the PAI-L assay represent TGF-β isoforms and not other super family members such as activin (Table 2).

Figure 4. *Luciferase assay*

Transfected Mink Lung Cells (TMLC) were plated at $1.6x10^5$ cells/ml in a 96 well plate in DMEM containing 10% fetal bovine serum. Cells were allowed to attach for 3 hours at 37°C in a 5% CO_2/95% air atmosphere incubator. After 3 hours incubation, wells were aspirated and serial dilutions of human TGF-β1 standards were added 100 µl per well in triplicates. The PAI-L assay shows a linear response (RLU) in the range between 0-40 pM of TGF-β1.

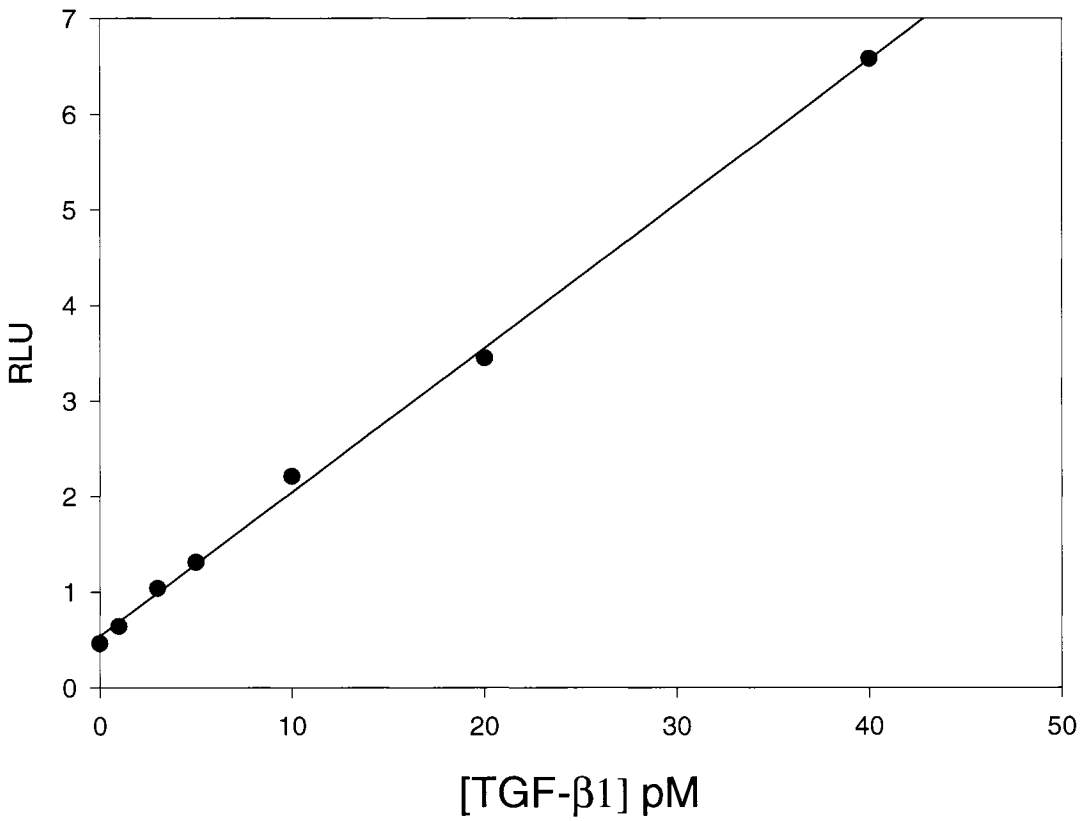


Figure. 4

Table 2. Analysis of active and total TGF-β in human primary skin fibroblasts in the presence of a pan anti TGF-β antibody (24 hours) that recognizes all three TGF-B isoforms using the PAI/L assay as described in Materials and Methods.

24 hours	TGF-β (pM)	
Antibody	absent	present
Active TGF-β	29.52 ± 4.9	undetectable
Total TGF-β	299.4 ± 63.17	undetectable

4.2.1.2. Modulation of TGF-β levels by hypoxia in human skin fibroblasts

4.2.1.2.1. Exposure to 2 hour hypoxia

To demonstrate that short term exposure to hypoxia has an effect on the expression of TGF-β, human primary skin fibroblasts were grown under hypoxic conditions for 2 hours and TGF-β expression was determined by the PAI/L assay. Figure 5 shows the concentration of TGF-β in human primary skin fibroblasts from the same pool that were grown at normoxia (N), hypoxia (H) for 2 hours, and subsequent re-exposure to normoxia for two hours (R). Hypoxia was generated using a Gas Pak system with a palladium catalyst and sodium borohydride tablet as was described in Materials and Methods.

The concentration of active TGF-β (TGF-β concentration in culture medium as it is) at hypoxic condition was significantly higher as compared to normoxic condition (Figure 5A, $P<0.01$). The concentration of TGF-β was decreased (but not statistically significant) during reoxygenation in comparison to hypoxic cells. No significant differences were observed between reoxygenation and normoxic cells.

The total TGF-β which includes the active and latent TGF-β was obtained by heating the test medium at 70°C for 10 minutes. This heating process activates the latent TGF-β. No significant differences were observed (Figure 5B) for the total TGF-β in hypoxic and reoxygenated cells as compared to normoxic cells. Hypoxia and reoxygenation (2 hours) resulted in a 4.5- and 2.9- fold increase, respectively, of active

Figure 5. *Detection of active and total TGF-β in human primary skin fibroblasts after 2 hour exposure to normoxia, hypoxia, and reoxygenated condition.*

Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior the incubation of cells at normoxia (N) (20% oxygen), or hypoxia (H) (< 0.2% oxygen), for 2 hours. For reoxygenated (R) condition, cells were grown at normoxia for 2 hours following a 2 hour hypoxic treatment. The conditioned medium was collected in siliconized tubes containing a protease inhibitor cocktail: 10ul phenylmethylsulfonyl fluoride (PMSF), and 20 µl protease inhibitor (PI/ml of medium). The concentration of active **(A)** and total **(B)** TGF-β in the conditioned medium was measured using the PAI/L assay as described in Materials and Methods in chapter 2. Measured values for the TGF-β concentration were corrected for variations in cell number by measuring total protein concentration using a Biorad assay. Hypoxic and reoxygenated conditions were each compared to cells grown at normoxic condition. ↕ $P < 0.01$.

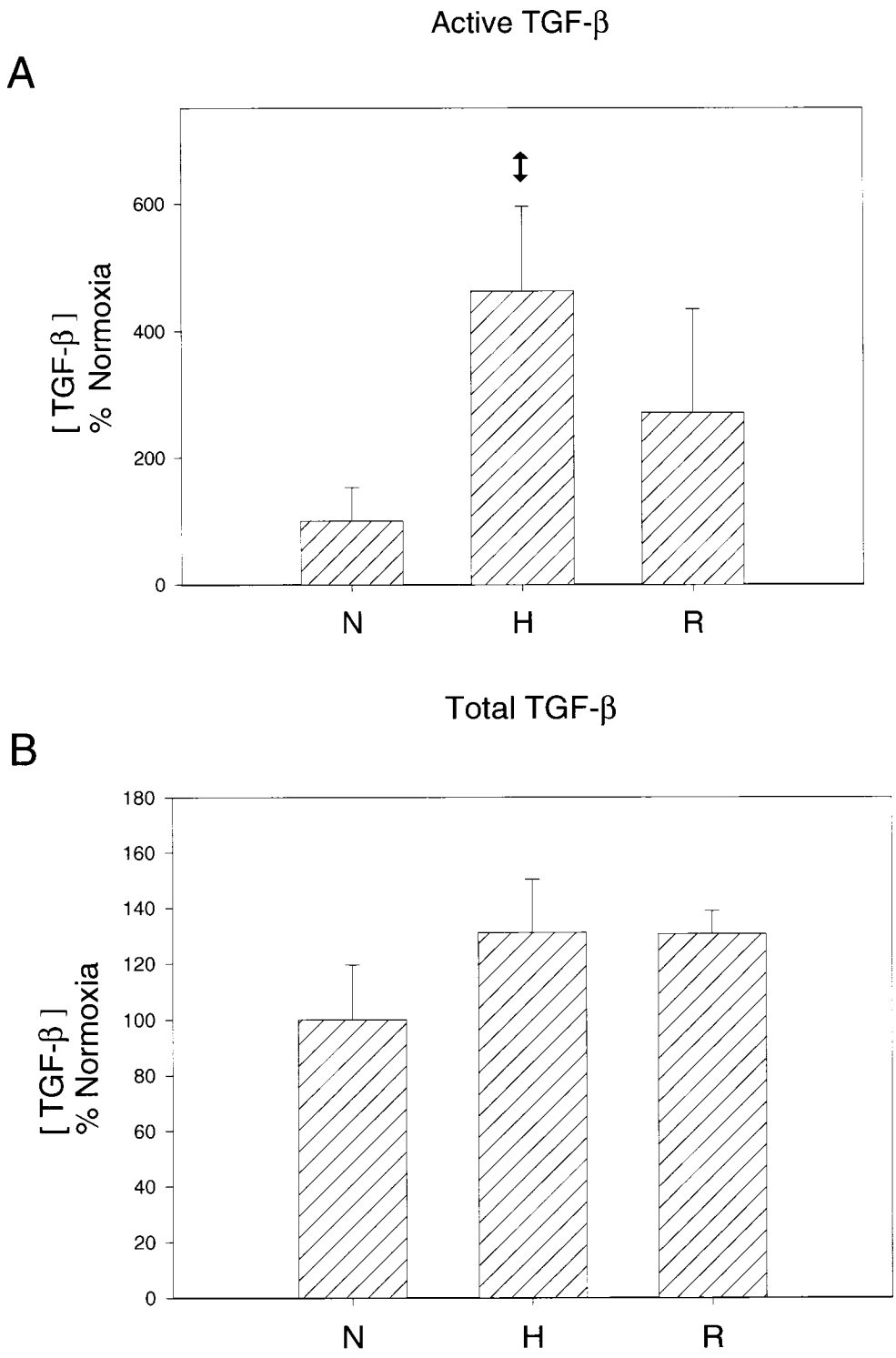

A
Active TGF-β
[TGF-β]
% Normoxia
600
400
200
0
N
H
R
B
Total TGF-β
[TGF-β]
% Normoxia
180
160
140
120
100
80
60
40
20
0
N
H
R

Figure. 5

TGF-β levels while total TGF-β concentration was increased 1.3- fold. This suggests that hypoxia greatly enhances the activation of TGF-β with almost no change in total TGF-β levels.

4.2.1.2.2. Exposure to 24 hour hypoxia

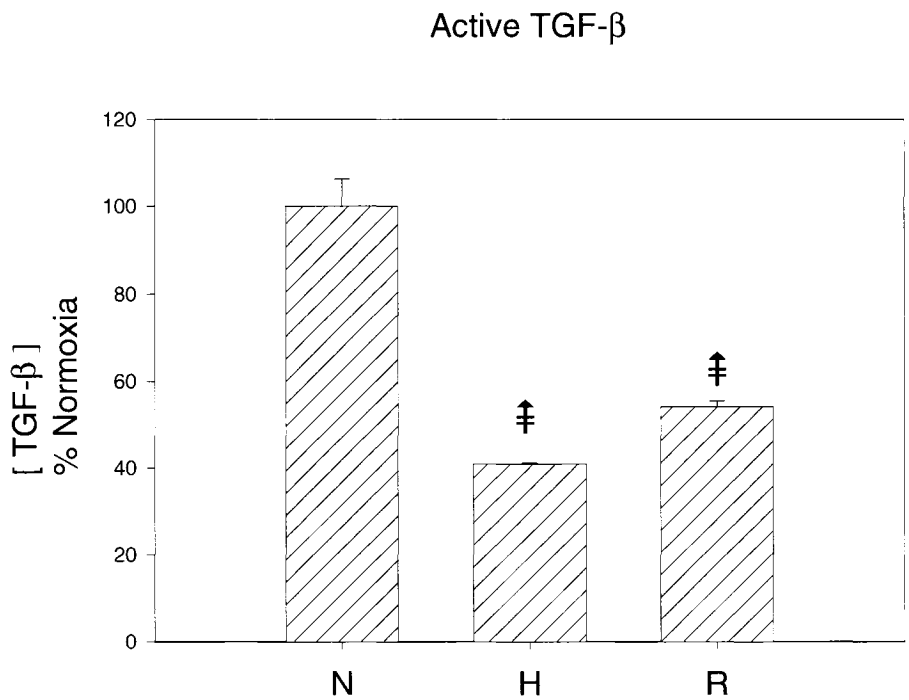
To further demonstrate that hypoxia has an effect on the expression of TGF-β in human primary skin fibroblasts at 24 hours, luciferase reporter assay using PAI-I/L assay was used again to determine the TGF-β levels. Figure 6 shows the concentration of TGF-β in human primary skin fibroblasts grown at normoxia (N), and hypoxia (H) for 24 hours and subsequently for two hours at reoxygenated (R) condition. According to Figure 6A the concentration of active TGF-β at hypoxic condition was significantly lower than the normoxic condition ($P<0.001$). The concentration of TGF-β was increased significantly ($P<0.001$) at reperfused condition as compared to hypoxic condition, but still significantly lower ($P<0.001$) as compared to normoxic condition. No significant changes were observed (Figure 6B) for the total TGF-β at hypoxic and reoxygenated condition as compared to normoxic condition.

Hypoxic, and reoxygenation treatment resulted in a 2.4- and 1.8- fold decrease, respectively, of active TGF-β levels while little change was observed for total TGF-β concentrations (1.3- and 1.1- fold). Comparison of active and total TGF-β at hypoxia as

Figure 6. *Detection of active and total TGF-β in human primary skin fibroblasts after 24 hours exposure to normoxia, hypoxia, and 2 hours of reoxygenation.*

Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior the incubation of cells at normoxia (N) (20% oxygen), or hypoxia (H) (< 0.2% oxygen), for 24 hours. For reoxygenated (R) condition, cells were grown at normoxia for 2 hours following a 24 hour hypoxic treatment. The conditioned medium was collected in siliconized tubes containing a protease inhibitor cocktail: 10ul phenylmethylsulfonyl fluoride (PMSF), and 20 µl protease inhibitor (PI/ml of medium). The concentration of active **(A)** and total **(B)** TGF-β in the conditioned medium was measured using the PAI/L assay as described in Materials and Methods in chapter 2. Measured values for TGF-β concentration were corrected for variations in cell number by measuring total protein concentration using a Biorad assay. Hypoxic and reoxygenated conditions were each compared to cells grown at normoxic condition. ‡ $P < 0.001$.

A


Active TGF-β
[TGF-β]
% Normoxia
120
100
80
60
40
20
0
N
H
R

B

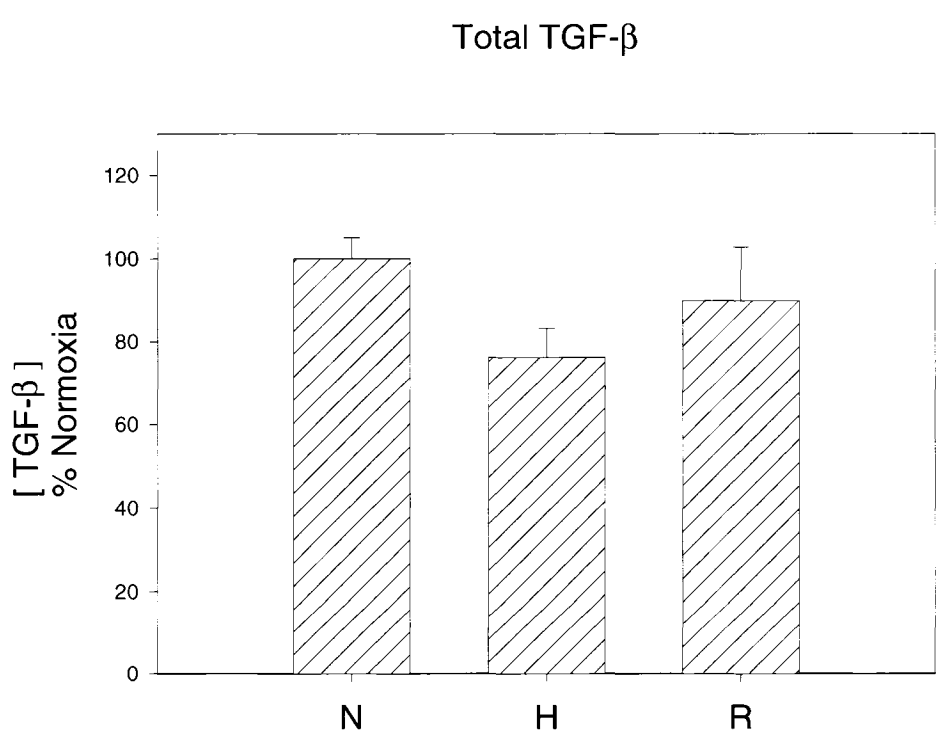

Total TGF-β
[TGF-β]
% Normoxia
120
100
80
60
40
20
0
N
H
R

Figure. 6

compared to normoxia, suggest that 24 hours exposure of cells to hypoxia decrease TGF-β activation.

4.2.1.3. Modulation of TGF-β receptor mRNAs expression by 2, 24, or 48 hour exposure to hypoxia and 2 hours reoxygenation in human skin fibroblasts and keratinocytes (HaCat).

4.2.1.3.1. Skin fibroblasts

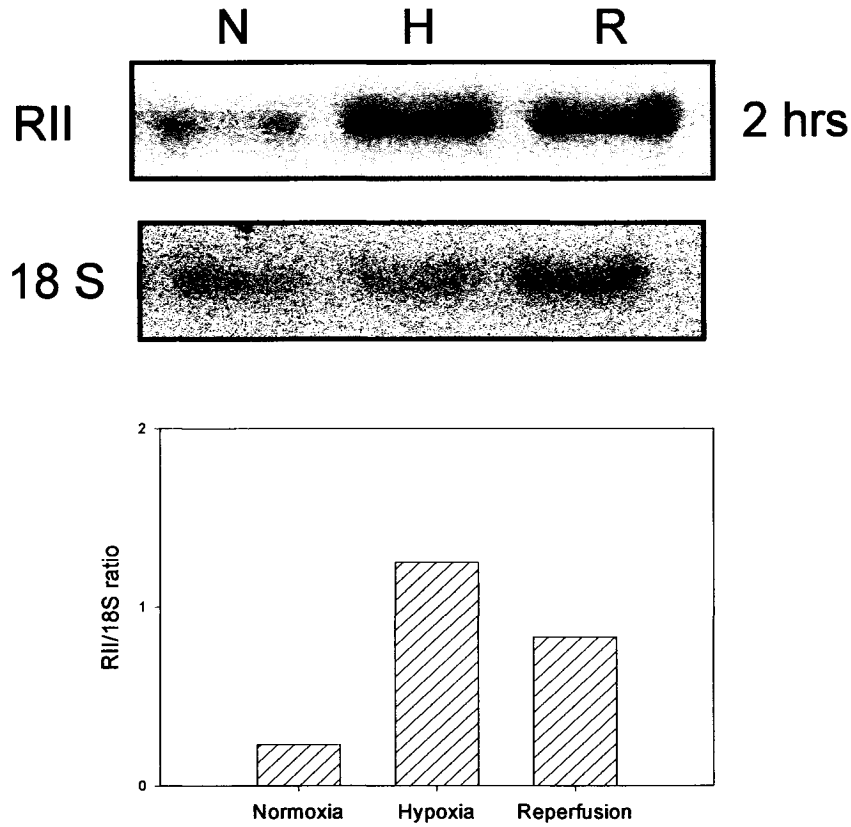
The roles of hypoxia and reoxygenation in regulating the expression of RI, and RII mRNAs in human primary skin fibroblasts were analyzed. As shown in Figure 7A, Northern blot analysis revealed that cells grown at hypoxic condition for 2 hours express higher level of RII mRNA as compared to normoxic condition. reoxygenation tended to return RII mRNA levels toward the original base level at normoxic condition. In contrast, cells that were exposed to hypoxia for 24 or 48 hours showed down regulation of RII mRNA at hypoxic condition as detected by Northern blot (Figure 7B, and Figure 7C) and RNase protection assay (data not shown). Reoxygenation again was tended to return the level of RII mRNA toward the level at normoxic condition. Interestingly, reoxygenation was able to counteract both the upregulatory and down regulatory effect of hypoxia on RII mRNA at 2 hours and 24-48 hours, respectively. The downregulation of RII mRNA at 24 hours might reflect a feed back loop associated with the early upregulation of RII at 2 hours of hypoxic treatment.

As shown in Figure 8, RNA protection assay analysis revealed that cells grown at hypoxic condition for 24 hours showed up regulation for RI mRNA. Reoxygenated

Figure 7. *Hypoxia (2, 24, and 48 hours) and reoxygenation (2 hours) modulate expression of RII mRNA in human primary skin fibroblasts.*

Northern blot analysis of RII mRNA in human primary skin fibroblasts at 2 (**A**), 24 (**B**), and 48 hours (**C**) indicate that RII mRNA expression is differentially regulated by hypoxia with respect to time in these cells. Skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior to the incubation of cells in normoxia (N) (20% oxygen) or hypoxia (H) (< 0.2% oxygen) for indicated times. For reoxygenated (R) condition, plates that were exposed to hypoxia (2, 24, or 48 hours) were then incubated at normoxic condition for 2 hours. Total RNA was extracted using acid guanidinium isothiocyanate-phenol-chloroform as described in Materials and Methods in chapter 2. From each sample 20 μg of RNA was analyzed by Northern blot using specific radiolabeled probes for RII mRNA and 18S ribosomal RNA. The 18S ribosomal RNA is used to normalize for the loading of RNA. The densitometric analysis of this data is shown at the bottom panel. The results shown are representative of three different experiments.

A


N
H
R
RII
2 hrs
18 S
RII/18S ratio
2
1
0
Normoxia
Hypoxia
Reperfusion

B

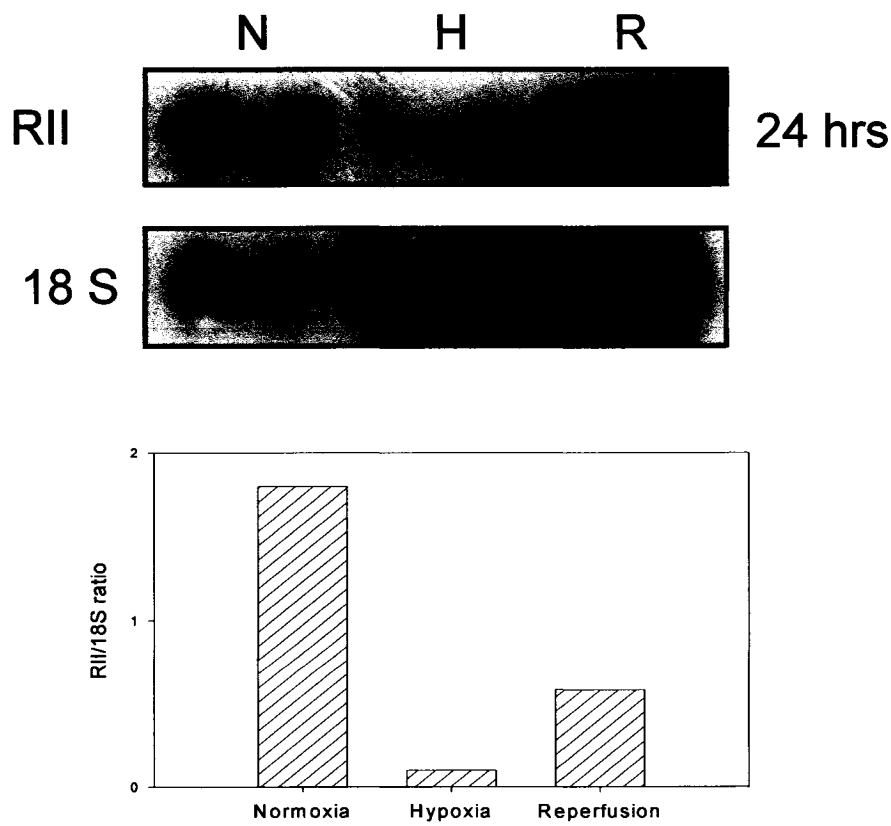

N
H
R
RII
24 hrs
18 S
RII/18S ratio
2
1
0
Normoxia
Hypoxia
Reperfusion

Figure. 7

C

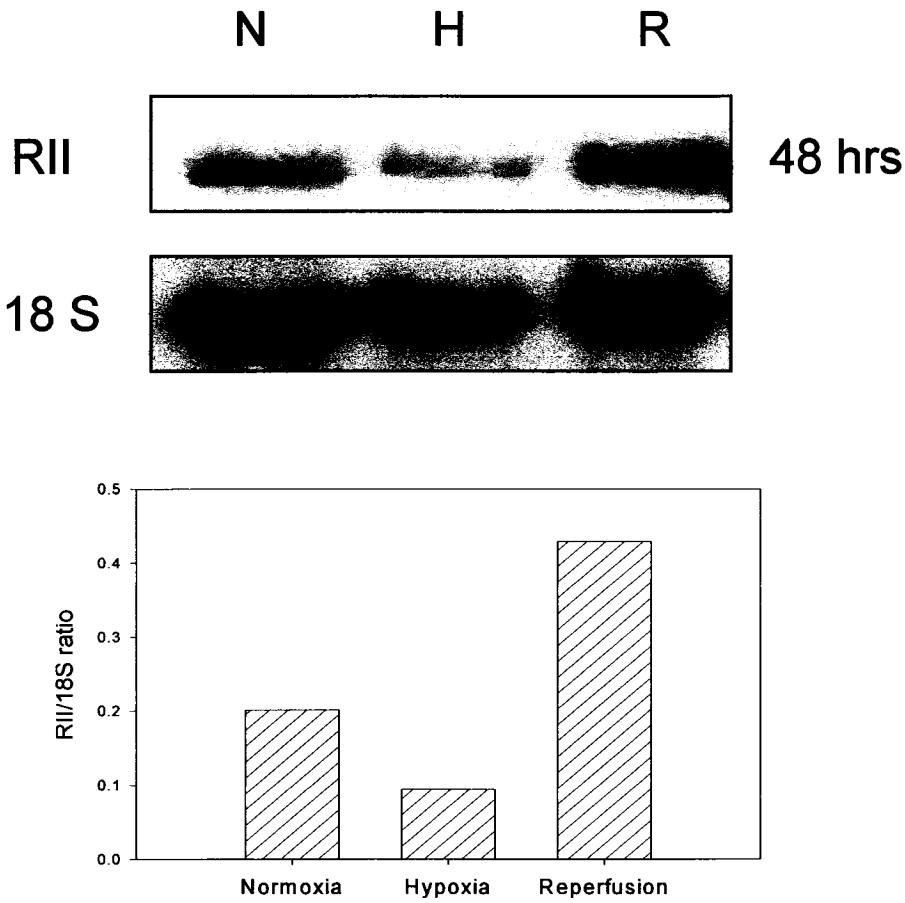

N
H
R
RII
48 hrs
18 S
RII/18S ratio
0.5
0.4
0.3
0.2
0.1
0.0
Normoxia
Hypoxia
Reperfusion

Figure. 7

Figure 8. *Hypoxia (24 hours) and reoxygenation (2 hours) modulate expression of RI mRNA in human primary skin fibroblasts.*

Ribonuclease protection assay (RPA) analysis indicates that expression of RI mRNA in human primary skin fibroblasts increased after 24 hours treatment with hypoxia. Skin fibroblasts were cultured with serum free medium over night. The medium was changed again with serum free medium at 3 hours prior to the incubation of cell in normoxia (N) (20% oxygen) or hypoxia (H) (< 0.2% oxygen). For reoxygenated (R) condition, plates were then incubated at normoxia for 2 hours following a 24 hour hypoxic treatment. Total RNA was extracted using TRIZOL reagent. From each sample 15 μg of RNA was analyzed by RPA for RI mRNA receptor and L32 mRNA expression. The L32 mRNA is shown to demonstrate equal loading of RNA (bottom panel). The densitometric analysis of this data is shown at the bottom panel.

A

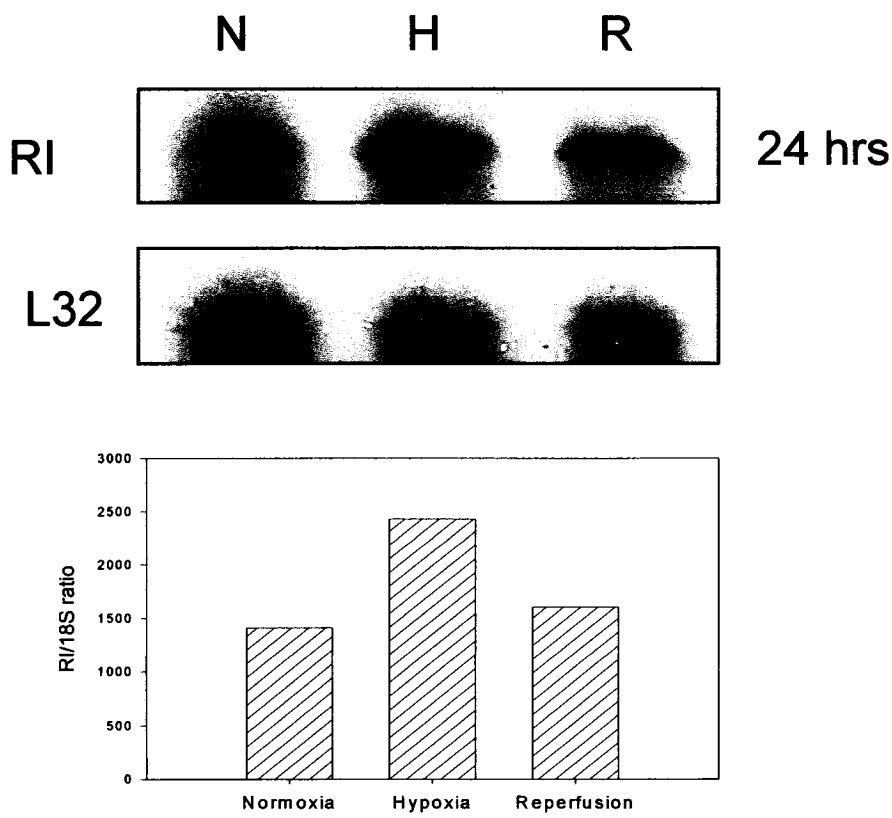

N
H
R
RI
24 hrs
L32
RI/18S ratio
3000
2500
2000
1500
1000
500
0
Normoxia
Hypoxia
Reperfusion

Figure. 8

Table 3. A representative expression (%) of RI and RII mRNAs in human primary skin fibroblasts exposed to hypoxia (H) for 2, 24, and 48 hours as compared to normoxia (N), using Northern blot and RNase protection assay as described in Materials and Methods.

	Hypoxia		
hours	2	24	48
RI (%)		172 ↑	
RII (%)	543 ↑	5 ↓	47 ↓

condition had down regulatory effect on the increased expression of RI receptor mRNA. The regulation of RI, and RII mRNA expressions by hypoxia is summarized in Table 3.

4.2.1.3.2. keratinocytes (HaCat)

To determine whether hypoxia has the same regulatory effect on expression of RI, and RII mRNAs in another skin cell type, HaCat cells (an immortalized human keratinocytes cell line) were grown at normoxia (N) or hypoxia (H) for 2, 4, 24, or 48 hours. As shown in Figure 9A, Northern blot analysis revealed that similar to skin fibroblasts HaCat cells grown at hypoxic condition for 2 hours express higher level of RII mRNA as compared to normoxic condition. Similar result was obtained using RNase protection assay for cells grown at hypoxia for 4 hours (Figure 9B). In contrast, exposure of keratinocytes to 24 hours or 48 hours hypoxia resulted in down regulation of RII mRNA, similar to what was observed in skin fibroblasts (Figure 9C and Figure 9D). As shown in Figure 9E, and 9F, RNA protection assay analysis revealed that RI mRNA was upregulated at 4, or 24 hours of hypoxia and down regulated at 48 hours of hypoxia (Figure 9G). The above observations are summarized in Table 4.

4.2.1.4. TGF-β receptor protein expression profiles in human skin fibroblasts

The TGF-β receptor profiles of human primary skin fibroblasts labeled with ^{125}I - TGF-β1 are illustrated in Figure 10A. Cells were labeled by incubation with ^{125}I-TGF-β1 at 4°C for 3 hours. The receptor-ligand complexes were then cross-linked with 1mM

Figure 9. *Hypoxia (2, 4, 24, or 48 hours) modulates expression of RI, and RII mRNAs in human keratinocyte (HaCat).*

HaCat cells were cultured with serum free medium over night. The medium was changed again with serum free medium at 3 hours prior to the incubation of cell in normoxia (N) (20% oxygen) or hypoxia (H) (< 0.2% oxygen) for 2 hours. Total RNA was extracted using TRIZOL reagent. From each sample 20 μg of RNA was analyzed for 2 hours hypoxic treatment by Northern blot using specific radiolabeled probes for RII mRNA and 18S ribosomal RNA. The 18S ribosomal RNA is shown to demonstrate equal loading of RNA (bottom panel). The results shown are representative of at least three different experiments. For cells treated with hypoxia for 4, 24, or 48 hours, RNA protection assay was used for detection of RI, RII, and L32 mRNAs. From each sample 15 μg of RNA was analyzed. The L32 mRNA is shown to demonstrate equal loading of RNA (bottom panel).

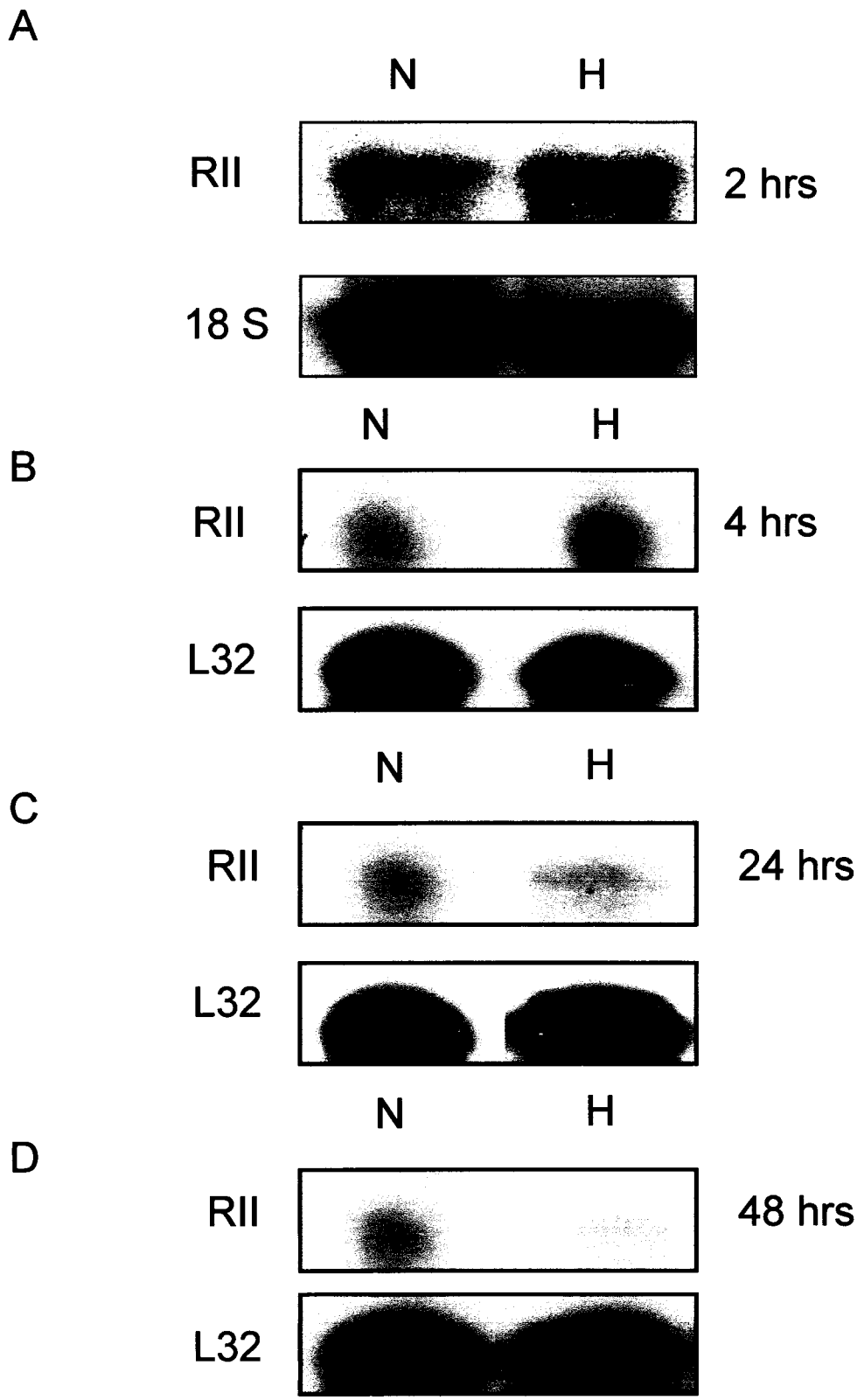

A
N
H
RII
2 hrs
18 S
B
N
H
RII
4 hrs
L32
C
N
H
RII
24 hrs
L32
D
N
H
RII
48 hrs
L32

Figure. 9

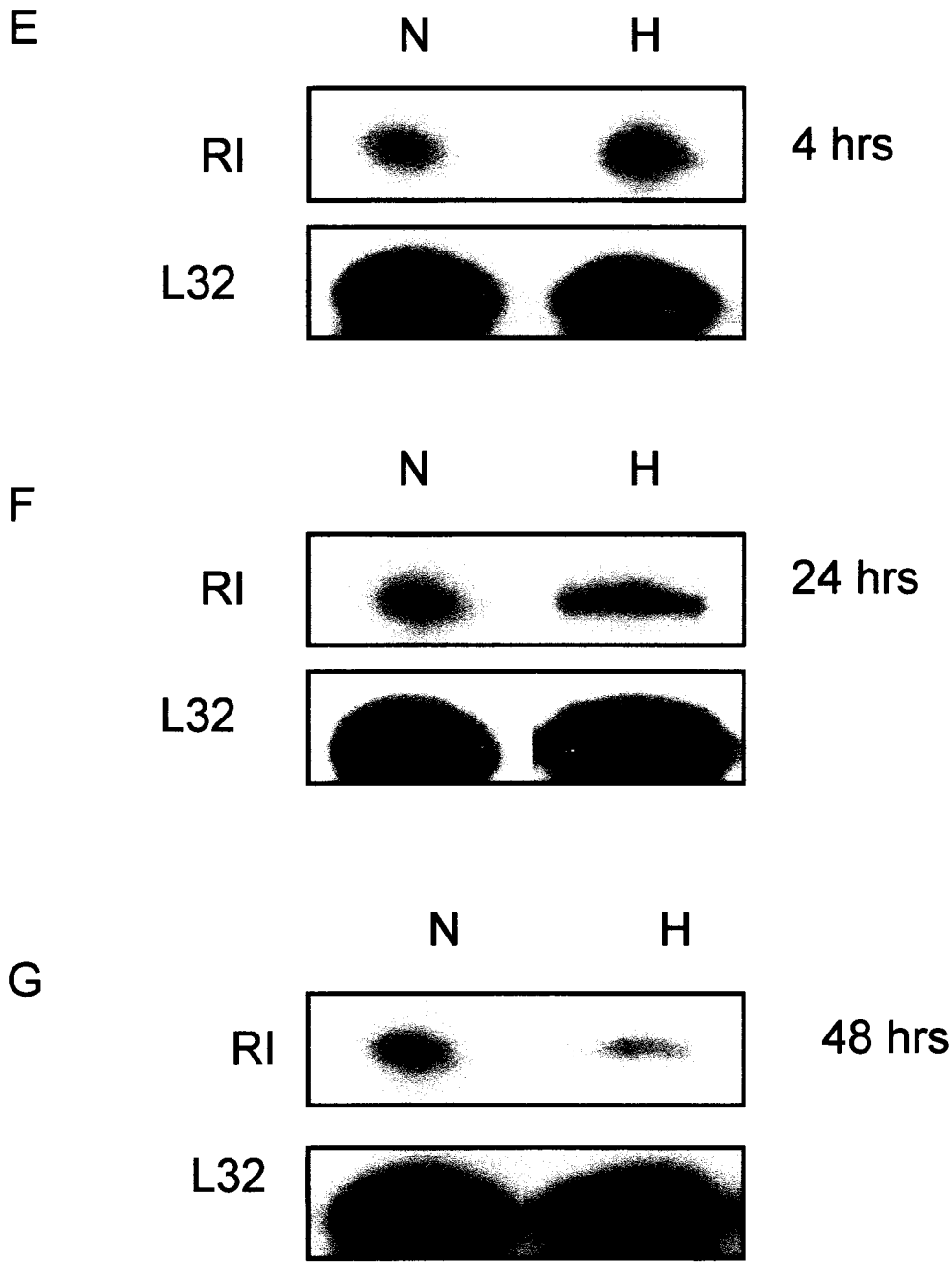


Figure. 9

Table 4. A representative expression (%) of RI and RII mRNAs in human keratinocytes (HaCat) exposed to hypoxia (H) for 2, 24, and 48 hours as compared to normoxia (N), using Northern blot and RNase protection assay as described in Materials and Methods.

	Hypoxia		
hours	2	24	48
RI (%)	144 ↑	136 ↑	71 ↓
RII (%)	188 ↑	93 ↓	52 ↓

Bis (Sulfocsuccinimidyl) suberate (BS3) which has a spacer arm length of 11.4 A°. Upon labeling of fibroblast cells with ^{125}I TGF-β1 with no addition of unlabeled TGF-β, three major binding complexes of relative molecular weights of 65 KDa, 85 kDa, and 200-300 kDa are observed when analyzed under reducing conditions. The observed binding complex corresponds to the cloned type I, II and III TGF-β receptors (Segarini et al, 1989). The binding affinity of the receptor types to TGF-β1 was studied by competition experiments using increasing concentrations of the unlabeled TGF-β1 isoform. The results indicate that TGF-β receptors: RI, RII and RIII proteins in human primary skin fibroblasts display high affinity for TGF-β1. Quantitative densitometry was done to create competition curves from these data (Figure 10B).

4.2.1.5. Regulation of TGF-β receptors proteins by hypoxia (24 hours) and reoxygenation (2 hours) in human skin fibroblasts

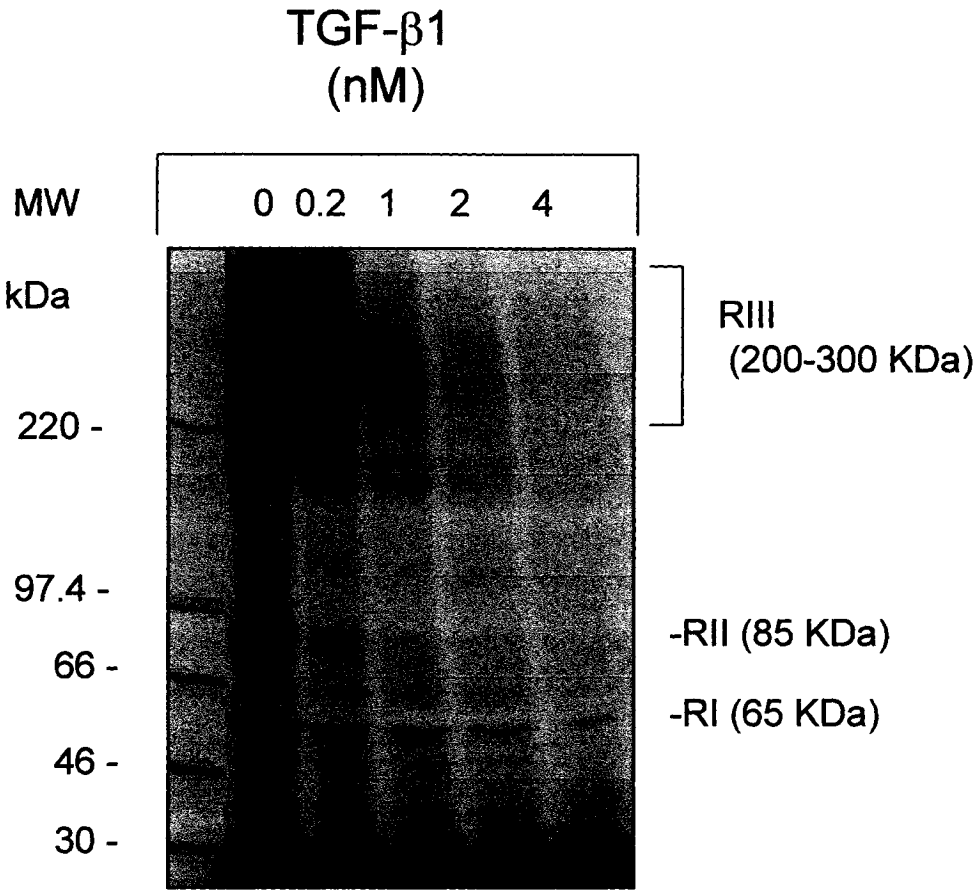
24 hour treatment of human primary skin fibroblasts with hypoxia decreased expression of TGF-β receptors: RI, RII, and RIII (Figure 11A). Quantitative densitometry was done to create comparison curves from these data (Figure 11B). RIII protein showed a dramatic decrease upon treatment with hypoxia. Reexposing the cells to oxygen (reoxygenated condition) for 2 hours reversed this decrease to a significant extent in expression. RI, and RII showed a clear down regulation at hypoxia as compared to normoxia. Two hours of reoxygenation was not able to reverse the down-

Figure 10. *Affinity labeling and competition curves of early passage of human primary skin fibroblasts for RI, RII, and RIII with ^{125}I-TGF-β1.*

Confluent monolayers were affinity labeled with 200 pM ^{125}I-TGF-β1 in the absence or presence of the indicated concentrations of unlabeled TGF-β1 (A). solubilized extracts of cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

The densitometric analysis of autoradiogram was performed to compare the expressions of TGF-β1 receptors: RI, RII, and RIII at different concentration of unlabeled TGF-β1 (bottom panel). The data for each binding component are expressed as a percentage of control (incubated with ^{125}I-TGF-β1 alone). The results shown are representative of at least three different experiments.

A


TGF-β1
(nM)
MW
0 0.2 1 2 4
kDa
220 -
97.4 -
66 -
46 -
30 -
RIII
(200-300 KDa)
-RII (85 KDa)
-RI (65 KDa)

B

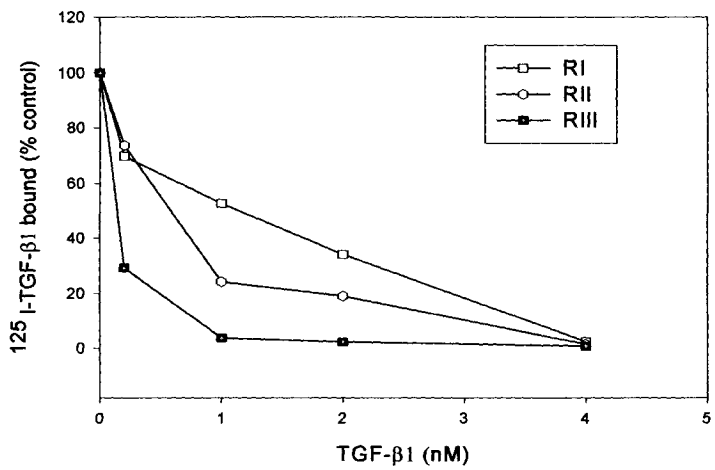

120
100
80
60
40
20
0
125 I-TGF-β1 bound (% control)
RI
RII
RIII
0
1
2
3
4
5
TGF-β1 (nM)

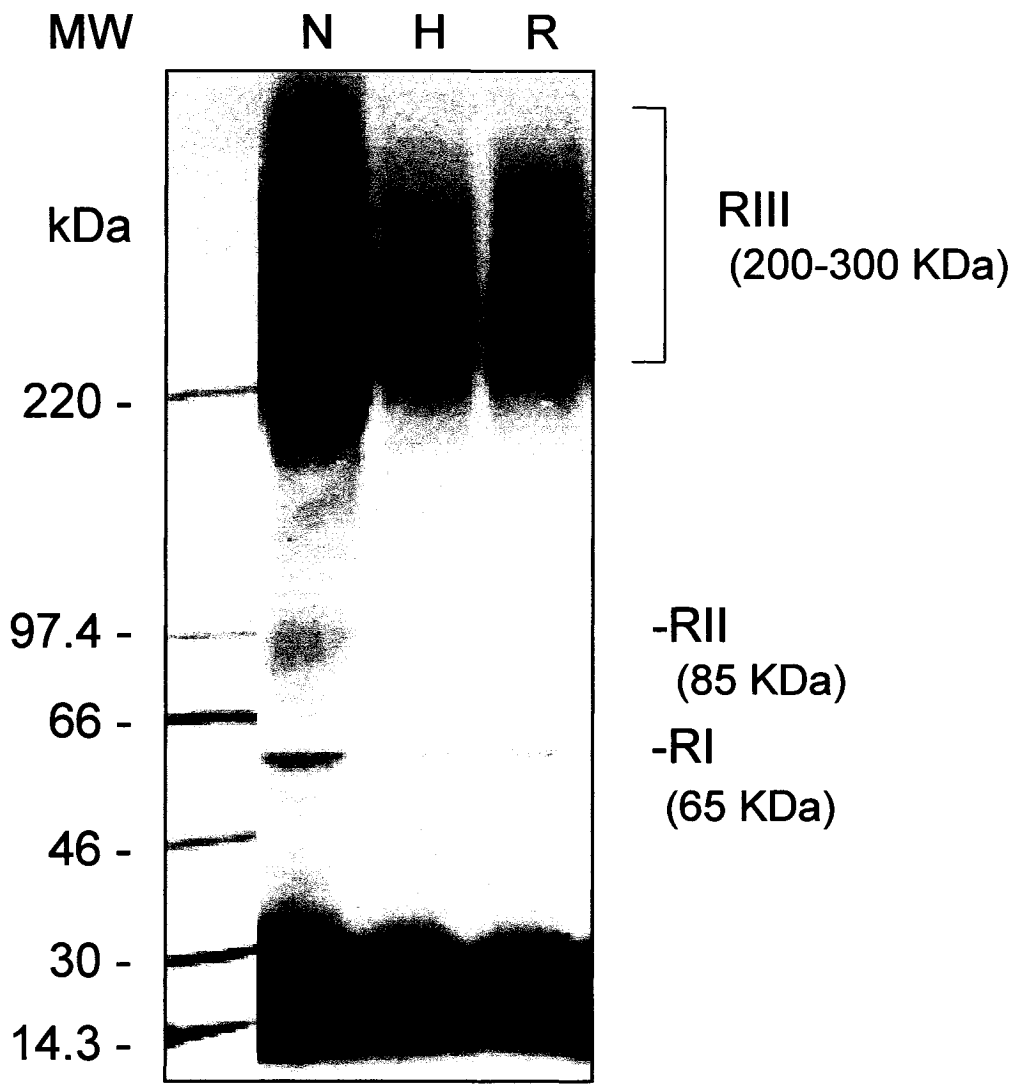
Figure. 10

Figure 11. *Affinity labeling and densitometric analysis of TGF-β receptors after exposure to hypoxia (24 hours) and reoxygenation (2 hours) in human primary skin fibroblasts.*

Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior the incubation of cells at normoxia (N) (20% oxygen), or hypoxia (H) (< 0.2% oxygen), for 24 hours. For reoxygenation (R), cells that were exposed to hypoxia for 24 hours were incubated in normoxic condition for an additional of 2 hours. Confluent monolayers were affinity labeled with 200 pM ^{125}I-TGF-β1. Solubilized cell extracts were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

The densitometric analysis of autoradiogram was performed to compare the expressions of TGF-β1 receptors: RI, RII, and RIII at Normoxia, Hypoxia, and reoxygenation (bottom panel). The data for each binding component are expressed as percent relative to the value in normoxic wells. The results shown are representative of at least three different experiments.

A


MW
N
H
R
kDa
220 -
97.4 -
66 -
46 -
30 -
14.3 -
RIII
(200-300 KDa)
-RII
(85 KDa)
-RI
(65 KDa)

B

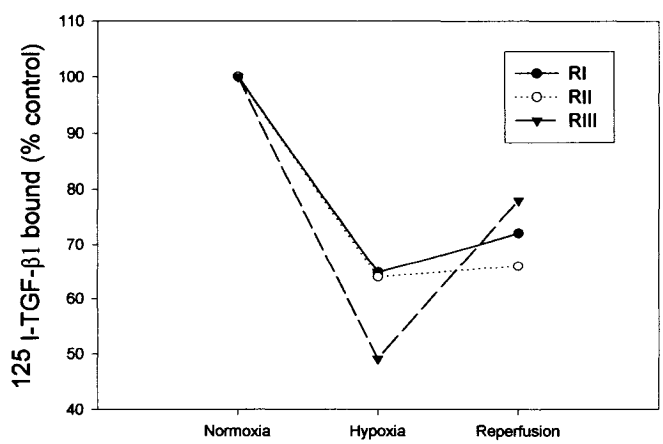

125 I-TGF-β1 bound (% control)
110
100
90
80
70
60
50
40
RI
RII
RIII
Normoxia
Hypoxia
Reperfusion

Figure. 11

regulatory effect of hypoxia to any significant extent. The data which indicate a decrease in RII at protein level by 24 hour exposure to hypoxia is consistent with the downregulation observed for RII mRNA at 24 hours.

4.2.1.6. Regulation of phosphorylated and total Smad2 and Smad3 by hypoxia (1hour, or 24 hours) and reoxygenation (2 hours) in human skin fibroblasts

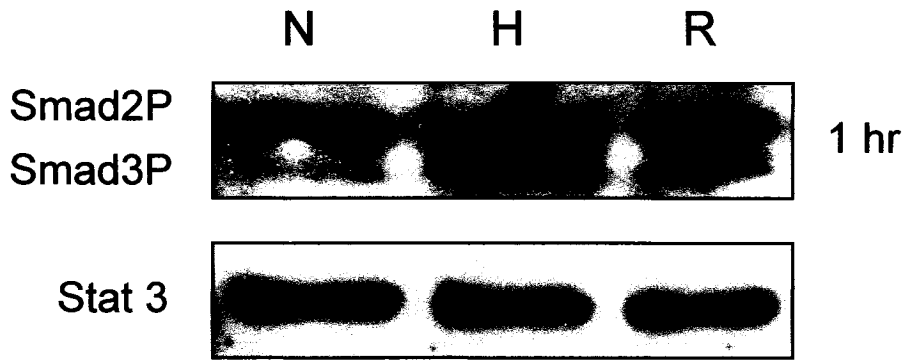
4.2.1.6. 1. Exposure to 1 hour hypoxia

To study the effect of hypoxia on down stream TGF-β signaling pathway, the phosphorylation state of Smad2 and Smad3, total Smad2 and total Smad3 were analyzed in human primary skin fibroblasts. As shown in Figure 12A, treatment of skin fibroblasts with hypoxia for 1 hour, leads to an increase in phosphorylated Smad2 (Smad2P) and phosphorylated Smad3 (Smad3P) and total Smad2 while total Smad3 remained unchanged. In reoxygenated cells, no changes were observed for Smad2P, Smad3P and total Smad2 as compared to hypoxic condition. However, total Smad3 increased significantly. As a result, Smad2P, Smad3P, and total Smad2 and total Smad3 in reoxygenated cells were still higher than in normoxic cells. Similar results were obtained after 30 minutes of hypoxia. The bottom panel shows Western blot for Stat3 to demonstrate equal loading of proteins.

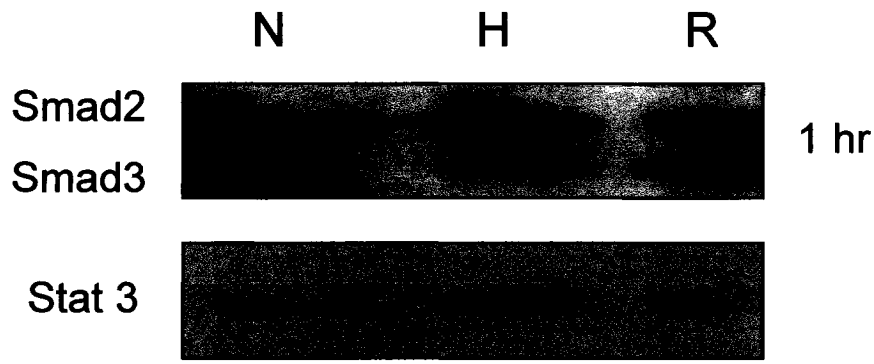
Figure 12. *Dynamic regulation of phosphorylated and total Smad2 and Smad3 by hypoxia (1 hour) and 2 hours of reoxygenation.*

Human primary skin fibroblasts were cultured in DMEM fetal bovine serum (FBS) free medium for 24 hours. The medium was changed again with serum free medium at 3 hours prior the incubation of cells at normoxia (N) (20% oxygen), or hypoxia (H) (< 0.2% oxygen) for 1 hour. For reoxygenated condition (R), cells were grown at normoxia for 2 hours following 1 hour hypoxic treatment. Total cell lysates were separated by SDS-polyacrylamide gel electrophoresis, and resolved proteins were transferred to a nitrocellulose membrane for Western blotting analysis. Immunoblotting was performed using a rabbit polyclonal antibody specific to the phosphorylated Smad2 (Smad2P) and Smad3 (Smad3P), or total Smad2 and Smad3 which recognizes both phosphorylated and unphosphorylated Smad2 and Smad3 (Santa Cruz). The same nitrocellulose membrane was reblotted with an anti-Stat3 antibody (Stat3) (Santa Cruz) to demonstrate equal protein loading. This data is representative of at least three different experiments.

A



B



	1 hr			
Smad/Stat3	Smad 2P	Smad 3P	Smad 2	Smad 3
H vs N	H↑	H↑	H↑	-
R vs N	R↑	R↑	R↑	R↑

↑ = increase
- = no change

Figure. 12

4.2.1.6.2. Exposure to 24 hour hypoxia

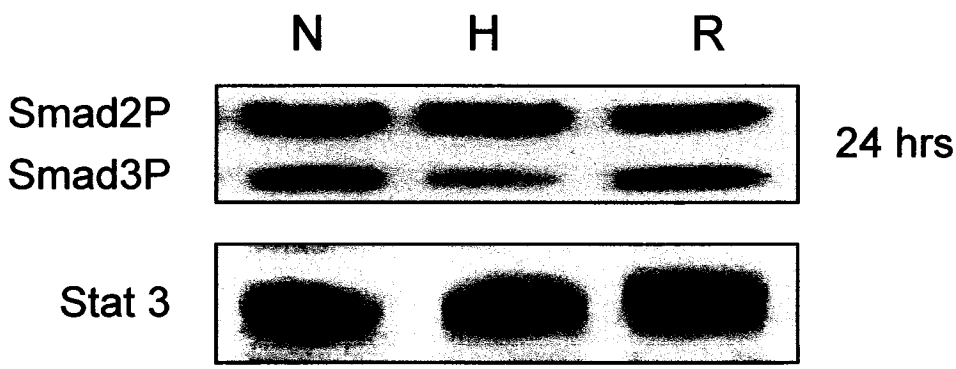
To study the effect of a longer period of hypoxia on phosphorylated and total Smad2 and Smad3, skin fibroblasts were treated with hypoxia for 24 hours with or without reoxygenation for 2 hours. 24 hour exposure to hypoxia decreased Smad3P with no change on Smad2P as compared to normoxia in skin fibroblasts (Figure 13A). There was a small increase in total Smad3 but not in total Smad2.

Reoxygenation counteracted the effect of 24 hour exposure of hypoxia on the Smad3P and total Smad3 and the levels of Smads proteins at reperfusion were similar to the values of Smads protein at normoxia. The bottom panel shows Western blot for Stat3 to demonstrate equal loading of protein.

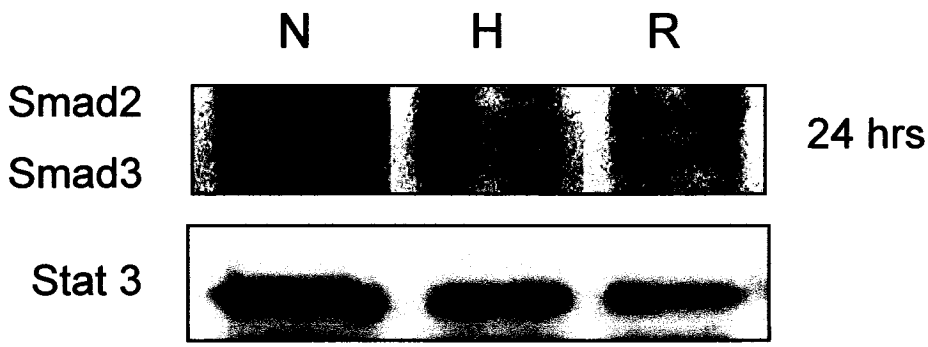
Figure 13. *Regulation of phosphorylated and total Smad2 and Smad3 by hypoxia (24 hour) and 2 hours of reoxygenation.*

Human primary skin fibroblasts were cultured in DMEM fetal bovine serum (FBS) free medium for 24 hours. The medium was changed again with serum free medium 3 hours prior the incubation of cells at normoxia (N) (20% oxygen), or hypoxia (H) (< 0.2% oxygen) for 24 hours. For reoxygenated condition (R), cells were grown at normoxia for 2 hours following a 24 hour hypoxic treatment. Immunoblotting was performed using a rabbit polyclonal antibody specific to the phosphorylated Smad2 (Smad2P) and Smad3 (Smad3P), or total Smad2 and Smad3 which recognizes both phosphorylated and unphosphorylated Smad2 and Smad3 (Santa Cruz). The same nitrocellulose membrane was reblotted with an anti-Stat3 antibody (Stat3) to demonstrate equal protein loading. The results shown are representative of at least three different experiments.

A



B



	24 hrs			
Smad/Stat3	Smad 2P	Smad 3P	Smad 2	Smad 3
H vs N	-	H↓	-	H↑
R vs N	-	-	-	-

↑ = increase
↓ = decrease
- = no change

Figure 13

4.3. Modulation of TGF-β Signaling Pathway by Steroids in Skin Cells

4.3.1. RESULTS

4.3.1.1. Steroids treatment (2, or 24 hours) regulate TGF-β levels

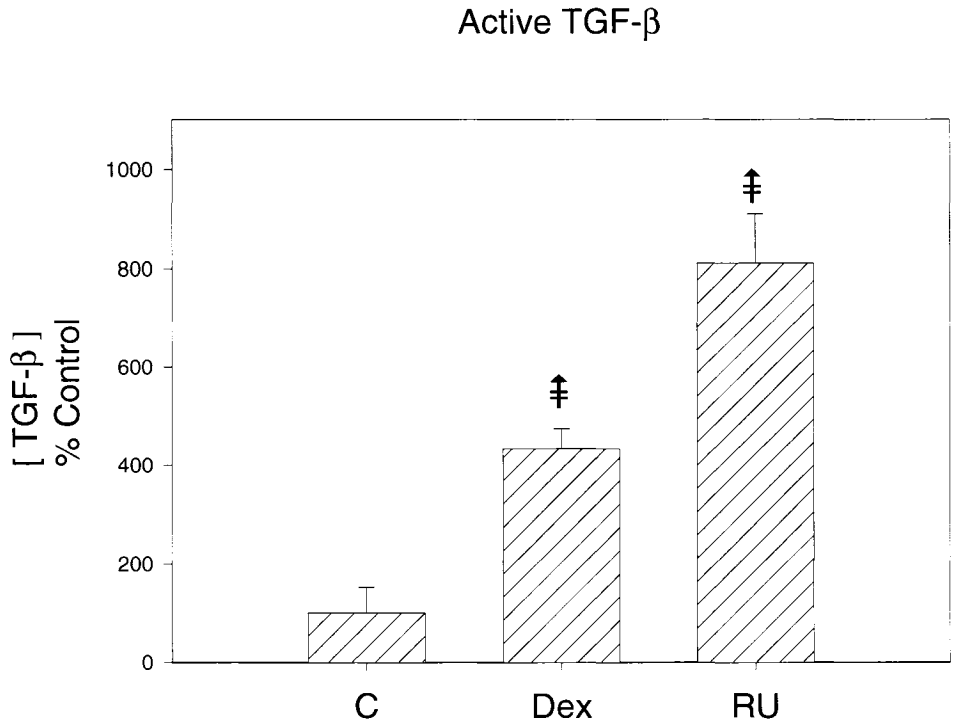
In order to determine if steroids have any effect on the concentration of TGF-β, human primary skin fibroblasts were treated at normoxia with 100 nM of steroids namely: dexamethasone (DEX), RU486 (RU), estrogen (EST), or tamoxifen (TAM) for 2 or 24 hours. As demonstrated in Figure 14A, there was a significant increase in concentration of active TGF-β in the cells treated with either dexamethasone ($P<0.001$), or RU486 ($P<0.001$) for 2 hours. The concentration of total (latent + active) TGF-β was also significantly upregulated at 2 hours of treatment with either dexamethasone, or RU486 ($P<0.005$) (Figure 14B). Dexamethasone treatment for 2 hours resulted in a 4.1-fold increase in active TGF-β while total TGF-β increased only by 2.4- fold. Similarly RU486 treatment resulted in a 7.6- fold increase in active TGF-β with a 2.5- fold increase in total TGF-β. These data indicate that dexamethasone and RU486 treatment result in enhanced activation of TGF-β although total production is also increased.

At 24 hours of treatment, as shown in Figure 14C, there was a significant decrease in concentration of active TGF-β in the cells treated with dexamethasone ($P<0.001$), or RU486 ($P<0.02$). Although there was a decrease in total TGF-β after 24 hour treatment with dexamethasone and RU486, this decrease was significant only for RU486 ($P<0.01$) (Figure 14D). Dexamethasone treatment for 24 hours resulted in a 1.5-fold decrease in active TGF-β and a 1.4- fold decrease in total TGF-β.

Figure 14. *Detection of active and total TGF-β in human primary skin fibroblasts after treatment with steroids (2, or 24 hours).*

Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior the treatment of cells with 100 nM of dexamethasone (Dex), RU486 (RU) (2 or 24 hours), estrogen (Est) **(E,F)**, or tamoxifen (Tam) **(G,H)** (24 hours) at normoxic condition. Control cells (C) received no treatment. The conditioned medium was collected in siliconized tubes containing a protease inhibitor cocktail: 10ul phenylmethylsulfonyl fluoride (PMSF), and 20 µl protease inhibitor (PI/ml of medium). The concentration of active **(A, C, E)** and total **(B, D, F)** TGF-β in the conditioned medium was measured using the PAI/L assay as described in Materials and Methods in chapter 2. Values for TGF-β concentration were corrected for variations in cell number by measuring total protein using a Biorad assay. Dexamethasone, RU486, estrogen, and tamoxifen treated cells were each compared to control cells grown at normoxia. * $P < 0.05$, ** $P < 0.02$, ↕ $P < 0.01$, ‡ $P < 0.005$, ‡ $P < 0.001$.

A


Active TGF-β
[TGF-β]
% Control
1000
800
600
400
200
0
C
Dex
RU

B

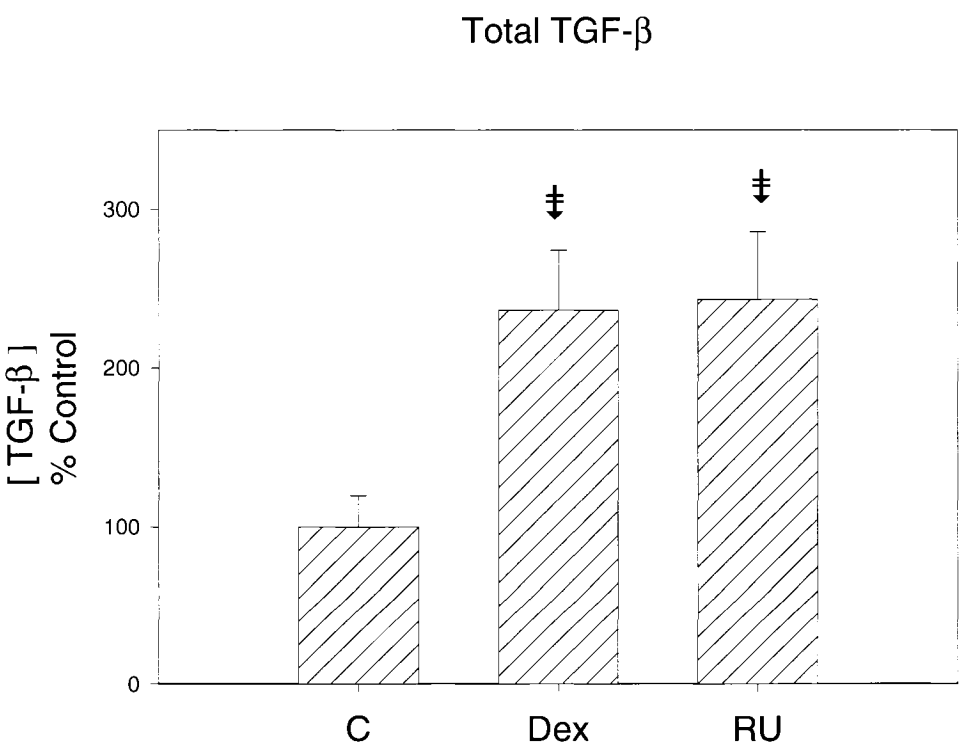
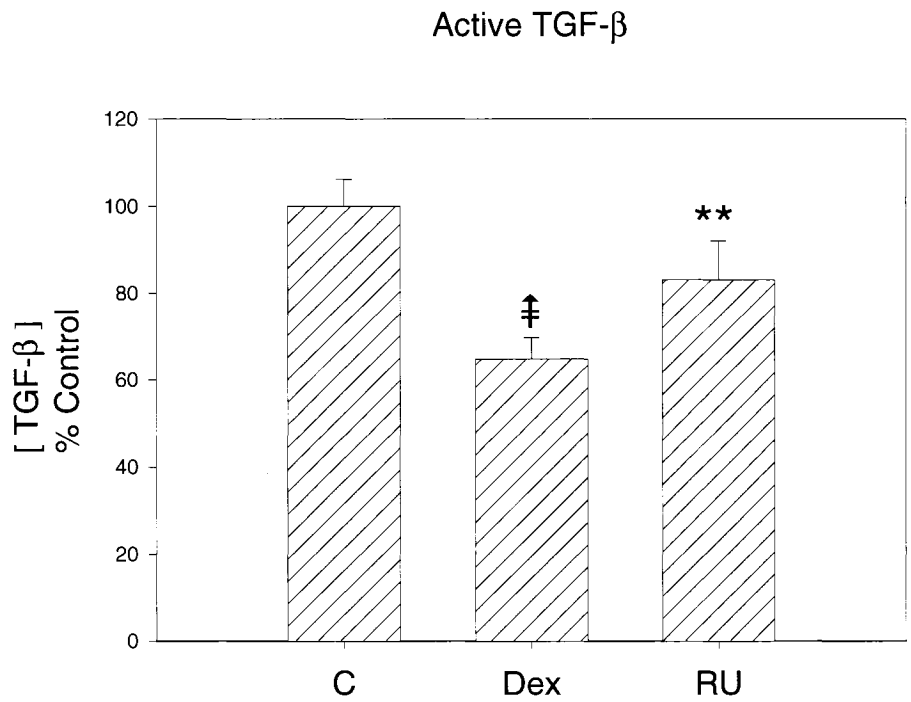

Total TGF-β
[TGF-β]
% Control
300
200
100
0
C
Dex
RU

Figure. 14

C


Active TGF-β
120
100
80
60
40
20
0
[TGF-β]
% Control
**
C
Dex
RU

D

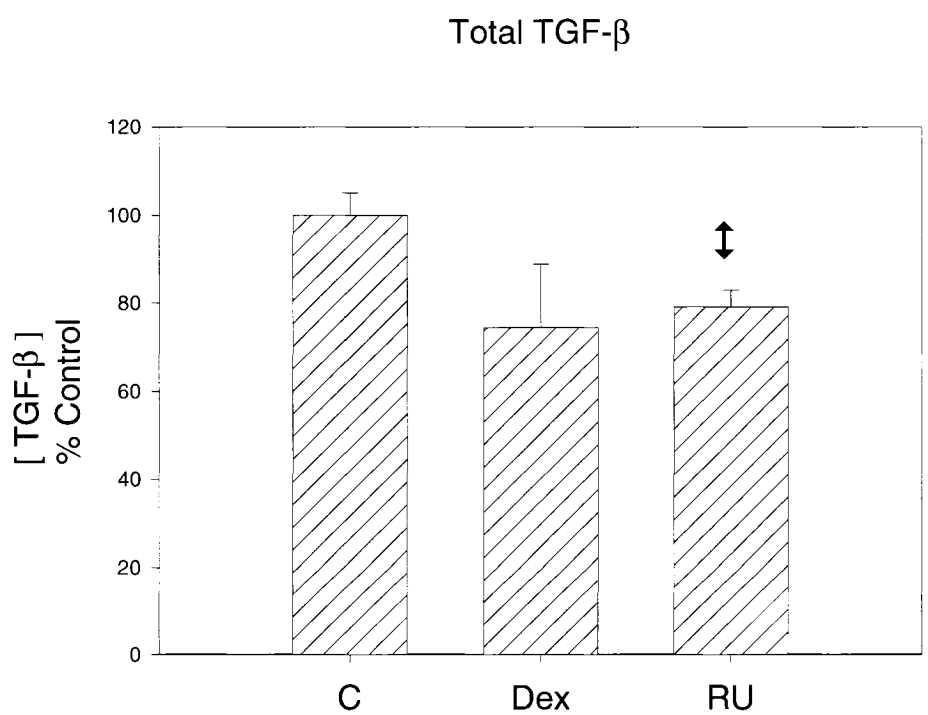
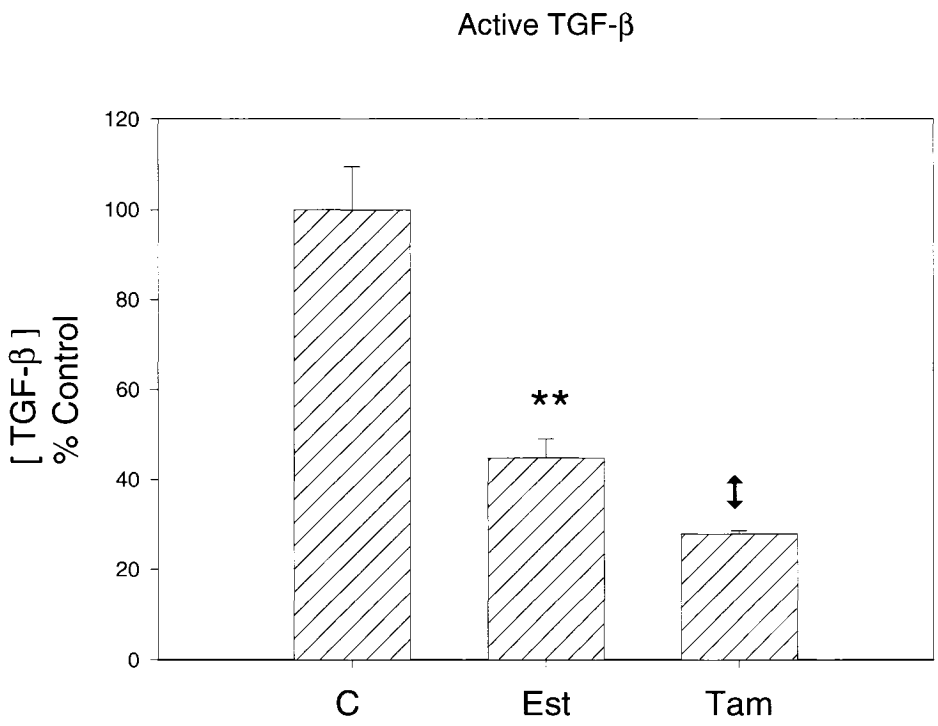

Total TGF-β
120
100
80
60
40
20
0
[TGF-β]
% Control
C
Dex
RU

Figure. 14

E


Active TGF-β
[TGF-β]
% Control
120
100
80
60
40
20
0
**
↕
C
Est
Tam

F

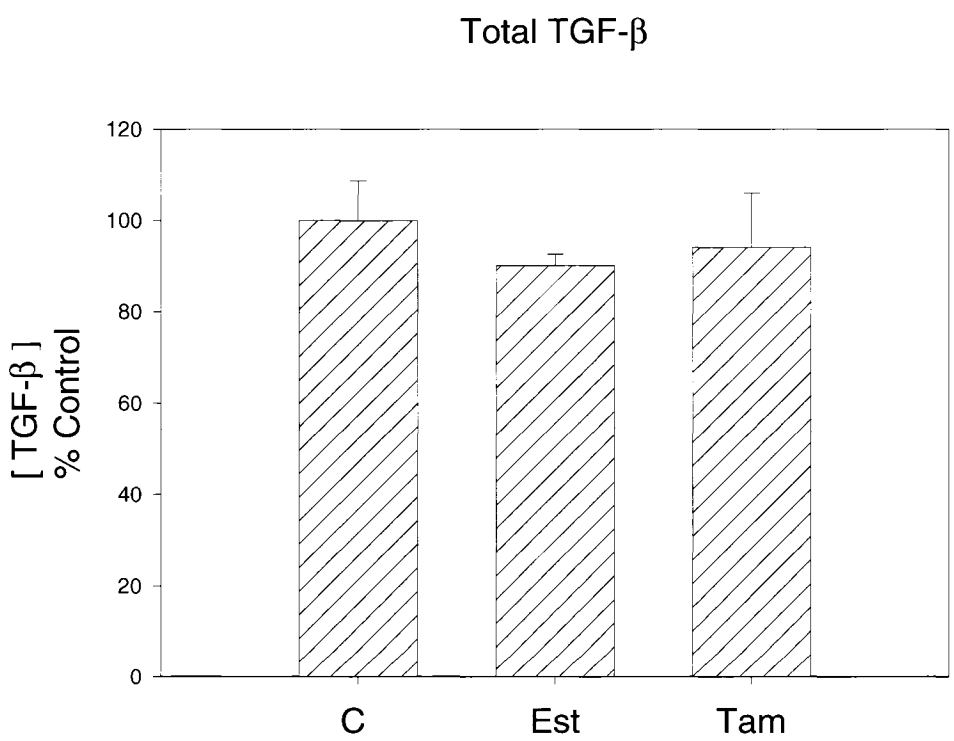

Total TGF-β
[TGF-β]
% Control
120
100
80
60
40
20
0
C
Est
Tam

Figure. 14

Similarly RU486 treatment resulted in a 1.2- fold decrease in active TGF-β with a 1.3-fold decrease in total TGF-β. These data indicate that dexamethasone and RU486 treatment result in inhibition of active and total TGF-β.

Estrogen and tamoxifen treatment resulted in a significant decrease in active TGF-β ($P< 0.02$ and $P<0.01$, respectively) as shown in Figure 14E, while total TGF-β remained unchanged (Figure 14F). Estrogen and tamoxifen treatment resulted in a 2.2- and 3.6- fold decrease, respectively, of active TGF-β levels while total TGF-β concentrations remained unchanged. This suggests that estrogen and tamoxifen inhibit TGF-β activation without changing total TGF-β levels.

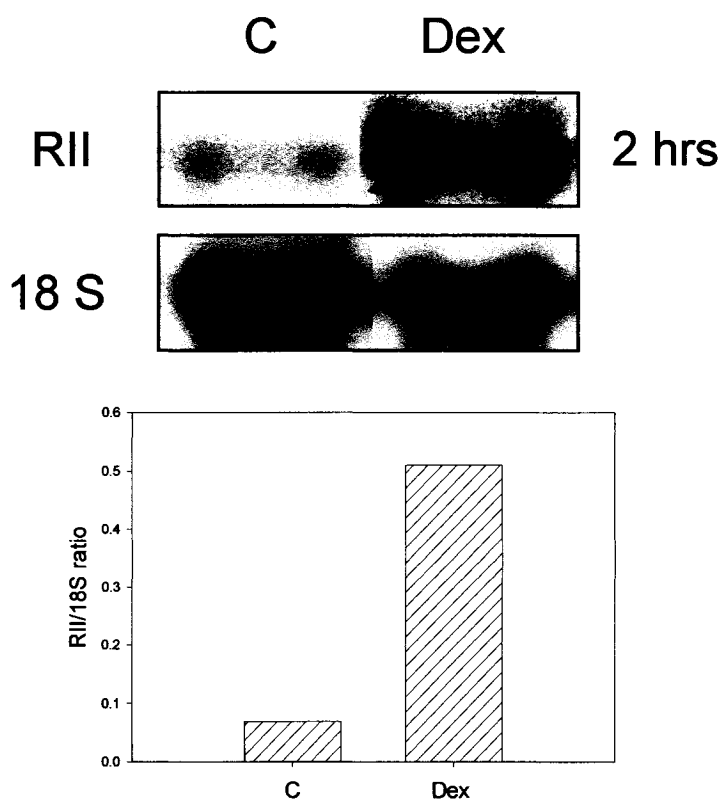
4.3.1.2 Dexamethasone or RU486 treatment (2 hours) regulate RII mRNA expression in human skin fibroblasts and keratinocytes (HaCat)

To determine whether short term exposure to steroids has a role in regulation of RII mRNA, 100 nM of dexamethasone (Dex), or RU486 (RU) was added to the human primary skin fibroblasts for 2 hours. The results indicated that within this short period of time, both dexamethasone, and RU486 were able to upregulate the expression of RII mRNA in human primary skin fibroblasts with dexamethasone having a higher upregulatory effect on expression of RII mRNA than RU486 (Figure: 15A & Figure 15B). Next we determine whether steroids have any similar regulatory effect on the expression of RII mRNA in another cell type, HaCat cells (an immortalized human

Figure 15. *Regulation of RII mRNA expression by dexamethasone or RU486 treatment (2 hours) in human primary skin fibroblasts and keratinocytes (HaCat).*

Both dexamethasone (Dex), and RU486 (RU) upregulated the RII mRNA expression when compared to untreated cells (control, C) under normoxic condition in skin fibroblasts and HaCat cells. Human primary skin fibroblasts and HaCat were cultured in fetal bovine serum (FBS) free medium for 24 hours. The medium was changed again with serum free medium at 3 hours prior to the addition of steroids. Cells were then incubated for 2 hours with 100 nM of steroid (Dex, or RU). Total RNA was extracted using the acid guanidinium isothiocyanate-phenol-chloroform as described in Materials and Methods in chapter 2. 20 μg of RNA from each sample was analyzed by Northern blot using specific radiolabeled probes for RII mRNA and 18S ribosomal RNA. The 18S ribosomal RNA was used to normalize for the loading of RNA (bottom panel). The densitometric analysis of this data is shown at the bottom panel. The results shown are representative of at least three different experiments.

A


C
Dex
RII
2 hrs
18 S
RII/18S ratio
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C
Dex

B

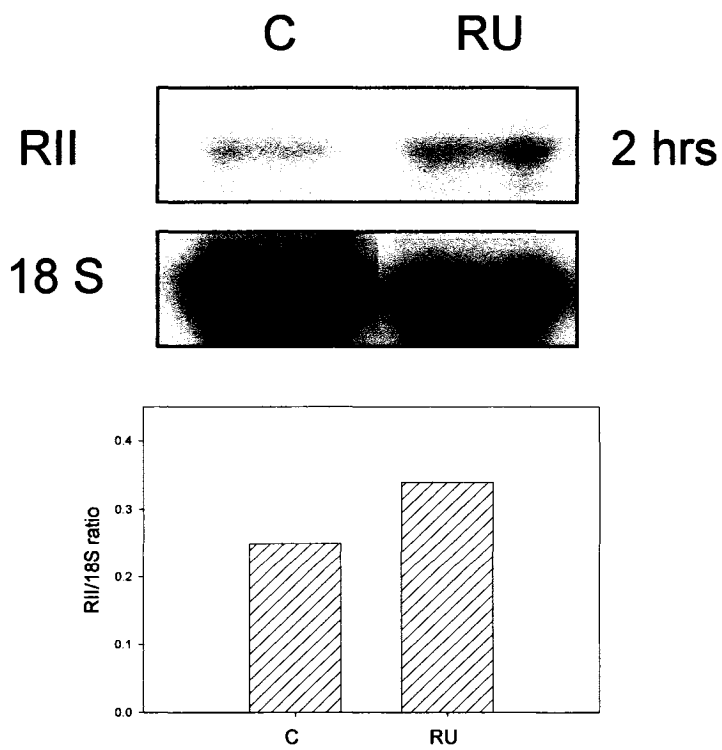

C
RU
RII
2 hrs
18 S
RII/18S ratio
0.4
0.3
0.2
0.1
0.0
C
RU

Figure. 15

C

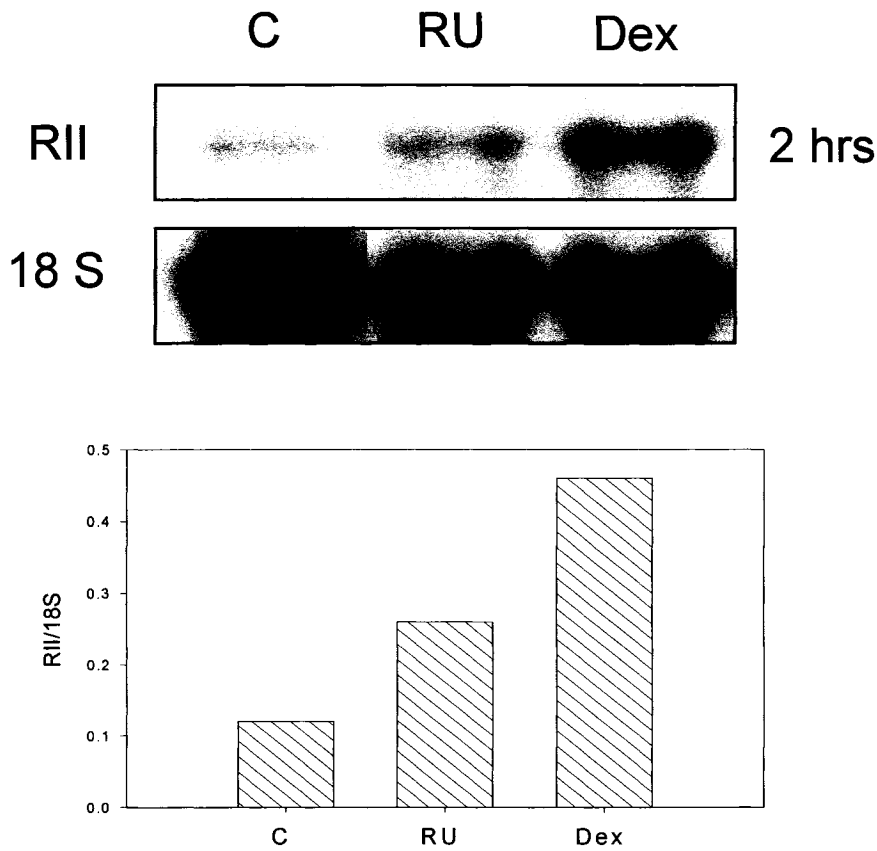


Figure. 15

keratinocyte cell line). Similar to the results obtained for skin fibroblasts, dexamethasone had higher ability to increase the expression of RII mRNA in HaCat cells as compared to RU486 (Figure 15C).

4.3.1.3. Steroids treatment (24 hours) regulate RII mRNA expression in human skin fibroblasts

To determine the effect of steroid treatment for a longer period of time, on the expression of RII mRNA, 100 nM of dexamethasone (Dex), RU486 (RU), estrogen (Est), tamoxifen (Tam), or Testosterone (Tes) was added to human primary skin fibroblasts for 24 hours. The results shown in Figure 16A, and Figure 16B, demonstrate that dexamethasone, RU486, estrogen, and tamoxifen increased the expression of RII mRNA, while, testosterone decreased the expression of RII mRNA (Figure 16C).

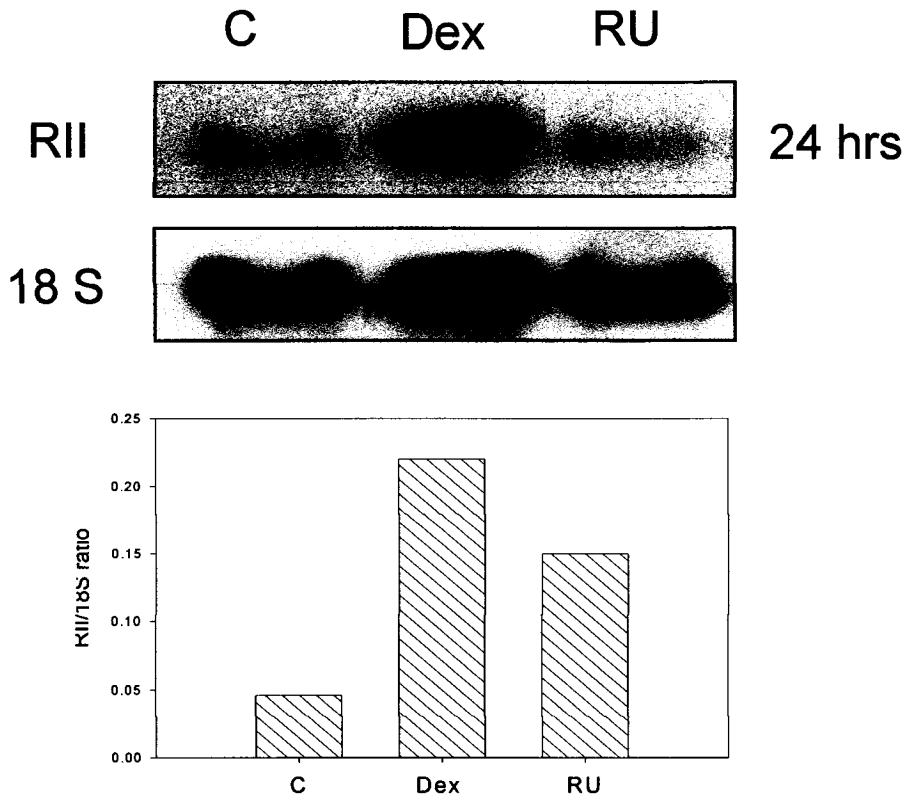
4.3.1.4. Dexamethasone, or RU486 (24 hours) modulate RI mRNA expression in human skin fibroblasts

We then determined the effect of dexamethasone and RU486 (24 hour treatment) on RI expression in human primary skin fibroblasts. RI mRNA was analyzed using RNase protection assay (RPA). As shown in Figure 17, RPA analysis revealed that dexamethasone and RU486 upregulated the expression of RI mRNA as compared to control untreated cells.

Figure 16. *Regulation of RII mRNA expression by dexamethasone, RU486, estrogen, tamoxifen, or testosterone treatment (24 hours) in human primary skin fibroblasts.*

Dexamethasone (Dex), RU486 (RU) (**A**), estrogen (Est), tamoxifen (Tam) (**B**), stimulated while testosterone (Tes) (**C**) inhibited the RII mRNA expression compared to untreated cells (control, C) under normoxic conditions in skin fibroblasts. Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior to the addition of steroids. Cells were then incubated for 24 hours with 100 nM of steroid. Total RNA was extracted using the acid guanidinium isothiocyanate-phenol-chloroform as described in Materials and Methods in chapter 2. 20 μg of RNA from each sample was analyzed by Northern blot using specific radiolabeled probes for RII mRNA and 18S ribosomal RNA. The 18S ribosomal RNA was used to normalize for loading of RNA (bottom panel). The densitometric analysis of this data is shown at the bottom panel. The results shown are representative of at least three different experiments.

A


C
Dex
RU
RII
24 hrs
18 S
RII/18S ratio
0.25
0.20
0.15
0.10
0.05
0.00
C
Dex
RU

B

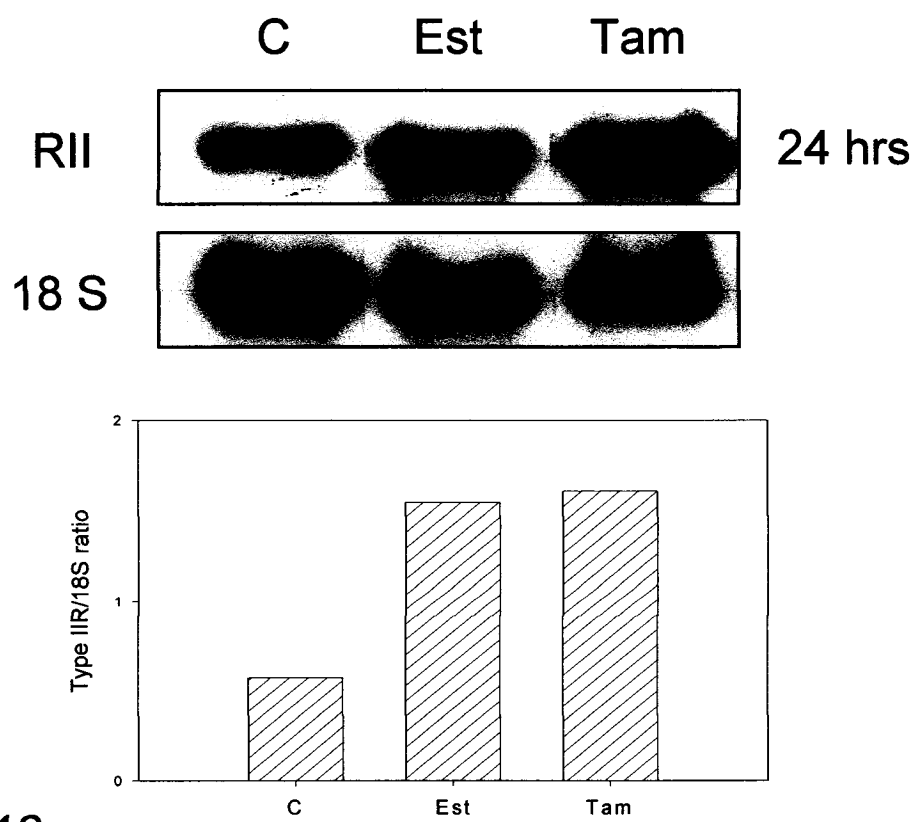

C
Est
Tam
RII
24 hrs
18 S
Type IIR/18S ratio
2
1
0
C
Est
Tam

Figure. 16

C

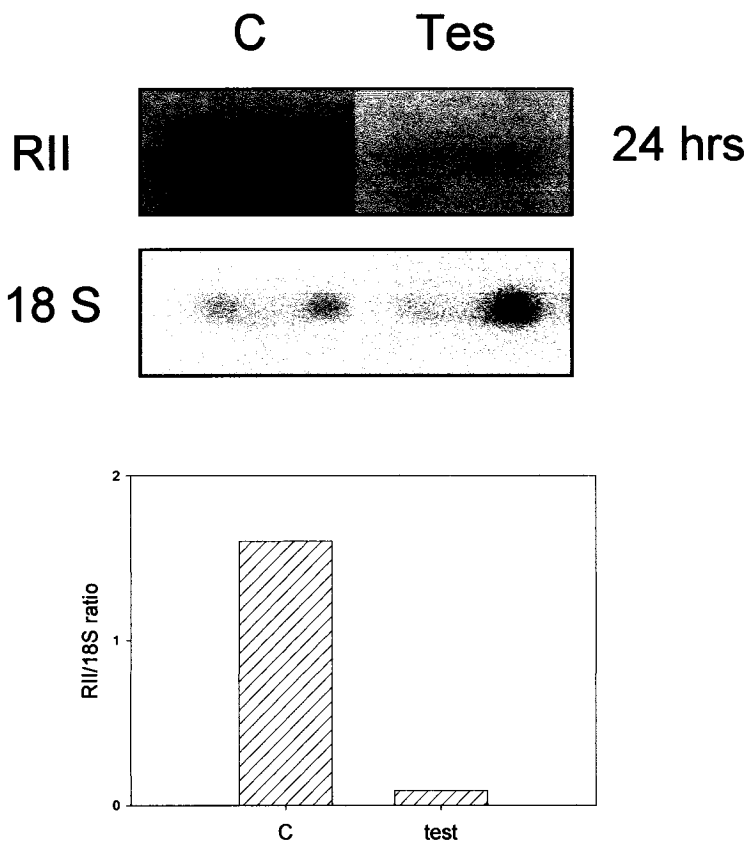


Figure. 16

Figure 17. *Modulation of expression of RI mRNA by dexamethasone or RU486 treatment (24 hours) in human primary skin fibroblasts.*

Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior the treatment of cells with 100 nM of dexamethasone (Dex) or RU486 (RU) for 24 hours at normoxic condition. Control (C) cells received no treatment. Total RNA was extracted using TRIZOL reagent. From each sample 15 μg of RNA was analyzed by Ribonuclease protection assay (RPA) for RI mRNA, and L32 mRNA expression. The L32 mRNA is shown to demonstrate equal loading of RNA (bottom panel). The densitometric analysis of this data is shown at the bottom panel.

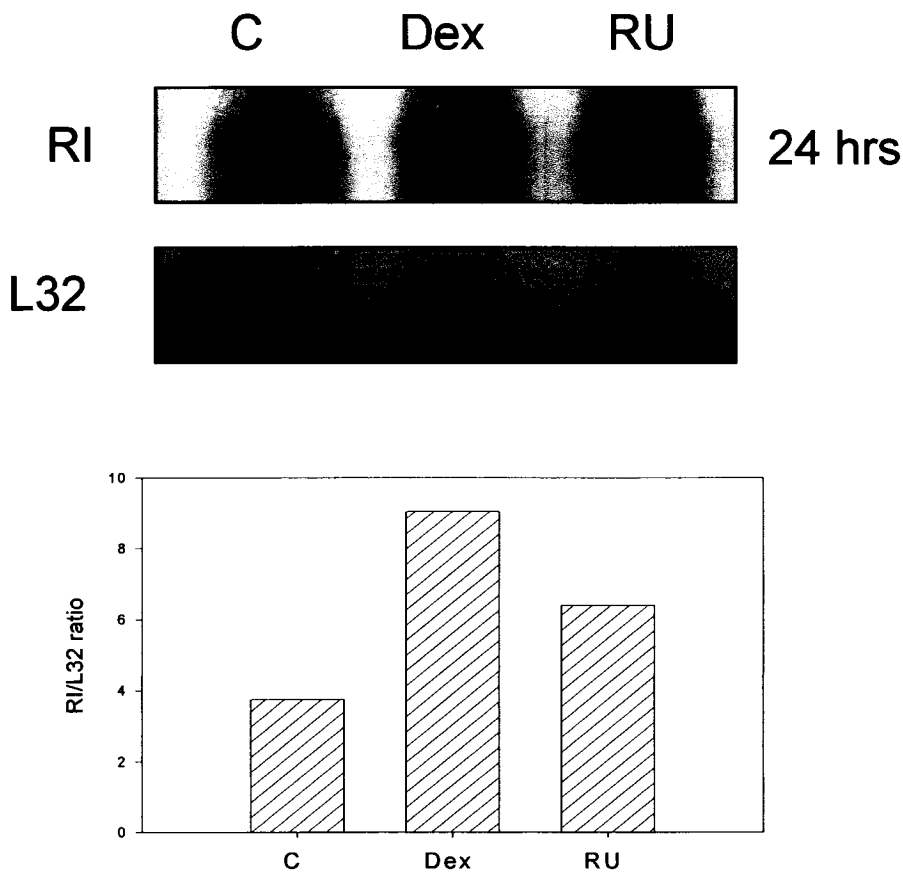


Figure. 17

4.3.1.5. Dexamethasone, or RU486 (24 hours) regulate TGF-β receptor proteins in human skin fibroblasts

Affinity labeling was used to study the effect of steroids (dexamethasone or RU486) on cell surface TGF-β receptor proteins. Human primary skin fibroblasts exposed to either dexamethasone (Dex), or RU486 (RU), for 24 hours showed higher expression for all the three major receptor complexes as compared to control untreated cells as detected by affinity labeling with ^{125}I-TGF-β1 (Figure 18A). Dexamethasone had a greater stimulatory effect on the expression of TGF-β type I, II, and III receptors when compared to RU486. Quantitative densitometry analysis from the above data is shown in Figure 18B.

4.3.1.6. Dexamethasone or RU486 regulate phosphorylated and total Smad2 and Smad3 in human skin fibroblasts.

4.3.1.6.1. Treatment for 1 hour

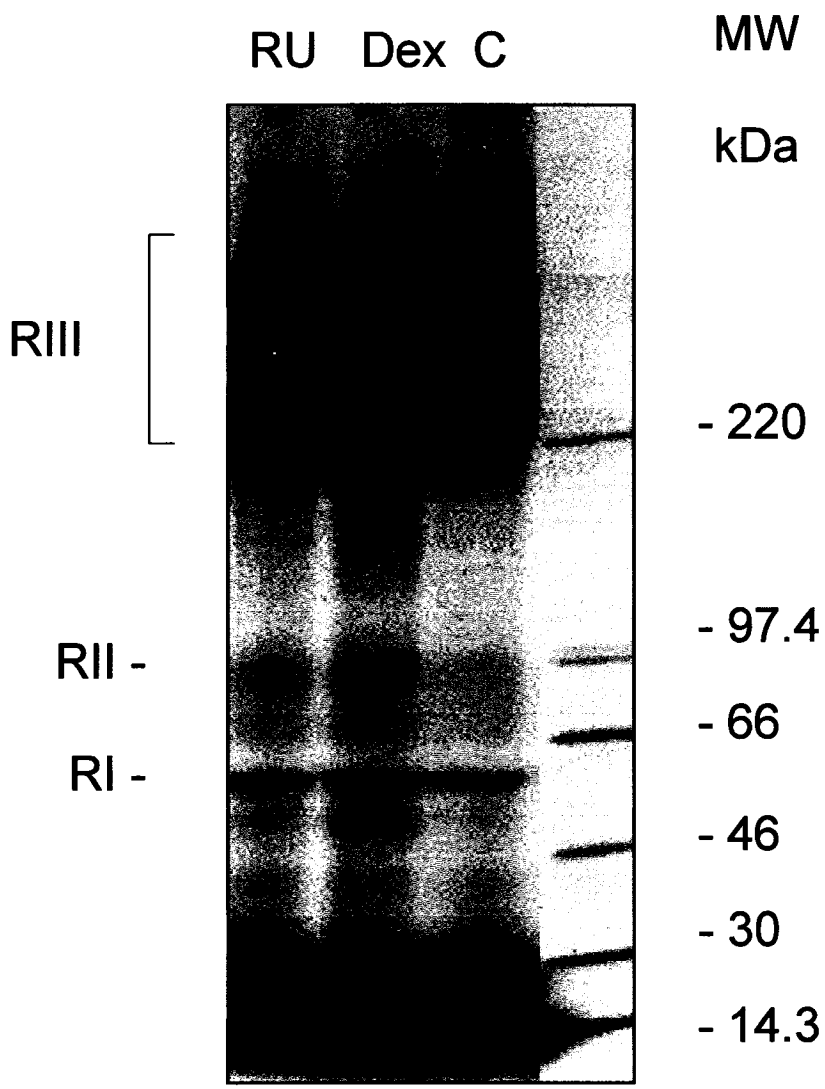
We then analyzed whether dexamethasone (Dex) or RU486 (RU) is able to regulate the active state of Smad2 and Smad3 or total Smad2 and Smad3 in human primary skin fibroblasts. As shown in Figure 19A and Figure 19B, 1 hour treatment of human primary skin fibroblasts with dexamethasone had no detectable effect on Smad2P and Smad3P but increased total Smad2 Smad3 as compared to control cells (C). On the

Figure 18. *Regulation of TGF-β receptor proteins by dexamethasone or RU486 treatment (24 hours) in human primary skin fibroblasts as detected by affinity labeling.*

Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior the treatment of cells with 100 nM of dexamethasone (Dex) or RU486 (RU) for 24 hours at normoxic condition. Control (C) cells received neither treatment. Cells were affinity labeled with ^{125}I-TGF-β1.

The densitometric analysis of autoradiogram was performed to compare the expressions of TGF-β1 receptors: RI, RII, and RIII in control, dexamethasone and RU486 treated cells (bottom panel). The data for each binding component are expressed as percent of control values. The results shown are representative of at least three different experiments.

A


RU
Dex
C
MW
kDa
RIII
RII -
RI -
- 220
- 97.4
- 66
- 46
- 30
- 14.3

B

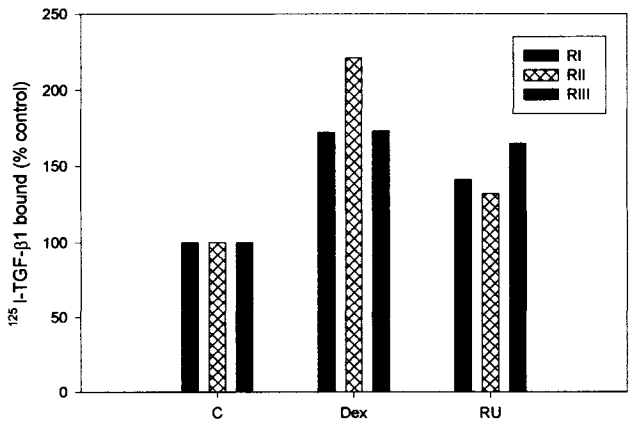
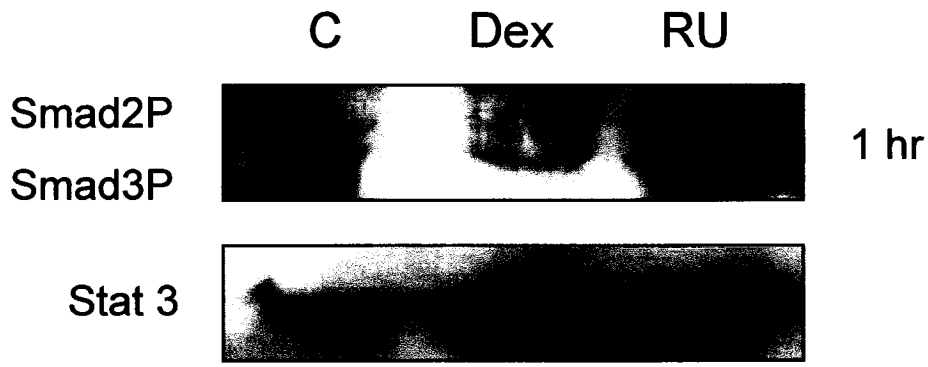

250
200
150
100
50
0
125I-TGF-β1 bound (% control)
RI
RII
RIII
C
Dex
RU

Figure. 18

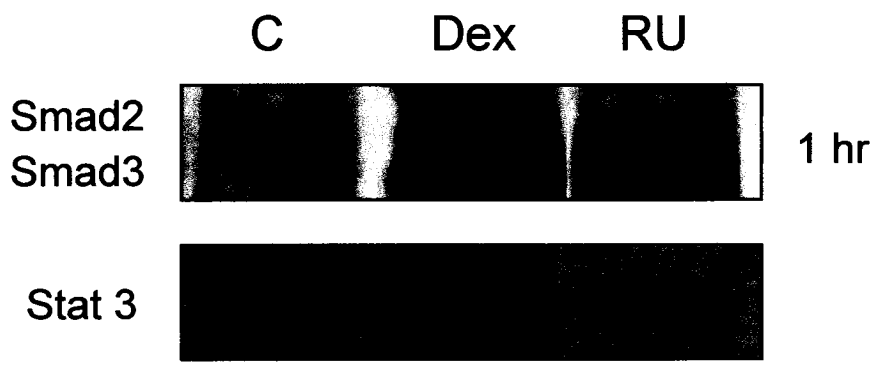
Figure 19. *Dynamic regulation of phosphorylated and total Smad2 and Smad3 by dexamethasone, or RU486 treatment (1 hour) in human primary skin fibroblasts.*

Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior the treatment of cells with 100 nM of dexamethasone (Dex) or RU486 (RU) for 1 hour at normoxic condition. Control (C) cells received no treatment. Immunoblotting was performed using a rabbit polyclonal antibody specific to the phosphorylated Smad2 (Smad2P) and Smad3 (Smad3P) or total Smad2 and Smad3 which recognizes both phosphorylated and unphosphorylated Smad2 and Smad3 (Santa Cruz). The same nitrocellulose membrane was reblotted with an anti-Stat3 antibody (Stat3) to demonstrate equal protein loading. The results shown are representative of at least three different experiments.

A



B



	1hr			
Smad/Stat3	Smad 2P	Smad 3P	Smad 2	Smad 3
Dex vs C	-	-	Dex↑	Dex↑
RU vs C	RU ↑	RU ↑	-	-

↑ = increase
- = no change

Figure. 19

other hand RU486 led to an increase in the Smad2P and Smad3P with no effect on the total Smad2 and Smad3.

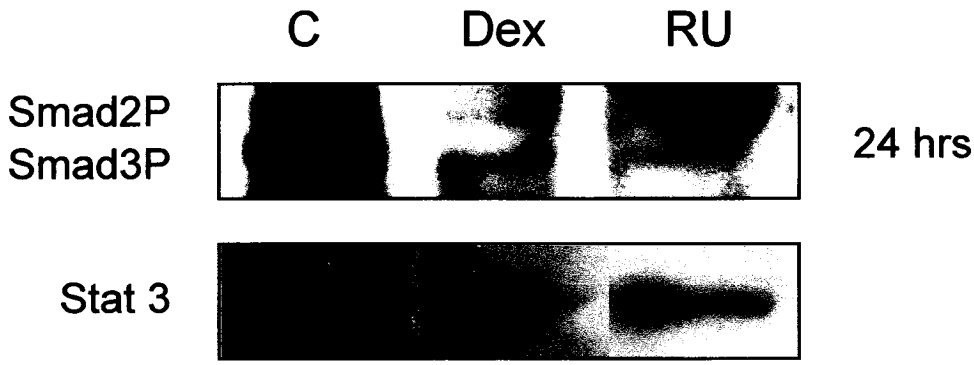
4.3.1.6.2. Treatment for 24 hours

Next we determined whether steroid treatment for 24 hours has any effect on the phosphorylated and total Smad2 and Smad3 in human skin fibroblasts. As shown in Figure 20A, and Figure 20B treatment of human primary skin fibroblasts with dexamethasone or RU486 for 24 hours decreased Smad2P and Smad3P and total Smad2 and total Smad3 as compared to control cells.

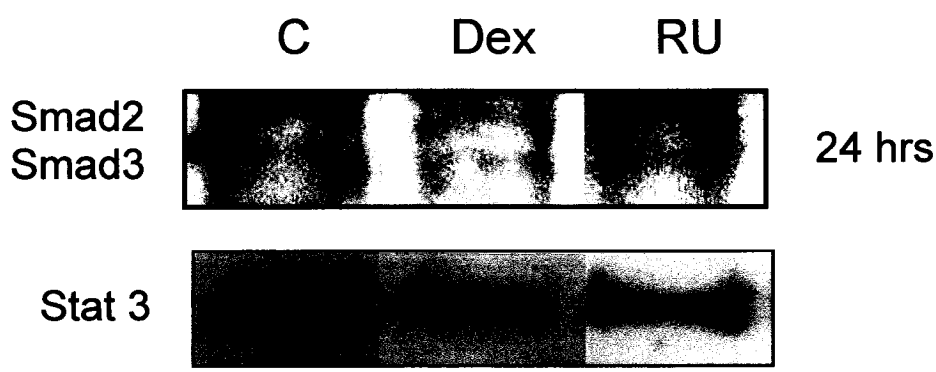
Figure 20. *Regulation of phosphorylated and total Smad2 and Smad3 by dexamethasone or RU486 treatment (24 hours) in human primary skin fibroblasts.*

Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior the treatment of cells with 100 nM of dexamethasone (Dex) or RU486 (RU) for 24 hours at normoxic condition. Control (C) cells received no treatment. Immunoblotting was performed using a rabbit polyclonal antibody specific to the phosphorylated Smad2 (Smad2P) and Smad3 (Smad3P) or total Smad2 and Smad3 which recognizes both phosphorylated and unphosphorylated Smad2 and Smad3 (Santa Cruz). The same nitrocellulose membrane was reblotted with an anti-Stat3 antibody (Stat3) to demonstrate equal protein loading. The results shown are representative of at least three different experiments.

A



B



	24 hr			
Smad/Stat3	Smad 2P	Smad 3P	Smad 2	Smad 3
Dex vs C	Dex↓	Dex↓	Dex↓	Dex↓
RU vs C	RU↓	RU↓	RU↓	RU↓

↓ = decrease

Figure. 20

4.4. Effect of Simultaneous Variation of Oxygen Tension and Steroid Status on the TGF-β Signaling Pathway in Skin Cells

4.4.1. RESULTS

4.4.1.1 The combined effect of 24 hour treatment of both hypoxia and steroids on TGF-β levels in human skin fibroblasts

4.4.1.1.1. Treatment with dexamethasone or RU486

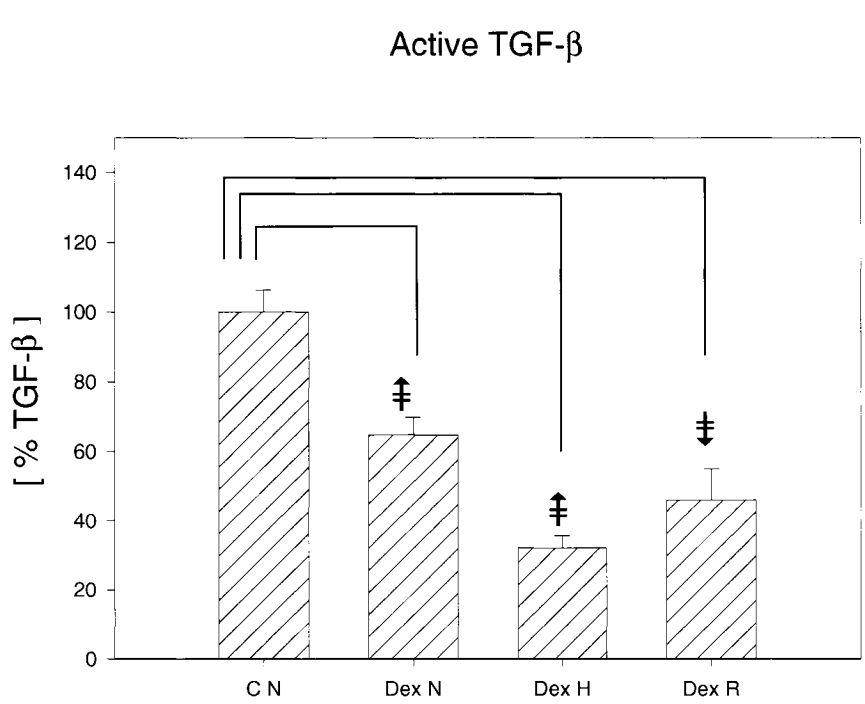
The combined effect of dexamethasone (Dex) or RU486 (RU) (24 hours) and hypoxia (24 hours) on the concentration of TGF-β was studied in human primary skin fibroblasts. As shown in Figure 21A and Figure 21C, there was a significant decrease ($P<0.001$) on the concentration of active TGF-β in cells treated with both hypoxia and steroids (dexamethasone (DEX H) or RU486 (RU H)) as compared to control untreated cells grown in normoxia (CN). Although the concentration of TGF-β increased after 2 hours of reoxygenation (DEX R, and RU R), the concentration of TGF-β was significantly lower as compared to control untreated cells grown at normoxia ($P<0.005$, and $P<0.02$ for Dex R and RU R respectively).

Interestingly, the same pattern was observed for the total TGF-β in human primary skin fibroblasts. There was a significant reduction in total TGF-β in dexamethasone ($P<0.02$) and RU486 ($P<0.02$) treated cells grown at hypoxic condition when compared to control (C) untreated cells. The concentration of TGF-β in reoxygenated condition was significantly lower ($P<0.05$) as compared to control untreated cells grown at normoxia (Figure 21B and Figure 21C).

Figure 21. *Detection of active and total TGF-β in human primary skin fibroblasts after 24 hour treatment with steroids (dexamethasone, RU486, estrogen, or tamoxifen) grown at normoxia, hypoxia, and 2 hours of reoxygenation*

Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior the treatment of cells with 100 nM of steroids: dexamethasone (Dex) **(A)**, RU486 (RU) **(C)**, estrogen (Est) **(E)**, or tamoxifen (Tam) **(G)**. Control cells (C) received no treatment. Cells were then grown at normoxia (N) (20% oxygen), or hypoxia (H) (< 0.2% oxygen) for 24 hours. For reoxygenated (R) condition, cells were grown at normoxia for 2 hours following a 24 hour hypoxic treatment. At 24 hours, conditioned medium were collected in siliconized tubes containing a protease inhibitor cocktail: 10ul phenylmethylsulfonyl fluoride (PMSF), and 20 µl protease inhibitor (PI/ml of medium). The concentration of active **(A, C, E, & G)** and total **(B, D, F, & H)** TGF-β in the conditioned medium was measured using the PAI/L assay as described in Materials and Methods in chapter 2. Values for TGF-β concentration were corrected for variations in cell number by measuring total protein using a Biorad assay. * $P < 0.05$, ** $P < 0.02$, ↕ $P < 0.01$, ǂ $P < 0.005$, ǂ $P < 0.001$.

A


Active TGF-β
[% TGF-β]
140
120
100
80
60
40
20
0
C N
Dex N
Dex H
Dex R

B

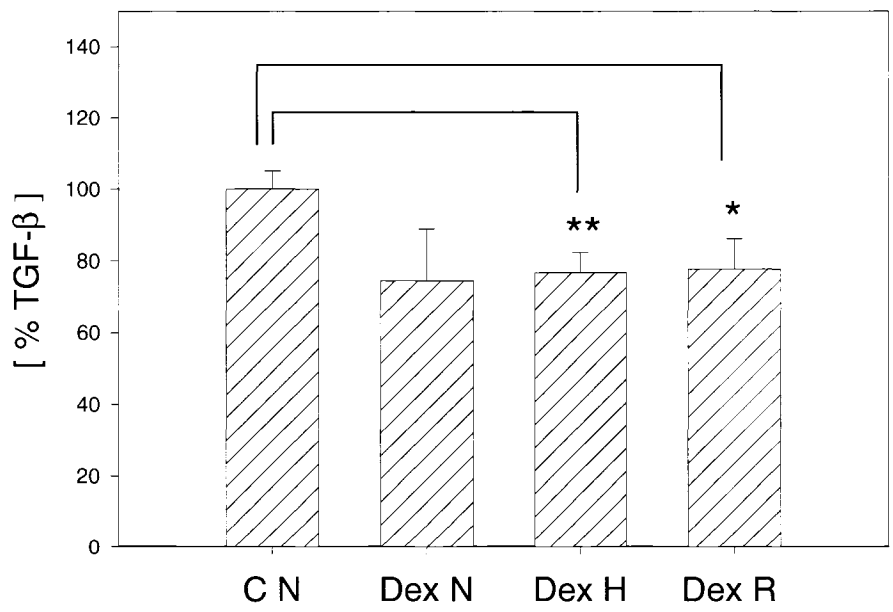
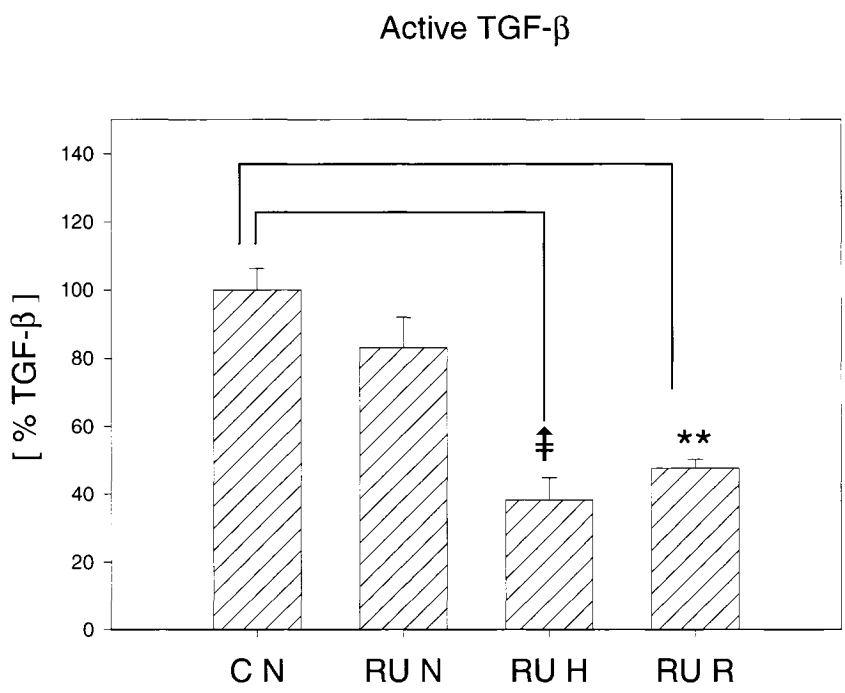

Total TGF-β
[% TGF-β]
140
120
100
80
60
40
20
0
**
*
C N
Dex N
Dex H
Dex R

Figure. 21

C


Active TGF-β
[% TGF-β]
140
120
100
80
60
40
20
0
**
C N
RU N
RU H
RU R

D

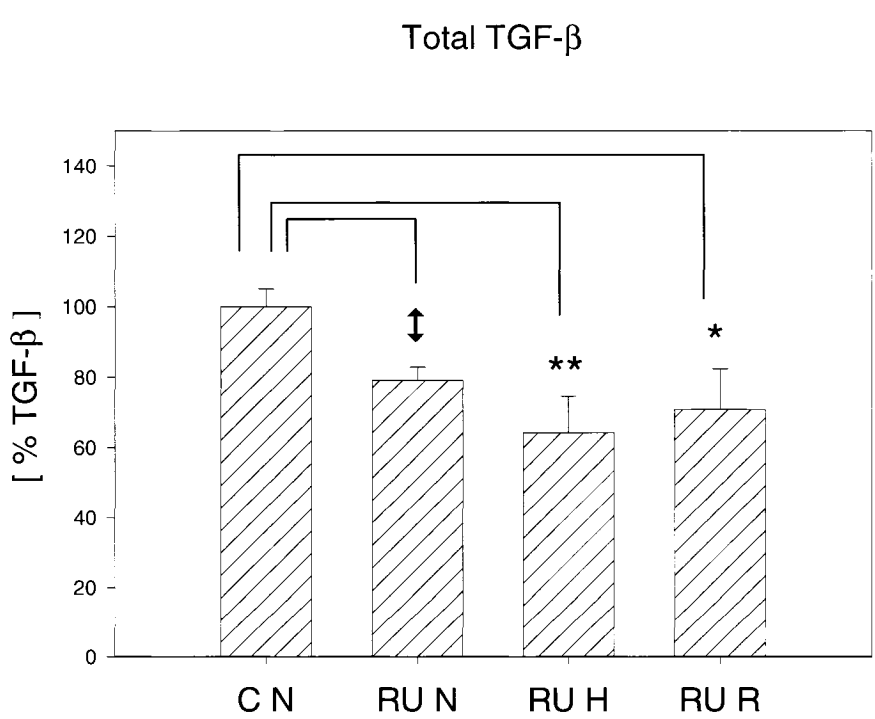

Total TGF-β
[% TGF-β]
140
120
100
80
60
40
20
0
**
*
C N
RU N
RU H
RU R

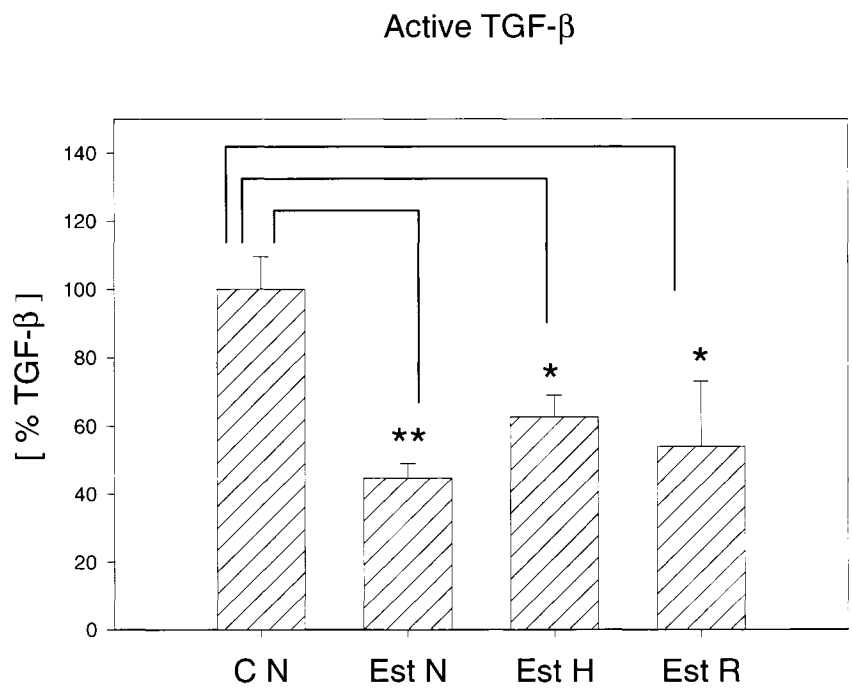
Figure. 21

Although, upon addition of dexamethasone ($P<0.001$) or RU486 ($P<0.001$) there was a significant decrease in the concentration of active TGF-β as compared to control cells, simultaneous treatment with hypoxia further decreased the concentration of active TGF-β (Figure 21A and Figure 21C). However, no significant differences were observed for the total TGF-β between cells treated with dexamethasone or RU486 in the presence and absence of hypoxia (Figure 21B and Figure 21D). Reoxygenation did not significantly increase active or total TGF-β as compared to hypoxic cells treated with dexamethasone or RU486.

4.4.1.1.2. Treatment with estrogen or tamoxifen

The effect of 24 hours of simultaneous exposure to estrogen or tamoxifen, and hypoxia on the concentration of TGF-β was studied in human primary skin fibroblasts. As shown in Figure 21E and Figure 21G, there was a significant decrease ($P<0.05$) in the concentration of active TGF-β in skin fibroblasts exposed to both hypoxia and estrogen (EST H) or tamoxifen (TAM H) (or estrogen ($P<0.02$) or tamoxifen ($P<0.01$) alone) as compared to control untreated cells grown at normoxia. Following two hours reoxygenation, cells treated with either estrogen ($P<0.05$) or tamoxifen ($P<0.05$) showed significant decease in concentration of TGF-β as compared to control untreated cells. No significant differences were observed for total TGF-β in cells that were treated with either estrogen or tamoxifen at hypoxia, and at reoxygenation (EST R or TAM R) as compared to control untreated cells (Figure 21F and Figure 21H).

E


Active TGF-β
[% TGF-β]
140
120
100
80
60
40
20
0
**
*
*
C N
Est N
Est H
Est R

F

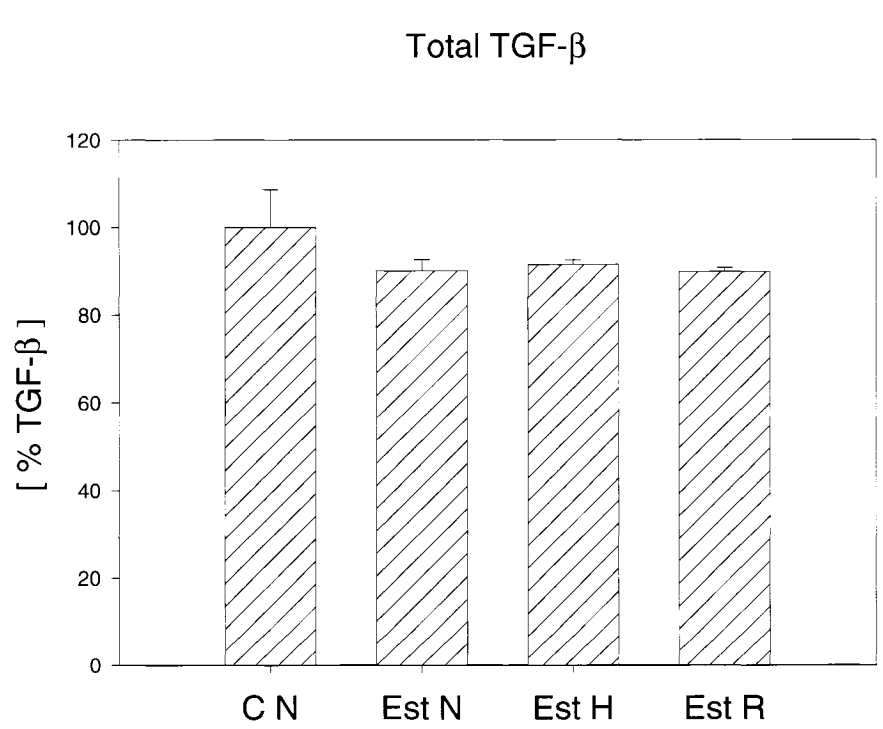
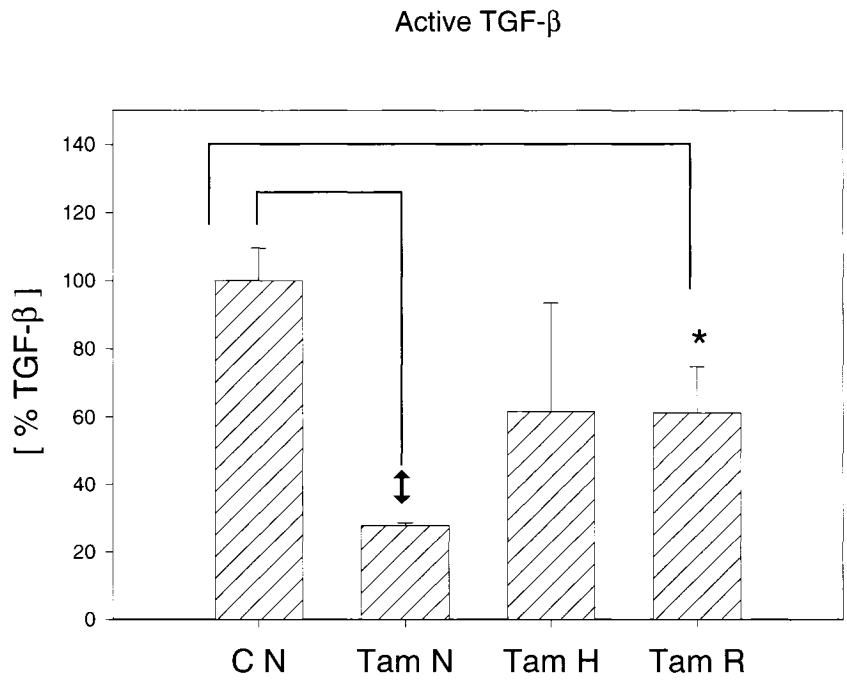

Total TGF-β
[% TGF-β]
120
100
80
60
40
20
0
C N
Est N
Est H
Est R

Figure. 21

G


Active TGF-β
[% TGF-β]
140
120
100
80
60
40
20
0
*
C N
Tam N
Tam H
Tam R

H

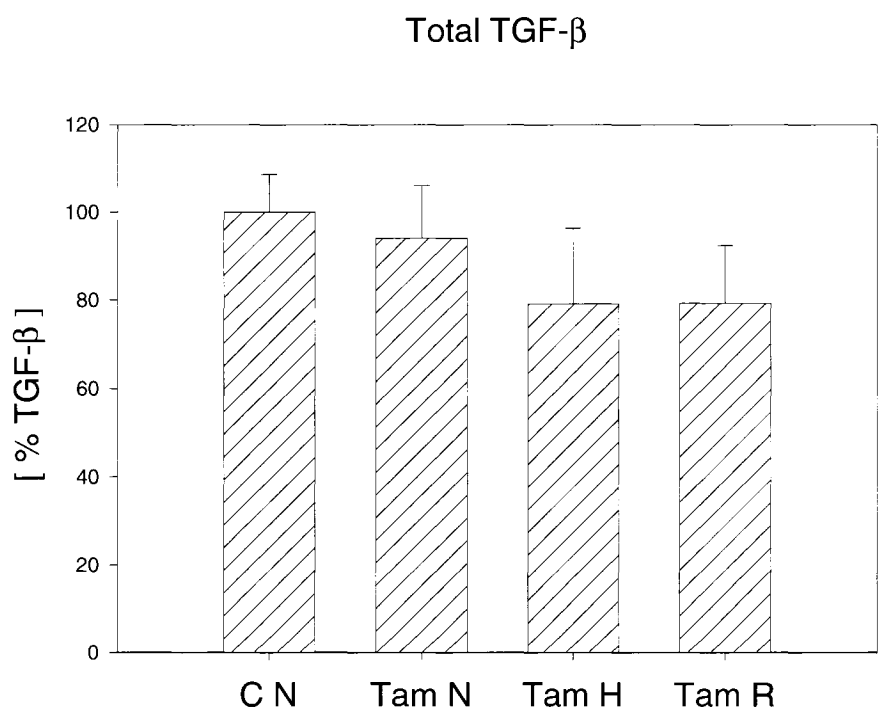

Total TGF-β
[% TGF-β]
120
100
80
60
40
20
0
C N
Tam N
Tam H
Tam R

Figure. 21

No significant changes were observed for the expression of both active and total TGF-β in cells treated with either estrogen or tamoxifen grown at hypoxia as compared to cells that were treated solely with estrogen (Figure 21E and Figure 21F) or tamoxifen (Figure 21G and Figure 21H). Furthermore, no significant differences were observed for the active and total TGF-β in reoxygenated cells as compared to hypoxic cells that were treated with estrogen or tamoxifen.

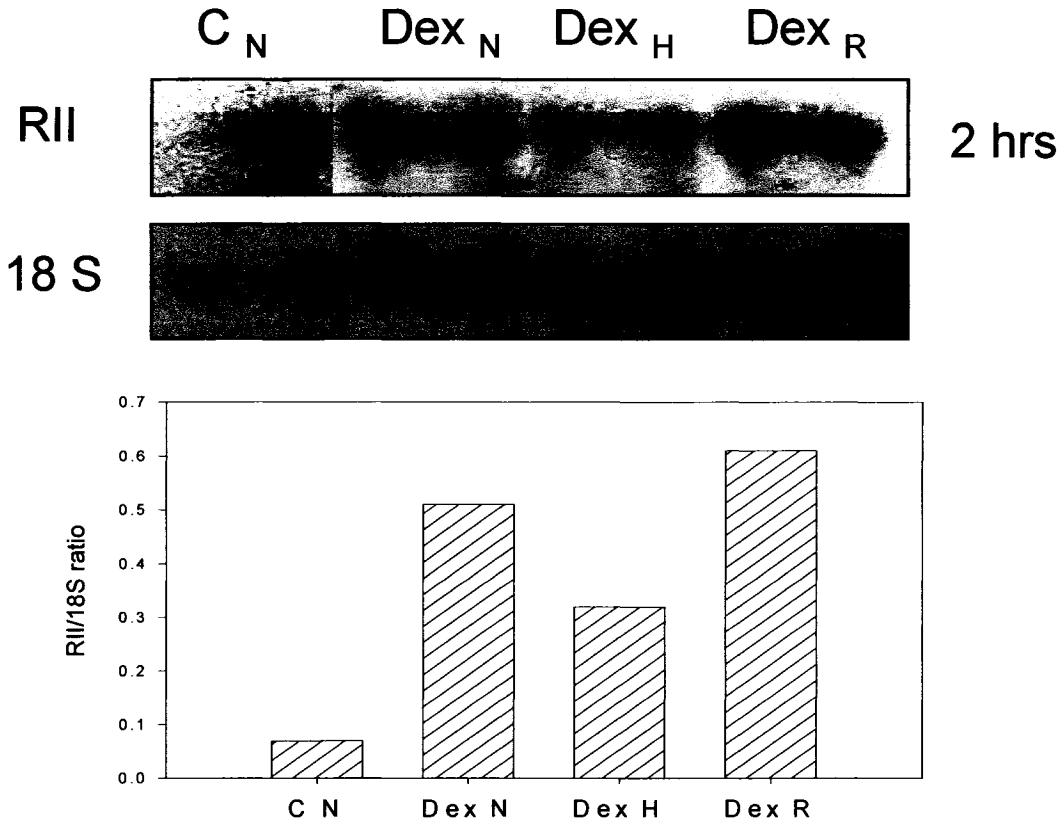
4.4.1.2. The combined effect of 2 hour treatment of both hypoxia and steroids (dexamethasone or RU486) on RII mRNA expression in human skin fibroblasts and keratinocytes (HaCat).

Next, we wanted to analyze the simultaneous effect of both steroids (dexamethasone or RU486) and hypoxia (2 hours) on the expression of RII mRNA in human primary skin fibroblasts, and human keratinocytes (HaCat). As shown in Figure 22A (human primary skin fibroblasts) and Figure 23A (HaCat cells), Northern blot analysis revealed that hypoxia inhibited dexamethasone-induced upregulation of RII mRNA in both cell types. Skin fibroblasts and HaCat cells treated with both hypoxia and dexamethasone still express higher level of RII mRNA as compared to control (N) untreated cells grown at normoxia. This effect of hypoxia was reversible since subsequent reoxygenation resulted in an increase in expression of RII mRNA. Both skin

Figure 22. *Regulation of RII mRNA expression in human primary skin fibroblasts treated with steroids (dexamethasone, or RU486) grown at normoxia, hypoxia and reoxygenation for 2 hours.*

Fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior to the addition of 100 nM of dexamethasone (Dex) (**A**), or RU486 (RU) (**B**). Control cells (C) received no treatment. Cells were then incubated for 2 hours under normoxia (N) (20% oxygen), or hypoxia (H) (< 0.2% oxygen). Reoxygenated (R) condition was achieved by incubating cells at normoxia for 2 hours following a 2 hour hypoxic treatment. Total RNA was extracted using TRIZOL reagent, and 20 μg of RNA from each sample was analyzed by Northern blot using specific radiolabeled probes for RII mRNA and 18S ribosomal RNA. The 18S ribosomal RNA is shown to demonstrate equal loading of RNA (bottom panel). The densitometric analysis of this data is shown at the bottom panel. The results shown are representative of at least three different experiments.

A


C N
Dex N
Dex H
Dex R
RII
2 hrs
18 S
RII/18S ratio
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C N
Dex N
Dex H
Dex R

B

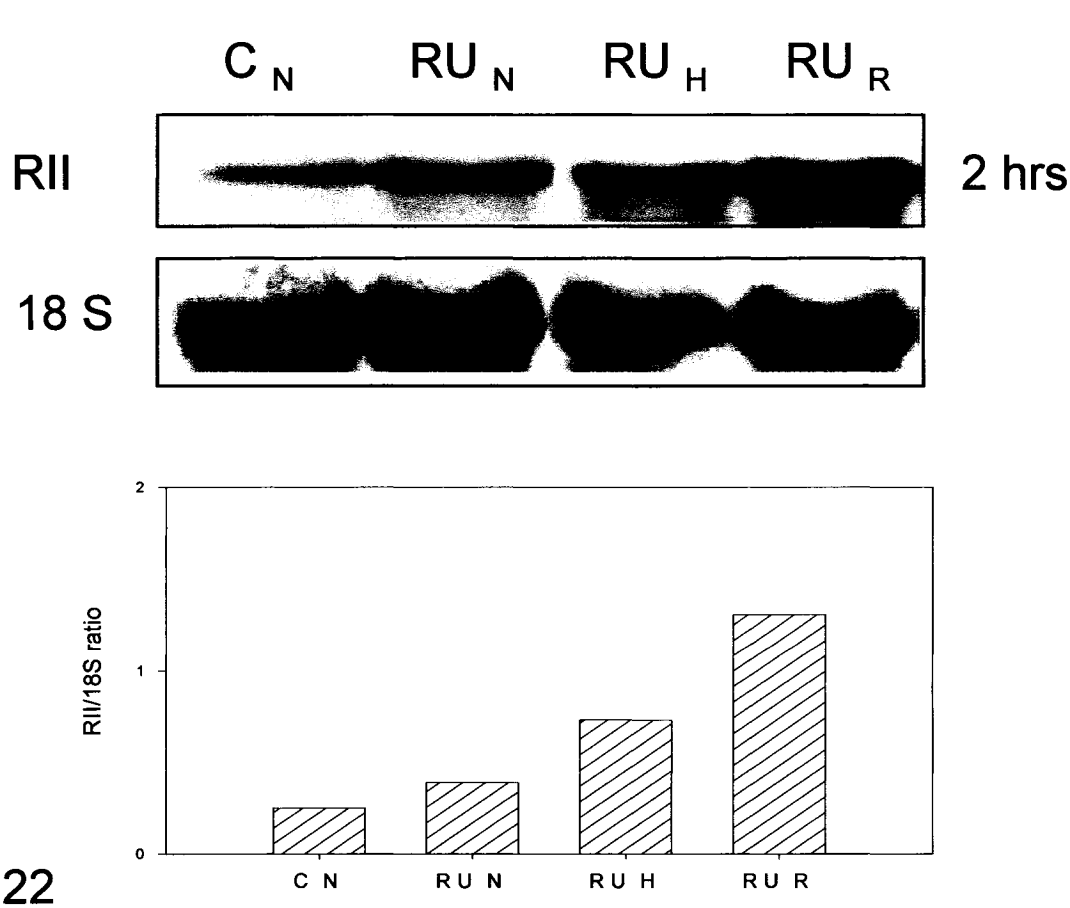
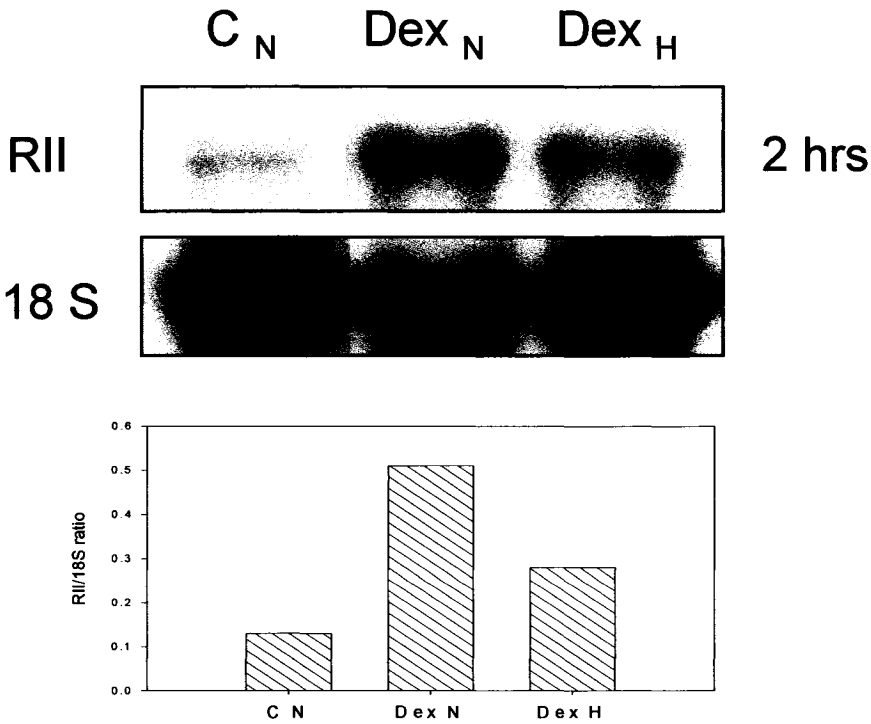

C N
RU N
RU H
RU R
RII
2 hrs
18 S
RII/18S ratio
2
1
0
C N
RU N
RU H
RU R

Figure. 22

Figure 23. *Regulation of RII mRNA expression in human keratinocytes (HaCat) treated with steroids (dexamethasone, or RU486) grown at normoxia and hypoxia for 2 hours.*

HaCat cells were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior to the addition of 100 nM of dexamethasone (Dex) **(A)**, or RU486 (RU) **(B)**. Control lane (C) received no treatment. Cells were then incubated for 2 hours under normoxia (N) (20% oxygen), or hypoxia (H) (< 0.2% oxygen). Total RNA was extracted, and 20 μg of RNA from each sample was analyzed by Northern blot using specific radiolabeled probes for RII mRNA and 18S ribosomal RNA. The 18S ribosomal RNA is shown to demonstrate equal loading of RNA (bottom panel). The densitometric analysis of this data is shown at the bottom panel. The results shown are representative of at least three different experiments.

A


C N
Dex N
Dex H
RII
2 hrs
18 S
RII/18S ratio
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C N
Dex N
Dex H

B

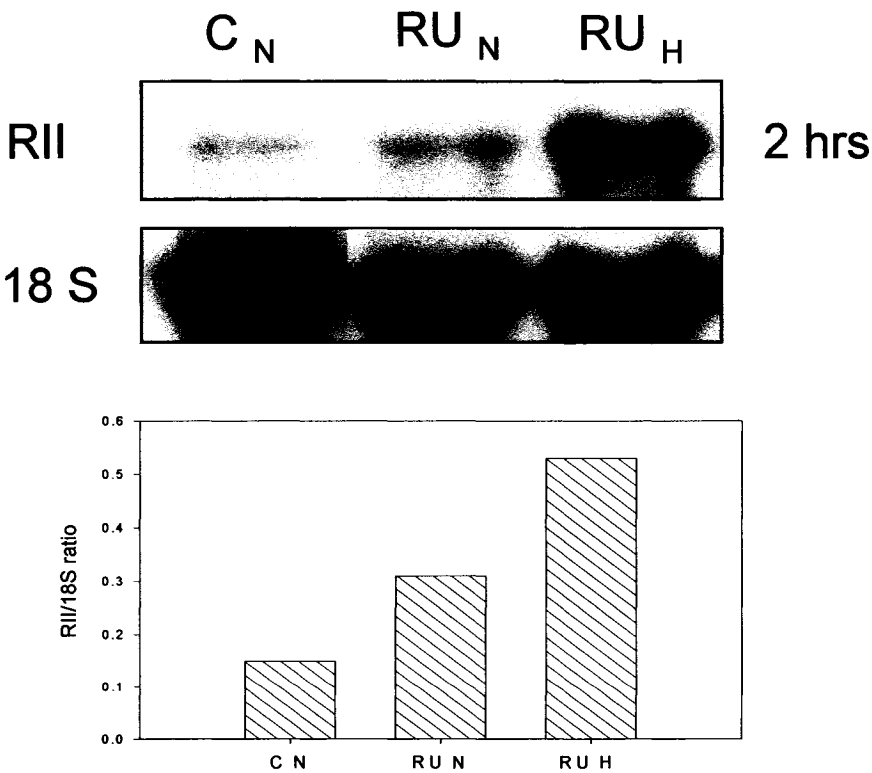

C N
RU N
RU H
RII
2 hrs
18 S
RII/18S ratio
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C N
RU N
RU H

Figure. 23

fibroblasts (Figure 22B) and HaCat cells (Figure 23B) exposed to RU486 for 2 hours showed increase in the expression of RII mRNA at normoxia. Hypoxia further increased the expression of RII mRNA in both fibroblasts and HaCat cells. Moreover, reoxygenation of RU486 treated fibroblasts was able to yet substantially increase the expression of RII mRNA

4.4.1.3. The combined effect of 24 hour treatment of both Hypoxia and steroids on regulation of RII mRNA expression in human skin fibroblasts

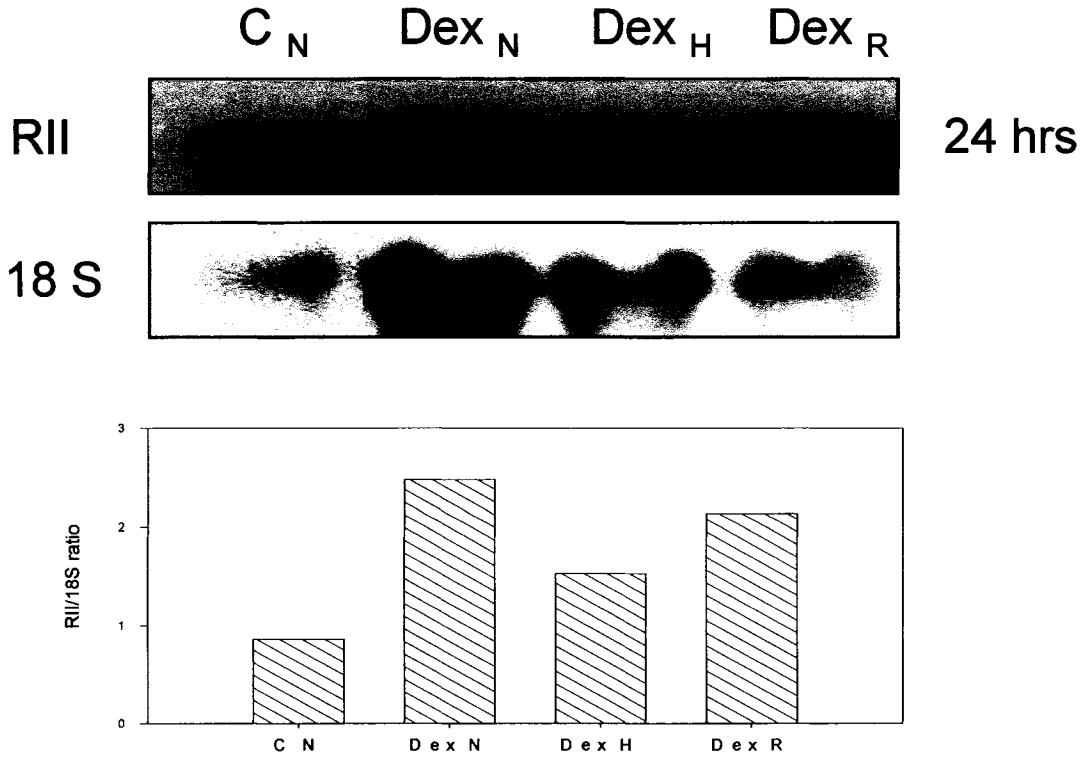
We then analyzed the effect of simultaneous treatment with both steroids and hypoxia for 24 hours on the expression of RII mRNA in human primary skin fibroblasts. Cells were treated with 100 nM of dexamethasone (Dex), RU486 (RU), estrogen (Est), tamoxifen (Tam), or testosterone (Tes). As shown in Figure 24, hypoxia inhibited dexamethasone (Figure 24A), RU486 (Figure 24B), estrogen (Figure 24C) and tamoxifen-induced (Figure 24D) upregulation of RII mRNA in human primary skin fibroblasts. The highest reduction of RII mRNA at hypoxia was observed for cells that were treated with tamoxifen. Hypoxia had little effect on testosterone-induced downregulation of RII mRNA (Figure 24E).

The down-regulatory effect of hypoxia was partially reversible for estrogen or dexamethasone treated cells. Subsequent reoxygenation resulted in an increase in the expression of RII mRNA in dexamethasone and estrogen treated cells as compared to both control (C) untreated cells and hypoxic treated cells (Figure 24A, and Figure 24C).

Figure 24. *Regulation of RII mRNA expression in human primary skin fibroblasts after 24 hour treatment with steroids (dexamethasone, RU486, estrogen, tamoxifen, or testosterone) grown at normoxia, hypoxia, and 2 hours of reoxygenation.*

Fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior to the addition of 100 nM of dexamethasone (Dex) **(A)**, RU486 (RU) **(B)**, estrogen (Est) **(C)**, tamoxifen (Tam) **(D),** or testosterone (Tes) **(E)**. Control lane (C) received no treatment. Cells were then incubated for 24 hours under normoxia (N) (20% oxygen), or hypoxia (H) (< 0.2% oxygen). Reoxygenation (R) was achieved by incubating cells at normoxia for 2 hours following a 24 hour hypoxic treatment. Total RNA was extracted using TRIZOL reagent. 20 μg of RNA from each sample was analyzed by Northern blot using specific radiolabeled probes for RII mRNA and 18S ribosomal RNA. The 18S ribosomal RNA is shown to demonstrate equal loading of RNA (bottom panel). The densitometric analysis of this data is shown at the bottom panel. The results shown are representative of at least three different experiments.

A


C N
Dex N
Dex H
Dex R
RII
24 hrs
18 S
RII/18S ratio
3
2
1
0
C N
Dex N
Dex H
Dex R

B

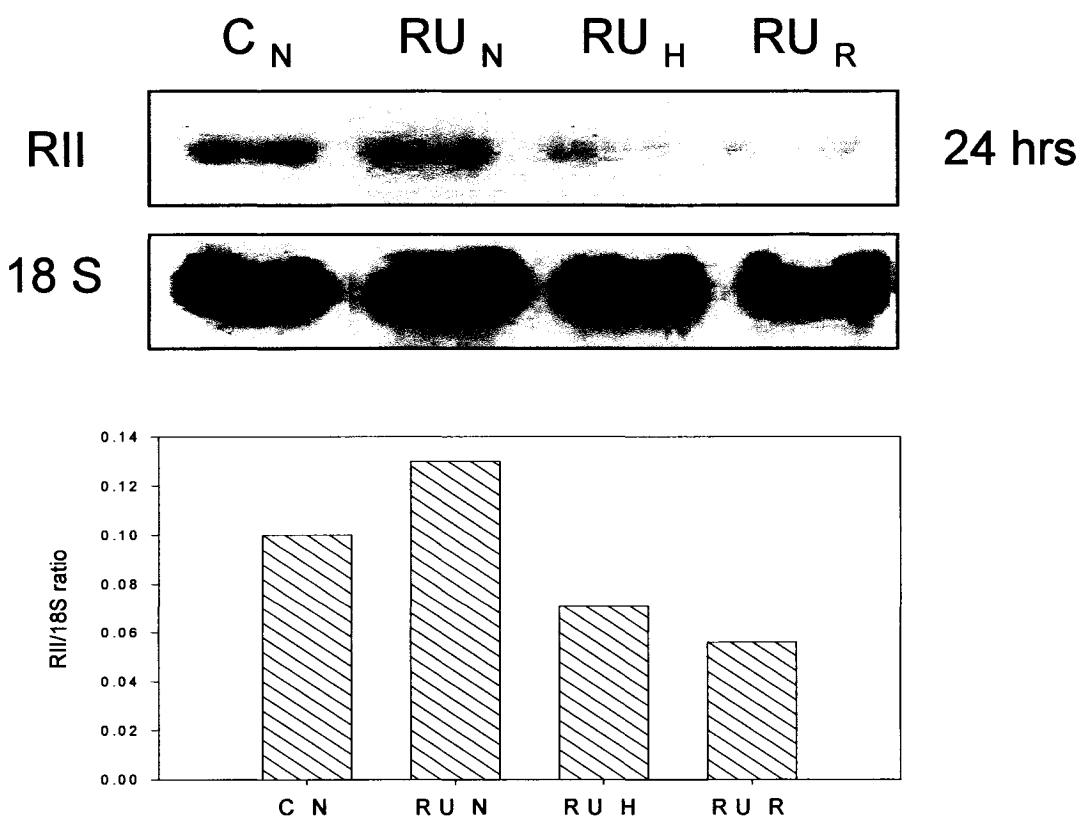
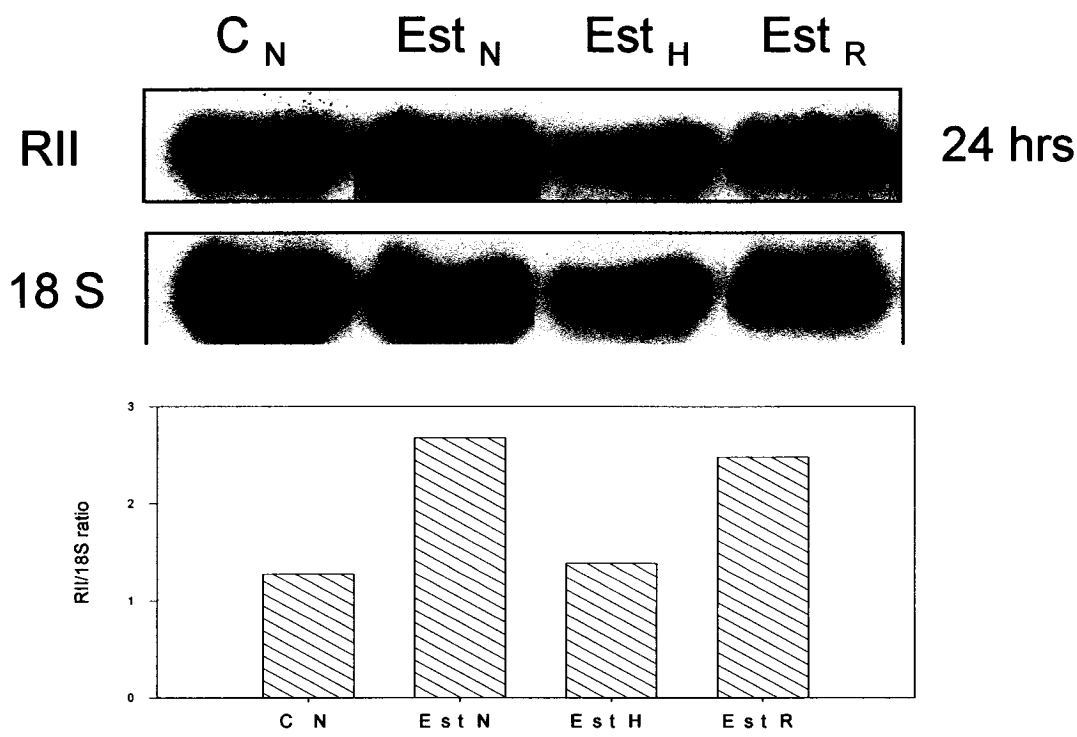

C N
RU N
RU H
RU R
RII
24 hrs
18 S
RII/18S ratio
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
C N
RU N
RU H
RU R

Figure. 24

C


C N
Est N
Est H
Est R
RII
24 hrs
18 S
RII/18S ratio
0
1
2
3
C N
Est N
Est H
Est R

D

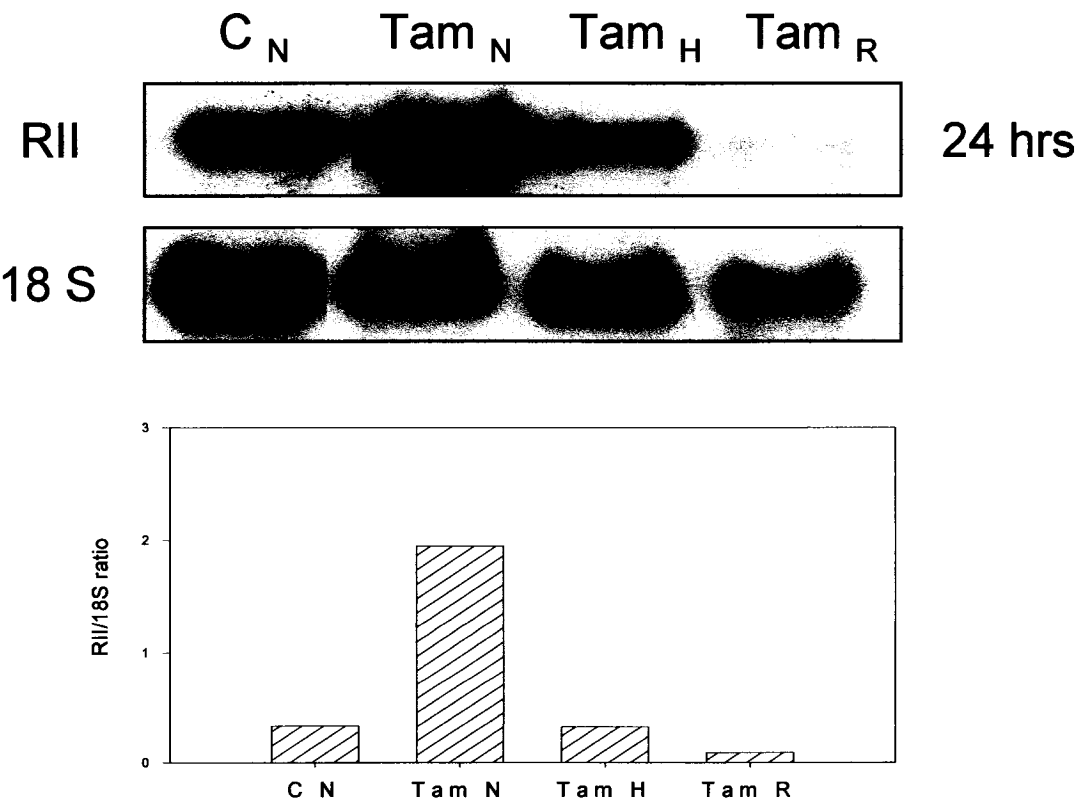

C N
Tam N
Tam H
Tam R
RII
24 hrs
18 S
RII/18S ratio
0
1
2
3
C N
Tam N
Tam H
Tam R

Figure. 24

E

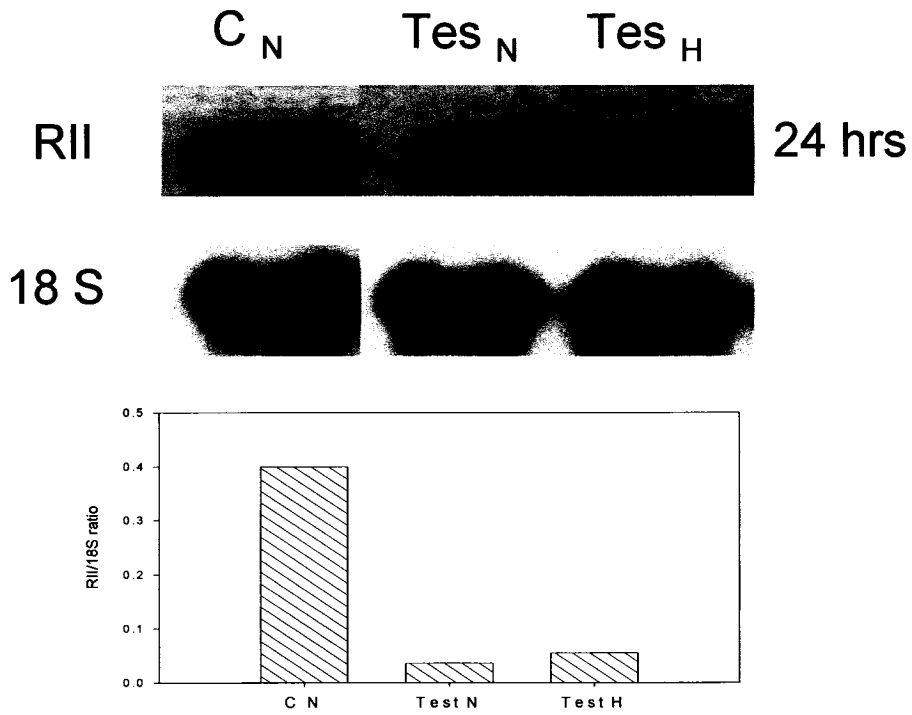

C N
Tes N
Tes H
RII
24 hrs
18 S
RII/18S ratio
0.5
0.4
0.3
0.2
0.1
0.0
C N
Test N
Test H

Figure. 24

However, reoxygenation did not reverse RII mRNA expression in RU486 (Figure 24B), and tamoxifen (Figure 24D) treated cells.

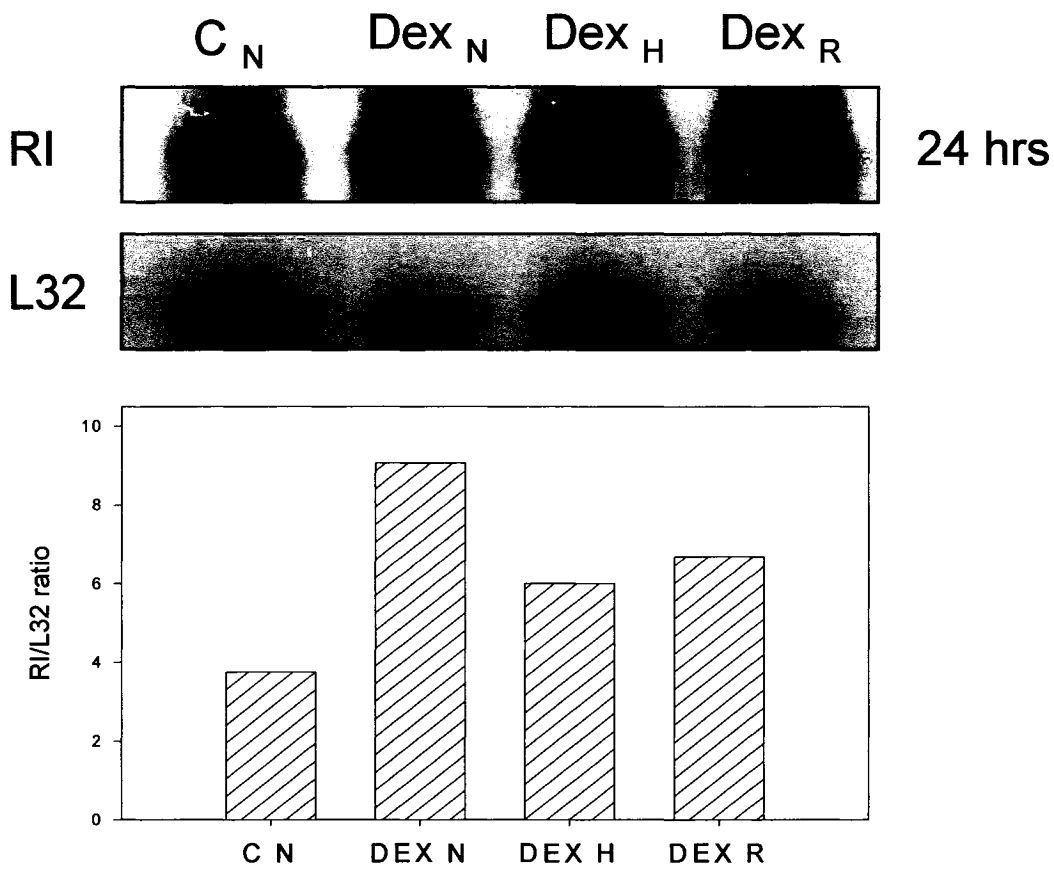
4.4.1.4. The combined effect of 24 hour treatment of both Hypoxia and steroids (dexamethasone or RU486) on modulation of RI mRNA expression in human skin fibroblasts

In parallel to our studies of RII mRNA, we examined the effect of 24 hour exposure to both hypoxia and steroids (dexamethasone or RU486) on the expression of RI mRNA in human primary skin fibroblasts using RNA protection assay. As shown in Figure 25A, treatment of cells with both hypoxia and dexamethasone (Dex H) for 24 hours decreased the expression of RI mRNA as compared to cells treated solely with dexamethasone. Reoxygenation did not result in any detectable changes in the expression of RI mRNA as compared to the cells treated with both hypoxia and dexamethasone. Similarly, cells that were exposed to hypoxia and RU486 for 24 hours (Figure 25B) showed down regulation of RI mRNA at hypoxia. Reoxygenation further decreased the expression of RI mRNA.

Figure 25. *Regulation of RI mRNA expression in human primary skin fibroblasts after 24 hour treatment with steroids (dexamethasone, or RU486) grown at normoxia, hypoxia, and 2 hours of reoxygenation.*

Ribonuclease protection assay (RPA) analysis revealed that there is a differential effect for the expression of RI mRNA in human primary fibroblasts that were treated with both hypoxia (H) (< 0.2% oxygen) and steroids (dexamethasone or RU486) for 24 hours as compared to normoxic (N) (20% oxygen) condition. Fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior the addition of 100 nM of dexamethasone (Dex) **(A)**, or RU486 (RU) **(B)**. Control lane (C) received no treatment. For reoxygenated (R) condition, cells that were exposed to hypoxia for 24 hours were then incubated at normoxia for 2 hours. Total RNA was extracted using TRIZOL reagent. From each sample 15 μg of RNA was analyzed by RPA for RI mRNA, and L32 mRNA expression. The L32 mRNA is shown to demonstrate equal loading of RNA (bottom panel). The densitometric analysis of this data is shown at the bottom panel.

A


C N
Dex N
Dex H
Dex R
RI
24 hrs
L32
RI/L32 ratio
10
8
6
4
2
0
C N
DEX N
DEX H
DEX R

B

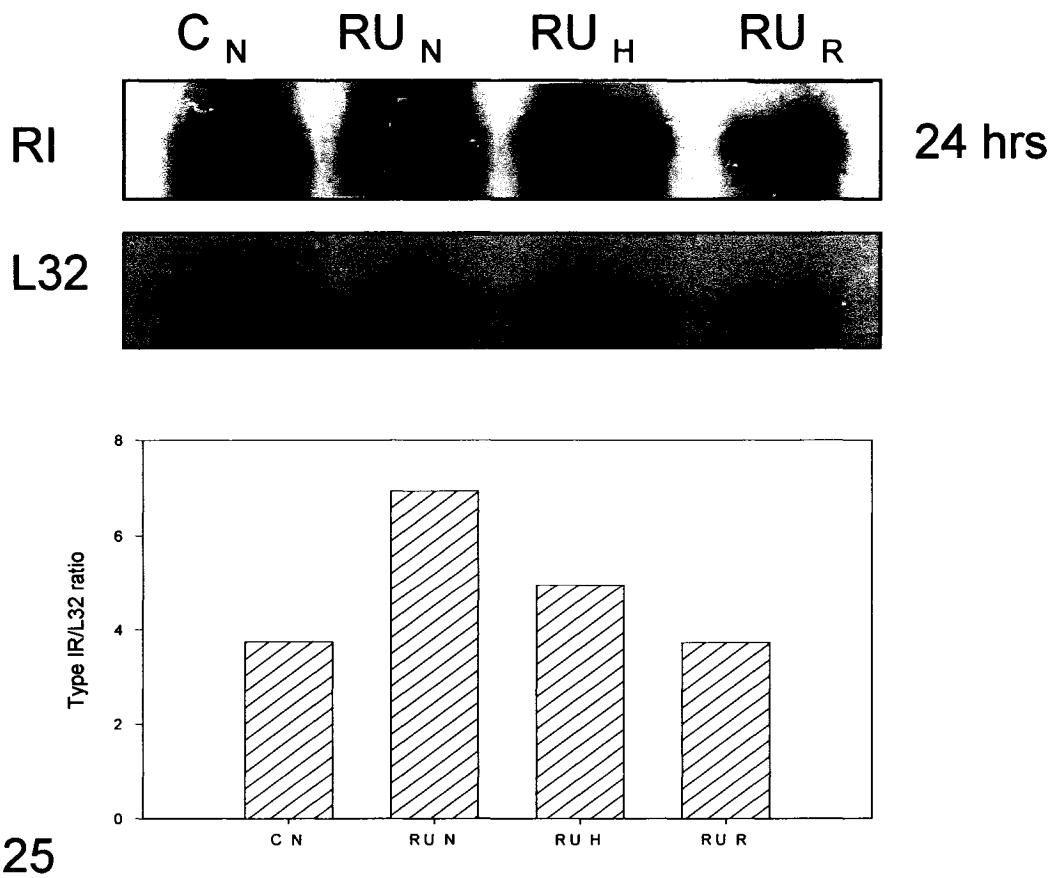

C N
RU N
RU H
RU R
RI
24 hrs
L32
Type IR/L32 ratio
8
6
4
2
0
C N
RU N
RU H
RU R

Figure. 25

4.4.1.5. The combined effect of both Hypoxia and steroids (1 or 24 hours) on regulation of phosphorylated and total Smad2 and Smad3 proteins in human skin fibroblasts

4.4.1.5.1. Treatment with dexamethasone

We then wanted to examine the combined effect of both hypoxia and dexamethasone treatment for 1 hour and 24 hours on the level of phosphorylation of Smad2 and Smad3, and total Smad2 and Smad3 in human primary skin fibroblasts. Using Western blot analysis, no changes were detected for the Smad2P and Samd3P for the cells that were treated with dexamethasone grown at normoxia or hypoxia for 1 hour (Figure 26A). Interestingly reoxygenation of the cells for 2 hours slightly increased the phosphorylated Smad2 as compared to control untreated cells.

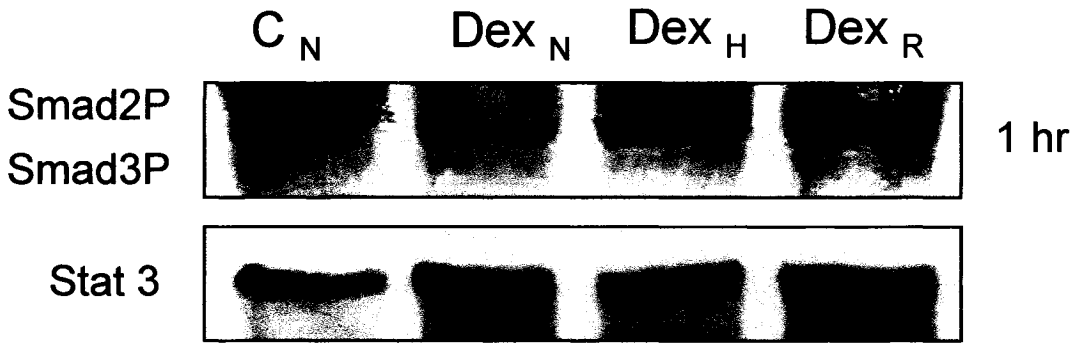
On the other hand, treatment of cells with dexamethasone grown at normoxia or hypoxia for 1 hour, increased the total Smad2 and Smad3 (Figure 26B). Hypoxia resulted in a considerable enhancement in the upregulation of the total Smad2 and Smad3. Reoxygenation for 2 hours, considerably reduced the levels of total Smad2 and Smad3 as compared to cells that were treated with both hypoxia and dexamethasone; however, the levels of total Smad2 and Smad3 were still higher than the control untreated cells.

24 hour treatment with dexamethasone down regulated the phosphorylated and total Smad2 and Smad3 (Figure 26C and Figure 26D). The combined effect of both

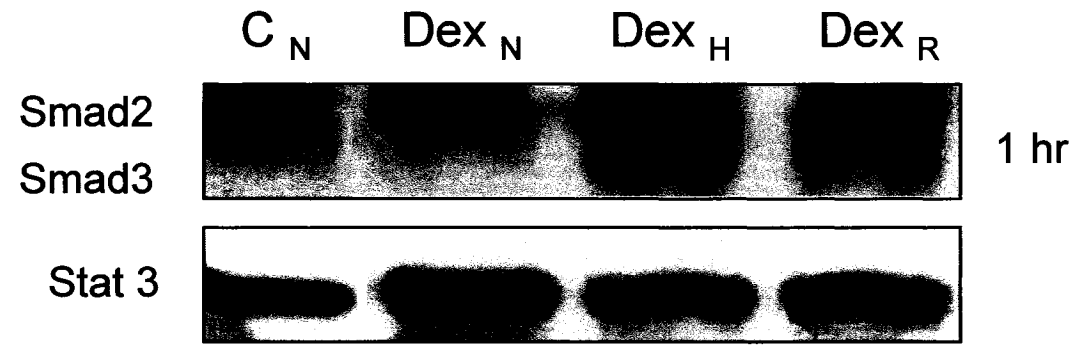
Figure 26. *Regulation of phosphorylated and total Smad2 and Smad3 in human primary skin fibroblasts after 1 or 24 hour treatment with dexamethasone grown at normoxia, hypoxia, and 2 hours of reoxygenation.*

Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior to the addition of 100 nM of dexamethasone (Dex). Cells were incubated at normoxia (N) (20% oxygen) or hypoxia (H) (<0.2% oxygen) for 1 hour **(A & B)** or 24 hours **(C & D)**. Reoxygenation (R) was achieved by incubating cells previously exposed to hypoxia for an additional exposure of 2 hours to normoxic condition. Immunoblotting was performed using a rabbit polyclonal antibody specific to the phosphorylated Smad2 (Smad2P) and Smad3 (Smad3P) or total Smad2 and Smad3 which recognizes both phosphorylated and unphosphorylated Smad2 and Smad3 (Santa Cruz). The same nitrocellulose membrane was reblotted with an anti-Stat3 antibody (Stat3) to demonstrate equal protein loading. The results shown are representative of at least three different experiments.

A



B

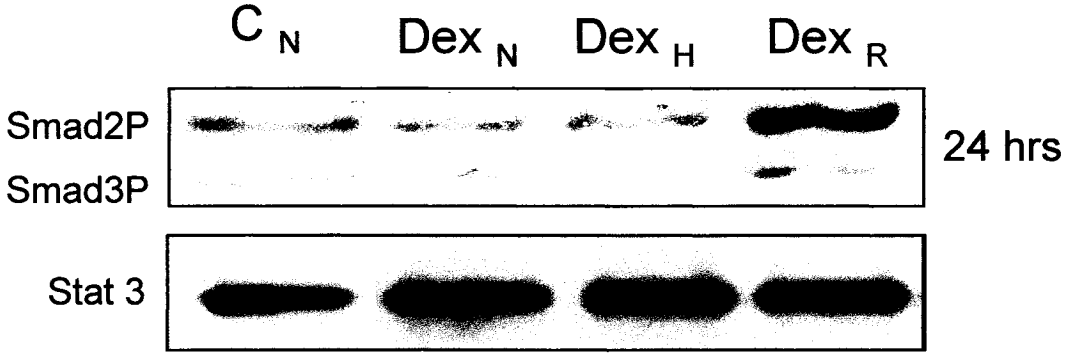


	1 hr			
Smad/Stat3	2P	3P	2	3
Dex_N vs C_N	-	-	Dex_N ↑	Dex_N ↑
Dex_H vs C_N	-	-	Dex_H ↑	Dex_H ↑
Dex_R vs C_N	Dex_R ↑	-	Dex_R ↑	Dex_R ↑

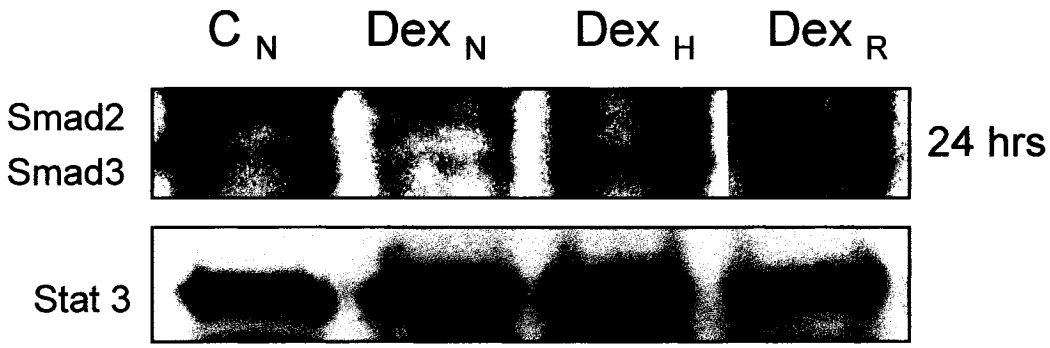
↑ = increase
↓ = decrease
- = no change

Figure. 26

C



D



	24 hrs			
Smad/Stat3	2P	3P	2	3
Dex_N vs C_N	Dex_N ↓	Dex_N ↓	Dex_N ↓	Dex_N ↓
Dex_H vs C_N	-	Dex_N ↓	Dex_H ↑	Dex_H ↑
Dex_R vs C_N	Dex_R ↑	Dex_R ↑	Dex_R ↑	Dex_R ↑

↑ = increase
↓ = decrease
- = no change

Figure. 26

hypoxia and dexamethasone had no effect on Smad2P but decreased Smad3P; however, total Smad2 and total Smad3 were upregulated. The fold induction of total Smad2 was less than total Smad3. The effect of 2 hours reoxygenation was quite pronounced. Reoxygenation increased phosphorylated and total Smad2 and Smad3 as compared to cells treated with both dexamethasone and hypoxia, and control (C) untreated cells.

4.4.1.5.2. Treatment with RU486

The regulation of phosphorylated and total Smad2 and Smad3 proteins by hypoxia and 100 nM of RU486 (1 and 24 hours) were studied in human primary skin fibroblasts. Phosphorylated Smad2 and Smad3 proteins were increased, while no change was detected for total Smad2 and Smad3 after 1 hour treatment with RU486 alone in cells grown at normoxia. The combined effect of RU486 and hypoxia for 1 hour had no observable effect on the Smad2P, but on the other hand it increased the Smad3P, total Smad2 and Smad3 as compared to control (C) untreated cells (Figure 27A and Figure 27B). Reoxygenation counteracted the effect of hypoxia on phosphorylated and total Smad2 and Smad3. However, compared to control untreated cells, Smad2P was lower and Smad3P was higher, and no difference was observed for total Smad2 and total Smad3 after 2 hours of reoxygenation.

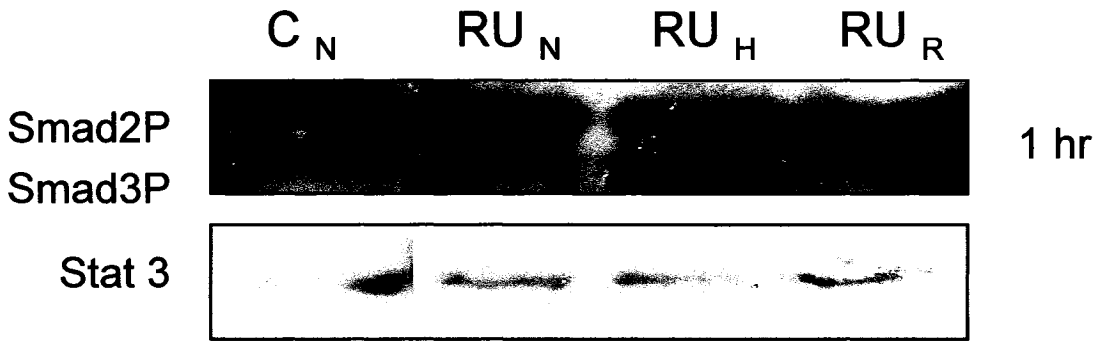
The treatment of cells with RU486 alone for 24 hours had downregulatory effect on the phosphorylated and total Smad2 and Smad3. The combined effect of RU486 and

hypoxia for 24 hours upregulated both phosphorylated and total Smad2 and Smad3 as compared to control untreated cells. Reoxygenation of cells for 2 hours counteracted the upregulatory effect of combined hypoxia and RU486. The levels of phosphorylated and total Smad2 and Smad3 were very close to the level of control (C) untreated cells (Figure 27C and Figure 27D).

Figure 27. *Regulation of phosphorylated and total Smad2 and Smad3 in human primary skin fibroblasts after 1 or 24 hour treatment with RU486 grown at normoxia, hypoxia, and 2 hours of reoxygenation.*

Human primary skin fibroblasts were cultured in fetal bovine serum (FBS) free DMEM for 24 hours. The medium was changed again with serum free medium at 3 hours prior the addition of 100 nM RU486 (RU). Cells were incubated at normoxia (N) (20% oxygen) or hypoxia (H) (<0.2% oxygen) for 1 hour **(A & B)** or 24 hours **(C & D)**. Reoxygenation (R) was achieved by incubating cells previously exposed to hypoxia for an additional exposure of 2 hours to normoxic condition. Immunoblotting was performed using a rabbit polyclonal antibody specific to the phosphorylated Smad2 (Smad2P) and Smad3 (Smad3P) or total Smad2 and Smad3 which recognizes both phosphorylated and unphosphorylated Smad2 and Smad3 (Santa Cruz). The same nitrocellulose membrane was reblotted with an anti-Stat3 antibody (Stat3) to demonstrate equal protein loading. This data is representative of three different experiments.

A



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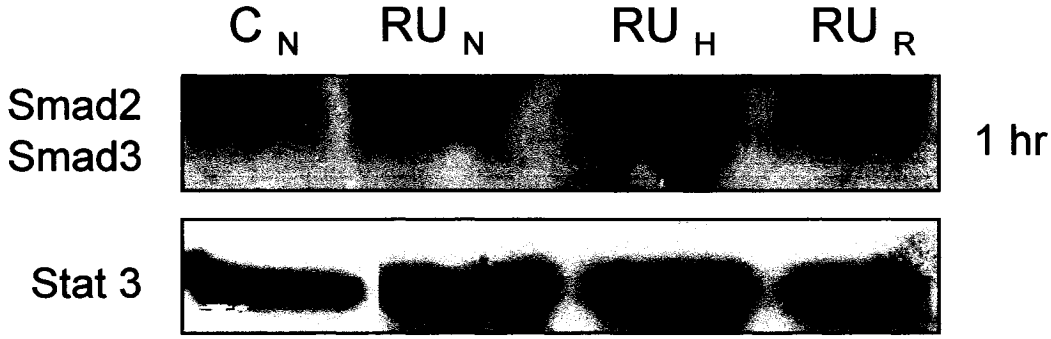
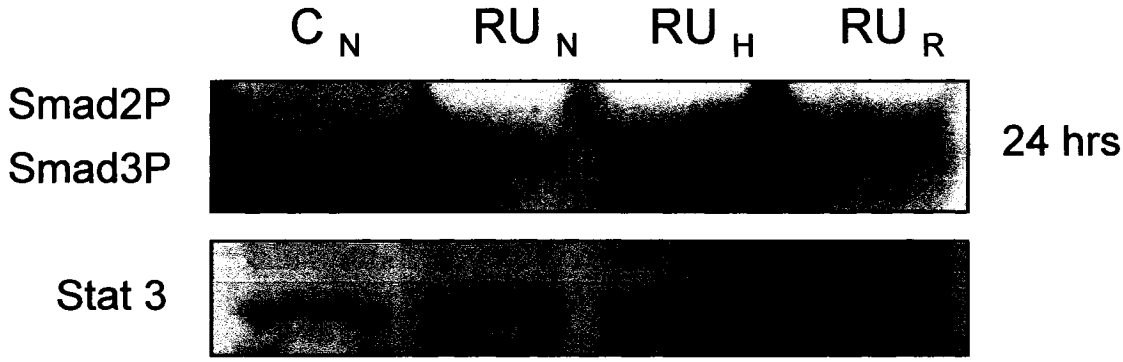


	1 hr			
Smad/Stat3	2P	3P	2	3
RU_N vs C_N	RU_N ↑	RU_N ↑	-	-
RU_H vs C_N	-	RU_H↑	RU_H ↑	RU_H ↑
RU_R vs C_N	RU_R ↓	RU_R ↑	-	-

↑ = increase
↓ = decrease
- = no change

Figure. 27

C



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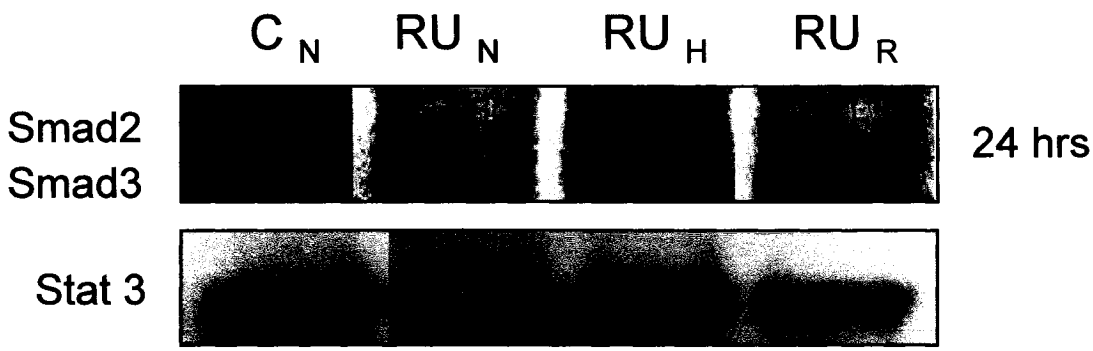

	24 hrs			
Smad/Stat3	2P	3P	2	3
RU_N vs C_N	$RU_N \downarrow$	$RU_N \downarrow$	$RU_N \downarrow$	$RU_N \downarrow$
RU_H vs C_N	$RU_H \uparrow$	$RU_H \uparrow$	$RU_H \uparrow$	$RU_H \uparrow$
RU_R vs C_N	-	-	-	-

↑ = increase
↓ = decrease
- = no change

Figure. 27

4.5. Summary of Results

Table 5. Analysis of active and total TGF-β in human primary skin fibroblasts exposed to normoxia (N), hypoxia (H) (2 hours & 24 hours), and reoxygenation (R) (2 hours) using the PAI/L assay as described in Materials and Methods. NS = not significant

tgf-β1			
2 hours		N vs H	N vs R
	Active	H ↑	NS
	Total	NS	NS
24 hours			
	Active	H ↓	R ↓
	Total	NS	NS

Table 6. Analysis of active and total TGF-β in human primary skin fibroblasts treated with steroids {dexamethasone (Dex), RU486 (RU), estrogen (Est), or Tamoxifen (Tam)} or left untreated (C) at normoxia for 2, and 24 hours using the PAI/L assay as described in Materials and Methods.

tgf-β1					
2 hours		C vs Dex	C vs RU	C vs Est	C vs Tam
	Active	Dex ↑	RU ↑		
	Total	Dex ↑	RU ↑		
24 hours					
	Active	Dex ↓	RU ↓	Est ↓	Tam ↓
	Total	NS	RU ↓	NS	NS

Table 7. Analysis of active and total TGF-β in human primary skin fibroblasts treated with both steroids {dexamethasone (Dex), RU486 (RU), estrogen (Est), or Tamoxifen (Tam)} and hypoxia (H) (24 hours) or reoxygenation (R) (2 hours) and fibroblasts that were left untreated under normoxia (C) (24 hours) using the PAI/L assay as described in Materials and Methods.

tgf-β1									
24 hours		C vs Dex		C vs RU		C vs Est		C vs Tam	
		H	R	H	R	H	R	H	R
	Active	Dex ↓	Dex ↓	RU ↓	RU ↓	Est ↓	NS	Tam ↓	NS
	Total	Dex ↓	Dex ↓	RU ↓	RU ↓	NS	NS	NS	NS

Table 8. Analysis of TGF-β receptors (RI, RII, and RIII), and Smad proteins {phospho Smad2 (Smad2P), phospho Smad3 (Smad3P), total Smad2 (Smad2), and total Smad3 (Smad3)} in human skin fibroblasts grown at normoxia (N), hypoxia (H), (2, 24, and 48 hours) and reoxygenation (R) (2 hours).

	N vs H					N vs R				
	mRNA			protein		mRNA			protein	
Hours	2	24	48	2	24	2	24	48	2	24
RI	H↑♦	H↑*	H↓♦		H↓		-			R↓
RII	H↑*	H↓*	H↓*		H↓	R↑	R↓	R↑		R↓
RIII					H↓					R↓
Hours				1	24				1	24
Smad2P				H↑	-				R↑	-
Smad3P				H↑	H↓				R↑	-
Smad2				H↑	-				R↑	-
Smad3				-	H↑				R↑	-

- = unchanged
* = same results were obtained for human keratinocytes (HaCat)
♦ = obtained for human keratinocytes (HaCat)

Table 9. Analysis of TGF-β receptors (RI, RII, and RIII), and Smad proteins {phospho Smad2 (Smad2P), phospho Smad3 (Smad3P), total Smad2 (Smad2), and total Smad3 (Smad3)} in human skin cells treated with steroids {dexamethasone (Dex), or RU486 (RU)}or left untreated (C) at normoxia for 2, and 24 hours.

	C vs Dex				C vs RU			
	mRNA		protein		mRNA		protein	
Hours	2	24	2	24	2	24	2	24
RI		Dex↑		Dex↑		RU↑		RU↑
RII	Dex↑*	Dex↑		Dex↑	RU↑*	RU↑		RU↑
RIII				Dex↑				RU↑
Hours			1	24			1	24
Smad2P			-	Dex↓			RU↑	RU↓
Smad3P			-	Dex↓			RU↑	RU↓
Smad2			Dex↑	Dex↓			-	RU↓
Smad3			Dex↑	Dex↓			-	RU↓

- = unchanged
* = same results were obtained for human keratinocytes (HaCat)

Table 10. Analysis of RII TGF-β receptor in human primary skin fibroblasts treated with steroids {estrogen (Est), Tamoxifen (Tam), or testosterone (Test)} or left untreated (C) at normoxia for 24 hours.

mRNA			
Hours	24		
	C vs Est	C vs Tam	C vs Testosterone
RII	Est↑	Tam↑	Test↓

Table 11. Analysis of TGF-β receptors (RI, RII, and RIII), and Smad proteins {phospho Smad2 (Smad2P), phospho Smad3 (Smad3P), total Smad2 (Smad2), and total Smad3 (Smad3)} in human skin cells treated with steroids {dexamethasone (Dex), or RU486 (RU)} or left untreated (C) exposed to normoxia (N), hypoxia (H) (2, and 24 hours), and reoxygenation (R) (2 hours).

	C vs Dex								C vs RU							
	mRNA				protein				mRNA				protein			
	H		R		H		R		H		R		H		R	
Hours	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24
RI		Dex ↑		Dex ↑						RU↑		RU↑				
RII	Dex ↑*	Dex ↑	Dex ↑	Dex ↓					RU↑	RU↓	RU↑ *	RU↓				
Hours					1	24	1	24					1	24	1	24
Smad2P					-	-	Dex ↑	Dex ↑					-	RU↑	RU↓	-
Smad3P					-	Dex ↓	-	Dex ↑					RU↑	RU↑	RU↑	-
Smad2					Dex ↑	Dex ↑	Dex ↑	Dex ↑					RU↑	RU↑	-	-
Smad3					Dex ↑	Dex ↑	Dex ↑	Dex ↑					RU↑	RU↑	-	-

- = unchanged
* = same results were obtained for human keratinocytes (HaCat)

Table 12. Analysis of RII TGF-β receptor in human skin cells treated with steroids {estrogen (Est), Tamoxifen (Tam), or testosterone (Test)} or left untreated (C) exposed to normoxia (N), hypoxia (H) (24 hours), and reoxygenation (R) (2 hours).

	mRNA				
Hours	24				
	C vs Est		C vs Tam		C vs Test
	H	R	H	R	H
RII	-	Est ↑	-	Tam ↓	Test↓

- = unchanged

4.6. Discussion:

The present study examined the regulation of TGF-β signaling by oxygen tension and steroids in human skin cells. The first part of the study dealt with the effect of hypoxia on TGF-β signaling pathway. Short term exposure to hypoxia (2 hours) increased the levels of active TGF-β while longer term exposure to hypoxia (24 hours) decreased the active TGF-β levels as compared to control untreated skin fibroblasts. It is well documented that TGF-β strongly enhance wound repair. Thus the increase in the active TGF-β at 2 hours of hypoxia is consistent with the notion that hypoxia during the early phase of wound healing facilitates the repair process. The observed down regulation of the active TGF-β at 24 hours of hypoxia in our experiment might provide an explanation for the inhibitory role of sustained hypoxia on wound healing as seen in patients with tissue ischemia.

The mechanism by which hypoxia may regulate TGF-β levels remains to be determined. During hypoxia changes in the expression of several genes have been documented (Melillo et al, 1995, Semenza, 2001). TGF-β is only functional when it is in its active state which is achieved through dissociation of TGF-β from the latent complexes by different mechanisms which may involve molecules such as TSP-1 and plasmin. Similar to our results, up-regulation of TGF-β activation by short term hypoxia has been shown in endothelial cells (Akman et al, 2001) and in perivascular glial cells of retina (Behzadian et al, 1998). Although, the mechanism of activation is not clear, it is

possible that the alteration in the active TGF-β by hypoxia could be indirect, involving the regulation of the activity of the molecules that activate of TGF-β. In fact, short term (2 hours) hypoxia has been shown to stimulate and longer term hypoxia (24 hours or 48 hours) has been demonstrated to inhibit the expression of TSP-1, a potent activator of TGF-β (Schultz-Cherry and Murphy-Ullrich, 1993; Tenan et al, 2000, Laderoute et al, 2000). Thus, the augmented levels of active TGF-β at 2 hours hypoxia and its decreased levels at 24 hour hypoxia observed in the present study may be mediated via TSP-1. Also, alpha2-antiplasmin, a liver produced inhibitor of plasmin is upregulated by hypoxia (Spranger et al, 1999), and may contribute to the decrease in active TGF-β after 24 hour exposure to hypoxia.

Our finding that the expression of RII (at mRNA level) was increased by short term hypoxia while it was decreased at longer exposure to hypoxia (at both mRNA and protein level), again, provide an explanation for the beneficiary role of initial hypoxia, and the inhibitory role of sustained hypoxia on wound healing, since RII expression is likely to dictate the responsiveness to TGF-β.

The observed upregulatory effect of long term hypoxia (24 hours) on the expression of RI (at mRNA level) in both fibroblasts and keratinocytes (HaCat cells) is intriguing. Interestingly, this increase in RI mRNA is not reflected at the protein level since RI protein is markedly decreased at 24 hours hypoxia as detected by affinity labeling. The fact that at 24 hours of hypoxia, there is a down regulation of RII mRNA while RI mRNA is increased may indicate that these two receptors are under distinct

regulatory controls and are affected differently by hypoxia at transcriptional level. The down regulation of both RI and RII proteins at 24 hour hypoxia may be due to the fact that the initial upregulation of TGF-β ligand by hypoxia (2 hours) and subsequent ligand-induced down regulation, at later time periods. In fact, pre-exposure of osteoblast cells to TGF-β1 causes a decrease in the receptor density (Centrella et al, 1996).

Short term hypoxia (1 hour), markedly increased Smad2P, Smad3P and total Smad2, and these increases were maintained during reoxygenation (2 hours). Although, no change was observed for total Smad3 after 1 hour of hypoxia, reoxygenation (2 hours) resulted in a dramatic increase in its levels. The increased phosphorylation of Smad2 and Smad3 is in accord with the augmentation of active TGF-β and signaling receptor levels during short term hypoxia, indicating the active induction of the TGF-β signaling machinery. The marked increase in total Smad2, and total Smad3 during the short time frame (1hour hypoxia and 1 hour hypoxia followed by 2 hours reoxygenation, respectively) is intriguing. This increase may reflect an inhibition of degradation rather than enhanced synthesis during this short period. Ubiquitination is an important regulatory mechanism which controls the steady-state of Smad2 and Smad3 (Lo and Massague, 1999; Fukuchi et al, 2001). The upregulation of total Smad2 and Smad3 observed in the present study may reside on the inhibition of their ubiquitination and their degradation by E3 ubiquitin ligases such as Smurfs. Hypoxia may interfere with the binding of Smurfs to Smads proteins and thus inhibiting their degradation as has

been suggested for the interaction between HIF-1α and the VHL component of E3 ubiquitin ligase (Pugh and Ratcliffe, 2003).

In contrast to short term hypoxia, long term hypoxia (24 hours) caused a down regulation of Smad3P with no change on Smad2P as compared to control untreated cells. The phosphorylation of Smad3 was exquisitely regulated by oxygen tension since reoxygenation fully reversed the effect of hypoxia. The decrease in the phosphorylation of Smad3 in skin fibroblasts as a function of time is in agreement with the result obtained from HUVEC cells (Zhang et al, 2003). The upregulation of total Smad3 suggests an increase in the unphosphorylated Smad3. This upregulation might be due to an increase in the rate of protein synthesis, or inhibition of its degradation or both. After reoxygenation for two hours, phosphorylated and total Smad2 and Smad3 were similar to that of control untreated cells grown at normoxia. Both long term hypoxia and Smad3 have been documented to have a negative role in wound healing (Siddiqui et al, 1996; Flanders et al, 2003). The observed upregulation of total Smad3 at 24 hour hypoxia, may suggest that one of the mechanism involved in delayed wound healing by hypoxia may include an increase in total Smad3.

The mechanism underlying the differential regulation of Smad2 and Smad3 during hypoxia remains to be determined. Total Smad2 and total Smad3 were distinctly regulated at 1 hour and 24 hours hypoxia and Smad2P and Smad3P were differentially modulated at 24 hour hypoxia. *In vivo* studies have shown that distinct functional roles exist for Smad2 and Smad3 in cells (Weinstein et al, 2000; Yang et al, 1999; Datto et al,

1999). Studies of Smad3 null mice and Smad3 heterozygous mice indicated that there is a dose-dependent effect on enhancing wound healing events such as epithelialization and reduced neutrophils numbers and fibrotic response (Flanders et al, 2003). Smad3 null mice show substantial protection from thickening of the epidermis, and hyperproliferation of cells in the hair follicle induced by irradiation as compared to wild type (Flanders et al, 2002). Thus, Smad3 may be the principal mediator of keratinocytes growth inhibition as well as of chemotaxis and TGF-β auto-induction in monocytes which chemotactically infiltrate the wound and produce cytokines that stimulate various functions, including inflammation. Moreover, the Smad3 null mice show defect in mucosal immunity, and in immune regulation (Yang et al, 1999). Thus, Smad3 appears to inhibit general aspects of wound healing. On the other hand, Smad2 promote wound healing by stimulating extracellular-matrix production in fibroblasts (Ashcroft et al, 1999b).

Steroids have important roles in skin homeostasis and wound healing. The deleterious effect of glucocorticoids on wound healing is well documented. Short term (2 hours) treatment with either dexamethasone or RU486 increased and longer term treatment (24 hours) decreased the active and total TGF-β in skin fibroblasts. The several fold greater magnitude of increase in active TGF-β levels as compared to that of total of concentration by dexamethasone and RU486 at 2 hours hypoxia suggests these steroids may induce activation of TGF-β. The mechanism by which dexamethasone and RU486 activate TGF-β after 2 hour of treatment is not known. Although, RU486 is

known to be a glucocorticoid antagonist, they displayed similar effects on TGF-β levels. However, RU486 was a more potent stimulator at 2 hour treatment and a less potent inhibitor at 24 hour treatment, of active TGF-β levels, as compared to dexamethasone.

TGF-β and glucocorticoid signaling pathways interact both positively and negatively in regulating a variety of physiologic and pathologic processes. The mechanism by which glucocorticoids inhibit wound healing involves the dysregulation of TGF-β signaling pathway although its anti-anabolic effect on collagen metabolism is also important (Hochberg, 2002). Glucocorticoids inhibit the TGF-β-induced expression of extracellular matrix proteins including fibronectin (Guller et al, 1995) and collagen (Slavin et al, 1994; Meisler et al, 1997), and proteinase inhibitors such as tissue inhibitors of metalloproteinase (Su et al, 1996). In the wounded skin, an increase in collagen synthesis occurs as early as 24 hours and might reflect qualitative or quantitative changes in extracellular matrix (Goforth and Gudas 1980). The differential modulation of TGF-β as a function of time by dexamethasone and RU486 is puzzling. Previously, Oxlund and co-workers (Oxlund et al, 1982) noted a biphasic effect of glucocorticoid treatment on rat skin, an initial increase in tensile strength associated with increased collagen crosslinking which is followed by decreased collagen synthesis resulting in decreased collagen content and thinner skin. A similar biphasic effect was also reported by Vogel (Vogel, 1974). Since TGF-β increases collagen synthesis in fibroblasts (Roberts et al, 1986), it is likely that the increase in the active TGF-β after

short term exposure mediates dexamethasone-induced collagen synthesis (Oxlund et al, 1982).

The observed decrease in the active TGF-β after 24 hour exposure to dexamethasone is in agreement with the inhibitory role of glucocorticoids on wound healing. Glucocorticoids have been reported to decrease activation and production of TGF-β1 in human skin fibroblasts (Wen et al, 2002). The biphasic effect of dexamethasone on the activation of TGF-β, at 2 hour and 24 hour of treatment observed in the present study is consistent with the biphasic role of dexamethasone on collagen deposition reported by Oxlund et al (1982) and Vogel (1974). This property of dexamethasone may have therapeutic relevance since it can potentially be used to regulate TGF-β levels to manipulate collagen synthesis and thus repair and scarring.

Little information is available on the mechanism of activation of TGF-β by dexamethasone. Dexamethasone may modulate TGF-β expression via the glucocorticoid response element (GRE) located in the promoter region of the human TGF-β1 gene (Parrelli et al, 1998). Furthermore, a recent study showed that dexamethasone was capable of inducing LTBP-1 at both protein and mRNA levels in human lung fibroblasts (Weikkolainen et al, 2003). Thus, dexamethasone can have an effect on the production and activation of TGF-β.

In postmenopausal women, estrogen replacement therapy is associated with prevention of loss of skin thickness and acceleration of skin tissue repair (Savaas et al,

1993) and an increase in TGF-β1 levels (Ashcroft et al, 1997). Whether estrogen regulates TGF-β action in the skin or during wound healing is not known. The observed decrease in the active TGF-β at 24 hours of exposure to estrogen or tamoxifen is in contrast with the beneficiary role of estrogen on both intact and wounded skin. The contradictory results may be attributable to the *in vitro* versus *in vivo* experimental conditions. In the present study, estrogen and tamoxifen potently decreased active TGF-β levels without significantly altering total TGF-β levels, suggesting that these steroids inhibit TGF-β activation The fact that both estrogen and its antagonist tamoxifen do not display opposing effects (both down regulate the active TGF-β) may be explained by the notion that tamoxifen is only a partial antagonist of estrogen. Interestingly, tamoxifen was a more potent inhibitor of TGF-β activation than estrogen.

Our results show that dexamethasone or RU486 increased the expression of TGF-β receptors (RI, RII, and RIII). This is not in accord with the known inhibitory effect of dexamethasone on wound healing *in vivo*. The discrepancy between our *in vitro* results and the *in vivo* observation emphasize the importance of cellular environment in determining TGF-β responses. The upregulation of RI by dexamethasone observed in the present study is consistent with the *in vivo* data of Frank et al (1996). However, in contrast to their results, the RII mRNA and protein was upregulated in our experiment. Complex interaction exists between dexamethasone and TGF-β receptors as has been documented in different cell types. Wickert et al. showed that after 8 hours of dexamethasone treatment RIII mRNA is increased in a time and dose-dependent manner

while the expression of RI and RII mRNAs is not altered in hepatic stellate cells (Wickert et al, 2004). Furthermore, dexamethasone decreased RI mRNA expression in matrix-producing bone cells (Chang et al, 1998). In that specific case, the transcriptional effect of dexamethasone seems to be mediated by Cbfa1, which is an osteoblast-specific transcription factor that is crucial for osteoblast differentiation and function. CBFa1 has a cis-acting element in the RI promoter that regulates its expression. Dexamethasone suppresses CBFa1 expression and indirectly represses RI expression (Chang et al, 1998; Ji et al, 1997). These observations show the complexity of the action of dexamethasone on TGF-β signaling, emphasizing the importance of cell types and experimental conditions.

Treatment of skin fibroblasts with estrogen and tamoxifen for 24 hours increased the expression of RII mRNA. Complex interaction exists between estrogen and TGF-β signaling. The estrogen effect is dependent on the state of cellular activation and is varied in different tissues. For example, estrogen inhibits TGF-β -induced collagen synthesis in renal mesangial cells (Lei et al, 1998; Silbiger et al, 1998), but how this is achieved is unclear. Recently Matsuda et al (2001) showed that ERs suppress TGF-β signaling by associating with, and acting as a transcriptional corepressor for Smad3. This may provide a molecular mechanism for the opposing effects of estrogen and TGF-β signaling in some disease states, such as renal injury, atherosclerosis, and breast cancer (Lei et al, 1998; Silbiger et al, 1998). RII promoter lacks a distinct TATA box, and Sp1 has been reported to play an important role in the initiation of transcription from

promoters lacking distinct TATA boxes (Pugh and Tjian, 1990). The molecular mechanism in upregulation of RII by estrogen may be due to an increase in the activity of Sp1 by estrogen in skin fibroblasts since suboptimal activity of Sp1 has been shown to result in transcriptional repression of RII.

Testosterone is the principal circulating androgenic hormone, and binds to the androgen receptor in target tissue to mediate its effects. Our data indicate that testosterone greatly reduces the expression of TGF-β receptor, and is consistent with the report of Kyprianou et al (1988) *in vivo* in the prostate. These results suggest an inhibitory effect of testosterone on TGF-β signaling and action in skin fibroblasts and thus in wound healing. Different reports have documented that males heal acute wounds more slowly than females and have an altered inflammatory response (Ashcroft et al, 2002; Ashcroft et al, 1999a; Taylor et al, 2002). Moreover, the male genotype is considered to be a positive risk factor for impaired healing in the elderly (Taylor et al, 2002).

Smad2 and Smad3 are important mediators of TGF-β signaling. The fact that the phosphorylation of Smad2 and Smad3 are not enhanced while the receptor levels are elevated in dexamethasone treated (2 hours and 24 hours) cells suggest that this steroid has an inhibitory effect on Smad phosphorylation. It is important to not here that glucocorticoid receptor directly interacts with Smad3 and inhibits its transcriptional activation (Song et al, 1999). Active TGF-β levels appear to parallel to total Smad2 and total Smad3 levels in dexamethasone treated (2 hour and 24 hours) cells, although it is

not as obvious in RU486 treated cells. It is possible that dexamethasone induced alteration in TGF-β activation and Smad synthesis are interdependent, but independent of Smad phosphorylation.

The total smad2 and smad3 proteins were increased after short term (1 hour) exposure to dexamethasone as compared to control untreated cells. The rapid upregulatory effect of dexamethasone on Smad proteins might be due to the inhibition of degradation of Smad proteins. Smad proteins remained unchanged by short term (1 hour) exposure to RU486 which stresses the fact that RU486 is only partial antagonist of dexamethasone. Glucocorticoids exert a deleterious effect on the wound healing process, which has been suggested to result from the anti-inflammatory action of these steroids. The fact that deletion of Smad3 is a positive factor in wound healing and our results showing that dexamethasone inhibits Smad phosphorylation and increases total levels of Smad3 provides a direct link between dexamethasone-induced impairment of healing and TGF-β signaling. Interestingly, glucocorticoid receptor has been suggested to inhibit TGF-β signaling by directly targeting Smad3 and Smad4 by blocking the activation function of Smad3/4 on target gene (Song et al, 1999)

Although RU486 is considered as an antagonist of dexamethasone, in our experiments, generally no opposing effect was observed between RU486 and dexamethasone treatment. The only difference observed between RU486 and dexamethasone was at the level of the magnitudes of the effect that they had on the regulation of Smad proteins. This might be due to the fact that RU486 is only a partial

antagonist of dexamethasone and may act as an agonist depending on the cellular context (Schulz et al, 2002). As such, RU486 may mimic some of the events related to GR activation, such as increase in GR translocation from the cytoplasm to the nucleus (Qi et al, 1990; Rupprecht et al, 1993; Jewell et al, 1995; Htun et al, 1996; Sackey et al, 1996) and binding to DNA (Schmidt, 1986; Beck et al, 1993). However, at the level of GR interaction, many differences exist between RU486 and dexamethasone: i) unlike dexamethasone, RU486 stabilizes the interaction between GR and hsps (Distelhorst and Howard, 1990; Beck et al, 1993); ii) the RU486-GR complex interacts with DNA with a much lower affinity than that of the Dexamethasone-GR complex (Bourgeois et al, 1984; Yu et al, 1995; Wagner et al, 1999); and iii) the RU486-GR complex is different from the agonists-GR complex in terms of recycling to the cytoplasm (Qi et al, 1990; Sackey et al, 1996).

Wound healing is a complex phenomenon that is under the regulation of different factors such as hypoxia and steroids. As mentioned earlier, glucocorticoids inhibit wound healing and estrogen promote wound repair. Recent studies have indicated that glucocorticoids have a role in the adaptation of cells to hypoxia. For example, the prophylactic administration of synthetic glucocorticoids dramatically prevents high mountain sickness (Johnson et al, 1984), and in rodents, the administration of glucocorticoids significantly reduces brain tissue damage after cerebral ischemia (Dardzinski et al, 2000). However, molecular mechanisms exist among steroids, hypoxia and TGF-β has not been investigated. To explore this, in the last part of study,

skin fibroblasts were treated with both hypoxia and steroids (dexamethasone or RU486) for 24 hours. Our results suggest that inhibitory effect on active TGF-β levels of 24 hours was additive, when dexamethasone (Figure 24A) or RU486 (Figure 24C) treatment was combined with hypoxia. For total TGF-β, however, no additive effect with hypoxia was detected for dexamethasone or RU486. The additive effect of hypoxia and steroids on inhibiting active TGF-β suggests that these agents may utilize distinct pathways for their inhibition. The inhibitory effect of dexamethasone and hypoxia on active TGF-β levels is consistent with the clinical observation of severe impairment of wound healing by these two agents (Tcacencu, 2002; Durmus et al, 2003)

In contrast with the results obtained with glucocorticoid and RU486, hypoxia was able to counteract the down regulation of active TGF-β by estrogen or tamoxifen. This is consistent with the beneficiary role of estrogen on wound healing. Hypoxia had no significant effect on the total TGF-β by estrogen and tamoxifen alone; indicating the observed increase in the active TGF-β is due to an increase in the activation of the existing latent TGF-β.

Hypoxia (2 or 24 hours) down regulated the dexamethasone-induced stimulation of RII and RI expression in both human skin fibroblasts and keratinocytes (HaCat); although the down regulatory effect of hypoxia was not strong enough to bring the RII to the level of control untreated cells. Thus, the combination of hypoxia and glucocorticoids enhances the mRNA expression of RII when compared with that

observed under hypoxia alone in skin fibroblasts and HaCat cells. Hypoxia could also partially reverse the upregulatory effect of both estrogen and tamoxifen on the expression of RII mRNA. Our results may suggest that hypoxia antagonize the effects for dexamethasone, estrogen, and tamoxifen on TGF-β signaling receptor expression.

The effect of short term (2 hours) hypoxia on RU486 treated cells was opposite to what was observed for dexamethasone treated cells. Hypoxia (2 hours) further upregulated RII in both types of skin (fibroblasts and keratinocytes) tested that were treated with RU486. However, 24 hours of hypoxia not only lowered the expression of RII in RU486 treated cells but also it further lowered from control untreated cells. The mechanism responsible for the differential regulation by hypoxia at 2 hours and 24 hours in RU486 treated cells is not clear. Nevertheless, our results suggest that hypoxia has a potent regulatory effect on factors modulating TGF-β receptor expression.

The potent effect of hypoxia on total Smad2 and total Smad3 in the presence of dexamethasone at 1 hour with little effect on their phosphorylated forms suggests that the nonphosphorylated and phosphorylated forms of Smads are distinctly regulated under these conditions. The differential effect of hypoxia alone on total Smad2 and total Smad3 (with marked increase in Smad2 at 1 hour and Smad3 at 24 hours imply distinct regulation of these two Smad pools. The potent effect of hypoxia on phosphorylated forms of Smad3 (1 hour and 24 hours) and Smad2 (24 hours) in RU486 treated cells but not dexamethasone treated cells suggest that the impact of hypoxia is critically dependent on the type of steroid present.

Thus, our results suggest that steroids and oxygen tension potentially regulate TGF-β signaling machinery including TGF-β levels, its receptors, and Smad proteins and therefore TGF-β action. In addition, we demonstrated that glucocorticoids and estrogens exert distinct effects on the TGF-β pathway and that their effects are critically dependent on oxygen tension in a steroid specific manner.

The *in vitro* methodological approach used in this study has limitations. The conclusions drawn should be confirmed by addressing the role of the hypoxia, and steroids, on the TGF-β signaling pathway within a more physiological context. Thus, *in vivo* studies should be done to verify the results. However, our studies done using individual cell types under controlled conditions provide important information based on which more complex questions may be asked. In our experiments, the concentration of 100 nM was used for each steroid. To establish the quantitative differences that exist in the effective range for each steroid, lower and higher concentrations of 100 nM should be tested.

In our study the expression of HIF-1 which is the master regulator of gene expression at hypoxic condition was not studied, and its effect on TGF-β signaling pathway in human primary fibroblast cells is not known. It is essential to study the possible modulation of TGF-β signaling pathway by HIF-1 with respect to our experimental conditions: i) hypoxia, ii) steroids, and iii) steroids and hypoxia. Moreover, it has been shown that co- activators, and co-repressors play an important role in both TGF-β and HIF-1 signaling pathway. It is pivotal to monitor the changes in the

expression of co-activators and co-repressors at various times under the above conditions to explore further the molecular mechanism involved in this process. This will allow the manipulation of these molecules to regulate specific TGF-β response.

Cross talk between TGF-β signaling and MAPK pathway has been described, but was not addressed in the present study. It is important to define the regulation of the MAP kinase pathway under our experimental conditions and to elucidate the interaction between the two pathways under those conditions. These information will assist in designing better strategies to manipulate TGF-β signaling and ultimately accelerating wound healing. It is interesting to note here that Mogford et al (2002) have shown that hypoxia have a major impact on TGF-β signaling pathway *in vivo*. Additionally, sex is a factor in determining the healing outcome in the human. The present study has used skin fibroblasts obtained from young women. Thus, it would be interesting to compare the results of the present study with those obtained with the skin fibroblasts from male subjects, and from older subjects.

Lastly, the effect of pre-exposure to hypoxia, and its impact on secondary exposure to hypoxia is an area which might provide further insight into the mechanism by which hypoxia regulate TGF-β action.

Chapter 5: General Conclusions

5.1. Conclusion:

TGF-β was discovered in 1981 as a factor that induced anchorage-independent growth in normal rat kidney fibroblast cells, and was recognized as a significant molecule that could play an important role in cellular function. For example, TGF-β regulates many aspects of cutaneous development, maintenance and repair (Roberts et al, 1986). TGF-β is a molecule with a relatively simple signaling pathway whose activity is modulated by cellular context, suggesting that the interaction of other signaling pathways with TGF-β signaling are important in determining TGF-β's effects. Steroids and oxygen tension are two elements that play important roles in wound healing. Although some studies have revealed the effects of these factors on TGF-β signaling, the mechanism of interactions is not clear. Thus, the present study used a pig skin flap model (*in vivo*) to investigate the role of ischemia and reperfusion on TGF-β and its receptors. In addition, we used an *in vitro* human skin cell (fibroblasts and keratinocytes (HaCat)) to study the effect of: i) hypoxia and reoxygenation, ii) steroids, and iii) steroids and hypoxia/ reoxygenation on TGF-β signaling (TGF-β ligand, its receptors, and their mediators, Smad2 and Smad3).

Our results demonstrate the occurrence of dynamic and cell type specific regulation of TGF-β receptors and TGF-β1 *in vivo* during ischemia-reperfusion, using the pig skin flap model. The most important finding in this present study is that global ischemia resulted in rapid (within 1hour) upregulation of types I, II and III TGF-β receptors on blood vessels (endothelial cells) and fibroblasts *in vivo*. While the

expression of the TGF-β receptors was increased on endothelial cells and fibroblasts within 1 hour of the induction of ischemia, subsequent reperfusion led to a significant decrease in the types I and II receptors in fibroblasts, but this decrease was not significant in blood vessels. The rapid upregulation of TGF-β receptors and TGF-β1, and thus enhanced TGF-β signal transducing machinery, in endothelial cells and fibroblasts during ischemia-reperfusion provide an explanation at the molecular level for the potent effect of TGF-β under these conditions, namely the tissue protective effect that TGF-β exerts against ischemia/reperfusion injury in several animal models.

Our *in vitro* results demonstrate that short term hypoxia (1-2 hours) activates the TGF-β signalling pathway (increases the active TGF-β, RII mRNA and phosphorylated Smad2 and Smad3, and total Smad2). Reoxygenated condition counteracts these effects and tends to normalize it to control untreated cells. On the other hand, long term hypoxia (24 hours) had inhibitory effect on TGF-β signalling by decreasing the active TGF-β, its receptors (RI, RII, and RIII) and Smad3P. Also, total Smad3 was increased and no change was observed in Smad2P and total Smad2. Our results show the strong responsiveness of TGF-β signalling molecules to hypoxia, and suggest potential inhibition of ubiquitination process in the upregulation of total Smad at 1 hour.

Short term exposure (2 hours) to glucocorticoids (dexamethasone and RU486) increased active and total TGF-β, RII in skin fibroblasts. Dexamethasone and RU486 differentially regulated Smad proteins. Dexamethasone had no effect and RU486 had upregulating effect on Smad2P and Smad3P. In contrast, RU486 had negligible effect

on total Smad2 and Smad3 while dexamethasone upregulated them. Long term exposure (24 hours) to all steroids tested decreased the active and total TGF-β, whereas TGF-β receptors (RI, and RII) mRNA and proteins were increased.

Interestingly, depending on the kind of steroid that skin cells were treated with, hypoxia (24 hours) had different effects on the TGF-β signalling components. Hypoxia counteracted the effects of estrogen and tamoxifen on active TGF-β. On the other hand, hypoxia displayed an additive effect on active TGF-β in dexamethasone and RU486 treated cells. At the receptor level, hypoxia decreased both RI and RII in cells treated with dexamethasone or RU486 for 24 hours, while it reversed the dexamethasone and RU486 induced downregulation of total Smad2 and total Smad3.

In summary, our results using skin cells in culture, demonstrate that regulatory factors such as hypoxia and steroids strongly modulate TGF-β signalling. Furthermore, our findings suggest that complex interactions exist between hypoxia and steroids in modulating TGF-β signalling. Our study clearly demonstrates that the TGF-β signalling pathway can be readily manipulated by steroids, hypoxia, or a combination of both.

Literature Cited

Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, and Riifkin DB. (1994). An assay for transfroming growth factor-B using cells transfected with a plasminogen activator inhibitor-1 Promoter-Luciferase construct. Analytical Biochemistry 216:276-284.

Adzick NS, Lorenz HP. (1994). Cells, matrix, growth factors, and the surgeon. The biology of scarless fetal wound repair. Ann Surg. 220(1):10-8.

Affinito P, Palomba S, Sorrentino C, Di Carlo C, Bifulco G, Arienzo MP, Nappi C. (1999). Effects of postmenopausal hypoestrogenism on skin collagen. Maturitas. 33(3):239-47.

Ahmed MM, Alcock RA, Chendil D, Dey S, Das A, Venkatasubbarao K, Mohiuddin M, Sun L, Strodel WE, Freeman JW. (2001). Restoration of transforming growth factor-beta signaling enhances radiosensitivity by altering the Bcl-2/Bax ratio in the p53 mutant pancreatic cancer cell line MIA PaCa-2. J Biol Chem. 277(3):2234-46.

Akhurst RJ, Lehnert SA, Faissner A, Duffie E. (1990). TGF beta in murine morphogenetic processes: the early embryo and cardiogenesis. Development. 108(4):645-56.

Akhurst RJ, Derynck R. TGF-beta signaling in cancer--a double-edged sword. (2001). Trends Cell Biol. 11(11):S44-51.

Akiyoshi S, Inoue H, Hanai J, Kusanagi K, Nemoto N, Miyazono K, Kawabata M. (1999). c-Ski acts as a transcriptional co-repressor in transforming growth factor-beta signaling through interaction with smads. J Biol Chem. 274(49):35269-77.

Akman HO, Zhang H, Siddiqui MA, Solomon W, Smith EL, Batuman OA. (2001). Response to hypoxia involves transforming growth factor-beta2 and Smad proteins in human endothelial cells. Blood. 98(12):3324-31.

Alevizopoulos A, Mermod N. (1997). Transforming growth factor-beta: the breaking open of a black box. Bioessays. 19(7):581-91.

Amendt C, Schirmacher P, Weber H, Blessing M. (1998). Expression of a dominant negative type II TGF-beta receptor in mouse skin results in an increase in carcinoma incidence and an acceleration of carcinoma development. Oncogene. 17(1):25-34.

Amento EP, Beck LS. (1991).TGF-beta and wound healing. Ciba Found Symp. 157:115-23; discussion 123-9.

Ammanamanchi S, Kim SJ, Sun LZ, Brattain MG. (1998). Induction of transforming growth factor-beta receptor type II expression in estrogen receptor-positive breast cancer cells through SP1 activation by 5-aza-2'-deoxycytidine. J Biol Chem. 1998 Jun 26;273(26):16527-34.

An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. (1998). Stabilization of wild-type p53 by hypoxia-inducible factor 1 alpha. Nature. 392(6674):405-8.

Anzano MA, Roberts AB, Smith JM, Sporn MB, De Larco JE. (1983). Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type alpha and type beta transforming growth factors. Proc Natl Acad Sci U S A. 80(20):6264-8

Arany Z, Huang LE, Eckner R, Bhattacharya S, Jiang C, Goldberg MA, Bunn HF, Livingston DM. (1996). An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci U S A. 93(23):12969-73.

Aschenbrenner JK, Sollinger HW, Becker BN, Hullett DA. (2001). 1,25-(OH(2))D(3) alters the transforming growth factor beta signaling pathway in renal tissue. J Surg Res. 100(2):171-5.

Ashcroft GS, Horan MA, Ferguson MW. (1995).The effects of ageing on cutaneous wound healing in mammals. J Anat. 187 (Pt 1):1-26.

Ashcroft GS, Dodsworth J, van Boxtel E, Tarnuzzer RW, Horan MA, Schultz GS, Ferguson MW. (1997). Estrogen accelerates cutaneous wound healing associated with an increase in TGF-beta1 levels. Nat Med. 3(11):1209-15

Ashcroft GS, Greenwell-Wild T, Horan MA, Wahl SM, Ferguson MW. (1999a). Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response. Am J Pathol. 155(4):1137-46.

Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JL, Mizel DE, Anzano M, Greenwell-Wild T, Wahl SM, Deng C, Roberts AB. (1999b). Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol. 1(5):260-6.

Ashcroft GS, Roberts AB. (2000). Loss of Smad3 modulates wound healing. Cytokine Growth Factor Rev. 11(1-2):125-31.

Ashcroft GS, Mills SJ. (2002). Androgen receptor-mediated inhibition of cutaneous wound healing. J Clin Invest. 110(5):615-24.

Ata KA, Lennmyr F, Funa K, Olsson Y, Terent A. (1999). Expression of transforming growth factor-beta1, 2, 3 isoforms and type I and II receptors in acute focal cerebral ischemia: an immunohistochemical study in rat after transient and permanent occlusion of middle cerebral artery. Acta Neuropathol (Berl) 97: 447-55.

Attisano L, Wrana JL. (2002). Signal transduction by the TGF-beta superfamily. Science. 296(5573):1646-7.

Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, Enomoto H, Simburger KS, Leitner ML, Araki T, Johnson EM Jr, Milbrandt J. (1998). Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. Neuron. 21(6):1291-302.

Bamberger AM, Bamberger CM, Gellersen B, Schulte HM. (1996). Modulation of AP-1 activity by the human progesterone receptor in endometrial adenocarcinoma cells. Proc Natl Acad Sci U S A. 93(12):6169-74.

Bandyopadhyay A, Zhu Y, Cibull ML, Bao L, Chen C, Sun L. (1999). A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells. Cancer Res. 59(19):5041-6.

Barnes PJ. (1998). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci (Lond). 94(6):557-72.

Basile DP, Rovak JM, Martin DR, Hammerman MR. (1996). Increased transforming growth factor-beta 1 expression in regenerating rat renal tubules following ischemic injury. Am J Physiol 270: F500-9.

Beck CA, Estes PA, Bona BJ, Muro-Cacho CA, Nordeen SK, Edwards DP. (1993). The steroid antagonist RU486 exerts different effects on the glucocorticoid and progesterone receptors. Endocrinology. 133:728-740.

Beck LS, Chen TL, Mikalauski P, Ammann AJ. (1990). Recombinant human transforming growth factor-beta 1 (rhTGF-beta 1) enhances healing and strength of granulation skin wounds. Growth Factors. 3(4):267-75.

Beck LS, Deguzman L, Lee WP, Xu Y, McFatridge LA, Gillett NA, Amento EP. (1991). Rapid publication. TGF-beta 1 induces bone closure of skull defects. J Bone Miner Res. 6(11):1257-65.

Beck LS, Deguzman L, Lee WP, Xu Y, McFatridge LA, Amento EP. (1991). TGF-beta 1 accelerates wound healing: reversal of steroid-impaired healing in rats and rabbits. Growth Factors. 5(4):295-304.

Beck LS, DeGuzman L, Lee WP, Xu Y, Siegel MW, Amento EP. (1993). One systemic administration of transforming growth factor-beta 1 reverses age- or glucocorticoid-impaired wound healing. J Clin Invest. 92(6):2841-9.

Becker A, Kuhnt T, Liedtke H, Krivokuca A, Bloching M, Dunst J. (2002). Oxygenation measurements in head and neck cancers during hyperbaric oxygenation. Strahlenther Onkol. 178(2):105-8

Behzadian MA, Wang XL, Shabrawey M, Caldwell RB. (1998). Effects of hypoxia on glial cell expression of angiogenesis-regulating factors VEGF and TGF-beta. Glia. 24(2):216-25.

Bellingham DL, Sar M, Cidlowski JA. (1992). Ligand-dependent down-regulation of stably transfected human glucocorticoid receptors is associated with the loss of functional glucocorticoid responsiveness. Mol Endocrinol. 6(12):2090-102.

Benhamou B, Garcia T, Lerouge T, Vergezac A, Gofflo D, Bigogne C, Chambon P, Gronemeyer H. (1992). A single amino acid that determines the sensitivity of progesterone receptors to RU486. Science. 255(5041):206-9.

Bennett NT, Schultz GS. (1993). Growth factors and wound healing: Part II. Role in normal and chronic wound healing. Am J Surg. 166(1):74-81.

Bentley JP, Brenner RM, Linstedt AD, West NB, Carlisle KS, Rokosova BC, MacDonald N. (1986). Increased hyaluronate and collagen biosynthesis and fibroblast estrogen receptors in macaque sex skin. J Invest Dermatol. 87(5):668-73.

Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, Oberhuber G. (2000). Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res. 60(17):4693-6.

Bitar MS, Labbad ZN. (1996). Transforming growth factor-beta and insulin-like growth factor-I in relation to diabetes-induced impairment of wound healing. J Surg Res. 61(1):113-9.

Bitar MS, Farook T, Wahid S, Francis IM. (1999). Glucocorticoid-dependent impairment of wound healing in experimental diabetes: amelioration by adrenalectomy and RU 486. J Surg Res. 82(2):234-43.

Bitar MS (2000). Insulin and glucocorticoid-dependent suppression of the IGF-I system in diabetic wounds. Surgery. 127(6):687-95.

Blancher C, Harris AL. (1998). The molecular basis of the hypoxia response pathway: tumour hypoxia as a therapy target. Cancer Metastasis Rev. 17(2):187-94.

Blanchere M, Saunier E, Mestayer C, Broshuis M, Mowszowicz I. (2002). Alterations of expression and regulation of transforming growth factor beta in human cancer prostate cell lines. J Steroid Biochem Mol Biol. 82(4-5):297-304.

Blobe GC, Schiemann WP, Pepin MC, 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(27):24627-37.

Bootcov MR, Bauskin AR, Valenzuela SM, Moore AG, Bansal M, He XY, Zhang HP, Donnellan M, Mahler S, Pryor K, Walsh BJ, Nicholson RC, Fairlie WD, Por SB, Robbins JM, Breit SN. (1997). MIC-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-beta superfamily. Proc Natl Acad Sci U S A. 94(21):11514-9

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol. 106(3):761-71

Boulanger J, Reyes-Moreno C, Koutsilieris M. (1995). Mediation of glucocorticoid receptor function by the activation of latent transforming growth factor beta 1 in MG-63 human osteosarcoma cells. Int J Cancer. 61(5):692-7.

Bourgeois S, Pfahl M, Baulieu EE. (1984). DNA binding properties of glucocorticosteroid receptors bound to the steroid antagonist RU-486. EMBO J. 3(4):751-5.

Boykin JV Jr. (2000). The nitric oxide connection: hyperbaric oxygen therapy, becaplermin, and diabetic ulcer management. Adv Skin Wound Care. 13(4 Pt 1):169-74.

Branton MH, Kopp JB. (1999). TGF-beta and fibrosis. Microbes Infect. 1(15):1349-65.

Brincat M, Kabalan S, Studd JW, Moniz CF, de Trafford J, Montgomery J. (1987). A study of the decrease of skin collagen content, skin thickness, and bone mass in the postmenopausal woman. Obstet Gynecol. 70(6):840-5.

Brincat MP. (2000). Hormone replacement therapy and the skin. Maturitas. 35(2):107-17.

Brizel DM, Scully SP, Harrelson JM, Layfield LJ, Bean JM, Prosnitz LR, Dewhirst MW. (1996). Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res. 56(5):941-3.

Brizel DM, Dodge RK, Clough RW, Dewhirst MW. (1999). Oxygenation of head and neck cancer: changes during radiotherapy and impact on treatment outcome. Radiother Oncol. 53(2):113-7

Brodin G, ten Dijke P, Funa K, Heldin CH, Landstrom M. (1999). Increased smad expression and activation are associated with apoptosis in normal and malignant prostate after castration. Cancer Res. 59(11):2731-8.

Brown CB, Boyer AS, Runyan RB, Barnett JV. (1999a). Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. Science. 283(5410):2080-2.

Brown JM, Giaccia AJ. (1998). The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res. 58(7):1408-16.

Brown JM. (1999b). The hypoxic cell: a target for selective cancer therapy--eighteenth Bruce F. Cain Memorial Award lecture. Cancer Res. 59(23):5863-70.

Brown JM. (2000). Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies. Mol Med Today. 6(4):157-62.

Brown PD, Wakefield LM, Levinson AD, Sporn MB. (1990). Physicochemical activation of recombinant latent transforming growth factor-beta's 1, 2, and 3. Growth Factors. 3(1):35-43.

Bruick RK. (2003). Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. Genes Dev. 17(21):2614-23.

Budtz PE. (1999). Role of proliferation and apoptosis in net growth rates of human breast cancer cells (MCF-7) treated with oestradiol and/or tamoxifen. Cell Prolif. 32(5):289-302.

Buisson A, Lesne S, Docagne F, Ali C, Nicole O, MacKenzie ET, Vivien D. (2003). Transforming growth factor-beta and ischemic brain injury. Cell Mol Neurobiol. 23(4-5):539-50.

Bullard KM, Cass DL, Banda MJ, Adzick NS. (1997). Transforming growth factor beta-1 decreases interstitial collagenase in healing human fetal skin. J Pediatr Surg. 32(7):1023-7.

Bunn HF, Poyton RO. (1996). Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev 76: 839-885.

Burger HG, Cahir N, Robertson DM, Groome NP, Dudley E, Green A, Dennerstein L. (1998). Serum inhibins A and B fall differentially as FSH rises in perimenopausal women. Clin Endocrinol (Oxf). 48(6):809-13.

Burnstein KL, Jewell CM, Cidlowski JA. (1990). Human glucocorticoid receptor cDNA contains sequences sufficient for receptor down-regulation. J Biol Chem. 265(13):7284-91.

Burnstein KL, Jewell CM, Sar M, Cidlowski JA. (1994). Intragenic sequences of the human glucocorticoid receptor complementary DNA mediate hormone-inducible receptor messenger RNA down-regulation through multiple mechanisms. Mol Endocrinol. 8(12):1764-73.

Cabral AL, Hays AN, Housley PR, Brentani MM, Martins VR. (2001). Repression of glucocorticoid receptor gene transcription by c-Jun. Mol Cell Endocrinol. 175(1-2):67-79.

Callens A, Vaillant L, Lecomte P, Berson M, Gall Y, Lorette G. (1996). Does hormonal skin aging exist? A study of the influence of different hormone therapy regimens on the skin of postmenopausal women using non-invasive measurement techniques. Dermatology. 193(4):289-94.

Camps JL, Chang SM, Hsu TC, Freeman MR, Hong SJ, Zhau HE, von Eschenbach AC, Chung LW. (1990). Fibroblast-mediated acceleration of human epithelial tumor growth in vivo. Proc Natl Acad Sci U S A. 87(1):75-9.

Cavailles V, Dauvois S, L'Horset F, Lopez G, Hoare S, Kushner PJ, Parker MG. (1995). Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. EMBO J. 14(15):3741-51.

Centrella M, Ji C, Casinghino S, McCarthy TL. (1996). Rapid flux in transforming growth factor-beta receptors on bone cells.J Biol Chem. 271(31):18616-22.

Chang DJ, Ji C, Kim KK, Casinghino S, McCarthy TL, Centrella M. (1998). Reduction in transforming growth factor beta receptor I expression and transcription factor CBFa1 on bone cells by glucocorticoid. J Biol Chem. 273(9):4892-6.

Chang H, Brown CW, Matzuk MM. (2002). Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev. 23(6):787-823.

Chau D, Mancoll JS, Lee S, Zhao J, Phillips LG, Gittes GK, Longaker MT. (1998). Tamoxifen downregulates TGF-beta production in keloid fibroblasts. Ann Plast Surg. 40(5):490-3.

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(22):10783-9.

Cheifetz S, Massague J. (1989). Transforming growth factor-beta (TGF-beta) receptor proteoglycan. Cell surface expression and ligand binding in the absence of glycosaminoglycan chains. J Biol Chem. 264(20):12025-8.

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(31):20767-72

Chen C, Pore N, Behrooz A, Ismail-Beigi F, & Maity A. (2001). Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J. Biol. Chem. 276, 9519-9525.

Chen CR, Kang Y, Siegel PM, Massague J. (2002). E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. Cell. 110(1):19-32.

Chen H, Tritton TR, Kenny N, Absher M, Chiu JF. (1996). Tamoxifen induces TGF-beta 1 activity and apoptosis of human MCF-7 breast cancer cells in vitro. J Cell Biochem. 61(1):9-17.

Chen JD, Evans RM. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature. 377(6548):454-7.

Chen RH, Ebner R, Derynck R. (1993). Inactivation of the type II receptor reveals two receptor pathways for the diverse TGF-beta activities. Science 260: 1335-1338.

Chen W, Wahl SM. (1999). Manipulation of TGF-beta to control autoimmune and chronic inflammatory diseases. Microbes Infect. 1(15):1367-80.

Chen YG, Liu F, Massague J. (1997). Mechanism of TGF beta receptor inhibition by FKBP12. EMBO J. 16(13):3866-76.

Chen, Y.G. and Massagué, J. (1999). Smad1 recognition and activation by the ALK1 group of transforming growth factor-β family receptors. J. Biol. Chem. 274, 3672-3677.

Chesnoy S, Lee PY, Huang L. (2003). Intradermal injection of transforming growth factor-beta1 gene enhances wound healing in genetically diabetic mice. Pharm Res. 20(3):345-50.

Chiarugi V, Magnelli L, Cinelli M. (1997). Complex interplay among apoptosis factors: RB, p53, E2F, TGF-beta, cell cycle inhibitors and the bcl2 gene family. Pharmacol Res. 35(4):257-61.

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(2):423-9.

Chomczynski P, Sacchi N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 162(1):156-9.

Chotnopparatpattara P, Panyakhamlerd K, Taechakraichana N, Tantivatana J, Chaikittisilpa S, Limpaphayom KK. (2001). An effect of hormone replacement therapy on skin thickness in early postmenopausal women. J Med Assoc Thai. 84(9):1275-80

Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature. 365(6449):855-9.

Clark RA. (1985). Cutaneous tissue repair: basic biologic considerations. I. J Am Acad Dermatol. 13(5 Pt 1):701-25.

Clark RA. (1988). Potential roles of fibronectin in cutaneous wound repair. Arch Dermatol. 124(2):201-6.

Cobb MH. (1999). MAP kinase pathways. Prog Biophys Mol Biol. 71(3-4):479-500.l

Cohen BJ, Danon D, Roth GS. (1987). Wound repair in mice as influenced by age and antimacrophage serum. J Gerontol. 42(3):295-301.

Colletta AA, Wakefield LM, Howell FV, van Roozendaal KE, Danielpour D, Ebbs SR, Sporn MB, Baum M. (1990). Anti-oestrogens induce the secretion of active transforming growth factor beta from human fetal fibroblasts. Br J Cancer. 62(3):405-9.

Cordeiro MF, Bhattacharya SS, Schultz GS, Khaw PT. (2000). TGF-beta1, -beta2, and -beta3 in vitro: biphasic effects on Tenon's fibroblast contraction, proliferation, and migration. Invest Ophthalmol Vis Sci. 41(3):756-63.

Corson GM, Chalberg SC, Dietz HC, Charbonneau NL, Sakai LY. (1993). Fibrillin binds calcium and is coded by cDNAs that reveal a multidomain structure and alternatively spliced exons at the 5' end. Genomics. 17(2):476-84.

Cotton SA, Herrick AL, Jayson MI, Freemont AJ. (1998). TGF beta-a role in systemic sclerosis? J Pathol. 184(1):4-6.

Cotran RS, Mayadas-Norton T. (1998). Endothelial adhesion molecules in health and disease. Pathol Biol (Paris) 46: 164-70.

Cowin AJ, Holmes TM, Brosnan P, Ferguson MW. (2001). Expression of TGF-beta and its receptors in murine fetal and adult dermal wounds. Eur J Dermatol. 11(5):424-31.

Cromack DT, Porras-Reyes B, Purdy JA, Pierce GF, Mustoe TA. (1993). Acceleration of tissue repair by transforming growth factor beta 1: identification of in vivo mechanism of action with radiotherapy-induced specific healing deficits. Surgery. 113(1):36-42

Cui W, Fowlis DJ, Cousins FM, Duffie E, Bryson S, Balmain A, Akhurst RJ. (1995). Concerted action of TGF-beta 1 and its type II receptor in control of epidermal homeostasis in transgenic mice. Genes Dev. 9(8):945-55.

Cui W, Fowlis DJ, Bryson S, Duffie E, Ireland H, Balmain A, Akhurst RJ. (1996). TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. Cell. 86(4):531-42.

Custodio JB, Dinis TC, Almeida LM, Madeira VM. (1994). Tamoxifen and hydroxytamoxifen as intramembraneous inhibitors of lipid peroxidation. Evidence for peroxyl radical scavenging activity. Biochem Pharmacol. 47(11):1989-98.

Custodio JB, Moreno AJ, Wallace KB. (1998). Tamoxifen inhibits induction of the mitochondrial permeability transition by Ca2+ and inorganic phosphate. Toxicol Appl Pharmacol. 152(1):10-7.

Czyzyk-Krzeska MF. (1997). Molecular aspects of oxygen sensing in physiological adaptation to hypoxia. Respir Physiol. 110(2-3):99-111.

Dabovic B, Chen Y, Colarossi C, Obata H, Zambuto L, Perle MA, Rifkin DB. (2002). Bone abnormalities in latent TGF-[beta] binding protein (Ltbp)-3-null mice indicate a role for Ltbp-3 in modulating TGF-[beta] bioavailability. J Cell Biol. 156(2):227-32.

Dallas SL, Miyazono K, Skerry TM, Mundy GR, Bonewald LF. (1995). Dual role for the latent transforming growth factor-beta binding protein in storage of latent TGF-beta in the extracellular matrix and as a structural matrix protein. J Cell Biol. 131(2):539-49.

Danon D, Kowatch MA, Roth GS. (1989). Promotion of wound repair in old mice by local injection of macrophages. Proc Natl Acad Sci U S A. 86(6):2018-20.

Dardzinski BJ, Smith SL, Towfighi J, Williams GD, Vannucci RC, Smith MB. (2000). Increased plasma beta-hydroxybutyrate, preserved cerebral energy metabolism, and amelioration of brain damage during neonatal hypoxia ischemia with dexamethasone pretreatment. Pediatr Res. 48(2):248-55.

Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF. (1995). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci U S A. 92(12):5545-9.

Datto MB, Frederick JP, Pan L, Borton AJ, Zhuang Y, Wang XF. (1999). Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. Mol Cell Biol. 19(4):2495-504.

de Caestecker MP, Piek E, Roberts AB. (2000). Role of transforming growth factor-beta signaling in cancer. J Natl Cancer Inst. 92(17):1388-402.

de Larco JE, Todaro GJ. (1978). Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci U S A. 75(8):4001-5

DeMarzo AM, Onate SA, Nordeen SK, Edwards DP. (1992). Effects of the steroid antagonist RU486 on dimerization of the human progesterone receptor. Biochemistry. 31(43):10491-501.

Deng X, Bellis S, Yan Z, Friedman E. (1999). Differential responsiveness to autocrine and exogenous transforming growth factor (TGF) beta1 in cells with nonfunctional TGF-beta receptor type III. Cell Growth Differ. 10(1):11-8.

Derynck R, Feng XH. (1997). TGF-beta receptor signaling. Biochim Biophys Acta. 1333(2):F105-50.

Derynck R, Akhurst RJ, Balmain A. (2001). TGF-beta signaling in tumor suppression and cancer progression. Nat Genet. 29(2):117-29.

Detmar M, Brown LF, Berse B, Jackman RW, Elicker BM, Dvorak HF, Claffey KP. (1997). Hypoxia regulates the expression of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) and its receptors in human skin. J Invest Dermatol 108: 263-8.

Dhandapani KM, Brann DW. (2003). Transforming growth factor-beta: a neuroprotective factor in cerebral ischemia. Cell Biochem Biophys. 39(1):13-22.

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 U S A 92:12215-9.

Distelhorst CW, Howard KJ. (1990). Evidence from pulse-chase labeling studies that the antiglucocorticoid hormone RU486 stabilizes the nonactivated form of the glucocorticoid receptor in mouse lymphoma cells. J Steroid Biochem. 36(1-2):25-31.

Dittmar KD, Pratt WB. (1997). Folding of the glucocorticoid receptor by the reconstituted Hsp90-based chaperone machinery. The initial hsp90.p60.hsp70-dependent step is sufficient for creating the steroid binding conformation. J Biol Chem. 272(20):13047-54.

Dong Y, Poellinger L, Gustafsson JA, Okret S. (1988). Regulation of glucocorticoid receptor expression: evidence for transcriptional and posttranslational mechanisms. Mol Endocrinol. 2(12):1256-64.

Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM. (1998). The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. Mol Endocrinol 12:1809–1817

Dumont A, Hehner SP, Schmitz ML, Gustafsson JA, Liden J, Okret S, van der Saag PT, Wissink S, van der Burg B, Herrlich P, Haegeman G, De Bosscher K, Fiers W. (1998). Cross-talk between steroids and NF-kappa B: what language?. Trends Biochem Sci. 23(7):233-5.

Dumont N, O'Connor-McCourt MD, Philip A. (1995). Transforming growth factor-beta receptors on human endometrial cells: identification of the type I, II, and III receptors and glycosyl-phosphatidylinositol anchored TGF-beta binding proteins. Mol Cell Endocrinol. 28;111(1):57-66.

Dumont N, Arteaga CL. (2000). Transforming growth factor-beta and breast cancer: Tumor promoting effects of transforming growth factor-beta. Breast Cancer Res. 2(2):125-32.

Durmus M, Karaaslan E, Ozturk E, Gulec M, Iraz M, Edali N, Ersoy MO. (2003). The effects of single-dose dexamethasone on wound healing in rats. Anesth Analg. 97(5):1377-80.

El-Rifai W, Powell SM. (2002). Molecular biology of gastric cancer. Semin Radiat Oncol.12(2):128-40.

Engle SJ, Hoying JB, Boivin GP, Ormsby I, Gartside PS, Doetschman T. (1999). Transforming growth factor beta1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. Cancer Res. 59(14):3379-86.

Enholm B, Paavonen K, Ristimaki A, Kumar V, Gunji Y, Klefstrom J, Kivinen L, Laiho M, Olofsson B, Joukov V, Eriksson U, Alitalo K. (1997). Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. Oncogene. 14(20):2475-83.

Eto Y, Tsuji T, Takezawa M, Takano S, Yokogawa Y, Shibai H. (1987). Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. Biochem Biophys Res Commun. 142(3):1095-103.

Evangelou A, Letarte M, Jurisica I, Sultan M, Murphy KJ, Rosen B, Brown TJ. (2003). Loss of coordinated androgen regulation in nonmalignant ovarian epithelial cells with BRCA1/2 mutations and ovarian cancer cells. Cancer Res. 63(10):2416-24.

Fabian C, Tilzer L, Sternson L. (1981). Comparative binding affinities of tamoxifen, 4-hydroxytamoxifen, and desmethyltamoxifen for estrogen receptors isolated from human breast carcinoma: correlation with blood levels in patients with metastatic breast cancer. Biopharm Drug Dispos. 2(4):381-90.

Falanga V, Qian SW, Danielpour D, Katz MH, Roberts AB, Sporn MB. (1991). Hypoxia upregulates the synthesis of TGF-beta 1 by human dermal fibroblasts. J Invest Dermatol. 97(4):634-7.

Falanga V, Martin TA, Takagi H, Kirsner RS, Helfman T, Pardes J, Ochoa MS. (1993). Low oxygen tension increases mRNA levels of alpha 1 (I) procollagen in human dermal fibroblasts. J Cell Physiol. 157(2):408-12.

Falanga V, Takagi H, Ceballos PI, Pardes JB. (1994). Low oxygen tension decreases receptor binding of peptide growth factors in dermal fibroblast cultures. Exp Cell Res. 213(1):80-4.

Fan JM, Ng YY, Hill PA, Nikolic-Paterson DJ, Mu W, Atkins RC, Lan HY. (1999). Transforming growth factor-beta regulates tubular epithelial-myofibroblast transdifferentiation in vitro. Kidney Int. 56(4):1455-67.

Feldser, D., Agani, F., Iyer, N.V., Pak, B., Ferreira, G. & Semenza, G.L. (1999). Reciprocal positive regulation of hypoxia-inducible factor 1^{α} and insulin-like growth factor 2. Cancer Res. 59, 3915-3918.

Feng XH, Derynck R. (1996). Ligand-independent activation of transforming growth factor (TGF) beta signaling pathways by heteromeric cytoplasmic domains of TGF-beta receptors. J Biol Chem 271: 13123-13129.

Feng XH, Derynck R. (1997). A kinase subdomain of transforming growth factor-beta (TGF-beta) type I receptor determines the TGF-beta intracellular signaling specificity. EMBO J. 16(13):3912-23.

Feng XH, Zhang Y, Wu RY, Derynck R. (1998). The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. Genes Dev. 12(14):2153-63.

Festuccia C, Bologna M, Gravina GL, Guerra F, Angelucci A, Villanova I, Millimaggi D, Teti A. (1999). Osteoblast conditioned media contain TGF-beta1 and modulate the migration of prostate tumor cells and their interactions with extracellular matrix components. Int J Cancer. 81(3):395-403.

Filvaroff E, Erlebacher A, Ye J, Gitelman SE, Lotz J, Heillman M, Derynck R. (1999). Inhibition of TGF-beta receptor signaling in osteoblasts leads to decreased bone remodeling and increased trabecular bone mass. Development. 126(19):4267-79.

Finkenzeller G, Technau A, Marme D. (1995). Hypoxia-induced transcription of the vascular endothelial growth factor gene is independent of functional AP-1 transcription factor. Biochem Biophys Res Commun. 208(1):432-9.

Flanders KC, Kim ES, Roberts AB. (2001). Immunohistochemical expression of Smads 1-6 in the 15-day gestation mouse embryo: signaling by BMPs and TGF-betas. Dev Dyn. 220(2):141-54.

Flanders KC, Sullivan CD, Fujii M, Sowers A, Anzano MA, Arabshahi A, Major C, Deng C, Russo A, Mitchell JB, Roberts AB. (2002). Mice lacking Smad3 are protected against cutaneous injury induced by ionizing radiation. Am J Pathol. 160(3):1057-68.

Flanders KC, Major CD, Arabshahi A, Aburime EE, Okada MH, Fujii M, Blalock TD, Schultz GS, Sowers A, Anzano MA, Mitchell JB, Russo A, Roberts AB. (2003). Interference with transforming growth factor-beta/ Smad3 signaling results in accelerated healing of wounds in previously irradiated skin. Am J Pathol. 163(6):2247-57.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol. 16(9):4604-13.

Fraire AE, Greenberg SD. (1973). Carcinoma and diffuse interstitial fibrosis of lung. Cancer. 31(5):1078-86.

Frank S, Madlener M, Werner S. (1996). Transforming growth factors beta1, beta2, and beta3 and their receptors are differentially regulated during normal and impaired wound healing. J Biol Chem. 271(17):10188-93.

Franzen P, ten Dijke P, Ichijo H, Yamashita H, Schulz P, Heldin CH, Miyazono K. (1993). Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. Cell 75: 681-92.

Frey RS, Mulder KM. (1997a). TGFbeta regulation of mitogen-activated protein kinases in human breast cancer cells. Cancer Lett.117(1):41-50.

Frey RS, Mulder KM. (1997b). Involvement of extracellular signal-regulated kinase 2 and stress-activated protein kinase/Jun N-terminal kinase activation by transforming growth factor beta in the negative growth control of breast cancer cells. Cancer Res. 57(4):628-33.

Friedman E, Gold LI, Klimstra D, Zeng ZS, Winawer S, Cohen A. (1995). High levels of transforming growth factor beta 1 correlate with disease progression in human colon cancer. Cancer Epidemiol Biomarkers Prev. 4(5):549-54.

Friess H, Yamanaka Y, Buchler M, Ebert M, Beger HG, Gold LI, Korc M. (1993). Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival. Gastroenterology. 105(6):1846-56.

Fukuchi M, Imamura T, Chiba T, Ebisawa T, Kawabata M, Tanaka K, Miyazono K. (2001). Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins. Mol Biol Cell. 12(5):1431-43.

Furukawa T, Konishi F, Shitoh K, Kojima M, Nagai H, Tsukamoto T. (2002). Evaluation of screening strategy for detecting hereditary nonpolyposis colorectal carcinoma. Cancer. 94(4):911-20.

Garrigue-Antar L, Munoz-Antonia T, Antonia SJ, Gesmonde J, Vellucci VF, Reiss M. (1995). Missense mutations of the transforming growth factor beta type II receptor in human head and neck squamous carcinoma cells. Cancer Res. 55(18):3982-7.

Geiser AG, Burmester JK, Webbink R, Roberts AB, Sporn MB. (1992). Inhibition of growth by transforming growth factor-beta following fusion of two nonresponsive human carcinoma cell lines. Implication of the type II receptor in growth inhibitory responses. J Biol Chem. 267(4):2588-93.

Gilmont RR, Dardano A, Engle JS, Adamson BS, Welsh MJ, Li T, Remick DG, Smith DJ Jr, Rees RS. (1996). TNF-alpha potentiates oxidant and reperfusion-induced endothelial cell injury. Surg Res 61: 175-182.

Giltay R, Kostka G, Timpl R. (1997). Sequence and expression of a novel member (LTBP-4) of the family of latent transforming growth factor-beta binding proteins. FEBS Lett. 411(2-3):164-8.

Gleizes PE, Munger JS, Nunes I, Harpel JG, Mazzieri R, Noguera I, Rifkin DB. (1997). TGF-beta latency: biological significance and mechanisms of activation. Stem Cells. 15(3):190-7.

Gold LI. (1999). The role for transforming growth factor-beta (TGF-beta) in human cancer. Crit Rev Oncog. 10(4):303-60.

Goforth P, Gudas CJ. (1980). Effects of steroids on wound healing: a review of the literature. J Foot Surg. 19(1):22-8.

Gorelik L, Flavell RA. (2000). Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity. 12(2):171-81

Gorlach, A., Diebold, I., Schini-Kerth, V.B., Berchner-Pfannschmidt, U., Roth, U., Brandes, R.P., Kietzmann, T. & Busse, R. (2001). Thrombin activates the hypoxia-inducible factor-1 signaling pathway in vascular smooth muscle cells: Role of the p22 (phox)-containing NADPH oxidase. Circ Res. 89, 47-54.

Gorsch SM, Memoli VA, Stukel TA, Gold LI, Arrick BA. (1992). Immunohistochemical staining for transforming growth factor beta 1 associates with disease progression in human breast cancer. Cancer Res. 52(24):6949-52.

Gougos A, Letarte M. (1990). Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. J Biol Chem. 265(15):8361-4.

Goumans MJ, Mummery C. (2000). Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. Int J Dev Biol. 44(3):253-65.

Graeber TG, Peterson JF, Tsai M, Monica K, Fornace AJ Jr, Giaccia AJ. (1994). Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. Mol Cell Biol. 14(9):6264-77.

Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature. 379(6560):88-91.

Groves RW, Schmidt-Lucke JA. (2000). Recombinant human GM-CSF in the treatment of poorly healing wounds. Adv Skin Wound Care. 13(3 Pt 1):107-12.

Gruschwitz M, Muller PU, Sepp N, Hofer E, Fontana A, Wick G.(1990). Transcription and expression of transforming growth factor type beta in the skin of progressive systemic sclerosis: a mediator of fibrosis? J Invest Dermatol. 94(2):197-203.

Guller S, Wozniak R, Kong L, Lockwood CJ. (1995). Opposing actions of transforming growth factor-beta and glucocorticoids in the regulation of fibronectin expression in the human placenta. J Clin Endocrinol Metab. 80(11):3273-8.

Guo Y, Kyprianou N. (1999). Restoration of transforming growth factor beta signaling pathway in human prostate cancer cells suppresses tumorigenicity via induction of caspase-1-mediated apoptosis. Cancer Res. 59(6):1366-71.

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(2):190-8

Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science. 271(5247):350-3.

Hamilton JA, Filonzi EL, Ianches G. (1993). Regulation of macrophage colony-stimulating factor (M-CSF) production in cultured human synovial fibroblasts. Growth Factors. 9(2):157-65.

Han SH, Yea SS, Jeon YJ, Yang KH, Kaminski NE. (1998). Transforming growth factor-beta 1 (TGF-beta1) promotes IL-2 mRNA expression through the up-regulation of NF-kappaB, AP-1 and NF-AT in EL4 cells. J Pharmacol Exp Ther. 287(3):1105-12.

Han MK, Kim JS, Park BH, Kim JR, Hwang BY, Lee HY, Song EK, Yoo WH. (2003). NF-kappaB-dependent lymphocyte hyperadhesiveness to synovial fibroblasts by hypoxia and reoxygenation: potential role in rheumatoid arthritis. J Leukoc Biol. 73(4):525-9.

Hannon GJ, Beach D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature. 371(6494):257-61.

Hartsough MT, Mulder KM. (1995). Transforming growth factor beta activation of p44mapk in proliferating cultures of epithelial cells. J Biol Chem. 270(13):7117-24.

Hartsough MT, Frey RS, Zipfel PA, Buard A, Cook SJ, McCormick F, Mulder KM. (1996). Altered transforming growth factor signaling in epithelial cells when ras activation is blocked. J Biol Chem. 271(37):22368-75.

Hartsough MT, Mulder KM. (1997). Transforming growth factor-beta signaling in epithelial cells. Pharmacol Ther. 75(1):21-41.

Hashiro M, Matsumoto K, Hashimoto K, Yoshikawa K. (1991). Stimulation of fibronectin secretion in cultured human keratinocytes by transforming growth factor-beta not by other growth inhibitory substances. J Dermatol. 18(5):252-7.

Hayes SA, Zarnegar M, Sharma M, Yang F, Peehl DM, ten Dijke P, Sun Z. (2001). SMAD3 represses androgen receptor-mediated transcription. Cancer Res. 61(5):2112-8.

Heck S, Kullmann M, Gast A, Ponta H, Rahmsdorf HJ, Herrlich P, Cato AC. (1994). A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. EMBO J. 13(17):4087-95.

Heine U, Munoz EF, Flanders KC, Ellingsworth LR, Lam HY, Thompson NL, Roberts AB, Sporn MB. (1987). Role of transforming growth factor-beta in the development of the mouse embryo. J Cell Biol. 105(6 Pt 2):2861-76.

Heldin CH, Miyazono K, ten Dijke P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature. 390(6659):465-71.

Hellwig-Burgel, T., Rutkowski, K., Metzen, E., Fandrey, J. & Jelkmann, W. (1999). Interleukin-1^{β} and tumor necrosis factor-$^{\alpha}$ stimulate DNA binding of hypoxia-inducible factor-1. Blood. 94, 1561-1567.

Herskowitz A, Choi S, Ansari AA, Wesselingh S. (1995). Cytokine mRNA expression in postischemic/reperfused myocardium. Am J Pathol 146: 419-428.

Hirshberg J, Coleman J, Marchant B, Rees RS. (2001). TGF-beta3 in the treatment of pressure ulcers: a preliminary report. Adv Skin Wound Care. 14(2):91-5.

Hochberg Z. (2002). Mechanisms of steroid impairment of growth. Horm Res.58 Suppl 1:33-8.

Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. (1996). Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res. 56(19):4509-15.

Hoeck W, Rusconi S, Groner B. (1989). Down-regulation and phosphorylation of glucocorticoid receptors in cultured cells. Investigations with a monospecific antiserum against a bacterially expressed receptor fragment. J Biol Chem. 264(24):14396-402.

Hogan BL. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. 10(13):1580-94.

Hojo M, Morimoto T, Maluccio M, Asano T, Morimoto K, Lagman M, Shimbo T, Suthanthiran M. (1999). Cyclosporine induces cancer progression by a cell-autonomous mechanism. Nature. 397(6719):530-4.

Hong H, Kohli K, Trivedi A, Johnson DL, Stallcup MR. (1996). GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. Proc Natl Acad Sci U S A. 93(10):4948-52.

Hong H, Kohli K, Garabedian MJ, Stallcup MR. (1997). GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. Mol Cell Biol. 17(5):2735-44.

Horlein AJ, Naar AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Soderstrom M, Glass CK, et al. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature. 377(6548):397-404.

Horvath LZ, Friess H, Schilling M, Borisch B, Deflorin J, Gold LI, Korc M, Buchler MW. (1996). Altered expression of transforming growth factor-beta S in chronic renal rejection. Kidney Int. 50(2):489-98.

Hosokawa R, Nonaka K, Morifuji M, Shum L, Ohishi M. (2003). TGF-beta 3 decreases type I collagen and scarring after labioplasty. J Dent Res. 82(7):558-64.

Hsing AY, Kadomatsu K, Bonham MJ, Danielpour D. (1996). Regulation of apoptosis induced by transforming growth factor-beta1 in nontumorigenic rat prostatic epithelial cell lines. Cancer Res. 56(22):5146-9.

Hsu M, Peled ZM, Chin GS, Liu W, Longaker MT. (2001). Ontogeny of expression of transforming growth factor-beta 1 (TGF-beta 1), TGF-beta 3, and TGF-beta receptors I and II in fetal rat fibroblasts and skin. Plast Reconstr Surg. 107(7):1787-94

Htun H, Barsony J, Renyi I, Gould DL, Hager GL. (1996). Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera. Proc Natl Acad Sci U S A. 93(10):4845-50.

Huang XZ, Wu JF, Cass D, Erle DJ, Corry D, Young SG, Farese RV Jr, Sheppard D. (1996). Inactivation of the integrin beta 6 subunit gene reveals a role of epithelial integrins in regulating inflammation in the lung and skin. J Cell Biol. 133(4):921-8.

Huse M, Chen YG, Massague J, Kuriyan J. (1999). Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. Cell. 96(3):425-36.

Huse M, Muir TW, Xu L, Chen YG, Kuriyan J, Massague J. (2001). The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. Mol Cell. 8(3):671-82.

Hutchison KA, Stancato LF, Owens-Grillo JK, Johnson JL, Krishna P, Toft DO, Pratt WB. (1995). The 23-kDa acidic protein in reticulocyte lysate is the weakly bound component of the hsp foldosome that is required for assembly of the glucocorticoid receptor into a functional heterocomplex with hsp90. J Biol Chem. 270(32):18841-7.

Iacopetta BJ, Soong R, House AK, Hamelin R. (1999). Gastric carcinomas with microsatellite instability: clinical features and mutations to the TGF-beta type II receptor, IGFII receptor, and BAX genes. J Pathol. 187(4):428-32.

Ignotz RA, Massague J. (1986). Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem. 261(9):4337-45.

Ignotz RA, Endo T, Massague J. (1987). Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-beta. J Biol Chem. 262(14):6443-6.

Issa LL, Leong GM, Eisman JA. (1998). Molecular mechanism of vitamin D receptor action. Inflamm Res. 47(12):451-75.

Itoh S, Itoh F, Goumans MJ, Ten Dijke P. (2000). Signaling of transforming growth factor-beta family members through Smad proteins. Eur J Biochem. 267(24):6954-67

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science. 292(5516):464-8

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim Av, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science. 292(5516):468-72.

Jackson TA, Richer JK, Bain DL, Takimoto GS, Tung L, Horwitz KB. (1997). The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. Mol Endocrinol. 11(6):693-705.

Janda E, Lehmann K, Killisch I, Jechlinger M, Herzig M, Downward J, Beug H, Grunert S. (2002). Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol. 156(2):299-313.

Janknecht R, Wells NJ, Hunter T. (1998). TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev. 12(14):2114-9.

Jaskoll T, Melnick M. (1999). Submandibular gland morphogenesis: stage-specific expression of TGF-alpha/EGF, IGF, TGF-beta, TNF, and IL-6 signal transduction in normal embryonic mice and the phenotypic effects of TGF-beta2, TGF-beta3, and EGF-r null mutations. Anat Rec. 256(3):252-68.

Jelaska A, Korn JH. (2000). Role of apoptosis and transforming growth factor beta1 in fibroblast selection and activation in systemic sclerosis. Arthritis Rheum. 43(10):2230-9.

Jennings MT, Kaariainen IT, Gold L, Maciunas RJ, Commers PA. (1994). TGF beta 1 and TGF beta 2 are potential growth regulators for medulloblastomas, primitive neuroectodermal tumors, and ependymomas: evidence in support of an autocrine hypothesis. Hum Pathol. 25(5):464-75.

Jewell CM, Webster JC, Burnstein KL, Sar M, Bodwell JE, Cidlowski JA. (1995). Immunocytochemical analysis of hormone mediated nuclear translocation of wild type and mutant glucocorticoid receptors. J Steroid Biochem Mol Biol. 55(2):135-46.

Jhappan C, Geiser AG, Kordon EC, Bagheri D, Hennighausen L, Roberts AB, Smith GH, Merlino G. (1993). Targeting expression of a transforming growth factor beta 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. EMBO J. 12(5):1835-45.

Ji C, Casinghino S, McCarthy TL, Centrella M. (1997). Multiple and essential Sp1 binding sites in the promoter for transforming growth factor-beta type I receptor. J Biol Chem. 272(34):21260-7

Ji C, Chen Y, McCarthy TL, Centrella M. (1999). Cloning the promoter for transforming growth factor-beta type III receptor. Basal and conditional expression in fetal rat osteoblasts. J Biol Chem. 274(43):30487-94.

Jiang B.H., Agani F, Passaniti A & Semenza G.L. (1997). V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. Can. Res. 57: 5328-5335.

Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance MA, Diamond A, Guttmacher AE, Jackson CE, Attisano L, Kucherlapati R, Porteous ME, Marchuk DA. (1996). Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. Nat Genet. 13(2):189-95

Johnson TS, Rock PB, Fulco CS, Trad LA, Spark RF, Maher JT. (1984). Prevention of acute mountain sickness by dexamethasone. N Engl J Med. 310(11):683-6.

Jonat C, Rahmsdorf HJ, Park KK, Cato AC, Gebel S, Ponta H, Herrlich P. (1990). Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell. 62(6):1189-204.

Jordan VC. (1992). The role of tamoxifen in the treatment and prevention of breast cancer. Curr Probl Cancer. 16(3):129-76.

Josien R, Douillard P, Guillot C, Muschen M, Anegon I, Chetritt J, Menoret S, Vignes C, Soulillou JP, Cuturi MC. (1998). A critical role for transforming growth factor-beta in donor transfusion-induced allograft tolerance. J Clin Invest. 102(11):1920-6.

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(4):415-21.

Kadin ME, Cavaille-Coll MW, Gertz R, Massague J, Cheifetz S, George D. (1994). Loss of receptors for transforming growth factor beta in human T-cell malignancies. Proc Natl Acad Sci U S A. 91(13):6002-6.

Kakonen SM, Selander KS, Chirgwin JM, Yin JJ, Burns S, Rankin WA, Grubbs BG, Dallas M, Cui Y, Guise TA. (2002). Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. J Biol Chem. 277(27):24571-8.

Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Gloss B, Lin SC, Heyman RA, Rose DW, Glass CK, Rosenfeld MG. (1996). A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell. 85(3):403-14.

Kanamaru Y, Nakao A, Mamura M, Suzuki Y, Shirato I, Okumura K, Tomino Y, Ra C. (2001). Blockade of TGF-beta signaling in T cells prevents the development of experimental glomerulonephritis. J Immunol. 166(4):2818-23.

Kanatani Y, Kasukabe T, Okabe-Kado J, Hayashi S, Yamamoto-Yamaguchi Y, Motoyoshi K, Nagata N, Honma Y. (1996). Transforming growth factor beta and dexamethasone cooperatively enhance c-jun gene expression and inhibit the growth of human monocytoid leukemia cells. Cell Growth Differ. 7(2):187-96.

Kang HY, Lin HK, Hu YC, Yeh S, Huang KE, Chang C. (2001). From transforming growth factor-beta signaling to androgen action: identification of Smad3 as an androgen receptor coregulator in prostate cancer cells. Proc Natl Acad Sci U S A. 98(6):3018-23.

Kanzaki T, Olofsson A, Moren A, Wernstedt C, Hellman U, Miyazono K, Claesson-Welsh L, Heldin CH. (1990). TGF-beta 1 binding protein: a component of the large latent complex of TGF-beta 1 with multiple repeat sequences. Cell. 61(6):1051-61.

Kedar RP, Bourne TH, Powles TJ, Collins WP, Ashley SE, Cosgrove DO, Campbell S. (1994). Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. Lancet. 343(8909):1318-21.

Kerrigan CL, Stotland MA. (1993). Ischemia reperfusion injury: a review. Microsurgery 14: 165-175.

Kerrigan CL, Zelt RG, Thomson JG, Diano E. (1986). The pig as an experimental animal in plastic surgery research for the study of skin flaps, myocutaneous flaps and fasciocutaneous flaps. Lab Anim Sci 36: 408-412.

Keski-Oja J, Koli K. (1992). Enhanced production of plasminogen activator activity in human and murine keratinocytes by transforming growth factor-beta 1. J Invest Dermatol. 99(2):193-200.

Khalil N, Corne S, Whitman C, Yacyshyn H. (1996). Plasmin regulates the activation of cell-associated latent TGF-beta 1 secreted by rat alveolar macrophages after in vivo bleomycin injury. Am J Respir Cell Mol Biol. 15(2):252-9.

Khanna AK, Cairns VR, Becker CG, Hosenpud JD. (1999). Transforming growth factor (TGF)-beta mimics and anti-TGF-beta antibody abrogates the in vivo effects of cyclosporine: demonstration of a direct role of TGF-beta in immunosuppression and nephrotoxicity of cyclosporine. Transplantation. 67(6):882-9.

Kim IY, Ahn HJ, Zelner DJ, Shaw JW, Sensibar JA, Kim JH, Kato M, Lee C. (1996). Genetic change in transforming growth factor beta (TGF-beta) receptor type I gene correlates with insensitivity to TGF-beta 1 in human prostate cancer cells. Cancer Res. 56(1):44-8.

Kim SJ, Letterio J. (2003). Transforming growth factor-beta signaling in normal and malignant hematopoiesis. Leukemia.17(9):1731-7

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(2-3):181-91.

Kingsley DM. (1994). The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev. 8(2):133-46.

Kloen P, Gebhardt MC, Perez-Atayde A, Rosenberg AE, Springfield DS, Gold LI, Mankin HJ. (1997). Expression of transforming growth factor-beta (TGF-beta) isoforms in osteosarcomas: TGF-beta3 is related to disease progression. Cancer. 80(12):2230-9.

Knisely JP, Rockwell S. (2002). Importance of hypoxia in the biology and treatment of brain tumors. Neuroimaging Clin N Am. 12(4):525-36.

Knuckey NW, Finch P, Palm DE, Primiano MJ, Johanson CE, Flanders KC, Thompson NL. (1996). Differential neuronal and astrocytic expression of transforming growth factor beta isoforms in rat hippocampus following transient forebrain ischemia. Brain Res Mol Brain Res 40: 1-14.

Koli KM, Ramsey TT, Ko Y, Dugger TC, Brattain MG, Arteaga CL. (1997). Blockade of transforming growth factor-beta signaling does not abrogate antiestrogen-induced growth inhibition of human breast carcinoma cells. J Biol Chem. 272(13):8296-302.

Koshikawa N, Iyozumi A, Gassmann M, Takenaga K. (2003). Constitutive upregulation of hypoxia-inducible factor-1alpha mRNA occurring in highly metastatic

lung carcinoma cells leads to vascular endothelial growth factor overexpression upon hypoxic exposure. Oncogene. 22(43):6717-24.

Koyama N, Koshikawa T, Morisaki N, Saito Y, Yoshida S. (1990). Bifunctional effects of transforming growth factor-beta on migration of cultured rat aortic smooth muscle cells. Biochem Biophys Res Commun. 169(2):725-9.

Kretzschmar M, Doody J, Massague J. (1997). Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. Nature. 389(6651):618-22.

Kretzschmar M, Doody J, Timokhina I, Massague J. (1999). A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. Genes Dev. 13(7):804-16.

Kuhn MA, Wang X, Payne WG, Ko F, Robson MC. (2002). Tamoxifen decreases fibroblast function and downregulates TGF (beta2) in dupuytren's affected palmar fascia. J Surg Res. 103(2):146-52.

Kuiper GG, Gustafsson JA. (1997). The novel estrogen receptor-beta subtype: potential role in the cell- and promoter-specific actions of estrogens and anti-estrogens. FEBS Lett. 410(1):87-90.

Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S. (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci U S A. 90(2):770-4

Kumar R, Baskakov IV, Srinivasan G, Bolen DW, Lee JC, Thompson EB. (1999). Interdomain signaling in a two-domain fragment of the human glucocorticoid receptor. J Biol Chem. 274(35):24737-41.

Kunz-Schughart LA, Knuechel R. (2002). Tumor-associated fibroblasts (part II): Functional impact on tumor tissue. Histol Histopathol. 17(2):623-37.

Kurokawa R, Soderstrom M, Horlein A, Halachmi S, Brown M, Rosenfeld MG, Glass CK. (1995). Polarity-specific activities of retinoic acid receptors determined by a co-repressor. Nature. 377(6548):451-4.

Kyprianou N, Isaacs JT. (1988). Identification of a cellular receptor for transforming growth factor-beta in rat ventral prostate and its negative regulation by androgens. Endocrinology. 123(4):2124-31.

Kyprianou N, Isaacs JT. (1989). Expression of transforming growth factor-beta in the rat ventral prostate during castration-induced programmed cell death. Mol Endocrinol. 3(10):1515-22.

Kyriakis SC, Tzika ED, Lyras DN, Tsinas AC, Saoulidis K, Sarris K. (1998). Effect of an inactivated Parapoxvirus based immunomodulator (Baypamun) on post weaning diarrhoea syndrome and wasting pig syndrome of piglets. Res Vet Sci. 64(3):187-90.

Labbe E, Silvestri C, Hoodless PA, Wrana JL, Attisano L. (1998). Smad2 and Smad3 positively and negatively regulate TGF beta-dependent transcription through the forkhead DNA-binding protein FAST2. Mol Cell. 2(1):109-20.

Lack J, O'Leary JM, Knott V, Yuan X, Rifkin DB, Handford PA, Downing AK. (2003). Solution structure of the third TB domain from LTBP1 provides insight into assembly of the large latent complex that sequesters latent TGF-beta. J Mol Biol. 334(2):281-91.

Laderoute KR, Alarcon RM, Brody MD, Calaoagan JM, Chen EY, Knapp AM, Yun Z, Denko NC, Giaccia AJ. (2000). Opposing effects of hypoxia on expression of the angiogenic inhibitor thrombospondin 1 and the angiogenic inducer vascular endothelial growth factor. Clin Cancer Res. 6(7):2941-50.

Laiho M, Saksela O, Keski-Oja J. (1987). Transforming growth factor-beta induction of type-1 plasminogen activator inhibitor. Pericellular deposition and sensitivity to exogenous urokinase. J Biol Chem. 262(36):17467-74.

Laiho M, Weis MB, Massague J. (1990). Concomitant loss of transforming growth factor (TGF)-beta receptor types I and II in TGF-beta-resistant cell mutants implicates both receptor types in signal transduction. J Biol Chem. 265(30):18518-24.

Lastres P, Letamendia A, Zhang H, Rius C, Almendro N, Raab U, Lopez LA, Langa C, Fabra A, Letarte M, Bernabeu C. (1996). Endoglin modulates cellular responses to TGF-beta 1. J Cell Biol. 133(5):1109-21.

Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol. 21(12):3995-4004.

LaVan FB, Hunt TK. (1990). Oxygen and wound healing. Clin Plast Surg. 17(3):463-72.

Le Douarin B, Zechel C, Garnier JM, Lutz Y, Tora L, Pierrat P, Heery D, Gronemeyer H, Chambon P, Losson R. (1995). The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. EMBO J. 14(9):2020-33.

Lefer AM, Tsao P, Aoki N, Palladino MA. (1990). Mediation of cardioprotection by transforming growth factor-beta. Science 249: 61-64.

Lei J, Silbiger S, Ziyadeh FN, Neugarten J. (1998). Serum-stimulated alpha 1 type IV collagen gene transcription is mediated by TGF-beta and inhibited by estradiol. Am J Physiol. 274(2 Pt 2):F252-8.

Leibovich SJ, Ross R. (1975). The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. Am J Pathol. 78(1):71-100.

Leof EB, Proper JA, Getz MJ, Moses HL. (1986). Transforming growth factor type beta regulation of actin mRNA. J Cell Physiol. 127(1):83-8.

Lerman OZ, Galiano RD, Armour M, Levine JP, Gurtner GC. (2003). Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia. Am J Pathol. 162(1):303-12.

Letterio JJ, Roberts AB. (1998). Regulation of immune responses by TGF-beta. Annu Rev Immunol. 16:137-61.

Levine JH, Moses HL, Gold LI, Nanney LB. (1993). Spatial and temporal patterns of immunoreactive transforming growth factor beta 1, beta 2, and beta 3 during excisional wound repair. Am J Pathol. 143(2):368-80.

Lewis KA, Gray PC, Blount AL, MacConell LA, Wiater E, Bilezikjian LM, Vale W. (2000). Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. Nature. 404(6776):411-4.

L'Horset F, Dauvois S, Heery DM, Cavailles V, Parker MG. (1996). RIP-140 interacts with multiple nuclear receptors by means of two distinct sites. Mol Cell Biol. 16(11):6029-36.

Li CY, Suardet L, Little JB. (1995). Potential role of WAF1/Cip1/p21 as a mediator of TGF-beta cytoinhibitory effect. J Biol Chem. 270(10):4971-4.

Lin HY, Wang XF, 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.

Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science. 260(5111):1130-2.

Ling WY, Johnston DW, Lea RH, Bent AE, Scott JZ, Toews MR. (1985). Serum gonadotropin and ovarian steroid levels in women during administration of a norethindrone-ethinylestradiol triphasic oral contraceptive. Contraception. 32(4):367-75.

Liu B, Dou CL, Prabhu L, Lai E. (1999). FAST-2 is a mammalian winged-helix protein which mediates transforming growth factor beta signals. Mol Cell Biol. 19(1):424-30.

Liu, X., Sun, Y., Weinberg, R. A. and Lodish, H. F. (2001). Ski/Sno and TGF-ß signaling. Cyt. Growth Factor Rev. 12, 1-8.

Lo RS, Massague J. (1999). Ubiquitin-dependent degradation of TGF-beta-activated smad2. Nat Cell Biol. 1999 Dec;1(8):472-8.

Lopez-Casillas F, Cheifetz S, Doody J, Andres JL, Lane WS, Massague J. (1991). Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. Cell. 67(4):785-95.

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

Lopez-Casillas F, Payne HM, Andres JL, Massague J. (1994). Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. J Cell Biol. 124(4):557-68.

Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, Carbone PP, DeMets DL. (1992). Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med. 326(13):852-6.

Luethviksson BR, Gunnlaugsdottir B. (2003).Transforming growth factor-beta as a regulator of site-specific T-cell inflammatory response. Scand J Immunol.58(2):129-38.

Luo K, Stroschein SL, Wang W, Chen D, Martens E, Zhou S, Zhou Q. (1999). The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling. Genes Dev. 13(17):2196-206.

Luukko K, Ylikorkala A, Makela TP. (2001). Developmentally regulated expression of Smad3, Smad4, Smad6, and Smad7 involved in TGF-beta signaling. Mech Dev. 101(1-2):209-12

Macias-Silva M, Abdollah S, Hoodless PA, Pirone R, Attisano L, Wrana JL. (1996). MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. Cell. 87(7):1215-24.

Maheux R, Naud F, Rioux M, Grenier R, Lemay A, Guy J, Langevin M. (1994). A randomized, double-blind, placebo-controlled study on the effect of conjugated estrogens on skin thickness. Am J Obstet Gynecol. 170(2):642-9.

Malonne H, Langer I, Kiss R, Atassi G. (1999). Mechanisms of tumor angiogenesis and therapeutic implications: angiogenesis inhibitors. Clin Exp Metastasis. 17(1):1-14.

Mandlekar S, Yu R, Tan TH, Kong AN. (2000). Activation of caspase-3 and c-Jun NH2-terminal kinase-1 signaling pathways in tamoxifen-induced apoptosis of human breast cancer cells. Cancer Res. 60(21):5995-6000.

Mandlekar S, Kong AN. (2001). Mechanisms of tamoxifen-induced apoptosis. Apoptosis. 6(6):469-77.

Mansbridge JN, Liu K, Pinney RE, Patch R, Ratcliffe A, Naughton GK. (1999). Growth factors secreted by fibroblasts: role in healing diabetic foot ulcers. Diabetes Obes Metab. 1(5):265-79.

Marchetti MC, Di Marco B, Cifone G, Migliorati G, Riccardi C. (2000). Dexamethasone-induced apoptosis of thymocytes: role of glucocorticoid receptor-associated Src kinase and caspase-8 activation. Blood. 101(2):585-93.

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(5215):1336-8.

Massague J. (1992a). Receptors for the TGF-beta family. Cell. 69(7):1067-70.

Massague J, Cheifetz S, Laiho M, Ralph DA, Weis FM, Zentella A. (1992b). Transforming growth factor-beta. Cancer Surv. 12:81-103.

Massague J. (1998). TGF-beta signal transduction. Annu Rev Biochem. 67:753-91.

Massague J, Blain SW, Lo RS. (2000a). TGFbeta signaling in growth control, cancer, and heritable disorders. Cell. 103(2):295-309.

Massague J, Chen YG. (2000b). Controlling TGF-beta signaling. Genes Dev. 14(6):627-44.

Massague J, Wotton D. (2000c). Transcriptional control by the TGF-beta/Smad signaling system. EMBO J. 19(8):1745-54.

Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, Ratcliffe PJ. (1997). Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci U S A. 94(15):8104-9.

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 399(6733):271-5.

McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, et al. (1994). Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet. 8(4):345-51.

McCord JM. (1985). Oxygen-derived free radicals in post ischemic tissue injury. N Engl J Med. 312: 159- 163.

McCune BK, Patterson K, Chandra RS, Kapur S, Sporn MB, Tsokos M. (1993). Expression of transforming growth factor-beta isoforms in small round cell tumors of childhood. An immunohistochemical study. Am J Pathol. 142(1):49-58

McDonald NQ, Hendrickson WA. (1993). A structural superfamily of growth factors containing a cystine knot motif. Cell. 73(3):421-4.

McKay LI, Cidlowski JA. (1998). Cross-talk between nuclear factor-kappa B and the steroid hormone receptors: mechanisms of mutual antagonism. Mol Endocrinol. 12(1):45-56.

McKay LI, Cidlowski JA. (1999). Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. Endocr Rev. 20(4):435-59.

McNeill H, Williams C, Guan J, Dragunow M, Lawlor P, Sirimanne E, Nikolics K, Gluckman P. (1994). Neuronal rescue with transforming growth factor-beta 1 after hypoxic-ischaemic brain injury. Neuroreport 5: 901-904.

Mehta JL, Yang BC, Strates BS, Mehta P. (1999). Role of TGF-beta1 in platelet-mediated cardioprotection during ischemia-reperfusion in isolated rat hearts. Growth Factors. 16: 179-190.

Meisler N, Keefer KA, Ehrlich HP, Yager DR, Myers-Parrelli J, Cutroneo KR. (1997). Dexamethasone abrogates the fibrogenic effect of transforming growth factor-beta in rat granuloma and granulation tissue fibroblasts. J Invest Dermatol. 108(3):285-9.

Melillo G, Musso T, Sica A, Taylor LS, Cox GW, Varesio L. (1995). A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. J Exp Med. 182(6):1683-93.

Mikulec AA, Hanasono MM, Lum J, Kadleck JM, Kita M, Koch RJ. (2001). Effect of tamoxifen on transforming growth factor beta1 production by keloid and fetal fibroblasts. Arch Facial Plast Surg. 3(2):111-4.

Milbrandt J, de Sauvage FJ, Fahrner TJ, Baloh RH, Leitner ML, Tansey MG, Lampe PA, Heuckeroth RO, Kotzbauer PT, Simburger KS, Golden JP, Davies JA, Vejsada R, Kato AC, Hynes M, Sherman D, Nishimura M, Wang LC, Vandlen R, Moffat B, Klein

RD, Poulsen K, Gray C, Garces A, Johnson EM Jr, et al. (1998). Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron. 20(2):245-53.

Minchenko A, Leshchinsky I, Opentanova I, Sang N, Srinivas V, Armstead V, Caro J. (2002). Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect. J Biol Chem. 277(8):6183-7.

Miyazono K, Olofsson A, Colosetti P, Heldin CH. (1991). A role of the latent TGF-beta 1-binding protein in the assembly and secretion of TGF-beta 1. EMBO J. 10(5):1091-101.

Miyazono K, Ichijo H, Heldin CH. (1993). Transforming growth factor-beta: latent forms, binding proteins and receptors. Growth Factors. 8(1):11-22.

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(5):378-85.

Mogford JE, Tawil N, Chen A, Gies D, Xia Y, Mustoe TA. (2002). Effect of age and hypoxia on TGFbeta1 receptor expression and signal transduction in human dermal fibroblasts: impact on cell migration. J Cell Physiol. 190(2):259-65.

Montesano R, Orci L. (1988). Transforming growth factor beta stimulates collagen-matrix contraction by fibroblasts: implications for wound healing. Proc Natl Acad Sci U S A. 85(13):4894-7.

Moren A, Olofsson A, Stenman G, Sahlin P, Kanzaki T, Claesson-Welsh L, ten Dijke P, Miyazono K, Heldin CH. (1994). Identification and characterization of LTBP-2, a novel latent transforming growth factor-beta-binding protein. J Biol Chem. 269(51):32469-78.

Morishima Y, Murphy PJ, Li DP, Sanchez ER, Pratt WB. (2000). Stepwise assembly of a glucocorticoid receptor.hsp90 heterocomplex resolves two sequential ATP-dependent events involving first hsp70 and then hsp90 in opening of the steroid binding pocket. J Biol Chem. 275(24):18054-60.

Moulin V. (1995). Growth factors in skin wound healing. Eur J Cell Biol. 68(1):1-7.

Moulin V, Auger FA, O'Connor-McCourt M, Germain L. (1997). Fetal and post natal sera differentially modulate human dermal fibroblast phenotypic and functional features in vitro. J Cell Physiol. 171: 1-10.

Moustakas A, Lin HY, Henis YI, Plamondon J, O'Connor-McCourt MD, Lodish HF. (1993). The transforming growth factor beta receptors types I, II, and III form hetero-oligomeric complexes in the presence of ligand. J Biol Chem 268: 22215-22218.

Moustakas A, Souchelnytskyi S, Heldin CH. (2001). Smad regulation in TGF-beta signal transduction. J Cell Sci. 114(Pt 24):4359-69.

Mukhopadhyay D, Tsiokas L, & Sukhatme V.P. (1998). High cell density induces vascular endothelial growth factor expression via protein tyrosine phosphorylation. Gene Expr. 7, 53-60.

Mulder KM. (2000). Role of Ras and Mapks in TGFbeta signaling. Cytokine Growth Factor Rev. 11(1-2):23-35

Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, Rifkin DB, Sheppard D. (1999). The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell. 96(3):319-28.

Muraoka RS, Dumont N, Ritter CA, Dugger TC, Brantley DM, Chen J, Easterly E, Roebuck LR, Ryan S, Gotwals PJ, Koteliansky V, Arteaga CL. (2002). Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. J Clin Invest. 109(12):1551-9.

Muro-Cacho CA, Anderson M, Cordero J, Munoz-Antonia T. (1999). Expression of transforming growth factor beta type II receptors in head and neck squamous cell carcinoma. Clin Cancer Res. 5(6):1243-8.

Murphy-Ullrich JE, Poczatek M. (2000). Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. Cytokine Growth Factor Rev. 11(1-2):59-69.

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-beta. Science. 237(4820):1333-6.

Muttukrishna S, Fowler PA, George L, Groome NP, Knight PG. (1996). Changes in peripheral serum levels of total activin A during the human menstrual cycle and pregnancy. J Clin Endocrinol Metab. 81(9):3328-34.

Naef M, Ishiwata T, Friess H, Buchler MW, Gold LI, Korc M. (1997). Differential localization of transforming growth factor-beta isoforms in human gastric mucosa and overexpression in gastric carcinoma. Int J Cancer. 71(2):131-7.

Nagarajan RP, Zhang J, Li W, Chen Y. (1999). Regulation of Smad7 promoter by direct association with Smad3 and Smad4. J Biol Chem. 274(47):33412-8.

Nagayama S, Onodera H, Toguchida J, Imamura M. (2002). Altered expression of the receptor and ligand in the TGF beta signaling pathway in diffusely infiltrating colon carcinoma. Anticancer Res. 22(6B):3545-54.

Nakajima Y, Miyazono K, Kato M, Takase M, Yamagishi T, Nakamura H. (1997). Extracellular fibrillar structure of latent TGF beta binding protein-1: role in TGF beta-dependent endothelial-mesenchymal transformation during endocardial cushion tissue formation in mouse embryonic heart. J Cell Biol. 136(1):193-204

Nakao A, Miike S, Hatano M, Okumura K, Tokuhisa T, Ra C, Iwamoto I. (2000). Blockade of transforming growth factor beta/Smad signaling in T cells by overexpression of Smad7 enhances antigen-induced airway inflammation and airway reactivity. J Exp Med. 192(2):151-8.

Nakashima R, Song H, Enomoto T, Murata Y, McClaid MR, Casto BC, Weghorst CM. (1999). Genetic alterations in the transforming growth factor receptor complex in sporadic endometrial carcinoma. Gene Expr. 8(5-6):341-52.

Nakayama H, Ichikawa F, Andres JL, Massague J, Noda M. (1994). Dexamethasone enhancement of betaglycan (TGF-beta type III receptor) gene expression in osteoblast-like cells. Exp Cell Res. 211(2):301-6.

Newfeld SJ, Wisotzkey RG, Kumar S. (1999). Molecular evolution of a developmental pathway: phylogenetic analyses of transforming growth factor-beta family ligands, receptors and Smad signal transducers. Genetics. 152(2):783-95.

Noble NA, Harper JR, Border WA. (1992). In vivo interactions of TGF-beta and extracellular matrix. Prog Growth Factor Res. 4(4):369-82.

Nordsmark M, Overgaard M, Overgaard J. (1996). Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol. 41(1):31-9.

Nordsmark M, Alsner J, Keller J, Nielsen OS, Jensen OM, Horsman MR, Overgaard J. (2001). Hypoxia in human soft tissue sarcomas: adverse impact on survival and no association with p53 mutations. Br J Cancer. 84(8):1070-5.

Nunes I, Shapiro RL, Rifkin DB. (1995). Characterization of latent TGF-beta activation by murine peritoneal macrophages. J Immunol. 155(3):1450-9.

Nunes I, Gleizes PE, Metz CN, Rifkin DB. (1997). Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. J Cell Biol. 136(5):1151-63.

Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. (1996). TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes Dev. 10(19):2462-77.

Oft M, Akhurst RJ, Balmain A. (2002). Metastasis is driven by sequential elevation of H-ras and Smad2 levels. Nat Cell Biol. 4(7):487-94.

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 U S A. 97(6):2626-31

O'Kane S, Ferguson MW. (1997). Transorming growth factor betas and wound healing. Int J Biochem Cell Biol 29: 63-78.

Olofsson A, Miyazono K, Kanzaki T, Colosetti P, Engstrom U, Heldin CH. (1992). Transforming growth factor-beta 1, -beta 2, and -beta 3 secreted by a human glioblastoma cell line. Identification of small and different forms of large latent complexes. J Biol Chem. 267(27):19482-8.

Onate SA, Tsai SY, Tsai MJ, O'Malley BW. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science. 270(5240):1354-7.

Osaki M, Tsukazaki T, Yonekura A, Miyazaki Y, Iwasaki K, Shindo H, Yamashita S. (1999). Regulation of c-fos gene induction and mitogenic effect of transforming growth factor-beta1 in rat articular chondrocyte. Endocr J. 46(2):253-61.

Overall CM, Wrana JL, Sodek J. (1991). Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta 1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. J Biol Chem. 266(21):14064-71.

Oxlund H, Sims T, Light ND. (1982). Changes in mechanical properties, thermal stability, reducible cross-links and glycosyl-lysines in rat skin induced by corticosteroid treatment. Acta Endocrinol (Copenh).101(2):312-20.

Panchenko MP, Williams MC, Brody JS, Yu Q. (1996). Type I receptor serine-threonine kinase preferentially expressed in pulmonary blood vessels. Am J Physiol. 270(4 Pt 1):L547-58.

Pandit S, Geissler W, Harris G, Sitlani A. (2002). Allosteric effects of dexamethasone and RU486 on glucocorticoid receptor-DNA interactions. J Biol Chem. 277(2):1538-43.

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(19):8772-6.

Park SH, Lee SR, Kim BC, Cho EA, Patel SP, Kang HB, Sausville EA, Nakanishi O, Trepel JB, Lee BI, Kim SJ. (2002). Transcriptional regulation of the transforming growth factor beta type II receptor gene by histone acetyltransferase and deacetylase is mediated by NF-Y in human breast cancer cells. J Biol Chem. 277(7):5168-74.

Parrelli JM, Meisler N, Cutroneo KR. (1998). Identification of a glucocorticoid response element in the human transforming growth factor beta 1 gene promoter. Int J Biochem Cell Biol. 30(5):623-7.

Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev. 22(2):153-83

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(6):1441-6.

Philibert D, Deraedt R, Teutsch G. (1981). RU486: A potent antiglucocorticoid in vivo. Tokyo, Japan: International Congress of Pharmacology, (Abstract 1463).

Picard D, Khursheed B, Garabedian MJ, Fortin MG, Lindquist S, Yamamoto KR. (1990). Reduced levels of hsp90 compromise steroid receptor action in vivo. Nature. 348(6297):166-8.

Picard O, Rolland Y, Poupon MF. (1986). Fibroblast-dependent tumorigenicity of cells in nude mice: implication for implantation of metastases. Cancer Res. 46(7):3290-4.

Pierce DF Jr, Johnson MD, Matsui Y, Robinson SD, Gold LI, Purchio AF, Daniel CW, Hogan BL, Moses HL. (1993). Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1. Genes Dev. 7(12A):2308-17.

Pierce DF Jr, Gorska AE, Chytil A, Meise KS, Page DL, Coffey RJ Jr, Moses HL. (1995). Mammary tumor suppression by transforming growth factor beta 1 transgene expression. Proc Natl Acad Sci U S A. 92(10):4254-8.

Pietenpol JA, Holt JT, Stein RW, Moses HL. (1990a). Transforming growth factor beta 1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. Proc Natl Acad Sci U S A. 87(10):3758-62.

Pietenpol JA, Stein RW, Moran E, Yaciuk P, Schlegel R, Lyons RM, Pittelkow MR, Munger K, Howley PM, Moses HL. (1990b). TGF-beta 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell. 61(5):777-85.

Pittelkow MR, Coffey RJ Jr, Moses HJ. (1988). Keratinocytes produce and are regulated by transforming growth factors. Ann N Y Acad Sci.548:211-24.

Podos SD, Hanson KK, Wang YC, Ferguson EL. (2001). The DSmurf ubiquitin-protein ligase restricts BMP signaling spatially and temporally during Drosophila embryogenesis. Dev Cell. 1(4):567-78.

Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. 8(1):9-22.

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(1):251-6.

Pouponnot C, Jayaraman L, Massague J. (1998). Physical and functional interaction of SMADs and p300/CBP. J Biol Chem. 273(36):22865-8.

Pratt WB, Toft DO. (1997). Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev. 18(3):306-60.

Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN, Ding J, Ferguson MW, Doetschman T. (1995). Transforming growth factor-beta 3 is required for secondary palate fusion. Nat Genet. 11(4):409-14.

Pu LL, Smith PD, Payne WG, Kuhn MA, Wang X, Ko F, Robson MC. (2000). Overexpression of transforming growth factor beta-2 and its receptor in rhinophyma: an alternative mechanism of pathobiology. Ann Plast Surg. 45(5):515-9.

Puchner MJ, Koppen JA, Zapf S, Knabbe C, Westphal M. (2002). The influence of tamoxifen on the secretion of transforming growth factor-beta2 (TGF-beta2) in glioblastomas: in vitro and in vivo findings. Anticancer Res. 22(1A):45-51.

Pugh BF, Tjian R. (1990). Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell. 61(7):1187-97.

Pugh CW, Ratcliffe PJ. (2003). The von Hippel-Lindau tumor suppressor, hypoxia-inducible factor-1 (HIF-1) degradation, and cancer pathogenesis. Semin Cancer Biol. 13(1):83-9

Puolakkainen PA, Twardzik DR, Ranchalis JE, Pankey SC, Reed MJ, Gombotz WR. (1995). The enhancement in wound healing by transforming growth factor-beta 1 (TGF-beta 1) depends on the topical delivery system. J Surg Res. 58(3):321-9.

Qi M, Stasenko LJ, DeFranco DB. (1990). Recycling and desensitization of glucocorticoid receptors in v-mos transformed cells depend on the ability of nuclear receptors to modulate gene expression. Mol Endocrinol. 4(3):455-64.

Quaglino D Jr, Nanney LB, Ditesheim JA, Davidson JM. (1991). Transforming growth factor-beta stimulates wound healing and modulates extracellular matrix gene expression in pig skin: incisional wound model. J Invest Dermatol. 97(1):34-42.

Ratcliffe PJ, O'Rourke JF, Maxwell PH, Pugh CW. (1998). Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. J Expt Bio 201: 1153-1162.

Reed SG. (1999). TGF-beta in infections and infectious diseases. Microbes Infect. 1(15):1313-25.

Refojo D, Liberman AC, Holsboer F, Arzt E. (2001). Transcription factor-mediated molecular mechanisms involved in the functional cross-talk between cytokines and glucocorticoids. Immunol Cell Biol. 79(4):385-94.

Reichardt HM, Umland T, Bauer A, Kretz O, Schutz G. (2000). Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. Mol Cell Biol. 20(23):9009-17.

Reiss M. (1997). Transforming growth factor-beta and cancer: a love-hate relationship?. Oncol Res. 9(9):447-57.

Reiss M. (1999). TGF-beta and cancer. Microbes Infect. 1(15):1327-47.

Reyes-Moreno C, Frenette G, Boulanger J, Lavergne E, Govindan MV, Koutsilieris M. (1995). Mediation of glucocorticoid receptor function by transforming growth factor beta I expression in human PC-3 prostate cancer cells. Prostate. 26(5):260-9.

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(15):1831-45.

Rhodes JM, Engelmyer E, Tilberg MS, Gifford R. (1995). Transforming growth factor β1 serves as an autocrine inhibitor of human endothelial cell/lymphocyte adhesion. J Surg Res. 59: 719-724.

Ribeiro SM, Poczatek M, Schultz-Cherry S, Villain M, Murphy-Ullrich JE. (1999). The activation sequence of thrombospondin-1 interacts with the latency-associated peptide to regulate activation of latent transforming growth factor-beta. J Biol Chem. 274(19):13586-93.

Richard, D.E., Berra, E. & Pouyssegur, J. (2000). Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. J. Biol. Chem. 275, 26765-26771.

Riesle E, Friess H, Zhao L, Wagner M, Uhl W, Baczako K, Gold LI, Korc M, Buchler MW. (1997). Increased expression of transforming growth factor beta s after acute oedematous pancreatitis in rats suggests a role in pancreatic repair. Gut. 40(1):73-9.

Roberts AB, Anzano MA, Lamb LC, Smith JM, Sporn MB. (1981). New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. Proc Natl Acad Sci U S A. 78(9):5339-43

Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, et al. (1986). Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci U S A. 83(12):4167-71.

Roberts CJ, Birkenmeier TM, McQuillan JJ, Akiyama SK, Yamada SS, Chen WT, Yamada KM, McDonald JA. (1988). Transforming growth factor beta stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. J Biol Chem 263: 4586-4592.

Roberts AB, Flanders KC, Heine UI, Jakowlew S, Kondaiah P, Kim SJ, Sporn MB. (1990a). Transforming growth factor-beta: multifunctional regulator of differentiation and development. Philos Trans R Soc Lond B Biol Sci. 327(1239):145-54.

Roberts AB; sporn MB. (1990b). In: Spron, M., Roberts, A. (Eds.), Handbook of Experimental Pharmacology Polypeptide Growth Factors and their Receptors. Springer, Berlin.

Roberts AB. (1995). Transforming growth factor-b: Activity and efficacy in animal models of wound healing. Wound Rep Reg. 3: 408-418.

Roberts AB. (1998). Molecular and cell biology of TGF-beta. Miner Electrolyte Metab 24:111-9.

Roberts AB, Derynck R. (2001a). Meeting report: signaling schemes for TGF-beta. Sci STKE. 2001(113):PE43.

Roberts AB, Piek E, Bottinger EP, Ashcroft G, Mitchell JB, Flanders KC. (2001b). Is Smad3 a major player in signal transduction pathways leading to fibrogenesis? Chest. 120(1 Suppl):43S-47S.

Roberts AB, Russo A, Felici A, Flanders KC. (2003). Smad3: a key player in pathogenetic mechanisms dependent on TGF-beta. Ann N Y Acad Sci. 995:1-10.

Rockey DC, Maher JJ, Jarnagin WR, Gabbiani G, Friedman SL. (1992). Inhibition of rat hepatic lipocyte activation in culture by interferon-gamma. Hepatology. 16(3):776-84.

Roelen BA, van Rooijen MA, Mummery CL. (1997). Expression of ALK-1, a type 1 serine/threonine kinase receptor, coincides with sites of vasculogenesis and angiogenesis in early mouse development. Dev Dyn. 209(4):418-30

Rofstad EK, Sundfor K, Lyng H, Trope CG. (2000). Hypoxia-induced treatment failure in advanced squamous cell carcinoma of the uterine cervix is primarily due to hypoxia-induced radiation resistance rather than hypoxia-induced metastasis. Br J Cancer. 83(3):354-9

Rooke HM, Vitas MR, Crosier PS, Crosier KE. (1999). The TGF-beta type II receptor in chronic myeloid leukemia: analysis of microsatellite regions and gene expression. Leukemia. 13(4):535-41.

Rosenblad C, Gronborg M, Hansen C, Blom N, Meyer M, Johansen J, Dago L, Kirik D, Patel UA, Lundberg C, Trono D, Bjorklund A, Johansen TE. (2000). In vivo protection of nigral dopamine neurons by lentiviral gene transfer of the novel GDNF-family member neublastin/artemin. Mol Cell Neurosci. 15(2):199-214.

Roy SK, Kole AR. (1995). Transforming growth factor-beta receptor type II expression in the hamster ovary: cellular site(s), biochemical properties, and hormonal regulation. Endocrinology. 136(10):4610-20.

Roy SK, Wang J, Yang P. (2003). Dexamethasone inhibits transforming growth factor-beta receptor (Tbeta R) messenger RNA expression in hamster preantral follicles: possible association with NF-YA. Biol Reprod. 68(6):2180-8.

Rupprecht R, Reul JM, van Steensel B, Spengler D, Soder M, Berning B, Holsboer F, Damm K. (1993). Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. Eur J Pharmacol. 247(2):145-54.

Ryan HE, Lo J, Johnson RS. (1998). HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J. 17(11):3005-15.

Sackey FN, Hache RJ, Reich T, Kwast-Welfeld J, Lefebvre YA. (1996). Determinants of subcellular distribution of the glucocorticoid receptor. Mol Endocrinol. 10(10):1191-205.

Saiki I, Murata J, Yoneda J, Kobayashi H, Azuma I. (1994). Influence of fibroblasts on the invasion and migration of highly or weakly metastatic B16 melanoma cells. Int J Cancer. 56(6):867-73.

Salomon GD, Kasid A, Bernstein E, Buresh C, Director E, Norton JA. (1990). Gene expression in normal and doxorubicin-impaired wounds: importance of transforming growth factor-beta. Surgery. 108(2):318-22; discussion 322-3.

Sanchez ER, Toft DO, Schlesinger MJ, Pratt WB. (1985). Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. J Biol Chem. 260(23):12398-401.

Sanchez ER, Meshinchi S, Tienrungroj W, Schlesinger MJ, Toft DO, Pratt WB. (1987). Relationship of the 90-kDa murine heat shock protein to the untransformed and transformed states of the L cell glucocorticoid receptor. J Biol Chem. 262(15):6986-91.

Sanchez ER. (1990). Hsp56: a novel heat shock protein associated with untransformed steroid receptor complexes. J Biol Chem. 265(36):22067-70.

Sanchez-Elsner T, Botella LM, Velasco B, Corbi A, Attisano L, Bernabeu C. (2001). Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression. J Biol Chem. 276(42):38527-35.

Sandau K.B, Faus H.G, & Brune B. (2000). Induction of hypoxia-inducible-factor 1 by nitric oxide is mediated via the PI 3K pathway. Biochem. Biophys. Res. Commun. 278, 263-267.

Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. (1997). TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development. 124(13):2659-70

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(22):13567-72

Santiago B, Galindo M, Rivero M, Pablos JL. (2001). Decreased susceptibility to Fas-induced apoptosis of systemic sclerosis dermal fibroblasts. Arthritis Rheum. 44(7):1667-76.

Sato Y, Tsuboi R, Lyons R, Moses H, Rifkin DB. (1990). Related Characterization of the activation of latent TGF-beta by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. J Cell Biol. 111(2):757-63.

Satterwhite DJ, Moses HL. (1994). Mechanisms of transforming growth factor-beta 1-induced cell cycle arrest. Invasion Metastasis. 14(1-6):309-18.

Sapolsky RM, Romero LM, Munck AU. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev. 21(1):55-89.

Savvas M, Bishop J, Laurent G, Watson N, Studd J. (1993). Type III collagen content in the skin of postmenopausal women receiving oestradiol and testosterone implants. Br J Obstet Gynaecol. 100(2):154-6.

Scherrer LC, Picard D, Massa E, Harmon JM, Simons SS Jr, Yamamoto KR, Pratt WB. (1993). Evidence that the hormone binding domain of steroid receptors confers hormonal control on chimeric proteins by determining their hormone-regulated binding to heat-shock protein 90. Biochemistry. 32(20):5381-6.

Schlunegger M.P, Grutter M.G. (1994). An unusual feature revealed by the crystal structure at 2.2° resolution of human transforming growth factor-beta2. Nature 358, 430-434.

Schmaltz C, Hardenbergh PH, Wells A, Fisher DE. (1998). Regulation of proliferation-survival decisions during tumor cell hypoxia. Mol Cell Biol. 18(5):2845-54.

Schmid P, Itin P, Cherry G, Bi C, Cox DA. (1998). Enhanced expression of transforming growth factor-beta type I and type II receptors in wound granulation tissue and hypertrophic scar. Am J Pathol. 152(2):485-93

Schmidt TJ. (1986). In vitro activation and DNA binding affinity of human lymphoid (CEM-C7) cytoplasmic receptors labeled with the antiglucocorticoid RU 38486. J Steroid Biochem. 24(4):853-63.

Schule R, Rangarajan P, Kliewer S, Ransone LJ, Bolado J, Yang N, Verma IM, Evans RM. (1990). Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell. 62(6):1217-26.

Schultz-Cherry S, Lawler J, Murphy-Ullrich JE. (1994a). The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-beta. J Biol Chem. 269(43):26783-8.

Schultz-Cherry S, Murphy-Ullrich JE. (1993). Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism. J Cell Biol. 122(4):923-32.

Schultz-Cherry S, Ribeiro S, Gentry L, Murphy-Ullrich JE. (1994b). Thrombospondin binds and activates the small and large forms of latent transforming growth factor-beta in a chemically defined system. J Biol Chem. 269(43):26775-82.

Schultz-Cherry S, Chen H, Mosher DF, Misenheimer TM, Krutzsch HC, Roberts DD, Murphy-Ullrich JE. (1995). Regulation of transforming growth factor-beta activation by discrete sequences of thrombospondin 1. J Biol Chem. 270(13):7304-10.

Schultz Jel J, Witt SA, Glascock BJ, Nieman ML, Reiser PJ, Nix SL, Kimball TR, Doetschman T. (2002). TGF-beta1 mediates the hypertrophic cardiomyocyte growth induced by angiotensin II. J Clin Invest. 109(6):787-96.

Schulz M, Eggert M, Baniahmad A, Dostert A, Heinzel T, Renkawitz R. (2002). RU486-induced glucocorticoid receptor agonism is controlled by the receptor N terminus and by corepressor binding. J Biol Chem. 277(29):26238-43.

Schutte M, Hruban RH, Hedrick L, Cho KR, Nadasdy GM, Weinstein CL, Bova GS, Isaacs WB, Cairns P, Nawroz H, Sidransky D, Casero RA Jr, Meltzer PS, Hahn SA, Kern SE. (1996). DPC4 gene in various tumor types. Cancer Res. 56(11):2527-30.

Schwarte-Waldhoff I, Volpert OV, Bouck NP, Sipos B, Hahn SA, Klein-Scory S, Luttges J, Kloppel G, Graeven U, Eilert-Micus C, Hintelmann A, Schmiegel W. (2000). Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. Proc Natl Acad Sci U S A. 97(17):9624-9.

Scortegagna M, Morris MA, Oktay Y, Bennett M, Garcia JA. (2003). The HIF family member EPAS1/HIF-2alpha is required for normal hematopoiesis in mice. Blood. 102(5):1634-40.

Segarini PR, Rosen DM, Seyedin SM. (1989). Binding of transforming growth factor-beta to cell surface proteins varies with cell type. Mol Endocrinol. 3(2):261-72.

Seki T, Yun J, Oh SP. (2003). Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. Circ Res. 93(7):682-9.

Sellheyer K, Bickenbach JR, Rothnagel JA, Bundman D, Longley MA, Krieg T, Roche NS, Roberts AB, Roop DR. (1993). Inhibition of skin development by overexpression of transforming growth factor beta 1 in the epidermis of transgenic mice. Proc Natl Acad Sci U S A. 90(11):5237-41.

Semenza GL , Wang GL. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol. 12(12):5447-54.

Semenza GL, Roth PH, Fang HM, Wang GL. (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem. 269(38):23757-63.

Semenza GL. (2000a). HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 88: 1474-1480.

Semenza GL. (2000b). Expression of hypoxia-inducible factor 1: mechanisms and consequences. Biochem Pharmacol. 59(1):47-53.

Semenza GL. (2001). HIF-1 and mechanisms of hypoxia sensing. Curr Opin Cell Biol. 13(2):167-71.

Senel O, Cetinkale O, Ozbay G, Achioglu F, and Bulan R. (1997). Oxygen free radicals impair wound healing in ischemic rat skin. Ann. Plast. Surg 39: 516-523.

Shah M, Foreman DM, Ferguson MW. (1995). Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. J Cell Sci. 108 (Pt 3):985-1002.

Shah MG, Maibach HI. (2001). Estrogen and skin. An overview. Am J Clin Dermatol. 2(3):143-50.

Shang Y, Brown M. (2002). Molecular determinants for the tissue specificity of SERMs. Science. 295(5564):2465-8.

Sheta E.A, Trout H., Gildea J.J, Harding M.A. & Theodorescu D. (2001). Cell density mediated pericellular hypoxia leads to induction of HIF-1 via nitric oxide and Ras/MAP kinase mediated signaling pathways. Oncogene. 20, 7624-7634.

Shi Y, Massague J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell. 113(6):685-700.

Shibuya H, Yamaguchi K, Shirakabe K, Tonegawa A, Gotoh Y, Ueno N, Irie K, Nishida E, Matsumoto K. (1996). TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. Science. 272(5265):1179-82.

Shihab FS, Yamamoto T, Nast CC, Cohen AH, Noble NA, Gold LI, Border WA. (1995). Transforming growth factor-beta and matrix protein expression in acute and chronic rejection of human renal allografts. J Am Soc Nephrol. 6(2):286-94.

Shin GT, Khanna A, Ding R, Sharma VK, Lagman M, Li B, Suthanthiran M. (1998). In vivo expression of transforming growth factor-beta1 in humans: stimulation by cyclosporine. Transplantation. 65(3):313-8.

Shipley GD, Pittelkow MR, Wille JJ Jr, Scott RE, Moses HL. (1986). Reversible inhibition of normal human prokeratinocyte proliferation by type beta transforming growth factor-growth inhibitor in serum-free medium. Cancer Res. 46(4 Pt 2):2068-71.

Shipley JM, Mecham RP, Maus E, Bonadio J, Rosenbloom J, McCarthy RT, Baumann ML, Frankfater C, Segade F, Shapiro SD. (2000). Developmental expression of latent transforming growth factor beta binding protein 2 and its requirement early in mouse development. Mol Cell Biol. 20(13):4879-87.

Shukla A, Meisler N, Cutroneo KR. (1999). Perspective article: transforming growth factor-beta: crossroad of glucocorticoid and bleomycin regulation of collagen synthesis in lung fibroblasts. Wound Repair Regen. 7(3):133-40.

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(6397):693-9

Siddiqui, A, Galiano, RD, Connors, D et al. (1996) "Differential effects of oxygen on human dermal fibroblasts: acute versus chronic hypoxia" Wound Repair Regen. 4: 211-218

Sieweke MH, Bissell MJ. (1994). The tumor-promoting effect of wounding: a possible role for TGF-beta-induced stromal alterations. Crit Rev Oncog. 5(2-3):297-311.

Silbiger S, Lei J, Ziyadeh FN, Neugarten J. (1998). Estradiol reverses TGF-beta1-stimulated type IV collagen gene transcription in murine mesangial cells. Am J Physiol. 274(6 Pt 2):F1113-8.

Silva CM, Powell-Oliver FE, Jewell CM, Sar M, Allgood VE, Cidlowski JA. (1994). Regulation of the human glucocorticoid receptor by long-term and chronic treatment with glucocorticoid. Steroids. 59(7):436-42.

Singer AJ, Clark RA. (1999). Cutaneous wound healing.N Engl J Med. 341(10):738-46

Skafar DF. (1991). Differences in the binding mechanism of RU486 and progesterone to the progesterone receptor. Biochemistry. 30(45):10829-32.

Slavin J, Unemori E, Hunt TK, Amento E. (1994). Transforming growth factor beta (TGF-beta) and dexamethasone have direct opposing effects on collagen metabolism in low passage human dermal fibroblasts in vitro. Growth Factors. 11(3):205-13.

Slavin J. (1996). The role of cytokines in wound healing. J Pathol. 178(1):5-10.

Smith CL, Onate SA, Tsai MJ, O'Malley BW. (1996). CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. Proc Natl Acad Sci U S A. 93(17):8884-8.

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(21):11776-81.

Souchelnytskyi S, ten Dijke P, Miyazono K, Heldin CH. (1996). Phosphorylation of Ser165 in TGF-beta type I receptor modulates TGF-beta1-induced cellular responses. EMBO J. 15(22):6231-40.

Spranger J, Meyer-Schwickerath R, Klein M, Schatz H, Pfeiffer A. (1999). Deficient activation and different expression of transforming growth factor-beta isoforms in active proliferative diabetic retinopathy and neovascular eye disease. Exp Clin Endocrinol Diabetes. 107(1):21-8

Srinivas V, Zhang LP, Zhu XH, Caro J. (1999). Characterization of an oxygen/redox-dependent degradation domain of hypoxia-inducible factor alpha (HIF-alpha) proteins. Biochem Biophys Res Commun. 260(2):557-61.

Stadelmann W, Digenis AG, Tobin GR. (1998). Impediments to wound healing. Am J Surg 176: 39S- 47S.

Steinbrech DS, Longaker MT, Mehrara BJ, Saadeh PB, Chin GS, Gerrets RP, Chau DC, Rowe NM, Gittes GK. (1999). Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation. J Surg Res. 84(2):127-33.

Steinbrech DS, Mehrara BJ, Rowe NM, Dudziak ME, Luchs JS, Saadeh PB, Gittes GK, Longaker MT. (2000). Gene expression of TGF-beta, TGF-beta receptor, and extracellular matrix proteins during membranous bone healing in rats. Plast Reconstr Surg. 105(6):2028-38

Sterner-Kock A, Thorey IS, Koli K, Wempe F, Otte J, Bangsow T, Kuhlmeier K, Kirchner T, Jin S, Keski-Oja J, von Melchner H. (2002). Disruption of the gene encoding the latent transforming growth factor-beta binding protein 4 (LTBP-4) causes abnormal lung development, cardiomyopathy, and colorectal cancer. Genes Dev. 16(17):2264-73

Stoica A, Saceda M, Fakhro A, Solomon HB, Fenster BD, Martin MB. (1997). The role of transforming growth factor-beta in the regulation of estrogen receptor expression in the MCF-7 breast cancer cell line. Endocrinology. 138(4):1498-505.

Stroschein SL, Wang W, Luo K. (1999). Cooperative binding of Smad proteins to two adjacent DNA elements in the plasminogen activator inhibitor-1 promoter mediates transforming growth factor beta-induced smad-dependent transcriptional activation. J Biol Chem. 274(14):9431-41.

Stroschein SL, Bonni S, Wrana JL, Luo K. (2001). Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN. Genes Dev. 15(21):2822-36.

Su S, Dehnade F, Zafarullah M. (1996). Regulation of tissue inhibitor of metalloproteinases-3 gene expression by transforming growth factor-beta and dexamethasone in bovine and human articular chondrocytes. DNA Cell Biol. 15(12):1039-48.

Sun L, Wu G, Willson JK, Zborowska E, Yang J, Rajkarunanayake I, Wang J, Gentry LE, Wang XF, Brattain MG. (1994). Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. Biol Chem. 269(42):26449-55.

Tacchini, L., Dansi, P., Matteucci, E. & Desiderio, M.A. (2001). Hepatocyte growth factor signalling stimulates hypoxia inducible factor-1 (HIF-1) activity in HepG2 hepatoma cells. Carcinogenesis. 22, 1363-1371.

Taipale J, Koli K, Keski-Oja J. (1992). Release of transforming growth factor-beta 1 from the pericellular matrix of cultured fibroblasts and fibrosarcoma cells by plasmin and thrombin. J Biol Chem. 267(35):25378-84.

Taipale J, Miyazono K, Heldin CH, Keski-Oja J. (1994). Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. J Cell Biol. 124(1-2):171-81.

Taipale J, Lohi J, Saarinen J, Kovanen PT, Keski-Oja J. (1995). Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. J Biol Chem. 270(9):4689-96.

Takehara K. (2000). Growth regulation of skin fibroblasts. J Dermatol Sci. 24 Suppl 1:S70-7.

Takeshita A, Yen PM, Misiti S, Cardona GR, Liu Y, Chin WW. (1996). Molecular cloning and properties of a full-length putative thyroid hormone receptor coactivator. Endocrinology. 137(8):3594-7.

Takeshita A, Imai K, Kato S, Kitano S, Hanazawa S. (1998). 1alpha,25-dehydroxyvitamin D3 synergism toward transforming growth factor-beta1-induced AP-1 transcriptional activity in mouse osteoblastic cells via its nuclear receptor. J Biol Chem. 273(24):14738-44.

Tam BYY, 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.

Tanaka H, Mori Y, Ishii H, Akedo H. (1988). Enhancement of metastatic capacity of fibroblast-tumor cell interaction in mice. Cancer Res. 48(6):1456-9.

Tang B, Bottinger EP, Jakowlew SB, Bagnall KM, Mariano J, Anver MR, Letterio JJ, Wakefield LM. (1998). Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency. Nat Med. 4(7):802-7.

Taylor RJ, Taylor AD, Smyth JV. (2002). Using an artificial neural network to predict healing times and risk factors for venous leg ulcers. J Wound Care. 11(3):101-5.

Tcacencu I. (2002). Mifepristone (RU-486) impairs post-surgical wound healing of the larynx. Med Sci Monit. 8(10):BR397-400.

Ten Dijke P, Yamashita H, Ichijo H, Franzen P, Laiho M, Miyazono K, Heldin CH. (1994a). Characterization of type I receptors for transforming growth factor-beta and activin. Science. 264(5155):101-4.

Ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K. (1994b). Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem. 269(25):16985-8.

Ten Dijke P, Goumans MJ, Itoh F, Itoh S. (2002). Regulation of cell proliferation by Smad proteins. J Cell Physiol. 191(1):1-16.

Tenan M, Fulci G, Albertoni M, Diserens AC, Hamou MF, El Atifi-Borel M, Feige JJ, Pepper MS, Van Meir EG. (2000). Thrombospondin-1 is downregulated by anoxia and suppresses tumorigenicity of human glioblastoma cells. J Exp Med. 191(10):1789-98.

Tetel MJ, Giangrande PH, Leonhardt SA, McDonnell DP, Edwards DP. (1999). Hormone-dependent interaction between the amino- and carboxyl-terminal domains of progesterone receptor in vitro and in vivo. Mol Endocrinol. 13(6):910-24.

Thenot S, Henriquet C, Rochefort H, Cavailles V. (1997). Differential interaction of nuclear receptors with the putative human transcriptional coactivator hTIF1. J Biol Chem. 272(18):12062-8.

Thompson TC, Truong LD, Timme TL, Kadmon D, McCune BK, Flanders KC, Scardino zPT, Park SH. (1992). Transforming growth factor beta 1 as a biomarker for prostate cancer. J Cell Biochem Suppl. 16H:54-61.

Thornton, R.D., Lane, P., Borghaei, R.C., Pease, E.A., Caro, J. & Mochan, E. (2000). Interleukin 1 induces hypoxia-inducible factor 1 in human gingival and synovial fibroblasts. Biochem. J. 350, 307-312.

Topper JN. (2000). TGF-beta in the cardiovascular system: molecular mechanisms of a context-specific growth factor. Trends Cardiovasc Med. 10(3):132-7.

Treanor JJ, Goodman L, de Sauvage F, Stone DM, Poulsen KT, Beck CD, Gray C, Armanini MP, Pollock RA, Hefti F, Phillips HS, Goddard A, Moore MW, Buj-Bello A, Davies AM, Asai N, Takahashi M, Vandlen R, Henderson CE, Rosenthal A. (1996). Characterization of a multicomponent receptor for GDNF. Nature. 382(6586):80-3.

Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell. 95(6):779-91.

Tyrone JW, Marcus JR, Bonomo SR, Mogford JE, Xia Y, Mustoe TA. (2000). Transforming growth factor beta3 promotes fascial wound healing in a new animal model. Arch Surg. 135(10):1154-9.

Ulloa L, Doody J, Massague J. (1999). Inhibition of transforming growth factor-beta/SMAD signalling by the interferon-gamma/STAT pathway. Nature. 397(6721):710-3.

Uzuka M, Nakajima K, Ohta S, Mori Y. (1980). The mechanism of estrogen-induced increase in hyaluronic acid biosynthesis, with special reference to estrogen receptor in the mouse skin. Biochim Biophys Acta. 627(2):199-206.

Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J. (1986). Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature. 321(6072):776-9

Van de Kerkhof PC, Van Bergen B, Spruijt K, Kuiper JP. (1994). Age-related changes in wound healing. Clin Exp Dermatol. 19(5):369-74.

Van Obberghen-Schilling E, Roche NS, Flanders KC, Sporn MB, Roberts AB. (1988). Transforming growth factor beta 1 positively regulates its own expression in normal and transformed cells. J Biol Chem. 263(16):7741-6.

Vassalli JD, Sappino AP, Belin D. (1991). The plasminogen activator/plasmin system. J Clin Invest. 88(4):1067-72.

Vegeto E, Allan GF, Schrader WT, Tsai MJ, McDonnell DP, O'Malley BW. (1992). The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. Cell. 69(4):703-13.

Vervoort M. (2000). hedgehog and wing development in Drosophila: a morphogen at work? Bioessays. 22(5):460-8.

Voegel JJ, Heine MJ, Zechel C, Chambon P, Gronemeyer H. (1996). TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. EMBO J. 15(14):3667-75.

Vogel HG. (1974). Correlation between tensile strength and collagen content in rat skin. Effect of age and cortisol treatment. Connect Tissue Res. 2(3):177-82.

Vorherr H. (1986). Fibrocystic breast disease: pathophysiology, pathomorphology, clinical picture, and management. Am J Obstet Gynecol. 154(1):161-79.

Wagner BL, Pollio G, Giangrande P, Webster JC, Breslin M, Mais DE, Cook CE, Vedeckis WV, Cidlowski JA, McDonnell DP. (1999). The novel progesterone receptor antagonists RTI 3021-012 and RTI 3021-022 exhibit complex glucocorticoid receptor antagonist activities: implications for the development of dissociated antiprogestins. Endocrinology. 140(3):1449-58.

Wahl SM, Hunt DA, Wakefield LM, McCartney-Francis N, Wahl LM, Roberts AB, Sporn MB. (1987). Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci U S A. 84(16):5788-92.

Wahl SM, McCartney-Francis N, Allen JB, Dougherty EB, Dougherty SF. (1990). Macrophage production of TGF-beta and regulation by TGF-beta. Ann N Y Acad Sci. 593:188-96.

Waikel RL, Wang XJ, Roop DR. (1999). Targeted expression of c-Myc in the epidermis alters normal proliferation, differentiation and UV-B induced apoptosis. Oncogene. 18(34):4870-8.

Wakefield LM, Roberts AB. (2002). TGF-beta signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev. 12(1):22-9.

Wall NA, Hogan BL. (1994). TGF-beta related genes in development. Curr Opin Genet Dev. 4(4):517-22.

Wan Y, Liu X, Kirschner MW. (2001). The anaphase-promoting complex mediates TGF-beta signaling by targeting SnoN for destruction. Mol Cell. 8(5):1027-39.

Wang GL, Semenza GL. (1993). General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci U S A. 90(9):4304-8.

Wang XF, Lin HY, Ng-Eaton E, Downward J, Lodish HF, Weinberg RA. (1991). Expression cloning and characterization of the TGF-beta type III receptor. Cell. 67(4):797-805.

Webster JC, Cidlowski JA. (1999). Mechanisms of Glucocorticoid-receptor-mediated Repression of Gene Expression. Trends Endocrinol Metab. 10(10):396-402.

Weigel PH, Hascall VC, Tammi M. (1997). Hyaluronan synthases. J Biol Chem. 272(22):13997-4000.

Weikkolainen K, Keski-Oja J, Koli K. (2003). Expression of latent TGF-beta binding protein LTBP-1 is hormonally regulated in normal and transformed human lung fibroblasts. Growth Factors. 21(2):51-60.

Weinstein M, Yang X, Deng C. (2000). Functions of mammalian Smad genes as revealed by targeted gene disruption in mice. Cytokine Growth Factor Rev. 11(1-2):49-58.

Wen FQ, Kohyama T, Skold CM, Zhu YK, Liu X, Romberger DJ, Stoner J, Rennard SI. (2002). Glucocorticoids modulate TGF-beta production. Inflammation. 26(6):279-90.

Wenger RH, Gassmann M. (1997). Oxygen(es) and the hypoxia-inducible factor-1. Biol Chem. 378(7):609-16.

Whitby DJ, Ferguson MW. (1991). Immunohistochemical localization of growth factors in fetal wound healing. Dev Biol. 147(1):207-15.

Wicke C, Halliday B, Allen D, Roche NS, Scheuenstuhl H, Spencer MM, Roberts AB, Hunt TK. (2000). Effects of steroids and retinoids on wound healing. Arch Surg. 135(11):1265-70

Wickert L, Abiaka M, Bolkenius U, Gressner AM. (2004). Corticosteroids stimulate selectively transforming growth factor (TGF)-beta receptor type III expression in transdifferentiating hepatic stellate cells. J Hepatol. 40(1):69-76.

Wiegers GJ, Reul JM. (1998). Induction of cytokine receptors by glucocorticoids: functional and pathological significance. Trends Pharmacol Sci. 19(8):317-21.

Wikstrom P, Westin P, Stattin P, Damber JE, Bergh A. (1999). Early castration-induced upregulation of transforming growth factor beta1 and its receptors is associated with tumor cell apoptosis and a major decline in serum prostate-specific antigen in prostate cancer patients. Prostate. 38(4):268-77.

Williams RL. (1997). Hyperbaric oxygen therapy and the diabetic foot. J Am Podiatr Med Assoc. 87(6):279-92.

Wissink S, van Heerde EC, vand der Burg B, van der Saag PT. (1998). A dual mechanism mediates repression of NF-kappaB activity by glucocorticoids. Mol Endocrinol. 12(3):355-63.

Woodley DT. (1996). Reepithelialization. In: Clark RAF, editor. The molecular and cellular biology of wound repair. New York: Plenum Press, 339-54.

Woo PL, Cha HH, Singer KL, Firestone GL. (1996). Antagonistic regulation of tight junction dynamics by glucocorticoids and transforming growth factor-beta in mouse mammary epithelial cells. J Biol Chem. 271(1):404-12.

Worby CA, Vega QC, Zhao Y, Chao HH, Seasholtz AF, Dixon JE. (1996). Glial cell line-derived neurotrophic factor signals through the RET receptor and activates mitogen-activated protein kinase. J Biol Chem. 271(39):23619-22.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. (1988). Novel regulators of bone formation: molecular clones and activities. Science. 242(4885):1528-34.

Wrana JL. (1998). TGF-beta receptors and signalling mechanisms. Miner Electrolyte Metab. 24(2-3):120-30.

Wu L, Mustoe TA. (1995). Effect of ischemia on growth factor enhancement of incisional wound healing. Surgery 117: 570-6.

Wu L, Wu Y, Gathings B, Wan M, Li X, Grizzle W, Liu Z, Lu C, Mao Z, Cao X. (2003). Smad4 as a transcription corepressor for estrogen receptor alpha. J Biol Chem. 278(17):15192-200

Xia YP, Zhao Y, Tyrone JW, Chen A, Mustoe TA. (2001). Differential activation of migration by hypoxia in keratinocytes isolated from donors of increasing age: implication for chronic wounds in the elderly. J Invest Dermatol. 116(1):50-6.

Xuan YT, Tang XL, Banerjee S, Takano H, Li RC, Han H, Qiu Y, Li JJ, Bolli R. (1999). Nuclear factor-kappaB plays an essential role in the late phase of ischemic preconditioning in conscious rabbits. Circ Res. 84(9):1095-109.

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(5244):2008-11.

Yamamoto T, Takagawa S, Katayama I, Nishioka K. (1999). Anti-sclerotic effect of transforming growth factor-beta antibody in a mouse model of bleomycin-induced scleroderma. Clin Immunol. 92(1):6-13.

Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, Smith JC, Heldin CH, Miyazono K. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. J Cell Biol. 130(1):217-26

Yanagi Y, Suzawa M, Kawabata M, Miyazono K, Yanagisawa J, Kato S. (1999). Positive and negative modulation of vitamin D receptor function by transforming growth factor-beta signaling through smad proteins. J Biol Chem. 274(19):12971-4.

Yanagisawa J, Yanagi Y, Masuhiro Y, Suzawa M, Watanabe M, Kashiwagi K, Toriyabe T, Kawabata M, Miyazono K, Kato S. (1999). Convergence of transforming growth factor-beta and vitamin D signaling pathways on SMAD transcriptional coactivators. Science. 283(5406):1317-21.

Yang L, Chan T, Demare J, Iwashina T, Ghahary A, Scott PG, Tredget EE. (2001). Healing of burn wounds in transgenic mice overexpressing transforming growth factor-beta 1 in the epidermis. Am J Pathol. 159(6):2147-57

Yang X, Letterio JJ, Lechleider RJ, Chen L, Hayman R, Gu H, Roberts AB, Deng C. (1999). Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. EMBO J. 18(5):1280-91.

Yang X, Chen L, Xu X, Li C, Huang C, Deng CX. (2001b). TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol. 153(1):35-46

Yang YA, Dukhanina O, Tang B, Mamura M, Letterio JJ, MacGregor J, Patel SC, Khozin S, Liu ZY, Green J, Anver MR, Merlino G, Wakefield LM. (2002). Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. J Clin Invest. 109(12):1607-15.

Yang-Yen HF, Chambard JC, Sun YL, Smeal T, Schmidt TJ, Drouin J, Karin M. (1990). Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell. 62(6):1205-15.

Yang ZZ, Zou AP. (2001). Transcriptional regulation of heme oxygenases by HIF-1alpha in renal medullary interstitial cells. Am J Physiol Renal Physiol. 281(5):F900-8.

Yao KS, Clayton M, O'Dwyer PJ. (1995). Apoptosis in human adenocarcinoma HT29 cells induced by exposure to hypoxia. J Natl Cancer Inst. 87(2):117-22.

Yaswen L, Kulkarni AB, Fredrickson T, Mittleman B, Schiffman R, Payne S, Longenecker G, Mozes E, Karlsson S. (1996). Autoimmune manifestations in the transforming growth factor-beta 1 knockout mouse. Blood. 87(4):1439-45.

Yin W, Smiley E, Germiller J, Mecham RP, Florer JB, Wenstrup RJ, Bonadio J. (1995). Isolation of a novel latent transforming growth factor-beta binding protein gene (LTBP-3). J Biol Chem. 270(17):10147-60.

Yu C, Warriar N, Govindan MV. (1995). Cysteines 638 and 665 in the hormone binding domain of human glucocorticoid receptor define the specificity to glucocorticoids. Biochemistry. 34(43):14163-73.

Yu F, White SB, Zhao Q, Lee FS. (2001). HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc Natl Acad Sci U S A. 98(17):9630-5.

Yu L, Border WA, Huang Y, Noble NA. (2003). TGF-beta isoforms in renal fibrogenesis. Kidney Int. 2003 Sep;64(3):844-56.

Yu Q, Stamenkovic I. (2000). Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev. 14(2):163-76.

Yue J, Buard A, Mulder KM. (1998). Blockade of TGFbeta3 up-regulation of p27Kip1 and p21Cip1 by expression of RasN17 in epithelial cells. Oncogene. 17(1):47-55.

Yue J, Mulder KM. (2001). Transforming growth factor-beta signal transduction in epithelial cells. Pharmacol Ther. 91(1):1-34.

Zatelli MC, Rossi R, degli Uberti EC. (2000). Androgen influences transforming growth factor-beta1 gene expression in human adrenocortical cells. J Clin Endocrinol Metab. 85(2):847-52.

Zelzer, E., Levy, Y., Kahana, C., Shilo, B.Z., Rubinstein, M. & Cohen, B. (1998). Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1$^{\alpha}$/ARNT. EMBO J. 17, 5085-5094.

Zhang H, Akman HO, Smith EL, Zhao J, Murphy-Ullrich JE, Batuman OA.(2003). Cellular response to hypoxia involves signaling via Smad proteins. Blood. 101(6):2253-60. Epub 2002 Oct 31

Zhang Y, Feng X, We R, Derynck R. (1996). Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. Nature. 383(6596):168-72.

Zhang Y, Feng XH, Derynck R. (1998). Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. Nature. 394(6696):909-13.

Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW. (1999). Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res. 59(22):5830-5.

Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu M.M, Simons JW. & Semenza GL. (2000). Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res. 60, 1541-1545.

Zhou G, Lee SC, Yao Z, Tan TH. (1999). Hematopoietic progenitor kinase 1 is a component of transforming growth factor beta-induced c-Jun N-terminal kinase signaling cascade. J Biol Chem. 274(19):13133-8.

Zhou L, Dey CR, Wert SE, Whitsett JA. (1996). Arrested lung morphogenesis in transgenic mice bearing an SP-C-TGF-beta 1 chimeric gene. Dev Biol. 175(2):227-38.

Zhu HJ, Burgess AW. (2001). Regulation of transforming growth factor-beta signaling. Mol Cell Biol Res Commun. 4(6):321-30.

Zhu Y, Richardson JA, Parada LF, Graff JM. (1998). Smad3 mutant mice develop metastatic colorectal cancer. Cell. 94(6):703-14.

Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, Gottschalk AR, Ryan HE, Johnson RS, Jefferson AB, Stokoe D, Giaccia AJ. (2000). Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev. 14(4):391-6.

and its receptors is regulated by acute ischemia and reperfusion in different cell types of the skin. Our results with a pig skin flap model show that ischemic conditions and reperfusion result in rapid and dynamic regulation of type I, II, and III TGF-β receptors and TGF-β1 in the skin. In addition, this regulation occurs in a cell type-specific manner, with endothelial cells and fibroblasts exhibiting the most marked alterations.

MATERIALS AND METHODS

Surgical preparation and experimental design. Six female White Landrace pigs (10–14 wk old) were housed in a temperature-controlled (20–22°C) animal holding room. The protocol for use of pigs in this experiment was approved by the McGill University Animal Care Committee. All the pigs were offered the same commercial pig diet and tap water ad libitum. Food was withheld the evening before surgery. Animals were sedated with intramuscular injections of ketamine (20 mg/kg), xylazine (2 mg/kg), and atropine (1 mg). Animals were anesthetized with an intravenous injection of pentobarbital sodium (6 mg/kg). They were then intubated, and general anesthesia was maintained with spontaneous inhalation of oxygen (8 l/min) and halothane (0.5–1.0%).

In each pig, bilateral buttock skin flaps (10 × 18 cm) based on the vascular pedicle of the superficial circumflex artery and its accompanying vena comitans as well as the lateral femoral cutaneous nerve were elevated and later returned and sutured to their beds with 3-0 nylon skin sutures (Fig. 1). The procedure used was described previously by Kerrigan et al. (16). Briefly, the flap on one side was randomly assigned to 1 h of arterial occlusion with the flap on the contralateral side acting as a nonischemic control. Arterial ischemia in these island flaps was created by clamp application on the artery, which mimics the clinical scenario of an ischemic free flap. Complete occlusion of the vascular pedicle was achieved by application of an Acland V2 microvascular clamp to the branch of the circumflex iliac artery supplying the buttock flap and was verified by application of 10% sodium fluorescein dye (15 mg/kg). Absence of fluorescein in the skin 15 min after dye injection indicated complete occlusion of the vascular pedicle. After 1 h of ischemia, the microvascular clamps were removed to allow reflow. From each pig, skin biopsies (4 × 8-mm pieces from the central portion in the proximal third of the flap) were taken at 1 h after the induction of ischemia and at 1 h after reflow from the experimental flap (ischemic) and at corresponding times from the control flap (nonischemic) and unoperated buttock skin. This experiment was repeated in all six pigs. Thus each group (unoperated skin, nonischemic control flap, ischemic flap, and reperfusion flap) represents 6 animals or 12 flaps. In a similar manner, an additional pair of bilateral skin flaps (thoracic area) was created on each of the six pigs (i.e., 12 additional flaps). Global arterial ischemia in these flaps was achieved by clamping the thoracodorsal artery. Biopsies were collected after 4 h of ischemia and at 4 h after reflow. No necrosis was observed in the flaps during the ischemia-reperfusion time period that we tested.

Preparation of skin tissue sections. Biopsies collected from the skin flaps and unoperated skin were fixed in 4% paraformaldehyde for 8 h followed by immersion in 15% sucrose for 30 h at 4°C. They were then embedded in Tissue Tek compound and frozen in liquid nitrogen. Serial sections of the frozen tissue were prepared with a cryostat. Each slide contained tissue sections (in duplicates or triplicates) from all four groups: *1*) unoperated skin, *2*) nonischemic control flap, *3*) ischemic flap, and *4*) reperfusion flap. A minimum of 15 slides each were analyzed for type I, II, and III receptors and TGF-β1 from each group.

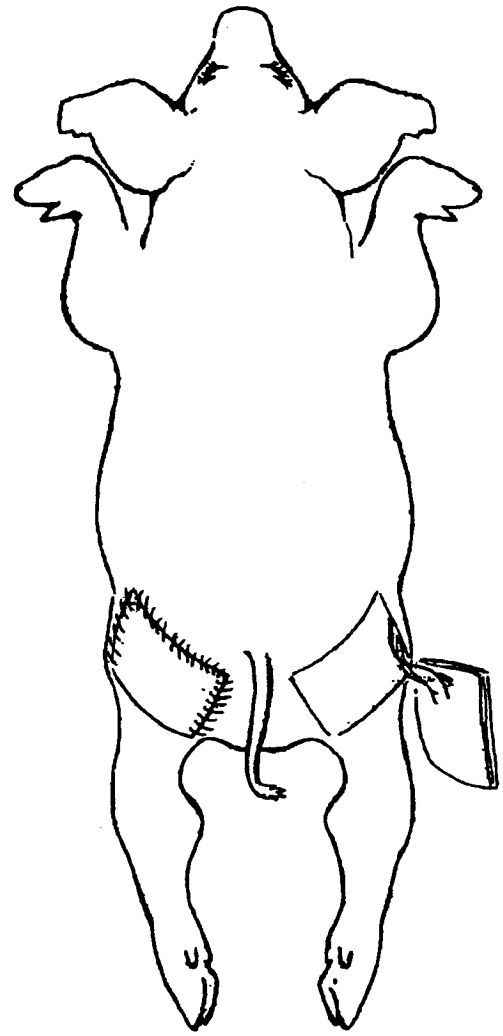

Fig. 1. Experimental model. In each pig, bilateral buttock skin flaps (10 × 18 cm) based on the vascular pedicle of the superficial circumflex artery and its accompanying vena comitans as well as the lateral femoral cutaneous nerve were elevated and later returned and sutured to their beds with 3-0 nylon skin sutures. The flap on one side was randomly assigned to 1 h of arterial occlusion, with the flap on the contralateral side acting as a nonischemic control. Arterial ischemia was created by clamp application on the artery. Complete occlusion of the vascular pedicle was achieved by application of an Acland V2 microvascular clamp to the branch of the circumflex iliac artery supplying the buttock flap and was verified by application of 10% sodium fluorescein dye (15 mg/kg). Absence of fluorescein in the skin 15 min after dye injection indicated complete occlusion of the vascular pedicle. After 1 h of ischemia, the microvascular clamps were removed to allow reflow. Skin biopsies (4 × 8 mm pieces from the central portion in the proximal third of the flap) were taken at 1 h after the induction of ischemia and at 1 h after reflow from the experimental flaps (ischemic or reperfused) and at corresponding times from the control flaps (nonischemic) and unoperated buttock skin.

Antibodies used. Expression of type I, II, and III TGF-β receptors and TGF-β1 in tissue sections was detected by

immunohistochemistry with their respective specific anti-peptide antibodies. The anti-type I and anti-type II receptor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were both rabbit polyclonal IgGs. The anti-type I antibody recognizes amino acids 158–179 of the precursor form of the type I TGF-β receptor (ALK5) of human origin, whereas the anti-type II antibody recognizes amino acids 550–565 of the precursor form of the human type II TGF-β receptor. The anti-type III antibody and the anti-TGF-β1 antibody were kind gifts from Dr. M. O'Connor-McCourt (Biotechnology Research Institute, Montreal, PQ, Canada). The peptide sequences used and the procedure employed for the preparation of the type III receptor antibody were exactly the same as described by Moustakas et al. (26). Briefly, polyclonal rabbit antisera were raised against a COOH-terminal epitope of human type III receptor, the immunoglobulins were prepared, and their ability to specifically immunoprecipitate the receptor was tested. In comparison studies, this antibody displayed the same specificity as the antibodies obtained from Dr. A. Moustakas (Whitehead Institute for Medical Research, Cambridge, MA). The procedure for the preparation of anti-TGF-β1 antibody involved coupling of TGF-β1 to keyhole limpet hemocyanin and injection of rabbits and was described previously (25). Normal rabbit IgG used as a negative control was obtained from Lipshaw Immunon (Pittsburgh, PA). The immunizing peptides or protein (same as used for the preparation of antibodies) that were used in control experiments to show the specificity of antibodies during the immunohistochemistry procedure were obtained from Santa Cruz Biotechnology (type I and II peptide), synthesized locally at Sheldon Biotechnology Institute (Montreal, PQ, Canada; type III peptide), or bought from R&D Systems (Minneapolis, MN; TGF-β1).

Immunohistochemistry. Immunohistochemical localization of type I, II, and III TGF-β receptors and TGF-β1 ligand was performed on 8-μm-thick cryostat sections of skin tissue placed onto gelatin-coated glass slides. The sections were washed and permeabilized three times for 15 min each with phosphate-buffered saline pH 7.5 (PBS) containing 0.1% Triton X-100. Endogenous peroxidase activity was then quenched by treating the sections with 1% H_2O_2 in 99% methanol for 45 min at room temperature. The sections were incubated in a humidified chamber for 3 h with blocking solution (PBS containing 1% normal goat serum, 0.3% Triton X-100, and 0.5% BSA) to block excess proteins and prevent nonspecific antibody binding. The primary antibodies diluted in the blocking solution were applied to the sections overnight at 4°C in a humidified chamber. The anti-type I and anti-type II antibodies were diluted 1:100 to a final concentration of 2 μg/ml, whereas the anti-type III antibody and anti-TGF-β1 antibody were diluted 1:400 and 1:250, respectively. The next day, slides were washed two times with PBS containing 0.1% Triton X-100 and once with PBS alone. The slides were incubated with biotinylated goat anti-rabbit secondary antibody (diluted to 0.5% in PBS containing 0.5% BSA and 1.5% normal goat serum) for 1 h. The sections were washed two times with PBS containing 0.1% Triton X-100 and once with PBS alone. This was followed by incubation with avidin-biotin complex (ABC; Vector Laboratories) diluted in PBS for 1 h. The slides were again washed two times with PBS containing 0.1% Triton X-100 and once with PBS alone. The brown color, indicating immunoreactivity, was developed with 0.05% of 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) in 0.1% H_2O_2 in PBS for 3 min. Sections were rinsed in distilled water. Finally, the slides were dehydrated and mounted with permount (Sigma). A corresponding set of slides was counterstained with Gill's hematoxylin before mounting. All tissue sections that were compared were treated at the same time and for the same length of time.

Normal rabbit IgG (Lipshaw Immunon) at the same concentration as the primary antibody was used as a negative control for the immunostaining protocol. Additional procedural control involved incubation without the primary antibody, with the rest of the protocol unchanged. Antibody specificity was proven by absorbing each antibody with a 50× excess of immunizing peptide or protein (see *Antibodies used*) to the antibody. Briefly, the peptide or protein was incubated with the primary antibody overnight at 4°C, centrifuged, and applied to the tissue samples instead of the unabsorbed antibody. Positive staining on tissue sections was not observed under any of the above circumstances.

Culture of skin fibroblasts. Early-passage skin fibroblasts were prepared from human skin tissue obtained at breast reduction surgery. The tissue was collected in Dulbecco's minimal essential medium (D-MEM), washed, and minced into pieces of <0.3 mm^3. The explants were distributed into 25-cm^2 tissue culture flasks (Costar, Cambridge, MA) and cultured in D-MEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (GIBCO, Burlington, ON, Canada). The cultures were maintained at 37°C in an atmosphere of 5% CO_2-95% air. Culture medium was changed every 3 days. Keratinocytes and other contaminating cells were removed by the first two or three subcultures. Cells from the fourth to tenth passages were used for experiments. The purity and homogeneity of the fibroblasts preparation were determined as described by Tam and Philip (39).

Culture of fibroblasts under hypoxic conditions. Skin fibroblasts growing as monolayers in 150-mm petri dishes (Starsted) and at ~70% confluence were exposed to hypoxia for 2 h with a Gas Pak system (BBL Gas Pak Plus system; Becton-Dickinson, Lincoln Park, NJ) at 37°C, as described by Detmar et al. (7). The Gas Pak system depletes oxygen by means of a palladium catalyst, and the oxygen concentration during hypoxia was <0.2%. Control cultures consisted of monolayers of fibroblasts at 70% confluence maintained under normoxic conditions (5% CO_2-95% air) at 37°C.

Northern blot analysis. Total RNA from skin fibroblasts cultured under normoxic and hypoxic conditions was isolated by homogenization in 4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5 M *N*-lauroylsarcosine and 0.1 mercaptoethanol (Sigma) as described previously by Chomczynski and Sacchi (5). RNA (20 ug/well) was electrophoresed on a 1% agarose formaldehyde gel, transferred to nylon membrane (Boehringer Mannheim), and ultraviolet cross-linked. Membranes were prehybridized overnight at 42°C and transferred to fresh hybridization solution containing ^{32}P-labeled probe. The type II TGF-β receptor probe was a 474-bp (521–995) fragment labeled with [^{32}P]dCTP with nick translation (GIBCO-BRL). After an overnight hybridization, membrane was washed twice, and the blot was exposed to Kodak X-ray film with an intensifying screen at −80°C for 94 h. Equivalent RNA loading and transfer were determined by subsequent reprobing with 18S rRNA that was radiolabeled as above. Scanning densitometry was performed to quantify relative mRNA abundance.

Evaluation of staining and statistical analysis. The results of immunohistochemistry studies were assessed in a blinded fashion by three separate investigators. The evaluation of positively staining skin structures (cell types) was performed semiquantitatively on an arbitrary scale ranging from 0 to 4 for each structure: 0, negative reaction; 1, positive reaction in

a few cells; 2, reaction in a moderate number of cells; 3, reaction in a large number of cells; 4, reaction in almost all cells. The Kruskal-Wallis test was used to analyze the differences between groups (unoperated buttock skin, nonischemic control flap, ischemic flap, and reperfusion flap) in the expression of type I, II, and III TGF-β receptors and TGF-β1. Differences with a P value of <0.05 was considered significant (2-sided test). Analysis of the data using a second nonparametric test, Monte Carlo estimates for the exact test, gave results similar to those obtained by the Kruskal-Wallis test. SAS version 8.0 statistical software (SAS Institute, 1999) was used for computation and analysis.

RESULTS

Immunohistochemical localization patterns of type I, II, and III TGF-β receptors and TGF-β1. To analyze the regulation of TGF-β receptors and TGF-β1 during ischemia-reperfusion injury, a pig skin flap model was used. Immunohistochemical localization of type I, II, and III TGF-β receptors and TGF-β1 ligand was performed in tissue sections prepared from nonischemic control flap, ischemic flap, reperfused flap, and unoperated buttock skin. Comparative analysis of immunostaining of type I, II, and III receptors revealed that expression of these receptors was increased in the nonischemic control flaps, ischemic flaps, and reperfused flaps compared with the unoperated skin (Figs. 2 and 3). The skin structures that showed the most dramatic increases were blood vessels (endothelial cells), fibroblasts, and the basal layer of the epidermis. The immunoreactivity of the three receptors were highest in the ischemic flap, with the nonischemic control flap showing significantly lower immunostaining. The expression of TGF-β1 in the ischemic flap, on the other hand, was not significantly different from that of the nonischemic control. Importantly, TGF-β1 expression in the nonischemic control was markedly higher than in the unoperated skin (Fig. 3). Semiquantitative evaluation of immunostaining and statistical analysis of the differences between groups (unoperated, nonischemic control, ischemic, and reperfused) for type I, II, and III receptors and TGF-β1 are shown in Table 1.

The skin structures immunostained for type I , II, and III TGF-β receptors and TGF-β1 ligand in the skin flaps were in general similar in the unoperated skin (Figs. 2 and 3). In the epidermis, no detectable immunostaining for type I, II, and III receptors and TGF-β1 was observed in the stratum corneum. The stratum lucidum showed the strongest immunostaining, whereas stratum granulosum and spinosum showed strong but less robust expression. However, these two layers displayed no dramatic alterations between groups. In the stratum basale, the immunostaining of the three receptors and TGF-β1 was moderate but showed marked differences in the expression of type I receptor and TGF-β1 under certain conditions. There was no significant difference in immunostaining patterns of type I, II, and III receptors and TGF-β1 between flaps exposed to 1 and 4 h of ischemia (data not shown). Data from flaps representing 1 h of ischemia and 1 h of reperfusion are shown in Table 1.

Type I and II TGF-β receptor expression. The immunoreactivity patterns of type I and II receptors are shown in Fig. 2. The expression of both type I and type II receptors was the highest in the ischemic flap (Fig. 2, c_I and c_{II}). The immunostaining of type I and II receptors on blood vessels ($P < 0.0004$ and $P < 0.01$, respectively) and fibroblasts ($P < 0.004$ and $P < 0.001$, respectively) in the ischemic flap (Fig. 2, c_I and c_{II}) was significantly higher compared with the nonischemic control flap (Fig. 2, b_I and b_{II}; Table 1). A higher magnification of the nonischemic control flap (e_I and e_{II} for types I and II, respectively) and ischemic flap (f_I and f_{II} for types I and II, respectively) is also shown in Fig. 2 to emphasize the effect of ischemia on receptor expression and to better depict the cell types involved. Although type I and II receptor staining was also increased in the stratum basale in the ischemic flap compared with the nonischemic control flap, it was significant ($P < 0.003$) only for the type I receptor. The immunoreactivity in the reperfused flap compared with ischemic flap was significantly lower for the type I (Fig. 2, d_I vs. c_I; $P < 0.01$) and type II (Fig. 2, d_{II} vs. c_{II}; $P < 0.01$) receptors on fibroblasts with no significant difference in blood vessels and stratum basale. The expression of type I and type II receptors in the nonischemic control flap (Fig. 2, b_I and b_{II}) compared with the unoperated skin (Fig. 2, a_I and a_{II}) was significantly increased in blood vessels ($P < 0.04$ and $P < 0.02$ for types I and II, respectively) and fibroblasts ($P < 0.005$ and $P < 0.03$ for types I and II, respectively) but not in stratum basale (Table 1). No immunoreactivity was observed in control experiments when the type I (Fig. 2*g*) and type II (Fig. 2*h*) antibodies preincubated with their respective immunizing peptides were used. The control slides (Fig. 2, *g* and *h*) were counterstained with Gill's hematoxylin before mounting to show the histology.

Type III TGF-β receptor and TGF-β1 expression. The immunoreactivity patterns of type III receptor and TGF-β1 ligand are shown in Fig. 3. As observed for type I and type II receptors, the expression of the type III receptor was highest in the ischemic flap (Fig. 3c_{III}), with the blood vessels ($P < 0.0009$) and fibroblasts ($P < 0.002$) of this flap showing markedly higher immunoreactivity than those of the nonischemic control flap (Fig. 3b_{III}; Table 1).

In the stratum basale, the type III immunostaining was not significantly different between the nonischemic control and ischemic flaps, as observed for type II but not type I in this cell layer. When the reperfused flap (Fig. 3d_{III}) was compared with the ischemic flap (Fig. 3c_{III}), the type III receptor immunoreactivity was unchanged in blood vessels, fibroblasts, and stratum basale. A higher magnification of the type III receptor immunostaining in the nonischemic control flap (e_{III}) and ischemic flap (f_{III}) is shown in Fig. 3 to highlight the alterations in immunoreactivity and cell types involved. When the type III receptor immunoreactivity in the nonischemic control flap (Fig. 3b_{III}) was compared with that of the unoperated skin (Fig. 3a_{III}), it was significantly increased in the control flap on blood

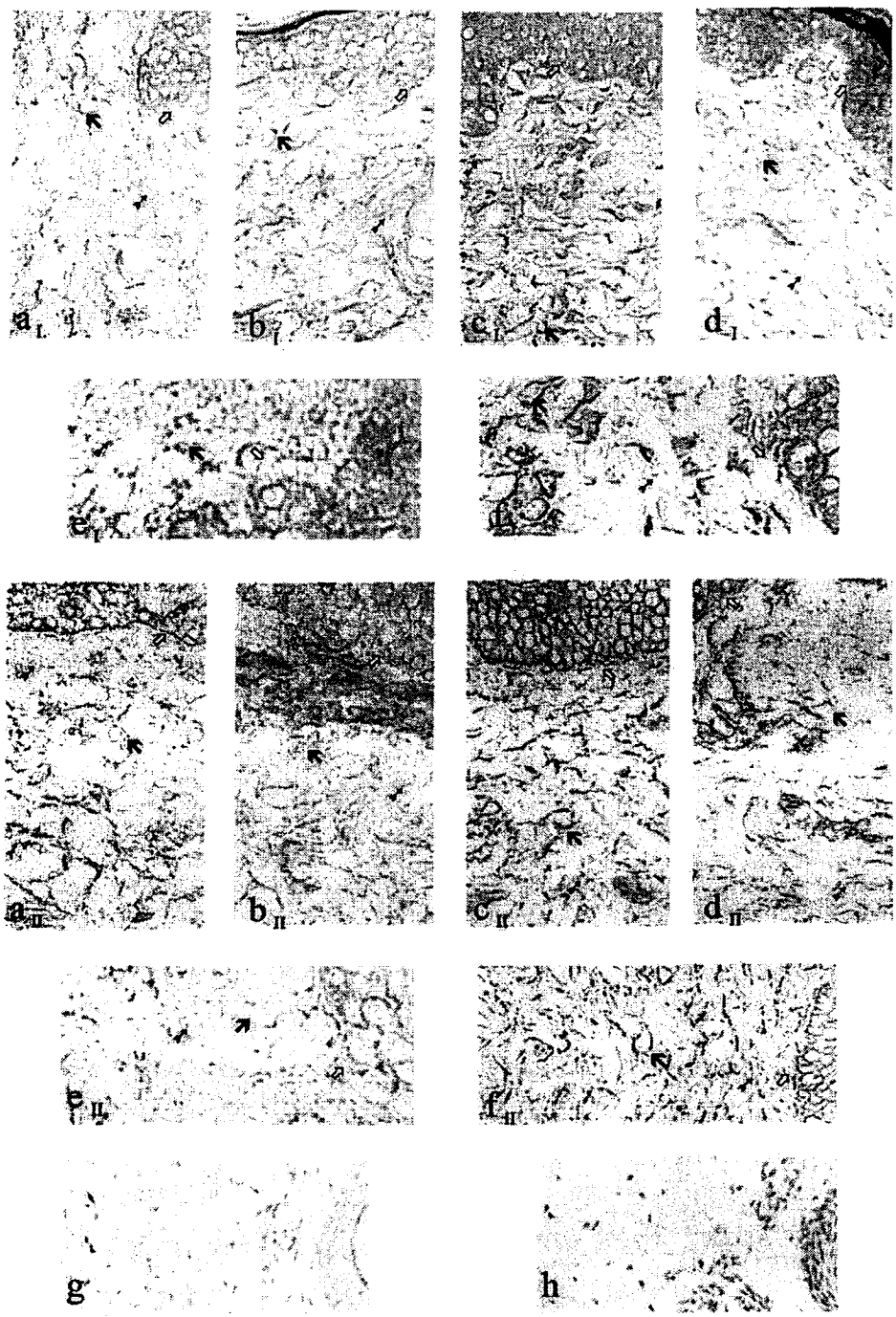

Fig. 2. Immunohistochemical localization of type I and II transforming growth factor (TGF)-β receptors in pig skin. Immunostaining patterns of type I and II receptors in unoperated skin (a_I and a_{II}, respectively), nonischemic control flap (b_I and b_{II}, respectively), ischemic flap (c_I and c_{II}, respectively), reperfused flap (d_I and d_{II}, respectively), nonischemic control flap (e_I and e_{II}, respectively; higher magnification, 40× original), and ischemic flap (f_I and f_{II}, respectively; higher magnification, 40× original) are shown. The immunostaining patterns show dynamic regulation of type I and II receptors in a cell type-specific manner. Significant alterations are observed in blood vessels (large filled arrows), fibroblasts (small filled arrows), and stratum basale (open arrows). Control experiments in which the primary antibodies—anti-type I (*g*) and anti-type II (*h*) receptor antibodies—were preincubated with their respective immunizing peptides do not show any immunoreactivity. *Sections g* and *h* were counterstained with hematoxylin before mounting. The result shown is representative of samples collected at 1 h after ischemia and at 1 h after reflow from experimental flaps and at corresponding times from control flaps and unoperated skin.

vessels ($P < 0.002$) and fibroblasts ($P < 0.0004$) but not in the stratum basale.

In contrast to what was observed for type I, II, and III receptors, the TGF-β1 immunoreactivity in the ischemic flap was not significantly different from that of nonischemic control in blood vessels, fibroblasts, or stratum basale. More importantly, however, TGF-β1 immunostaining in the nonischemic control flap (Fig. $3b_{\beta I}$) was markedly higher than that of the unoperated skin (Fig. $3a_{\beta I}$) on blood vessels ($P < 0.0005$) and fibroblasts ($P < 0.005$). Stratum basale showed no significant difference. Interestingly, TGF-β1 immunostaining in the reperfused flap (Fig. $3d_{\beta I}$) compared with the ischemic flap (Fig. $3c_{\beta I}$) was significantly higher in stratum basale ($P < 0.05$), with no significant difference in blood vessels and fibroblasts. In control experiments in which the anti-type III receptor (Fig. 3*g*) and anti-TGF-β1 (Fig. 3*h*) antibodies preincubated with their respective immunizing peptides were used, no detectable immunoreactivity was seen. The control

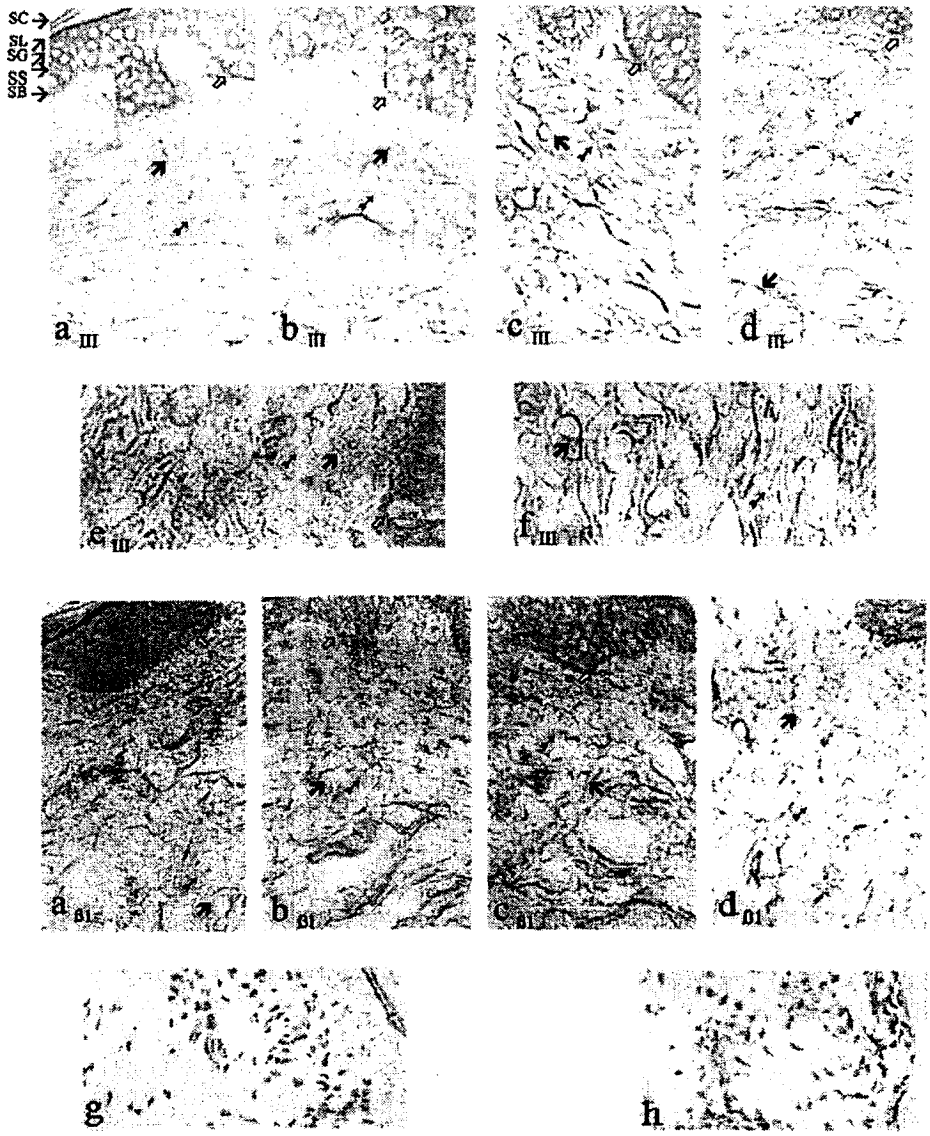

Fig. 3. Immunohistochemical localization of type III TGF-β receptors and TGF-β1 in pig skin. Immunostaining of type III receptors and TGF-β1 in unoperated skin (a_{III} and $a_{\beta 1}$, respectively), nonischemic control flap (b_{III} and $b_{\beta 1}$, respectively), ischemic flap (c_{III} and $c_{\beta 1}$, respectively), and reperfused flap (d_{III} and $d_{\beta 1}$, respectively) is shown. The immunostaining patterns show dynamic regulation of type III receptors and TGF-β1 in a cell type-specific manner. Marked alterations are observed in blood vessels (large filled arrows), fibroblasts (small filled arrows), and stratum basale (open arrows). Control experiments in which the primary antibodies—anti-type III receptor (*g*) and anti-TGF-β1 (*h*) antibodies—were preincubated with their respective immunizing peptides do not show any immunoreactivity. *Sections g* and *h* were counterstained with hematoxylin before mounting. The result shown is representative of samples collected at 1 h after ischemia and at 1 h after reflow from experimental flaps and at corresponding times from control flaps and unoperated skin. SC, stratum corneum; SL, stratum lucidum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale.

Table 1. *Semiquantitative analyses of immunoreactivity of type I, II, and III TGF-β receptors and TGF-β1 in unoperated skin, nonischemic control flap, ischemic flap, and reperfused flap*

	Type I R	Type II R	Type III R	TGF-β1
Blood vessels				
Cont vs. Ischem	Ischem ↑ (0.0004)	Ischem ↑ (0.01)	Ischem ↑ (0.0009)	NS
Ischem vs. Reper	NS	NS	NS	NS
Unop vs. Cont	Cont ↑ (0.04)	Cont ↑ (0.02)	Cont ↑ (0.002)	Cont ↑ (0.0005)
Fibroblast				
Cont vs. Ischem	Ischem ↑ (0.004)	Ischem ↑ (0.001)	Ischem ↑ (0.002)	NS
Ischem vs. Reper	Ischem ↑ (0.01)	Ischem ↑ (0.01)	NS	NS
Unop vs. Cont	Cont ↑ (0.005)	Cont ↑ (0.03)	Cont ↑ (0.0004)	Cont ↑ (0.005)
Stratum basale				
Cont vs. Ischem	Ischem ↑ (0.003)	NS	NS	NS
Ischem vs. Reper	NS	NS	NS	Reper ↑ (0.05)
Unop vs. Cont	NS	NS	NS	NS

Data represent values from 6 pigs (12 flaps) analyzed by Kruskal-Wallis test on means ± SD of samples collected at 1 h of ischemia and 1 hr after reflow from experimental (ischemic or reperfused) flaps and from control (nonischemic) flaps (Cont) and unoperated skin (Unop). TGF, transforming growth factor; R, receptor; Ischem, ischemic flap; Reper, reperfused flap. Nos. in parentheses are *P* values; NS, not significant.

slides were counterstained with Gill's hematoxylin to show the histology (Fig. 3, *g* and *h*).

Northern blot analysis of type II TGF-β receptor expression. To determine whether hypoxia is able to regulate the expression of type II TGF-β receptors in skin fibroblasts in vitro, early-passage human skin fibroblasts were subjected to hypoxic conditions for 2 h while the control cells remained under normoxic conditions. Expression of the type II receptor was determined by Northern blot analysis. The results shown in Fig. 4 demonstrate that exposure to hypoxic condition for 2 h markedly increased the abundance of type II receptor mRNA in early-passage skin fibroblasts. This observation illustrates that acute exposure to hypoxia leads to an increase in the expression of type II receptor in early-passage skin fibroblasts and supports the in vivo immunohistochemistry results presented above.

DISCUSSION

The pathophysiological mechanisms involved in ischemia-reperfusion injury, a common denominator in a variety of clinical conditions from myocardial infarction and cerebral ischemia to tissue transplantation and free tissue transfer, are poorly understood. TGF-β has been shown to provide protection against ischemia-reperfusion injury in many organ systems and is known to be a key regulator of the tissue repair process. However, little is known about the regulation of TGF-β receptors during ischemia and reperfusion. Our results demonstrate the occurrence of dynamic and cell type-specific regulation of TGF-β receptors and TGF-β1 in vivo during ischemia-reperfusion in a pig skin flap model. This model was designed to study the early dynamic changes (1 and 4 h) during ischemia as well as reperfusion. This time frame was chosen because it represents the clinically significant period during which intervention with therapeutic agents might have a beneficial effect on the deleterious effects of injury. Thus the model we have used does not allow us to evaluate the late effects of ischemia-reperfusion injury. The bilateral skin flap design, with the contralateral flap acting as the control, avoids animal-to-animal variation and allows us to tease out the effect of ischemia from that of wounding (creation of the flap), which is also associated with ischemia, on the regulation of the TGF-β/TGF-β receptor system in vivo.

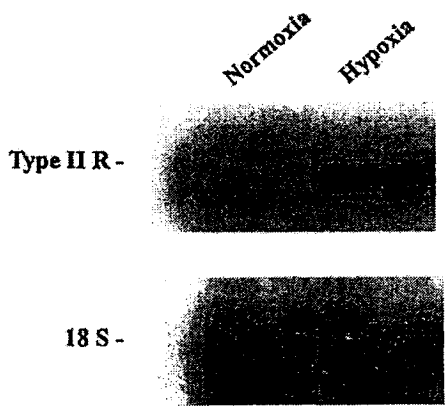

Fig. 4. Hypoxia upregulates type II TGF-β receptor (R) mRNA expression in skin fibroblasts in vitro. Early-passage human skin fibroblasts were cultured and subjected to 2 h of hypoxia as described in MATERIALS AND METHODS. Total RNA was extracted, and Northern blot was done with a cDNA probe for the type II TGF-β receptor. Type II receptor mRNA expression under normoxia (20% oxygen) and hypoxia (< 0.2% oxygen) conditions is shown (*top*). The 18S ribosomal RNA is shown to demonstrate equal loading of RNA (*bottom*).

The most important finding in the present study is that global ischemia resulted in rapid (within 1 h) upregulation of type I, II, and III TGF-β receptors on blood vessels (endothelial cells) and fibroblasts. Immunostaining of TGF-β receptors in those cell types was markedly higher in the ischemic skin flap (subjected to global ischemia) than in the nonischemic control flap. This increase was maintained at 4 h of ischemia (data not shown). Importantly, creation of the flap (which involves wounding and partial ischemia) resulted in an increase in the expression of all three TGF-β receptors and TGF-β1 in endothelial cells and fibroblasts, as shown by significantly higher immunostaining in the nonischemic control flap than in the unoperated skin. The absence of a further increase in TGF-β1 expression in endothelial cells (blood vessels) and fibroblasts after the induction of global ischemia is intriguing. It is possible that the expression of TGF-β1 is already maximal because of the partial ischemia and wounding induced by the creation of the flap, and subsequent global ischemia may have no further effect.

Although the expression of the TGF-β receptors was increased on endothelial cells and fibroblasts within 1 h of the induction of ischemia, subsequent reperfusion led to a significant decrease in type I and type II receptors in fibroblasts, but this decrease was not significant in blood vessels. The rapid upregulation of TGF-β receptors and TGF-β1, and thus enhanced TGF-β signal transducing machinery, in endothelial cells and fibroblasts during ischemia-reperfusion provides an explanation at the molecular level for the potent effect of TGF-β under these conditions, namely the tissue protective effect that TGF-β exerts against ischemia-reperfusion injury in several animal models (18, 23, 24). The endothelial cell is the principal cell type involved in the development of ischemia reperfusion injury, and TGF-β has been shown to be a key regulator of several endothelial responses important in attenuating ischemia reperfusion injury. For example, TGF-β has been demonstrated to potently inhibit endothelial adhesiveness to polymorphonuclear leukocytes (18, 29) to inhibit free radical generation and preserve vasomotor tone (19, 24).

Although the middle layers of the epidermis (granulosum, spinosum, and lucidum) showed strong immunoreactivity for type I, II, and III receptors and TGF-β1, it was the stratum basale (basal keratinocytes) that exhibited significant differences in immunoreactivity, specifically that for the type I receptor during ischemia and that for TGF-β1 after reflow. However, it is possible that the high expression of the receptors and the

ligand in the middle layers precluded the detection of alterations in immunoreactivity. The significance of the upregulation of type I receptors but not type II and III receptors or TGF-β1 by global ischemia (nonischemic control vs. ischemia) and the enhanced expression of TGF-β1 but not type I, II, and III receptors after reperfusion on basal keratinocytes is not known.

Although sustained ischemia results in the failure of wounds to heal and hyperbaric oxygen has been documented to enhance wound healing, ischemia in the initial phase of the wound healing process stimulates fibroplasia and angiogenesis. Therefore, occlusive dressings that create hypoxia have been shown to promote wound healing (38). Interestingly, it was demonstrated recently that TGF-β was capable of accelerating wound healing under both nonischemic and ischemic conditions, whereas fibroblast growth factor and platelet-derived growth factors were ineffective under ischemic conditions (42). That TGF-β was effective under ischemic conditions would be predicted from our results, which show that the expression of the TGF-β/TGF-β receptor system is enhanced during ischemia. Thus the impaired wound healing observed in ischemic tissue is not likely due to decreased TGF-β action but may be accounted for by the reduced supply of nutrients and immune cells, increased production of oxygen free radicals (37), decreased action of other growth factors (42), or all of the above. Limited information is available on the regulation of TGF-β receptors in skin cells under ischemic conditions. In in vitro culture studies with skin fibroblasts, hypoxia has been reported to induce TGF-β1 expression (9) and to decrease TGF-β receptor mRNA and binding (10). The decreased expression of TGF-β receptors in vitro during hypoxia reported by Falanga et al. (10) is not consistent with our results in vivo (Table 1) and in vitro (Fig. 4) or with those of Ata et al. (1) in vivo in the brain. Differences in experimental conditions may explain this discrepancy.

Although our study does not allow us to determine the precise temporal relationship between the induction of ligand and receptors, the rapid upregulation of TGF-β1 or all three TGF-β receptors by partial or global ischemia suggests that ischemia may have a direct effect on the expression of these receptors and ligand. Our in vitro results demonstrating the upregulation of type II receptor mRNA at 2 h of hypoxia in skin fibroblasts (Fig. 4) support this conclusion. In the only other study that examined the regulation of TGF-β receptors under ischemic conditions, receptor expression was analyzed only at later time periods, namely, on days 1 and 3 after ischemia (1). Regulation of gene expression by low oxygen concentration is now a well-documented phenomenon (3, 28). Whether hypoxia-inducible factor (HIF)-1, a master regulator of oxygen homeostasis (36, 40), is involved in the induction of the TGF-β/TGF-β receptor system during ischemia remains to be determined. It is interesting to note in this regard that hypoxia and TGF-β were recently reported to synergistically cooperate to induce vascular endothelial growth factor expression (34) and that this cooperation may involve a physical association between HIF-1α and Smad3, a central mediator of TGF-β action.

The cellular distribution of the three types of receptors was similar to that of TGF-β1. The concomitant expression profiles and the colocalization of the TGF-β/TGF-β receptor system in the same cell types is consistent with numerous studies showing the heteromeric complex formation of the three receptors and their high affinity for the TGF-β1 ligand. The colocalization and the synchrony in the regulation of the type I and type II TGF-β receptors on endothelial cells and fibroblasts are not consistent with the notion that activation of these receptors leads to distinct TGF-β signaling pathways performing independent functions (4, 11) but suggest that they cooperate to initiate the TGF-β signaling cascade.

In summary, our results demonstrate that the TGF-β/TGF-β receptor system is dynamically regulated during ischemia-reperfusion in a cell type-specific manner in the skin. The data presented define an increase in the expression of TGF-β receptors and TGF-β1 as a rapid adaptive response to partial or global ischemia. Together, our results indicate that TGF-β action is enhanced under ischemic conditions and provide an explanation at the molecular level for the potent effects of TGF-β under these conditions. The augmented TGF-β responsiveness may be an important determinant for the tissue protective effect of TGF-β against ischemia-reperfusion injury.

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REFERENCES

1. **Ata KA, Lennmyr F, Funa K, Olsson Y, and Terent A.** Expression of transforming growth factor-beta1, 2, 3 isoforms and type I and II receptors in acute focal cerebral ischemia: an immunohistochemical study in rat after transient and permanent occlusion of middle cerebral artery. *Acta Neuropathol (Berl)* 97: 447–455, 1999.
2. **Basile DP, Rovak JM, Martin DR, and Hammerman MR.** Increased transforming growth factor-β1 expression in regenerating rat renal tubules following ischemic injury. *Am J Physiol Renal Fluid Electrolyte Physiol* 270: F500–F509, 1996.
3. **Bunn HF and Poyton RO.** Oxygen sensing and molecular adaptation to hypoxia. *Physiol Rev* 76: 839–885, 1996.
4. **Chen RH, Ebner R, and Derynck R.** Inactivation of the type II receptor reveals two receptor pathways for the diverse TGF-beta activities. *Science* 260: 1335–1338, 1993.
5. **Chomczynski P and Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987.
6. **Cotran RS and Mayadas-Norton T.** Endothelial adhesion molecules in health and disease. *Pathol Biol (Paris)* 46: 164–170, 1998.

7. **Detmar M, Brown LF, Berse B, Jackman RW, Elicker BM, Dvorak HF, and Claffey KP.** Hypoxia regulates the expression of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) and its receptors in human skin. *J Invest Dermatol* 108: 263–268, 1997.
8. **Diebold RJ, Eis MJ, Yin M, Ormsby I, Boivin GP, Darrow BJ, Saffitz JE, and Doetschman T.** Early-onset multifocal inflammation in the transforming growth factor beta 1-null mouse is lymphocyte mediated. *Proc Natl Acad Sci USA* 92: 12215–12219, 1995.
9. **Falanga V, Qian SW, Danielpour D, Katz MH, Roberts AB, and Sporn MB.** Hypoxia up-regulates the synthesis of TGF-β1 by human dermal fibroblasts. *J Invest Dermatol* 97: 634–637, 1991.
10. **Falanga V, Takagi H, Ceballos PI, and Pardes JB:.** Low oxygen tension decreases receptor binding of peptide growth factors in dermal fibroblast cultures. *Exp Cell Res* 213: 80–84, 1994.
11. **Feng XH and Derynck R.** Ligand-independent activation of transforming growth factor (TGF) beta signaling pathways by heteromeric cytoplasmic domains of TGF-beta receptors. *J Biol Chem* 271: 13123–13129, 1996.
12. **Franzen P, ten Dijke P, Ichijo H, Yamashita H, Schulz P, Heldin CH, and Miyazono K.** Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. *Cell* 75: 681–692, 1993.
13. **Gilmont RR, Dardano A, Engle JS, Adamson BS, Welsh MJ, Li T, Remick DG, Smith DJ Jr, and Rees RS.** TNF-alpha potentiates oxidant and reperfusion-induced endothelial cell injury. *J Surg Res* 61: 175–182, 1996.
14. **Herskowitz A, Choi S, Ansari AA, and Wesselingh S.** Cytokine mRNA expression in postischemic/reperfused myocardium. *Am J Pathol* 146: 419–428, 1995.
15. **Kerrigan CL and Stotland MA.** Ischemia reperfusion injury: a review. *Microsurgery* 14: 165–175, 1993.
16. **Kerrigan CL, Zelt RG, Thomson JG, and Diano E.** The pig as an experimental animal in plastic surgery research for the study of skin flaps, myocutaneous flaps and fasciocutaneous flaps. *Lab Anim Sci* 36: 408–412, 1986.
17. **Knuckey NW, Finch P, Palm DE, Primiano MJ, Johanson CE, Flanders KC, and Thompson NL.** Differential neuronal and astrocytic expression of transforming growth factor beta isoforms in rat hippocampus following transient forebrain ischemia. *Brain Res Mol Brain Res* 40: 1–14, 1996.
18. **Lefer AM, Ma XL, Weyrich AS, and Scalia R.** Mechanism of the cardioprotective effect of TGF-β1 in feline myocardial ischemia and reperfusion. *Proc Natl Acad Sci USA* 90: 1018–1022, 1993.
19. **Lefer AM, Tsao P, Aoki N, and Palladino MA.** Mediation of cardioprotection by transforming growth factor-β. *Science* 249: 61–64, 1990.
20. **Lin HY, Wang XF, Ng-Eaton E, Weinberg RA, and Lodish HF.** Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. *Cell* 68: 775–785, 1992.
21. **Lopez-Casillas F, Wrana JL, and Massague J.** Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* 73: 1435–1444, 1993.
22. **McCord JM.** Oxygen-derived free radicals in post ischemic tissue injury. *N Engl J Med* 312: 159–163, 1985.
23. **McNeill H, Williams C, Guan J, Dragunow M, Lawlor P, Sirimanne E, Nikolics K, and Gluckman P.** Neuronal rescue with transforming growth factor-beta 1 after hypoxic-ischaemic brain injury. *Neuroreport* 5: 901–904, 1994.
24. **Mehta JL, Yang BC, Strates BS, and Mehta P.** Role of TGF-beta1 in platelet-mediated cardioprotection during ischemia-reperfusion in isolated rat hearts. *Growth Factors* 16: 179–190, 1999.
25. **Moulin V, Auger FA, O'Connor-McCourt M, and Germain L.** Fetal and postnatal sera differentially modulate human dermal fibroblast phenotypic and functional features in vitro. *J Cell Physiol* 171: 1–10, 1997.
26. **Moustakas A, Lin HY, Henis YI, Plamondon J, O'Connor-McCourt MD, and Lodish HF.** The transforming growth factor beta receptors types I, II, and III form hetero-oligomeric complexes in the presence of ligand. *J Biol Chem* 268: 22215–22218, 1993.
27. **O'Kane S and Ferguson MW.** Transforming growth factor βs and wound healing. *Int J Biochem Cell Biol* 29: 63–78, 1997.
28. **Ratcliffe PJ, O'Rourke JF, Maxwell PH, and Pugh CW.** Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. *J Exp Biol* 201: 1153–1162, 1998.
29. **Rhodes JM, Engelmyer E, Tilberg MS, and Gifford R.** Transforming growth factor β1 serves as an autocrine inhibitor of human endothelial cell/lymphocyte adhesion. *J Surg Res* 59: 719–724, 1995.
30. **Roberts AB.** Transforming growth factor-β: activity and efficacy in animal models of wound healing. *Wound Repair Regen* 3: 408–418, 1995.
31. **Roberts AB.** Molecular, and cell biology of TGF-beta. *Miner Electrolyte Metab* 24: 111–119, 1998.
32. **Roberts AB, Flanders KC, Heine UI, Jakowlew S, Kondaiah P, Kim SJ, and Sporn MB.** Transforming growth factor-beta: multifunctional regulator of differentiation and development. *Philos Trans R Soc Lond B Biol Sci* 327: 145–154, 1990.
33. **Roberts CJ, Birkenmeier TM, McQuillan JJ, Akiyama SK, Yamada SS, Chen WT, Yamada KM, and McDonald JA.** Transforming growth factor beta stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. *J Biol Chem* 263: 4586–4592, 1988.
34. **Sanchez-Elsner T, Botella LM, Velasco B, Corbi A, Attisano L, and Bernabeu C.** Synergistic cooperation between hypoxia and transforming growth factor-β pathways on human vascular endothelial growth factor gene expression. *J Biol Chem*: 38527–38535, 2001.
35. **Seekamp A, Warren JS, Remick DG, Till GO, and Ward PA.** Requirements for tumor necrosis factor-alpha and interleukin-1 in limb ischemic/reperfusion injury and associated lung injury. *Am J Pathol* 143: 453–463, 1993.
36. **Semenza GL.** HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol* 88: 1474–1480, 2000.
37. **Senel O, Cetinkale O, Ozbay G, Achioglu F, and Bulan R.** Oxygen free radicals impair wound healing in ischemic rat skin. *Ann Plast Surg* 39: 516–523, 1997.
38. **Stadelmann W, Digenis AG, and Tobin GR.** Impediments to wound healing. *Am J Surg* 176: 39S–47S, 1998.
39. **Tam BYY and Philip A.** 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, 1988.
40. **Wang GL and Semenza GL.** General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci USA* 90: 4304–4308, 1993.
41. **Wang XF, Lin HY, Ng-Eaton E, Downward J, Lodish HF, and Weinberg RA.** Expression cloning and characterization of the TGF-beta type III receptor. *Cell* 67: 797–805, 1991.
42. **Wu L and Mustoe TA.** Effect of ischemia on growth factor enhancement of incisional wound healing. *Surgery* 117: 570–576, 1995.

access to RI and RII. Betaglycan is a proteoglycan that presents TGF-β to RII, increasing binding of TGF-β to RII (12,14). Endoglin is a transmembrane protein that binds TGF-β 1 and 3 with high affinity (15,16). It is a homodimeric glycoprotein that has been shown to form a heteromeric complex with RI and RII (14,17). It has a strong homology with betaglycan in the transmembrane intracellular domain (18,19). It is thought to modulate TGF-β signaling and responses through interactions on cell surfaces with TGF-β receptors. It is highly expressed on endothelial cells of tissues undergoing angiogenesis, such as tumours, healing wounds, psoriasis, and embryonic and stroke tissue (20-25). Endoglin's preferential expression in the vasculature of malignant tumors is of particular interest given its potential diagnostic and therapeutic applications. Endoglin has been localized to human chromosome 9 (26) and is the target gene for hemorrhagic telangiectasia type-1 (HHT-1), or Osler-Weber-Rendue disease (27,28).

Since ischemia-induced changes in tissue are in part regulated by TGF-β, it is important to elucidate the precise mechanism by which they occur if we are to influence the effects of ischemia on angiogenesis. We have previously demonstrated that TGF-β and its receptors type I, II, and III are upregulated in ischemic injury *in vivo*. This upregulation of TGF-β function in ischemia might play a factor in the ischemia-induced angiogenesis seen in ischemic tissue. Since endoglin is one of the receptors involved in regulating TGF-β signaling, we tested whether endoglin expression will increase in response to ischemic injury. In the present study we demonstrate an increased expression of endoglin, and its ligand TGF-β1 in the epidermal layer, blood vessels, and fibroblasts in ischemic skin flaps as compared to non-ischemic control skin flaps.

MATERIALS AND METHODS

Animal Management and Surgical Preperations

The protocol for use of pigs in this experiment was approved by the McGill Universtiy Animal Care Committee. Six female, White Lancrace pigs (10-14 weeks old) were housed in a temperature-controlled (20-22°C) animal holding room. All the pigs were offered the same commercial pig diet and tap water *ad libitum*. Food was withheld the evening of the surgery. Animals were sedated with intramuscular injections of ketamine (20 mg/kg) and xylazine (2 mg/kg) and then received 1mg Atropine. Intravenous dose of sodium pentobarbital (6 mg/kg) was administered. General anesthesia was maintained during all procedures with spontaneous inhalation of oxygen (8 l/min) and halothane (0.5-1.0%). A bilateral 10 cm x 18 cm flap design connected through a vascular pedicle that contains the superficial circumflex artery and its accompanying vena comitantes as well as the lateral femoral cutaneous nerve was used as a skin flap (29).

Arterial ischemia in island flaps was created by clamp application on the artery and used to mimic the clinical scenario of an ischemic free flap. The flap on one side was assigned to 1 hour of arterial occlusion with the contralateral side acting as a nonischemic control. Complete occlusion of the vasculature was achieved by application of an Acland V2 microvascular clamp to the branch of the circumflex iliac artery supplying buttock flaps. Ischemia was verified by application of 10% sodium fluorescein (15 mg/kg) dye. Absence of fluorescein in the skin 15 minutes after dye injection indicated complete occlusion of the vascular pedicle. Samples were taken at 1 hour from flaps.

Tissue samples

Skin biopsies collected from pig flaps were fixed in 4% paraformaldehyde for 8 hours followed by 15% sucrose solution for 30 hours at 4°C. They were embedded in Tissue Tek compound and frozen in liquid nitrogen. Frozen tissues were cut using a cryostat. Each slide had at least two copies of the two different sets of tissues namely control non-ischemic flap and ischemic flap. This helped to control for variations in intensity of staining as might occur during the procedure, e.g. during the peroxidase reaction (see below).

Polyclonal antibodies

The antibody against Endoglin EG (591-609) was a gift from Dr. Miyazono (Ludwig Institute for Cancer Research, Uppsala, Sweden); the method of preparation has been described previously (18). The antibody against TGF-β1 was a gift from Dr. O'Connor-McCourt (Biotechnology Research Institute, Montreal, Quebec, Canada).

Immunohistochemistry Staining Procedures

Staining was performed using an indirect immunoperoxidase technique. Negative control (absent primary antibody) stainings were included. In brief, 8 mm thick Cryosections on gelatin coated glass slides were washed and permeabilized three times at room temperature each for 15 min with phosphate-buffered saline (PBS), 0.1% triton X-100, pH 7.5. Then they were treated for 10 min at room temperature with 3% H_2O_2 in 99% methanol, to block endogenous peroxidase activity. They were incubated in a humidifier chamber for 10 min at room temperature with 10% normal goat serum, 0.3% triton, and 0.5% bovine serum albumin (BSA) in PBS. Anti-endoglin was diluted to a final dilution of 1:400 in blocking

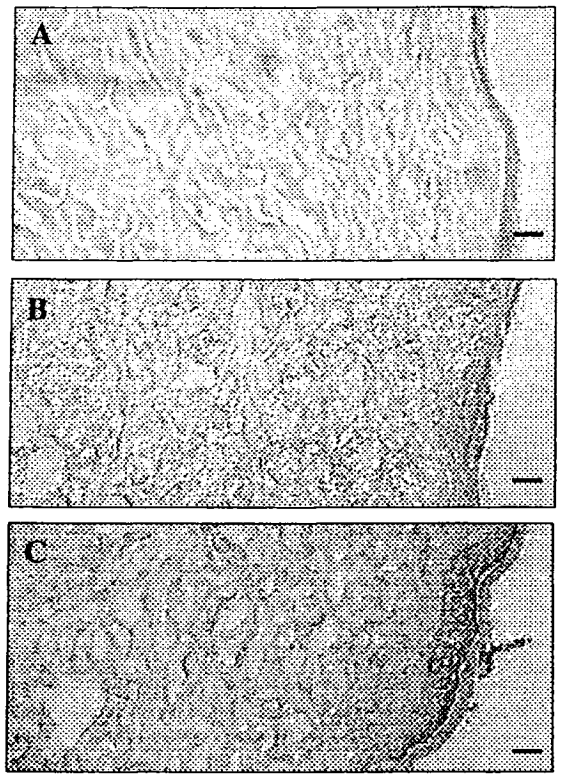

Figure 1. Endoglin expression in skin. A: Control for immunostaining (no primary antibody); B: Non-ischemic tissue; C: Ischemic tissue. Scale bar = 40 μm

solution. Anti-TGF-β1 was diluted to a final dilution of 1:160. These primary antibodies were applied to the sections for a duration of 1 hour at room temperature. The slides were then incubated in biotinylated goat anti-rabbit secondary antibody for 10 min. and in streptavidin-peroxidase (HRP) for 10 min. The slides were stained with 3-amion-9-ethyl-carbazole 200 mg/l in sodium acetate buffer and 0.01% H_2O_2 (AEC solution). The slides were washed between each incubation with PBS. The slides were finally washed with demineralized water, counterstained with Mayer's haematoxylin (Sigma, St. Louis, MO) and mounted in glycerine gelatine.

Histological Examination

Slides were analyzed by light microscopy. Using a 4-point scale, the staining level was assessed at the epidermal region, in the blood vessels, and in the skin fibroblasts in a blinded fashion, without knowledge of whether the flaps were from control or ischemic groups. The structures with the most staining were assigned 4 points, where the ones with the least staining were assigned 1 point. The p values were determined by a non-parametric Wilcoxon Signed Rank Test (30,31).

Table 1. Immunostaining for TGF-β1 and endoglin in control versus ischemic tissues.

Tissue	Expression Levels TGF-β1	Endoglin
Epidermis		
Control	1.00	1.00
Ischemia	2.50	2.97
Blood Vessels		
Control	1.00	1.37
Ischemia	2.25	3.00
Fibroblasts		
Control	1.00	1.70
Ischemia	2.25	3.00

RESULTS

Immunohistochemical localization of TGF-β1 and endoglin was performed in tissue sections prepared from buttock skin flaps of pigs. The expression profiles of these proteins were analyzed in non-ischemic control flaps and ischemic flaps. The skin structures immunostained for the ligand TGF-β1 and for the receptor endoglin were in general similar between ischemic and non-ischemic flaps (Figure 1). Comparative analysis of immunostaining for TGF-β1 and endoglin revealed that the expression of both these proteins was increased in the ischemic flaps compared to the non-ischemic flaps (Table 1). The skin structures that showed the most dramatic increases were the blood vessels (endothelial cells), fibroblasts, and the basal layer of the epidermis (basal keratinocytes) (Figure 1).

Epidermis

There was a significant increase in immunostaining for TGF-β1 in the epidermis of ischemic flaps (2.50) as compared with control (1.00) ($p < 0.031$). We observed a cytoplasmic granular staining in the keratinocytes of the epidermis of endoglin-stained tissue. This is the first report of keratinocyte-expression of the endoglin receptor. There was a significant increase in immunostaining for endoglin in the epidermis of ischemic flaps (2.97) compared to control flaps (1.00) ($p < 0.0026$).

Blood Vessels

There was a significant increase in immunostaining for TGF-β1 in the blood vessels of ischemic flaps (2.25) compared to control flaps (1.0) ($p < 0.031$). There was also a significant increase in immunostaining for endoglin in the blood vessels of ischemic flaps (3.00) compared to control flaps (1.37) ($p < 0.0026$).

Fibroblasts

There was a significant increase in immunostaining for TGF-β1 in the fibroblasts of ischemic flaps (2.25) compared to control flaps (1.00) ($p < 0.031$). There was

also a significant increase in immunostaining for endoglin in the fibroblasts of ischemic flaps (3.00) compared with control flaps (1.70) ($p < 0.007$).

DISCUSSION

We used a pig skin flap model to study the effect of ischemia on the expression of endoglin and its ligand TGF-β1. In our comparison, the effect of wounding itself on the flap was minimized by comparing ischemic skin flaps to non-ischemic control flaps rather than to unoperated skin. Thus, any changes observed were solely due to the effect of ischemia on the flap. The major finding of the study was the observation of an increased expression of endoglin, and its ligand TGF-β1 in the epidermal layer, blood vessels, and fibroblasts in ischemic skin flaps compared to non-ischemic control skin flaps.

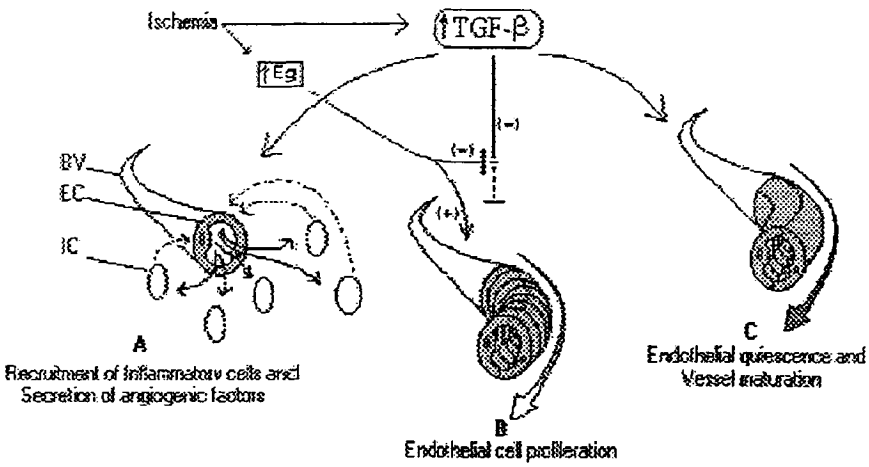

Figure 2. Proposed model for effects of TGF-β and endoglin in angiogenesis. Ischemia causes increased levels of TGF-β. The immediate effect is to recruit inflammatory cells which, in turn, secrete angiogenic factors (A). Endoglin levels then rise and reduce the inhibitory effects of TGF-β on angiogenesis, thereby allowing new blood vessels to form (B). As endoglin levels wane, TGF-β acts to control angiogenesis and return the blood vessels to a quiescent state. BV: blood vessels; EC: endothelial cells; IC: inflammatory cells; Eg: endoglin.

The cellular TGF-β receptor system consists of several membrane receptors including receptor type I (RI), II (RII), III (RIII or betaglycan) and endoglin. Receptors type I and II are responsible for TGF-β's signal transduction which is in turn modulated by betaglycan and endoglin, both of which have no direct function in signal transduction. Our data indicate that the expression of TGF-β increases under ischemic conditions. Given the role of TGF-β in angiogenesis, this may represent a mechanism by which ischemia stimulates angiogenesis. Although TGF-β1 inhibits endothelial cell proliferation *in vitro*, it strongly induces angiogenesis in *in vivo* assays such as the chick chorioallantoic membrane assay (5). *Ex vivo* studies in human tissue showed that TGF-β1 is present in tissues undergoing neovascularization (24). An explanation for the conflicting findings of *in vitro* versus *in vivo* studies is that the role of TGF-β is to recruit inflammatory cells into the immediate vicinity of the applied stimulus, and these cells will subsequently secrete positive mediators of angiogenesis, such as vascular endothelial growth factor (VEGF) (5). TGF-β may therefore be an indirect, but necessary player in angiogenesis *in vivo*.

Letamendia *et al.* found that endoglin inhibits the TGF-β1-dependent responses of cellular proliferation (32,33). Endoglin binds TGF-β1 much more weakly than betaglycan, and only in the presence of RII, as opposed to betaglycan which can bind TGF-β1 independently of RII. Endoglin's potentiation of TGF-β1's binding to its receptors remains hard to reconcile with its inhibitory effect on TGF-β signaling. Letamendia *et al.* proposed a model where the RII-induced ligand binding to endoglin is actually a deflection of the ligand from the signaling core and thus may represent an inhibition of TGF-β signaling.

TGF-β clearly inhibits endothelial cell growth, migration, and capillary formation *in vitro* (34-36). Endoglin, on the other hand, weakens TGF-β's inhibitory effect on endothelial cells, resulting in an increased cell migration. By antagonizing TGF-β's effects on endothelial cells, endoglin may contribute to the proliferation, migration, and capillary formation of endothelial cells (37). This explains the defective vascular development in the endoglin-knockout mouse (38), as well as the symptoms seen in HHT-1, where endoglin is the affected gene.

This explanation is also consistent with our results where both TGF-β and endoglin are upregulated in ischemia. We believe that both of these factors are needed in angiogenesis. We propose a model for the role of TGF-β in angiogenesis (Figure 2) where TGF-β first rises under the direct influence of ischemia, and acts by recruiting inflammatory cells which secrete the necessary growth factors acting on endothelial cells, resulting in angiogenesis. Subsequently, TGF-β signaling is inhibited by endoglin which permits endothelial cells to escape TGF-β's negative influence and proliferate to form new blood vessels. Subsequently, TGF-β acts on the formed blood vessels to induce endothelial quiescence and vessel maturation.

This final step is not well characterized, but is necessary if the endothelial cells are not to proliferate without inhibition, therein becoming malignant.

We are gaining more insight into the complexities of angiogenesis: We now realize that angiogenesis is not an event, but a multi-step heterogeneous process. The idea that certain factors 'stimulate angiogenesis' is being replaced by the concept that certain factors or their receptors may stimulate or inhibit very specific steps in angiogenesis. With this idea, it is possible to reconcile much of the confusion and the contradictions related to the role of TGF-β and endoglin in angiogenesis.

Much work remains to be done to delineate the exact mechanisms by which TGF-β acts and the precise role of endoglin in this complex cascade. Understanding how to regulate the actions of TGF-β by manipulating endoglin's expression may provide a therapeutic avenue given the importance of TGF-β in tumour angiogenesis.

REFERENCES

1. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other diseases. Nature Medicine 1995; 1: 27-31.
2. Kumar P. CD 105 and angiogenesis. Journal of Pathology 1996; 178: 363-366.
3. Roberts AB, Sporn MB. Physiological actions and clinical applications of transforming growth factor-β (TGF-β). Growth Factors 1993; 8: 1-9.
4. Roberts AB, Spom MB, Assoian RK, et al. Transforming growth factor β: rapid induction of fibrosis and angiogenesis in vivo. Proceedings of the National Academy of Science USA 1986; 83: 4167-4171.
5. Yang EY, Moses HL. Transforming growth factor β1-induced changes in cell migration, proliferation and angiogenesis in the chick choriallantoic membrane. Journal of Cell Biology 1990; 111: 731-741.
6. Lefer AM, Ma XL, Weyrich AS, Scalia R. Mechanism of the cardioprotective effect of transforming growth factor beta 1 in feline myocardial ischemia and reperfusion. Proceedings of the National Academy of Sciences USA 1993; 90: 1018-1022.
7. McNeill H, Williams C, Guan J, et al. Neuronal rescue with transforming growth factor-beta 1 after hypoxic-ischaemic brain injury. Neuroreport 1994; 5: 901-904.
8. Mehta JL, Yang BC, Strates BS, Mehta P. Role of TGF-β1 in platelet-mediated cardioprotection during ischemia-reperfusion in isolated rat hearts. Growth Factors. 1999; 16: 179-190.
9. Miyazono K, Ten-Dijke P, Ichijo H, Heldin CH. Receptors for transforming growth factor-β. Advances in Immunology 1994; 55: 181-220.
10. Massague J, Attisano L, Wrana JL. The TGF-β family and its composite receptors. Trends in Cell Biology 1994; 4: 172-178.
11. Wrana JL, Attisano L, Wieser R, et al. Mechanism of activation of the TGF-β receptor. Nature 1994; 370: 341-347.
12. ten Kijke T, Yamashita H, Ichijo H, et al. Characterization of type I receptors for transforming growth factor-beta and activin. Science 1994; 264: 101-104.
13. Moustakas A, Lin HY, Henis YI, et al. The transforming growth factor beta receptors type I, II, and III form hetero-oligomeric complexes in the presence of ligand. Journal of Biological Chemistry 1993: 268: 22215-22218.
14. Lopez-Casillas F, Wrana JL, Massague J. Betaglycan presents ligand to the TGF-β signaling receptor. Cell 1993; 73: 1435-1444.
15. Gougos A, Letarte M. Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. Journal of Biological Chemistry 1990; 265: 8361-8364.
16. Cheifetz S, Bellón T, Calés C, et al. Endoglin is a component of the transforming growth factor-β receptor system in human endothelial cells. Journal of Biological Chemistry 1992; 267: 19027-19030.
17. Yamashita H, Ichijo H, Grimsby S, et al. Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor-β. Journal of Biological Chemistry 1994; 269: 1995-2001.
18. Lopez-Casillas F, Chiefetz S, Doody J, et al. Structure and expression of the membrane proteolgycan betaglycan, a component of the TGF-β receptor. Cell 1991; 67: 785-95
19. Wang XF, Lin HY, Ng-Eaton E, et al. Expression, cloning and characterization of the TGF-β type III Receptor. Cell 1991; 67: 797-805.
20. Letarte M, Breaves A, Vera S. CD 105 (endoglin). In: Scholossman SF et al., eds. Leucocyte Typing V. Oxford: Oxford University Press, 1995: 1756-1759.
21. Westphal JR, Willems HW, Schalkwijk, CJM, et al. Characteristics and possible function of endoglin. A TGF-β binding protein. Behring Institute Mitteilungen 1993; 92: 15-22.
22. Gougos A, St-jacques S, Greaves A, et al. Identification of distinct epitopes of endoglin, an RGD-containing glycoprotein of endothelial cells, leukaemic cells and syncytiotrophoblasts. International Immunology 1992: 4: 83-92.
23. Krupinski J, Kumar P, Kumar S, Kaluza J. Increased expression of TGF-β1 in brain tissue after ischemic stroke in humans. Stroke 1996; 27: 852-857.
24. Wang JM, Kumar S, Pye D, et al. Breast carcinoma: comparative study of tumor vasculature using two endothelial cell markers. Journal of the National Cancer Institute 1994; 86: 386-388.
25. Wang JM, Kumar S, van Agthoven AJ, et al. Irradiation induces up-regulation of E9 protein (CD105) in human vascular endothelial cells. International Journal of Cancer 1995; 62: 791-796.
26. Lopez-Casillas F, Payne HM, Andres JL, Massague J. Betaglycan can act as a dual modulator of TGF-β access to signaling receptors: mapping of ligand binding and GAG attachment sites. Journal of Cell Biology 1994; 124: 557-568.
27. McAllister KA, Grogg KM, Johnson DW, et al. Endoglin, a TGF-β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nature Genetics 1994; 8: 345-351.
28. McAllister KA, Baldwin MA, Thukkani AK, et al. Six additional mutations in the endoglin gene in HHT1 suggest a dominant-negative effect of receptor function. Human Molecular Genetics 1995; 4: 1983-1985.
29. Picard-Ami LA Jr, Thomson JG, Kerrigan CL. Critical ischemia times and survival patterns of experimental pig flaps. Plastic and Reconstructive Surgery 1990; 86: 739-743.
30. Conover WJ. Practical Nonparametric Statistics, 2nd ed. New York: Wiley, 1980.
31. Lehmann EL. Nonparametrics: Statistical Methods Based on Ranks. San Francisco: Holden and Day, 1975.
32. Letamendia A, Lastres P, Botella LM, et al. Role of endoglin in cellular responses to transforming growth factor-β. Journal of Biological Chemistry 1998; 273: 33011-33019.
33. Lastres P, Letamendia A, Zhang H, et al. Endoglin modulates cellular responses to TGF-β1. Journal of Cell Biology 1996; 133: 1109-1121.
34. Sutton AB, Canfield AE, Schor SL, et al. The response of endothelial cells to TGF-β1 is dependent upon cell shape,

proliferative state and the nature of the substratum. Journal of Cell Science 1991; 99: 777-787.

35. Klein-Soyer C, Archipoff G, Beretz A, Cazenave JP. Opposing effects of heparin with TGF-ß or aFGF during repair of a mechanical wound of human endothelium. Biology of the Cell 1992; 75: 155-162.
36. Takashima S, Klagsbrun M. Inhibition of endothelial cell growth by macrophage-like U-937 cell-derived oncostatin M, leukemia inhibitory factor, and transforming growth factor β1. Journal of Biological Chemistry 1996; 271: 24901-24906.
37. Li C, Hampson IN, Hampson L, et al. CD105 antagonizes the inhibitory signaling of transforming growth factor beta1 on human vascular endothelial cells. FASEB Journal 2000; 14: 55-64.
38. Li DY, Sorensen LK, Brook BS, et al. Defective angiogenesis in mice lacking endoglin. Science 1999; 284: 1534-1537.

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