Mechanisms of action of transforming growth factor beta and activin in haematopoietic cells

by

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Your file Votre référence
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Dedicated to my family,

To the memory of my Father, Ing. Hector Valderrama-Elizalde

and my Grandfather Alejandro Carvajal Hernandez

To México

For a job well done

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Tlazohcamati huel miec.

Preface
&
Contribution of Authors

I have chosen to present the current thesis as a dissertation, which takes the format of a collection of research papers. In order to inform the external examiner of the faculty regulation concerning this format of thesis preparation, I have reproduced in full form the guideline for thesis preparation of McGill Faculty of Graduate Studies.

"Candidates have the option of including as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly duplicate text of **one** or more published papers. These texts must be bound as an integral part of the thesis and reprints of published papers can include in appendices. The thesis must be written as a cohesive unit with a logical progression from one chapter to the next rather than as a collection of manuscripts. Connecting texts that provide logical bridges between the different papers are mandatory. The thesis must still confirm with all other requirements of the "Guidelines for thesis preparation". The thesis must include a table of contents, an abstract in both French and English, an introduction which clearly states the rational and objectives of the research, a review of literature, a final conclusion or summary, and a comprehensive reference list. Additional material must be provided, where appropriate (e.g. in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of research reported in the thesis. In the case of co-author papers, the candidate is required to make an explicit statement in the thesis as who contributed to such work and to what extent. Supervisors must attest to the accuracy of this statement at the doctoral oral defence. Since the task of experiments is made more difficult in

these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the authors of the co-authors papers."

The data presented in this thesis has been published or is to be submitted to peer-reviewed journals for publication. This thesis contains the texts adopted from the following papers:

Chapter 2:

Activin/TGFβ induce apoptosis through Smad-dependent expression of the lipid phosphatase SHIP.

VALDERRAMA-CARVAJAL Héctor, et al. (2002).
Nat Cell Biol. Dec; 4(12): 963-9

*** ***Original Paper included in Appendix 1.***

Chapter 3:

Activin and TGF-β regulate expression of the phosphatidyl inositol 5' phosphatase SHIP-1 through Smads and AP-1.

VALDERRAMA-CARVAJAL Héctor, and Lebrun JJ (2004).
(Manuscript to be submitted)

The candidate performed 95% of all the research included in this thesis. Contributions of other authors to this work are described below:

Dr. Jean-Jacques LEBRUN from Hormones and Cancer Research Unit, Department of Medicine, Royal Victoria Hospital, McGill University, Montreal, Canada, was supervisor of the work presented in this thesis and contributed intellectual support, research funding during my experimental work and manuscript writing process. Dr. JJ LEBRUN conceived of the studies, co-ordinated its design and helped drafts and finalized the two manuscripts presented in this thesis.

Dr. Gerald KRYSTAL from the Terry Fox Laboratory, Vancouver, British Columbia, donated generously the SHIP-1 wt and SHIP-1 -/- macrophages used in chapter 2. Dr. KRYSTAL helped in reviewing the manuscript presented in chapter 2.

Dr. S. Ali from Department of Medicine, Division of Hematology, Molecular Oncology Group, Royal Victoria Hospital, McGill University, Montreal, QC, Canada, contributed intellectually and reviewing the manuscript presented in chapter 2.

From Dr. Jean-Jacques LEBRUN's laboratory at the Hormones and Cancer Research Unit at the Royal Victoria Hospital, Cocolakis E, carried out the experiments shown in chapter 2 figures 2.1b and 2.1c, which described the apoptotic level by Annexin V labelling and FACS analysis. Lee EH helped in reproducing the different luciferase assays in chapter 2 in figure 2.3. VALDERRAMA-CARVAJAL H performed the data presented in chapter 3.

In addition to the papers included in this thesis, I also contributed with different experiments included into the following papers (1999-2005), which have been published or submitted:

1. Valverde-Franco G, Liu H, Davidson D, Chai S, **Valderrama-Carvajal H**, Goltzman D, Ornitz DM, and Henderson JE. (2004). Defective bone mineralization and osteopenia in young adult FGFR3-/- mice.
   Hum. Mol. Genet. Feb 1:13(3): 271-84.
2. Soros V, **Valderrama-Carvajal H.**, Richard S, and Cochrane, A. (2001). Inhibition of Human Immunodeficiency Virus type 1 Rev function by a dominant-negative mutant of Sam68 through sequestration of unspliced RNA at perinuclear bundles.
   J. Virol. Sept; 75(17): 8203-15.
3. Chen T, Cote J, **Carvajal HV**, and Richard S. (2001). Identification of Sam68 Arginine Glycin-Rich Sequence Capable of Conferring Non-specific RNA Binding to the GSG domain.
   J. Biol. Chem. Aug 17: 276(33): 30803-11.
4. Derry JJ, Richard S, **Valderrama-Carvajal H**, Ye X, Vasioukhin V, Cochrane AW, Chen T, Tyner AL. (2000). Sik (BRK) phosphorylates Sam68 in the nucleus and negatively regulates its RNA binding ability.
   Mol Cell Biol. 2000 Aug; 20(16): 6114-26.

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List of Abbreviations

ActRIA	Activin receptor type IA (ALK2)
ActRIIB	Activin receptor type IB (ALK4)
Akt/PKB	Protein Kinase B
ALK	Activin receptor-like kinase
AMH	Anti-Mullerian hormone
AMHR	Anti-Mullerian hormone receptor
ARE	Activin responsive element
ART	Artemin
BAMBI	BMP and activin membrane-bound inhibitor
BLAST	Basic local alignment search tool
BMP	Bone Morphogenetic Protein
BMPRIA	BMP receptor type IA (ALK3)
BMPRIB	BMP receptor type IB (ALK6)
BMPRII	BMP receptor type II
cdk	Cyclin-dependent kinase
DN	Dominant negative
DPC4	Deleted in pancreatic carcinoma locus 4
Dpp	Decapanteplegic
DVR	Dpp and Vg1 related TGFb superfamily
E	Embryonic day (embryonic day 0 = day of coitus)
EGF	Epidermal growth factor
ES cell	Embryonic stem cell
FAST-1	Forkhead activin signal transducer 1
FKBP12	FK506 binding protein, 12 kDa
FRAP	FKBP12-rapamycin-associated protein
FSH	Follicle-stimulating hormone
FYVE zinc finger domain	FYVE zinc finger domain **F**ab1, **Y**OTB/ZK632.12, **V**ac1 and EE1 Domain
GDF	Growth/differentiation factor
GDNF	Glial cell line-derived neurotrophic factor
GFRa	GDNF family receptor a
GPI	Glycosyl phosphatidylinositol
GS Domain	Glycine/Serine domain
GSK	Glycogen synthase kinase
HA	Hemaglutinin
HGF	Hepatocyte Growth Factor
INF-γ	Interferon-gamma
IL	Interleukin
JNK	Jun N-terminal Kinase
LAP	Latency associated peptide
LPM	Lateral plate mesoderm
LTBP	Latent TGFb binding protein
MAD	Mothers against Dpp
MAPK	Mitogen-activated protein kinase

MDF	Modifier derived factor
MDR	Modifier derived receptor
MEN	Multiple endocrine neoplasia
MH1/2	MAD homology 1 and 2
MIS	Mullerian inhibiting substance
Mix.2	Mixer 2
MSP	Maximal segment pair
NBRF	National Biomedical Research Foundation
NGF	Nerve growth factor
NTN	Neurturin
eop	One-eyed pinhead
P	Postnatal day (postnatal day 0 = day of birth)
P.A.G.E.	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PCR	Polymerase chain reaction
PDK1/2	3-Phosphoinositide-Dependent Protein Kinase1/2
PH	Pleckstrin homology domain
PI3K	Phosphinositol-3-kinase
PLC	Phospholipase C
PNS	Peripheral nervous system
PSP	Persephin
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and TENsin homolog
PTU	Propylthiuracil
RAFT	FKBP 12-rapamycin-associated protein
Rb	Retinoblastoma
Ret	Rearranged in transformation
RPA	Ribonuclease protection assay
RSTK	Receptor serine/threonine kinase
RTK	Receptor tyrosine kinase
rtTA	Reverse tetracycline-controlled transactivator
SARA	Smad Anchor for Receptor Activation
sax	Saxophone
SBE	Smad binding elements
SDS	Sodium dodecyl sulfate
sma	Small phenotype
Smad	Sma/MAD related
Smurf	Smad ubiquitination regulatory factor
TβRI	TGFβ receptor type I (ALK5)
TβRII	TGFβ receptor type I
TβRIII	TGFβ receptor type I (β-Glycans)
TGFβ	Transforming Growth Factor β
TGIF	Transforming Growth Factor β-Interacting Factor
TRE	Transforming Growth Factor β-Responsive Element
Vgr1	Vegetal receptor-1
Xnr	*Xenopus laevis* nodal-related

ABSTRACT

The aim of this work was to investigate the role of TGFβ family members in the induction of cell growth arrest and apoptosis in immune cell types. The TGFβ superfamily is a large group of evolutionary conserved polypeptide growth factors, involved in different physiological processes. Any deregulation of the different components of the TGFβ signaling pathway, has been largely implicated in multiple human critical disorders including cancer. Activin, originally isolated from gonadal fluid, and more recently described as an antiproliferative and proapoptotic factor in different cell types has been implicated in different immune functions. In particular, activin and TGFβ play an important role in the haematopoietic tissue. They are critical death inducers in the immune system contributing to the elimination of different activated immune cell types. Control of immune cell proliferation, activation and subsequent elimination of activated cell populations by cell growth arrest and apoptosis are critical events for controlling infections and preventing autoimmune disease. However, very limited information about the downstream target genes and their signaling mechanism that relay on the inhibitory effects on cell growth by activin and TGFβ ligands. Using a screen for genes that are differentially regulated by activin and TGFβ in haematopoietic cells, we found that the phospholipids phosphatase SHIP-1 was strongly upregulated by activin and TGFβ. Thus, we hypothesized that TGFβ and activin induce cell growth arrest in immune cells through up-regulation of SHIP-1 with a significant and subsequent decrease in PtdIns 3,4,5-P3 levels affecting cell survival. Furthermore, we attempted to characterize the different intracellular signalling pathways downstream of these serine/threonine kinase receptors

that lead to SHIP-1 overexpression as well as the transcription factors involved in the mediation of transcriptional regulation of the SHIP-1 gene promoter.

Chapter 1 provides a broad introduction to the field of TGFβ signaling focusing on TGFβ-induced apoptosis in immune cell types and the biology of inositol phosphatases involved in phospholipids metabolism, mainly focused on SHIP-1. Chapter 2 contains data demonstrating that the activin/TGFβ-induced cell growth arrests and apoptosis through expression of SHIP-1. Data in chapter 3 proved evidences about the cross-talk between the Smad and JNK MAP kinase signalling pathways and their role in the transcriptional regulation of the SHIP-1 gene promoter. Finally, chapter 4, is focused on the discussion and the propose model of how activin/TGFβ-induced SHIP-1 expression blocks induction of cell survival signals. As a dynamic cellular and molecular process the induction of SHIP-1 by TGFβ ligands might be in co-association with other apoptosis molecules leading to cell growth arrest and apoptosis in different cell populations of the immune system.

RESUMÉ

Le but de ce travail était d'étudier le rôle des membres de la famille des facteurs de croissance, TGFβ dans l'induction de l'arrêt de croissance des cellules et de l'apoptose dans les cellules immunitaires. La superfamille de TGFβ est un grand groupe de facteurs de croissance qui sont conservés durant l'évolution. Ils sont impliqués dans différents processus physiologiques. Une déréglementation des différents composants de la voie du signal de TGFβ, a été en grande partie impliquée dans plusieurs désordres important chez l'humain, comme le cancer. L'activin, originellement identifiée dans le fluide gonadal a, plus récemment été décrit comme facteur antiprolifératif et proapoptotique dans différentes types et fonctions des cellules immunitaires. En particulier, les membres de la famille TGFβ, jouent un rôle important au niveau du tissu hématopoïétique. Ils sont les inducteurs cruciaux de la mort cellulaire dans le système immunitaire, contribuant à l'élimination de différentes populations immunitaires des cellules activées. Le contrôle de la prolifération et de l'activation des cellules immunitaire l'élimination qui s'en suit par l'arrêt de la croissance cellulaire et l'apoptose, sont des événements critiques dans la curabilité des infections et la prévention des maladies auto-immunitaires. Cependant, l'information concernant les gènes impliqués et leurs mécanismes de signalisation est très limitée. Nous avons utilisé un criblage pour les gènes ayant une expression différencier par l'activin et TGFβ dans des cellules hématopoietiques. Nous avons constaté que les niveaux d'expression de la phosphatase des phospholipides, SHIP-1, étaient fortement augmentés par l'activin et le TGFβ. Ainsi, nous avons proposé que le TGFβ et l'activin induisent l'arrêt de croissance des cellules immunitaires par l'augmentation des niveaux

d'expression de la protéine SHIP-1. Ceci est suivi d'une diminution significative des niveaux de PtdIns 3,4,5-P3 qui ultimement affecterait la survie de cellules. En outre, nous avons essayé de caractériser les différentes voies de signalisation intracellulaire en aval de ces récepteurs à activité serine/thréonine-kinase qui mènent à l'augmentation de l'expression de SHIP-1 ainsi que les facteurs de transcription impliqués dans la régulation du contrôle transcriptionel du gène SHIP-1 dans les différents groupes de cellules immunitaires.

Le chapitre 1 fournit une introduction circonstanciée de la voie de signalisation du TGFβ, principalement se concentrant sur l'apoptose induite par TGFβ et l'activin et la biologie des phosphatases d'inositol impliquées dans le métabolisme des phospholipides, avec une emphase particulière sur SHIP-1.

Le chapitre 2 regroupe des données qui démontrent que l'arrêt de la croissance cellulaire et l'apoptose se fait par l'intermédiaire de l'augmentation de l'expression de SHIP-1 dans les cellules immunitaires.

Les résultats présentés au chapitre 3 démontrent la coopération entre les signalisation des Smads et JNK dans la régulation de la transcription du gène SHIP-1. Pour conclure, le chapitre 4 présente une discussion des résultats obtenus et propose un modèle expliquant comment l'induction de l'expression de la phosphatase SHIP-1 par l'activin et TGFβ bloque l'induction des signaux de survie dans differents groupes de cellules immunitaires. L'induction de SHIP-1 par les ligands de la famille de TGFb, pourrait exister en la association avec d'autres molécules d'apoptose et faire partie d'un processus cellulaire et moléculaire dynamique,

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. – General Introduction

Cellular communication is of central importance to prevent multicellular organisms from being a shapeless pile of individual cells. This communication is executed through an elaborate inter- and intracellular signaling network, which relays on the continuous information flow between different cell groups and their environment. Any disruption between these signaling mechanisms may affect essential biological processes for the organism, resulting ultimately in pathological conditions [1-7].

Cells are a simple but yet complex system that communicate through direct cell-cell interaction, cell contact with extracellular matrix components or responding to different signaling molecules such as neurotransmitters, growth factors, hormones and among others. The extracellular signal carried by these molecular mediators is directed transformed into an intracellular message by binding to cellular receptors, group of transmembranal proteins, predominantly located at the plasma membrane level, with certain degree of specificity inducing a significant change in either the activity or conformation of the receptor [8, 9]. The outcome of these molecular actions is the activation of downstream signaling cascades, which modulate differents intracellular functions [10-15].

Growth factors are highly conserved molecules, found in both invertebrate and vertebrate organisms. They control a wide range of physiological events during the lifetime of the organism. Among these events are: embryonic development, cell division regulation and proliferation, cell

migration, differentiation and cell death. The importance of these molecules is clearly illustrated through evolution by their conservation level [1, 16-22].

Cytokines are a group of inducible growth factors, central to the maintenance of cellular homeostasis. While cytokines affect the functions of different cell populations, they are of particular importance in modulating the immune system cells [23-25]. Cytokines regulate critical processes such as immune cell growth and activation, as well as the inflammatory response. regulation. One class of cytokines with a central role in immune function is the *Transforming Growth Factor beta* (TGFβ) superfamily [15, 26-32].

Growth Factors of the TGFβ family encompass a large group of multifunctional cytokines. The family is comprised of TGFβs, activins, bone morphogenic proteins (BMPs), and others. This large group of cytokines regulate cell growth, differentiation and apoptosis, in nearly all cell types where their receptors are expressed [33]. Due to their important role in the cell proliferation regulation, whether they are epithelial, endothelial or haematopoietic cells, disruption in the signaling of TGFβ superfamily members can contribute to carcinogenesis. In later cancer stages, the growth-inhibitory role of TGFβ is often replaced by a stimulatory role. By stimulating angiogenesis, the expression of matrix metalloproteinases, and by inhibiting the immune defence, this promotes metastasis [34, 35]. These observations highlight the dual role of TGFβ as both a tumor suppressor and a tumor-promoting agent, depending on the temporal and cellular context [36-38]. Given the critical role of the TGFβ superfamily of growth factors in the regulation of cell growth and apoptosis, we chose to explore the signal transduction mechanisms downstream of the activin/TGFβ receptors that regulate cell growth arrest/apoptosis in immune cells.

The aim of this investigation was to characterize the molecular mechanisms by which activin and TGFβ induce cell growth arrest and apoptosis in immune cells. The research

presented in this thesis has uncovered previously undescribed signaling pathways involved in its actions leading to cell growth arrest and apoptosis.

This thesis is divided in four chapters. I will first review the literature covering the different TGFβ superfamily growth factors and their biology. In Chapter 2, I will demonstrate that the TGFβ family members regulate apoptosis in haematopoietic cells through the expression of the inositol phosphatase SHIP-1, which is a central regulator of a phospholipid metabolism. In addition, I will demonstrate the requirement of the Smad pathway in the transcriptional regulation of the SHIP-1 gene promoter. In Chapter 3, I explore the transcriptional regulation of the SHIP-1 gene promoter more detailed. This section illustrates that not only the Smad pathway, but also the stress JNK MAP kinase pathway, is involved at the transcriptional level. The findings presented in Chapters 2 and 3 are unified during the last chapter, which consists of a general discussion. Our results link for the first time phospholipid metabolism to activin/TGFβ-mediated apoptosis. Our results define and suggest that the TGFβ ligands as potent inducers of the SHIP-1 expression in immune cell types.

1.2. General Features

The TGFβ related growth factors belong to a large family of cytokines that have been implicated with a multitude of cellular responses, ranging from development to cell-cycle regulation and induction of apoptosis [19, 22, 26, 39-43]. Studies in the last fifteen years have identified homologous TGFβ family members in humans and all animal organisms explored [44-46]. The complex and, at times, paradoxical behaviour of the TGFβ family members in promoting or inhibiting cell function, is critical to normal physiology and homeostasis. This makes the TGFβ ligands family, a particularly intriguing group of molecules to study. Much information has been gained from the study of transgenic mouse models displaying targeted

disruptions at different levels of the TGFβ expression and signaling. The essential functions and biology of the TGFβ family members elucidated by these models are detailed in Table 1.I. (pages 16-17).

TGFβ ligands are dimeric molecules composed of two subunits linked by a disulfide bridge. They bind to a specific group of transmembranal serine/threonine receptor kinases known as TGF-β Receptor (TβR) type I, type II and type III. The signaling pathways activated by these growth factors modulate the expression of different target genes, either through transcriptional activation or repression [47-49]. Their effects are mediated by a group of intracellular signaling molecules called Smads. These mediators are divided in three categories: Receptor activated Smads (R-Smads), Common mediator Smad (Co-Smad) and the Inhibitory Smads (I-Smads) [15, 26, 32, 43, 50-55]. Of the immediate responses triggered by TGFβ ligands in a given cell, over half result in activation and the rest result in the repression of target genes.

The TGFβ superfamily of growth factors and cytokines was first recognized in the late 1980s [29, 56-59]. Since then, from *Drosophilae melanogaster* to *Homo sapiens*, more than 50 family members have been identified, constituting the largest family of cytokines ever described [60-65]. A phylogenetic tree of representative members of the TGFβ superfamily is presented in Figure 1.1 (pages 6-7). Furthermore, the TGFβ superfamily members and the different components of their signaling pathways have been highly conserved through out evolution. [63, 65-69]. Different types of evidence have demonstrated that close related family members have the ability to perform the functions of their homologues when expressed in distant species, further supporting their high conservation level [70, 71]. The intracellular TGFβ signaling pathways are also highly conserved. Figure 1.2 summarizes the different general steps that are involved in TGFβ signaling (pages 8-9).

In summary, the various biological functions of members of the TGFβ superfamily are illustrated in Figure 1.3 (pages 10-11). These functions highly depend on the cellular and temporal context. These include regulation of early embryonic development [72], cell-cycle control [73] and arrest, cell proliferation [74-76] and differentiation [77], extracellular matrix formation [78-81], angiogenesis [82], and immune functions such as haematopoiesis [83], T/B cell differentiation [84], inflammation and wound healing among others [34, 85-89]. Given these many essential functions, any disruption in the TGFβ signaling or loss responsiveness to TGFβ can result in pathological conditions, in particular malignant diseases [88, 90-92]. Figure 1.3, illustrates the actions of activin and TGFβ in different targeted cells (pages 10-11).

Figure 1.1. Transforming Growth Factor β superfamily. A schematic representation of a dendrogram that shows the evolutionary relationship among the different related members of the TGFβ superfamily [93]. Relationships have been based upon amino acid sequence (not shown) particularly in the ligand regions.

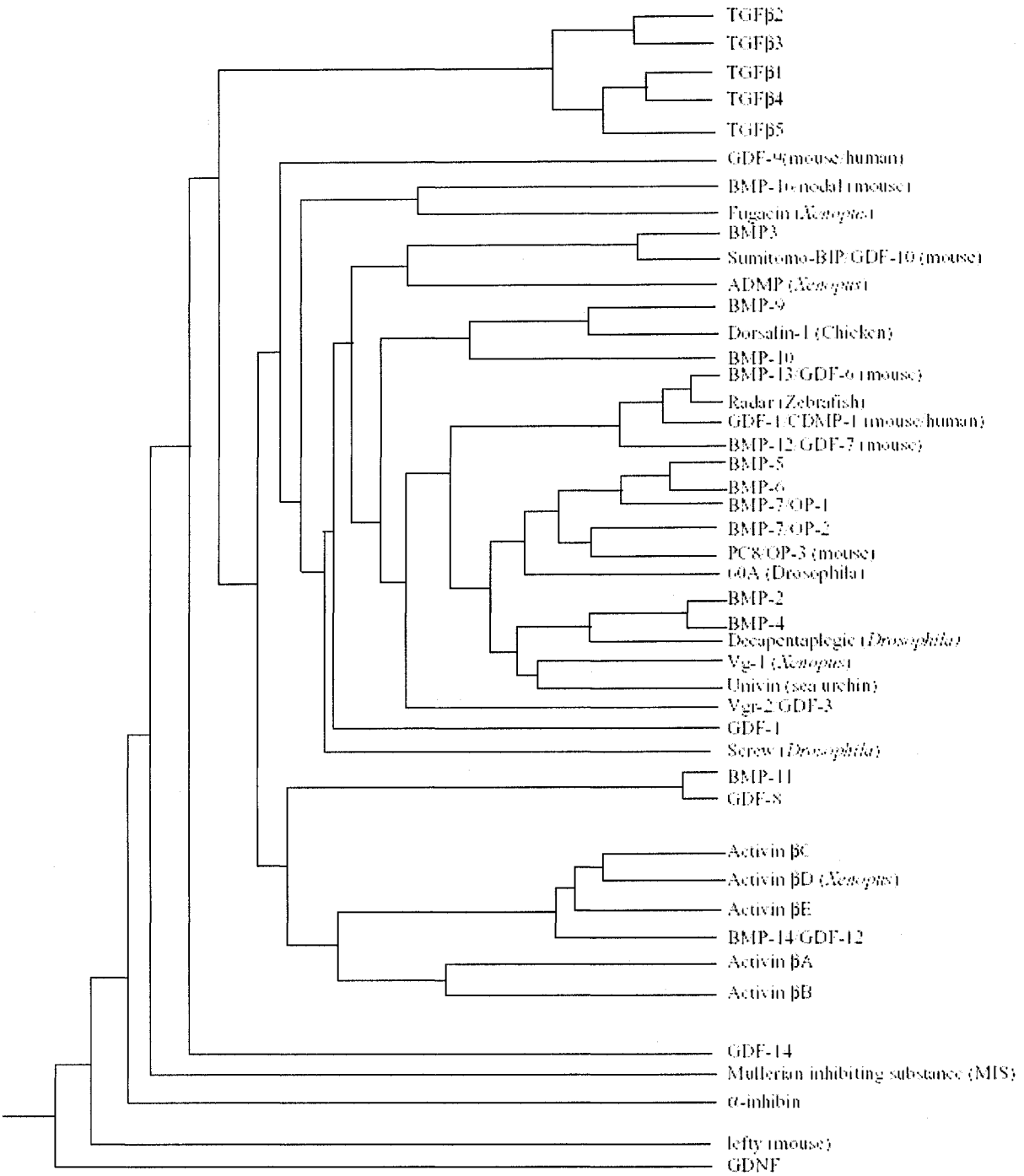

TGFβ2
TGFβ3
TGFβ1
TGFβ4
TGFβ5
GDF-9(mouse/human)
BMP-16(nodal)(mouse)
Fugacin (*Xenopus*)
BMP3
Sumitomo-BIP/GDF-10 (mouse)
ADMP (*Xenopus*)
BMP-9
Dorsalin-1 (Chicken)
BMP-10
BMP-13/GDF-6 (mouse)
Radar (Zebrafish)
GDF-1/CDMP-1 (mouse/human)
BMP-12/GDF-7 (mouse)
BMP-5
BMP-6
BMP-7/OP-1
BMP-7/OP-2
PC8/OP-3 (mouse)
60A (Drosophila)
BMP-2
BMP-4
Decapentaplegic (*Drosophila*)
Vg-1 (*Xenopus*)
Univin (sea urchin)
Vgr-2/GDF-3
GDF-1
Screw (*Drosophila*)
BMP-11
GDF-8
Activin βC
Activin βD (*Xenopus*)
Activin βE
BMP-14/GDF-12
Activin βA
Activin βB
GDF-14
Mullerian inhibiting substance (MIS)
α-inhibin
lefty (mouse)
GDNF

Figure 1.1

Figure 1.2. The TGFβ signaling pathway. Schematic representation of the general steps involved in TGFβ signaling in cellular systems. In the TGFβ signaling machinery, inductive signals may be either TGFβ or non-TGFβ molecules that can initiate the TGFβ production, which is subjected to different post-translational modification and protein maturation. Then the mature and active TGFβ ligands exert their biological effects by binding themselves to their respective expressed-receptors in different cellular systems.

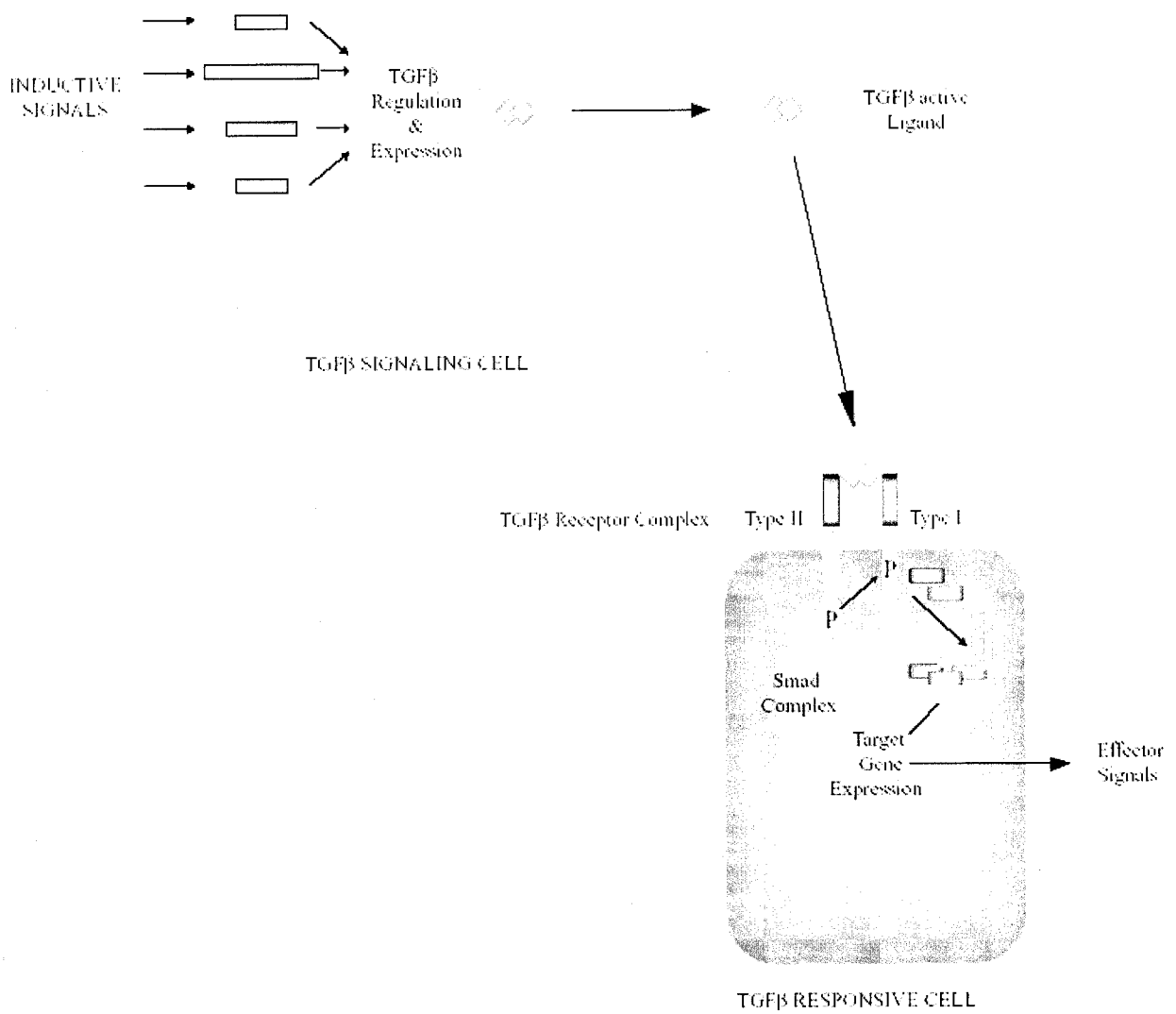

INDUCTIVE SIGNALS
TGFβ Regulation & Expression
TGFβ active Ligand
TGFβ SIGNALING CELL
TGFβ Receptor Complex
Type II
Type I
P
P
Smad Complex
Target Gene Expression
Effector Signals
TGFβ RESPONSIVE CELL

Figure 1.2.

Figure 1.3. Effects of the TGFβ and activin in different targeted tissues and cellular types. Schematic representation of the different TGFβ and activin effects in different biological systems. The roles of these growth factors have been implicated in different biological and physiological processes, as illustrated.

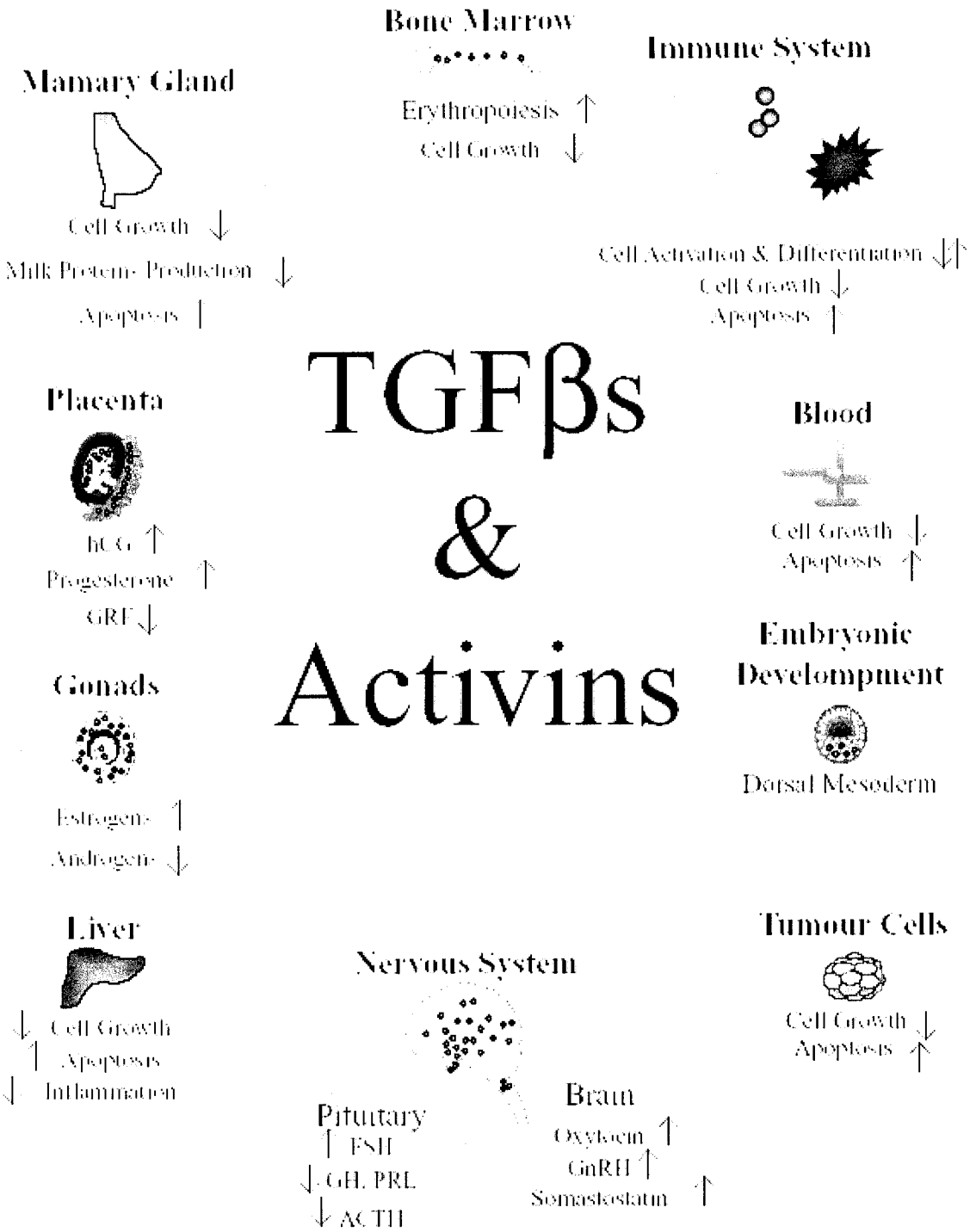

Bone Marrow
Erythropoiesis ↑
Cell Growth ↓
Immune System
Mamary Gland
Cell Growth ↓
Milk Protein Production ↓
Apoptosis ↑
Cell Activation & Differentiation ↓↑
Cell Growth ↓
Apoptosis ↑
TGFβs
&
Activins
Placenta
hCG ↑
Progesterone ↑
GRF ↓
Blood
Cell Growth ↓
Apoptosis ↑
Embryonic Development
Dorsal Mesoderm
Gonads
Estrogens ↑
Androgens ↓
Liver
↓ Cell Growth
↑ Apoptosis
↓ Inflammation
Nervous System
Pituitary
↑ FSH
↓ GH, PRL
↓ ACTH
Brain
Oxytocin ↑
GnRH ↑
Somatostatin ↑
Tumour Cells
Cell Growth ↓
Apoptosis ↑

Figure 1.3.

This group of growth factors has been related to oncogenesis for many years [42]. Originally, several groups were named TGFβ in adifferent way. For example, TGFβ1 was also considered as the Cartilage Inducing Factor [94], glioblastoma immunosuppressive factor [95, 96], myoblast differentiation inhibitor factor [97, 98] and epithelial growth inhibitor [99]. All these observations demonstrated that the different molecules that were measured, end up being the TGFβ protein [73, 76, 100-102].

The multifunctional effects of TGFβ endow it as both a tumor suppressor and tumor promoting activity, depending on the stage of carcinogenesis and the responsiveness of the tumor cell. TGFβ ligands have the ability to inhibit epithelial cell growth through the induction of apoptosis and cell cycle arrest, thus contributing to a tumor suppression during the early stages of carcinogenesis [103-107]. However, later in their development, many tumor cells lose their responsiveness to the growth inhibitory actions of the TGFβ [69]. The loss of the TGFβ-growth inhibition is associated with increased invasiveness and metastasis of tumor cells [108-114]. In addition, cancer cells have developed numerous molecular mechanisms for escaping the host's immune response, either by successfully evading a functional immune system or by suppressing the immune attack. Recent evidence suggest that the TGFβ family members are key cytokines used to escape the host's immune response [115, 116]. As mentioned before, the TGFβ superfamily consists of several structurally related cytokines including the TGFβs, activins and bone morphogenic proteins (BMPs). I will now discuss these different family members in detail.

1.3. The TGFβ Superfamily

1.3.a. TGFβ Subfamily

TGFβ is the prototype and founding member of one of the largest families of structurally related growth factors and polypeptides ever reported [58]. The TGFβ subfamily is composed of five isoforms. Three of them are found in mammals: TGFβ1 [32], TGFβ2 -also known as *polyergin* [117-120], and TGFβ3 [121-125]. In addition, the chicken TGFβ4 [126] and *Xenopus laevis* (TGFβ5), both homologues of TGFβ1, are included in this family. All TGFβ isoforms are encoded by distinct genes, located on different chromosomes [127-130].

TGFβ ligands can form homo- and hetorodimers. These heterodimeric associations can occur between TGFβ1 and TGFβ2 and between TGFβ2 and TGFβ3 [131, 132] . The dimers are linked through disulfide bond and have a molecular mass of approximately 25000 Da (25 kDa). Based on their amino acid sequence, TGFβ ligands have been found to be highly conserved through evolution, and have pleotropic effects. Their effect on different cell types ultimately depends on the molecular and temporal context in which they act [26, 133, 134]. The various TGFβ isoforms have been shown to bind to the same group of TGFβ receptors type II *in vitro*, and have similar actions. However, the different TGFβ ligands present distinct expression levels *in vivo*. Knockout models featuring selective ablation of TGFβ1, TGFβ2 or TGFβ3 support these findings. While there is an overlap between the phenotypes of the various knockouts, subtle differences indicate many specific functions of the TGFβ1, 2 and 3 [64, 135, 136]. The TGFβ1 and TGFβ2 appear to have similar functions. Yet it has been suggested that the TGFβ1 plays a more important role in certain immune functions, while the TGFβ2 regulates mesodermal induction [137, 138]. Transcriptional analysis of the different TGFβ ligands indicates, that each

TGFβ isoforms is regulated in a independent manner [139]. Table 1.I (page 14-17) summarizes the different murine knockout phenotypes of the different components involved in the TGFβ signaling pathways.

In addition, TGFβ ligands have a central role in the inhibition of cell cycle progression in epithelial and haematopoietic cell populations. They also control the proliferation and differentiation of mesenchymal cells; TGFβ ligands are considered to be strong inducers of extracellular matrix deposition. This feature makes them critical players in wound healing, beyond their contribution to control the injury immune response [64, 88, 140]. The action sites of the various TGFβ isoforms are illustrated in Figure 1.3 (pages10-11).

1.3.b. Inhibin and Activin Subfamily

Inhibins and activins are members of the TGFβ superfamily of growth factors. These growth factors, were initially identified as important growth factors because of their role in the regulation of the anterior pituitary gland [141, 142]. Inhibins were isolated in 1985 from bovine and porcine follicular fluid [143-146]. These hormones were found to be produced in two forms through dimeric association linked by a disulfide bridge between an α subunit of 18kDa and one of two related β subunits, βA and βB, with an approximately 14kDa molecular weight. Inhibin A is composed by an α-βA and inhibin B of α and βB subunits [143, 145, 147, 148]. Inhibins have the ability to suppress the synthesis and secretion of the FSH [145, 147].

Activins are related to inhibins. They form dimers composed of two β subunits [146, 149, 150]. Activins were first discovered and characterized for their ability to regulate the production of FSH from anterior pituitary cells. They were originally isolated from gonadal fluid in 1986 [151, 152]. Two groups of activins were identified: either homo- or heterodimers that include an

inhibin β subunit [145, 153]. Until now, five β subunits have been reported: βA, βB, βC, βE and βD, but only a single α subunit has been identified [146, 149, 154]. The activins isoforms represent an array of possible dimers: activin A (βaβa), activin B (βbβb) and activin AB (βaβb) [155]. The three remaining activin-related β subunits βC and βE in mammals and βD in *Xenopus laevis*, do not appear to play an important role in FSH regulation [149, 156-162].

Activins are not only important for different reproductive functions [163-167], but are also involved in the early stages of development, where they promote mesodermal induction and erythroid cell differentiation as documented in *Xenopus sp.* [141, 142, 152, 168-174]. In addition, activins are important negative regulators of cell growth. The antiproliferative and the more recently discovered proapoptotic, effects of these growth factors have been reported in many different tissues and cell types such as erythroleukemia cells [175, 176], capillary endothelial cells [177, 178], hepatocytes [179, 180] and breast cancer cells [181], as well as in various immune cell types [182-184] such as plasmocytoma cells [84]. Interestingly, mutations in the different components of the activin signaling pathway have also been found in different types of cancer. These observation are consistent with the critical role of TGFβ in cell growth regulation in cancer development [185-195].

Table 1.I. TGFβ and related family members knockout (-/-) phenotypes.

GENE/Ligand	Phenotype	Ref.
TGF β1	Present defective yolk sac hematopoiesis and severe vascular endothelial differentiation. Lethal phenotype at E10.5 or around P21. Dual phenotype that survives by maternal TGFβ1 transference or rescued by TGFβ administration.Born normal but dies at P20-28 from an excessive multifocal inflammatory disease assicauted with tissue necrosis.	50 440 441
TGF β2	Perinatal death. Presents multiple several defects in different anatomical structures such as: heart, lung, craniofacial, limb, spinal column, eye, inner ear, urogenital system.	51
TGF β3	Lethal phenotype, which dies before P1 due to lung development delayed and with defective palatogenesis. Mice do not present craniofacial defects.	52 53
Activin βA	Lethatl phenotype, which dies before P1. Lack of whiskers and lower incisors related with significant craniofacial defects including cleft palate. The newborns are not capable to suckle.	76
Activin Bb	Newborns with severe failure of eyelid fusion and profoundly impaired reproductive ability of the females with perinatal lethality incidence. Completely viable and fertile; incompletely penetrance eyelid fusion defects.	77 78
Activin βA/βB	The combined phenotype of the single mutants.	76
Type I receptors	Phenotype	Ref.
T βRI	They die at midgestation stage of severe defects in vascular development of the yolk sac and placenta.	26
ActRIA	They present a significant arrest at early gastrulation. Severe abnormal visceral endoderm associated with a critical and severe disruption of mesoderm formation.	126
ActRIB	During development there is a severe disruption of epiblast and extraembryonic ectoderm. The embryos show growth arrest at egg cylinder stage, before going to gastrulation.	127
ALK1	Organisms die at midgestation of severe vascular abnormalities. Formation of cavernous vessels associated with hyperdilation of large vessels. This observed phenotype is due to a deficient differentiation and recruitment of vascular smooth muscle cells.	123
Type II Receptors	Phenotype	Ref.
T βRII	Embryonically lethal around E10.5. Lethal phenotype due to defects in yolk sac hematopoiesis and vasculogenesis processes.	25
ActRIIA	Approximatelly 22% present defect at the skeletal and facial levels such as cleft palate, eyelid closure defects. The organisms dies shortly after birth; the remaining organisms show no phenotype except a suppressed FSH secretion and reduced fertility.	198
ActRIIB	Multiple defects related to left-right axis formation. Similar defect on the anteroposterior patterning observing randomized heart position, malposition of the great arteries, ventricular and atrial septal defects, right pulmonary isomerism, splenic abnormalities, and vertebral pattern anomalies. Perinatally lethal a few days after birth.	199
ActRIIA/B	Embryonically lethal at E7.5. Gastrulation failure and primitive streak formation is observed at the same embryonic time (E7.5).	175

Table 1.I. (Cont...) TGFβ and related family members knockout (-/-) phenotypes.

Type III Receptors.	Phenotype	Ref.
Endoglin	The phenotype observed is embryonic lethal at 10.0-11.5. The embryos present an impaired vascular development and maturation, and cardiac defects.	124, 125, 201
Smads	Phenotype	Ref.
Smad1	Embryonically lethal at E9.5. Fail to establish chorion-allantoic circulation.	165
Smad2	Embryonically lethal at E7.5-8.5. Do not form an egg cylinder or a primitive streak. Lack mesoderm and fail to gastrulate.	166, 167, 168
Smad3	They are viable and fertile. Develop severe metastatic colorectal adenocarcinomas at 4 to 6 months of age. Die between 1 and 8 months due to a defective immune function: thymic involution, enlarged lymph nodes, and formation of bacterial abscesses adjacent to mucosal surfaces. Small but viable; early forelimb malformation; survive until adulthood. Accelerated wound healing: increased rate of re-epithelialization and reduced local infiltration of monocytes.	169, 170, 171, 172
Smad4	The organisms are embryonic lethal at E6.5-8.5.They fail to gastrulate or form mesoderm related with severe abnormal visceral development.	173, 174
Smad5	They die at E10.5-11.5 due to defects in angiogenesis with massive apoptosis of mesenchymal cells. Lethal phenotype. Die at E9.5-11.5; defects in yolk sac vasculature, gut, heart, neural tube, left-right asymmetry. They present severe defects in left-right asymmetry; low *lefty1* expression; symmetrical *nodal*, *lefty2* and *Pitx2* expression.	176, 177, 178
Smad6	This phenotype presents multiple cardiovascular abnormalities including hyperplasia of cardiac valves and aortic ossification.	153

1.3.c. BMPs Subfamily

The bone morphogenic proteins (BMPs) constitute the largest subfamily within the TGFβ superfamily with over 20 members (Figure 1.2, page 7). Initially the first BMPs were isolated from bone extracts. These new molecules had a strong bone and cartilage formation inducers [196]. Since their characterization, BMPs have been widely implicated in several early developmental events, which are constantly maintained as later organogenesis occurs in both invertebrates and vertebrates [197, 198]. Mutation analysis performed on *Drosophila sp.* have shown various BMPs phenotypes. For example, the ablation of the decaplentaplegic (dpp) gene shows a particular phenotype which presents a set of various deficiencies and duplications of different structures of the fly [199]. Furthermore, two mammalian homologues of dpp, BMP2 and BMP4, have been identified as inducing factors that are responsible for the ectopic cartilage formation [200]. BMPs play an important role as a critical factors involved during different developmental stages in *Xenopus sp*, [201]. During these embryonic events, BMPs affect the patterning of the mesoderm, dorsalization and the formation of eyes and wings in *Drosophila sp.*, development [174, 200, 202, 203]. Within the BMPs subfamily the BMP5 subgroup has important physiological roles. In this family, the BMP5, BMP6/Vgr1, BMP7/OP1, and BMP8/OP2 are included members were all identified as bone-inducing proteins [204-207]. Interestingly, in association with the BMP2 and BMP4, this subfamily has been shown to be involved in the development of almost every anatomical structure and they have important roles in neuronal development [203, 208]. In addition, two other related members have been identified in a search for mammalian homologues for Vgr1, which was previously discover in *Xenopus sp.*, oocytes [209] and BMP6/Vgr1 [210]. Whatron and colleagues identified the 60A family member in the *Drosophila sp*, during the searching for homologues of the TGFβ [211].

1.3.d. GDNF subfamily

The Glial cell-Derived Neurotrophic Factor (GDNF) subfamily encompasses GDNF, neurturin [212], artemin (ART), and persephin (PSP) factors [213-223]. Originally GDNF was isolated as a factor able to induce survival in midbrain dopaminergic neurons [221]. GDNF promotes the survival of other peripheral and central neuronal types but also affects kidney development [224].

Based on these features, it has been proposed that this ligand may be a promising candidate to be used as a treatment for different nervous diseases [225, 226]. The *GDNF -/-* mice phenotype is embryonically lethal. These mice die at early stages due to severe developmental defects wich affecting the formation of that neural structure, kidneys and stomach [226]. The severe lethality of this phenotype does not permit the evaluation of its role in neuronal and organelle development. Nevertheless, the generation of conditional knockout mice for GNDF has helped to resolve this problem as well as to study the actions of this neuronal factor [227, 228].

Interestingly, it has been reported that GDNF ligands, signal through a completely different receptor system. This signaling pathway is mediated via the Ret tyrosine kinase receptor which is a completely different signaling receptor complex than the rest of the other TGFβ ligands superfamily [106, 229]. Briefly, the GDNF signaling pathways are activated by the binding of a GDNF dimer to a multicomponent receptor complex, which is composed of a pair of two subunits of glycosylphosphatidylinositol-linked ligand-binding protein named GFRα1, which induces the receptor dimerization with two subunits of Ret. Following this, the two Ret molecules are transphosphorylated on specific tyrosine residues in their tyrosine kinase (TK) domains. This phosphorylation event leads to the activation of intracellular downstream signaling pathways. Interestingly, it has been reported that the GDNF can signal in a Ret-independent

manner [221]. In this scenario, a GDNF/GFRα1 dimer-receptor complex is able to interact with the neural cell adhesion molecule NCAM, inducing GDNF signaling in the absence of Ret tyrosine receptor, which induces the activation of the Src-like kinase, Fyn, targeting downstream phosphorylation events of different intracellular targets [230, 231].

1.3.e. Other divergent members

1.3.e.*i*. Mullerian Inhibiting Substance (MIS)

Another important group included in the TGFβ superfamily is the Müllerian inhibiting substance (MIS, or anti-Müllerian hormone, AMH). This subgroup is composed of the MIS and the lefty1 and 2 [232]. It has been shown that the Sertoli cells of the testis synthesize MIS, inducing the regression of the Müllerian duct in male embryos during embryogenesis. These observations are supported by the *MIS*$^{-/-}$ male mouse model (or male MIS receptor deficient mice, *AMHR*$^{-/-}$), which results in internal pseudohermaphroditism with uterine and ovarian tissue bedding [233, 234]. Inactivating mutations of the MIS (or AMHR) have been described in humans, which prove the Müllerian duct syndrome (PMDS) in humans [233, 234].

1.3.e.*ii*. NODAL

Within the TGFβ superfamily, the Nodal has been considered as an intermediate member involved in the axial mesoderm induction as well as in the left-right asymmetry during development [208, 235, 236]. In similar context, the Dorsalin and GDF8 are considered intermediate family members as well. It has been shown that these two family members are important regulators for cell differentiation of the neural structure such as the neural tube, as well as in the inhibition of skeletal muscle growth respectively [231, 237-239].

1.4. TGFβ Signaling Pathway

The TGFβ signaling pathway can be divided into four general regulatory steps. First, an active TGFβ ligand is generated after the transcription, translation and secretion from a signaling cell. The ligand then traverses through the intercellular space where it can either be stored, associated to various extracellular matrix proteins, or it can interact with a complex of receptors on the surface of target cells. Upon association of ligand with this receptor complex, specific intracellular messengers known as Smads are activated. Finally, the activated Smad complex translocates to the nucleus, where it associates with different binding partners, regulating either positively or negatively the expression of specific target genes (Figure 1.4 pages 22-23). Similar to other signal transduction pathways, each step of the TGFβ signaling pathway is under strict regulation [26, 55, 69, 240].

I.4.a. TGFβ Serine/Threonine Kinase Receptors

I.4.a.*i.* Type I and Type II TGFβ Receptors

TGFβ and activin interact with a receptor complex formed of two different receptors, Type I and Type II. These two serine/threonine receptors consist of approximately 500-aminoacid sequences. They contain an extracellular domain, a single transmembranal region and a large intracellular domain, which includes the serine/threonine kinase domain.

Upon ligand binding to the type II receptor, the type I receptor is recruited into the complex [241-243]. Under basal conditions, type II receptors for TGFβ and activin are phosphorylated on serine and threonine residues but type I receptors are not [244-249]. Following ligand-induced receptor heterodimerization, the type I receptor is rapidly transphosphorylated by the kinase domain of the Type II receptor [245, 250-252].

Figure 1.4. TGFβ signaling pathway. Members of the TGFβ ligands associate with a specific group of serine/threonine kinase receptor that subsequently recruit and activated the downstream molecule known as Smads. These molecules are the canonical signaling molecules, which modulate at the transcriptional level, according to the TGFβ ligands reponse. Once in the nucleus the regulation

of different regulated target genes takes place with the specific associated co-repressors or co-activators in different cell types [253, 254].

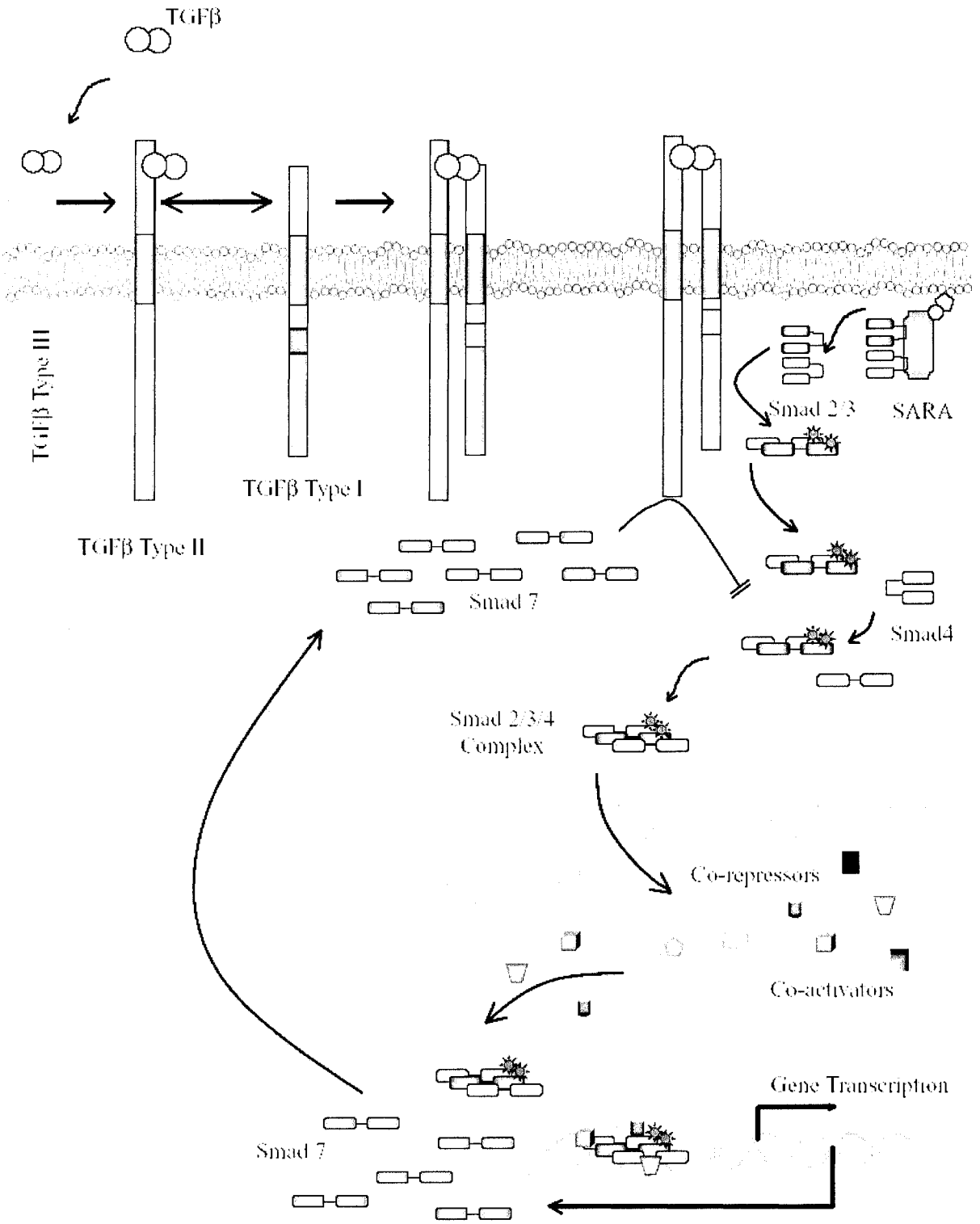

TGFβ
TGFβ Type III
TGFβ Type I
TGFβ Type II
Smad 2/3
SARA
Smad 7
Smad4
Smad 2/3/4
Complex
Co-repressors
Co-activators
Gene Transcription
Smad 7

Figure 1.4.

Phosphorylation of the type I receptor occurs in the juxtamembrane domain of the receptor, a region rich in glycine and serine residues known as the GS box [245]. This domain has been found to be highly conserved among all type I receptors of the superfamily. Ligand-induced phosphorylation of the serine and threonine residues in the GS box is required for a full activation of signaling by the TGFβ and activin type I receptors [245, 255, 256].

Within the GS box of type I TGFβ receptor, the penultimate residue at the boundary of the kinase domain is always a threonine or glutamine residue. Mutation of these residues to aspartate or glutamate endows the receptor with elevated kinase activity *in vitro*, and constitutive signaling activity in the cell. Mutation of the threonine residue to aspartate, known as TΔD, generates a constitutively active receptor fully capable of transmitting a signal in the absence of a ligand or type II receptor activation [250, 256]. Figure 1.5 illustrates the main regions of the two types of TGFβ receptors (pages 26-27). The top figure indicates the three main major regions of the TGFβ type II and type I receptors. The bottom figure illustrates in a more detailed way the different regions and domains of the TGFβ receptors.

I.4.a.*ii*. Type III TGFβ Receptors

An additional class of receptors, known as type III receptors, was discovered using ligand cross-linking experiments. This group of receptors consists of two related proteins, beta-glycan (β-glycan) and endoglin [257, 258]. It has been observed that these receptors lack of intrinsic signaling activity: therefore, it has been suggested that they regulate and stabilize TGFβ access to the signaling receptors. In addition, β-glycans have been reported to bind to all three TGFβ ligands with high affinity [259, 260], and to facilitate ligand binding to type II receptors [51, 258]. In contrast, endoglin has been shown to bind to TGFβ1 and TGFβ3, but not to TGFβ2 [261].

Figure 1.5. TGFβ Receptors. A schematic representation of the different regions of the TGFβ type II and type I receptors. The top figure illustrates the three main regions contained in the two types of receptors. The bottom figure illustrates in more detailed way the different domains and regulatory regions that determine the regulation of the receptor at the ligand binding and kinase activity respectively.

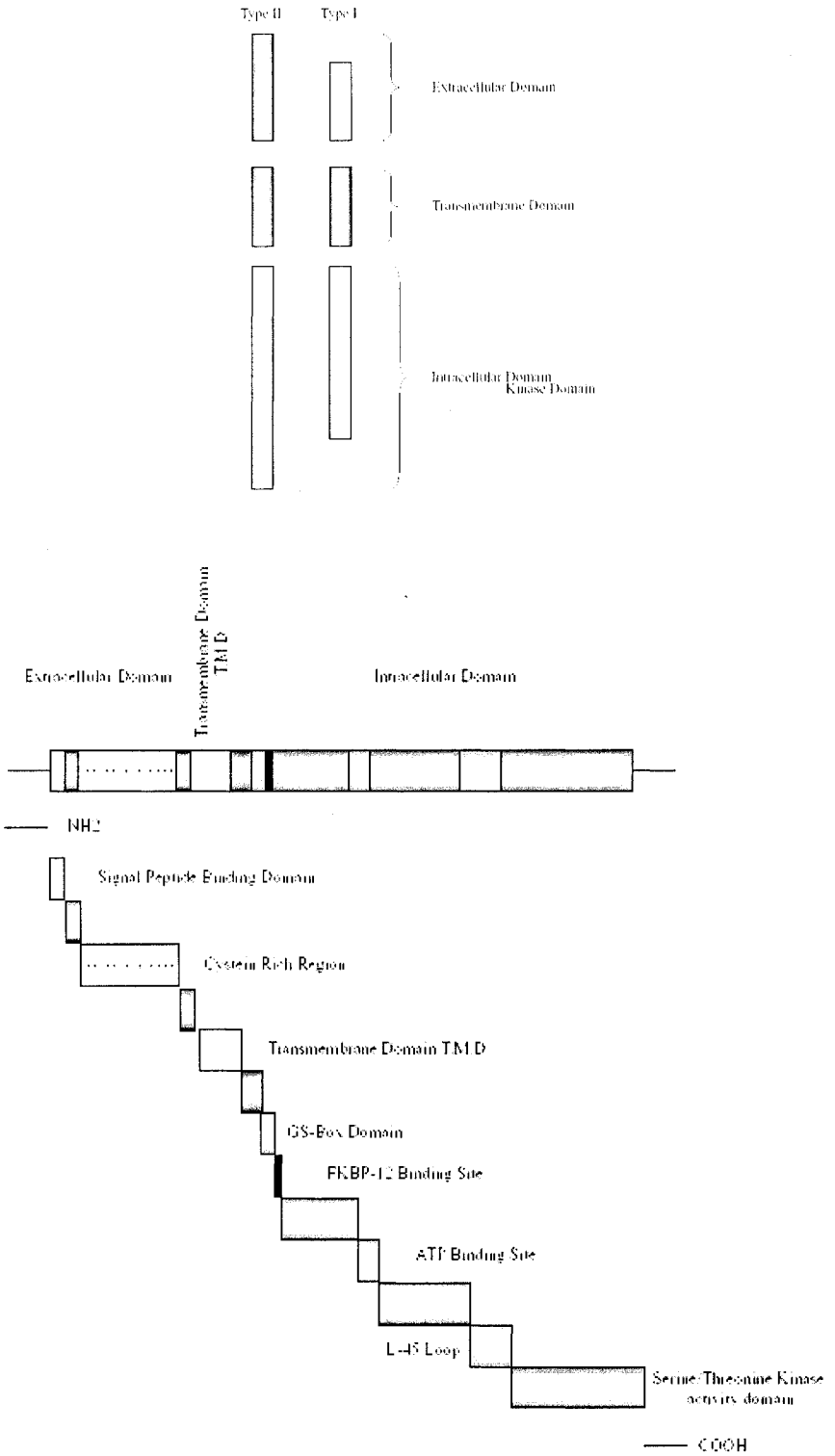

Type II
Type I
Extracellular Domain
Transmembrane Domain
Intracellular Domain
Kinase Domain
Transmembrane Domain
TM D
Extracellular Domain
Intracellular Domain
NH2
Signal Peptide Binding Domain
Cystein Rich Region
Transmembrane Domain TM D
GS-Box Domain
FKBP-12 Binding Site
ATP Binding Site
L-45 Loop
Serine/Threonine Kinase
COOH

Figure 1.5.

1.5. TGFβ Intracellular Signaling Mediators

Upon ligand binding to the constitutively phosphorylated Type II receptor, both TGFb and activin initiate their singaling pathway. The type II receptor then recruits the type I receptor, and through phophorylation in the GS domain, induces activation of the type I receptor kinase. The activated receptor complex then recruits the Smad proteins, the canonical downstream signaling molecules for these serine/threonine kinase receptors [58, 63, 65-67, 69, 262-264].

I.5.a. Smads

I.5.a.*i.* Classification of Smads

Based on their different functions, Smads are divided into three families: (1) the receptor-regulated Smads (R-Smads), which are found to be the direct substrates of the TGFβ type I receptors, (2) the common-partner Smad (Co-Smad) that heterodimerizes with the R-Smads upon ligand stimulation, and [265] the inhibitory Smads (I-Smads). The last ones are responsible for inhibiting signaling by the other two groups of Smads [15, 62].

The R-Smads Smad2 and Smad3 mediate the signaling from the TGFβ and activin ligands through the TβR-I/Alk5 and ActR-IB receptors, respectively [266-268]. The BMP signaling is mediated through the R-Smads 1, 5 and 8 which become phosphorylated and activated by the ActR-I, BMPR-IA or BMPR-IB receptors [269]. The Co-Smad, Smad4 (also known as DPC4, deleted in pancreatic carcinomas), appears to be the critical player on both BMP- and TGFβ/activin-mediated pathways. The affinity of the Smad4 for the different R-Smad molecules is significantly increased through phosphorylation of the C-terminal part of R-Smads leading to the formation of a complex [270, 271].

The third class of Smads, I-Smads, includes members such as the Daughters against dpp (Dad) in *Drosophila* [272] and Smad6 and Smad7 in vertebrates [273, 274]. The I-Smads are characterized as inhibitors of the TGFβ/activin and signaling pathways. It has been shown that the TGFβ, activin and BMP ligands directly induce the expression of the I-Smads. Based on this last feature, the I-Smads have been proposed to function as negative feedback loops, thus limiting the signal transduction pathway induced by TGFβ ligands [275].

Different studies have described the mechanism by which the I-Smad blocks vary the TGFβ's signaling pathways [273, 276, 277]. Briefly, I-Smads are capable of interacting stably with the type I receptor, thus competing with R-Smads and blocking their further activation [273, 276, 277]. Interestingly, an alternative mechanism of inhibitory actions for the Smad6 has been described. In this scenario, the Smad6 seems to compete with the Smad4 for binding to the Smad1, thereby preventing the formation of a functional heteromeric Smad1/Smad4 complex [274]. On the other hand, the Smad7 is widely considered as the general inhibitor for the TGFβ superfamily-induced response whereas the Smad6 is a blocker of the BMP-mediated signaling; [278] although to date this conclusion remains controversial [276].

I.5.a.*ii.* Identification of Smads

Smads were initially identified when screening for second-site mutations which enhanced the severity of mutations in the TGFβ family members. The first Smad to be characterized was the *Mothers against dpp* (Mad) in *Drosophila*. Mad mutations were identified by their ability to act as dominant maternal enhancers of dpp mutant phenotypes [279, 280]. Subsequently the sma-2, sma-3 and sma-4 were identified in the *C. elegans* based on their ability to replicate some daf-4 (TGFβ Type II receptor) mutant phenotypes [281]. Subsequent, sequence analysis of the *sma* genes with Mad shows extensive amino acid conservation. The conserved sequences were used

to identify similar proteins in vertebrates. The vertebrate homologues of the *mad*- and *sma*-genes were dubbed *smads* [279-283].

I.5.a.*iii*. Structure of Smads

Structurally, the Smad proteins present three important domains. The N-terminal and C-terminal regions of the R-Smads and the Co-Smads display a great deal of homology and have been designated as the Mad homology 1 (MH1) and Mad homology 2 (MH2) domains, respectively. In addition, a middle proline-rich linker domain exists between the MH1 and MH2 domain, known as a linker domain [53, 55, 284].

The MH1 domain is known to bind DNA. It is also involved in determining the association with different transcription factors. On the other hand, the MH2 domain participates in protein-protein interactions and houses the well-characterized SSXS phosphorylation site [285, 286]. The R-Smads, but not the Co- or I-Smads, contain a C-terminal SSXS motif which is phosphorylated upon the receptor's interaction [287]. The MH2 domain is involved in establishing both receptor-Smad and Smad-Smad interactions [288]. In addition, structural analysis has shown that the specificity between the R-Smads and their interaction with either TGFβ/activin or BMP receptors, is determined by the L3 loop region within the MH2 domain [289-292]. Figure 1.6 illustrates the general molecular structure of Smads (pages 31-32).

Figure 1.6. General Structure of the Smads. This model shows the different key location, structures and functions associated with the different regions of the R-Smads. R-Smads are composed of three regions as indicated: Two main domains that are highly conserved known as Mad Homology domains (MH1 and MH2) located at the NH_2 and COOH-terminal respectively. In addition, a non-conservative, middle linker region. In different molecular models it has been observed that in R-Smads, the MH1 domain regulates the association with DNA and contains a nuclear localization signal (NLS). The MH2 domain is a multifunctional region that is involved for different intra- and intermolecular interactions associated with different transcriptional transactivation activities. Smads are phosphorylated in response to the TGFβ ligands on critical serine residues at the C-terminus.

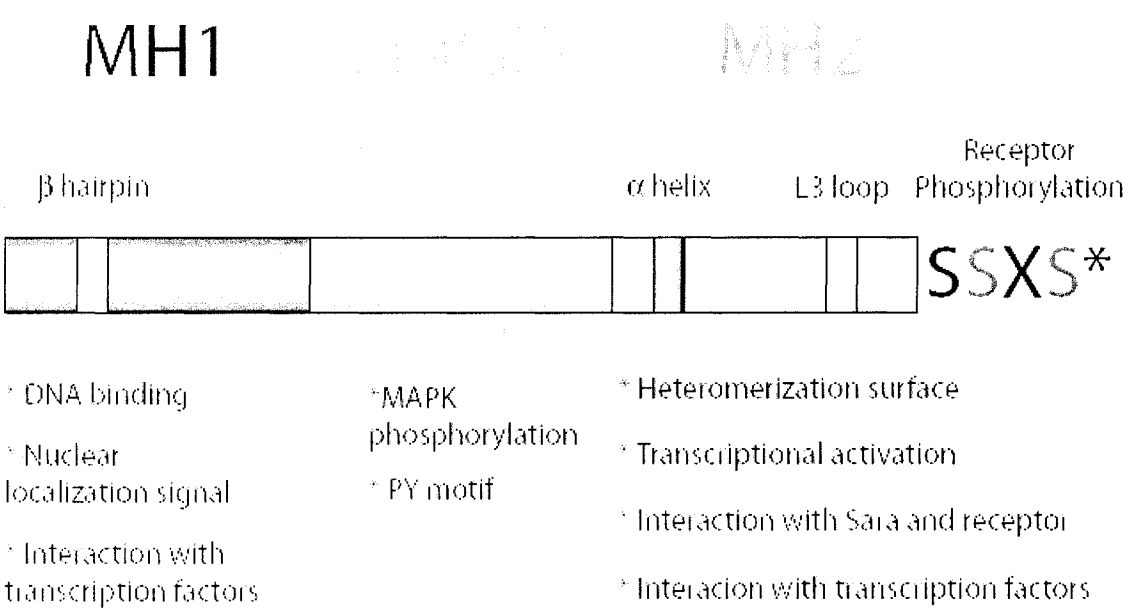

MH1
MH2
β hairpin
α helix
L3 loop
Receptor Phosphorylation
SSXS*
* DNA binding
* Nuclear localization signal
* Interaction with transcription factors
*MAPK phosphorylation
* PY motif
* Heteromerization surface
* Transcriptional activation
* Interaction with Sara and receptor
* Interacion with transcription factors

Figure 1.6.

1.5.b. The Smad Signaling Pathway

TGFβ stimulation induces the receptor heterodimerization and activation, followed by the recruitment and activation of R-Smads by the activated Type I receptor [15, 52, 62]. Interestingly, R-Smads have been found to be in association with cytoskeletal elements such as the microtubules. Different studies have demonstrated that the microtubules play an important role in guiding the Smads to the plasma membrane [293, 294]. These results showed that a particular FYVE-domain containing protein known as "Smad Anchor for Receptor Activation" SARA, modulated the proper location of the R-Smads at the plasma membrane. The FYVE-domain module is a protein structure found in another group of proteins, which are widely associated to the endosome formation [293]. The FYVE-domain protein is able to interact directly with Smad2 and Smad3. The role of SARA is to recruit the R-Smads by controlling the subcellular localization of R-Smads and by interacting with the TGFβ receptor complex when the signaling pathway is initiated. However, while the receptor-mediated phosphorylation and activation of the R-Smads induces their dissociation from SARA, they are able to bind to the Smad4 and translocate to the nucleus [293, 295]. Mutations of SARA induce the mislocalization of R-Smads, resulting in decreased TGFβ-dependent transcriptional responses. These observations suggest that the proper regulation of the Smad localization is crucial for TGFβ signaling. Therefore, these observations consider SARA as an essential component of the TGFβ pathway that brings the R-Smad substrate to the activated TGFβ receptor complex.

Phosphorylation of the R-Smads upon ligand activation occurs at the C-terminal region, specifically on the serine residues of the SSXS motif. This phosphorylation causes the release of the auto-inhibitory intramolecular interaction between the Mad-homology 1 (MH1) and Mad-homology 2 (MH2) domains of the R-Smads. This allows hetorodimerization with the common

partner Smad4 [41]. Subsequently, the Smad complex translocates to the nucleus, where it can associate with different co-activators and/or co-repressors of transcription, regulating the expression of various target genes in a differential way [15, 33, 55, 68, 69, 73, 296].

Interactions between Smad2 and Smad3 and their Type I receptors are mediated through SARA proteins in human and in *Xenopus* [293]. No counterparts have been identified to date for Sara for Dpp/BMP signaling Smads. Co-Smads, including *Drosophilae medea* and vertebrate Smad4, act in association with R-Smads from multiple pathways. Co-Smads lack the SSXS phosphorylation sequence in their MH2 domain, making them unresponsive to direct Type I receptor activation [297]. However, R-Smads and Co-Smads form multimeric protein complexes with each other [298]. R-Smad activation is necessary for a Co-Smad accumulation within the nucleus [299]. Promoter gene regions of various TGFβ-regulated target genes containing R-Smads binding sites also house Co-Smad binding sites [300]. These observations suggest that multimeric protein complex formation is required for Co-Smads to exert an effect on target genes, while R-Smads have the ability to function in the nucleus with or without Co-Smads. Figure 1.7 illustrates the general activated Smad signaling through TGFβ ligands (pages 35-36).

Figure 1.7. Smad signaling pathway. Smad molecules play a central part in the mediation of the TGF family member signal transduction. Once the receptor's complexes are activated, the affinity of the Receptor Smads, R-Smad2/3 for SARA diminish and they are recruited by the activated Type I receptor, which subsequently phosphorylates them at the C-terminus. Phosphorylated R-Smads2/3 translocate to the cytoplasm in order to associate with the common-Smad, Co-Smad Smad4. Once the Smad2/3/4 complex is formed, it travels towards the nucleus where in association of different co-activators or co-repressors, they regulate the transcription of several target genes.

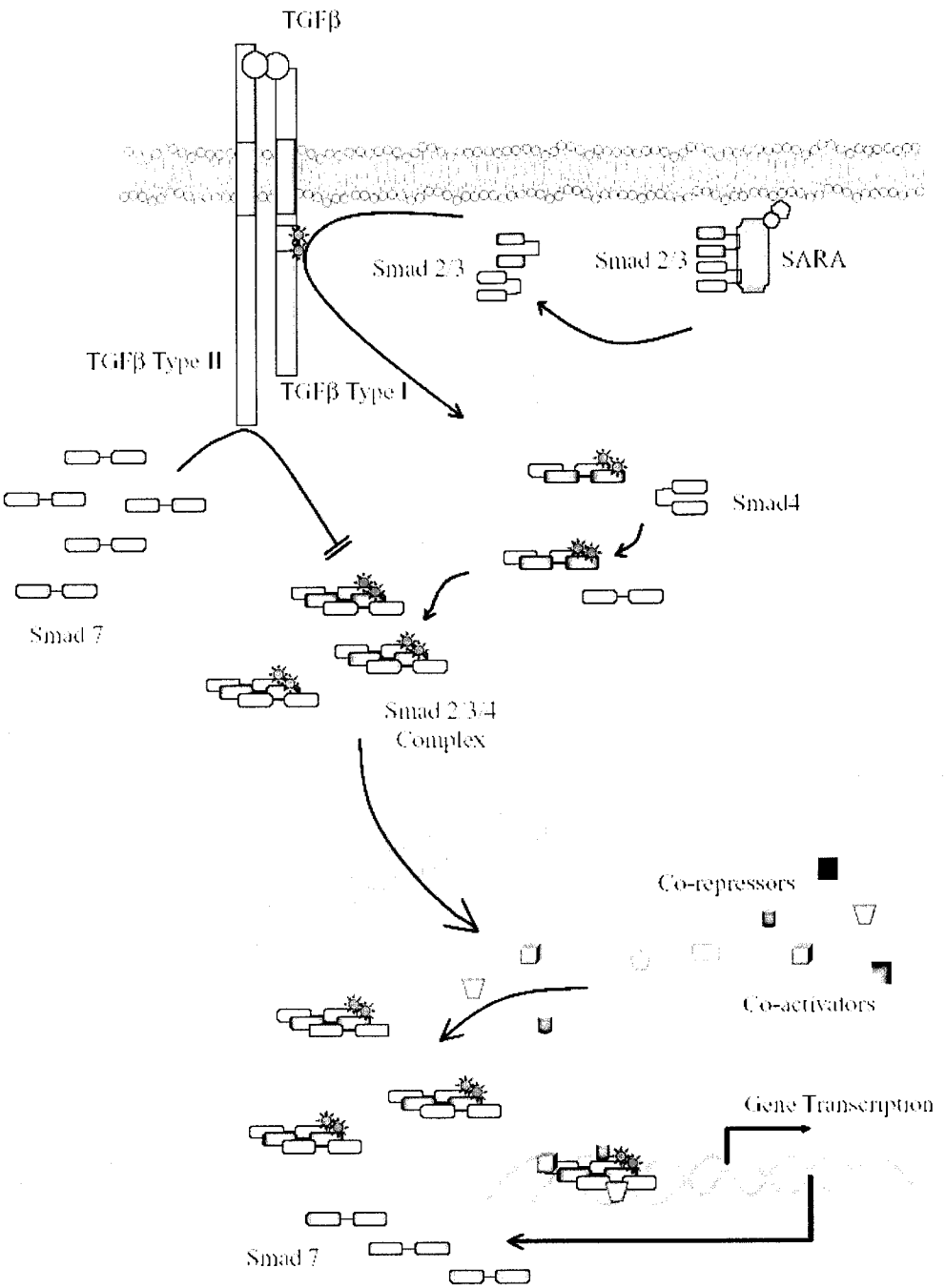

TGFβ
Smad 2/3
Smad 2/3
SARA
TGFβ Type II
TGFβ Type I
Smad4
Smad 7
Smad 2/3/4
Complex
Co-repressors
Co-activators
Gene Transcription
Smad 7

Figure 1.7.

I.5.c. Cross-talk between Smad and MAP kinase pathway.

1.5.c.i. Non-Smad Pathways

Smads are important key players in the TGFβ's family members signaling pathways. However, accumulating new evidence has shown that other signaling pathways are also activated downstream of these growth factor receptors. Different reports indicate that both TGFβ and BMP could send signals through the Jun N-terminal kinase (JNK) and p38 MAPK pathways [86, 181, 301-304]. To date, the different molecular and biochemical links between TGFβ receptor and the different downstream MAP kinase signaling pathways has not been completely elucidated. Based on different studies, one potential candidate has been suggested, one who can link these two signaling cascade systems, the TGFβ activated kinase, TAK1, member of the MAPK kinase kinase (MAPKKK) family [305-307].

The TGFβ can rapidly activate the JNK signaling pathway via MKK4 (MAPK kinase 4) [302]. In a similar context the p38 is activated by the TGFβ via the p38 upstream MAPK kinase 3 (MKK3) [303]. These activated signal pathways lead to the transcriptional activation of the downstream target genes. Moreover, activin can induce the p38 signaling and phosphorylation of the transcription factor ATF2 in human breast cancer cells [181]. These results support the observations made by Sano and colleagues, which revealed an interaction between the basic leucine zipper region of ATF2 and the MH1 domain of Smad3 and Smad4 [301, 303]. Recently, our lab demonstated that both the p38 MAP kinase and Smad pathways are required for activin-mediated cell growth inhibition in breast cancer cells. These results clearly highlight the complexity of activin signaling leading to gene activation [181]. In addition, the possible involvement of other molecular factors involved in the linking of TGFβ and MAPK cannot be ruled out. In this context, other factors such as TAB1 and XIAP might be located further

upstream in the signaling pathway affecting the TAK1 activation [308]. During the last decade, a large amount of information has accumulated, describing the cross-talk between the several components of the TGFβ signaling pathway with those of the MAP kinases signaling pathways. For instance, the Erk-activated Ras pathway is able to exert a modulatory effect at different levels of the TGFβ signaling cascade [309-313].

In this scenario, the Erk has been shown to modulate the TGFβ signaling pathway by decreasing the expression of a TGFβ receptor in H-Ras transformed rat's intestinal epithelial cells [314]. In addition, the TGFβ and BMP signaling pathways are negatively regulated by the oncogenic Ras, which induces a decrease in the accumulation of Smads in the nucleus. This Smad cytoplasmic accumulation has been observed upon EGF and HGF stimulation that induces phosphorylation of Smad1, Smad2 and Smad3 on MAPK and Erk consensus sites located in the linker region [315-317]. This phosphorylation event leads to strong cytoplasmic retention of the R-Smad, reducing significantly the transcriptional activation and regulation of different Smad-regulated target genes. Mutation of the Erk phosphorylation sites of Smad3, result in a significant Ras-resistance rescue of the growth inhibitory effects of the TGFβ's in the Ras-transformed cell types. In addition, the EGF stimulation that induces a less extensive phosphorylation and cytoplasmic retention of the Smad2 and Smad3, compared to the oncogenic Ras, which might explain the silencing effect of anti-mitogenic TGFβ functions of the effect of hyperactive Ras in different cancer cell populations [316]. Therefore, the Erk MAP kinase pathway is not only implicated in the cytoplasmic Smad retention, but also in the modulation of the Smad transcriptional activity. In this context, it has been reported that the expression of different Smad-interacting co-repressors is upregulated in response to activation of the Erk [318].

Figure 1.8 is a general schematic representation of the different signaling cascades that interact and regulated TGFβ signaling pathway (pages 40-41).

I.5.d. Specificity of the Smad signaling pathway.

A vast body of studies have clearly shown that the different branches of the TGFβ family members can regulate several sets of target genes, via their respective downstream modulators such as the Smads [288]. Both R- and Co-Smads complexes are capable of interacting with distinct DNA sequences known as Smad Binding Elements (SBE). Among these SBE, CAGAC, GTCTAGAC and other GC rich domains which are widely well and significantly recognized by the Smads [33, 319, 320]. The nature of interaction between Smads and their recognized binding sequences have been shown to be weak, suggesting that the Smads need the assistance of other proteins for correct binding [33]. However, it has also been shown that a specific pentameric CAGAC DNA-binding motif occurs significantly in a high frequency within the genome and highly recognized by Smads, indicating the possibility of non-specific and promiscuous binding of the Smads with DNA can occur. Thus, the participation and involvement of other factors are therefore required for increased specificity and a proper target selection.

Such co-factors are structurally diverse proteins that share the ability to associate with Smad molecules and a neighboring DNA sequences [296]. Cell type specific response could be explained by the fact that certain cofactor combinations are expressed differentially in cell or tissue context [300, 321].

FIGURE 1.8 - TGFβ and crosstalk between other signaling pathways. TGFβ ligands can mediate their intracellular signals not only activating the Smad signaling pathway, but also they can activate other downstream cascades in particular MAP kinase signaling pathways, like the Erk, the p38 stress-activated or the Jun N-terminal kinase, JNK, MAP kinase pathways. The activation of these pathways lead to increase the activity of different transcription factors that regulate the transcriptional regulation of different target genes.

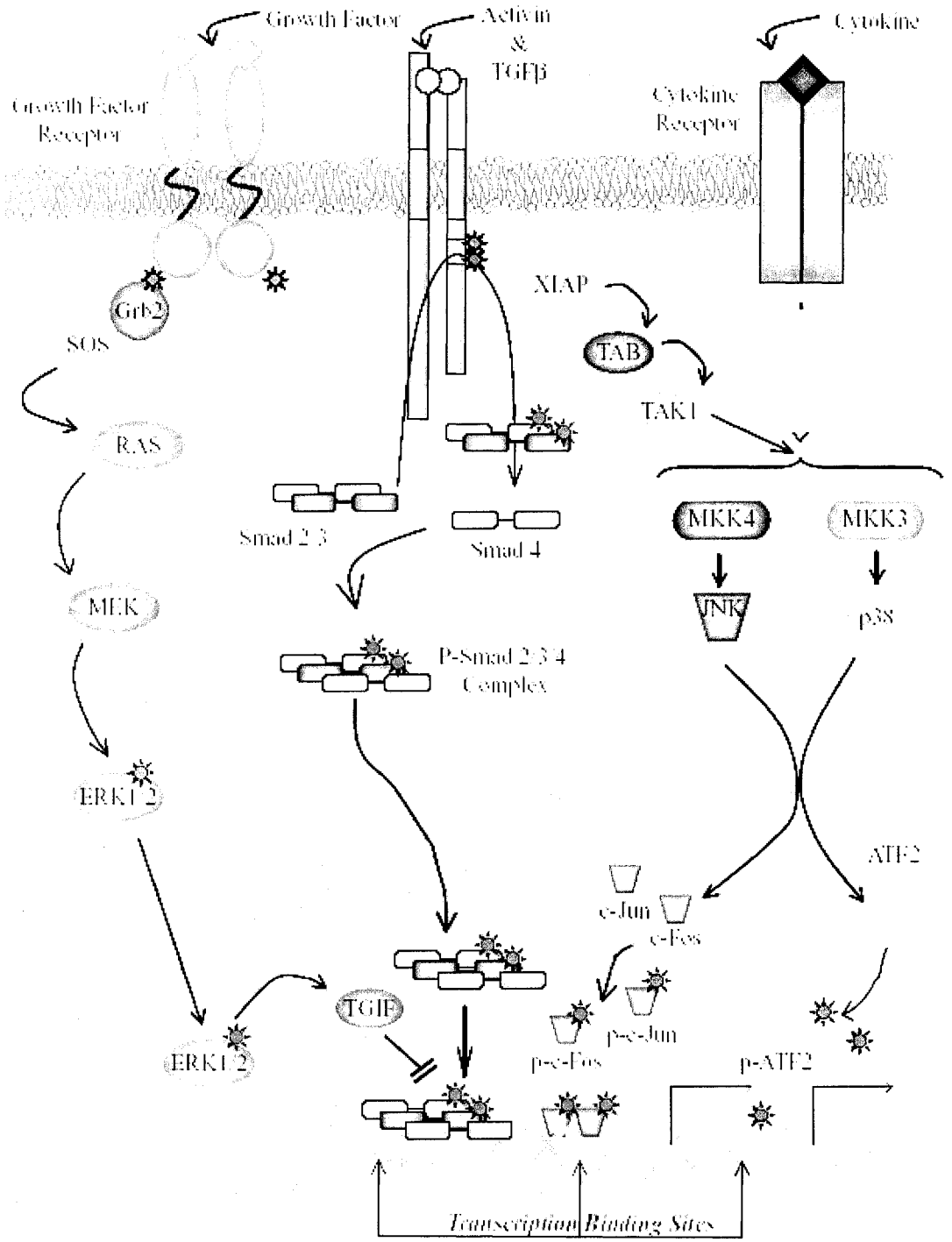

Growth Factor
Activin & TGFβ
Cytokine
Growth Factor Receptor
Cytokine Receptor
Grb2
SOS
RAS
MEK
ERK1/2
XIAP
TAB
TAK1
MKK4
MKK3
JNK
p38
Smad 2/3
Smad 4
P-Smad 2/3/4 Complex
TGIF
ERK1/2
c-Jun
c-Fos
p-c-Jun
p-c-Fos
ATF2
p-ATF2
Transcription Binding Sites

Figure 1.8.

The existence of different Smad-associated co-factors has already been shown [322, 323]. This raises the question of how the specificity in a Smad signal transduction is achieved. At first, the most important groups of Smad associated partners are the molecules named *adaptor molecules*, which are required for proper DNA-Smad interactions [322, 323]. In this context, it has been proposed that in order to get a proper DNA binding, Smads, particularly Smad1, associates with the olf-associated zing finger, OAZ [321]. Likewise Smad2 binds to the forkhead activin signal transducer, FAST, and Mixer [322, 323]. On the other hand, it has been shown that the different adaptor proteins OAZ, FAST and Mixer do not have intrinsic transcriptional activity. In this case, structural studies have indicated that the recognition between the Smad molecules and the adaptor protein requires a specific bulging α-helix 2 region located in the MH2 domain in the Smad proteins and a Smad-interaction domain, which is conserved in the FAST and Mixer but not in the OAZ [288, 321, 323].

Transcription factors are another important and essential group of Smad-interacting proteins [322, 323]. It has been demonstrated that different types of transcription factors can associate and form various functional complexes with Smad proteins as it has been demonstrated with the for JunB [324], transcription factor binding to immunoglobulin heavy constant μ enhancer 3', TFE3 [325], core-binding factor A/acute myeloneus leukemia (CBFA/AML) proteins [326, 327] and lymphoid enhancer binding factor 1/T-cell specific factor, LEF1/TCF [328, 329]. Furthermore, it has been shown that the LEF1/TCF is the main mediator for the WNT/β-catenin signaling pathway. The LEF1/TCF is able to co-operate with Smads in the activation of the *Xenopus* twin, Xtwn, in response to the Nodal related signals [329]. Smads associate and recruit repressors as well as transcriptional activators. Smads can associate with repressors such as the TGFβ-interacting factor, TGIF [330], Sloan-Kettering Institute proto-

oncogene (Ski) [331] and Ski-related novel gene N, SnoN [332]. These molecules act as repressors and they can bind to histone deacetylases, HDAC, which are generally implicated in chromatin condensation and transcriptional silencing. The HDAC binding counteracts the effect of histone acetyltransferase, HAT activity, which is in tight association with the co-activators CBP and p300, which are responsible for transcriptional activation [296]. However, the relative balance between the levels of co-repressors versus co-activators is thought to be the crucial event that determines the final outcome of the transcription.

I.5.e. Smad interacting partners

Smads are known to be the canonical molecules that modulate the TGFβ signaling pathways [133, 333-335]. Smad3 and Smad4 bind to DNA but with low affinity [33]. Generally, these Smads can achieve higher affinity and DNA interaction by physically associating with different DNA binding partners [296, 336]. Based on these observations, it has been suggested that Smads require a specific association with an interacting partner's proteins that can act as co-activators or co-repressors, which are differentially expressed in different cell types [286, 330, 334, 335, 337-340]. The interaction of the Smads with the different transcription factors is directly mediated through their MH1 or MH2 domains in a cell or tissue dependent way. This diversity in molecular association patterns of Smads in reponse to the TGFβ ligands provides a base for the observed complexity and cellular dependence of transcriptional regulation [300, 321, 341]. The interaction between Smad's and its different binding proteins is well documentated. Examples of Smad co-activators include the AP-1 family members [342], the core-binding factors/acute myeloneous leukemia (CBFA/AML) proteins [327, 343-345] DBP/p300 family members [345-347], the 1,25-dihydroxyvitamin D3 [348], and the transcription factor ATF2 [181, 301] among others. Known Smad transcriptional co-repressors include SKI and SnoN [331, 349]

and the TGFβ-Interacting Factor (TGIF) that bind to Smad2 and Smad3 in competition with p300 [350]. Figure 1.9 illustrates a summary of the different groups of Smad's interacting partners (pages 45-46).

I.6. TGFβ effects on Cell Cycle

I.6.a. Cell Cycle

The eukaryotic cell cycle is a biological process, which is essential for any type of cell in order to promote cellular division. In general the cell cycle can be divided in two main phases, the interphase and Mitosis. The interphase includes three subphases: G1, S and G2. During the G1 phase, the cell prepares itself and all the molecular machinery for a DNA replication [335, 351]. Subsequently, the replication of the genetic material occurs during the S phase. Following the DNA replication, the cell goes through the next cell cycle phase known as a gap period, also known as G2, where the cell is ready to progress into mitosis. During the mitosis, the genetic material is condensed into segregated chromosomes, therefore allowing cytokinesis to occur. In addition, it is known that during the development, differentiation, or growth-factor withdrawal, cells can enter an inactive period defined as G0, before returning to G1 [351]. This natural process is tightly regulated at different levels. In order to achieve this regulation, a precise control along the different transitory events between the different cell cycle phases is essential. The transition from the G1 to the S phase depends on two types of proteins: cyclins and cyclin-dependent kinases (cdks) [352-357].

Figure 1.9. Smads and their interacting associated partners. RSmad–Smad4 complexes are able to interact with several DNA binding co-repressors or co-activators in different ways. These protein-protein associations allow the formation of the transcription complex that will differentially regulate the expression of target specific genes.

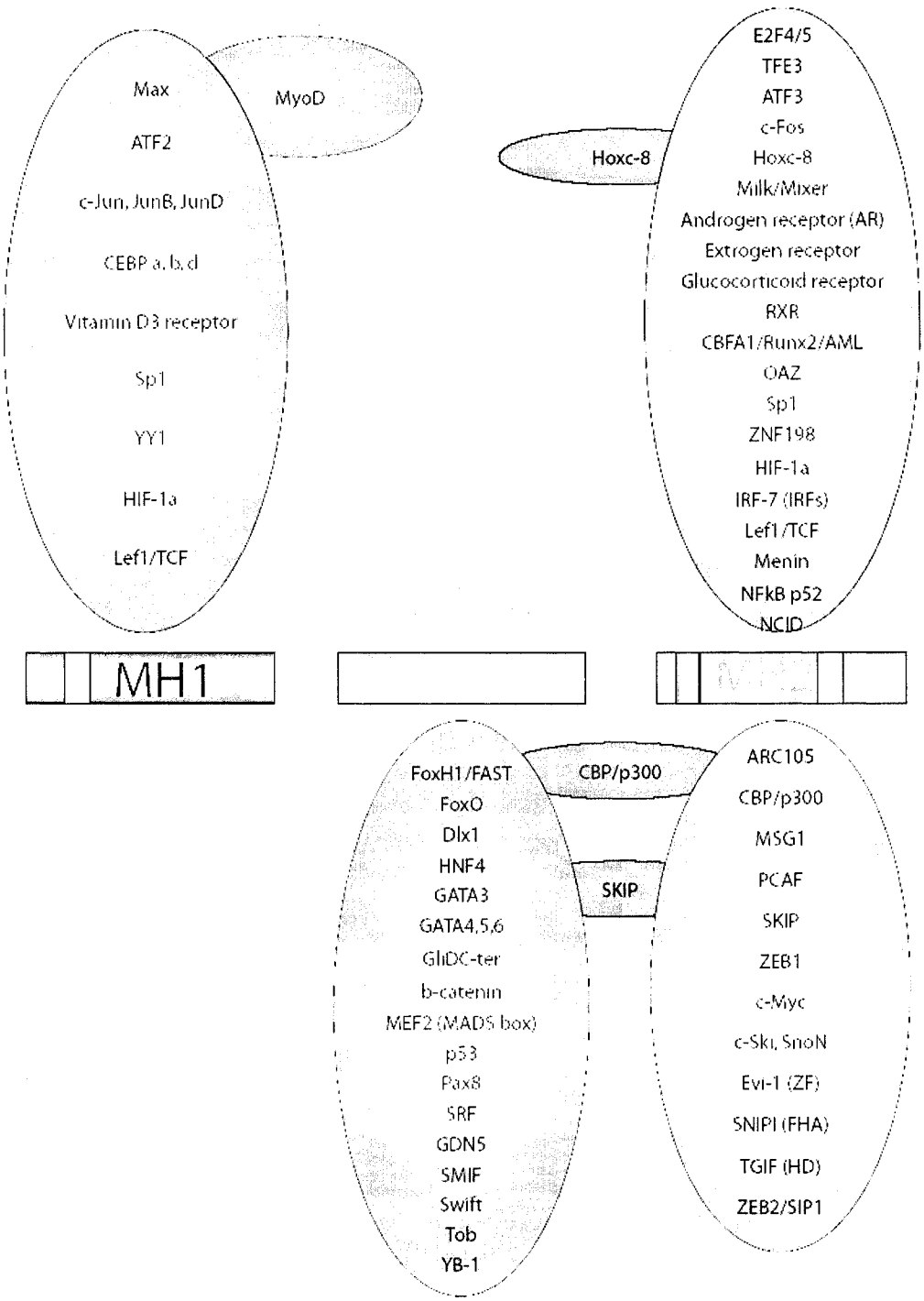

Max
ATF2
c-Jun, JunB, JunD
CEBP a, b, d
Vitamin D3 receptor
Sp1
YY1
HIF-1a
Lef1/TCF
MyoD
Hoxc-8
E2F4/5
TFE3
ATF3
c-Fos
Hoxc-8
Milk/Mixer
Androgen receptor (AR)
Extrogen receptor
Glucocorticoid receptor
RXR
CBFA1/Runx2/AML
OAZ
Sp1
ZNF198
HIF-1a
IRF-7 (IRFs)
Lef1/TCF
Menin
NFkB p52
NCID
MH1
FoxH1/FAST
FoxO
Dlx1
HNF4
GATA3
GATA4,5,6
GliDC-ter
b-catenin
MEF2 (MADS box)
p53
Pax8
SRF
GDN5
SMIF
Swift
Tob
YB-1
CBP/p300
SKIP
ARC105
CBP/p300
MSG1
PCAF
SKIP
ZEB1
c-Myc
c-Ski, SnoN
Evi-1 (ZF)
SNIP1 (FHA)
TGIF (HD)
ZEB2/SIP1

Figure 1.9.

I.6.b. TGFβ-induced cell growth arrest

One of the critical roles of the TGFβ is to inhibit the epithelial, endothelial and haematopoietic cells growth [74-76, 358]. Cancer cells have developed different mechanisms to escape cell growth inhibition induced by the TGFβ growth factors [26, 37, 104]. Furthermore, it has been shown that the TGFβ family members have the ability to block the cell cycle progression at an early G1 phase [359, 360]. TGFβ actions on cell cycle inhibition are achieved by controlling several important regulators of the cell cycle. In this context, it has been reported that the first target of TGFβ happens to be the retinoblastoma (Rb). TGFβ has the ability to significantly inhibit the hypophosphorylation of the Rb during the cell growth arrest [361, 362]. The TGFβ-dependent cell growth inhibition also requires the regulation of the cyclin-dependent kinases (CDK) activity. The TGFβ effects on the CDK have been observed at different levels such as their expression [363], or via blocking the cyclin/CDK complex activities as well as the upregulation of specific CDK inhibitors [74-76].

1.6.c. Cyclins and CDKi as TGFb target genes.

During cell cycle arrest, TGFβ promotes rapidly the expression of $p15^{Ink}$4b (p15), which associates to the cyclin D-CDK4/6 complex. The cyclin D-CDK4/6 complex has been described to act as a mitogen sensor at early G1 phases [42]. The complex formed by Cyclin D-CDK4/6 can also sequester CDK inhibitors of the Cip/Kip family of proteins, $p21^{Waf/Cip}$ (p21) and $p27^{Kip1}$ (p27).

Induction of an additional TGFβ-regulated protein, p15, results in p21 and p27 displacement from the cyclin D-CDK4/6 complexes, which then become available to target cyclin E/CDK2 and Cyclin A/CDK2 complexes [42]. In addition, the TGFβ-induced increase

expression of p21, promoting its enhanced association with the p27, inactivating the cyclin E/CDK2 and cyclin A/CDK2 complexes. The above responses to TGFβ lead to a complete abrogation of the G1 phase cyclin/CDK complexes. Along with those responses, the TGFβ secures the increasing of the CDK4 levels at the transcriptional levels [103]. Moreover, the TGFβ stimulation has been shown to downregulate the Cdc25A, a member of the Cdc26 family of tyrosine phosphatases, which removes the tyrosine phosphorylation from the CDKs. The outcome of this event leads to the further inhibition of the cyclin/CDK complexes [364].

Another cellular response to TGFβ that has been described is the transcriptional repression of the cell cycle modulating factor c-Myc [365]. This response occurs upstream of the CDK inhibition by the TGFβ, both temporally and spatially [366, 367]. In addition, the overexpression of c-Myc renders cells resistant to the antiproliferative effects of the TGFβ by blocking p15 and p21 transcriptional induction [368, 369]. Moreover, it has been described that the repression of c-Myc is achieved by a protein complex that includes p107, a member of the Rb protein family, along with the E2F4, E2F5 and Smad proteins. It has been reported that the TGFβ not only targets the function of the Rb proteins by inhibiting the CDKs, but also utilizes some of the Rb proteins as signaling effectors to exert its growth inhibitory pathway [370]. Transcriptional induction of the p15 and repression of c-Myc has, so far, been reported in a handful of cell types [351, 353, 356, 358, 365-369]. On the other hand, the TGFβ and related ligands induce the p21 transcription in various normal epithelial and carcinoma cell lines [296, 371-374]. Figure 1.10 presents a schematic representation of the main effects of the TGFβ ligands on the cell cycle progression (pages 48-49).

Figure 1.10. TGFβ effects on the regulation of cell cycle progression. General effects of the TGFβ lignads during the the different phases of the cell cycle. Member of the TGFβ superfamily of growth factors exert their effects in cell cycle arrest at the level of signal transduction events or Smad-cofactor complexes converging on the target genes that mediate cell cycle arrest.

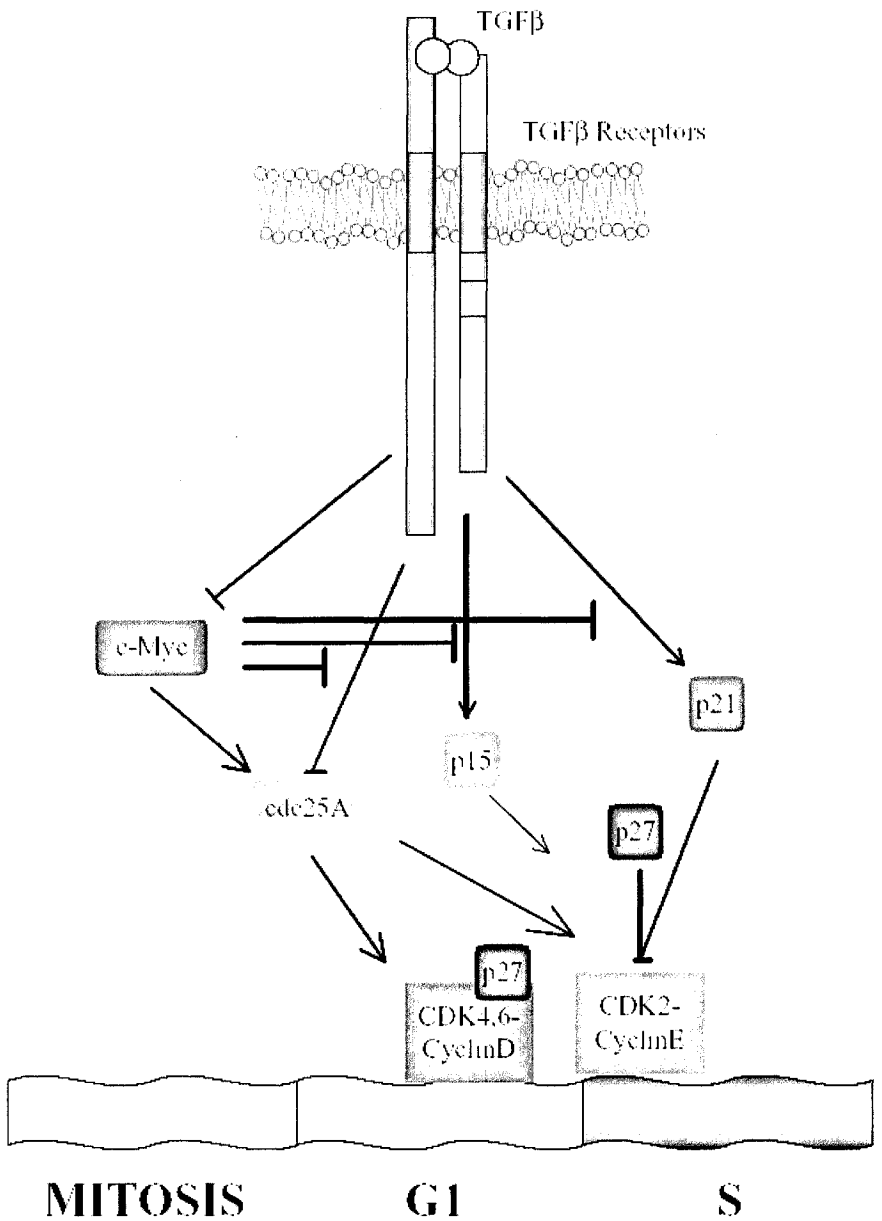

TGFβ
TGFβ Receptors
c-Myc
p21
p15
cdc25A
p27
p27
CDK4,6-
CyclinD
CDK2-
CyclinE
MITOSIS
G1
S

Figure 1.10.

I.6.c. TGFβ ligands and Apoptosis

Currie and colleagues originally adopted the term apoptosis in 1972 to describe their observation of apoptosis or programmed cell death [375]. The apoptotic process results in proteolytic cleavage of over a 100 substrates in mammalian cells, usually mediated by aspartate-directed cysteine proteases called caspases [376, 377]. Two main pathways are described in the programmed cell death: the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic).

The final balance between the survival, proliferation and death of a cell, is crucial to many physiological processes. Deregulation of this balance has been associated with various disease processes. Certain chronic degenerative diseases and immunodeficiencies result due to excessive apoptosis, whereas insufficient apoptosis contribute to the development of cancer and autoimmunity [378, 379, 629]. During apoptosis development it is required for the elimination of damaged, immature or abnormal cells. In the immune system, apoptosis is extremely important as it tightly regulates the selection of different T cell populations. During the immune response, the regulation and later depletion of activated T cells is also dictated by apoptosis [377, 380, 381, 629].

Nevertheless, other signaling pathways have been reported to be involved in the induction and modulation of different apoptotic events. Some examples of these signaling pathways are: the MAP kinase signaling cascades, NF-kB, PKB/Akt and extracellular signals like TGFβ among others [308, 377, 382-386, 629]. As previously mentioned, one of the most important death inducers are the different members of the TGFβ superfamily of growth factors [69, 387-389, 629]. Research has demonstrated that the TGFβ is a crucial regulator of the balance between cell growth and apoptosis. Interestingly, the apoptotic events regulated by TGFβ ligands are cell type

and context-dependent. In fact, the TGFβ may provide signals for either cell survival or apoptosis, making it a molecule with a dual role [15, 63, 387-393, 629]. Until this date, the different molecular mechanisms underlying the role of the TGFβ family members in apoptosis are not completely understood. The Smad signaling is the principal pathway activated by TGFβ ligands. Nevertheless, it has been shown that TGFβ signaling is able to cooperate with other signaling pathways such as the death receptor apoptotic pathway induced by the Fas-ligand and TNFα, stress and apoptotic MAP kinase pathways regulated by the JNK and p38, Akt, NF-kB and the mitocondrial apoptotic pathway which is mediated by the Bcl-2 family members. Furthermore, TGFβ signaling has been shown to lead to oxidative process as well. These findings have allowed integrating TGFβ signaling cascade as a part of the apoptotic pathways [330, 333, 334, 338-340, 350, 394-397, 629]. The existence of these different interactions and the balance between the various stimuli provides the basis for the pro- or anti-apoptotic output of the TGFβ ligands signaling in a given cell system. TGFβ ligands are considered as an immunosuppressor and although the mechanisms are activated by the TGFβ in immunosuppresion, they are not completely understood. The inhibition of apoptosis through TGFβ ligands participate in this process. It has been demonstrated that the TGFβ is able to either suppress or positively modulate the apoptotic events in different T cell populations [69, 398-401, 629]. Treatment with TGFβ can suppress or induce the sensitivity to apoptosis induced by FasL as well as TNF-α in activated T cells [400, 401, 629]. Therefore, based on these indications, TGFβ seems to be an important player in the inhibition of the Fas/FasL and TNF-α-induced apoptosis in immune cells [402-406, 629]. Figure 1.11 illustates the different survival and apoptotic signaling pathways, which interact with the TGFβ signaling pathway (shown in pages 53-54).

FIGURE 1.11. Interactions of TGFβ signaling cascade with apoptotic extrinsic and intrinsic pathways. Schematic representation of the extrinsic and intrinsic apoptotic-induced signals in different cell types.

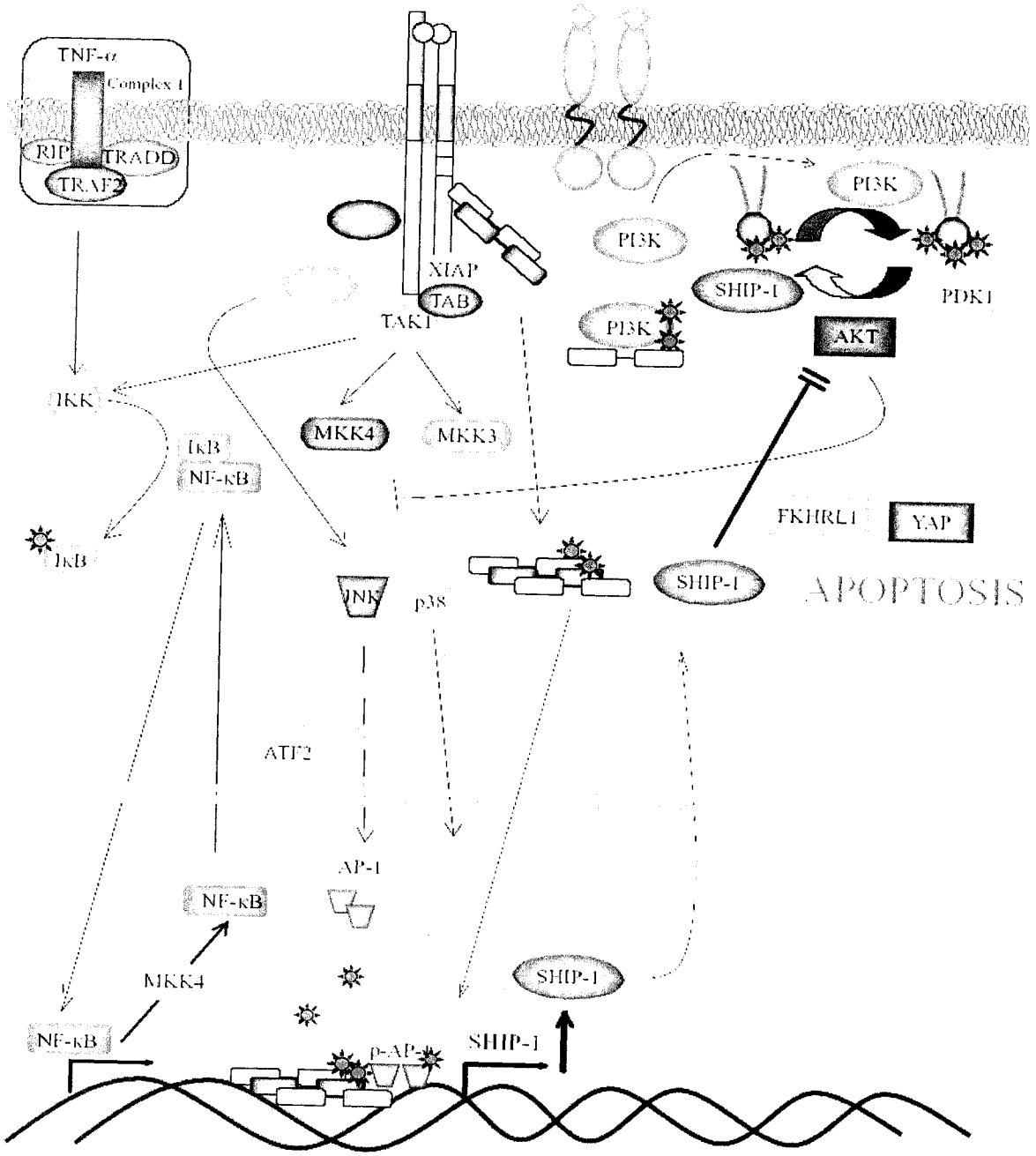

TNF-α
Complex I
RIP
TRADD
TRAF2
XIAP
TAB
TAK1
PI3K
PI3K
PI3K
SHIP-1
PDK1
AKT
IKK
MKK4
MKK3
IκB
NF-κB
IκB
FKHRL1
YAP
JNK
p38
SHIP-1
APOPTOSIS
ATF2
AP-1
NF-κB
MKK4
SHIP-1
NF-κB
SHIP-1

Figure 1.11.

1.7. TGFβ family members in pathological conditions

Homeostasis is understood as the physiological process that requires intractely balanced interactions between cells and the network of secreted factors in the organism. Some of these factors such as growth factors, hormones, cytokines among others, are the responsible to keep the proper function of the biological systems. Thus, any disruption or alterations at the different levels of the signaling pathways regulated by these growth factors have been linked to different pathological processes This is clearly evident in the different molecular and cellular events regulated by different TGFβ family members [63, 407, 408]. Different evidences have demonstrated that either increase or decrease in the production of TGFβ links to numerous diseases states (Blobe GC et al., 2000). In addition, any mutations in the genes for TGFβ ligands, its receptors, or intracellular signaling pathways associated with these ligands have also been involved in the pathogenesis of different diseases including cancer. In this context TGFβ signaling pathway has been described that confer resistance to growth inhibition by TGFβ, thus allowing uncontrolled proliferation of the cells [409-416]

1.7.a. Role of TGFβ in Cancer

Large amount of information indicates that in cancer cells, the production of TGFβ is significant decrease, followed by a significant increase in the invasiveness capacity of the cells by increasing their proteolytic activity and promoting their binding to several cell-adhesion molecules. Oncogenesis due to unrestricted growth results from defects in the negative regulatory control of TGFβ family members. Indeed, resistance to the antiproliferative effects of TGFβ ligands is often observed in different types of human cancers [417]. In early stages of

tumorigenesis, a cell loses its responsiveness to TGFβ mediated cell growth inhibition as a result of mutation or loss of expression of some components involved and required in the TGFβ signaling pathway. This has been reported in the case of pancreatic and colon cancer, which have several mutations affecting at least one component of the TGFβ signaling pathway [418-420]. In addition, Smad4, initially identified as deleted in pancreatic cancer 4 (DPC4), is found to be mutated in more than 50 percent of pancreatic cancer and 30 percent of colorectal cancers [419, 420]. Different groups have shown that normally both alleles are mutated or deleted in these types of cancer, thus defining Smad4 as a tumor-suppressor gene [421, 422]. Similar mutations have been described in other pathologies such as familial juvenile polyposis, an autosomal dominant disorder characterized by a predisposition of hematomatous polyps and cancers of the gastrointestinal track. Interestingly, no mutations in Smad3 have been found in human cancers, although loss of heterozygosity at this locus has been detected in some colorectal cancers. Thus, these findings clearly indicate that TGFβ signaling pathway has an essential role in tumorigenesis [421, 422].

1.7.b. Role of TGFβ in Tumor Progression

Once tumor cells acquired resistance to growth inhibition by TGFβ, both the tumor cells and the normal stromal cells within the tumors often increase their production of TGFβ. Subsequently, in response to increased production of TGFβ, the tumor cells become more invasive and metastize to disistant organs, at least in part as a result of TGFβ-mediated stimulation of angiogenesis and cell motility, suppression of the immune system, and increased interaction of tumor cells with the extracellular matrix. In the later stages of various cancers, increased production of TGFβ has been associated with increased invasiveness. Thus, resistance to TGFβ leads to tumor formation and to great invasiveness of these tumor cells.

1.7.c. Fibrosis

Perhaps the most widely studied biological function of TGFβs have been in the areas of fibrosis and wound healing [423-425]. It has been shown that elevated levels of TGFβ are often accompanied by fibrosis and inflammation in the heart, liver, and kidneys. Similarly, lung and liver fibrosis sometimes occurs as a life-threatening complication following bone marrow transplantation. High levels of TGFβ detected pretransplant may be predictive for the development of these complications. These observations have suggested that the subsequent fibrotic effects may be associated with overproduction of TGFβ. [426-428]. Thus, the continued presence of high systemic levels of TGFβ results in uncontrolled stimulation of normal biological processes that ultimately results in severe disease pathologies.

1.7.d. Immunity

The pleotropism of TGFβs is also manifested in its ability to upregulate or downregulate cellular functions such as activation of different immune cell populations. The downregulating effects can be either beneficial or deleterious depending on the circumstances. Such downregulated events are likely to be beneficial in situation where a successful immune response needs to become quiescent. However, the inappropriate production of TGFβ during crucial phases of immune response generation may also blunt the development of beneficial responses and lead to disease progression [34, 35, 62, 63, 408, 429-434]. Hematopoiesis is regulated by the proper balance of self-renewal (cellular proliferation), differentiation and apoptosis of different hematopoietic progenitor cells. It has been demonstrated that the effects of TGFβ on hematopoietic cells and hematopoiesis are both cell and context specific [435]. In many cases opposing effects of TGFβ on the proliferation, differentiation and survival of hematopoietic

progenitor cells have been reported in vitro and in vivo. Moreover, TGFβ signaling pathway represent a major antiproliferative and differentiation signal for hematopoietic progenitor cells, effectively preventing progression through the cell cycle and promoting differentiation. I t has also been shown that treatment with TGFβ1 induces cell growth arrest and inhibition in different immune cell types [435]. Recently, studies performed in human hematopoietic cells in vitro support an important role for TGFβ in the regulation of different hematopoietic-cell proliferation, differentiation, and apoptosis [399, 435].

1.8. Regulation of the immune system by TGFβ

1.8.a. TGFβ ligands in Immune Cell Survival

The existence of a strict control of the different immune cell populations is required and essential to allow normal and correct immune response, preventing unexpected and undesirable self-targeted responses [436]. TGFβ is a member of a large family of evolutionary conserved proteins. Several of the TGFβ-family receptor as well as their ligands are practically expressed in every cell and tissue in the body. The three closely related members of the superfamily, have been implicated as important players in the regulation of immune cell populations [437-439].

The different knockout mice models have highlighted the importance of these growth factors in different biologicals and physiologicals processes (Table 1.1, page 15-16). Some of the different reported phenotypes present multifocal inflammatory response in the pups that are born alive [440, 441]. TGFβ has also been implicated in T-cell differentiation. This has been suggested by the early discovery of the anti-prolifeartive effect of this growth factor on T cells in vitro, as well as the inhibition of interleukin-2 (IL-2), production, and a cytokine essential for T-cell proliferation through an autocrine mechanism [442-446].

In addition, TGFβ and related members, in particular activin, has been shown to have an important effect in inhibiting proliferation and cell cycle progression in immune cell types through a variety of mechanisms, that include up regulation of cell-cycle inhibitors $p15^{INK4B}$ [180, 447], p21 [448, 449] and/or p27 [450, 451] and by the downregulation of c-myc (see Figure 1.10, pages 49-50).

On the other hand, activation of naïve T cells during the immune response leads to their differentiation into two main effectors T-cell subsets of cell populations. The first population is the responsible for the cytotoxic activity, defined as CD8+ T cells and the other which performs a helper functions, involving CD4+ T cells. The molecular and cellular mechanisms that differentiates naïve T cells to these specific subpopulations that involves the triggering of a specific programme of molecular and epigenetic changes, result in the stable expression of a specific T-cell phenotype. Although certain cytokines and growth factors can aid T-cell differentiation, TGFβ inhibits the acquisition of most, if not all, effectors functions by naïve T cells: CD8+ T cells activated in the presence of TGFβ do not acquire CTL functions and CD4+ T cells fail to become T-cell helper, Th1 or Th2 cells [400, 452].

TGFβ is also is involved in regulating T-cell differentiation [453]. In the case of inhibition of T-helper-cell differentiation, even though TGFβ can inhibit both Th1 and Th2 differentiation, the development of Th2 cells seems to be more sensitive to TGFβ than differentiated Th1 cells [453]. TGFβ has the ability to completely block the T-cell differentiation towards Th2 under the all-experimental conditions tested. On the other hand, the ability of TGFβ to inhibit Th1 differentiation, seems to depend on the strain of mice and strength of T-cell co-stimulation [387, 388, 454, 455]. It has been shown that IFN-γ that is produced by differentiated Th1 cells can block the TGFβ inhibitory effects. One potential mechanism by which IFN-γ

signaling can block TGFβ inhibitory effects is through the up regulation of Smad7, an inhibitor of TGFβ signaling. Nevertheless, it has been reported that IFN-γ only protects naïve T cells from TGFβ, since that fully differentiated Th1 cells do not express the IFN-γ receptors [454].

Consistent with this observation, TGFβ can inhibit IFN-γ production by fully differentiated Th1 cells *in vivo* [456] and *in vitro* [457]. The possible existence of other mechanisms that can block the inhibitory effects of TGFβ on T cells can not be ruled out as fully differentiated Th2 cells that do not make any IFN-γ seem to be insensitive to TGFβ inhibition of Th2 cytokine secretion.

1.8.b. Role of TGFβ and activin in immune cells

TGFβ and activin are crucial regulators of cell growth and apoptosis of the immune system. The different cell populations in the immune system are constantly travelling to distant target sites in the organism. When no longer required for immune defense, they can represent a considerable threat to tissue integrity: the elimination of activated immune cells by apoptosis prevents tissue damage.

Activin and TGFβ, as cell death inducers in the haematopoietic tissue compartment, are crucial to the proper elimination of activated lymphocytes and the maintenance of peripheral tolerance, thereby preventing autoimmune disease [34, 85, 86, 304, 440, 458-464].

The antiproliferative effects, and more recently discovered proapoptotic effects, of activin have been observed in peripheral blood granulocyte-macrophage colony-forming unit progenitors [463], as well as B cell leukemia [464], erythroleukemia [183], and plasmocytoma cells [465]. TGFβ, also a critical regulator of immune cell growth arrest, [86, 440, 458] induces cell growth inhibition and apoptosis in primary cultured lymphocytes [459, 460, 466]. Consistent with this

observation, TGFβ1 deficient mice develop extensive lymphocytic hyperproliferation with a significant increase in production antibodies. [85, 440, 458].

1.8.c. TGFβ in Inhibition of Antitumor Immunity

The balance between proper and adequate immune response to several stimuli, such as an antigen or pathogen and tolerance, is required for normal immune homeostasis and the well being of the host. In this scenario, the complex self-regulation as well as multiple mechanisms has been implicated in the immune tolerance networks, involving apoptosis, anergy, and active suppression. TGFβ ligands are also potent immunoregulatory cytokines that contribute to the function and generation of different immune cell population [388, 467]. Moreover, many cellular effects of TGFβ ligands facilitate tumour establishment as well as its growth and metastasis [69]. It has been reported that both lymphocytes and tumor cells express TGFβ receptors. In contrast to immune cells, tumour cells eventually cease to be responsive to the inhibitory effects of TGFβ and acquire invasive and/or metastatic phenotype [468-470]. Thus, TGFβ is no longer able to inhibit tumor growth progression, but induces immunosuppression in patients with different advanced or metastatic tumors. In addition, TGFβ is a strong inducer of angiogenesis, a biological process widely used for different tumours to promote metastasis [471].

Animal studies have suggested that TGFβ-mediated immunosuppression mechanism is the most important of these effects and that the presence of activated TGFβ in the tumor microenvironment protects tumor cells from recognition by the immune system [439]. In addition, TGFβ also has a strong effect in inactivation of natural killer (NK) and lymphokine activated killer (LAK) cells. This has been attributed to possible effect of TGFβ inhibiting TNF-α and-β secretion [472-475]. Moreover, it has been reported that TGFβs is a key player in the activation of CD8+ T-suppressor cells, attenuating significantly the production of IgG *in vitro* [476-480].

All these results strongly indicate that TGFβ has the ability to interfere with both the recognition and destruction of tumor cells by the immune system. Thus, these findings suggest that tumor growth and metastasis, are not critically affected by TGFβ, thus, indicating that the primary TGFβ's target in vivo is the immune system and not the tumor itself.

Lastly, although hematologic malignancies represent a broad spectrum of disease, the involvement of the TGFβ signaling pathway in these diseases can be characterized by several themes. In several immune diseases, resistance to the growth-inhibitory and apoptosic effects of TGFβ induces clonal expansion and suggests and early role for the TGFβ signaling pathway in diseases pathogenesis. On the other hand, in certain hematopoetic diseases such as Chronic myeloid leukemia (CML) disease-specific oncoproteins interfere with TGFβ signaling after disease initiation and are associated with disease progression, thus these observations suggests a role for disrupted TGFβ signaling in disease progression. Finally, elevated levels of TGFβ ligand appear to be an essential mediator of different types of leukemias [413, 435].

Although these studies clearly suggest a critical role for TGFβ signaling cascade in aspect of the pathogenesis of different hematologic malignancies, major callenges in the TGFβ signaling remain to be elucidated. It will be extremely interesting to evaluate the different signaling pathways involved in mediating the cell- and context-dependent effects of TGFβ ligands. Thus, the of other signaling cascades that TGFβ both signals through and crosstalk with, (ie: MAP kinase, Rho and AKT/PI-3 kinase pathways), and the mechanism for signaling thorugh these pathways. This will allow to both target and use TGFβ signaling casacade as a potential target for therapies to treat different hematologic malignancies.

1.8.d. Signaling pathways in immune cell populations

During immune response, the activation of different cell populations as well as the beginning of several molecular/cellular processes, are tightly regulated and well-orchestrated processes. In particular, signal transduction through different group of receptors presented in lymphocytes determines the fate of these cellular populations [212, 481-497]. Additionally, activation of different signal transduction pathways in T/B cells occurs via the specific T-cell Receptor and B-cell receptor, TCR and BCR, respectively. Any deffects of these types of receptors can lead to different immunodeficiency, autoimmune/lymphoproliferative disorders, leukemias or lymphomas [493-497]. Interestingly, various events are known to activate by ligation of the BCR; however the critical parameters determining the biological outcome of the signal transduction are not fully understood. During the activation events, the involvement of different important molecules that act on phospholipid metabolism. BCR signaling results from the initial clustering of receptor-associated signaling elements, protein tyrosine kinase, transphosphoryaltion events and subsequently recruitment of signaling molecules from the cytoplasm to the membrane-associated complexes [498-517]. Besides the different protein components recruited here, the membrane phospholipids undergo phosphorylation/dephosphorylation changes during this signal transduction cascade, thereby ensuring the proper assembly of signaling complexes and the transduction of the signal from the plasma membrane, to the cytoplasm and into the nucleus. Phosphatidylinositols (PtdIins) are key components of the membrane and they exist in a wide variety of phosphorylated forms. The different types of generated phosphatidylinositols are regulated by kinases and phosphatases. In particular, it has been shown that the Phospholipase Cγ, (PLCγ), which participates in BCR signal transduction, is involved in the hydrolysis of phosphatidylinositl-4,5-biphosphatase, PI-3,

5-P_2 resulting in the production of soluble inositol-1, 4,5-triphosphate (IP_3) and membrane-anchored diacylglycerol (DAG). In addition, these molecules have been shown to act as second messenger in well characterized biological events [498, 499, 518-522]. IP_3 is able to stimulate and increase cytosolic free Ca^{2+} by activating release from the endoplasmic reticulum through IP_3-gated calcium channels and binding to IP_3R, DAG is able to binds and activates many protein kinase C (PKC) isoforms [523-526]. On the other hand, as with any other signaling cascade, the proper regulation of this signaling pathway is required and essential to maintain a proper cellular and molecular regulation. The phosphatidyl inositol 3-kinase (PI3K) is considered as a key player in the various survival and differentiation event during activation and regulation in lymphocyte cell populations [455, 485, 491]. The activation of the PI3K begins at the membrane level where different tyrosine kinase receptor complexes initiate their downstream cascades events through PI3K [516]. PI3K is a heterodimer complex molecule composed of a 110-kDa catalytic subunit (p110) and an 85-kDa (p85). Once activated, PI3K induces phosphoryaltion at the D-3' position of phosphoinositides (PtdIns), producing two specific phosphatidylinositol substrates: phosphatidylinositol-3, 4-biphosphate, PI-3, 4,P2 and phosphatidylinositol-3, 4,5-triphosphate, PI-3, 4,5-P_3 [527-533]. Interestingly, it has been demonstrated that PtdIns acts as second messenger serving as substrate for the recruitment of several proteins that require plasma membrane translocation for their activated. In the basal state, the levels of PtdIns are generally low, but they increase rapidly after activation of the different tyrosine kinase receptors by growth factors [534, 535]. In this context, signaling proteins such as Akt and Btk are recruited to the plasma membrane, via their pleckstrin homology (PH) domains, which can recognise and bind to PI (3,4,5) P3 with high affinity [536-538]. For example, Akt has highly affinity for PtdIns 3,4,5-P3 and recruited to the plasma membrane proximity, where it can interact with other kinases such as PDK1 and PDK2 resulting in Akt phosphorylation and activation. Once phosphorylated, Akt

is released from the plasma membrane to activate other specific downstream target signaling molecules involved in different biological events such as GSK-3 [536-538]. Moreover, some other PH-containing molecules, such as TAPP1/2, have more binding affinity for PI-3,4-P2 [539-543]. Through the action of specific lipid phosphatases, the attenuation of growth factor or cytokine-induced activation takes place [544]. This process is achieved by the actions of kinases and phosphatases that degrade the main signals generated by PI lipids [516]. Among these molecules, the two-inositol phosphatases have been implicated in the metabolism of PI-3,4,5-P3. The 54-kDa tumor suppressor PTEN, which is ubiquitously expressed is responsible for the hydrolysis of PI-3,4,5-P3 to PI-4,5-P2, whereas the 145-kDa haematopoietic-restricted SH2-containing inositol 5`-phosphatase SHIP (also known as SHIP-1), metabolizes PI-3,4,5-P3 to PI-3,4-P2, have been subject of large investigation for their crucial role in maintaining the proper balance in different cell types [499, 507, 545-549].

1.9. SHIP-1

1.9.a. SH2-containing inositol-5'-phosphatase 1, SHIP-1

Phospholipid metabolism has been extensively studied in the past year due to its critical role in regulating cell proliferation, differentiation, migration, morphology and apoptosis. The role and importance of the phosphatidylinositols in signal transduction is critical and is further illustrated by the complex pathways interconnecting the kinases and phosphatases that metabolize phosphatidylinositols [342, 399, 550-555].

The src homology 2 (**SH**2) domain-containing-5'-**I**nositol **P**hosphatase 1, SHIP-1, is known as an important negative regulator of proliferation, survival and cell activation in several haematopoietic cell types [545]. SHIP-1 is a 145 kDa intracellular protein that specifically hydrolyzes the D-5' position phosphtate of two known substrates: PtdIns-3,4,5-P3 and the inositol 1,3,4,5-tetrakisphosphate [342, 399, 556, 557].

SHIP-1 expression is found in all blood lineages, to various degrees of expression and it appears to change during haematopoietic cell development [399, 550-555]. SHIP-1 5' phosphatase activity is not regulated by tyrosine phosphorylation or by interaction with adaptor proteins but by its localization at the plasma membrane in proximity to phospholipids [558]. By breaking down PtdIns 3,4,5-P3 to PtdIns 3,4-P2, SHIP inhibits the activation of PH domain-containing proteins such as the survival kinase AKT (PKB). Thus a better understanding of the different roles that inositol phosphates play in immune cell functions is crucial. SHIP-1 is a important player during different cellular states such as survival, differentiation, prolifeation and apoptosis among others [399, 558]. See Figure 1.12 in pages 67-68.

Figure 1.12 Model of the phosphatase activity of SHIP and its effects in different downstream signaling pathways. This figure represents the role of SHIP-1, and its role in different cellular pathways. The different cascades represented in this figure indicate the putative signaling affected downstream SHIP-1's phospholids products: PI-3,4,5-P3 and PI-3,4-P2.

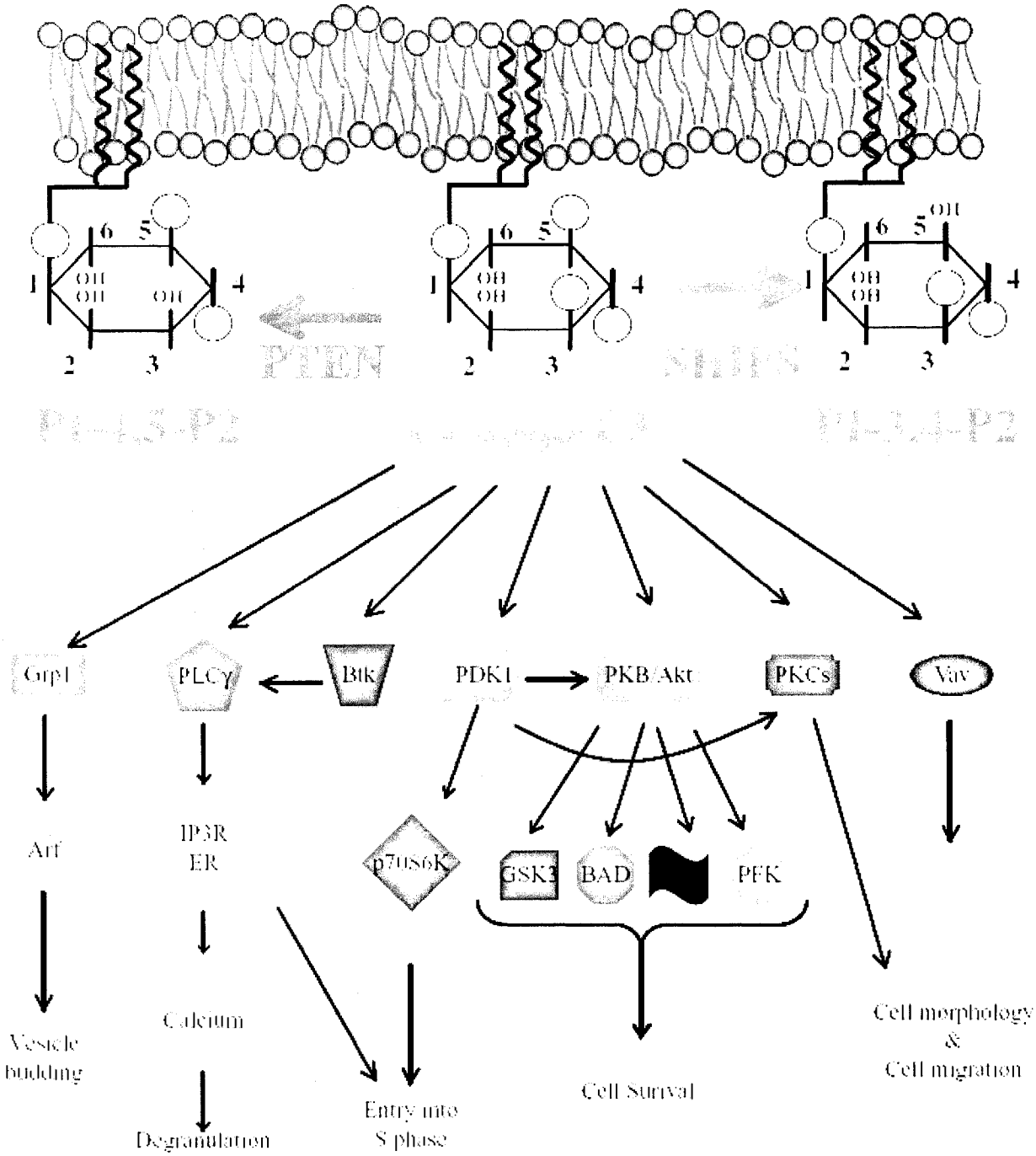

PTEN
SHIP
PI-4,5-P2
PI-3,4-P2
Grp1
PLCγ
Btk
PDK1
PKB/Akt
PKCs
Vav
Arf
IP3R
ER
p70S6K
GSK3
BAD
PFK
Vesicle budding
Calcium
Degranulation
Entry into S phase
Cell Survival
Cell morphology & Cell migration

Figure 1.12.

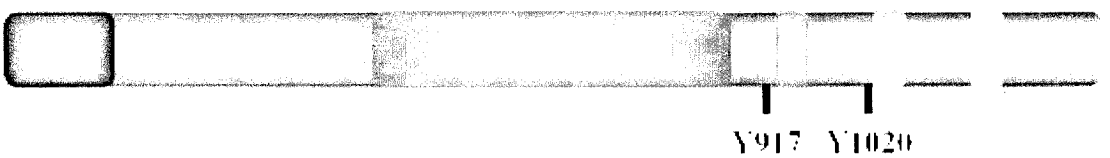
1.9.b. Identification and Structure of SHIP-1

Initially, SHIP-1 was originally identified by several groups in the early 1990's as a 145 kDa protein that was tyrosine phosphorylated by different stimuli in immune cells [545, 559]. In 1996, the cDNA encoding SHIP-1 was cloned [560-563]. Gene structure analysis has revealed that the human SHIP-1 contains 1188 amino acids, sharing approximately 87.2% sequence identity with the murine SHIP-1, composed of 1190 amino acids. This molecule contains several motifs that are used to sustain different protein-protein interactions with molecules such as Grb-2 and Shc [558-561, 564]. The NH2 terminus is the most evident motif spanning approximately the first 100 amino acids and it binds with high affinity to the conserved pY(Y/D)X(L/I/V) sequence in different intracellular regions of several immune receptors. In addition a well-defined phosphatase domain that regulates its enzymatic activity. This is a particular feature of the different members of the SHIP family of inositol 5' phosphatases. This is the catalytic domain, which carries the phosphatase activities of this molecule. SHIP-1 dephosphorylates inositol rings at the 5' position specifically [565, 566]. In addition SHIP-1 also posseses two NPXY sequences that bind when phosphorylated to different proteins bearing PTB domains. Finally, the C-terminus of the molecule contains several regions including three putative SH3 interacting motifs, and two potential phosphotyrosine binding (PTB) domain binding sites [558, 560, 561, 564]. A schematic representation of the general structure of the phosphatydilinositol 5' phosphatase SHIP-1 is shown in Figure 1.13 (pages 69-70).

Figure 1.13. Schematic representation of the molecular structure of SHIP-1. The SH2 domain inositol 5'-phosphatase domain and tyrosine in the C-terminus are depicted. The proline-rich sequences in the C-terminus are shown. SHIP-1 (alson known as SHIPα) has an apparent molecular mass of 145 kDa and its expression has been detected only in haematopoietic cell types.

5' IPase Domain:

Central Catalytic domain which
catalyzes the conversion of
the specfic following substrates:
PI-3,4,5-P3 to PI-3,4-P2
&
I-1,3,4,5-P4 to I-1,3,4-P3



SH2 domain:

Protein-protein interaction domain
(NH2-terminus)
Regulates the interaction with
different phosphorylated
domains of TTMS, TEAMS of the
FCγRIIB1 and different cytoplasmic
proteins:
Shc, p26 dok, Dok-3, Dab-1,Grb2, c-Abl

Phoshotyrosine mediate
interaction with
PTB & SH2 domains.
Proline-rich regions
interact with SH3 domains

Figure 1.13.

1.9.c. SHIP family of proteins

SHIP-1-related proteins have been identified [559]. Western blot analysis against SHIP-1 has revealed the existence of at least seven different potential immunoreactive species. The diversity of different detectable proteins vary depending on the cell type, cell/tissue maturity and expression time that are being analysed. The existence of several SHIP isoforms suggests the possibility of specific and/or overlaping roles for each one of these isoforms on a special-tempo manner [399, 559, 567].

The expression of the different SHIP family members (SHIPα, β and δ) is detectable mainly in haematopoietic cell types [399, 545, 549, 556, 557, 559, 568, 569]. Two related SHIP-1 isoforms SHIPβ (135 kDa) and SHIPδ (110 kDa) have been identified. SHIPβ has a 183 base pair frame deletion at the C-terminus, resulting in a protein of 918 amino acids that lacks one of the proline-rich regions [558, 568, 570]. The expression level of SHIPβ has been observed in different myeloid cell as well as A20 B cell lines [399, 559, 568]. SHIPδ is the other family member, which lacks 167 base pairs region. This molecule shows a significant shift in the reading frame, producing 41 unique amino acids before a premature stop codon, resulting in a complete abscense of the COOH-terminus of the phosphatase. Thus, SHIPδ has a sequence of 918 amino acids, and obviously lacks the tyrosine 1020 and the proline-rich regions.

Recently a new member of the SHIP family has been identified, SHIP-2, which is coded by a different gene. In addition, SHIP2 expression is more ubiquitous than that SHIP-1 and detectable in more somatic tissues and cell types [571, 572]. SHIP-2 shares a high homology with SHIP-1 especially in the SH2 domains, indicating the possibility of divergence of their binding partners. The inositol phosphatase domains also share high level of similarities, and both hydrolize the 5' position of the inositol ring from PtdIns-3,4,5-P3 and I-1,3,4,5-P4 respectively.

Both TGFβ and activin, induce SHIP-1 expression in different immune cell types [399], whereas SHIP-2 expression increases in B cells after a specific cell activation [573, 574]. Interestingly, it has been observed that SHIP-2 has only one NPYX motif and several proline-rich domains. Thus, SHIP family members are important in controlling and modulating phospholipid metabolism, in particular keeping the proper balance between phosphorylated products PIP3/IP4 and unphosphorylated PIP2/IP3. The outcome of this phospholipid metabolism ensures proper regulation of cell proliferation and survival, apoptosis as well as cell migration among others.

1.9.d. SHIP-1-associated signaling molecules

SHIP-1 is an intracellular phosphatase that is tyrosine phosphorylated by different members of the Src kinase family [510, 558, 568, 570, 575-578]. As mentioned previously SHIP-1 phosphatase contains different regulatory as well as catalytic regions in its structure suggesting an important role for this phosphatase as an adaptor protein as well as an enzyme.

Furthermore, several proteins that interact with SHIP-1 regulate different cellular and molecular responses. SHIP-1 is required for the attenuation of activated T and B cell populations [579, 580]. In this scenario, it has been shown that SHIP-1 is recruited to the plasma membrane by its SH2 domains interaction with the immune receptor inhibitory motif (ITIM) localized in the tail of different inhibitory receptors [579, 580]. Since SHIP-1 has been considered as a negative regulator of cellular signaling, its interaction with ITIMs is expected. SHIP was found to directly interact with FcγRIIB1, which is the inhibitory low affinity receptor for IgG antibodies [581-583].

SHIP-1 was originally identified as an interacting partner of immune receptor tyrosine based activating motif [175] of the β and γ subunits of FcεRI, through its SH2 domain, particularly with the which is a high affinity receptor for IgE antibodies [562]. SHIP-1 can also interact via its SH2 to the ITAM motif of CD3 γ, δ and ε chains as well as the T cell receptor ζ

chain, c-Met [584], CDw150/SLAM and the erythropoietin receptor [585-587]. SHIP-1 also interacts with gr49b1, inhibitory co-receptor family member, and cytoplasmic proteins like Shc [585], p62dok, Dok-3, and Dab-1, p85 subunit of the PI3K [507, 575, 588, 589]. These interactions are regulated via the different tyrosines presented at the C-terminus of SHIP-1 and the different SH2 domains of the interacting molecules. In addition, SHIP-1 presents proline-rich regions in the C-terminus, which enables it to interact with different SH3-containing molecules. In this context Grb2 and Src associate with SHIP-1 through its SH3 domain [590, 591].

1.9.e. Biology of SHIP-1

It has been shown that SHIP-1 becomes tyrosine phosphorylated after stimulation of immune cells by several types of cytokines and associates with Shc [556, 557]. SHIP-1 is directly recruited to T and B cell antigen receptor through out the association of T cell co-stimulatory receptor such as CD28 in immune cell types [545, 551, 561, 592-594]. In addition, it has been reported that the FcγRIIb co-receptor enables the B cell to distinguish between free antigen and immune complexes composed of antigen bound to IgG antibodies during humoral immune response. The co-ligation of the B cells has served as an excellent model to study the role of SHIP-1 in attenuating this signaling pathway in immune cells. This mechanism suggests that FcγRIIB is able to inhibit calcium influx and downstream responses that are regulated by these immune receptors [498, 499, 509, 559, 571, 573, 574, 581, 582, 595-599].

SHIP-1 is involved in the phospholipid metabolism since it is able to hydrolyze specifically two substrates, the phosphatydilinositol-3,4,5-P3, (PtdIns-3, 4,5-P3), and the inositol 1,3,4,5-tetrakisphosphate (IP4) *in vitro* [342, 399, 556, 557]. These two specific inositols act as a

second messenger in different growth factor mediated signaling pathways. It has been reported that the phosphatase activity of SHIP-1, does not change upon growth factor stimulation [399].

Additionally, SHIP-1 inhibits receptor activation in myeloid cells, mast cells and B cells by binding to the tyrosine phosphorylated immunoreceptor tyrosine-based inhibitory motif [ITIM; Py(Y/D)X(L/I/V)], of the inhibitory co-receptor FcγRIIB via its SH2 motif [545, 559, 561, 563, 582, 583, 594, 595, 600]. SHIP-1 translocation to the proximity of the plasma membrane, allows the phosphatase to interact with the SH2 domains of the intracellular regions of TCR or BCR via the interaction between ITIM. Once at the plasma membrane, SHIP-1 generates PIP2 from PIP3 to PIP2 and also prevents any membrane translocation of PH domain containing proteins such as Akt, BTK or PDK1 [84, 498, 601-606]. SHIP-1 gene disruption (SHIP-$1^{-/-}$gene) in mice leads to a myeloproliferative disorder due to a increased sensitivity of the haematopoietic cells for several cytokines including GM-CSF and IL-3 [556, 569, 607]. These observations confirm the important role of this inositol phosphatase in the immune regulation and physiology [554, 607].

I.9.f. Synthesis and Degradation of SHIP-1

The proper balance in protein synthesis and degradation is crucial for a proper homeostasis of the organism. It has been observed that SHIP-1 is detectable at the 7.5 day in mice embryos [558]. In addition, SHIP-1 expression is restricted the haematopoietic tissue. Moreover, pulse-chase experiments have revealed that the different isoforms of SHIP are generated that display similar half lives of about 10h [557, 558, 567]. Thus, these results have suggested the possibility that different smaller forms can be generated by translational modification. Interestingly, reduced expression of SHIP-1 occurs in different patients with leukemia [608]. Its decreased expression correlates with increased expression of BCR-ABL [591, 599, 608-611]. Taken together these results suggest that SHIP-1 could be considered as a tumor

suppression gene during myelopoiesis, since its downregulation seems to be required for the development of different leukemias particularly chronic myeloid leukemia.

1.9.g. SHIP-1 and Immune cell signaling attenuation

The role and importance of the phosphatidyl inositols in signal transduction are critical as illustrated by the different complex pathways that are inter-connect the kinases and phosphatases that metabolize phosphatidyl inositols [342]. A key and central player is PtdIns 3,4,5-P3 that acts as a second messenger in response to a large array of extracellular signals [342]. PtdIns 3,4,5-P3, like PtdIns 3,4-P2, are the products by the enzyme PI3' kinase (PI3'K), which is one of the more relevant kinases involved in the regulation and induction of cell survival in different cell types. Indeed, survival signaling pathways usually trigger activation of the PI3' kinase through transmembrane receptor, which contain intrinsic tyrosine kinase activity, recruit cytoplasmic tyrosine kinases or couple to seven transmembrane G protein-couple receptors [612-616]. Both types of phospholipids, PtdIns 3,4-P2 and PtdIns 3,4,5-P3 act as signaling intermediates and regulate downstream signal transduction cascades required for the proper maintenance of the cell [617]. In addition, it has been shown that they can activate a number of cellular intermediates and modulate different downstream molecules in particular, PH-containing domain proteins such as AKT, (PBK), Btk, PDK1, GRP-1, Vav, and PLCγ-1. These molecules need to be targeted to the plasma membrane for their activation (see figure 1.12, pages 70-71) [545, 557, 618-621]. One of these kinases, AKT, has been implicated in the control of cell survival. Binding of the phospholipids to AKT results in translocation of this kinase from the cytoplasm to the plasma membrane that is critical for its complete activation [622, 623]. Relocation of AKT to the plasma membrane brings it in proximity to its regulatory kinases such as PDK1 and PDK2. These latter are the responsible of AKT phosphorylation and its activation [624-626]. This phosphorylation

evets occurs specifically on Thr308 and Ser473, which are required for fully kinase activation [606].

1.9.h. SHIP-1 and apoptosis in immune cells

SHIP-1 is a 145 kDa intracellular protein that recognizes and hydrolyzes the D-5' phosphate of PtdIns 3,4,5-P3 [545, 561, 563, 594]. It has been shown that the expression of SHIP-1 is haematopoietic-restricted. In addition, SHIP-1 expression varies in different immune cellular events as well as during haematopoiesis development [627]. The SHIP-1 knockout mice, are viable and fertile but have a shortened lifespan due to myeloid infiltration of vital organs [553, 607, 628]. The increased myeloid cell proliferation observed in the SHIP-1 knockout mice has been associated with a significant increase in the levels PtdIns 3,4,5-P3 as well as AKT activation. These observations clearly illustrate the critical role played by this phosphatase in apoptosis of different immune cell types. In addition, our group has demonstrated that the SHIP-1 expression and its activity are directly regulated in a Smad-dependent way in different haematopoietic cell types. This increase in expression and phosphatase activity is related with TGFβ-induced cell growth arrest and apoptosis [84]. This study, described in chapter2, demonstrates that TGFβ1-induced SHIP-1 expression inhibited the activation of Akt leading to apoptosis, even after treatment with survival factors such as IL-6 that activate Akt [498, 551].

1.12. SUMMARY

Control of immune cell proliferation, activation and subsequent elimination by cell growth arrest and apoptosis is critical for controlling infections and preventing autoimmune disease. The TGFβ family is a large family of widespread and evolutionarily conserved polypeptide growth factors that regulate growth, differentiation, embryogenesis and apoptosis in nearly all cell types. Based on the critical roles of the different family members in different biological processes, deregulation of their signaling has been implicated in multiple human disorders including cancer. Extensive observations and experimental data built through over the last two past decades of research have indicated that the TGFβ and activin family of growth factors play an important role in controlling apoptosis in various cell types of the immune system. In addition, abnormal expression of TGFβ receptors has been described in several immune disorders. However, the regulation of TGFβ target genes and their signaling mechanisms involved in such immune disorders is not completely known.

Thus, this is the core hypothesis tested through the research presented in this thesis. These findings should help to establish a new link between two important signaling pathways machinery, TGFβ signaling cascade and the phospholipids metabolism. Better understanding of this link will allow for the development of disease mechanism-targeting solutions for disordes involving different disregulation or uncontrolled cellular process in the immune system.

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CHAPTER 2

Activin/TGF-β induce apoptosis through Smad-dependent expression of the lipid phosphatase SHIP-1

2.1. Prologue

Control of immune cell proliferation, activation and subsequent elimination by cell growth arrest and apoptosis is critical for controlling infections and preventing autoimmune disease. The TGFβ and activin family of growth factors play an important role in controlling apoptosis in various cell types of the immune system. However, there is very limited information regarding their target genes and their signaling mechanisms.

Using a screen for genes that are differentially regulated by TGFβ ligands in haematopoietic cells, I found that SHIP-1 is strongly up regulated by activin and TGFβ. Based on these observations, I analyzed the ability of TGFβ and activin to induce cell growth arrest in immune cells through up-regulation of SHIP-1. In addition, I demonstrated that activin/TGFβ-induced SHIP-1 was related with a decrease in PtdIns3,4,5-P3 levels with a direct inhibition of the survival kinase Akt. Thus, the results presented in this chapter demonstrated for the first time a link for the first time TGFβ-induced apoptosis to phospholipid metabolism and enlarge our knowledge of the mechanism of action of the activin/TGFβ serine kinase receptors.

2.2. ABSTRACT

Members of the TGFβ family regulate fundamental physiological processes, such as cell growth, differentiation and apoptosis in nearly all cell types [1]. As a result, defects in their signaling pathways have been linked to uncontrolled cell proliferation and carcinogenesis [1]. Given their critical role, here we explored the signal transduction mechanisms, downstream of the activin/TGFβ receptors, leading to cell growth arrest/apoptosis. We show that TGFβ family members regulate apoptosis in hematopoietic cells through expression of the inositol phosphatase SHIP-1, a central regulator of phospholipids metabolism [2]. We further demonstrated the requirement of the Smad pathway in the transcriptional regulation of the SHIP-1 gene. Activin/TGFβ-induced SHIP-1 expression and activity leads to intracellular changes in the pool of phospholipids, as well as in inhibition of the phosphorylation of the protein kinase AKT and cell survival. Our results link for the first time phospholipids metabolism to activin/TGFβ-mediated apoptosis and define TGFβ family members as potent inducers of SHIP-1 expression.

2.3. INTRODUCTION

TGFβ and activin belong to a large family of ubiquitous and evolutionary conserved polypeptide growth factors that play a critical role in controlling cell growth and apoptosis. TGFβ and its receptors are expressed by almost every cell in the body and deregulation of its signaling pathways have been implicated in multiple human disorders including cancer [1]. Similarly, activin originally isolated from gonadal fluid [3] is also an important regulator of cell growth and apoptosis of various cell types [4]. TGFβ and activin signal through a complex of two serine kinase receptors that upon ligand stimulation recruit and phosphorylate Smad2 and Smad3, the canonical downstream effector molecules for these receptors. Once activated Smad2 and Smad3 heterodimerize with the tumor suppressor protein Smad4 and translocate to the nucleus where they participate in transcriptional activation of target genes. However, the nature of these genes and the downstream biochemical processes that they regulate to control cell growth remain largely unknown.

2.4. RESULTS AND DISCUSSION

TGFβ and activin induce growth inhibition and apoptosis of both cultured and primary lymphocytes [4-6]. Down-regulation of Bcl2 family members [7-9], NF-Kappa B inhibition [6], up-regulation of the proapoptotic factor Bax [10], or activation of caspase proteases [11] have all been reported in response to TGFβ and activin in various myeloid leukemia and B-lymphoma cell types. It was also recently shown that the death adaptor protein Daxx could mediate TGFβ-induced apoptosis in B-cell lymphomas [12]. To begin to elucidate the mechanism of action of these growth factors we initially screened for genes that were differentially regulated by activin and TGFβ by gene profiling using gene

DNA chip technology. We used murine plasmocytoma, MPC-11, hybridomas, B9, myeloma, M1 as well as purified human primary lymphocytes in which cell viability was potently decreased by 40 to 50 percent in response to activin and TGFβ respectively (Fig.2.4.1.a, pages 115-116). The effect of activin and TGFβ is observed primarily on apoptosis as shown by Annexin V labeling in B9 hybridoma cells (Fig.2.4.1.b, see pages 117-118) and flow cytometry analysis (Fig.2.4.1.c, see page 119-120).

SHIP-1 is a 145 kD intracellular phosphatase that hydrolyzes the D-5' position phosphate of both phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P_3) and 1,3,4,5-tetrakisphosphate (IP_4) [13] and plays a critical role in cell growth regulation [2]. As shown in Fig. 2.4.2.a, activin induces a clear and rapid increase in SHIP-1 mRNA in MPC-11 cells and exert its effect through a direct transcriptional regulatory mechanism, as it is not affected by the translational inhibitor cycloheximide. The same result was obtained in B9 cells and M1 cells treated with activin or TGFβ (data not shown) (Fig.2.4.3.a, pages 123-124).

Figure 2.4.1.a Activin and TGFβ induce apoptosis in hematopoietic cells.

Cell viability colorimetric assay (MTT) [3-(4,5-Dimethylthiazolyl-2)-2,5 Diphenyltetrazoliumbromided (MTT)] in triplicate of plasmocytoma MPC-11, hybridoma B9, myeloma M1 cells and purified human lymphocytes, stimulated or not with activin or TGFβ respectively for seventy-two hours. Values are expressed in arbitrary units.

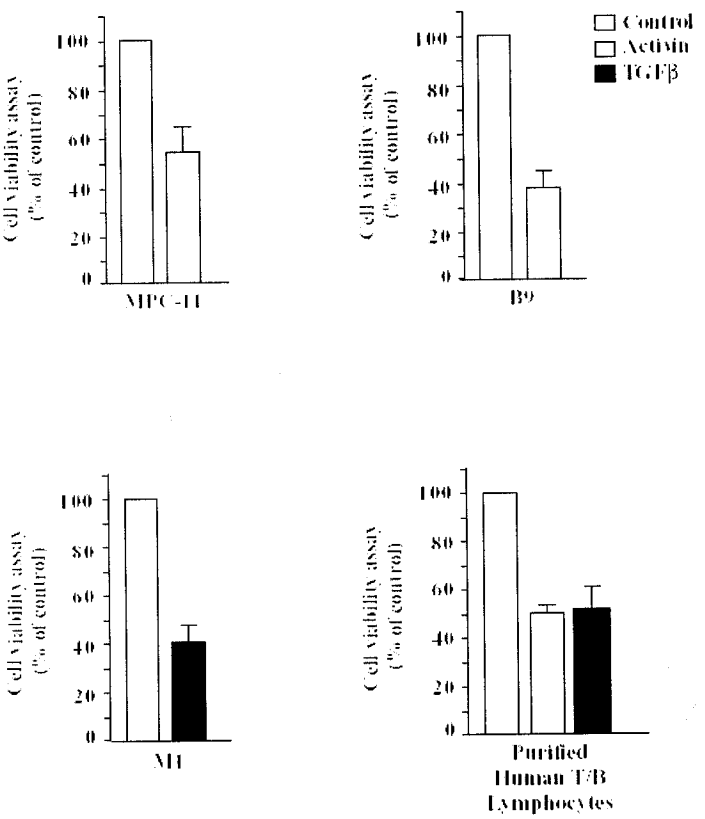
Control
Activin
TGFβ
Cell viability assay
(% of control)
100
80
60
40
20
0
MPC-11
B9
MH
Purified
Human T/B
Lymphocytes

Figure 2.4.1.a.

Figure 2.4.1.b. Activin and TGFβ induce apoptosis in hematopoietic cells.

B9 cells were stimulated for different periods of time with activin as indicated. The level of apoptosis obtained was analyzed by Annexin V labelling. The treated cells were analyzed by direct immunofluorescence.

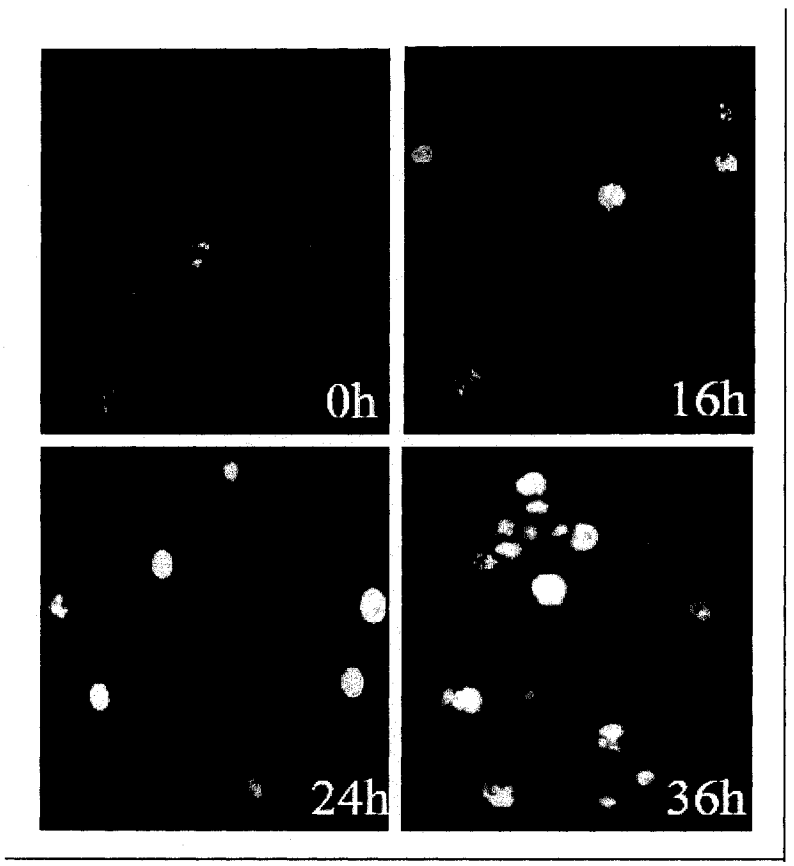

Figure 2.4.1.b.

Figure 2.4.1.c. Activin and TGFβ induce apoptosis in hematopoietic cells.

MPC11, B9 and M1 cells were stimulated or not with activin and TGFβ respectively for 72 hours. The distribution of cells in the cell cycle was quantified by analysis of propidium iodide stained cells using flow cytometry.

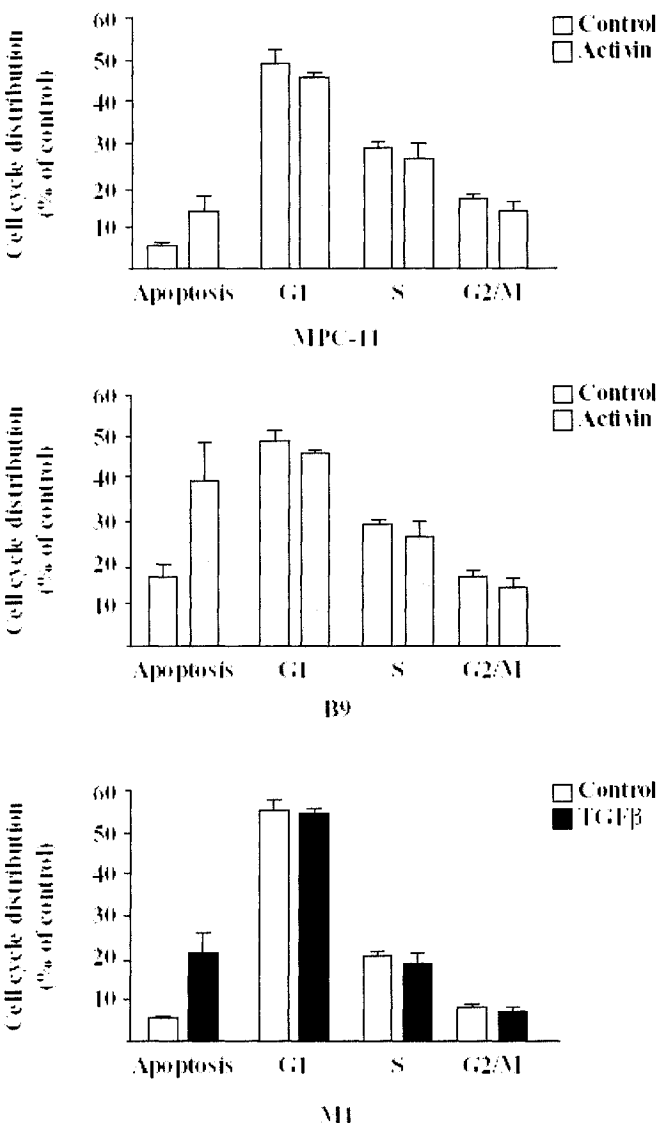
Cell cycle distribution
(% of control)
60
50
40
30
20
10
Control
Activin
Apoptosis
G1
S
G2/M
MPC-11
Control
Activin
B9
Control
TGFβ
M1

Figure 2.4.1.c.

Figure 2.4.2.a. Activin and TGFβ induce SHIP-1 mRNA levels.

Total mRNA extracted from MPC-11 cells that were stimulated with activin for 0, 8 and 24 hours. Affymetrix Gene Chip Mu11kSubB microarray was performed using this mRNA. Reverse transcription reactions were performed to confirm the microarray results using oligo-dT and cDNAs were amplified using specific oligonucleotides to SHIP-1 and GAPDH as a control. The results were analyzed by densitometry and values are expressed as fold-induction compared to the control after normalization to the GAPDH mRNA levels.

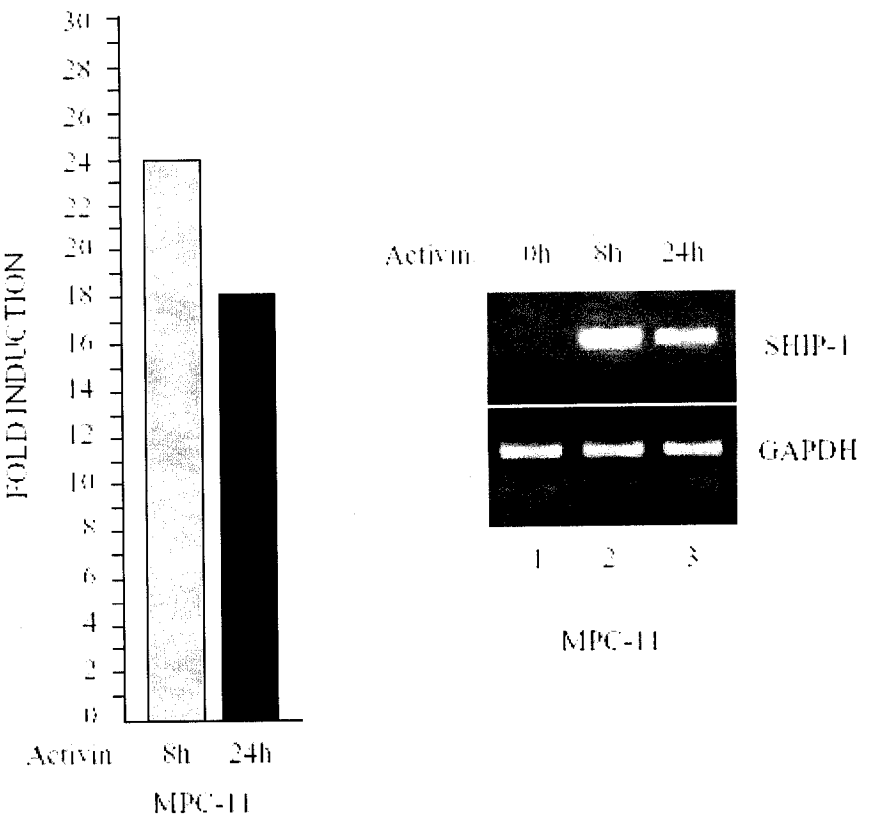
FOLD INDUCTION
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Activin
8h
24h
MPC-11
Activin
0h
8h
24h
SHIP-1
GAPDH
1
2
3
MPC-11

Figure 2.4.2.a.

Figure 2.4.3.a. Activin and TGFβ induce SHIP-1 mRNA levels. MPC-11, B9 and M1 cells were stimulated with activin for 0, 15', 30', 1h, 2h, 4h, 8h, 16h, and 24 hours with or without cycloheximide pretreatment (10ug/ml for 3h). Reverse transcription reactions were performed using oligo-dT and cDNAs were amplified using specific oligonucleotides to SHIP-1 and GAPDH as a control. The results were analyzed by densitometry and values are expressed as fold-induction compared to the control after normalization to the GAPDH mRNA levels.

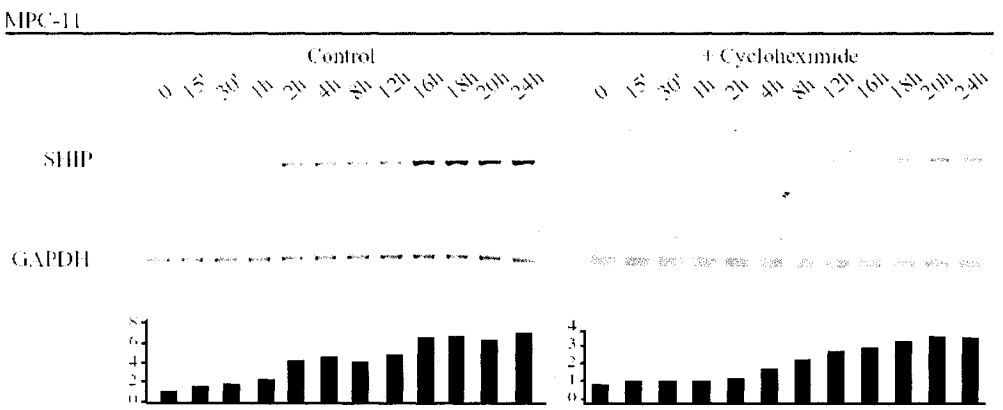
MPC-11
Control
+ Cyclohexmide
0 15' 30' 1h 2h 4h 8h 12h 16h 18h 20h 24h
SHIP
GAPDH

Figure 2.4.3.a.

To determine if the activin-induced increase in SHIP-1 mRNA level was followed by an increase in SHIP-1 protein expression, MPC-11, B9, M1 cells and purified human lymphocytes were stimulated with activin or TGFβ for different periods of time and total cell lysates analyzed by Western blots using a specific monoclonal antibody to SHIP-1. As shown in Fig.2.4.3.b (pages 126-127), while there was little or no detectable expression of SHIP-1 in non-stimulated cells, a clear increase in SHIP-1 protein expression was observed when cells were treated with activin/TGFβ. This effect was observed within 4 to 8 hours following stimulation and was further increased at 16 and 24 hours. The anti-SHIP-1 antibody recognized two forms of SHIP-1 in our cell lines, a predominant 145 kDa form and a much lower expressed 135 kDa form (Fig.2.4.3.b, pages 126-127). This is in agreement with that observed in human bone marrow cell and peripheral blood mononuclear cells where high levels of the 145 kDa and lower levels of a 135 kDa form, generated by alternative splicing, are expressed [14]. As a control, the blots were stripped and reprobed with an antibody directed against Stat3 to demonstrate equal loading (Fig.2.4.3.b., lower panels, pages 126-127).

Together our results highlight TGFβ family members as direct positive transcriptional regulators of SHIP-1 expression and define a novel-signaling pathway for these growth regulatory factors. Interestingly, the 3' lipid phosphatase PTEN, also known as TEP1 (TGFβ-regulated and epithelial-cell-enriched phosphates) is also regulated by TGFβ [15], suggesting that these two phosphatases share a common mechanism of action in regulating cell death in response to TGFβ family members.

Figure 2.4.3.b. Activin and TGFβ induce SHIP-1 protein expression.

MPC-11, B9, M1 cells and human purified lymphocytes were stimulated with activin or TGFβ for 0, 4, 8, 16, and 24h. Total cell lysates were obtained and analyzed by immunoblot using a specific monoclonal antibody against SHIP-1 (upper panel). The blot was stripped and reprobed with an anti-Stat3 antibody to confirm equal loading (lower panel).

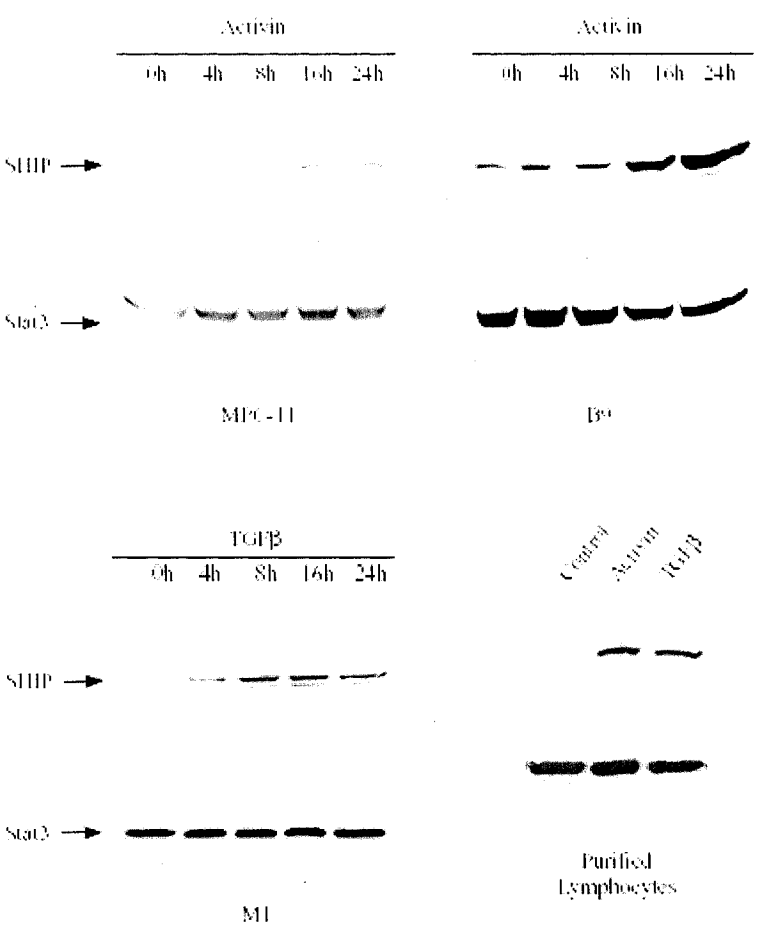
Activin
0h 4h 8h 16h 24h
SHIP
Stat3
MPC-11
Activin
0h 4h 8h 16h 24h
B9
TGFβ
0h 4h 8h 16h 24h
SHIP
Stat3
M1
Control
Activin
TGFβ
Purified
Lymphocytes

Figure 2.4.3.b.

To then characterize the transcriptional mechanisms by which activin/TGFβ induce expression of SHIP-1 mRNA, we cloned a 1.4 kb fragment from the 5' regulatory sequence of the mouse SHIP-1 gene, based on the genomic structure of the SHIP-1 gene [16]. The SHIP-1 gene promoter was then subcloned upstream of the firefly luciferase gene in PGL3 basic vector (SHIP-1-Lux) and its activity was strongly induced by activin and TGFβ (Fig. 2.4.4.a, pages 130-131).

To investigate the role of the Smad pathway in activin/TGFβ-induced SHIP-1 gene promoter activation, we used murine embryonic fibroblasts (MEFs) established from Smad4 knock-out mice, in which the Smad pathway is inactivated [17]. Wild type $MEFs^{(+/+)}$ and $Smad4^{(-/-)}$ MEFs were transfected with either SHIP-1 -Lux or ARE-Lux, another activin/TGFβ responsive promoter construct, used as a control. While both gene promoter constructs were strongly activated by activin and TGFβ in $MEFs^{(+/+)}$, this effect was abolished in the $Smad4^{(-/-)}$ MEFs but fully restored when Smad4 was co-transfected, confirming the requirement and critical role played by the Smad pathway in the mediation of these effects (Fig.2.4.4.b, pages 132-133).

To further investigate the specific role played by Smad2 and Smad3 in activin/TGFβ-mediated SHIP-1 gene promoter induction, 293 cells were co-transfected with the SHIP-1-Lux reporter construct and cDNAs encoding for Smad2, Smad3, Smad4, the inhibitory Smad7 [18] or the dominant negative forms of Smad2 and Smad3 (ΔNSmad2 and ΔNSmad3) [19, 20]. The 293 cells express relatively low levels of TGFβ receptors but they do respond to both activin and TGFβ (Fig. 2.4.4.a, see pages 130-131) and transient transfection of these cells is usually achieved at very high efficiency. Overexpression of Smad2, Smad3 or Smad4 significantly increased activin/TGFβ-

mediated activation of the SHIP-1 promoter, while expression of Smad7, ΔNSmad2 and ΔNSmad3 completely abolished these effects (Fig.2.4.4.c. lower panel, see pages 136-137). Together these results demonstrate the requirement of the Smad pathway downstream of the activin and TGFβ receptor signaling cascade for activation of the SHIP-1 gene promoter.

Figure 2.4.4.a. Activin/TGFβ-induced SHIP-1 transcription requires Smad2, Smad3 and Smad4.

293 cells were transfected with the SHIP-1 -Lux reporter construct or empty luciferase vector (pGL3) and the β-galactosidase expression plasmid. Cells were stimulated with either activin or TGFβ for 18h. The luciferase activity was normalized to β-galactosidase values. Results represent means and standard deviations of three independent experiments.

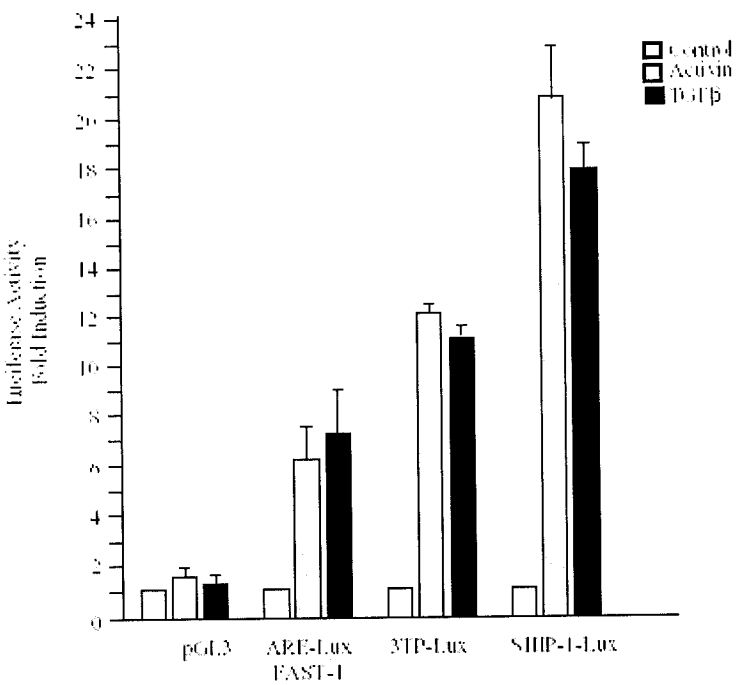

Figure 2.4.4.a.

Figure 2.4.4.b. Activin/TGFβ-induced SHIP-1 transcription requires Smad signaling pathway.

Mouse embryonic fibroblasts (MEFs) established from the Smad4 knockout mice $^{(-/-)}$ or from wild type mice $^{(+/+)}$ were transfected with the SHIP-1 -Lux and ARE-Lux/Fast1 promoter constructs with or without an expression vector encoding Smad4. The activin/TGFβ response was measured using a luciferase assay. The Luciferase activity was normalized to b-galactosidase values. Results represent means and standard deviations of three independent experiments.

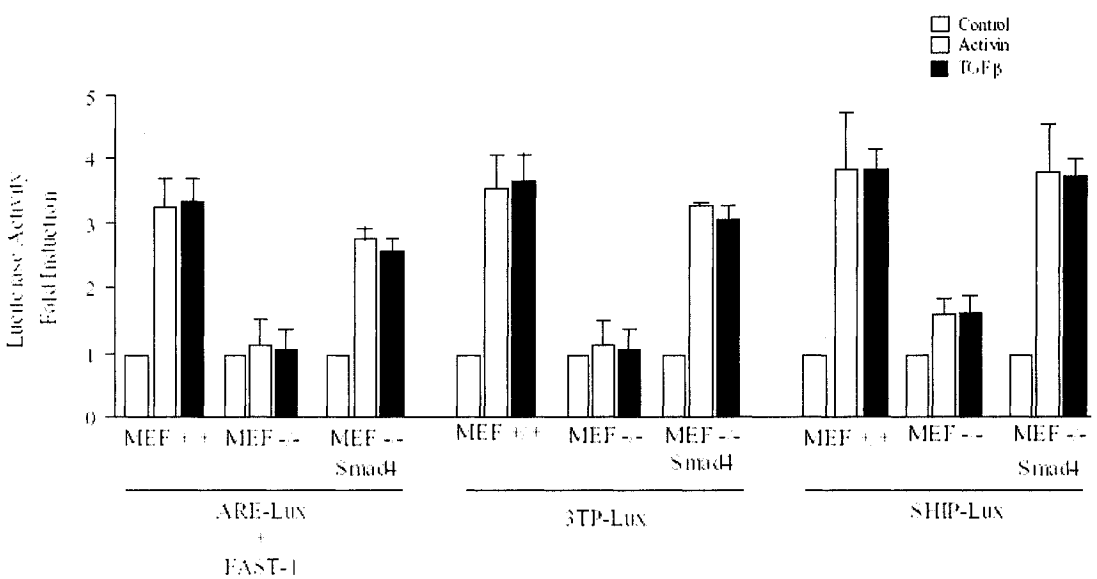
Control
Activin
TGFβ
Luciferase Activity
Fold Induction
0
1
2
3
4
5
MEF + +
MEF - -
MEF - -
Smad4
ARE-Lux
+
FAST-1
3TP-Lux
SHIP-Lux

Figure 2.4.4.b.

SHIP-1's 5'phosphatase activity does not change significantly following cytokine stimulation [21], suggesting that its effects are regulated by the level of expression [2]. To determine whether activin/TGFβ-induced SHIP-1 expression is followed by a concomitant increase in SHIP-1 phosphatase activity, we measured the intracellular levels of SHIP-1's two substrates, IP_4 and PtdIns3,4,5-P3, as described in Methods. As shown in Fig.2.4.5.a (see pages 138-139), activin and TGFβ induced a marked increased in SHIP-1 expression and a clear decrease in IP_4 levels while the levels of IP_3 were concomitantly increased. This is interesting since SHIP-1 's ability to hydrolyze IP_4 has only been clearly demonstrated *in vitro* using 5'-phosphatase assays [13], and no report of any changes in IP_3 and IP_4 levels have been reported with cells from SHIP-$1^{-/-}$ mice [22]. Therefore, our findings provide the first evidence demonstrating that SHIP-1 functions as an IP_4 phosphatase *in vivo*. It will be interesting to determine in future studies if the changes in IP_3/IP_4, resulting from TGFβ/activin-induced SHIP-1 expression and activity, affects the entry of extracellular calcium and subsequent activation of calcium dependent protein kinases since these events are tightly regulated by IP_3 [23].

To measure PtdIns3,4,5-P3 levels, total phosphatidylinositols from B9 cells, stimulated or not with activin for different periods of time were extracted, separated by thin layer chromatography and analyzed by autoradiography. As shown in Fig. 2.4.5.b., (pages 140-141), treatment of the cells with activin led to a marked decrease in PtdIns3,4,5-P3 levels after 6 to 24 hours of treatment, correlating with the activin effect on SHIP-1 -increased expression (Fig.2.4.3.b, pages 126-127). The same effects were observed in MPC-11 and M1 cells stimulated with activin or TGFβ (Figure 2.4.5.b., lower panel, pages 140-141). The observed decrease in PtdIns3,4,5-P3 is unlikely due to a

change in PTEN activity as no change in PTEN mRNA was observed in our Affymetrix experiment (1.15 fold-induction) or in PTEN protein expression in MPC-11 cells treated with activin (Fig.2.4.6.a, pages 148-149), confirming that this effect is specifically due to activin/TGFβ-mediated SHIP-1 expression. Together, our data clearly indicate that activin/TGFβ-mediated SHIP-1 protein expression is followed by an increase in SHIP-1 lipid phosphatase activity. Phospholipid metabolism plays a critical role in regulating cell growth and apoptosis.

Figure 2.4.4.c. Smad2, Smad3 and Smad4 are required and enhanced the Activin/TGFβ-induced SHIP-1 transcription gene promoter.

293 cells were transiently transfected with the SHIP-1-Lux reporter construct in the presence of various Smad expression plasmids, as indicated, stimulated with either activin or TGFβ. The promoter activation was assessed for luciferase activity. The Luciferase activity was normalized to β-galactosidase values. Results represent means and standard deviations of three independent experiments.

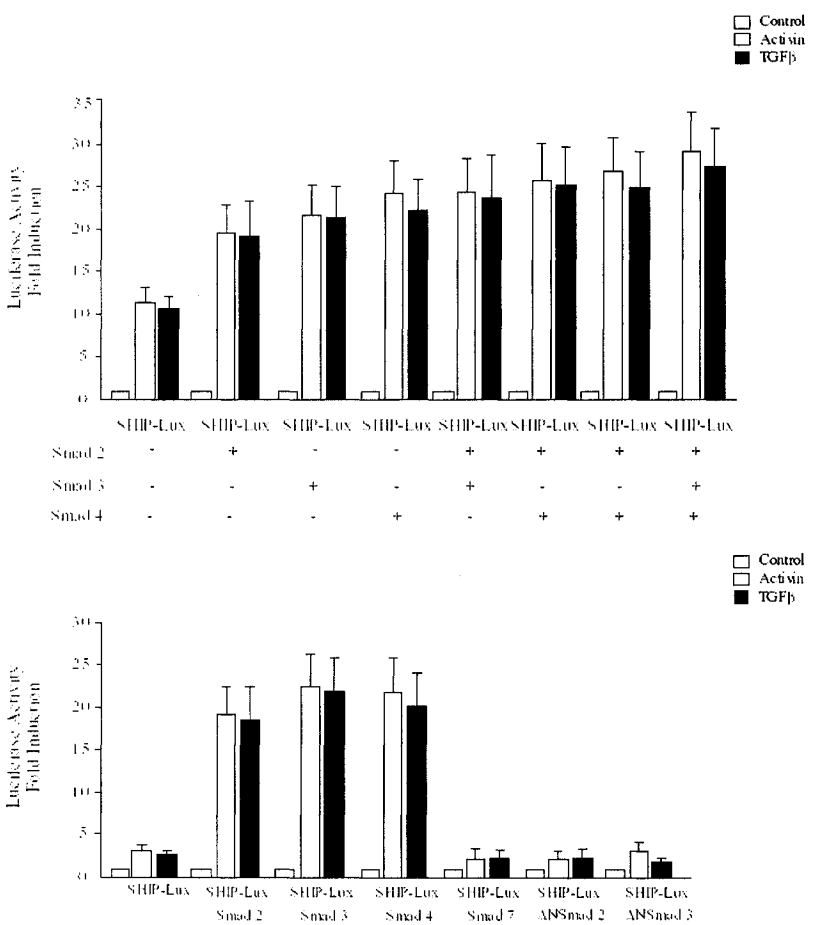
Control
Activin
TGFβ
Luciferase Activity
Fold Induction
SHIP-Lux
Smad 2
Smad 3
Smad 4
Smad 7
ΔNSmad 2
ΔNSmad 3

Figure 2.4.4.c.

Figure 2.4.5.a. Activin/TGFβ-induced SHIP-1 expression and activity lead to change in phospholipids metabolism. MPC-11, B9 and M1 cells were treated or not with activin and TGFβ respectively, were labeled with Myo-[2-^{3}H(N)]-Inositol. Total radiolabeled phospholipids were extracted and the IP3 and IP4 separated by differential elution buffers. Levels of IP4 (left panel) and IP3 (middle panel) are expressed in cpm. SHIP-1 protein expression levels were assessed by Western blot (right panel).

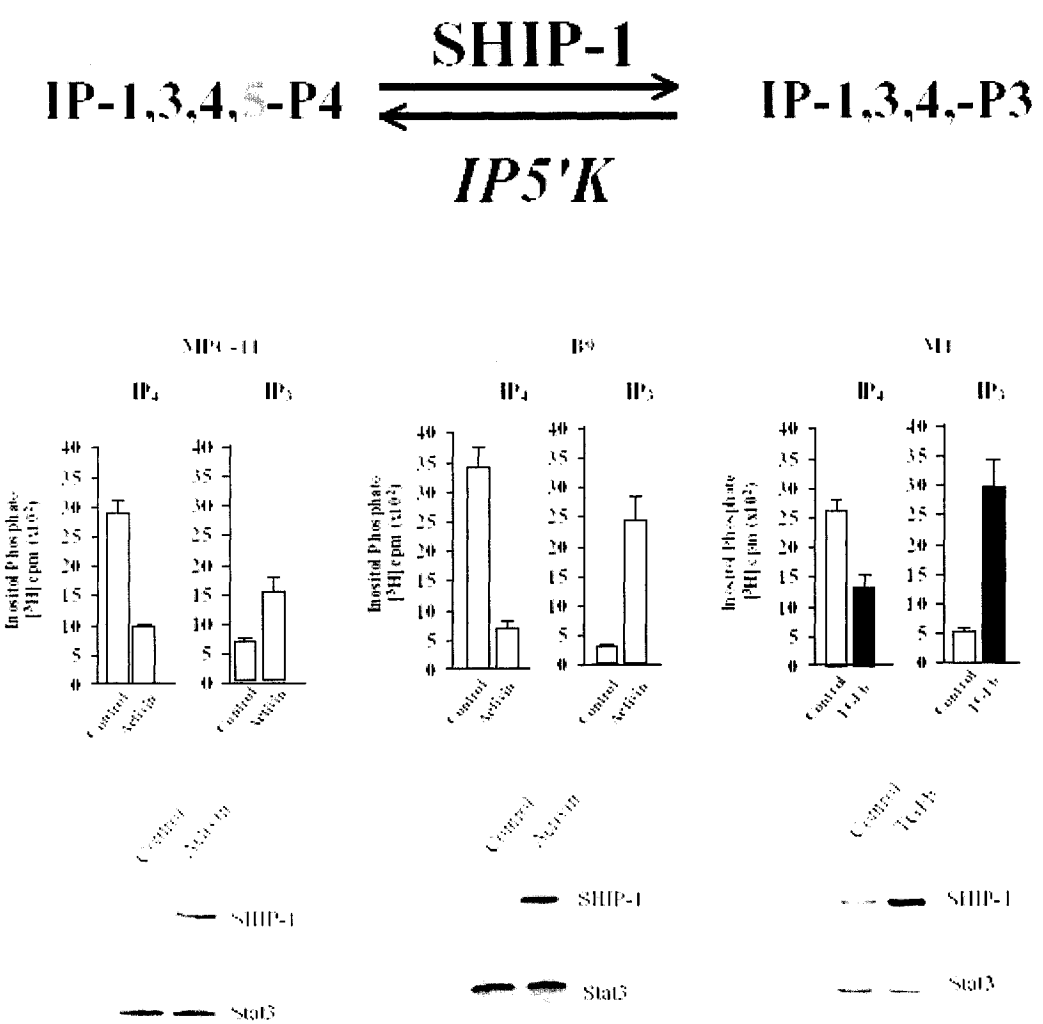
SHIP-1
IP-1,3,4,5-P4
IP-1,3,4,-P3
IP5'K
B9
IP4
IP3
Control
Activin
SHIP-1
Stat3

Figure 2.4.5.a.

Figure 2.4.5.b. Activin/TGFβ-induced SHIP-1 expression and activity lead to inhibition of AKT phosphorylation.

B9 cells treated with activin for different periods of time were metabolically labeled with ^{32}P-orthophosphate. PtdIns3,4,5-P3 were separated by thin layer chromatography, analyzed by autoradiography and quantified by densitometry.

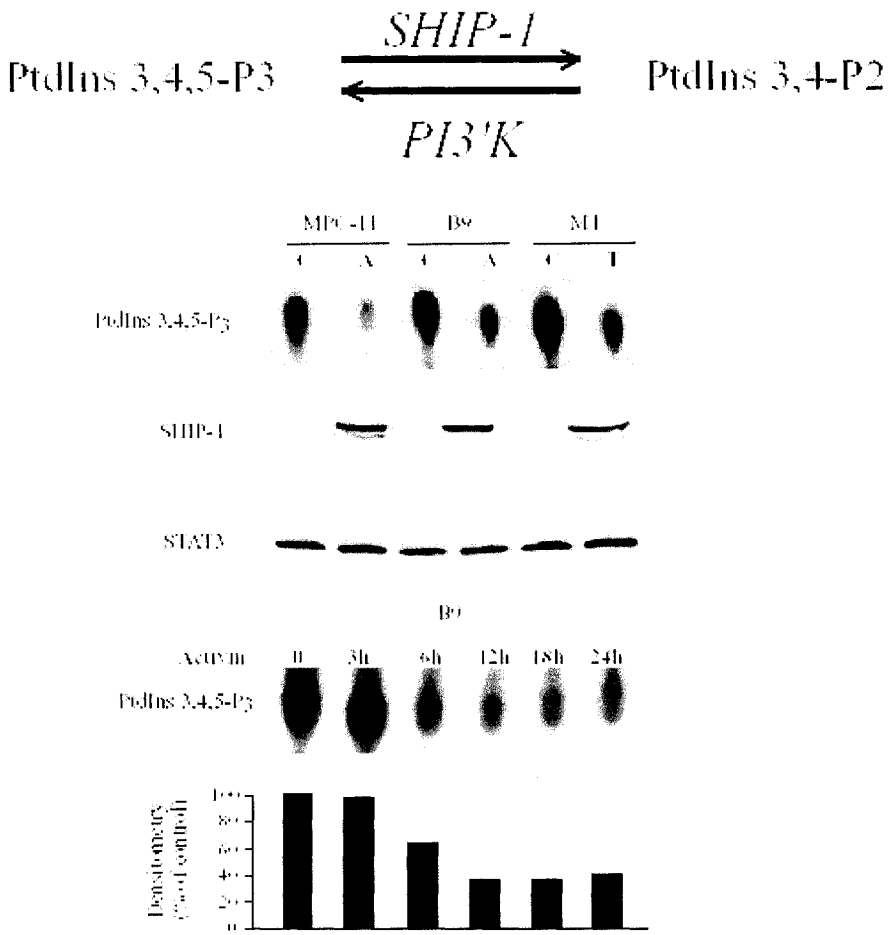
SHIP-1
PtdIns 3,4,5-P3
PtdIns 3,4-P2
PI3'K
MPC-11
B9
M1
PtdIns 3,4,5-P3
SHIP-1
STAT3
B9
Activin
0
3h
6h
12h
18h
24h
PtdIns 3,4,5-P3

Figure 2.4.5.b

The second messenger PtdIns3,4,5-P3 is produced by the enzyme PI3 kinase and regulates activation and phosphorylation of the pleckstrin homology domain-containing protein kinase AKT [24]. By breaking down PtdIns3,4,5-P3 to PtdIns 3,4-P_2, SHIP-1 terminates the activation of the kinase AKT [25]. As AKT is a central regulator of cell growth and survival, we focused on analyzing the role of activin/TGFβ-induced SHIP-1 expression on AKT activation. As shown in Fig.2.4.5.c. (pages 143-144), treatment of MPC-11 cells with sodium pervanadate for 15 min led to activation of the PI3 kinase pathway and subsequent phosphorylation of AKT on Thr^{308}. Interestingly, this effect was largely decreased in cells pretreated with activin and TGFβ for 24 hours. The levels of AKT were shown to be similar in the different samples by stripping and reprobing the blot with an anti-AKT antibody and increased SHIP-1 expression in response to activin was assessed by anti-SHIP-1 immunoblot (Fig. 2.4.5.c, pages 143-144). The same effect was observed in B9 and M1 cells treated with activin or TGFβ and no direct activin/TGFβ effect was observed on AKT phosphorylation (Fig. 2.4.5.c, pages 143-144).

To add more physiological relevance to our results, we examined the effects of TGFβ-induced SHIP-1 expression on AKT phosphorylation in response to IL-6, a natural cell survival factor in immune cells. Interestingly, the IL-6-induced phosphorylation of AKT on Ser^{473} and Thr^{308} was blocked in M1 cells overexpressing SHIP-1 in response to TGFβ (Fig.2.4.5.d, pages 145-146) and the same effect was observed in B9 cells pretreated with activin. Together, these results indicate that activin/TGFβ-induced expression of the inositol phosphatase SHIP-1 is coupled with a decrease in cell survival stimuli-induced AKT activation and provide a phospholipid-dependent mechanism of action for these growth factors in the mediation of apoptosis.

Figure 2.4.5.c. Activin/TGFβ-induced SHIP-1 expression and activity lead to inhibition of AKT phosphorylation.

Treatment of MPC-11, B9 and M1 cells with activin and TGFβ for 24 hours results in an increase in SHIP-1 expression (upper panel) and antagonizes AKT phosphorylation on Thr^{308} in response to 0.2 mM Na pervanadate (middle panel). Reprobing of the blot with anti-AKT confirmed equal loading (lower panel).

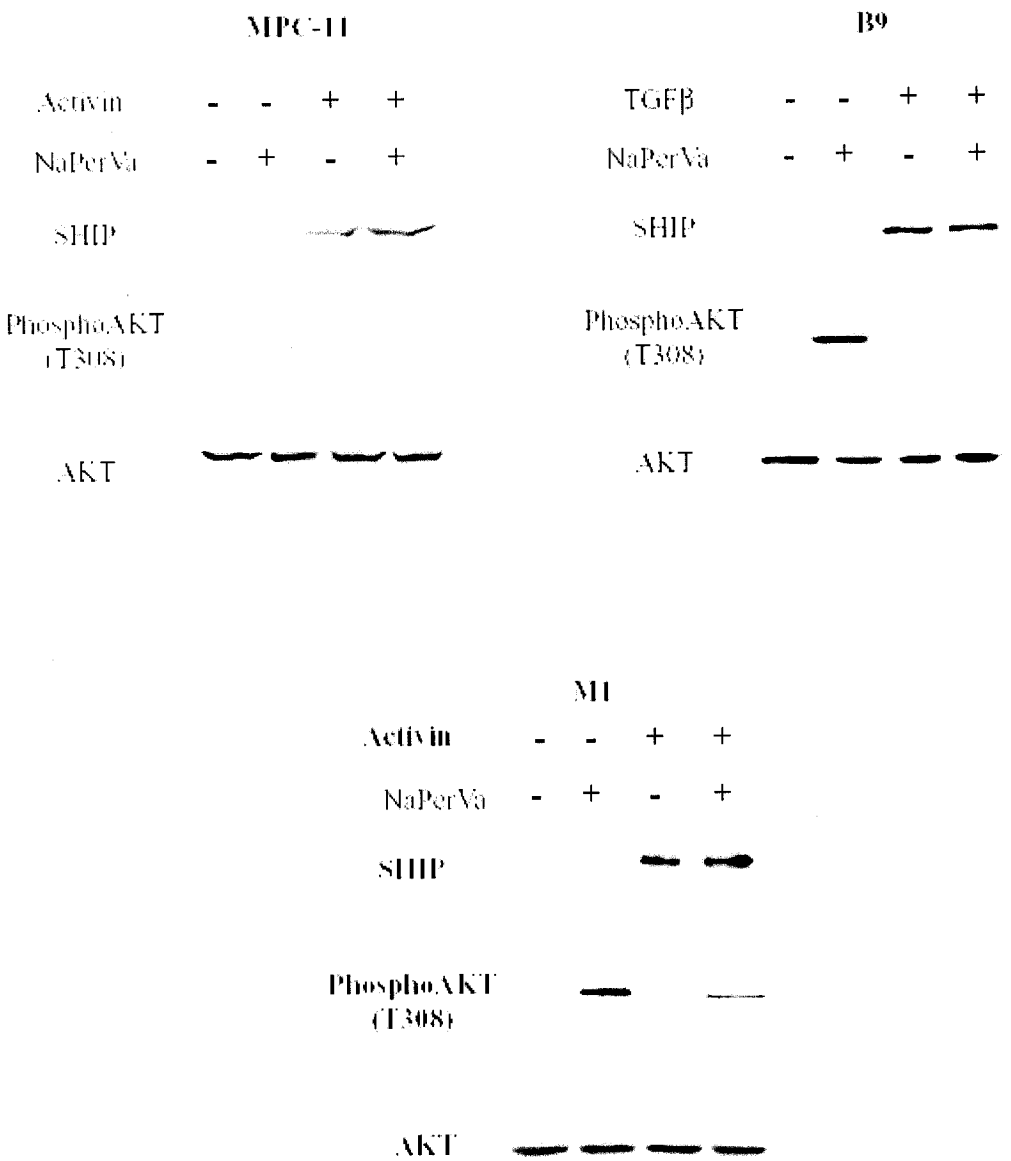
MPC-11
Activin - - + +
NaPerVa - + - +
SHIP
PhosphoAKT (T308)
AKT
B9
TGFβ - - + +
NaPerVa - + - +
SHIP
PhosphoAKT (T308)
AKT
M1
Activin - - + +
NaPerVa - + - +
SHIP
PhosphoAKT (T308)
AKT

Figure 2.4.5.c.

Figure 2.4.5.d. Activin/TGFβ-induced SHIP-1 expression and activity lead to inhibition of AKT phosphorylation.

Activin/TGFβ-induced SHIP-1 expression in B9 and M1 cells antagonizes AKT phosphorylation in response to IL-6 on both Thr308 and Ser 473 (middle panels). Reprobing of the blot with anti-AKT confirmed equal loading (lower panel). Increased SHIP-1 expression in response to TGFβ was monitored by anti-SHIP-1 immunoblot (upper panel).

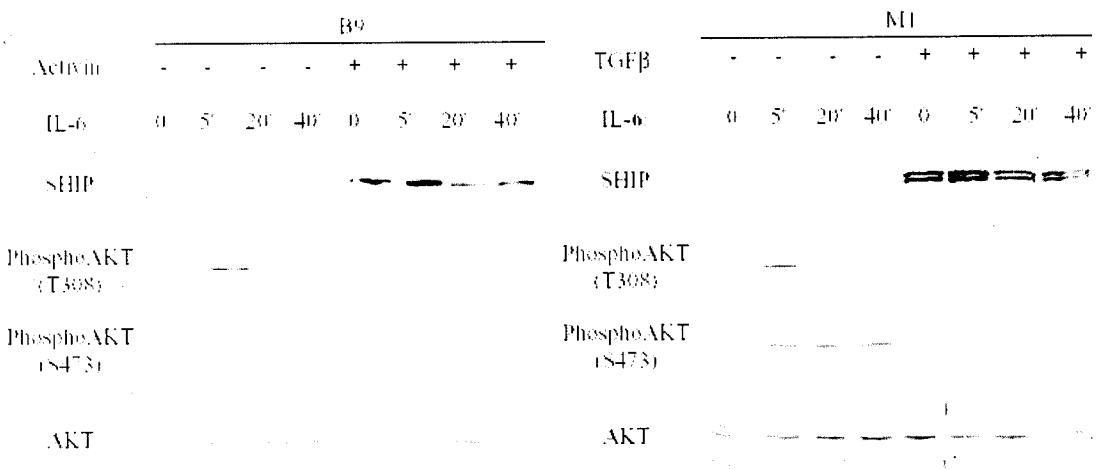
B9
Activin
IL-6
SHIP
PhosphoAKT (T308)
PhosphoAKT (S473)
AKT
M1
TGFβ
IL-6
SHIP
PhosphoAKT (T308)
PhosphoAKT (S473)
AKT

Figure 2.4.5.d.

The SHIP-1 knockout mouse, which is viable and fertile, has a shortened lifespan due to myeloid cell overproduction and infiltration of vital organs [26], highlighting the critical role played by this phosphatase in the apoptosis of immune cells. To determine the contribution of SHIP-1 to activin/TGFβ-mediated proapoptotic effects, we examined their effects in the absence of SHIP-1. We used phosphorothioate antisense oligonucleotides to the 5'-coding sequence of SHIP-1 (SHIP-1 -AS1 and SHIP-1 -AS2) or a scrambled sequence as a control (CTL-1). As shown in Fig. 2.4.6.a. (pages 148-149), pretreatment of MPC-11 cells with the antisense oligonucleotide to SHIP-1 inhibited activin-induced expression of SHIP-1 while not affecting PTEN and Stat3 protein levels. Interestingly, blocking expression of basal endogenous level of SHIP-1 also led to an increase in basal AKT phosphorylation, independently of activin stimulation of the cells, further demonstrating the critical role played by SHIP-1 in regulating AKT activity (Fig. 2.4.6.a, pages 148-149). Interestingly, activin-mediated growth inhibition of MPC-11 cells was clearly antagonized and almost completely reversed in the presence of 50 μM of anti-SHIP-1 antisense oligonucleotides while not affected by the control oligonucleotide (Fig. 2.4.6.b, pages 150-151). The same effects were observed in B9 and M1 cells treated with activin and TGFβ respectively as well as in human purified lymphocytes (Fig. 2.4.6.c, pages 152-153).

Figure 2.4.6.a. Inhibition of expression of the lipid phosphatase SHIP-1 prevents activin and TGFβ-induced apoptosis.

SHIP and PTEN protein expression levels as well as AKT phosphorylation in MPC-11 cells treated or not with the antisense oligonucleotide to SHIP mRNA (SHIP-AS2) were measured by Western blot in cells stimulated or not with activin for 24h. For loading controls the blot was reprobed with an anti-Stat3 antibody.

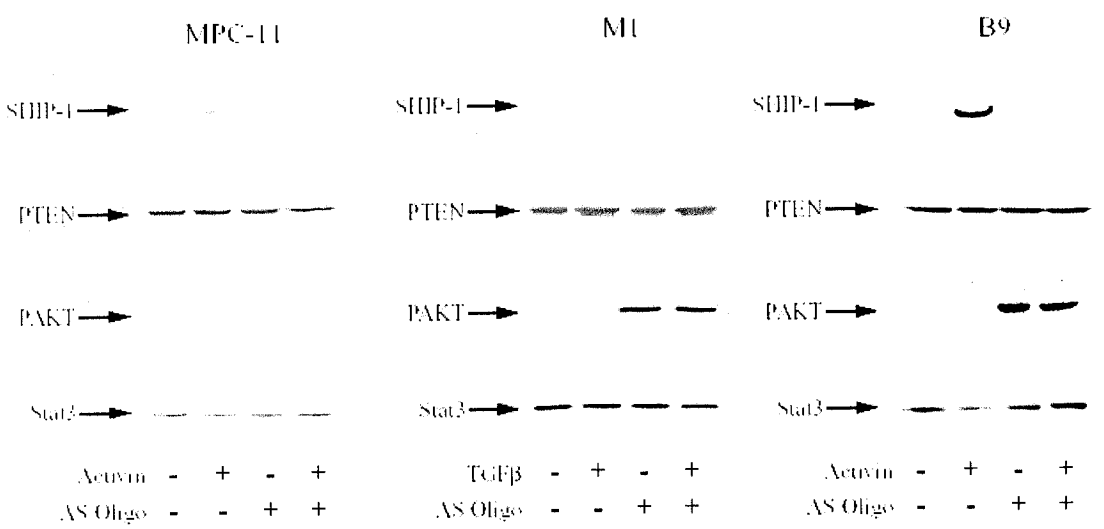
MPC-11
M1
B9
SHIP-1
PTEN
PAKT
Stat3
Activin - + - +
AS Oligo - - + +
TGFβ - + - +
AS Oligo - - + +
Activin - + - +
AS Oligo - - + +

Figure 2.4.6.a.

Figure 2.4.6.b. Inhibition of expression of the lipid phosphatase SHIP-1 prevents activin and TGFβ-induced apoptosis.

MPC-11 cells were treated with 50 μM of phosphorothioate antisense oligonucleotide to SHIP mRNA (SHIP-AS1 and SHIP-AS2) or a with a control oligonucleotide (CTL-1) for 10h before being stimulated with activin. Cell viability was assessed after 72h by MTT colorimetric assays carried out in triplicate. Values are expressed in arbitrary units.

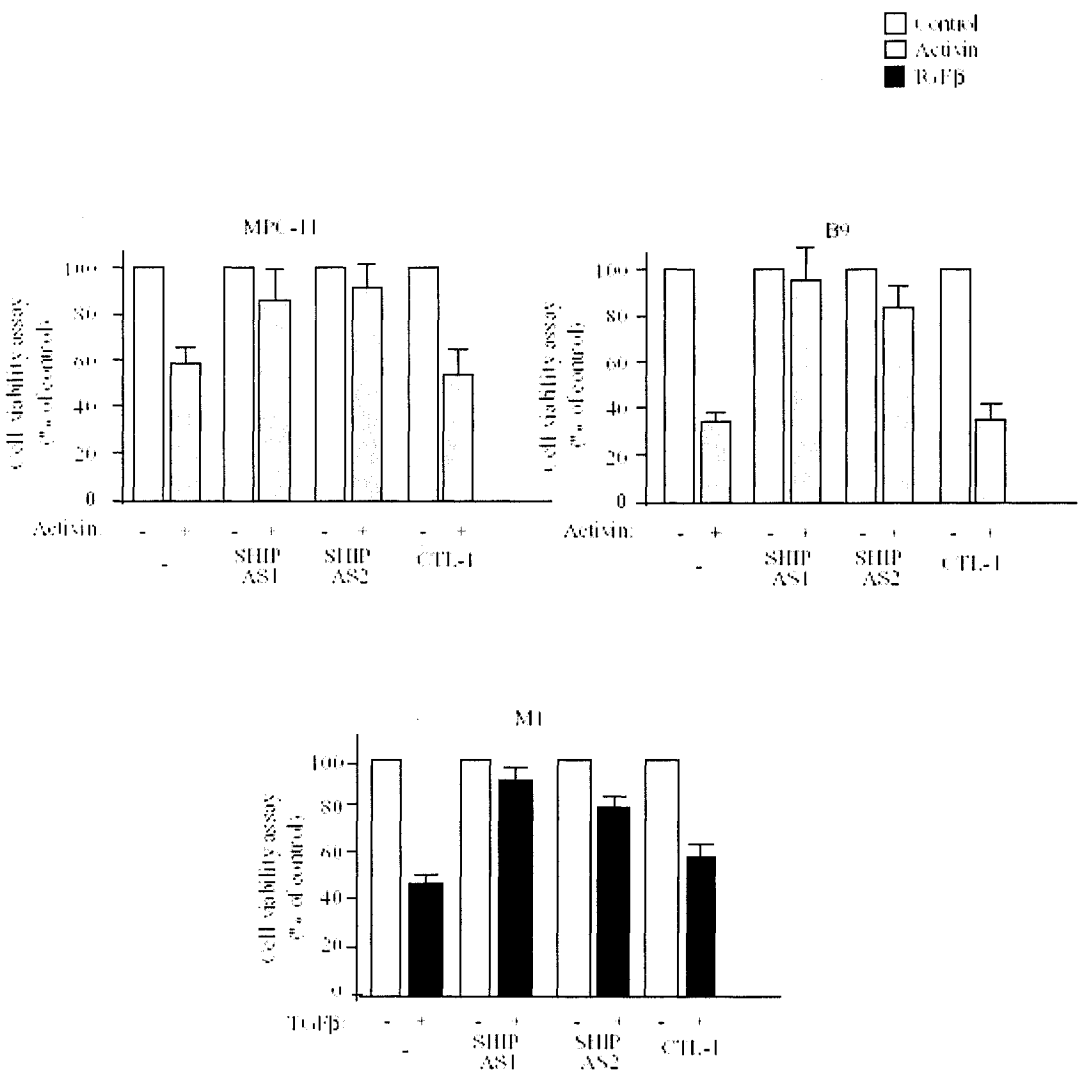
Control
Activin
TGFβ
MPC-11
B9
M1
cell viability assay
(% of control)
Activin
TGFβ
-
+
SHIP AS1
SHIP AS2
CTL-1
0
20
40
60
80
100

Figure 2.4.6.b.

Figure 2.4.6.c. Inhibition of expression of lipid phosphatase SHIP-1 prevents activin and TGFβ-induced apoptosis in human purified lymphocytes.

Human purified lymphocytes were treated with 50 μM of each phosphorothioate antisense oligonucleotide to SHIP mRNA (SHIP-AS1 and SHIP-AS2) or a with a control oligonucleotide (CTL-1) for 10h before being stimulated with activin and TGFβ. Cell viability was assessed after 72h by MTT colorimetric assays carried out in triplicate. Values are expressed in arbitrary units.

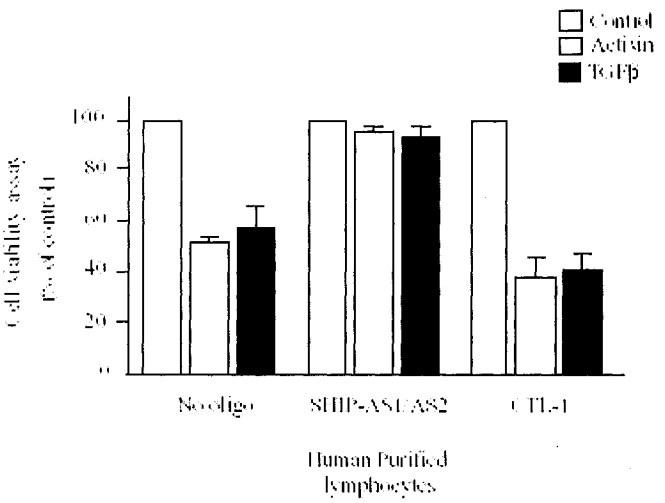
Control
Activin
TGFβ
100
80
60
40
20
0
Cell viability assay
Fold of control
No oligo
SHIP-AS1/AS2
CTL-1
Human Purified
lymphocytes

Figure 2.4.6.c.

To further demonstrate the requirement of SHIP-1 in activin/TGFβ-mediated apoptosis, we used chicken B-lymphocytes (DT-40) as well as DT-40 cells in which either the SHIP-1 gene (SHIP-1 $^{-/-}$) or the SHP-1 gene (SHP-1$^{-/-}$) were deleted by homologous recombination [27]. As shown in Fig. 2.6.d (left panel, pages 155-156), in parental DT-40 and DT-40 deficient in SHP-1 cells, activin and TGFβ inhibited cell viability. However, DT-40 cells deficient in SHIP-1 were resistant to these effects of activin/TGFβ. Finally, to determine whether SHIP-1 was also important for the apoptosis of normal primary cells in response to TGFβ family members, cell viability of bone marrow derived macrophages from wild type mice (+/+) and SHIP-1 -deficient mice (-/-) was examined. As shown in Fig. 2.6.d (lower panel, pages 155-156), activin/TGFβ treatment of wild type macrophages led to a 70% reduction in cell viability while there was only a 20% reduction in SHIP-1 -/- macrophage survival (Fig. 2.6.d, lower panel, pages 155-156). To explain the residual loss of viability in SHIP-1 -/- macrophages it is conceivable that activin and TGFβ utilizes alternate pathways to induce cell death in immune cells. This is consistent with a recent finding showing that TGFβ could mediate apoptosis through the adapter protein Daxx and the JNK pathway [12]. Similar results were obtained with two different preparations of bone marrow derived macrophages.

Figure 2.4.6.d. Inhibition of expression of the lipid phosphatase SHIP-1 prevents activin and TGFβ-induced apoptosis in chicken and mouse immune cell types. Parental chicken B lymphocytes (DT-40), DT-40 SHIP-deficient (SHIP -/-) cells, DT-40 SHP-1 deficient (SHP-1 -/-) cells as well as bone marrow derived macrophages from normal mice (+/+) and SHIP-deficient mice (-/-) were stimulated or not with activin or TGFβ for 72h and cell viability assessed by MTT assays carried out in triplicate. Values are expressed in arbitrary units and represent the average and standard deviation of 4 separate experiments.

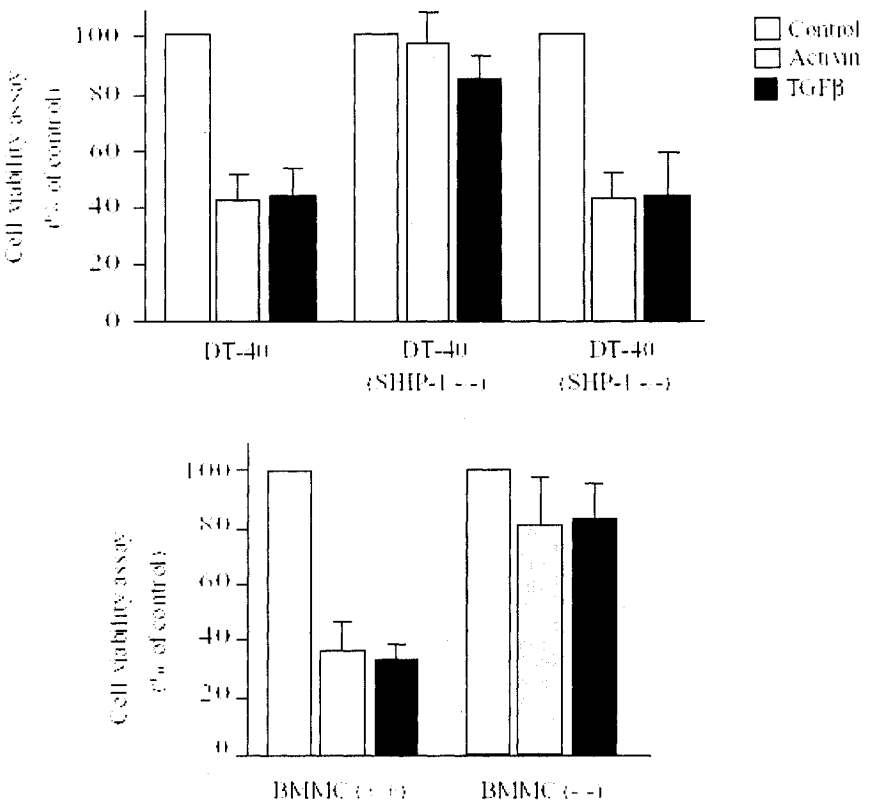
Control
Activin
TGFβ
100
80
60
40
20
0
DT-40
DT-40
(SHIP-1 - -)
DT-40
(SHIP-1 - -)
100
80
60
40
20
0

Figure 2.4.6.d.

Together, our results demonstrate that regulation of SHIP-1 expression by TGFβ family members is critical and required for their pro-apoptotic effects in both normal and cancer cells. Based on SHIP-1's critical role as a cell death regulator in immune cells, one might expect abnormalities in its expression in pathological conditions such as leukemia and autoimmune diseases. The human SHIP-1 gene is located on chromosome 2 at 2q36-37 and even though mutations or deletions in this region do no represent a hallmark of human diseases, aberrant translocations and sporadic abnormalities at this chromosomal location have been detected in several leukemias [28]. Finally, while the involvement of phospholipid metabolism in cellular homeostasis has been widely documented, little is known regarding the regulation of expression of lipid kinases and phosphatases that maintain the intracellular pool of phospholipids. Here we demonstrate that TGFβ family members directly regulate the expression and activity of the lipid phosphatase SHIP-1. Furthermore, our results link for the first time TGFβ-induced apoptosis to phospholipid metabolism and enlarge our knowledge of the mechanism of action of the activin/TGFβ serine kinase receptors.

2.5 Material and Methods

2.5.1. Cell Culture. - MPC-11cells, 293, CHO, wild type and Smad4 knock-out (Smad4-/-) mouse embryonic fibroblasts (MEFs) were cultured in DMEM containing 10% FCS, M1 cells were cultured in RPMI containing 10% FCS. B9 were cultured in RPMI containing 10% FCS supplemented with 50 μM β-mercaptoethanol. DT-40 wild type, DT-40 (SHIP-1 -/-) and DT-40 (SHP-1 -/-) were cultured in DMEM, 10% FCS, 2% chicken serum. Bone marrow derived SHIP-1 +/+ and -/- macrophages were obtained as described [29] and maintained in IMDM containing 10% FCS and 1000U of M-CSF/ml.

2.5.2. Cloning and generation of SHIP-1 reporter construct and luciferase assays. - The 1.4 kb sequence of the SHIP-1 gene promoter was generated by PCR from MPC-11 genomic DNA. The amplified promoter fragment was digested by XhoI and HindIII and cloned into the pGL3 Luciferase basic reporter vector to generate the 1.4 kb SHIP-1 -Lux reporter construct. For luciferase assays, the SHIP-1 -Lux and ARE-Lux/Fast1 constructs were co-transfected by calcium phosphate in 293 cells with an expression vector encoding for β-galactosidase gene, in the presence or absence or various Smad expression plasmids, a described in the figure legend. Transfections of the MEFs were performed using Lipofectamine Plus (Invitrogen Life Technologies) according to the manufacturer's instructions. One day after transfection, cells were serum starved for 12 hours and treated with or without activin (0.5 nM) or TGFβ (0.2 nM)- for 18 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 μl of cell lysate (EG&G Berthold Luminometer) and normalized to the β-galactosidase activity.

2.5.3. Reverse Transcription Polymerase Chain Reaction (RT-PCR). - MPC-11, B9 and M1 were treated with activin and TGFβ for different periods of time and total RNA extracted using Trizol reagents (Life Technologies, GibcoBRL, Gaithersburg, MD). Reverse transcription (RT) of total cellular RNA and amplification of DNA products for SHIP-1 and GAPDH were carried out using Superscript First Strand Synthesis System for RT-PCR (Invitrogen Life Technologies). Primer sequences used for SHIP-1 amplification were as follows: Sense: 5'-CCTCCAACCCCTCCCTCCCAACCA-3'; and antisense 5'-AACGCCGGCGGCATGGCAGTCCTGCCAA-3'. Densitometric analysis was performed using Alpha Innotech Corporation Fluorochem 8000 software version 3.04.

2.5.4.Cell viability assay (MTT). - Cells were plated in triplicate at 5000 cells/100 μl in RPMI medium with 2% FCS. Cells were stimulated or not with activin (0.5 nM) or TGFβ (0.2 nM) and incubated over a 3-day period. Cell growth was assessed using the non-radioactive MTT cell growth 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. DT-40 parental and knockout (SHIP-1 -/- and SHP-1 -/-) cell lines were plated in triplicate at 5000 cells/100 μl in 2% FCS; 1% chicken serum DMEM. SHIP-1 +/+ and -/- macrophages were plated in triplicate at 5000 cells/100 μl in IMDM medium with 10% FCS and 2% M-CSF. Cells were stimulated with activin or TGFβ for three days before being assessed by MTT assay

2.5.5.Flow Cytometry. - Cells were plated in triplicates at 300 000 cells/1 ml in RPMI medium with 2% FCS. Cells were stimulated or not with activin (0.5 nM) or TGFβ (0.2 nM) and incubated over a 3-day period. Cells were subsequently washed in PBS and fixed in 70% ethanol overnight. Cellular DNA was then labeled with 50ng/ml

Propidium Iodide (PI) in PBS, 1.6% Triton and incubated overnight at 4°C in the presence of 50mg/ml RNAseA. The next day flow cytometry was carried out in an EPICS XL series flow cytometer (Beckman Coulter, Miami, FL). Fluorescence was excited by an argon-ion air-cooled 15mW continuous laser power at 488nm. PI emission peak was at 620nm and excitation peak was at 536nm. At least 20 000 gated events were recorded for each sample, and the data were analyzed by Multi-cycle software for Windows (Phoenix Flow Systems).

2.5.6.Annexin V labeling. - Cells were plated in RPMI containing 2% FCS and stimulated with activin for 0, 16, 24 and 36 hours. Cells were then collected, washed, stained with annexin V coupled to fluorescein isothiocyanate (Roche #828681) in accordance with the manufacturer's instructions and analyzed by immunofluorescence (Nikon Eclipse E600) using the MetaImaging Series-Metamorph software (Universal Imaging Corporation).

2.5.7.Western Blot Analysis. - Cells were plated in RPMI containing 2% FCS and stimulated or not with activin, TGFβ Il-6 or sodium pervanadate for the indicated times. Cells were lysed on ice in lysis buffer (50mM HEPES pH7.5, 150 mM NaCl, 100 mM Na fluoride, 10 mM Na pyrophosphate, 5mM EDTA, 10% Glycerol, 0.5 % NP40, 0.5% Na deoxycholate) supplemented with 100 mM Na vanadate, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 2 µg/ml pepstatin. Total cell extracts were then separated on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose and incubated with the indicated specific antibodies overnight at 4°C (anti-SHIP-1 , Santa Cruz sc-8425; anti-PTEN, Santa Cruz sc-7974; anti-Stat3 sc-8019; anti-AKT, NEB 9272; anti-phospho-Thr^{308}-AKT, NEB 9275S; anti-phospho-Ser^{473}-AKT, NEB 9276S).

2.5.8. Intracellular phosphoinositols (IP_3, IP_4) measurements. - Cells were plated at 5 $\times 10^6$ cells/ml in inositol free medium containing 1 mCi Myo-[2-^{3}H(N)]-Inositol and stimulated or not with activin and TGFβ for 16h. Cells were washed three times with Krebs HEPES (146 mM NaCl; 4.2 mM KCl; 0.5 mM $MgCl_2$; 1.0 mM $CaCl_2$; 10mM HEPES pH 7.4; 20 mM LiCl; 5.9 mM Glucose) and lysed with 5% of perchloric acid. The supernatant were collected in 500 µl of HEPES-KOH buffer (HEPES 75 mM and KOH 1.5M), adjusted to pH 7 with 5% perchloric acid and phospholipids were separated using DOWEX AG 1X-8 (200-400 mesh) column. IP_1 and IP_2 were first removed from the column by washing with ammonium formate (400 mM) and elution of the inositol phosphates IP_3 and IP_4 was performed using specific buffers (IP_3: 700 mM ammonium formate, 100 mM formic acid (HCOOH); IP_4: 1000 mM ammonium formate, 100 mM formic acid (HCOOH)). Measurement and quantification of each isoform of inositol phosphate were then performed using a WinSpectral 1414 Liquid Scintillation Counter.

2.5.9. Phosphatidylinositol (PtdIns3,4,5-P3) measurement.- Cells were plated at 5 $\times 10^6$ cells/ml in phosphate free RPMI medium, pretreated or not with activin and TGFβ for 24h or the indicated times for the activin time course experiment. Cells were collected and labeled with 100 µCi ^{32}P orthophosphate (PerkinElmer Inc. NEX053) for 3h in incubation medium (0.1% BSA in phosphate free medium) at 37°C. Cells were washed three times with phosphate free medium and cells were resuspended with 500 µl HCl:EtOH buffer. Phosphatidilinositols were extracted with chloroform, lyophilized, resuspended in 25 µl of chloroform, separated by thin layer chromatography and analyzed

by autoradiography. Densitometric analysis was performed using Alpha Innotech Corporation Fluorochem 8000 software version 3.04.

2.5.10. Antisense oligonucleotides treatment. - Cells were plated in 96-well plates at 5 $\times 10^3$ cells/well in 2% FCS and treated or not with antisense oligonucleotides for SHIP-1 mRNA (SHIP-1 -AS1 and SHIP-1 -AS2) or a control antisense oligonucleotide (CTL-1) at 50 μM. After 12 hours the cells were stimulated with activin (0.5 nM) or TGFβ (0.2 nM). Seventy two hours following ligand stimulation cell growth was measured using the MTT assay as described above. We used the following phosphorothioate oligonucleotides: SHIP-1 -AS1 5'-CAGGGACCATGGCAGGCATG-3'; SHIP-1 -AS2 5'-GGGTGCATTACCCATGTTCC-3'. The sequence of the control oligonucleotide (CTL-1) was 5'- TCAGACTGGCTCTCTCCATG -3'.

2.6. Acknowledgements.

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CHAPTER 3

Activin and TGFβ regulate expression of the phosphatidyl inositol 5' phosphatase SHIP-1 through Smads and AP-1

3.1. Prologue

Activin and TGFβ are potent cell growth inhibitors and proapoptotic factors in immune cells, but the intracellular target genes that relay their signals remain unknown. In a screen for downstream-regulated target genes for TGFβ ligands in different immune cell types, we found that the Src homology 2 [1] domain-containing 5' inositol phosphatase, SHIP-1, was significantly upregulated. The Smad signaling pathway plays a central role in the mediation of the TGFβ family members signal transduction. However, accumulating evidence indicates that other signaling pathways also participate in the mediation of TGFβ intracellular signals. TGFβ family members can activate and signal through several MAPK pathways, such as the ERK, the p38 stress-activated kinase or the Jun N-terminal kinase (JNK). Our previous studies have demonstrated that TGFβ family members induced cell growth arrest and apoptosis through Smad-dependent increase of the SH2-containing Inositol Phosphatase 5', SHIP-1, in different immune cell types. Activin/TGFβ-induced SHIP-1 correlated with a decrease in phospholipids (PtdIns-3, 4, 5-P3) and inhibition of the survival kinase Akt and led to increase in cell growth arrest and apoptosis. These results suggest new mechanisms by which these growth factors modulate the phospholipid products and their metabolism to induce cell growth-arrest and apoptosis in immune cells.

In order to better understand the mechanism by which TGFβ and activin regulate SHIP-1 expression and to further dissect the downstream transcriptional events involved in this process, we extended our studies to delineate the potential signaling pathways involved at the transcriptional level that regulates the SHIP-1 gene promoter as well as the identification of the different DNA binding sites and the transcriptional factors that mediate activin and TGFβ effects in immune cell types. Furthermore, we analysed the involvement not only of Smad signaling pathway but also MAP kinase signaling pathway in the transcriptional regulation of the SHIP-1 gene promoter in immune cell types.

3.2. ABSTRACT

Members of the TGFβ family of growth factors are highly conserved during evolution. TGFβ family members have unique immunoregulatory properties. Almost all immune cells produce them. The function of TGFβ ligands in the haematopoietic tissue is critical as like any death inducers in this compartment, since they prevent tumorogenesis and contribute to the elimination of activated lymphocytes and the maintenance of peripheral tolerance. However, the mechanisms by which these growth factors exert their growth-inhibitory effects remain unclear. In this study, we show that TGFβ ligands regulate positively the expression of the SH2-containing inositol 5' phosphatase, SHIP-1, at the transcriptional level through the Smad pathway as well as the stress MAPK signaling pathways. Our results show that the association between Smad4 and AP-1 transcription factors regulates in a conformation-dependent the transcription regulation for the SHIP-1 gene promoter in immune cells types.

3.3. INTRODUCTION

Cancer cells have developed numerous molecular mechanisms for escaping and avoiding the host's immune response. These actions take place, either by successfully evading a fully functional immune system or by actively suppressing the immune attack. Due to these actions, the cancer cells are not longer recognized and effectively eliminated by the host's defence system. Current evidence strongly suggests that an active cell-mediated immunosuppressor mechanism is initiated via the secretion of immunosuppressor cytokines. Among these type of molecules, the Transforming Growth Factor β (TGFβ) seems to be one of the key cytokines widely used to escape the immune response generated by the host's defence system. TGFβ super family of cytokines is a large category of ubiquitous and highly conserved polypeptide growth factors that has been referred as regulators of a plethora of biological processes including cell growth, differentiation, embryogenesis and apoptosis in nearly all cell types [2]. This family of growth factors includes the TGFβs, the activins and the morphogenetic proteins (BMPs) among others [3-6]. However, deregulation, disruption or mutation involved in TGFβ signaling pathway components have been correlated with several physiological disorders, diseases and oncogenic processes [5, 7-11].

Activin, a member of this family, considered as a molecular element that participates in the anterior pituitary function, was isolated from the gonads based on its ability to control and regulate the secretion levels of follicle-stimulating hormone (FSH) from this anatomical structure [12-14]. As a member of the TGFβ family of growth factors, activin plays an important role in regulating cell growth and differentiation of numerous cell types [15]. Since its molecular identification, the antiproliferative and proapoptotic

effects of activins have been observed in many tissues and cell types. [16]. The immunoregulatory properties of the TGFβ family members have been widely studied and has shown that these growth factors are produced by every leukocyte lineage and control differentiation, proliferation, survival and state of activation of immune cells [8, 17]. The physiology of activin and TGFβ in the haematopoietic tissue is essential in this tissue. They prevent tumorogenesis and contribute to the elimination of activated lymphocytes and the maintenance of peripheral tolerance [17, 18].

Despite their critical role in the elimination of damaged or abnormal cells in the immune system, the mechanisms by which these growth factors induce apoptosis remain largely unexplored. Recent evidence has shown that some of the effects of activin on programmed cell death may be mediated through suppression of Bcl2 expression as described in hybridoma cells or up-regulation of the proapoptotic factor Bax and subsequent caspase activation as described in myeloid leukemia cells [19].

Down regulation of Bcl2 family members or activation of caspase activity has also been reported in response to TGFβ [20]. Recently it was shown that the death adaptor protein Daxx could mediate TGFβ-induced apoptosis through the JNK pathway in B-cell lymphomas [21]. This Smad-independent effect is mediated through a direct interaction of Daxx and the type II receptor for TGFβ and indicates that other signaling pathways are involved in the mediation of the activin and TGFβ effects on programmed cell death in immune cell types. Furthermore, we have recently reported that activin and TGFβ regulate cell growth arrest/apoptosis in both normal and cancer haematopoietic cells through expression of the inositol phosphatase SHIP-1, a central regulator of phospholipids metabolism in immune cell types [22].

Inhibition of growth arrest is signalled by the binding of TGFβ to the constitutively activated kinase Type II receptor, its oligomerization with and sequential activation of the Type I receptor. This occurs with the immediate transphosphorylation of a juxtamembrane glycine and serine-rich domain of the receptor (GS box), that is highly conserved among all type I receptors of the TGFβ family [23-25]. Once activated this receptor functions as a tetrameric complex, with the type I receptor transmitting the intracellular signals by recruiting and phosphorylating downstream molecules known as the receptor-associated Smad proteins (R-Smads: Smad2 and Smad3). R-Smads containing two serine residues located at the carboxy-terminus, complexes with the common Smad or co-Smad, Smad4 forming a complex (Smad2/3/4). The Smad complex, then translocates to the nucleus where it regulates the transcriptional activation or repression of target genes alone or with DNA-associated binding proteins partners of different [26-29].

Smad signaling pathway is essential for most, but not all, TGFβ-mediated gene regulation [30]. TGFβ has been shown to activate and signal through other downstream signaling pathways such as the extracellular-signal-regulated kinase 1 and 2 (ERK1, ERK2), the p38 or c-Jun amino-terminal kinase (JNK) mitogen-activated protein kinase (MAPKs) in various cell lines and this can proceed rapidly and independently of Smads [29, 31, 32]. Activation of the p38 and JNK signaling pathways leads to increased activity of different transcription factors such as AP-1 (Jun-Fos) and ATF2, also known as CRE-binding protein (CREBP1), which regulate the transcription of gene promoter using AP-1 (Jun-Fos) and CREBP1/ATF-transcriptional binding sites [33, 34]. Recently we have reported that activin and TGFβ induce cell growth arrest through Smads, p38 kinase and the transcription factor ATF2 in breast cancer cells [35]. Similar results were observed in liver cell types treated with TGFβ ligands. In this context, both, activin and TGFβ were found

capable of inducing cell growth inhibition in human hepatocarcinoma cells through the increased gene expression of the cyclin-dependent kinase inhibitor (CDKI) p15INK4B in a Smad-dependent the involvement of Sp1 in promoting $p15^{INK4B}$ gene activation [36]. In addition, different studies have demonstrated that TGFβ-mediated transcriptional activation of several target genes requires the participation of the AP-1 transcription factor family members. The expression of many AP-1 proteins is induced as an early response to TGFβ in a cell type-specific manner. Molecular studies in *Drosophilae melanoganster* have revealed direct overlap between AP-1 and TGFβ signaling, suggesting a conserved convergence of these pathways that is evolutionarily conserved. Thus, these results demonstrate the existence of a strong regulated link between TGFβ signaling pathway and AP-1 in regulating the TGFβ expression of target genes. Moreover, TGFβ and BMP also send signals through the JNK and p38 signaling modules [37-40]. In some cases, TGFβ has also been reported to signal through an ERK 1/2-dependent mechanism [41]. In summary, activin/TGFβ signal transduction is mediated through the canonical Smad pathway but also requires distinct signaling cascades that provide tissue specificity, highlighting the complexity of activin and TGFβ signaling leading to gene activation. Taken together, these studies demonstrate the existence of a strong link between TGFβ signaling and AP-1 in the regulation of the expression of different target genes.

Here, we have explored the transcriptional mechanisms of the mouse SHIP-1 gene promoter regulation by activin and TGFβ signaling pathway. We show that this promoter contains several regulatory sequences. The binding of Smads and AP-1 transcription factors to specific sequence elements is necessary for efficient basal promoter activity and induction by activin and TGFβ. Moreover, promoter-mapping analysis indicates, that in addition to the Smads, activin and TGFβ ligands requires the transcription factor AP-1 to

regulate SHIP-1 gene expression. Together, our results define the SHIP-1 as an important target for TGFβ family members in immune cells, thus highlighting the critical role for these growth factors in regulating immune cell growth and apoptosis.

3.4. RESULTS

3.4.1. Loss of expression of the inositol phosphatase SHIP-1 blocks TGFβ ligands-mediated growth arrest:

We have previously shown that TGFβ and activin induce growth inhibition through the regulation of the phosphatidyl inositol phosphatase SHIP-1 expression [22]. SHIP-1 expression has been demonstrated to be restricted to haematopoietic cells where it plays a critical role in immune cell growth regulation[42]. To further investigate the signaling pathways and mechanisms used by TGFβ and activin to regulate SHIP-1 expression and their role in immune cell growth arrest, we used chicken B lymphocyte cells (DT40) as a model system. We examined the TGFβ ligands effects in wild type DT40 cells and in DT40 cell lines in which SHIP-1 gene (SHIP-$1^{-/-}$) was deleted by homologous recombination [43]. As a control, we used DT40 cell in which another tyrosine phosphatase SHP-1 was deleted as previously reported [43]. Cells were stimulated with activin or TGFβ for 24h and the expression level of SHIP-1 in these cell lines was determined by western blot analysis using a specific monoclonal antibody. As shown in Fig.3.4.1.a (pages 174-175), while there is no or little detectable expression of SHIP-1 in non-stimulated cells, a marked increase is seen in cell exposed to activin or TGFβ. We observed that the activin or TGFβ treatment did not affect the expression of other proteins such as the tyrosine phosphatase SHP-1 and the 3' phosphatidyl inositol PTEN as shown in figure 3.4.1.a (middle panel, pages 174-175). As a loading control the membranes were stripped and reprobed with a specific monoclonal antibody directed against Stat3 showing equal loading (Fig.3.4.1.a, lower

panel, pages 174-175). Thus, these results are in agreement with our previous observation showing that activin and TGFβ can induce SHIP-1 expression in various immune cell lines where these growth factors induce cell growth arrest and apoptosis [22].

Figure 3.4.1.a. Activin and TGFβ induce SHIP-1 protein expression in chicken B-lymphocytes.

Chicken B-lymphocytes DT40, SHIP-1 -/- and SHP-1 -/- were stimulated or not with activin [0.5 nM] and TGFβ [0.2 nM] for 24h. Total cell lysates were obtained and examined by western blot analysis using a specific monoclonal antibody against SHIP-1 (upper panel). The blot was stripped and reprobed with an anti-SHP-1, anti-PTEN. For loading control, the membranes were stripped and reprobed anti-tubulin antibody (lower panel).

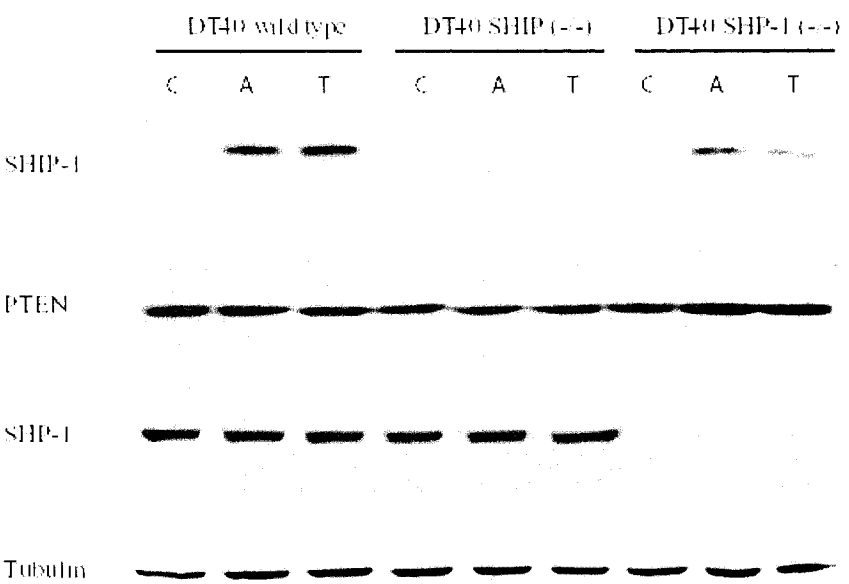
DT40 wild type
DT40 SHIP (- -)
DT40 SHP-1 (- -)
C A T C A T C A T
SHIP-1
PTEN
SHP-1
Tubulin

Figure 3.4.1.a.

We then investigated the role of SHIP-1 in TGFβ ligands-mediated growth arrest in these B cell lymphocytes. Wild type DT40 cells, (SHIP-$1^{-/-}$) and (SHP-$1^{-/-}$) cells, cultured in the presence of 2% foetal bovine serum (FBS) and 0.5% chicken serum (CS), were stimulated with activin or TGFβ for different period of time. As shown in Fig.3.4.1.b (upper panel, pages 177-178), activin strongly inhibits growth of these chicken B lymphocyte cells. This effect increases with time of stimulation to reach 60% growth inhibition after 5 days. However, this effect is completely abolished in the (SHIP-$1^{-/-}$) cells (Fig.3.4.1.b, middle panel, pages 177-178). In the control (SHP-$1^{-/-}$) cells, activin retains its growth inhibitory effect similar to what was observed in the wild type cells (Fig.3.4.1.b, right panel, pages 177-178). The same esults were obtained in cells stimulated with TGFβ (data not shown). As a control the cell growth arrest effect was compared to MPC-11 and M1 cell lines, which have been previously shown to response to Activin and TGFβ respectively. Thus, these results indicate that activin exerts a strong effect in cell growth inhibitory effects in lymphocytes in a SHIP-1 dependent manner.

Figure 3.4.1.b. Activin and TGFβ induce cell growth arrest in haematopoietic cells. Cell viability colorimetric assay non-radioactive (MTT) [3-(4,5-Dimethylthiazolyl-2)-2,5 Diphenyltetrazoliumbromided (MTT)] was performed in triplicate using B-lymphocytes cells (DT40), SHIP-1-/-, SHP-1-/-. Plasmocytoma MPC-11, and myeloma M1 cells we used as a reference control,. The different cell lines were then stimulated or not with activin [0.5 nM] and TGFβ [0.2 nM] respectively for a 5-days period. Absorbance was measured at 570 nm with a reference wavelength at 450nm, and O.D. values were quantified. The absorbance values represent the average of 3 independent experiments.

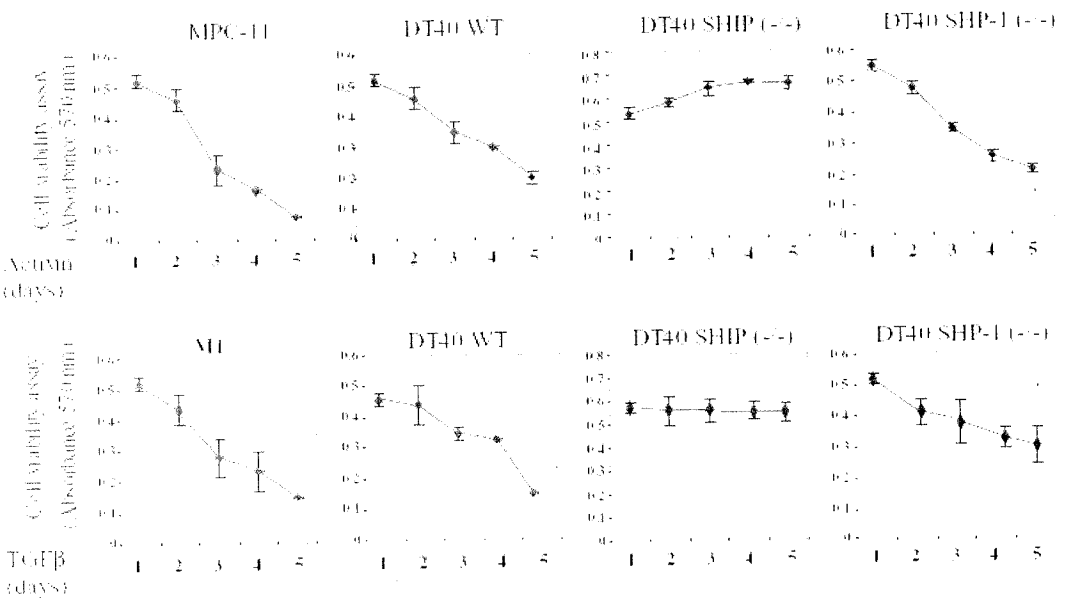
MPC-11
DT40 WT
DT40 SHIP (-/-)
DT40 SHIP-1 (-/-)
Cell viability (ΔAbsorbance 570-690)
Nocodazole (days)
MPC-11
DT40 WT
DT40 SHIP (-/-)
DT40 SHIP-1 (-/-)
Cell viability (ΔAbsorbance 570-690)
TGFβ (days)

Figure 3.4.1.b.

3.4.2. TGFβ ligands activate and signal through the Smad and stress-activated signaling pathways in immune cells.

Smads are the downstream key players in the activin/TGFβ signaling pathways. However, depending on the target tissue, other signaling cascades such as MAPK, can also be activated downstream of these growth factor receptors. As reported previously, both activin and TGFβ are critical regulators of haematopoietic cell growth and survival [44]. We investigated the effect of activin and TGFβ on these signaling pathways in immune cells. We used DT40 wt cells but also myeloma (M1) and plasmocytoma (MPC-11) cells in which, as we previously showed, activin/TGFβ-mediated SHIP-1 expression is clearly correlated with a strong induction of proapoptotic/growth inhibitory effect in these cell lines [44]. To pursue this goal, cells were plated in starvation media overnight and then stimulated for different periods of time with either activin (DT40, MPC-11) or TGFβ (DT40, M1). We initially evaluated the activation of the Smad signaling pathway in these different immune cell types reflected by the phosphorylation levels of Smad2 in response to TGFβ ligands. Cell lysates were then subjected to a western blot analysis using phospho-Smad2 specific antibody. As shown in Fig. 3.4.2.a (upper panel, pages 181), the exposure of all three cell lines to activin/TGFβ lead to a clear and rapid increase in Smad2 phosphorylation. Equal loading of the proteins was demonstrated by reprobing the membrane with an anti-Smad2/3 antibody (Fig.3.4.2.a, lower panel, page 181). These results clearly indicate that the Smad pathway is induced in immune cells in response to both activin and TGFβ ligands.

Figure 3.4.2.a. TGFβ ligands activate the Smad signaling pathways in immune cells. Treatment with activin and TGFβ induces activation of Smad signaling pathway in immune cell types. Western blot analysis of the levels of phosphorylation of Smad2/3 following treatment with activin [0.5 nM] and TGFβ [0.2 nM] to MPC-11, M1 and DT40 as indicated. Membranes were stripped and reprobed with total anti-Smad2/3 to confirm equal loading (lower panel).

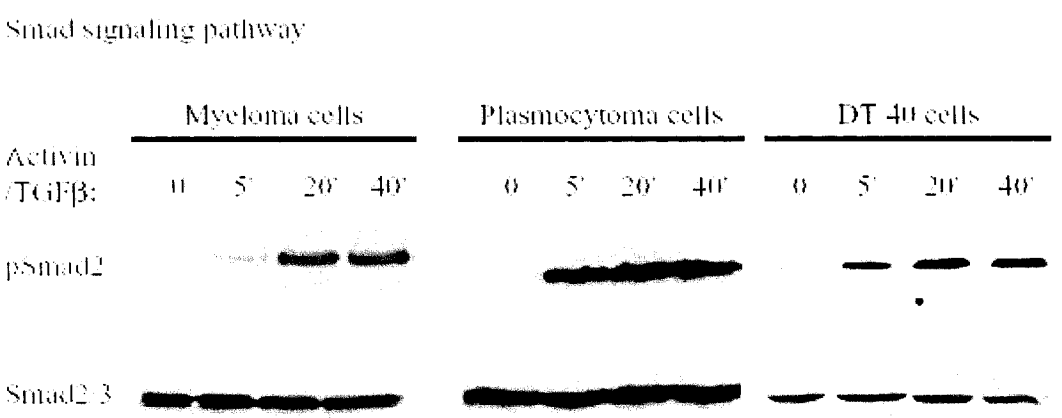
Smad signaling pathway
Myeloma cells
Plasmocytoma cells
DT 40 cells
Activin
/TGFβ:
0
5'
20'
40'
pSmad2
Smad2/3

Figure 3.4.2.a.

We next determined whether the stimulation with TGFβ ligands, have an effect on the p38 signaling pathway. To investigate this, lysates of control and treated-cells were subjected to western blot analysis using a specific phospho-p38 antibody. As shown in Fig. 3.4.2.b (pages 183-184), both activin and TGFβ induce phosphorylation of the p38 kinase. In order to confirm the kinase p38 signaling pathway is well activated by TGFβ ligands, we determine whether the upstream MAP kinase MMK3 [45] and downstream transcription factor ATF2, target of the p38 MAPK, were activated upon treatment with these growth factors. To address this issue, we used phosphospecific antibodies directed to MKK3 and ATF2. Figure 3.4.2.b (pages 183-184), clearly demonstrates that treatment with the ligands (activin and TGFβ), resulted in a rapid and clear increase in the phosphorylation levels of the p38 upstream kinase MKK3, which paralleled the increase in phosphorylation for ATF2, downstream target of the p38 kinase, (Figure 3.4.2.b. lower panel, pages 183-184) [46]. As loading controls, membranes were stripped and reprobed with antibodies to tubulin, p38 and ATF2 respectively (Fig. 3.4.2.b, pages 183-184).

Figure 3.4.2.b. TGFβ ligands activate the stress-activated kinase p38 pathway in immune cells.

Treatment with activin and TGFβ induces activation of stress p38 MAP kinase signaling pathway in immune cell types. Western blot analysis indicates the levels of phosphorylation of MKK3, p38 AND ATF2, following treatment with activin [0.5nM] and TGFβ [0.2nM] to MPC-11, M1 and DT40 at different periods of time as indicated. Membranes were stripped and reprobed with total anti-tubulin, anti-p38 and anti-ATF2 respectively to confirm equal loading (lower panel).

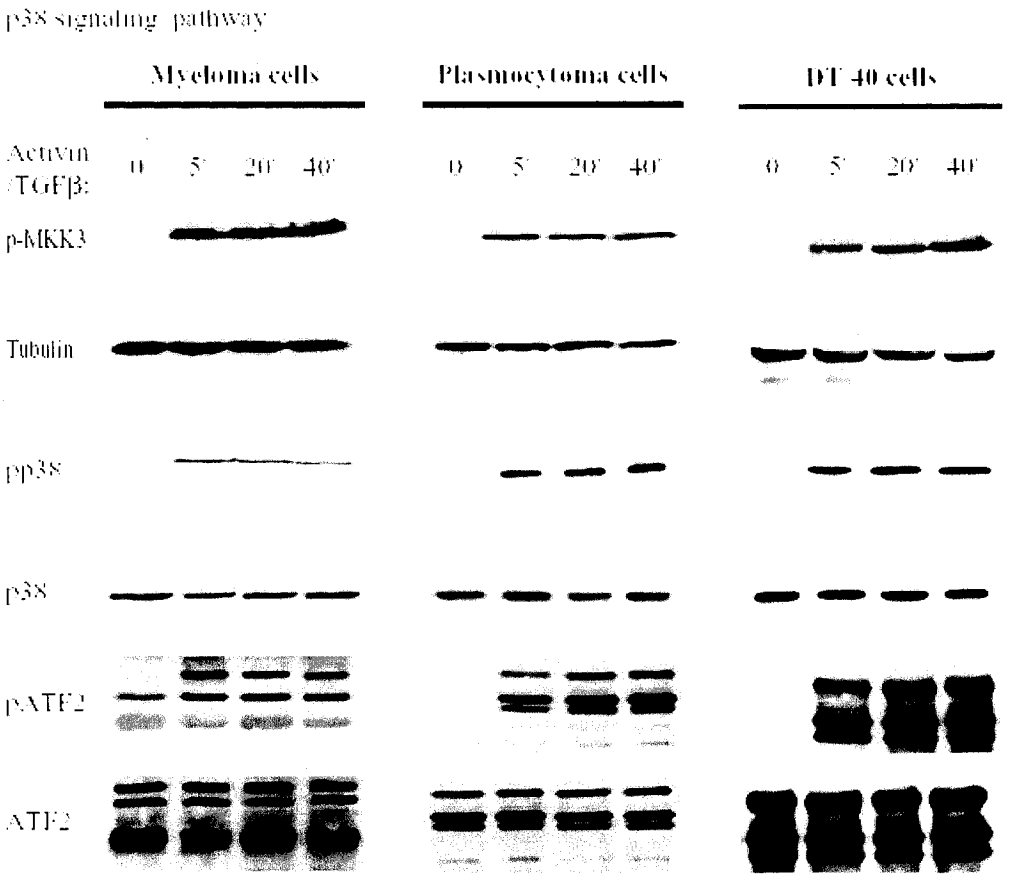
p38 signaling pathway
Myeloma cells
Plasmocytoma cells
DT 40 cells
Activin /TGFβ:
0
5'
20'
40'
p-MKK3
Tubulin
pp38
p38
pATF2
ATF2

Figure 3.4.2.b.

We next evaluated the effects of activin/TGFβ on the JNK signaling pathway. Using a phospho-specific anti-JNK antibody that recognizes both activated isoforms of JNK (42 kDa, 44 kDa) [47-49] we found that TGFβ ligands can activate the JNK kinases (Fig.3.4.2.c, lower panel, see page 187-188). Interestingly, specific JNK isoforms seem to be activated in response to activin and TGFβ stimulation in a cell-type specific manner. While TGFβ activates both 42 and 44 kDa JNK isoforms in M1, cell, activin clearly activates the 44 kDa in DT40 and 42 kDa in plasmocytoma cells (Figure 3.4.2.c, pages 187-188). The significance of these findings remains unknown. The transcription factor c-Jun, a JNK downstream target, is also phosphorylated in response to activin/TGFβ in the three cell lines. The membranes were stripped and reprobed with antibodies for total JNK and c-Jun respectively in the three different cell lines in order to ascertain equal loading of samples (Figure 3.4.2.c, bottom panel, pages 187-188). Thus, taken together these results, provide evidence demonstrating that both the canonical Smad pathway and stress-activated kinases, p38 and JNK, are activated in response to TGFβ ligands in immune cells, suggesting that these pathways may play a role in integrating the effects of activin/TGFβ-mediated on SHIP-1 expression and cell growth arrest.

To further identify the DNA sequences on the SHIP-1 promoter that confer activin/TGFβ responsiveness we cloned 2,143 bp upstream of the initiation start site on the mouse SHIP-1 gene. Primers were designed based on the 5' end region of the mouse SHIP-1 gene previously reported (GenBank accession number: U39203) [50]. This upstream region was then subcloned into the luciferase reporter vector pGL3-Basic. To test the responsiveness of this promoter construct CHO cells were transiently transfected with the 2,143bp construct or a responsive promoter construct (3TP-Lux) as a control and stimulated or not with activin and TGFβ for 18h. As shown in Figure 3.4.3.a (pages 189-190), both

growth factors strongly induce SHIP-1 gene promoter activity as well as 3TP-lux activity. This indicate that activin/TGFβ regulatory sequences are present in the 2,1 Kb upstream section of the SHIP-1 gene.

Analysis of this 5'-flanking regions using MATInspector program (http://www.genomatix.de/cgi-bin/matinspector/matinspector.pl) did not result in the predication of a core promoter region. No TATA-box and no GC-box were fund in the environment of the transcription start site. Despite the lack of these important elements, we could identify several putative transcription factor-binding sites in the mouse SHIP-1/2143bp gene promoter region, including several Smad, ATF2 and AP-1 DNA binding elements (Figure 3.4.3.b, see pages 191-192).

Figure 3.4.2.c. TGFβ ligands activate and signal through the JNK signaling pathway in immune cells.

Treatment with activin and TGFβ induces activation of the JNK MAP kinase-signaling pathway in immune cell types. Western blot analysis indicates the levels of phosphorylation of JNK and a downstream target molecules c-Jun following treatment with activin [0.5nM] and TGFβ [0.2nM] to MPC-11, M1 and DT40 at different periods of time as indicated. Membranes were stripped and reprobed with total anti-JNK and anti-tubulin, respectively to confirm equal loading (lower panel).

JNK kinase pathway

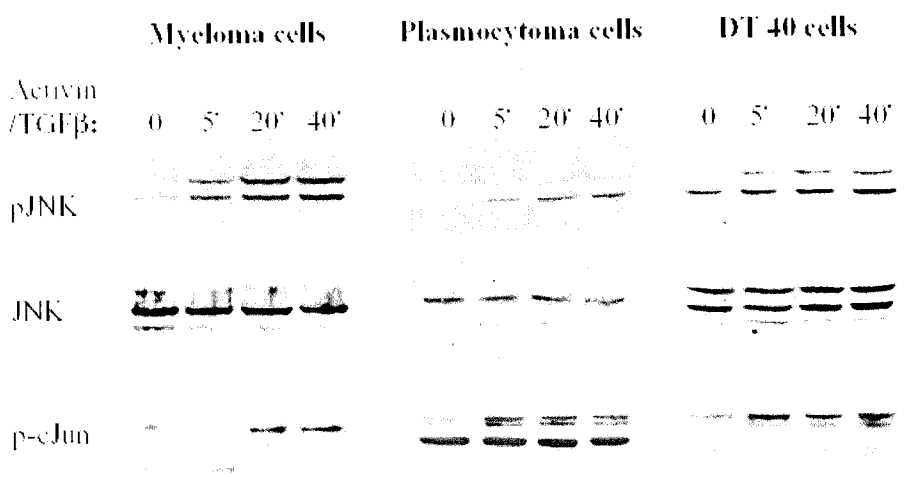

Figure 3.4.2.c.

Figure 3.4.3.a. Activin and TGFβ treatment induce activation of the SHIP-1 2143 bp gene promoter.

CHO cells were transfected with the 3TP-Lux and SHIP-1 2143-Lux reporter constructs or empty luciferase vector (pGL3) and the b-galactosidase expression plasmid. Cells were stimulated with either activin [0.5nM] or TGFβ [0.2nM] for 18h. The luciferase activity was normalized to β-galactosidase values. Results represent means and standard deviations of three independent experiments.

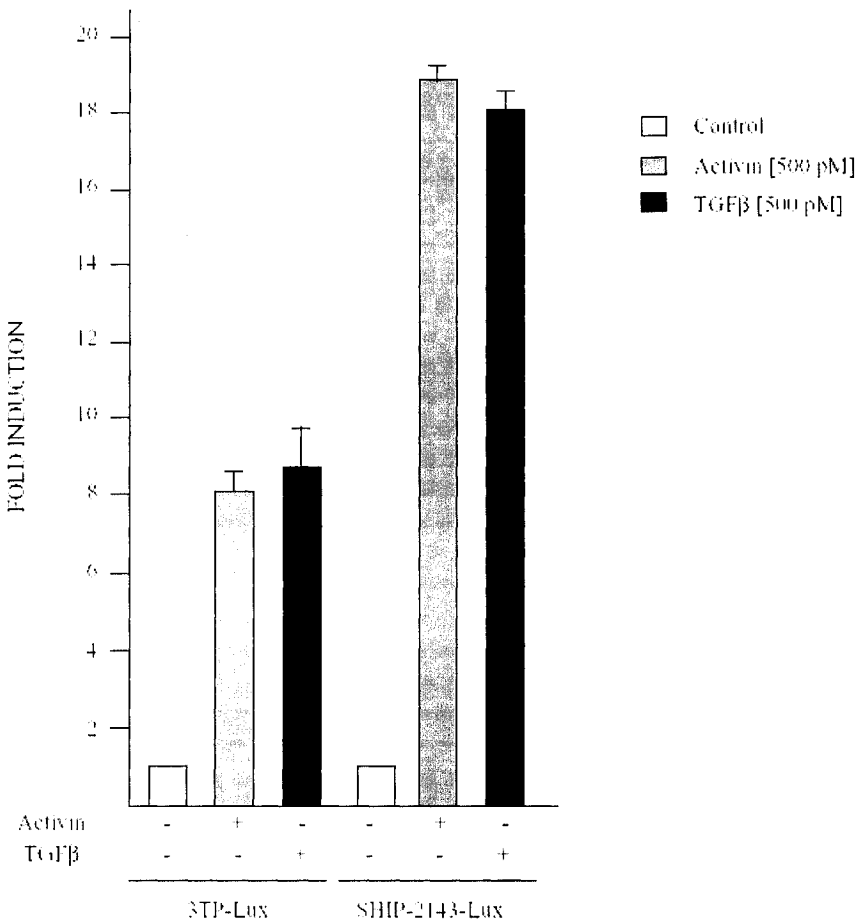
FOLD INDUCTION
20
18
16
14
12
10
8
6
4
2
Control
Activin [500 pM]
TGFβ [500 pM]
Activin
TGFβ
3TP-Lux
SBIP-2143-Lux

Figure 3.4.3.a.

Figure 3.4.3.b. Analysis of the 5' UTR regions of the SHIP-1 gene promoter. A schematic representation of the different potential transcriptional binding sites identified within the SHIP-1 gene promoter. These different sequences were identified by MATInspector program: http://www.genomatix.de/cgi-bin/matinspector/matinspector.pl.

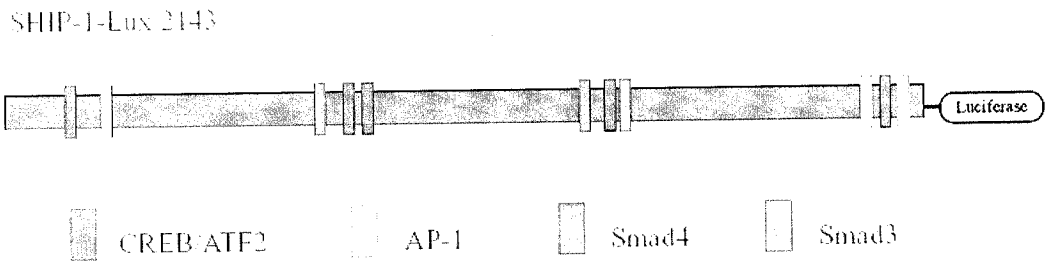

Figure 3.4.3.b.

To better characterize specific regulatory sequences within the 2.1kb section of the SHIP-1 promoter, progressive truncation mutants were generated by PCR using 5' specific sense primers. These truncated promoter regions were then subcloned in pGL3-basic vector respectively (Fig.3.4.3.c. pages 195-196) and their responsiveness to activin and TGFβ was assessed as described above for the 2,143 bp construct. As shown in Figure 3.4.3.b (page 191-192) removal of the first 1,112 base pair (SHIP-1-2143-Lux, mutants SHIP-1-1651-Lux, SHIP-1-1231-Lux, SHIP-1-1031-Lux) did not alter activin and TGFβ effect. When the next 200bp were removed, we observed a significantly 50% decrease in the activin/TGFβ response (mutant SHIP-1831-Lux). Further truncation of the next 200bp induced no change in the activin/TGFβ response, while removing the next 200bp also resulted in 50% decrease in activity (mutant SHIP–431-Lux). Finally, removal of the 100bp proximal to the start site (mutant SHIP-1-131-Lux) completely abolished the residual activity. Together these results indicate that SHIP-1 gene promoter contains three important domains (I, II and III) that are necessary for mediating activin/TGFβ induces transcriptional activation (Figure 3.4.3.d. see pages 197-198).

Based on our results from the signaling pathways activated downstream of activin/TGFβ in these cells (Fig.3.4.2, pages 180-181; 184-185; 187-188) and the deletion analysis (Figure 3,4,3,c, pages 195-196), we focused our attention to putative Smad, ATF2 and AP-1 binding sites within these three domains. Sequence analysis revealed a Smad4 binding consensus site found in domain I, and two AP-1 binding consensus sites, which are located in the third domain (Fig.3.4.3.c, pages 191-192). No ATF2 DNA binding site was found in any of the three domains nor was any binding site for Smad4 and AP-1 found in domain II.

3.4.4. Role of Smad4 and AP-1 transcription factors in the transcriptional regulation of the SHIP-1 gene promoter.

To confirm the importance of these three domains in activin/TGFβ-mediated SHIP-1 gene promoter activity, we generated specific internal deletion mutants within the full-length (2,143-Lux) promoter construct, in which each of the three domains were removed individually or in combination (Fig.3.4.4.a, see pages 200-201). These deletion constructs were fused to the basic luciferase reporter gene (pGL3) and the response to TGFβ ligands was evaluated.

Figure 3.4.3.c. SHIP-1 2.1 kb gene promoter progressive truncation analysis. Different progressive truncation analysis of the SHIP-1 2.1 kb gene promoter were transfected in CHO cell for at least 18 h. Cells then were stimulated with or without activin [0.5 nM] and TGFβ [0.2 nM] respectively for 18h and their responsiveness were assessed by luciferase assay. The luciferase activity was normalized to β-galactosidase values. Results represent means and standard deviations of three independent experiments.

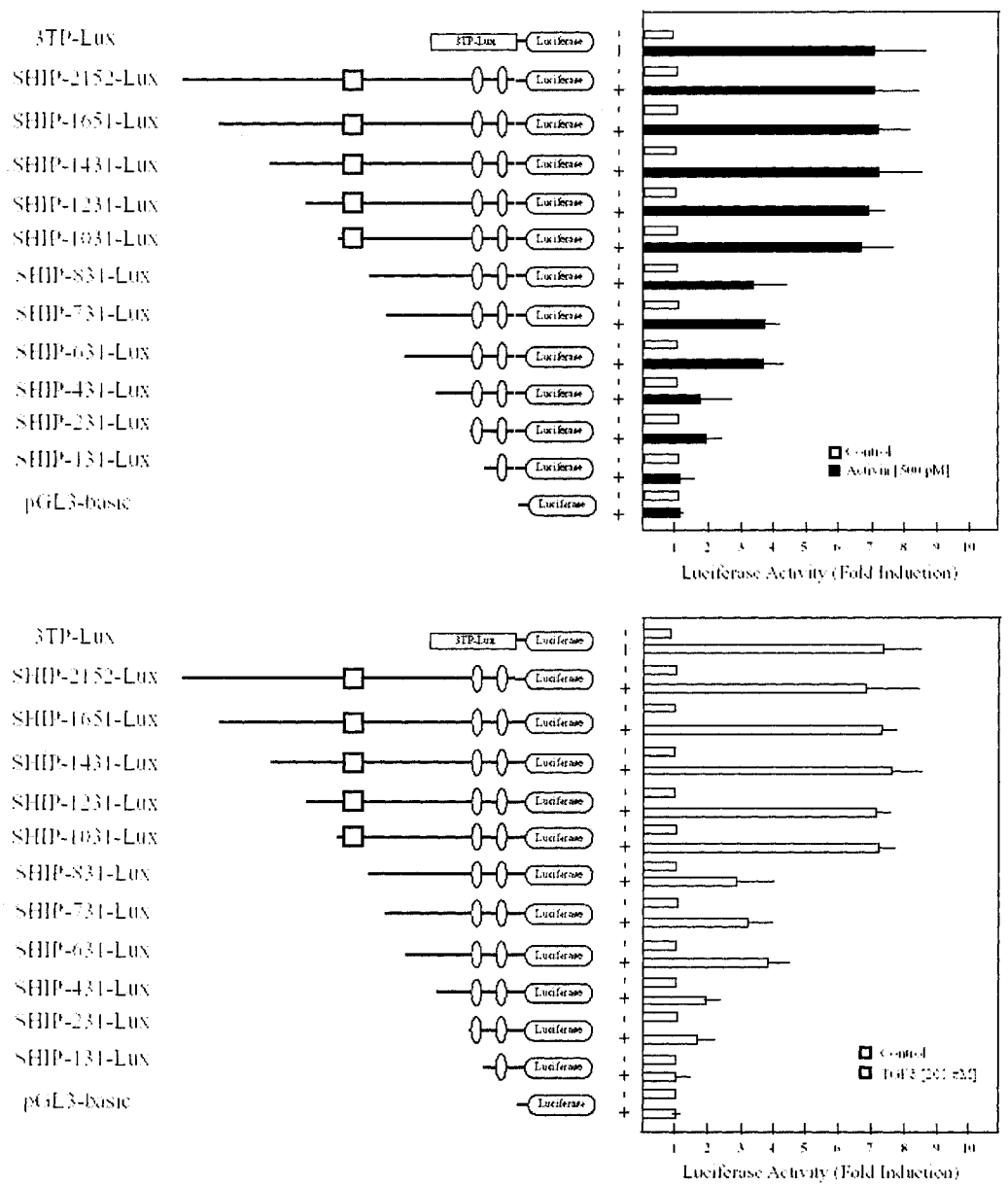
3TP-Lux
SHIP-2152-Lux
SHIP-1651-Lux
SHIP-1431-Lux
SHIP-1231-Lux
SHIP-1031-Lux
SHIP-831-Lux
SHIP-731-Lux
SHIP-631-Lux
SHIP-431-Lux
SHIP-231-Lux
SHIP-131-Lux
pGL3-basic
Luciferase
Control
Activin [500 pM]
1 2 3 4 5 6 7 8 9 10
Luciferase Activity (Fold Induction)
3TP-Lux
SHIP-2152-Lux
SHIP-1651-Lux
SHIP-1431-Lux
SHIP-1231-Lux
SHIP-1031-Lux
SHIP-831-Lux
SHIP-731-Lux
SHIP-631-Lux
SHIP-431-Lux
SHIP-231-Lux
SHIP-131-Lux
pGL3-basic
Luciferase
Control
TGF-β [200 pM]
1 2 3 4 5 6 7 8 9 10
Luciferase Activity (Fold Induction)

Figure 3.4.3.c.

Figure 3.4.3.d. Schematic representation of the SHIP-1 promoter and the three potential regulatory domains involved in the transcriptional activation.

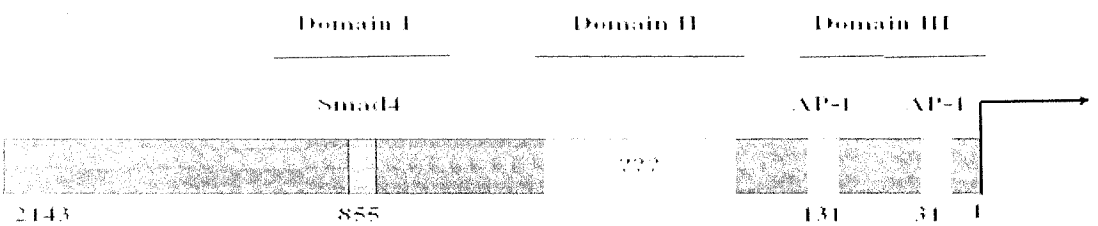

Figure 3.4.3.d.

As shown in Figure 3.4.4.a removal of each single domain leads to more of 50% decrease in luciferase activity (Figure 3.4.4.a, constructs B, C, and D, see page 200-2001). These results suggest that each of these domains participate in the transcriptional regulation of the SHIP-1 gene in response to TGFβ ligands. Next, we removed these three domains in combination (Fig. 3.4.4.a; constructs E, F, G and H, pages 200-201). We then evaluated the ability of these to respond to TGFβ ligands and we observed that the activation is severely reduced if not completely abolished in response to activin and TGFβ as expected (Fig. 3.4.4.a, pages 200-201). These results clearly indicate that these three domains play an important role in the activin/TGFβ-induction of the SHIP-1 gene promoter. The involvement of these domains seems to be crucial for the proper transcriptional conformation and regulation of the SHIP-1 gene promoter region in response to TGFβ ligands. As mentioned above, we identified a Smad4 binding site in domain I, and two AP-1 binding sites within domain III. In order to evaluate the role of these potential transcription-binding sites, we modified the Smad and AP-1 binding sequences in the SHIP-1-2143-Lux construct. For this purpose, we generated a series of reporter constructs that contained specific site-direct mutations targeting these transcription binding sites within in domain I and domain III (Fig.3.4.4.b, see pages 202-203). As expected, each of these constructs produced the same effect of the deletion of the separate domain, inducing more that 50% decrease in ligand-induced activity (Fig. 3.4.4.b, see pages 202-203).

Figure 3.4.4.a. Internal deletion analysis of SHIP-1 gene promoter regulation.

The different individual 200 bp internal deletions of the SHIP-1 2.1 kb gene promoter constructs were transfected in CHO cells for 18h. Cell were synchronized by starvation for 12h and then stimulated for at least 18h with or without activin [0.5nM] or TGFβ [0.2nM] for 18h respectively. Their responsiveness to activin/TGFβ was assessed by luciferase activity. The luciferase activity was normalized to β-galactosidase values. Results represent means and standard deviations of three independent experiments.

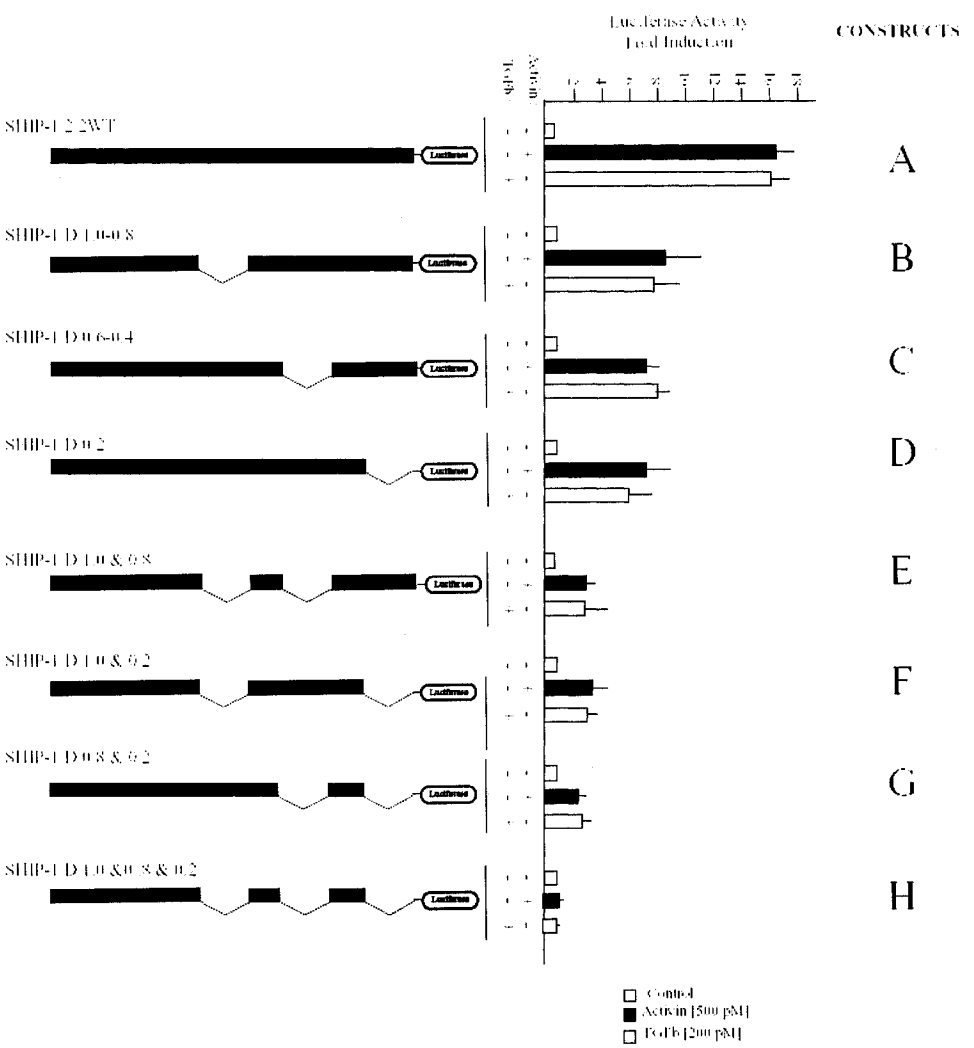
Luciferase Activity
Fold Induction
CONSTRUCTS
A
B
C
D
E
F
G
H
Control
Activin [500 pM]
FGFb [200 pM]

Figure 3.4.4.a

Figure 3.4.4.b. Effect of internal deletions in Smad4 and AP-1 binding sequence on SHIP-1 gene promoter activity. The different individual specific point mutations for Smad4 and AP-1 binding sequences respectively of the SHIP-1 2.1 kb gene promoter constructs were generated and then transfected in CHO cells for 18h. Cell were synchronized by starvation for 12h and then stimulated for at least 18h with or without TGFβ. Their responsiveness to activin/TGFβ was assessed by luciferase activity. The luciferase activity was normalized to β-galactosidase values. Results represent means and standard deviations of three independent experiments.

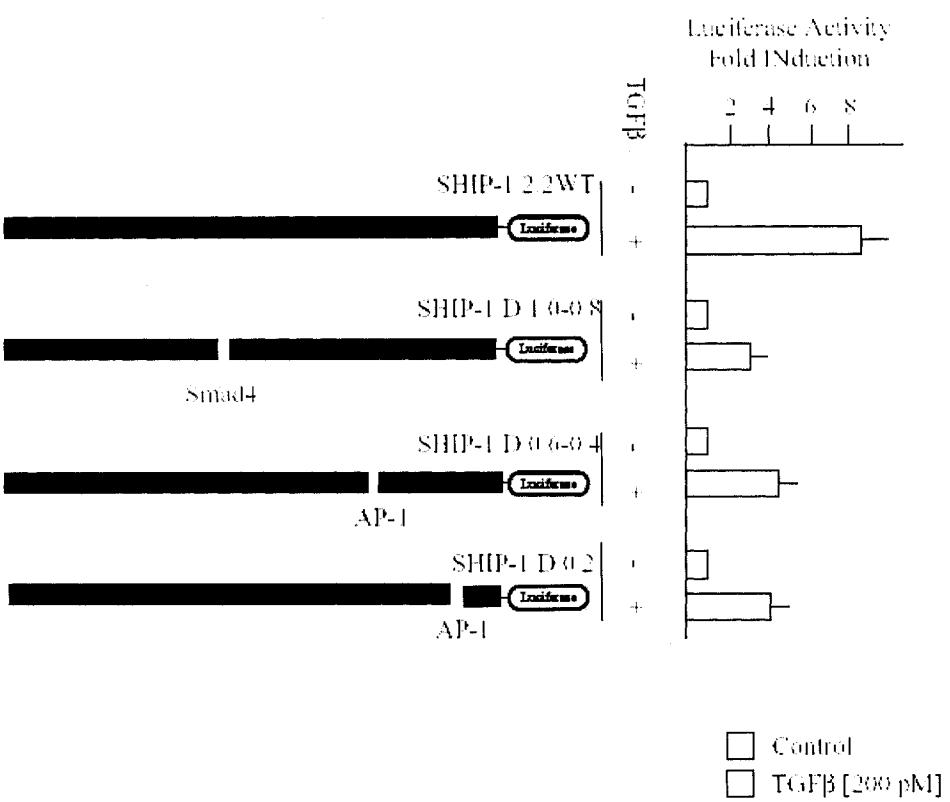
Luciferase Activity
Fold INduction
2
4
6
8
TGFβ
SHIP-1 2.2WT
Luciferase
+
SHIP-1 D1 0-0 8
Luciferase
+
Smad4
SHIP-1 D 0 6-0 4
Luciferase
+
AP-1
SHIP-1 D 0 2
Luciferase
+
AP-1
Control
TGFβ [200 pM]

Figure 3.4.4.b.

These results indicate that regulation of the SHIP-1 gene promoter by TGFβ family members requires the presence of a Smad4 DNA binding site at position -855, and two AP-1 DNA binding sites in the proximal region of the promoter position –151 and -31. Our results also indicate that a third region located between domain I and II also plays an important role, even though it does not contain any Smad, ATF2 or AP-1 binding regulatory sites.

3.4.5. Activin and TGFβ induce Smad4 and AP-1 binding to the mouse SHIP-1 gene promoter.

We performed electro-mobility shift assays (EMSA) using nuclear cell extracts from cells stimulated either with activin or TGFβ in an attempt to characterize the DNA-binding activity of the Smad4 and AP-1 transcription factors that specifically bind to the SHIP-1 gene promoter (Smad4: 5'-AAC ATA GCC ATG TCT AGC CAC AGA GGG AAT 3'; AP-1 (AP-1#1: 5'-AAA CAG GAA GTC AGT CAG TTA AGC TGG TAG-3' and AP-1 #2: 5'-TGG GAG TGG CTG CTG ACC CAG TCC AGG AGA-3'). Nuclear extracts were prepared as previously described [51] from non-stimulated or TGFβ-stimulated M1 cells for different periods of time: while, maximum binding occurred at 45 min the complex could be clearly detected after 10 min. (Fig. 3.4.5.a, pages 206-209). This suggests that de novo protein synthesis is not necessary and that a preexisting transcriptional complex is rapidly and post-translationally modified or translocated into the nucleus. As shown in Fig. 3.4.5.a, (pages 206-207; 208-209) while no shift is observed in the absence of stimulation, a specific protein-DNA complex showed retarded gel mobility in cells stimulated with TGFβ for the Smad4 and the two AP-1 probes (lanes 1 and 2 for each panel, pages 206-207 and 208-209 respectively). The specificity of the retarded

protein complex was verified by co-incubating the reaction with cold competitor probes [100X] that induced complete disappearance of the signal (lanes 3 and 4 for each panel). This DNA-binding complex formation is specific since this excess of the non-radiolabelled oligonucleotide probes, displace the corresponding bands (Fig. 3.4.5.a., see pages 206-209).

In order to determine whether our 24 bp oligonucleotide probes used in the mobility shift assays could interfere with transcription factor other than Smad4 and AP-1, we generated mutant probes in which the wildtype sequence for Smad4 or AP-1 binding sites were specifically changing disrupting the specific recognition sequence for these transcription factor respectively. These specific sequences are: MUTSmad4: 5'-GCA AAC ATA GCC ATA CTC AGC CAC AGA GGG -3'; MUTAP-1 #1: 5'-GCC TGA AAC AGG AAG TAA AAC AGT TAA GCT GGT AGG-3'; MUTAP-1 # 2: 5'-GAG TGG CTG CTG TTT TAG TCC AGG AGA C-3'). The mutant and wild type probes were then used in shift assays using nuclear extracts from M1 myeloma cells stimulated or not with TGFβ.

As shown in Figure 3.4.5.a and b (see pages 206-209 respectively), incubation of the nuclear extracts with the mutants probes for Smad4 and the two AP-1, were unable to induce any retarded protein complex (lanes 1 and 2 in each panel) as compared to the wild type probes (lanes 3 and 4 in each panel). These results show that the interaction is sequence-specific as mutant probes are unable to induce any complex formation. Thus, the presence of a Smad4 binding site and two AP-1 binding sites are required for TGFβ ligands to induce the SHIP-1 promoter and prevention of Smad4 and AP-1 to bind to their respective sites abolishes TGFβ effect. Similar effects were obtained using nuclear extracts from MPC-11 plasmocytoma cell lines stimulated with activin.

Figure 3.4.5.a. Activin/TGFβ treatment induces Smad4 DNA binding to the SHIP-1 gene promoter. Smad 4 binds to SHIP-1 gene promoter in upon activin and TGFβ stimulation. Nuclear extracts from non-stimulated M1 (lane 1) or stimulated with TGFβ [0.2nM] (lane 2) were incubated with a ^{32}P labeled Smad4 probe and separated by electromobility shift assay (EMSA). In lane 3 and 4 the specificity on the retarded protein complex was verified by co-incubating the reaction with cold probes [100X]. In addition, a specific mutant probes for Smad4 biding site was used to confirm that the probes used during these experiments were not interfering with other proteins (lanes 5 and lane 6). In lane 7 and 8, the specificity on the retarded protein complex was verified by co-incubating the reaction with cold mutant probe for Smad4 [100X].

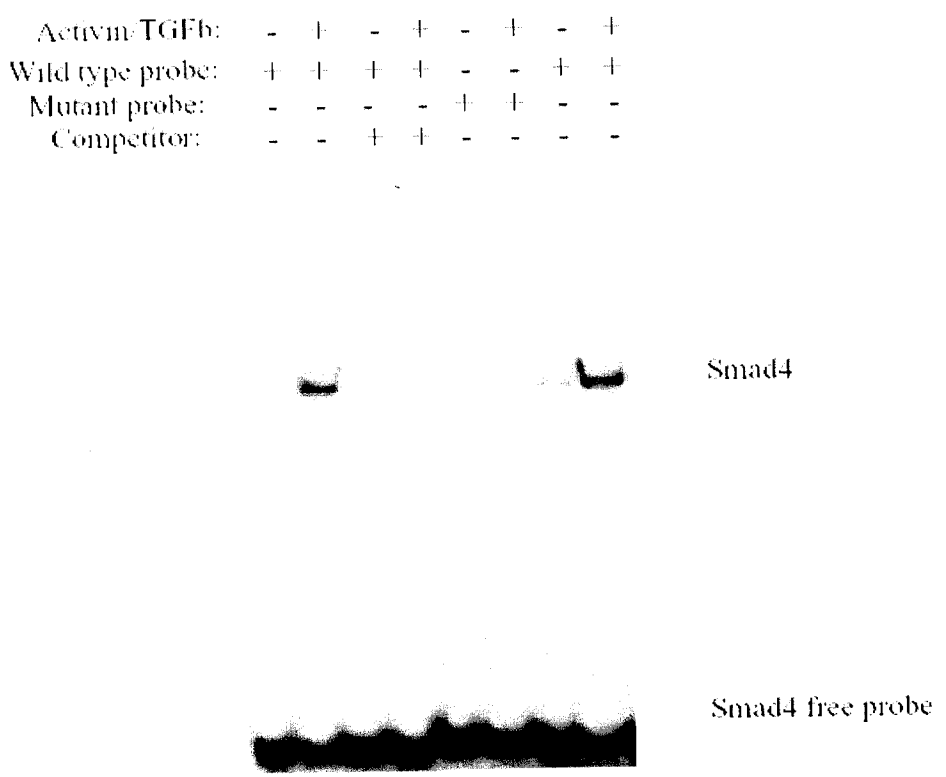
Activin/TGFb: - + - + - + - +
Wild type probe: + + + + - - + +
Mutant probe: - - - - + + - -
Competitor: - - + + - - - -
Smad4
Smad4 free probe

Figure 3.4.5.a.

Figure 3.4.5.b. Activin/TGFβ treatment induces AP-1 DNA binding to the SHIP-1 gene promoter. AP-1 binds to SHIP-1 gene promoter in upon activin and TGFβ stimulation. Nuclear extracts from non-stimulated M1 (lane 1) or stimulated with TGFβ (lane 2) were incubated with a ^{32}P labeled AP-1#1 and AP-1#2 probes respectively they were separated by electromobility shift assay (EMSA). In lane 3 and 4 the specificity on the retarded protein complex was verified by co-incubating the reaction with cold probes [100X]. In addition, a specific mutant probes for AP-1 biding site was used to confirm that the probes used during these experiments were not interfering with other proteins (lanes 5 and lane 6). In lane 7 and 8, the specificity on the retarded protein complex was verified by co-incubating the reaction with cold mutant probe for AP-1 #1 and AP-1 #2 [100X].

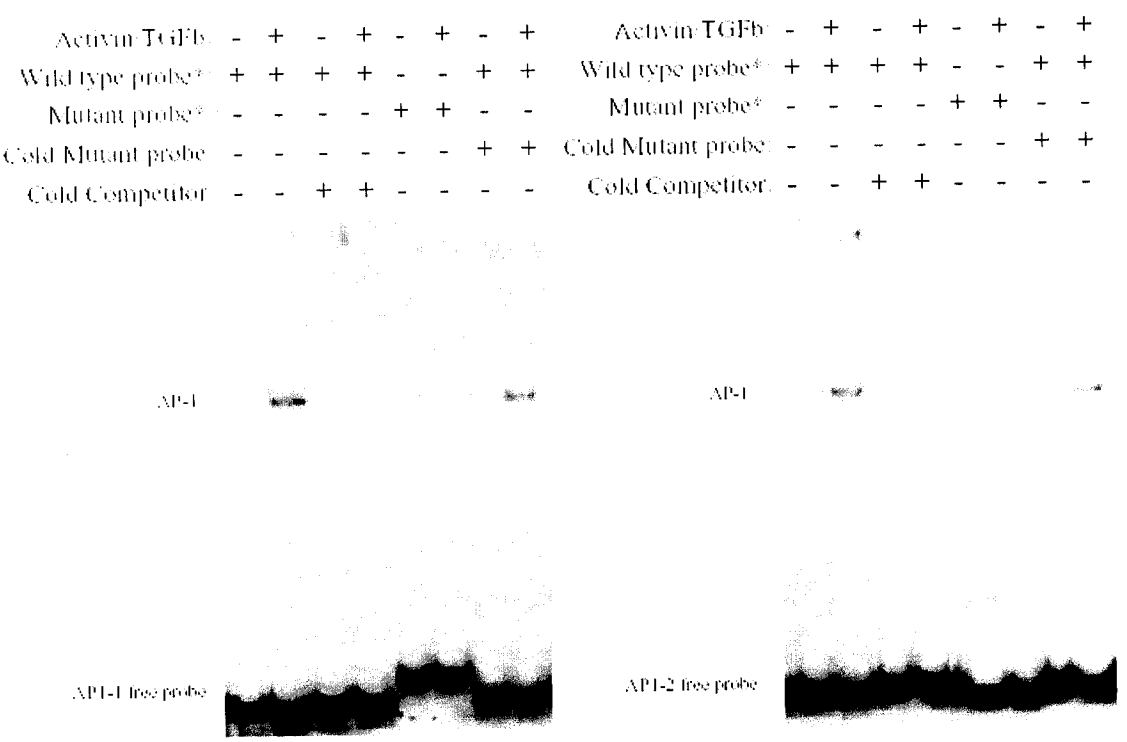
Activin/TGFb - + - + - + - +
Wild type probe* + + + + - - + +
Mutant probe* - - - - + + - -
Cold Mutant probe - - - - - - + +
Cold Competitor - - + + - - - -
Activin/TGFb - + - + - + - +
Wild type probe* + + + + - - + +
Mutant probe* - - - - + + - -
Cold Mutant probe - - - - - - + +
Cold Competitor - - + + - - - -
AP-1
AP-1
AP1-1 free probe
AP1-2 free probe

Figure 3.4.5.b.

We next extended our analysis to determine whether Smad 4 could directly interact with AP-1 as previously observed [52]. To demonstrate the physical presence of Smad4 and AP-1 on the DNA of their respective binding sites, **DNA Affinity Precipitation** assay (**DNAP**) experiments were performed. Briefly, we used a 3' biotinylated oligonucleotides corresponding to the Smad4 (Smad4: 5'-AAC ATA GCC ATG TCT AGC CAC AGA GGG AAT 3'-Biot and 3'-TTG TAT GGC TAC AGA TCG GTG TCT CCC TTA-5') and the two AP-1 (AP-1#1: 5'-AAA CAG GAA GTC AGT CAG TTA AGC TGG TAG-3'-Biot and 3'-TTT GTC CTT CAG TCA GTC AAT TCG ACC ATC-5'; and AP-1 #2: 5'-TGG GAG TGG CTG CTG ACC CAG TCC AGG AGA-3'-Biot and 3'-GCC CTC ACC GAC GAC TGG GTC AGG TCC TCT-5').

The different biotinylated oligonucleotides were then incubated and mixed with 100-200 μg of nuclear cell extracts from unstimulated and stimulated MPC-11 plasmocytoma cell lines and incubated with activin at 4°C for 2 hr. After this, 30 μl of resuspended Streptavidin beads were added to each reaction-mixtured and kept at 4°C overnight with shaking. The protein complexes associated with the beads were washed 3 times and subjected to a western blot analysis. We used anti-Smad4 and anti-c-Jun specific monoclonal antibodies to determine the presence of these transcription factors in the immunoprecipitated DNA-protein complexes. As is shown in Fig 3.4.5.c (pages 212-213), formation of activin/TGFβ-induced Smad4-DNA complex was detected. (Fig. 3.4.5.c., left panel, pages 212-213). Similar results were obtained when we evaluated the presence of any member involved in the formation of the AP-1 complex, c-Jun [53]. In addition, Figure 3.4.5.c, right panel, (pages 212-213) demonstrate that activin and TGFβ, are able to induce the recruitment of AP-1 sites of the SHIP-1 promoter. Nuclear extracts from MPC-11 cells

incubated with or without activin for 45 min were treated with the biotynilated probe for 2 hrs. After washing, the DNA-protein complexes were subjected to electrophoresis and western blot analysis respectively. As shown in Fig.3.4.5.c (left panel, pages 212-213), activin clearly induces the DNA binding of Smad4 on its specific binding site. Similarly, activin is able to induce DNA binding of c-Jun on the two proximal AP-1 sites (Figure.3.4.5.c right panel, pages 212-213). Interestingly, both 49KDa and 47KDa but not 43 kDa isoforms of c-Jun was able to bind DNA. Equal loading and purity for the nuclear extracts were confirmed by using an anti- TFIIB antibody. As shown in Fig.3.4.5.c (bottom panel, pages 212-213), whereas there is no detectable presence of TFIIB in the cytoplasmic fractions, a significant and equal amount of this protein was observed in the nuclear extracts prepared of both non-stimulated and stimulated samples. (Fig. 3.4.5.c bottom panel, pages 212-213).

Figure 3.4.5.c. Binding of Smad 4 and c-Jun to SHIP-1 gene promoter. M1 cells were starved for 24 h in serum-free culture medium and then treated with TGFβ for 45 min. The nuclear extracts were prepared and subjected to DNA affinity precipitation assay. Wild-type [54] Smad4 and c-Jun 3′-biotinated oligonucleotides corresponding to domain I and III in the SHIP-1 gene promoter regions were used as probes for DNA affinity precipitation assay. DNA-proteins complexes bound were incubated with sepharose beads. Subsequently, these complexes were eluted and then resolved by 10% SDS-PAGE for immunoblot analysis with anti-Smad4 and anti-c-Jun antibodies respectively. Western blot analysis was done as described above. Equal loading was revealed by using a mouse monoclonal antibody against the nuclear Pol II transcription factor II B, TFIIB (bottom panel).

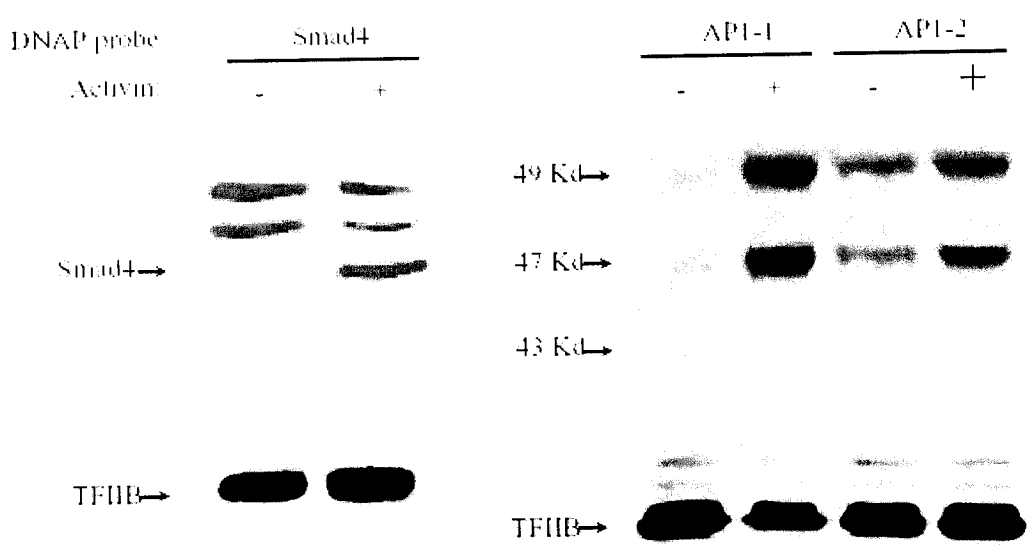
DNAP probe
Smad4
Activin
-
+
Smad4
TFIIB
AP1-1
AP1-2
-
+
-
+
49 Kd
47 Kd
43 Kd
TFIIB

Figure 3.4.5.c.

Smad4 and AP-1 co-operation has been previously reported in other TGFβ responsive systems [53]. Interestingly, it appears that Smad4/AP-1 association occurs once these transcription factors are phosphorylated and activated followed by a specific molecular association that subsequently induces their respective binding to the specific binding site sequences (Figure 3.4.5.c., pages 212-213). Thus, these results demonstrated that Smad4 and AP-1 are being recruited to their specific binding sites the domain I, and domain III of the SHIP-1 gene promoter in a ligand-dependent manner.

3.4.6. Activin and TGFβ signaling induces complex formation between Smad4 and AP-1.

To determine if Smad4 and AP-1 physically interact, co-immunoprecipitation experiments were performed. MPC-11 and M1 cells were stimulated with activin or TGFβ respectively to different time points. Total cell lysates were incubated with an anti-Smad4 or anti-c-Jun antibodies. These immunoprecipitated complexes were then revealed by Western blot with the c-Jun or anti-Smad4 specific monoclonal antibodies respectively. As shown in Figure 3.5.6.a (pages 215-216), both Smad4 and c-Jun associate with each other in a ligand-dependent manner. As a control, total lysates were immunoblotted with the anti-Smad4 or anti-c-Jun antibody to show equal expression levels of endogenous proteins. The outcome of these experiments indicates that activin and TGFβ signaling pathways lead to complex formation between the Smads and AP-1 transcription factors in immune cell types.

Figure 3.4.6.a. Activin and TGFβ induce Smad4 and c-Jun interaction in an activin/TGFβ-dependent manner. Co-immunoprecipitation analysis using anti-Smad4 and anti-c-Jun antibody (Santa Cruz), from MPC-11 and M1 cell pretreated with activin and TGFβ respectively, were immunoblotted for endogenous Smad4 and c-Jun (top panel). Total lysates revealed the interaction between Smad and c-Jun in a ligand-dependent manner. Total lysates revealed for anti-Smad4 and anti-c-Jun demonstrate equal protein expression (bottom panel).

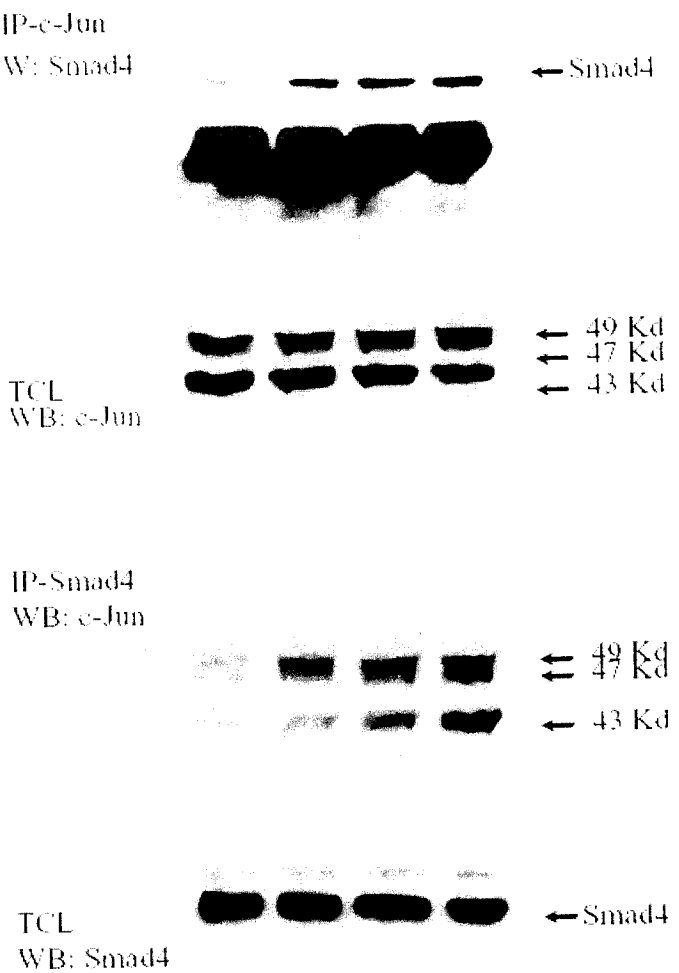
IP-c-Jun
W: Smad4
←Smad4
49 Kd
47 Kd
43 Kd
TCL
WB: c-Jun
IP-Smad4
WB: c-Jun
49 Kd
47 Kd
43 Kd
TCL
WB: Smad4
←Smad4

Figure 3.4.6.a.

3.5. Discussion.

TGFβ ligands are involved in the regulation of a large variety of biological processes such as cell cycle arrest and apoptosis [1]. Both activin and TGFβ up-regulate cell cycle arrest inducing factors $p15^{INK4B}$ [2] and $p21^{CIP1/WAF1}$ [3, 4]. Activin and TGFβ also down-regulate growth-promoting transcription factors like c-myc [5], inhibitors of DNA binding (ID)-1 and ID-2 [6] and the tyrosine phosphatase Cdc25A [7].

Even though activin and TGFβ act as a potent inhibitor of cell growth, their effects on cell cycle arrest and apoptosis regulators in immune cell types have not been fully characterized. In this study we demonstrated that both activin and TGFβ exert a strong growth inhibitory effect in immune cells and that their effects are mediated through transcriptional activation of SHIP-1. Our findings also revealed for the first time that both Smad and the AP-1 family of transcription factors are required for maximal induction of SHIP-1 gene by activin and TGFβ.

Using the B lymphocyte cell line DT40 we first elucidated the mechanisms involved in the induction of inositol phosphatase SHIP-1 expression by activin/TGFβ. We confirmed the involvement and activation of Smad pathway as expected, and additionally demonstrated the involvement also of JNK/AP-1 signaling pathway.

Our findings clearly demonstrated that activin and TGFβ induce Smad-AP-1 complex formation that is required for their synergistic actions are in agreement with similar findings in immune cell types [8, 9]. Sequence and deletion analysis of the promoter region of SHIP-1 revealed that Smad4 consensus binding site resides between -1031 to -831 bp, whereas two AP-1 response elements are situated in the region of -131 to -1 bp upstream of the initiation site [8, 9].

Deletion analysis of the different mutants of SHIP-1 promoter-luciferase constructs revealed that removal of either domain I or domain III significantly attenuates the stimulatory effects of activin or TGFβ by approximately 50%. Surprisingly, deletion of the promoter sequence between these two domain sequences, domain II (-830 to –232 bp), significantly attenuated the normal effects to a comparable extent.

Interestingly, our results from the sequence analysis of the full-length SHIP-1 gene promoter indicated that domain II did not appear to contain any activin/TGFβ-regulated response elements previously reported.

These observations raise the question of how does TGFβ induced association between Smads (Smad4) and AP-1 transcription factors (c-Jun and/or c-Fos), allowing them interact with their respective regulatory sites in domain I, and domain III, which are quite separated?

Our promoter sequence analysis provides a possible answer to this question. We identified the existence of an intermediate promoter region (domain II), situated –830 bp to –232 bp between domain I, and domain III. This promoter region seems to be required and involved in the activin/TGFβ-induced SHIP-1 gene promoter activation.

Removal of domain II results in a significant decrease in promoter activity by approximately 50%. Moreover, the activin/TGFβ-induced promoter activity was almost if not completely abolished, when these regions were deleted in different combination (Constructs A-H, Figure 3.4.4.a, and page 201). Interestingly, sequence analysis of the domain II in the SHIP-1 gene promoter, indicate the absence of potential consensus binding sequences which could be activin/TGFβ-transcriptional regulated.

This suggests that this promoter regions is involved in a ligand-independent regulatory transcriptional regulation indicating that the full-length promoter (-2143 to –1 bp) is required for synergizing the inductive effects of Smads and AP-1.

The most characterized intracellular mediators of the activin/TGFβ signaling pathways are the two pathway-specific Smads: Smad2 and Smad3. Both Smad2 and Smad3 heterodimerize with the common Smad partner Smad4 in a ligand-dependent manner. Once the Smad complex is formed and activated, it translocates into the nucleus where it participates in the activation of target genes. However, it has been shown recently that the Smad proteins do not act in seclusion, but rather interact with various other signaling molecules, required for specific activin/TGFβ-induced target gene activation in different cell context.

Our results clearly indicate that activin/TGFβ-mediated SHIP-1 expression requires both the Smad pathway and the transcription factor c-Jun. It has been reported that TGFβ anti-proliferative effects in immune cells also involves both the Smad and AP-1 [9]. Furthermore, work from others as well as data presented herein clearly indicate that Smad proteins physically interact with the transcription factors c-Jun and c-Fos upon activin or TGFβ stimulation [9]. Thus, this could represent a general mechanism of cell growth inhibition by TGFβ family members in their various target tissue.

Different reports have demonstrated that the role of Smad2 and Smad3 in mediating both activin and TGFβ effects on gene transcription of target genes is clearly complex. For example, activin/TGFβ effects on the reporter construct 3TP-Lux that contains a section of the plasminogen activator inhibitor-1 (PAI-1) display a significant increase in response of TGFβ as well as in the presence of Smad 2 or Smad3 [10-15].

However, other activin and TGFβ responsive promoters such as the goosecoid promoter, respond in a completely different manner to Smad2 and Smad3, with Smad2 exerting a positive effect and Smad3 an inhibitory effect [16, 17]. This could presumably be due to a competition between Smad2 and Smad3 for their common DNA binding site on the goosecoid promoter [18].

These observations clearly suggest that while Smad2 acts as a positive regulator, Smad3 plays a dual role depending on the nature of the promoter.

Our findings reported in Chapter 2 indicate that with respect to the SHIP-1 gene promoter activation, both Smad2 and Smad3 act as a positive regulators of the activin and TGFβ response [15]. We also showed that Smad2 and Smad3 in the presence of Smad4 increased the SHIP-1 gene promoter activation in response to activin and TGFβ.

In addition, we also previously reported that while both Smad2 and Smad3 act as positive regulators, Smad7 completely abolishes the activin/TGFβ-induced SHIP-1 gene promoter activity, confirming the role of Smad7 as a strong inhibitor of the activin/TGFβ signaling pathways [15, 19].

Several promoters, which are transcriptionally activated by TGFβ ligands, require the association and interaction of Smad protein with other transcription factors such as SP-1 [20], CREB/p300, myocardin (Myocd) [21], hairy/enhancer-of-split-related transcription repressor (Hey) [22], PKA, GA-binding protein (GABP) [23], glucocorticoid receptor (GR) [23-26], ATF2 [27], ATF3 [6], hepatocyte nuclear factor 4 (HNF-4) [28], TGFβ stimulated factor 1 (TSF1) [29], NFkB [30, 31], p53 [32] and AP-1 [9, 33, 34].

For this latter group of transcription factors, transcriptional cooperation between Smads (Smad4) and AP-1 (c-Jun) correlates with a physical interaction between these proteins required for the transcriptional activation of different TGFβ-regulated promoters [9]. AP-1 family member, c-Jun and c-Fos, can form homo- or heterodimers in order to activate transcription in different promoter regions of several target genes through their ability to interact directly with the AP-1 binding site [35, 36].

To date different DNA consensus binding sequences in the promoter regions of several TGFβ-induced target genes have been described [35, 36]. For example, the 12-O-tetradecanoyl-

13-acetate (TPA)-responsive gene promoter elements (TRE) are involved in the transcriptional responses of several TGFβ-regulated target genes. Interestingly, it has been described that members of the AP-1 transcription factors family members, c-Jun and c-Fos bind to and regulate transcription from TREs, which are therefore known as AP-1 binding sites [9]. In this experimental system, Smad proteins interact directly with the TRE in a ligand-dependent manner.

Association of Smad3 with Smad4 can activate TGFβ-inducible transcription from the TRE in both c-Jun/c-Fos-dependent and -independent manner [9]. Thus, these protein-protein associations complement interactions between c-Jun and c-Fos, and between Smad3 and Smad4 [9].

Interestingly, the proposed mechanism of transcriptional activation by TGFβ, through functional and physical interactions between Smad3-Smad4 and c-Jun-c-Fos, strongly suggests that Smad signalling and MAPK/JNK signalling converge at AP-1-binding sites on different promoter of target genes [9].

Our finding lead us to propose a new transcriptional model in which domain II might play a promoter conformation role, which is necessary for the activin/TGFβ-mediated transcriptional activation of the SHIP-1 promoter. Domain II brings into proximity domain I, and domain III thereby permitting the Smad/AP-1 complex to exert maximal activation of SHIP-1 promoter activity.

In this scenario, the direct interaction between Smad4 and c-Jun will maximize the activin/TGFβ-induced transcriptional activation of the SHIP-1 gene promoter in immune cell types. Thus, collectively our results propose a novel transcriptional mechanism by which activin and TGFβ regulate the transcriptional activation of the SHIP-1 gene promoter.

Additionally, this transcriptional regulation requires the direct association between the activated Smads and AP-1 (c-Jun) transcriptional factors acquired by a promoter conformational change regulated by domain II.

In this study, I also provided data showing that activin and TGFβ activate and signal through the p38 module of MAP kinase pathway, which specifically regulates a specific downstream transcription factor, ATF2/CREBP1 as previously reported [37-43]. Our promoter analysis data did not reveal the presence of any ATF2/CREBP1 binding sequences in the three different promoter regions (domain I, II and III), which are involved in the transcriptional regulation of the SHIP-1 gene promoter.

The precise role of p38 signalling cascade in activin/TGFβ-induced SHIP-1 expression remains to be elucidated. It will therefore be interesting in future studies to determine whether the Smads physically interact with members of the p38 MAP kinase cascade to regulate the transcriptional activation of the SHIP-1 gene promoter.

The results presented here do not imply that the activin/TGFβ-induced SHIP-1 expression is the only molecular mechanism used by TGFβ family members to induce and regulate growth arrest and apoptosis in immune cells. The existence of other regulated genes and signaling pathways in immune cells potentially modulated by any TGFβ ligand cannot be disregarded. This statement is supported based on the recent observations that demonstrate the involvement of DAXX and DAPK signaling pathways in TGFβ-induce cell growth arrest and apoptosis [45]. Collectively, taken together the above findings, we can conclude that the mechanism described here by which activin and TGFβ exert their cell growth inhibitory and apoptotic effect in immune cell system is essential to keep the proper balance in cellular functions which is reflected in the host's homeostasis.

In summary the data presented in this chapter demonstrate that Smads and c-Jun interact with distinct domains of SHIP-1 gene promoter. In addition, these transcription factors contribute equally to the inductive effect of activin and TGFβ. The effects of Smad4 and c-Jun are synergistic, but only when the intervening region connecting Smad4 and c-Jun response binding elements is presented. This coupled with the fact that Smads and AP-1 physically associate, suggest that the domain connecting these group of transcription factors, response domain provide a proper conformational folding of the promoter to bring the two elements in close proximity.

3.6. Material and Methods

3.7.1. Cell culture: Cell lines were obtained from the American Type Culture Collection (ATCC). MPC-11, CHO, 293, wild type and Smad4 knock-out ($Smad4^{-/-}$) mouse embryonic fibroblasts (MEF) cells were cultured in culture with DMEM media containing 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2mM L-Glutamine (complete medium). M1 cells were cultured in RPMI containing 10% FCS supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 2mM L-Glutamine (complete medium). DT40 cells were cultured in DMEM containing 10% FCS, 2% Chicken serum (CS), supplemented with 50mM b-mercaptoethanol, 100 IUs/ml penicillin, 100 μg/ml streptomycin and 2mM L-Glutamine (complete medium). All the cell lines were kept at 37° C in a humidified 5% CO_2: 95% air atmosphere.

3.6.2. Cloning and generation of SHIP-1-1 2.2 kb reporter constructs: The 2.2-kb sequence of the SHIP-1-1 gene promoter was generated by PCR from genomic DNA extracted from MPC-11. The amplified promoter fragment was digested by XhoI and Hind III and cloned into the pGL3 luciferase basic reporter vector (lacking promoter/enhancer sequences) to generate the 2.2-kb SHIP-1-Lux reporter construct. Furthermore, the 5' SHIP-1 deletion constructs were generated by PCR. The sequences of the oligonucleotides cloned are: **SHIP-1-2143 (2.2)**: 5'-CCG CTC GAG GGA TCC CAG ACA TTG GAC GG-3'; **SHIP-1-1651 (1.7)**: 5'-CCG CTC GAG GCT CTA TAA GAG AGC AGG C-3'; **SHIP-1-1231 (1.2)**: 5'-CCG CTC GAG CAA GCT CCT GCC ATG GAT GA-3'; **SHIP-1-1031 (1.0)**: 5'-CCG CTC GAG AGA TGA AGG AGT GAA CAA TCA-3'; **SHIP-1-831 (0.8)**: 5'-CCG CTC GAG TGA CTG GCC ATT TGG GCT TC-3'; **SHIP-1-731 (0.7)**: 5'-CGC CTC GAG TTT TAG CTA GGG AT AGT GTT GCT CAC

AGT-3'; **SHIP-1-631 (0.6)**: 5'-CCG CTC GAG ATT AAA ACA ACC CCT ACA GAT GT-3'; **SHIP-1-431 (0.4)**: 5'-CGC CTC GAG CCT ATT CAC AAA TGT TGG GCC-3'; **SHIP-1-231 (0.2)**: 5'-CCG CTC GAG CCC CCC CCC CAC TTG GTT TCT-3'; **SHIP-1-131 (0.1)**: 5'-TAA GCT GGT AGG AGC AGC AGA GGC AAT TTC T-3'; and the complementary strand used for all the PCR reactions: 5'-CCC AAG CTT ATG CCR GCC ATG GTC CCT-3' OR 5'-CCC AAG CTT GGG TCT CCT GGA CTG GGT C-3'. SHIP-1 promoter internal deletions are: **SHIP-1-2.2 Δ 1.0-0.8**: 5'-CAT CTA ACG TGA CAA GGT TCA TGA CTG GCC ATT TGG GCT TCT-3' and its complementary strand: 5'-AGA AGC CCA AAT GGC CAG TCA TGA ACC TTG TCA CGT TAG ATG-3'; **SHIP-1Δ 0.6-0.4**: 5'-TTC TGA CAT CAA CTA GCT TCA CCT ATT CAC AAA TGT TGG GCC-3'and its complementary strand: 5'-GGC CCA ACA TTT GTG AAT AGG GAA GCT AGT TGA TGT CAG AAG-3'; **SHIP-1 Δ 0.2-0.0**:5'-GGT GTC ACA TCT AAC GTG ACA AGG TTC AGA GGT GTC ACA TCT AAC-3' and its complementary strand: 5'-TCT GAA CCT TGT CAC GTT AGA TGT GAC AAC TCT GAA CCT TGT CAC GTT-3'. The amplified promoter fragment was digested by XhoI and Hind III and cloned into the pGL3 luciferase basic reporter vector (lacking promoter/enhancer sequences). Each PCR mixture consisted of 25mM MgSO4, dNTPs (10mM each), 1X PCR reaction buffer, primer (10μM each) and 1.25 U Vent Taq Polymerase, performed in a reaction volume of 50 μl. All the constructs were sequence-checked. The site-directed mutagenesis in the mouse SHIP-1 gene promoter was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocols. For this the sequences of the mutant oligonucleotides cloned are: SHIP-1 gene promoter mutants are: **SHIP-1-2.2 mutSmad4Binding Site (BS)**: 5'- GCA AAC ATA GCC ATA

CTC AGC CAC AGA GGG -3' and its complementary strand: 5'-CCC TCT GTG GCT GAG TAT GGC TAT GTT TGC-3'; **SHIP-1-2.2 mutAP1 #1BS**: 5'-GCC TGA AAC AGG AAG TAA AAC AGT TAA GCT GGT AGG-3' and its complementary strand: 5'-CCT ACC AGC TTA ACT GTT TTA CTT CCT GTT TCA GGC-3'; **SHIP-1-2.2 mutAP1 # 2BS**: 5'-GAG TGG CTG CTG TTT TAG TCC AGG AGA C-3' and its complementary strand: 5'-GTC TCC TGG ACT AAA ACA GCA GCC ACT C-3'

3.6.3. Transfection and Luciferase report assay: The 2.2-kb SHIP-1-Lux, 5' deletion constructs and 3TP-Lux constructs were transiently co-transfected by Lipofectamine Plus according to the manufacturer's instructions (Invitrogen Life Technologies) in CHO cells with a CMVβ-gal expression vector encoding for β-galactosidase gene used as an internal control. One day after transfection, cells were synchronized by serum starvation for 12 hrs and treated with or without activin (0.5nM) of TGFβ (0.2nM) for 18 hrs. Then, cells were washed once with PBS and Lysed in 250 µl of lysis buffer (1% Triton X-100; 15mM MgSO4; 4mM EGTA; 1mM DTT; 25mM glycyglycine) on ice. The luciferase activity of each different sample was measured using in combination 45 µl of cell lysates and 5 µl of cocktail assay reagent (EG&G Berthold Luminometer) and normalized to the relative β-galatosidase activity.

3.6.4. Western blot analysis: Cells were plated in DMEM and RPMI (MPC-11 and M1 respectively) starvation media for at least 12 h. After this starvation time period, the cells were stimulated in a time dependent manner either with or without activin (0.5nM) or TGFβ (0.2nM). Cells were lysed on ice in lysis buffer (50mM Hepes at pH 7.5, 150mM sodium chloride, 100mM sodium fluoride, 10mM sodium pyrophosphate, 5mM EDTA, 10% glycerol, 0.5% NP40 and 0.5 % sodium deoxycholate). Supplemented

with 100mM sodium vanadate, 1mM phenyl methylsuphonyl fluoride (PMSF), 10 mg ml-1 aprotinin, 10 mg ml^{-1} leupeptin and 2 mg ml^{-1} pepstatin. Total cell extracts were then separated on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel, transferred to nitrocellulose. Electrophoresis was performed in 500 ml of 1X TGS, using the Bio-Rad Protean electrobotting apparatus. Proteins on the membrane were visualized by Red Ponceaus S staining. Nitrocellulose membranes were washed with TBS-T (TBS with 0.05% Tween-20) for 10 min, at room temperature. Non-specific sites were blocked by soaking the membrane in TBB buffer (1X TBS containing 5% non-fat milk, 0.05% Tween-20) for 1h at room temperature. Western blotting was performed with a 1:3000 dilution of different antibodies in (antibody buffer) overnight at 4° C (anti-SHIP-1 sc-8425, anti-phospho-Smad2 Sc-6829, anti-Smad2/3 sc-8332, anti-Smad4 sc-7966, anti-phospho-p38 NEB9211S, anti-p38 NEB 9212, anti-phospho-MKK3 sc-8407, anti-phospho ERK1/2 NEB 9106, anti-ERK1/2 NEB 9102, anti-phospho JNK RD System MAB17761, anti-JNK sc-1648, anti-phospho-ATF2 sc-8398, anti-phospho-C-Jun sc-16312-R, anti-Tubulin Sigma T4026). As a secondary antibody, we used anti-mouse or anti-rabbit horseradish peroxidase-conjugated (HRP), in a 1:10,000 dilution in TBB buffer, for 1 h at room temperature. After 3 washes of 15 min with TBS-T at room temperature, bands were visualized by enhanced chemiluminescente (Lumi-Light Plus Western blotting substrate, Boehringer) according to the manufacturer's instruction and detected using an Alpha Innotech Fluorochem Imaging system (Packard Camberra).

3.6.5. Nuclear Extracts Preparation: MPC-11, M1 and CHO cells were seeded (15 X 10^6 cells/reaction) in starvation media for at least 12h. Right after, cells were rinse with 1X PBS and then, stimulated with or without activin (0.5nM) of TGFβ (0.2nM) for

45 min at 37° C in a humidified 5% CO2 atmosphere. After washing, cells were resuspended in a hypotonic buffer containing 10mM Hepes-KOH pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 1mM Na_3VO_4, 20mM NaF, 1mM PMSF and protein inhibitors. The cell lysates were incubated for 15 min on ice and centrifuged at 14000 rpm for 15 min at 4°C. The pellet obtained was resuspended in a hypertonic buffer containing: 20mM Hepes-KOH pH 7.9, 25% glycerol, 420mM NaCl, 1.5mM MgCl2, 0,2mM EDTA, 1Mm Na_3VO_4, 20mM NaF and protein inhibitors. After and additional incubation for 20 min was performed on ice, and then vortex for 30 min. The suspension was centrifuged again at 14,000 rpm for 5 min and the supernatant containing the nuclear extracts were measured by MSA to determine their protein concentration. The nuclear extracts were stored at -80°C until they were used.

3.6.6. Electromobility-retardation shift assays (EMSAs): Electrophoretic mobility shift assays (EMSAs) were performed by incubation of 10 μg of nuclear extracts proteins for 45 min on ice with the ^{32}P specific labelled probes corresponding to the Smad4 and the two AP1 sites for 20 min at room temperature. Samples were analyzed by electrophoresis on a 5% polyacrylamide nonreducing gel. The gels were pre-run in 0.5 X TBE buffer for 1 h). For supershift experiments, 1 μg of antibody (anti-Smad4 and anti-c-Jun both from Santa Cruz) were added to 10 μg of nuclear extracts and incubated for 1 hr prior adding the ^{32}P-labeled oligonucleotide probes on ice. The oligonucleotides used were based on the putative Smad4 and AP1 binding sites in the SHIP-1-1 gene promoter, as predicted by the MATInspector Professional (http://genomatrix.gsf.de/cgi-bin/matinspector/matinspector.pl), transcription factor search programs we found Smad4 and the two AP1 binding sites (wild type and mutant respectively). The used

oligonucleotides were: 5'-AAA CAG GAA GTC AGT CAG TTA AGC TGG TAG-3' (Smad4 WT); 5'-GCA AAC ATA GCC ATA CTC AGC CAC AGA GGG-3' (Smad4 mut); 5'-GCA AAC ATA GCC ATA CTC AGC CAC AGA GGG-3' (AP1 1 WT); 5'-GCC TGA AAC AGG AAG TAA AAC AGT TAA GCT GGT AGG-3' (AP1 1 mut); 5'-GCC TGA AAC AGG AAG TAA AAC AGT TAA GCT GGT AGG-3' (AP1 2 WT) and 5'-GAG TGG CTG CTG TTT TAG TCC AGG AGA C-3' (AP1 2 mut). Double stranded oligonucleotides were obtained by mixing the single stranded oligo with their complement in a molar ration 1:1, incubating them for 10 min at 95 °C, and cooling down slowly to 4°C. Gels were dried and bands were visualized by autoradiography.

3.6.7. DNA affinity precipitation assay –DNAP-: MPC-11 and M1 cells were seeded at 20 X106 cells/ 100 cm2 plate and cultured for 24h. Cells were treated with 0.5nM Activin or 0.2nm TGFβ respectively for 1 h, washed twice with PBS and collected in PBS with a cell lifter. After cells were pelleted at 735 X g and resuspended in DNA affinity precipitation (DNAP) buffer containing 25mM Tris [pH 7.5], 80mM NaCl, 35mM KCl, 5 Mm mGcL2, 10% glycerol, 1mM DTT, and 0.1% IPEGAL CA-630, with the previously described mixture of phosphatase and protease inhibitors and the addition of 0.5mM NaF). The cells then were disrupted for 10 s three times at setting 3 on a Heat System ultrasonic processor XL and rotated for 30 min at 4 °C. The extracted whole-cell lysates were cleared by centrifugation at 10,000-x g for 20 min at 4°C.

3.6.8. Immunoprecipitation and co-immunoprecipitation analysis: The following day, cells were harvested, immunoprecipitated with anti-myc or anti-flag antibodies (Santa Cruz) and western blot for endogenous Smad4 and/or Ap1. Total cell lysates revealed for anti-myc used as a control to demonstrate equal protein loading.

3.6.9. Cell viability assay [3-(4, 5-Dimethulthiazolyl-2)-2, t Diphenyltetrazoliumbromide (MTT)]:

CHO cells were plated in triplicate at 5000 cells /100 µl density in DMEM medium with 2% FCS. Cells were stimulated or not with activin (0.5nM) or TGFβ (0.2nM) and incubated over a 3-day period. Cell growth was assessed using the non-radioactive MTT cell growth 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. DT-40 parental and knock-out H7-11 (SHIP-$1^{-/-}$) and Y9-3 (SHP-1) cell lines were plated in triplicate at 5000 cells/100 µl in 2% FCS; 1% CS DMEM. Cells were stimulated with activin and TGFβ for three days before being assessed by MTT assay.

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CHAPTER 4

GENERAL DISCUSSION

4.1.1. Activin and TGFβ-induced apoptosis in immune cell types.

Members of the activin/TGFβ family of growth factors are important mediators of cell growth arrest and apoptosis in different cell types including those ones of immune origin. A vast amount of information has demonstrated that activin and TGFβ are potent inducers of apoptosis and G1 arrest [1-6]. In addition, these growth factors also are capable of modifying the activity and expression of cell cycle molecules such as cyclin-dependent kinases inhibitors, in example, p15 [7, 8], and down regulation of proto-oncogenic factors, such as c-myc [9].

The main objective of the work described in this thesis was to elucidate the intracellular signaling pathways and their downstream target genes that relay activin and TGFβ cell growth inhibitory effects in immune cells. For this purpose, I explored these downstream mechanisms through the TGFβ and activin receptor in different immune cell lines as well as human primary lymphocytes.

Gene expression profiles revealed SHIP-1 as a potential signaling target for TGFβ, leading us to postulate that activin and TGFβ exert potent growth regulatory effects in immune cells. Our screening of activin/TGFβ-regulated genes revealed that several genes were differentially regulated. In the study presented in Chapter 2, we found that the Src-homology 2 (SH2) domain-containing 5' Inositol phosphatase, SHIP-1, is strongly up

regulated by TGFβ family members. Increased in SHIP-1 mRNA was directly correlated with increase in SHIP-1 expression at the protein level. These results were confirmed using other cell lines such as the hybridoma cell lines (B9), mouse myeloid B lymphocyte cells M1, acute promyelocitic leukemia HL-60 and the B-cell chicken lymphocytes DT40. Our study described in Chapter 2 defines a critical role for SHIP-1 with activin and TGFβ signaling and revealed for the first time an important biological link between TGFβ signaling and phospholipids metabolism.

The phosphatidyl inositols, PtdIns 3, 4, 5-P3 and PtdIns 3, 4-P2, participate in the activation process of Akt/PKB. The ability of these phospholipid products to recruit PDK1 and then to activate Akt at the plasma membrane has been well documented [10-14]. The full activation of Akt requires PtdIns-3,4,5-P3-mediated phosphorylation of Thr308 and Ser473 [10-15]. Interestingly, phosphorylation of Thr308 residue within the PH domain by PI3K has unmasks the activation site of Akt [15]. These findings prompted up to investigate the effect of activin/TGFβ-induced SHIP-1 expression on the phosphorylation and activation status of Akt [15-18]. Our data clearly demonstrated that the Akt activation was significantly antagonized by activin/TGFβ-induced SHIP-1, followed by a significant increase in PtdIns-3, 4-P2 and a decrease in PtdIns-3, 4, 5-P3 levels at the same time of the increase in SHIP-1 expression in activin and TGFβ stimulated cells. SHIP-1 expression significantly reduced Thr308 and Ser473 phosphorylation in Akt modifying significantly its activity. Our findings concur with the model proposed by Scheid et al [15], which suggested that only phosphorylation of the Thr308 residue of Akt is PtdIns 3,4,5-P3 dependent and is sufficient for full activation of Akt [15].

The generation of PtdIns-3,4,5-P3 is a transient event as these phospholipids are rapidly broken down by the lipid phosphatases [19-22]. Survival signaling pathways lead to activation of the PI3 kinase, production of PtdIns-3, 4, 5-P3 and Akt activation. Deregulation of the PI3 kinase pathway, has been related to many human diseases. Over-activation of tyrosine kinase signaling pathways involving subsequent increased PI3 kinase and Akt activities is a significant occurrence in oncogenesis. Also, increased Akt activity has been widely reported in breast and other types of cancers, such as leukemia [23-27]. Thus, the data presented in chapter 2, highlights the role of growth factors from the TGFβ superfamily in regulating cell growth arrest and apoptosis through SHIP-1-mediated inhibition of phosphoinositol signaling, our findings reveal, a novel SHIP-1 dependent mechanism by which TGFβ family members and their serine kinase receptors regulate phospholipids and induce cell death in the immune system cell growth inhibition and apoptosis in haematopoietic cell lines. These findings provide a mechanistic insight into the regulation of the immune function by activin/TGFβ-induced cell growth arrest and apoptosis [2, 4, 28-33]. Lastly, our findings suggest that activin and TGFβ are key modulators of the expression levels of SHIP-1 in both normal and cancer immune cell populations.

4.2.1. Transcriptional Regulation of the SHIP-1 gene promoter by activin and TGFβ in immune cells.

In Chapter 3, certain data was provided demonstrating the effects of activin and TGFβ on the regulation of the SHIP-1 gene promoter at the transcriptional level. In addition, our results presented in this section clearly indicate that activin/TGFβ-mediated

effects on the SHIP-1-induced expression are specifically restricted to the immune system. As we previously mentioned, TGFβ are involved in a large variety of biological processes ranging from development, cell growth arrest, apoptosis as well as in a broad range of pathogenic mechanisms involving primary effects on immune cells [5, 34]. Moreover, different studies revealed that activin and TGFβ ligands induce cell cycle arrest by up-regulating important molecular factors involved in cell cycle arrest such as the cyclin-dependent kinase inhibitors, $p15^{INK4B}$ [35], $p21^{CIP1/WAF1}$ [36, 37] in different cell types. TGFβ ligands also down-regulate growth-promoting transcription factors like c-myc [38], inhibitor of DNA binding (ID)-1 and ID-2 [39] and the tyrosine phosphatase Cdc25A [40] in different cell types. Even activin and TGFβ act as potent cell growth inhibitors, their effects on cell cycle arrest and apoptosis regulators in immune cell types have been less well characterized. The data presented in this study demonstrated that both activin and TGFβ significantly mediated the transcriptional regulation of the promoter region of inositol phosphatase SHIP-1 in immune cell types. In addition, we provided several evidences that indicate for the first time that the Smad and the AP-1 family of transcription factors are required for transducing activin and TGFβ signals to increase specifically SHIP-1 expression and cell growth arrest/apoptosis in immune cell types. We also showed that SHIP-1 transcription is controlled through three important promoter elements (domains). Interestingly, we identified the presence of some DNA consensus binding sites for TGFβ-mediated transcription factors only in the domain I and domain III which seem to be required for the proper activin/TGFβ-mediated SHIP-1 gene promoter transcripitional activation. Therefore, these observations clearly indicate that the SHIP-1 expression is regulated by on activin and TGFβ signaling cascade in immune cells.

In the chicken B lymphocyte cell line DT40, we demonstrated the involvement and activation of the Smad pathway as well as the JNK/AP-1 pathways. We also showed that Smad4 and c-Jun interact in a ligand-dependent manner. This ligand-dependent interaction seems to be involved in the enhancement of the transcriptional activation of the SHIP-1 gene promoter in a synergistic manner in different immune cell types. These findings concur with certain previous observations demonstrating that activin and TGFβ can activate Smad and JNK signaling pathways, inducing a physical association of the Smad complex with the AP-1 in a ligand dependent fashion in different immune cell types. It has been reported that the Smad3 can, through its MH1 and MH2 domains, interact directly with the c-Jun and the c-Fos upon TGFβ stimulation [41]. AP-1 DNA binding sites can be activated either by direct binding of the Smad3 to the specific DNA binding consensus site or by interaction of the hetoromeric TGFβ-induced Smad3/Smad4 complex and the AP-1 transcriptional factor complex, in such a manner as to provide additional stability to the complex [41, 42]. Therefore, these observations suggest that this transcriptional complex not only provides transcriptional diversity to the system, but also suggests a cross-talk base between MAP kinase cascades, JNK kinase and Smad signaling pathways in the transcriptional activation of the TGFβ-regulated downstream target genes, in addition to direct phosphorylation of Smad proteins from receptor tyrosine kinases which activate the MEK1 or a signal downstream [42, 43].

In addition, our results identified three putative promoter regions (domains I, II and III) involved in the transcriptional activation of the SHIP-1 gene promoter by activin and TGFβ in immune cells. Deletion analysis of the SHIP-1 promoter (-2143 to –1 bp), demonstrated the presence of a potential putative Smad4 consensus binding element at the

–855 bp position, located within domain I (-1031 to –831) upstream of the initiation site, whereas two additional AP-1 (c-Jun) responsive consensus-binding sites were located at the –131 bp and –31 bp positions respectively, in domain III (-231 to –1), which is located close to the proximal to the start site.

The Smad binding specificity depends on the differential expression of different Smad-associated partners in a cell-dependent context [2]. Clarifying how this transcriptional plasticity is attained, it is essential to understand the embryonic development and cancer. In this context, it has been reported that most carcinomas have selectively lost the growth arrest response and gained metastatic abilities in response to the TGFβ. Some of these regulators appear to act in parallel to the Smad signal transduction cascade and converge at the target gene expression level [44]. However, we also observed that both, activin and TGFβ significantly promote the recruitment of Smad4 and c-Jun at their respective consensus response elements presented in distinctive regions of the SHIP-1 gene promoter regions respectively in a ligand dependent way. We demonstrated that the activin/TGFβ-induced Smad4/c-Jun association is required to the promote SHIP-1 promoter activation. Our findings concur with previous reports, that demonstrate the cross-talk between Smad and JNK signaling pathways as essential for the transcriptional regulation of different activin/TGFβ-induced target genes [41, 45]. As previously discussed, the analysis of the SHIP-1 promoter-luciferase construct revealed that the deletion of either domain I or domain III significantly attenuate the stimulatory effects of activin or TGFβ by approximately 50%. Surprisingly, the elimination of the promoter sequence between these domains, specifically domain II (-830 to –232 bp) also attenuated significantly its stimulatory effects to a comparable extent. Interestingly, our analysis sequence results from the full-length SHIP-1 gene promoter indicate that the

domain II did not appear to contain any activin/TGFβ-regulated response elements previously reported. Collectively these results, indicate that the full-length promoter (-2143 to –1 bp) is required for synergizing the inductive effects of Smads and AP-1.

Activin/TGFβ-mediated induction of the Smad proteins (Smad4) and the AP-1 (c-Jun) transcription factors led to their physical association and cooperatively enhance transcriptional activation of the SHIP-1 gene promoter in immune cell types. Therefore, our findings clearly indicate that the TGFβ ligands, activin and TGFβ itself, can activate both Smad and JNK signaling pathways, inducing physical association of the Smad complex with AP-1 (c-Jun), promoting SHIP-1 expression via Smad4 and c-Jun response elements presented in distinct regions of the SHIP-1 gene promoter. The most characterized intracellular mediators of the activin/TGFβ signaling pathways are the two receptor-specific Smads: Smad2 and Smad3. Both Smad2 and Smad3 heterodimerize with the common Smad partner Smad4 in a ligand-dependent manner and then, this complex translocates into the nucleus where it activate different target genes. However, it has been shown that the Smad proteins do not act in seclusion, but rather interact with other various signaling molecules, required for specific activin/TGFβ-induced target gene activation. Our results clearly indicate that activin/TGFβ-mediated SHIP-1 expression requires both the Smad pathway and the transcription factor c-Jun. [41]. The combined activation of these transcription factors could represent a general mechanism of cell growth inhibition by the TGFβ. The involvement of the Smad2 and Smad3 in mediating both activin and TGFβ effects on gene transcription of target genes is clearly complex [2, 3, 42, 46, 47]. Both activin and TGFβ are capable of activating a specific promoter reporter construct 3TP-Lux, which contains a section of the plasminogen activator inhibitor-1 (PAI-1) and where responsiveness to activin and TGFβ is enhanced by Smad 2 or Smad3 [33, 48-52].

However, it has also been shown that other activin and TGFβ responsive promoters such as the goosecoid promoter, respond in a completely different manner to Smad2 and Smad3 [53, 54]. In this context, Smad2 significantly increase the TGFβ-induced goosecoid promoter gene activity, meanwhile Smad3 has an inhibitory effect [53, 54]. This may be due to a competition between Smad3 and Smad4 for their common DNA binding site on the goosecoid promoter [55]. These observations clearly suggest that while the Smad2 acts as a positive regulator, the Smad3 plays a dual role depending on the nature of the promoter. Our data indicates that concerning the SHIP-1 gene promoter activation, both the Smad2 and Smad3 act as positive regulators of the activin and TGFβ action [33]. Meanwhile, when the Smad2 and Smad3 are in the presence of the Smad4, they increase the SHIP-1's gene promoter activation in response to activin and TGFβ [33]. In addition, we also previously reported that while both Smad2 and Smad3 act as positive regulators, the Smad7 completely abolishes the activin/TGFβ-induced SHIP-1 gene promoter activity, confirming the role of the Smad7 as a strong inhibitor of the activin/TGFβ's signaling pathways [2, 3, 33, 42, 46, 47]. Transcriptional activation of several promoter elements by TGFβ ligands, depended on the interaction and association of the Smad proteins with other transcription factors such as SP-1 [56], CREB/p300 , myocardin (Myocd) [57], hairy/enhancer-of-split-related transcription repressor (Hey) [58], PKA, GA-binding protein (GABP) [59], glucocorticoid receptor (GR) [59-62], ATF2 [63], ATF3 [39], hepatocyte nuclear factor 4 (HNF-4) [64], TGFβ stimulated factor 1 (TSF1) [65], NFkB [66, 67], p53 [68] and AP-1 [41, 69, 70]. For the AP-1 family of transcription factors, it has been demonstrated that the transcriptional cooperation between Smads (Smad4) and AP-1 (c-Jun) correlates with a physical interaction between these proteins required for the transcriptional activation of different TGFβ-regulated

promoters [41]. AP-1 family members, c-Jun and c-Fos, which can form homo- or heterodimers in order to activate transcription in different promoter regions of several target genes through their ability to interact directly with the AP-1 binding site [71, 72]. The TGFβ-receptor activation induces the formation of a DNA-protein complex. As previously reported by Roberts and others [42, 73], receptor-activated Smads (R-Smads), Smad2 and Smad3, required Smad4 to form a heterotrimeric complex to induce transcriptional activation, even though it has been described that these transcription factors can travel into to the nucleus in the absence of the Smad4 [42, 73]. It has been described by Zhang et al. [41], that the Smad3 can interact directly with the c-Jun and c-Fos through its MH1 and MH2 domain. This association, clearly leads to the suggestion that the AP-1 DNA binding consensus sites can be activated either by direct binding of Smad3 and Smad4 to the DNA elements itself or by a specific interaction of the heteromeric Smad3/Smad4 complex and the AP-1 transcription factor complex, in such a manner as to provide additional stability to the complex. Besides providing transcriptional diversity, this mechanism also suggests a cross-talk base between the MAP kinase cascade and the JNK kinase and talk between the JNK MAP kinase and the TGFβ/Smad signaling pathway, in addition to direct phosphorylation of the Smad proteins through signals from the tyrosine kinase receptor, which activates the MAP kinase signaling pathway or a downstream kinases [41-43, 45, 73].

The Smad3 with the Smad4 association can activate the TGFβ-inducible transcription from the TRE in both c-Jun/c-Fos-dependent and -independent manner [41]. Thus, these protein-protein associations complement the interactions between c-Jun and c-Fos, and between the Smad3 and Smad4 [41]. Transcriptional activation, through

functional and physical interactions between Smad3-Smad4 and c-Jun-c-Fos, strongly suggest that the Smad signaling and MAPK/JNK signaling converge at the AP-1-binding sites on different promoters of target genes [41, 45].

It is required to date the different DNA consensus binding sequences in the promoter regions of several TGFβ-induced target genes that have been described [71, 72]. For example, the 12-O-tetradecanoyl-13-acetate (TPA)-responsive gene promoter elements (TRE) are involved in the transcriptional responses of several TGFβ-regulated target genes. Members of the AP-1 transcription factors family members, c-Jun and c-Fos bind to and regulate transcription from TREs, which are therefore known as AP-1 binding sites [41]. Smad proteins interact directly with the TRE, particularly which the Smad3 in a ligand-dependent manner. The TGFβ-receptor activation induced the formation of a DNA-protein complex. The association of the Smad3 with the Smad4 can activate the TGFβ-inducible transcription from the TRE in both c-Jun/c-Fos-dependent and -independent manner [41]. Furthermore, we physically demonstrated the association between Smads and c-Jun/AP-1. These and related findings [41], suggest that these protein-protein associations complement the interactions between c-Jun and c-Fos, and between Smad3 and Smad4 [41]. Therefore transcriptional activation by TGFβ's involves functional and physical interactions between the Smad3-Smad4 and c-Jun-c-Fosx, this strongly suggests that Smad signaling and MAPK/JNK signaling converge at the AP-1-binding promoter sites [41].

We also provided data showing that activin and TGFβ activate and signal through the p38 module of the MAP kinase pathway, which specifically regulates a certain downstream transcription factor, ATF2/CREBP1 as previously reported [8, 74-76]. The

SHIP-1 promoter does not appear to contain putative ATF2/CREBP1 binding sequences in the three different promoter regions (domain I, II and III), which are involved in the transcriptional regulation of the SHIP-1 gene promoter. The precise role of the p38 signaling cascade in activin/TGFβ-induced SHIP-1 expression remains to be elucidated. Future studies should help clarify whether or not Smads physically interact with members of the p38 MAP kinase cascade to regulate the transcriptional activation of the SHIP-1 gene promoter.

In our initial deletion analysis of the SHIP-1 promoter gene, we observed that when either of these three promoter domains were removed individually there was approximately a 50% decrease in the promoter activation. Interestingly enough, the elimination of these domains in different combination or all together, significantly abolished almost if not completely the activin/TGFβ-induced SHIP-1 promoter activity. Our promoter analysis of the three domains revealed the presence of a specific Smad binding consensus site (the Smad4 binding site), domain I (- 1031 bp to - 831 bp). In addition, we also found two AP-1 consensus binding sites located within domain III (-231 bp to -1 bp) upstream of the transcription initiation site in the SHIP-1 gene. Thus, our results suggest that these three domains play an important part in the transcriptional regulation of the SHIP-1 gene promoter. Moreover, our result demonstrate that both the Smad4 and AP-1 are specifically recruited in, domains I and III respectively. These observations raise the question of: **How does the TGFβ induced association between Smad (Smad4) and AP-1 transcription factors (c-Jun and/or c-Fos), allow them to interact with their respective regulatory sites in domain I, and domain III, which are separated by domain II situated at -830 bp to -232 bp region upstream of the transcription initiation site?**. Our promoter sequence analysis provides a possible

answer to this question. This promoter region seems to be required and involved in the activin/TGFβ-induced SHIP-1 gene promoter activation. The removal of domain II, led to a significant decrease in the promoter activity by approximately 50%. Additionally, combined deletions within the SHIP-1 gene promoter, resulted in a significant inhibition of the activin/TGFβ-induced promoter activity. As far as we could determine there were no known binding sequences for activin/TGFβ-regulated transcription factors. Thus domain II participates in a ligand-independent regulatory transcriptional role.

Based on our observations, we propose a new transcriptional model in which domain II might play a promoter conformation role, which is necessary for the activin/TGFβ-mediated transcriptional activation of the SHIP-1 promoter. The conformational change occurring in the SHIP-1 gene promoter mediated by domain II, make it likely to bring domain I and III into proximity, which contain specific Smad4 and AP-1 binding sequences respectively. We have also demonstrated that these groups of transcription factors associated in a ligand-dependent manner. Once these transcription factors are recruited in their DNA binding sequences in a activin/TGFβ-dependent manner, domain II induces a specific change in the conformation that permits the direct contact between the Smad4 and AP-1 (c-Jun) complexes to their domains I and III respectively. In this scenario, the direct interaction between Smad4 and c-Jun will maximize the activin/TGFβ-induced transcriptional activation of the SHIP-1 gene promoter in immune cell types.

Lastly, it is well established that the Smads bind to DNA with low affinity, and it is due to this that they require the interaction with other co-activator or co-repressors to mediate DNA-binding more efficient in the specific SBE located in the promoter regions of the several target genes [2]. We have identified a potential putative p53 consensus

binding site overlapping with the Smad4 binding site located in domain I (-1031 bp to -831 bp) in the SHIP-1 gene. It was been previously reported that the p53 is required for the TGFβ gene responses by cooperating with Smad [68, 77]. In addition, the p53 requires the Smad's activity to perform its TGFβ-like effects [68, 78, 79]. Since the p53 acts in a selective manner on a subset of TGFβ target promoters, rather than as a general enhancer of the TGFβ responses [68, 78, 79]. Moreover, it has been demonstrated that p53 associates with Smad2 and Smad3 in vivo in a TGFβ-dependent manner, but at the same time, p53 is recruited to its own consensus binding site on a promoter to enhance and maximize a TGFβ-induced transcription [68, 79]. In addition, p53 contains the binding domain for other transcription factors, such as JunD, member of the AP-1 transcription factors family. The AP-1 proteins have been implicated in cell proliferation, apoptosis as well as in tumorigenesis [80-83]. Thus, these observations suggest that these other transcription factors may modulate and stabilize the interaction between p53 and Smads. While the p53/Smad2 complex regulates the transcriptional regulation of the Mix.2 [43, 84, 85], the Smad2 MH2 region is free to interact with FAST-1 (Forkhead Activin Signal Transducer 1) and Smad4. Based on these observations, it has been suggested in this experimental system that the Smad2 may bridge the DNA-bound of the p53 and FAST-1 leading to the assemblement of a more stable and specific multifactorial complex [86]. Interestingly, p53 can be an important player for TGFβ-induced growth arrest in mammalian cells [86]. In addition, p53 pathway also contributes to TGFβ-mediate activation of the PAI-1 and Mix.2 genes that bear a functional p53 binding-element, suggesting the existence of a putative co-regulated mechanism controled by TGFβ and p53 signaling pathways. Moreover, it has been suggested that only two cellular responses

might be under a general control of p53 family members and TGFβ, particularly, cell growth arrest and extracellular matrix remodelling and attachment [79]. Thus, it is possible that p53 could participate in the activin/TGFβ-mediated transcriptional regulation of the SHIP-1 in association with Smad4 and AP-1 complex in immune cells. The model that we proposed here, suggests the involvement of p53 in that the transcriptional regulation of the activin/TGFβ-induced SHIP-1 transcriptional activation cannot be ruled out. The results presented here should not be taken to mean solely as an indicative that the regulation of the SHIP-1 expression is the only molecular mechanism used by TGFβ family members to induce and regulate growth arrest and apoptosis in immune cells. The existence of other regulated genes and signaling pathways in immune cells potentially modulated by any TGFβ ligand cannot be disregarded. This statement is supported based on the recent observations that demonstrate the involvement of DAXX and DAPK signaling pathways in TGFβ-induced cell growth arrest and apoptosis [87]. Collectively, these pathways may govern the extent to which activin and TGFβ ligands exert their effects of cell growth inhibition and apoptosis in immune cell system.

In summary the data presented in Chapter 3 demonstrates that the Smad and c-Jun interact with distinct domains of the SHIP-1 gene promoter. In addition, these transcription factors contribute equally to the inductive effect of activin and TGFβ. The effects of Smad4 and c-Jun are cooperative, but only when the intervening region connecting Smad4 and c-Jun response binding elements are presented. This coupled with the fact that Smad and AP-1 physically associate, suggesting that the domain connecting these transcription factor groups, response domain provide a proper conformational folding of the promoter to bring the two elements into close proximity.

Finally, the advances made since the initial identification and cloning of the type II activin receptor in 1991 have led to the characterization of over a dozen related receptor serine kinases as well as, to the discovery of the Smad proteins and have improved our knowledge of the mechanism of action of activin and TGFβ. The identification of the activin/TGFβ-regulated target genes and intracellular signaling pathways that lead to cell growth arrest and apoptosis in immune cells will be of high interest for the development of therapeutic approaches towards cancer and autoimmune diseases. The different evidences presented in this doctoral thesis have addressed the role of the inositol phosphtase SHIP-1 in the mediation of the proapoptotic effect of the TGFβ family members, to define the contribution of the different signaling pathways involved in these effects and to characterize the transcriptional machinery that relay these signals.

4.3. - A proposed model for the possible transcriptional regulation of the SHIP-1 gene promoter by activin and TGFβ in immune cells.

The TGFβ superfamily of growth factors and cytokines has been involved in different biological processes. The TGFβ signaling pathway has been largely related with a plethora of human conditions, including fibrosis, cancer and different autoimmune diseases. Based on the evidence presented in this thesis and a number of different studies, a model is proposed to explain how activin and TGFβ mediates apoptosis and cell growth arrest through the transcriptional activation of the SHIP-1 gene promoter (Figure 4.3.1. pages 255-256).

Figure 4.3.1. A proposed model for the activin/TGFβ-induced SHIP-1 expression in immune cell types. Upon ligand stimulation, the activin/TGFβ signaling pathway is activated which leads to the activation of the Smad cascade that goes into the nucleus and in association with the AP-1 regulates the transcriptional activation of the SHIP-1 gene in immune cells.

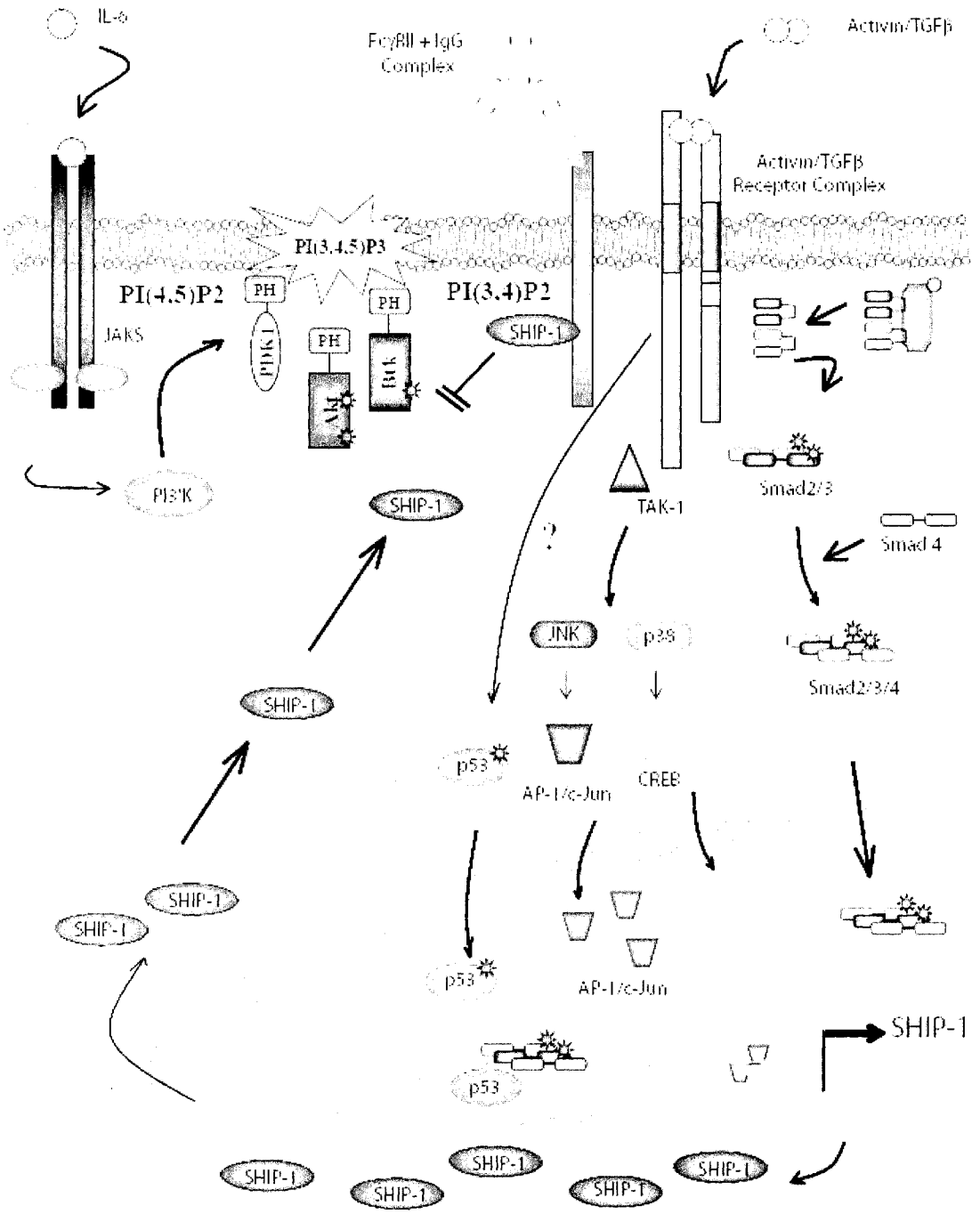
IL-6
FcγRII + IgG Complex
Activin/TGFβ
Activin/TGFβ Receptor Complex
PI(3,4,5)P3
PI(4,5)P2
PI(3,4)P2
PH
JAKS
SHIP-1
PI3'K
TAK-1
Smad2/3
Smad 4
Smad2/3/4
JNK
p38
p53
AP-1/c-Jun
CREB
SHIP-1

Figure 4.3.1.

While the TGFβ signaling cascade participates in parallel with other signaling pathways in a cellular dynamic context, the general process by which activin/TGFβ-induced SHIP-1 expression can be divided into several sequential steps. First, activin and TGFβ signaling is initiated by the ligand binding to two transmembrane spanning activin type II receptors (ActRII) at the cell-surface. Consecutively, this leads to the recruitment and phosphorylation of the activin type I receptor [88]. Once activated the TGFβ receptor complex, the ALK4 phosphorylates the main downstream intracellular mediator the receptor Smad2 and Smad3 on two C-terminal serine residues (SSxS motif) [42, 48]. Once phosphorylated, the Smad2 and Smad3, then hetoro-oligomerize in the cytoplasm with the common Smad, Smad4, and translocate to the nucleus where they interact with several transcription factors, co-activator or co-repressors to regulate the SHIP-1 gene promoter transcriptional activation in a specific manner. In our model although the Smad pathway represents the canonical signaling pathway used by activin and TGFβ, other intracellular pathways are known to mediate signaling by the activin and TGFβ growth factors. In this case the MAP Kinase JNK is activated in response to activin and TGFβ in the immune cells downstream of the TGFβ receptor complex. In this model we also proposed that p53 pathway is also activated in response to activin and TGFβ. Following receptor activation by activin and TGFβ, Smad, JNK and p53 signaling cascades are activated. Therefore the Smad complex (Smad2/Smad3/Smad4), c-Jun and p53 can move into the nucleus. Once these transcription factors are recruited at their specific consensus binding sites in the SHIP-1 promoter, Smad complex (Smad2/Smad3/Smad4)/p53 in domain I and c-Jun in the domain III respectively. Once recruited these growth factors, there is a conformational change induce by the intermediate promoter region, domain II,

which brings the Smad complex close to c-Jun (Figure 4.3.2.). In this scenario, the interaction of the Smads/p53 complex with c-Jun, in particular Smad3/Smad4 could stabilize the transcriptional complex, enhancing the activin/TGFβ-induced activation of the SHIP-1 gene promoter. Thus, the participation of the activin/TGFβ-induced p53/Smad (Smad2/Smad3/Smad4)/AP-1 (c-Jun) complex in the transcriptional regulation of the SHIP-1 gene promoter may induce a more stable transcriptional complex maximizing the SHIP-1 expression (Figure 4.3.2.)

Once SHIP-1 is expressed, it translocates to the plasma membrane where it associates via its SH2 domain with the phosphotyrosine residues located in the intracellular fraction of the FcγRII, receptors involved in the attenuation of proliferating signals in immune cells. Subsequently, SHIP-1's phosphatase activity increases directly with its expression level. Therefore, it is recruited into the plasma membrane, SHIP-1, metabolizes the PtdIns, 3, 4, 5-P3, to PtdInd, 3, 4-P2. Moreover, with these decreased levels of PIP3, the recruitment of the PH-domain containing proteins such as PKB/Akt, involved in proliferation, is significantly attenuated, followed by a clear induction of cell growth arrest and apoptosis. Thus, with this model we propose a new mechanism in which the TGFβ ligands, activin and TGFβ, induce and regulate cell growth arrest and apoptosis via the regulation at the transcriptional and expression levels of the inositol phosphatase SHIP-1 in cell populations of the immune system (Figure 4.3.2, pages 259-260).

Figure 4.3.2. A proposed model for the transcriptional activation of the SHIP-1 gene promoter regulated by Smads and AP-1 upon activin and TGFβ stimulation in immune cell types.

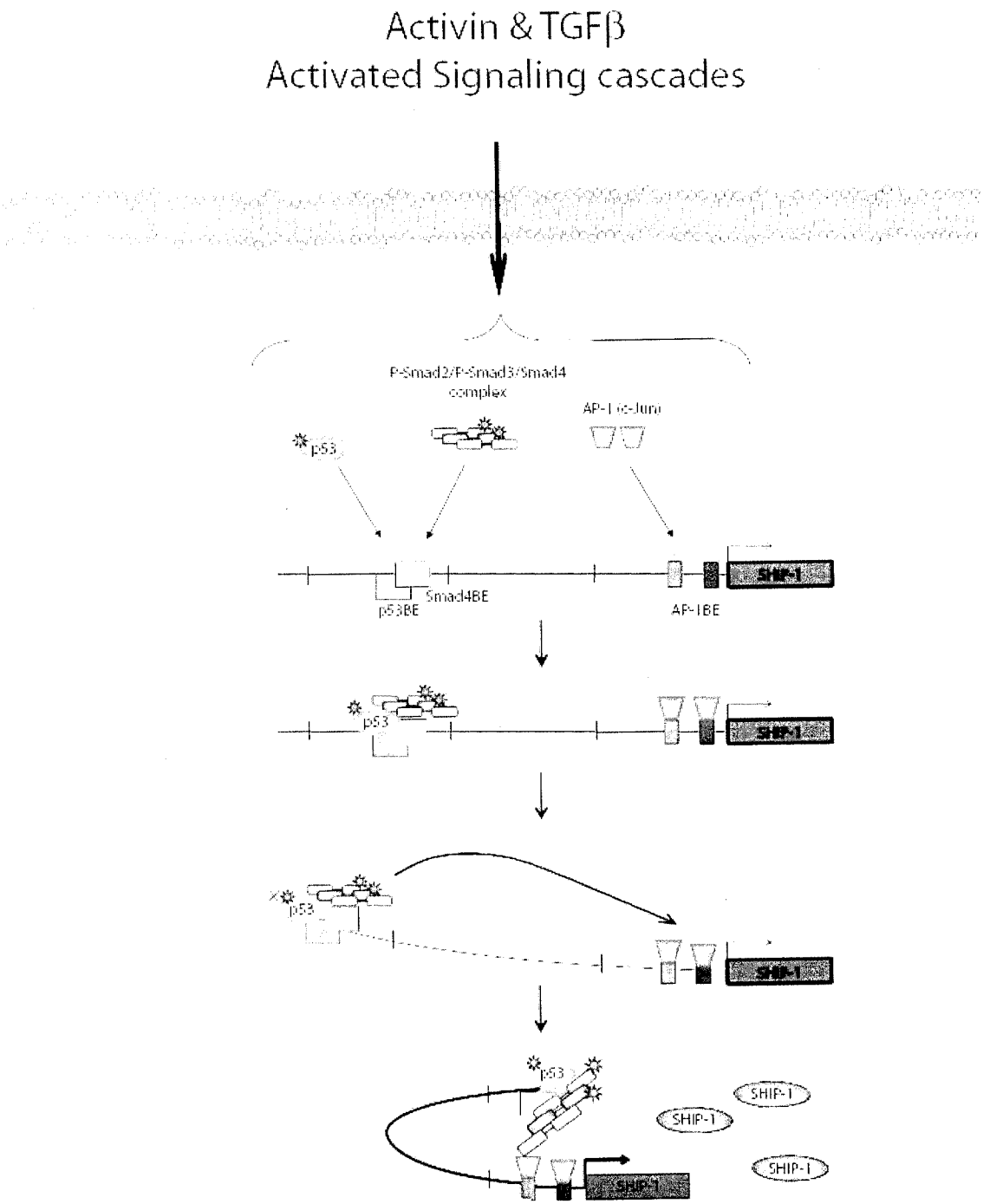
Activin & TGFβ
Activated Signaling cascades
P-Smad2/P-Smad3/Smad4
complex
AP-1 c-Jun
p53
Smad4BE
p53BE
AP-1BE
SHIP-1
p53
SHIP-1
p53
SHIP-1
p53
SHIP-1
SHIP-1
SHIP-1

Figure 4.3.2.

4.4. Conclusion Remarks

The TGFβ superfamily of growth factors has been significantly expanding over the last several years. There is accumulating evidence which clearly indicates that this category of growth factors and cytokines mediate different biological functions including those immune-related through the expression of different target genes. In the future, more work needs to be done in order to elucidate the different mechanisms used by the TGFβ's involved in several aspects of cell growth arrest and apoptosis. To achieve this, it is essential to identify those physiological TGFβ-mediated target genes. Transcriptional activity, expression and phosphatase activity of the SHIP-1 is directly regulated by activin and TGFβ signaling pathways in immune cells. SHIP-1 is a critically important regulator of cell death in immune cells, due to its role in different signaling pathways. Importantly, linking for the first time the TGFβ-induced apoptosis to phospholipids metabolism helped to expand our understanding of how activin and TGFβ serine kinase receptors functions in the regulation of immune cell proliferation and apoptosis.

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Claims of originality

5.1. Claims of originality

The core of the research presented in this thesis is the original data contained in Chapter2, has been published in *Nature Cell Biology* journal (Appendix 1). The data contained in Chapter 3, a complete manuscript ready to be submitted for publication. Thus, the work presented in chapters 2 and 3 of this thesis has provided several novel and original contribution to the existing body of the scientific knowledge in TGFβ superfamily of growth factors. These include:

1. Identification of the phosphatydilinositol phosphatase SHIP-1 as a novel and direct activin/TGFβ-regulated target gene in immune cells.
2. Demonstration that TGFβ family members regulate cell growth arrest and apoptosis through the expression of SHIP-1.
3. Demonstration of a novel link between phospholipids metabolism and to activin/TGFβ-mediated apoptosis.
4. Demonstration that absence of the activin/TGFβ-induced SHIP-1 expression significantly diminishes the inhibitory cell growth and proapoptotic effects of activin and TGFβ.
5. Demonstration that in the activin/TGFβ-induced transcriptional activation of SHIP1, Smad and JNK MAP kinase signaling pathways are involved in a synergistic manner.

Finally, the concluding discussion in Chapter 4 contains intellectual contribution in terms of the generation of a novel hypothesis on the role of activn/TGFb-induced SHIP-1 expression involved in cell growth arrests and apoptosis in the immune system. This is an issue particularly lacking data prior to this thesis, particularly the effects of these growth factors in the transcriptional regulation and expression of the inositol phosphtase SHIP-1 in immune cell types.

APPENDIX 1

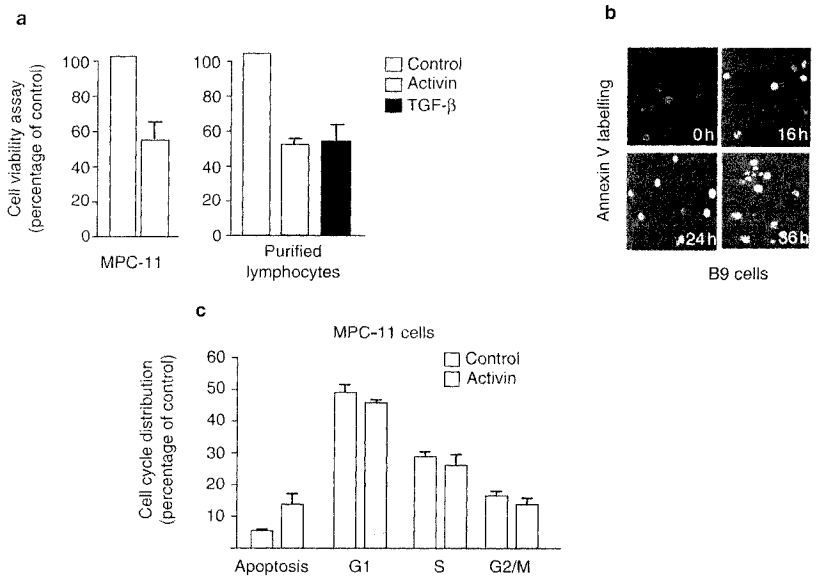

Figure 1 **Activin and TGF-β induce apoptosis in haematopoietic cells. a**, Cell viability colorimetric assay (MTT) of MPC-11 cells and purified human lymphocytes, treated with or without activin or TGF-β for 72 h. Experiments were performed in triplicate and values are expressed in arbitrary units. **b**, For annexin V labelling, B9 cells were stimulated for different times with activin and analysed by direct immunofluorescence. **c**, MPC11 cells were treated with or without activin for 72 h. The distribution of cells in the cell cycle were quantified by propidium iodide staining of cells using flow cytometry.

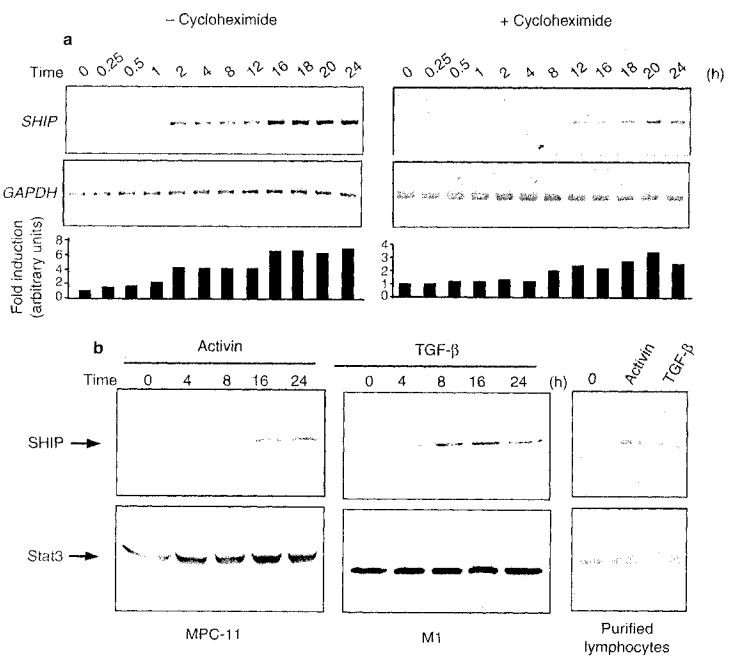

Figure 2 **Activin and TGF-β induce *SHIP* expression. a**, MPC-11 cells were stimulated with activin for various times with or without pretreatment with cycloheximide (10 μg ml^{-1} for 3 h). RT-PCR reactions were performed using oligo-dT and cDNAs were amplified using oligonucleotides specific to *SHIP*. RT-PCR of *GAPDH* was used as a control. The results were analysed by densitometry and values are expressed as fold-induction compared with control after normalization to the *GAPDH* mRNA levels. **b**, MPC-11 cells, M1 cells and human purified lymphocytes were stimulated with activin or TGF-β for various times. Total cell lysates were analysed by immunoblotting using a specific monoclonal antibody against SHIP (top). Equal loading was confirmed by stripping and reprobing the blot with an anti-Stat3 antibody (bottom).

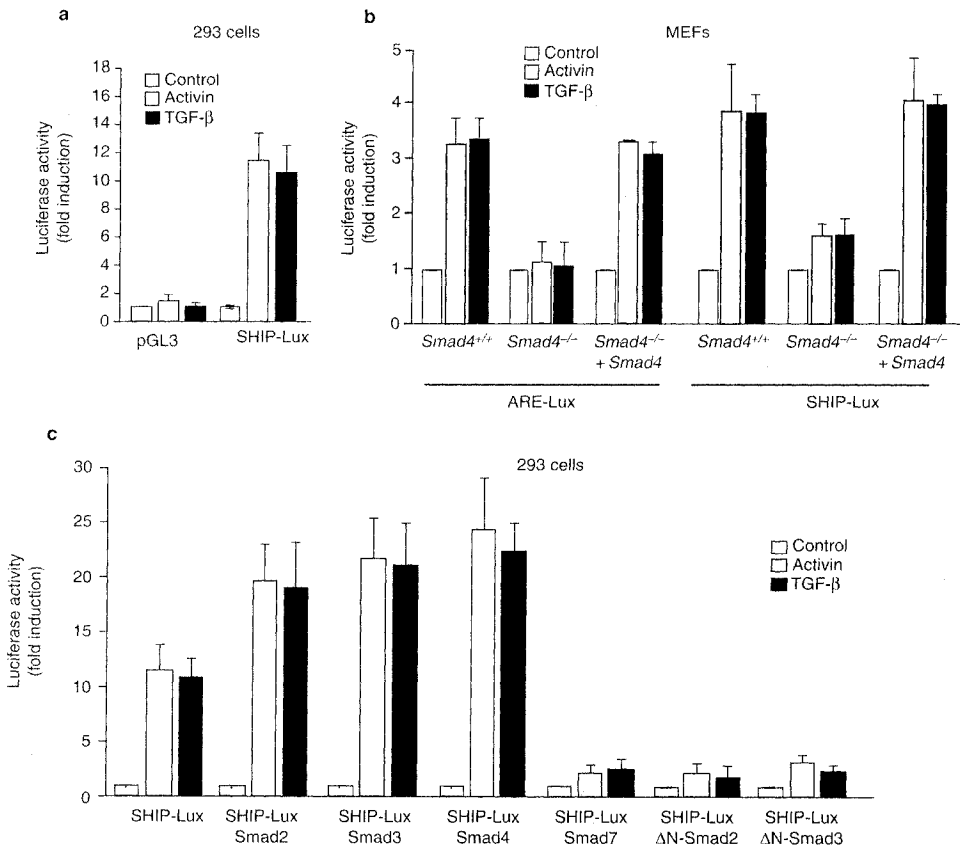

Figure 3 **Activin/TGFβ-induced transcription of *SHIP* requires Smad2, Smad3 and Smad4. a**, 293 cells were transfected with the SHIP-Lux reporter construct or empty luciferase vector (pGL3) and the β-galactosidase expression plasmid. Cells were then stimulated with activin or TGF-β for 18 h. The luciferase activity was normalized to β-galactosidase values. Results represent mean and standard deviations of three independent experiments. **b**, MEFs established from the *Smad4*$^{-/-}$ or *Smad4*$^{+/+}$ were transfected with the SHIP-Lux and ARE-Lux/Fast1 promoter constructs, with or without an expression vector encoding Smad4. The activin/TGF-β response was measured using a luciferase assay. **c**, 293 cells were transfected with the SHIP-Lux reporter construct and various Smad expression plasmids, as indicated. Cells were then stimulated with activin or TGF-β and assessed for luciferase activity.

Interestingly, the 3′ lipid phosphatase PTEN, also known as TEP1 (TGF-β-regulated and epithelial-cell-enriched phosphates), is also regulated by TGF-β (ref. 15). This suggests that these two phosphatases share a common mechanism for regulating cell death in response to stimulation by TGF-β family members.

To characterize the transcriptional mechanisms by which activin/TGF-β induce expression of *SHIP* mRNA, we cloned a 1.4-kb fragment from the 5′ regulatory sequence of the mouse *SHIP* gene on the basis of the genomic structure of the *SHIP* gene[16]. The *SHIP* gene promoter was then subcloned upstream of the firefly luciferase gene in the PGL3 basic vector (SHIP-Lux). We observed that promoter activity was strongly induced by activin and TGF-β treatment (Fig. 3a). To investigate the role of the Smad pathway in activin/TGF-β-induced activation of the *SHIP* gene promoter, we used murine embryonic fibroblasts (MEFs) established from *Smad4* knockout mice, in which the Smad pathway is inactivated[17]. Wild-type and *Smad4*$^{-/-}$ MEFs were transfected with either SHIP-Lux or ARE-Lux, another control activin/TGF-β-responsive promoter construct. Although both gene promoter constructs were strongly activated by activin and TGF-β in wild-type MEFs, this effect was abolished in the *Smad4*$^{-/-}$ MEFs, and fully restored when Smad4 was cotransfected (Fig. 3b). This confirms that the Smad pathway is critical for mediating these effects.

To further investigate the function of Smad2 and Smad3 in activin/TGF-β-mediated induction of the *SHIP* gene promoter, 293 cells were cotransfected with the SHIP reporter construct and cDNAs encoding Smad2, Smad3, Smad4, the inhibitory Smad7 (ref. 18) or the dominant-negative forms of Smad2 and Smad3 (ΔN-Smad2 and ΔN-Smad3; refs 19,20). 293 cells express relatively low levels of TGF-β receptors, but do respond to treatment with activin and TGF-β (Fig. 3a). In addition, transient transfection of these cells is usually achieved at very high efficiency. Overexpression of Smad2, Smad3 or Smad4 significantly increased activin/TGF-β-mediated activation of the *SHIP* promoter, whereas expression of Smad7, ΔN-Smad2 or ΔN-Smad3 completely abolished these effects (Fig. 3c). Together, these results demonstrate that the Smad pathway is required downstream of the activin and TGF-β receptor signalling cascade for activation of the *SHIP* gene promoter.

The 5′ phosphatase activity of SHIP does not change significantly after cytokine stimulation[21], suggesting that its effects are regulated by the level of expression[2]. To determine whether activin/TGF-β-induced expression of *SHIP* results in a concomitant increase in SHIP phosphatase activity, we measured the intracellular levels of the two SHIP substrates, $InsP_4$ and $PtdInsP_3$ (see Methods). Activin induced a marked increase in *SHIP* expression and a clear decrease in $InsP_4$ levels, whereas the level of $InsP_3$ was

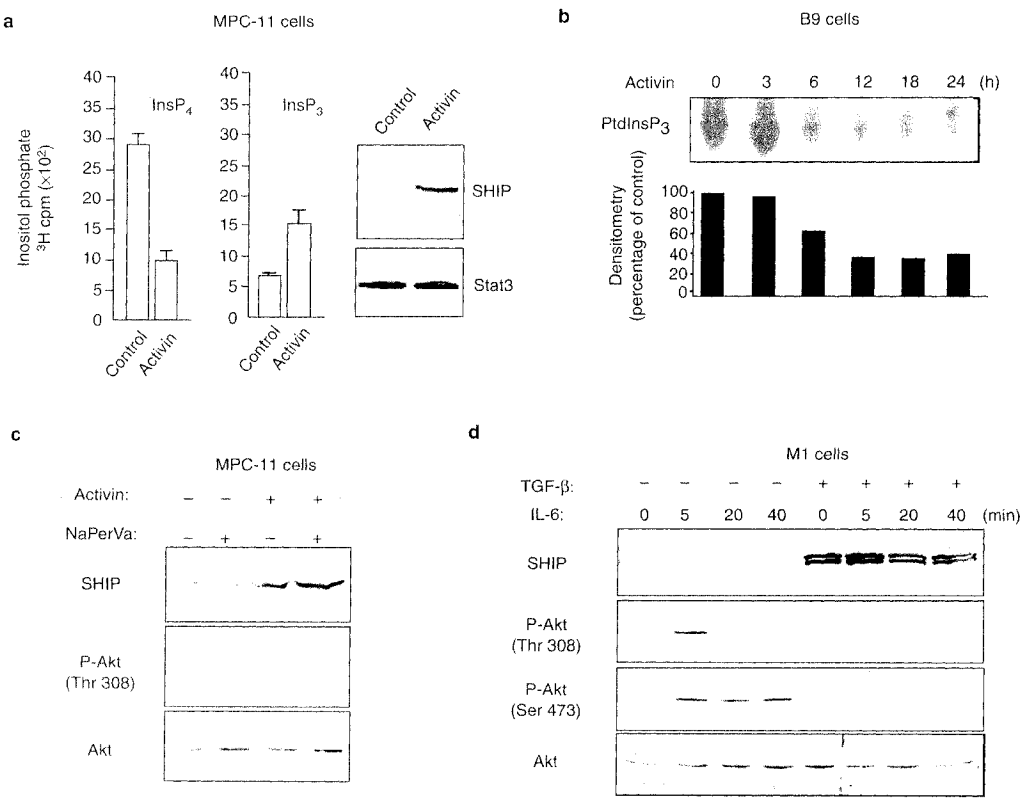

Figure 4 **Activin/TGF-β-induced SHIP expression and activity inhibit Akt phosphorylation. a**, MPC-11 cells treated with or without activin were labelled with Myo-2-^{3}H(N)-inositol. Total radiolabelled phospholipids were extracted and the $InsP_3$ and $InsP_4$ were separated by differential elution. Levels of $InsP_4$ (left) and $InsP_3$ (middle) are expressed in counts per minute (cpm). SHIP protein levels were assessed by western blot (right). **b**, B9 cells treated with activin for different times were metabolically labelled with ^{32}P-orthophosphate. $PtdInsP_3$ was separated by thin layer chromatography, analysed by autoradiography and quantified by densitometry. **c**, Treatment of MPC-11 cells with activin for 24 h increases *SHIP* expression (top) and antagonizes Akt phosphorylation of Thr 308 in response to 0.2 mM sodium pervanadate (middle). Equal loading was confirmed by reprobing the blot with an anti-Akt antibody (bottom). **d**, TGF-β-induced *SHIP* expression in M1 cells antagonizes Akt phosphorylation in response to IL-6 on both Thr 308 and Ser 473 (middle). Equal loading was confirmed by reprobing the blot with an anti-Akt antibody (bottom). Increased *SHIP* expression in response to TGF-β was monitored by immunoblotting with an anti-SHIP antibody (top).

concomitantly increased. This is interesting, as the ability of SHIP to hydrolyse $InsP_4$ has only been clearly demonstrated *in vitro* using 5′-phosphatase assays[13], and no report of any changes in $InsP_3$ and $InsP_4$ levels have been reported with cells from *SHIP*$^{-/-}$ mice[22]. Therefore, our findings provide the first evidence that SHIP functions as an $InsP_4$ phosphatase *in vivo*. It will be interesting to determine if the changes in the levels of $InsP_3$/$InsP_4$ resulting from TGF-β/activin-induced *SHIP* expression and activity affects the entry of extracellular calcium and subsequent activation of calcium-dependent protein kinases, as these events are tightly regulated by $InsP_3$ (ref. 23).

To measure $PtdInsP_3$ levels, B9 cells were either left untreated or were stimulated with activin for various times, before extraction of total phosphatidylinositols. Samples were then separated by thin layer chromatography and analysed by autoradiography. Treatment of the cells with activin resulted in a marked decrease in $PtdInsP_3$ levels after 6–24 h of treatment (Fig. 4b), correlating with the effect of activin on SHIP-increased expression (Fig. 2b). The same effects were observed in MPC-11 and M1 cells stimulated with activin or TGF-β (data not shown). The observed decrease in $PtdInsP_3$ levels is probably not caused by a change in PTEN activity, as no change in *PTEN* mRNA was observed in our gene chip microarray experiment (1.15-fold induction) or in the level of PTEN protein in MPC-11 cells treated with activin (Fig. 5a). This confirms that the effect is caused specifically by activin/TGF-β-mediated expression of *SHIP*. Together, our data clearly indicate that activin/TGF-β- increased *SHIP* expression is associated with an increase in SHIP lipid phosphatase activity.

Phospholipid metabolism is critical for the regulation of cell growth and apoptosis. The second messenger $PtdInsP_3$ is produced by the enzyme phosphatidylinositol-3-OH kinase (PI(3)K) and regulates activation and phosphorylation of the pleckstrin homology domain-containing protein kinase Akt (ref. 24). By breaking down $PtdInsP_3$ to $PtdInsP_2$, SHIP terminates the activation of Akt (ref. 25). As Akt is a central regulator of cell growth and survival, we focused on analysing the role of TGF-β/activin-induced *SHIP* expression on Akt activation. Treatment of MPC-11 cells with sodium pervanadate for 15 min resulted in activation of the PI(3)K pathway and subsequent phosphorylation of Akt on Thr 308 (Fig. 4c). Interestingly, this effect was largely inhibited in cells pretreated with activin for 24 h. Equal loading of Akt was confirmed by stripping and reprobing the blot with an anti-Akt antibody, and increased *SHIP* expression in response to activin was assessed by immunoblotting with an anti-SHIP antibody (Fig. 4c). The same effect was observed in B9 and M1 cells treated with activin or TGF-β, and no direct activin/TGF-β effect was observed on Akt

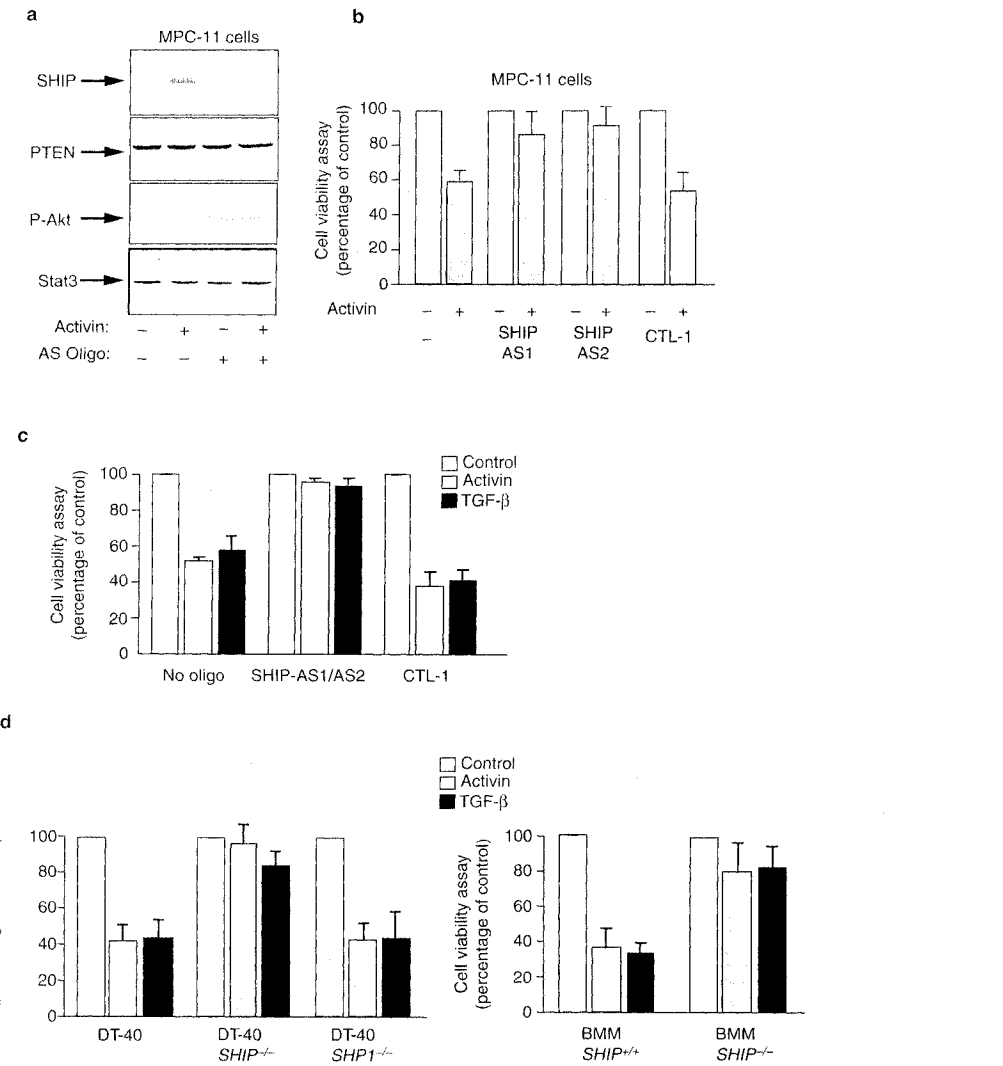

Figure 5 **Inhibition of *SHIP* expression prevents activin- and TGF-β-induced apoptosis. a**, SHIP and PTEN protein levels were measured by western blotting of cells stimulated in the presence or absence of activin for 24 h. Similarly, phosphorylation of Akt in MPC-11 cells treated with or without SHIP-AS2 was assessed by western blotting. As a loading control, the blots were stripped and reprobed with an anti-Stat3 antibody. **b**, MPC-11 cells were treated for 10 h with 50 μM SHIP-AS1 and SHIP-AS2, or with CTL-1, before stimulation with activin. Cell viability was assessed after 72 h by MTT colorimetric assays. Experiments were performed in triplicate and values are expressed in arbitrary units. **c**, Human purified lymphocytes were treated for 10 h with 50 μM SHIP-AS1 and SHIP-AS2, or with CTL-1, before stimulation with activin and TGF-β. Cell viability was assessed after 72 h by MTT colorimetric assays. Experiments were performed in triplicate and values are expressed in arbitrary units. **d**, Parental, *SHIP*$^{-/-}$ and *SHP1*$^{-/-}$ DT-40 cells, as well as bone marrow-derived macrophages (BMM) from *SHIP*$^{+/+}$ and *SHIP*$^{-/-}$ mice, were stimulated in the presence or absence of activin or TGF-β for 72 h. Cell viability was assessed by MTT assays . Experiments were performed in triplicate. Values are expressed in arbitrary units and represent the average and standard deviation of four separate experiments.

phosphorylation (data not shown). To add more physiological relevance to our results, we examined the effects of TGF-β-induced *SHIP* expression on Akt phosphorylation in response to interleukin-6 (IL-6), a natural cell survival factor in immune cells. IL-6-induced phosphorylation of Akt on Ser 473 and Thr 308 was blocked in M1 cells expressing *SHIP* in response to TGF-β (Fig. 4d), and the same effect was observed in B9 cells pretreated with activin (see Supplementary Information, Fig. S2). Together, these results indicate that activin/TGF-β-induced expression of SHIP is coupled to a decrease in cell survival stimuli-induced activation of Akt, and provide a phospholipid-dependent mechanism of action for these growth factors in mediating apoptosis.

The *SHIP* knockout mouse (which is viable and fertile), has a shortened lifespan that is caused by overproduction of myeloid cells and infiltration of vital organs[20], highlighting the importance of this phosphatase in the apoptosis of immune cells. To determine the contribution of SHIP to activin/TGF-β-mediated pro-apoptotic effects, we examined their effects in the absence of SHIP. We used phosphorothioate antisense oligonucleotides to the 5′-coding sequence of *SHIP* (SHIP-AS1 and SHIP-AS2) or a scrambled sequence as a control (CTL-1). Pretreatment of MPC-11 cells with the antisense oligonucleotide to SHIP inhibited activin-induced

expression of *SHIP* (Fig. 5a), although it did not affect the levels of PTEN and Stat3. Blocking expression of the basal endogenous level of SHIP also resulted in an increase in basal Akt phosphorylation independently of activin stimulation, further demonstrating the importance of SHIP in regulating Akt activity (Fig. 5a). Activin-mediated growth inhibition of MPC-11 cells was antagonized and almost completely reversed in the presence of 50 μM anti-SHIP antisense oligonucleotides, although it was unaffected by the control oligonucleotide (Fig. 5b). The same effects were observed in M1 cells treated with TGF-β (see Supplementary Information, Fig S3), as well as in human purified lymphocytes (Fig. 5c).

To further demonstrate the requirement of SHIP in activin/TGF-β-mediated apoptosis, we used parental chicken B lymphocytes (DT-40), as well as DT-40 cells in which either the *SHIP* gene (*SHIP*$^{-/-}$) or the tyrosine phosphatase *SHP1* gene (*SHP1*$^{-/-}$) were deleted by homologous recombination[27]. In both parental and *SHP1*-deficient DT-40 cells, activin and TGF-β inhibited cell viability. However, *SHIP*$^{-/-}$ DT-40 cells were resistant to the effects of activin/TGF-β. Finally, to determine whether SHIP is also important for the TGF-β-induced apoptosis of normal primary cells, the cell viability of bone marrow-derived macrophages from wild-type and *SHIP*-deficient mice was examined. Activin/TGF-β treatment of wild-type macrophages resulted in a 70% reduction in cell viability, whereas only a 20% reduction in *SHIP*$^{-/-}$ macrophage survival was recorded (Fig. 5d, right). To explain the residual loss of viability in *SHIP*$^{-/-}$ macrophages, it is conceivable that activin and TGF-β utilize alternate pathways to induce cell death in immune cells. This is consistent with recent studies showing that TGF-β can mediate apoptosis through the adaptor protein Daxx and the JNK (c-Jun N-terminal kinase) pathway[12]. Similar results were obtained with two different preparations of macrophages derived from bone marrow. Our results demonstrate that regulation of *SHIP* expression by TGF-β family members is critical and required for their pro-apoptotic effects in both normal and cancer cells.

As SHIP is a critically important regulator of cell death in immune cells, one might expect to find abnormalities in its expression in pathological conditions, such as leukaemia and auto-immune diseases. The human *SHIP* gene is located on chromosome 2 at position 2q36–37, and although mutations or deletions in this region do not represent a hallmark of human diseases, aberrant translocations and sporadic abnormalities at this chromosomal location have been detected in several leukaemias[28]. Finally, although the involvement of phospholipid metabolism in cellular homeostasis has been widely documented, little is known about the regulation of lipid kinase and phosphatase expression that maintains the intracellular pool of phospholipids. Here, we demonstrate that the expression and activity of SHIP is regulated directly by TGF-β family members. Furthermore, our results link TGF-β-induced apoptosis to phospholipid metabolism and expand our understanding of how activin/TGF-β serine kinase receptors function. □

Methods

Cell culture

MPC-11, 293, CHO, and wild-type and *Smad4*$^{-/-}$ MEFs were cultured in DMEM containing 10% foetal calf serum (FCS). M1 cells were cultured in RPMI containing 10% FCS. B9 cells were cultured in RPMI containing 10% FCS supplemented with 50 μM β-mercaptoethanol. Wild-type, *SHIP*$^{-/-}$ and *SHP1*$^{-/-}$ DT-40 cells were cultured in DMEM containing 10% FCS and 2% chicken serum. Bone marrow-derived *SHIP*$^{+/+}$ and *SHIP*$^{-/-}$ macrophages were obtained as described[29] and maintained in IMDM containing 10% FCS and 1000 U ml^{-1} of macrophage colony-stimulating factor (MCSF).

Cloning and generation of SHIP reporter construct and luciferase assays

The 1.4-kb sequence of the *SHIP* gene promoter was generated by PCR from MPC-11 genomic DNA. The amplified promoter fragment was digested by *Xho*I and *Hin*dIII and cloned into the pGL3 luciferase basic reporter vector to generate the 1.4-kb SHIP-Lux reporter construct. For luciferase assays, the SHIP-Lux and ARE-Lux/FastI constructs were cotransfected by calcium phosphate in 293 cells with an expression vector encoding for β-galactosidase gene, in the presence or absence or various Smad expression plasmids, as described in the legend of Fig. 3. MEFs were transfected with Lipofectamine Plus (Invitrogen, Carlsbad, CA). One day after transfection, cells were serum-starved for 12 h and treated with or without activin (0.5 nM) or TGF-β (0.2 nM) for 18 h. Cells were then washed once with PBS and lysed in 250 μl of lysis buffer (1% Triton X-100, 15 mM magnesium sulphate, 4 mM EGTA, 1 mM dithiothreitol and 25 mM glycylglycine) on ice. The luciferase activity of each sample was measured in an EG&G Berthold Luminometer using 45 μl of cell lysate and normalized to the β-galactosidase activity.

Reverse-transcription PCR

MPC-11, B9 and M1 cells were treated with activin and TGF-β for different times and total RNA was extracted using Trizol reagents (Invitrogen). Reverse transcription of total cellular RNA and amplification of DNA products for SHIP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were carried out using Superscript First Strand Synthesis System for RT-PCR (Invitrogen). Primer sequences used for SHIP amplification were as follows (sense: 5′-CCTCCAACCCCCTCCCTCCCAACCA-3′ and antisense 5′-AACGCCGGCGGCATGGCAGTCCTGCCAA-3′). Densitometric analysis was performed using Alpha Innotech Corporation (San Leandro, CA) Fluorochem 8000 software version 3.04.

Cell viability assay (MTT)

Cells were plated in triplicate at 5,000 cells per 100 μl in RPMI containing 2% FCS. Cells were stimulated in the presence or absence of activin (0.5 nM) or TGF-β (0.2 nM) and incubated over a three-day period. Cell growth was assessed using the non-radioactive MTT cell growth assay for eukaryotic cells (Cell Titer 96, G4000; Promega, Madison, WI). Absorbance was measured at 570 nm with a reference wavelength at 450 nm using a Biotek (Winooski, VT) microplate reader. Wild-type, *SHIP*$^{-/-}$ and *SHP1*$^{-/-}$ cell lines were plated in triplicate at 5,000 cells per 100 μl in DMEM containing 2% FCS and 1% chicken serum. *SHIP*$^{+/+}$ and *SHIP*$^{-/-}$ macrophages were plated in triplicate at 5,000 cells per 100 μl in IMDM medium containing 10% FCS and 2% MCSF. Cells were stimulated with activin or TGF-β for three days before being assessed by MTT assay.

Flow cytometry

Cells were plated in triplicate at 300,000 cells per ml in RPMI containing 2% FCS. Cells were stimulated in the presence or absence of activin (0.5 nM) or TGF-β (0.2 nM) and incubated over a three-day period. Subsequently, cells were washed in PBS and fixed overnight in 70% ethanol. Cellular DNA was then labelled with 50 ng ml^{-1} propidium iodide (PI) in PBS, 1.6% Triton X-100 and incubated overnight at 4 °C in the presence of 10 μg ml^{-1} RNAse A.

The next day, cells were analysed in an EPICS XL series flow cytometer (Beckman Coulter, Miami, FL). Fluorescence was excited by an argon–ion air-cooled 15-mW continuous laser power at 488 nm. PI emission peak was at 620 nm and excitation peak was at 536 nm. At least 20,000 gated events were recorded for each sample and the data were analysed by Multi-cycle software for Windows (Phoenix Flow Systems, San Diego, CA).

Annexin V labelling

Cells were plated in RPMI containing 2% FCS and stimulated with activin for 0, 16, 24 and 36 h. Cells were then collected, washed, stained with annexin V–fluorescein isothiocyanate (828681; Roche) in accordance with the manufacturer's instructions and analysed by immunofluorescence microscopy (Eclipse E600; Nikon, Montreal, Canada) using the MetaImaging Series-Metamorph software (Universal Imaging Corporation, Downingtown, PA).

Western blot analysis

Cells were plated in RPMI containing 2% FCS and stimulated in the presence or absence of activin, TGF-β, IL-6 or sodium pervanadate for the indicated times. Cells were lysed on ice in lysis buffer (50 mM Hepes at pH 7.5, 150 mM sodium chloride, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 5 mM EDTA, 10% glycerol, 0.5% NP40 and 0.5% sodium deoxycholate) supplemented with 100 mM sodium vanadate, 1 mM phenyl methylsulphonyl fluoride (PMSF), 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin and 2 μg ml^{-1} pepstatin. Total cell extracts were then separated on a 10% SDS–polyacrylamide gel electrophoresis (PAGE) gel, transferred to nitrocellulose and incubated with the indicated specific antibodies overnight at 4 °C (anti-SHIP sc-8425, anti-PTEN sc-7974, anti-Stat3 sc-8019, Santa Cruz Biotechnology, Santa Cruz, CA; anti-Akt 9272, anti-phospho-Thr 308-Akt 9275S, anti-phospho-Ser 473-Akt 9276S, New England Biolabs, Beverly, MA).

Intracellular phosphoinositol ($InsP_3$, $InsP_4$) measurements

Cells were plated at 5×10^6 cells per ml in inositol-free medium containing 1 mCi Myo-2-^{3}H-(N)-inositol and stimulated in the presence or absence of activin and TGF-β for 16 h. Cells were washed three times with Krebs–Hepes (146 mM sodium chloride, 4.2 mM potassium chloride, 0.5 mM magnesium chloride, 1.0 mM calcium chloride, 10 mM Hepes at pH 7.4, 20 mM lithium chloride and 5.9 mM glucose) and lysed with 5% perchloric acid. The supernatant was collected in 500 μl of Hepes–potassium hydroxide buffer (75 mM Hepes and 1.5 M potassium hydroxide), adjusted to pH 7 with 5% perchloric acid and phospholipids were separated using a DOWEX AG 1X-8 (200–400 mesh) column.

$InsP_1$ and $InsP_2$ were first removed from the column by washing with ammonium formate (400 mM) and elution of $InsP_3$ and $InsP_4$ was performed using specific buffers ($InsP_3$, 700 mM ammonium formate and 100 mM formic acid; $InsP_4$, 1 M ammonium formate and 100 mM formic acid). Measurement and quantification of each inositol phosphate isoform was then performed using a WinSpectral (Perkin Elmer, Boston, MA) 1414 liquid scintillation counter.

Phosphatidylinositol ($PtdInsP_3$) measurement

Cells were plated at 5×10^6 cells per ml in phosphate-free RPMI medium, pretreated in the presence or absence of activin and TGF-β for 24 h or the indicated times for the activin time-course experiment. Cells were collected and labelled with 100 μCi ^{32}P-orthophosphate (NEX053; PerkinElmer) for 3 h in incubation medium (0.1% BSA in phosphate-free medium) at 37 °C, washed three times with phosphate-free medium and resuspended with 500 μl of HCl:EtOH buffer. Phosphatidylinositols were extracted with chloroform, lyophilized, resuspended in 25 μl of chloroform, separated by thin layer chromatography and analysed by autoradiography. Densitometric analysis was performed using Alpha Innotech Corporation Fluorochem 8000 software version 3.04.

Antisense oligonucleotide treatment

Cells were plated in 96-well plates at 5×10^3 cells per well in 2% FCS and treated in the presence or absence of antisense oligonucleotides for *SHIP* mRNA (SHIP-AS1 and SHIP-AS2) or a control antisense oligonucleotide (CTL-1) at 50 μM. After 12 h, cells were stimulated with activin (0.5 nM) or TGF-β (0.2 nM). Cell growth was measured 72 h after ligand stimulation using the MTT assay, as

described above. We used the following phosphorothioate oligonucleotides: SHIP-AS1 5′-CAGGGAC-CATGGCAGGCATG-3′; SHIP-AS2 5′-GGGTGCATTACCCATGTTCC-3′. The sequence of the control oligonucleotide (CTL-1) was 5′-TCAGACTGGCTCTCTCCATG-3′.

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Supplementary Information accompanies this paper on www.nature.com/naturecellbiology.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

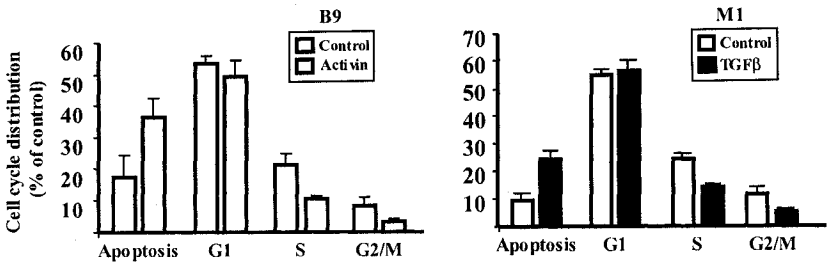

Fig.S1 Activin and TGFβ induce apoptosis in hematopoietic cells. B9 and M1 cells were stimulated or not with activin or TGFβ for 72 hours. The distribution of cells in the cell cycle were quantified by analysis of propidium iodide stained cells using flow cytometry.

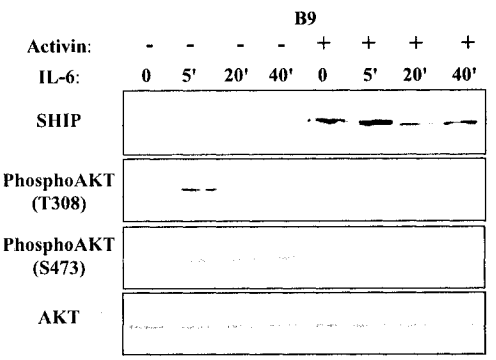

Fig.S2 Activin-induced SHIP expression in B9 cells antagonizes AKT phosphorylation in response to IL-6 on both Thr308 and Ser 473 (middle panels). Reprobing of the blot with anti-AKT confirmed equal loading (lower panel). Increased SHIP expression in response to activin was monitored by anti-SHIP immunoblot (upper panel).

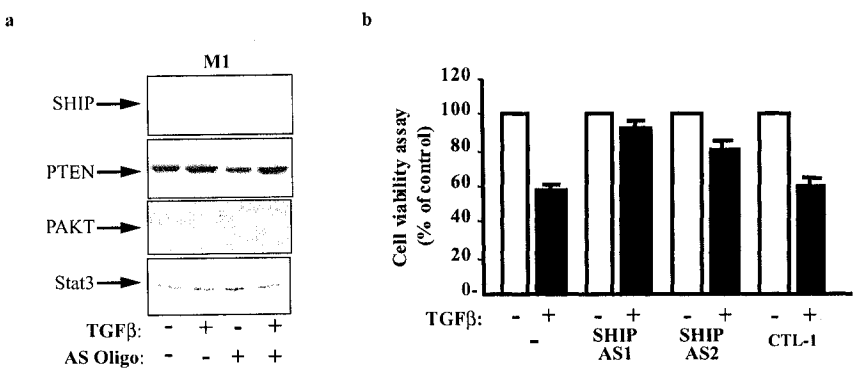

Fig.S3 Inhibition of expression of the lipid phosphatase SHIP prevents TGFβ-induced apoptosis. a, SHIP and PTEN protein expression levels as well as AKT phosphorylation in M1 cells treated or not with the antisense oligonucleotide to SHIP mRNA (SHIP-AS2) were measured by Western blot in cells stimulated or not with TGFβ for 24h. For loading controls the blot was reprobed with an anti-Stat3 antibody. b, M1 cells were treated with 50 ?M of phosphorothioate antisense oligonucleotide to SHIP mRNA (SHIP-AS1 and SHIP-AS2) or a with a control oligonucleotide (CTL-1) for 10h before being stimulated with TGFβ. Cell viability was assessed after 72h by MTT colorimetric assays carried out in triplicate. Values are expressed in arbitrary units.