# NOTE TO USERS

# The Effect of Activin/TGFβ Signaling

# in Mammary Epithelial and Breast Cancer Cells

by

**Eftihia Cocolakis**

**Department of Medicine**

**Division of Experimental Medicine**

**McGill University**

**Montreal, Quebec, Canada**

February 2007

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

*Your file Votre référence*
*ISBN: 978-0-494-32343-4*
*Our file Notre référence*
*ISBN: 978-0-494-32343-4*

## TABLE OF CONTENTS

## ABSTRACT

Activin and TGFβ, members of the TGFβ superfamily, are pluripotent cytokines that are expressed in virtually every cell of the body. These factors play diverse roles in the body such as regulating early development of the embryo, differentiation, extracellular matrix formation, hematopoiesis, angiogenesis and immune functions. TGFβ superfamily signaling is transduced by heteromeric serine/threonine kinase receptors at the cell surface and the intracellular mediator, the Smad complex. Following activation of the receptors, there is recruitment and phosphorylation the Smads. As a result the Smad proteins accumulate in the nucleus, bind co-activators or repressors and elicit or suppress transcription of target genes.

To date, the molecular signaling mechanisms for activin/TGFβ in mammary gland growth and differentiation have not been fully elucidated. Our data identify a novel regulatory crosstalk mechanism by which activin/TGFβ induced Smad signaling acts to antagonize Stat5 transactivation in mammary epithelial cells. We demonstrate an inhibitory effect of activin/TGFβ on milk protein expression, specifically βcasein. We further show that activin/TGFβ inhibitory effect upon βcasein expression is not due to changes in either Stat5 phosphorylation, translocation to the nucleus or binding on the Stat5 response element. We finally demonstrate that the Smads are required to block Stat5 transactivation by activin/TGFβ and show that they are important mediators in activin/TGFβ inhibitory response upon Stat5 target gene expression, in particular βcasein and cyclin D1. Finally, we unveil the mechanism by which these two signaling

cascades antagonize their effects and find that activated Smads inhibit Stat5 association with its co-activator CBP, thus blocking Stat5 transactivation of its target genes. Thus, we define a novel crosstalk mechanism between two divergent signaling pathways that are involved in regulating mammary gland growth and differentiation

Whereas the role of TGFβ signaling in breast cancer has been well characterized, we sought out to study the role and mechanism of action of activin in the human breast cancer T47D cells. We found that activin treatment of T47D cells leads to a potent inhibition of cell growth. We further show that activin induces the Smad, the p38-mitogen activated kinase pathways and the p38 downstream target ATF2. Finally, using specific inhibitors to block p38 MAPK, activin-mediated cell growth inhibition is completely abolished. Together, these results define a novel signaling mechanism induced by activin in breast cancer cells.

Finally in an attempt to identify genes regulated by activin in breast cancer cells, we discover the death adaptor molecule RAIDD as a novel target of activin signaling. We show that RAIDD mRNA and protein levels are potently upregulated by activin. Using antisense-oligos directed against RAIDD, we show that RAIDD expression is necessary in mediating activin inhibition in breast cancer cells. Hence, we define the involvement of a new player in activin mediated cell growth inhibition.

Collectively, these studies reveal novel mechanisms of the activin/TGFβ signaling cascade in normal mammary epithelial cells and breast cancer cells.

## RESUME

Activine et TGFβ, membres de la superfamille TGFβ, sont des cytokines pluripotentes. Ces facteurs jouent des rôles variés dans le corps tels que la régulation des étapes précoces du développement de l'embryon, la différenciation, la formation de la matrice extracellulaire, l'hématopoïèse, l'angiogénèse et les fonctions immunitaires. La voie de signalisation médiée par activine/TGFβ est initiée suite à la fixation d'un ligand à un complexe de deux récepteurs de la famille des sérine/thréonine kinases. Après transactivation des récepteurs, les médiateurs intracellulaires Smads sont recrutés et phosphorylés. Il en résulte une accumulation des protéines Smads dans le noyau où elles vont se fixer à des coactivateurs ou des répresseurs pour activer ou réprimer la transcription de gènes cibles.

A ce jour, les mécanismes moléculaires de la voie de signalisation impliquant activine/TGFβ dans la croissance de la glande mammaire et sa différenciation n'ont pas été complètement élucidés. Nos résultats identifient un nouveau mécanisme de régulation par «cross talk» au cours duquel activine/TGFβ induisent la voie de signalisation des protéines Smads en antagonisant la transactivation de Stat5 dans les cellules épithéliales mammaires. Nous démontrons que activine/TGFβ a un effet inhibiteur sur l'expression des protéines de lait, spécifiquement sur l'expression de la β caséine. De plus, nous montrons que l'effet inhibiteur de activine/TGFβ en réponse a l'expression de la β caséine n'est pas du à des changements de phosphorylation de Stat5, de sa translocation dans le noyau ou de sa fixation sur ses éléments de réponses. Finalement, nous démontrons que Smad2, Smad3 et Smad4 sont requis afin de bloquer la transactivation de Stat5 en réponse à activine/TGFβ; par ailleurs nous montrons qu'ils

sont des médiateurs importants de la réponse inhibitrice de activine/TGFβ en réponse à l'expression des gènes cibles de Stat5, en particulier β caséine et cycline D1. Ainsi, nous définissons un nouveau mécanisme de «cross talk » entre deux voies de signalisation divergentes qui sont impliquées dans la régulation de la croissance et de la différenciation de la glande mammaire par lequel la voie de signalisation des protéines Smad inhibe l'activation transcriptionnelle médiée par Stat5 dans les cellules épithéliales mammaires en inhibant l'association de Stat5 avec le cofacteur CBP.

Bien que le rôle de la voie de signalisation TGFβ dans le cancer du sein a été bien caractérisé, nous avons voulu étudier le rôle et le mécanisme d'action d'activine dans la lignée de cellules T47D du cancer du sein. Nous avons trouvé qu'un traitement des cellules T47D par activine conduit à une forte inhibition de la croissance cellulaire. De plus, nous montrons qu'activine induit Smad, les voies des kinases activées par les mitogènes ($p38^{MAPK}$) ainsi que ATF2, la cible en aval de $p38^{MAPK}$. Finalement, l'inhibition de $p38^{MAPK}$ par des inhibiteurs spécifiques abolit totalement l'inhibition de la croissance cellulaire médiée par activine. Ces résultats définissent un nouveau mécanisme de signalisation induit par activine dans les cellules du cancer du sein.

Afin d'identifier les gènes impliquées dans l'inhibition par l'activine dans le cancer du sein, nous avons mis en evidence la protéine RAIDD comme une nouvelle cible de l'activine. En effet, l'activine augmente l'ARNm ainsi que le niveaux protéique de RAIDD. De plus, en utulisant des oligos antisenses pour RAIDD, nous avons montré que son expression est nécessaire à l'inibition par l'activine dans les cellules T47D. Avec ces resultats, nous mettons en evidence l'implication d'un nouvel acteur, la proteine RAIDD, dans l'inhibition cellulaire par l'activine.

L'ensemble de nos résultats mettent à jour de nouveaux mécanismes dans la cascade de signalisation activine/TGFβ dans les cellules épithéliales mammaires normales ainsi que dans les cellules du cancer du sein.

## PREFACE AND CONTRIBUTIONS OF AUTHORS

"As an alternative to the traditional thesis format, the thesis can consist of a collection of papers of which the student is an author or co-author." (Quoted from McGill University guidelines for submitting a doctoral thesis at: http//www.mcgill.ca/gps/programs/thesis/guidelines/preparation/)
In accordance with the above guidelines for thesis preparation and submission, I have included the following papers as the core of my thesis (CHAPTER II, III and IV).

- **Cocolakis E.**, Drolet L., Ho W.Y.J., Haines E., Ali S. and Lebrun J.J. Smad Signaling Antagonizes Stat5-Mediated Growth And Differentiation Of Mammary Epithelial Cells. (manuscript to be submitted)
- **Cocolakis E.**, Lemay S., Ali S. and Lebrun J.J. (2001) The p38 MAP Kinase Pathway Is Required For Cell Growth Inhibition Of Human Breast Cancer Cells In Response To Activin. J. Biol. Chem. *276*: 18430-6.
- **Cocolakis E.** and Lebrun J.J. The Death Adaptor Molecule RAIDD is as a Novel Activin Target Necessary for Activin-Induced Cell Growth Inhibition. (manuscript in preparation)

I have included as the first chapter, an extensive literature review as an introduction to the thesis. CHAPTER II, Smad Signaling Antagonizes Stat5-Mediated Growth and Differentiation of Mammary Epithelial Cells, is a first authored manuscript in which I performed all the experiments and wrote the manuscript in collaboration with my supervisor Dr. Lebrun. This paper will soon be submitted for publication. CHAPTER III, The p38 MAP Kinase Pathway is required for Cell Growth Inhibition of Human Breast Cancer Cells in Response to Activin, is a first authored manuscript that I wrote and performed all the experiments under the supervision of Dr. Lebrun. This paper is

reproduced from The Journal of Biological Chemistry by copyright permission from The American Society for Biochemistry and Molecular Biology Inc. publisher. A reprint copy is included in the appendix. CHAPTER IV, The Death Adaptor Molecule RAIDD is as a Novel Activin Target Necessary for Activin-Induced Cell Growth Inhibition is a manuscript in preparation that I wrote and performed all the experiments for under the supervision of Dr. Lebrun. Finally, CHAPTER V, includes a detailed discussion of the findings presented in the thesis. In order to maintain the focus of this thesis on the effect of activin/TGFβ signaling in mammary epithelial and breast cancer cells, I have not included two publications to which I have made a substantial contribution.

- Valderrama-Carvajal H, **Cocolakis E**, Lacerte A, Lee EH, Krystal G, Ali S, Lebrun JJ. Activin/TGFβ induce apoptosis through Smad-dependent expression of the lipid phosphatase SHIP. Nat Cell Biol. 2002 Dec;4(12):963-9.
- Ho J, **Cocolakis E**, Dumas VM, Posner BI, Laporte SA, Lebrun JJ. The G protein-coupled receptor kinase-2 is a TGFβ-inducible antagonist of TGFβ signal transduction. EMBO J. 2005 Sep 21;24(18):3247-58.

For the first paper, I performed all the biological assays such as the Flow Cytometry experiments, the MTT assays and Annexin V staining. For the EMBOJ paper, I performed the Flow Cytometry assays and the RT-PCR experiments. I will discuss the implication of these papers to my thesis work in the final chapter.

The following is how the co-authors contributed to the papers in this thesis:

Loren Drolet helped reproduce some of the luciferase assays perfomed, CHAPTER II Figure 10, for statistical analysis and helped me with the cyclin D1 western blots. Joanne Wing Yee Ho performed some co-immunoprecipitation experiments that were presented in CHAPTER II discussion as "figure not shown" and contributed to useful discussions about the manuscript. Dr. Suhad Ali provided multiple reagents and invaluable insight for both manuscripts. Dr. Serge Lemay performed the RNAse protection assay Figure 2 CHAPTER III.

## ACKNOWLEDGEMENTS

This was an incredible journey and the lessons learned were invaluable. Firstly, I'd like to thank my mentor Dr. Jean-Jacques Lebrun for his patience, guidance and for allowing me the opportunity to develop my research skills. Alongside my supervisor, I extend my gratitude to the members of the Lebrun lab past and present for their help, support and friendship, especially Joanne Ho. I would also like to express my appreciation to Dr. Suhad Ali for her continuous counsel and advice. Furthermore, I wish to recognize all the students and staff at the Royal Victoria Hospital and more specifically the Hormones and Cancer Research Unit. In addition, my studies and work would not have been made possible without the continuing support of the Division of Experimental Medicine and the Experimental Medicine Graduate Student Society.

I am especially grateful to my wonderful parents and sister, without their love and continuous encouragement, none of this would have been possible. Finally, I would like to thank the love of my life, Billy Andriopoulos; you have been my pillar of strength throughout the final chapter of my PhD.

## CLAIM TO ORIGINALITY

The present thesis, consisting of five chapters, was written by myself, Eftihia Cocolakis, under the supervision of my thesis director, Dr. Jean-Jacques Lebrun. The manuscript presented in CHAPTER II, will be submitted for publication and consists of original and unpublished data. CHAPTER III contains a refereed published paper consisting at the time of original and unpublished findings. CHAPTER IV is a manuscript in preparation composed of novel unpublished data.

The major novel findings of the presented thesis are as follows:

1. Activin/TGFβ induced Smad signaling inhibits Stat5 transactivation and gene upregulation in mammary epithelial cells.
2. Activin signaling via the Smad and the p38 MAPK pathways inhibits the growth of human breast cancer cells, T47D.
3. The death adaptor molecule RAIDD is a novel target of activin and is necessary in the inhibition of cell growth by activin in T47D cells.

# CHAPTER I.

# GENERAL INTRODUCTION

## 1. Activin/TGFβ Signaling

Activins and transforming growth factor βs (TGFβs) belong to a group of pluripotent polypeptide growth factors, called the TGFβ superfamily. These proteins exert diverse roles on different target tissues throughout the body and control a plethora of biological functions including cell growth, differentiation, apoptosis, migration, adhesion, and embryogenesis.

### 1.1. Ligand

#### 1.1.1. TGFβ

TGFβ1 is the prototype founding family member of the superfamily. TGFβ1 was initially discovered in combination with TGF-α (EGF) as a factor called sarcoma growth factor (SGF). This factor was shown to transform rat fibroblasts and induce their growth in soft agar[1, 2]

##### 1.1.1.1. TGFβ isoforms, sites of synthesis and knockouts

To date the three TGFβ isoforms identified in mammals are TGFβ1, TGFβ2, and TGFβ3. They are highly homologous and arise from distinct genes on different chromosomes. TGFβ is primarily produced by T cells[3], however, platelets, neutrophils, macrophages, bone, placenta, kidneys and the endometrium are also rich sources of TGFβs. Although the three TGFβ isoforms have similar biological functions *in vitro*, their *in vivo* patterns of expression differ. Indeed, this is consistent with the dissimilar

phenotypes in TGFβ knockout mice models. Greater than 50% of TGFβ1 null mice die *in utero* due to vasculogenesis and hematopoiesis defects. The surviving TGFβ1 knockouts develop inflammatory disease and pass away within a month after birth[4-7 8]. In contrast, mice lacking TGFβ2 die perinatally due to malformations of tissues and organs during development[9]. Finally, TGFβ3 knockouts die from abnormal lung development and cleft palate after they are born[10, 11].

### 1.1.1.2. TGFβ structure

Mature TGFβs are 25 kilodalton (kDa) homodimers composed of two 12.5 kDa polypeptides. TGFβs are initially synthesized as large precursor proteins, the 55kDa pre-proTGFβs. Shortly after synthesis, the preproTGFβs dimerize. The pre-proprotein contains a hydrophobic signal peptide, an N-terminal pro-domain and a biologically active peptide at the C-terminus[12, 13]. The biologically active peptide contains 7-9 cysteines that form intramolecular disulfide bonds which form the cysteine knot that is characteristic to almost all the TGFβ family members. Upon removal of the signal peptide, the biologically active peptide is cleaved by a furin-like endoproteinase[14]. This pro-peptide also known as the latency associated peptide (LAP), remains non-covalently attached to the active peptide and prevents binding of mature TGFβ to its receptors[13, 15]. This way TGFβs can be maintained in large inactive extracellular reserves. LAP can associate with other latent TGFβ associated proteins (LTBPs) such as fibroblast growth factor receptor[16-20]. LTBPs function to enhance secretion by targeting the complex to the extracellular matrix or to the cell surface where activation takes place. Futhermore,

LTBPs stabilize the TGFβ -LAP complex and ensure that TGFβ is folded properly[21, 22]. TGFβs are rendered active once LAP dissociates. The precise mechanism by which active TGFβs are released is not completely understood.

### 1.1.2. Activin

Activin was first purified from porcine follicular fluid based on its ability to stimulate FSH secretion from the pituitary[23, 24]. Since then, multiple functions have been assigned to activin in a wide range of tissue. Activin's roles throughout the body are diverse and include the regulation of; embryo development, stem cell biology, reproductive biology, erythroid differentiation, systemic inflammation, cell death, wound healing and fibrosis.

#### 1.1.2.1. Activin isoforms, sites of synthesis and knockouts

Located on different chromosomes, five isoforms of the activin monomeric subunits (βA, βB, βC, βD, and βE) have been identified; however the precise biological function for the latter three is not clear[25, 26]. The β subunit was initially attributed to activin's antagonist, inhibin. Inhibin is composed of both β and α subunits, giving rise to inhibin A (α βA) and inhibin B (α βB). The β subunits of activins form homo or heterodimers giving rise to activin A (βAβA), activin AB (βAβB), activin B (βBβB), etc. Amongst all activins, activin A has been most extensively studied.

The βA and βB subunits are expressed in most tissues and cell types, whereas the βC and βE are mostly found in the liver[27, 28 29]. Knockout studies of the differenct activin

subunits have highlighted their importance *in vivo*. Whereas mice lacking the activin βC and βE subunits have no obvious abnormalities[30], the βA knockouts die perinatally of craniofacial defects[31,32]. On the other hand, βB knockout mice are viable and display defective eyelids, reproductive anomalies, incomplete mammary gland development and an absence of lactation[33, 34].

### 1.1.2.2. Activin structure

Similarly to TGFβ, activin β subunits are produced as pre-proprotein containing an N-terminal signal peptide, a prodomain and the C-terminal biologically active peptide. The biologically active peptide contains 8 cysteine residues forming the cysteine knot. After cleavage at a furin recognition site, the biologically active 25kDa activin is made up of two monomer subunits held together by a disulfide bond[35]. Dimerization and processing of activins occurs within the cell before their secretion[36]. Unlike TGFβ, which is secreted in its latent form, activins are secreted in their dimeric form as biologically active peptides[37].

## 1.1.3. Other Ligands of the TGFβ Superfamily

The superfamily consists of more than 42 human encoded-members, 7 in *Drosophila melanogaster* and 4 in the nematode *Caenorhabditis elegans*. In addition to both TGFβs and activins, the superfamily consists of inhibins, nodal, leftys, myostatin, bone-morphogenic proteins (BMPs), growth/differentiation factors (GDFs) and Mullerian inhibiting substance with pleitropic roles throughout the body. For example, myostatin

is an important regulator of muscle growth (reviewed in [38]). As the name suggests, the BMPs play a critical role in bone development, mesoderm formation, heart and cartilage development (reviewed in [39]). Mullerian inhibiting substance causes the regression of the Mullerian ducts which otherwise gives rise to the fallopian tubes, uterus, and upper vagina (reviewed in [40]).

### 1.2. Receptors

Activin and TGFβ signal through transmembrane serine threonine kinase receptors[41]. Based on homology of the TGFβ superfamily of receptors, they are subdivided into two classes; the type I receptors and the type II receptors. These receptors exist as homodimers at the cell surface in the absence of ligand. Each are glycoproteins of approximately 55 and 70kDa respectively. Type III receptors are accessory receptors that lack any intrinsic kinase activity. Whereas in humans there are approximately 42 TGFβ superfamily ligands, there are only 7 type I receptors (activin receptor-like kinases, ALKs 1-7) and 5 type II receptors (TβRII, ActRIIA, ActRIIB, BMPRII, MISRII). Multiple ligands interact with the few receptor complexes that exist, allowing for binding promiscuity. Depending on which receptor the ligands bind to, determines the intracellular signaling that is induced.

#### 1.2.1. Type I and Type II Receptors

Different names have been attributed to the type I receptors depending on the groups that cloned them simultaneously. The activin type I receptor is known as ALK4 (activin-receptor like kinase 4)[42] or ActRIB (activin receptor type IB)[43]. Activin A mainly binds

ALK4. Recently, evidence suggests that activins B and AB, preferentially bind ALK7[44]. Receptors for the βC or βE subunits are largely unknown. The TGFβ type I receptor is known as ALK5[45] and TβRI[46]. TGFβ can also signal through another type I receptor, ALK1[42, 46, 47]. Whereas ALK4 and ALK5 are expressed in different cells, ALK1 is mainly expressed at sites of angiogenesis, with highest expression in endothelial cells[47]. One TGFβ type II receptor exists, TβRII[48], and there are two activin type II receptors, ActRIIA and ActRIIB[49, 50]. Activin A binds ActRIIA with a three to four fold lower affinity in comparison to ActRIIB.

#### 1.2.1.1. Structure of the Receptors

Both receptor types consist of three domains: the extracellular, transmembrane and intracellular domains. The major distinction between the two types of receptors is the conserved amino acid sequence within the glycine and serine rich (GS) juxtamembrane domain and the kinase domain within the intracellular domain of the type I receptors.

The extracellular domains of the type I and II receptors are approximately 150 amino acids and are glycosylated. The fold of the receptors is controlled by 10 or more cysteines where three cysteines form a distinct cluster near the transmembrane domain[51, 52].

Within the intracellular region of the type I receptor exist two very important domains, the GS and the kinase domain. The GS domain contains a repeated glycine and serine sequence directly preceding the kinase domain. Upon binding to ligand, the type II receptor can interact with the type I receptor. Heterocomplex formation will allow for the constitutively active type II receptor to transphosphorylate the type I receptor. More

specifically transphosphorylation occurs within the GS domain, on the serine and threonine residues,[53] which subsequently activates signaling. The kinase domain contains a L45 loop sequence that is critical for signal specificity and recruitment of the intracellular mediators, the Smad proteins[54-56].

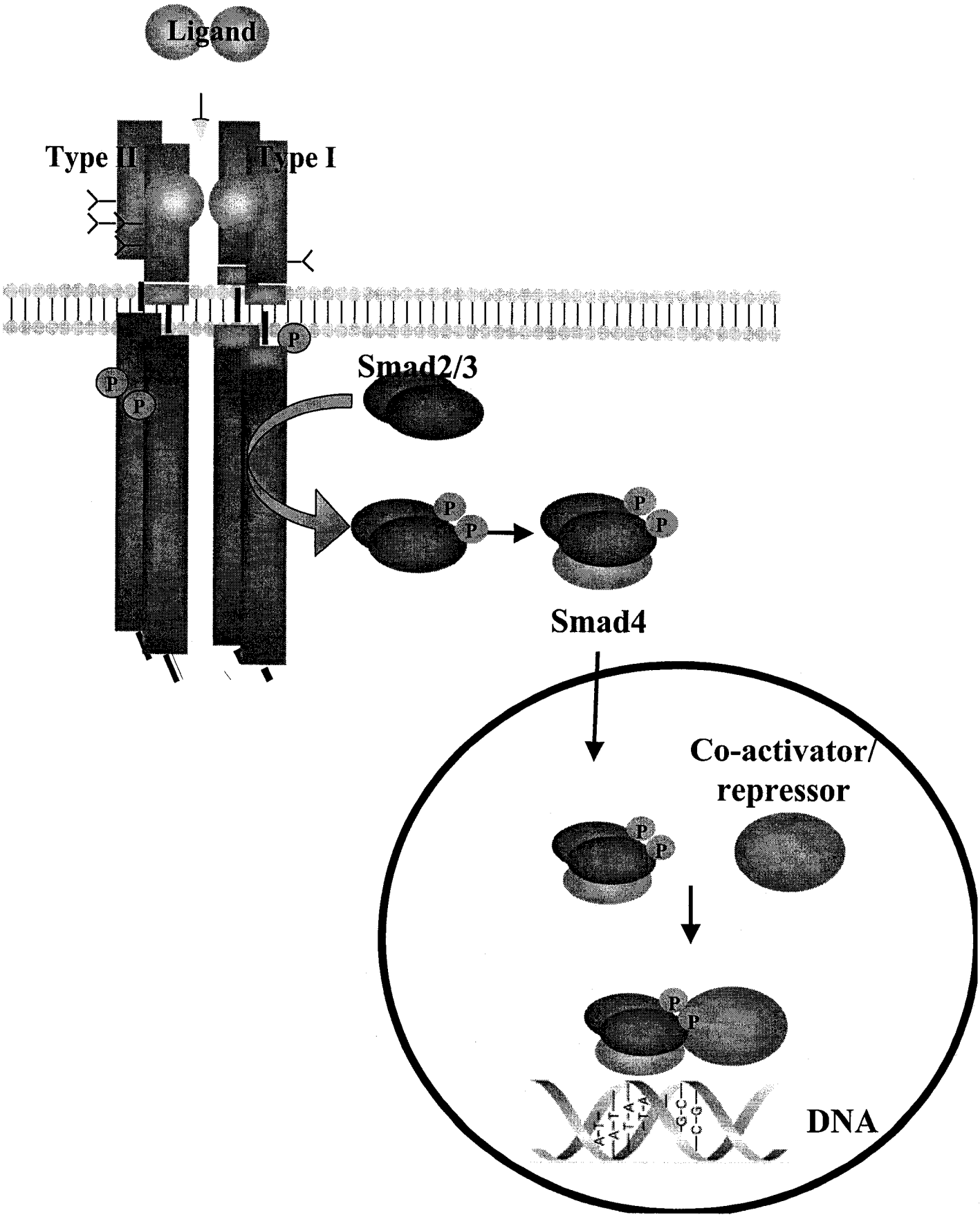
### 1.2.2. Type III Receptor

In addition to type I and II receptors, ligand crosslinking methods revealed the existence of additional TGFβ binding proteins. In comparison to the type I and II, the type III receptors are higher in molecular weight. Betaglycan and endoglin (or CD105) are two examples of type III receptors. They are both single transmembrane proteins that exist as homodimers. Betaglycan, a proteoglycan containing membrane glycoprotein, binds TGFβ and facilitates its interaction with the type II receptor[57, 58]. Specifically, betaglycan has higher affinity to TGFβ2 than TGFβ1 and TGFβ3[57, 59]. Betaglycan has also been shown to interact with inhibin which promotes interaction with the activin type II receptor. Signaling is henceforth blocked because it competes with activin for receptor binding[60]. Endoglin is abundant in endothelial cells and contains a transmembrane region and a cytoplasmic tail homologous to betaglycan[61]. Endoglin may interact with several members of the TGFβ superfamily including TGFβ1, TGFβ3 and activin[62, 63].

### 1.3. Core Pathway - The Smads

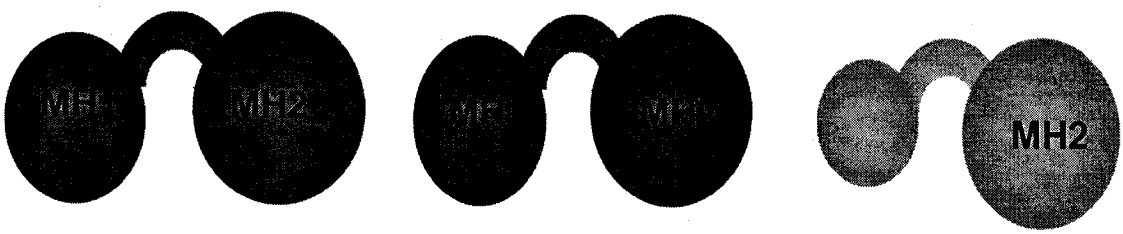
In the TGFβ superfamily, the Smad proteins are the key propagators of the intracellular signal, Figure 1. They act directly downstream of the receptors at the plasma membrane and then translocate to the nucleus and interact with various co-factors to modulate the transcription of target genes. In total, 8 mammalian Smads, 4 *Drosophila* Smads, and 3 Smads in *C.elegans* are known to exist. Smads may be subdivided into three classes: the receptor Smads (R-Smads), the common-partner Smads (Co-Smads) and the inhibitory Smads (I-Smads), Figure 2. There are 5 mammalian R-Smads in which Smad1, Smad5 and Smad8 act downstream of the BMP and anti-Mullerian receptors, and Smad2 and Smad3 propagate the signal for the TGFβ, activin and Nodal receptors. There is 1 Co-Smad, Smad4 that binds the R-Smads, and 2 I-Smads, Smad6 and Smad7, that are involved in signal downregulation.

# Figure 1. Activin/TGFβ Signaling



# Figure 2.
# Smad proteins and their structural elements

| R-Smads | Co-Smad | I-Smad |
|---|---|---|
| Smad1, Smad2, Smad3,Smad5, Smad8 | Smad4 | Smad6, Smad7 |




Adapted from: Massague J. et al. Genes Dev. 2005; 19: 2783-2810

### 1.3.1. Smad cloning

The first member of the Smad family to be discovered through genetic screens was the *Drosophila* Mothers against Decapentaplegic (DPP) gene (Mad), which is required for the DPP/TGFβ response[64] . Along with the discovery of Mad, a separate group discovered additional components of the TGFβ family called the Sma genes in *C.elegans*[65]. Henceforth arose the Smad, a contraction of "Sma" and "Mad" to describe the TGFβ superfamily signal mediators[66]. Subsequently, the Smads were either cloned by screening cDNA libraries or by searching EST based databases[67]. Smad4 was initially identified by screening pancreatic cancer for gene mutations and was termed deleted in pancreatic carcinoma locus 4 (DPC4)[68].

### 1.3.2. Smad structure

Smads have an approximate length of 500 amino acids, consisting of two major domains joined together by a linker region. The N-terminus contains a Mad-homology 1 domain (MH1) whereas in the C-terminus there exists a Mad-homology 2 domain (MH2), Figure 2. The MH1 domain is highly conserved amongst all Smads with the exception of Smad6 and Smad7. On the other hand, the linker domain varies across to all Smads, whereas the MH2 domain is conserved. The MH1 domain is involved in nuclear importing, interaction with nuclear proteins and binding to DNA. Interestingly, a 30 base pairs insert encoded by exon 3 in the MH1 domain of Smad2 inhibits DNA binding[69]. The MH2 domain mediates type I receptor recognition, Smad oligomerization and interaction with cytoplasmic retention proteins, nuclear pore complex proteins and DNA-binding cofactors. The L3 loop in the R-Smad MH2 domain

and the L45 loop of the type I receptor governs the specificity of interaction between R-Smad and type I receptor. The L45 loop of the TGFβ and activin type I receptors are identical which permits activation of the same Smads, Smad2 and Smad3[55, 56]. The interaction between R-Smad and type I receptor is stabilized by the phosphorylated GS motif of the type I receptor binding to the Smad sequence downstream of the L3 loop[70]. At the extreme C-terminal region of the MH2 domain, the R-Smads contain a Serine-X-Serine motif that is phosphorylated by the type I receptor. Once phosphorylated, the R-Smads (Smad2 and Smad3 for activin/TGFβ signaling) whose MH1 and MH2 domains are initially associated, unfold and subsequently interact with Co-Smad (Smad4), forming heterocomplexes[71].

### 1.3.3. Activation Requires Smad Accessory Proteins

Efficient activation and receptor interaction upon activin and TGFβ stimulation requires the Smad anchor for receptor activation (SARA)[72, 73]. SARA is a FYVE domain protein that is anchored to the plasma membrane through interactions with phosphatidylinositol-3-phosphate. Initiation of the TGFβ signal occurs through binding of Smad2/3 to SARA and the receptors in early endosomes[74, 75 76].

The TGFβ receptor Smad complex is also stabilized through its interaction with Disabled-2 (Dab2)[77]. Furthermore, the ras GTPase activating protein (GAP)-binding protein Dok-1 has been shown to interact with the activin receptor. Upon activin stimulation, Dok-1 associates with Smad3 and is required for signaling[78].

### 1.3.4. Smad Regulation

Smad proteins can be regulated through various forms of post-translational modifications such as phosphorylation, acetylation, sumoylation and ubiquitination.

#### 1.3.4.1. Smad Phosphorylation

Phosphorylation of R-Smads by the type I receptor permits Smad2 and Smad3 to bind their common partner Smad4 and accumulate in the nucleus. Recently, multiple kinases have been shown to attenuate the Smad. Indeed, the MAP kinase extracellular signal-regulated (ERK), protein kinase C (PKC), calmodulin kinase II (CamKII), G1 cyclin dependent kinases Cdk2/3 and G-coupled receptor kinase 2 (GRK2) may phosphorylate either Smad2 and/or Smad3 in their linker domains or their MH1 domains and consequently block signal transduction[79-83]. Furthermore, no effect or enhancement of the Smad transcriptional effect occurs when the Smad2/3 linker domain is phosphorylated by c-Jun terminal kinase (JNK), Rho/ROCK and p38 mitogen activating protein kinase (MAPK)[84-86].

#### 1.3.4.2. Smad Dephosphorylation

Whereas phosphorylation is critical in activation of the Smad pathway and in the mediation of the TGFβ biological effect, downregulation of the signal is also vital in maintaining a normal cellular response. R-Smad dephosphorylation induces dissociation of the R-Smad-Smad4 complex and export out of the nucleus into the cytoplasm. Recently, the intensely sought after Smad phosphatase, protein phosphatase 1-alpha

(PPM1A/PP2Cα) was identified[87]. PPM1A dephosphorylation of Smad2/3 induces nuclear export and is imperative in the attenuation of the TGFβ signal[87].

### 1.3.4.3. Smad Acetylation, Ubiquitination and Sumoylation

Acetylation is yet another post-translational modification of proteins in which there are various functional consequences. The functional effects of protein acetylation include modulation of protein-DNA and protein-protein interactions and inhibition of nuclear export. p300, an acetyltransferase, has been shown to acetylate the MH2 domain of Smad3 which subsequently upregulates transcriptional activity[88]. Furthermore, p300 may also acetylate Smad7 at sites where ubiquitin moieties attach and thus prevent receptor induced ubiquitination[89].

Ubiquitination may target proteins for degradation or it may act as a signal similar to phosphorylation. Smad2, Smad3, Smad4, and Smad7 are polyubiquitinated and targeted to the 26S proteosome for degradation[90-92]. The Homologous to the E6-accessory protein C-terminus (HECT)-domain E3 ligases Smad ubiquitin regulatory factors (Smurf1 and Smurf2), the ubiquitin ligases Tiul1, SCF/Roc1, NEDD4/2, HxN3, SCF b-TrCP1, Ectodermin, and Arkadia[92-96] target the Smads for degradation. In addition, the ubiquitin ligase Itch may add only limited ubiquitin moieties to improve its interaction with the TGFβ type I receptor[97].

Multiple reports claim that Smad4 sumoylation may influence its activity depending on the factor bound to it[98-102].

### 1.3.5. Smad Nuclear and Cytoplasmic Shuttling

The distribution of Smads within a cell consists of shuttling between the cytoplasm and nucleus. Smad2 and Smad3 can be imported into the nucleus through a transport receptor independent mechanism. A hydrophobic corridor in their MH2 domains interact with the nucleoporins Nup153 and Nup214[103, 104]. However Smad3 has additionally been reported to interact with the transport receptor importin. Smad3 has a nuclear localization sequence (NLS) within its MH1 domain which interacts with importins[105, 106]. In contrast to Smad3, Smad2 is unable to interact with importins due to the extra insert in its MH1 domain. In addition, nuclear export of Smad2 and Smad3 is also thought to be due to their interaction with Nup153 and Nup214[103]. Interestingly they possess a nuclear export sequence (NES) but do not interact with the nuclear exporter CRM1[107]. On the other hand, a NES within Smad4's linker region interacts with CRM1 and exports Smad4 to the cytoplasm[108]. Upon Smad4 interaction with the phosphorylated Smad2/3, the NES is masked causing the complex to remain nuclear. Once the R-Smads are dephosphorylated, Smad4 NES is uncovered and Smad4 is subsequently exported from the nucleus by CRM1[109, 110].

### 1.3.6. Smad Nuclear Signaling

All Smads possess the ability to induce transcription. Whereas Smad2 cannot bind DNA due to its extra exon, Smad3 and Smad4 may interact weakly with DNA. The β-hairpin loop within the MH1 domain allows for Smad3 and Smad4 to bind the Smad Binding Element (SBE), 5'-CAGAC-3'[69]. At natural promoters, Smad interaction with transcription factors that bind DNA adjacent to the SBE, allows for higher affinity binding for both factors. GC-rich motifs in certain gene promoters may also bind Smad3

and Smad4[111]. Smads interactions with various co-activators, co-repressors or transcription factors allow for widespread flexibility and bestow specificity of the TGFβ/activin signal within the cell. Therefore, the intracellular DNA binding partners that interact with the Smads will determine what genes will be transcribed and henceforth the final biological outcome.

### 1.3.6.1. Smads and Transcription Factors

Smads interact with a wide range of DNA transcription factors through either their MH1 or MH2 domains, Table 1. The interaction between Smads and transcription factors is cell context specific and grants the versatility of the activin/TGFβ signal. Smad2, Smad3 and Smad4 interact with various transcription factors including the bHLH, bZIP, Forkhead, Homeodomain, nuclear receptor, Runx and zinc finger protein families, amongst others (reviewed in [112]). Through these interactions they either positively or negatively regulate a multitude of genes.

## Table 1. Smad-Binding Transcription Factors

| Smad-binding partners | Interacting Smad and domains | Features/mechanisms of action | References |
|---|---|---|---|
| *bHLH family* | | | |
| E2F4/5 | Smad3 (MH2) | Recruitment of p107 to Smad3 to repress the c-myc gene | 107 |
| Max | Smad3 (MH1) | Max inhibits transcription activation by Smad3 | 108 |
| MyoD | Smad3 (MH1-linker) | Interference of MyoD/E protein/DNA complex formation | 109 |
| TFE3 | Smad3/4 | Synergistic cooperation on TGF-β target genes such as PAI-1, Smad7 | 110-112 |
| *bZIP family* | | | |
| ATF2 | Smad3/4 (MH1) | Stimulation of ATF2 transactivation | 113 |
| ATF3 | Smad3 (MH2) | Repression of the Id1 promoter | 114 |
| c-Fos | Smad3 (MH2) | Cooperation on AP-1-dependent TGF-β target genes | 115 |
| c-Jun, JunB, JunD | Smad3 ,Smad4 | Positively and negatively regulate Smad activity | 115,116 |
| CEBPα, β, δ | Smad3 (MH1) | Smad3 inhibits CEBP's transactivation | 117,118 |
| *Forkhead family* | | | |
| FoxH1/FAST | Smad2/3 | Formation of activin-responsive factors on the activin-responsive promoters | 105,119,120 |
| FoxO | Smad2/3 | Regulation of p21Cip1 | 121 |
| *Homeodomain protein* | | | |
| Dlx1 | Smad4 | Inhibits Smad4 signaling | 122 |
| Milk/Mixer | Smad2 (MH2) | Recruitment of Smad2/Smad4 activators to the activin-responsive complex | 120,123 |
| *Nuclear receptor family* | | | |
| Androgen receptor (AR) | Smad3 (MH2) | Reciprocal inhibition of Smad3 DNA-binding activity and of AR activity | 124-126 |
| Estrogen receptor | Smad3/4(MH2) | Repression of Smad target genes | 126,127 |
| Glucocorticoid receptor | Smad3 (MH2) | Inhibition of Smad3 transactivation activity | 128 |
| HNF4 | Smad3/4 | Cooperative activation | 129 |

| | | | |
|---|---|---|---|
| RXR | Smad3 (MH2) | | 130 |
| Vitamin D3 receptor | Smad3 (MH1) | Coactivation of ligand-induced transactivation of vitamin D receptor | 131 |
| *Runx family* | | | |
| CBFA1/Runx2/AML | Smad2/3 (MH2) | Regulation of immune responses | 132-134 |
| *Zinc finger protein family* | | | |
| GATA3 | Smad3 | Recruits Smad3 to GATA sites to cooperatively activate transcription | 135 |
| GliΔC-ter | Smad2/3/4 | Unknown | 136 |
| OAZ | Smad4 (MH2) | Formation of BMP-responsive activator complex | 137 |
| Sp1 | Smad2 (MH1)Smad4 (MH2) | Cooperative activation of TGF-β target genes, e.g., p15Ink4B, p21Cip1, Smad7, PAI-1, and collagen | 133,138 |
| YY1 | Smad4 (MH1) | Complex with Smads and GATA | 139,140 |
| ZNF198 | Smad3 (MH2) | Unknown | 141 |
| *Others* | | | |
| β-catenin | Smad4 | Wnt-dependent activation of LEF1 target genes | 142-144 |
| HIF-1α | Smad3 (MH1, MH2) | Cooperation of TGF-β with hypoxia pathway and angiogenesis | 145 |
| IRF-7 (IRFs) | Smad3 (MH2) | Smad3 activation of IRF-7 transactivation function | 146 |
| Lef1/TCF | Smad2/3/4 (MH1, MH2) | Smad coactivation of LEF1 signaling | 142,147,148 |
| MEF2 (MADS box) | Smad3 | Smad3 represses the transcription activity of MEF2 | 149 |
| Menin | Smad2/3 (MH2) | Facilitate Smad DNA binding | 150 |
| NFκB p52 | Smad3 | Coactivation of κB site | 151 |
| NICD | Smad3 (MH2) | Coactivation of NICD-RBP-Jk complex to regulate the Notch targets | 135,152 |
| p53 | Smad2/4 | Synergism and antagonism | 153-155 |
| Pax8 | Smad3 | Smad3 reduces Pax8 DNA binding | 156 |
| SRF | Smad3 | Mediate TGF-β-induced SM22α transcription | 157 |

Adapted from [106].

### 1.3.6.2. Smads interactions with Co-activators and Co-repressors

In addition to cooperating with transcription factors, Smads may also recruit co-activators or co-repressors to their transcriptional complexes to further control activin/TGFβ signaling. Co-activators, such as the CREB binding protein (CBP) and p300, enhance transcription by bridging the RNA polymerase II complex with the transcription factor/Smad complex. In contrast, co-repressors such as TG3-interacting factor (TGIF), Sloan-Kettering Institute proto-oncogene (Ski) and Ski-related novel gene (SnoN) repress activin/TGFβ mediated gene activation by binding Smads. More specifically, suppression of gene activation occurs by Smads/co-repressor complex either on their own or through their binding to chromatin-condensing histone deacetylases, (reviewed in [112]).

### 1.3.7. Inhibitory Smads

Smad6 and Smad7 are the two inhibitory Smads in vertebrates. Whereas Smad6 inhibits the BMP pathway, Smad7 inhibits both the activin/TGFβ and BMP signaling[164,165, 166]. The MH2 domain in I-Smads is highly homologous to that of R-Smad and Smad4. Their MH1 domain is disparate and is dissimilar to other Smads. However, the I-Smad MH2 domain does not possess the SXS motif of phosphorylation that the R-Smad contains. Smad7 inhibits the activin/TGFβ signal through three mechanisms. First, Smad7 competes for binding with Smad2/3 to the activated type I receptor therefore blocking signal mediation by the R-Smads[165, 166]. Second, Smad7 is involved in targeting receptor degradation by the recruitment of the ubiquitin ligase Smurf[167, 168].

Another recent role for Smad7 is induction of receptor dephosphorylation and degradation by recruiting the protein phosphatase PP1/GADD34 to the activated receptor complex[169]. A negative feedback loop is involved in the downregulation of the activin/TGFβ signal via upregulation of Smad7 gene transcription. More specifically, the upregulation occurs upon activation of R-Smad/Smad4 by activin/TGFβ induction. Furthermore, TGFβ signaling induces translocation of Smad7 from the nucleus to the cytoplasm where upon receptor binding signaling is attenuated[170]. Finally, evidence for Smad7's role in transcriptional regulation is based on its transactivation potential, interaction, and acetylation by p300[89]. In addition to activin/TGFβ induction of Smad7, its expression is also increased by interferon-γ STAT signaling, TNF-α induced NF-κB, epidermal growth factor receptor (EGFR) and other tyrosine kinases[171, 172].

### 1.3.8. Smad knockouts

The Smad2 and Smad4 knockout mice are embryonic lethal[173-176]. While the Smad2 deficient mice die *in utero* due to a lack of anteriorposterior axis formation and no mesoderm formation, the Smad4 knockout's lethality is due to defective visceral endoderm leading to gastrulation defects. The Smad3 knockout phenotype is less severe. Although the mice are viable, they display reduced body size, metastatic colorectal tumor development, accelerated wound healing, impaired mucosal immunity and massive inflammation[177-180]. Although it appears that Smad2 and Smad3 are functionally similar, besides their differences in DNA binding, the phenotype of their respective knockout mice are dissimilar. This suggests that Smad2 may play a more pertinent role in embryo development. Recently, the functional role of Smad7 *in vivo*

was elucidated through the generation of mice lacking exon I of Smad7. The Smad7 exon I knockout mice are viable displaying a reduction in body size, and an impaired immune system[181].

### 1.4. Crosstalk with Other Signaling Pathways

It has become increasingly apparent that TGFβ responses are not only regulated by the Smads but also other signaling pathways. The TGFβ receptors activate Smad-independent pathways that can directly crosstalk with the Smad pathway but can also allow for Smad-independent biological responses by TGFβ. Besides the mitogen-activated protein kinases (MAPKs) which will be covered in detail in CHAPTER III, TGFβ can signal through the phosphatidyl inositol-3-kinase (PI3K)/Akt pathway and Rho family members. The PI3K pathway plays an important role in regulating anti-apoptotic/survival signals, epithelial to mesenchymal transition (EMT) and protein synthesis. For example, activation of the PI3K/Akt inhibits TGFβ-induced apoptosis in primary mammary epithelial cells and in the HC11 mouse mammary epithelial cell line[182]. Interestingly, inhibition of the PI3K pathway reduces phosphorylated Smad2 by TGFβ which consequently prevents EMT, a process that is important in mediating tumor cell invasion to surrounding tissue in which TGFβ plays a prominent role[183]. TGFβ activation of the small GTPases, RhoA, Rac and Cdc42 may also lead to EMT by affecting membrane ruffling, stress fiber formation and lamellipodia formation[184]. In summary, TGFβ crosstalk mechanisms lead to the regulation of the final biological outcome.

## 2. Biological Functions of Activin/TGFβ

Activin and TGFβ regulate a plethora of biological activities including cellular proliferation, differentiation, adhesion, apoptosis, extracellular matrix production, immune regulation, embryogenesis, hormonal control, tumor suppression, angiogenesis and tumor promotion. The physiological effects of these factors are manifested in a cellular specific manner. Although activin and TGFβ share identical signaling machinery, targeted *in vivo* disruption of the ligands and their receptors result in mice with varying phenotypes. However, a study has revealed a similar transcriptome regulated by both signaling pathways[185]. Using cell lines overexpressing the activated form of both the activin type I (ALK4) and TGFβ type I (ALK5) receptors, the transcriptomes induced by each signaling pathway were investigated. Interestingly, no differences between the two pathways induced by these genes *in vitro* were observed[185]. These findings allude to a functional redundancy between both pathways in the regulation of the biological effect.

### 2.1. Cellular Proliferation

One of the most important biological functions of activin and TGFβ is their ability to induce cell cycle arrest in certain cells. Indeed, in the last few years a tremendous amount of knowledge on the cytostatic effect of these factors has been gathered. Activin and TGFβ play a vital role in growth inhibition of epithelial, hematopoietic, neuronal and endothelial cells. TGFβ mediates cell growth inhibition by directly affecting cell cycle components, particularly those involved in the G1 phase of the cell cycle, and

through repression of mitogenic transcription factors[186-191]. Activin and TGFβ signaling upregulate expression of the cyclin dependent kinase inhibitors, $p15^{INK4B}$ and $p21CIP1^{/WAF1}$, which through a tightly controlled mechanism leads to the inactivation of G1 phase cyclin dependent kinases[127, 144, 187, 188, 192, 193]. Additionally, TGFβ rapidly represses expression of the mitogen c-myc which is involved in inhibition of $p15^{INK4B}$ and $p21CIP1^{/WAF1}$ [113]. Besides c-myc, both activin and TGFβ signaling downregulate the differentiation transcription factors Id1, Id2, and Id3 [120]. Id proteins (Id1–Id4) are negative regulators of basic helix–loop–helix (bHLH) transcription factors. They promote cell growth, inhibit differentiation, and play critical roles in development and cancer[194]. Although today, it is widely accepted that TGFβ inhibits growth of multiple cell types, TGFβ was initially discovered as a factor that induced anchorage-independent growth of fibroblasts[195, 196]. Thus, TGFβ mitotic ability is still observed in certain transformed cells and immortalized fibroblasts[197].

## 2.2. Differentiation

Both activin and TGFβ can regulate the differentiation of a wide spectrum of cells including immune, erythroid, neuronal, mesenchymal, keratinocytes, bronchial epithelial and colon carcinoma cells. For instance, during early pregnancy activin regulates the differentiation of human cytotrophoblasts[198]. Furthermore, activin is involved in the regulation of branching morphogenesis in the lung, kidney and prostate (reviewed in [199]). Differentiation by TGFβ and activin is controlled either directly by modulating differentiation-specific genes, or by influencing cell adhesion or proliferation (reviewed

in [200]). For example, activin can cause differentiation of erythroleukemic cells, induction of hemoglobin synthesis and inhibition of cellular division[201-203].

## 2.3. Apoptosis

The apoptotic function of activin and TGFβ has been investigated in many cell types and in most cases their signaling pathways are pro-apoptotic. Indeed, they have been shown to induce apoptosis in epithelial cells, endothelial cells, hematopoietic stem cells, lymphocytes, hepatocytes, and neurons, as well as in breast cancer, gastric cancer, hepatic cancer, lymphoma, ovarian cancer, and prostate cancer cells (reviewed in [204]). TGFβ and activin induced apoptosis is frequently a Smad-mediated process. Through Smad signaling, they may induce the expression of pro-apoptotic genes such as the TGFβ-inducible early-response gene (TIEG1), death associated protein kinase (DAPK) and SH2-domain containing inositol-5-phosphate (SHIP)[205-207]. Furthermore, TGFβ may upregulate proapoptotic factors (ie, Bax), and/or downregulate antiapoptotic factors (ie, Bcl-2 and Bcl-xL) and activate caspases that partake in the apoptotic effector system[204]. On the other hand, other factors may cooperate with TGFβ to induce death in different types of cells. For instance, TGFβ and tumor necrosis factor α (TNF-α) may synergize to induce apoptosis in transformed fibroblasts by reducing levels of Bcl-2 and Bcl-xL[208]. TGFβ may also antagonize pro-survival signals and by doing so induces apoptosis. For example, Smad3 binds to the pro-survival kinase Akt allowing for TGFβ to induce its apoptotic effect[209, 210]. Moreover their role in inducing apoptosis *in vivo* is critical for proper organ development. For example, after lactation, mammary glands display elevated TGFβ3 levels which subsequently induces programmed cell death

during involution[211]. Depending on the cellular system, activin and TGFβ are death-inducing agents, however, the precise mechanisms involved in their pro-apoptotic function are still poorly defined and they are cell and context specific.

## 2.4. Extracellular Matrix Production and Adhesion

Tissue homeostasis and function depends on the regulation of extracellular matrix production and turnover. Activin/TGFβ signaling tightly controls the production of major constituents of the extracellular matrix and matrix regulatory enzymes such as collagens, fibronectin and protease inhibitors such as plasminogen activator inhibitor I (PAI-1), and tissue inhibitor of metalloproteinases (TIMPs)[212]. For instance, Smads in cooperation with other transcription factors regulate the expression of PAI-1[116, 212-215]. In addition, JNK activation by TGFβ regulates fibronectin expression[216]. During tumorigenesis, TGFβ may cause the degradation of basement membrane components which subsequently leads to a loss of cellular-matrix adhesion. TGFβ mediates this effect through direct activation of proteolytic enzymes such as matrix metalloproteases 2 and 9 (MMP2 and MMP9). This leads to degradation of type IV collagen in the basement membrane[217, 218]. TGFβ and activin induced loss of cell-to-cell adhesion properties is characterized by its ability to decrease E-cadherin and regulate the expression of other cytoskeletal proteins[219]. Loss of adhesion caused by TGFβ induces epithelial to mesenchymal transition (EMT) allowing cells to become more motile and invasive. This morphogenic response is imperative in TGFβ's role in regulating embryogenesis and the pathology of cancer.

## 2.5. Immune Regulation

Activin and TGFβ signaling play a critical role in regulating the immune system. Indeed, TGFβ has been characterized as the most powerful immunosuppressor in mammals[220]. TGFβ's essential role in maintaining proper immune regulation is evident from the TGFβ1 knockout mice. These mice die from immune deregulation complications[5, 221]. Furthermore at the cellular level, TGFβ inhibits the proliferation and functional differentiation of immune cells including, natural killer cells, T lymphocytes, neutrophils, macrophages, and B cells. Additionally, TGFβ's immunosuppressive capabilities have a direct effect on tumor progression. More specifically, TGFβ derived from tumors suppress T-cell activation allowing tumors to escape immune surveillance (reviewed in [222]).

## 2.6. Development

TGFβ, activin and their signaling components play a crucial role in embryo development. The imperative role of these signaling components is obvious from lethal phenotypes of the knockout models. TGFβ1, ALK4, TβRII, ActRIIA-ActRIIB double knockout, Smad2 and Smad4 knockout mice die during embryo development[4, 175, 223-226]. Among the multiple defects in development TGFβ2 and TGFβ3 null mice have pronounced lung development impairment leading to perinatal death[9, 10]. Activin βA knockout mice die postnatally from craniofacial defects[31]. In *xenopus* embryos, truncated activin receptor inhibits mesoderm induction[227]. In all, TGFβ superfamily signaling members play a regulatory role in early post implantation mouse embryonic

and extraembryonic angiogenesis, vasculogenesis and development of the eyes, heart, left-right asymmetry, craniofacial, nervous and gonads (reviewed in [228]).

## 2.7. Hormonal Regulation

Activin's role as an important regulator of hormones was evident upon its original identification as a stimulator of FSH production in the pituitary[23, 24, 229]. Recently, activin has not only been shown to regulate FSH but also LH production via Smad signaling[230-232]. Furthermore, *in vitro* data suggest that activin strongly inhibits growth hormone and corticotrophin (ACTH) secretion from the pituitary[233-235]. On the other hand, activin may stimulate progesterone, GnRH and hCG production in placental cells[236].

## 2.8. Angiogenesis

Activin/TGFβ has an imperative biological effect on angiogenesis, a process by which blood vessels deliver nutrients and oxygen to cells. TGFβ signaling component's vital role in angiogenesis is evident in the TGFβ1 and TβRII knockout mice that die *in utero* due to defective angiogenesis and vasculogenesis[224]. Moreover, mice with null mutations in genes encoding ALK1, ALK5 and endoglin have defective angiogenic phenotypes[237-240]. TGFβ1 may stimulate fibroblasts and epithelial cells to produce a vital angiogenic growth factor called vascular endothelial growth factor (VEGF). VEGF acts directly on endothelial cells to induce proliferation and migration[241]. Interestingly, two TGFβ signaling pathways are stimulated in endothelial cells. Not only is the Smad2/3 pathway induced through ALK5, but due to high expression of ALK1 in

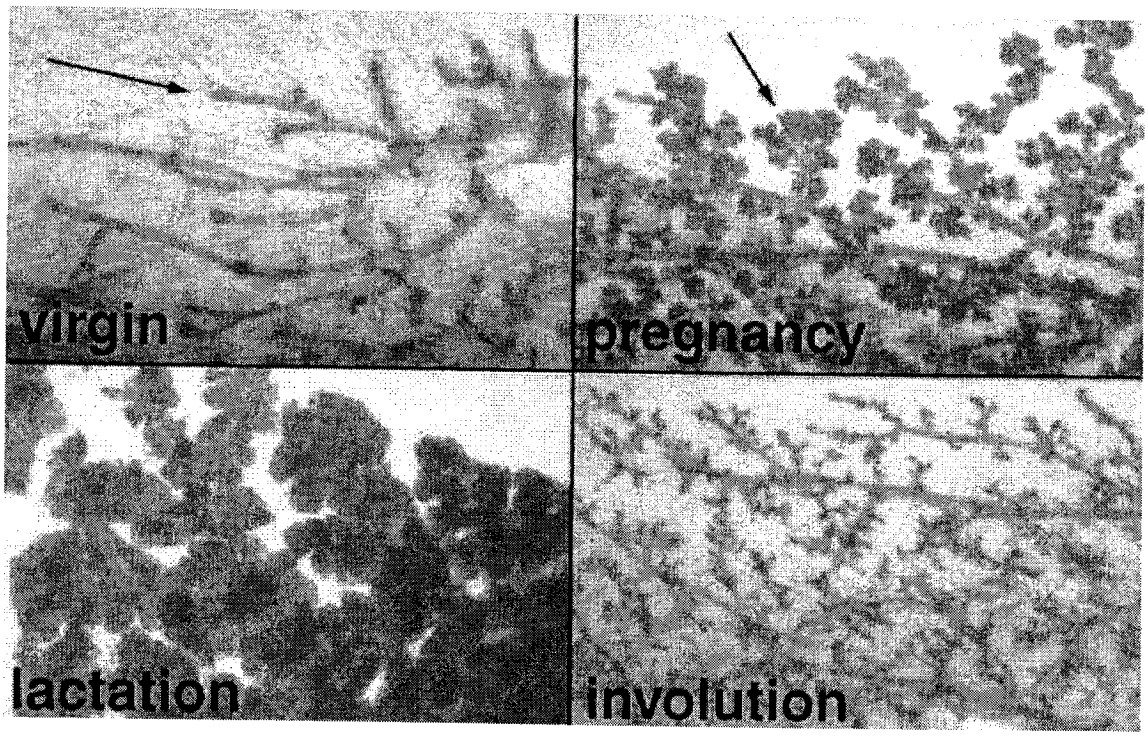
endothelial cells[47], the Smad1/5 pathway is also activated. While TGFβ binding to ALK5 seems to play a role in vascular maturation, ALK1 binding stimulates endothelial cell proliferation[242]. TGFβ's ability to balance signaling between ALK1 and ALK5 allows for the regulation of vascular homeostasis. A major role for activin and TGFβ is to induce angiogenesis of tumor cells which is crucial for tumor growth and invasion leading to metastasis.

### 3. Mammary Gland Development

Mammary gland development is an ongoing process throughout the lifetime of a reproductive female. This continuing process is under the control of multiple growth factors such as estrogen, progesterone, prolactin (PRL), TGFβ and activin. Development of the mammary gland begins in the embryo where the rudimentary mammary epithelium is formed, Figure 3. At puberty, a network of ductal branches invades the fat pad forming terminal end buds (TEBs) under the control of ovarian hormones. During pregnancy, reproductive hormones induce massive epithelial proliferation which allows for lateral branching of the ducts forming lobuloalveolar structures. Alveoli are lined with milk secreting epithelial cells surrounded by myoepithelial cells that envelop a circular lumen. Upon lactation, terminally differentiated lobular alveoli secrete milk. Once suckling ceases, two stages of involution occur. The first is induced because of milk stasis where there is extensive remodeling of the alveolar structures due to apoptosis. It is a stage that is characterized by regulation of apoptotic and anti-apoptotic genes and downregulation of milk protein gene expression[243-245]. One of the main signaling molecules that is upregulated and

responsible for inducing apoptosis during the initial phase of involution is TGFβ3. The second stage of involution is defined by the destruction of the lobuloaveolar structures due to a loss of circulating hormones and induction of protease genes[244, 245].

**Figure 3. Mammary Gland Development**




**Mammary Gland Development Images taken from [246]**

### 3.1. Hormonal control of Ductal and Alveolar Development

Mammary growth from birth to the onset of puberty is minimal and proportional to that of the growth of the body. Genetic and tissue transplant experiments have implicated the requirement of certain genes during the initial fetal development of the mammary gland including Lef-1, Msx1, Msx2 and PTHrP[247] [248]. The sex steroid hormones estrogen and progesterone are critical for mammary ductal and alveolar development. Evidence from the estrogen receptor and progesterone receptor knockouts confirm their necessity for ductal development and for lobuloalveolar development respectively. During early puberty, estrogen, growth hormone and local production of insulin like growth factor-1, IGF-1, induces the growth of mammary ductal epithelial cells[249]. Initial alveolar proliferation is due to the synergistic action of prolactin and progesterone[250]. Other factors such as several cytokines including RANK-L, and members of the epidermal growth factor, EGF, family are also important for lobuloalveolar development[251, 252]. During pregnancy and lactation, prolactin, placental lactogens, progesterone and local growth factors influence extensive proliferation of the lobuloalveolar epithelium. Prior to lactation, both progesterone and placental lactogen levels fall. During lactation, the lobuloalveolar epithelium becomes secretory, allowing for the synthesis of milk proteins and lactogenic enzymes.

## 4. Activin/TGFβ signaling in Mammary Gland Development

The mammary gland is a major endocrine target tissue for activin and TGFβ. They appear to play a role in the regulation of mammary epithelium growth and differentiation at all stages of the gland's development including involution. The role of TGFβ in this gland has been studied more extensively in comparison to activin, due to the generation of multiple transgenic mice. The importance of activin in mammary gland development stems from the expression of activin subunits and more specifically the phenotype of activin βB subunit knockout mice.

### 4.1. Ligand and Signaling Components Expression in the Breast

#### 4.1.1. TGFβs and TGFβ receptors

TGFβ isoforms are expressed in the mammary gland at almost all physiological stages except lactation. During lactation, although there is extensive downregulation of all isoforms, there are trace levels of TGFβ2 mRNA[211, 253, 254]. In virgin mice, TGFβ1 surrounds the ducts and accumulates in the periductal extracellular matrix where it maintains the spaces between the ducts[253]. Functional *in vivo* studies reveal that pellets slowly releasing TGFβ1/2/3 that are implanted in the mammary end buds of mice, inhibit ductal growth[253, 255]. Similar studies performed with TGFβ implants did not effect lobuloalveolar development[256]. During pregnancy, low levels of TGFβ1 and even lower levels of TGFβ2 are found in alveoli and in the ducts whereas TGFβ3 is highly expressed in these structures[253]. At involution, an initial increase of TGFβ3 is due to milk stasis and a subsequent increase in TGFβ1 and TGFβ2. At this stage of mammary

gland development, TGFβ induces massive apoptosis resulting in restructuring and restoration of the gland back to its virgin state.

The type I and type II TGFβ receptors are expressed in both the ductal epithelial and stromal cells in the virgin, pregnant, and involuting breast suggesting that these aforementioned cells are responsive to TGFβ[257].

### 4.1.2. Activin and activin receptors

Both activin and its receptors are expressed in mammary tissue[258-260]. Functionally, activin inhibits the growth of both primary and transformed mammary epithelial cells. In addition activin suppresses tubule formation observed in human mammary organoids in response to hepatocyte growth factor/scatter factor (HGF/SF), suggesting a role for activin in regulating mammary cell growth and morphogenesis[258]. Furthermore, the presence of activin in its dimeric form in the cystic fluid of fibrocystic disease implies a local production and secretion of this protein in the human breast.

The expression of activin components is elevated during mid to late lactation and depressed during both involution in the mammary epithelium and stroma[261]. Elevated levels of phosphorylated Smad3 during lactation in mammary epithelial cells are indicative of activin signaling[261]. It has beens suggested that the downregulation of TGFβ during lactation is to avoid apoptosis and that activin signaling permits proper lactation while restricting hyperproliferation of the tissue[261].

### 4.1.3. The Smads

A study in which the role for Smad3 in mammary gland development is investigated, found that all Smads are expressed throughout all stages of development[178]. Although

still present, expression of all Smads decreases during lactation in the gland. For Smad2 and Smad4, the decrease is sustained at early stages of involution and is restored at day 6 of involution[178]. Furthermore, when Smad3 null glands and Smad3 expressing glands are transplanted into the fat pads of nude mouse hosts, apoptosis occurs during involution more in the Smad3 expressing glands. This suggests that Smad3 contributes to the induction of apoptosis during involution[178].

### 4.2. Knowledge Gained from Mouse Models

The expression pattern of TGFβ and activin in the mouse suggests that they may have roles in regulating branching morphogenesis, lactation, and involution. An animal model system developed to elucidate the *in vivo* role of activin has highlighted its importance in mammary gland development. In particular, deletion of the activin βB subunit, ablating three of the dimeric β molecules (activin B, activin AB and inhibin B), causes incomplete mammary development and an absence of lactation[262]. Interestingly, when the mammary glands of βB knockout mice are transplanted into wild-type mice, they can develop normally[262]. However, in order for the gland to undergo proper development, it requires that the site of transplantation of the mammary epithelium be an active stromal tissue such as the fat pad. This suggests that stromal βB production can compensate for the epithelial deficiency[262].

Invaluable insight in understanding the function of TGFβ in the mammary gland has been achieved via gain of function and loss of function transgenic animals. The first TGFβ transgenic mouse to be developed was a constitutively active TGFβ1 from the mouse mammary tumor virus (MMTV) promoter. This mouse resulted in the inhibition

of ductal development without affecting alveolar growth[263]. On the other hand, when TGFβ1 was expressed using a milk protein specific promoter, whey acidic protein (WAP)-TGFβ1 alveolar development was significantly inhibited which resulted in the suppression of lactogenesis[264]. Interestingly, transplantation of the transgenic mouse's mammary epithelium into the fat pad of wildtype mice barred normal mammary development. This observation implies that the TGFβ1 effect on lobuloalveolar development is due to an autocrine rather than a paracrine effect[265]. This means that TGFβ1 is not affecting the production of a factor in the stroma that may be inducing the (WAP)-TGFβ1 phenotype. In summary, these transgenic mouse models, using developmentally restricted promoters, illustrate that TGFβ inhibits proliferation within the mammary epithelium in response to either the hormones of puberty or pregnancy however the molecular mechanisms in inducing this effect are unknown.

Loss of function studies further reveal the role of TGFβ signaling in the breast. Dominant negative TβRII receptor expression via a MMTV promoter, resulted in precocious alveolar hyperplasia and lactation in virgin mice[266]. Recently, a conditional knockout of TβRII in fibroblasts revealed reduced ductal elongation[267]. Furthermore, these mice lacked both mature ducts and terminal endbuds highlighting the importance of stromal TGFβ on gland development[267]. Hence loss of TGFβ signaling in the stroma, altered paracrine signaling to the mammary epithelium. Finally, overexpression of a constituvely active TGFβ type I receptor (ALK5TD) mutant using the MMTV promoter in mice exhibited depressed lobuloaveolar development during pregnancy[268]. The importance of TGFβ in the mammary gland is further illustrated by TGFβ1-null heterozygous mice. These animals display accelerated ductal outgrowth during puberty

and alveolar expansion during pregnancy due to an increase in mammary epithelial cell proliferation[269]. This suggests that TGFβ normally acts as an inhibitor of ductal elongation and branching.

Whereas, as mentioned earlier, Smad2 and Smad4 knockouts are lethal, Smad3-null mutant mice survive postnatally. Analysis of the Smad3 knockout mice reveals that the mammary gland of virgins show delayed development[178]. However, when given appropriate hormonal stimulation, the Smad3 null mammary epithelium develops normally and undergoes full functional differentiation[178]. Future conditional knockouts will further address the relative contribution of each Smad in mammary gland development.

### 4.3. Summary

It is clear from a variety of studies looking at TGFβ/activin signaling components expression, within various stages of mammary gland development, that they induce a myriad of effects. *In vivo* studies using exposure to exogenous sources or transgenic expression of constitutively active protein have shown what TGFβ can do in the mammary gland. These have generally led to the conclusion that TGFβ has a prominent role in regulating pattern formation by the epithelium, perhaps via interactions with the stroma, and is involved in fate decisions by individual cells. Although no transgenic mice models have been generated to investigate activin signaling within the mammary gland, the activin βB knockout mouse alludes to an important function activin in the regulation of mammary differentiation and lactation. However, the signaling pathways

activated by activin and TGFβ leading to these aforementioned effects are not yet fully characterized.

## 5. Activin/TGFβ and breast cancer

TGFβ signaling has been described as a double-edged sword in cancer[270]. This description was coined due to its dual and contrasting role in tumor biology. In early stages of tumorigenesis TGFβ is a potent inhibitor of tumor cell growth where its signaling components have been characterized as tumor suppressive. In contrast, TGFβ also has the potential to drive malignant progression, invasion, and metastasis. Although activin's increasingly vital role in breast cancer pathogenesis and metastasis emerges exponentially, most of the focus to date has been on TGFβ.

### 5.1. Ligand expression and regulation

In primary human breast cancers, TGFβ localization concentrates around epithelial tumor cells, while the flanking stromal cells are negative. Multiple studies have found a positive correlation between TGFβ expression by primary breast cancer and advanced tumor progression[271-274]. Furthermore, many breast cancers synthesize and secrete high levels of TGFβ[275]. These studies implicate TGFβ as a promoter of breast cancer progression rather than an inhibitor of tumorigenesis.

Initial systemic localization studies of activin subunits display positive expression in breast tissue whereas malignant breast lesions are negative[259]. This suggests that the absence of activins in carcinoma tissue may facilitate abnormal cell proliferation. Furthermore, there is a downregulation of activin and its signaling components in high

grade breast cancers, suggesting a role for activin in breast tumor development[276]. However, subsequent reports have also correlated increased activin expression with increased bone metastasis. Fascinatingly, activin may thus be implicated in the progression of breast cancer metastasis[277].

### 5.2 Receptor expression and regulation

Interestingly, two studies have reported diminished TβRII expression in early and late stages of human breast cancer[278]. This decreased expression of TβRII correlates with an increase risk of developing invasive breast cancer[278]. ALK5 expression was observed in epithelial cell cultures derived from malignant breast tissue[279]. *In vitro*, numerous breast carcinoma cell lines, express all TGFβ and activin signaling components. Some exceptions include the MDA-MB-468 which lacks Smad4, and ZR-75-1 and T47D, where TβRII is not detected[280].

### 5.3. Smad signaling

#### 5.3.1. Cytostatic Response

Escape of cellular proliferation inhibition is a defining hallmark of cancer. TGFβ mediated inhibition of cellular proliferation is associated with its ability to block tumorigenesis. TGFβ/Smad signaling increases expression of $p21CIP1^{/WAF1}$ in the human mammary epithelial cell line MCF-10A[281]. In breast cancer cells, TGFβ growth inhibititory response could be restored once the TβRII is reintroduced into the cells through the induction of cell cycle inhibitors $p21CIP1^{/WAF1}$ and p27[282]. Interestingly, it

has been reported that TGFβ receptors and Smad activity are still present when breast cancer cells lose their cytostatic response[283]. The inability for TGFβ to induce the cytostatic response is apparent in breast cancer metastasis[284, 285]. Indeed, the loss of TGFβ cytostatic response in breast cancer cells has been associated with a defective repression of c-myc[284] and induction of the cell cycle inhibitor p15 by the Smads[285]. Furthermore, the transcription factor C/EBPβ is central in regulating c-myc and $p15^{INK4B}$ by TGFβ signaling[285]. In metastatic breast cancer, TGFβ's inability to regulate cell growth inhibition may be due to an increase in the inhibitory isoform LIP of C/EBPβ[285].

A limited study on MCF7 cells showed that, as well as expressing the activin receptors, these cells produced activin subunit proteins[260]. Indeed, activin was found to cause G1 cell cycle arrest and hence inhibit MCF7 cell growth. Later studies have shown that activin inhibits cellular proliferation by increasing expression of $p15^{INK4B}$ which reduced expression of cyclin A and subsequently leading to retinoblastoma protein phosphorylation[286].

The effects of activin on a panel of breast cancer cell lines that were ER positive or negative were investigated[287]. The ER-positive cell lines in the study were inhibited by activin, whereas the ER-negative cell lines were not. In two of the ER-negative cell lines, resistance to the growth-inhibitory effects of activin were explained by down-regulation of the activin receptors. In two other ER-negative cell lines, MDA-MB-231 and MDA-MB-468, activin insensitivity was not due to reduced activin receptor levels. Instead, the failure of the MDA-MB-468 cell line to respond to activin was explained by loss of Smad4 expression in these cells. Transfection of Smad4 into these cells rendered

them sensitive to inhibition by activin. The other activin resistance/ER-negative cell line, MDA-MB231, expressed both Smad4 and Smad2. In this case, additional studies revealed that these cells lacked a functional ActRI[287]. These studies demonstrate that there is a loss of activin signaling components in more aggressive estrogen receptor negative cell lines suggesting a role for this pathway in tumor progression.

### 5.3.2. Apoptosis

Cancer cells ability to evade apoptotic signals allows for their survival. Resistance to TGFβ-induced apoptosis may be an essential component of tumorigenesis, particularly for cancers arising from tissues in which TGFβ is a prominent regulator of apoptosis. Indeed, TGFβ mediated apoptosis is important in restructuring the mammary epithelium during involution[211]. TGFβ downregulates the mitochondrial anti-apoptotic factors Bcl-2 and Bcl-$X_L$ in mouse mammary epithelial cells inducing apoptosis[288, 289]. As mentioned previously, TGFβ is a death-inducing factor in breast cancer cells. TGFβ has been shown to induce cell death in MCF7 breast cancer cells through a substantial downregulation of Bcl-2 and Bcl- $X_L$ mRNA[290]. In addition, TGFβ may sensitize cells for other pro-apoptotic stimuli. For example, when dominant negative TβRII was introduced in the breast cancer cell line MCF7, these cells became resistant to TNF-α-induced cell death[291]. Furthermore, breast cancer cells treated with Tamoxifen lead to an upregulation of TGFβ and subsequent cell death[292]. In addition, the ability of TGFβ to induce apoptosis in lymphocytes may be a critical component for the immunosuppressive effect of TGFβ during tumorigenesis which will be reviewed further

in subsequent sections. Although a well-documented phenomenon, the biochemical mechanism responsible for TGFβ induced apoptosis in breast cancer remains elusive.

### 5.4. Receptor and Smad mutations

TGFβ and activin signaling component mutations are often associated with human cancer. Indeed, the TβRII and the ActRII genes contain a poly-adenine tract in their kinase domains that is often mutated in human cancers such as gastric, pancreatic, lung, and brain (reviewed in [293]). Cumulative point mutations in the kinase domain of TβRII are present only in advanced recurrent breast cancers and not in primary breast tumors[294]. While there are no reports of ALK4 mutations in breast cancer, conflicting data on the incidence of mutations in ALK5 exist. One particular study reveals that metastatic breast cancers possess a point mutation in the kinase domain of ALK5. The authors hence suggest that the loss of ALK5 signaling via TGFβ is imperative in the progression of metastasis[295]. Yet another study has challenged these finding having reported after examining metastatic breast cancers that no such mutations in ALK5 were detected[283].

Mutations of the gene encoding Smad2 occur in a limited number of colon, lung, liver and cervical carcinomas[296-298]. On the other hand, there is only one reported case where Smad3 is highly downregulated in gastric cancer[299]. Smad4 is the most frequently mutated Smad gene in cancer, especially of pancreatic origin[300] and is the only known Smad mutation in breast cancer[301]. Furthermore, Smad4 is mutated and deleted in hepatocellular, bladder, biliary tract, ovarian, intestinal, colorectal, lung, prostate and cervical cancers[301]. In addition, Smad4 is absent in breast cancer cell lines and invasive

ductal carcinomas[302, 303]. These studies support a role for Smad4 in the development of breast cancer

Taken together, the low number of reported mutations in TGFβ/activin signaling components in breast cancer may be due to the dual role of these factors to not only inhibit tumorigenesis but also induce metastasis.

### 5.5. Mouse models in breast cancer

Evidence of TGFβ having prominent tumor suppressive properties in the mammary gland is supported by transgenic mice studies. An increase in TGFβ1 expression in the breast effectively decreases the number of tumors when MMTV-TGFβ1 mice are crossed with a tumor forming MMTV-TGFα mouse[263]. Moreover, MMTV-TGFβ1 inhibits mammary tumor development in mice challenged with the chemical carcinogen 7,12-dimethylbenz(α)anthracene (DMBA)[263]. In the same line of evidence, the incidence, number and size of developing tumors increases dramatically in the breast when dominant negative TβRII is overexpressed in mouse epithelium (MMTV-DNIIR) when challenged with DMBA[304]. The MMTV-neu mouse is a well characterized model that develops adenocarcinomas that metastasize to the lung[305]. Introduction of the activated form of the TGFβ type I receptor (ALK5TD) fused to an MMTV promoter in neu expressing mice in the mammary epithelium (MMTV-neu) developed fewer tumors. On the other hand, MMTV-DNIIR administration into the neu mice lead to shortened tumor latency[268]. While having no effect on normal mammary gland development, the conditional knockout of Smad4, $Smad4^{MMTVcreKO}$, resulted in hyperproliferative

mammary epithelial cells. These cells subsequently differentiated to a squamous epithelial phenotype[306].

The above mouse models clearly define TGFβ signaling as functioning to inhibit tumor formation. In contrast, evidence exists characterizing TGFβ as having a defining role in promoting cancer and metastasis. This is observed in the MMTV- ALK5TΔD/MMTV-neu mice, where there is an increase in tumor metastasis to the lung[268]. On the other hand, blocking TGFβ signaling with MMTV-DNIIR in the neu mice resulted in fewer metastases[268]. TGFβ's ability to promote metastasis was further demonstrated when mice with inducible polyomavirus middle-T-tumors were crossed against mice with inducible expression of TGFβ1. More specifically, the progeny displayed an elevated lung metastasis phenotype[307].

### 5.6. Summary

TGFβ has an important role in normal mammary biology as a potent regulator of mammary epithelial proliferation, mammary ductal and alveolar development, and postlactation involution of the mammary gland. Hence, it is not surprising that TGFβ signaling plays an important role in mammary carcinogenesis. Mulitple studies that have been described support TGFβ's ability to inhibit breast tumorigenesis. TGFβ can potently inhibit breast cancer cell growth. There mutation in or loss of expression of members of the TGFβ signaling pathway including TβRII and ALK5 in some human breast cancers, indicating that human breast cancers may develop resistance to TGFβ's antiproliferative effects. Furthermore, there is decreased breast cancer formation in human patients with elevated levels of TGFβ. Through upregulation of TGFβ, the

antiestrogen tamoxifen inhibits breast cancer cell growth. Furthermore, *in vitro* studies presented suggest that the mechanisms for resistance to the growth-inhibitory effects of TGFβ are key cellular events during mammary carcinogenesis. Activin signaling components are expressed in breast tissue and a loss of their expression correlates with increasing breast cancer grade. Moreover, activin is known to induce cell cycle arrest n breast cancer cells however, the precise signaling mechanisms are not fully elucidated.

## 6. TGFβ and breast cancer metastasis

Recent evidence has attributed a novel role for TGFβ, not only as a tumor suppressor in early stages of tumorigenicity but also as promoter of metastasis in advanced cancer. A cell model was developed to demonstate how TGFβ can switch from a tumor suppressor to a prometastastic factor in the course of carcinogenic progression[308]. In this model, the TGFβ induced switch occurred at the transition from histologically low to high grade breast cancer [308]. Genetic or epigenetic changes that occur during this transition alter the cellular interpretation of the TGFβ signal[308]. TGFβ has also been shown to be a major effector of breast tumor metastasis *in vivo*. Indeed, when TGFβ signaling was blocked in the metastatic breast cancer cell line MDA-MB-231 and injected into immuno-deficient mice, less tumors metastasized to the bone[309]. Furthermore, parathyroid hormone-releasing peptide (PTHrP), which is associated with metastatic disease, was induced in tumor cells by TGFβ[309]. Groups of genes directly involved in mediating bone metastasis have been revealed in the MDA-MB-231 cell line[310]. Two genes, *IL11* and *CTGF*, are induced by TGFβ through Smad signaling, contributing to osteolytic bone metastases[310].

## 6.1. Tumor Promoting Effects of TGFβ

The stroma and microenvironment directly influence the initiation, promotion, or progression of a cancer. Human breast cancers are characterized by desmoplasia, fibroblast proliferation and remodeling of the extracellular matrix[311]. TGFβ's pro-tumorigenic role is defined by its ability to affect both the carcinoma cell directly and the surrounding stroma. The importance of TGFβ signaling on stroma is displayed in an *in vivo* mouse model. TβRII knockout in mammary gland fibroblasts leads to a promotion of tumor growth and invasiveness. In conjunction, there is an increase in hepatocyte growth factor (HGF), macrophage stimulating protein (MSP) and TGF-α mediated signaling networks[267]. This study suggests that TGFβ regulates various growth factors that promote breast cancer in the stroma[267].

TGFβ tumor promoting effects are due to its ability to induce immune suppression, angiogenesis, tissue invasion, extracellular matrix deposition and epithelial to mesenchymal transition.

### 6.1.1. Immune suppression/evasion

Tumor cells express tumor-specific antigens that are detected by the body's immune system which leads to their elimination. By acquiring the ability to evade immunosurveillance, cancer cells can develop and progress. TGFβ secretion is a major mechanism by which the tumor can suppress the immune system. TGFβ immunosuppressive role in cancer progression is supported by the fact that cancer cells

are able to produce and secrete TGFβ[312]. Experimental models and specimens from cancer patients contain elevated levels of TGFβ[313]. *In vivo* and *in vitro* evidence associate TGFβ as a potent immunosuppressor. TGFβ produced by T cells can blocks production of interleukin 2 (IL-2) to inhibit IL-2-dependent proliferation of T cells[3]. TGFβ also inhibits the differentiation of T cells, and prevents naïve T cells from acquiring cytotoxic or helper functions[220]. TGFβ has been implicated in *in vivo* tumorigenesis through its effect on natural killer (NK) cell activity[314]. Neutralizing antibodies against TGFβ block MDA-MB-231 local tumor and metastasis formation in NK-competent mice but not in NK-deficient mice[314]. This study suggests that TGFβ allows for the highly metastatic breast cancer cell line MDA-MB-231 to metastasize by inhibiting NK cells, further supporting the role for tumor secreted TGFβ as a suppressor of the host immune surveillance[314].

### 6.1.2. Angiogenesis

Angiogenesis is necessary for proper nutrient and oxygen supply to the tumor. The formation of new microvasculature by capillary sprouting is a prerequisite for tumor growth. This process allows for intravasation of the blood system which paves the way for metastasis. In human breast tumors, increased TGFβ is associated with an augmentation of microvessel density, correlating with poor patient prognosis[315]. A potent inducer of angiogenesis is vascular endothelial growth factor (VEGF) whose expression is regulated by TGFβ in breast cancer cells[316]. Through TGFβ's direct effect on the formation of a new vasculature to the tumor, it promotes tumorigenesis.

### 6.1.3. Tissue invasion/Cellular adhesion

Cancer cell invasiveness requires a motile cellular phenotype and the ability for tumor cells to degrade their extracellular milieu. TGFβ induction of the homeobox transcription factor CUTL1, a poor prognosis factor for metastatic breast cancer, regulates the expression of multiple genes regulating cell motility, tumor cell invasiveness, and extracellular deposition[317]. Additionally, both Smad dependent and independent mechanisms are involved with TGFβ-induced tumor cell invasiveness. This includes a PI3K/Akt dependent and Smad independent mechanism by which TGFβ induces motility in metastatic breast cancer cells[318].

In normal situations, TGFβ increases production of the extracellular matrix proteins collagen and fibrinogen while decreasing matrix degradation enzymes such as collagenase. During breast tumorigenesis, TGFβ augments these extracellular matrix degrading enzymes, increasing proteolytic activity of cancer cells. One of these factors is urokinase-type plasminogen activator (uPA), which plays a key role in tumor matrix degradation and promotes tumor progression[319]. By increasing its expression, TGFβ allows for the invasiveness of breast cancer cells. Furthermore, TGFβ activation is enhanced by the production of proteases and plasmin by tumor cells[320].

In summary, by increasing the proteolytic activity and decreasing the adhesiveness of cancer cells, TGFβ acts to promote breast tumor invasion.

### 6.1.4. Epithelial to Mesenchymal Transition

Potential lethality arising from solid tumors stems from their ability to invade surrounding tissue and metastasize to target organs. In order for the tumors to invade they must undergo the aforementioned process of dedifferentiation EMT. This transitional process is characterized by a loss of cell-cell junction proteins such as E-Cadherin and gain of mesenchymal proteins such as vimentin. Furthermore, this process is typified by a loss of cell polarity, repression of epithelial markers, and cytoskeletal reorganization. Motility of these cells may only occur upon transition from their initial epithelial origin to fibroblastoid cells. The newly mobile cells are now invasive and capable of degrading the basement membrane and interstitial matrix. Therefore, alterations in stromal and growth will lead to eventual metastasis.

In both normal and transformed mammary epithelial cells, TGFβ may induce morphological changes characteristic of EMT[219, 321, 322]. These morphological changes include reorganization of the actin cytoskeleton, downregulation of adhesion and cytoskeletal molecules and expression of mesenchymal markers[219, 321]. For example, TGFβ may downregulate expression of the epithelial markers E-Cadherin, ZO-1, vinculin and keratin. In contrast, it may upregulate expression of the mensenchymal markers vimentin and N-Cadherin. These specific aforementioned changes induce alterations in cell morphology and motility[184, 219, 323].

Cooperation between the TGFβ Smad pathway and other pathways such as activated Ras, ERK MAPK, p38 MAPK, NFκB and PI3K are all involved in EMT (reviewed in [324]). For example, in Ras transformed breast carcinomas, Smad signaling cooperates with NFκB contributing to EMT *in vitro* and metastasis *in vivo*[325]. TGFβ may also

induce EMT in a Smad independent mechanism. Through ALK5 interaction with the polarity protein Par6, TGFβ stimulation results in dissolution of the actin cytoskeleton and tight junctions through degradation of RhoA[326]. Smad3 and Smad4 have been shown to act cooperatively with Rho and p38 to induce stress fibre formation thus inducing EMT. Transcriptional regulation of EMT by TGFβ involves multiple targets such as Id genes, the SNAIL family of zinc finger proteins, two-handed zinc finger/homeodomain proteins, basic helix-loop-helix proteins, and high motility group box-containing proteins (reviewed in [324]). Therefore, through multiple mechanisms TGFβ induces EMT allowing for tumors to invade surrounding tissue leading to their metastasis.

### 6.2. Summary

TGFβ inhibits primary tumor development and growth by inducing cell cycle arrest and apoptosis during the early phase of epithelial tumorigenesis. When tumor cells become resistant to growth inhibition by TGFβ due to inactivation of the TGFβ signaling pathway or aberrant regulation of the cell cycle in later stages of tumor progression, the role of TGFβ becomes one of tumor promotion. This suggests a pro-oncogenic role for TGFβ in addition to its tumor suppressor role. Through various alterations in the complex TGFβ signaling and cell cycle pathways, human cancers resist TGFβ-mediated inhibition of proliferation. As we have seen, TGFβ can exert effects on tumor and stromal cells as well as alter the responsiveness of tumor cells to TGFβ to stimulate invasion, angiogenesis, and metastasis, and to inhibit immune surveillance. Furthermore, when epithelial cells lose autocrine TGFβ responsiveness they procure a growth advantage leading to malignant progression.

## 7. Hypothesis and objectives of this work

Activin and TGFβ signaling are pluripotent cytokines with diverse roles within different organs throughout the body, with the mammary gland being one of the key target tissues. In fact their signaling components are regulated throughout mammary gland development. Animal model systems developed to elucidate the *in vivo* role of activin and TGFβ have further highlighted their importance in mammary gland development. Collectively, these *in vitro* and *in vivo* findings emphasize a role of these TGFβ family members in negatively regulating mammary growth and differentiation. However, the precise signaling mechanism of action of activin and TGFβ in the breast remains to be dissected.

A plethora of studies evaluating the effects of TGFβ in breast carcinogenesis have been performed, defining TGFβ as a potent inhibitor of growth in early stages of tumorigenesis and a promoter of metastasis. Evidence of activin's anti-proliferative effects in breast cancer are highlighted by studies looking at expression of activin signaling subunits in high grade breast cancers. These studies reveal that a loss of activin signaling correlates with high grade breast cancer suggesting it may play a role in breast cancer development. The limited data obtained from few studies mentioned in CHAPTER I describing the localization of activin and its effects in breast cancer cells suggest that resistance to the growth-inhibitory effects of activin involve changes to the activin signaling pathway. Hence, the contribution of activin, and the activin signaling pathway to breast tumorigenesis have not been defined.

In order to better understand the role of activin/TGFβ signaling pathway in normal mammary gland biology and the pathogenesis of breast cancer, the objectives of my

doctoral thesis work were as follows. The first objective was to elucidate the role of activin and TGFβ signaling in the mediation of growth and differentiation of the mammary gland. This study was performed in mammary epithelial cells where I looked at the activin/TGFβ signaling effect on the prolactin Jak/Stat pathway which is one of the central hormones regulating the development of the breast. The second objective of my project was to analyze the effect of activin in breast cancer and to dissect the molecular mechanism governing activin's role in breast carcinogenesis. Hence, I unveiled for the first time the activation of a Smad-independent pathway downstream of activin, the p38 MAPK. Furthermore, I showed that activin is a potent inhibitor of breast cancer cell growth and this effect is mediatiated through activin induced p38 MAPK activation. Finally, the third objective of my doctoral work was to identify the target genes involved in activin mediated cell growth inhibition of human breast cancer cells. As such, I have found a novel activin target gene, the death adaptor protein RAIDD, which is potently upregulated upon activin signaling. Moreover, I show that RAIDD expression is necessary in inducing activin mediated cell growth inhibition.

# CHAPTER II.

## Smad Signaling Antagonizes Stat5-Mediated Growth And Differentiation Of Mammary Epithelial Cells

Eftihia Cocolakis , Loren Drevet , Joanne Wing Yee Ho , Eric Haines , Suhad Ali , and

Jean-Jacques Lebrun

## PREFACE

As described in CHAPTER I, multiple studies underlining the relevance of activin and TGFβ signaling in the breast exist. However, the precise signaling mechanisms regulated have not been defined. Therefore, in this study, we sought out to elucidate the effect of activin and TGFβ signaling on mammary gland growth and differentiation. In the following paper, we dissected the molecular mechanism regulated by activin/TGFβ signaling in mammary epithelial cells. One of the key signaling pathways involved in mammary gland differentiation and proliferation is the prolactin Jak-Stat pathway. As such, I have included as a prelude to this paper, a detailed account of prolactin Jak/Stat signaling and its role in the biology of the mammary gland. Finally, I have described prolactin Jak/Stat's role in breast carcinogenesis in order to understand the implications of the work presented in this chapter.

### 1. The Role of the Prolactin (PRL) Jak-Stat Pathway in Proliferation and Differentiation of the Mammary Gland

#### 1.1. PRL and PRL Receptor

PRL is a polypeptide hormone that is synthesized and secreted mainly by cells in the pituitary. PRL exerts a vast array of biological functions and is studied extensively for its effects on mammary gland development and lactation. PRL's imperitive role in this gland is well illustrated by a lack of lobuloaveolar development in the PRL knockout mouse[327]. Therefore PRL deficiency results in a mammary gland resembling that at early puberty.

The PRL receptor (PRLR) belongs to the class I cytokine receptor superfamily that includes among others the receptors for multiple interleukins, growth hormones and erythropoietin[328]. PRLR knockout mice at puberty lack ductal branching which further confirms the necessity of this cytokine in mammary gland development[329]. Furthermore, PRLR heterozygote animals ($PRLR^{+/-}$) are not able to lactate due to reduced ductal branching during puberty and depressed lobuloalveolar development during pregnancy. Moreover, during pregnancy there is a complete failure of lobuloalveolar development when $PRLR^{-/-}$ mammary epithelium is transplanted into the fat pads of a normal mouse[329].

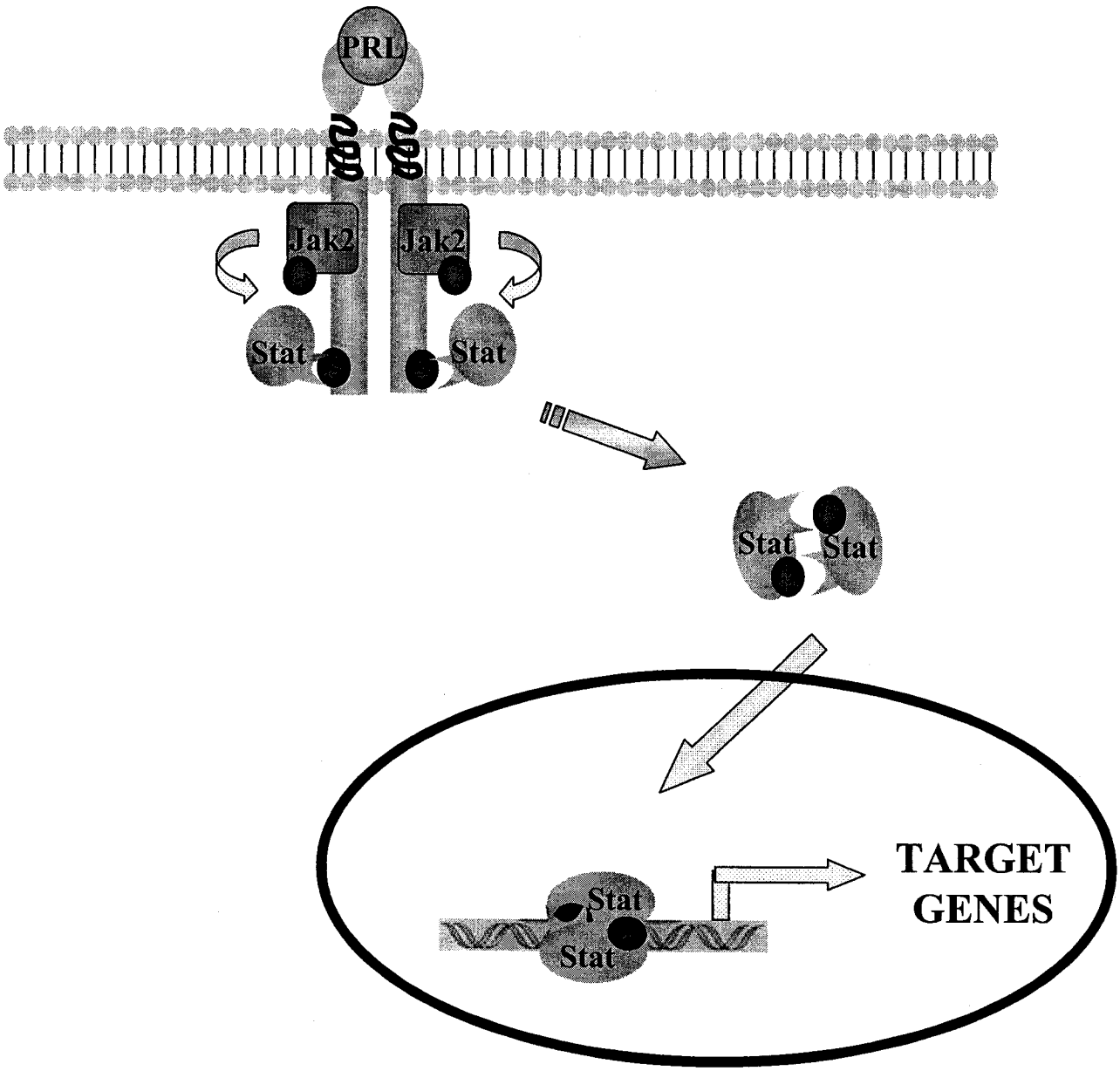
### 1.2. Jak-Stat signaling

The Jak-Stat pathway has emerged as the main transducer of the PRL signal. PRL signaling commences with ligand binding to the PRLR, Figure 4. Because the receptor lacks any intrinsic kinase domain it utilizes the cytoplasmic tyrosine kinase, Janus kinase-2 (Jak2) to transduce its signal[330]. Jak2 is constitutively associated to the PRLR. PRL induction of PRLR oligomerization brings two Jak2 molecules in close proximity so they may transphosphorylate each other[331]. Once activated, Jak2 tyrosine phosphorylates the receptor[332] allowing for recruitment of SH2-domain containing proteins such as the transcription factor: signal transducer and activator of transcription (Stat) family[333]. Jak2 subsequently phosphorylates Stat5 on a conserved tyrosine residue (Tyr694) which leads to receptor dissociation and dimerization of the Stat5 molecules[334]. Stat5 dimers then translocate into the nucleus and bind to a specific sequence known as the γ-interferon-activated sequence (GAS) motif. Sequences containing the GAS motif

include those expressed in milk protein genes. Stat5 exists in two isoforms, Stat5a and Stat5b. The two unique isoforms are encoded by separate genes and share approximately a 90% homology. Stat5a was originally identified as a mammary gland factor (MGF) in sheep mammary glands activated by PRL[335].

A definite role for Stat5 in mammary gland development was demonstrated via gene targeting. The Stat5a knockouts have impaired lactation and lobuloalveolar development[336]. Furthermore, they display a reduction in alveoli number and a failure of alveoli functional differentiation[336]. The Stat5b knockout phenotype also exhibits diminished lobuloalveolar development but to a lesser extent than Stat5a. In contrast, a more dramatic phenotype occurs in the Stat5a/b double knockout in comparison to that of the single knockouts[337]. Therefore, knockout studies done thus far suggest that Stat5 plays a vital role in alveolar proliferation, differentiation and expansion in the mammary gland.

# Figure 4. Prolactin Induced JAK-STAT Signaling



### 1.3. Stat5 structure

Stat5 belongs to the Stat family of latent cytoplasmic proteins that range from a molecular weight of 90 to 100 kDa. The Stat family contains 8 members; Stat1 (α and β), Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6. Stats contain six domains that are functionally and structurally conserved. More specifically, they include the amino-terminal, coiled-coil, DNA-binding, linker, SH2, and transactivation domains. The amino-terminal domain is the major site of protein-protein interaction. Furthermore, it is implicated in both Stat nuclear translocation[338] and interaction with nuclear co-activators such as CBP/p300[339]. The coiled-coil domain is made up of four α-helices forming a hydrophilic surface allowing for protein interaction[340, 341]. Moreover, the coiled-coil domain is involved in receptor binding, activation and nuclear export[342, 343]. The linker domain separates the DNA-binding domain with the SH2 domain and is able to mediate transcription[344]. The SH2 domain represents the most highly conserved Stat motif[333] and is essential for receptor recognition of the phosphotyrosine docking sites[345, 346 347]. Furthermore the SH2 domain is required for Stat dimerization and determines the specificity of dimer formation[346, 348]. The carboxyl-terminus transactivation domain is the least conserved and thus most variable domain amongst the Stats[349]. Truncated isoforms of Stats that lack the transactivation domain are not able to drive transcription[350]. This domain mediates the interaction of a variety of nuclear co-activators facilitating chromatin modification and transcriptional activity[351]. Stat5 transactivation domain interacts directly with CBP/p300 and enhances PRL mediated transcriptional activity[352]. Importantly, serine residues on the transactivation domain

have also been shown to regulate transcriptional activity[353, 354]. For example, serine phosphorylation of Stat5 has been to shown to promote its stability[355].

### 1.4. Regulation of Stat5 activity

Downregulation of Stat5 activity exists at multiple levels of the signaling cascade such as the receptor, Jak2 and Stat5 itself. Various phosphatases such as the SH2 domain-containing tyrosine phosphatase (Shp-2)[356, 357], cytoplasmic protein-tyrosine phosphatase 1B (PTP1B)[358], serine/threonine protein phosphatase 2A (PTP2A)[359] and T-cell protein-tyrosine phosphatase (TC-PTP)[360] are capable of regulating Stat5 activity. For example, TC-PTP dephosphorylates and deactivates PRL induced Stat5 in the nucleus[360]. Stat5 activity is also regulated by the protein inhibitor of activated Stats (PIAS) family[361]. PIAS function as small ubiquitin-related modifier 1-tethering protein ligases which may regulate localization, stability, and activity of a variety of proteins. For example, PIAS3 is capable of specifically inhibiting Stat5. Suppressors of cytokine signaling (SOCS) proteins also possess the capability to modulate Stat5 activity.

Eight members of this family exist including SOCS1-7 and cytokine-inducible SH2 protein (CIS). These proteins are characterized by a central SH2 domain and a unique carboxyl terminus motif called the SOCS box. This unique box is implicated specifically in the binding machinery of the ubiquitin-proteosomal system. Amongst other cytokines, PRL is able to induce expression of SOCS proteins[362]. As such, SOCS-3 is able to inhibit PRL-induced Stat5 activation. Furthermore, SOCS-1 interacts directly with Jak2 and subsequently targets it for degradation[363]. Another level of Stat5

regulation is through the ubiquitin proteosomal degradation system. More specifically, Stat5 ubiquitination and targeted degradation downregulates Jak-Stat signaling[364].

### 1.5. Stat5 gene regulation

The main effect of activated Stat5 lies in its ability to bind DNA and induce transcription of target genes. Stat5 dimers recognize and bind to a canonical gamma interferon (IFN-γ)-activated (GAS)-like site with a general palindromic sequence of 8 base pairs (TTCNNNGA). Stat5's capacity to activate transcription is determined by the nuclear co-activators it recruits and their ability for basic transcriptional machinery interaction and chromatin modification. Stat5 has been shown to interact with numerous transcription factors and co-regulators such as the glucocorticoid receptor[365], centrosomal P4.1-associated protein (CPAP)[366], N-myc interactor (Nmi)[367], Stat5 and receptor corepressor (SMRT)[368], p100[369], ERK[370] and CBP/p300[352], all of which act as either co-activators or co-repressors to Stat5 mediated gene modulation. For example, interaction of Stat5 with the glucocorticoid receptor protects Stat5 from inactivation by dephosphorylation, and enhances its activation [365, 371]. CBP/p300 bind the transactivation domain of Stat5 and is essential for its activation[339, 372-374].

In response to PRL induced activation of Stat5, milk gene proteins such as βcasein, whey acidic protein, and β lactoglobulin are upregulated[375]. Stat5 is also necessary in inducing expression of the cell cycle regulator cyclin D1 by PRL in mammary epithelial cells[376].

## 2. PRL Jak-Stat pathway in breast cancer

Whereas the role of PRL in mammary gland development, growth, and differentiation is obvious, the involvement of PRL in the induction and progression of human breast cancer is subject of great debate. In humans, some studies claim that high PRL levels correlate with the risk of breast cancer in postmenopausal women, while others claim that there is no correlation[377-379]. In rodents however, evidence of an association between increased PRL secretion and tumorigenesis via chemical carcinogens exists[380]. Furthermore, mammary tumors develop in transgenic mice overexpressing PRL[380]. Both PRL and its receptor are present in breast cancer cell lines and mammary tumors, creating an autocrine-paracrine effect of PRL[381, 382]. Interestingly, the use of various PRL blocking techniques interferes with the autocrine-paracrine loop in the human breast cancer cell line T47D and subsequently suppresses cellular proliferation[381, 383, 384]. In contrast, PRL has also been implicated as an inhibitor of breast cancer due to its ability to upregulate the tumor suppressor Breast Cancer 1 (BRCA1) in breast cancer cells[385].

The exact role for Stat5 in the growth and survival of mammary tumor cells remains to be elucidated. Importantly, in leukemias and lymphomas, Stat5a and Stat5b are constitutively phosphorylated[386, 387]. Moreover, a high proportion of human breast cancers have reported Stat5 to be activated and localized in the nucleus[388]. At a molecular level, the aforementioned observations may be explained by Stat5's ability to regulate cell cycle progression and survival. More specifically, this occurs through regulation of the cell cycle regulatory proteins cyclin D1 and the anti-apoptotic factor Bcl-$X_L$ [376, 389]. However, no reports link regulation of these proteins by Stat5 to breast

cancer development. Clinically, patients whose tumors displayed elevated levels of active Stat5 resulted in a favorable prognosis[390].

### 3. PRL Jak-Stat pathway in breast cancer metastasis

The major cause of death in patients with breast cancer is due to metastasis. PRL has recently been defined as an invasion suppressor hormone in breast cancer cells[391]. More specifically, PRL activation of Jak2 plays a critical role in the inhibition of breast cancer cell invasion capacity. This vital inhibition may stem from a negative regulation of both the MAPK (ERK1/2) and Smad pathways[391]. This is further supported by Stat5's capability to suppress breast cancer cell invasion[392]. Excitingly, Stat5's invasive suppression potential is the biological link where elevated Stat5 activation leads to improved breast cancer patient prognosis[390].

### 4. Purpose of this study

In order to elucidate the effect of activin and TGFβ signaling on mammary gland growth and differentiation, we looked at activin/TGFβ induced Smad crosstalk with prolactin induced Jak/Stat pathway.

In this study, we define a novel crosstalk mechanism in which activin/TGFβ induced Smad signaling antagonizes one of the key players involved in mammary gland growth and differentiaion, Stat5.

## ABSTRACT

Two critical signaling cascades within the mammary epithelial tissue are the TGFβ/Smad and the Jak/Stat pathway. Interestingly, opposing physiological effects between these two signaling pathways are prominent in the regulation of mammary gland development and breast carcinogenesis. However, the exact nature of the biological network existing between the Smad and Stat signal transduction pathways has remained elusive. Our results identify a novel regulatory crosstalk mechanism by which activin/TGFβ-induced Smad signaling acts to antagonize prolactin-mediated Jak/Stat signaling. While these two pathways appear to signal in a linear manner in the cytoplasm they converge to antagonize their effects on target gene expression within the nucleus. Our data show that ligand-induced activation of Smad2, 3 and 4 leads to a direct inhibition of Stat5 transactivation, leading to inhibition of Stat5-mediated target gene expression, such as βcasein and cyclin D1, thereby blocking vital processes for mammary gland growth and differentiation. Finally, we unveiled the mechanism by which these two signaling cascades antagonize their effects and found that activated Smads inhibit Stat5 association with its co-activator CBP, thus blocking Stat5 transactivation of its target genes.

## INTRODUCTION

Mammary gland growth and differentiation is a complex process regulated by steroids, polypeptide hormones, and growth factors. Among those, prolactin and TGFβ family members, play a major role in the regulation mammary gland development. Prolactin is required for lobuloalveolar formation of the mammary ducts during pregnancy and lactation. On the other hand, TGFβ has an opposite effect, inducing apoptosis during mammary gland involution and inhibiting milk protein expression [211, 393]. Transgenic mouse models developed to investigate the *in vivo* response to TGFβ indicate that TGFβ inhibits proliferation in response to the hormones that regulate mammary gland development[264, 394, 395]. The effect of activin on the development of the mammary gland stems from the activin bB subunit knockout mouse. Deletion of the activin βb subunit, through ablation of three of the dimeric β molecules (activin B, activin AB and inhibin B), results in mice with the phenotype of incomplete mammary development and an absence of lactation[262].

Prolactin signal transduction is induced by formation of a homodimeric complex of two molecules of prolactin receptor (PRLR), which lack intrinsic kinase activity but are constitutively associated with the intracellular tyrosine kinase Jak2 [332, 396, 397]. Prolactin-induced receptor homodimerization brings their associated Jak2 molecules in close proximity allowing for their transactivation as well as phosphorylation of the PRLR [331]. These phospho-tyrosine residues on both Jak2 and the PRLR then create docking sites for the recruitment and activation of the transcription factor Stat5 via its SH2 domain [398]. Once phosphorylated, Stat5 homodimerizes and translocates into the nucleus to bind

response elements on target gene promoters, such as those encoding milk gene proteins and cell growth regulators [399, 376]. The importance of Stat5a in the mammary gland development is further highlighted by the Stat5a knockout mouse which show no lobuloalveolar development during pregnancy and a complete absence of lactation [336]. The physiological role and importance of Stat5 has been subsequently extended, as many cytokines including growth hormone, erythropoietin, thrombopoietin, granulocyte-macrophage-colony-stimulating factor and most interleukins also signal through Stat5 [400-405].

TGFβ family members transduce their signals by binding to serine/threonine kinase receptors (type I and type II). Following ligand binding to the type II receptor, the type I receptor is recruited to the complex and transphosphorylated by the type II kinase domain. This in turn will lead to activation and phosphorylation of the Smad proteins, the main downstream effector molecules for these receptors. The activated type I receptor phosphorylates receptor-regulated Smads (R-Smads), Smad2 and 3, on their carboxyl-terminal serine residues (SxS motif). Phosphorylation allows for heterotrimerization of two phosphorylated R-Smad subunits with one common partner Smad4 subunit [406-408]. Subsequently, the Smad heterotrimer translocates into the nucleus where it associates with various transcription factors, co-activators or co-repressors to regulate expression of target genes in a cell and tissue specific manner [409]. Activin and TGFβ share a common signaling pathway through activation of Smad2, 3 and 4. While TGFβ inhibits milk protein synthesis and secretion in explants isolated from mid-pregnant mice [253, 410, 411], no role for activin in the regulation of lactation has yet been

established. In this study, we show that both activin and TGFβ, through the Smad pathway, potently regulate this process by antagonizing prolactin/Stat5 target gene expression.

The Stat5 and Smad pathways play a critical role in regulating mammary gland development. These two signaling pathways oppose their effects and the resulting balance between these two antagonistic pathways allows for proper regulation of mammary epithelial cell growth and differentiation. However, the mechanisms by which these two signaling cascades regulate each other remain unknown. This led us to investigate the molecular crosstalk mechanisms existing between the Smad and Stat signal transduction pathways. We identified a novel regulatory crosstalk mechanism by which activin/TGFβ-induced Smad signaling led to direct inhibition of Stat5-mediated gene transcription regulating differentiation and growth. Furthermore, we showed that activin/TGFβ signaling greatly reduced Stat5 interaction with its co-activator CBP, thereby inhibiting Stat5 transactivation capacity.

## MATERIAL AND METHODS

**Reagents:** Ovine prolactin (Sigma-Aldrich), human TGFβ1 (PeproTech, Inc), Activin A (Dr. Y Eto and Ajinomoto Co.). Monoclonal antibodies to STAT5 (BD Transduction Laboratories), to phospho-Stat5Y-694 (Intermedico), to cyclin D1 (NeoMarkers, Fremont CA), to Stat5A (Intermedico), to phosphoSmad2 (Cell Signaling), to hemagglutin (HA) (Roche Diagnostics), to phosphoSmad3 (Chemicon), to Smad2/3 (Santa Cruz), to βtubulin (Sigma), to Jak2 (Upstate Biotechnologies); goat anti-mouse horseradish peroxidase (Santa Cruz), to ERK1/2 (Cell Signaling) goat anti-rabbit horseradish peroxidase (Sigma-Aldrich); Lumi-light plus kit (Roche Diagnostics). Actinomycin D (Sigma-Aldrich). Anti-HA affinity matrix clone 3F10 (Roche Diagnostics).

**Plasmid constructs:** Expression plasmid encoding MGF-Stat5 (pXM-MGF/STAT5) and the rat βcasein promoter (-344/-1) were obtained from Dr. Bernd Groner (Institute for Biomedical Research, Frankfurt am Main, Germany). The promoter construct 3TPluc was obtained from Dr. Joan Massague (Howard Hughes Medical Institute, Memorial Sloan-Kettering). The promoter construct XPAL7 was obtained from Dr. Bernard Turcotte (Hormones and Cancer Research Unit, Montreal, Canada). Cytomegalovirus expression plasmid pR/CMV vector (Invitrogen) containing cDNA encoding for the PRLR long form was obtained from Dr. Paul Kelly (INSERM Endocrinologie Moleculaire, Faculté de Médecine Necker, Paris, France). GAL4-STAT5a was obtained form Dr. Bernard Callus. The cyclin D1 (D1-944) luciferase promoter construct was generously provided by Dr. Linda Schuler (Department of

Comparative Biosciences, University of Wisconsin-Madison, Madison, USA). Dominant negative Smad2 (DNSmad2) and dominant negative Smad3 (DNSmad3) were generated as previously described[412]. Activin type I receptor mutant (ALK4mL45) was generated as previously described[413]. The 5XSTAT5-luc reporter was made by cloning a double-stranded oligonucleotide (5'-CTAGCTGTGGACTTCTTGGAATTAAGGGACTTTTGTGTGGACTTCTTGGAATTAAGGGACTTTTGTGTGGACTTCTTGGAATTAAGGGACTTTTGTGTGGACTTCTTGGAATTAAGGGACTTTTG TGTGGACTTCTTGGAATTAAGGGACTTTTGC-3'), containing 5X Stat5 binding sites from the bovine βcasein promoter, into pTA-luc (Clontech Laboratories).

**Cell Culture and transfections:** HC11 cells (obtained from Dr. Nancy Hynes, Friedrich Miescher Institute, Basel, Switzerland and Dr. Bernd Groner, Institute for Biomedical Research, Frankfurt am Main, Germany) were cultured for 2 days in RPMI 1640 supplemented with 10% fetal bovine serum, 10ng/ml epidermal growth factor, 5μg/ml insulin, 50units/ml penicillin/streptomycin. At confluency HC11 cells were induced for 2 days RPMI 1640 supplemented with 10% fetal bovine serum, 1μM hydrocortisone, 5μg/ml insulin, 50units/ml penicillin/streptomycin. Cells were starved overnight in RPMI supplemented 0.5mg/ml fetuin and 10 μg/ml transferrin and stimulated with 5μg/ml ovine prolactin, 1μM hydrocortisone and 5μg/ml insulin (HIP) to produce βcasein. Mouse embryonic fibroblasts (MEF, MEFSmad4(-/-)) were obtained from Dr. Christian Sirard, Brain Tumor Research Center, McGill University, Montreal,

Canada. CHO and MEF cells were maintained in 10% fetal bovine serum, 50units/ml penicillin/streptomycin in DMEM.

**Cell lysis and Western blotting:** Total cell extracts were collected as follows. Cells were lysed in 300 µl of lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10% (v/v) glycerol, 0.5% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, 5 mg/ml aprotinin) for 5 min at 4 °C. The lysates were then centrifuged at 14,000 rpm for 10 min at 4 °C.

Cytoplasmic and nuclear extracts were prepared as follows. The cells were lysed with a hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM $Na_3VO_4$, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, and 2 µg/ml leupeptin) and vortexed for 1 min. Cells were pelleted at 14,000 rpm for 15 min at 4 °C, and the supernatant was collected (cytoplasmic fraction). The pellet was then washed 3× with phosphate-buffered saline and lysed with a high salt buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM $Na_3VO_4$, 20 mM NaF, 5 µg/ml aprotinin, and 2 µg/ml leupeptin). Vortexed at 4 C for 30 min, centrifuged at 14,000 rpm for 15 min at 4 °C and the supernatant was collected (nuclear fraction).

Cell extracts were separated on polyacrylamide gels, transferred onto nitrocellulose and incubated with specific antibodies as indicated. Immunoreactivity was revealed by chemiluminescence (Lumi-Light Plus Western Blotting substrate, Roche) according to

the manufacturer's instructions and measured using an Alpha Innotech Fluorochem Imaging system (Packard Canberra).

**Northern blot analysis:** HC11 cells were cultured to differentiate and then treated or not with HIP, 0.5nM Activin A or both. Total RNA was extracted using Trizol (Invitrogen). Each sample (20μg) was then separated on agarose gels (1% agarose in 0.04M 3-(N-morpholino)propanesulfonic acid; 0.01M sodium acetate; 10mM EDTA, pH 8.0; and 2.5M formaldehyde) and transferred to nylon membranes. A probe for mouse βcasein was labeled using the Random Priming Kit (Roche) and added to the hybridization solution (0.5 NaPO4, pH 7.2, 1mM EDTA, pH 8.0, 7% SDS, 1% BSA, and 200mg/ml salmon sperm DNA). Results were revealed using a phosphorimager Cyclone Storage Phosphorscreen (Packard Canberra).

**RT PCR:** Differentiatied HC11 cells were treated for 24 hours with HIP and subsequently with or without Actinomycin D, Actinomycin D and 0.5 nM activin or Actinomycin D and 100pM TGFb from 0 to 24 hours. Total RNA was extracted using Trizol reagents (Invitrogen). Reverse transcription of total cellular RNA using random primers was carried out using Superscript Reverse Transcriptase (Invitrogen) as per the manufacturer's instruction. Subsequently, amplification of cDNA to obtain products for βcasein and GAPDH was performed. The PCR conditions were as follows: 30 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 1 min 30 s).

**EMSA:** EMSA were performed as previously described[414]. 6μg of nuclear extracts were incubated with the Stat5 binding site from the bovine βcasein promoter (5'-

AGATTTCTAGGAATTCAATCC-3')[335] (20,000 cpm) for 30 min on ice in 20 µl of electrophoretic mobility shift assay (EMSA) buffer (10 mM HEPES (pH 7.6), 2 mM $NaHPO_4$, 0.25 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 80 mM KCl, 2% glycerol, 100-µg/ml poly(dI-dC)). Supershift experiments were performed by adding anti-Stat5a 30 min prior to the binding reaction at a dilution of 1:500. A 4% polyacrylamide gel that was prerun for 2 h at 200 V in 0.25× TBE buffer (22.5 mM Tris-borate (pH 8.0), 0.5 mM EDTA). The samples were run on gel for 1 h at 200 V in 0.25xTBE at room temperature. The gels were dried and revealed using a phosphorimager Cyclone Storage Phosphorscreen (Packard Canberra).

**Luciferase assays:** CHO and MEFs were transciently co-transfected with 2 µg of different promoter constructs (3TP-luc, βcaseinlux, 5XSTAT5-luc, pXPAL7) and with varying combinations and concentrations of cDNAs encoding PRLR, MGF-STAT5, Smad2, Smad3, Smad4, GAL4-STAT5a, DNSmad2, DNSmad3, ALK4, ALK4mL45 , and the βgalactosidase and 3TP-Luc gene reporter construct. CHO cells were transfected using lipofectamine 2000 reagent as per the manufacturers instructions (Invitrogen) and MEFs were transfected with lipofectamine Plus as per the manufacturers instructions (Invitrogen). Luciferase activity was measured (EG&G Berthold Luminometer) and normalized to the relative βgalactosidase activity.

**Statistical Analysis:** Results are expressed as mean ± SD of three or more separate independent experiments. Statistical analysis was assessed by one-way ANOVA or the unpaired *t* test, as indicated in figure legends, using GraphPad Prism 4 software (GraphPad Software, Inc.). Statistical analyses were meant to compare fold induction

(percentage of control) of TGFβ/activin-treated samples among themselves within each experiment. Additional post-ANOVA tests were performed when necessary to compare all data with HIP-treated control Bonferroni's Multiple Comparison test GraphPad Prism. For all statistical analyses and tests, a p value $< 0.05$ was considered significant and is indicated on the top of the error bars by an asterisk.

## RESULTS

**Activin and TGFβ inhibit lactogenic hormone-induced milk gene:** The signals that regulate mammary gland development also coincide with those that are involved in milk protein production [415]. Originally derived from a mammary epithelial cell line isolated from mid-pregnant BALB/c mouse mammary gland tissue, HC11 cells can be differentiated with lactogenic hormone stimulation to synthesize the milk protein βcasein [416, 417]. HC11 cells have often been used as a model system to study mammary gland differentiation [399].

To investigate the role of activin/TGFβ on mammary gland differentiation we examined the effects of these two growth factors on the regulation of expression of the milk protein βcasein. HC11 cells were differentiated and subsequently treated or not with either prolactin, activin, or a combination of both for different periods of time and βcasein mRNA level was assessed using Northern blot analysis. As shown in Fig.1a, prolactin induced a strong up-regulation of βcasein mRNA levels at all time points analyzed while activin alone had no effect. However, when cells were treated with both activin and prolactin, simultaneously, βcasein mRNA levels were strongly suppressed (Fig. 1a). The same results were obtained in cells treated with TGFβ (data not shown).

To then investigate whether the decrease in prolactin-induced βcasein mRNA levels upon activin treatment was followed by a decrease in βcasein protein levels, HC11 cells were treated or not with prolactin, activin or a combination of both factors and total cell lysates were analyzed by Western blot using an anti-βcasein antiserum. As shown in

Fig.1b (upper panel), while prolactin significantly increased βcasein protein levels, this effect was almost completely blocked in the presence of activin. Reprobing of the membrane with an anti-Erk1/2 antibody showed equal loading of proteins (Fig.1b, lower panel). Previous studies have shown that *in vitro* optimal Stat5-mediated βcasein gene expression is achieved upon treatment of mammary epithelial cells with a combination of lactogenic hormones containing glucocorticoid (such as hydrocortisone), insulin and prolactin[418, 419]. As such, we treated differentiated HC11 cells with hydrocortisone, insulin and prolactin (HIP) for 24 hours, in either the presence or absence of TGFβ or activin and subsequently examined the effect upon βcasein protein expression. Whereas HIP induced a robust increase in βcasein protein levels, both activin and TGFβ completely inhibited its expression (Fig.1c, upper panel). The membrane was stripped and reprobed with anti-Erk1/2 antibody to show equal loading of the proteins (Fig.1c, lower panel). Together these results indicate that activin and TGFβ are strong inhibitors of prolactin or HIP-mediated βcasein gene expression in mammary epithelial cells, suggesting the existence of a direct cross-talk mechanism between these two growth factor family signaling pathways.

**Jak/Stat Expression Levels and βcasein Protein Stability are not affected by Activin/TGFβ:** To rule out that the inhibitory activin/TGFβ effect observed on prolactin-induced βcasein expression was merely due to a change in Jak or Stat expression level we examined the expression of Jak2 and Stat5 in HC11 cells upon activin/TGFβ treatment from 4 to 24 hours. As shown in Fig.1d, we observed no change

in their expression, indicating that activin/TGFβ regulation of βcasein expression is not a result of changes in Jak2 or Stat5 expression levels.

Lactogenic hormone treatment can augment the rate of βcasein mRNA transcription two to four fold. However, this is not sufficient to explain the drastic increase in βcasein mRNA which can rise seventeen to twenty five fold above basal levels [420]. This net increase is in fact due to a direct transcriptional activation of the βcasein gene coupled to stabilization of βcasein mRNA [421]. To then examine whether activin/TGFβ could also affect βcasein mRNA stability, HC11 cells were treated for 24 hours with lactogenic hormones (HIP) to increase βcasein mRNA levels and subsequently treated with the transcriptional inhibitor actinomycin D. βcasein mRNA levels were then examined by RT PCR analysis. As shown in Fig.2 (left panel), in control HC11 cells treated with actinomycin D alone, we observed a significant decrease in βcasein mRNA levels after 16 hrs, while the levels remained constant at earlier time points. Activin (middle panel) or TGFβ (right panel) treatment of the cells did not affect βcasein mRNA stability as degradation rate was identical to that observed for the control cells (left panel). GAPDH mRNA levels were analyzed in parallel as a negative control (Fig.2). These results indicate that activin/TGFβ do not affect βcasein mRNA stability but rather act at the transcriptional level.

**Activin/TGFβ Does Not Affect Lactogenic Hormone Induced Phosphorylation and Nuclear Translocation of Stat5:** Following phosphorylation by the Jak2 kinase, Stat5 rapidly accumulates in the nucleus to regulate transcription of target genes. To determine

whether activin/TGFβ signaling could affect Stat5 phosphorylation and nuclear translocation, HC11 cells were treated or not with HIP in the presence or absence of activin for 0 to 60 min. Cytoplasmic and nuclear fractions were purified and analyzed by Western blot analysis using a specific phospho-Stat5 antibody. As shown in Fig.3a (upper panel), HIP rapidly induced Stat5 phosphorylation and this effect was unaltered by activin treatment. Equal loading of the proteins was assessed by reprobing the membrane with an anti-Stat5 antibody (Fig.3a, bottom panel). Similarly, Stat5 nuclear translocation in response to HIP treatment was not antagonized by activin (Fig.3b). The nuclear fraction membranes were reprobed with an anti-TBP for equal loading (Fig.3b, lower panel). Proper activin signaling in these cells was verified by reprobing of the membrane with a phospho-Smad3 antibody. As shown in Fig.3c, activin rapidly induced Smad3 phosphorylation in a time dependent manner. These results indicate that activin does not affect HIP-induced phosphorylation and nuclear accumulation of Stat5 and suggests that the antagonistic effect observed for activin on HIP-induced βcasein expression takes place at a step further downstream within the nucleus.

**Activin/TGFβ Treatment does not affect Stat5 binding to the βcasein Promoter:** βcasein gene expression is mediated through direct binding of Stat5 to the βcasein gene promoter. We then investigated whether activin/TGFβ could interfere with Stat5 DNA binding, using electromobility shift assay (EMSA). HC11 cells were treated from 0 to 120 minutes with HIP, activin or both, nuclear fractions were isolated and EMSA was performed using the Stat5 binding site (TTCNNNGAA) from the βcasein promoter as a

probe. As illustrated in Fig.4a, HIP induced a very rapid and transient DNA/protein complex formation while activin alone had no effect.

Interestingly, co-treatment of the cells with HIP and activin did not affect HIP-induced Stat5 DNA binding. The same results were observed in response to TGFβ (Fig.4b). The presence of Stat5 in the HIP-induced DNA-protein complex was ensured by supershift experiment using a polyclonal antibody against Stat5. As shown in Fig.4b, (lanes 5, 6), addition of anti-Stat5a resulted in a supershift of the complex in HIP-treated extracts. Together, these results indicate that the decrease of βcasein mRNA and protein levels by activin/TGFβ is not due to an inhibition of Stat5 binding to the βcasein promoter.

**Activin/TGFβ Inhibit Activation of the βcasein gene Promoter in response to lactogenic hormones:** In trying to understand the mechanism by which activin/TGFβ act to inhibit βcasein synthesis, we next investigated their effects at the level of transcription of the βcasein gene promoter. For this, HC11 cells stably expressing the βcasein gene promoter fused to the luciferase gene were treated from 4 to 24 hours with either lactogenic hormones, activin, TGFβ or a combination of both. As shown in Fig.5, whereas HIP increased βcasein luciferase activity in a time dependent manner, this effect was strongly inhibited by activin and TGFβ (Fig.5). Activin and TGFβ treatments alone did not affect βcasein luciferase activity (Fig.5). These results demonstrate that activin and TGFβ robustly block lactogenic hormone induction of the βcasein gene promoter activity.

**Stat5 Mediated Transcription is Blocked by Activin/TGFβ Treatment:** The βcasein gene promoter construct encompasses the proximal part of the promoter that includes Stat5 binding sites, but also contains regulatory regions for other transcription factors such as the CCAAT enhancer binding protein C/EBPβ, Oct1 [422-424]. To delineate if the changes in βcasein expression are a result of activin/TGFβ induced alterations in Stat5 specific activation of the βcasein gene promoter, we made an artificial promoter containing five tandem repeats of the Stat5 response elements fused to the luciferase gene (5XStat5-luc) and generated HC11 stable cell lines expressing this 5XStat5-luc (HC11-5XStat5-luc). As shown in Fig.6a, the three positive clones that were selected all strongly responded to the HIP treatment, as measured by the luciferase activity. Interestingly, while TGFβ alone had no effect on this reporter construct, it strongly inhibited HIP-induced 5XStat5 luciferase activity, demonstrating the TGFβ antagonizes Stat5-mediated transcriptional activity (Fig.6a).

To further extend our results to human mammary epithelial cells, we transfected MCF10A cells with the 5XStat5-luc reporter construct and examined the TGFβ response on HIP-induced Stat5 transactivation. As shown in Fig.6b, HIP treatment induced a strong increase of 5X-Stat5-luc promoter activity and this effect was also robustly antagonized by TGFβ. Together these results indicate that TGFβ signaling directly block HIP-induced Stat5 activation in both mouse and human mammary epithelial cells, thereby leading to inhibition of milk gene protein expression.

**Smad signaling blocks Stat5-induced gene transcription:** To then address the molecular mechanisms by which activin/TGFβ antagonizes Stat5 signaling, we next investigated the role of the Smad pathway, the main regulatory pathway downstream of activin/TGFβ. For this, CHO cells were co-transfected with the βcasein luciferase reporter construct, cDNAs encoding Stat5 and PRLR in the presence or absence of varying combinations of cDNAs encoding Smad2, Smad3, and Smad4. Cells were then stimulated overnight with HIP or TGFß alone or in combination. As shown in Fig.7a, TGFβ significantly repressed HIP-induced βcasein (left panel) and 5XStat5 (right panel) luciferase activity. Interestingly, overexpression of Smad3 alone or in combination with Smad4 significantly potentiated the TGFβ inhibitory effect on HIP-induced luciferase activity for both promoter constructs (Figs.7b, c). Furthermore, as shown in Fig.7c (middle and left panels), a similar result was obtained in response to activin and with overexpression of Smad2. Together, these data indicate that the Smad proteins play a critical role in mediating activin/TGFβ inhibition of lactogenic hormone-induced βcasein gene promoter activation.

Having demonstrated that overexpression of the Smads could potentiate activin/TGFβ mediated inhibition of prolactin/Stat5 βcasein gene promoter activation, we next examined the effect of blocking Smad signaling on the activin/TGFβ response. For this we used dominant negative Smad2 and dominant negative Smad3, in which the carboxyl-terminus serine residues were mutated to alanine and hence cannot be phosphorylated by the receptor [412]. Likewise, we also used a mutant form of the activin type I receptor ((ALK4mL45) in which the three critical residues for interaction site with

Smad2 and Smad3 (N265, D267 and N268 within the L45 loop of the receptor) were mutated to alanine. The resulting mutant receptor, fails to recruit the Smads and is unable to mediate activin signaling [412].

The efficiency of the dominant negative Smads (DNSmads) and the mutant receptor (ALK4mL45) was first assessed using the activin/TGFβ responsive promoter construct 3TPluc. As shown in Fig.7d, DN Smad2, DN Smad3 and the mutant receptor (ALK4mL45) all strongly inhibited activin-induced 3TPluc activity. We then assessed the effect of overexpressing DNSmad2, DNSmad3 or ALK4mL45 in CHO cells transfected with cDNAs encoding the PRLR, and Stat5, along with βcasein or 5XStat5-luc reporter constructs. Whereas in the control transfected cells HIP/activin treatment led to inhibition of βcasein-luc and 5XStat5-luc luciferase activity, overexpression of DNSmad2, DNSmad3 and ALK4mL45 completely reversed these effects clearly highlighting the important role played by the Smads in mediating the inhibitory effect of activin/TGFβ on Stat5 driven promoters (Fig.7e).

Smad signaling in response to activin/TGFβ is centrally controlled by the common partner Smad4. Hence to further demonstrate the involvement of Smad pathway in mediating activin/TGFβ inhibition of βcasein gene expression and Stat5 activation we used murine embryonic fibroblasts (MEFs) established from wild type or Smad4 knockout mice [425]. Inactivation of Smad4 expression has been shown to prevent activin/TGFβ signaling and target gene transcriptional activation [207, 425]. Wild-type and Smad4 null mutant MEFs were co-transfected with either βcasein promoter or 5XStat5

promoter, Stat5 and PRLR. Subsequently cells were treated or not with HIP, TGFβ or HIP/TGFβ. Although in the wild type MEFs, HIP-induced βcasein promoter and 5XStat5 luciferase activity was antagonized by TGFβ, this effect was completely abolished in the Smad4 deficient MEFs (Fig.7f). Furthermore, restoring Smad4 expression in the Smad4 null mutant MEFs also restored the TGFβ inhibitory effect on HIP-induced βcasein gene expression and Stat5 activation, clearly indicating the importance of the Smad pathway in repressing Stat5 mediated gene activation (Fig.7f).

Collectively these results demonstrate that the Smad pathway strongly suppresses Stat5-mediated gene activation. Furthermore we show that the Smad pathway is necessary in mediating activin/TGFβ antagonistic effect on Stat5 transcriptional activation.

**The Transactivation capacity of Stat5 is Repressed Potently by the Smads:** The transactivation domain of Stats is essential to drive transcription.[426]. To evaluate the involvement of activin/TGFβ signaling via the Smads on Stat5 transactivation ability we co-transfected CHO cells with a GAL4 responsive promoter fused to the luciferase gene (pXPAL7) with a chimeric construct containing the GAL4 DNA binding domain and the carboxyl-terminus Stat5 transactivation domain (GAL4-Stat5a) [427]. The cells were also transfected with or without increasing concentrations of Smad3/4. As shown in Fig.8, whereas TGFβ induced a 27% decrease in GAL4-Stat5a induced luciferase activity, increasing concentrations of Smad proteins led to a parallel increasing inhibition of the luciferase activity by TGFβ to reach 55% repression at the highest Smad concentration.

This finding demonstrates that Smad signaling efficiently blocks Stat5 transactivation ability leading to inhibition of milk gene protein production.

**Stat5 Target Gene Cyclin D1 is Inhibited by Activin/TGFβ:** In the mammary gland Stat5 regulates milk gene protein production and cell differentiation but also controls expression of genes involved in cell cycle progression and survival. Interestingly, prolactin/Stat5 and activin/TGFβ/Smad signaling have opposed effects on mammary epithelial cell growth and survival. Having shown that activin/TGFβ-induced Smad signaling inhibited Stat5 transactivation and βcasein production we next investigated whether Smad could also antagonize Stat5-mediated gene expression of other target genes, in particular those involved in Stat5-induced cell growth, such as cyclin D1. Indeed, prolactin signaling via Stat5 has been shown to induce cyclin D1 proximal promoter and increase in cyclin D1 protein expression in the mammary epithelial cells PRE-1 [376, 428]. To investigate whether the lactogenic stimulation (HIP) could also induce an increase in cyclin D1 protein expression in HC11 cells, we treated differentiated HC11 cells from 0 to 8 hours with HIP. As seen in Fig.9a, HIP induced a very rapid and transient increase in cyclin D1 protein levels peaking at 4 hours and returning back to basal levels at 8 hours. The membrane was then probed with anti-βtubulin as a loading control. We then sought to evaluate the effect of activin/TGFβ on HIP induced cyclin D1 protein levels. Differentiated HC11 cells were treated for 2 hours with or without HIP, TGFβ or both. Whereas HIP induced an increase in cyclin D1 protein, TGFβ was able to completely reverse HIP induction of cyclin D1 back to basal levels (Fig.9b). To then evaluate whether the TGFβ antagonistic effect was mediated at the transcriptional level,

we used the proximal cyclin D1 promoter fused to luciferase (cyclinD1-944), which has previously been shown to be activated by prolactin in a Stat5 dependent manner [376]. CHO cells were transfected with the cyclinD1-944 reporter construct, Stat5, PRLR and βgalactosidase cDNAs and stimulated overnight with HIP, TGFβ or both. As shown in Fig.9c, HIP treatment induced cyclin D1 gene promoter activity and this effect was significantly reversed in the presence of TGFβ.

Finally to evaluated the role of the Smad pathway in antagonizing Stat5-mediated cyclin D1 gene promoter activation. CHO cells were transiently co-transfected with cyclinD1-944, Stat5, PRLR, βgal with or without a combination of Smad2, Smad3 and Smad4. As shown in Fig.9d, in the absence of overexpressed Smad proteins TGFβ induced a 20% reversal effect of HIP-induced cyclin D1 gene promoter activity. Interestingly, overexpression of the Smad proteins resulted in a clear potentiation of the TGFβ antagonistic effect to approximately 50% reversal of HIP-induced cyclin D1 gene promoter activity (Fig.9d). These results demonstrate that activin/TGFβ-inhibition of Stat5-induced cyclin D1 protein expression requires the Smad pathway. In all, these findings reveal that another Stat5 target gene, cyclin D1, activation is repressed by activin/TGFβ mediated Smad signaling.

**Activin/TGFβ signaling reduces Stat5 interaction with CBP:** We next wanted to elucidate the mechanism by which activin/TGFβ-mediated Smad signaling blocked Stat5 transactivation of its target genes. It was previously shown that the co-activator CBP/p300 physically interacts with Stat5 to regulate Stat5-mediated gene expression [352].

In fact, CBP/p300 binds the transactivation domain of various Stat family members and is essential for their activation [339, 372-374]. Henceforth, to determine whether activin/TGFβ would reduce Stat5 interaction with CBP, CHO cells were transfected with PRLR, Stat5 and HA-tagged CBP. Cells were subsequently treated for 25 minutes with or without HIP, TGFβ or both, before being lysed and immunoprecipitated with an anti-HA antibody, affinity conjugated to matrix beads and revealed by western blot analysis with the anti-Stat5 antibody. As shown in Fig.10, HIP treatment of the cells induced increased association between HA-CBP and Stat5. However, when the cells were treated with TGFβ this interaction was significantly diminished. Equal level of protein levels were ensured by reprobing membranes with anti-HA and ant-Stat5 antibodies (Fig.10). Furthermore, total cell lysates were probed with anti-phosphoStat5 and anti-phosphoSmad3, to ensure proper ligand stimulation (Fig.10). These results indicate that activin/TGFβ inhibit Stat5 interaction with the co-activator CBP.

# DISCUSSION

Mammary gland growth and differentiation is regulated by an assembly of signaling networks in response to various hormones and growth factors and alterations within these signaling cascades represent underlying causes of a variety of human diseases, such as breast cancer. Interestingly, the final outcome of mammary epithelial cell behavior and biological responses are in fact controlled by the way these pathways interact with each other to form signaling networks, a mechanism referred as crosstalk. Two critical signaling cascades within the mammary epithelial tissue are the activin/TGFβ/Smad signaling and the Jak/Stat pathway. These two pathways oppose their physiological effects and are eminent in the regulation of mammary gland development and breast carcinogenesis. However, the exact nature of the biological network existing between the Smad and Stat signal transduction pathways has remained elusive. Our results underlined a novel crosstalk mechanism in which the intracellular mediators of activin/TGFβ signaling, the Smad proteins, inhibit Stat5 regulated gene expression. These antagonistic effects take place in the nucleus and we showed that while activin/TGFβ-mediated Smad signaling does not prevent Stat5 phosphorylation, translocation and DNA binding, it blocks Stat5 association with the co-activator CBP, thereby inhibiting Stat5 transactivation.

Crosstalk mechanisms between distinct signaling pathways are key in providing an integrated response and regulating homeostasis. A previous study indicated that interferon-γ signaling through the Jak/Stat pathway could downregulate TGFβ signaling [171]. In fact, the existence of a direct effect of the Jak/Stat pathway towards TGFβ

signaling is also supported by the fact that prolactin inhibits TGFβ-induced apoptosis in primary mammary epithelial cells. Inhibition of TGFβ-induced apoptosis is carried out by induction of the kinase Akt in a PI3K-dependent manner [182]. However, the reciprocal effect of the TGFβ/Smad signaling on Stat signaling has to date remained inconclusive. For instance, in mouse lymphocytes, TGFβ immunosuppressive effects are partly mediated through negative inhibition of IL-2 and IL-12 induced phosphorylation of Jak/Stat proteins [429, 430]. However, in human T cells and natural killer cells, TGFβ had no effect on IL-2 and IL-12 induced Jak/Stat phosphorylation [431]. Pregnant mice overexpressing the whey-acidic protein (WAP) promoter-driven transforming growth factorβ cDNA displayed increased apoptosis in pregnant and lactating mammary glands associated with decreased lactation [264, 265, 432]. Added to the fact that all three isoforms of TGFβ are upregulated during involution [211, 254, 433], it has been suggested that reduced milk protein expression may be due to a decrease in lobuloaveoli. However, our findings demonstrate that the TGFβ/Smad signaling pathway tightly regulate Jak/Stat signaling in mammary gland through a direct crosstalk mechanism leading to inhibition of Stat5 transactivation of its target genes by Smad signaling.

In this study, we show that both βcasein and cyclin D1 induced expression are regulated by activin/TGFβ signaling. Cyclin D1 is an important cell cycle regulator that controls lobuloalveolar proliferation. Furthermore, cyclin D1 knockout mice fail to undergo proper lobuloalveolar proliferation during pregnancy, a phenotype shared by PRL and PRLR knockout mice [399, 434, 435], consistent with the fact PRL regulates cyclin D1 gene promoter through Stat5 signaling [428]. TGFβ's antagonistic effect on lactogenic hormone

induction of cyclin D1, implicates TGFβ in a regulatory role of *in vivo* mammary gland development. This regulatory role allows for the differentiation phase of late pregnancy to occur by regulating proliferation of the lobuloaveoli. Moreover, activin/TGFβ Smad signaling inhibition on cyclin D1 suggests that the effect of activin/TGFβ Smad signaling is broad, potentially encompassing all Stat5 target genes and biological systems.

We observed that activin/TGFβ signaling significantly reduces Stat5 association with the co-activator CBP. Interestingly, CBP/p300 can bind Smad2, Smad3 and Smad4, and contribute to their full activation [436-438]. Thus, our data suggest that the Smads may compete with Stat5 for CBP/p300 binding, thereby inhibiting Stat5 transactivation ability. Such mechanism is reminiscent of Stat1α competition with Smad3 for CBP/p300 binding. Stat1α binding to CBP/p300 allows interferon-γ to block TGFβ induced transcription of type I collagen by inhibiting Smad activities[439]. The competition model by which the Smads are sequestering CBP/p300 away from Stat5 can potentially be applied to other Stat molecules where CBP/p300 binding is essential for their activation.

Our findings unveiled strong antagonistic crosstalk mechanism between the Smad and Stat signaling pathway in regulating mammary gland. The potent tumor suppressor and growth inhibitory effects of activin/TGFβ pathway are well established in mammary epithelial and breast cancer cells [440, 441]. This is consistent with an antagonistic role played by the Smad towards prolactin signaling which is known to induce proliferation and survival in breast cancer cells [376, 383, 442]. TGFβ also displays a dual role in cancer

where as the tumor progresses TGFβ acts to promote tumor metastasis [171]. Interestingly, prolactin also exhibit a dual role, as in later stages of tumor development prolactin acts as an invasion suppressor hormone [391]. Thus, once again our data are consistent with these observations and suggest that the antagonistic crosstalk mechanism existing between the two pathways is critical to mammary cell growth and differentiation, and also regulates breast cancer formation and tumor metastasis.

The importance of molecular crosstalk mechanisms and their final biological outcomes are emphasized in this paper. Understanding how two prominent pathways, the activin/TGFβ/Smad and prolactin Jak/Stat pathways act to antagonize their effects has provided insight into their relative contributions to mammary gland differentiation. Future studies may thus address whether they are applicable to other biological systems.

## ACKNOWLEDGEMENTS

We are thankful to Dr. J. Massague for the 3TP-luc construct, Dr. N. Hynes for the βcasein HC11 stable cell line, the polyclonal antibody to milk proteins, Dr. B. Groner for MGF-STAT5 (pXM-MGF/STAT5) and the rat βcasein promoter (-344/-1), Dr. B. Turcotte for XPAL7 promoter construct, Dr. P Kelly for the long form of the prolactin receptor (long PRLR), Dr. B Callus for GAL4-STAT5a, Dr. L. Schuler for the cyclin D1 promoter (D1-944), Dr. XF Yang for HA-CBP and Dr. Y. Eto and Ajinomoto Co., Inc., for activin A.

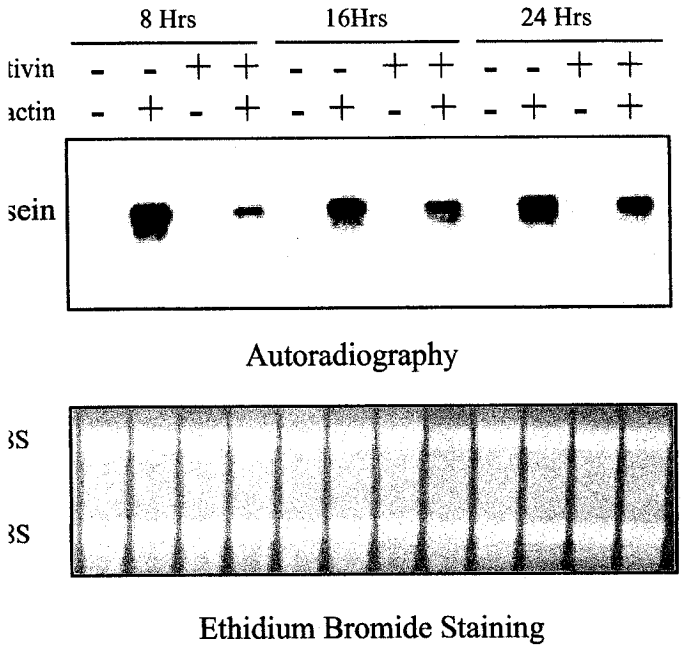
## FOOTNOTES

J.J.L. and S.A. are Research Scientist of the National Cancer Institute of Canada supported with funds provided by the Canadian Cancer Society, EC is a recipient of a McGill University Health Center and Department of Medicine at McGill University scholarship. JH is a recipient of studentship from the NCIC. This work was supported by a grant from the Canadian Institutes for Health Research (MOP-53141 to J.J.L.).

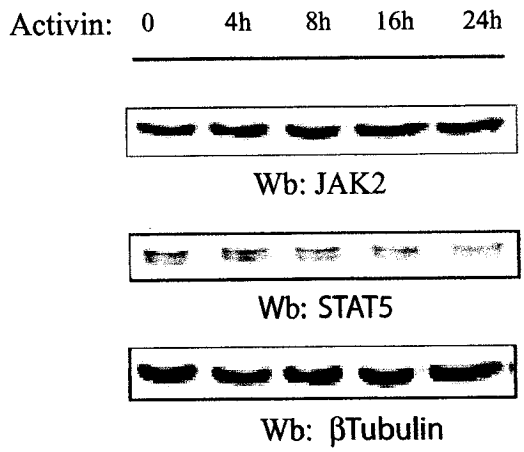
## FIGURES

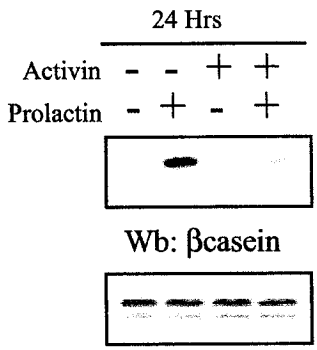
**Figure 1.** Activin/TGFβ inhibits lactogenic hormone induced βcasein expression in mouse mammary epithelial cells. (**A**) HC11 cells were differentiated and subsequently treated for 8, 16 and 24 hours with prolactin, activin or prolactin/activin, and total RNA was analyzed by Northern blot using a specific probe for βcasein (upper panel). Equal loading was assessed by ethidium bromide staining (lower panel). (**B**) Differentiated HC11 cells were treated with prolactin, activin, prolactin/activin for 24 hours and total cell lysates were analyzed by Western blot assay using a specific antibody against βcasein (upper panel) or ERK1/2 (lower panel) as a loading control. (**C**) Differentiated HC11 cells were treated with or without HIP, activin, HIP/activin, TGFβ, HIP/TGFβ for 24 hours and total cell lysates were analyzed by Western blot assay using a specific antibody against βcasein (upper panel) or ERK1/2 (lower panel) as a loading control. (**D**) HC11 cells were treated with activin from 0, 4, 8, 16 and 24 hours and total cells lysates were analyzed by Western blot using specific antibodies against Jak2 (upper panel), Stat5 (middle panel) and βtubulin (lower panel).

A




D



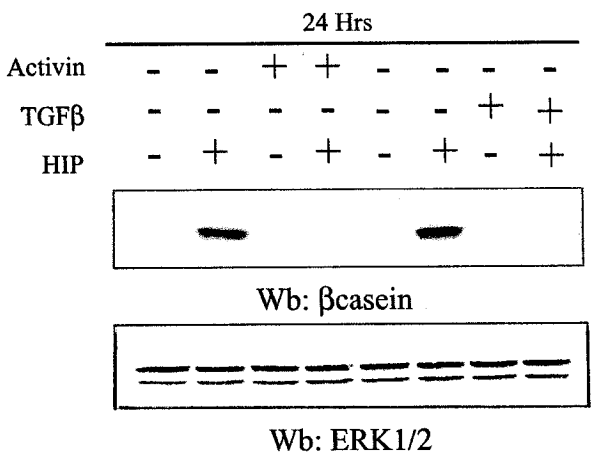




**Fig. 1Activin/TGFβ inhibits lactogenic hormone induced βcasein expression in mouse mammary epithelial cells**

**Figure 2. Activin/TGFβ do not affect milk protein mRNA stability.**

Following treatment for 24 with HIP, differentiated HC11 cells were treated with either actinomycin D, actinomycin D/activin or actinomycin D/TGFβ. Cells were then collected from 0 to 24 hours and total RNA was isolated. Reverse transcription reactions were performed using random primers and cDNAs were amplified for 30 cycles using specific oligonucleotides to βcasein and GAPDH as a control.

**Figure 3. Stat5 phosphorylation and translocation to the nucleus is unaffected by activin/TGFβ signaling.**

**(A and B)** HC11 cells were treated with HIP or HIP/activin from 0 to 60 minutes and cytoplasmic and nuclear fractions were isolated. Western blots were performed using specific antibodies against phosphoStat5, Stat5, and TBP. **(C)** HC11 cells were treated for the indicated time periods with activin or HIP/activin. Nuclear fractions were analyzed by Western blot using specific antibodies against phosphoSmad3 and TBP.

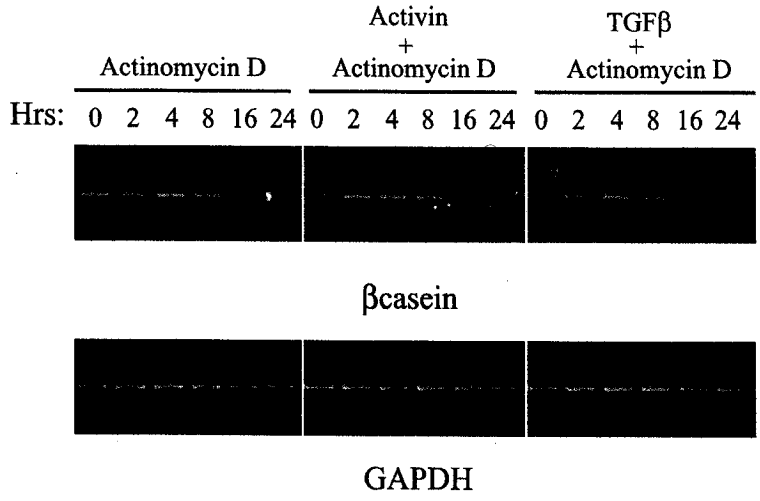



**Fig. 2 Activin/TGFβ do not effect milk protein mRNA stability**

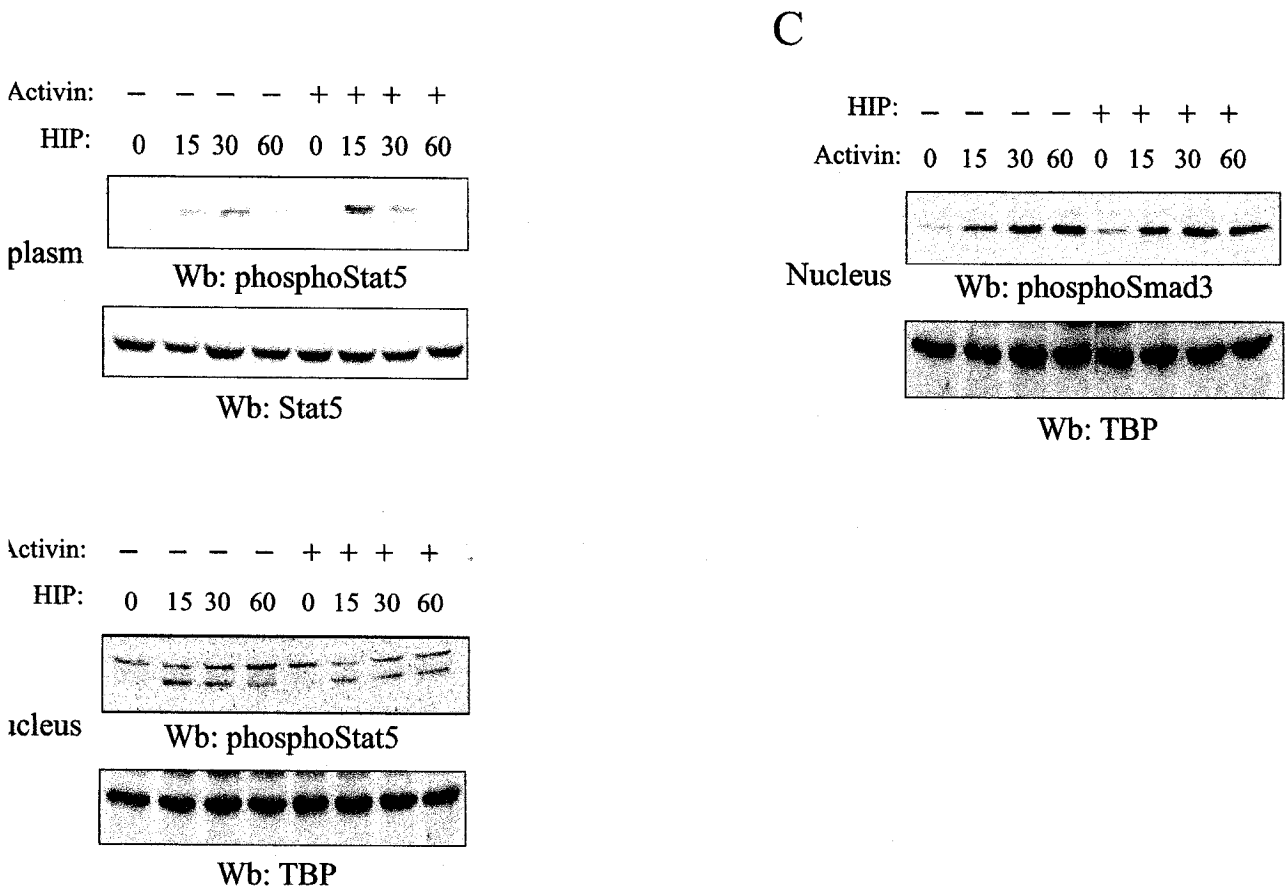
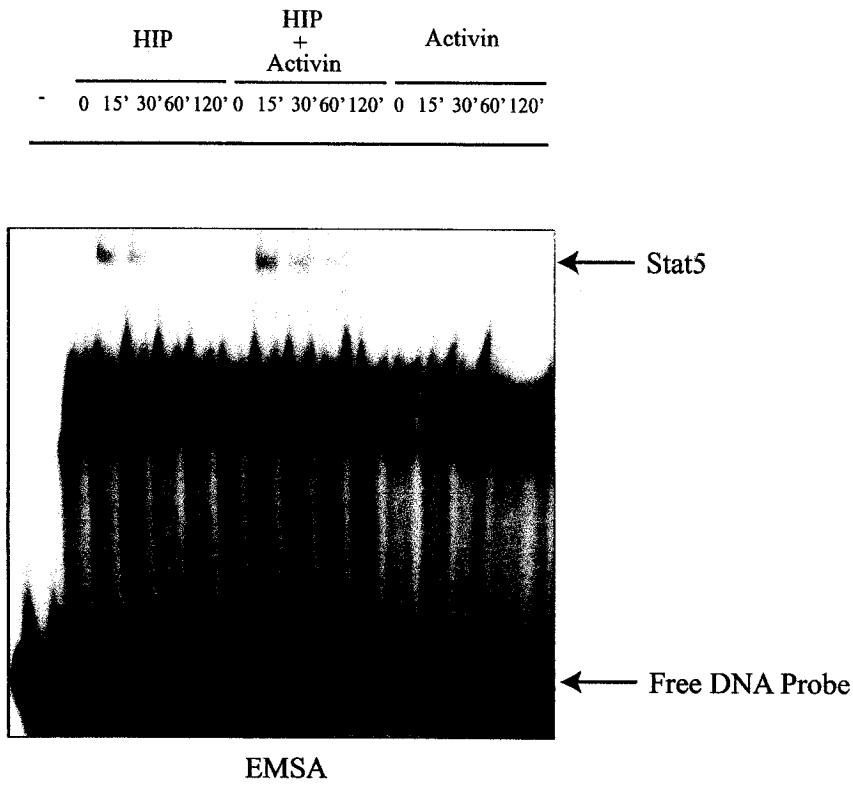



**Fig. 3 Stat5 phosphorylation and translocation to the nucleus is unaffected by activin/TGFβ signaling**

**Figure 4. Activin/TGFβ signaling do not inhibit Stat5 binding to the Stat5 binding element on the βcasein promoter.**

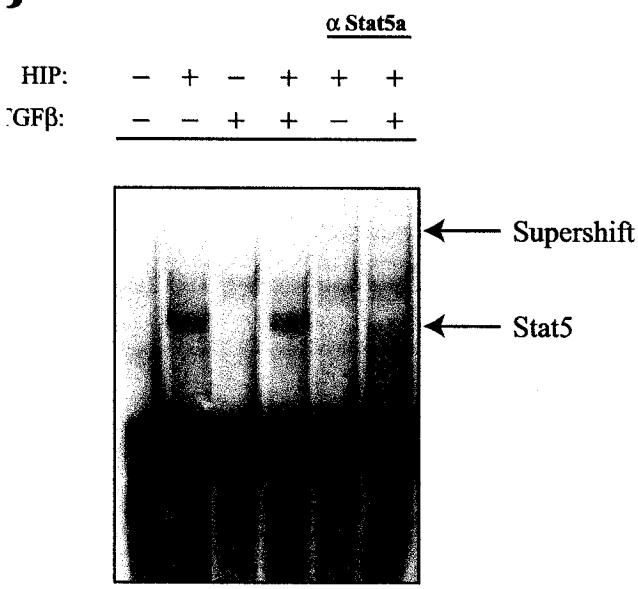
**(A)** Nuclear extracts from HC11 cells were isolated after treatment with HIP, HIP/activin or activin from 0 to 120 minutes. Electromobility shift assays were performed, using a 21 base pairs double stranded oligonucleotide encompassing the Stat5-binding site of the bovine βcasein promoter as a probe. **(B)** EMSA on nuclear extracts treated or not with HIP, HIP/TGFβ or TGFβ for 20 minutes were performed. Supershifts were performed with a polyclonal antibody against Stat5a (lanes 5-6).

A




EMSA

B




EMSA

**Fig. 4 Activin/TGFβ signaling do not inhibit STAT5 binding to the STAT5 binding element on the βcasein promoter**

**Figure 5. βcasein promoter activation is potently inhibited by activin/TGFβ.**

HC11 cells which are stably transfected with the rat βcasein promoter (-344/-1) were treated with or without HIP, activin or HIP/activin (left panel) or HIP, TGFβ or HIP/TGFβ (right panel) for 4, 8, 16 and 24 hours. Luciferase were subsequently performed. For statistical analysis, Two-way ANOVA was performed with a post-ANOVA Bonferroni's Multiple Comparison test. **$p<0.01$ as compared with HIP alone treatment. *** $p<0.001$ as compared with HIP alone.

**Figure 6. Activin/TGFβ block Stat5-mediated transcription.**

**(A)** HC11 clonal cells expressing a luciferase reporter driven by five Stat5 binding sites (5XStat5-luc) clone #2, clone #10, and clone #15 were treated or not with HIP, TGFβ, HIP/TGFβ for 16 hours and the response was measured by luciferase assay. **(B)** Human mammary epithelial cells, MCF10A cells, were co-transfected with 5XSta5-luc, long PRLR, MGF/Stat5a and βgalactosidase and luciferase assays were performed after 16 hour treatment with or without HIP, TGFβ or the combination of the two. For statistical analysis, an One-way ANOVA with a post-ANOVA Bonferroni's Multiple Comparison test (A and B), ***$p<0.001$ versus HIP treatment alone.

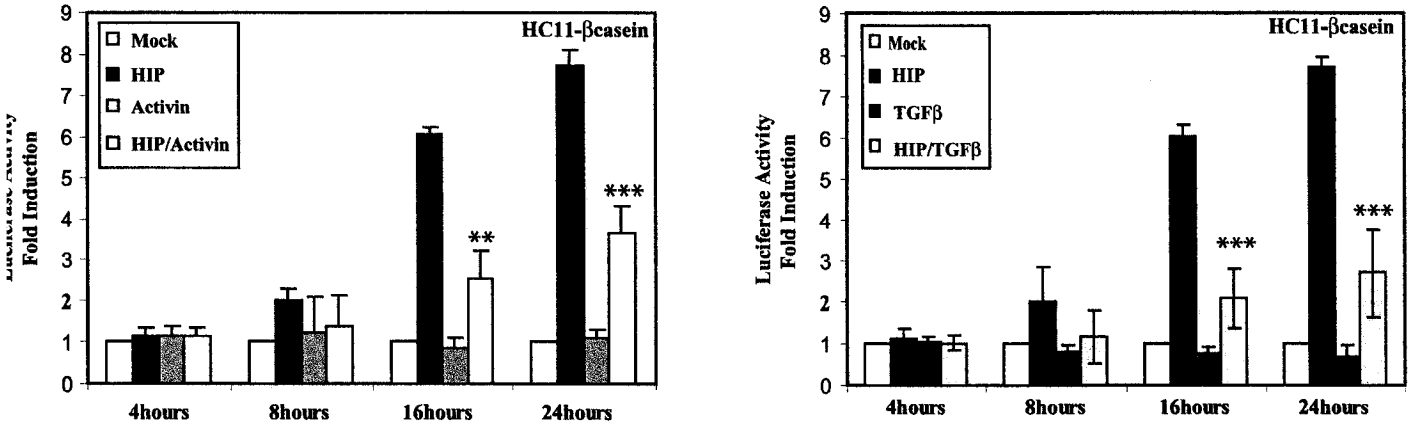



**Fig. 5 βcasein promoter activation is potently inhibited by activin/TGFβ.**

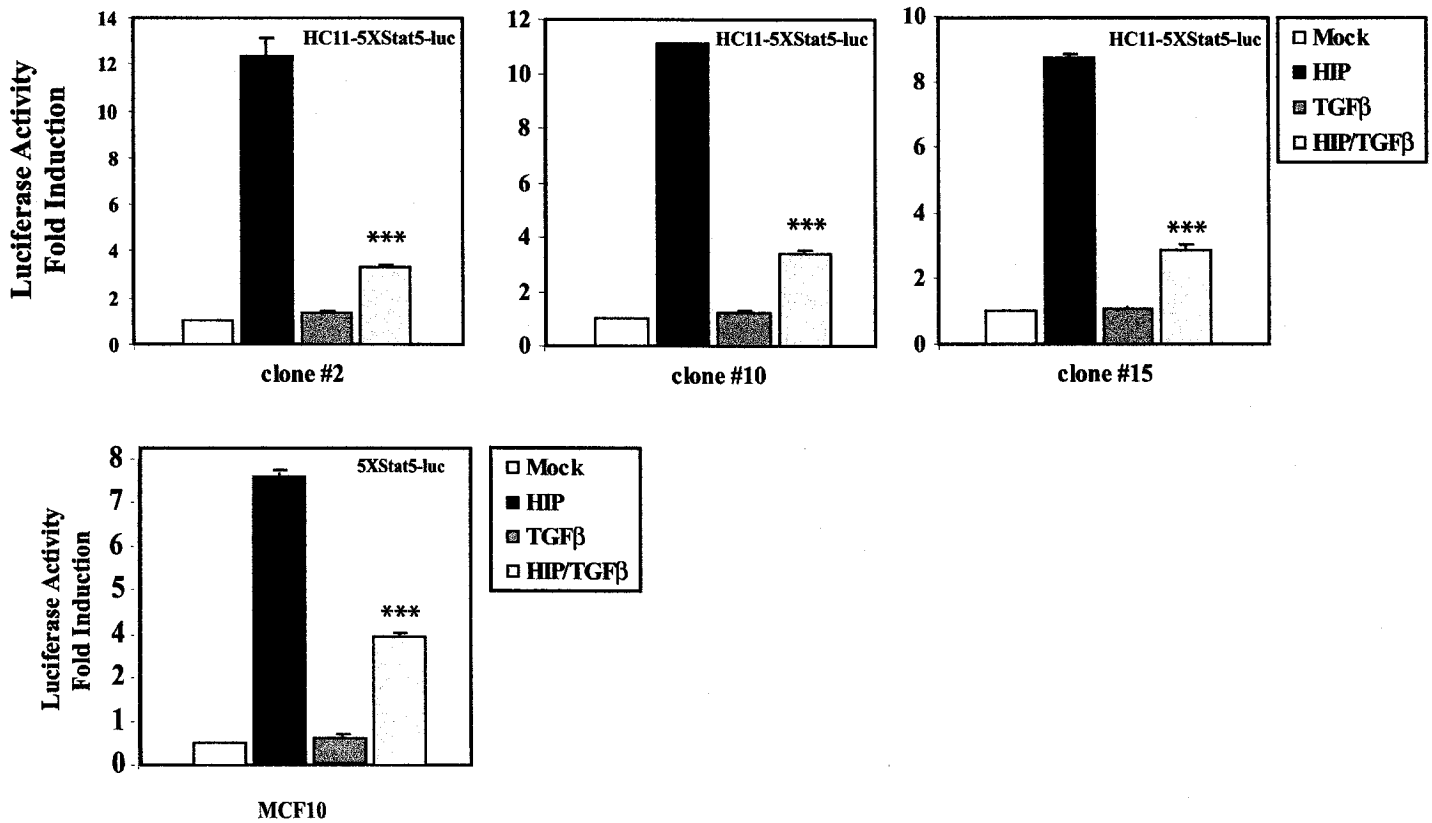



**Fig. 6 Activin/TGFβ block STAT5-mediated transcription**

**Figure 7. Smads regulate activin/TGFβ inhibition of Stat5-mediated transcription.**

(**A, B, C, E**) CHO cells were transciently transfected with either a 344 base pairs of the proximal βcasein promoter fused to the luciferase gene (βcasein-lux) or 5XStat5-luc and long PRLR, MGF/Stat5, βgalactosidase. (**A**) Transfected CHO cells were treated with or not HIP, activin or HIP/activin (left panels) or HIP, TGFβ, HIP/TGFβ (right panels) for 16 hours and luciferase assays were performed (**B and C**) In addition CHO cells were also co-transfected with or without various combinations of full length Smad2, Smad3 and Smad4 as indicated. Subsequently, cells were treated with either HIP or HIP/activin (left panels) and HIP/TGFβ (right panels). Luciferase activity is represented as % induction of the indicated promoter βcasein-lux (B panel) and 5XStat5-luc (C panel) as compared to HIP only treated samples. (**D**) CHO cells were transfected with 3TPluc, βgalactosidase and either dominant negative Smad2 (DNSmad2), dominant negative Smad3 (DNSmad3) or the activin type I receptor mutant (ALK4mL45) cDNAs. After treatment with activin for 16 hours luciferase activity was measured. (**E**) CHO cells were co-transfected with βcasein-lux (left panel) or 5XStat5-luc (right panel) and DNSmad2 or DNSmad3 or ALK4mL45. After 16 hours treatment with HIP alone or HIP/activin, luciferase assays were performed. Luciferase activity is assessed by % induction of the indicated promoter as compared to HIP only treated samples. (**F**) Smad4(+/+) MEFs (left panel) and Smad4(-/-) MEFs (middle and right panel) were transfected with βgalactosidase, long PRLR, MGF-Stat5, βgalactosidase and βcasein-luc (upper panel) or 5XStat5-luc (lower panel) with or without Smad4. The HIP, TGFβ and HIP/TGFβ response was measured after 16 hours by luciferase assay. For statistical

analysis, One-way ANOVA with a post-ANOVA Bonferroni's Multiple Comparison test was performed, *$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to HIP only treatments.

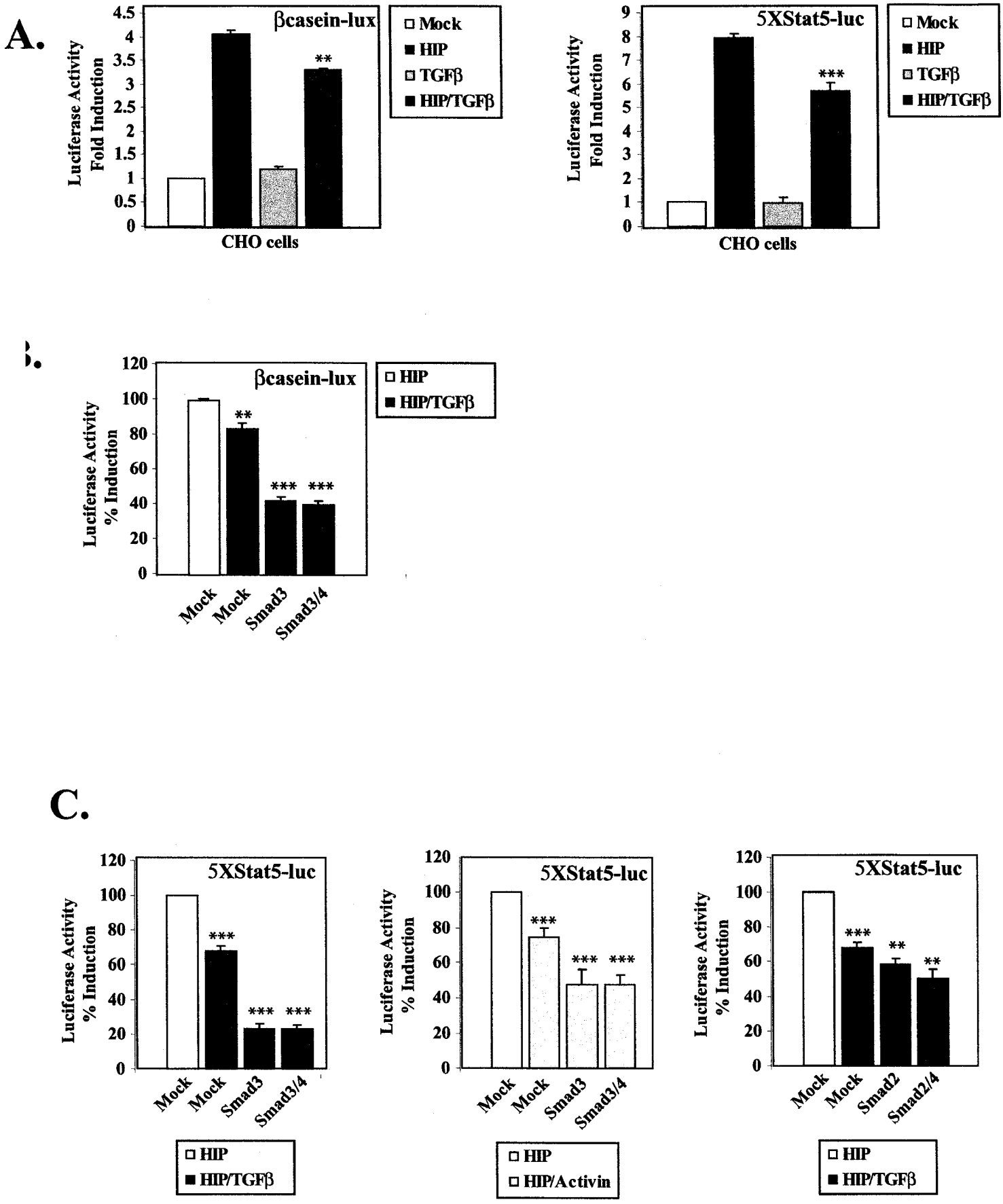
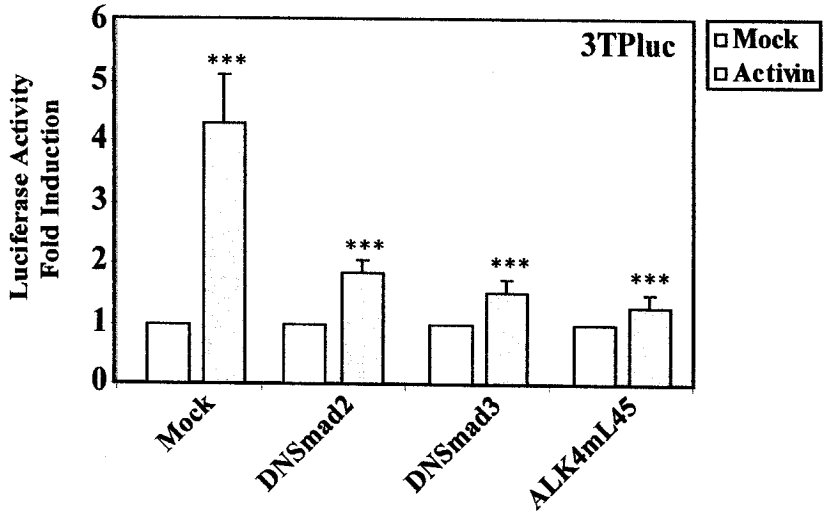
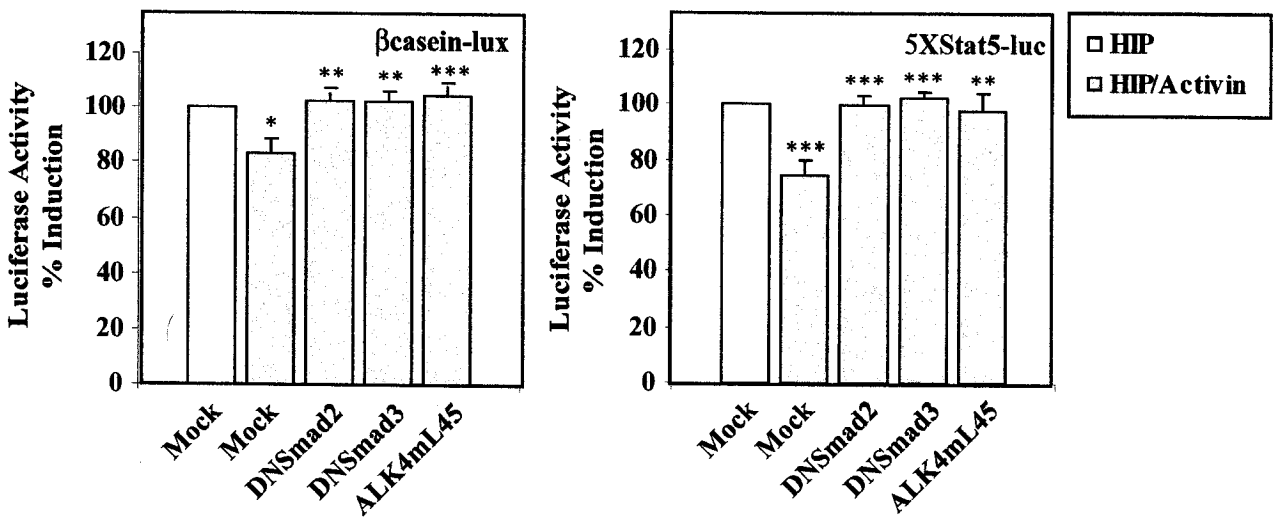



**Fig. 7 Smads regulate activin/TGFβ inhibition of STAT5-mediated transcription**

D


3TPluc
Mock
Activin
Luciferase Activity
Fold Induction
Mock
DNSmad2
DNSmad3
ALK4mL45


βcasein-lux
5XStat5-luc
HIP
HIP/Activin
Luciferase Activity
% Induction
Mock
Mock
DNSmad2
DNSmad3
ALK4mL45

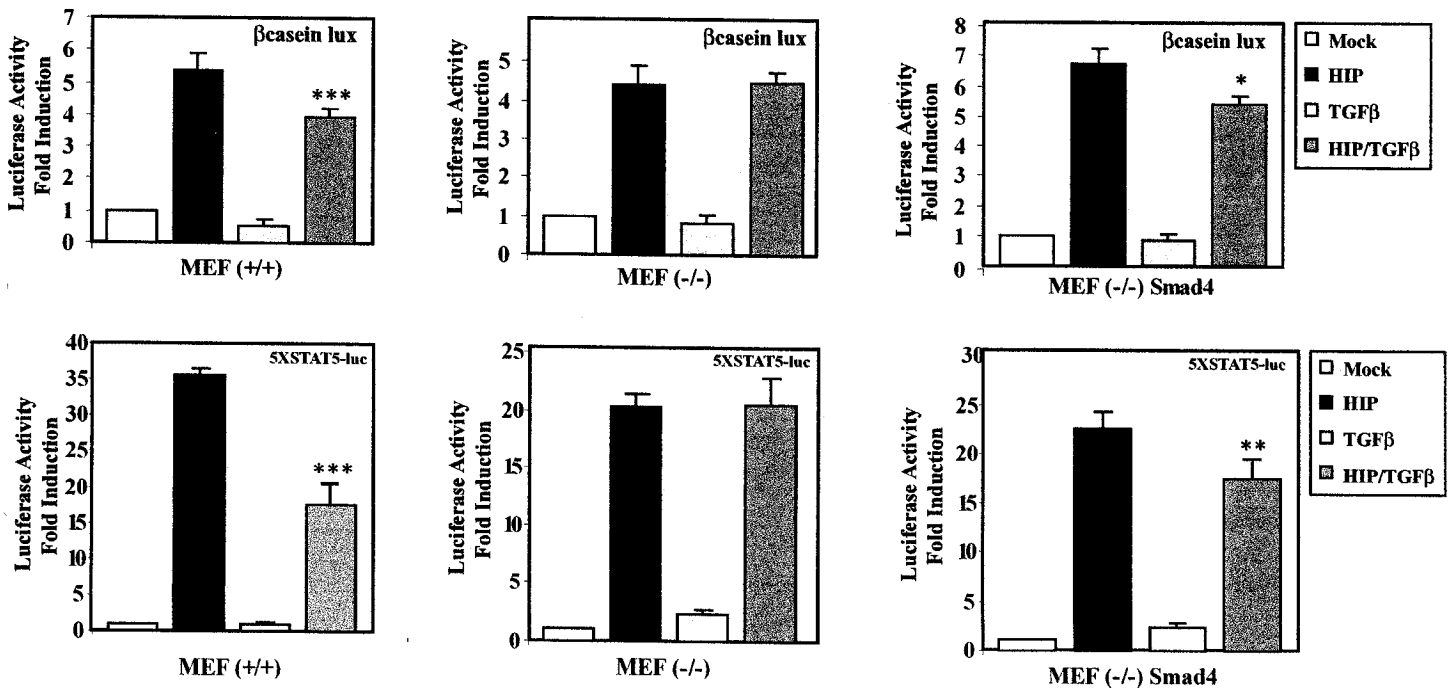

βcasein lux
5XSTAT5-luc
Mock
HIP
TGFβ
HIP/TGFβ
Luciferase Activity
Fold Induction
MEF (+/+)
MEF (-/-)
MEF (-/-) Smad4

**Figure 8. Stat5 transactivation is blocked by the Smads.**

CHO cells were transfected with βgalactosidase, XPal7, GAL4-STAT5TAD and with or without Smad3, Smad4 or Smad2, Smad3, Smad4 from 1μg to 4μg. Cells were treated with HIP or HIP/TGFβ overnight and luciferase assays were performed. Values are represented as % repression as compared to non-TGFβ treated samples. For statistical analysis, One-way ANOVA followed by post-test for linear trend were performed, $p<0.001$ for increasing Smad concentrations.

**Figure 9. Stat5 target gene cyclin D1 induction is blocked by Activin/TGFβ Smad signaling.**

(**A**). Differentiated HC11 cells were treated from 0 to 8 hours with HIP and western blot analysis was performed using cyclin D1 antibody (upper panel) and βtubulin (lower panel). (**B**) Differentiated HC11 cells were treated with or without HIP, TGFβ, HIP/TGFβ for 2 hours and total cell lysates were analyzed by Western blot assay using a specific antibody against cyclin D1 (upper panel) or βtubulin (lower panel) as a loading control. (**C**) CHO cells were transfected with the 944 base pairs proximal cyclin D1 promoter (cyclinD1-944) along with long PRLR, MGF/Stat5, βgalactosidase. Cells were treated overnight with or without HIP, TGFβ, HIP/TGFβ. Luciferase activity was assessed. (**D**) CHO cells were transfected with cyclinD1-944, long PRLR, MGF/Stat5 and bgalactosidase with or without Smad2, Smad3, and Smad4 cDNAs. Cells were treated for 16 hours with or without HIP or HIP/TGFβ. Luciferase activity was assessed. Values are represented as % repression as compared to HIP alone treatment. For

statistical analysis, One-way ANOVA with a post-ANOVA Bonferroni's Multiple Comparison test was performed (C), ***$p<0.001$ versus HIP treatment alone. An unpaired t test was performed (D), **$p<0.01$ versus mock transfected cells.

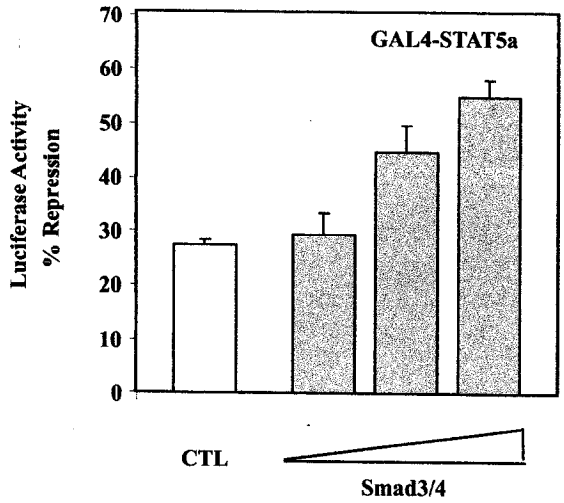



**Fig. 8. STAT5 transactivation is blocked by the Smads**

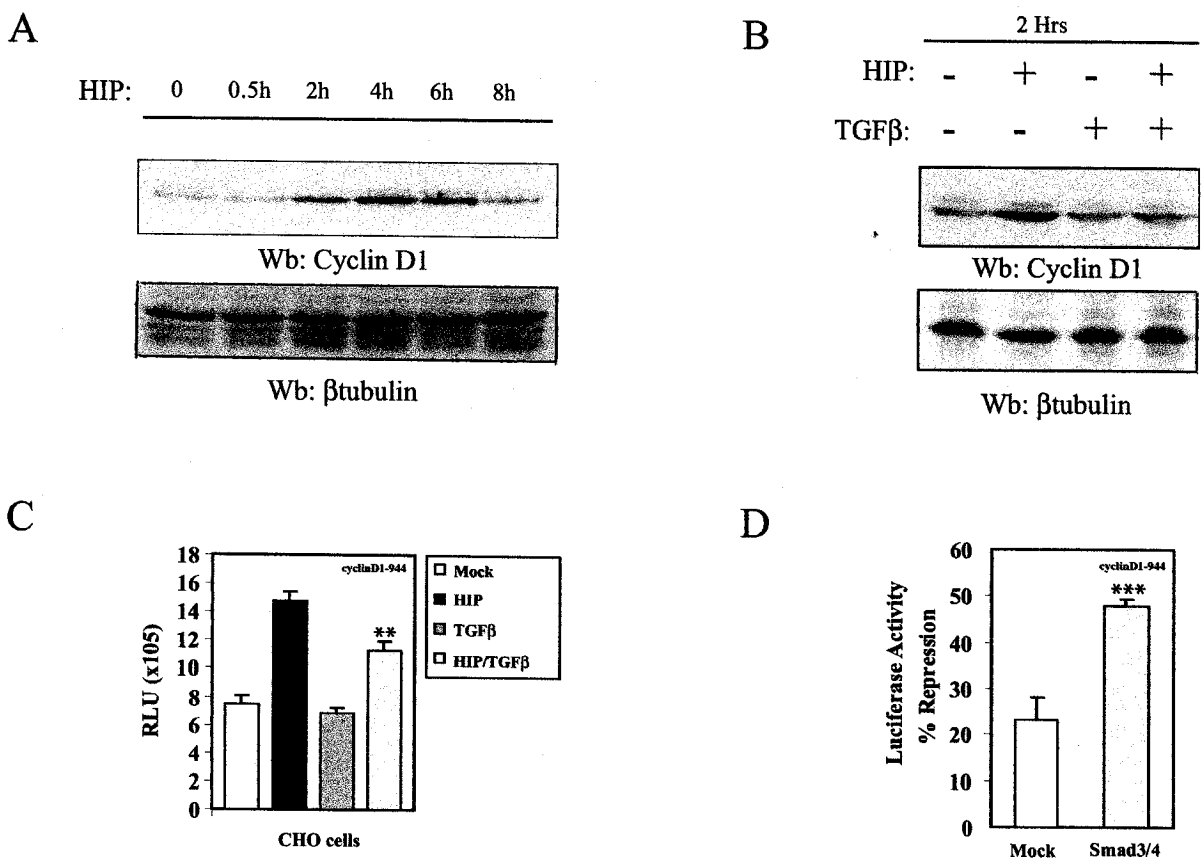



**Fig.9. Stat5-induced cyclin D1 expression is blocked by activin/TGFβ Smad signaling**

**Figure 10. Activin/TGFβ Smad signaling inhibits Stat5 interaction with CBP.**

CHO cells were transfected with PRLR, MGF/Stat5, and HA-CBP. Cells were subsequently treated and total cell lysates were extracted. Cell lysates from CHO cells stimulated with or without HIP, TGFβ, HIP/TGFβ for 25 minutes were immunoprecipitated with an anti-HA affinity matrix and analyzed by Western blot with an anti-Stat5 antibody (top panel) and an anti-HA antibody (second panel). Total cell lysates representing 10% of the immunoprecipitating input were analyzed by Western blot using antibodies to phosphoStat5, phosphoSmad3, Stat5A and HA as controls.

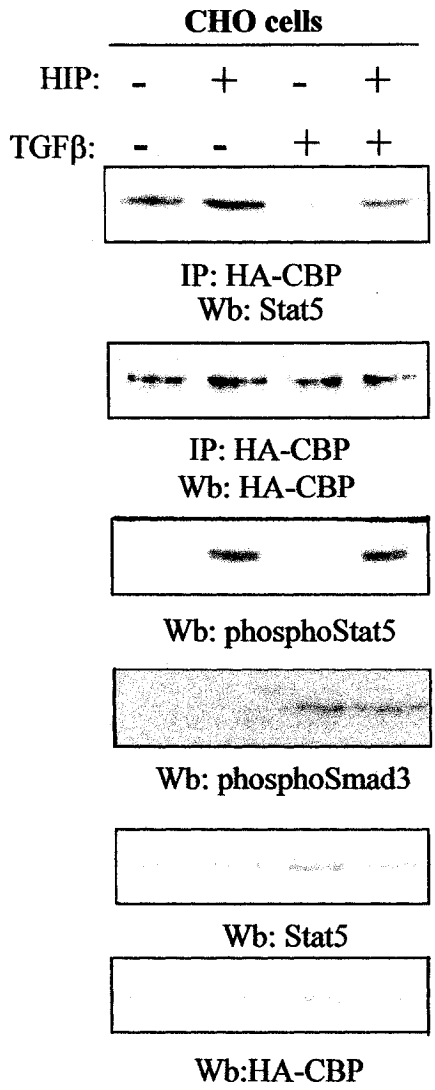



**Fig. 10. Activin/TGFβ Smad signaling inhibits Stat5 interaction with CBP**

## CHAPTER III.

# The p38 MAP Kinase Pathway is required for Cell Growth Inhibition of Human Breast Cancer Cells in Response to Activin

Eftihia Cocolakis, Serge Lemay, Suhad Ali, and Jean-Jacques Lebrun

Published in the Journal of Biological Chemistry, vol. 276: 18430-6 (2001)

## PREFACE

As we defined in CHAPTER II a novel mechanism by which TGFβ and activin regulate mammary gland development, we wanted to investigage the effect of these family members in the diseased state of this organ. Whereas many studies have been conducted evaluating TGFβ signaling mechanisms in breast cancer, as seen in CHAPTER I, activin induced signaling pathways in mammary carcinogenesis remained elucive. Furthermore, while Smads are key players in TGFβ signaling, accumulating evidence indicate that other pathways are also induced downstream of their receptors. As such, some key signaling pathways that are activated downstream of TGFβ family members are the mitogen activating protein kinases (MAPKs). Hence, I have included an introduction on MAPK signaling and crosstalk with TGFβ pathways in order to better understand the paper presented in this chapter.

### 1. MAPK signaling: A brief Overview

MAPK signal transduction is induced in response to a wide array of extracellular stimuli. Classically, the MAPK signaling cascade involves three activation steps. The MAPK kinase kinase (MAPKK) will activate the MAPK kinase (MAPKK) which will in turn activate the MAPK. MAPK phosphorylate proteins on serine and threonine residues. Hence, MAPK rapidly target a multitude of substrates including protein kinases and transcription factors. The members of this family are subdivided into three groups: the extracellular-signal regulated kinases (ERKs), the stress activated kinases JNK and p38.

### 1.1. The p38 MAPK pathway

The p38 MAPK pathway is activated in response to physiological stress, osmotic stress, inflammatory stimuli, and ultraviolet exposure[443]. To date, five p38 isoforms have been identified (p38α, β, β2, γ and δ)[444-448]. Shortly after the discovery of the first p38 isoform, a target for an anti-inflammatory drug (SB203580) which binds pyridinyl imidazole derivatives was termed cytokine suppressive anti-inflammatory drugs binding protein (CSBP) which was identical to p38[449]. The p38 inhibitor, SB203580, binds to the ATP pocket of p38 and thus inhibits its enzymatic activity[450]. p38α and β are ubiquitously expressed whereas p38γ is found mostly in skeletal muscle. p38δ is predominantly expressed in lung, kidneys, testis, pancreas and small intestine. The MAPKKK that are known to be involved in the cascade are MEKK1-4, MLK2/3, DLK, ASK1, Tpl2, and TAK1. The MAPKK are MKK3 and MKK6 (reviewed in [451]). Some substrates for p38 include the serine threonine specific kinases, the MAP-kinase activated protein kinases MAPKAPK2 and MAPKAPK3, which in turn phosphorylate the small heat shock protein HSP27, ATF1, MNK. Furthermore, the transcription factor ATF2, CREB, MEF2C, CHOP, Elk-1 and SAP-1 are also substrates of p38 (reviewed in [452]). ATF2 and CREB once activated bind to cAMP response (CRE) elements on the promoters of target genes[453]. The p38 MAPK pathway is involved in the regulation of inflammation, apoptosis, proliferation, development, cellular differentiation, and tumor suppression.

## 2. TGFβ signaling crosstalk with the MAPK pathways

The integration of complex signaling cascades contributes to the final TGFβ biological effect. Increasing evidence has highlighted the importance of other signal pathways either being activated by TGFβ or having a direct effect on Smad signaling. In addition, TGFβs not only signal through the canonical Smad pathway but induction may occur through pathways such as the ERK mitogen activating protein kinase (MAPK) pathway, the JNK pathway and the p38 MAPK pathway. MAPK pathway activation by TGFβ is cell specific.

### 2.1. MAPK Activation by TGFβ

Multiple examples in the literature exist where TGFβ activates the ERK, JNK and p38 MAPK pathways. Depending on the kinetics of TGFβ induced MAPK activation determines the involvement of the Smad pathway. For instance, TGFβ activation of the MAPK pathways with slow kinetics suggests, in some instances, a Smad-dependent transcriptional response. For example, TGFβ activation of Smads leads to upregulation of GADD45b which activates MKK4 and subsequently p38[454]. On the other hand, TGFβ activation of MAPKs with quick kinetics implies that they are a direct non-transcriptional target of this signaling pathway[455]. This observation is supported by studies performed using Smad4-deficient cells, dominant-negative Smads or studies performed using mutated TGFβ type I receptors, defective in Smad activation[455]. Using Smad4-deficient cells, TGFβ stimulation can still activate JNK[456]. Also, TGFβ may still induce p38 phosphorylation when using a mutant TGFβ type I receptor that cannot phosphorylate Smad2/3[457]. Finally, there is also evidence demonstrating convergence of

the MAPKs and the Smads. For instance, the downstream target of p38 MAPK, activating-transcription factor 2 (ATF2), interacts with Smad3 and Smad4, leading to a synergistic effect of both pathways[119]. Furthermore, both the Smad and JNK pathway synergize their effects via their interaction between c-Jun and Smad3[121, 458, 459].

Activation of the p38 MAPK and JNK pathways, by TGFβ, occurs via various MAPKKK, amongst others. The biochemical link between the TGFβ receptors and the MAPK cascade has not been sufficiently defined. It has been suggested that XIAP (X-linked inhibitor of apoptosis) may be the link between the receptor and TGFβ induced activation of TAK1, but direct interaction has yet to be determined[460]. TGFβ induced activation of MAPKKK TGFβ activated kinase 1 (TAK1)[461] which is enhanced by the scaffold protein TAK1 activator (TAB1)[462], positively regulates JNK and p38 MAPK[461]. TAB1 may also interact with Smad7 leading to p38 activation by TAK1[463]. Evidence also indicates that TGFβ activates JNK by a hematopoietic progenitor kinase (HPK-1) that phosphorylates TAK1 activating JNK[464]. TGFβ can further activate JNK and subsequently c-Jun through MKK4[458]. Through TGFβ type II receptor interaction with the death adaptor protein Daxx, TGFβ activates the MAPKKK ASK-1 then JNK and induces apoptosis[465]. Finally, depending on the cell line, TGFβ can regulate the Rho-like GTPases, Rac and Cdc42, inducing activation of JNK and p38 pathways. As illustrated by these multiple examples, TGFβ may activate the MAPKs through a variety of kinases. The biological consequences of TGFβ induced MAPK activation remain poorly characterized.

## 3. Purpose of Study

The aim of this study was to determine the effect of activin in breast cancer cells and to delineate the signaling pathways triggered downstream of activin. We demonstrate for the first time that activin induced an inhibition of cell growth in human breast cancer cells through activation of the p38 MAPK pathway. Moreover, at the time when this study was undertaken there were no published cases by which activin could convey its signal through a Smad-independent process. Hence, this was one of the first reports published demonstrating that activin can signal through the p38 MAPK pathway. We further showed that the p38 MAPK pathway is necessary for activin mediated cell growth repression.

## ABSTRACT

Activin, a member of the TGFβ family inhibits cell growth in various target tissues. Activin interacts with a complex of two receptors that upon activation phosphorylate specific intracellular mediators, the Smad proteins. The activated Smads interact with diverse DNA binding proteins and co-activators of transcription in a cell specific manner, thus leading to various activin biological effects. In this study, we investigated the role and mechanism of action of activin in the human breast cancer T47D cells. We found that activin treatment of T47D cells leads to a dramatic decrease in cell growth. Thus activin appears as a potent cell growth inhibitor of these breast cancer cells. We show that activin induces the Smad pathway in these cells but also activates the p38-mitogen activated kinase pathway, further leading to phosphorylation of the transcription factor ATF2. Finally, specific inhibitors of the p38 kinase (SB202190, SB203580, and PD169316) but not an inactive analogue (SB202474) or the MEK-1 inhibitor PD98059 completely abolish the activin-mediated cell growth inhibition of T47D cells. Together, these results define a new role for activin in human breast cancer T47D cells and highlight a new pathway utilized by this growth factor in the mediation of its biological effects in cell growth arrest.

## INTRODUCTION

Activin, a member of the TGFβ family, regulates cell growth of various cell types. Activin interacts with a complex of two receptors (type I & type II), both containing an extra-cellular domain, a single transmembrane region and a large intracellular domain that contain a serine/threonine kinase domain. The type II receptor which is constitutively phosphorylated [466] transphosphorylates the type I receptor (ALK4) upon ligand stimulation, on serine and threonine residues [53, 467, 468]. The activated receptor complex then recruits the two receptor-regulated Smad2 and Smad3 [469-472]. Following binding and phosphorylation by the activin type I receptor, Smad2 and Smad3 are released to the cytoplasm where they associate with the common-partner Smad4 before being translocated to the nucleus [472-475].

Both Smad3 and Smad4 but not Smad2 can directly bind DNA elements (Smad binding element, SBE) and activate the transcription of the target genes [476]. However, the DNA binding affinity of the Smads is low [69] and they usually require the presence of other DNA binding proteins to efficiently interact with the promoters of their responsive target genes. As a result, the SBE elements are often found close to the DNA binding element of other transcription factors. Among those are the FAST family members, FAST1 [125] and FAST2 [111], TFE3 [213], fos and Jun [121], Sp1 [477], CBP/p300 [478], Evi-1 [479], ATF2 [119].

The Smad proteins are central elements in the activin receptor signaling pathway but are not the sole pathway activated by this receptor complex. Other members of the TGFβ super family have been shown to activate different signaling pathways, in addition to the

Smads. TGFβ itself can activate a member of the MAPKKK family of kinases, TAK1 (TGF activated kinase) [461]. TAK1 then activates the stress-activated kinase p38 and the transcription factor ATF2, a member of the b-ZIP family of DNA binding proteins [119]. *In vitro* studies also suggested that the transcription factor ATF2 could interact with the MH1 domains of two activin responsive Smads, Smad3 and Smad4 [119, 480]. Both TGFβ and the Müllerian inhibiting substance (MIS) were also shown to mediate some of their biological effects through an NFkB-mediated pathway [440, 481]. It is therefore conceivable that activin also utilizes other signaling pathways to transduce its signals.

Activin, its receptors and the Smads are expressed in myoepithelial cells as well as in a certain number of human breast cancer cell lines [258, 260], suggesting a role for this growth factor and its downstream effectors, the Smads, in mammary cell growth and differentiation. Several reports have recently implicated TGFβ family members or their downstream signaling pathways in the regulation of breast cancer cell growth. Indeed, Smad4 can restore cell growth arrest in MDA-MB-468 cells, a breast cancer cell line in which the Smad4 gene is deleted [482]. Genetic mutations or loss of expression of the activin and TGFβ receptors is also found in human breast cancers [259, 295]. Finally, TGFβ and MIS mediates cell growth arrest in breast cancer by reducing NFkB DNA binding activity [440, 481] and activin itself can modulate cell growth of the breast cancer cells MCF7 [258].

In the present study, we investigated the role and mechanism of action of activin in breast cancer cells. We show here for the first time that activin strongly inhibits cell

growth of the human breast cancer cell line T47D. In addition, our results indicate that activin induce the Smad pathway in these cells but also activate the p38 MAP kinase pathway. Activation of this pathway further leads to phosphorylation of the transcription factor ATF2. Furthermore, we show that specific inhibitors of the p38 MAP kinase pathway fully antagonize the activin-mediated cell growth arrest in T47D cells. Thus, this highlights for the first time the involvement of this p38 kinase pathway downstream of the activin receptor signal transduction pathways leading to cell growth arrest.

## MATERIALS & METHODS

**Cell culture and Proliferation Assay:** T47D cells were cultured in Dulbeco Modified Eagles Medium (DMEM) in the presence of 10% fetal calf serum. For proliferation assay, cells were plated in triplicates in 96-well dishes, at 5,000 cells/100ul in 2% FCS serum. Cells were treated with stimulated or no with activin (0.5 nM) and grown over a 5 day period. Cell proliferation was assessed using direct cell counting and the non-radioactive MTT cell proliferation assay for eukaryotic cells (Cell Titer 96, Promega G 4000). Absorbance was measured at 570 nm with a reference wavelength at 450 nm, using a Bio-tek Microplate reader.

**Transfection and Reporter Assay:** T47D cells ($10^7$ cells) were transfected by electroporation (Biorad Gene Pulser II) in 500 µl of PBS (240 Volts and 975 µF) with 10 µg of each of the indicated cDNAs. Following transfection, cells were plated in 6 well dishes in DMEM (10% FCS) for 24 hours recovery. The following day, cells were starved overnight in DMEM without serum and stimulated or no with activin (0.5nM) for 16 hours. Then, cells were washed once with PBS and lysed in 250 µl of lysis buffer (1% Triton X-100; 15 mM $MgSO_4$; 4 mM EGTA; 1 mM DTT; 25 mM glycylglycine) on ice. The luciferase activity of each sample was measured using 45 ul of cell lysate (EG&G Berthold Luminometer) and normalized to the relative β-galactosidase activity.

**RNAse Protection Assay:** RNAse protection assay was performed using the hcc-2 template set and RiboQuant kit from Pharmingen (San Diego, CA) according to the manufacturer's instructions, with minor modifications. Radiolabeled antisense RNA

probes were prepared by in vitro transcription of the hcc-2 templates with T7 RNA polymerase in the presence of $\alpha$-$^{32}$P UTP (NEN, Boston, MA). After DNAse I digestion, phenol-chloroform extraction, and ethanol precipitation, the probes were quantified. RNA samples (5 μg) were dried in a vacuum centrifuge and resuspended in 20 μl hybridization buffer containing 8x105 cpm of radiolabeled probes. Hybridization (overnight at 56 C), RNAse A/T1 digestion (1 hour at 30 C), proteinase K digestion, phenol-chloroform extraction, ethanol precipitation and gel resolution (5% polyacrylamide, 8 M urea sequencing gel) were carried out according to the instructions contained in the RiboQuant RNAse protection assay kit. A yeast tRNA-only reaction was included as a negative control to ensure complete RNAse digestion. Undigested RNA probes were also resolved on each gel to ensure their integrity and to serve as size markers. The cell cycle genes represented in the assays and the size of corresponding protected probe/mRNA duplexes were as follows: p130, 400 bp; Rb, 352 bp; p107, 317 bp; p53, 283 bp; p57, 252 bp; p27, 227 bp; p21, 202 bp; p19, 182 bp; p16, 163 bp; p14/p15, 133 bp; L32 riboprotein (L32, used as a housekeeping control), 113 bp; and GAPDH, 96 bp. The dried gel was exposed to X-Ray film. The positions of the protected probes were confirmed by plotting on a semi-log graph.

**Western blot analysis:** T47D cells were plated at $10^6$ cells/ml in 6 well dishes in DMEM (10% FCS). The following day, cells were starved for an overnight period and stimulated or no with activin for different period of time as indicated. Total cell extracts were prepared from these cells were then separated on a polyacrylamide gel, transferred onto nitrocellulose and incubated with the indicated specific antibody overnight at 4°C (p38 (NEB, cat#9212), phosphop38 (NEB, cat#9210), ATF2 (NEB, cat# 9222),

phosphoATF2 (Santa Cruz, cat#8398), Smad2/3 (Santa Cruz, cat# 8332), phosphoSmad2 (UBI, cat# 6829), Smad4 (Santa Cruz, cat# 7966), ERK1/2 (NEB, cat#9102), phosphoERK1/2 (NEB, cat#9101). Following incubation, the membranes were washed twice for 10 min in washing buffer (50 mM Tris-HCl pH 7.6; 200 mM NaCl; 0.05% Tween 20) and incubated with a secondary antibody coupled to peroxidase (Santa Cruz at a 1/10,000 dilution) for 1 hour at room temperature. Then, the membranes were washed four times for 15 min in the washing buffer and immunoreactivity was normalized by chemiluminescence (Lumi-Light Plus Western Blotting substrate, Boehringer) according to the manufacturer's instructions and revealed using an Alpha Innotech Fluorochem (Packard Canberra).

## RESULTS

**Activin Inhibits T47D Human Breast Cancer Cell Growth:** Although activin and its receptors are expressed in a number of breast cancer cell lines, the role of activin in the regulation of breast cancer cell growth has not been fully investigated yet. To analyze the role of activin in regulating growth of human breast cancer cells, we utilized the human breast cancer cell line T47D that endogenously expresses the activin-responsive Smad2, Smad3 and Smad4 [280]. Using a cell growth and viability assay (MTT assay), we show that activin treatment of T47D cells leads to a significant inhibition in their growth, apparent as early as day 2 and reaching 40% inhibition at day 3 (Fig.1A). To verify that activin affects cell growth and not the metabolic rate of the cell, direct cell counting was also performed. As shown in Fig.1B, activin stimulation of T47D cells for three days also results in clear cell growth inhibition. Therefore, activin appears as a potent cell growth inhibitor for T47D breast cancer cells.

**Activin Modulates Cell Cycle Regulators in Breast Cancer Cells:** TGFβ family members regulate cell growth through different mechanisms. They often mediate cell cycle arrest through up regulation of the three cyclin dependent kinase inhibitors $p15^{INK4B}$, $p21CIP1^{/WAF1}$ and p27 [186, 188, 483]. Since activin exerts a strong effect on cell growth in T47D (Fig.1), we analyzed its effects in modulating gene expression levels of different cyclin dependent kinase inhibitors as well as of other cell cycle regulatory genes. For this, we examined the level of mRNA species of different cell cycle regulators, using a highly sensitive and specific ribonuclease protection assay (RPA). As shown in Fig.2, T47D cells were stimulated for different periods of time with activin (0.5

nM). Total RNA from un-stimulated or stimulated cells were extracted and hybridized with multiple antisense probes for human cell cycle regulators (p15, p16, p18, p19, p21, p27, as well as for p53, p57, p107 p130, the Retinoblastoma protein (Rb) and the two house keeping genes L32 and GAPDH). As shown in Fig.2, a modest but reproducible ligand-dependent increase in the mRNA level of $p21^{CIP1/WAF1}$ was observed. This is consistent with a micro-array analysis of T47D cells treated for 8 hours with activin, that shows a 1.7 fold increase in $p21^{CIP1/WAF1}$ mRNA level (JJL, unpublished data). mRNA levels for $p15^{INK4B}$ were also consistently increased upon activin treatment, though at a lower level than $p21^{CIP1/WAF1}$. This experiment was repeated three times and showed consistent results. None of the other cell cycle regulators (p130, Rb, p107, p53, p57, p27, p19, p18, p16) or house keeping genes (L32, GAPDH) mRNA levels showed any significant or reproducible difference in response to activin (Fig.2). Our attempts to detect $p15^{INK4B}$ and $p21^{CIP1/WAF1}$ protein levels in these cells were unsuccessful, probably due to low level of expression of theses two proteins. This suggests that, at least part of the activin effect on cell growth arrest in T47D cells is mediated through up-regulation of $p15^{INK4B}$ and $p21^{CIP1/WAF1}$.

**Activin Induces Smad2 Phosphorylation in T47D Cells:** To then analyze the role of the Smad pathway in T47D cells, we first examined the activation state of Smad2, following activin stimulation. Cells were starved for an overnight period and treated with 0.5 nM activin for different period of time, as indicated in Fig.3. Total cell lysates were separated by SDS-PAGE electrophoresis and resolved proteins were transferred to a nitrocellulose membrane for western blotting analysis. The membrane was probed with a specific antibody to phospho-Smad2 that recognizes the two phosphorylated serine

residues in the C-terminal end of the MH2 domain of Smad2 (SSXS). As shown in Fig.3, upper panel, activin treatment of T47D cells leads to a clear phosphorylation of Smad2, as early as 15 min. following ligand stimulation of the cells. The membrane was stripped and reprobed with a polyclonal antibody that recognizes both Smad2 and Smad3 (Fig.3, middle panel) and subsequently with a monoclonal antibody to Smad4 (Fig.3, lower panel) and shows equal levels of all Smads in all samples. This data indicates that the Smad pathway is functional in T47D cells and is activated in response to activin stimulation.

**Activin Induces 3tplux and ARE-Lux Promoters in T47D Cells:** To further examine the activation of the activin receptor/Smad pathway in T47D cells, we analyzed the ability of activin to induce two activin receptor/Smad responsive promoter constructs (3TPLux and ARE-Lux). T47D cells were transiently co-transfected as shown in material and methods with the promoter construct 3TPLux or ARE-Lux and an expression vector encoding the co-activator Fast1. As shown in Fig.4A, activin treatment of T47D cells led to a 2.6 and 2.7 fold induction for 3TPLux and ARE-Lux respectively. Furthermore, T47D cells were also co-transfected with 3TPLux or ARE-Lux/Fast1, and an expression vector encoding a constitutively active form of the activin type I receptor (ALK4 TΔD). This point mutation replaces threonine 206 by an aspartic acid and renders the receptor constitutively active even in the absence of ligand or type II receptor [53]. As shown in Fig.4B, ALK4 TΔD mimics activin effects on the activation of the two promoter constructs, leading to a 3.2 and 2.7 fold induction for 3TPlux and ARE-Lux/Fast1 respectively. Finally, to confirm the involvement of the Smad pathway, a

dominant negative form of Smad3, which lacks the MH2 domain (Smad3ΔC) was transfected in T47D cells together with 3TPLux. Deletion of the C-terminal domain of Smad3 results in the loss of homo and heterodimerization with the wild type Smad4 as well as in its ability to induce a reporter construct [484]. Cells were stimulated or not with activin and as seen in Fig.4C, over expression of Smad3ΔC completely blocks activin-induced 3TPLux activity. Together, these results confirm that the activin receptor/Smad pathway is functional in breast cancer cells.

**Activin Activates The p38 Kinase Pathway in T47D Cells:** Recently, the p38 mitogen-activated protein kinase (MAPK) pathway was shown to regulate gene expression in response to TGFβ [480]. To assess the role of this pathway in activin-mediated cell growth inhibition of breast cancer cells, T47D cells were starved overnight and stimulated with 0.5 nM of activin for different periods of time as indicated in Fig.5A. Total cell lysates were analyzed by immunoblot using specific antibodies directed against the phosphorylated form of the p38 kinase (pp38) and the normal form of p38 (p38). As shown in Fig.5A, upper panel, activin treatment of the T47D cells results in a clear increase in p38 phosphorylation in a time dependent manner. Activin effect is maximal at 20 to 40 min. and then decreases to return to basal level. The membrane was stripped and reprobed with an antibody directed against p38 and shows equal amount of the p38 kinase in all lanes (Fig.5A, lower panel).

We then analyzed the activin effects on the phosphorylation of the transcription factor ATF2, a downstream target of the p38 kinase. Total cell lysates were analyzed by immunoblot using specific antibodies directed the phosphorylated ATF2 (pATF2) or

ATF2 (ATF2). As shown in Fig.5B (upper panel), there is a time-dependent phosphorylation of the transcription factor ATF2 following activin treatment of the cells. The phosphorylation of ATF2 correlates with the activation of the p38 MAP kinase and shows a maximum phosphorylation at 40 min. The membrane was stripped and reprobed with an anti-ATF2 antibody and shows equal amount of the transcription factor in all lanes (Fig 5B. lower panel). Together, these data demonstrate that the p38 MAP kinase/ATF2 pathway is activated in T47D cells in response to activin.

**The p38 Kinase Inhibitor PD169316 Blocks Activin-Induced p38 and ATF2 Phosphorylation:** To further confirm the involvement of the p38 MAP kinase pathway downstream of the activin receptor, T47D cells were treated with a specific p38 kinase inhibitor (PD169316) or DMSO as a control. Cells were then stimulated or not with activin for 30 min. and total cell lysates were analyzed by Western blotting using different antibodies directed against phosphop38 or p38, phospho-ATF2 or ATF2. As shown in Fig. 6A (upper panel), in the presence of DMSO, activin induces phosphorylation of the kinase p38, confirming that previously seen (Fig.5). However, in the presence of the specific p38 kinase inhibitor (PD169316), this activin effect on p38 phosphorylation is abolished (Fig. 6A, upper panel). The membrane was stripped and reprobed with an antibody directed against p38 and shows equal amount of proteins in all lanes (Fig. 6A, lower panel). Similarly, as shown in Fig.6B, upper panel, activin induces phosphorylation of the transcription factor ATF2 in the presence of DMSO but this activin-induced effect is abolished in the presence of the p38 kinase inhibitor (PD169316). Equal amount of protein in all lanes was ensured by stripping and reprobing of the membrane with an anti-ATF2 antibody (Fig.6B, lower panel).

**The p38 Kinase Inhibitors Antagonize Activin-Induced Cell Growth Arrest in Breast Cancer Cells:** In order to evaluate the contribution of the p38 kinase pathway in activin-mediated cell growth inhibition in T47D cells, we used different p38 kinase specific inhibitors (SB202190, SB203580, PD169316), or an inactive analog (SB202474) and the MEK1/ERK1/2 inhibitor (PD98059) as controls, in both MTT (Fig.7A) and direct cell counting assays (Fig.7B). T47D cells were cultured in DMEM, 2% serum for three days and stimulated or not with 0.5 nM of activin in the presence or the absence of the different inhibitors. As shown in Fig.7A and 7B, after three days, cell growth is reduced by 40% in activin-treated cells as compared to untreated cells, similar to that previously observed in Fig.1A and 1B. However, in the presence of each the three specific p38 kinase inhibitors (SB202190, SB203580, PD169316), the inhibitory effect of activin on cell growth is abolished. On the other hand, the activin effect on cell growth inhibition is maintained in samples treated with the inactive form of the p38 kinase inhibitor (SB202474), or with the MEK1/ERK1/2 inhibitor (PD98059). Our results indicate that p38 kinase specific inhibitors nearly completely reverse the activin effect. As p38 kinase inhibitors could affect TGFβ receptor activity [485] we examined their effect on activin-induced Smad2 phosphorylation. As shown in Fig.7C, while activin-induced p38 phosphorylation is inhibited by pretreatment of the cells with all three active forms of p38 kinase inhibitors, we observed no significant inhibitory effect on Smad2 phosphorylation under the same conditions. Thus it is likely that the antagonistic effect exerted by the p38 inhibitors on activin-induced cell growth arrest is mediated through inhibition of the p38 kinase pathway downstream of the activin receptor.

As activin is potent cell growth inhibitor in many different cell lines, the effect of the p38 kinase inhibitors were also analyzed in several activin-responsive cell line such as K562, CHO and MCF7. Interestingly, the activin inhibitors could reverse the activin effects on cell growth arrest in all cell line tested (data not shown). This indicates that the contribution of the p38 MAP kinase pathway to activin-mediated cell growth arrest is critical.

**Activin Effect on Cell Growth Arrest is Not Mediated Through the MEK1/ERK1/2 MAP Kinase Pathway:** The absence of effect of the MEK1/ERK1/2 inhibitor (PD98059) on activin-mediated cell growth arrest (Fig.7) suggests that activin does not modulate the MAP kinase MEK1/ERK1/2 pathway to arrest cell growth. The MEK1/ERK1/2 pathway is known to be involved in cell proliferation in response to various growth factors. To confirm that activin does not modulate or inhibits activation of this pathway in response to growth factors, T47D cells were starved overnight and stimulated with EGF (20 ng/ml) for different periods of time in the absence or presence of 1 nM of activin (Fig.8). Total cell lysates were then analyzed by Western blotting using an antibody directed against phospho-ERK1/2 (α-PERK). As shown in Fig.8 (upper panel), EGF very rapidly and transiently induces the phosphorylation of ERK1/2 (p42/p44). However, activin co-stimulation of the cells does not affect EGF-induced ERK1/2 phosphorylation. The membrane was stripped and reprobed with an anti-ERK antibody and shows equal amount of MAP kinase in all samples. Together this indicates that the activin effect on cell growth arrest in T47D cells is not mediated through the MEK1/ERK1/2 MAP kinase pathway.

## DISCUSSION

Members of the TGFβ family of growth factors are important factors in regulating cell growth inhibition hence it is critical to characterize their intracellular signaling mechanisms. While it is known that activin signals through activation of Smad proteins, the activation of other intracellular signaling pathways and their contribution to activin-mediated cell growth inhibition remain to be characterized. In this paper we have examined the role of activin in mediating cell growth inhibition of breast cancer cells. Our results indicate that activin induce the Smad pathway in T47D cells and emphasize the involvement of the p38 MAP kinase pathway in activin-induced cell growth inhibition of these breast cancer cells.

Abnormalities in the signaling pathways of activin/TGFβ have been clearly linked to various cancers, including breast cancer [486]. We analyzed activin effects on the regulation of cell growth of human breast cancer cells. Using the human breast cancer cell line T47D, we found that activin has a profound and significant effect on the growth of these cells. We further investigated how activin triggers its effects in this cell line. Activin treatment of T47D cells leads to rapid phosphorylation of the receptor-regulated Smad2. Furthermore, both activin or the constitutively active form of the activin type I receptor (ALK4TΔD) induce the two promoter constructs 3TPLux and ARE-Lux and this effect is completely abolished in the presence of an over expressed dominant negative form of Smad3 (Smad3ΔC). All together, these results suggest that the activin receptor/Smad pathway is activated and can regulate the activin response in breast

cancer cells, confirming the central role played by the Smad proteins in the mediation of the activin response.

The p38 MAP kinase is involved in regulating cellular responses to stress and cytokines [444, 449, 487, 488]. p38 kinase is activated and phosphorylated at the Thr (180)-Tyr (182) site by the two closely related dual specificity protein kinases MKK3 and MKK6 [489, 490]. The activated p38 kinase has been shown to phosphorylate several transcription factors such as ATF2 [443] and Max [491], Elk-1 [492] and indirectly CREB via activation of Nrf2 [493], Stat1 [494], MEF-2 [495]. The p38 pathway is activated in response to TGFβ in C2C12, Mv1LU and 293 cells [119, 480]. TGFβ can induce phosphorylation of both p38 and the transcription factor ATF2 in these cell lines. In addition, p38 and ATF2 can contribute to the activation of the synthetic reporter construct 3TPLux in these cells but the physiological significance of this pathway in the mediation of the TGFβ effects remains unclear. We show here that activin induces the p38 kinase pathway in T47D cells leading to phosphorylation of both the p38 kinase and the transcription factor ATF2. Furthermore, we show that the p38/ATF2 pathway is required to transduce the activin effects on cell growth inhibition. Indeed, different specific p38 kinase inhibitors, but not their inactive analog or the MEK inhibitor can totally reverse the activin effect on cell growth inhibition. This highlights a new role for the p38 kinase pathway in the control of cell growth and proliferation downstream of the activin/TGFβ super family of growth factors. TGFβ family members often require the presence of parallel or synergistic pathways to the Smads to carry on their full biological effects of these growth factors and diversity of the Smad-interacting partners may contribute to signal specificity [496]. In

future studies, it will be interesting to examine the level of interaction between the Smad and the p38 kinase pathways in response to activin in T47D cells, as *in vitro* studies have suggested that the Smads could physically interact with the transcription factor ATF2 [119, 480].

Signaling by the MAPK family is organized hierarchically in three different steps. MAPK, such as p38, are phosphorylated by MAPK-kinases (MAPKKS), such as MKK3 and MKK6 in the case of p38. The MAPKKs are themselves activated and phosphorylated by the MAPKK-kinases (MAPKKKs), such as MLK, TAK and ASK1 kinases act as MAPKKKs. Finally, the MAPKKKs are regulated by cell surface receptors or other external stimuli [497, 498]. It will be interesting to identify the upstream kinases and other partner proteins involved in the activin-mediated p38 activation that are acting between the activin receptor complex and the p38 kinase in the signaling cascade. Recent reports indicated TAK-1 a member of the MAPKKK family is activated by several cytokines including TGFβ [461] and the bone morphogenetic protein [460]. TAK1 is a potent activator of the p38 kinase [499]. It will, therefore, be interesting to determine whether or not TAK1 also lies downstream of the activin receptor complex signaling cascade.

It was also recently shown that the Müllerian Inhibitory substance (MIS) represses the growth of breast cancer cells by regulating the NFkB pathway [481]. TGFβ effect on cell growth inhibition of breast cancer has also been shown to be associated with a reduced

NFkB activity [440]. This suggests that different member of the TGFβ super family may regulate cell growth by utilizing different signaling pathways in the same target tissues. Interestingly, TAK1 was also shown to lead to NFkB activation [480], suggesting a potential role for this factor downstream of the activin receptor.

Our results indicate that activin strongly represses the cell growth of the breast cancer cells T47D. Further characterization of the downstream target genes that are modulated in T47D cells in response to activin will greatly enhance our understanding of its mechanism of action on cell growth regulation. Our data suggest that at least some of these targets could be the cyclin dependent kinase inhibitors. However, it will remain to determine if other cell cycle regulators as well as apoptosis regulators are also regulated by activin in these cells. Indeed, identification of the target genes, involved in the regulation of cell cycle and/or apoptosis will be of importance to shed light on the activin receptor mechanism of action in breast cancer cells.

## ACKNOWLEDGMENTS

The authors are thankful to Wylie Vale and the National Hormone & Pituitary program & Dr. Parlow for providing activin, to Dr. J. Massagué for providing the 3TPLux construct, to Dr. B. Volgestein for the Fast-1 construct and Drs J Wrana and L. Attisano for ARE-Lux construct.

## FOOTNOTES

This work was supported by the Medical Research Council of Canada (MRC grant# 24836) and the American Concern Foundation for Cancer Research. JJ. Lebrun is an MRC scholar and S. Ali is an FRSQ scholar (Foundation pour la Recherche en Sante du Quebec).

**Abbreviations:** TGFβ, transforming growth factor β; bp, base pair; ALK, activin receptor like kinase; CDK, cyclin dependent kinase; CDKI, cyclin dependent kinase inhibitor; Fast1, forkhead activin signal transducer-1.

## FIGURES

**Fig. 1**: **Activin induces cell growth arrest of the breast cancer cell line T47D.** T47D cells were grown in 2% FCS DMEM over a 5 day period in the presence or the absence of 0.5 nM activin. Cell proliferation was assessed by (A) MTT colorimetric assay in triplicates and (B) direct cell counting. Values are expressed in arbitrary units.

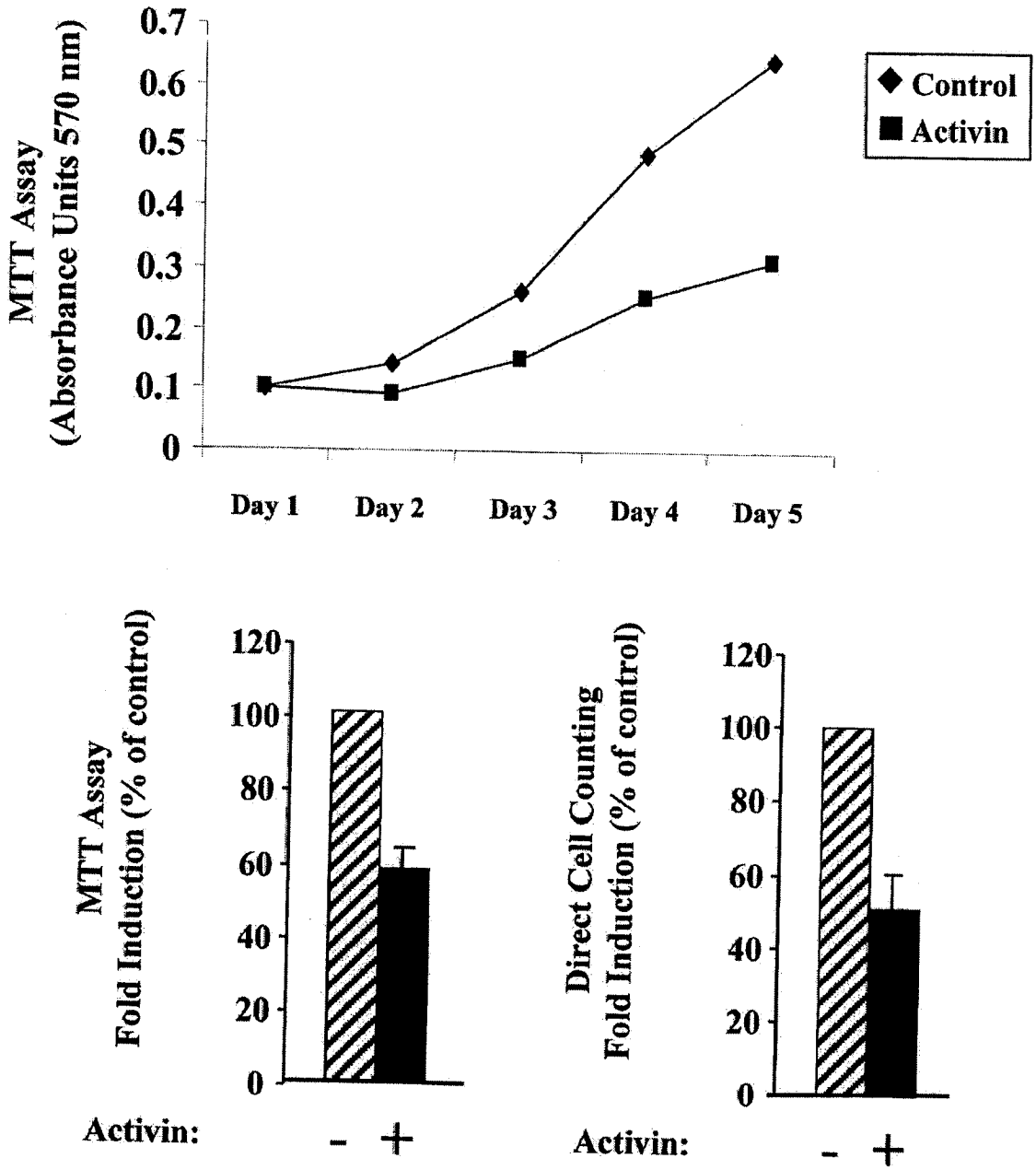



Figure 1. Activin induces cell growth arrest of the breast cancer cell line T47D

**Fig. 2: Expression of cell cycle genes in T47D cells in response to activin.** Total RNA (5 μg) obtained from T47D cells treated for 0, 4, 8, 16 or 24 hours with activin (0.5 nM). RNase protection assay was performed with radiolabeled probes for the indicated human cell cycle genes and 2 housekeeping control genes (L32 and GAPDH), as described in Materials and Methods. The positions of the protected probes are shown to the left of the autoradiography. Yeast tRNA (lane 6) is shown as a negative control. Undigested probes (lane 7) were used as size standards.

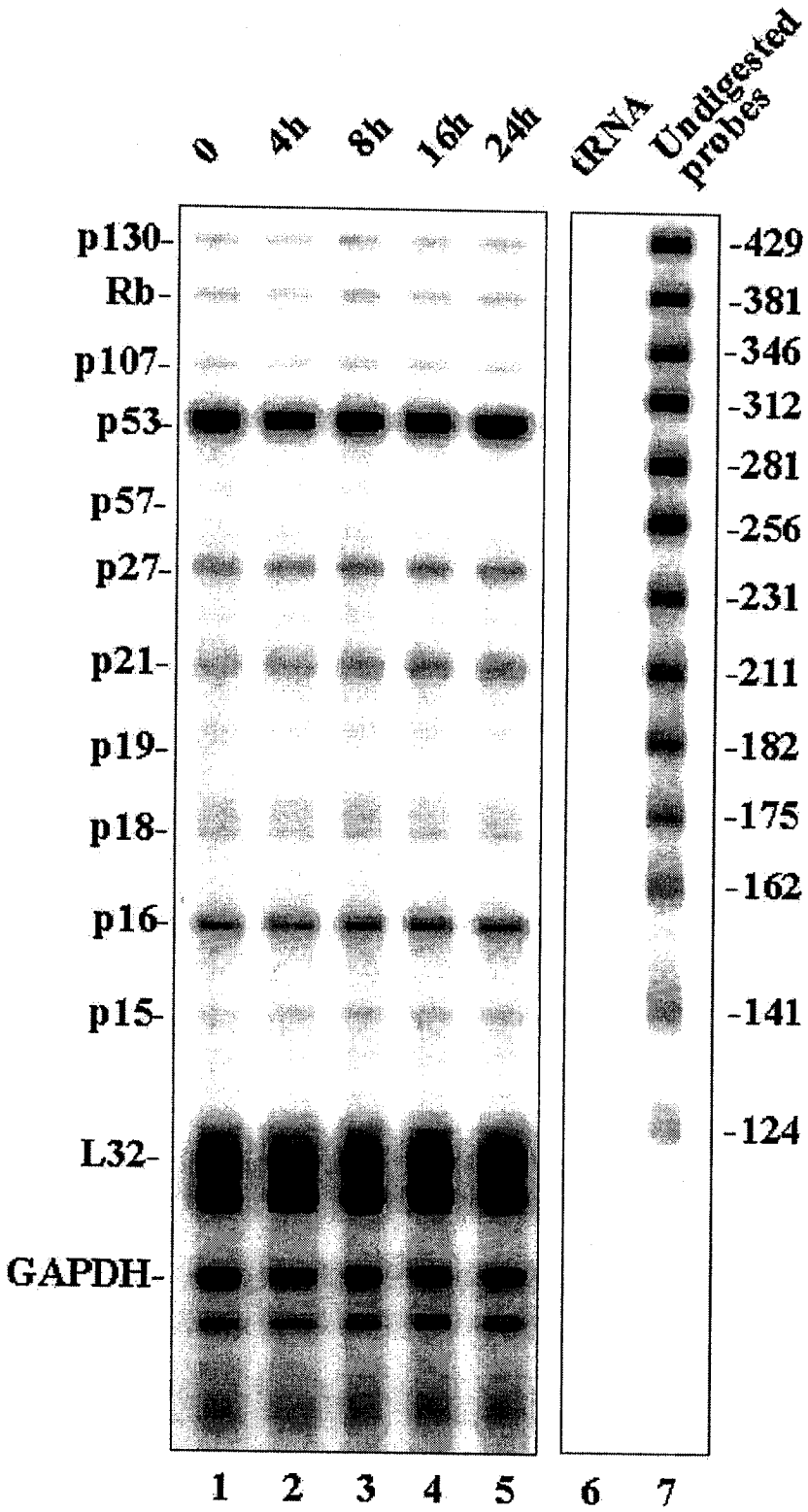



Figure 2. Expression of cell cycle genes in T47D cells in response to activin

**Fig. 3**: **Activin induces Smad2 phosphorylation in T47D cells**: T47D cells were treated with 0.5 nM activin for 0, 15, 30 and 60 min. Cell lysates were analyzed by Western blot using a specific antibody to phospho-Smad2 (Upper panel). The membrane was stripped and reprobed with an anti-Smad2/3 antibody (middle panel) and an anti-Smad4 antibody (lower panel).

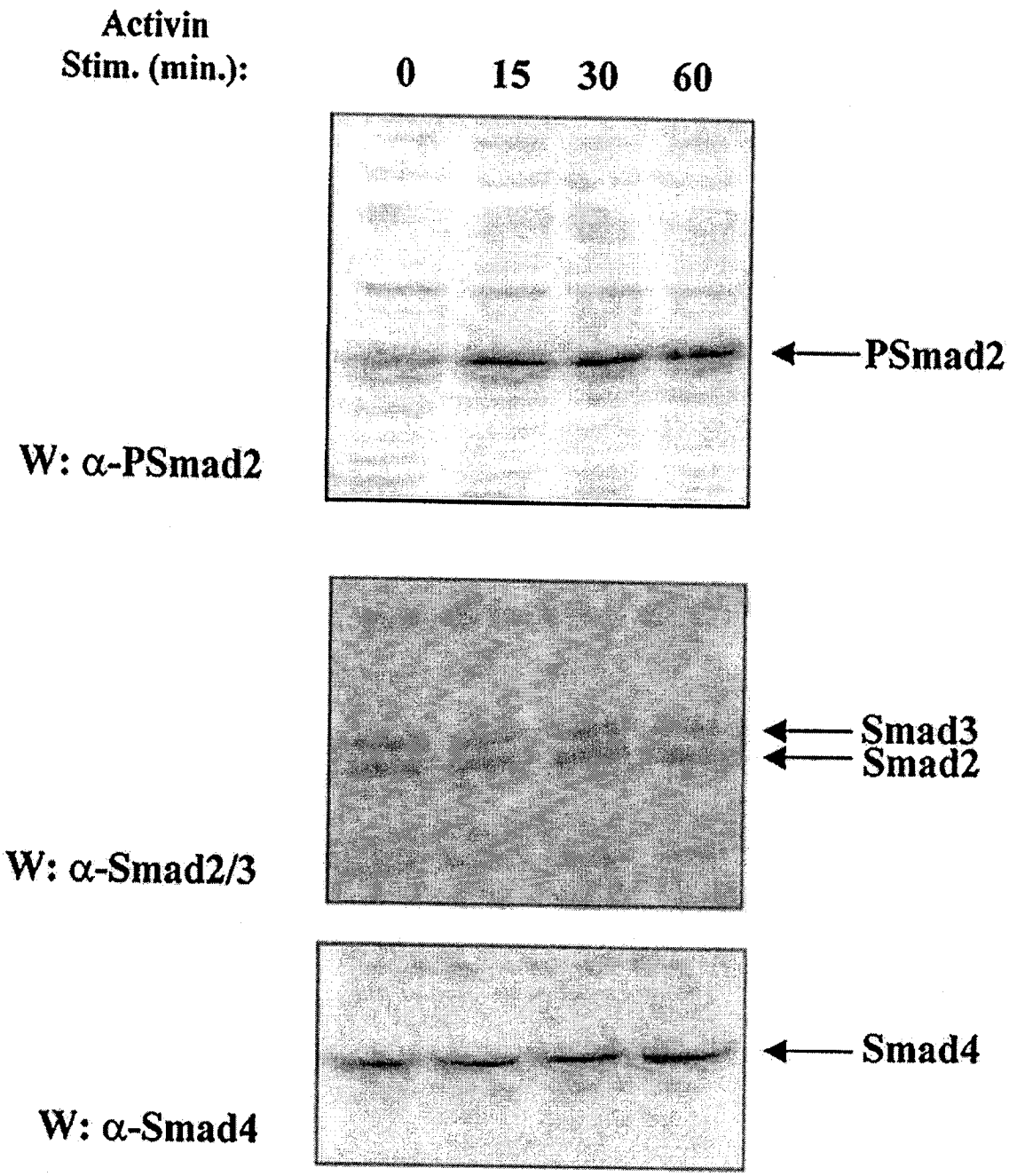



Figure 3. Activin induces Smad2 phosphorylation in T47D cells

**Fig. 4: Activin induces 3TPLux and ARE-Lux promoters in T47D cells**: **(A)** T47D cells transfected with the activin receptor/Smad responsive promoter constructs 3TP-Lux or ARE-Lux/Fast1 reporter constructs were stimulated with activin 16 hours. The luciferase activity was normalized to the relative β-galactosidase values. Results represent means and standard deviations of three independent experiments. **(B)** T47D cells transfected with the promoter constructs 3TP-Lux or ARE-Lux/Fast1 reporter constructs in the presence or the absence of the constitutively active form of the activin type I receptor (ALK4 TΔD). The luciferase activity was normalized to the relative β-galactosidase values. Results represent means and standard deviations of three independent experiments. **(C)** T47D cells were transfected with the promoter construct 3TP-Lux in the presence or the absence of the truncated form of Smad3 (Smad3ΔC) and stimulated with activin 16 hours. The luciferase activity was normalized to the relative β-galactosidase values. Results represent means and standard deviations of three independent experiments.

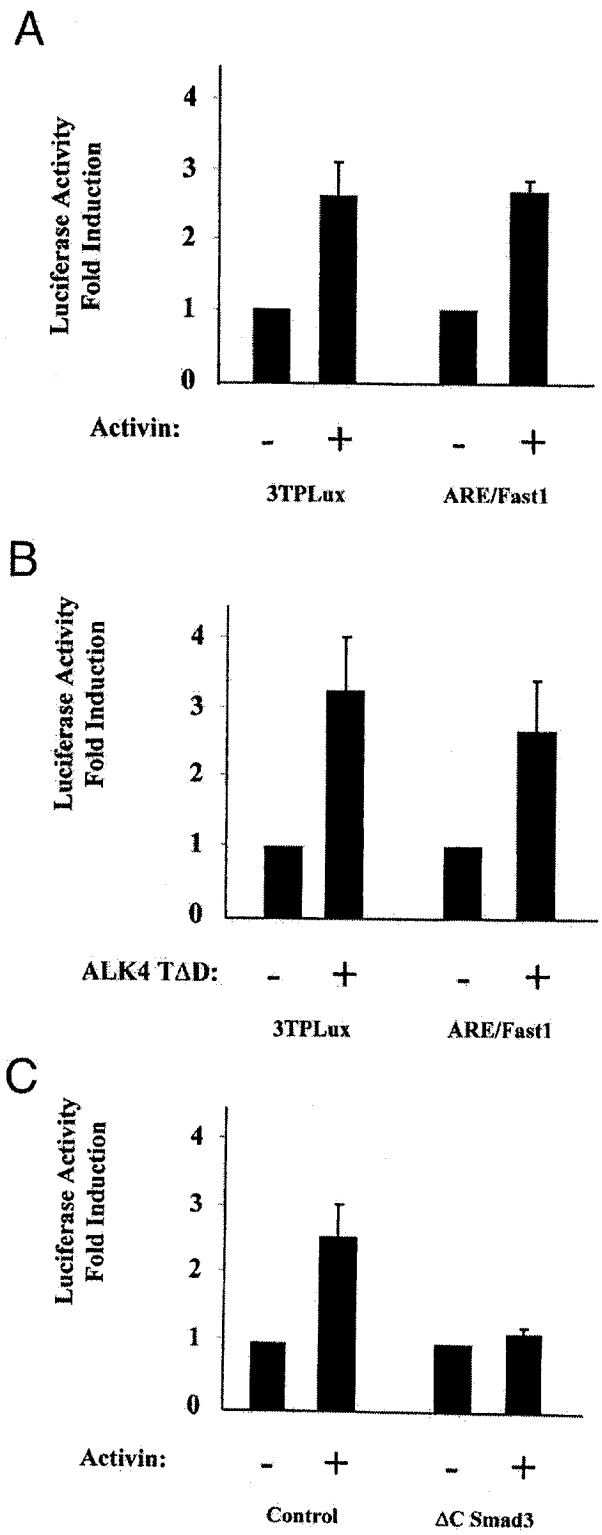



Figure 4. Activin induces 3Tplux and ARE-Lux promoters in T47D cells

**Fig. 5: Activin induces the p38 kinase pathway in T47D cells:** T47D cells were starved overnight and stimulated with 0.5 nM of activin for 0, 5, 15, 30, 60 and 90 min. **(A)** Total cell lysates were analyzed by immunoblot using specific antibodies directed against the phosphorylated form of the p38 kinase (pp38) (upper panel). The membrane was stripped and reprobed with an anti-p38 (p38) antibody (lower panel). **(B)** Similarly, total cell lysates were analyzed by immunoblot using a specific antibody directed against the phosphorylated ATF2 (pATF2) (upper panel). The membrane was stripped and reprobed with an anti-ATF2 (ATF2) antibody (lower panel).

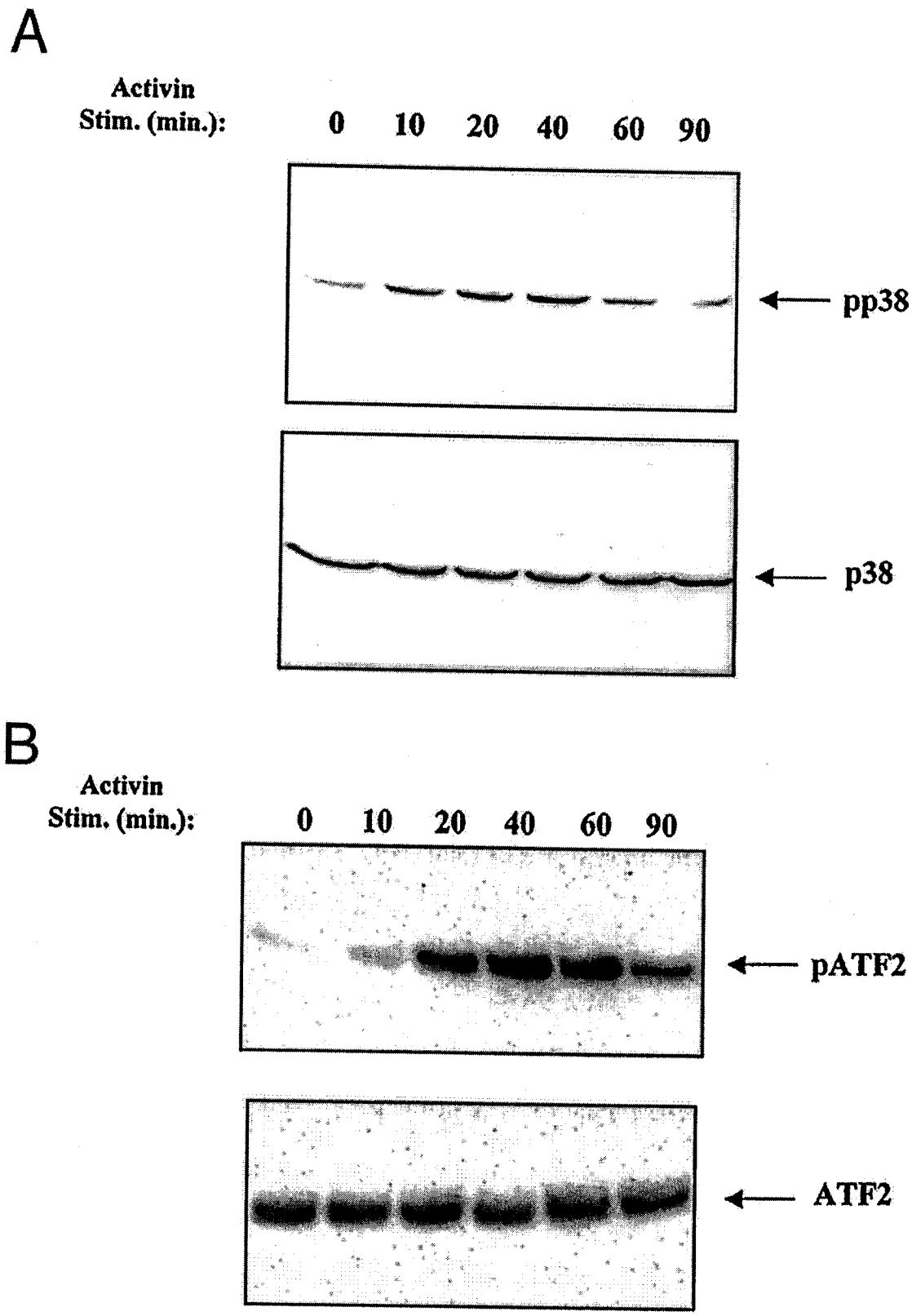



Figure 5. Activin induces the p38 kinase pathway in T47D cells

**Fig. 6: The p38 kinase inhibitor PD169316 blocks activin-induced p38 and ATF2 phosphorylation:** T47D cells were pretreated with DMSO or with a specific p38 inhibitor (PD169316) at 10 μM for 1 hour before being stimulated with activin for 30 min. **(A)** Total cell lysates were analyzed by immunoblot using a specific antibody directed against the phosphorylated form of the p38 kinase (pp38) (upper panel). The membrane was stripped and reprobed with an anti-p38 (p38) antibody (lower panel). **(B)** Similarly, total cell lysates were analyzed by immunoblot using a specific antibody directed against the phosphorylated ATF2 (pATF2) (upper panel). The membrane was stripped and reprobed with an anti-ATF2 (ATF2) antibody (lower panel).

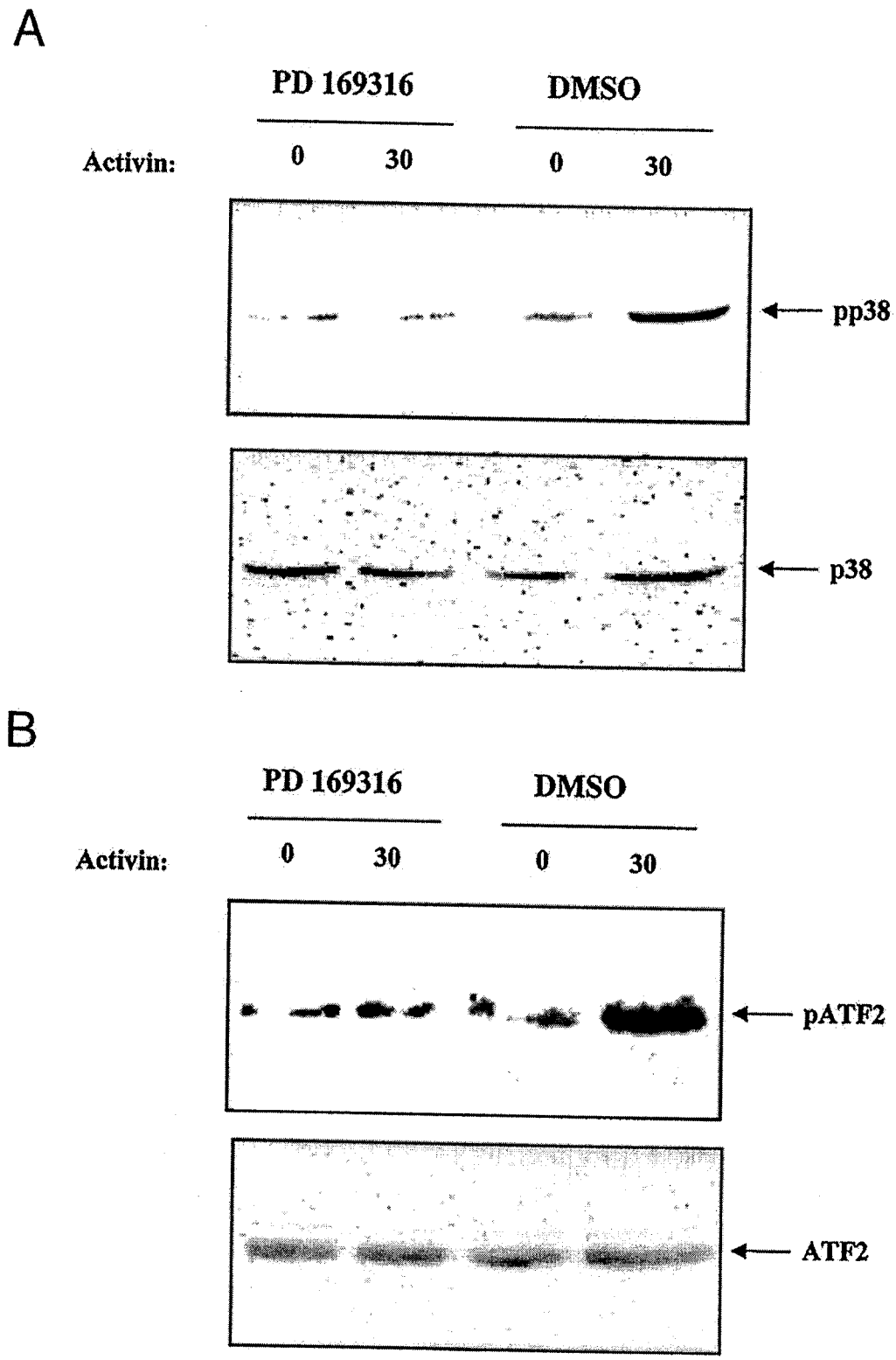



Figure 6. The p38 kinase inhibitor PD169316 blocks activin-induced p38 and ATF2 phosphorylation

**Fig. 7: p38 kinase inhibitors antagonize activin-induced cell growth arrest in breast cancer cells:** T47D cells were cultured in DMEM, 2% serum for three days and stimulated or no with 0.5 nM of activin in the presence or the absence of 10 uM of the different p38 kinase specific inhibitors (SB202190, SB203580, PD169316), or an inactive analog (SB202474) and the MEK1/ERK1/2 inhibitor (PD98059) as controls. Cell growth was assessed by **(A)** MTT colorimetric assay in triplicates and **(B)** direct cell counting. Values represent means and standard deviations of five separate experiments and are expressed as percentage of inhibition compared to the control. **(C)** T47D cells were pretreated with DMSO or with the p38 inhibitors (SB202190, PD169316 or SB203580) at 10 μM for 45 min. before being stimulated with activin for 15 min. Total cell lysates were analyzed by immunoblot using specific antibodies directed against the phosphorylated form of Smad2 (pSmad2, upper panel), the phosphorylated form of p38 kinase (pp38, middle panel) or p38 kinase (p38, lower panel).

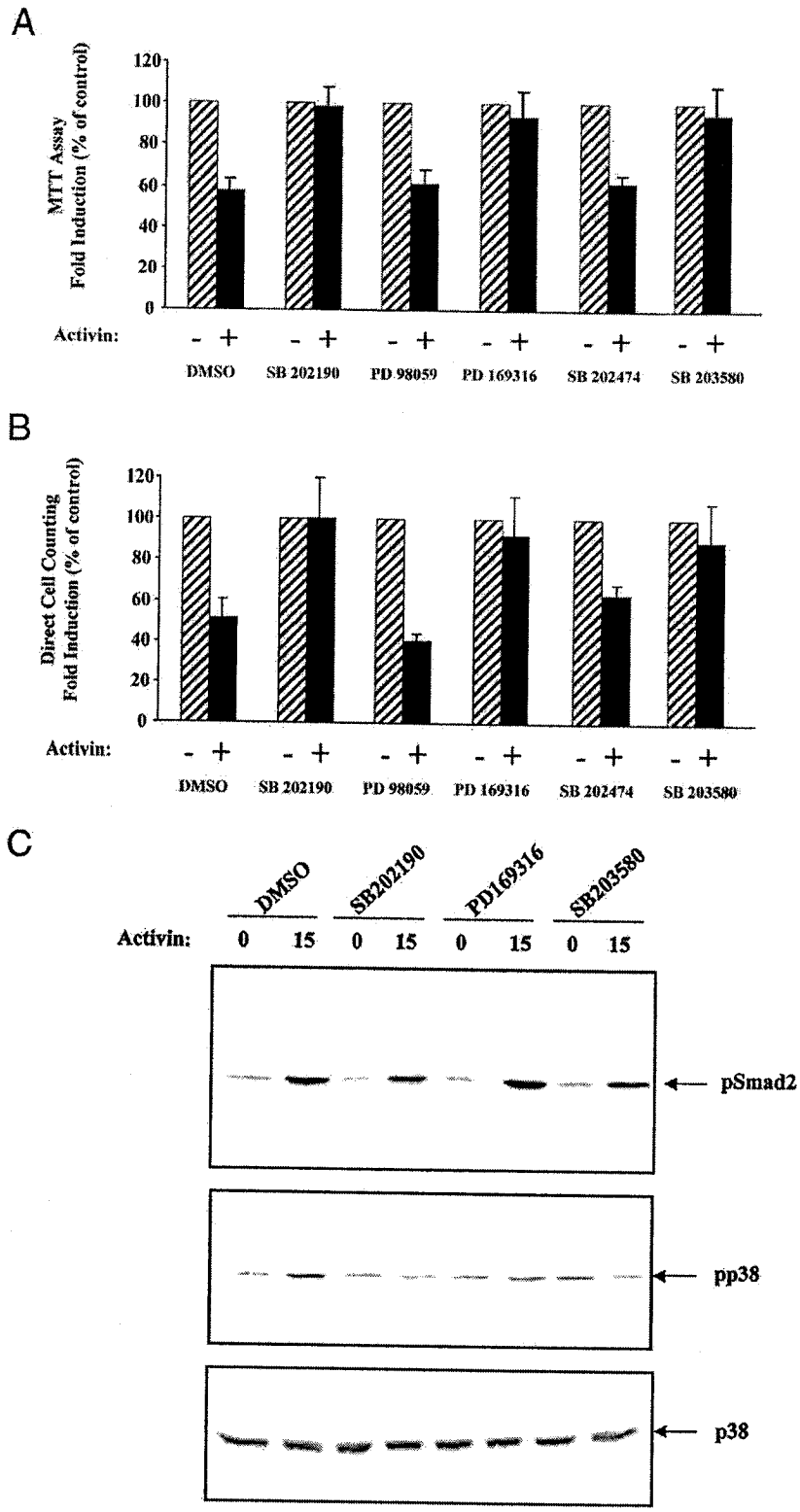



Figure 7. p38 kinase inhibitors antagonize activin-induced cell growth arrest in breast cancer cells

**Fig. 8: Activin effect on cell growth arrest is not mediated through the MAP Kinase pathway:** T47D cells were starved overnight and stimulated with EGF (20 ng/ml) for different period of times in the absence or presence of 1 nM of activin. Total cell lysates were then analyzed by Western blotting using an antibody directed against phospho-ERK1/2 (upper panel). The membrane was stripped and reprobed with an anti-ERK1/2 antibody (lower panel).

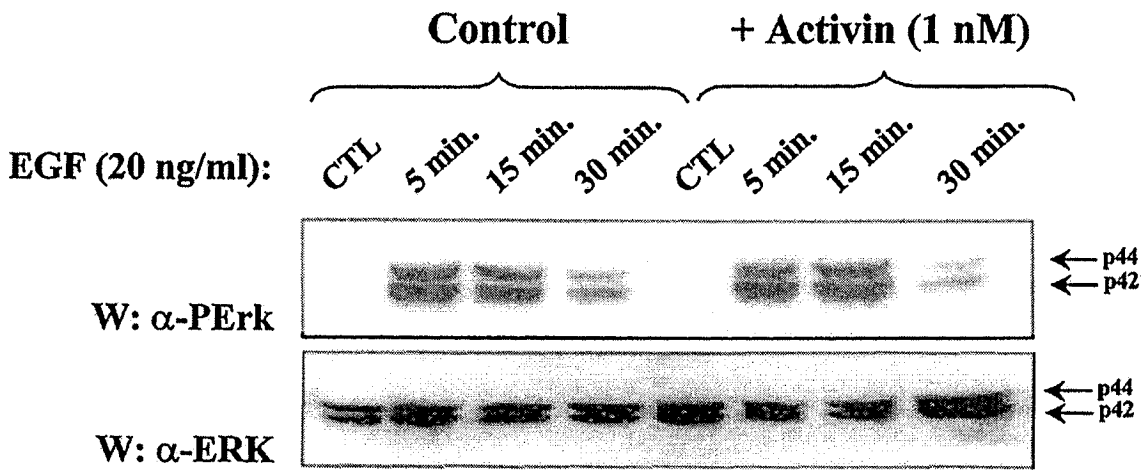



Figure 8. Activin effect on cell growth arrest is not mediated through the MAPK pathway

## CHAPTER IV.

# The Death Adaptor Molecule RAIDD is as a Novel Activin Target Necessary for Activin-Induced Cell Growth Inhibition

Eftihia Cocolakis and Jean-Jacques Lebrun

## PREFACE

As a direct continuation of CHAPTER III, we decided to characterize the intracellular target genes that relay activin effects on cell growth inhibition in human breast cancer cells by gene profiling using affymetrix DNA microarrays. As such, we identified the death adaptor molecule RAIDD as a novel target of activin signaling. I have included a brief introduction on RAIDD in order to better comprehend the study that follows.

### 1. TGFβ and Death Receptor Signaling

The prototypic death receptor pathway involves TNF-α and FasL signaling through their respective receptors TNF-RI (tumor necrosis factor receptor I) and Fas, leading to intracellular signaling with an end biological outcome of apoptosis. An important pathway activated by FasL is through the death adaptor molecule Daxx that directly interacts with Fas. Indeed, Daxx interaction with Fas leads to ASK-1 (apoptosis signal-regulating kinase 1) activation and subsequent activation of JNK, leading to apoptosis[500, 501]. Interestingly, a yeast-two hybrid screening has identified Daxx as a binding partner of the TGFβ type II receptor. Daxx interacts with the cytoplasmic tail of the receptor, and its carboxyl-terminus was also shown to act as a dominant-negative inhibitor of TGFβ-induced apoptosis and to regulate TGFβ-induced JNK activation[465]. Furthermore, antisense oligonucleotides to Daxx inhibit TGFβ-induced apoptosis[465]. This study was the first to link a death adaptor protein to a member of the TGFβ superfamily and define a novel pathway in the induction of apoptosis. To date, no death adaptor molecules have been shown to be involved within the activin signaling pathway.

## 2. The Death Adaptor RAIDD

The death adaptor molecule RAIDD (for RIP-associated ICH-1/CED-3-homologous protein with a death domain) was initially identified in the NCBI GenBank expressed-sequence tag (EST) data base based on its homology with the prodomain of the human ICE-like protease ICH-1 or procaspase-2[502]. Subsequent to the initial discovery, another group idenfied RAIDD as the molecule CRADD (for caspase and RIP adaptor with death domain) through a similar database screening[503]. RAIDD is constitutively expressed, with highest expression in the adult heart, testis, liver, skeletal muscle, fetal liver and kidney. Located on chromosome 22, the RAIDD gene encodes for a 200 amino acid protein with a relative molecular weight of approximately 22 kDa.

RAIDD is composed of a N-terminal CARD (caspase activation and recruitment domain) which shares high homology with caspase-2. The C-terminus is composed of a DD (death domain) whose sequence is similar to that of other DD-containing proteins involved in death receptor signaling. Through homotypic interactions, the DD of RAIDD interacts with the DD of RIP (receptor interacting protein). RIP is a serine threonine kinase involved in the death receptor pathway. Similarly, through homotypic interactions via its CARD domain, RAIDD recruits and activates caspase-2 zymogens[502]. RAIDD was found to promote apoptosis by recruiting caspase-2 to TNF-RI in the presence of RIP and the death adaptor molecule TRADD. Dominant negative forms of RAIDD, however, did not abrogate TNF-α-mediated cell death, suggesting that TNF-α signaling does not require RAIDD to induce apoptosis[502].

## 3. Purpose of study

Based on our previous study in which we showed that activin induces cell growth arrest in human breast cancer cells through the p38 MAPK pathway, we sought out to investigate the target genes donwnstream of activin signaling. We show that activin induces a strong upregulation of RAIDD mRNA and protein levels in T47D breast cancer cells. Furthermore, RAIDD is required for activin mediated cell growth inhibition of these cells.

## ABSTRACT

Activin, a prominent member of the transforming growth factor-β (TGFβ) superfamily of pluripotent cytokines plays a crucial role in growth control of multiple several cell types. Here we identify a novel activin target gene encoding RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain), a death adaptor protein involved in TNF-RI (tumor necrosis factor receptor 1) induced signaling that mediates programmed cell death by TNF-α. We reveal that activin upregulates RAIDD protein levels in human breast cancer cells. We further show that RAIDD increase in protein levels is not limited to breast cancer cells but also to human hepatocarcinoma cells. Finally, using antisense oligonucleotides to RAIDD, we demonstrate that RAIDD expression is necessary for activin mediated cell growth arrest.

## INTRODUCTION

The transforming growth factor β (TGFβ) superfamily of pluripotent growth factors consists of over 42 members including activins, TGFβs and bone morphogenic proteins (BMPs) among others, regulating a vast array of physiological and pathophysiological processes throughout the body. These polypeptides affect cell growth, differentiation and apoptosis in almost all cell types, including those of epithelial, endothelial and hematopoietic origin[504].

Activin, their receptors and the Smads are widely expressed in all tissues and the regulatory role played by these growth factors is of central importance to human diseases. Indeed, mutations or deletions within these genes are often the underlying basis for human cancers. Interestingly, the involvement of these growth factors in human cancer is multifaceted. While they initially contribute tumor suppression by efficiently inhibiting cell proliferation, the TGFβ growth inhibitory responses in cancer cells are often replaced by invasive and pro-metastatic responses as tumors progress, highlighting the dual role of TGFβ as both a tumor suppressor and tumor promoting agent.

Activin signal transduction begins with ligand binding to a single-transmembrane-spanning, constitutively auto-phosphorylated serine/threonine kinase, type II receptor. Upon ligand binding, the type II receptor recruits and transphosphorylates the type I receptor within a juxtamembrane glycine and serine-rich region, rendering type I's kinase active. The activated type I receptor then phosphorylates the intracellular mediators known as the receptor-regulated Smads (R-Smads) Smad2 and Smad3, on serine residues with their carboxyl-terminus. Activation of the R-Smads allows for heterodimerization with common partner Smad4. Subsequently, the Smad

heterocomplex translocates to the nucleus where it can associate with either co-activators or co-repressors to elicit or repress transcription of target genes[504].

In the present study we describe a novel target gene of activin signaling, the death adaptor molecule RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain). We show that RAIDD mRNA and protein levels are upregulated in response to activin in human breast cancer (T47D) cells and human heptocarcinoma (HUH7) cells. Finally we demonstrate that RAIDD expression is necessary in mediating activin cell growth inhibition. Our data, implicate for the first time RAIDD as target in the mediation of activin's anti-proliferative effect.

## MATERIALS and METHODS

**Cell culture and Proliferation Assay:** T47D and HUH7 cells were cultured in Dulbeco Modified Eagles Medium (DMEM) in the presence of 10% fetal calf serum. For proliferation assay, cells were plated in triplicates in 96-well dishes, at 5,000 cells/100ul in 2% FCS serum. Cells were treated with stimulated or no with activin (0.5 nM) and grown over a 3 day period. Cell proliferation was assessed the non-radioactive MTT cell proliferation assay for eukaryotic cells (Cell Titer 96, Promega G 4000). Absorbance was measured at 570 nm with a reference wavelength at 450 nm, using a Bio-tek Microplate reader.

**Reverse-transcription PCR:** T47D cells were treated with or without 0.5 nM activin from 2 to 8 hours, and total RNA was extracted using Trizol reagents (Invitrogen). Reverse transcription of total cellular RNA using oligo-dT primers was carried out using Stratacript Reverse Transcriptase (Stratagene) as per the manufacturer's instruction. Subsequently, amplification of cDNA to obtain products for RAIDD and GAPDH was performed. The PCR conditions were as follows: 30 cycles (94°C for 30 s, 58°C for 30 s, 72°C for 1 min).

**Western analysis:** Cells were cultured in DMEM, 2% fetal calf serum and stimulated or not with 0.5 nM activin for different periods of time as indicated in the figures. Total cell extracts were separated on a 12% polyacrylamide gel, transferred onto nitrocellulose and incubated with the indicated specific antibodies (anti-RAIDD, Santa-Cruz; anti-tubulin, Sigma). Immunoreactivity was revealed by chemiluminescence (Lumi-Light Plus

Western Blotting substrate, Roche) according to the manufacturer's instructions and measured using an Alpha Innotech Fluorochem Imaging system (Packard Canberra).

**Antisense Oligonucleotide (AS-Oligo) Treatment:** Phosphorothioate-derivatized antisense RAIDD and control oligonucleotides (C-oligos) (20 bp) were synthesized. The AS-oligo was 5'-GGGCGGCCTTCAGCCCCATG-3', and the C-oligo was 5'-TCAGACTGGCTCTCTCCATG-3'. Cells were plated in the presence or absence of 50 μM RAIDD AS-oligo or control C-oligo for 12 h and then stimulated with 0.5 nM activin. MTT assays were performed 72 h of ligand stimulation.

**Statistical Analysis:** Results are expressed as mean ± SD of three or more separate independent experiments. Statistical analysis was assessed by one-way ANOVA as indicated in the figure legend, using GraphPad Prism 4 software (GraphPad Software, Inc.). Statistical analyses were meant to compare fold induction of activin-treated samples among themselves within each experiment. For all statistical analyses and tests, a p value $< 0.05$ was considered significant and is indicated on the top of the error bars by an asterisk.

## RESULTS

**RAIDD is novel target gene of Activin signaling in human breast cancer cells T47D**: We have previously shown that activin induces cell-growth inhibition in human breast cancer (T47D) cells[441]. To identify novel activin target genes which may be responsible for mediating the growth-inhibitory effect, we performed Affymetrix human Gene Chip U95A microarray experiments using activin-treated T47D cells. From our microarray experiments, we found the mRNA level of the death adaptor protein RAIDD to be significantly increased T47D cells treated for 2 and 8 hours with activin (5.8 and 5.3, respectively), Fig.1a. The activin-induced increase in RAIDD mRNA levels was verified by RT–PCR using primers specific for RAIDD. As shown in Fig.1b, activin induces an increase in RAIDD mRNA levels at 2 hours and is maintained at 8 hours. Hence, the micro-array data was confirmed demonstrating an increase in the RAIDD transcript by activin treatment.

**Activin increases RAIDD protein levels**: To then investigate whether the activin induced increase in RAIDD mRNA levels was followed by an increase in RAIDD protein levels, T47D cells were treated or not with activin for 2 to 24 hours and total cell lysates were analyzed by Western blot using an anti-RAIDD antibody. As shown in Fig.2a (upper panel), activin induced an increase in RAIDD protein levels starting at 8 hours and maintained until 24 hours. Reprobing of the membrane with an anti-βtubulin antibody showed equal loading of proteins (Fig.2a, lower panel). We also wanted to investigate whether the effects of activin on RAIDD protein levels could be extended to other cell types. Therefore, we treated the human hepatocarcinoma cell line HUH7 with activin from 0 to 24 hours and evaluated RAIDD expression by Western blot analysis.

Activin was able to induced RAIDD protein levels in HUH7 cells as seen in Fig.2b (upper panel). The blot was reprobed with a βtubulin antibody as a control for equal loading Fig.2b (lower panel). Thus our findings demonstrate that activin is a key regulator of RAIDD expression in both breast and liver cancer cells.

**RAIDD is necessary for activin induced cell growth inhibition:** In order to evaluate the contribution of the RAIDD in activin-mediated cell growth inhibition in T47D cells, we used anti-sense phosphorothioated oligos targeted towards RAIDD mRNA or a scrambled oligo as a negative contro in MTT assays, Fig.3. T47D cells were cultured in DMEM, 2% serum for three days and stimulated or not with 0.5 nM of activin in the presence or the absence of the antisense oligos. As shown in Fig. 3, after three days, cell growth is reduced by 40% in activin-treated cells as compared to untreated cells. However, in the presence of the RAIDD antisense oligo (AS-RAIDD), the inhibitory effect of activin on cell growth is abolished. On the other hand, the activin effect on cell growth inhibition is maintained in samples treated with the control scrambled oligo (AS-CTL). Our results indicate that antisense RAIDD completely reverse the activin effect. These data demonstrate that activin acts as a potent cell growth inhibitor and that this effect requires the death adaptor protein, RAIDD.

## DISCUSSION

In this study, we demonstrate that RAIDD is a novel activin target gene and that its expression is necessary for mediating activin's growth inhibitory effect in breast cancer cells. The essential role of activin in inducing cell growth inhibition in a variety of cells and physiological conditions is well documented; however, the molecular mechanisms enabling this process are far from being clear. The identification of RAIDD as a target gene of activin signaling and its involvement in the inhibition of cell growth now provides a novel explanation for activin-induced cell growth inhibition.

The widespread expression of the activin-receptors, and RAIDD suggest that this mechanism may be a common method by which activin inhibits cell growth throughout the body. Indeed, we show that activin also upregulates RAIDD expression in human hepatocarcinoma cells. Activin has also been shown to induce apoptosis in various liver cells. It would be interesting to investigate whether RAIDD is involved in this biological process in other activin target tissues such as the liver.

Many types of tumour cells develop a resistance to activin-induced growth inhibition and concomitantly acquire the ability to release activin[505]. Once released, activin- may induce apoptosis in nearby lymphocytes, enabling the tumour cells to evade destruction by the cellular components of the immune system. Furthermore, there is a downregulation of activin and its signaling components in high grade breast cancers, suggesting a role for activin in breast tumor development[276]. As such, a loss of response to activin-induced cell growth inhibition may be crucial in the development of breast cancer. Observations such as these highlight the importance of activin-induced cell growth inhibition in many different pathological processes.

## ACKNOWLEDGMENTS

We are thankful to Dr Y Eto and Ajinomoto Co., Inc. for activin A. JJL is Research Scientist of the National Cancer Institute of Canada, supported with funds provided by the Canadian Cancer Society, SAL holds a Canadian research chair in Molecular Endocrinology, EC is a recipient of a McGill University Health Center and Faculty of Medicine scholarship. This work was supported by grants from the CIHR (MOP-53141 to JJL and MOP-49447 to SAL).

## FIGURES

**Figure 1.** Transcriptional Regulation of RAIDD Gene by Activin. Total RNA from T47D cells stimulated with activin for the indicated periods. (**A**) Results from Affymetrix human Gene Chip U95A microarray for RAIDD mRNA levels. (**B**) RT-PCR reactions were performed using oligo-dT and cDNAs were amplified using oligonucleotides specific to RAIDD and GAPDH.

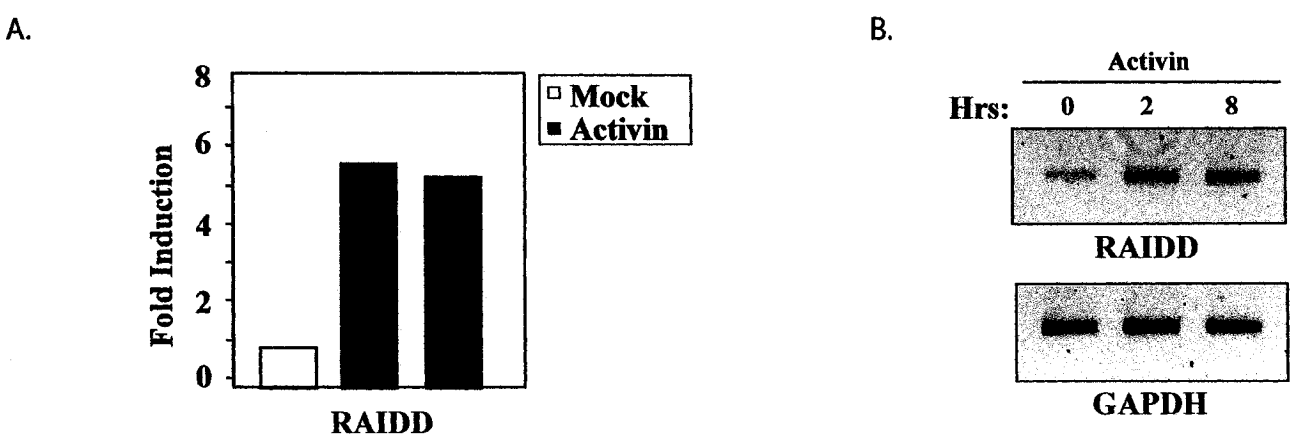



**Figure 1. Activin increases RAIDD mRNA levels in T47D human breast cancer cells.**

**Figure 2.** Activin induces RAIDD protein expression. (**A**) T47D and (**B**) HUH7 cells were stimulated with activin for various times. Total cell lysates were analysed by immunoblotting using a specific polyclonal antibody against RAIDD (top). Equal loading was confirmed with an anti-βtubulin antibody (bottom).

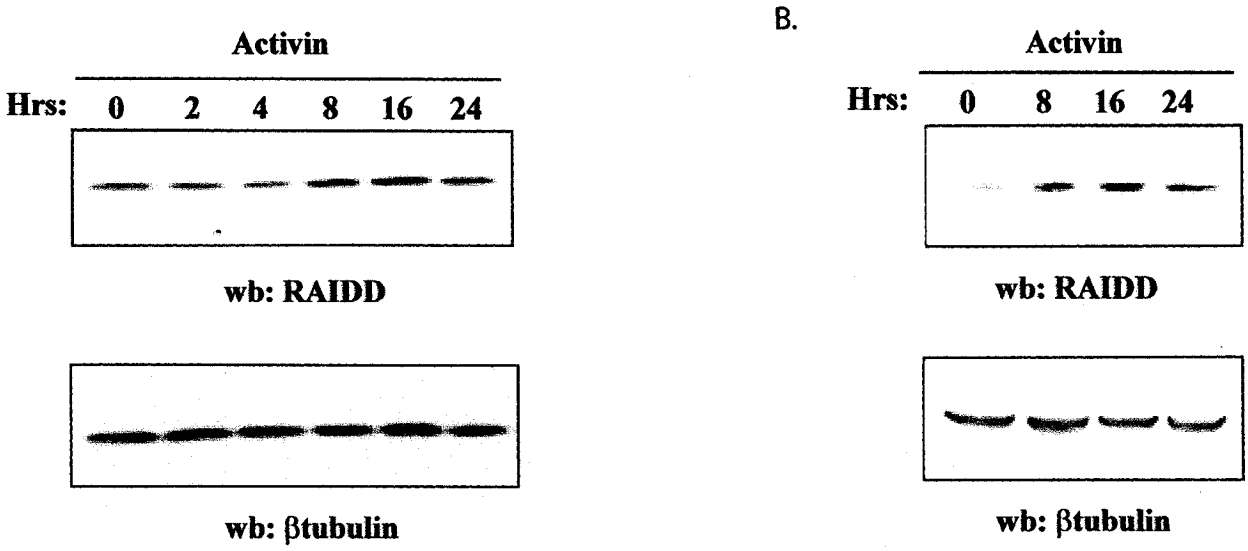



**Figure 2. Activin increases RAIDD protein levels.**

**Figure 3.** Inhibition of RAIDD expression prevents activin induced cell growth inhibition. T47D cells were treated for 12 hours with or without 50μM AS-RAIDD or AS-CTL, prior to activin stimulation. Cell viability was assessed 72 hours after 0.5nM activin treatment via MTT assays. For statistical analysis, One-way ANOVA was performed with ** $p<0.05$.

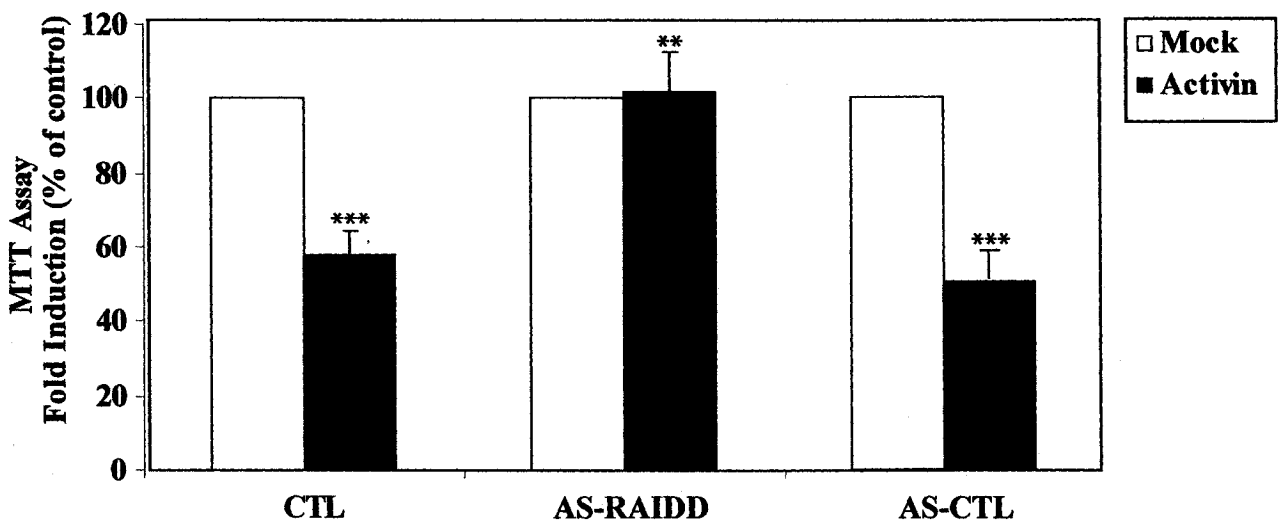



**Figure 3. Antisense-RAIDD antagonizes activin induced cell growth inhibition in breast cancer cells .**

# CHAPTER V.

# GENERAL DISCUSSION

## 1. Defining the Effect of Activin/TGFβ signaling on Mammary Gland Development

Activin and TGFβ signaling components are present throughout the development of the mammary gland, playing a crucial role in maintaining cellular homeostasis. Control of cellular homeostasis within the mammary gland by activin/TGFβ is thought to be due to its anti-proliferative and apoptotic effects. A critical regulator in breast development is the lactogenic hormone prolactin. Prolactin has a positive role in mammary gland biology. Through its main signaling effecter, Stat5, prolactin induces ductal and lobuloalveolar proliferation and differentiation. The final effect of prolactin signaling induced mammary differentiation is its ability to stimulate milk protein production through a direct transcriptional mechanism. It is therefore not surprising that activin/TGFβ and prolactin signaling pathways crosstalk with each other, resulting in antagonistic physiological effects. We have defined a novel crosstalk mechanism by which activin/TGFβ induced Smad activation results in the inhibition of lactogenic hormone induced Stat5 transactivation by decreasing Stat5 interaction with the co-activator CBP. The discovery of this novel crosstalk mechanism in mammary epithelial cells between the Smad and Stat pathway has allowed us to better understand the role of activin/TGFβ signaling in mammary gland development.

### 1.1. Other Potential Mechanism by which Smads Inhibit Stat5 Transactivation

Although, activin/TGFβ signaling appears to inhibit Stat5 interaction with CBP, TGFβ treatment does not completely prevent Stat5 and CBP interaction, CHAPTER II Figure 10. Therefore, additional mechanisms by which Smad2/3/4 may inhibit the transactivation of Stat5 are possible. Upon activin/TGFβ stimulation, the Smads may recruit a Stat5 co-repressor to the promoter and subsequently induce its inhibition. Otherwise, upon activin/TGFβ signaling, Smad2/3/4 may compete for other co-activators of Stat5 transactivation, similar to what we have shown for CBP.

Smad recruitment of a co-repressor to the Stat5 complex may be visualized by EMSA. More specifically, an additional shift in the EMSA is achieved using the Stat5 binding site of βcasein promoter, Chapter II Figure 4, in the lanes that were treated with HIP/activin or HIP/TGFβ. However, we do not see such a shift. On the other hand, we cannot exclude the possibility that the Smads are recruiting a co-repressor to both the βcasein and cyclin D1 promoters at a location other than the Stat5 binding site. However, a strong repression by Smad signaling on the artificial promoter construct containing five tandem repeats of the Stat5 binding site may be observed. The inhibitory effect of TGFβ is abolished when using the Smad4 knockout MEFs and the dominant negative Smads, Chapter II Figure 7. The effect of Smad signaling appears to be specific for Stat5 transactivation and may be due to Smads recruitment of a Stat5 repressor such as a histone deacetylase. Indeed, Smad3 interaction with HDAC4 and 5, leads to repression of gene expression[506]. The other possibility is that Smad2/3/4 may recruit away a co-activator from the Stat5 complex which may subsequently affect its transactivation.

Stat5 has been shown to interact with numerous transcription factors and co-regulators such as the glucocorticoid receptor[365], cetrosomal P4.1-associated protein (CPAP)[366], N-myc interactor (Nmi)[367], STAT5 and receptor corepressor (SMRT)[368], p100[369], ERK[370], CBP/p300[352], STAP-2/BKS[507], NCoA-1/SRC-1[508], Oct-1[509], and ERα[510]. All of the aforementioned act as either co-activators or co-repressors to Stat5 mediated gene modulation. To date, we are unable to observe any interactions between Stat5 and Smad2, Smad3 or Smad4 in our mammary epithelial cell system. Aside from CBP/p300, the Smads share other Stat5 binding partners such as the glucocorticoid receptor and NCoA-1/SRC-1. Perhaps, competition for binding with some of the essential binding partners is another mechanism by which the Smads are inhibiting Stat5 transactivation. For example, interaction of Stat5 with the glucocorticoid receptor protects Stat5 from inactivation by dephosphorylation and enhances its activation [365, 371]. Interestingly, the carboxyl-terminus domain of Smad3 may interact with the glucocorticoid receptor and inhibit Smad3 transactivation[134]. However, if Smad3 was recruiting away the glucocorticoid receptor from Stat5, a decrease in nuclear Stat5 phosphorylation would be observed. Since this is not the case, a mechanism by which glucocorticoid receptor is regulated by the Smads in order to inhibit Stat5-mediated gene activation is unlikely.

Another potential candidate that may be regulated by activin/TGFβ/Smad signaling is the nuclear receptor co-activator1 (NCoA-1) or steroid receptor co-activator 1 (SRC-1). Initially discovered as a factor that interacts with nuclear receptors by enhancing their transcription capabilities[511], NCoA-1/SRC-1 may interact with the transactivation domain of Stat5, augmenting PRL-induced βcasein activation[508]. Furthermore, NCoA-

1/SRC-1 may bind Smad3, enhancing the functional link between Smad3 and CBP/p300[512]. This interaction potentiates TGFβ-induced Smad3-mediated transcription[512]. Henceforth, NCoA-1/SRC-1 may also be competed away from Stat5 by Smad signaling, allowing for the inhibition of HIP induced Stat5 target gene activation.

### 1.2. Tight Regulation of Stat5 Necessary for Maintaining Proper Mammary Gland Development

Opposing physiological effects between activin/TGFβ signaling and the prolactin Jak-Stat pathway are prominent in the regulation of mammary gland development. We demonstrate a novel mechanism in which the intracellular mediators of activin/TGFβ signaling, the Smad proteins, inhibit Stat5 regulated gene induction by the inhibition of Stat5 transactivation. Our results highlight a novel crosstalk mechanism between two important signal transducing pathways. Interestingly, constitutively active Stat5 expression in the mammary gland leads to an increase in cellular proliferation during pregnancy in mice accompanied by shrunken alveoli during late pregnancy and early lactation[513]. The phenotype of the forced activation of Stat5 mouse highlights the importance of tight regulation of Stat5 activity in the mammary gland in order to prevent an abnormal phenotype. Our study, CHAPTER II, emphasizes the significance of activin and TGFβ signaling within the breast to maintain proper Stat5 gene activation. It is possible that activin and TGFβ induced inhibition of Stat5 transactivation is the mechanism by which Stat5 levels are monitored to avoid over-proliferation of the mammary epithelium and premature lactation.

## 2. Mode of Inhibition of Activin in Breast Cancer Cells

Although it appears that activin and TGFβ activate similar intracellular signaling cascades, the ligands and their distinct receptors differ greatly in their pattern of expression. Many *in vivo* and *in vitro* studies define possible roles for TGFβ in mammary gland development, breast cancer and breast cancer metastasis, activin induced signal transduction in this tissue remains elusive.

### 2.1. Activin induction of the p38 MAPK pathway

In our second study, we evaluate the effect of activin signaling in breast cancer cells. We have found that along with the canonical Smad pathway, the p38 MAPK pathway is induced by activin. Furthermore, we show that the p38 MAPK pathway is required for activin mediated cell growth inhibition. Using three specific p38 MAPK inhibitors we completely reverse activin inhibition in the breast cancer cell line T47D. A subsequent study performed to investigate activin's antiproliferative effects in breast cancer cells demonstrated that activin induced G1 arrest in T47D cells through enhanced expression of p15, reduced cyclin A expression, and reduced phosphorylation of the retinoblastoma (Rb) protein[286]. These authors were not able to see p38 MAPK activation by activin treatment. It is possible that their experimental conditions were dissimilar to ours and that although we both used T47D cells, inconsistencies among the same cell lines often exist. Hence, one must always be cautious with results obtained when limiting themselves to only one cell line. In fact, we were able to see p38 MAPK activation in other cell lines and another breast cancer cell line MCF7 (data not shown). Furthermore, we were able to see reversal of activin induced cell growth inhibition in MCF7 cells using the p38 MAPK pathway inhibitors.

We were the first to unveil that activin signals through Smad-independent pathway, the p38 MAPK pathway. Interestingly, studies performed subsequent to ours also observe activin induced activation of p38 MAPK pathway. For example, in the erythroleukemia cell line K562, activin induces p38 phosphorylation, mediating growth inhibition and hemoglobin synthesis[514]. Furthermore, activation of p38 MAPK pathway by activin in lactotrophs inhibits the pituitary transcription factor Pit-1[412]. Suggesting that activin induced activation of p38 MAPK is not limited to breast cancer cells.

### 2.2. Contribution of the Smad and p38 MAPK Pathways in Activin Mediated Cell Growth Inhibition in Breast Cancer Cells

Activin induces p38 MAPK activation and is involved in inducing cell growth inhibition in T47D breast cancer cells. This complete reversal by the p38 MAPK inhibitors suggests that the p38 MAPK pathway is central in mediating cell growth inhibition by activin in T47D cells. However, we still observe phosphorylation of Smad2 and activation of activin receptor/Smad responsive promoter constructs 3TP-Lux and ARE-Lux/Fast1 constructs, Chapter III Figure 3 and 4. A subsequent study has revealed that the Smad pathway is involved in mediating cell cycle arrest by activin in T47D cells[286]. Thus it would be interesting to investigate the relative contribution of the Smad and p38 MAPK pathways in provoking cell growth arrest in breast cancer cells. Interestingly, *in vitro* studies reveal that interaction between the p38 downstream target ATF2 and Smad3/4, mediate transcriptional activation by TGFβ[119, 480]. Therefore in T47D cells, a similar mechanism may exist which entails a point of convergence of the p38 and Smad pathway through ATF2.

Activin stimulation increases the mRNA of the cell cycle dependant inhibitors, $p15^{INK4B}$ and $p21CIP1^{/WAF1}$, Chapter III Figure 2. This suggests that the activin inhibition of cell growth might be due in part to upregulation of $p15^{INK4B}$ and $p21CIP1^{/WAF1}$. Indeed, the activin/TGFβ cytostatic response in multiple cell lines is mediated through a Smad dependent upregulation of $p15^{INK4B}$ and $p21CIP1^{/WAF1}$ [127, 144, 186, 187, 192, 515, 516]. Furthermore, TGFβ activation of p38 MAPK has also been shown to stabilize $p21CIP1^{/WAF1}$ levels[517]. This implies that both the Smad and p38 MAPK pathways may be involved in regulating $p15^{INK4B}$ and $p21CIP1^{/WAF1}$ expression. As a consequence, this may mediate activin induced cell growth arrest in T47D breast cancer cells.

### 2.3. Contribution of Novel Target Genes Regulated by Activin Signaling to the Activin and TGFβ Signaling Pathways

In the fourth chapter of my thesis, we discovered a novel activin target gene that is necessary in the anti-proliferative effect of activin in T47D cells. RAIDD, a death adaptor molecule, is upregulated by activin treatment, and its expression is imperative in blocking cell growth of breast cancer cells by activin. Another death adaptor molecule, Daxx, as described earlier, is involved in mediating TGFβ to induce apoptosis through direct interaction with the TβRII and activation of the JNK pathway[465]. Thus, we investigated RAIDD interaction with the activin receptors but we were unable to detect binding. What remains to be determined is the mechanism by which it mediates its anti-proliferative effects in breast cancer cells.

Interestingly, we have also characterized two other novel target genes, the inositol phosphatase Src homology 2 (SH2) domain-containing 5' inositol phosphatase (SHIP) and the protein G-coupled receptor kinase 2 (GRK2) that are strongly upregulated by

both activin and TGFβ. SHIP is an inositol lipid phosphatase that hydrolyses the D-5' phosphate position of both phosphatidylinosytol-3,4,5-triphosphate and inositol-1,3,4,5-tetrakisphosphate, which is critical in the regulation of cell growth[518]. Activin and TGFβ induced Smad signaling results in a robust increase in SHIP levels in immune cells. Furthermore, this increase in SHIP expression is necessary in mediating apoptosis in these cells through the regulation of lipid metabolism. Based on these results we wanted to examine the role of SHIP in the regulation of cell growth arrest by activin in breast cancer cells. However, SHIP expression is restricted to the hematopoietic system thus we investigated activin's effects on the far more ubiquitously expressed homologue SHIP2[519-521]. Unfortunately, we were not able to see any regulation of SHIP2 by activin in breast cancer cells (data not shown). Perhaps, TGFβ/activin-induced up-regulation of SHIP may be limited to hematopoietic cells and not to breast cancer cells. Most likely, in epithelial derived cancers, such as that of the breast, target genes like RAIDD, as we have demonstrated in CHAPTER IV, play an imperative role in controling activin's anti-proliferative effects.

We've also identified GRK2, a kinase involved in the desensitization of G protein-coupled receptors (GPCR), as a downstream target and regulator of the TGFβ-signaling cascade. We show that GRK2 expression levels are upregulated in response to activin/TGFβ signaling. Furthermore, GRK2 physically interacts with the MH1 and MH2 domains of the receptor-regulated Smads and phosphorylates their linker region on a specific single serine/threonine residue. GRK2-induced Smad phosphorylation blocks activin/TGFβ-induced Smad activation, nuclear translocation and target gene expression. The net effect of GRK2 on activin/TGFβ responses leads to an inhibition of their

antiproliferative and proapoptotic functions. Interestingly, activin was also able to induce GRK2 protein expression levels in MCF7 breast cancer cells. This study is particularly exciting due to the potential development of antagonists for anticancer therapies to activin/TGFβ Smad signaling. Recently, many studies have highlighted the therapeutic potential of various TGFβ antagonists as antimetastatic drugs[522, 523]. As such, a useful method in treating breast cancer metastasis may be through the modulation of GRK2 function.

### 3. Impact of Thesis Discoveries on Breast Cancer

Breast cancer is the most common cancer in women. One in every nine Canadian women will develop breast cancer in a lifetime and one in twenty-seven will die. In 2006, an estimated 22,200 Canadian women will be diagnosed with breast cancer and 5,300 will die of it. Furthermore, approximately 160 men will be diagnosed with breast cancer and 45 will die of it[524]. Deciphering the crosstalk mechanisms and key signaling pathways in breast tumorigenesis and metastasis is critical for the development of new therapies to treat human breast cancer.

It would not be surprising that Smad antagonism on Stat5 mediated gene activation is a common mode of action for activin/TGFβ to block prolactin biological effects. Henceforth, a tight regulation of activin/TGFβ signaling components in mammary gland development and carcinogenesis is necessary to sustain normal homeostasis. A high proportion of human breast cancers have reported Stat5 to be activated and localized in the nucleus[388]. Overexpressing Smads in these specific types of breast cancers would

block Stat5 activation, as we have shown *in vitro* in CHAPTER II, which could lead to a potential treatment of this disease. On the other hand, in more advanced stages of the disease, TGFβ signaling promotes cancer progression. We have shown in a collaborative study that blocking prolactin signaling triggers the TGFβ pathway, causing breast cancer cells to become more invasive[391]. Hence, activin/TGFβ mediated inhibition of Stat5 transactivation may be another means by which activin/TGFβ may induce breast cancer metastasis by blocking prolactin anti-invasive signaling.

Besides the antagonistic crosstalk-mechanism between Smads and Stat5, we have identified two major players in activin induced breast cancer cell growth inhibition, the p38 MAPK pathways and the death adaptor RAIDD. The process by which mammary tissues become cancerous must involve a multistep component where genes and pathways are misregulated resulting in malignancy. An extensive analysis of the precise signaling mechanisms, crosstalk pathways and genes that are regulated by activin and TGFβ signaling pathways will allow us to decipher their relative contributions to the development, differentiation, transformation and tumor progression of the mammary gland. Targeting specific pathways such as the p38 MAPK or particular genes like RAIDD would pave the way for a mechanism-based design of anti-cancer drugs that would be beneficial in the treatment of patients with breast cancer.

## REFERENCES


1. Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M. & Sporn, M.B. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proc Natl Acad Sci U S A* **78**, 5339-43 (1981).
2. Anzano, M.A., Roberts, A.B., Smith, J.M., Sporn, M.B. & De Larco, J.E. 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**, 6264-8 (1983).
3. Kehrl, J.H. et al. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* **163**, 1037-50 (1986).
4. Dickson, M.C. et al. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* **121**, 1845-54 (1995).
5. Shull, M.M. et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* **359**, 693-9 (1992).
6. Kulkarni, A.B. et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A* **90**, 770-4 (1993).
7. Kulkarni, A.B. & Karlsson, S. Transforming growth factor-beta 1 knockout mice. A mutation in one cytokine gene causes a dramatic inflammatory disease. *Am J Pathol* **143**, 3-9 (1993).
8. Diebold, R.J. et al. 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 (1995).
9. Sanford, L.P. et al. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* **124**, 2659-70 (1997).
10. Kaartinen, V. et al. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* **11**, 415-21 (1995).
11. Proetzel, G. et al. Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* **11**, 409-14 (1995).
12. Lawrence, D.A., Pircher, R., Kryceve-Martinerie, C. & Jullien, P. Normal embryo fibroblasts release transforming growth factors in a latent form. *J Cell Physiol* **121**, 184-8 (1984).
13. Gentry, L.E., Lioubin, M.N., Purchio, A.F. & Marquardt, H. Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. *Mol Cell Biol* **8**, 4162-8 (1988).
14. Dubois, C.M., Laprise, M.H., Blanchette, F., Gentry, L.E. & Leduc, R. Processing of transforming growth factor beta 1 precursor by human furin convertase. *J Biol Chem* **270**, 10618-24 (1995).

15. Gentry, L.E. & Nash, B.W. The pro domain of pre-pro-transforming growth factor beta 1 when independently expressed is a functional binding protein for the mature growth factor. *Biochemistry* **29**, 6851-7 (1990).
16. Olofsson, A. et al. Latent transforming growth factor-beta complex in Chinese hamster ovary cells contains the multifunctional cysteine-rich fibroblast growth factor receptor, also termed E-selectin-ligand or MG-160. *Biochem J* **324 ( Pt 2)**, 427-34 (1997).
17. Saharinen, J., Taipale, J., Monni, O. & Keski-Oja, J. Identification and characterization of a new latent transforming growth factor-beta-binding protein, LTBP-4. *J Biol Chem* **273**, 18459-69 (1998).
18. Giltay, R., Kostka, G. & Timpl, R. Sequence and expression of a novel member (LTBP-4) of the family of latent transforming growth factor-beta binding proteins. *FEBS Lett* **411**, 164-8 (1997).
19. Yin, W. et al. Isolation of a novel latent transforming growth factor-beta binding protein gene (LTBP-3). *J Biol Chem* **270**, 10147-60 (1995).
20. Wang, L., Clutter, S., Benincosa, J., Fortney, J. & Gibson, L.F. Activation of transforming growth factor-beta1/p38/Smad3 signaling in stromal cells requires reactive oxygen species-mediated MMP-2 activity during bone marrow damage. *Stem Cells* **23**, 1122-34 (2005).
21. Miyazono, K. & Heldin, C.H. Structure, function and possible clinical application of transforming growth factor-beta. *J Dermatol* **19**, 644-7 (1992).
22. Taipale, J., Saharinen, J., Hedman, K. & Keski-Oja, J. Latent transforming growth factor-beta 1 and its binding protein are components of extracellular matrix microfibrils. *J Histochem Cytochem* **44**, 875-89 (1996).
23. Vale, W. et al. Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* **321**, 776-9 (1986).
24. Ling, N. et al. A homodimer of the beta-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. *Biochem Biophys Res Commun* **138**, 1129-37 (1986).
25. Oda, S., Nishimatsu, S., Murakami, K. & Ueno, N. Molecular cloning and functional analysis of a new activin beta subunit: a dorsal mesoderm-inducing activity in Xenopus. *Biochem Biophys Res Commun* **210**, 581-8 (1995).
26. Fang, J., Yin, W., Smiley, E., Wang, S.Q. & Bonadio, J. Molecular cloning of the mouse activin beta E subunit gene. *Biochem Biophys Res Commun* **228**, 669-74 (1996).
27. Vejda, S. et al. Expression and dimerization of the rat activin subunits betaC and betaE: evidence for the ormation of novel activin dimers. *J Mol Endocrinol* **28**, 137-48 (2002).
28. O'Bryan, M.K. et al. Cloning and regulation of the rat activin betaE subunit. *J Mol Endocrinol* **24**, 409-18 (2000).
29. Gold, E.J. et al. Cell-specific expression of betaC-activin in the rat reproductive tract, adrenal and liver. *Mol Cell Endocrinol* **222**, 61-9 (2004).
30. Lau, A.L., Kumar, T.R., Nishimori, K., Bonadio, J. & Matzuk, M.M. Activin betaC and betaE genes are not essential for mouse liver growth, differentiation, and regeneration. *Mol Cell Biol* **20**, 6127-37 (2000).
31. Matzuk, M.M., Kumar, T.R. & Bradley, A. Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature* **374**, 356-60 (1995).

32. Ferguson, C.A. et al. Activin is an essential early mesenchymal signal in tooth development that is required for patterning of the murine dentition. *Genes Dev* **12**, 2636-49 (1998).
33. Schrewe, H., Gendron-Maguire, M., Harbison, M.L. & Gridley, T. Mice homozygous for a null mutation of activin beta B are viable and fertile. *Mech Dev* **47**, 43-51 (1994).
34. Vassalli, A., Matzuk, M.M., Gardner, H.A., Lee, K.F. & Jaenisch, R. Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. *Genes Dev* **8**, 414-27 (1994).
35. Huylebroeck, D. et al. Expression and processing of the activin-A/erythroid differentiation factor precursor: a member of the transforming growth factor-beta superfamily. *Mol Endocrinol* **4**, 1153-65 (1990).
36. Gray, A.M. & Mason, A.J. Requirement for activin A and transforming growth factor--beta 1 pro-regions in homodimer assembly. *Science* **247**, 1328-30 (1990).
37. Todorovic, V. et al. Latent TGF-beta binding proteins. *Int J Biochem Cell Biol* **37**, 38-41 (2005).
38. Cheng, J. et al. TGF-beta1 stimulates monocyte chemoattractant protein-1 expression in mesangial cells through a phosphodiesterase isoenzyme 4-dependent process. *Am J Physiol Cell Physiol* **289**, C959-70 (2005).
39. Chen, D., Zhao, M. & Mundy, G.R. Bone morphogenetic proteins. *Growth Factors* **22**, 233-41 (2004).
40. Teixeira, J., Maheswaran, S. & Donahoe, P.K. Mullerian inhibiting substance: an instructive developmental hormone with diagnostic and possible therapeutic applications. *Endocr Rev* **22**, 657-74 (2001).
41. Mathews, L.S. & Vale, W.W. Expression cloning of an activin receptor, a predicted transmembrane serine kinase. *Cell* **65**, 973-82 (1991).
42. ten Dijke, P. et al. Characterization of type I receptors for transforming growth factor-beta and activin. *Science* **264**, 101-4 (1994).
43. Carcamo, J. et al. Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor beta and activin. *Mol Cell Biol* **14**, 3810-21 (1994).
44. Tsuchida, K. et al. Activin isoforms signal through type I receptor serine/threonine kinase ALK7. *Mol Cell Endocrinol* **220**, 59-65 (2004).
45. Franzen, P. et al. Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. *Cell* **75**, 681-92 (1993).
46. Attisano, L. et al. Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* **75**, 671-80 (1993).
47. Roelen, B.A., van Rooijen, M.A. & Mummery, C.L. 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**, 418-30 (1997).
48. Lin, H.Y., Wang, X.F., Ng-Eaton, E., Weinberg, R.A. & Lodish, H.F. Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. *Cell* **68**, 775-85 (1992).
49. Attisano, L., Wrana, J.L., Cheifetz, S. & Massague, J. Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. *Cell* **68**, 97-108 (1992).

50. Mathews, L.S., Vale, W.W. & Kintner, C.R. Cloning of a second type of activin receptor and functional characterization in Xenopus embryos. *Science* **255**, 1702-5 (1992).
51. Pepin, M.C., Beauchemin, M., Plamondon, J. & O'Connor-McCourt, M.D. Scanning-deletion analysis of the extracellular domain of the TGF-beta receptor type II. *Biochem Biophys Res Commun* **220**, 289-93 (1996).
52. Herpin, A., Favrel, P. & Cunningham, C. Gene structure and expression of cg-ALR1, a type I activin-like receptor from the bivalve mollusc Crassostrea gigas. *Gene* **301**, 21-30 (2002).
53. Attisano, L., Wrana, J.L., Montalvo, E. & Massague, J. Activation of signalling by the activin receptor complex. *Mol Cell Biol* **16**, 1066-73 (1996).
54. Chen, Y.G. et al. Determinants of specificity in TGF-beta signal transduction. *Genes Dev* **12**, 2144-52 (1998).
55. Chen, Y.G. & Massague, J. Smad1 recognition and activation by the ALK1 group of transforming growth factor-beta family receptors. *J Biol Chem* **274**, 3672-7 (1999).
56. Feng, X.H. & Derynck, R. A kinase subdomain of transforming growth factor-beta (TGF-beta) type I receptor determines the TGF-beta intracellular signaling specificity. *Embo J* **16**, 3912-23 (1997).
57. Lopez-Casillas, F. et al. Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. *Cell* **67**, 785-95 (1991).
58. Lopez-Casillas, F., Wrana, J.L. & Massague, J. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* **73**, 1435-44 (1993).
59. Wang, X.F. et al. Expression cloning and characterization of the TGF-beta type III receptor. *Cell* **67**, 797-805 (1991).
60. Lewis, K.A. et al. Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. *Nature* **404**, 411-4 (2000).
61. Gougos, A. & Letarte, M. Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. *J Biol Chem* **265**, 8361-4 (1990).
62. Lastres, P. et al. Endoglin modulates cellular responses to TGF-beta 1. *J Cell Biol* **133**, 1109-21 (1996).
63. Barbara, N.P., Wrana, J.L. & Letarte, M. Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-beta superfamily. *J Biol Chem* **274**, 584-94 (1999).
64. Newfeld, S.J., Chartoff, E.H., Graff, J.M., Melton, D.A. & Gelbart, W.M. Mothers against dpp encodes a conserved cytoplasmic protein required in DPP/TGF-beta responsive cells. *Development* **122**, 2099-108 (1996).
65. Savage, C. et al. Caenorhabditis elegans genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor beta pathway components. *Proc Natl Acad Sci U S A* **93**, 790-4 (1996).
66. Liu, F. et al. A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature* **381**, 620-3 (1996).
67. Massague, J. TGF-beta signal transduction. *Annu Rev Biochem* **67**, 753-91 (1998).
68. Hahn, S.A. et al. Allelotype of pancreatic adenocarcinoma using xenograft enrichment. *Cancer Res* **55**, 4670-5 (1995).

69. Shi, Y. et al. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. *Cell* **94**, 585-94 (1998).
70. Wu, J.W. et al. Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-beta signaling. *Mol Cell* **8**, 1277-89 (2001).
71. Wu, J.W., Fairman, R., Penry, J. & Shi, Y. Formation of a stable heterodimer between Smad2 and Smad4. *J Biol Chem* **276**, 20688-94 (2001).
72. Tsukazaki, T., Chiang, T.A., Davison, A.F., Attisano, L. & Wrana, J.L. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* **95**, 779-91 (1998).
73. Wu, G. et al. Structural basis of Smad2 recognition by the Smad anchor for receptor activation. *Science* **287**, 92-7 (2000).
74. Di Guglielmo, G.M., Le Roy, C., Goodfellow, A.F. & Wrana, J.L. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol* **5**, 410-21 (2003).
75. Hayes, S., Chawla, A. & Corvera, S. TGF beta receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2. *J Cell Biol* **158**, 1239-49 (2002).
76. Panopoulou, E. et al. Early endosomal regulation of Smad-dependent signaling in endothelial cells. *J Biol Chem* **277**, 18046-52 (2002).
77. Hocevar, B.A., Smine, A., Xu, X.X. & Howe, P.H. The adaptor molecule Disabled-2 links the transforming growth factor beta receptors to the Smad pathway. *Embo J* **20**, 2789-801 (2001).
78. Yamakawa, N., Tsuchida, K. & Sugino, H. The rasGAP-binding protein, Dok-1, mediates activin signaling via serine/threonine kinase receptors. *Embo J* **21**, 1684-94 (2002).
79. Kretzschmar, M., Doody, J., Timokhina, I. & Massague, J. A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev* **13**, 804-16 (1999).
80. Yakymovych, I., Ten Dijke, P., Heldin, C.H. & Souchelnytskyi, S. Regulation of Smad signaling by protein kinase C. *Faseb J* **15**, 553-5 (2001).
81. Wicks, S.J., Lui, S., Abdel-Wahab, N., Mason, R.M. & Chantry, A. Inactivation of smad-transforming growth factor beta signaling by Ca(2+)-calmodulin-dependent protein kinase II. *Mol Cell Biol* **20**, 8103-11 (2000).
82. Matsuura, I. et al. Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* **430**, 226-31 (2004).
83. Ho, J. et al. The G protein-coupled receptor kinase-2 is a TGFbeta-inducible antagonist of TGFbeta signal transduction. *Embo J* **24**, 3247-58 (2005).
84. Mori, S. et al. TGF-beta and HGF transmit the signals through JNK-dependent Smad2/3 phosphorylation at the linker regions. *Oncogene* **23**, 7416-29 (2004).
85. Yoshida, K. et al. Transforming growth factor-beta and platelet-derived growth factor signal via c-Jun N-terminal kinase-dependent Smad2/3 phosphorylation in rat hepatic stellate cells after acute liver injury. *Am J Pathol* **166**, 1029-39 (2005).
86. Kamaraju, A.K. & Roberts, A.B. Role of Rho/ROCK and p38 MAP kinase pathways in transforming growth factor-beta-mediated Smad-dependent growth inhibition of human breast carcinoma cells in vivo. *J Biol Chem* **280**, 1024-36 (2005).

87. Lin, X. et al. PPM1A functions as a Smad phosphatase to terminate TGFbeta signaling. *Cell* **125**, 915-28 (2006).
88. Inoue, Y. et al. Smad3 is acetylated by p300/CBP to regulate its transactivation activity. *Oncogene* (2006).
89. Gronroos, E., Hellman, U., Heldin, C.H. & Ericsson, J. Control of Smad7 stability by competition between acetylation and ubiquitination. *Mol Cell* **10**, 483-93 (2002).
90. Lo, R.S. & Massague, J. Ubiquitin-dependent degradation of TGF-beta-activated smad2. *Nat Cell Biol* **1**, 472-8 (1999).
91. Zhu, H., Kavsak, P., Abdollah, S., Wrana, J.L. & Thomsen, G.H. A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature* **400**, 687-93 (1999).
92. Koinuma, D. et al. Arkadia amplifies TGF-beta superfamily signalling through degradation of Smad7. *Embo J* **22**, 6458-70 (2003).
93. Izzi, L. & Attisano, L. Ubiquitin-Dependent Regulation of TGFbeta Signaling in Cancer. *Neoplasia* **8**, 677-88 (2006).
94. Seo, S.R. et al. The novel E3 ubiquitin ligase Tiul1 associates with TGIF to target Smad2 for degradation. *Embo J* **23**, 3780-92 (2004).
95. Wan, M. et al. Smad4 protein stability is regulated by ubiquitin ligase SCF beta-TrCP1. *J Biol Chem* **279**, 14484-7 (2004).
96. Dupont, S. et al. Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. *Cell* **121**, 87-99 (2005).
97. Bai, Y., Yang, C., Hu, K., Elly, C. & Liu, Y.C. Itch E3 ligase-mediated regulation of TGF-beta signaling by modulating smad2 phosphorylation. *Mol Cell* **15**, 825-31 (2004).
98. Lee, P.S., Chang, C., Liu, D. & Derynck, R. Sumoylation of Smad4, the common Smad mediator of transforming growth factor-beta family signaling. *J Biol Chem* **278**, 27853-63 (2003).
99. Lin, X. et al. Activation of transforming growth factor-beta signaling by SUMO-1 modification of tumor suppressor Smad4/DPC4. *J Biol Chem* **278**, 18714-9 (2003).
100. Ohshima, T. & Shimotohno, K. Transforming growth factor-beta-mediated signaling via the p38 MAP kinase pathway activates Smad-dependent transcription through SUMO-1 modification of Smad4. *J Biol Chem* **278**, 50833-42 (2003).
101. Long, J., Wang, G., He, D. & Liu, F. Repression of Smad4 transcriptional activity by SUMO modification. *Biochem J* **379**, 23-9 (2004).
102. Chang, C.C., Lin, D.Y., Fang, H.I., Chen, R.H. & Shih, H.M. Daxx mediates the small ubiquitin-like modifier-dependent transcriptional repression of Smad4. *J Biol Chem* **280**, 10164-73 (2005).
103. Xu, L., Kang, Y., Col, S. & Massague, J. Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus. *Mol Cell* **10**, 271-82 (2002).
104. Xu, L., Alarcon, C., Col, S. & Massague, J. Distinct domain utilization by Smad3 and Smad4 for nucleoporin interaction and nuclear import. *J Biol Chem* **278**, 42569-77 (2003).

105. Xiao, Z., Liu, X. & Lodish, H.F. Importin beta mediates nuclear translocation of Smad 3. *J Biol Chem* **275**, 23425-8 (2000).
106. Kurisaki, A., Kose, S., Yoneda, Y., Heldin, C.H. & Moustakas, A. Transforming growth factor-beta induces nuclear import of Smad3 in an importin-beta1 and Ran-dependent manner. *Mol Biol Cell* **12**, 1079-91 (2001).
107. Fornerod, M., Ohno, M., Yoshida, M. & Mattaj, I.W. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-60 (1997).
108. Pierreux, C.E., Nicolas, F.J. & Hill, C.S. Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus. *Mol Cell Biol* **20**, 9041-54 (2000).
109. Watanabe, M., Masuyama, N., Fukuda, M. & Nishida, E. Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal. *EMBO Rep* **1**, 176-82 (2000).
110. Inman, G.J., Nicolas, F.J. & Hill, C.S. Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. *Mol Cell* **10**, 283-94 (2002).
111. Labbe, E., Silvestri, C., Hoodless, P.A., Wrana, J.L. & Attisano, L. Smad2 and Smad3 positively and negatively regulate TGF beta-dependent transcription through the forkhead DNA-binding protein FAST2. *Mol Cell* **2**, 109-20 (1998).
112. Feng, X.H. & Derynck, R. Specificity and versatility in tgf-beta signaling through Smads. *Annu Rev Cell Dev Biol* **21**, 659-93 (2005).
113. Chen, C.R., Kang, Y., Siegel, P.M. & Massague, J. E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. *Cell* **110**, 19-32 (2002).
114. Grinberg, A.V. & Kerppola, T. Both Max and TFE3 cooperate with Smad proteins to bind the plasminogen activator inhibitor-1 promoter, but they have opposite effects on transcriptional activity. *J Biol Chem* **278**, 11227-36 (2003).
115. Liu, D., Black, B.L. & Derynck, R. TGF-beta inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3. *Genes Dev* **15**, 2950-66 (2001).
116. Hua, X., Miller, Z.A., Wu, G., Shi, Y. & Lodish, H.F. Specificity in transforming growth factor beta-induced transcription of the plasminogen activator inhibitor-1 gene: interactions of promoter DNA, transcription factor muE3, and Smad proteins. *Proc Natl Acad Sci U S A* **96**, 13130-5 (1999).
117. Huse, M. et al. The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. *Mol Cell* **8**, 671-82 (2001).
118. Kawata, Y. et al. bcn-1 Element-dependent activation of the laminin gamma 1 chain gene by the cooperative action of transcription factor E3 (TFE3) and Smad proteins. *J Biol Chem* **277**, 11375-84 (2002).
119. Sano, Y. et al. ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-beta signaling. *J Biol Chem* **274**, 8949-57 (1999).
120. Kang, Y., Chen, C.R. & Massague, J. A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* **11**, 915-26 (2003).
121. Zhang, Y., Feng, X.H. & Derynck, R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature* **394**, 909-13 (1998).

122. Frederick, J.P., Liberati, N.T., Waddell, D.S., Shi, Y. & Wang, X.F. Transforming growth factor beta-mediated transcriptional repression of c-myc is dependent on direct binding of Smad3 to a novel repressive Smad binding element. *Mol Cell Biol* **24**, 2546-59 (2004).
123. Choy, L. & Derynck, R. Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. *J Biol Chem* **278**, 9609-19 (2003).
124. Coyle-Rink, J. et al. Interaction between TGFbeta signaling proteins and C/EBP controls basal and Tat-mediated transcription of HIV-1 LTR in astrocytes. *Virology* **299**, 240-7 (2002).
125. Chen, X. et al. Smad4 and FAST-1 in the assembly of activin-responsive factor. *Nature* **389**, 85-9 (1997).
126. Randall, R.A. et al. Recognition of phosphorylated-Smad2-containing complexes by a novel Smad interaction motif. *Mol Cell Biol* **24**, 1106-21 (2004).
127. Seoane, J., Le, H.V. & Massague, J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* **419**, 729-34 (2002).
128. Chiba, S. et al. Homeoprotein DLX-1 interacts with Smad4 and blocks a signaling pathway from activin A in hematopoietic cells. *Proc Natl Acad Sci U S A* **100**, 15577-82 (2003).
129. Germain, S., Howell, M., Esslemont, G.M. & Hill, C.S. Homeodomain and winged-helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. *Genes Dev* **14**, 435-51 (2000).
130. Chipuk, J.E. et al. The androgen receptor represses transforming growth factor-beta signaling through interaction with Smad3. *J Biol Chem* **277**, 1240-8 (2002).
131. Hayes, S.A. et al. SMAD3 represses androgen receptor-mediated transcription. *Cancer Res* **61**, 2112-8 (2001).
132. Malek, D., Gust, R. & Kleuser, B. 17-Beta-estradiol inhibits transforming-growth-factor-beta-induced MCF-7 cell migration by Smad3-repression. *Eur J Pharmacol* **534**, 39-47 (2006).
133. Matsuda, T., Yamamoto, T., Muraguchi, A. & Saatcioglu, F. Cross-talk between transforming growth factor-beta and estrogen receptor signaling through Smad3. *J Biol Chem* **276**, 42908-14 (2001).
134. Song, C.Z., Tian, X. & Gelehrter, T.D. Glucocorticoid receptor inhibits transforming growth factor-beta signaling by directly targeting the transcriptional activation function of Smad3. *Proc Natl Acad Sci U S A* **96**, 11776-81 (1999).
135. Chou, W.C. et al. Mechanism of a transcriptional cross talk between transforming growth factor-beta-regulated Smad3 and Smad4 proteins and orphan nuclear receptor hepatocyte nuclear factor-4. *Mol Biol Cell* **14**, 1279-94 (2003).
136. Pendaries, V., Verrecchia, F., Michel, S. & Mauviel, A. Retinoic acid receptors interfere with the TGF-beta/Smad signaling pathway in a ligand-specific manner. *Oncogene* **22**, 8212-20 (2003).
137. Yanagisawa, J. et al. Convergence of transforming growth factor-beta and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science* **283**, 1317-21 (1999).

138. Hanai, J. et al. Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter. *J Biol Chem* **274**, 31577-82 (1999).
139. Pardali, K. et al. Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. *J Biol Chem* **275**, 29244-56 (2000).
140. Zhang, Y. & Derynck, R. Transcriptional regulation of the transforming growth factor-beta -inducible mouse germ line Ig alpha constant region gene by functional cooperation of Smad, CREB, and AML family members. *J Biol Chem* **275**, 16979-85 (2000).
141. Blokzijl, A. et al. Cross-talk between the Notch and TGF-beta signaling pathways mediated by interaction of the Notch intracellular domain with Smad3. *J Cell Biol* **163**, 723-8 (2003).
142. Liu, F., Massague, J. & Ruiz i Altaba, A. Carboxy-terminally truncated Gli3 proteins associate with Smads. *Nat Genet* **20**, 325-6 (1998).
143. Hata, A. et al. OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. *Cell* **100**, 229-40 (2000).
144. Feng, X.H., Lin, X. & Derynck, R. Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-beta. *Embo J* **19**, 5178-93 (2000).
145. Kurisaki, K. et al. Nuclear factor YY1 inhibits transforming growth factor beta- and bone morphogenetic protein-induced cell differentiation. *Mol Cell Biol* **23**, 4494-510 (2003).
146. Lee, K.H., Evans, S., Ruan, T.Y. & Lassar, A.B. SMAD-mediated modulation of YY1 activity regulates the BMP response and cardiac-specific expression of a GATA4/5/6-dependent chick Nkx2.5 enhancer. *Development* **131**, 4709-23 (2004).
147. Warner, D.R., Roberts, E.A., Greene, R.M. & Pisano, M.M. Identification of novel Smad binding proteins. *Biochem Biophys Res Commun* **312**, 1185-90 (2003).
148. Hu, M.C. & Rosenblum, N.D. Smad1, beta-catenin and Tcf4 associate in a molecular complex with the Myc promoter in dysplastic renal tissue and cooperate to control Myc transcription. *Development* **132**, 215-25 (2005).
149. Hussein, S.M., Duff, E.K. & Sirard, C. Smad4 and beta-catenin co-activators functionally interact with lymphoid-enhancing factor to regulate graded expression of Msx2. *J Biol Chem* **278**, 48805-14 (2003).
150. Lei, S., Dubeykovskiy, A., Chakladar, A., Wojtukiewicz, L. & Wang, T.C. The murine gastrin promoter is synergistically activated by transforming growth factor-beta/Smad and Wnt signaling pathways. *J Biol Chem* **279**, 42492-502 (2004).
151. Sanchez-Elsner, T. et al. Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression. *J Biol Chem* **276**, 38527-35 (2001).
152. Qing, J. et al. Transforming growth factor beta/Smad3 signaling regulates IRF-7 function and transcriptional activation of the beta interferon promoter. *Mol Cell Biol* **24**, 1411-25 (2004).

153. Labbe, E., Letamendia, A. & Attisano, L. Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and wnt pathways. *Proc Natl Acad Sci U S A* **97**, 8358-63 (2000).
154. Nishita, M. et al. Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer. *Nature* **403**, 781-5 (2000).
155. Liu, D., Kang, J.S. & Derynck, R. TGF-beta-activated Smad3 represses MEF2-dependent transcription in myogenic differentiation. *Embo J* **23**, 1557-66 (2004).
156. Kaji, H., Canaff, L., Lebrun, J.J., Goltzman, D. & Hendy, G.N. Inactivation of menin, a Smad3-interacting protein, blocks transforming growth factor type beta signaling. *Proc Natl Acad Sci U S A* **98**, 3837-42 (2001).
157. Lopez-Rovira, T., Chalaux, E., Massague, J., Rosa, J.L. & Ventura, F. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. *J Biol Chem* **277**, 3176-85 (2002).
158. Komuro, A. et al. Negative regulation of transforming growth factor-beta (TGF-beta) signaling by WW domain-containing protein 1 (WWP1). *Oncogene* **23**, 6914-23 (2004).
159. Cordenonsi, M. et al. Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. *Cell* **113**, 301-14 (2003).
160. Takebayashi-Suzuki, K. et al. Interplay between the tumor suppressor p53 and TGF beta signaling shapes embryonic body axes in Xenopus. *Development* **130**, 3929-39 (2003).
161. Wilkinson, D.S. et al. A direct intersection between p53 and transforming growth factor beta pathways targets chromatin modification and transcription repression of the alpha-fetoprotein gene. *Mol Cell Biol* **25**, 1200-12 (2005).
162. Costamagna, E., Garcia, B. & Santisteban, P. The functional interaction between the paired domain transcription factor Pax8 and Smad3 is involved in transforming growth factor-beta repression of the sodium/iodide symporter gene. *J Biol Chem* **279**, 3439-46 (2004).
163. Qiu, P., Feng, X.H. & Li, L. Interaction of Smad3 and SRF-associated complex mediates TGF-beta1 signals to regulate SM22 transcription during myofibroblast differentiation. *J Mol Cell Cardiol* **35**, 1407-20 (2003).
164. Hata, A., Lagna, G., Massague, J. & Hemmati-Brivanlou, A. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev* **12**, 186-97 (1998).
165. Hayashi, H. et al. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* **89**, 1165-73 (1997).
166. Nakao, A. et al. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* **389**, 631-5 (1997).
167. Kavsak, P. et al. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* **6**, 1365-75 (2000).
168. Suzuki, C. et al. Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. *J Biol Chem* **277**, 39919-25 (2002).
169. Shi, W. et al. GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor. *J Cell Biol* **164**, 291-300 (2004).

170. Itoh, S. et al. Transforming growth factor beta1 induces nuclear export of inhibitory Smad7. *J Biol Chem* **273**, 29195-201 (1998).
171. Ulloa, L., Doody, J. & Massague, J. Inhibition of transforming growth factor-beta/SMAD signalling by the interferon-gamma/STAT pathway. *Nature* **397**, 710-3 (1999).
172. Bitzer, M. et al. A mechanism of suppression of TGF-beta/SMAD signaling by NF-kappa B/RelA. *Genes Dev* **14**, 187-97 (2000).
173. Waldrip, W.R., Bikoff, E.K., Hoodless, P.A., Wrana, J.L. & Robertson, E.J. Smad2 signaling in extraembryonic tissues determines anterior-posterior polarity of the early mouse embryo. *Cell* **92**, 797-808 (1998).
174. Weinstein, M. et al. Failure of egg cylinder elongation and mesoderm induction in mouse embryos lacking the tumor suppressor smad2. *Proc Natl Acad Sci U S A* **95**, 9378-83 (1998).
175. Sirard, C. et al. The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev* **12**, 107-19 (1998).
176. Yang, X. et al. Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension. *Circ Res* **96**, 1053-63 (2005).
177. Zhu, Y., Richardson, J.A., Parada, L.F. & Graff, J.M. Smad3 mutant mice develop metastatic colorectal cancer. *Cell* **94**, 703-14 (1998).
178. Yang, Y.A. et al. Smad3 in the mammary epithelium has a nonredundant role in the induction of apoptosis, but not in the regulation of proliferation or differentiation by transforming growth factor-beta. *Cell Growth Differ* **13**, 123-30 (2002).
179. Datto, M.B. et al. Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. *Mol Cell Biol* **19**, 2495-504 (1999).
180. Ashcroft, G.S. et al. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nat Cell Biol* **1**, 260-6 (1999).
181. Li, R. et al. Deletion of exon I of SMAD7 in mice results in altered B cell responses. *J Immunol* **176**, 6777-84 (2006).
182. Bailey, J.P. et al. Prolactin and transforming growth factor-beta signaling exert opposing effects on mammary gland morphogenesis, involution, and the Akt-forkhead pathway. *Mol Endocrinol* **18**, 1171-84 (2004).
183. Bakin, A.V., Tomlinson, A.K., Bhowmick, N.A., Moses, H.L. & Arteaga, C.L. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* **275**, 36803-10 (2000).
184. Bhowmick, N.A. et al. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* **12**, 27-36 (2001).
185. Ryu, B. & Kern, S.E. The essential similarity of TGFbeta and activin receptor transcriptional responses in cancer cells. *Cancer Biol Ther* **2**, 164-70 (2003).
186. Hannon, G.J. & Beach, D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* **371**, 257-61 (1994).

187. Reynisdottir, I., Polyak, K., Iavarone, A. & Massague, J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes Dev* **9**, 1831-45 (1995).
188. Datto, M.B. et al. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci U S A* **92**, 5545-9 (1995).
189. Iavarone, A. & Massague, J. Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. *Nature* **387**, 417-22 (1997).
190. Coffey, R.J., Jr. et al. Growth modulation of mouse keratinocytes by transforming growth factors. *Cancer Res* **48**, 1596-602 (1988).
191. Laiho, M., DeCaprio, J.A., Ludlow, J.W., Livingston, D.M. & Massague, J. Growth inhibition by TGF-beta linked to suppression of retinoblastoma protein phosphorylation. *Cell* **62**, 175-85 (1990).
192. Staller, P. et al. Repression of p15INK4b expression by Myc through association with Miz-1. *Nat Cell Biol* **3**, 392-9 (2001).
193. Seoane, J. et al. TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nat Cell Biol* **3**, 400-8 (2001).
194. Lasorella, A., Uo, T. & Iavarone, A. Id proteins at the cross-road of development and cancer. *Oncogene* **20**, 8326-33 (2001).
195. Roberts, A.B. et al. Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. *Proc Natl Acad Sci U S A* **77**, 3494-8 (1980).
196. Moses, H.L., Branum, E.L., Proper, J.A. & Robinson, R.A. Transforming growth factor production by chemically transformed cells. *Cancer Res* **41**, 2842-8 (1981).
197. Alexandrow, M.G. & Moses, H.L. Transforming growth factor beta 1 inhibits mouse keratinocytes late in G1 independent of effects on gene transcription. *Cancer Res* **55**, 3928-32 (1995).
198. Caniggia, I., Lye, S.J. & Cross, J.C. Activin is a local regulator of human cytotrophoblast cell differentiation. *Endocrinology* **138**, 3976-86 (1997).
199. Ball, E.M. & Risbridger, G.P. Activins as regulators of branching morphogenesis. *Dev Biol* **238**, 1-12 (2001).
200. Moses, H.L. & Serra, R. Regulation of differentiation by TGF-beta. *Curr Opin Genet Dev* **6**, 581-6 (1996).
201. Murata, M., Onomichi, K., Eto, Y., Shibai, H. & Muramatsu, M. Expression of erythroid differentiation factor (EDF) in Chinese hamster ovary cells. *Biochem Biophys Res Commun* **151**, 230-5 (1988).
202. Eto, Y. et al. Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. *Biochem Biophys Res Commun* **142**, 1095-103 (1987).
203. Yu, J. et al. Importance of FSH-releasing protein and inhibin in erythrodifferentiation. *Nature* **330**, 765-7 (1987).
204. Schuster, N. & Krieglstein, K. Mechanisms of TGF-beta-mediated apoptosis. *Cell Tissue Res* **307**, 1-14 (2002).

205. Tachibana, I. et al. Overexpression of the TGFbeta-regulated zinc finger encoding gene, TIEG, induces apoptosis in pancreatic epithelial cells. *J Clin Invest* **99**, 2365-74 (1997).
206. Jang, C.W. et al. TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nat Cell Biol* **4**, 51-8 (2002).
207. Valderrama-Carvajal, H. et al. Activin/TGF-beta induce apoptosis through Smad-dependent expression of the lipid phosphatase SHIP. *Nat Cell Biol* **4**, 963-9 (2002).
208. Schulz, A. & Bauer, G. Selective effect of tumor necrosis factor on transformed versus nontransformed cells: nonselective signal recognition but differential target cell response. *Anticancer Res* **20**, 3435-42 (2000).
209. Conery, A.R. et al. Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. *Nat Cell Biol* **6**, 366-72 (2004).
210. Remy, I., Montmarquette, A. & Michnick, S.W. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. *Nat Cell Biol* **6**, 358-65 (2004).
211. Nguyen, A.V. & Pollard, J.W. Transforming growth factor beta3 induces cell death during the first stage of mammary gland involution. *Development* **127**, 3107-18 (2000).
212. Dennler, S. et al. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *Embo J* **17**, 3091-100 (1998).
213. Hua, X., Liu, X., Ansari, D.O. & Lodish, H.F. Synergistic cooperation of TFE3 and smad proteins in TGF-beta-induced transcription of the plasminogen activator inhibitor-1 gene. *Genes Dev* **12**, 3084-95 (1998).
214. Hua, X., Miller, Z.A., Benchabane, H., Wrana, J.L. & Lodish, H.F. Synergism between transcription factors TFE3 and Smad3 in transforming growth factor-beta-induced transcription of the Smad7 gene. *J Biol Chem* **275**, 33205-8 (2000).
215. Song, C.Z., Siok, T.E. & Gelehrter, T.D. Smad4/DPC4 and Smad3 mediate transforming growth factor-beta (TGF-beta) signaling through direct binding to a novel TGF-beta-responsive element in the human plasminogen activator inhibitor-1 promoter. *J Biol Chem* **273**, 29287-90 (1998).
216. Hocevar, B.A., Brown, T.L. & Howe, P.H. TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *Embo J* **18**, 1345-56 (1999).
217. Strutz, F. et al. Role of basic fibroblast growth factor-2 in epithelial-mesenchymal transformation. *Kidney Int* **61**, 1714-28 (2002).
218. Li, J.H., Huang, X.R., Zhu, H.J., Johnson, R. & Lan, H.Y. Role of TGF-beta signaling in extracellular matrix production under high glucose conditions. *Kidney Int* **63**, 2010-9 (2003).
219. Miettinen, P.J., Ebner, R., Lopez, A.R. & Derynck, R. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J Cell Biol* **127**, 2021-36 (1994).
220. Gorelik, L. & Flavell, R.A. Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol* **2**, 46-53 (2002).
221. Christ, M. et al. Immune dysregulation in TGF-beta 1-deficient mice. *J Immunol* **153**, 1936-46 (1994).

222. de Visser, K.E. & Kast, W.M. Effects of TGF-beta on the immune system: implications for cancer immunotherapy. *Leukemia* **13**, 1188-99 (1999).
223. Gu, Z. et al. The type I activin receptor ActRIB is required for egg cylinder organization and gastrulation in the mouse. *Genes Dev* **12**, 844-57 (1998).
224. Oshima, M., Oshima, H. & Taketo, M.M. TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev Biol* **179**, 297-302 (1996).
225. Song, J. et al. The type II activin receptors are essential for egg cylinder growth, gastrulation, and rostral head development in mice. *Dev Biol* **213**, 157-69 (1999).
226. Nomura, M. & Li, E. Smad2 role in mesoderm formation, left-right patterning and craniofacial development. *Nature* **393**, 786-90 (1998).
227. Sokol, S.Y. & Melton, D.A. Interaction of Wnt and activin in dorsal mesoderm induction in Xenopus. *Dev Biol* **154**, 348-55 (1992).
228. Chang, H., Brown, C.W. & Matzuk, M.M. Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr Rev* **23**, 787-823 (2002).
229. Ling, N. et al. Pituitary FSH is released by a heterodimer of the beta-subunits from the two forms of inhibin. *Nature* **321**, 779-82 (1986).
230. Yamada, Y. et al. Differential activation of the luteinizing hormone beta-subunit promoter by activin and gonadotropin-releasing hormone: a role for the mitogen-activated protein kinase signaling pathway in LbetaT2 gonadotrophs. *Biol Reprod* **70**, 236-43 (2004).
231. Bernard, D.J. Both SMAD2 and SMAD3 mediate activin-stimulated expression of the follicle-stimulating hormone beta subunit in mouse gonadotrope cells. *Mol Endocrinol* **18**, 606-23 (2004).
232. Coss, D., Thackray, V.G., Deng, C.X. & Mellon, P.L. Activin regulates luteinizing hormone beta-subunit gene expression through Smad-binding and homeobox elements. *Mol Endocrinol* **19**, 2610-23 (2005).
233. Kitaoka, M., Kojima, I. & Ogata, E. Activin-A: a modulator of multiple types of anterior pituitary cells. *Biochem Biophys Res Commun* **157**, 48-54 (1988).
234. Billestrup, N., Gonzalez-Manchon, C., Potter, E. & Vale, W. Inhibition of somatotroph growth and growth hormone biosynthesis by activin in vitro. *Mol Endocrinol* **4**, 356-62 (1990).
235. Bilezikjian, L.M., Blount, A.L., Campen, C.A., Gonzalez-Manchon, C. & Vale, W. Activin-A inhibits proopiomelanocortin messenger RNA accumulation and adrenocorticotropin secretion of AtT20 cells. *Mol Endocrinol* **5**, 1389-95 (1991).
236. Petraglia, F., Vaughan, J. & Vale, W. Inhibin and activin modulate the release of gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone from cultured human placental cells. *Proc Natl Acad Sci U S A* **86**, 5114-7 (1989).
237. Oh, S.P. et al. 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**, 2626-31 (2000).
238. Urness, L.D., Sorensen, L.K. & Li, D.Y. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat Genet* **26**, 328-31 (2000).
239. Larsson, J. et al. Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *Embo J* **20**, 1663-73 (2001).

240. Li, D.Y. et al. Defective angiogenesis in mice lacking endoglin. *Science* **284**, 1534-7 (1999).
241. Pertovaara, L. et al. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem* **269**, 6271-4 (1994).
242. Goumans, M.J. et al. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *Embo J* **21**, 1743-53 (2002).
243. Heermeier, K. et al. Bax and Bcl-xs are induced at the onset of apoptosis in involuting mammary epithelial cells. *Mech Dev* **56**, 197-207 (1996).
244. Lund, L.R. et al. Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development* **122**, 181-93 (1996).
245. Ossowski, L., Biegel, D. & Reich, E. Mammary plasminogen activator: correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. *Cell* **16**, 929-40 (1979).
246. Hennighausen, L. (2006).
247. Satokata, I. et al. Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nat Genet* **24**, 391-5 (2000).
248. Foley, J. et al. Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development. *Development* **128**, 513-25 (2001).
249. Ruan, W., Catanese, V., Wieczorek, R., Feldman, M. & Kleinberg, D.L. Estradiol enhances the stimulatory effect of insulin-like growth factor-I (IGF-I) on mammary development and growth hormone-induced IGF-I messenger ribonucleic acid. *Endocrinology* **136**, 1296-302 (1995).
250. Traurig, H.H. A radioautographic study of cell proliferation in the mammary gland of the pregnant mouse. *Anat Rec* **159**, 239-47 (1967).
251. Fata, J.E. et al. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* **103**, 41-50 (2000).
252. Long, W. et al. Impaired differentiation and lactational failure of Erbb4-deficient mammary glands identify ERBB4 as an obligate mediator of STAT5. *Development* **130**, 5257-68 (2003).
253. Robinson, S.D., Silberstein, G.B., Roberts, A.B., Flanders, K.C. & Daniel, C.W. Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development. *Development* **113**, 867-78 (1991).
254. Faure, E., Heisterkamp, N., Groffen, J. & Kaartinen, V. Differential expression of TGF-beta isoforms during postlactational mammary gland involution. *Cell Tissue Res* **300**, 89-95 (2000).
255. Silberstein, G.B. & Daniel, C.W. Reversible inhibition of mammary gland growth by transforming growth factor-beta. *Science* **237**, 291-3 (1987).
256. Daniel, C.W., Silberstein, G.B., Van Horn, K., Strickland, P. & Robinson, S. TGF-beta 1-induced inhibition of mouse mammary ductal growth: developmental specificity and characterization. *Dev Biol* **135**, 20-30 (1989).
257. Joseph, H., Gorska, A.E., Sohn, P., Moses, H.L. & Serra, R. Overexpression of a kinase-deficient transforming growth factor-beta type II receptor in mouse

mammary stroma results in increased epithelial branching. *Mol Biol Cell* **10**, 1221-34 (1999).
258. Liu, Q.Y. et al. Inhibitory effects of activin on the growth and morpholgenesis of primary and transformed mammary epithelial cells. *Cancer Res* **56**, 1155-63 (1996).
259. Di Loreto, C. et al. Human mammary gland and breast carcinoma contain immunoreactive inhibin/activin subunits: evidence for a secretion into cystic fluid. *Eur J Endocrinol* **141**, 190-4 (1999).
260. Ying, S.Y. & Zhang, Z. Expression and localization of inhibin/activin subunits and activin receptors in MCF-7 cells, a human breast cancer cell line. *Breast Cancer Res Treat* **37**, 151-60 (1996).
261. Jeruss, J.S., Santiago, J.Y. & Woodruff, T.K. Localization of activin and inhibin subunits, receptors and SMADs in the mouse mammary gland. *Mol Cell Endocrinol* **203**, 185-96 (2003).
262. Robinson, G.W. & Hennighausen, L. Inhibins and activins regulate mammary epithelial cell differentiation through mesenchymal-epithelial interactions. *Development* **124**, 2701-8 (1997).
263. Pierce, D.F., Jr. et al. Mammary tumor suppression by transforming growth factor beta 1 transgene expression. *Proc Natl Acad Sci U S A* **92**, 4254-8 (1995).
264. Jhappan, C. et al. Targeting expression of a transforming growth factor beta 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. *Embo J* **12**, 1835-45 (1993).
265. Kordon, E.C. et al. Ectopic TGF beta 1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. *Dev Biol* **168**, 47-61 (1995).
266. Gorska, A.E., Joseph, H., Derynck, R., Moses, H.L. & Serra, R. Dominant-negative interference of the transforming growth factor beta type II receptor in mammary gland epithelium results in alveolar hyperplasia and differentiation in virgin mice. *Cell Growth Differ* **9**, 229-38 (1998).
267. Cheng, N. et al. Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks. *Oncogene* **24**, 5053-68 (2005).
268. Siegel, P.M., Shu, W., Cardiff, R.D., Muller, W.J. & Massague, J. Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci U S A* **100**, 8430-5 (2003).
269. Ewan, K.B. et al. Latent transforming growth factor-beta activation in mammary gland: regulation by ovarian hormones affects ductal and alveolar proliferation. *Am J Pathol* **160**, 2081-93 (2002).
270. Akhurst, R.J. & Derynck, R. TGF-beta signaling in cancer--a double-edged sword. *Trends Cell Biol* **11**, S44-51 (2001).
271. Walker, R.A. & Gallacher, B. Determination of transforming growth factor beta 1 mRNA expression in breast carcinomas by in situ hybridization. *J Pathol* **177**, 123-7 (1995).
272. Wong, S.T. et al. The TGF-alpha precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. *Cell* **56**, 495-506 (1989).

273. Dalal, B.I., Keown, P.A. & Greenberg, A.H. Immunocytochemical localization of secreted transforming growth factor-beta 1 to the advancing edges of primary tumors and to lymph node metastases of human mammary carcinoma. *Am J Pathol* **143**, 381-9 (1993).
274. Gorsch, S.M., Memoli, V.A., Stukel, T.A., Gold, L.I. & Arrick, B.A. Immunohistochemical staining for transforming growth factor beta 1 associates with disease progression in human breast cancer. *Cancer Res* **52**, 6949-52 (1992).
275. Teicher, B.A. Malignant cells, directors of the malignant process: role of transforming growth factor-beta. *Cancer Metastasis Rev* **20**, 133-43 (2001).
276. Jeruss, J.S., Sturgis, C.D., Rademaker, A.W. & Woodruff, T.K. Down-regulation of activin, activin receptors, and Smads in high-grade breast cancer. *Cancer Res* **63**, 3783-90 (2003).
277. Leto, G. et al. Activin A circulating levels in patients with bone metastasis from breast or prostate cancer. *Clin Exp Metastasis* (2006).
278. Gobbi, H. et al. Transforming growth factor-beta and breast cancer risk in women with mammary epithelial hyperplasia. *J Natl Cancer Inst* **91**, 2096-101 (1999).
279. Chakravarthy, D., Green, A.R., Green, V.L., Kerin, M.J. & Speirs, V. Expression and secretion of TGF-beta isoforms and expression of TGF-beta-receptors I, II and III in normal and neoplastic human breast. *Int J Oncol* **15**, 187-94 (1999).
280. Pouliot, F. & Labrie, C. Expression profile of agonistic Smads in human breast cancer cells: absence of regulation by estrogens. *Int J Cancer* **81**, 98-103 (1999).
281. Bachman, K.E. et al. p21(WAF1/CIP1) mediates the growth response to TGF-beta in human epithelial cells. *Cancer Biol Ther* **3**, 221-5 (2004).
282. Ammanamanchi, S. & Brattain, M.G. Restoration of transforming growth factor-beta signaling through receptor RI induction by histone deacetylase activity inhibition in breast cancer cells. *J Biol Chem* **279**, 32620-5 (2004).
283. Anbazhagan, R., Bornman, D.M., Johnston, J.C., Westra, W.H. & Gabrielson, E. The S387Y mutations of the transforming growth factor-beta receptor type I gene is uncommon in metastases of breast cancer and other common types of adenocarcinoma. *Cancer Res* **59**, 3363-4 (1999).
284. Chen, C.R., Kang, Y. & Massague, J. Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. *Proc Natl Acad Sci U S A* **98**, 992-9 (2001).
285. Gomis, R.R., Alarcon, C., Nadal, C., Van Poznak, C. & Massague, J. C/EBPbeta at the core of the TGFbeta cytostatic response and its evasion in metastatic breast cancer cells. *Cancer Cell* **10**, 203-14 (2006).
286. Burdette, J.E., Jeruss, J.S., Kurley, S.J., Lee, E.J. & Woodruff, T.K. Activin A mediates growth inhibition and cell cycle arrest through Smads in human breast cancer cells. *Cancer Res* **65**, 7968-75 (2005).
287. Kalkhoven, E. et al. Resistance to transforming growth factor beta and activin due to reduced receptor expression in human breast tumor cell lines. *Cell Growth Differ* **6**, 1151-61 (1995).
288. Nass, S.J., Li, M., Amundadottir, L.T., Furth, P.A. & Dickson, R.B. Role for Bcl-xL in the regulation of apoptosis by EGF and TGF beta 1 in c-myc overexpressing mammary epithelial cells. *Biochem Biophys Res Commun* **227**, 248-56 (1996).

289. Amundadottir, L.T., Merlino, G. & Dickson, R.B. Transgenic mouse models of breast cancer. *Breast Cancer Res Treat* **39**, 119-35 (1996).
290. Chen, H., Tritton, T.R., Kenny, N., Absher, M. & Chiu, J.F. Tamoxifen induces TGF-beta 1 activity and apoptosis of human MCF-7 breast cancer cells in vitro. *J Cell Biochem* **61**, 9-17 (1996).
291. Tobin, S.W. et al. Inhibition of transforming growth factor beta signaling in MCF-7 cells results in resistance to tumor necrosis factor alpha: a role for Bcl-2. *Cell Growth Differ* **12**, 109-17 (2001).
292. Perry, R.R., Kang, Y. & Greaves, B.R. Relationship between tamoxifen-induced transforming growth factor beta 1 expression, cytostasis and apoptosis in human breast cancer cells. *Br J Cancer* **72**, 1441-6 (1995).
293. Levy, L. & Hill, C.S. Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev* **17**, 41-58 (2006).
294. Lucke, C.D. et al. Inhibiting mutations in the transforming growth factor beta type 2 receptor in recurrent human breast cancer. *Cancer Res* **61**, 482-5 (2001).
295. Chen, T., Carter, D., Garrigue-Antar, L. & Reiss, M. Transforming growth factor beta type I receptor kinase mutant associated with metastatic breast cancer. *Cancer Res* **58**, 4805-10 (1998).
296. Eppert, K. et al. MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* **86**, 543-52 (1996).
297. Yakicier, M.C., Irmak, M.B., Romano, A., Kew, M. & Ozturk, M. Smad2 and Smad4 gene mutations in hepatocellular carcinoma. *Oncogene* **18**, 4879-83 (1999).
298. Maliekal, T.T., Antony, M.L., Nair, A., Paulmurugan, R. & Karunagaran, D. Loss of expression, and mutations of Smad 2 and Smad 4 in human cervical cancer. *Oncogene* **22**, 4889-97 (2003).
299. Han, S.U. et al. Loss of the Smad3 expression increases susceptibility to tumorigenicity in human gastric cancer. *Oncogene* **23**, 1333-41 (2004).
300. Hahn, S.A. et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* **271**, 350-3 (1996).
301. Schutte, M. et al. DPC4 gene in various tumor types. *Cancer Res* **56**, 2527-30 (1996).
302. Dai, J.L., Bansal, R.K. & Kern, S.E. G1 cell cycle arrest and apoptosis induction by nuclear Smad4/Dpc4: phenotypes reversed by a tumorigenic mutation. *Proc Natl Acad Sci U S A* **96**, 1427-32 (1999).
303. Zhong, D. et al. Homozygous Deletion of SMAD4 in Breast Cancer Cell Lines and Invasive Ductal Carcinomas. *Cancer Biol Ther* **5**, 601-7 (2006).
304. Bottinger, E.P. et al. Expression of a dominant-negative mutant TGF-beta type II receptor in transgenic mice reveals essential roles for TGF-beta in regulation of growth and differentiation in the exocrine pancreas. *Embo J* **16**, 2621-33 (1997).
305. Muller, W.J., Ho, J. & Siegel, P.M. Oncogenic activation of Neu/ErbB-2 in a transgenic mouse model for breast cancer. *Biochem Soc Symp* **63**, 149-57 (1998).
306. Li, W. et al. Squamous cell carcinoma and mammary abscess formation through squamous metaplasia in Smad4/Dpc4 conditional knockout mice. *Development* **130**, 6143-53 (2003).

307. Muraoka, R.S. et al. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. *Mol Cell Biol* **23**, 8691-703 (2003).
308. Tang, B. et al. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* **112**, 1116-24 (2003).
309. Yin, J.J. et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* **103**, 197-206 (1999).
310. Kang, Y. et al. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. *Proc Natl Acad Sci U S A* **102**, 13909-14 (2005).
311. Erickson, A.C. & Barcellos-Hoff, M.H. The not-so innocent bystander: the microenvironment as a therapeutic target in cancer. *Expert Opin Ther Targets* **7**, 71-88 (2003).
312. Bodmer, S. et al. Immunosuppression and transforming growth factor-beta in glioblastoma. Preferential production of transforming growth factor-beta 2. *J Immunol* **143**, 3222-9 (1989).
313. Hersey, P. Impediments to successful immunotherapy. *Pharmacol Ther* **81**, 111-9 (1999).
314. Arteaga, C.L., Dugger, T.C., Winnier, A.R. & Forbes, J.T. Evidence for a positive role of transforming growth factor-beta in human breast cancer cell tumorigenesis. *J Cell Biochem Suppl* **17G**, 187-93 (1993).
315. de Jong, J.S., van Diest, P.J., van der Valk, P. & Baak, J.P. Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. *J Pathol* **184**, 53-7 (1998).
316. Donovan, D. et al. TGF beta-1 regulation of VEGF production by breast cancer cells. *Ann Surg Oncol* **4**, 621-7 (1997).
317. Michl, P. et al. CUTL1 is a target of TGF(beta) signaling that enhances cancer cell motility and invasiveness. *Cancer Cell* **7**, 521-32 (2005).
318. Dumont, N., Bakin, A.V. & Arteaga, C.L. Autocrine transforming growth factor-beta signaling mediates Smad-independent motility in human cancer cells. *J Biol Chem* **278**, 3275-85 (2003).
319. Hildenbrand, R. et al. Transforming growth factor-beta stimulates urokinase expression in tumor-associated macrophages of the breast. *Lab Invest* **78**, 59-71 (1998).
320. Andreasen, P.A., Kjoller, L., Christensen, L. & Duffy, M.J. The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int J Cancer* **72**, 1-22 (1997).
321. Oft, M. et al. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* **10**, 2462-77 (1996).
322. Hosobuchi, M. & Stampfer, M.R. Effects of transforming growth factor beta on growth of human mammary epithelial cells in culture. *In Vitro Cell Dev Biol* **25**, 705-13 (1989).
323. Piek, E., Moustakas, A., Kurisaki, A., Heldin, C.H. & ten Dijke, P. TGF-(beta) type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. *J Cell Sci* **112 ( Pt 24)**, 4557-68 (1999).

324. Zavadil, J. & Bottinger, E.P. TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* **24**, 5764-74 (2005).
325. Huber, M.A. et al. NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. *J Clin Invest* **114**, 569-81 (2004).
326. Ozdamar, B. et al. Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science* **307**, 1603-9 (2005).
327. Horseman, N.D. et al. Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. *Embo J* **16**, 6926-35 (1997).
328. Bazan, J.F. A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor beta-chain. *Biochem Biophys Res Commun* **164**, 788-95 (1989).
329. Ormandy, C.J., Binart, N. & Kelly, P.A. Mammary gland development in prolactin receptor knockout mice. *J Mammary Gland Biol Neoplasia* **2**, 355-64 (1997).
330. Kishimoto, T. Signal transduction through homo- or heterodimers of gp130. *Stem Cells* **12 Suppl 1**, 37-44; discussion 44-5 (1994).
331. Finidori, J. & Kelly, P.A. Cytokine receptor signalling through two novel families of transducer molecules: Janus kinases, and signal transducers and activators of transcription. *J Endocrinol* **147**, 11-23 (1995).
332. Rui, H., Lebrun, J.J., Kirken, R.A., Kelly, P.A. & Farrar, W.L. JAK2 activation and cell proliferation induced by antibody-mediated prolactin receptor dimerization. *Endocrinology* **135**, 1299-306 (1994).
333. Rane, S.G. & Reddy, E.P. JAKs, STATs and Src kinases in hematopoiesis. *Oncogene* **21**, 3334-58 (2002).
334. Gouilleux, F., Wakao, H., Mundt, M. & Groner, B. Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. *Embo J* **13**, 4361-9 (1994).
335. Wakao, H., Gouilleux, F. & Groner, B. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *Embo J* **13**, 2182-91 (1994).
336. Liu, X. et al. Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev* **11**, 179-86 (1997).
337. Teglund, S. et al. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell* **93**, 841-50 (1998).
338. Strehlow, I. & Schindler, C. Amino-terminal signal transducer and activator of transcription (STAT) domains regulate nuclear translocation and STAT deactivation. *J Biol Chem* **273**, 28049-56 (1998).
339. Zhang, J.J. et al. Two contact regions between Stat1 and CBP/p300 in interferon gamma signaling. *Proc Natl Acad Sci U S A* **93**, 15092-6 (1996).
340. Chen, X. et al. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. *Cell* **93**, 827-39 (1998).
341. Becker, S., Groner, B. & Muller, C.W. Three-dimensional structure of the Stat3beta homodimer bound to DNA. *Nature* **394**, 145-51 (1998).
342. Zhang, T., Kee, W.H., Seow, K.T., Fung, W. & Cao, X. The coiled-coil domain of Stat3 is essential for its SH2 domain-mediated receptor binding and

subsequent activation induced by epidermal growth factor and interleukin-6. *Mol Cell Biol* **20**, 7132-9 (2000).
343. Begitt, A., Meyer, T., van Rossum, M. & Vinkemeier, U. Nucleocytoplasmic translocation of Stat1 is regulated by a leucine-rich export signal in the coiled-coil domain. *Proc Natl Acad Sci U S A* **97**, 10418-23 (2000).
344. Yang, E., Wen, Z., Haspel, R.L., Zhang, J.J. & Darnell, J.E., Jr. The linker domain of Stat1 is required for gamma interferon-driven transcription. *Mol Cell Biol* **19**, 5106-12 (1999).
345. Greenlund, A.C., Farrar, M.A., Viviano, B.L. & Schreiber, R.D. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). *Embo J* **13**, 1591-600 (1994).
346. Heim, M.H., Kerr, I.M., Stark, G.R. & Darnell, J.E., Jr. Contribution of STAT SH2 groups to specific interferon signaling by the Jak-STAT pathway. *Science* **267**, 1347-9 (1995).
347. Barahmand-Pour, F., Meinke, A., Groner, B. & Decker, T. Jak2-Stat5 interactions analyzed in yeast. *J Biol Chem* **273**, 12567-75 (1998).
348. Shuai, K. et al. Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell* **76**, 821-8 (1994).
349. Darnell, J.E., Jr. STATs and gene regulation. *Science* **277**, 1630-5 (1997).
350. Schindler, C. & Strehlow, I. Cytokines and STAT signaling. *Adv Pharmacol* **47**, 113-74 (2000).
351. Horvath, C.M. STAT proteins and transcriptional responses to extracellular signals. *Trends Biochem Sci* **25**, 496-502 (2000).
352. Pfitzner, E., Jahne, R., Wissler, M., Stoecklin, E. & Groner, B. p300/CREB-binding protein enhances the prolactin-mediated transcriptional induction through direct interaction with the transactivation domain of Stat5, but does not participate in the Stat5-mediated suppression of the glucocorticoid response. *Mol Endocrinol* **12**, 1582-93 (1998).
353. Decker, T. & Kovarik, P. Serine phosphorylation of STATs. *Oncogene* **19**, 2628-37 (2000).
354. Kovarik, P. et al. Specificity of signaling by STAT1 depends on SH2 and C-terminal domains that regulate Ser727 phosphorylation, differentially affecting specific target gene expression. *Embo J* **20**, 91-100 (2001).
355. Beuvink, I. et al. Stat5a serine phosphorylation. Serine 779 is constitutively phosphorylated in the mammary gland, and serine 725 phosphorylation influences prolactin-stimulated in vitro DNA binding activity. *J Biol Chem* **275**, 10247-55 (2000).
356. Chen, Y. et al. Identification of Shp-2 as a Stat5A phosphatase. *J Biol Chem* **278**, 16520-7 (2003).
357. Chughtai, N., Schimchowitsch, S., Lebrun, J.J. & Ali, S. Prolactin induces SHP-2 association with Stat5, nuclear translocation, and binding to the beta-casein gene promoter in mammary cells. *J Biol Chem* **277**, 31107-14 (2002).
358. Aoki, N. & Matsuda, T. A cytosolic protein-tyrosine phosphatase PTP1B specifically dephosphorylates and deactivates prolactin-activated STAT5a and STAT5b. *J Biol Chem* **275**, 39718-26 (2000).

359. Yokoyama, N., Reich, N.C. & Miller, W.T. Involvement of protein phosphatase 2A in the interleukin-3-stimulated Jak2-Stat5 signaling pathway. *J Interferon Cytokine Res* **21**, 369-78 (2001).
360. Aoki, N. & Matsuda, T. A nuclear protein tyrosine phosphatase TC-PTP is a potential negative regulator of the PRL-mediated signaling pathway: dephosphorylation and deactivation of signal transducer and activator of transcription 5a and 5b by TC-PTP in nucleus. *Mol Endocrinol* **16**, 58-69 (2002).
361. Kotaja, N., Karvonen, U., Janne, O.A. & Palvimo, J.J. PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. *Mol Cell Biol* **22**, 5222-34 (2002).
362. Chen, X.P., Losman, J.A. & Rothman, P. SOCS proteins, regulators of intracellular signaling. *Immunity* **13**, 287-90 (2000).
363. Ungureanu, D., Saharinen, P., Junttila, I., Hilton, D.J. & Silvennoinen, O. Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1. *Mol Cell Biol* **22**, 3316-26 (2002).
364. Chen, Y., Dai, X., Haas, A.L., Wen, R. & Wang, D. Proteasome-dependent down-regulation of activated Stat5A in the nucleus. *Blood* **108**, 566-74 (2006).
365. Stocklin, E., Wissler, M., Gouilleux, F. & Groner, B. Functional interactions between Stat5 and the glucocorticoid receptor. *Nature* **383**, 726-8 (1996).
366. Peng, B. et al. CPAP is a novel stat5-interacting cofactor that augments stat5-mediated transcriptional activity. *Mol Endocrinol* **16**, 2019-33 (2002).
367. Zhu, M., John, S., Berg, M. & Leonard, W.J. Functional association of Nmi with Stat5 and Stat1 in IL-2- and IFNgamma-mediated signaling. *Cell* **96**, 121-30 (1999).
368. Nakajima, H., Brindle, P.K., Handa, M. & Ihle, J.N. Functional interaction of STAT5 and nuclear receptor co-repressor SMRT: implications in negative regulation of STAT5-dependent transcription. *Embo J* **20**, 6836-44 (2001).
369. Paukku, K., Yang, J. & Silvennoinen, O. Tudor and nuclease-like domains containing protein p100 function as coactivators for signal transducer and activator of transcription 5. *Mol Endocrinol* **17**, 1805-14 (2003).
370. Pircher, T.J., Petersen, H., Gustafsson, J.A. & Haldosen, L.A. Extracellular signal-regulated kinase (ERK) interacts with signal transducer and activator of transcription (STAT) 5a. *Mol Endocrinol* **13**, 555-65 (1999).
371. Wyszomierski, S.L., Yeh, J. & Rosen, J.M. Glucocorticoid receptor/signal transducer and activator of transcription 5 (STAT5) interactions enhance STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation. *Mol Endocrinol* **13**, 330-43 (1999).
372. Bhattacharya, S. et al. Cooperation of Stat2 and p300/CBP in signalling induced by interferon-alpha. *Nature* **383**, 344-7 (1996).
373. Horvai, A.E. et al. Nuclear integration of JAK/STAT and Ras/AP-1 signaling by CBP and p300. *Proc Natl Acad Sci U S A* **94**, 1074-9 (1997).
374. Paulson, M. et al. Stat protein transactivation domains recruit p300/CBP through widely divergent sequences. *J Biol Chem* **274**, 25343-9 (1999).
375. Groner, B. Transcription factor regulation in mammary epithelial cells. *Domest Anim Endocrinol* **23**, 25-32 (2002).

376. Brockman, J.L., Schroeder, M.D. & Schuler, L.A. PRL activates the cyclin D1 promoter via the Jak2/Stat pathway. *Mol Endocrinol* **16**, 774-84 (2002).
377. Clevenger, C.V. & Plank, T.L. Prolactin as an autocrine/paracrine factor in breast tissue. *J Mammary Gland Biol Neoplasia* **2**, 59-68 (1997).
378. Couse, J.F. & Korach, K.S. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* **20**, 358-417 (1999).
379. Hankinson, S.E. et al. Plasma prolactin levels and subsequent risk of breast cancer in postmenopausal women. *J Natl Cancer Inst* **91**, 629-34 (1999).
380. Wennbo, H., Kindblom, J., Isaksson, O.G. & Tornell, J. Transgenic mice overexpressing the prolactin gene develop dramatic enlargement of the prostate gland. *Endocrinology* **138**, 4410-5 (1997).
381. Ginsburg, E. & Vonderhaar, B.K. Prolactin synthesis and secretion by human breast cancer cells. *Cancer Res* **55**, 2591-5 (1995).
382. Clevenger, C.V. et al. Expression of prolactin and prolactin receptor in human breast carcinoma. Evidence for an autocrine/paracrine loop. *Am J Pathol* **146**, 695-705 (1995).
383. Llovera, M. et al. Human prolactin (hPRL) antagonists inhibit hPRL-activated signaling pathways involved in breast cancer cell proliferation. *Oncogene* **19**, 4695-705 (2000).
384. Chen, W.Y., Ramamoorthy, P., Chen, N., Sticca, R. & Wagner, T.E. A human prolactin antagonist, hPRL-G129R, inhibits breast cancer cell proliferation through induction of apoptosis. *Clin Cancer Res* **5**, 3583-93 (1999).
385. Favy, D.A. et al. Prolactin-dependent up-regulation of BRCA1 expression in human breast cancer cell lines. *Biochem Biophys Res Commun* **258**, 284-91 (1999).
386. Spiekermann, K., Biethahn, S., Wilde, S., Hiddemann, W. & Alves, F. Constitutive activation of STAT transcription factors in acute myelogenous leukemia. *Eur J Haematol* **67**, 63-71 (2001).
387. Spiekermann, K. et al. Constitutive activation of STAT3 and STAT5 is induced by leukemic fusion proteins with protein tyrosine kinase activity and is sufficient for transformation of hematopoietic precursor cells. *Exp Hematol* **30**, 262-71 (2002).
388. Cotarla, I. et al. Stat5a is tyrosine phosphorylated and nuclear localized in a high proportion of human breast cancers. *Int J Cancer* **108**, 665-71 (2004).
389. Socolovsky, M., Fallon, A.E., Wang, S., Brugnara, C. & Lodish, H.F. Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* **98**, 181-91 (1999).
390. Nevalainen, M.T. et al. Signal transducer and activator of transcription-5 activation and breast cancer prognosis. *J Clin Oncol* **22**, 2053-60 (2004).
391. Nouhi, Z. et al. Defining the role of prolactin as an invasion suppressor hormone in breast cancer cells. *Cancer Res* **66**, 1824-32 (2006).
392. Sultan, A.S. et al. Stat5 promotes homotypic adhesion and inhibits invasive characteristics of human breast cancer cells. *Oncogene* **24**, 746-60 (2005).
393. Mieth, M., Boehmer, F.D., Ball, R., Groner, B. & Grosse, R. Transforming growth factor-beta inhibits lactogenic hormone induction of beta-casein expression in HC11 mouse mammary epithelial cells. *Growth Factors* **4**, 9-15 (1990).

394. Pierce, D.F., Jr. et al. Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1. *Genes Dev* **7**, 2308-17 (1993).
395. Jackson, J.A. et al. Gastrointestinal nematode infection is associated with variation in innate immune responsiveness. *Microbes Infect* (2005).
396. Rui, H., Kirken, R.A. & Farrar, W.L. Activation of receptor-associated tyrosine kinase JAK2 by prolactin. *J Biol Chem* **269**, 5364-8 (1994).
397. Dusanter-Fourt, I. et al. Identification of JAK protein tyrosine kinases as signaling molecules for prolactin. Functional analysis of prolactin receptor and prolactin-erythropoietin receptor chimera expressed in lymphoid cells. *Embo J* **13**, 2583-91 (1994).
398. Taniguchi, T. Cytokine signaling through nonreceptor protein tyrosine kinases. *Science* **268**, 251-5 (1995).
399. Schmitt-Ney, M., Doppler, W., Ball, R.K. & Groner, B. Beta-casein gene promoter activity is regulated by the hormone-mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor. *Mol Cell Biol* **11**, 3745-55 (1991).
400. Gouilleux, F. et al. Prolactin, growth hormone, erythropoietin and granulocyte-macrophage colony stimulating factor induce MGF-Stat5 DNA binding activity. *Embo J* **14**, 2005-13 (1995).
401. Ruff-Jamison, S., Chen, K. & Cohen, S. Epidermal growth factor induces the tyrosine phosphorylation and nuclear translocation of Stat 5 in mouse liver. *Proc Natl Acad Sci U S A* **92**, 4215-8 (1995).
402. Pallard, C. et al. Interleukin-3, erythropoietin, and prolactin activate a STAT5-like factor in lymphoid cells. *J Biol Chem* **270**, 15942-5 (1995).
403. Pallard, C. et al. Thrombopoietin activates a STAT5-like factor in hematopoietic cells. *Embo J* **14**, 2847-56 (1995).
404. Ripperger, J.A., Fritz, S., Hocke, G.M. & Fey, G.H. Purification of the interleukin-6-inducible complex II reveals two proteins capable of binding to the IL-6-response element. *Ann N Y Acad Sci* **762**, 459-61 (1995).
405. Ripperger, J.A. et al. Transcription factors Stat3 and Stat5b are present in rat liver nuclei late in an acute phase response and bind interleukin-6 response elements. *J Biol Chem* **270**, 29998-30006 (1995).
406. Jayaraman, L. & Massague, J. Distinct oligomeric states of SMAD proteins in the transforming growth factor-beta pathway. *J Biol Chem* **275**, 40710-7. (2000).
407. Chacko, B.M. et al. The L3 loop and C-terminal phosphorylation jointly define Smad protein trimerization. *Nat Struct Biol* **8**, 248-53 (2001).
408. Chacko, B.M. et al. Structural basis of heteromeric smad protein assembly in TGF-beta signaling. *Mol Cell* **15**, 813-23 (2004).
409. Derynck, R. & Zhang, Y.E. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577-84 (2003).
410. Robinson SD, R.A., and Daniel CW. TGF beta suppresses casein synthesis in mouse mammary explants and may play a role in controlling milk levels during pregnancy. *Journal of Cell Biology* **120**, 245-251 (1993).
411. Sudlow, A.W., Wilde, C.J. & Burgoyne, R.D. Transforming growth factor-beta 1 inhibits casein secretion from differentiating mammary-gland explants but not from lactating mammary cells. *Biochem J* **304 ( Pt 2)**, 333-6 (1994).

412. de Guise, C. et al. Activin Inhibits the Human Pit-1 Gene Promoter through the p38 Kinase Pathway in a Smad-Independent Manner. *Endocrinology* **147**, 4351-62 (2006).
413. Itoh, S. et al. Elucidation of Smad requirement in transforming growth factor-beta type I receptor-induced responses. *J Biol Chem* **278**, 3751-61 (2003).
414. Cella, N., Groner, B. & Hynes, N.E. Characterization of Stat5a and Stat5b homodimers and heterodimers and their association with the glucocortiocoid receptor in mammary cells. *Mol Cell Biol* **18**, 1783-92 (1998).
415. Topper, Y.J. & Freeman, C.S. Multiple hormone interactions in the developmental biology of the mammary gland. *Physiol Rev* **60**, 1049-106 (1980).
416. Ball, R.K., Friis, R.R., Schoenenberger, C.A., Doppler, W. & Groner, B. Prolactin regulation of beta-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. *Embo J* **7**, 2089-95 (1988).
417. Danielson, K.G., Oborn, C.J., Durban, E.M., Butel, J.S. & Medina, D. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. *Proc Natl Acad Sci U S A* **81**, 3756-60 (1984).
418. Doppler, W., Groner, B. & Ball, R.K. Prolactin and glucocorticoid hormones synergistically induce expression of transfected rat beta-casein gene promoter constructs in a mammary epithelial cell line. *Proc Natl Acad Sci U S A* **86**, 104-8 (1989).
419. Doppler, W., Hock, W., Hofer, P., Groner, B. & Ball, R.K. Prolactin and glucocorticoid hormones control transcription of the beta-casein gene by kinetically distinct mechanisms. *Mol Endocrinol* **4**, 912-9 (1990).
420. Guyette, W.A., Matusik, R.J. & Rosen, J.M. Prolactin-mediated transcriptional and post-transcriptional control of casein gene expression. *Cell* **17**, 1013-23 (1979).
421. Choi, K.M., Barash, I. & Rhoads, R.E. Insulin and prolactin synergistically stimulate beta-casein messenger ribonucleic acid translation by cytoplasmic polyadenylation. *Mol Endocrinol* **18**, 1670-86 (2004).
422. Doppler, W., Welte, T. & Philipp, S. CCAAT/enhancer-binding protein isoforms beta and delta are expressed in mammary epithelial cells and bind to multiple sites in the beta-casein gene promoter. *J Biol Chem* **270**, 17962-9 (1995).
423. Senyuk, V. et al. The leukemia-associated transcription repressor AML1/MDS1/EVI1 requires CtBP to induce abnormal growth and differentiation of murine hematopoietic cells. *Oncogene* **21**, 3232-40 (2002).
424. Inman, C.K., Li, N. & Shore, P. Oct-1 counteracts autoinhibition of Runx2 DNA binding to form a novel Runx2/Oct-1 complex on the promoter of the mammary gland-specific gene beta-casein. *Mol Cell Biol* **25**, 3182-93 (2005).
425. Sirard, C. et al. Targeted disruption in murine cells reveals variable requirement for Smad4 in transforming growth factor beta-related signaling. *J Biol Chem* **275**, 2063-70 (2000).
426. Ebert, K.M. et al. Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. *Biotechnology (N Y)* **9**, 835-8. (1991).
427. Callus, B.A. & Mathey-Prevot, B. Hydrophobic residues Phe751 and Leu753 are essential for STAT5 transcriptional activity. *J Biol Chem* **275**, 16954-62 (2000).

428. Brockman, J.L. & Schuler, L.A. Prolactin signals via Stat5 and Oct-1 to the proximal cyclin D1 promoter. *Mol Cell Endocrinol* **239**, 45-53 (2005).
429. Bright, J.J., Kerr, L.D. & Sriram, S. TGF-beta inhibits IL-2-induced tyrosine phosphorylation and activation of Jak-1 and Stat 5 in T lymphocytes. *J Immunol* **159**, 175-83 (1997).
430. Bright, J.J. & Sriram, S. TGF-beta inhibits IL-12-induced activation of Jak-STAT pathway in T lymphocytes. *J Immunol* **161**, 1772-7 (1998).
431. Sudarshan, C., Galon, J., Zhou, Y. & O'Shea, J.J. TGF-beta does not inhibit IL-12- and IL-2-induced activation of Janus kinases and STATs. *J Immunol* **162**, 2974-81 (1999).
432. Smith, G.H. TGF-beta and functional differentiation. *J Mammary Gland Biol Neoplasia* **1**, 343-52 (1996).
433. Strange, R., Li, F., Saurer, S., Burkhardt, A. & Friis, R.R. Apoptotic cell death and tissue remodelling during mouse mammary gland involution. *Development* **115**, 49-58 (1992).
434. Sadarangani, A. et al. In vivo and in vitro estrogenic and progestagenic actions of Tibolone. *Biol Res* **38**, 245-58 (2005).
435. Sicinski, P. & Weinberg, R.A. A specific role for cyclin D1 in mammary gland development. *J Mammary Gland Biol Neoplasia* **2**, 335-42 (1997).
436. Feng, X.H., Zhang, Y., Wu, R.Y. & Derynck, R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. *Genes Dev* **12**, 2153-63 (1998).
437. Wang, G., Long, J., Matsuura, I., He, D. & Liu, F. The Smad3 linker region contains a transcriptional activation domain. *Biochem J* **386**, 29-34 (2005).
438. Mark P. de Caestecker, T.Y., David Wang, W. Tony Parks, Shixia Huang, Caroline S. Hill, Toshi Shioda, Anita B. Roberts, and Robert J. Lechleider The Smad4 Activation Domain (SAD) Is a Proline-rich, p300-dependent Transcriptional Activation Domain. *J Biol Chem* **275**, 2115-2122 (2000
).
439. Ghosh, A.K., Yuan, W., Mori, Y., Chen, S. & Varga, J. Antagonistic regulation of type I collagen gene expression by interferon-gamma and transforming growth factor-beta. Integration at the level of p300/CBP transcriptional coactivators. *J Biol Chem* **276**, 11041-8 (2001).
440. Sovak, M.A., Arsura, M., Zanieski, G., Kavanagh, K.T. & Sonenshein, G.E. The inhibitory effects of transforming growth factor beta1 on breast cancer cell proliferation are mediated through regulation of aberrant nuclear factor-kappaB/Rel expression. *Cell Growth Differ* **10**, 537-44 (1999).
441. Cocolakis, E., Lemay, S., Ali, S. & Lebrun, J.J. The p38 MAPK pathway is required for cell growth inhibition of human breast cancer cells in response to activin. *J Biol Chem* **276**, 18430-6 (2001).
442. Acosta, J.J. et al. Src mediates prolactin-dependent proliferation of T47D and MCF7 cells via the activation of focal adhesion kinase/Erk1/2 and phosphatidylinositol 3-kinase pathways. *Mol Endocrinol* **17**, 2268-82 (2003).
443. Raingeaud, J. et al. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem* **270**, 7420-6 (1995).

444. Han, J., Lee, J.D., Bibbs, L. & Ulevitch, R.J. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265**, 808-11 (1994).
445. Jiang, Y. et al. Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *J Biol Chem* **271**, 17920-6 (1996).
446. Lechner, C., Zahalka, M.A., Giot, J.F., Moller, N.P. & Ullrich, A. ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation. *Proc Natl Acad Sci U S A* **93**, 4355-9 (1996).
447. Goedert, M., Cuenda, A., Craxton, M., Jakes, R. & Cohen, P. Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. *Embo J* **16**, 3563-71 (1997).
448. Jiang, Y. et al. Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta. *J Biol Chem* **272**, 30122-8 (1997).
449. Lee, J.C. et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372**, 739-46 (1994).
450. Tong, L. et al. A highly specific inhibitor of human p38 MAP kinase binds in the ATP pocket. *Nat Struct Biol* **4**, 311-6 (1997).
451. Kyriakis, J.M. & Avruch, J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* **81**, 807-69 (2001).
452. Cohen, P.S. et al. The critical role of p38 MAP kinase in T cell HIV-1 replication. *Mol Med* **3**, 339-46 (1997).
453. Smeal, T., Hibi, M. & Karin, M. Altering the specificity of signal transduction cascades: positive regulation of c-Jun transcriptional activity by protein kinase A. *Embo J* **13**, 6006-10 (1994).
454. Takekawa, M. et al. Smad-dependent GADD45beta expression mediates delayed activation of p38 MAP kinase by TGF-beta. *Embo J* **21**, 6473-82 (2002).
455. Massague, J. How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* **1**, 169-78 (2000).
456. Engel, M.E., McDonnell, M.A., Law, B.K. & Moses, H.L. Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J Biol Chem* **274**, 37413-20 (1999).
457. Yu, L., Hebert, M.C. & Zhang, Y.E. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. *Embo J* **21**, 3749-59 (2002).
458. Atfi, A., Djelloul, S., Chastre, E., Davis, R. & Gespach, C. Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor beta-mediated signaling. *J Biol Chem* **272**, 1429-32 (1997).
459. Qing, J., Zhang, Y. & Derynck, R. Structural and functional characterization of the transforming growth factor-beta -induced Smad3/c-Jun transcriptional cooperativity. *J Biol Chem* **275**, 38802-12 (2000).
460. Yamaguchi, K. et al. XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. *Embo J* **18**, 179-87 (1999).

461. Yamaguchi, K. et al. Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. *Science* **270**, 2008-11 (1995).
462. Shibuya, H. et al. TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. *Science* **272**, 1179-82 (1996).
463. Edlund, S. et al. Transforming growth factor-beta1 (TGF-beta)-induced apoptosis of prostate cancer cells involves Smad7-dependent activation of p38 by TGF-beta-activated kinase 1 and mitogen-activated protein kinase kinase 3. *Mol Biol Cell* **14**, 529-44 (2003).
464. Wang, W., Zhou, G., Hu, M.C., Yao, Z. & Tan, T.H. Activation of the hematopoietic progenitor kinase-1 (HPK1)-dependent, stress-activated c-Jun N-terminal kinase (JNK) pathway by transforming growth factor beta (TGF-beta)-activated kinase (TAK1), a kinase mediator of TGF beta signal transduction. *J Biol Chem* **272**, 22771-5 (1997).
465. Perlman, R., Schiemann, W.P., Brooks, M.W., Lodish, H.F. & Weinberg, R.A. TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nat Cell Biol* **3**, 708-14 (2001).
466. Mathews, L.S. & Vale, W.W. Characterization of type II activin receptors. Binding, processing, and phosphorylation. *J Biol Chem* **268**, 19013-8 (1993).
467. Attisano, L., Wrana, J.L., Lopez-Casillas, F. & Massague, J. TGF-beta receptors and actions. *Biochim Biophys Acta* **1222**, 71-80 (1994).
468. Willis, S.A., Zimmerman, C.M., Li, L.I. & Mathews, L.S. Formation and activation by phosphorylation of activin receptor complexes. *Mol Endocrinol* **10**, 367-79 (1996).
469. Baker, J.C. & Harland, R.M. A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. *Genes Dev* **10**, 1880-9 (1996).
470. Chen, Y., Lebrun, J.J. & Vale, W. Regulation of transforming growth factor beta- and activin-induced transcription by mammalian Mad proteins. *Proc Natl Acad Sci U S A* **93**, 12992-7 (1996).
471. Zhang, Y., Feng, X., We, R. & Derynck, R. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature* **383**, 168-72 (1996).
472. Lebrun, J.J., Takabe, K., Chen, Y. & Vale, W. Roles of pathway-specific and inhibitory Smads in activin receptor signaling. *Mol Endocrinol* **13**, 15-23 (1999).
473. Lagna, G., Hata, A., Hemmati-Brivanlou, A. & Massague, J. Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways. *Nature* **383**, 832-6 (1996).
474. Macias-Silva, M. et al. MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* **87**, 1215-24 (1996).
475. Nakao, A. et al. TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *Embo J* **16**, 5353-62 (1997).
476. Zawel, L. et al. Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell* **1**, 611-7 (1998).
477. Moustakas, A. & Kardassis, D. Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members. *Proc Natl Acad Sci U S A* **95**, 6733-8 (1998).

478. Nakashima, R. et al. Genetic alterations in the transforming growth factor receptor complex in sporadic endometrial carcinoma. *Gene Expr* **8**, 341-52 (1999).
479. Kurokawa, M. et al. The oncoprotein Evi-1 represses TGF-beta signalling by inhibiting Smad3. *Nature* **394**, 92-6 (1998).
480. Hanafusa, H. et al. Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. *J Biol Chem* **274**, 27161-7 (1999).
481. Segev, D.L. et al. Mullerian inhibiting substance inhibits breast cancer cell growth through an NFkappa B-mediated pathway. *J Biol Chem* **275**, 28371-9 (2000).
482. de Winter, J.P., Roelen, B.A., ten Dijke, P., van der Burg, B. & van den Eijnden-van Raaij, A.J. DPC4 (SMAD4) mediates transforming growth factor-beta1 (TGF-beta1) induced growth inhibition and transcriptional response in breast tumour cells. *Oncogene* **14**, 1891-9 (1997).
483. Zauberman, A., Oren, M. & Zipori, D. Involvement of p21(WAF1/Cip1), CDK4 and Rb in activin A mediated signaling leading to hepatoma cell growth inhibition. *Oncogene* **15**, 1705-11 (1997).
484. Wu, R.Y., Zhang, Y., Feng, X.H. & Derynck, R. Heteromeric and homomeric interactions correlate with signaling activity and functional cooperativity of Smad3 and Smad4/DPC4. *Mol Cell Biol* **17**, 2521-8 (1997).
485. Eyers, P.A., Craxton, M., Morrice, N., Cohen, P. & Goedert, M. Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution. *Chem Biol* **5**, 321-8 (1998).
486. Massague, J., Blain, S.W. & Lo, R.S. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* **103**, 295-309 (2000).
487. Rouse, J. et al. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**, 1027-37 (1994).
488. Freshney, N.W. et al. Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* **78**, 1039-49 (1994).
489. Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B. & Davis, R.J. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol Cell Biol* **16**, 1247-55 (1996).
490. Derijard, B. et al. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* **267**, 682-5 (1995).
491. Zervos, A.S., Faccio, L., Gatto, J.P., Kyriakis, J.M. & Brent, R. Mxi2, a mitogen-activated protein kinase that recognizes and phosphorylates Max protein. *Proc Natl Acad Sci U S A* **92**, 10531-4 (1995).
492. Price, M.A., Cruzalegui, F.H. & Treisman, R. The p38 and ERK MAP kinase pathways cooperate to activate Ternary Complex Factors and c-fos transcription in response to UV light. *Embo J* **15**, 6552-63 (1996).
493. Alam, J. et al. Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor. *J Biol Chem* **275**, 27694-702 (2000).

494. Gohda, E. et al. Biological and immunological properties of human hepatocyte growth factor from plasma of patients with fulminant hepatic failure. *Biochim Biophys Acta* **1053**, 21-6 (1990).
495. Yang, S.H., Galanis, A. & Sharrocks, A.D. Targeting of p38 mitogen-activated protein kinases to MEF2 transcription factors. *Mol Cell Biol* **19**, 4028-38 (1999).
496. ten Dijke, P., Miyazono, K. & Heldin, C.H. Signaling inputs converge on nuclear effectors in TGF-beta signaling. *Trends Biochem Sci* **25**, 64-70 (2000).
497. Herlaar, E. & Brown, Z. p38 MAPK signalling cascades in inflammatory disease. *Mol Med Today* **5**, 439-47 (1999).
498. Ichijo, H. From receptors to stress-activated MAP kinases. *Oncogene* **18**, 6087-93 (1999).
499. Moriguchi, T. et al. Purification and identification of a major activator for p38 from osmotically shocked cells. Activation of mitogen-activated protein kinase kinase 6 by osmotic shock, tumor necrosis factor-alpha, and H2O2. *J Biol Chem* **271**, 26981-8 (1996).
500. Yang, X., Khosravi-Far, R., Chang, H.Y. & Baltimore, D. Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell* **89**, 1067-76 (1997).
501. Chang, H.Y., Nishitoh, H., Yang, X., Ichijo, H. & Baltimore, D. Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science* **281**, 1860-3 (1998).
502. Duan, H. & Dixit, V.M. RAIDD is a new 'death' adaptor molecule. *Nature* **385**, 86-9 (1997).
503. Ahmad, M. et al. CRADD, a novel human apoptotic adaptor molecule for caspase-2, and FasL/tumor necrosis factor receptor-interacting protein RIP. *Cancer Res* **57**, 615-9 (1997).
504. Shi, Y. & Massague, J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700 (2003).
505. Chen, Y.G. et al. Activin signaling and its role in regulation of cell proliferation, apoptosis, and carcinogenesis. *Exp Biol Med (Maywood)* **231**, 534-44 (2006).
506. Kang, J.S., Alliston, T., Delston, R. & Derynck, R. Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. *Embo J* **24**, 2543-55 (2005).
507. Sekine, Y. et al. Physical and functional interactions between STAP-2/BKS and STAT5. *J Biol Chem* **280**, 8188-96 (2005).
508. Litterst, C.M., Kliem, S., Marilley, D. & Pfitzner, E. NCoA-1/SRC-1 is an essential coactivator of STAT5 that binds to the FDL motif in the alpha-helical region of the STAT5 transactivation domain. *J Biol Chem* **278**, 45340-51 (2003).
509. Magne, S., Caron, S., Charon, M., Rouyez, M.C. & Dusanter-Fourt, I. STAT5 and Oct-1 form a stable complex that modulates cyclin D1 expression. *Mol Cell Biol* **23**, 8934-45 (2003).
510. Wang, Y. & Cheng, C.H. ERalpha and STAT5a cross-talk: interaction through C-terminal portions of the proteins decreases STAT5a phosphorylation, nuclear translocation and DNA-binding. *FEBS Lett* **572**, 238-44 (2004).
511. Onate, S.A., Tsai, S.Y., Tsai, M.J. & O'Malley, B.W. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**, 1354-7 (1995).

512. Dennler, S. et al. The steroid receptor co-activator-1 (SRC-1) potentiates TGF-beta/Smad signaling: role of p300/CBP. *Oncogene* **24**, 1936-45 (2005).
513. Iavnilovitch, E., Groner, B. & Barash, I. Overexpression and forced activation of stat5 in mammary gland of transgenic mice promotes cellular proliferation, enhances differentiation, and delays postlactational apoptosis. *Mol Cancer Res* **1**, 32-47 (2002).
514. Huang, H.M., Chang, T.W. & Liu, J.C. Basic fibroblast growth factor antagonizes activin A-mediated growth inhibition and hemoglobin synthesis in K562 cells by activating ERK1/2 and deactivating p38 MAP kinase. *Biochem Biophys Res Commun* **320**, 1247-52 (2004).
515. Datto, M.B., Yu, Y. & Wang, X.F. Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/p21 promoter. *J Biol Chem* **270**, 28623-8 (1995).
516. Ho, J. et al. Activin induces hepatocyte cell growth arrest through induction of the cyclin-dependent kinase inhibitor p15INK4B and Sp1. *Cell Signal* **16**, 693-701 (2004).
517. Kim, G.Y. et al. The stress-activated protein kinases p38 alpha and JNK1 stabilize p21(Cip1) by phosphorylation. *J Biol Chem* **277**, 29792-802 (2002).
518. Damen, J.E. et al. The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase. *Proc Natl Acad Sci U S A* **93**, 1689-93 (1996).
519. Huber, M. et al. The role of SHIP in growth factor induced signalling. *Prog Biophys Mol Biol* **71**, 423-34 (1999).
520. Pesesse, X., Deleu, S., De Smedt, F., Drayer, L. & Erneux, C. Identification of a second SH2-domain-containing protein closely related to the phosphatidylinositol polyphosphate 5-phosphatase SHIP. *Biochem Biophys Res Commun* **239**, 697-700 (1997).
521. Muraille, E., Pesesse, X., Kuntz, C. & Erneux, C. Distribution of the src-homology-2-domain-containing inositol 5-phosphatase SHIP-2 in both non-haemopoietic and haemopoietic cells and possible involvement of SHIP-2 in negative signalling of B-cells. *Biochem J* **342 Pt 3**, 697-705 (1999).
522. Bandyopadhyay, A. et al. Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. *Cancer Res* **62**, 4690-5 (2002).
523. Muraoka, R.S. et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* **109**, 1551-9 (2002).
524. Society, C.C. (2006).

# APPENDIX

## ABBREVIATIONS

ACTH, adrenocorticotropin hormone

ActRIB, activin receptor type IB

ActRII, activin type II receptor

ALK, activin-receptor like kinase

ASK, apoptosis-regulating-signal kinase

ATF-2, activating-transcription factor-2

ATP, adenosine tri-phosphate

bHLH, basic helix-loop-helix

BMP, bone-morphogenic protein

BRCA1, breast cancer 1

b-TrCP1, [beta]-transducin repeat-containing protein

bZIP, basic leucine zipper domain protein

CamKII, calmodulin kinase II

CBP, CREB binding protein

Cdk, cyclin dependent kinase

CIS, cytokine-inducible SH2 protein

Co-Smad, common-partner Smad

CPAP, cetrosomal P4.1-associated protein

CREB, cyclic AMP response element binding protein

CRM1, chromosomal region maintenance 1

CSBP, cytokine suppressive anti-inflammatory drugs binding protein

CTGF, connective tissue growth factor

Dab2, Disabled-2

DAPK, death associated protein kinase

DLK, dual leucine zipper-bearing kinase

DMBA, 7,12-dimethylbenz[α]anthracene

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

EMT, epithelial to mesenchymal transition

ERK, extracellular signal-regulated

FSH, follicular stimulating hormone

GAS, γ-interferon-activated sequence

GDF, growth/differentiation factor

GnRH, Gonadotropin-Releasing Hormone

GRK2, G-coupled receptor kinase 2

GS, glycine serine

hCG, Human Chorionic Gonadotropin

HIP, hydrocortisone insulin prolactin

HECT, homologous to the E6-accessory protein C-terminus

HGF/SF, hepatocyte growth factor/scatter factor

HPK, hematopoietic progenitor kinase

ICH-1, Ice/ced-3

ICE, interleukin-1beta converting enzyme

IGF-1, insulin like growth factor-1

IL11, interleukin-11

I-Smad, inhibitory Smad

Jak, Janus kinase

JNK, c-Jun terminal kinase

LAP, latency associated peptide

Lef-1, Lymphoid enhancer factor-1

LH, luteinizing hormone

LTBP, latent TGFβ associated protein

Mad, Mothers against DPP gene

MAPK, mitogen activating protein kinase

MAPKAPK, MAP-kinase activate protein kinase

MAPKK, MAPK kinase

MAPKKK, MAPKK kinase

MEF2C, myocyte enhancer factor 2C

MEK, mitogen activated and extracellular signal-regulated kinase

MEKK, mitogen activated and extracellular signal-regulated kinase kinase

MGF, mammary gland factor

MH1, Mad-homology 1 domain

MH2, Mad-homology 2 domain

MLK, mixed-lineage kinase

MMP, matrix metalloprotease

MMTV, mouse mammary tumor virus

MSP, macrophage stimulating protein

Msx, muscle segment homeobox

NICD, Notch intracellular domain

NEDD4/2, neural precursor cell expressed, developmentally down-regulated 4-2

NES, nuclear export sequence

NFκB, nuclear factor-kappaB

NK, natural killer

NLS, Nuclear localization sequence

Nmi, N-myc interactor

Nup, nucleoporin

PAI, plasminogen activator inhibitor

PI3K, phosphatidyl inositol-3-kinase

PIAS, protein inhibitor of activated Stats

PKA, protein kinase A

PP2A, protein phosphatase 2A

PP2C, protein phosphatase 2C

PPM1A, protein phosphatase 1alpha

PRL, prolactin

PRLR, PRL receptor

PTHrP, parathyroid hormone-releasing peptide

PTP, protein-tyrosine phosphatase

RAIDD, RIP-associated ICH-1/CED-3-homologous protein with a death domain

RIP, receptor interacting protein

ROCK, Rho-associated kinase

R-Smad, receptor Smad

SAP-1, sphingolipid activator protein

SAPK, stress activated protein kinase

SARA, Smad anchor for receptor activation

SCF, Skp1/Cullin1/F-box protein

Roc1, regulator of Cullins 1

SGF, sarcoma growth factor

SHIP, SH2-domain containing inositol-5-phosphate

Shp-2, SH2 domain-containing tyrosin phosphatase

Ski, Sloan-Kettering Institute proto-oncogene

SMRT, Stat5 and receptor corepressor

Smurf, Smad ubiquitin regulatory factor

SnoN, Ski-related novel gene

SOCS, Suppressors of cytokine signaling

STAT, signal transducer and activator of transcription

TAB, TAK1 activator

TAK1, TGFβ activated kinase 1

TCF, T cell-specific factor

TC-PTP, T-cell protein-tyrosine phosphatase

TEB, terminal end bud

TGF-α, transforming growth factor

TGFβ, transforming growth factor β

TGIF, TG3-interacting factor

TIEG1, TGFβ-inducible early-response gene

TIMP, Tissue Inhibitor of Metalloproteinase

Tiul1, for TGIF interacting ubiquitin ligase 1

TNF-α, tumor necrosis factor-α

TβRI, TGFβ type I receptor

TβRII, TGFβ type II receptor

uPA, urokinase plasminogen activator

VEGF, vascular endothelial growth factor

WAP, whey acidic protein

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 276, No. 21, Issue of May 25, pp. 18430–18436, 2001
*Printed in U.S.A.*

# The p38 MAPK Pathway Is Required for Cell Growth Inhibition of Human Breast Cancer Cells in Response to Activin*

Received for publication, November 29, 2000, and in revised form, February 12, 2001
Published, JBC Papers in Press, February 20, 2001, DOI 10.1074/jbc.M010768200

**Eftihia Cocolakis, Serge Lemay, Suhad Ali‡, and Jean-Jacques Lebrun§**

*From the Department of Medicine, Royal Victoria Hospital, Molecular Endocrinology Laboratory, McGill University, Montreal H3A 1A1, Canada*

**Activin, a member of the TGFβ family inhibits cell growth in various target tissues. Activin interacts with a complex of two receptors that upon activation phosphorylate specific intracellular mediators, the Smad proteins. The activated Smads interact with diverse DNA binding proteins and co-activators of transcription in a cell-specific manner, thus leading to various activin biological effects. In this study, we investigated the role and mechanism of action of activin in the human breast cancer T47D cells. We found that activin treatment of T47D cells leads to a dramatic decrease in cell growth. Thus activin appears as a potent cell growth inhibitor of these breast cancer cells. We show that activin induces the Smad pathway in these cells but also activates the p38-mitogen-activated protein kinase pathway, further leading to phosphorylation of the transcription factor ATF2. Finally, specific inhibitors of the p38 kinase (SB202190, SB203580, and PD169316) but not an inactive analogue (SB202474) or the MEK-1 inhibitor PD98059 completely abolish the activin-mediated cell growth inhibition of T47D cells. Together, these results define a new role for activin in human breast cancer T47D cells and highlight a new pathway utilized by this growth factor in the mediation of its biological effects in cell growth arrest.**

Activin, a member of the TGFβ[1] family, regulates cell growth of various cell types. Activin interacts with a complex of two receptors (types I and II), both containing an extracellular domain, a single transmembrane region, and a large intracellular domain that contain a serine/threonine kinase domain. The type II receptor, which is constitutively phosphorylated (1) transphosphorylates the type I receptor (ALK4) upon ligand stimulation, on serine and threonine residues (2–4). The activated receptor complex then recruits the two receptor-regulated Smad2 and Smad3 (5–8). Following binding and phosphorylation by the activin type I receptor, Smad2 and Smad3 are released to the cytoplasm where they associate with the common-partner Smad4 before being translocated to the nucleus (8–11).

Both Smad3 and Smad4 but not Smad2 can directly bind DNA elements (Smad binding element) and activate the transcription of the target genes (12). However, the DNA binding affinity of the Smads is low (13), and they usually require the presence of other DNA binding proteins to efficiently interact with the promoters of their responsive target genes. As a result, the Smad binding elements are often found close to the DNA binding element of other transcription factors. Among those are the FAST family members, FAST1 (14) and FAST2 (15), TFE3 (16), Fos and Jun (17), Sp1 (18), CBP/p300 (19), Evi-1 (20), and ATF2 (21).

The Smad proteins are central elements in the activin receptor signaling pathway but are not the sole pathway activated by this receptor complex. Other members of the TGFβ superfamily have been shown to activate different signaling pathways, in addition to the Smads. TGFβ itself can activate a member of the MAPKKK family of kinases, TAK1 (TGF-activated kinase) (22). TAK1 then activates the stress-activated kinase p38 and the transcription factor ATF2, a member of the b-ZIP family of DNA binding proteins (21). *In vitro* studies also suggested that the transcription factor ATF2 could interact with the MH1 domains of two activin responsive Smads, Smad3 and Smad4 (21, 23). Both TGFβ and the Müllerian inhibiting substance (MIS) were also shown to mediate some of their biological effects through an NFκB-mediated pathway (24, 25). It is therefore conceivable that activin also utilizes other signaling pathways to transduce its signals.

Activin, its receptors, and the Smads are expressed in myoepithelial cells as well as in a certain number of human breast cancer cell lines (26, 27), suggesting a role for this growth factor and its downstream effectors, the Smads, in mammary cell growth and differentiation. Several reports have recently implicated TGFβ family members or their downstream signaling pathways in the regulation of breast cancer cell growth. Indeed, Smad4 can restore cell growth arrest in MDA-MB-468 cells, a breast cancer cell line in which the Smad4 gene is deleted (28). Genetic mutations or loss of expression of the activin and TGFβ receptors is also found in human breast cancers (29, 30). Finally, TGFβ and MIS mediates cell growth arrest in breast cancer by reducing NFκB DNA binding activity (24, 25), and activin itself can modulate cell growth of the breast cancer cells MCF7 (26).

In the present study, we investigated the role and mechanism of action of activin in breast cancer cells. We show here for the first time that activin strongly inhibits cell growth of the

* This work was supported in part by the Medical Research Council of Canada (Grant 24836) and by the American Concern Foundation for Cancer Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ A Foundation pour la Recherche en Sante du Quebec scholar.

§ A Medical Research Council scholar. To whom correspondence should be addressed: Dept. of Medicine, Royal Victoria Hospital, Molecular Endocrinology Laboratory, 687 Pine Ave. West, McGill University, Montreal H3A 1A1, Canada. Tel.: 514-842-1231 (ext. 4846); Fax: 514-982-0893; E-mail: JJ.Lebrun@MUHC.McGill.CA.

[1] The abbreviations used are: TGFβ, transforming growth factor β; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; TAK1, TGF-activated kinase 1; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; MIS, Müllerian inhibiting substance; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; L32, L32 riboprotein; pp38, phosphorylated form of p38 kinase; EGF, epidermal growth factor; bp, base pair(s); ALK, activin receptor-like kinase; Fast1, forkhead activin signal transducer-1; STAT, signal transducers and activators of transcription.

human breast cancer cell line T47D. In addition, our results indicate that activin induces the Smad pathway in these cells but also activates the p38 MAPK pathway. Activation of this pathway further leads to phosphorylation of the transcription factor ATF2. Furthermore, we show that specific inhibitors of the p38 MAPK pathway antagonize the activin-mediated cell growth arrest in T47D cells. Thus, this highlights for the first time the involvement of this p38 kinase pathway downstream of the activin receptor signal transduction pathways leading to cell growth arrest.

## MATERIALS AND METHODS

*Cell Culture and Proliferation Assay*—T47D cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) in the presence of 10% fetal calf serum. For proliferation assay, cells were plated in triplicates in 96-well dishes, at 5000 cells/100 μl in 2% FCS. Cells were stimulated or not with activin (0.5 nM) and grown over a 5-day period. Cell proliferation was assessed using direct cell counting, and the non-radioactive MTT cell proliferation assay for eukaryotic cells (Cell Titer 96, Promega G 4000). Absorbance was measured at 570 nm with a reference wavelength at 450 nm, using a Bio-tek Microplate reader.

*Transfection and Reporter Assay*—T47D cells ($10^7$ cells) were transfected by electroporation (Bio-Rad Gene Pulser II) in 500 μl of phosphate-buffered saline (240 V and 975 microfarads) with 10 μg of each of the indicated cDNAs. Following transfection, cells were plated in 6-well dishes in DMEM (10% FCS) for 24-h recovery. The following day, cells were starved overnight in DMEM without serum and stimulated or not with activin (0.5 nM) for 16 h. Then, cells were washed once with phosphate-buffered saline and lysed in 250 μl of lysis buffer (1% Triton X-100, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, 25 mM glycylglycine) on ice. The luciferase activity of each sample was measured using 45 μl of cell lysate (EG&G Berthold luminometer) and normalized to the relative β-galactosidase activity.

*RNase Protection Assay*—RNase protection assay was performed using the hcc-2 template set and RiboQuant kit from PharMingen (San Diego, CA) according to the manufacturer's instructions, with minor modifications. Radiolabeled antisense RNA probes were prepared by *in vitro* transcription of the hcc-2 templates with T7 RNA polymerase in the presence of [α-$^{32}$P]UTP (PerkinElmer Life Sciences, Boston, MA). After DNase I digestion, phenol-chloroform extraction, and ethanol precipitation, the probes were quantified. RNA samples (5 μg) were dried in a vacuum centrifuge and resuspended in 20 μl of hybridization buffer containing $8 \times 10^5$ cpm of radiolabeled probes. Hybridization (overnight at 56 °C), RNase A/T1 digestion (1 h at 30 °C), proteinase K digestion, phenol-chloroform extraction, ethanol precipitation, and gel resolution (5% polyacrylamide, 8 M urea sequencing gel) were carried out according to the instructions contained in the RiboQuant RNase protection assay kit. A yeast tRNA-only reaction was included as a negative control to ensure complete RNase digestion. Undigested RNA probes were also resolved on each gel to ensure their integrity and to serve as size markers. The cell cycle genes represented in the assays and the size of corresponding protected probe/mRNA duplexes were as follows: p130, 400 bp; Rb, 352 bp; p107, 317 bp; p53, 283 bp; p57, 252 bp; p27, 227 bp; p21, 202 bp; p19, 182 bp; p16, 163 bp; p14/p15, 133 bp; L32 riboprotein (L32, used as a housekeeping control), 113 bp; and glyceraldehyde-3-phosphate dehydrogenase, 96 bp. An exposure was made of the dried gel onto x-ray film. The positions of the protected probes were confirmed by plotting on a semi-log graph.

*Western Blot Analysis*—T47D cells were plated at $10^6$ cells/ml in 6-well dishes in DMEM (10% FCS). The following day, cells were starved for an overnight period and stimulated or not with activin for different periods of time as indicated. Total cell extracts prepared from these cells were then separated on a polyacrylamide gel, transferred onto nitrocellulose, and incubated with the indicated specific antibody overnight at 4 °C (p38 (New England BioLabs, catalogue no. 9212), phosphop38 (New England BioLabs, catalogue no. 9210), ATF2 (New England BioLabs, catalogue no. 9222), phosphoATF2 (Santa Cruz Biotechnologies, catalogue no. 8398), Smad2/3 (Santa Cruz, catalogue no. 8332), phosphoSmad2 (Upstate Biotechnology Inc., catalogue no. 6829), Smad4 (Santa Cruz, catalogue no. 7966), ERK1/2 (New England BioLabs, catalogue no. 9102), and phosphoERK1/2 (New England BioLabs, catalogue no. 9101)). Following incubation, the membranes were washed twice for 10 min in washing buffer (50 mM Tris-HCl, pH 7.6; 200 mM NaCl; 0.05% Tween 20) and incubated with a secondary antibody coupled to peroxidase (from Santa Cruz; at a 1/10,000 dilution) for 1 h at room temperature. Then, the membranes were washed four times for 15 min in the washing buffer, and immunoreactivity was normalized by chemiluminescence (Lumi-Light Plus Western blotting substrate, Roche Molecular Biochemicals) according to the manufacturer's instructions and revealed using an Alpha Innotech Fluorochem imaging system (Packard Canberra).

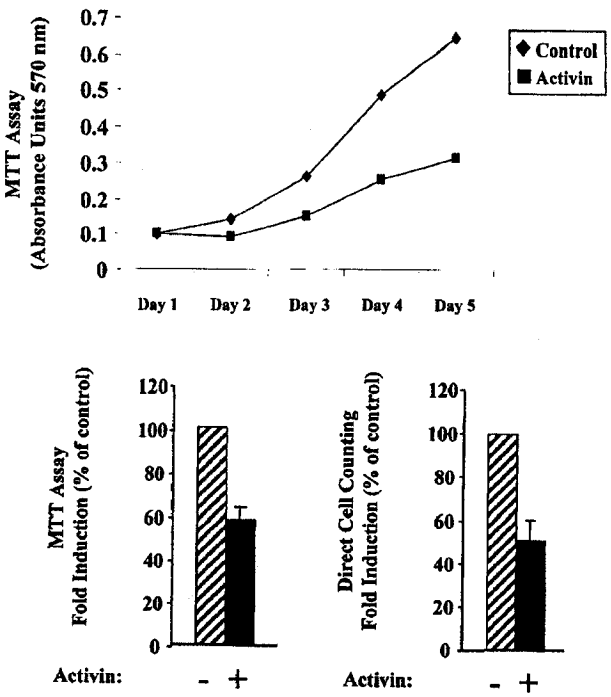



FIG. 1. **Activin induces cell growth arrest of the breast cancer cell line T47D.** T47D cells were grown in 2% FCS DMEM over a 5-day period in the presence or absence of 0.5 nM activin. Cell proliferation was assessed by (*A*) MTT colorimetric assay in triplicates and (*B*) direct cell counting. Values are expressed in arbitrary units.

## RESULTS

*Activin Inhibits T47D Human Breast Cancer Cell Growth*—Although activin and its receptors are expressed in a number of breast cancer cell lines, the role of activin in the regulation of breast cancer cell growth has not yet been fully investigated. To analyze the role of activin in regulating growth of human breast cancer cells, we utilized the human breast cancer cell line T47D, which endogenously expresses activin-responsive Smad2, Smad3, and Smad4 (31). Using a cell growth and viability assay (MTT assay), we show that activin treatment of T47D cells leads to a significant inhibition in their growth, apparent as early as day 2 and reaching 40% inhibition at day 3 (Fig. 1*A*). To verify that activin affects cell growth and not the metabolic rate of the cell, direct cell counting was also performed. As shown in Fig. 1*B*, activin stimulation of T47D cells for 3 days also results in clear cell growth inhibition. Therefore, activin appears as a potent cell growth inhibitor for T47D breast cancer cells.

*Activin Modulates Cell Cycle Regulators in Breast Cancer Cells*—TGFβ family members regulate cell growth through different mechanisms. They often mediate cell cycle arrest through up-regulation of the three cyclin-dependent kinase inhibitors $p15^{INK4B}$, $p21CIP1^{WAF1}$, and p27 (32–34). Because activin exerts a strong effect on cell growth in T47D (Fig. 1), we analyzed its effects in modulating gene expression levels of different cyclin-dependent kinase inhibitors as well as of other cell cycle regulatory genes. For this, we examined the level of mRNA species of different cell cycle regulators, using a highly sensitive and specific ribonuclease protection assay. As shown in Fig. 2, T47D cells were stimulated for different periods of

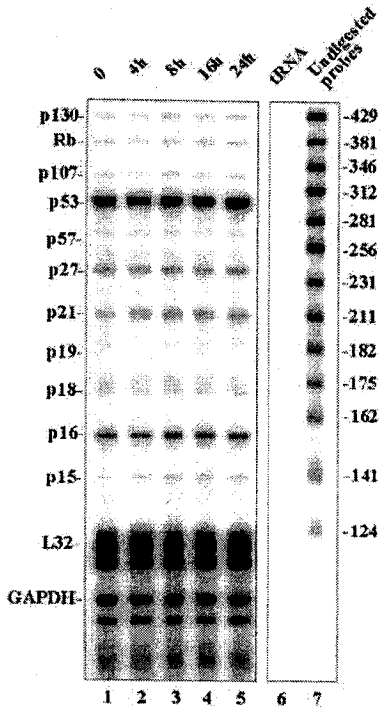



FIG. 2. **Expression of cell cycle genes in T47D cells in response to activin.** Total RNA (5 μg) obtained from T47D cells treated for 0, 4, 8, 16, or 24 h with activin (0.5 nM). An RNase protection assay was performed with radiolabeled probes for the indicated human cell cycle genes and two housekeeping control genes (L32 and glyceraldehyde-3-phosphate dehydrogenase), as described under "Materials and Methods." The positions of the protected probes are shown to the *left* of the autoradiography. Yeast tRNA (*lane 6*) is shown as a negative control. Undigested probes (*lane 7*) were used as size standards.

time with activin (0.5 nM). Total RNA from unstimulated or stimulated cells were extracted and hybridized with multiple antisense probes for human cell cycle regulators (p15, p16, p18, p19, p21, p27, as well as for p53, p57, p107 p130, the retinoblastoma protein (Rb), and the two housekeeping genes L32 and glyceraldehyde-3-phosphate dehydrogenase). As shown in Fig. 2, a modest but reproducible ligand-dependent increase in the mRNA level of $p21^{CIP1/WAF1}$ was observed. This is consistent with a microarray analysis of T47D cells treated for 8 h with activin, which shows a 1.7-fold increase in $p21^{CIP1/WAF1}$ mRNA level.[2] mRNA levels for $p15^{INK4B}$ were also consistently increased upon activin treatment, although at a lower level than $p21^{CIP1/WAF1}$. This experiment was repeated three times and showed consistent results. None of the other cell cycle regulators (p130, Rb, p107, p53, p57, p27, p19, p18, or p16) or housekeeping genes (L32 or glyceraldehyde-3-phosphate dehydrogenase) mRNA levels showed any significant or reproducible difference in response to activin (Fig. 2). Our attempts to detect $p15^{INK4B}$ and $p21^{CIP1/WAF1}$ protein levels in these cells were unsuccessful, probably due to the low level of expression of theses two proteins. This suggests that at least part of the activin effect on cell growth arrest in T47D cells is mediated through up-regulation of $p15^{INK4B}$ and $p21^{CIP1/WAF1}$.

*Activin Induces Smad2 Phosphorylation in T47D Cells*—To then analyze the role of the Smad pathway in T47D cells, we first examined the activation state of Smad2, following activin stimulation. Cells were starved for an overnight period and treated with 0.5 nM activin for a different period of time, as indicated in Fig. 3. Total cell lysates were separated by SDS-polyacrylamide gel electrophoresis, and resolved proteins were transferred to a nitrocellulose membrane for Western blotting analysis. The membrane was probed with a specific antibody to phospho-Smad2 that recognizes the two phosphorylated serine residues in the C-terminal end of the MH2 domain of Smad2 (SSXS). As shown in Fig. 3, *upper panel*, activin treatment of T47D cells leads to a clear phosphorylation of Smad2, as early as 15 min following ligand stimulation of the cells. The membrane was stripped and reprobed with a polyclonal antibody that recognizes both Smad2 and Smad3 (Fig. 3, *middle panel*) and subsequently with a monoclonal antibody to Smad4 (Fig. 3, *lower panel*) and shows equal levels of all Smads in all samples. These data indicate that the Smad pathway is functional in T47D cells and is activated in response to activin stimulation.

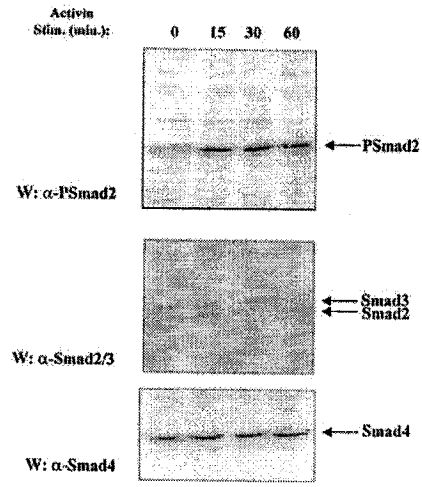



FIG. 3. **Activin induces Smad2 phosphorylation in T47D cells.** T47D cells were treated with 0.5 nM activin for 0, 15, 30, and 60 min. Cell lysates were analyzed by Western blot using a specific antibody to phospho-Smad2 (*upper panel*). The membrane was stripped and reprobed with an anti-Smad2/3 antibody (*middle panel*) and an anti-Smad4 antibody (*lower panel*).

*Activin Induces 3TPLux and ARE-Lux Promoters in T47D Cells*—To further examine the activation of the activin receptor/Smad pathway in T47D cells, we analyzed the ability of activin to induce two activin receptor/Smad-responsive promoter constructs (3TPLux and ARE-Lux). T47D cells were transiently co-transfected as shown under "Materials and Methods" with the promoter construct 3TPLux or ARE-Lux and an expression vector encoding the co-activator Fast1. As shown in Fig. 4*A*, activin treatment of T47D cells led to a 2.6- and 2.7-fold induction of 3TPLux and ARE-Lux, respectively. Furthermore, T47D cells were also co-transfected with 3TPLux or ARE-Lux/Fast1 and an expression vector encoding a constitutively active form of the activin type I receptor (ALK4 TΔD). This point mutation replaces threonine 206 by an aspartic acid and renders the receptor constitutively active even in the absence of ligand or type II receptor (4). As shown in Fig. 4*B*, ALK4 TΔD mimics activin effects on the activation of the two promoter constructs, leading to a 3.2- and 2.7-fold induction of 3TPlux and ARE-Lux/Fast1, respectively. Finally, to confirm the involvement of the Smad pathway, a dominant negative form of Smad3, which lacks the MH2 domain (Smad3ΔC), was transfected in T47D cells together with 3TPLux. Deletion of the C-terminal domain of Smad3 results in the loss of homo- and heterodimerization with the wild type Smad4 as well as in its ability to induce a reporter construct (35). Cells were stimulated or not with activin and, as seen in Fig. 4*C*, overexpression of Smad3ΔC completely blocks activin-induced 3TPLux activity. Together, these results confirm that the activin receptor/

[2] J.-J. Lebrun, unpublished data.

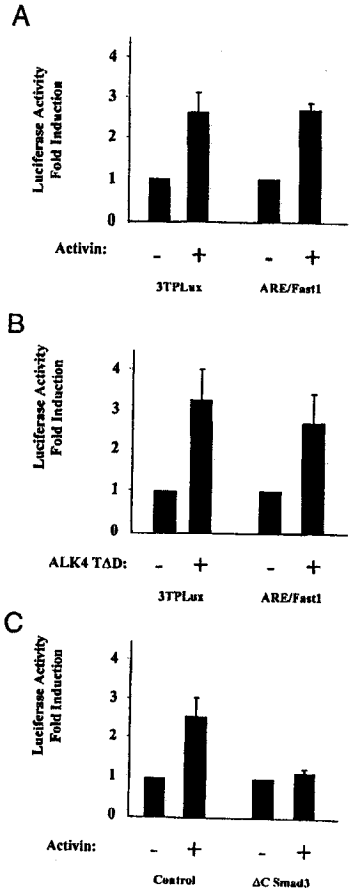



FIG. 4. **Activin induces 3TPLux and ARE-Lux promoters in T47D cells.** *A*, T47D cells transfected with the activin receptor/Smad responsive promoter constructs 3TP-Lux or ARE-Lux/Fast1 reporter constructs were stimulated with activin 16 h. The luciferase activity was normalized to the relative β-galactosidase values. Results represent means and standard deviations of three independent experiments. *B*, T47D cells transfected with the promoter constructs 3TP-Lux or ARE-Lux/Fast1 reporter constructs in the presence or the absence of the constitutively active form of the activin type I receptor (*ALK4 TΔD*). The luciferase activity was normalized to the relative β-galactosidase values. Results represent means and standard deviations of three independent experiments. *C*, T47D cells were transfected with the promoter construct 3TP-Lux in the presence or absence of the truncated form of Smad3 (*Smad3ΔC*) and stimulated with activin 16 h. The luciferase activity was normalized to the relative β-galactosidase values. Results represent means and standard deviations of three independent experiments.

Smad pathway is functional in breast cancer cells.

*Activin Activates the p38 Kinase Pathway in T47D Cells*—Recently, the p38 mitogen-activated protein kinase (MAPK) pathway was shown to regulate gene expression in response to TGFβ (23). To assess the role of this pathway in activin-mediated cell growth inhibition of breast cancer cells, T47D cells were starved overnight and stimulated with 0.5 nM activin for different periods of time as indicated in Fig. 5*A*. Total cell lysates were analyzed by immunoblot using specific antibodies directed against the phosphorylated form of the p38 kinase (*pp38*) and the normal form of p38 (*p38*). As shown in Fig. 5*A*, *upper panel*, activin treatment of the T47D cells results in a clear increase in p38 phosphorylation in a time-dependent manner. Activin effect is maximal at 20–40 min and then decreases to return to basal level. The membrane was stripped and reprobed with an antibody directed against p38 and shows an equal amount of the p38 kinase in all lanes (Fig. 5*A*, *lower panel*).

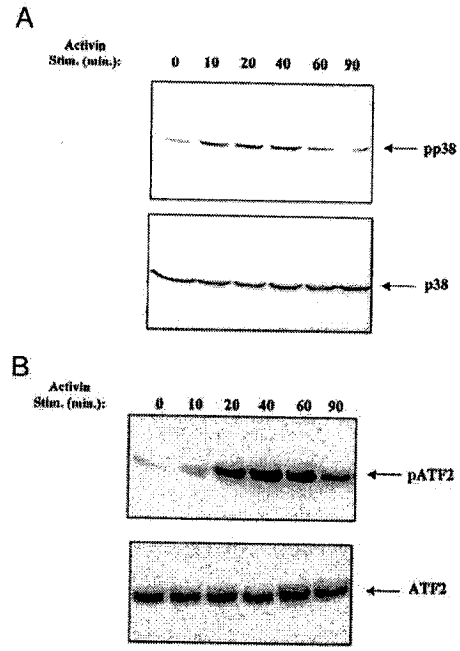



FIG. 5. **Activin induces the p38 kinase pathway in T47D cells.** T47D cells were starved overnight and stimulated with 0.5 nM of activin for 0, 5, 15, 30, 60, and 90 min. *A*, total cell lysates were analyzed by immunoblot using specific antibodies directed against the phosphorylated form of the p38 kinase (*pp38*) (*upper panel*). The membrane was stripped and reprobed with an anti-p38 (*p38*) antibody (*lower panel*). *B*, similarly, total cell lysates were analyzed by immunoblot using a specific antibody directed against the phosphorylated ATF2 (*pATF2*) (*upper panel*). The membrane was stripped and reprobed with an anti-ATF2 (*ATF2*) antibody (*lower panel*).

We then analyzed the activin effects on the phosphorylation of the transcription factor ATF2, a downstream target of the p38 kinase. Total cell lysates were analyzed by immunoblot using specific antibodies directed against the phosphorylated ATF2 (*pATF2*) or ATF2 (*ATF2*). As shown in Fig. 5*B* (*upper panel*), there is a time-dependent phosphorylation of the transcription factor ATF2 following activin treatment of the cells. The phosphorylation of ATF2 correlates with the activation of the p38 MAPK and shows a maximum phosphorylation at 40 min. The membrane was stripped and reprobed with an anti-ATF2 antibody and shows an equal amount of the transcription factor in all lanes (Fig. 5*B*, *lower panel*). Together, these data demonstrate that the p38 MAPK/ATF2 pathway is activated in T47D cells in response to activin.

*The p38 Kinase Inhibitor PD169316 Blocks Activin-induced p38 and ATF2 Phosphorylation*—To further confirm the involvement of the p38 MAPK pathway downstream of the activin receptor, T47D cells were treated with a specific p38 kinase inhibitor (PD169316) or $Me_2SO$ as a control. Cells were then stimulated or not with activin for 30 min, and total cell lysates were analyzed by Western blotting using different antibodies directed against phosphop38 or p38, phospho-ATF2 or ATF2. As shown in Fig. 6*A* (*upper panel*), in the presence of $Me_2SO$, activin induces phosphorylation of the kinase p38, confirming the previously seen results (Fig. 5). However, in the presence of the specific p38 kinase inhibitor PD169316, this activin effect on p38 phosphorylation is abolished (Fig. 6*A*, *upper panel*). The membrane was stripped and reprobed with an antibody directed against p38 and shows an equal amount of proteins in all lanes (Fig. 6*A*, *lower panel*). Similarly, as shown in Fig. 6*B*, *upper panel*, activin induces phosphorylation of the transcription factor ATF2 in the presence of $Me_2SO$, but this activin-induced effect is abolished in the presence of the p38

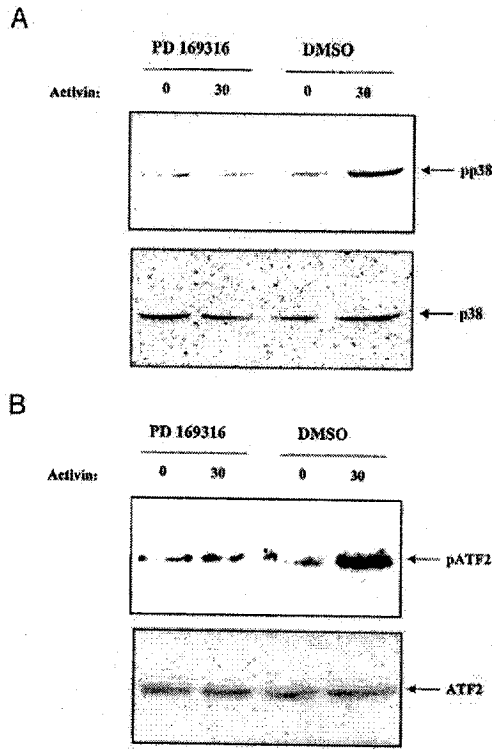



FIG. 6. **The p38 kinase inhibitor PD169316 blocks activin-induced p38 and ATF2 phosphorylation.** T47D cells were pretreated with $Me_2SO$ or with the specific p38 inhibitor PD169316 at 10 $\mu$M for 1 h before being stimulated with activin for 30 min. *A*, total cell lysates were analyzed by immunoblot using a specific antibody directed against the phosphorylated form of the p38 kinase (*pp38*) (*upper panel*). The membrane was stripped and reprobed with an anti-p38 (*p38*) antibody (*lower panel*). *B*, similarly, total cell lysates were analyzed by immunoblot using a specific antibody directed against the phosphorylated ATF2 (*pATF2*) (*upper panel*). The membrane was stripped and reprobed with an anti-ATF2 (*ATF2*) antibody (*lower panel*).

kinase inhibitor PD169316. An equal amount of protein in all lanes was ensured by stripping and reprobing of the membrane with an anti-ATF2 antibody (Fig. 6*B*, *lower panel*).

*The p38 Kinase Inhibitors Antagonize Activin-induced Cell Growth Arrest in Breast Cancer Cells*—To evaluate the contribution of the p38 kinase pathway in activin-mediated cell growth inhibition in T47D cells, we used different p38 kinase-specific inhibitors (SB202190, SB203580, PD169316) or an inactive analogue (SB202474) and an MEK1/ERK1/2 inhibitor (PD98059) as controls, in both MTT (Fig. 7*A*) and direct cell counting assays (Fig. 7*B*). T47D cells were cultured in DMEM, 2% serum for 3 days and stimulated or not with 0.5 nM activin in the presence or absence of the different inhibitors. As shown in Fig. 7, *A* and *B*, after 3 days cell growth is reduced by 40% in activin-treated cells as compared with untreated cells, similar to that previously observed in Fig. 1, *A* and *B*. However, in the presence of each the three specific p38 kinase inhibitors (SB202190, SB203580, PD169316), the inhibitory effect of activin on cell growth is abolished. On the other hand, the activin effect on cell growth inhibition is maintained in samples treated with the inactive form of the p38 kinase inhibitor SB202474 or with the MEK1/ERK1/2 inhibitor PD98059. Our results indicate that p38 kinase-specific inhibitors nearly completely reverse the activin effect. Because p38 kinase inhibitors could affect TGF$\beta$ receptor activity (36), we examined their effect on activin-induced Smad2 phosphorylation. As shown in Fig. 7*C*, although activin-induced p38 phosphorylation is inhibited by pretreatment of the cells with all three active forms of

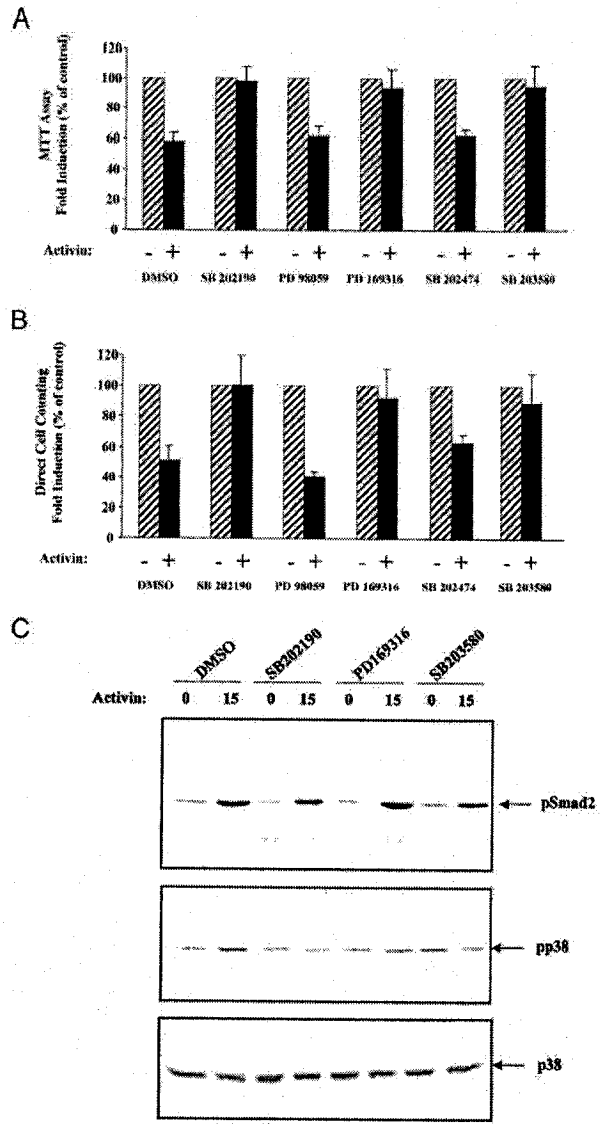



FIG. 7. **p38 kinase inhibitors antagonize activin-induced cell growth arrest in breast cancer cells.** T47D cells were cultured in DMEM, 2% serum for 3 days and stimulated or not with 0.5 nM activin in the presence or absence of 10 $\mu$M of the different p38 kinase-specific inhibitors, SB202190, SB203580, and PD169316, or the inactive analogue SB202474 and the MEK1/ERK1/2 inhibitor PD98059 as controls. Cell growth was assessed by (*A*) MTT colorimetric assay in triplicates and (*B*) direct cell counting. Values represent means and standard deviations of five separate experiments and are expressed as percentage of inhibition compared with the control. *C*, T47D cells were pretreated with $Me_2SO$ or with the p38 inhibitors (SB202190, PD169316, or SB203580) at 10 $\mu$M for 45 min before being stimulated with activin for 15 min. Total cell lysates were analyzed by immunoblot using specific antibodies directed against the phosphorylated form of Smad2 (*pSmad2*, *upper panel*), the phosphorylated form of p38 kinase (*pp38*, *middle panel*), or p38 kinase (*p38*, *lower panel*).

p38 kinase inhibitors, we observed no significant inhibitory effect on Smad2 phosphorylation under the same conditions. Thus it is likely that the antagonistic effect exerted by the p38 inhibitors on activin-induced cell growth arrest is mediated through inhibition of the p38 kinase pathway downstream of the activin receptor.

As activin is potent cell growth inhibitor in many different cell lines, the effect of the p38 kinase inhibitors were also analyzed in several activin-responsive cell lines such as K562, Chinese hamster ovary, and MCF7. Interestingly, the activin

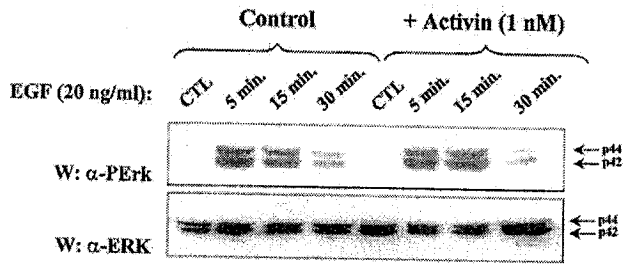



FIG. 8. **Activin effect on cell growth arrest is not mediated through the MAPK pathway.** T47D cells were starved overnight and stimulated with EGF (20 ng/ml) for different period of times in the absence or presence of 1 nM activin. Total cell lysates were then analyzed by Western blotting using an antibody directed against phospho-ERK1/2 (*upper panel*). The membrane was stripped and reprobed with an anti-ERK1/2 antibody (*lower panel*).

inhibitors could reverse the activin effects on cell growth arrest in all cell lines tested (data not shown). This indicates that the contribution of the p38 MAPK pathway to activin-mediated cell growth arrest is critical.

*Activin Effect on Cell Growth Arrest Is Not Mediated through the MEK1/ERK1/2 MAPK Pathway*—The absence of effect of the MEK1/ERK1/2 inhibitor PD98059 on activin-mediated cell growth arrest (Fig. 7) suggests that activin does not modulate the MAPK MEK1/ERK1/2 pathway to arrest cell growth. The MEK1/ERK1/2 pathway is known to be involved in cell proliferation in response to various growth factors. To confirm that activin does not modulate or inhibit activation of this pathway in response to growth factors, T47D cells were starved overnight and stimulated with EGF (20 ng/ml) for different periods of time in the absence or presence of 1 nM activin (Fig. 8). Total cell lysates were then analyzed by Western blotting using an antibody directed against phospho-ERK1/2 (α-PERK). As shown in Fig. 8 (*upper panel*), EGF very rapidly and transiently induces the phosphorylation of ERK1/2 (p42/p44). However, activin co-stimulation of the cells does not affect EGF-induced ERK1/2 phosphorylation. The membrane was stripped and reprobed with an anti-ERK antibody and shows an equal amount of MAPK in all samples. Together this indicates that the activin effect on cell growth arrest in T47D cells is not mediated through the MEK1/ERK1/2 MAPK pathway.

## DISCUSSION

Members of the TGFβ family of growth factors are important factors in regulating cell growth inhibition; hence, it is critical to characterize their intracellular signaling mechanisms. Although it is known that activin signals through activation of Smad proteins, the activation of other intracellular signaling pathways and their contribution to activin-mediated cell growth inhibition remain to be characterized. In this paper we have examined the role of activin in mediating cell growth inhibition of breast cancer cells. Our results indicate that activin induces the Smad pathway in T47D cells and emphasize the involvement of the p38 MAPK pathway in activin-induced cell growth inhibition of these breast cancer cells.

Abnormalities in the signaling pathways of activin/TGFβ have been clearly linked to various cancers, including breast cancer (37). We analyzed activin effects on the regulation of cell growth of human breast cancer cells. Using the human breast cancer cell line T47D, we found that activin has a profound and significant effect on the growth of these cells. We further investigated how activin triggers its effects in this cell line. Activin treatment of T47D cells leads to rapid phosphorylation of the receptor-regulated Smad2. Furthermore, both activin or the constitutively active form of the activin type I receptor (ALK4TΔD) induce the two promoter constructs 3TPLux and ARE-Lux, and this effect is completely abolished in the presence of an overexpressed dominant negative form of Smad3 (Smad3ΔC). All together, these results suggest that the activin receptor/Smad pathway is activated and can regulate the activin response in breast cancer cells, confirming the central role played by the Smad proteins in the mediation of the activin response.

The p38 MAPK is involved in regulating cellular responses to stress and cytokines (38–41). p38 kinase is activated and phosphorylated at the $Thr^{180}$-$Tyr^{182}$ site by the two closely related dual specificity protein kinases MKK3 and MKK6 (42, 43). The activated p38 kinase has been shown to phosphorylate several transcription factors such as ATF2 (44), Max (45), and Elk-1 (46) and indirectly cAMP-response element-binding protein via activation of Nrf2 (47), STAT1 (48), and MEF-2 (49). The p38 pathway is activated in response to TGFβ in C2C12, Mv1LU, and 293 cells (21, 23). TGFβ can induce phosphorylation of both p38 and the transcription factor ATF2 in these cell lines. In addition, p38 and ATF2 can contribute to the activation of the synthetic reporter construct 3TPLux in these cells, but the physiological significance of this pathway in the mediation of the TGFβ effects remains unclear. We show here that activin induces the p38 kinase pathway in T47D cells leading to phosphorylation of both the p38 kinase and the transcription factor ATF2. Furthermore, we show that the p38/ATF2 pathway is required to transduce the activin effects on cell growth inhibition. Indeed, different specific p38 kinase inhibitors, but not their inactive analogue or the MEK inhibitor can totally reverse the activin effect on cell growth inhibition. This highlights a new role for the p38 kinase pathway in the control of cell growth and proliferation downstream of the activin/TGFβ superfamily of growth factors. TGFβ family members often require the presence of parallel or synergistic pathways to the Smads to carry out their full biological effects and diversity of the Smad-interacting partners may contribute to signal specificity (50). In future studies, it will be interesting to examine the level of interaction between the Smad and the p38 kinase pathways in response to activin in T47D cells, because *in vitro* studies have suggested that the Smads could physically interact with the transcription factor ATF2 (21, 23).

Signaling by the MAPK family is organized hierarchically in three different steps. MAPK, such as p38, are phosphorylated by MAPK kinases (MAPKKS), such as MKK3 and MKK6, in the case of p38. The MAPKKs are themselves activated and phosphorylated by the MAPKK kinases (MAPKKKs), such as MLK, TAK, and ASK1 kinases, which act as MAPKKKs. Finally, the MAPKKKs are regulated by cell surface receptors or other external stimuli (51, 52). It will be interesting to identify the upstream kinases and other partner proteins involved in the activin-mediated p38 activation that are acting between the activin receptor complex and the p38 kinase in the signaling cascade. Recent reports indicated that TAK1, a member of the MAPKKK family, is activated by several cytokines, including TGFβ (22) and bone morphogenetic protein (53). TAK1 is a potent activator of p38 kinase (54). It will be interesting, therefore, to determine whether or not TAK1 also lies downstream of the activin receptor complex signaling cascade.

It was also recently shown that the Müllerian Inhibitory substance (MIS) represses the growth of breast cancer cells by regulating the NFκB pathway (25). The TGFβ effect on cell growth inhibition of breast cancer has also been shown to be associated with a reduced NFκB activity (24). This suggests that different members of the TGFβ superfamily may regulate cell growth by utilizing different signaling pathways in the same target tissues. Interestingly, TAK1 was also shown to lead to NFκB activation (55), suggesting a potential role for this

factor downstream of the activin receptor.

Our results indicate that activin strongly represses the cell growth of T47D breast cancer cells. Further characterization of the downstream target genes that are modulated in T47D cells in response to activin will greatly enhance our understanding of its mechanism of action on cell growth regulation. Our data suggest that at least some of these targets could be the cyclin-dependent kinase inhibitors. However, it will remain to be determined whether other cell cycle regulators as well as apoptosis regulators are also regulated by activin in these cells. Indeed, identification of the target genes, involved in the regulation of cell cycle and/or apoptosis, will be important in shedding light on the activin receptor mechanism of action in breast cancer cells.

*Acknowledgments*—We are thankful to Wylie Vale and the National Hormone & Pituitary program and Dr. Parlow for providing activin, to Dr. J. Massagué for providing the 3TPLux construct, to Dr. B. Volgestein for the Fast-1 construct, to Dr. Y. Chen for the Smad3ΔC construct, and to Drs. J. Wrana and L. Attisano for ARE-Lux construct.

REFERENCES

1. Mathews, L. S., and Vale, W. W. (1993) *J. Biol. Chem.* **268,** 19013–19018
2. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) *Nature* **370,** 341–347
3. Willis, S. A., Zimmerman, C. M., Li, L. I., and Mathews, L. S. (1996) *Mol. Endocrinol.* **10,** 367–379
4. Attisano, L., Wrana, J. L., Montalvo, E., and Massague, J. (1996) *Mol. Cell. Biol.* **16,** 1066–1073
5. Baker, J. C., and Harland, R. M. (1996) *Genes Dev.* **10,** 1880–1889
6. Chen, Y., Lebrun, J. J., and Vale, W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 12992–12997
7. Zhang, Y., Feng, X., We, R., and Derynck, R. (1996) *Nature* **383,** 168–172
8. Lebrun, J. J., Takabe, K., Chen, Y., and Vale, W. (1999) *Mol. Endocrinol.* **13,** 15–23
9. Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massague, J. (1996) *Nature* **383,** 832–836
10. Macias-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attisano, L., and Wrana, J. L. (1996) *Cell* **87,** 1215–1224
11. Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) *EMBO J.* **16,** 5353–5362
12. Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kern, S. E. (1998) *Mol. Cell* **1,** 611–617
13. Shi, Y., Wang, Y. F., Jayaraman, L., Yang, H., Massague, J., and Pavletich, N. P. (1998) *Cell* **94,** 585–594
14. Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G., and Whitman, M. (1997) *Nature* **389,** 85–89
15. Labbe, E., Silvestri, C., Hoodless, P. A., Wrana, J. L., and Attisano, L. (1998) *Mol. Cell* **2,** 109–120
16. Hua, X., Liu, X., Ansari, D. O., and Lodish, H. F. (1998) *Genes Dev.* **12,** 3084–3095
17. Zhang, Y., Feng, X. H., and Derynck, R. (1998) *Nature* **394,** 909–913
18. Moustakas, A., and Kardassis, D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6733–6738
19. Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K., and Taga, T. (1999) *Science* **284,** 479–482
20. Kurokawa, M., Mitani, K., Irie, K., Matsuyama, T., Takahashi, T., Chiba, S., Yazaki, Y., Matsumoto, K., and Hirai, H. (1998) *Nature* **394,** 92–96
21. Sano, Y., Harada, J., Tashiro, S., Gotoh-Mandeville, R., Maekawa, T., and Ishii, S. (1999) *J. Biol. Chem.* **274,** 8949–8957
22. Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995) *Science* **270,** 2008–2011
23. Hanafusa, H., Ninomiya-Tsuji, J., Masuyama, N., Nishita, M., Fujisawa, J., Shibuya, H., Matsumoto, K., and Nishida, E. (1999) *J. Biol. Chem.* **274,** 27161–27167
24. Sovak, M. A., Arsura, M., Zanieski, G., Kavanagh, K. T., and Sonenshein, G. E. (1999) *Cell Growth Differ.* **10,** 537–544
25. Segev, D. L., Ha, T. U., Tran, T. T., Kenneally, M., Harkin, P., Jung, M., MacLaughlin, D. T., Donahoe, P. K., and Maheswaran, S. (2000) *J. Biol. Chem.* **275,** 28371–28379
26. Liu, Q. Y., Niranjan, B., Gomes, P., Gomm, J. J., Davies, D., Coombes, R. C., and Buluwela, L. (1996) *Cancer Res.* **56,** 1155–1163
27. Ying, S. Y., and Zhang, Z. (1996) *Breast Cancer Res. Treat.* **37,** 151–160
28. de Winter, J. P., Roelen, B. A., ten Dijke, P., van der Burg, B., and van den Eijnden-van Raaij, A. J. (1997) *Oncogene* **14,** 1891–1899
29. Di Loreto, C., Reis, F. M., Cataldi, P., Zuiani, C., Luisi, S., Beltrami, C. A., and Petraglia, F. (1999) *Eur. J. Endocrinol.* **141,** 190–194
30. Chen, T., Carter, D., Garrigue-Antar, L., and Reiss, M. (1998) *Cancer Res.* **58,** 4805–4810
31. Pouliot, F., and Labrie, C. (1999) *Int. J. Cancer* **81,** 98–103
32. Hannon, G. J., and Beach, D. (1994) *Nature* **371,** 257–261
33. Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., and Wang, X. F. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5545–5549
34. Zauberman, A., Oren, M., and Zipori, D. (1997) *Oncogene* **15,** 1705–1711
35. Wu, R. Y., Zhang, Y., Feng, X. H., and Derynck, R. (1997) *Mol. Cell. Biol.* **17,** 2521–2528
36. Eyers, P. A., Craxton, M., Morrice, N., Cohen, P., and Goedert, M. (1998) *Chem. Biol.* **5,** 321–328
37. Massague, J., Blain, S. W., and Lo, R. S. (2000) *Cell* **103,** 295–309
38. Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A. R. (1994) *Cell* **78,** 1027–1037
39. Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., and Saklatvala, J. (1994) *Cell* **78,** 1039–1049
40. Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., *et al.* (1994) *Nature* **372,** 739–746
41. Han, J., Lee, J. D., Bibbs, L., and Ulevitch, R. J. (1994) *Science* **265,** 808–811
42. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. (1996) *Mol. Cell. Biol.* **16,** 1247–1255
43. Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) *Science* **267,** 682–685
44. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) *J. Biol. Chem.* **270,** 7420–7426
45. Zervos, A. S., Faccio, L., Gatto, J. P., Kyriakis, J. M., and Brent, R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10531–10534
46. Price, M. A., Cruzalegui, F. H., and Treisman, R. (1996) *EMBO J.* **15,** 6552–6563
47. Alam, J., Wicks, C., Stewart, D., Gong, P., Touchard, C., Otterbein, S., Choi, A. M., Burow, M. E., and Tou, J. (2000) *J. Biol. Chem.* **275,** 27694–27702
48. Goh, K. C., Haque, S. J., and Williams, B. R. (1999) *EMBO J.* **18,** 5601–5608
49. Yang, S. H., Galanis, A., and Sharrocks, A. D. (1999) *Mol. Cell. Biol.* **19,** 4028–4038
50. ten Dijke, P., Miyazono, K., and Heldin, C. H. (2000) *Trends Biochem. Sci.* **25,** 64–70
51. Herlaar, E., and Brown, Z. (1999) *Mol. Med. Today* **5,** 439–447
52. Ichijo, H. (1999) *Oncogene* **18,** 6087–6093
53. Yamaguchi, K., Nagai, S., Ninomiya-Tsuji, J., Nishita, M., Tamai, K., Irie, K., Ueno, N., Nishida, E., Shibuya, H., and Matsumoto, K. (1999) *EMBO J.* **18,** 179–187
54. Moriguchi, T., Toyoshima, F., Gotoh, Y., Iwamatsu, A., Irie, K., Mori, E., Kuroyanagi, N., Hagiwara, M., Matsumoto, K., and Nishida, E. (1996) *J. Biol. Chem.* **271,** 26981–26988
55. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) *Nature* **398,** 252–256

# Copyright Permission Policy

**ASBMB Journals**
*Journal of Biological Chemistry*
*Molecular and Cellular Proteomics*
*Journal of Lipid Research*
*Biochemistry and Molecular Biology Education*
*ASBMB Today*

## ASBMB does not charge for and grants use without requiring your copyright permission request for:

- Original authors wanting to reproduce portions of their own work; or to republish their material in not-for-profit formats or venues.
- Students wanting to reproduce or republish their work for educational purposes.
- Students using other authors' material for their theses.
- Reproduction or republication of abstracts only.
- Photocopying up to 5 copies for personal use.
- Non-profit educational institutions making multiple photocopies of articles for classroom use; all such reproduction must utilize institutionally owned equipment for this purpose.

***Use of copyrighted material requires proper citation.***

## For all other uses, contact Copyright Clearance Center.