Role of the G protein-coupled receptor kinase 2 in mediating Transforming Growth Factor beta and G protein-coupled receptor signaling and crosstalk mechanisms

by

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ABSTRACT

Transforming growth factor β (TGFβ) and Angiotensin II (AngII) signaling occurs through two distinct receptor superfamilies, the serine/threonine kinase and G protein-coupled receptors (GPCRs). Through diametric actions, TGFβ and AngII regulate various biological responses, including cell proliferation and migration. Previously, we identified the G protein-coupled receptor kinase 2 (GRK2), which acts through a negative feedback loop mechanism to terminate Smad signaling. To investigate the impact of TGFβ-induced GRK2 expression on GPCR signaling, we examined its effect on AngII signaling in vascular smooth muscle cells (VSMCs). We show that activation of the TGFβ signaling cascade results in increased GRK2 expression levels, consequently inhibiting AngII-induced ERK phosphorylation and antagonizing AngII-induced VSMC proliferation and migration. The inhibitory effect of TGFβ on AngII signaling occurs at the MEK-ERK interface and is abrogated when an anti-sense oligonucleotide directed against GRK2 is used. Thus, we conclude that TGFβ signaling antagonizes AngII-induced VSMC proliferation and migration through the inhibition of ERK phosphorylation. GRK2 is a key factor in mediating this crosstalk.

RÉSUMÉ

Le TGFβ et l'Angiotensine II (AngII) exercent des effets opposés sur la prolifération et la migration cellulaire dans les cellules musculaires lisses (VSMC). Nous avons identifié la protéine kinase GRK2 (*G protein-coupled receptor kinase 2*) comme cible directe du TGFβ. Afin de comprendre l'impact de l'augmentation d'expression de GRK2 induite par le TGFβ sur la voie signalétique de l'AngII, nous avons étudié son effet en aval du récepteur à l'AngII dans les VSMC. Nos résultats indiquent que l'augmentation de GRK2, en réponse au TGFβ, antagonise les effets de l'AngII sur la phosphorylation des MAP kinases ainsi que sur ses effets prolifératifs et pro-migratoires. Les effets inhibiteurs du TGFβ en aval de l'AngII dépendent de GRK2, puisqu'ils disparaissent lorsque l'expression de celle-ci est bloquée au moyen d'un oligonucléotide antisense. En conclusion, GRK2 apparaît donc comme un facteur primordial dans la régulation de la prolifération et la migration des VSMC en réponse au TGFβ et à l'AngII.

ABBREVIATIONS

$^{-/-}$: knockout mouse model

~: approximately

Ψ: hydrophobic residue

aa: amino acid

ACE: angiotensin converting enzyme

ActRII: activin type II receptor

AHM: anti-Müllerian hormone

Akt: protein kinase B

ALK: activin receptor-like kinase

AngII: angiotensin II

AP-1: activation protein

ARF6: ADP-ribosylation factor 6

Arg (R): arginine

ARNO: ARF nucleotide-binding site opener

Asn (N): asparagine

AS-Oligo: antisense oligonucleotide

Asp (D): aspartic acid

AT_1R: angiotensin II type I receptor

ATP: adenosine 5'-triphosphate

βAR: β-adrenergic receptor

βARK1: β-adrenergic receptor kinase 1, also known as GRK2

BMP(R): bone morphogenic protein (receptor)

bp: base pair

Ca^{2+}: calcium

CaMKII: calcium-calmodulin-dependent protein kinase II

CBP: CREB binding protein

CDK(I): cyclin dependent kinase (inhibitor)

cDNA: complementary DNA

C-oligo: control oligonucleotide

COS7: SV40 transformed African Green Monkey kidney cells

Co-Smad: common partner Smad

CPD: carboxypeptidase D

CREB: cAMP response element-binding protein

C-terminal: carboxyl-terminal

CTGF: connective-tissue growth factor

CTKD: C-terminal kinase domain

D domain: docking domain
DAG: 1,2-diacylglycerol
DCD: dermcidin
DGCR6: DiGeorge syndrome critical region gene 6
DMEM: Dulbecco's Modified Eagles Medium
DNA: deoxyribonucleic acid
E1: ubiquitin-activating enzyme
E2: ubiquitin-conjugating enzyme
E3: ubiquitin ligase
ECM: extracellular matrix
EDTA: ethylenediamine tetra-acetic acid
eEF1A1: eukaryotic translation elongation factor 1 α 1
EGF(R): epidermal growth factor (receptor)
EMT: epithelial-to-mesenchymal transition
eNOS: endothelial nitric oxide synthase
ERK: extracellular signal-related kinase
F-actin: filamentous actin
Fast-1: forkhead activin signal transducer 1
FBS: fetal bovine serum
FHA domain: forkhead-associated domain
FoxH1: forkhead box H1
FoxO: forkhead box, sub-group O
G protein: guanine nucleotide-binding protein
g: gram
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GC-rich regions: guanine-cytosine rich regions
GDF: growth and differentiation factor
GDNF: glial cell-derived neurotrophic factor
GDP: guanosine diphosphate
GIT proteins: G protein-coupled receptor kinase-interacting protein
Glu (E): glutamic acid
GMP: guanosine monophosphate
GPCR: G protein-coupled receptor
GRK: G protein-coupled receptor kinase
GS box: glycine-serine-rich domain
GTP: guanosine triphosphate
GTPase: guanosine triphosphatase

HDAC: histone deacetylase
HECT: homologous to E6AP C-terminal
HEK-293: human embryonic kidney-293 cells
HeLa: Henrietta Lacks cells
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF1α: hypoxia-inducible factor 1α
His (H): histidine
hrs: hours
HUH7: human hepatocarcinoma cells
IGF(R): insulin growth factor (receptor)
Ile (I): isoleucine
IP3: inositol 1,4,5-trisphosphate
I-Smads: inhibitory Smads
JNK: c-Jun amino-terminal kinase
kDa: kilo Dalton
LAMC1: lamininγ-1 chain
LAP: latent-associated peptide
LC/MS/MS: liquid chromatography/mass spectrometry/mass spectrometry (Tandem)
Leu (L): leucine
LTBP: latent TGFβ binding protein
Lys (K): lysine
Mad: Mothers Against Decapentaplegic
MAGUK: membrane-associated guanylate kinase
MAP(K): mitogen-activated protein (kinase)
MCF7: Michigan Cancer Foundation 7; human caucasian breast adenocarcinoma
Met (M): methionine
mg: milligram
MH1: Mad homology 1
MH2: Mad homology 2
min: minutes
MIS: Müllerian inhibiting substance
MK: MAPK-activated protein kinase
mM: milliMolar
MMP: matrix metalloproteinase
mRNA: messenger RNA
MSK: mitogen- and stress-activated kinase
NAD(PH): nicotinamide adenine dinucleotide (phosphate)

NES: nuclear export signal
NLS: nuclear localization signal
nM: nano Molar
NPRTase/PP3856: nicotinate phosphoribosyltransferase
NSF: N-ethylmaleimide-sensitive fusion protein
N-terminal: amino-terminal
NTKD: N-terminal kinase domain
p42-44 MAPK: ERK pathway
PAK1: p21-activated kinase 1
PBS: phosphate buffered saline
PH domain: pleckstrin homology domain
Phe (F): phenylalanine
PI3K: phosphoinositide-3-kinase
PIF-CP: proteolysis-inducing factor-core peptide
PIP2: phosphatidylinositol 4,5-bisphosphate
PIP3: phosphatidylinositol-3,4,5-triphosphate
PKA: protein kinase A
PKC: protein kinase C
PLC: phospholipase C
PMSF: phenylmethylsulfonyl fluoride
PP3856/ NPRTase: nicotinate phosphoribosyltransferase
Pro (P): proline
pS/pT: phosphoserine/phosphothreonine
RAS: renin-angiotensin system
RGS: regulator of G protein signaling
RH domain: arginine-histidine domain
RNA: ribonucleic acid
ROS: reactive oxygen species
rRNA: ribosomal RNA
RSK: p70 ribosomal S6 kinase
R-Smads: receptor-regulated Smads
RTK: receptor tyrosine kinase
RT-PCR: reverse-transcription polymerase chain reaction
RUNX: runt-related transcription factor
SAPK: stress-activated protein kinase
SARA: Smad anchor for receptor activation
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser (S): serine
shRNA: short hairpin RNA
SIM motif: single input motif
Sma: Small body size
SMC: smooth muscle cell
Smurf: Smad ubiquitination regulatory factor
Sp1: specificity protein 1
STAT: signal transducer and activator of transcription
STRAP: serine/threonine kinase receptor-associated protein
SUMO: small ubiquitin-like modifer
$t_{1/2}$: half life
TAK1: TGFβ-activated kinase-1
TGFβ: transforming growth factor β
Thr (T): threonine
TRIP: TGFβ receptor-interacting protein-1
tRNA: transfer RNA
Trp (W): tryptophan
Tyr (Y): tyrosine
TβRI: TGFβ type I receptor
µg: micro gram
µL: micro Litre
µM: micro Molar
Val (V): valine
VEGF: vascular endothelial growth factor
VSMC: vascular smooth muscle cell
WD domain: tryptophan-aspartic acid domain
Xaa (X): unknown or 'other' amino acid
Zn^{2+}: Zinc

LIST OF FIGURES

CONTRIBUTION OF THE AUTHORS

"As an alternative to the traditional thesis format, the thesis can consist of a collection of papers of which the student is an author or co-author."

[quoted from the McGill University guidelines for Thesis Preparation, http://www.mcgill.ca/gps/current/programs/thesis/guidelines/preparation/]

In accordance with the above guidelines for thesis preparation and submission, I have included the following paper as the core of my thesis (Chapter 2):

> **Mancini J.,** Chen H., Ho J., Laporte S., Richard D.E., and Lebrun J.J.
> TGFβ-induced GRK2 expression attenuates AngII-regulated Vascular Smooth Muscle Cell proliferation and migration. (Manuscript to be submitted)

The following individuals have contributed as co-authors of the paper which I have included my thesis:

All experiments currently in the manuscript have either been repeated by myself, or have been performed by me. Hui Chen performed some of the RT-PCR and MTT experiments, and assisted with the scratch assays. Dr. Joanne Ho contributed to the scientific discussions and helped with critical reading of the manuscript. Dr. Stéphane Laporte contributed to the scientific discussions and provided assistance with his knowledge of GPCR/GKR2. Finally, Dr. Darren E. Richard performed the HIF-1α experiment.

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CHAPTER 1: INTRODUCTION

1.1 HYPOTHESIS & RATIONALE

The founding member of the Transforming Growth Factor β (TGFβ) superfamily of regulatory proteins, TGFβ1, was discovered over a quarter of a century ago [1]. The TGFβ superfamily and its constituent members are widely expressed in various cell types and play essential regulatory roles, best illustrated by the variety of human diseases in which deregulation of TGFβ signaling components have been implicated. Although several key members and targets of the TGFβ signaling pathway have been identified and characterized, much remains unknown and/or poorly understood.

Previously, our laboratory identified the G protein-coupled receptor kinase 2 (GRK2) as a downstream target of the TGFβ signaling cascade. GRK2 was found to act through a negative feedback loop mechanism, resulting in the termination of TGFβ-induced signaling, thus inhibiting the tumor suppressive effects of TGFβ. Initially, this effect was studied in liver carcinomas. However, when the analysis was extended to other cell types, such as breast cancer cells and VSMCs, it was found that the effect of TGFβ-induced GRK2 upregulation was not tissue specific. These results suggested a potentially important regulatory role for GRK2, as well as suggested the potential for being a key component in crosstalk mechanisms with other signaling pathways. In particular, GRK2 is known to play a critical role downstream of G protein-coupled receptor (GPCR) signaling. As such, we sought to investigate whether increased TGFβ-induced GRK2 levels, in response to TGFβ stimulation, would affect GPCR signaling. In fact, studies have shown the TGFβ and the angiotensin II (AngII) signaling pathways to be intricately intertwined, as expression of both TGFβ and of its type I receptor are reportedly induced upon AngII stimulation [2].

In order to examine the effects of both the TGFβ and of the GPCR signaling pathways, we chose to work with VSMCs, as they are a well-described model system possessing both TGFβ receptors (TβR) and AngII type 1 receptors (AT_1R) [3]. Altered cellular behavior and function in VSMCs leads to the induction of vascular diseases, including chronic pulmonary hypertension, atherosclerosis and cardiovascular pathologies. Interestingly, both TGFβ and AngII have been implicated in vascular diseases. Moreover, TGFβ and AngII are known to exert opposite effects on VSMC proliferation and survival [4].

Given that GRK2 is an important regulatory GPCR kinase and that GPCR signaling leads to the regulation of cell proliferation and migration, we hypothesize that ***TGFβ-***

induced upregulation of GRK2 may be involved in mediating the crosstalk between the TGFβ and AngII signaling pathways, thus regulating cell proliferation and migration.

The following thesis examines the role TGFβ-induced GRK2 expression on serine/threonine kinase receptors and on GPCR crosstalk signaling mechanisms, as it pertains to vascular and cardiac diseases. The introduction provides a general overview of the TGFβ superfamily of signaling proteins, followed by an overview of the ubiquitously expressed GPCR family. Other signaling pathways and their role in signal crosstalk are explored, paying close attention to the roles of the TGFβ ligand and of the GPCR kinase, GRK2. Finally, we will look at the crosstalk between TGFβ and the GPCR ligand, AngII.

The second chapter presents the first draft of our manuscript describing how TGFβ-induced GRK2 expression attenuates AngII-regulated VSMC proliferation and migration. Chapter three proposes future directions for our study, particularly in characterizing the regulation and crosstalk mechanism of GRK2. Preliminary results are discussed.

Finally, chapter four ends the thesis with a final discussion regarding the implications of GRK2 as the mediator of TGFβ and GPCR signaling and crosstalk mechanisms.

1.2 OVERVIEW OF THE TGFβ SIGNALING PATHWAY

1.2.0 Preface

The TGFβ superfamily is comprised of a large group of structurally related pluripotent polypeptides comprised of TGFβs, activins, inhibins, bone morphogenic proteins (BMPs) and other growth and differentiation factors (GDFs) [2, 5], each capable of regulating a fascinating array of cellular processes. TGFβ and its receptors are widely expressed in various cell types and the regulatory role they play is of central importance to the control and development of human disease. Loss of TGFβ signaling often results in hyperproliferative disorders and has been linked to cancer development as well as to inflammatory and autoimmune disease. However, supersensitivity to TGFβ signaling has been implicated in immunosuppression and tumor metastasis [6, 7].

Binding of the TGFβ ligand to a single transmembrane spanning TGFβ type II receptor (TβRII) initiates TGFβ signaling. Recruitment of the TGFβ type I receptor (TβRI) to TβRII ensues, and TβRI is transphosphorylated by the kinase domain of TβRII. This modification induces a conformational change in the TβRI, thereby activating its kinase domain. Once activated, TβRI phosphorylates the primary intracellular mediators of the TGFβ signaling cascade, the receptor-regulated Smads (R-Smads), Smad2 and 3, on their carboxyl-

terminal (C-terminal) domain serine residues. Consequently, this phosphorylation event allows for the recruitment of common partner Smad (Co-Smad), Smad4, to the phosphorylated Smad2 and 3. This heterotrimeric complex can then translocate to the nucleus, where it can associate with various co-activators or co-repressors to regulate gene transcription in a cell-dependent manner. In fact, cell type and regulated gene expression, allows for the multifaceted regulatory roles executed by the TGFβ signaling pathway.

Although the Smads are the primary mediators of the TGFβ signaling pathway [5], they are not the sole mediators of its regulatory effects. Through several crosstalk and feedback mechanisms, the signaling network monitors and modifies the activity of constituent pathways, thus determining the nature and timing of the signals. TGFβ has reportedly been involved in crosstalk with several other signaling pathways, namely the extracellular signal-related kinase (ERK), the mitogen-activated protein kinase (MAPK), the stress-activated protein kinases (SAPK), phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) pathway and the small Rho-like guanosine triphosphatases (GTPases) [3, 8, 9]. These pathways can be induced by TGFβ and can regulate the activity of the Smads. As such, deregulation of the TGFβ signaling pathway is often associated with human diseases ranging from autoimmune disease, fibrotic disorders, cancer and tumor metastases [3].

Since the identification of TGFβ1, scientists have been trying to understand the role of TGFβ in both normal and diseased states. Although some of the key players and their roles have been identified, much remains to be learned.

1.2.1 TGFβ Superfamily

The TGFβ superfamily is composed of more than 40 structurally related members including TGFβs, activins, inhibins, BMPs and GDFs [2, 3, 10]. Found in species ranging from worms to mammals [11], each factor is capable of regulating a fascinating array of cellular processes including proliferation, lineage determination, differentiation, extracellular matrix (ECM) production, tissue homeostasis, embryogenesis, motility, adhesion and death [3, 5] in both a time- and tissue-specific manner. Receptor specificity, tissue distribution and expression levels contribute to the resulting cellular response [12].

Ligands of the TGFβ superfamily contain a characteristicly-spaced pattern of 7-9 cysteine residues [12-16]. Ligands from different subfamilies share a sequence identity of ~30-50%, whereas factors within the same subfamily are 60-80% similar [13, 16]. Most members are synthesized in the same way, and their mature forms secreted as homo or

heterodimers with each monomer in a pre-proprotein form. Bioactive forms of these ligands are generated through cleavage of the pro-form's C-terminal end [12, 13, 17].

1.2.1.1 TGFβ Subfamily

Discovery and expression: TGFβ1 was the first member of the TGFβ superfamily to be characterized after isolation from human platelets, human placenta and bovine kidney, and was termed transforming growth factor for its ability to induce anchorage independent growth of fibroblasts [13]. Since, these factors have been shown to be active from the earliest stages of embryo development through adulthood [18].

Presently, the TGFβ subfamily includes five isoforms (TGFβ1-5) that have been characterized in higher vertebrates and share a close sequence homology. Three isoforms have been identified in mammals, TGFβ1-3, showing between 70-80% homology. Although these three members reportedly have similar properties *in vitro*, TGFβ1 is the most abundantly and universally expressed isoform and thus, is the most studied [19].

Structure: The mature active form of TGFβ is composed of homo or heterodimers in which 6 of the characteristicly-spaced 7-9 cysteine residues form three intra-subunit disulfide bonds, which are important for structural integrity. The remaining cysteines form a disulfide bond with the other subunit in order to stabilize the dimer interface [12]. Crystal analysis of TGFβ2 (Fig.1) describes each 12-15 kDa monomer [10] as two pairs of antiparallel β-strands projecting away from a long α-helix, forming a "four-digit hand" [10]. Each β-strand is likened to a "finger" and the helix to a "wrist". The variable "fingertip" regions are responsible for forming contacts with the receptors.

The conserved characteristic cysteine pattern suggests three-dimensional structure similarity and crystallography of TGFβ1 and 3 identified no marked differences between the tertiary structures. However, minor sequence variations were attributed to determining receptor-ligand affinities and specificities [13, 20]. Despite overall structure similarity, the three isoforms are associated with similar yet distinct non-overlapping functions.

Synthesis: Ligands of the TGFβ superfamily are secreted as inactive precursors, which are cleaved into mature ligands. Alternatively, they can be secreted as mature active dimmers that are inhibited by circulating agonists. Inactive precursors are secreted as homo or heterodimers, where each monomer is synthesized in a latent pre-proprotein form [5, 18, 21] and is composed of a hydrophobic signaling peptide, a variable amino-terminal (N-terminal) pro-domain and a smaller, biologically active C-terminal peptide. After directing the precursor molecule to the secretory pathway, the signal peptide is cleaved and the pro-

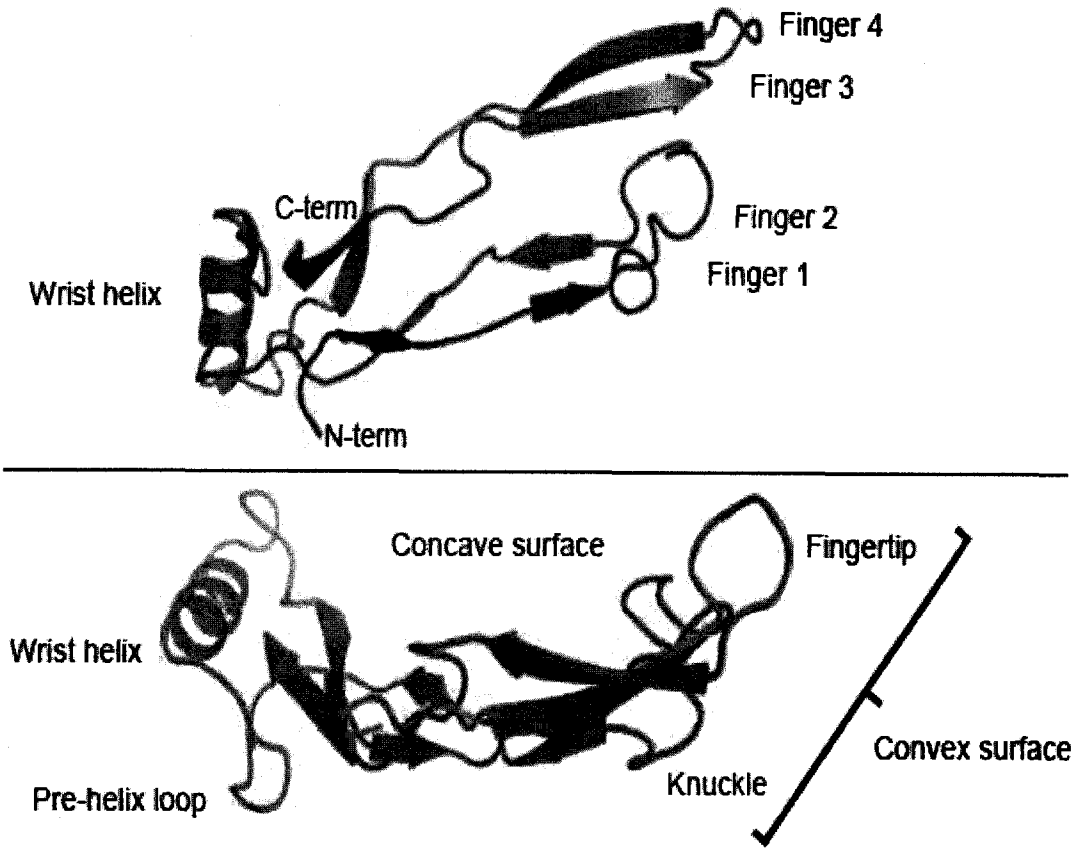

Fig.1: The TGFβ fold

A typical TGFβ monomer consists of a cysteine knot motif with two pairs of antiparallel β-strands (fingers) extending from an α-helix (wrist region). The β strands are curved to form both a concave and a convex surface for receptor interaction (Lin, Lerch *et al.* 2006)

form of the ligand is targeted to the Golgi apparatus [13]. In the *trans*-Golgi, TGFβs are proteolytically cleaved at a conserved dibasic RXXR site by furin-type enzymes [21]. Upon secretion, the resulting pro-domain, termed latent-associated peptide (LAP), remains non-covalently associated with its C-terminal bioactive portion, known as the latent TGFβ binding protein (LTBP). LTBPs are, in fact, not required to maintain TGFβ-latency but rather to facilitate the secretion, storage, or activation of TGFβ-LAP [5, 22].

The extracellular concentration of active TGFβ is primarily regulated by the conversion of latent TGFβ into active TGFβ [2]. Latent TGFβs are stored in the extracellular matrix (ECM), where they are anchored to the matrix by the LTBP. At any given time, tissues contain significant amounts of latent TGFβ. Activation of only a small portion of latent TGFβ is required to generate a maximal cellular response [21]. Various triggers have been proposed for activation, including proteases such as plasmin or cathepsin [23], matrix metalloproteinases (MMP-2 and -9) [23], binding of LAP to the mannose-6-phosphate receptor [23], αVβ6 integrin [23], acidic cellular environments [2, 23], thrombospondin-1 [2, 23], and reactive oxygen species (ROS) [2]. MMPs are often found in malignant tumors that secrete high levels of active TGFβ. Plasmins release LTBP by acting on its protease-sensitive hinge. Similarly, thrombospondin-1 and αVβ6 can bind LAP, causing a conformational change in the latent complex and unmasking the receptor binding site. This diversity of activation methods suggests a highly controlled and cell-specific response mechanism, which can account for the plethora of biological roles TGFβ performs.

1.2.1.2 Activin/Inhibin Subfamily

Activins and inhibins were first identified as components of gonadal fluids that had the ability to stimulate or to suppress follicle-stimulating hormone secretion from pituitary gonadotropes, respectively [24]. Moreover, activin was identified as an endocrine regulator of pituitary function and, independently, as an inducer of mesoderm in frogs [5]. Since their discovery, these two widely expressed opposing factors have been shown to play a vital role in both paracrine and autocrine regulation of growth and differentiation [24]. Although activins are mainly produced in the pituitary and act locally to regulate the function of its targets, inhibins are more established as endocrine feedback modulators. However, they have been shown to act locally as autocrine and paracrine factors [24].

Activins exist either as homo or as heterodimers comprised of two β subunits linked together by disulfide bonds. Currently, five homologous β genes (β_A-β_E) have been identified of which only β_A and β_B appear to have a biological function. However, activin

β_C transcripts have been found abundantly in the liver [25]. Interestingly, activin β_A and β_C subunits display reciprocal expression, suggesting an antagonistic relationship [13, 25].

Composed of one α and of one β subunit linked together by disulfide bonds, inhibins function as potent activin-antagonists and sometimes as agonists of certain BMPs. Bound to betaglycan, inhibin blocks activin from binding to its type II receptor. To date, no receptor has been validated for inhibin [14]. Activin, on the other hand, signals through activin receptor-like kinases (ALKs) 2, 4 and 7. ALK4 is the type I receptor shared by all activins. They also signal through activin type II receptors (ActRII) A and B [14].

1.2.1.3 Nodal

First cloned from a 7.5 day post-coitum mouse embryo cDNA library [26], Nodal is involved in cell differentiation, playing an essential role in mesoderm formation and axis specification. In addition to being pro-apoptotic and growth-inhibitory effects, it has been identified as a critical regulator of early vertebrate development, involved in the induction of dorsal mesoderm, anterior patterning and formation of left-right asymmetry [26]. The Nodal knockout model is embryonic lethal, due to defects in primitive streak formation.

Nodal signals through ALK4 and 7, but preferentially uses ALK7 as it shows enhanced binding in the presence of co-receptor, cripto [14]. ActRIIA and B are its type II receptors.

Despite having similar kinase domains, ALK7's extracellular domain is unique to that of ALK4. In fact, ALK7 is not able to bind TGFβ, activin or BMP7, even in the presence of type II receptors. Signaling initiated by Nodal activates Smad2 and 3, which interact with Smad4 and translocate to the nucleus, where they bind *cis*-acting elements in the promoter region of target genes and modulate gene transcription.

While Nodal, activin AB and activin B share the same type I receptor, TGFβs, Nodal, Activin, GDF9 and Lefty activate the same R-Smads, suggesting that crosstalk between Nodal and other members of TGFβ family can occur [26].

1.2.1.4 BMP/GDF Subfamily

First identified in the 1960s as bone repair factors, it was not until the 1980s that the proteins responsible for bone induction were purified and cloned. Over 20 BMPs have been identified and characterized, including many GDFs due to their sequence similarities.

BMP family members are involved in diverse biological processes including cell differentiation, cell-fate determination, cell growth, neurogenesis, morphogenesis, apoptosis and embryonic development, in various cell types. In particular, during early or

late embryonic events, BMPs have been implicated in dorsoventral pattern formation, mesodermal induction, organ and tissue development, and limb formation [27].

BMPs are synthesized as large precursor molecules, active as both homo and heterodimers held together by a single disulfide bond. Dimerization, after which they undergo proteolytic cleavage to generate mature dimmers, is required for bone induction. The N-terminal region determines the stability of the processed mature protein and the downstream sequence adjacent to the cleavage site determines the efficiency of cleavage [28]. Signaling, triggered by BMPs, is transduced through the type I and type II serine/threonine kinase receptors, leading to activation of the Smad proteins.

1.2.1.5 Distant Members

There exists a group of distant TGFβ family members that share weak homologies with other members of the TGFβ superfamily. For instance, the glial cell-derived neurotrophic factor (GDNF) family is composed of GDNF, neuturin, artemin and persephin [29]. It was purified and characterized in 1993 as a growth factor promoting the survival of embryonic dopaminergic neurons in the midbrain [29]. Although it contains the 7 characteristic cysteine residues, it remains the most divergent of the TGFβ family members, showing less than 20% sequence homology to the other members. GDNF signals through the receptor tyrosine kinase (RTK), Ret [5], unlike the other TGFβ members.

The next most divergent is the Anti-Müllerian Hormone (AHM), also known as Müllerian Inhibiting Substance (MIS). MIS is a glycoprotein expressed early in gonadal differentiation of the male [30] and is involved in the regression of the Müllerian duct.

TGFβ and MIS exhibit weak homology, particularly around the characteristic cysteine residues, and MIS also exhibits similar growth inhibiting properties as TGFβ. Like the other memebers, MIS may be biologically active as a disulfide-linked dimer.

Lefty, distantly related for its lack of cysteine residues required for homodomerization, is a negative regulator of TGFβ and Nodal signaling. It inhibits the activation of the primary mediators of the TGFβ signaling cascade, the Smads [31].

1.2.2 TGFβ Receptor Superfamily

With the cloning of the first activin receptor in 1991 [32], it was discovered that ligands of the TGFβ superfamily signal through a family of single transmembrane spanning serine/threonine kinase receptors, with the exception of GDNF. Based on their structural and functional properties, the TGFβ receptor family is divided into two

subfamilies: the type I receptors (55kDa) and the type II receptors (70kDa) [5, 13]. In contrast to the numerous TGFβ superfamily ligands, only five type II receptors and seven type I receptors have been described [11]. Each ligand uses a distinct receptor, but many share receptor subunits, creating competition at the level of receptor binding. Finely tuned ligand-receptor expression patterns, differing in affinity and specificity, allow for the transmission of highly diverse and distinct signaling responses [12].

The more divergent type III receptor does not have intrinsic signaling function. However, it appears to regulate TGFβ access to the signaling receptors [5].

Type I and Type II Receptors: To date, seven members of TβRI family have been identified and characterized [11]. Members of the type I receptor family express a higher degree of sequence similarity than do the members of the type II receptor family, particularly within the kinase domain [5]. In the basal state, TβRI is unphosphorylated and relatively inactive. It has low affinity for circulating ligand and instead has high intrinsic affinity for TβRII, to which it is immediately recruited. Only then does TβRI recognize bound ligand [14], and in response, phosphorylate the Smads. Within the heteromeric receptor complex, it is the TβRI that determines signaling specificity [11].

As a result of being simultaneously cloned by different groups, most type I receptors have received different names. One practice has been to use the neutral nomenclature, Activin-receptor Like Kinase (ALK), and to adopt a more descriptive name when the physiological ligand becomes known [5]. Originally, TβRI was known as ALK5. Recently, ALK-1 was identified as an endothelial specific TβRI [11].

The TβRII is a constitutively active transmembrane receptor. It has different degrees of affinity for circulating ligand. Upon ligand binding, it exerts its only known function, which is to recruit and transphosphorylate the type I receptor, thereby activating it [9].

In mammals, five different TβRIIs have been identified and found to have varying affinity for to circulating ligand. Consequently, the specificity of the ligand-induced response is mediated by the type II receptor. Nomencalture dictates that type II receptors are named after the primary ligands they bind, thus TβRII, BMPRII, AMHRII and ActRIIA/B, selectively bind TGFβs, BMPs, MISs, and both BMPs/GDFs, respectively [5].

1.2.2.1 Structure

Receptors for this superfamily are single transmembrane spanning serine/threonine kinase receptors. In addition to the conserved sequences these receptors share within their kinase domains, they are characterized by the presence of a glycine-serine-rich

juxtamembrane domain (GS box) in the type I receptor and by the constitutively active nature of the type II receptor, due to its ability to autophosphorylate.

The TβR superfamily is described as having a "three-finger toxin fold", in reference to a class of neurotoxins known as the "three-finger toxins". This fold is comprised solely of β-strands, stabilized by four disulfide bonds formed by eight conserved cysteine residues. A ligand-binding surface is created by three pairs of antiparallel β-strands. Despite the common architecture and the conserved cysteine residues, very little sequence identity and no functional overlap exist between the two types of receptors [12].

Both the type I and the type II receptors are composed of several domains, some of which play a critical role in signal transduction. The relatively short 150 amino acid (aa) extracellular domain of both receptors is N-glycosylated and contains 10 or more cysteine residues that are thought to determine the general fold of this region. Near the transmembrane, a characteristic cluster is formed by three cysteines, whereas spacing of the others varies and is more conserved in type I receptors than in the type II receptors [5]. Although generally similar in structure, the type I receptor has no C-terminal extensions following the kinase domain, whereas the type II receptors maintain a short sequence.

The kinase region of both the type I and type II receptor conforms to the canonical sequence of a serine/threonine protein kinase domain [5]. However, unique to the type I receptor is the GS domain, also known as the GS box. The GS box represents a key regulatory region that controls the catalytic activity of the TβRI kinase as well as its interaction with receptor substrates [5, 9, 11]. Immediately after the GS box, all type I receptors have a leucine-proline motif that serves as a binding site for immunophilin FKBP12, a negative regulator of receptor signaling. Most importantly, located in the juxtamembrane region, immediately preceding the kinase domain, this highly conserved 30 aa region contains a characteristic TTSGSGSG sequence. Transphosphorylation of the TβRI GS region is performed by the constitutively active TβRII, which activates TβRI in a ligand-dependent manner, allowing it to phosphorylate the R-Smads on their C-terminal serines. Therefore, one can conclude that the TβRI acts downstream of the TβRII. Moreover, TβRI has been shown to determine Smad-isoform activation specificity via the L45 loop within its kinase domain [11]. Phosphorylation by the TβRII, on the other hand, appears to selectively modulate the intensity of different TGFβ responses.

1.2.2.2 Ligand-Receptor Interactions

The various TGFβ superfamily ligands, in addition to the smaller number of receptors and the plethora of distinct regulatory roles performed by these proteins, suggests that signaling is controlled by a set of finely tuned critical ligand-receptor interactions.

Biologically active ligands of the TGFβ superfamily are secreted as dimers linked together by a disulfide bond. The dimeric nature of these ligands suggests that they bind pairs of type I and II receptors, forming heterotetrameric receptor complexes where each receptor binds only one monomer of the dimeric ligand [5, 13].

Two general models of ligand binding have been described: (i) sequential binding and (ii) cooperative binding. Sequential binding is a two-step model in which the ligand first interacts with the type II receptor and then, sequentially, recruits the type I receptor. Based on studies involving TGFβ-resistant cell mutants, this binding model was found to be characteristic of TGFβ/activin receptors. In contrast, the cooperative model involves high affinity ligand binding to both the type I and the type II receptors when expressed together, but with low affinity when expressed separately. This model is typical of BMPs.

1.2.2.3 Mechanism of Receptor Activation

In the basal state, the type I receptor is unphosphorylated and, therefore, inactive. Conversely, the type II receptor is constitutively active due to the autophosphorylation of various serine residues within its juxtamembrane region and kinase domain. Autophosphorylation is ligand independent, whereas activation of the type I receptor is highly dependent on ligand binding. Formation of the ligand-induced receptor type II complex rapidly leads to the transphosphorylation and to the activation of the GS region on the type I receptor kinase, allowing for signaling to occur. Deletion studies have revealed that not all phosphorylated residues are important in mediating type II receptor signaling. Nevertheless, S213 and S409, in the type II receptor, are essential for receptor signaling.

Overexpression studies and *in vitro* co-incubation experiments demonstrated spontaneous association of the type I and of the type II receptor, confirming their intrinsic affinity for one another. In the absence of ligand, the two receptors maintained the ability to form homodimers, an interaction mediated at least in part by the cytoplasmic regions of the receptors. However, in cells expressing moderate levels of TGFβ receptors, formation of heterotetrameric complexes and activation of the type I receptor appeared to be highly dependent on ligand binding.

Formation of the heterotetrameric TGFβ receptor complex is essential for signal transduction. Chimeric receptor constructs containing both receptor kinase domains in various configurations demonstrated that signaling is only achieved when the type I and type II receptor kinase domains are brought together [5]. *In vitro* studies have also shown that ligand binding does not increase the overall phosphorylation of the type II receptor or its kinase activity. Instead it promotes recruitment of the type I receptor for heterocomplex formation. Thus, it appears that type II receptors might be constitutively active kinases that require the ligand to interact with the type I receptor as a substrate [5].

Transphosphorylation of the type I receptor occurs on five clustered serine/threonine residues contained within its GS box and is required for signal transduction. Mutation of some residues results in partial loss of signaling, whereas mutation of four or all five residues leads to complete receptor inactivation [5, 13]. Typically, the type I receptor is kept inactive by a wedge-shaped GS region that presses against the kinase domain, dislocating its catalytic centre. Phosphorylation within the GS region results in a conformational change, releasing this self-inhibitory activity and allowing the binding of ATP to drive Smad phosphorylation.

1.2.2.4 Receptor Interacting Proteins

Several proteins have been found to interact with receptors of the TGFβ superfamily. Although their precise roles in signal transduction remain unclear, they have been associated with the regulation of receptor-mediated signaling, particularly in the prevention of "leaky" signaling. A few examples are listed below.

Type I Receptor Interacting Proteins: FKBP12, an abundant 12kDa cytosolic protein, is known to bind the type I receptor and inhibit TGFβ signaling. Interaction of FKBP12 is mediated by the binding of its active site to a conserved Leu-Pro motif contained within the juxtamembrane of the type I receptor, adjacent to the phosphorylation sites of the GS domain. FKBP12 binds the receptor in the basal state and sterically inhibits type II receptor phosphorylation of the type I receptor. However, upon TGFβ-induced receptor complex formation, FKBP12 is released. Thus, it has been postulated that the role of FKBP12 may be to guard against spurious activation of TGFβ signaling by ligand-independent encounters of type I and type II receptors [5, 22].

Another type I receptor binding protein is the serine/threonine kinase receptor-associated protein (STRAP). STRAP recruits Smad7 to the TβRI receptor, preventing R-Smad phosphorylation and, thus potentiating the inhibitory effects of Smad7 [11].

Finally, BMP and activin membrane-bound inhibitor (BAMBI), a pseudoreceptor, forms inactive dimers with type I receptors, interfering with their activation [22, 33].

Type II Receptor Interacting Proteins: The TGFβ receptor-interacting protein-1 (TRIP-1), a WD40 repeat containing protein, binds to TβRII in a ligand-independent manner, which requires the receptor kinase activity, and results in the phosphorylation of TRIP-1. TRIP-1 down-regulates TβRII-mediated transcriptional activity.

Although it functions mostly at the level of TβRI, STRAP can also interact with TβRII.

1.2.2.5 Mechanism of Receptor Internalization and Endosomal Trafficking

Only recently has internalization of ligand-bound receptors been associated with serine/threonine kinase receptors. Traditionally, this role in receptor-dependent signaling regulation was attributed to the GPCRs and RTKs. To date, two mechanisms of internalization have been described (Fig.2): (i) Clathrin-dependent internalization and, (ii) Clathrin-independent/Caveolae-mediated internalization. Recent data suggest that TGFβ receptors are internalized via both mechanisms. In internalization through clathrin-dependent mechanisms, TGFβ signal propagation is promoted through the recruitment of specific signaling components into endocytic compartments along with the activated receptors. However, lipid raft-mediated internalization dampens TGFβ receptor-dependent signals through the degradation of the receptor complex [34].

Clathrin-Dependent Internalization: Clathrin-dependent internalization involves the recruitment of clathrin from the cytoplasm to the plasma membrane. There it forms a polygonal lattice encaging the cargo to be internalized into a clathrin-coated pit, which can then bud and pinch off into clathrin-coated vesicles. Activation protein-2 (AP-2) initiates internalization by binding to clathrin and to the cargo, on tyrosine or di-leucine motifs. Dynamin then positions itself around the invaginating membranes and promotes fission of the clathrin coated pits. After internalization, receptors are either recycled back to the cell surface or targeted for degradation in the lysosome [13, 34].

Clathrin-mediated endocytosis is thought to be mediated through TβRIII interaction, as it can directly bind β-arrestin2 [35]. β-arrestins are key mediators of GPCR internalization and desensitization, since they recruit clathrin and AP-2 to the activated receptor complex.

TGFβ receptor internalization may be a necessary process required for the activation of the Smad substrates. Smad anchor for receptor activation (SARA) localizes Smad2 to the type I receptor to be phosphorylated. Interestingly, SARA contains an FYVE domain [9], which binds to the phosphatidyl inositol-3'-phosphate of early endosomes [18].

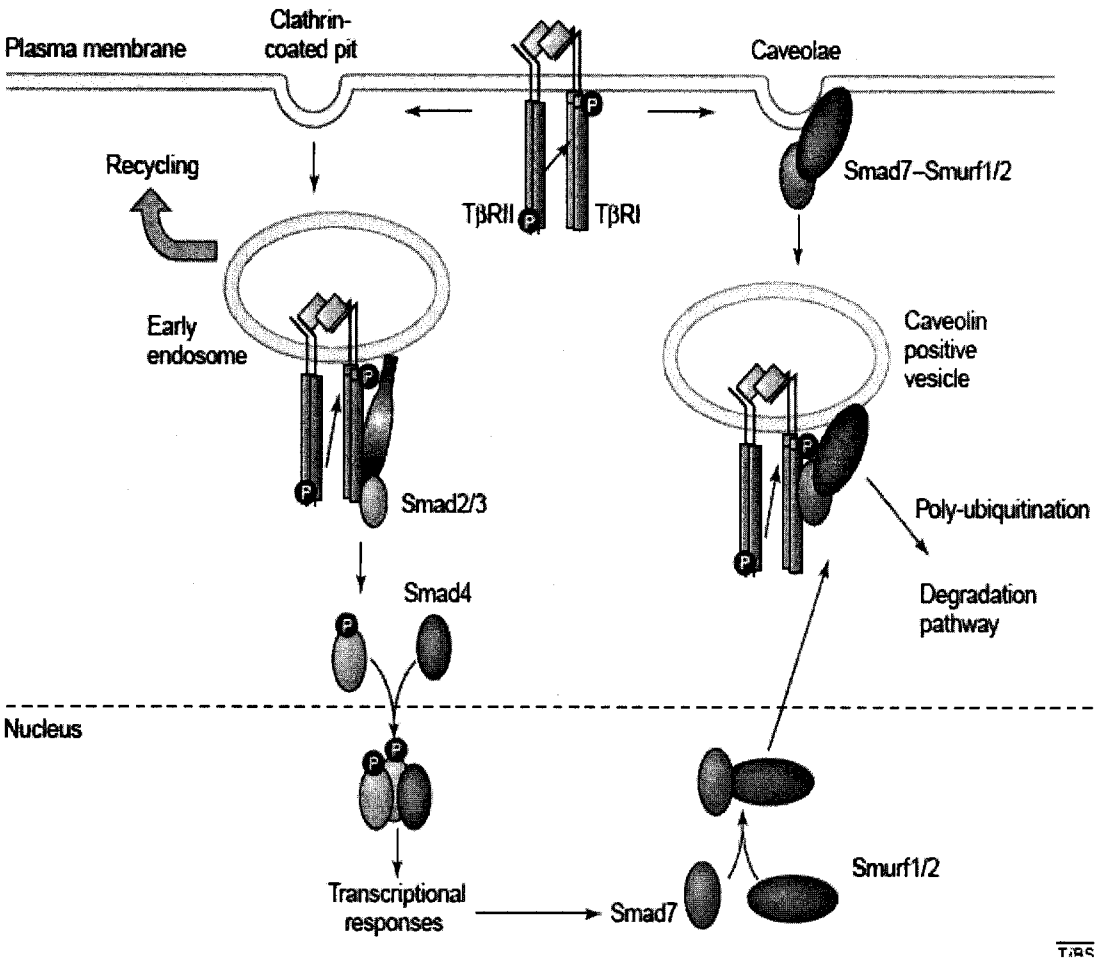

Fig.2: Clathrin-mediated internalization vs. caveolae-mediated internalization

Illustrated, is a model of two distinct internalization routes for TGFβ receptors that predetermines whether receptors will induce a signaling response or be downregulated. Clathrin-dependent internalization into early endosomes promotes TGFβ receptor signaling, whereas caveolae-mediated internalization is required for the degradation of TGFβ receptors (ten Dijke and Hill 2004)

Caveolae-mediated Internalization: Less understood is the mechanism of clathrin independent internalization. The cholesterol depletion sensitivity of clathrin independent internalization routes first led to the notion of lipid-dependent internalization [13]. Lipid rafts are microdomains rich in many kinds of lipids, such as cholesterol, glycolipids, sphingolipids, and signaling molecules, present in the cell membrane. Although little is known about the machinery involved, it is believed that caveolin-1, a palmitoylated protein that binds fatty acids, is important in the formation of caveolae [34]. This route is thought to lead to receptor interaction with ubiquitin ligase (E3), Smad ubiquitination regulatory factor 2 (Smurf2), that targets the receptor for inactivation [18].

1.2.3 The Smads

The word Smad is derived from the founding members of this protein family: the *Caenorhabditis elegans* protein, Small body size (Sma) and the *Drosophila* protein, Mothers Against Decapentaplegic (Mad) [9, 36].

The Smad family of cytoplasmic transcription factors are the primary mediators of the TGFβ signaling pathway. Through a series of interactions beginning at the TβRI and ending in the nucleus,where they bind DNA, the Smads convert, regulate and integrate extracellular signals transduced by the receptors and control target gene expression.

1.2.3.1 Classes of Smads

To date, eight different members of the Smad family (Smad1-8) of signal transduction molecules have been identified in mammals. Based on structure and function, the Smads have been classified into three groups (Fig.3): (i) Receptor-regulated Smads (R-Smads), (ii) Common partner Smad (Co-Smad), and (iii) Inhibitory Smads (I-Smads) [9, 18, 37].

R-Smads: Composed of Smad1, 2, 3, 5 and 8, R-Smads are the only Smads that are directly phosphorylated and activated by the type I receptor kinase. R-Smads are predominantly present as cytoplasmic monomers [11]. Receptor-induced phosphorylation occurs on the two serines of a conserved C-terminal SSXS motif, which releases the Smads from cytoplasmic anchors [9], promoting association and heterocomplex formation with Smad4. The heterocomplex then translocates to the nucleus, where it can directly bind DNA or where it associates with co-activators and/or co-repressors to regulate target gene expression. Smad2 and 3 transmit TGFβ/activin signals, whereas Smad 1, 5 and 8 are activated by the BMP subfamily.

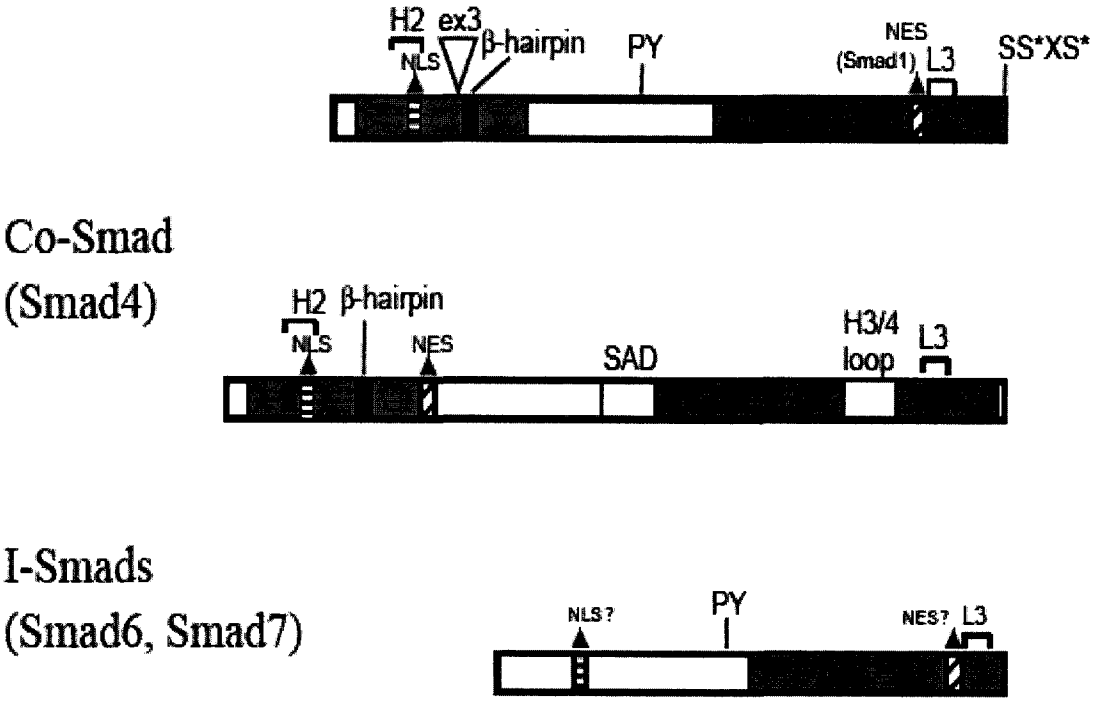

Fig.3: Classes and structural features of Smads

Diagrammatic representation of the three Smad subfamilies. The protein diagrams are arbitrarily aligned relative to their C-termini. The MH1 domain is colored in blue and the MH2 domain in green. Selected domains and sequence motifs are indicated as follows: α-helix H2, L3 and H3/4 loops, β-hairpin, the unique exon 3 of Smad2 (ex3), NLS and NES motifs or putative (?) such motifs, the proline-tyrosine (PY) motif of the linker that is recognised by the Hect domain of Smurfs, the unique SAD domain of Smad4 and the SSXS motif of R-Smads, with asterisks indicating the phosphorylated serine residues (Moustakas, Souchelnytskyi *et al.* 2001)

Co-Smad: Co-Smad, Smad4, (also known as DPC4, deleted in pancreatic carcinoma locus 4) functions as a shared partner of the R-Smads, forming a heterotrimeric complex which then translocates to the nucleus. Although the overall structure of Smad4 is similar to that of R-Smads, Smad4 contains an insertion within its Mad Homology 2 (MH2) domain and is normally not phosphorylated in response to agonists [5], as it lacks the necessary C-terminal residues targeted by the type I receptor kinase. Although Smad4 is required for Smad2- and 3-dependent growth inhibitory responses in mammalian cells, some Smad4-independent TGFβ responses have been described. Certain Smad4-deficient cell lines display a limited responsiveness to TGFβ, such as induction of fibronectin expression and TGFβ-induced cell cycle arrest. However, these responses may be mediated, in part, by TGFβ-induced activation of c-Jun N-terminal Kinase (JNK) and ERK1/2 MAPK pathways. As a result, activation of alternative pathways downstream of the TGFβ receptors may occur, bypassing the Smad-dependent pathways [13].

I-Smads: Primarily located in the nucleus [38], Smad6 and 7 are structurally divergent members of the Smad family and are believed to act in an autocrine negative feedback loop to control the intensity and duration of TGFβ signaling responses [11]. By interfering with phosphorylation of the R-Smads, Smad6 preferentially inhibits BMP signaling, whereas Smad7 can inhibit both TGFβ/activin and BMP signaling. These Smad proteins contain a characteristic MH2 domain, but their N-terminal domains share little homology with the typical MH1 domain. Like Smad4, I-Smads lack a C-terminal SSXS motif. It has been suggested that this may, in fact, stabilize I-Smad interaction with the type I receptor, thus enabling them to interfere with R-Smad activation. Smad7 has also been found to recruit E3 ubiquitin ligases, Smurf1 and 2, to the activated type I receptor, resulting in receptor ubiquitination and degradation and termination of signaling [36, 39, 40].

1.2.3.2 Structural Features of Smads

Smad proteins are conserved across species, with homology mainly in the N-terminal Mad Homology 1 (MH1) and C-terminal Mad Homology 2 (MH2) domains ('Mad homology' in reference to the first identified family member, the *Drosophila* Mad gene product) [9, 11, 36, 41]. The MH1 and MH2 domains form globular structures separated by a less well conserved proline-rich linker region of variable length.

MH1 domain: Located in the N-terminal region of the Smad, the MH1 domain is ~130 aa and is highly conserved between the R-Smads and Smad4. In the basal state, the MH1 domain interacts with its own MH2 domain, inhibiting transcriptional and biological

activities. Upon phosphorylation and subsequent activation, the R-Smads have the ability to translocate to the nucleus. However, they require association with Smad4 for nuclear accumulation. Nuclear import is mediated by a nuclear localization signal (NLS) or by a lysine-rich nuclear localization-like sequence located in the MH1 domain. These regions allow interaction with importin-β, which is responsible for mediating the transport of cargo across the nucleopore complex [11, 18], allowing nuclear accumulation to occur.

In the nucleus, the MH1 domain enables sequence-specific binding of the Smads to DNA, with the exception of Smad2, which contains a β-hairpin insert adjacent to the DNA-binding element of the MH1 domain, thereby preventing direct contact with DNA [9, 11, 18]. Similarly, I-Smads express only short segments of MH1 homology in their N-terminal region and are thus unable to bind DNA.

Linker: The linker region is a poorly conserved proline-rich region of variable size and length that links together the MH1 and MH2 domains of the Smads. It contributes to the formation of Smad homo-oligomers and is also an important site of TGFβ signaling regulation, as it contains recognition sites for diverse kinases, particularly MAPKs [5, 9]. Phosphorylation of the MAPK residues inhibit nuclear translocation of the Smads. Recently, another phosphorylation site was discovered within the Smad2/3 linker region. The GRK2 was shown to physically interact with and phosphorylate R-Smad2 and 3 at a single serine/threonine residue [42]. This GRK2-induced Smad phosphorylation blocks TGFβ-induced Smad activation and subsequent nuclear translocation, consequently inhibiting the biological effects of TGFβ. These phosphorylation sites are suspected of mediating crosstalk between Smad proteins and their environment, serving as potential integration sites for other regulatory signaling pathways.

Also contained within the R-Smad and I-Smad linker region is a PY motif, which acts as a recognition site for Smurf1 [9, 18, 38]. The PY motif determines Smad stability by mediating the interaction with ubiquitin ligases that target Smads for degradation.

In Smad4, the linker region contains a nuclear export signal (NES) [18].

MH2 domain: The C-terminal MH2 domain is 200 aa long and is highly conserved in all Smad isoforms. This region is indispensable for Smad homodimerization and for association with Smad4, DNA-binding factors and transcriptional co-activators and/or co-repressors. Most importantly, this region contains the SSXS motif. For activation to occur, the MH2 domain of the R-Smads must transiently associate with and be phosphorylated by the type I receptor kinase on this C-terminal motif. Phosphorylation of the R-Smads

induces a conformational change in their structure, relieving the inhibitory contact between the MH1 and MH2 regions, and allowing for hetero-oligomerization with Smad4 [13]. Mediating the interaction between the R-Smads and Smad4 is the L3 loop. The L3 loop is a short conserved 17 aa sequence that interacts with a specific region of the type I receptor kinase, known as the L45 loop. The L3/L45 loop interaction (Fig.4) defines the specific downstream signaling that occurs after ligand binding. Interestingly, the L3 loop sequence differs only by two amino acids between the Smad1, 5, 8 BMP subgroup and the Smad2, 3 TGFβ/activin subgroups. This difference in surface structure is sufficient for Smad discrimination by the receptor [9]. In addition to providing a platform for receptor interaction, the L3 loop also allows for Smad trimerization to occur through associations of the L3 loop and the phosphorylated C-terminal residues of the trimeric partner [13].

Unlike the R-Smads, Smad4 lacks the C-terminal SSXS motif, and thus is not a substrate for the type I receptor kinase. Similarly, I-Smads also lack the C-terminal SSXS motif. This motif is believed to stabilize I-Smad interaction with the type I receptor, creating competition for R-Smad activation.

1.2.3.3 Signaling Through Smads

The most compelling evidence that Smads function downstream of TGFβ receptors came from the observation that in response to TGFβ and related agonists, Smads are phosphorylated, accumulate in the nucleus and become transcriptionally active [5] (Fig.5). However, the ability of Smad proteins to accumulate in the nucleus in response to agonist stimulus was also one of the key observations placing Smads downstream of the TGFβ receptors [18]. Originally, in the absence of ligand, Smads were thought to be static cytoplasmic proteins. Now, it is widely accepted that Smad proteins possess nucleocytoplasmic shuttling abilities. Their nuclear accumulation results from receptor-mediated phosphorylation events that decrease the affinity of R-Smads for cytoplasmic anchors and increase their affinity for nuclear factors [18]. Dephosphorylation of the R-Smads allows them to return to the cytoplasm, where they can repeat the process.

Smad Nucleocytoplasmic Dynamics: Traditionally, nuclear translocation of cytoplasmic proteins in response to regulatory signals was controlled by the interaction of importins with an NLS in the target cargo proteins. Cargo-bound importin-α binds importin-β, which directly interacts with the nucleoporins, allowing passage of the importin-β-importin-α-cargo complex into the nucleus. However, Smad2, 3, and 4 appear to interact directly with the nucleoporins through a hydrophobic corridor located within the

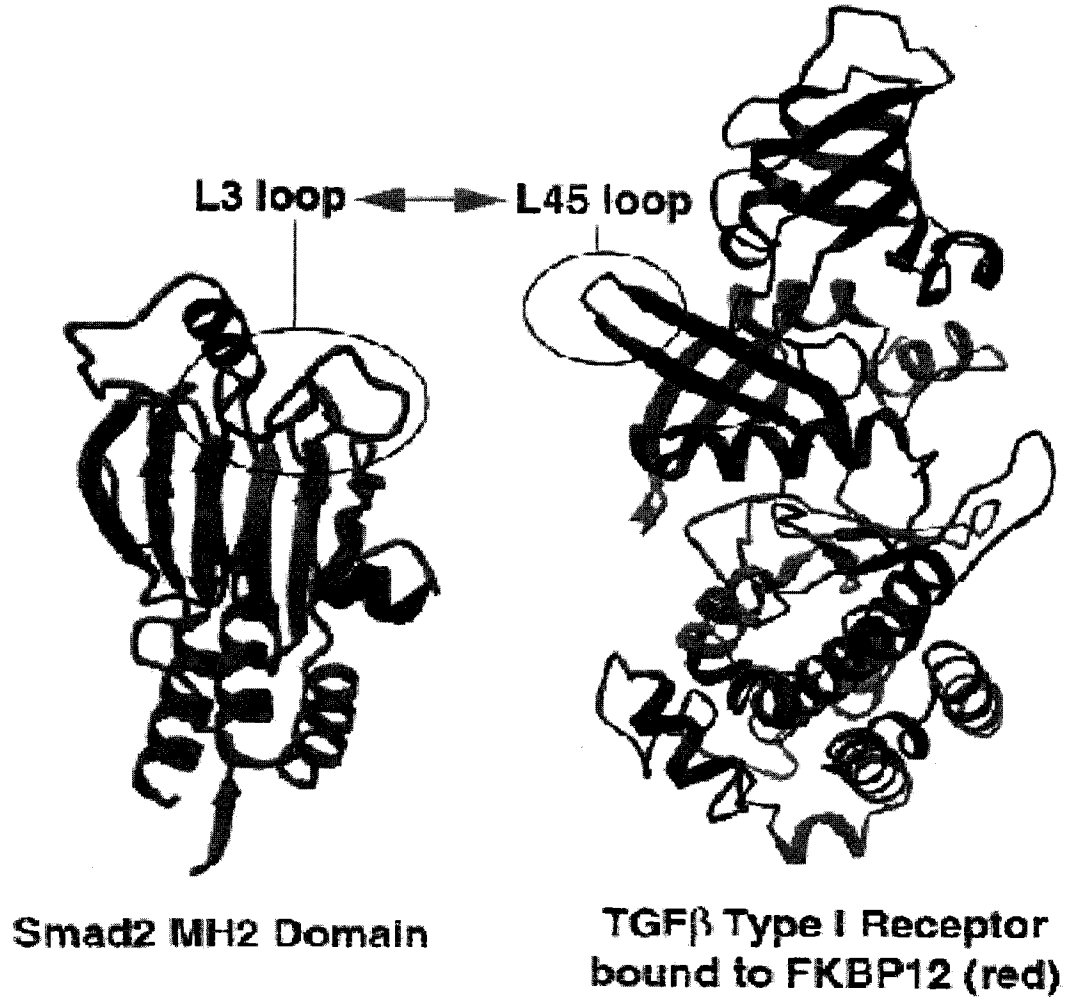

Fig.4: TGFβ receptor regulation and Smad interaction

In the basal state, the type I receptor is maintained inactive by the GS domain (green), which presses against and dislocates the catalytic center of the kinase domain (blue). The immunophilin FKBP12 (red) binds to the GS domain, occluding its phosphorylation sites. Phosphorylation of the GS domain by the type II receptor in the ligand-induced complex is predicted to remove the inhibitory constraint. The specificity of receptor-Smad recognition is dictated by the L45 loop region on the receptor and the L3 loop region on the MH2 domain of Smad (Massague and Chen 2000)

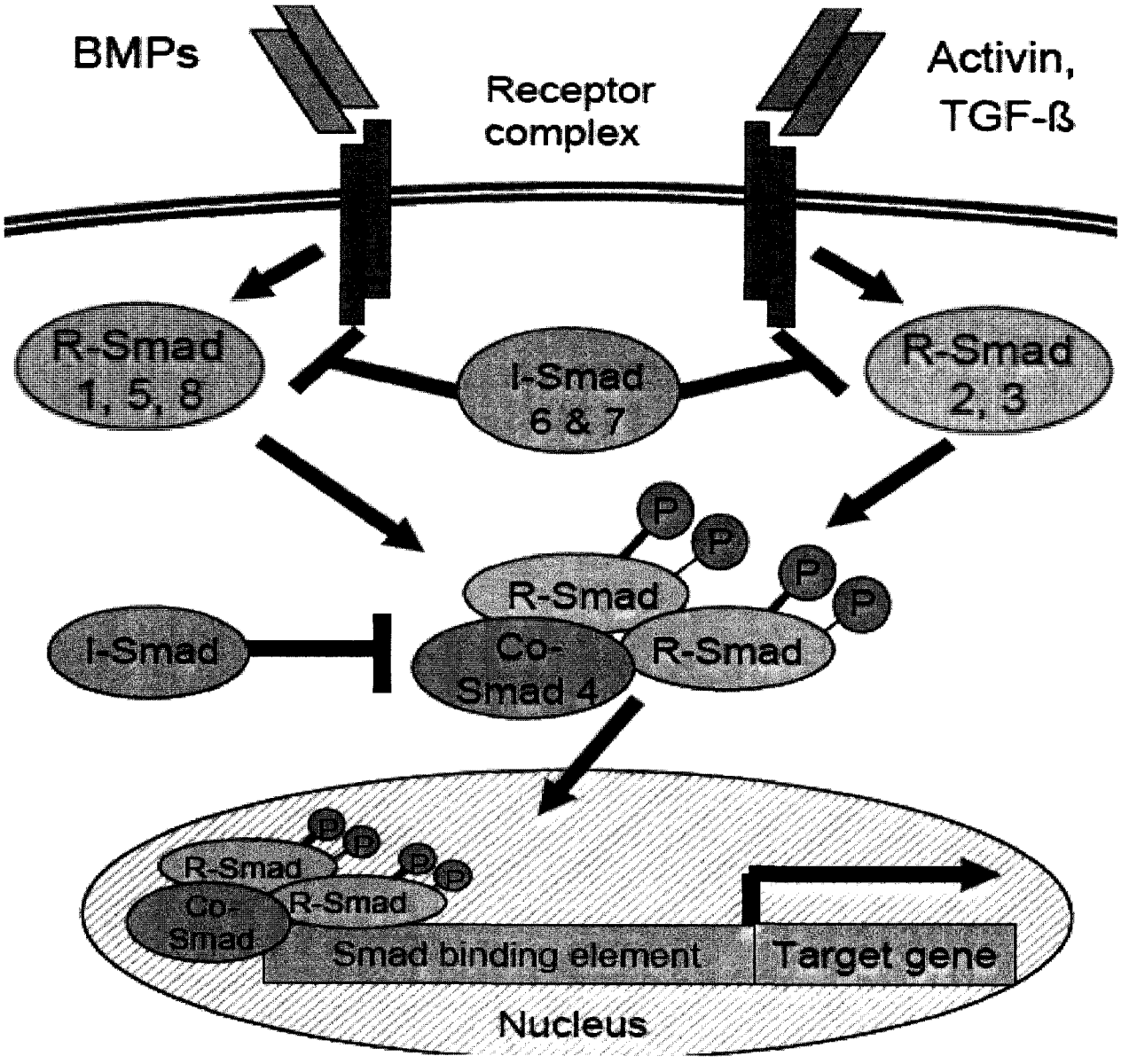

Fig.5: TGFβ superfamily signaling through Smads

Ligands of the TGFβ superfamily bind to and activate cell surface receptors. Smad2 and Smad3 (activin/TGFβ) or Smad1, Smad5 and Smad8 (BMPs) are phosphorylated by activated receptors, form a heterocomplex with Smad4 and translocate to the nucleus where they regulate gene transcription. Inhibitory Smads, Smad6 and 7, antagonize TGFβ superfamily signaling (Itman, Mendis *et al.* 2006)

Smad MH2 domain, and thus can translocate into the nucleus independently of the importin complex. Other studies have described a different kind of importin interaction between Smad3 and 4, but not Smad2, and a lysine-rich sequence within the MH1 domain resembling an NLS. The exon 3 encoded insert in the MH1 domain is believed to interfere with the interaction between importin and Smad2. Thus, it appears that Smad2, 3, and 4 undergo nuclear import by means of direct interaction with nucleoporins. However, this process may be aided by importin-β, in the case of Smad3 and 4 [18].

Smad4 is widely distributed in the cell and undergoes continuous nucleocytoplasmic shuffling in the basal state. It is believed that R-Smad phosphorylation creates a binding site for Smad4, resulting in its recruitment to the activated complex followed by nuclear translocation. Although Smad4 contains a nuclear export signal (NES), it is believed that heterocomplex formation masks this sequence, permitting nuclear accumulation.

Smad Subcellular Retention Mechanisms: Basal state R-Smads are concentrated in the cytoplasm [5, 18] despite their inherent ability to translocate to the nucleus. This phenomenon is believed to be due to interaction with cytoplasmic retention factors.

The best described retention factor for Smad2 and 3 is the membrane-associated SARA protein. SARA-Smad interaction is mediated by an 80 aa Smad binding domain (SBD) and an FYVE phospholipid-binding domain. Through a hydrophobic corridor on the MH2 domain, the SBD of SARA binds to R-Smads, preventing their translocation to the nucleus and, instead, localizing them at the plasma membrane. Receptor-mediated C-terminal phosphorylation of the R-Smads results in decreased affinity for SARA and subsequent release of the R-Smads, which leads to unmasking of the NLS and to nuclear translocation.

Smad Adaptors for Receptor Interactions: Several proteins facilitating the interaction of R-Smads with the receptor complexes have been described. The best characterized has been SARA, which localizes Smads2/3 to the plasma membrane, where they can more easily interact with the type I receptor. Another FYVE domain protein, Hgs, has been found to cooperate with SARA in Smad phosphorylation [18].

More recently, a cytoplasmic isoform of the promyelocytic leukemia protein (cPML) has been identified as a critical SARA-, Smad2/3- and TGFβ receptor-interacting protein. However, unlike embryonic lethal Smad2 or TGFβ receptor knockout models, PML-deficient mice develop normally, suggesting that its association may not be critical.

TGFβ-receptor-associated protein-1 (TRAP-1) and TRAP-1-like protein have been identified as adaptor proteins permitting formation of Smad2/3-Smad4 complexes.

Cytoskeletal proteins are often overlooked for their role in signal transduction. Diverse extracellular signals are coupled at the plasma membrane to intracellular signal transduction pathways and to the cytoskeleton [43]. As such, Smads are shuffled along cytoskeletal networks and scaffolding proteins. Microtubules bind unphosphorylated Smads2/3 and the interaction is dissociated upon ligand stimulation. Chemically-induced disruption of the microtubule network results in enhanced Smad2 phosphorylation [44]. Filamin, an actin crosslinking factor and scaffolding protein, also associates with Smads, positively regulating Smad signal transduction. Interestingly, cells defective in filamin expression display impaired TGFβ signaling and Smad2 phosphorylation [36, 45].

Smads as Receptor Substrates: The TGFβ ligand binds to the constitutively phosphorylated serine/threonine TβRII and recruits the TβRI, forming a heterotetrameric receptor complex. The TβRII kinase domain transphosphorylates the TβRI GS domain, thereby inducing a conformational change in TβRI, which leads to the activation of its kinase domain. Recognition of the unphosphorylated Smads is mediated by the receptor L45 loop and by the L3 loop and α-helix regions located in the R-Smad MH2 domain [9].

Activation of the type I receptor kinase leads to the phosphorylation of the R-Smads on a C-terminal SSXS motif. Although the kinetics of this phosphorylation are relatively slow ($t_{1/2}$ ~5min) when transfected Smads are used, evidence shows that Smads are the direct substrates of the receptors [5]. In contrast to the R-Smads, C-terminal phosphorylation does not occur in Smad4 or in the I-Smads, as they lack the necessary motif. Mutational analysis shows that phosphorylation of this motif is required for Smad activation [5].

Smad Transcriptional Complexes: The ability of Smads to activate transcription was first observed using Gal4-Smad fusion contructs [5]. The MH2 domain was fused to a Gal4-DNA binding domain (GBD), and demonstrated that transcriptional activity of the GBD-Smad fusion protein could be induced by the coexpression of Smad4 [18].

Smad Heterocomplexes: Following R-Smad phosphorylation by TβRI, biochemical and structural evidence suggest that their phosphorylated C-terminal tail specifically interacts with the L4 loop of another Smad, causing oligomerization to occur [36].

Smad proteins exist primarily as monomers, only forming oligomers upon phosphorylation. The stoichiometry of oligomerization has been the subject of much debate [18]. Substitutional analysis, whereby the Smad C-terminal serines were replaced by acidic residues, suggested that the Smads form heterotrimers of two phospho-R-Smad and of one Smad4 molecule [18]. Similarly, heterocomplex formation was suggested to

occur first by the formation of an R-Smad homo-oligomer, which quickly would convert to a hetero-oligomer containing the Co-Smad, Smad4 [36]. Oligomerization is assisted by extensive contacts between the loop-helix region of one subunit and the three-helix bundle of another, areas containing many evolutionarily conserved residues [36]. Inactive cytoplasmic Smads are intrinsically auto-inhibited by an intramolecular interaction between the MH1 and MH2 domain [46]. Smad4 also contains a unique loop in its MH2 domain that prevents spontaneous oligomerization in the absence of signaling [46].

The formation of heterodimers and of heterotrimers is believed to involve interactions with other transcription factors and target genes. For example, the Mix2 promoter might be targeted by Smad2/2/4 complexes bound to forkhead activin signal transducer-1 (Fast-1) or -3, whereas the JunB promoter may be targeted by a Smad3/4 heterodimer [13, 18].

Whether Smad4 is required in Smad transcriptional complexes remains an issue of much debate. To date, all endogenous Smad complexes described have been shown to contain Smad4 and all target genes characterized by chromatin immunoprecipitation show Smad4 binding with the R-Smads [18, 47]. Smad4-dificient tumor cells and fibroblasts from Smad4-deficient mice still display some TGFβ gene responses [18]. Certain pancreatic carcinoma cells lacking Smad4 contain high levels of phosphorylated R-Smads and respond to TGFβ signaling with increased motility [18]. However, TGFβ receptors could signal some of these responses in a Smad-independent manner via MAPK, PI3K, protein phosphatase 2A or Rho family members [18, 47, 48].

Smad DNA Binding: Once in the nucleus, the Smad heterocomplex is able to bind DNA with low affinity and specificity. However, the Smads rely on interactions with various DNA binding partners to regulate gene transcription in a cell-specific manner [13]. Despite the many TGFβ-responsive targets in a given cell, only a select few are activated by the given R-Smad/Smad4/DNA binding partner combination. These Smad binding partners allow for specificity of target gene, pathway, cell type and of specific transcriptional effects in response to TGFβ signaling [18, 47].

A vast array of DNA-binding transcription factors have been identified as Smad-interacting, providing a basis for the breadth of TGFβ transcriptional responses. The first identified Smad-interacting transcription factor was the forkhead family member, forkhead box H1 (FoxH1), also known as Fast-1 [11, 18, 49]. FoxH1 cooperatively binds the activin-response element on the Mix2 promoter region. Two separate sequences on FoxH1, a proline-rich SIM domain and a FoxH1-specific FM motif, interact with the MH2 domain

of Smad2 and 3. The SIM motif is also found in the Mix family of homeodomain transcription factors, Mixer and Milk, which partner with Smad in the regulation of *Xenopus goosecoid* [18]. Other DNA-binding partners include *Vent2,* three members of the RUNX family (RUNX1-3), Activation Protein-1 (AP-1), E2F family members and Sp1.

Found within many Smad-responsive gene promoters is the specific DNA sequence 5'-CAGAC-3', known as the Smad-binding element (SBE) [11, 50]. SBE is recognized by the β-hairpin domain located within the MH1 domain of the R-Smads. Because the affinity of Smad binding is too low to support binding of the Smad heterocomplex on a single SBE, only promoters containing multiple repeats are able to promote transcription. One such candidate is Smad7, which contains two palindromic SBEs. Nevertheless, it requires AP-1 or Sp1 cooperation with the Smad complex for full activation. Similarly, the $p21^{Cip1}$ promoter contains up to four consecutive SBEs in the TGFβ responsive region, but still requires the participation of FoxO as a Smad partner [18].

A subset of promoters has been found to be capable of binding GC-rich regions, in addition to SBEs, in order to promote ligand-induced transcription. Thus, high affinity Smad-DNA binding can be achieved through the binding of different transcription factors to their specific DNA sequences in the vicinity of one or more SBEs or GC-rich regions, enabling selective ligand-induced target gene expression [11, 47].

Smad-Dependent Transcriptional Activation and Repression: Smads positively or negatively regulate target gene expression in a cell-specific manner. In addition to interacting with DNA-binding transcription factors, the Smads also bind co-activators and/or co-repressors to help regulate transcription. The functional role of these cofactors resides in their ability to remodel chromatin. For instance, the CBP/p300 co-activator complex acetylates histones, causing chromatin relaxation and transcription to occur. Conversely, Smad transcriptional complexes contain histone deacetylases (HDACs), which act on either the specific target gene or the chromatin-associated protein [47].

Smads can also regulate transcription by competitively binding promoters, co-activators and/or co-repressors, displacing them from the target gene promoter region. For example, Smad3 binds the myogenic differentiation transcription factor, myoD. By binding to myoD, Smad3 prevents its binding to E-box responsive elements and induction of genes critical for myogenic differentiation [13].

Further regulation of Smad transcriptional complexes involves the binding of transcriptional co-repressors that interfere with the ability of the Smads to associate with

co-activators. The proto-oncogenes Ski and SnoN were originally isolated as Smad4 interacting proteins, and were also shown to bind Smad2 and 3 [18]. Ski competes with Smads2 and 3 for binding of the MH2 region on Smad4, preventing the formation of an active Smad complex. A similar model is proposed for SnoN.

Ski can also mediate repression of TGFβ signaling by modulating transcription of target genes via recruitment of nuclear transcriptional co-repressors and HDACs, in addition to interfering with Smad-mediated binding to the transcriptional co-activator, p300/CBP [51]. Despite being expressed at relatively low levels, Ski and SnoN can undergo Smad-targeted ubiquitination and degradation [18]. Finally, SnoN is upregulated by TGFβ and, thus acts in a negative feedback loop to control TGFβ signaling.

1.2.3.4 Regulation of Smad Signaling

Ligand binding leads to TGFβ receptor activation, which can remain active for 3-4 hrs. Continuous receptor activation is required to maintain nuclear localization of activated Smads [48]. Consequently, precise regulation of the Smad signaling molecules is required to modulate duration and intensity of the signal. In the presence of stimulus, Smads undergo phosphorylation, followed by nuclear shuttling and dephosphorylation, after which they return to the cytoplasm where they can repeat the process. Several forms of Smad modification have been described to modify the TGFβ signaling cascade.

Dephosphorylation of the Smads: When the Smads were discovered as substrates for the type I receptor kinase, it was conceivable to assume a role for a potential phosphatase. Evidence for the existence of R-Smad regulation by a phosphatase was first observed when TGFβ receptor kinase activity was blocked, causing a rapid decrease in the amount of phospho-Smad2/3, followed by a return of Smad2/3 to the cytoplasm [52]. Recently, a genomic approach was taken to screen the catalytic subunits of 39 phosphatases in order to identify a Smad2/3-interacting phosphatase. This approach led to the identification of a member of the metal ion-dependent phosphatases, PPM1A/PP2Cα, as the sole Smad phosphatase responsible for terminating TGFβ/activin signaling [13]. PPM1A was shown to regulate phosphorylation, oligomerization and nuclear export of both Smad2 and 3, and when depleted using short hairpin RNA (shRNA), resulted in enhanced antiproliferative and transcriptional responses to TGFβ [13]. It is possible that these two mechanisms operate to different extents on different pools of phosphorylated Smad complexes that mediate acute or prolonged responses [18].

Regulation of Smads by Ubiquitination and Acetylation: Also contributing to the decline of phosphorylated R-Smads, following TGFβ stimulation, is the slow-acting process of ubiquitin-dependent proteasome-mediated degradation [18, 22].

***Ubiquitination*:** Ubiquitination is a three-step process involving the conjugation of a ubiquitin moiety to the lysine side chains of the target protein. First, the inactive ubiquitin precursor is activated by the ubiquitin-activating enzyme (E1) and transferred to a reactive cysteine residue of the ubiquitin-conjugating enzyme (E2). E2 then transfers the activated ubiquitin to the substrate either directly or via E3. Ubiquitin-dependent degradation of activated mediators may ensure a swift elimination of their signals or it may selectively remove the surplus of activated Smad from the nucleus by targeting Smads that are not bound to target promoters or to other partners [22].

Smurfs: Smad ubiquitination regulatory factor-1 is a member of the homologous to E6AP C-Terminal (HECT) subclass of E3 ubiquiting ligases that target substrates for classical degradation via the 26S proteasome [11, 22]. The Smurf family members contain a HECT domain, an N-terminal C2-phospholipid, a calcium (Ca^{2+}) binding domain and 2-4 WW protein interaction domains, through which they interact with the PY motif contained within the R-Smad linker region [18]. Smad4 does not contain a PY motif and thus can only be targeted for ubiquitination when associated with R-Smads. In addition to Smads, Smurfs target TGFβ receptors, transcriptional co-repressors and I-Smads for degradation. Smurf-1 targets BMP Smads, whereas Smurf-2 targets both BMP and TGFβ Smads.

Itch: Interestingly, despite polyubiquitination by E3, Itch promotes TGFβ signaling by forming a better interaction between Smad2 and the TGFβ receptor complex [18]. How Itch polyubiquitination differs from Smad2 polyubiquitination by Smurf-2 is unclear.

***Acetylation*:** It is believed that substrate susceptibility to ubiquitination may be controlled by acetylation of the same lysine residues [18]. Acetylation increases the level of transcriptional activity by loosening highly compacted chromatin.

Smurfs: Smurf-mediated ubiquitination of the I-Smads involves a competition for the same lysine residues, with acetylation. Nuclear Smad7 can be acetylated at two N-terminal lysines, preventing receptor-induced ubiquitination of Smad7 by Smurf [18].

SnoN: SnoN, a member of the Ski family of proto-oncogenes, acts as a negative regulator of Smad transcription through the recruitment of co-repressors and HDACs. During TGFβ signaling, Smad2 interacts with both Smurf2 and SnoN, enabling Smurf2 to

target SnoN for ubiquitination and proteasomal degradation, thereby relieving TGFβ target genes from co-repressor restraints and allowing ligand-induced expression [13].

Regulation of Smads by Sumoylation: Small ubiquitin-like modifer (SUMO) controls protein targeting within the cell, leading to decreased transcriptional activity [18].

Smad4 hosts two sumoylation consensus motifs, one in the MH1 domain and the other in the linker region. The sumoylation motif, ψKxE (where ψ is hydrophobic, x is variable and K is the lysine to be modified) is targeted by the protein inhibitor of activated STAT family of SUMO E3 ligases and displays two opposing transcriptional effects [18].

Regulation of Smad Signaling by Non-TGFβ Kinases: The highly variable R-Smad linker region is the primary target of regulatory phosphorylation by a large number of non-TGFβ kinases. This region contains multiple serine/threonine consensus sites for ERK/MAPK and for proline-directed kinases [47].

ERKs/MAPKs: Activated in response to mitogenic growth factors, these kinases phosphorylate the linker region of Smad1, 2 and 3. Some studies suggest that ERK1/2 phosphorylation of these R-Smads inhibits ligand-induced nuclear accumulation and thus blocks TGFβ antiproliferative responses [53].

CDKs: The cyclin dependent kinases (CDKs) 2 and 4 are responsible for cell cycle progression from G_1 to S phase. They can phosphorylate Smad3 at a site within the MH1 domain (T8) and at two sites within the linker region (T178 and S212), one of which is shared with ERK (T178) [54]. Inhibition of TGFβ-induced transcriptional activity and antipoliferative function are inhibited by CDK-mediated phosphorylation of Smad3 [54].

CaMKII: The Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII), targets the SSXS motif, where X is preferentially a hydrophobic residue. Despite having five such motifs, only S240 within the Smad2 linker region is reportedly phosphorylated by CaMKII. Phosphorylation blocks Smad2 nuclear accumulation, induces heterodimerization of a signaling-incompetent Smad2/4 complex independent of type I receptor kinase C-terminal phosphorylation, and prevents TGFβ-dependent Smad2/3 heterodimerization, leading to an overall inactivation of TGFβ signaling [13, 55]. CaMKII is activated in response to Ca^{2+} mobilization in response to RTK signaling.

JNK: In response to mitogenic and stress signals, JNK phosphorylates Smad3 at a region distinct from its C-terminal activation site, causing enhanced activation and nuclear accumulation [56]. MAPK1, an upstream activator of ERK and JNK, can phosphorylate Smad2, enhancing Smad2/4 heterodimerization as well as nuclear translocation [13].

PKC: Protein kinase C (PKC)-mediated phosphorylation has been observed within the MH1 domain of Smad3, a site which may serve as a point of integration for various signaling pathways. Phosphorylation of this site reportedly prevents DNA binding [57]. PKC is typically activated downstream of various RTKs, GPCRs and ion channels.

Akt: Protein kinase B/Akt acts as junction between the insulin and the TGFβ signaling pathways to regulate sensitivity to TGFβ-induced apoptosis through a kinase-independent method [58]. Akt associates with Smad3, sequestering it in the cytoplasm, thus preventing its C-terminal phosphorylation, heterocomplex formation with Smad4 and nuclear translocation. This association is promoted by insulin and inhibited by TGFβ.

GRK2: Recently, GRK2 was shown to physically interact with and phosphorylate a single serine/threonine residue (T197 in Smad2 and S157 in Smad3) within the R-Smad linker region [42]. Phosphorylation of the linker blocks TGFβ-induced Smad activation and subsequent nuclear translocation, thereby inhibiting the biological effects of TGFβ.

1.2.4 TGFβ Signaling in Disease

The TGFβ superfamily of growth factors is responsible for regulating a plethora of cellular processes. Both TGFβ and its receptors are widely expressed in various cell types [5], as TGFβ has a crucial role in tissue homeostasis. Disruption of this critical pathway can have severe physiological consequences and has been implicated in many human diseases, including cancer, autoimmune, fibrotic and cardiovascular disease [3]. Loss of TGFβ signaling often results in hyperproliferative disorders and has been linked to cancer development and to inflammatory and autoimmune disease. However, supersensitivity to TGFβ signaling has been implicated in immunosuppression and tumor metastasis [6, 7].

1.2.4.1 TGFβ Knockout Mouse Models

As the *in vitro* information we collect on the intricately complex mediators and pathways involved in the TGFβ signaling network becomes more abundant, it is becoming increasingly important to verify these effects *in vivo*. Consequently, knockout mouse models as well as transgenic mice have been developed in order to characterize the role of the primary signaling component of the TGFβ signaling cascade.

TGFβ: In mice, TGFβ loss-of-function studies have revealed its critical role during embryonic development and in maintaining tissue homeostasis during adult life [11]. Over 50% of TGFβ1 knockout ($TGF\beta1^{-/-}$) mice die during embryogenesis, whereas the survivors develop severe inflammatory disorders and typically die within 1 month. $TGF\beta2^{-/-}$ mice,

particularly females, develop various craniofacial and skeletal deformities, in addition to retinal hyperplasia and heart and renal defects. TGFβ3$^{-/-}$ mice often exhibit cleft palates and delayed lung development. As such, both TGFβ2$^{-/-}$ and 3$^{-/-}$ mice die perinatally [59].

TGFβ Receptors: The type I and type II receptor knockout mice are embryonic lethal, both displaying defects in vascular development. Moreover, mice lacking the type I receptor exhibit an absence of circulating red blood cells.

Smads: Gene disruption in mice has revealed the many specific developmental and physiological functions of Smads. Mice deficient in either Smad2 or 4 are embryonic lethal, where deletion of Smad2 results in failure of egg cylinder elongation, mesoderm formation and gastrulation, and deletion of Smad4 demonstrates retarded growth, no mesoderm formation and no gastrulation [11]. Gastrulation was rescued when a mutant embryo was surrounded by wild-type embryonic tissue, suggesting that Smad2 and 4 first function in the extraembryonic tissue, signaling to the embryo proper for development to proceed. However, substantial developmental defects were observed later in development. Heterozygous Smad2 animals showed severe gastrulation defects in 20% of the embryos, later lacking mandibles or eyes.

Smad3$^{-/-}$ mice survive but exhibit impaired immunity, chronic infection, and are prone to developing metastatic colorectal cancer, often dying within 1 to 8 months. Although Smad2 and 3 have similar functions with respect to signaling, the viability of Smad3$^{-/-}$ *vs.* the lethality of Smad2$^{-/-}$, suggests that Smad2 may play a more vital role in development.

Smad5$^{-/-}$ mice die between 10.5-11.5 days of embryogenesis due to circulatory system defects, including enlarged vessels and low numbers of smooth muscle cells (SMC).

A knockout model for Smad6, but not Smad7, has been reported [11]. Most mice lacking Smad6 survive, but show severe defects in the formation of cardiac valves and septation. Moreover, they have high blood pressure.

1.2.4.2 Role of TGFβ in Cancer

The role of TGFβ in human cancer is multifaceted. Initially TGFβ contributes to tumor suppression by inhibiting cell proliferation. As the tumor progresses, the TGFβ growth-inhibitory responses are often replaced by invasive, pro-metastatic effects, particularly by inducing an epithelial-to-mesenchymal transition (EMT) in pre-malignant cells and by subsequently promoting metastasis [60-63]. Prevention, and/or control of tumor metastasis, is critical for cancer patient survival as it is often the leading cause of patient mortality.

Cell Cycle Arrest: Normally TGFβ is a potent mediator of growth inhibition, and tissue homeostasis. In mammalian cells, the cell cycle is carefully regulated by CDKs, which act sequentially to phosphorylate target substrates, such as retinoblastoma (Rb), allowing for G_1/S transition and cell cycle progression [64]. The negative regulatory effects of TGFβ on cell proliferation include induction of G_1 arrest, promotion of terminal differentiation and activation of cell death mechanisms. Disruption of these effects could predispose to or cause cancer [5]. Important TGFβ targets involved in cell cycle regulation are the cyclin dependent kinase inhibitors (CDKI) $p15^{INK4B}$, $p21^{CIP1}$ and $p27^{KIP1}$[40], that prevent cell cycle progression by binding to CDKs, blocking Rb phosphorylation. TGFβ also inhibits c-myc expression, allowing TGFβ-mediated CDKI induction to occur and preventing the cell from developing resistance to TGFβ-induced growth suppression [40].

Apoptosis: TGFβ is a pro-apoptotic factor which exerts its regulatory effects through caspase activation, through the upregulation of pro-apoptotic factors such as Bax and/or through the downregulation of anti-apoptotic factors such as Bcl-2 and $Bcl\text{-}_{XL}$. A loss of TGFβ-mediated apoptosis may allow for the accumulation of premalignant cells [7].

Mutations/Inactivations/Deletions: In cancer, the TGFβ signaling network is often disrupted by missense mutations, nonsense mutations, small deletions, frameshift mutations, or loss of the entire chromosomal region [5] in Smad2 and 4. In fact, support for a Smad tumor suppressor role came with frequent mutation/inactivation or homozygous deletion of Smad4 in pancreatic cancers [11]. However, Smad4 is infrequently mutated in breast, ovarian, head and neck, prostatic, esophageal, lung and gastric cancers [5, 11]. Although infrequent, Smad2 mutations are found in some cases of lung and colorectal cancers [5, 11, 65]. Most often, it is the MH2 domain that is mutated, which can disrupt the formation of R-Smad homo and heteromeric complex formation, it may also block receptor dependent R-Smad phosphorylation or result in unstable Smad proteins [5, 11].

Frequently mutated in esophageal, gastric, colorectal, endometrial and heptocellular cancer [5, 66] is the TβRII. Insertions or deletions within the extracellular domains generate truncated or inactive receptors. Mutations in the TβRI have been found in ovarian, breast and pancreatic cancer, as well as in T-cell lymphoma [62].

Tumor Promoting Effects: As tumors increase in aggressivity, the TGFβ tumor-suppressive effects are lost, promoting tumor growth and invasive effects.

Immunosuppression: TGFβ is a critical negative regulator of the immune system, as it inhibits interleukin-2 production, which prevents T-cell proliferation. It also inhibits naïve

T-cell development and deactivates macrophages by suppressing nitric oxide and reactive oxygen intermediates [1, 40]. However, most impressive is its ability to evade immunosurveillance. Active cancer cells secrete large amounts of TGFβ, thus allowing tumor cells to escape cytotoxic T lymphocyte-mediated clearance [13].

Angiogenesis: The ability of tumor cells to induce new blood vessel formation from pre-existing vasculature is essential in supplying the metabolic needs of a growing tumor [40, 62]. TGFβ can induce the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) and connective-tissue growth factor (CTGF). It can also create a favorable environment for the growth and maintenance of new blood vessels by upregulating MMP-2 and -9 that degrade ECM [40, 62].

EMT/Metastasis: The ability of cells to metastasize requires the loss of cell-cell adhesions and acquisition of fibroblastic characteristics, a process called EMT [40, 62, 67]. In fact, 90% of all cancer deaths arise from the metastatic spread of primary tumors [68]. TGFβ is a key regulator of cellular adhesion, motility and ECM. Increased expression of Snail and SIP1, both transcriptional factors known to repress the adhesion junction protein E-cadherin, leads to loss of cellular adhesion. Cancer cells then break-off the tumor and travel through the vasculature where they implant and form secondary tumors [69].

Summary: The role of TGFβ in cancer is both complex and dualistic. While initially acting as a growth inhibitory and pro-apoptotic cytokine, TGFβ undergoes a mechanistic switch, changing from an epithelial to a mesenchymal phenotype and becoming a pro-metastatic, pro-angiogenic tumor-promoting factor. What triggers this mechanistic switch currently remains unknown. Further studies regarding the signaling pathways, crosstalk networks and cellular regulations involved need to be undertaken.

1.2.4.3 Role of TGFβ in Pulmonary Fibrosis

TGFβ1 is a multifunctional cytokine. It plays a profibrotic role in progressive lung fibrosis, enhancing fibroblast chemotaxis and proliferation, as well as inducing ECM synthesis [8]. Several pulmonary fibrosis mouse models and patients with idiopathic pulmonary fibrosis, chronic lung disease of prematurity or forms of acute and chronic adult lung disease, show increased TGFβ1 production in the lung [8].

1.2.4.4 Role of TGFβ in Hepatic Fibrosis

Liver fibrogenesis is characterized by excessive accumulation of ECM due to increased synthesis, deposition of newly formed components and decreased degradation of ECM. These factors ultimately lead to cirrhosis and complications such as portal hypertension,

liver failure, and hepatocellular carcinoma [70]. Increased levels of TGFβ1 have been associated with such fibrotic pathologies, particularly due to its role in stimulating ECM and in downregulating MMP production. In addition to regulating the ECM, TGFβ1 can also alter the expression of integrins, potentially enhancing their adhesion to the ECM.

Of note is the role Smad3 has in hepatic fibrosis. Smad3 has been identified as an intracellular mediator of the fibrogenic process acting via the TGFβ, p38, MAPK, and ERK/MAPK signaling pathways [70]. Smad3 mediates the expression of collagen Iα1 and its overexpression increases fibronectin promoter activity. Moreover, loss of Smad3 was shown to interfere with TGFβ-mediated induction of EMT, as well as with the expression of collagen genes. Taken together, these results identified Smad3 as a pivotal mediator for TGFβ in hepatic fibrosis, branding it as a potential target for therapeutic strategies.

1.2.4.5 Role of TGFβ in Renal Fibrosis

TGFβ plays a key role in the progression of renal fibrosis. Recent studies have demonstrated that Smad signaling is also a critical pathway for renal fibrosis induced by other pro-fibrotic factors [2]. In fact, a 9000-gene chip microarray analysis revealed Smad3 to be essential for TGFβ signaling. Most collagen genes were found to have Smad3 binding sequences, possibly contributing to Smad3-mediated TGFβ-induced ECM expression, which contributes to the excessive connective tissue accumulation within the organ. Consistent with this observation, $Smad3^{-/-}$ mice were found to be protective against renal fibrosis induced by AngII, diabetic kidney disease and ureteral obstructive nephropathy [2]. In addition to TGFβ-induction, renal fibrosis is also induced via the ERK/p38 MAPK-Smad signaling crosstalk pathway. The role of Smad2 remains elusive.

Interestingly, secreted TGFβ in its latent form was suggested to play an important role in controlling renal inflammation [2].

1.2.4.6 Role of TGFβ in Cardiovascular Disease

Cardiovascular disease, currently the leading cause of death and illness in developed countries [71], is characterized by an ongoing inflammatory response [71, 72]. TGFβ is an important regulatory cytokine with anti-inflammatory and profibrotic properties, and has been identified at the site of various diseases. Originally it was considered the "protective cytokine", as it played an important role in maintaining normal vessel wall structure and in controlling the balance between inflammation and ECM deposition. However TGFβ participates in the pathogenesis of many cardiovascular diseases, including hypertension, restenosis, atherosclerosis, cardiac hypertrophy, and heart failure [3]. As in cancer, the role

of TGFβ can change for the worse. Loss of its protective effects is attributed to changes in TGFβ receptor profiles and regulated by local levels of TGFβ, which contribute to the development of atherosclerosis. In the diseased vessel, TβRI is upregulated and TGFβ stimulates ECM production, which can promote early fatty streak lesion formation [3]. In fact, TGFβ is believed to be the most important ECM regulator, both increasing production and preventing degradation. It increases the synthesis of ECM proteins, such as fibronectin and collagens, even at low concentrations [3].

The mechanisms involved in TGFβ-mediated vascular fibrosis are complex and include the activation of Smad proteins, protein kinases, production of mediators, regulatory complexes and crosstalk between various pathways. TGFβ stimulation promotes the formation of various regulatory complexes that, in the right cellular context, can either up or downregulate genes involved in cardiovascular diseases. For instance, TGFβ stimulation activates Smad and AP-1, which mediate enhanced expression of other TGFβ-responsive genes such as collagen, c-Jun, endothelin-1, or peroxisome proliferator activated receptor gamma. Each of these genes exhibit important cardiovascular functions, particularly in ventricular remodeling as a result of cardiac fibrosis or vascular angiogenesis [38].

Another transcriptional binding factor, Sp1, showed increased collagen expression upon TGFβ stimulation. Smads are assumed to act as bridging molecules between Sp1 and AP-1, enhancing collagen synthesis, which enhances cardiac stiffness [38]. Moreover, Sp1/Smad also interacts with CDKIs to influence cardiomyocyte growth and differentiation. Integrins, previously shown to participate in cardiac hypertrophy, are also mediated by this complex. Finally, hypoxia-inducible factor 1α (HIF1α)/Smad cooperation was shown to stimulate angiogenesis of ischemic tissues due to VEGF induction.

Taken together, TGFβ-induced Smads play an important role in gene regulation, which leads to cardiac remodeling. The absence or inhibition of Smads appears to correlate with conditions found in hyperotrophic growth processes, whereas the activation of Smad2, 3, and 4 by the TGFβ/activin subfamily contributes to cardiac fibrosis and apoptosis [38].

1.2.5 TGFβ Crosstalk with other Signaling Pathways

It is becoming increasingly clear that signaling pathways are not insulated devices but rather complex networks of carefully timed and precisely regulated interactions. Although the Smads are the only known direct receptor substrates of the TGFβ signaling pathway, they represent, by no means, the sole pathway by which TGFβ regulates cellular function.

Several other signaling pathways share protein targets and have been shown to regulate the Smad proteins, which serve as sites of signal integration, making them mediators of crosstalk between various signaling pathways [9].

MAPKs: Both the MAPK and the TGFβ pathways have a reputed history of crosstalk. Initial evidence supporting Smad-independent activation of the MAPK pathway by TGFβ came from studies using Smad4-deficient cells or dominant negative Smads, in which TGFβ-dependent transcription could still be observed [56]. Nevertheless, the effects of TGFβ on the MAPKs vary extensively in kinetics magnitude and kinase subtype, and are present in only some of many cell lines surveyed [9]. Similarly, MAPKs have also been implicated in the regulation of Smad-mediated signaling. Contained within the Smad linker region is a MAPK recognition sequence. Despite the various studies investigating the interaction between TGFβ and MAPKs, the mechanism of their crosstalk remains unclear and the biological consequences of these activations poorly characterized.

ERK: The ERK-mediated pathways are primarily involved in cell proliferation and differentiation and are generally considered to be anti-apoptotic. TGFβ has been shown to induce activation of the ERK pathway, resulting in an induction of TGFβ expression, which leads to an amplification of the initial TGFβ response [73]. Contrarily, these activated ERKs can then inhibit TGFβ signaling through phosphorylation of the MH1 domain of Smad2 and of the Smad1, 2, and 3 linker regions [9, 11, 53, 74]. In response to epidermal growth factor (EGF) and at low levels of TGFβ stimulation, these phosphorylations reduce nuclear accumulation of activated Smads. Although higher levels of TGFβ reverse this effect, several Smad-responses are still altered by ERK activation [9].

Phorbol-12-myristate-13-acetate reportedly stimulates ERK-mediated phosphorylation of Smad3, thereby activating Smad3 signaling. Similarly, Ras-mediated activation of ERK is said to enhance TGFβ/Smad1-mediated responses [11]. Alternatively, activation of MAPK pathways by TGFβ can also affect transcriptional responses through direct effects on Smad-interacting transcription factors [13]. TGFβ has been shown to activate activating transcription factor 2 (ATF2) and AP-1 proteins downstream of the MAPK cascade, which enables these transcriptional complexes to cooperate with Smads. Together they regulate TGFβ-induced transcription at TGFβ-responsive AP-1 or ATF2 promoter binding sites, serving as a point of convergence for the TGFβ-induced Smad and MAPK pathways [48].

SAPK: The JNK and p38 signaling pathways are often associated with TGFβ-induced apoptosis [75, 76]. Both these pathways regulate transcriptional activity of the R-Smads in

different ways. TGFβ-induced activation of JNK leads to the phosphorylation of Smad3, promoting its nuclear translocation and transcriptional activity [56]. JNK is also known to inhibit Smad2-dependent transcription through the formation of complexes between the co-repressor, TGF-induced factor (TGIF), and Smad2 [77]. Although the JNK response to TGFβ may take several hours, suggesting that JNK is not a primary transducer of TGFβ signals in these cells [5], it is clearly involved in signaling crosstalk with TGFβ.

Similarly, p38 can either promote the association of Smad3 with the co-activator p300 to induce TGFβ responsive target gene expression [78], or it can enhance Smad4-dependent transcription through the regulation of Smad4 sumoylation [79]. Both pathways can also modulate TGFβ signaling through regulation of Smad7 expression [65, 80].

PI3K/Akt: Both TGFβ and the PI3K/Akt pathway play a critical role in a number of cellular responses including cell growth, protein synthesis and anti-apoptosis/survival. However, TGFβ induces both apoptosis and cell-cycle arrest in some cell lines, but only growth arrest in others. It has been reported that sensitivity to TGFβ-induced apoptosis is regulated by crosstalk between the Akt/PKB serine/threonine kinase and Smad3 [58], whereby Akt directly interacts with unphosphorylated Smad3 to sequester it outside the nucleus, preventing its phosphorylation and nuclear translocation. It was also demonstrated that the ratio of Smad3 to Akt correlates with the sensitivity of cells to TGFβ-induced apoptosis, clearly illustrating an important role for crosstalk between PI3K/Akt and TGFβ.

Moreover, TGFβ-mediated activation of the PI3K/Akt pathway is involved in the regulation of cell migration, survival, and EMT [81]. Akt directly phosphorylates FoxO transcription factors, which excludes them from the nucleus. As a result, they cannot form transcriptional complexes with Smad3/4 or elicit FoxO-dependent gene responses [13, 81].

Others: TGFβ-mediated activation of small GTP-binding proteins is required for membrane ruffling, lamellipodia formation, stress-fiber formation and for the promotion of EMT [11, 13]. A direct link between TGFβ receptors and Rho-like GTPases, capable of regulating cytoskeletal organization, provides a novel mechanism through which TGFβ can directly modulate EMT [41, 82] and other signaling pathways [83, 84]. Smad3 and 4 act cooperatively with Rho and p38 to induce the expression of various proteins involved in the formation of actin stress fibers, favoring EMT transdifferentiation [85, 86].

Also of note is the role of the ERK-activated Ras pathway, which can modify TGFβ signaling in various ways. Hyperactive Ras, associated with many cancers, counteracts the

anti-proliferative activity of TGFβ through the downregulation of TGFβ receptors, attenuation of Smad accumulation in the nucleus and perhaps by other mechanisms [9].

Summary: The intense network of crosstalk mechanisms that regulate and/or are regulated by TGFβ have yet to be fully understood. Many intracellular proteins function as mediators of crosstalk or feedback [9], increasing the complexity of this signaling network.

1.3 OVERVIEW OF THE GPCR SIGNALING PATHWAY

1.3.0 Preface

Found in all mammalian cells, GPCRs constitute a large superfamily of heptahelical transmembrane-spanning receptors. Numbering in the thousands, it is not surprising that these receptors are responsible for a myriad of biological functions and initiate virtually every physiological response we experience. And, it is no wonder that GPCRs represent the most widely targeted pharmacological protein class.

These receptors transmit extracellular stimuli through intracellular secondary messengers via coupling to heterotrimeric guanine nucleotide-binding proteins (G proteins) and the subsequent regulation of effector molecules. Agonist-binding, triggers a conformational change in the receptor, as well as dissociation of the Gβγ subunits, which leads to the association of an important family of regulatory kinases, the GRKs, with the receptor. In fact, agonist-induced responses can be terminated within milliseconds to minutes, through a process initiated by receptor phosphorylation. Recruitment of a family of cytoplasmic proteins, the arrestins, leads to receptor desensitization and/or subsequent downregulation via receptor internalization.

The following section describes the GPCR superfamily, its constituents and their physiological roles. GPCR agonist, AngII, and kinase, GRK2, will be the main focus of the section, as both are implicated in a variety of diseases, most notably vascular diseases.

1.3.1 The G Protein-Coupled Receptors

GPCRs are seven-transmembrane proteins that make up the largest superfamily of cell surface receptor proteins [87, 88]. More than 800 distinct GPCRs are present in the human genome, as they are found on the surface of all the cells of multicellular organisms, and are known to respond to hormones, ions, neurotransmitters, chemokines, odorants, or tastants [35, 87]. It is no surprise that GPCRs regulate a part of nearly every physiological function and that they represent the most widely targeted pharmacological protein class [87].

Upon ligand binding, the intracellular domain of the receptor undergoes a conformational change, allowing for association with heterotrimeric G proteins to take place. These proteins, composed of membrane anchored βγ subunits and a GDP-bound α subunit, then exchange GDP for GTP – the mechanism for which they are named. This conformational change and the liberation of Gβγ subunits trigger the association of GRKs with the receptor. These agonist-bound GPCRs are phosphorylated by GRKs on various serine and/or threonine residues contained within the C-terminal tail and within the third intracellular loop. Phosphorylation creates high affinity sites for arrestin binding [13], which prevents receptors from signaling by recruiting various endocytic components, such as clathrin and β2-adaptin, and initiating receptor internalization. After internalization, receptors are either recycled back to the plasma membrane or targeted for degradation.

GPCR responsiveness is determined by a tightly regulated balance between receptor signaling, desensitization and resensitization [88]. Receptors then couple to a subset of the 16 heterotrimeric G protein subtypes, which are functionally grouped into four broad classes: G_s, G_i, G_q, andG_{12} [87]. Some receptors couple to a single G protein, whereas others bind to all four, thereby activating several signaling pathways at once. Some receptors even signal independently of their G proteins.

Receptor sensitivity varies based on the amount of signaling the receptor has done. In general, receptors adjust their sensitivity to the range of agonist concentrations to which they are exposed [87]. Desensitization not only functions to terminate receptor signaling, but also to resensitize the receptor after prolonged or repeated exposure to an agonist. In addition to mediating receptor internalization, the GRK-arrestin pathway also terminates GPCR signaling, sometimes while still in the presence of the activating agonist.

1.3.1.1 Signaling

GPCRs represent the largest family of transmembrane signaling molecules in the human genome. Their primary mode of cellular activation occurs through heterotrimeric G proteins, which in turn can activate a wide spectrum of effector molecules, including phosphodiesterases, phospholipases, adenylyl cyclases and ion channels [88].

Ligands for these receptors include large glycoprotein hormones, a multitude of peptides, bioactive lipids, amino acids and their metabolites such as dopamine and norepinephrine, small molecules such as acetylcholine and sucrose, Ca^{2+}, and even photons [87]. Upon ligand binding to the heptahelical receptor, the intracellular domain of the receptor undergoes a conformational change, allowing for G proteins association to occur.

These G proteins exchange GDP for GTP, leading to the dissociation of the G protein into Gα and Gβγ subunits, both of which modulate different effector systems [89].

The conformational change of the receptor and the liberation of Gβγ subunits have been suggested to trigger the association between GRKs and the receptor. GRKs then phosphorylate the agonist-bound GPCR on various serine and/or threonine residues, creating high affinity binding sites for β-arrestins [13]. Arrestin-binding terminates receptor signaling by recruiting the endocytic components and initiating receptor internalization, after which receptors are either recycled or targeted for degradation.

In 2005, Lefkowitz and Shenoy revealed a "newly appreciated" signaling mechanism involving the GRK/β-arrestin protein families. Arrestins serve as multifunctional adaptor and scaffolding proteins, recruiting a broad spectrum of signaling molecules and assemblies to the receptors in a strictly activation-dependent fashion [35]. In 2006, Yang and Xia confirmed this by showing that after internalization, the GPCR-β-arrestin complex can form a signalosome that activates signaling proteins, such as ERK1/2, p38 MAPK, and JNK [88]. The β-arrestin scaffolds served to connect the activated GPCR with the tyrosine kinase c-Src, as well as with the PI3K/Akt and the NF-κB pathways.

1.3.1.2 Internalization

The concept of GPCR internalization originated from the observation that β-adrenergic agonist treatment resulted in a loss of β-adrenergic receptor (βAR) recognition sites on the surface of frog erythrocytes. Differential sedimentation located internalized hydrophobic and hydrophilic β2-adrenergic receptor (β2AR) in a "light vesicular" fraction, separate from cell surface receptors in the "heavy vesicular" plasma membrane fraction [88].

GRK2 and arrestins directly participate in receptor endocytosis, internalization, intracellular trafficking, resensitization, and in the modulation of MAPK cascades by GPCRs [88, 89]. In fact, the binding of arrestin following receptor phosphorylation is critical for the internalization of receptors, as β-arrestin1 and 2 mediate the recruitment of clathrin and β2-adaptin. Kinetic differences suggest that GPCR internalization can be mediated by multiple endocytic mechanisms and/or that structural heterogeneity between receptor subtypes modulates their relative affinities to bind endocytic adaptor [88].

Aside from clathrin, β-arrestins also interact with endocytic elements, including AP-2 [90], the small GTPase ARF6 [91] and its guanine nucleotide exchange factors ARNO and NSF. They also bind and are ubiquitinated by the E3 ligase, Mdm2, an event required for

β-arrestin-mediated endocytosis [35]. Endocytosis recycles resensitized receptors back to the plasma membrane. However receptors can be downregulated through ubiquitination.

In addition to receptor internalization, β-arrestins also act as scaffolding molecules, bridging receptors and signaling proteins. The scaffold facilitates GPCR activation of the ERK/MAPK cascade, bringing these molecules closer to the receptor complex [89].

1.3.1.3 Desensitization

Receptor desensitization, the waning of GPCR responsiveness to the agonist with time, is an important physiological feedback mechanism that protects against acute and chronic receptor overstimulation. The GRK and arrestin protein families play a pivotal role in the process of agonist-activated GPCR desensitization [88].

Agonist stimulation triggers a complex regulatory mechanism following recruitment of specific GRKs, which includes phosphorylation of the receptor. GRKs phosphorylate the intracellular loops and/or C-terminal tail of the receptor, a process that enhances receptor affinity for cytosolic arrestin proteins [88, 89]. Binding of the arrestins leads to uncoupling of the GPCR from its bound G protein and termination of receptor signaling. The GRK-arrestin pathway promotes clathrin-mediated internalization, a process that plays an important role in regulating cellular activity both by mediating long-term desensitization through the degradation of receptors, and by recycling desensitized receptors back to the cell surface to initiate additional rounds of signaling after resensitization [88].

Receptor desensitization can also result from prolonged or repeated exposure to high concentrations of agonist. GRKs recognize and phosphorylate the activated receptor. In addition to mediating receptor internalization, the GRK-arrestin pathway can also terminate GPCR signaling while still in the presence of the activating agonist. As a result, the endocytosed receptor remains agonist- and activation-free until it is ready to be degraded or recycled to the plasma membrane, where it can signal again. Desensitization is a regulatory mechanism by which GPCRs ensure optimal response and function.

1.3.1.4 Regulators of the GPCR Signaling Pathway

Arrestins: β-arrestins are a family of scaffolding proteins and/or signal transducers that connect GPCRs to diverse signaling pathways within the cell [35, 92]. In mammals, 4 arrestins divided into two families have been identified, (i) the visual arrestins, composed of arrestin-1 and -4, which are restricted to photoreceptor cells and (ii) the ubiquitously expressed β-arrestin1 and 2, required for GPCR internalization and desensitization [35, 87, 88, 92]. Most receptors in the body are regulated by β-arrestin1 or 2.

β-arrestins have two distinct domains held together by a 12-residue "hinge" region. The domains are held intact by intramolecular interactions defined by buried polar residues, as well as by the "three-element interface" that composed of the "buried" C tail, β strand I, and α helix I [35]. Although β-arrestins interact with many different proteins, they do not contain any specialized protein-protein interaction domains. Instead, they display higher affinity for phosphorylated forms of their binding proteins.

Originally located in the cytoplasm, arrestins translocate to the plasma membrane upon GRK phosphorylation of the GPCR, where they can bind to the phosphorylated receptors. This association results in the uncoupling of the G protein-dependent receptor signaling complex. Arrestins then recruit endocytic machinery to promote receptor internalization. This mechanism regulates aspects of cell motility, chemotaxis, apoptosis, and likely of other cellular functions, through a rapidly expanding list of signaling pathways [35].

Interestingly, arrestins have also been located in the nucleus, suggesting a role in transcriptional regulation. In fact, Kang *et al.* (2005) showed that in transfected fibroblasts, β-arrestin1 translocates to the nucleus in response to activation of two prototypical GPCRs, the κ- and δ-opioid receptors. They also suggested that β-arrestin1 acts as a nuclear scaffold, recruiting p300 to the cAMP response element-binding protein (CREB). Recruitment leads to histone H4 acetylation and to chromatin recognition, increasing gene expression [92]. However, receptors have not been described in the nucleus, raising the possibility that arrestins may act as molecular scaffolds in the absence of receptors. In fact, β-arrestins have been shown to act as scaffolds for multiple signaling kinases, some of which, like ERK and Akt, undergo nuclear translocation [92]. Actually, β-arrestin-dependent GPCR signaling lasts longer than conventional G protein-dependent signaling. It is characterized by slower onset, greater persistence, retention of the activated ERK in cytosolic endocytic vesicles, and absence or paucity of transcriptional regulation [35, 92].

In addition to mediating GPCR endocytosis, after phosphorylation by TβRII, β-arrestin 2 also mediates the endocytosis of TβRIII, downregulating antiproliferative signaling [35].

β-arrestins both terminate and act as junctions for signaling pathways. Thus, it is no surprise that they are suspected of playing a role in signal crosstalk.

β-Adrenergic Receptors: In the mid 1980's adenylyl cyclase-coupled β2AR was found to share structural and regulatory features with the visual sensing protein rhodopsin. Further studies revealed that G protein-mediated signaling was attenuated by a highly conserved process involving phosphorylation of the activated receptors by specific protein

kinases, such as rhodopsin kinase (now known as GRK1) and the β-adrenergic receptor kinase 1 (βARK1, currently known as GRK2) [35].

In the heart, catecholamines bind to βARs, which modulate cardiac responses by coupling to and activating G_s proteins of the GPCR signaling cascade. βAR signaling, which is compromised in many cardiac diseases, is regulated by GRKs [93]. Interestingly, levels of the most abundant cardiac GRK, GRK2, are elevated in heart failure.

G Protein-Coupled Receptor Kinases: GRKs belong to a family of seven mammalian serine/threonine protein kinases that specifically recognize and phosphorylate agonist-activated GPCRs, promoting arrestin binding [88, 93-95]. Originally identified in the mid-1980's as the critical initial step for the uncoupling of receptor from G proteins, resulting in attenuated or desensitized GPCR signaling [94], GRKs are cytosolic and demonstrate a high degree of substrate selectivity in phosphorylating agonist-occupied GPCRs [93, 94].

GRK family members can be classified into three main groups based on sequence homology, localization, substrate specificity, or mechanism of action [87, 88, 93, 95] (Fig.6): (i) Rhodopsin kinase or visual GRK subfamily (GRK1 and 7), (ii) the βAR-adrenergic receptor kinases subfamily (GRK2/3) and, (iii) the GRK4 subfamily (GRK4, 5 and 6). These kinases share a central catalytic domain that is homologous to other serine/threonine kinases, flanked by an N-terminal domain and a variable-length C-terminal domain that contains specific regulatory sites [93, 95].

Although these kinases share some structural characteristics, they are distinct enzymes with specific regulatory properties. GRK2, 3, 5 and 6 are ubiquitously expressed in mammalian tissues [95]. GRK2 and 3 share a pleckstrin homology (PH) domain that controls phosphatidylinositol 4,5-bisphosphate (PIP2) and G protein βγ subunit-mediated translocation of these kinases to the inner leaflet of the plasma membrane, near activated receptor substrates [87, 93]. Association of GRK2 and 3 with the plasma membrane, as evidenced both *in vitro* and *in vivo*, is determined by the GPCR present [93].

Due to a lack of plecktrin homology (PH) domain, GRK4, 5, and 6 directly bind PIP2 and/or undergo covalent lipid modification with palmitate to primarily reside at the plasma membrane. GRK4 has limited distribution, found mainly in the testis. GRK5 is involved in cardiac disease, however in a manner distinct from GRK2. Other GRKs display a restricted expression pattern, like GRK1 in the retina and pineal gland, and GRK7 in the cones [89].

GRKs are key contributors of the phosphorylation-dependent GPCR desensitization pathway. They are also important modulators of intracellular GPCR signaling cascades due

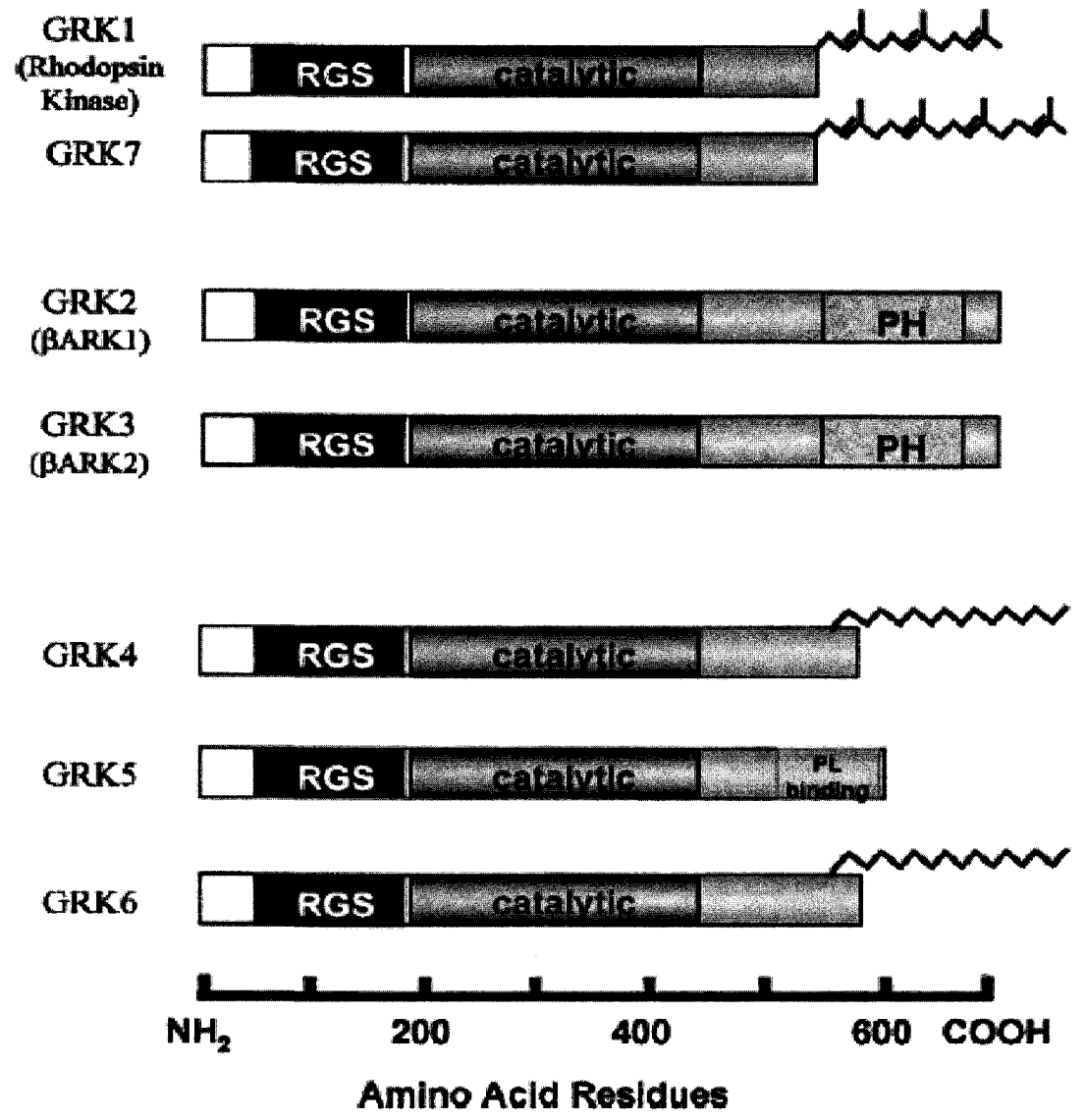

Fig.6: Classes and domain architecture of GRKs

The three classes and structural organization of the seven known mammalian GRKs are represented. RGS, regulators of G protein signaling homology domain; PH, pleckstrin homology domain; PL, phospholipid. Splice variants of GRK1, 4, and 6 have been identified (Penn, Pronin *et al.* 2000)

to their ability to interact with a variety of proteins involved in signaling and trafficking. Emerging evidence indicates that GRK activity is tightly regulated by certain mechanisms, including phosphorylation by different kinases and by interaction with several cellular proteins, including non-receptor proteins, pointing to novel cellular roles for GRK [95].

1.3.2 G Protein-Coupled Receptor Kinase 2

1.3.2.1 Structural & Functional Features of the GRK family

In general, the GRK kinase domain is well conserved among the different subfamilies, showing ~45% sequence identity. Flanked by an N-terminal RH domain and a C-terminus of variable length, this catalytic domain is homologous to other serine/threonine kinases.

The N-terminal RH domain is composed of 183-188 aa, which includes a region of homology to regulators of G protein signaling (RGS) proteins. It displays weak homology (~27%) with the other kinases, leading to speculation that this region may be important for receptor recognition. The RGS domain provides a potential mechanism by which GRKs can regulate GPCR signal transduction via phosphorylation-independent mechanisms [88].

The C-termini have little or no sequence homology within the kinase family. However, they appear to contain key determinants for their localization and/or translocation to the membrane by means of post translational modification or via sites of interaction with lipids or membrane proteins [89]. Consequently, membrane associated GRK1 and 7 are isoprenylated, whereas GRK4 and 6 are post-translationally palmitoylated on one or more cysteine residues to ensure exclusive membrane-associated localization. GRK2 and 3 bear an extended C-terminus, containing a 125 aa PH domain involved in the modulation of kinase targeting to the membrane. GRK5, also predominantly membrane-bound, binds to membrane phospholipids via positively charged amino acid clusters [88, 89].

GRK2 and 3 are 85% similar in primary structure and 95% similar with respect to their catalytic domain. However, their C-termini share only 52% similarity, accounting for their differential affinities for specific GPCRs [93]. Recently, a second binding site for Gβγ-subunits has been identified within the first 53 aa of GRK2, suggesting that either the N- or the C-terminal regions might be sufficient to allow GRK2 targeting to the membrane [88].

RH Domain: Composed of nine α-helices analogous to other RGS domains and of two additional α-helices derived from a region between the kinase and the PH domain, the RH domain of GRK2 can interact with both the kinase and the PH domain, suggesting an important role in the regulation of kinase activity [95].

Recent studies have implicated GRK2 in phosphorylation-independent desensitization of various GPCRs, notably type 1A AngII [95]. As such, the function of the RH domain provides an additional mechanism of receptor signaling regulation for GRK2 at the G protein level. The RH domain has been shown to specifically interact with Gαq family members, although it does not stimulate the GTPase activity of Gαq as efficiently as other classical RGS proteins do [95]. Although GRK2 has a poor Gαq-GTPase activating protein activity, the RH domain may shield the interaction between G protein and GPCR. This means the RH domain is able to inhibit Gαq-mediated phospholipase C (PLC) activity, independently of receptor phosphorylation. Thus, it can be concluded that the GRK2-RH domain binds Gαq in a manner more similar to an effector-like interaction than to an RGS-like one, suggesting for GRK2 the role of Gαq signaling-mediated pathway effector [95].

The tyrosine kinase, c-Src, can phosphorylate GRK2 at several places within the RH domain. Phosphorylation enhances GRK2-Gαq interaction, increasing both GRK2 kinase activity towards GPCRs and its specific interaction with Gαq subunits. Such an increase leads to the rapid swith-off of Gαq-mediated signaling [95].

Kinase Domain: The GRK kinase domain is relatively well conserved among the different subfamilies, showing similarity to other serine/threonine kinases. Activation of GRK2 and 3 requires the activation and dissociation of a heterotrimeric G protein, such that the kinases are activated by free Gβγ subunits [96]. When complexed with Gβγ subunits, the kinase appears to lie in a resting conformation through its association with the RH domain. These interactions may have a regulatory role in GRK2 activation [95].

PH Domain: Consisting of seven β-strands and one C-terminal α-helix, the GRK2 PH domain is involved in phospholipid binding and membrane targeting. Both the RH and PH domains interact, such that changes in the conformation of either domain, due to protein-protein interaction or phosphorylation, can lead to changes in catalytic activity via their interface with the kinase domain.

The PH domains of GRK2 and 3 can also directly interact with PIP2 and other acidic phospholipids, which have clear effects on kinase activity. The fact that PIP2 appears to bind to the N-terminus of the PH domain suggests that Gβγ and lipids contribute synergistically to GRK2 localization and activation [89].

Gβγ subunits: The kinase activity of GRK2 and 3 toward receptor substrates is enhanced by Gβγ subunits. The Gβγ subunit dependence links GRK kinase activity to the activation of a heterotrimeric G-protein [96]. Free Gβγ subunits bind to GRK2 with high

affinity and are required, in reconstituted systems, for association of GRK2 to lipid vesicles and for GPCR phosphorylation [89].

In the C-terminus, the PH domain of both GRK2 and 3 partially overlap with a Gβγ-binding region. Through multi-site contact with Gβγ of the PH domain, GRK2 activity is enhanced via GPCR-mediated allosteric activation. It has also been suggested that Gβγ interaction with GRK2 and 3 targets the kinases to membrane sites, where GPCRs are being activated, and thus determines their substrate specificity.

Interestingly, GRK2 activity can also be regulated through the modulation of the Gβγ subunit. In fact, GRK2 activity is inhibited when phosphorylation by MAPK hampers Gβγ binding. In contrast, Protein Kinase A (PKA)-mediated phosphorylation of GRK2 has the opposite effect, as it facilitates contacts with Gβγ subunits and kinase activation [95].

1.3.2.2 Regulation of GRK2 activity

It has become increasingly apparent that GRK function is a highly regulated process. Precise localization and time-specific expression is necessary for proper function. It is perhaps due to this tight regulation (Fig.7) that the currently identified seven GRKs can control the responsiveness of the numerous GPCRs in a given cell.

The mechanisms by which GRK activity is regulated are by subcellular localization, by alterations in intrinsic kinase activity, and by alterations in GRK expression levels [94].

Subcellular localization & Alterations in intrinsic kinase activity

GPCRs: GPCR activation immediately recruits GRKs to the plasma membrane, where they phosphorylate the GPCR tail in order to stop signaling. In fact, Chen *et al.* (1993) demonstrated that the presence of activated receptor increased both the half maximal effective concentration and the V_{max} of GRK2-mediated phosphorylation of synthetic peptides [94]. Although the mechanism by which interaction with the receptor increases catalytic activity is unclear, it is presumed that receptor-GRK interaction induces a conformational change in the GRK, releasing an auto-inhibitory constraint in a manner similar to that demonstrated for numerous other kinases [94]. Moreover, the ability of GRKs to interact with activated GPCR and Gαq subunits also provides a mechanism for phosphorylation-independent termination of GPCR signal transduction. Signal termination occurs by interfering with the association of stimulated GPCR and Gα subunits with their cellular effectors. This regulation appears to involve both the N- and C-terminal domains of the kinase, as well as various intracellular targets. Disruption of such intramolecular

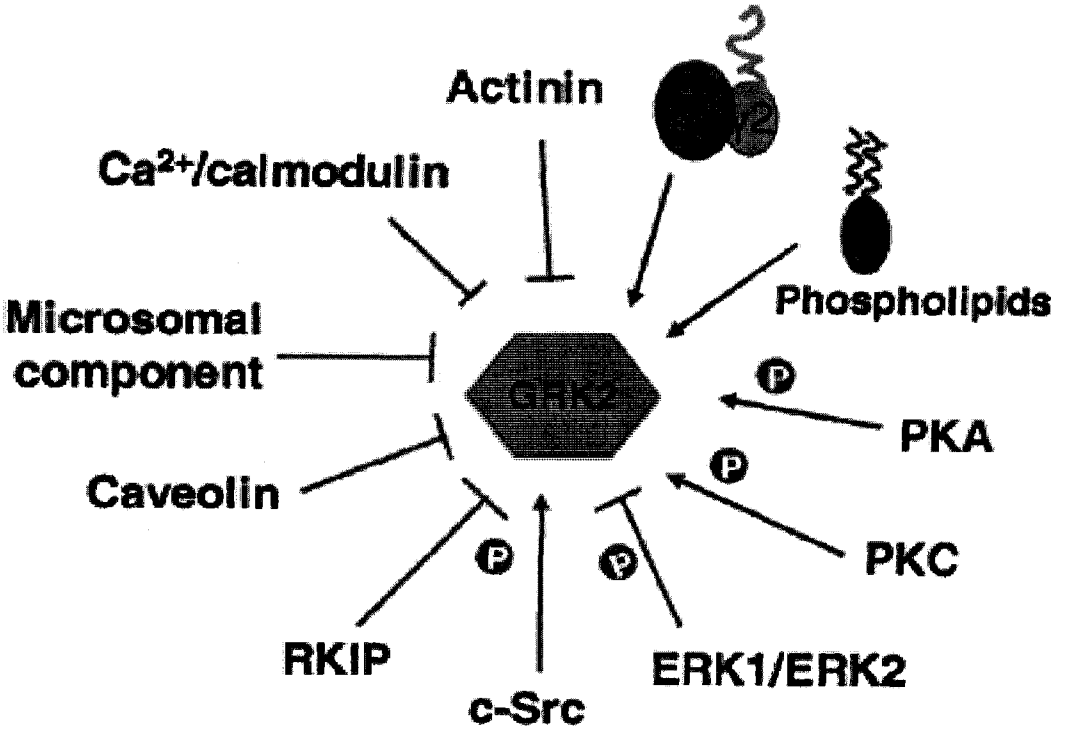

Fig.7: Regulators of GRK2 activity

Diagram showing the positive regulators of GRK2 activity, phospholipids, PKA, PKC, c-Src, as well as the negative regulators of GRK2 activity, Actinin, Ca^{2+}/calmodulin, caveolin, RKIP. The various GRK2 regulators contribute its ability to confer a wide array of physiological responses (Ribas, Penela *et al.* 2007)

contacts would promote conformational changes in the kinase, simultaneously leading to GRK2 translocation and activation [89].

G proteins: Upon agonist binding and GPCR-G protein activation, dissociation of Gβγ and Gα occurs. GRK2 binds free Gβγ and translocates to the plasma membrane. Mutations in the Gβγ binding domain fail to bind GRK2 and, thus inhibit GPCR phosphorylation.

In 1999, Carman *et al.* showed that GRK2 interaction with activated Gαq effectively inhibited Gαq-mediated PLC [94]. Affinity for activated Gα was shown to either influence GRK-receptor specificity or provide a potential mechanism for efficient signal termination.

Phospholipids: Through direct binding to phospholipids, GRK2 can promote membrane localization, and increase catalytic activity of the kinase. Recent studies demonstrate that phospholipid-binding is critical for GRK2 function in cells [94].

Caveolin: Caveolins are vesicular invaginations of the plasma membrane composed of cholesterol, glycosphingolipids, and caveolin. They act as scaffolds for a variety of signaling molecules including GPCRs, different MAPKs, and G proteins [94, 95], which help to limit and/or compartmentalize signaling.

Binding of caveolin to GRK2 greatly reduces its catalytic activity, suggesting that caveolae play an important role in compartmentalizing GRKs, potentially providing a mechanism for rapid activation/inactivation of GRKs that may be necessary in certain signaling situations [94]. Caveolin binding motifs on GRK2 are located in the PH and in the N-terminal domain. In actual fact, the N-terminal domain mediates caveolin interaction with GRKs lacking the PH domain [89, 94, 95].

Calcium-binding proteins: Cellular Ca^{2+} levels regulate GRK2 activity through Ca^{2+}-sensing protein interactions with GRK2. For instance, calmodulin interacts with GRK2 at sites located within its N- and C-terminal domains, thus inhibiting phosphorylation of GPCRs by GRKs in a Ca^{2+}-dependent manner. Interestingly, calmodulin's potency of inhibition varies as a function of GRK subtype, with GRK2 having the lowest affinity [89, 94]. In this case, the potential for calmodulin-mediated inhibition of GRK2 may exist only in cells where calmodulin is highly expressed, as in neurons. As such, it has been reported that GRK2 interacts with the neuronal calcium sensor-1 (NCS-1), resulting in the modulation of GRK2-mediate desensitization of D2 dopamine receptors [89, 95].

Phosphorylation by Kinases: Phosphorylation of GRKs at different sites and by a variety of protein kinases has emerged as an important regulatory mechanism for their

activity, protein interaction and even protein stability. Such phosphorylation-dependent mechanisms allow for feedback and crosstalk mechanisms to occur [89].

PKC: PKC is activated by Ca^{2+} and diacylgycerol (DAG) upon receptor stimulation of PLC, and can phosphorylate numerous substrates including GPCRs, G proteins, RTKs and other proteins [94]. PKC activates GRK2 by enhancing its translocation to the plasma membrane. In fact, when multiple GPCRs are simultaneously activated in a given cell, the net effect of PKC and Ca^{2+}/calmodulin is to preferentially enhance GRK2 activity and desensitize GRK2 receptor substrates [94]. PKC-mediated phosphorylation of GRK2 leads to enhanced receptor phosphorylation but not of soluble peptides, suggesting that PKC phosphorylation stimulates GRK2 translocation without affecting catalytic activity [89].

PKA: The ability of PKA to directly phosphorylate GRK2, leading to enhanced GRK2 activity on β2AR, is dependent on the presence of GRK2-bound Gβγ and on PKA tethering to the receptor. As in the case of PKC, PKA phosphorylation enhances GRK2 binding to Gβγ subunits without affecting the kinase activity, thereby promoting membrane targeting.

p42-44 MAPK, Src: The regulation of GRKs by kinases activated downstream of heterotrimeric G proteins represents classical feedback regulation [94]. In 1999, Pitcher *et al.* demonstrated feedback inhibition of GRK2 as a result of p44 MAPK phosphorylation of GRK2 on S670, which dramatically reduced GRK2 activity and Gβγ sensitivity.

GPCRs can trigger the modulation of another non-RTK, c-Src. c-Src has been shown to phosphorylate and regulate GRK2, increasing its activity towards both receptor and non-receptor substrates. Tyrosine phosphorylation also appears to enhance the interaction of GRK2 with Gαq, thereby promoting its degradation by the proteasome pathway.

Collectively, these studies suggest that p42/p44 MAPK and Src are physiologically relevant regulators of GRK activity, and point to additional modes of cross-regulation between RTK and GPCR signaling pathways [94].

Raf Kinase Inhibitor Protein (RKIP): After GPCR stimulation, PKC phosphorylation of RKIP increases PKC binding affinity to GRK2, as well as dissociation from known target, Raf-1. These effects prolong ERK activation and block kinase activity [95].

PI3K: Direct protein-protein interaction between PI3Kγ and GRK2 is mediated by the phosphoinositide kinase (PIK) domain. Overexpression of the PIK domain inhibits PI3K-GRK2 interaction, markedly attenuating β2AR endocytosis, which is characteristic of heart failure [95]. This experiment illustrates the importance of the PI3K and GRK2 interaction.

Akt: Akt, a serine-threonine kinase, directly associates with the C-terminus of GRK2 in an agonist-dependent manner [95], thereby inhibiting Akt phosphorylation. Interestingly, GRK2 upregulation occurs in vascular diseases where inhibition of Akt has been observed.

GRK-Interacting (GIT) Proteins: GIT proteins are ubiquitous multifunctional proteins that can interact with a variety of signaling molecules involved in cellular processes such as cytoskeletal dynamics, membrane trafficking, cell adhesion and signal scaffolding.

Endogenous GITs are strictly required for receptor internalization, whereas overexpression strongly impair endocytosis of several GPCRs. It has been proposed that GRKs may function as anchoring proteins for GIT molecules, recruiting them to the vicinity of receptor complexes that undergo endocytosis. However, the functional consequences of the GRK/GIT interaction remain to be elucidated.

Clathrin: In addition to enhancing β-arrestin recruitment to the plasma membrane, clathrin binds GRK2 through a clathrin box located in the C-terminal domain of the kinase. This interaction appears to be involved in agonist-promoted GPCR internalization via a dynamin dependent mechanism [95]. In fact, GRK2 has been detected in endosomal vesicles, consistent with a role in receptor internalization. As clathrin can bind and regulate GRK2, presumably it can do the same to the growing list of GRK2 non-receptor substrates.

MEK/ERK interface: In 2007 Ribas, Penela *et al.* described GRK2-transfected cells in which elevated levels of GRK2 were found to inhibit chemokine-mediated induction of ERK activity. Correspondingly, decreased levels of GRK2 promoted a more robust ERK activation upon agonist treatment. Neither the GRK2 kinase activity nor its interaction with G protein subunits was necessary for this inhibition and no change in MEK activation was observed. Interestingly, GRK2 and MEK1 have been described in the same multimolecular complex and correlated with inhibition of ERK activation. It has been proposed GRK2-MEK binding can interfere with MEK association to proteins important for its cellular compartmentalization, internalization, or activity, such as MEK-ERK scaffolds [95].

Heat shock protein 90 (Hsp90): Hsp90 is a protein chaperone that binds to a variety of kinases, GPCRs and G proteins, assisting their folding and maturation. Although its role in GPCR signaling is unknown, several GRKs have been shown to interact with Hsp90.

Alterations in GRK expression levels: GRKs are proteins not only tightly regulated at the level of kinase activity or of subcellular localization, but also at their expression level. Generally, GRKs are expressed at relatively low levels in the cell. However, changes in GRK expression, particularly of GRK2, are associated with altered GPCR signaling in a

variety of diseases including hypothyroidism, rheumatoid arthritis, hypertension, cardiac hypertrophy, congestive heart failure, myocardial ischemia/infarction, ventricular overload disease and cystic fibrosis [89]. Altered levels of GRK2 may result from an imbalance between transcriptional and post-transcriptional mechanisms, as is discussed below.

Transcriptional level: Transcriptional regulation of GRK2 expression is cell specific. In vascular cells, expression of GRK2 is controlled at the transcriptional level by the crosstalk of different signaling pathways. Alterations in any one of these pathways can result in altered expression of GRK2, and can lead to severe pathologies. For instance, in aortic SMCs, transcriptional activity of the GRK2 gene promoter is increased, leading to physiological vasoconstriction and hypertrophy.

GRK degradation: GRK2 is a short-lived protein, with $t_{1/2}$ ~1h. Generally, it undergoes polyubiquitination and is degraded by the proteasome.

Counter intuitively, agonist stimulation enhances kinase turnover. Similarly, sustained GPCR stimulation results in the downregulation of steady-state kinase levels. However, interfering with GRK2 degradation increases GPCR desensitization, thus it appears that the kinase activity is necessary for regulated protein degradation. This retarded degradation was observed in the presence of an inactive mutant, GRK2-K220R. Interestingly, increasing the level of β-arrestin1 or 2 promoted turnover in the mutant, indicating that β-arrestin plays an essential role in GRK2 degradation. In fact, the effect of β-arrestin in GRK2 degradation was found to be mediated by the recruitment of c-Src to a specific subcellular context. Tyrosine phosphorylation of GRK2 by c-Src is essential for GRK2 degradation via the proteasome pathway [89]. However, tyrosine phosphorylation is not the only signal that can target this protein for degradation. MAPK-mediated phosphorylation can also target GRK2 degradation via the proteasome pathway.

1.3.3 Receptor Transactivation Cascades/Interaction with other Receptors

GRKs and arrestins also interact with non-GPCRs, namely TβRs, epidermal growth factor receptors (EGFRs) and insulin growth factor receptors (IGFRs), among others. Hence, GRK/arrestin-mediated regulation of GPCR signaling may be indirectly affecting signaling of, or may be indirectly affected by, such growth factor receptors.

1.3.3.1 Epidermal Growth Factor Receptor (EGFR)

AngII can transactivate the EGFR, leading to the activation of the Ras/ERK cascade, Akt, p38-MAPK and to the subsequent growth and migration of vascular smooth muscle

cells (VSMCs). This activation can occur in various ways and possibly even through an intracellular-signal-dependent cascade. In fact, phosphorylation of AT_1R on Y319 is reportedly required for EGFR transactivation in AngII-induced cardiac hypertrophy [97].

In VSMCs, AngII-induced EGFR transactivation was shown to regulate migration and hypertrophy. Metalloprotease inhibition attenuated AngII-induced ERK activation, and blocked growth and migration of AngII-stimulated VSMCs [97]. It was also shown that ERK and p38-MAPK exist downstream of EGFR transactivation induced by AngII.

1.3.3.2 Platelet Derived Growth Factor Receptor (PDGFR)

PDGFR and AngII comprise a complex signaling network, as PDGFR transactivation can be induced via the AT_1R in some cells. PDGFR transactivation mediates AngII-induced ERK activation in mesangial cells and ligand independent AngII-induced PDGFR transactivation occurs in VSMCs.

1.3.3.3 Insulin-like Growth Factor-1 Receptor (IGF-1R)

Touyz *et al.* (2003) suggested that AngII induces ROS production partially through IGF-1R transactivation, leading to p38-MAPK and ERK5 activation in VSMCs [97]. AngII-induced tyrosine phosphorylation of IGF-1R has been reported in VSMCs and it hs been suggested that IGF-1R transactivation is required for PI3K activation by AngII.

1.3.3.4 Rho/ROCK

In addition to its primary role in cytoskeletal reorganization and SMC Ca^{2+} sensitization, Rho has been implicated in cardiovascular remodeling associated with hypertension, atherosclerosis and other cardiovascular diseases [97].

In VSMCs, the AT_1R is coupled to $G_{12/13}$ and to Gq. In addition to activating the AT_1R, AngII also increases GTP-bound RhoA. Gq may participate in Rho activation. Activation of the Rho/ROCK pathway by AngII occurs in parallel with EGFR transactivation in VSMCs. Interestingly, activation of Rho/ROCK is specifically required for AngII-induced JNK activation and subsequent VSMC migration [97].

1.3.4 Role of GRK2 in Disease

1.3.4.1 GRK-Arrestin Knockout Mouse Models

Most of what we know about receptor regulation and signaling via GRK-arrestin pathways has been determined using model cell systems [87]. Although *in vitro* studies are indispensable tools, it is often unclear whether such discoveries can successfully be transferred into specific organs and elicit the same effects or have detectable physiological

consequences. Consequently, studies using knockout and transgenic mice, viral-mediated overexpression or shRNA knockdown, and genetic studies in human patients have recently begun to probe the physiological roles of GRKs and arrestins [87].

Genetic deletion of GRK2 in mice results in embryonic lethality after 15.5 days of gestation due to defects in cardiac development and function [13, 87, 93, 98]. This lethality highlights the critical role of GRK2, as no other kinase can compensate for its function. In general, it was found that GRK isoforms could not complement one another [95].

GRK2-heterozygous mice exhibit altered cardiac function, in contrast to knockout models for GRK3 or 5 that were viable without observable alteration in cardiac function. Hata and Koch (2003) performed extensive studies involving GRK2 overexpression in the hearts of mice and demonstrated that changes in GRK2 levels significantly influenced cardiac performance. They highlighted GRKs as potential targets for the treatment of heart failure, but emphasized that of the three GRKs studied in the heart (i.e., GRK2, 3, and 5), GRK2 is the most important for myocardial development and function.

Due to the ubiquitous nature of their expression, it is impossible to infer β-arrestin specificity based on their expression patterns. Knockout models did not exhibit visible abnormalities or phenotypes and most importantly, both β-arrestins could not be simultaneously knocked out, as embryonic lethality would ensue [98]. Nevertheless, it was shown that β-arrestin1 is critical for βAR desensitization, as increasing concentrations of heart-infused β-agonist isoproterenol resulted in enhanced βAR signaling.

β-arrestin2 knockouts have been used to study the *in vivo* desensitization of the opioid and chemokine CXCR4 receptors [98]. Studies showed that analgesic effect of morphine were potentiated and prolonged without developing tolerance, but developing dependence.

Both knockout and transgenic mouse models have confirmed the importance of GRK2 and β-arrestins in the regulation of cardiac physiology and of receptor desensitization, respetively. Results of these studies invite the further study and development of novel therapeutic strategies for diseases with GPCR deregulation [88, 93, 98].

1.3.4.2 Role of GRK2 in Disease

Altered GRK activity and expression have been found in cardiovascular diseases such as congestive heart failure and hypertension, in immune and inflammatory diseases such as rheumatoid arthritis or multiple sclerosis, in thyroid gland pathologies, opioid addiction, retinitis pigmentosa, ovarian cancer and in cystic fibrosis, among other pathologies [95]. Due to their widespread nature, it is not surprising that GRKs play a critical role in the

maintenance of various regulatory processes. As demonstrated by the embryonic lethality of $GRK2^{-/-}$ mouse models, and the cardiac dysfunction associated with heterozygous GRK2 expression model, GRK2 is one of, if not the, most important of the GRKs.

The following section briefly describes the role of GRKs in various systemic diseases, paying particular attention to the role of GRK2.

Immune System: Cells of the immune system have particularly high expression of GRK2 and 6. However, expression levels are altered in diseased states. For instance, lymphocytes from humans with rheumatoid arthritis or rats with acute adjuvant arthritis have decreased levels of GRK2 and 6 expression. Induced allergic encephalomyelitis and humans with hypertension demonstrate increased lymphocytic GRK2 expression, whereas cultured human T lymphocytes exposed to oxidative stress through H_2O_2 or by coculture with activated neutrophils have reduced GRK2 levels.

Studies using GRK2-heterozygous mice, show that T lymphocytes display increased chemotaxis toward the CCR5 receptor ligands CCL4 and 5, and toward the CCR1 ligand CCL3. The CCR5 receptor exhibits reduced phosphorylation in lymphocytes with reduced GRK2, but also exhibits enhanced signaling, which is consistent with a decrease in receptor desensitization [87]. Another GRK2-heterozygous mouse model system, this time for multiple sclerosis, illustrates how GRK2 can have both deleterious and protective effects. These mice display a more rapid onset of lymphoctye infiltration into the brain. However, unlike wild-type mice, they fail to undergo relapse.

Respiratory System: Mice lacking GRK3, but not GRK2-heterozygous mice, exhibit improved airway smooth muscle response and sensitivity to cholinergic agonist. Thus, suggesting that GRK3 play an important role in airway smooth muscle physiology [87].

Skeletal System: Osteoblasts express GRK2 and β-arrestin1. But, GRK2 C-terminal inhibitor-expressing mice demonstrate enhanced age-dependent bone remodeling [87].

Nervous System: GRK2 has widespread expression in the brain, where more than 90% of known GPCRs are located. Expression of GRK2 is developmentally regulated, with a marked increase during the second postpartum week. Alterations in its expression have been described in several disorders and/or following pharmacological treatments [87].

GRK2 has also been implicated in the development of opioid tolerance and dependence, in the regulation of corticotrophin releasing factor receptor type 1 in the anterior pituitary gland, in the regulation of NCS-1 expression in schizophrenia and in bipolar disorder, and in hypoxia-induced brain damage [87].

Hypertension: GRK2 expression in blood lymphocytes is positively correlated with blood pressure and negatively correlated with β2AR-mediated adenylyl cyclase activity, suggesting that GRK2 may reduce β2AR responsiveness in a hypertensive state. Hypertensive rats reportedly have increased VSMC expression of GRK2 [87]. Another study identified PKC-activating agents as potential inducers of GRK2 in hypertension [94].

Cardiovascular System: GPCRs are critical regulators of cardiovascular integrity. By extension, so are GRKs and arrestins, as they control the sensitivity of receptor responses. Consequently, GRKs are implicated in the pathophysiology of human diseases.

Studies in transgenic mice have helped establish GPCR-GRK specificity in the heart. Using overexpression or knockout constructs, a critical role for GRK2 in the regulation of both GPCR and myocardial function was found [94]. In fact, a study by Ungerer *et al.* (1993) first involved GRK2 in the link between βAR desensitization and heart failure. This study found elevated GRK2 mRNA and activity levels in end-stage failing heart samples.

In the early stages of heart failure, the sympathetic nervous system has been shown to stimulate GRK2 expression in response to stress, suggesting that GRK2 expression *in vivo* is subject to regulation by circulating catacholamines. Catecholamines (i.e. the sympathetic neurotransmitter norepinephrine and the adrenal hormone epinephrine) bind to myocardial β1- and β2AR, leading to a conformational change, and thus permitting coupling with Gs proteins. The heterotrimeric Gs protein dissociates into two subunits, the GTP-binding Gα subunit that stimulates adenylyl cyclase (hence the designation Gs) and the Gβγ heterodimer involved in downstream effector activities that regulate cardiac function. Recruitment of GRKs and the subsequent phosphorylation of βAR lead to the recruitment of β-arrestins in order to mediate receptor internalization and desensitization. However, in heart failure, the loss of receptor signaling is associated with an approximate threefold elevation in myocardial GRK2 expression and GRK activity [93]. In fact, GRK2 upregulation commonly occurs in the early pathogenesis of heart failure, often preceding the development of clinical symptoms. Use of a GRK2 overexpressing its C-terminus restored βAR signaling, as well as basal GRK2 activity in myocytes, suggesting that GRK2 represents a potentially powerful therapy for the rescue of failing cardiomycytes [93].

However, controversy surrounds the protective *versus* maladaptive roles that GRKs play in the heart. Originally, elevated GRK2 levels were thought to be cardioprotective. Experimental models have proven otherwise, showing that inhibition of GRK2 activity, by blockade of the Gβγ interaction, reverses βAR desensitization and improves cardiac

performance [93]. Consequently, current therapies for heart failure include βAR-antagonists and angiotensin converting enzyme (ACE) inhibitors in order to decrease GRK2 expression and prevent βAR desensitization.

Some effects on the cardiovascular system are mediated through other organs. For instance, GRK2 has a quite unexpected role in the liver, where it is an important regulator of portal blood pressure [87]. GRK2-heterozygous mice were found to be resistant to liver injury-induced portal hypertension. Normally, Akt phosphorylates endothelial nitric oxide synthase (eNOS) through a G protein βγ-subunit-activated PI3K-dependent mechanism, which increased nitric oxide production.GRK2 then phosphorylated and desensitized the endothelin receptors. Instead, GRK2 bound Akt, inhibiting its kinase activity and reducing eNOS phosphorylation as well as nitric oxide release. Thus, GRK2 acts in a noncatalytic manner to reduce Akt-mediated signaling. It is possible that this GRK2-mediated inhibition of Akt may be a prominent regulatory mechanism in a variety of diseases.

1.4 ACTIVATION OF SIGNALING PATHWAYS & CROSSTALK MECHANISMS

1.4.1 MAPK Families

MAPK pathways are widespread in eukaryotic cells, regulating diverse biological activities such as gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation [99-102]. Five mammalian groups have been characterized: (i) ERK1/2, (ii) JNK1, 2, and 3, (iii) p38 isoforms α, β, γ, and δ, (iv) ERK3 and 4, and (v) ERK5.

MAPKs can be activated by various stimuli. ERK1/2 are preferentially activated by growth factors and phorbol esters, while JNK and p38 kinase are more responsive to stress stimuli, such as osmotic shock, ionizing radiation and cytokines [99, 100].

Interestingly, each MAPK family is composed of a set of three evolutionarily conserved, sequentially acting kinases: MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK). MAPKKKs are serine/threonine kinases [99] that are activated through phosphorylation and/or as a result of their interaction with small GTP-binding proteins of the Ras/Rho family, in response to extracellular stimuli. Its activation leads to the phosphorylation and activation of MAPKK, which then stimulates MAPK activity through dual phosphorylation on threonine and tyrosine residues located in the activation loop of the kinase subdomain VIII. Once activated, MAPKs phosphorylate target substrates on serine or threonine residues followed by a proline [100]. However, substrate selectivity is often conferred by specific interaction motifs located on physiological

substrates, whereas cascade specificity is also mediated through interaction with scaffolding proteins, such as β-arrestins [99]. Scaffold proteins organize pathways into specific modules through the simultaneous binding of several components.

MAPKs are activated by substrate phosphorylation, including phospholipases, transcription factors, and cytoskeletal proteins. They also catalyze the phosphorylation and activation of several protein kinases, termed MAPK-activated protein kinases (MKs).

A MAPKKK of note is TGFβ-activated kinase-1 (TAK1). Together with the Smads, it plays a critical role in the transduction of TGFβ1 signals in cardiomyocyte differentiation and cardiac development [103].

1.4.1.1 ERK1/2

Properties: Known as the classical mitogen kinase cascade, ERK1/2, is composed of MAPKKKs (A-, B-Raf, and Raf-1), MAPKKs (MEK1/2), and MAPKs (ERK1/2). Widely expressed, they are strongly activated by growth factors, phorbol esters, serum and to a lesser extent by GPCR ligands, cytokines, osmotic stress, and microtubule disorganization [100]. ERK signaling is facilitated by a β-arrestin scaffold bound to FilaminA [99].

Activation Mechanisms: GPCRs and RTKs transmit activating signals to the Raf/MEK/ERK cascade through different isoforms of the small GTP-binding protein, Ras. Recruitment of the Ras-activating guanine nucleotide exchange factor, Son of Sevenless (SOS), activates membrane-associated Ras, which then interacts with a wide range of downstream effector proteins, including isoforms of the serine/threonine kinase, Raf. Regulation of both Ras and Raf is crucial for proper maintenance of cell proliferation, as activating mutations in these genes often leads to oncogenesis [100].

Activated Raf binds to and phosphorylates the dual-specificity kinases MEK1/2, which in turn phosphorylate ERK1/2 within a conserved TEY motif in their activation loop. Amplification through the signaling cascade is such that activation of solely 5% of Ras molecules is sufficient to induce full activation of ERK1/2 [100, 104].

The GPCR ligand, AngII, has been shown to transactivate ERK signaling [105], obtaining a maximal response within 5min in VSMCs [106] and 2min in HEK-293s [99]. ERK activation leads to various responses in cell growth and proliferation [107]. As such, TGFβ has also been shown to activate ERK signaling [3].

ERK-activation can also occur via Ras-independent mechanisms, as in the presence of high extracellular Ca^{2+} levels. Calcium stimulation leads to the downreagulation of Ras activity, rendering the cell temporarily insensitive to mitogenic ERK activation [108].

Schmidt *et al.* (2000) propose that ERK activation, in response to Ca^{2+} stimuation, is mediated by activation of both MEK and Raf isoforms [108].

Substrates and Functions: ERK1/2 are distributed throughout quiescent cells, but accumulate in the nucleus upon stimulation caused by nuclear retention, dimerization, phosphorylation, and release from cytoplasmic anchors. Activated ERK1/2 phosphorylate numerous substrates in all cellular compartments, including various membrane proteins (Syk and calnexin), nuclear substrates (c-Fos, c-Myc, and STAT3), cytoskeletal proteins (neurofilaments and paxillin), and several MKs.

ERK1/2 signaling has been implicated as a key regulator of cell proliferation, as it activates cell cycle progression protein, Elk-1 [99]. Consequently, ERK pathway inhibitors are entering clinical trials as potential anticancer agents [100]. ERK has also been associated with AngII-induced TGFβ-independent Smad activation in renal fibrosis [2].

1.4.1.2 MAPK-Activated Protein Kinase (MKs)

The MK family comprises 11 members activated by mitogens acting through the ERK1/2 kinase cascade (RSK1-4, and MNK2), stress stimuli acting through the p38 kinase cascade (MK2/3, and possibly MK5), or both (MSK1/2 and MNK1) [100]. Based on sequence homology, MKs are classfied into five subgroups, the RSKs, MSKs, MNKs, MK2/3, and MK5.

RSK: RSK, MSK, and MNK represent three kinase subfamilies of ERK1/2 substrates. RSK family members are exclusively activated by ERKs [100].

Discovery: Discovered in *Xenopus laevis,* RSK1 was the first MK identified. Today, homologues called p90 ribosomal S6 kinases (RSK) are present in most vertebrates.

The RSK family contains four human isoforms (RSK1-4), showing 73-80% sequence identity. Homologues are particular among the serine/threonine kinases in that they contain two distinct and functional kinase domains, the C-terminal kinase domain (CTKD) and the N-terminal kinase domain (NTKD). The RSK CTKD belongs to the Ca^{2+}- and calmodulin-dependent kinase (CaMK) group and the RSK NTKD belongs to the AGC kinase family, which includes PKA, PKC, Akt, and p70 ribosomal S6 kinases 1/2 (S6K1 and -2) [100].

Structure and Expression: All four human RSK isoforms have a similar structure, with both kinase domains joined by a linker region. Although NTKD is responsible for substrate phosphorylation, both kinase domains can autophosphorylate sites important for RSK1/2 activation. All RSK isoforms also contain a C-terminally located docking domain (D domain) responsible for docking and activation by ERK1/2.

In cells, RSKs and ERK1/2 are known to interact but dissociate upon activation. In quiescent cells, RSK1-3 and ERK1/2 are usually cytoplasmic, but upon stimulation, they translocate to the nucleus of activated HeLa, COS-7, and HEK-293 cells. RSK3 is the only human isoform to possess a potential NLS but it is unclear whether this domain is functional or if other regions are required. It appears likely that the RSKs are widespread within the cell and that their localization is regulated by multiple mechanisms.

Activation Mechanisms: All RSK isoforms contain six phosphorylation sites shown to be responsive to mitogen stimulation in both RSK1/2. Of these, phosphorylation of the turn motif (S363) in RSK1/2, is essential for kinase activity. Interestingly, *in vivo*, PKA and C have been shown to autophosphorylate on the homologous site. This site is modified by a heterologous kinase in Akt, suggesting that the NTKD of the RSKs or a membrane-associated kinase may also phosphorylate the RSK isoforms at this site.

The serine/threonine kinase, 3-phosphoinositide-dependent kinase 1 (PDK1), phosphorylates the NTKD activation loop of the RSKs, thereby activating the NTKD. PDK1 is also required for the activation of many AGC kinases, as mitogen stimulation of PDK1-null embryonic stem cells did not lead to the activation of either RSK1, Akt, or S6K1 [100]. Phosphorylation of the CTKD activation loop was thought to be mediated by ERK1/2, but mutation of this site only partially reduced RSK1/2 activation. Interestingly, mitogen-activated ERK1/2 may promote RSK1 activation by mediating its translocation to the plasma membrane, where it can be activated by membrane-associated kinases.

Finally, the catalytic activity of RSK1 is negatively regulated by its interaction with 14-3-3β at a phosphoserine-containing motif. Because RSKs have been shown to interact with upstream activators (ERK1/2 and PDK1), various downstream substrates, and regulatory proteins such as 14-3-3β, they appear to function as scaffold proteins that allow multiple proteins to come together and form a signaling network [100].

Substrates and functions: Although RSK1 was first discovered as a kinase that phosphorylated the ribosomal subunit protein S6, this protein is not a major physiological target of RSK. Substrate specificity for target phosphorylation was found to require the minimum sequence Arg/Lys-Xaa-Arg-Xaa-Xaa-pSer/Thr or Arg-Arg-Xaa-pSer/Thr. It appears that RSK1 prefers to phosphorylate Ser rather than Thr residues by a factor of 5. However, it remains unclear whether all RSK isoforms share the same target substrates. Most RSK functions, such as transcriptional regulation, cell cycle regulation, and cell survival, can be determined by the nature of their substrates.

Transcriptional regulation by RSK: Immediately after activation and translocation, RSKs and ERKs are capable of phosphorylating multiple transcription factors that contribute to the induction of immediate early (IE) genes, namely the transcription factors serum response factor (SRF) and CREB and possibly the chromatin-associated histone H3. Additionally, many IE gene products, such as c-Fos and c-Jun, are also phosphorylated by the RSKs, providing a dual mechanism of IE gene control. In fact, some IE genes act as molecular sensors for ERK and RSK nuclear localization, signal duration, and signal strength, such that they can enhance IE product stability, promoting further phosphorylation events and increasing physiological effects.

Stimulation of the ERK pathway promotes the interaction between RSK1 and the transcriptional coactivator CBP. CBP and p300 are known to associate with several transcription factors that also known RSK1/2 substrates, such as CREB, c-Fos, c-Jun, ER81, and NF-κB. These associations suggest that RSK1 binding to CBP may provide a second mechanism for transcriptional control [100]. In fact, ER81 performs many essential functions in homeostasis, signaling response, and development. Transcription initiator factor (TIF)-1A also becomes phosphorylated by ERK1/2 and RSK2, following stimulation. TIF-1A is required for RNA polymerase I transcription and for rRNA synthesis. Interaction with such regulatory proteins suggests that both RSK1/2 and ERK1/2 are also involved in these regulatory processes.

RSK and cell cycle control: In addition to contributing to IE gene response during the G_0/G_1 phase, RSK1/2 may also promote G_1-phase progression through the phosphorylation and subsequent inhibition of the CDKI, $p27^{kip1}$ [100]. Phosphorylation of $p27^{kip1}$ promotes its association with 14-3-3, preventing its translocation to the nucleus.

ERK1/2 and RSKs may also regulate progression through the G_2 phase, through the phosphorylation and inhibition of Myt1 kinase, reducing its ability to inhibit the kinase activity of Cdc2/cyclin B1 complexes.

RSK2 also regulates the cell cycle through phosphorylation of histone H3. This process requires activation of the MAPK-RSK pathway, which contributes to chromatin remodeling and to increased transcriptional regulation of several genes.

RSK promotes survival: Studies indicate that RSK1/2 regulate survival in proliferating and differentiated cells. Survival can be promoted through transcription-dependent and independent methods, through the creation of a caspase-inhibitory box that binds and inhibits caspase-1 and -8, or through the activation of the NF-κB transcription factors.

Other targets of RSK: RSKs phosphorylate many other targets, such as polyribosomes, the Na^+/H^- exchanger isoform 1 (NHE-1), the tumor suppressor LKB1, the cell adhesion molecule L1, and the cytoskeleton-associated protein Filamin A, suggesting that it play a regulatory role in both cell proliferation and cytoskeletal remodeling.

Proper regulation of the Ras/ERK pathway through phosphorylation-mediated negative feedback occurs at many levels. In fact, $RSK2^{-/-}$ mouse fibroblasts displayed higher and more sustained ERK phosphorylation, suggesting that RSK2 inhibits the ERK1/2 signaling cascade [100]. Interestingly, EGF-mediated Akt stimulation was also higher in $RSK2^{-/-}$ cells, suggesting that Akt may compensate for the loss of RSK2 or that RSK2 is involved in feedback inhibition of the PI3K/Akt pathway through an unknown mechanism [100].

PI3K/Akt: AngII activates PI3K/Akt via EGFR transactivation [105]. PI3K catalyzes the phosphorylation of PIP2 to phosphatidylinositol-3,4,5-triphosphate (PIP3) and, when activated, promotes cell growth and inhibits apoptosis [99].

In human lung fibroblasts, the PI3K/Akt pathway regulates cell viability by mediating the β1-integrin interaction with the ECM. It is also responsible for stimulation of collagen synthesis in cultured human dermal fibroblasts. Moreover, a recent study has shown that synchronous activation of the PI3K/Akt pathway is required for collagen and ECM production in cultured keloid-derived fibroblasts. These findings suggest a crucial role in the development of fibrosis and keloids [105].

Akt has been shown to inhibit Smad translocation to the nucleus by directly binding to their MH2 domain and linker region, sequestering them in the cytoplasm [38], and thus inhibiting Smad-mediated apoptosis. Moreover, several studies suggest that TGFβ directly modulates the PI3K signaling pathway [8]. It was shown that TGFβ-stimulated PI3K/Akt pathway activation resulted in increased activation of Smad3, leading to transcriptional activation of collagen I expression [8], finally leading to the development of tissue fibrosis.

1.4.1.3 p38

Properties: The model member of the second MAPK-related pathway in mammalian cells is p38. This group consists of MEKK1-4, MEK3 and 6, and the known p38 isoforms (α, β, γ, and δ) [38, 99, 100]. Although p38α has 50% amino acid identity with ERK2, the p38 isoforms are strongly activated by environmental stresses and inflammatory cytokines, unlike the ERKs [99, 100]. In fact, most stimuli that activate p38 also activate JNK.

Activation Mechanisms: MEK3 and 6 show a high degree of specificity for p38, as they do not activate ERK1/2 or JNK. However, MEK4, a known JNK kinase, possesses

some MAPKK activity toward p38, suggesting that it might act as a possible site of signal integration between the p38 and JNK pathway [100]. While MEK6 activates all p38 isoforms, MEK3 preferentially phosphorylates the p38α and β isoforms.

Recently, the κ-opioid receptor was shown to activate p38 by a mechanism involving GRK3 phosphorylation and β-arrestin2 [99], demonstrating GCPR-mediated signaling interaction. Similarly, AngII was shown to regulate p38 through redox-sensitive signaling cascades in VSMCs [106]. Recent studies also showed that TAK1 can activate p38, which results in the phosphorylation of the Smad2/3 linker region. These results located p38-MAPK upstream of Smad signaling [8] and suggest that it can modulate Smad activity.

Substrates and Functions: p38 regulates cytokine expression, transcription factors MEF2, ATF-2, ELK-1, Ets-1, p53 [8] and cell surface receptors. Thus, p38 is critical for normal immune and inflammatory responses. Found in the nucleus and in the cytoplasm of quiescent cells, precise localization after cell stimulation is not well understood [100].

1.4.1.4 JNK

Properties: The JNK/SAPK family was originally isolated from rat livers injected with cycloheximide. It consist of JNK1-3, ubiquitously expressed in 10 or more spliced forms.

Activation Mechanisms: JNKs are strongly activated in response to cytokines, UV irradiation, growth factor deprivation, DNA-damaging agents, and to a lesser extent by some GPCRs, serum, and growth factors [100]. Like ERK1/2 and p38, JNK activation requires dual phosphorylation on tyrosine and threonine residues within a conserved TPY motif. MEK 4 and 7 are the MAPKKs that catalyze this reaction [100]. AngII, on the other hand, regulates p38-MAPK through redox-sensitive signaling cascades in VSMCs [106]. It also activates p21-activated kinase 1 (PAK1), which subsequently mediates JNK activation and hypertrophy [97]. However, JNK3, which is present primarily in the brain [100], is activated by β-arrestin2 signaling pathways [99].

Substrates and Functions: Targets of JNK include the transcription factors c-Jun [100], ATF-2, ELK-1, and p53 [8]. However, due to limited nuclear translocation following stimulation, JNK has an increasing list of cytoplasmic substrates. As a result, JNK can phosphorylate SMADs, enhancing heterocomplex formation and nuclear translocation [38]. Interestingly, JNK-Smad interaction has been attributed to accelerated apoptosis in ischemic hearts [38]. Alternatively, JNK appears to be indispensable for AngII-induced VSMC migration [97].

1.4.2 Docking Interactions in the MAPK Cascade

1.4.2.1 Description of Docking Sites

D domains: Efficiency and specificity of signaling within the MAPK cascade is achieved through specialized docking motifs present in scaffold proteins, MAPKKs, MAPKs, and their substrates [100]. These D domains are characterized by a cluster of positively charged residues surrounded by hydrophobic residues and are located either upstream or downstream of the phospho-acceptor site on MAPK substrates.

Docking Site for ERK and FXFP (DEF domains): This class of MAPK docking sites is recognized only by ERK1/2 and typically lies C-terminal to the phospho-acceptor site. DEF domains are required for efficient substrate phosphorylation by ERK1/2 [100].

Common Docking (CD) and ERK Docking (ED) motifs: A conserved C-terminal CD motif, located outside of the ERK, p38, and JNK catalytic domain, was found to mediate MAPK interactions with upstream activators and downstream substrates. The CD motif contains acidic hydrophobic residues necessary for interaction with positively charged hydrophobic residues of the D domains. Similarly, the ED motif, located opposite the MAPK active center, is thought to regulate binding specificity. Exchange of only two residues within the ED site of ERK2 altered its binding specificity to that of p38 [100].

The N-terminal domain of MAPKs are involved in docking specificity, however the exact requirements remain to be determined.

1.4.2.2 Docking Interactions with MKs

Properties: Almost all MKs possess a D domain motif necessary for ERK1/2 docking to RSKs, for ERK1/2 and p38 binding to MSK2, and for p38 docking to MK5. The number and location of hydrophobic residues within the D domain regulates MAPK specificity.

Regulation of docking: MKs, such as RSK, bind ERK1/2 and/or p38 in quiescent cells and dissociate following stimulation. Although the mechanisms involved in complex dissociation are currently unknown, mutational analysis revealed that autophosphorylation of a residue near the RSK1 D domain was required for regulated release of ERK1/2 [100].

1.5 GPCR LIGAND: ANGIOTENSIN II

The octapeptide hormone, AngII, is the major effector molecule of the renin-angiotensin system (RAS) [97, 109]. Classically viewed solely as a hormonal circulating system involved in the regulation of blood pressure cardiovascular homoeostasis, salt and fluid homeostasis, the RAS is now seen as autocrine/paracrine endocrine system [97, 110].

Renin, produced by the kidneys in response to decreased intra-renal blood pressure or decreased delivery of Na^+ or Cl^-, cleaves the peptide bond between Leu-Val residues on angiotensinogen. Cleavage of liver-produced angtiotensinogen results in AngI, which does not appear to have a physiological role aside from being the precursor to AngII. Through the action of ACE, which is present on the luminal surface of the vascular endothelium, AngI is converted to AngII by the cleavage of the terminal Phe-His bond [110].

AngII plays a central role in the control of blood pressure through its actions on vascular smooth muscle contractility, aldosterone secretion from adrenal glomerulosa cells, ion transport in renal tubular cells, and dipsogenic responses in the brain [109]. Because AngII regulates cell growth, inflammation, and fibrosis, it plays a central role in various cardiovascular diseases, such as hypertension, atherosclerosis, restenosis after angioplasty and heart failure [23, 97]. In fact, among the various factors involved in vascular fibrosis, AngII has a predominant role. AngII can activate the Smad pathway, independently of TGFβ, and shares many intracellular signals implicated in fibrosis with TGFβ [23].

There are at least two seven transmembrane GPCRs known to mediate AngII function in mammalian tissue, the AT_1R and the AT_2R [97, 109]. The AT_1R mediates most of the physiological and pathophysiological actions of AngII in VSMCs, as it is predominantly expressed in cardiovascular cells [97]. However, some of the functions of the AT_1R on the cardiovascular system are mediated through other organs. In addition to the vasculature, AT_1R is expressed in the kidney, heart, adrenal gland, brain, lung and adipose tissues.

Upon binding to AT_1R, AngII activates various signaling molecules, including small G proteins Ras, Rho and Rac, which play important roles in cardiovascular remodeling. Via EGFR/ErbB1 transactivation, AngII-mediated Ras activation causes vascular hypertrophy and hyperplasia, and subsequently induces the Raf/ERK and PI3K/Akt pathways. Rac is an important component of the reduced NADPH oxidase complex. In VSMCs, it is involved in AngII-mediated PAK1 activation, which leads to JNK activation and hypertrophy.

1.5.1 Receptors

1.5.1.1 AT_1R

AT_1R is the most predominantly expressed receptor in cardiovascular cells, and thus mediates most of the physiological and pathophysiological actions of AngII in VSMCs. Via the AT_1R, AngII activates a number of cytoplasmic signaling pathways that contribute

to vasoconstriction, SMC motility and growth, aldosterone secretion, and vascular remodeling. It also induces VSMC hypertrophy, hyperplasia and migration [35, 97].

Receptor stimulation promotes interaction with multiple heterotrimeric G proteins, including $G_{q/11}$, G_i, G_{12} and G_{13}. However, the main signal transduction pathway of AT_1R, involves the activation of PLC through the $G_{q/11}$ family of G proteins. These interactions produce second messengers, inositol trisphosphate (IP3), DAG, ROS, Ca^{2+} signals and PKC activation, which then activate their own respective signaling pathways [97, 109].

AngII binding also causes rapid desensitization and internalization of AT_1R. Agonist stimulation of AT_1R leads to GRK2 phosphorylation of the C-terminal tail of the receptor and β-arrestin recruitment. β-arrestin does not dissociate from the receptor and accompanies it into the cell, where the complex may reside for extended periods of time in endosomal vesicles before being sorted to lysosomes or slowly recycled [35]. Recently AT_1-receptor-associated protein (ATRAP) was identified as a negative regulator of AT_1R signal transduction. ATRAP binds to the C-terminal domain of AT_1R and promotes AT_1R recycling to the plasma membrane [97]. Structurally, the intracellular third loop of AT_1R is essential for receptor-G protein coupling. Also essential for signaling, desensitization and internalization is the AT_1R receptor C-terminal cytoplasmic domain, which directly associates with several non-G protein signaling molecules, such as JAK2 and PLCγ-1. The C-terminal tail also provides a binding site to form AT_1R homo and hetero-dimers [97].

Endothelial Signaling of AT_1R: Not much is known about AngII signaling in endothelial cells (EC). However, recent evidence suggests that AngII-mediated AT_1R activation is a critical factor in endothelial dysfunction caused by oxidative stress, possibly caused by an alternative of nitric oxide (NO) function and by an induction of vascular insulin resistance [97]. Dysfunctional EC are characterized by less NO production, causing accelerated vasoconstriction, SMC proliferation, inflammation and a prothrombotic state.

1.5.1.2 AT_2R

The second subtype of AngII receptors expressed in mammalian tissues is AT_2R, which shares ~30% sequence identity with AT_1R [109]. In contrast to the lifelong expression of the AT_1R, AT_2R is abundantly and transiently expressed in fetal and neonatal tissues. Although it is present only at low levels in adult tissues, it is upregulated in certain pathophysiological conditions such as vascular injury, myocardial infarction, heart failure and skin wound healing [105].

Reportedly, AT_2R activation counteracts the actions of AT_1R [97, 105], although the precise mechanisms involved are unknown. However, it has been suggested that AT_2R expressed in the endothelium may counteract the effects of AT_1R to prevent endothelial dysfunction, possibly through NO production [97]. In fact, eNOS phosphorylation through AT_2R was also reported in aortic ECs [97].

A significant role for AT_2R has been described in human skin fibroblasts. In the fibroblasts of patients with diffuse cutaneous systemic sclerosis (SSc), the AT_2R is upregulated and inhibits AngII-mediated procollagen production [105], counteracting the effects of AT_1R signaling. Moreover, Liu, Cheng *et al.* demonstrated that AngII regulates the PI3K/Akt pathways via a negative crosstalk between the two receptors.

AT_2R has also been implicated in cell growth inhibition, renal inflammatory cell recruitment [23], as well as in AngII-induced gene expression of ECM [103].

1.6 CROSSTALK BETWEEN ANGIOTENSIN II & TGFβ

As the complex TGFβ signaling pathway is deciphered, it is becoming increasingly evident that crosstalk mechanisms between TGFβ and other signaling pathways are involved in the progression of human disease. In fact, the crosstalk between AngII and TGFβ is well established [2, 23, 103]. It has been reported that AngII upregulates TGFβ expression via activation of the AT_1R in various cells, including cardiac myocytes [103], myofibroblasts [103], fibroblasts [103], VSMCs [3], mesangial cells [3], and in the lung [8] via direct or indirect mechanisms. In fact, AngII regulates TGFβ expression at the transcriptional level, as induction of TGFβ expression does not occur in the presence of actinomycin D [103]. Recently, AngII-dependent TGFβ upregulation was found to be mediated by NAD(P)H oxidase, and by the subsequent activation of PKC, p38-MAPK, and nuclear AP-1 binding activity [103].

Originally described as a potent vasoconstrictor, AngII is now recognized as a multifunctional hormone, influencing many cellular processes including cell growth, apoptosis, migration, inflammation, and fibrosis. It has therefore been postulated that AngII may not directly stimulate fibrosis but may do so indirectly by inducing the expression of growth factors such as TGFβ, which then act via auto/paracrine mechanisms. In fact, it has been well documented that AngII and many other factors induce renal, pulmonary and cardiac fibrosis by stimulating TGFβ production [2, 8, 103]. These studies suggest crosstalk between various pathways such as the TGFβ signaling cascade, which

appears to act downstream of AngII, promoting remodeling of the cellular environment and progression of fibrosis. It appears that induction of TGFβ is absolutely required for Ang II-induced cardiac hypertrophy in vivo [103]. AngII can directly activate transcription of collagens and fibronectin. Strong evidence supports the notion that the long term *in vitro* and *in vivo* profibrotic effects of AngII are mediated by paracrine release of the "most potent profibrotic cytokine identified to date", TGFβ [111].

Although TGFβ can counteract the proliferative effect of AngII on VSMCs, the increasing number of diseased systems expressing high levels of both AngII and TGFβ prompted a gene expression profiling experiment, that identified the AT_1R gene as a novel TGFβ target [8]. TGFβ stimulation activates AT_1R gene transcription (Fig.8), leading to upregulation of steady-state mRNA levels of AT_1R and increased AT_1R protein densities. Currently, very little information is known regarding the mechanisms by which TGFβ1 enhances AT_1R gene expression [8]. However, it has been postulated that TGFβ treatment enhances AT_1R expression by the synergistic interaction between the Smads and specific kinase signaling pathways that are simultaneously activated by TβRI [8]. These effects were observed in lung fibroblasts, adrenal cells, and trophoblasts [8].

Use of ACE inhibitors and AT_1R antagonists, to inhibit AngII, was shown to reduce expression of TGFβ and Smad activity in tissues, which resulted in reduced myocardial hypertrophy/fibrosis in *in vivo* rat models [23, 38, 103]. Similarly, use of TGFβ neutralizing antibodies or truncated TβRII [3], diminished AngII-induced cardiac and vascular fibrosis, and prevented hypertension-induced end-organ damage in hypertensive rat models and in age-induced cardiac fibrosis [111]. In addition, blockade of TGFβ also diminished AngII-induced ECM production. Interestingly AngII and TGFβ share some intracellular mechanisms involved in fibrosis, including activation of protein kinases, production of growth factors and activation of the Smad pathway [3, 23]. Interestingly, AngII has been shown to activate an early Smad signal, independently of TGFβ, via the ERK1/2 MAPK signaling pathway [3, 4, 8]. TGFβ activation has been linked to long-term exposure and chronic infection [4].

Also of note, the p38-MAPK, JNK, and PI3K signaling pathways are also involved in TGFβ-induced increase in AT_1R density, as elimination of any of these signaling pathways attenuates the potential of TGFβ to stimulate AT_1R expression [8]. It was shown that the Smad and kinase signaling pathways do not act independently but involve some level of intracellular crosstalk or scaffold, supporting the hypothesis that a self-potentiating loop

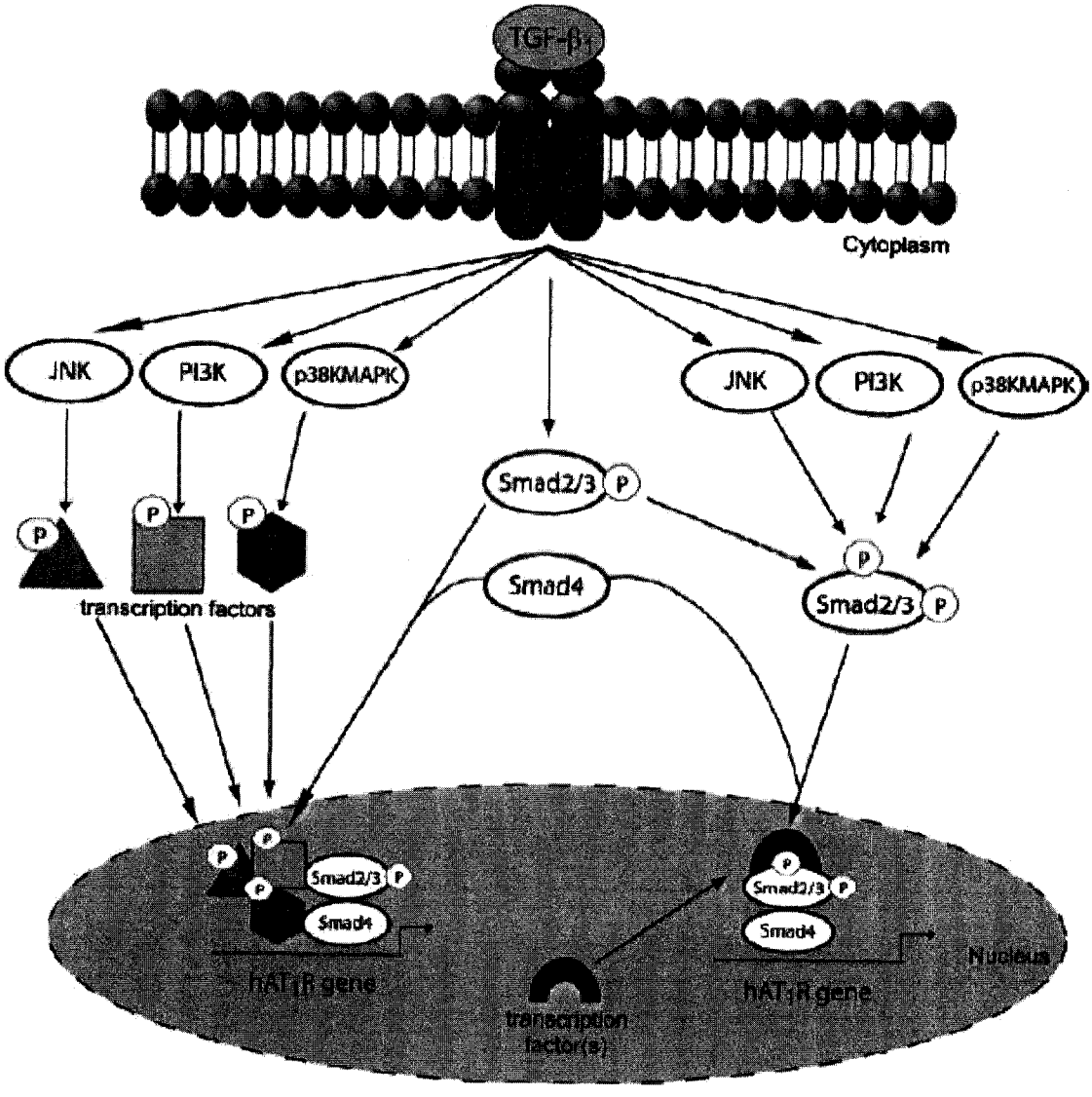

Fig.8: Proposed model for the synergistic activation of TGFβ-mediated activation of AT_1R gene expression

Fig.8: Proposed model for the synergistic activation of TGFβ-mediated activation of AT_1R gene expression

This figure represents a model of the proposed synergistic interaction among TGFβ activation of the Smads, the PI3K, the p38, and the JNK signaling pathways, to augment hAT_1R gene expression. Traditionally, TGFβ signaling is initiated by ligand binding to the transmembrane receptors, TβRI and TβRII. The activated TβRI subsequently phosphorylates Smad2 and 3 within their conserved C-terminal SSXS motif. These activated Smad proteins, together with Smad4, translocate to the nucleus and regulate the transcription of target genes. Our study demonstrates that there is intracellular crosstalk between the described Smad pathway and the PI3K, p38K, and JNK signaling pathways. It has been proposed by Martin, Buckenberger *et al.* (2007) that activation of PI3K, p38K, and JNK by TβRI/TβRII leads to phosphorylation (P) of Smad2/3 at additional ser/thr sites located in the linker region of these proteins. The hyperphosphorylated Smad2/3, together with Smad4, are translocated to the nucleus, specific co-activators are recruited to the transcriptional complex, and hAT_1R gene expression is stimulated. Alternatively, TGFβ activation of PI3K, p38K, and JNK may result in the direct or indirect phosphorylation of distinct transcription factors, which translocate to the nucleus and merge their signals with the activated R-Smad/Smad4 complex, and hAT_1R gene expression is subsequently activated (Martin, Buckenberger *et al.* 2007)

exists between AngII and TGFβ [2, 8]. In fact, Smads have been proposed to be the major point of signal integration [2]. Activation of Smad3 but not Smad2 was found to be a key mechanism by which AngII mediates arteriosclerosis [4]. Contrarily, Euler-Taimor and Herger (2006) showed that idiopathic hypertrophic cardiomyopathy remodeling and fibrosis was dependent on ERK and Smad2 activation [38]. Nevertheless, actual crosstalk between AngII and TGFβ has been located at the postreceptor level of Smad signaling.

TAK1 couples extracellular stimuli to gene transcription and has been shown to transduce TGFβ signaling along with the Smads [103]. Interestingly, AngII shares many intracellular signals implicated in fibrosis with TGFβ. Many of these fibrotic factors involve the activation of MAPK, RhoA/Rho-kinase, and redox mechanisms [3]. Activation of these pathways leads to numerous heterogeneous downstream events that play essential roles in the biological activities of AngII, such as cell growth, inflammation, migration, ECM production, apoptosis and stimulation of transcriptional activity via AP-1 binding [3, 4, 106]. Nevertheless, the Smad pathway is believed to mediate both TGFβ- and AngII-induced CTGF and ECM overexpression [2, 3, 23, 103], particularly in VSMCs and cardiac cells. Moreover,TGFβ activates TAK1 [103], which may serve as a mediator of AngII/TGFβ-associated cardiac remodeling, coupling AngII and TGFβ stimuli to fibrotic gene transcription, enhanced by AP-1. The AP-1 complex is involved in AngII-mediated TGFβ induction, whereby AngII activates AP-1 via transactivation of EGF, leading to increased expression of TGFβ mRNA and ERK activation [103].

Pleiotropic actions of AngII stimulation include vasoconstriction, cell growth, fibrogenesis, migration, and inflammation, all processes that are associated with activation of complex signaling pathways and dynamic remodeling of the actin cytoskeleton [106]. AngII-mediated ROS formation and activation of p38-MAPK and JNK, but not ERK1/2, require an intact actin cytoskeleton [106]. Disruption of the actin cytoskeleton with cytochalasin B results in the inhibition of AngII-mediated activation of p38-MAPK and JNK, but not of ERK1/2 [106]. These observations suggest that the cytoskeleton may be a central point of crosstalk in growth- and redox-signaling pathways by AngII [106].

An alternative explanation for the role of intracellular crosstalk amongst the Smad, PI3K, p38 MAPK, and JNK pathways in TGFβ stimulated AT1R expression is that each kinase pathway phosphorylates specific transcriptional coactivators that are necessary for activation of the AT_1R gene [8]. Based on various studies, there is a clear interdependence between Smad signaling and the specific pathways that regulate Smad signaling through

phosphorylation of the linker region [8], providing further evidence that targeting Smad signaling may be a more effective therapeutic strategy [2].

Both AngII and TGFβ are key mediators of fibrosis, particularly in the kidney and in the heart. Although this functional connection has been well characterized in recent years, only limited information is currently available on how they elicit cardiac growth responses [103]. Recently, the discovery of TGFβ-induced AT_1R upregulation has provided evidence of a positive feedback loop between AngII and TGFβ, resulting in the amplification of profibrotic effects [8]. A better understanding of the mechanisms involved in mediating this important crosstalk can provide novel therapeutic approaches for fibrotic diseases.

CHAPTER 2: TGFβ-INDUCED GRK2 EXPRESSION ATTENUATES ANGII-REGULATED VASCULAR SMOOTH MUSCLE CELL PROLIFERATION AND MIGRATION

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Manuscript to be submitted

2.1 ABSTRACT

Signaling initiated by the Transforming Growth Factor β (TGFβ) and angiotensin II (AngII) occurs through two structurally and functionally distinct receptor superfamilies, the serine/threonine kinase and the G protein-coupled receptors (GPCRs), respectively. Through diametric actions, TGFβ and AngII play important roles in regulating various biological responses such as cell proliferation and migration. Previously, we identified the G protein-coupled receptor kinase 2 (GRK2), a key regulatory factor in the desensitization of GPCRs, as a direct downstream target of the TGFβ signaling cascade. GRK2 acts through a negative feedback loop mechanism to terminate TGFβ-induced Smad signaling. To investigate the impact of TGFβ-induced GRK2 expression on GPCR signaling, we examined its effect on AngII signaling in vascular smooth muscle cells (VSMCs). In this study, we show that activation of the TGFβ signaling cascade in VSMCs results in increased GRK2 expression levels. This expression consequently inhibits AngII-induced ERK phosphorylation and antagonizes AngII-induced VSMC proliferation and migration. Moreover, the inhibitory effect of TGFβ on AngII signaling occurs at the MEK-ERK interface and is abrogated when an anti-sense oligonucleotide directed against GRK2 is used. Thus, we conclude that TGFβ signaling antagonizes AngII-induced VSMC proliferation and migration through the inhibition of ERK phosphorylation and that GRK2 is a key factor in mediating the crosstalk between these two receptor superfamilies.

2.2 INTRODUCTION

The transforming growth factor β (TGFβ) is a pleiotropic growth factor that regulates a diverse array of biological responses such as cell proliferation, survival and migration in a wide variety of cell types, including cardiac lineages [1]. Vascular smooth muscle cells (VSMCs) represent a unique myogenic lineage, as unlike their cardiac and skeletal counterparts, VSMCs do not terminally differentiate. Instead, they usually undergo cell cycle arrest, only to progress through cell division at a very low frequency [2, 3]. Under the pathophysiological conditions of many vascular diseases, such as atherosclerosis, quiescent VSMCs can, however, re-enter the cell cycle and mitosis [4, 5]. TGFβ signaling inhibits the proliferation and migration of VSMCs [6, 7]. In fact, TGFβ signaling induces VSMCs to undergo cell cycle arrest in the G_2 phase of the cycle [8], which is mediated by the activation of the protein kinase C-related kinase-1 (PRK-1). Subsequently, Cdc25C is inhibited in rat pulmonary arterial VSMCs [9, 10]. Disruption of the TGFβ signaling

pathway has been implicated in numerous human diseases, such as cancer, autoimmune, fibrotic and cardiovascular diseases [11].

TGFβ-induced signal transduction is initiated upon ligand binding to the homodimer of a single spanning transmembrane serine/threonine kinase type II receptor. Binding results in the recruitment of the type I receptor, forming a ligand-bound heterotetrameric receptor complex. Activation of the type I receptor kinase activity is induced upon its phosphorylation by the kinase domain of the type II receptor. Once activated, the type I receptor kinase phosphorylates the primary intracellular mediators of the TGFβ signaling cascade, Smad2 and Smad3, on a carboxyl-terminal serine motif. Phosphorylated Smad2 and Smad3 can then associate with the common partner, Smad4, and translocate into the nucleus where the heterocomplex associates with transcriptional co-activators or co-repressors, resulting in the subsequent activation or repression of various target genes [12]. While other signaling pathways have been shown to regulate Smad-mediated TGFβ signaling [13-16], relatively less is known about the impact of TGFβ signal transduction on signaling from other receptor superfamilies. Recently, we demonstrated that TGFβ signaling directly results in increased expression of the G protein-coupled receptor kinase 2 (GRK2) [17]. GRK2 is a key regulatory kinase involved in the early initiating steps towards the desensitization of G protein-coupled receptors (GPCRs). Minor alterations in GRK2 expression level have been linked to various pathological conditions such as rheumatoid arthritis, multiple sclerosis and hypertension [18].

AngiotensinII (AngII) is the predominant peptide hormone of the rennin-angiotensin system, which initiates signaling by binding to a seven transmembrane spanning Gq-coupled AngII type I receptor (AT_1R). AngII exerts opposite effects to those of TGFβ on VSMC cell growth and induces cell proliferation and migration. AngII binding to the Gq-coupled AT_1R leads to an elevation in intracellular calcium levels and to the activation of protein kinase C (PKC), thus stimulating growth and migration of endothelial cells [19, 20]. These mitogenic and migratory responses of AngII are mediated through multiple intracellular signaling pathways, including activation of phospholipase C (PLC), inositol 1,4,5-trisphosphate (IP3), 1,2-diacylglycerol (DAG) cascade and mitogen-activated protein (MAP) kinases [21]. AngII signaling is thought to contribute to the pathogenesis of various cardiovascular diseases, due to its growth-promoting effects on VSMCs.

In the present study, we set out to examine the crosstalk mechanisms between the TGFβ and AngII receptor families. We found that the TGFβ-mediated increase of GRK2

expression in VSMCs is time dependent and further leads to the inhibition of AngII-mediated extracellular signal-regulated kinase (ERK) phosphorylation, proliferation and migration of VSMCs. Thus, GRK2 appears to be a key factor in regulating the crosstalk mechanisms, as well as the antagonistic effects, between TGFβ and AngII in VSMCs.

2.3 MATERIALS & METHODS

Cell culture: Rat VSMCs were cultured in low glucose Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Invitrogen), 100units/ml penicillin, 10mg/ml streptomycin, and 2mM glutamine. VSMCs were grown to 80% confluence and then growth arrested, overnight, in serum-free DMEM supplemented with 100units/ml penicillin, 10mg/ml streptomycin, and 2mM glutamine.

Reverse transcription-PCR: VSMCs were treated with 0.2nM of TGFβ for 0, 4, 8, 16, 24hrs and total RNA was extracted using Trizol reagent (Invitrogen). Reverse transcription of total cellular RNA, using oligo-dT primers, was carried out using Stratascript Reverse Transcriptase (Stratagene) as per the manufacturer's instruction. Subsequent amplification of cDNA was performed in order to obtain products for GRK2 and GAPDH. The primer sequences for GRK2 were as follows: sense 5'-CGAGGTGGACCTTTGAGAAG-3', and antisense 5'-CACTCTTCGAGAAGGGATGC-3'. Using PCR conditions: 30 cycles (94°C for 30s, 60°C for 30s, 72°C for 1min30s).

Western analysis: VSMCs were cultured in 10% heat-inactivated FBS-supplemented low glucose DMEM. Overnight starvation in serum-free low glucose DMEM was followed by treatment with or without 1μM AngII in starvation media. Cells were collected and lysed with lysis buffer [50mM Hepes, pH7.5; 150mM sodium chloride; 100mM sodium fluoride; 10mM sodium pyrophosphate; 5mM EDTA, pH 8.0; 10% glycerol; 0.5% Nonidet P40; 0.5% sodium deoxycholate] supplemented with protease inhibitors 1mM phenylmethylsulfonyl fluoride (PMSF), 10μg/ml aprotinin, 10μg/ml leupeptin, 2μg/ml pepstatin (BioShop). Total cell lysates were resolved on polyacrylamide gels, transferred onto nitrocellulose and incubated with specific antibodies. Immunoreactivity was revealed by chemiluminescence (Lumi-Light Plus Western blot substrate, Roche) and measured using an Alpha Innotech Fluorochem Imaging system (Packard Canberra). Densitometric analysis was performed using Fluorochem 8000 software (Alpha Innotech).

Antisense Oligonucleotide (AS-Oligo) Treatment: 20bp phosphorothioate-derivatized antisense GRK2 and control oligonucleotides (C-oligos) were synthesized. The sequences of the oligos are as follows: AS-oligo 5'-ACCGCCTCCAGGTCCGCCAT-3', and C-oligo 5'-TCAGACTGGCTCTCTCCATG-3'. VSMCs were transfected with 100nM of the GRK2 antisense oligo, using Lipofectamine 2000 (Invitrogen). Cells were pretreated or not overnight with TGFβ (0.2nM) and split the following day. After allowing the cells to adhere, they were stimulated or not with AngII, collected and lysed as previously described. Western blot was performed with specific antibodies.

Cell viability assay (MTT): $3x10^5$ VSMCs were plated in 6-well dishes in 10% heat-inactivated FBS-supplemented low glucose DMEM and starved overnight. Cells were stimulated with or without TGFβ in serum-free low glucose DMEM for 24hrs. The following day, the cells were split and plated in triplicate (2,500 cells per 50μl) in 10% heat-inactivated FBS-supplemented low glucose DMEM, in 96-well plates for 2hrs. Media was then changed to 0.1% heat-inactivated FBS-supplemented low glucose DMEM for 20min, after which another 50μl low glucose DMEM containing a 0.1% FBS supplement with or without AngII was added. Cell growth was assessed using the MTT cell proliferation assay for eukaryotic cells (Cell Titer 96, Promega G4000) after 24hrs. Absorbance was measured at 570nm using a Bio-tek Microplate reader.

Scratch assay: For migration studies, VSMCs were plated in a 6-well dish to achieve 100% confluence. The cell monolayer was then scratched gently, using a sterile blunt wooden stick, to produce a cell-free zone bordered by a straight wound edge. To remove debris, cells were washed with 1X PBS and replaced with fresh starvation media, with or without TGFβ. Plates were incubated and images of the same scratch region were taken at 24, 48 and 72hrs.

Statistical Analysis: Results are expressed as a mean ± SD of three or more separate independent experiments performed in triplicate. Statistical analyses were assessed using the t-test. Statistical analyses were meant to compare fold induction (% of control) of TGFβ-treated samples among themselves, within each experiment. For all statistical analysis and tests, a p value of < 0.05 was considered significant and indicated above the error bars by an asterisk. Blots shown are representative of at least 3 experiments.

2.4 RESULTS

TGFβ increases GRK2 levels in VSMCs: We previously found the GRK2 kinase to be upregulated in hepatocarcinoma cells and to act in a negative feedback loop to terminate Smad signaling [17]. As GRK2 also plays a critical role downstream of GPCR signaling, we sought to investigate whether increased GRK2 levels, in response to TGFβ, would affect GPCR signaling. Crosstalk mechanism between these two families of growth factor receptors was previously suggested, as expression of TGFβ itself and its type I receptor have been shown to be induced upon AngII stimulation [6, 22, 23].

VSMCs play a critical role in maintaining vascular integrity. Deregulation of VSMC migration, proliferation, release of cytokines or production of extracellular matrix proteins induced by pro-inflammatory stimuli are and have been implicated as key contributors to the development of various vascular diseases, including chronic pulmonary hypertension and atherosclerosis [24]. Interestingly, TGFβ and AngII have opposite effects on VSMC proliferation and survival. To first assess the TGFβ effect on GRK2 expression, VSMCs were stimulated for various periods of time, as indicated in Fig.9 and GRK2 mRNA and protein levels were analyzed by RT-PCR and Western blot respectively. Blots shown are representative of at least 3 experiments. Fig.9a shows TGFβ stimulation rapidly increasing GRK2 mRNA expression, followed by a progressive increase in GRK2 protein levels, showing its highest expression at 24hrs (Fig.9b). These results indicate that GRK2 mRNA and protein expression are regulated by TGFβ in a time-dependent manner in VSMCs.

TGFβ pretreatment of VSMCs antagonizes AngII-mediated ERK phosphorylation independently of MEK and p38 activation: AngII is a well known inducer of cellular proliferation and migration. These effects are mediated through multiple signaling cascades, including the ERK and the stress-activated kinase p38 pathway [25, 26]. We first examine the effects of AngII on MEK1/2, ERK1/2 and p38 phosphorylation in VSMCs. Cells were treated with AngII for various periods of time, as shown in Fig.10a. Total cell lysates were analyzed by Western blot using phospho-specific antibodies against pMEK1/2, pERK1/2 and pp38. Blots are representative of at least 3 experiments. Fig.10a showed that all three kinases were strongly phosphorylated in response to AngII treatment.

To then assess whether TGFβ could antagonize AngII signaling, VSMCs pretreated or not with TGFβ, were stimulated or not with AngII for 3min. Fig.10b and c showed that AngII-mediated phosphorylation of MEK1/2 and p38 were not affected by TGFβ

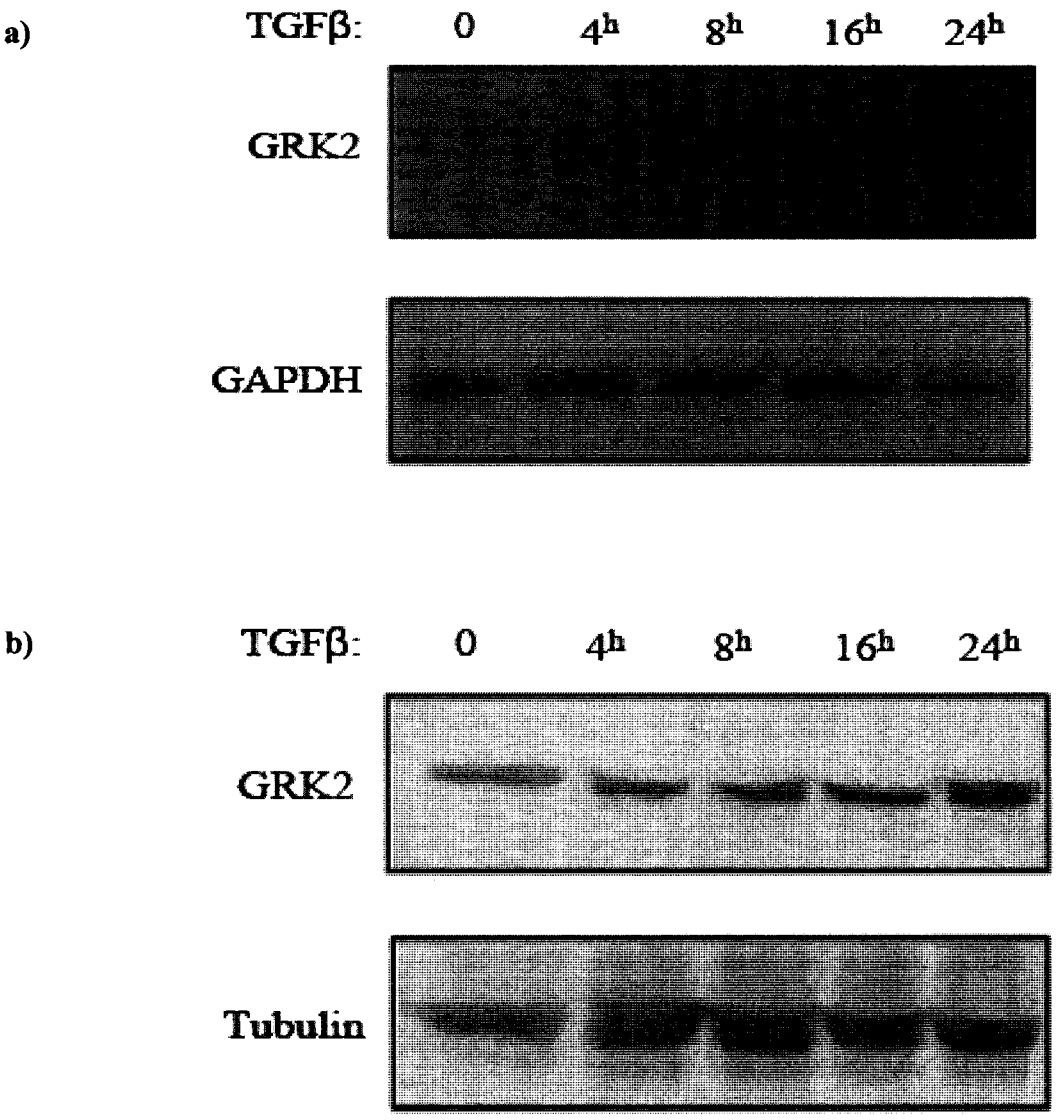


Fig.9: TGFβ induces upregulation of GRK2 in VSMCs

(a) VSMCs were stimulated with TGFβ for 0, 4, 8, 16 and 24hrs and total mRNA was analyzed by RT-PCR using specific primers for GRK2 (upper panel). Reverse transcription reactions were performed using oligo-dT. cDNA were amplified using specific primers for GRK2 and GAPDH as a control (lower panel). (b) VSMCs were stimulated with TGFβ for 0, 4, 8, 16 and 24hrs and total cell lysates were analyzed by Western blot using a specific monoclonal antibody against GRK2 (upper panel) or β-tubulin (lower panel). Blots shown are representative of at least 3 experiments.

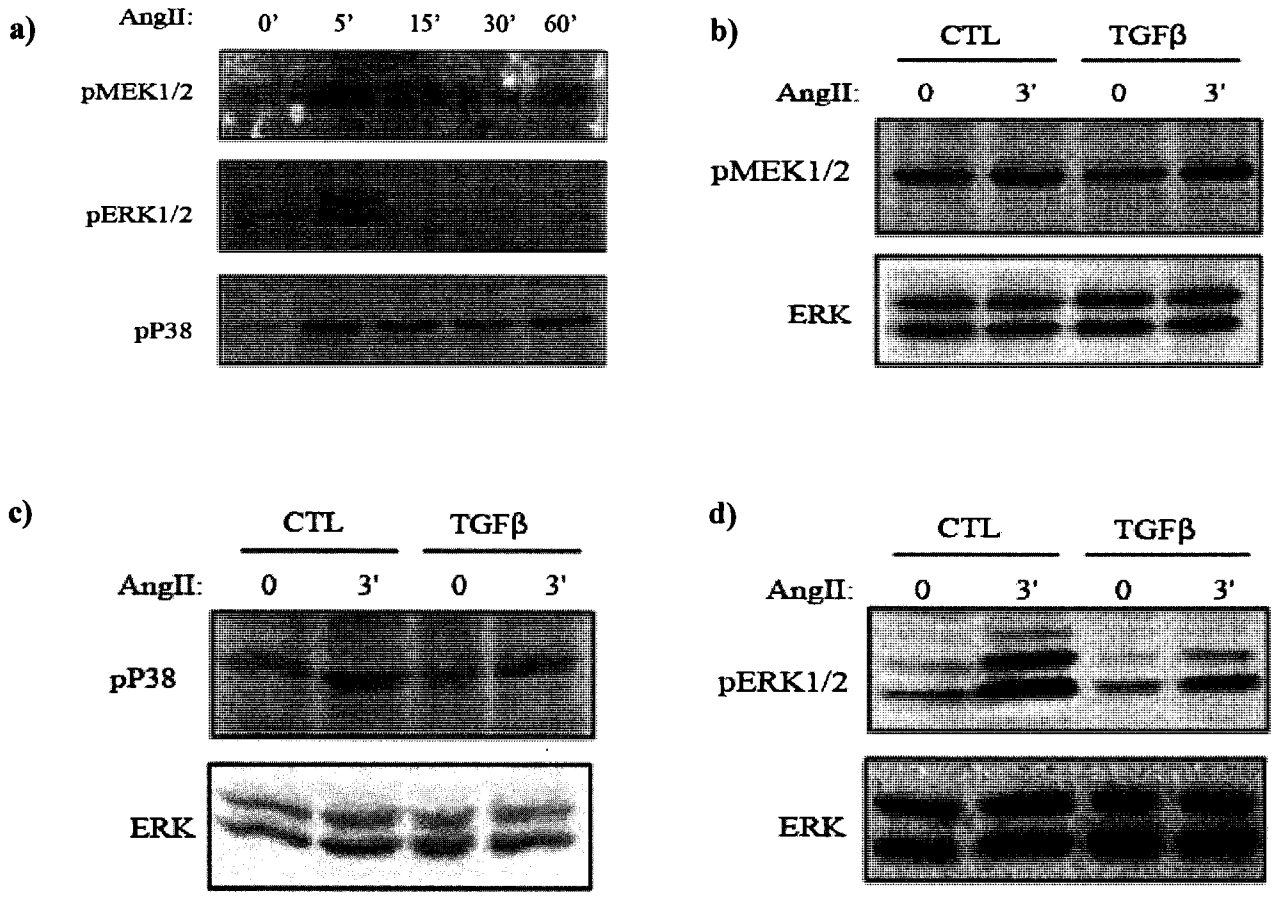


Fig.10: TGFβ pretreatment of VSMCs antagonizes AngII-mediated ERK phosphorylation independently of MEK and p38 activation

(a) VSMCs were treated with AngII for 0, 5, 15, 30, 60min. Total cell lysates were analyzed by Western blot using phospho-specific antibodies directed against pMEK1/2, pERK1/2 and pp38. All three kinases were strongly phosphorylated in response to the AngII treatment, as early as 5min after stimulation (b) VSMCs were pretreated or not with TGFβ, overnight. The following day, the cells were stimulated or not with AngII for 3min. AngII-mediated phosphorylation of MEK1/2 was not affected by TGFβ pre-treatment (c) similarly, AngII-mediated phosphorylation of p38 was not affected by TGFβ pre-treatment. However (d) TGFβ pretreatment of the VSMCs almost completely abolished AngII-mediated phosphorylation of ERK1/2. Results are representative of at least 3 experiments.

pretreatment. However, Fig.10d showed that TGFβ pretreatment of the VSMCs almost completely abolished AngII-mediated phosphorylation of ERK1/2.

TGFβ pretreatment of VSMCs antagonizes AngII-mediated HIF-1α expression: The transcription factor hypoxia-inducible factor (HIF) -1α is involved in hypoxia-induced enhancement of VSMC proliferative responses [27]. It has also been reported that HIF-1α is induced by non-hypoxic stimuli, like AngII in VSMC [28]. AngII modulates HIF-1α mRNA transcription through the activation of DAG-sensitive PKC and reactive oxygen species (ROS) production. It then activates the phosphoinositide-3-kinase (PI3K) pathway, which surpasses hypoxic induction and leads to the activation of HIF-1α-responsive genes, such as vascular endothelial growth factor (VEGF) [29]. As HIF-1α transcriptional activity is partially dependent on the ERK pathway [30], we examined the effect of TGFβ pretreatment on AngII-induced expression of HIF-1α. Blots are representative of at least 3 experiments. Fig.11 shows that HIF-1α protein expression levels were strongly increased in cells treated with AngII. However, this effect was blocked in VSMCs pretreated with TGFβ, suggesting that TGFβ inhibits AngII-mediated transcriptional activity.

HIF-1α migration has a diffuse migration pattern due to varying degrees of post-translational phosphorylation and closely follows the activation of pERK [31]. The two bands observed in Fig.11 are due to this modification.

GRK2 antisense oligonucleotide reverses the inhibitory effect of TGFβ pretreatment on AngII-mediated ERK phosphorylation: To determine whether the antagonistic effects of TGFβ on AngII-induced ERK phosphorylation were dependent on TGFβ-induced GRK2 expression, VSMCs were transfected or not with an antisense oligonucleotide directed against GRK2. Fig.12 (upper panel) shows increasing levels of GRK2 in response to TGFβ pre-treatment (lanes 3 and 4), as compared to unstimulated lanes 1 and 2. This effect was efficiently reversed in cells transfected with the antisense oligonucleotide and pre-treated with TGFβ (lanes 5 and 6). In fact, TGFβ pretreatment of the VSMCs led to a clear inhibition of AngII-induced ERK1/2 phosphorylation (middle, lanes 3 and 4) and this effect was fully antagonized in cells transfected with the GRK2 antisense oligonucleotide (middle, lanes 5 and 6). These results indicate that TGFβ-mediated inhibition of AngII-mediated ERK1/2 phosphorylation is dependent on TGFβ-induced GRK2 expression. The third panel shows equal loading using total ERK. All blots shown are representative of at least 3 experiments. However, another experiment involving transfection of the C-oligo remains to be performed in order to validate our results.

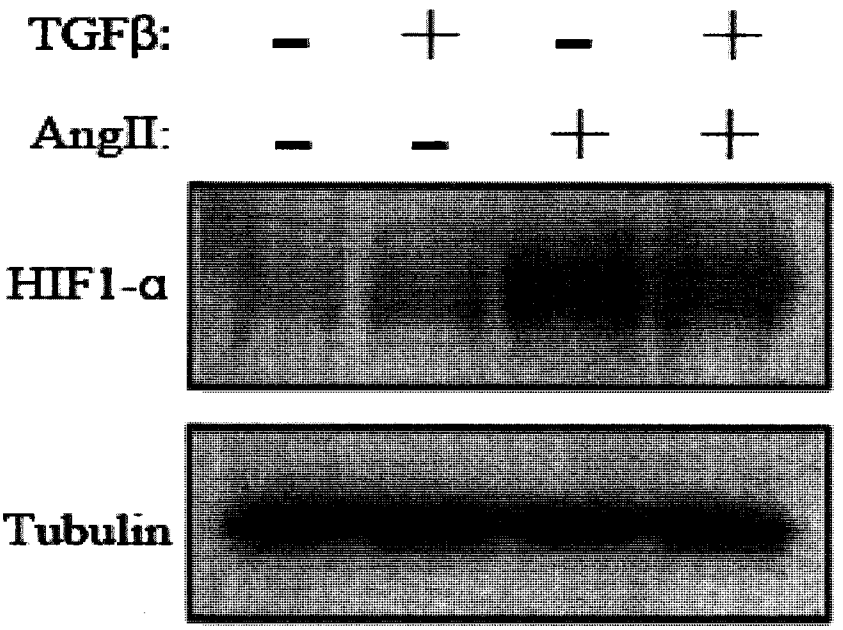


Fig.11: TGFβ pretreatment of VSMCs antagonizes AngII-mediated HIF-1α expression

VSMCs were pretreated with or without TGFβ and then stimulated with AngII. Cells were collected and the total cell lysates were analyzed by Western blot using antibodies for HIF-1α and β-Tubulin. HIF-1α protein expression levels were strongly increased in cells treated with AngII. However, this effect was blocked in VSMCs pretreated with TGFβ, suggesting that TGFβ inhibits AngII-mediated transcriptional activity. The two bands observed are due to post-translational phosphorylation of HIF-1α, which contributes to its diffuse migration pattern.

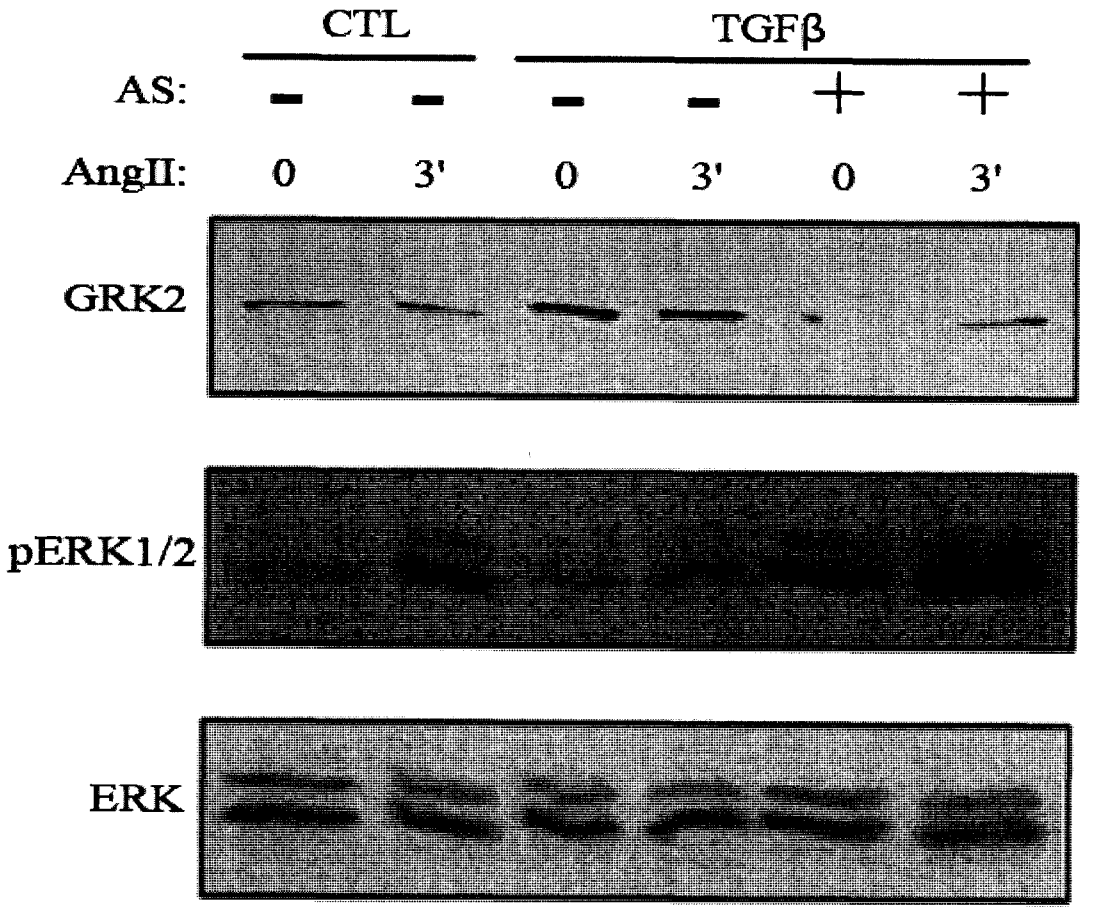


Fig.12: GRK2 antisense oligonucleotide reverses the inhibitory effect of TGFβ pretreatment on AngII-mediated ERK phosphorylation

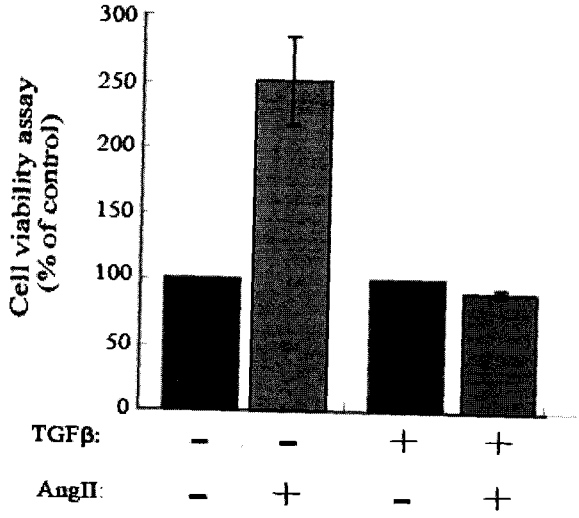
VSMCs were transfected with a GRK2 antisense oligonucleotide (100nM). Cells were split and pretreated or not, overnight, with TGFβ. The following day, the cells were stimulated or not with AngII. The upper panel shows that GRK2 protein levels were increased in response to TGFβ pre-treatment (lanes 3, 4), as compared to the non-stimulated cells (lanes 1, 2). This effect was efficiently reversed in cells transfected with the antisense oligonucleotide (lanes 5, 6). The second panel shows that TGFβ pre-treatment led to a clear inhibition of AngII-induced ERK1/2 phosphorylation (lanes 3, 4) and that this effect was fully antagonized in cells transfected with the GRK2 antisense oligonucleotide (lanes 5, 6). The final panel of total ERK served as a loading control. Another experiment involving transfection with a C-oligo remains to be performed in order to validate our results. Western blots are representative of at least 3 experiments.

TGFβ pretreatment of VSMCs antagonizes AngII-induced proliferation and migration: AngII regulates cell growth and migration in various cell types, including VSMCs [19, 20]. Thus, to assess the potential of TGFβ to antagonize AngII-mediated cell growth and migration, VSMCs were treated or not with TGFβ for 24hrs prior to being stimulated with AngII for another 24hrs. As shown in Fig.13a, in the absence of TGFβ, AngII strongly induced cell growth as measured by the MTT cell viability assay in three separate experiments. However, in cells treated with TGFβ, the AngII proliferative effect was completely abolished.

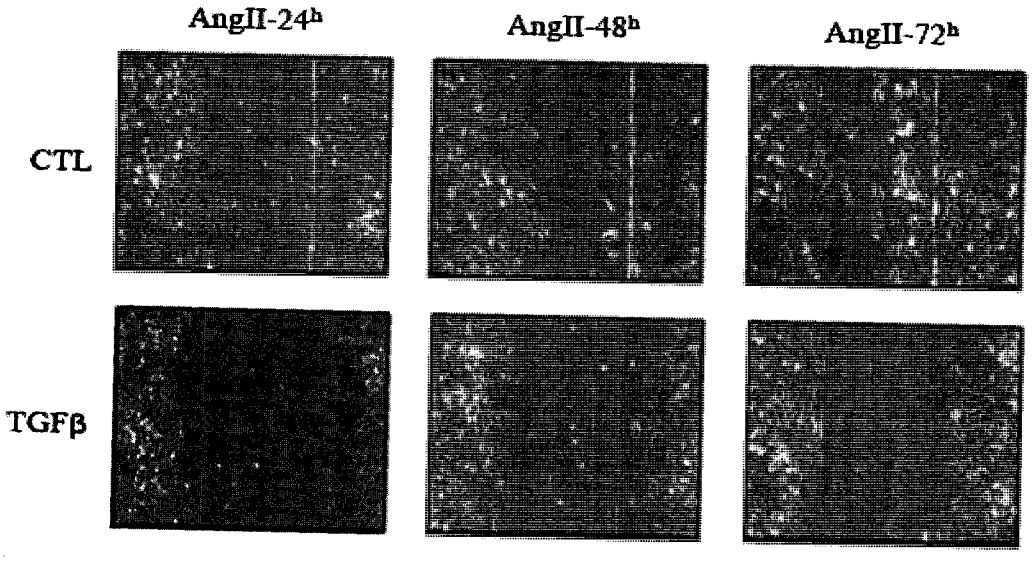
Similarly, we examined the effect of AngII on cell migration, using the scratch/wound healing assay. As shown in Fig.13b, VSMCs stimulated with AngII showed a time-dependent closure of the wound (upper panels). However, in cells pretreated with TGFβ, the migration rate of the cells was significantly retarded, as shown by the delayed wound closure (lower panels). Migration distance was measured and quantified in three separate experiments and showed a significant delay in wound closure when cells were pretreated with TGFβ (Fig.13c). Together, these results indicate that TGFβ acts as a potent antagonist of the proliferative and promigratory effects of AngII stimulation in VSMCs.

TGFβ antagonistic effect on AngII-induced cell proliferation and migration is GRK2-dependent: As demonstrated in Fig.12, the antagonistic effect of TGFβ on AngII-induced ERK1/2 phosphorylation is dependent on GRK2. Activation and phosphorylation of ERK1/2 is critical for AngII-mediated cell proliferation and migration. Thus, we next assessed whether TGFβ-induced inhibition of AngII-mediated cell proliferation and migration was also dependent on GRK2 expression. VSMC cell growth was measured using the MTT cell viability assay, as previously described, in cells treated with or without TGFβ, in the absence or presence of overexpressed GRK2 antisense oligonucleotide. As shown in Fig.14a, AngII-mediated cell growth was blocked in cells treated with TGFβ and this effect was completely reversed in cells transfected with the antisense oligonucleotide. Similarly, VSMC cell migration was measured using the scratch assay. Fig.14b, shows that the AngII-mediated promigratory effect was strongly inhibited by TGFβ and significantly reversed in cells transfected with the antisense oligonucleotide. These results indicate that TGFβ-mediated increase of GRK2 expression is required for TGFβ to fully antagonize AngII-mediated ERK phosphorylation, cell growth and migration. Results shown are representative of at least 3 experiments.

a)


Cell viability assay
(% of control)
300
250
200
150
100
50
0
TGFβ: - - + +
AngII: - + - +

b)


AngII-24h
AngII-48h
AngII-72h
CTL
TGFβ

c)

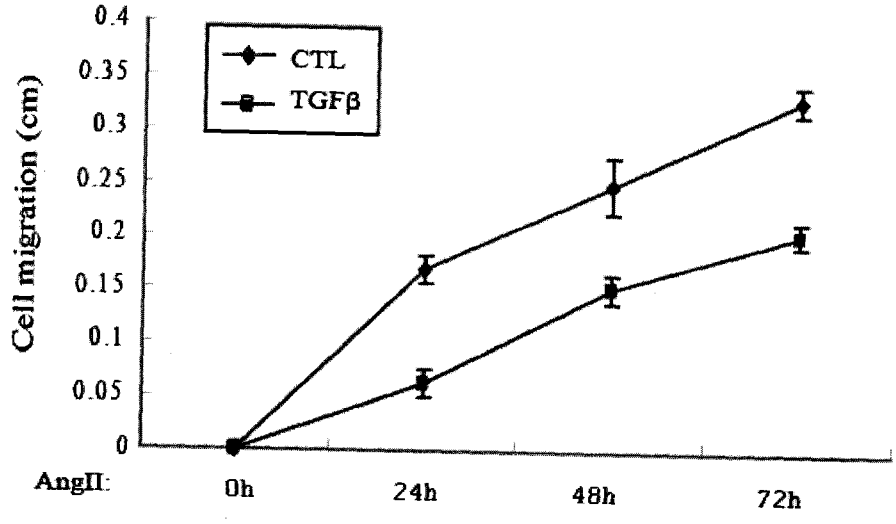
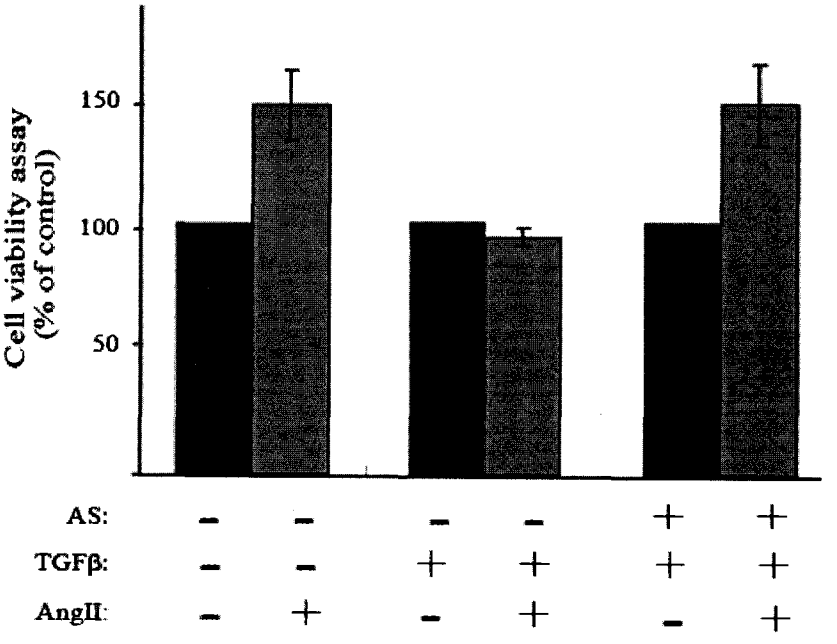

Cell migration (cm)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
CTL
TGFβ
AngII:
0h
24h
48h
72h

Fig.13: TGFβ pretreatment of VSMCs antagonizes AngII-induced proliferation and migration

(a) VSMCs were prestimulated with TGFβ overnight. The following day, they were split and plated in triplicate, in 96-well plates. After allowing them to adhere, cells were stimulated or not with AngII. Growth was assessed using the MTT assay and absorbance was measured at 570nm using a Bio-tek Microplate reader. In the absence of TGFβ, AngII strongly induced cell growth, as measure by three separate MTT cell viability assays. However, in cells treated with TGFβ, the AngII proliferative effect was completely abolished (b) VSMCs were grown to 90% confluence and were pretreated or not with TGFβ, overnight. The following day, cells were scratched with a sterile blunt object and stimulated or not with AngII. Migration was observed at 24, 48 and 72hrs. VSMCs stimulated with AngII showed a time-dependent closure of the wound (upper panels). However, in cells pretreated with TGFβ, the cell migration was significantly retarded, as shown by the delayed wound closure (lower panels) (c) Migration distance was measured and quantified, in three separate experiments, and showed a significant delay in wound closure when cells were treated with TGFβ

a)


Cell viability assay
(% of control)
150
100
50
AS: - - - - + +
TGFβ: - - + + + +
AngII: - + - + - +

b)

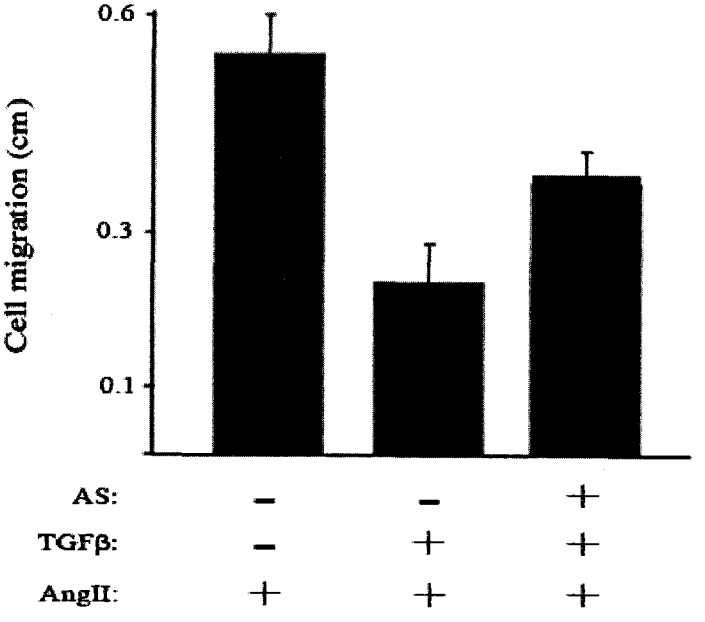

Cell migration (cm)
0.6
0.3
0.1
AS: - - +
TGFβ: - + +
AngII: + + +

Fig.14: TGFβ antagonistic effect on AngII-induced cell proliferation and migration is GRK2-dependent

(a) VSMCs were transfected with 100nM of oligo, using Lipofectamine 2000, and then pretreated or not with TGFβ, overnight. The following day, they were split and plated in triplicate in 96-well plates. After allowing the cells to adhere, they were stimulated or not with AngII. Growth was assessed using the MTT assay, and absorbance was measured at 570nm. AngII-mediated cell growth was blocked in cells treated with TGFβ and this effect was completely reversed in cells transfected with the antisense oligonucleotide. (b) VSMCs were transfected with 100nM of oligo, using Lipofectamine 2000, and grown to 90% confluence, after which they were pretreated or not with TGFβ overnight. The following day, cells were scratched with a sterile blunt object and stimulated or not with AngII. Migration was observed. The AngII-mediated promigratory effect was strongly inhibited by TGFβ and significantly reversed in cells transfected with the antisense oligonucleotide. Results are representative of at least 3 experiments.

2.5 DISCUSSION

In this study, we show that activation of the TGFβ signaling cascade in VSMCs results in a time-dependent increase of GRK2 kinase expression. Consequently, this upregulation leads to an inhibition of AngII-induced ERK phosphorylation and inhibition of AngII-mediated VSMC proliferation and migration. We found the antagonistic effect of TGFβ on AngII-mediated responses to be specifically dependent on TGFβ-induced GRK2 expression, as these effects are fully reversed when GRK2 expression is blocked using a GRK2 antisense oligonucleotide. Moreover, this inhibitory effect appears to occur at the interface between MEK and ERK rather than at the AngII receptor level. Together, our results define a novel regulatory and antagonistic mechanism between two distinct families of growth factors (serine/threonine kinase and GPCR) and highlight the important role of GRK2 as the main mediator of the crosstalk between these two signaling pathways.

The TGFβ and AngII signaling pathways are intricately intertwined and regulate various biological responses, including cell proliferation, survival and migration. Crosstalk mechanisms between TGFβ and AngII were previously suggested by studies showing that AngII could stimulate TGFβ mRNA expression and promote its conversion into its biologically active form in VSMCs [32]. Moreover, AngII increases Smad2 and Smad4 protein levels both *in vitro* and *in vivo* [33]. TGFβ is expressed in endothelial cells, myofibroblasts, VSMCs, macrophages and hematopoietic cells [34]. TGFβ regulates cell proliferation, apoptosis, differentiation and migration in cardiovascular cells and has been shown to participate in the pathogenesis of many cardiovascular diseases, including hypertension, atherosclerosis, cardiac hypertrophy, and heart failure [2]. As such, an AngII-mediated increase in active TGFβ may contribute to the pathogenesis of vascular disorders. Similarly, the antagonistic effect of TGFβ signaling on AngII-mediated cell proliferation and migration may impact the proper vascular function. Thus, a complete understanding of how these different signaling pathways interconnect and communicate with each other, to regulate VSMC biological responses such as proliferation and migration, will prove useful in the design of therapies for vascular disorders. AngII signaling is known to increase atherosclerotic disease. In fact, inhibition of the angiotensin converting enzyme (ACE), which is responsible for AngII production, has already proven to be useful in reducing ischemic heart diseases, reducing the size of atherosclerotic lesions in a different animal models of the disease, without major changes on blood pressure [35].

Thus, therapies based on the use of TGFβ-signaling-mimetics, as potent antagonists of AngII-mediated cell signaling, may prove useful in this context.

We previously identified GRK2 as a direct downstream target of the TGFβ signaling cascade [17]. GRK2 acts through a negative feedback loop, terminating TGFβ-induced signaling and thereby inhibiting its tumor suppressive effects in normal hepatocytes and in human hepatocarcinoma cells [17]. The TGFβ effect on GRK2 expression is not restricted to the liver, as we show here that TGFβ strongly upregulates GRK2 expression in VSMCs. This upregulation occurs in a time-dependent manner, at both the mRNA and protein level.

GRK2 is a critical downstream component of GPCR signaling, including the AngII pathway. The kinase activity of GRK2 is capable of desensitizing a wide array of G protein-coupled receptors [36]. In primary smooth muscle cells (SMCs), GRK2 overexpression diminished SMC receptor signaling assessed by second messenger synthesis, DNA synthesis, and/or SMC proliferation in response to AngII, endothelin-1, thrombin, thromboxane A2, platelet-derived growth factor (PDGF), and FBS [37]. The ERK cascade is central to AngII-induced proliferation and migration [25]. Considering the importance of GRK2 downstream of AngII signaling, we investigated whether TGFβ-mediated increase of GRK2 expression could affect the regulatory effects of AngII on p38/MAPK/ERK-induced cell proliferation and migration in VSMCs. Our results defined GRK2 as a central regulator, at the crossroad between these two signaling cascades.

TGFβ-induced increase in GRK2 expression leads to the inhibition of AngII-mediated ERK activation, highlighting the preponderant role played by GRK2 in MAPK regulation and activation. Our results are also consistent with recent studies showing that in the presence of low levels of GRK2, such as in splenocytes from the GRK2 hemizygous mice, an increased and enhanced ERK activation is observed in response to chemokine stimulation [38]. Similarly, reduced GRK2 levels observed in T cells are associated with an increase in MAPK activation and in chemokine-induced cell migration [39].

HIF is one of many possible major transcriptional regulators potentially involved in hypoxia-induced enhancement of VSMC proliferative responses [27]. The HIF-1 transcription factor is a heterodimer composed of HIF-1α and HIF-1β. Although the HIF-1β protein is readily found in all cells, HIF-1α is virtually undetectable in normal conditions. In contrast to hypoxia, it has been reported that HIF-1α is induced by non-hypoxic stimuli, such as angiotensin, in VSMCs. AngII modulates two different pathways to increase HIF-1α protein expression levels. The first involves an increase in the rate of

HIF-1α mRNA transcription through a mechanism requiring the activation of the DAG-sensitive PKC. The second involves increasing the rate of translation of this newly produced HIF-1α mRNA by activating ROS production, which subsequently activates the PI3K pathway. Together, these two pathways increase VSMC HIF-1α protein expression to levels surpassing those of hypoxic-induction and lead to the activation of HIF-1-responsive genes such as VEGF [29]. Importantly, HIF-1 transcriptional activity is partially dependent on the ERK pathway [30].

Interestingly, TGFβ-mediated increase of GRK2 expression blocked AngII-mediated ERK phosphorylation and activation of its downstream target HIF-1α, without affecting MEK phosphorylation. These results suggest that the GRK2-mediated inhibitory effects are independent of receptor phosphorylation and internalization, and directly act on ERK activation. However, one cannot completely exclude the possibility that GRK2 is functioning at the level of receptor desensitization. Nevertheless, our results agree with a previous study showing that GRK2 and MEK are found in the same multimolecular complex and that chemokine-driven MEK stimulation is not blocked in HEK-293 cells overexpressing GRK2 [38]. Increasing GRK2 levels simulates several diseased states, most notably cardiovascular disease. Thus, it is conceivable that the increased levels of GRK2 can interact with MEK, thereby preventing ERK phosphorylation. Our results also suggest this, and delineate an important role for GRK2 in the control of ligand-induced ERK activation at the level of the MEK-ERK interface [38].

AngII exerts a proliferative, anti-apoptotic function on various cell types. AngII-induced proliferative effects are mediated through the ERK pathway, mainly by the transactivation of the EGF receptor [40]. Other signaling pathways, such as PI3K/Akt are also activated by AngII and implicated in AngII-mediated cell proliferation, migration and survival. Interestingly, the AngII receptor is often overexpressed in tumor cells, suggesting a potential role for AngII in tumor progression [41, 42]. TGFβ, on the other hand, acts as a potent tumor suppressor by inhibiting cell proliferation, inducing apoptosis and preventing cell immortalization [43]. Thus, the inhibitory effects exhibited by TGFβ, on AngII-mediated cell signaling, account for and explain its strong tumor suppressive effects. Indeed, not only does TGFβ directly act to induce apoptosis and block cell proliferation but, it also antagonizes AngII proliferation and AngII survival effects. Thus, blocking AngII signaling using specific antagonists may prove useful in the development of novel antitumoral compounds.

2.6 REFERENCES

1. Massague, J., S.W. Blain, and R.S. Lo, *TGFbeta signaling in growth control, cancer, and heritable disorders.* Cell, 2000. **103**(2): p. 295-309.
2. Gordon, D., et al., *Cell proliferation in human coronary arteries.* Proc Natl Acad Sci U S A, 1990. **87**(12): p. 4600-4.
3. Halayko, A.J. and J. Solway, *Molecular mechanisms of phenotypic plasticity in smooth muscle cells.* J Appl Physiol, 2001. **90**(1): p. 358-68.
4. Braun-Dullaeus, R.C., M.J. Mann, and V.J. Dzau, *Cell cycle progression: new therapeutic target for vascular proliferative disease.* Circulation, 1998. **98**(1): p. 82-9.
5. Ross, R., *The pathogenesis of atherosclerosis--an update.* N Engl J Med, 1986. **314**(8): p. 488-500.
6. Goodman, L.V. and R.A. Majack, *Vascular smooth muscle cells express distinct transforming growth factor-beta receptor phenotypes as a function of cell density in culture.* J Biol Chem, 1989. **264**(9): p. 5241-4.
7. Morisaki, N., et al., *Effects of transforming growth factor-beta 1 on growth of aortic smooth muscle cells. Influences of interaction with growth factors, cell state, cell phenotype, and cell cycle.* Atherosclerosis, 1991. **88**(2-3): p. 227-34.
8. Owens, G.K., et al., *Transforming growth factor-beta-induced growth inhibition and cellular hypertrophy in cultured vascular smooth muscle cells.* J Cell Biol, 1988. **107**(2): p. 771-80.
9. Deaton, R.A., et al., *Transforming growth factor-beta1-induced expression of smooth muscle marker genes involves activation of PKN and p38 MAPK.* J Biol Chem, 2005. **280**(35): p. 31172-81.
10. Su, C., et al., *PKN activation via transforming growth factor-beta 1 (TGF-beta 1) receptor signaling delays G2/M phase transition in vascular smooth muscle cells.* Cell Cycle, 2007. **6**(6): p. 739-49.
11. Ruiz-Ortega, M., et al., *TGF-beta signaling in vascular fibrosis.* Cardiovasc Res, 2007. **74**(2): p. 196-206.
12. Derynck, R. and Y.E. Zhang, *Smad-dependent and Smad-independent pathways in TGF-beta family signalling.* Nature, 2003. **425**(6958): p. 577-84.
13. Kretzschmar, M., J. Doody, and J. Massague, *Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1.* Nature, 1997. **389**(6651): p. 618-22.
14. Kretzschmar, M., et al., *A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras.* Genes Dev, 1999. **13**(7): p. 804-16.

15. Matsuura, I., et al., *Cyclin-dependent kinases regulate the antiproliferative function of Smads.* Nature, 2004. **430**(6996): p. 226-31.
16. Wicks, S.J., et al., *Inactivation of smad-transforming growth factor beta signaling by Ca(2+)-calmodulin-dependent protein kinase II.* Mol Cell Biol, 2000. **20**(21): p. 8103-11.
17. Ho, J., et al., *The G protein-coupled receptor kinase-2 is a TGFbeta-inducible antagonist of TGFbeta signal transduction.* Embo J, 2005. **24**(18): p. 3247-58.
18. Penn, R.B., A.N. Pronin, and J.L. Benovic, *Regulation of G protein-coupled receptor kinases.* Trends Cardiovasc Med, 2000. **10**(2): p. 81-9.
19. Burnier, M. and H.R. Brunner, *Angiotensin II receptor antagonists.* Lancet, 2000. **355**(9204): p. 637-45.
20. Schmidt-Ott, K.M., S. Kagiyama, and M.I. Phillips, *The multiple actions of angiotensin II in atherosclerosis.* Regul Pept, 2000. **93**(1-3): p. 65-77.
21. Touyz, R.M., *Intracellular mechanisms involved in vascular remodelling of resistance arteries in hypertension: role of angiotensin II.* Exp Physiol, 2005. **90**(4): p. 449-55.
22. Stouffer, G.A. and G.K. Owens, *Angiotensin II-induced mitogenesis of spontaneously hypertensive rat-derived cultured smooth muscle cells is dependent on autocrine production of transforming growth factor-beta.* Circ Res, 1992. **70**(4): p. 820-8.
23. Fukuda, N., et al., *Angiotensin II upregulates transforming growth factor-beta type I receptor on rat vascular smooth muscle cells.* Am J Hypertens, 2000. **13**(2): p. 191-8.
24. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.
25. Zhao, Y., et al., *Role of Ras/PKCzeta/MEK/ERK1/2 signaling pathway in angiotensin II-induced vascular smooth muscle cell proliferation.* Regul Pept, 2005. **128**(1): p. 43-50.
26. Seay, U., et al., *Transforming growth factor-beta-dependent growth inhibition in primary vascular smooth muscle cells is p38-dependent.* J Pharmacol Exp Ther, 2005. **315**(3): p. 1005-12.
27. Schultz, K., B.L. Fanburg, and D. Beasley, *Hypoxia and hypoxia-inducible factor-1alpha promote growth factor-induced proliferation of human vascular smooth muscle cells.* Am J Physiol Heart Circ Physiol, 2006. **290**(6): p. H2528-34.
28. Touyz, R.M., G. Yao, and E.L. Schiffrin, *Role of the actin cytoskeleton in angiotensin II signaling in human vascular smooth muscle cells.* Can J Physiol Pharmacol, 2005. **83**(1): p. 91-7.
29. Page, E.L., et al., *Induction of hypoxia-inducible factor-1alpha by transcriptional and translational mechanisms.* J Biol Chem, 2002. **277**(50): p. 48403-9.

30. Minet, E., et al., *Transduction pathways involved in Hypoxia-Inducible Factor-1 phosphorylation and activation.* Free Radic Biol Med, 2001. **31**(7): p. 847-55.
31. Richard, D.E., et al., *p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1.* J Biol Chem, 1999. **274**(46): p. 32631-7.
32. Weigert, C., et al., *Angiotensin II induces human TGF-beta 1 promoter activation: similarity to hyperglycaemia.* Diabetologia, 2002. **45**(6): p. 890-8.
33. Ikedo, H., et al., *Smad protein and TGF-beta signaling in vascular smooth muscle cells.* Int J Mol Med, 2003. **11**(5): p. 645-50.
34. Annes, J.P., J.S. Munger, and D.B. Rifkin, *Making sense of latent TGFbeta activation.* J Cell Sci, 2003. **116**(Pt 2): p. 217-24.
35. Daugherty, A. and L. Cassis, *Angiotensin II-mediated development of vascular diseases.* Trends Cardiovasc Med, 2004. **14**(3): p. 117-20.
36. Peppel, K., et al., *Overexpression of G protein-coupled receptor kinase-2 in smooth muscle cells reduces neointimal hyperplasia.* J Mol Cell Cardiol, 2002. **34**(10): p. 1399-1409.
37. Peppel, K., et al., *Overexpression of G protein-coupled receptor kinase-2 in smooth muscle cells attenuates mitogenic signaling via G protein-coupled and platelet-derived growth factor receptors.* Circulation, 2000. **102**(7): p. 793-9.
38. Jimenez-Sainz, M.C., et al., *G protein-coupled receptor kinase 2 negatively regulates chemokine signaling at a level downstream from G protein subunits.* Mol Biol Cell, 2006. **17**(1): p. 25-31.
39. Vroon, A., et al., *Reduced GRK2 level in T cells potentiates chemotaxis and signaling in response to CCL4.* J Leukoc Biol, 2004. **75**(5): p. 901-9.
40. Fischer, O.M., et al., *EGFR signal transactivation in cancer cells.* Biochem Soc Trans, 2003. **31**(Pt 6): p. 1203-8.
41. Deshayes, F. and C. Nahmias, *Angiotensin receptors: a new role in cancer?* Trends Endocrinol Metab, 2005. **16**(7): p. 293-9.
42. Suganuma, T., et al., *Functional expression of the angiotensin II type 1 receptor in human ovarian carcinoma cells and its blockade therapy resulting in suppression of tumor invasion, angiogenesis, and peritoneal dissemination.* Clin Cancer Res, 2005. **11**(7): p. 2686-94.
43. Wahl, S.M., *Transforming growth factor-beta: innately bipolar.* Curr Opin Immunol, 2007. **19**(1): p. 55-62.

CHAPTER 3: PERSPECTIVES

3.1 PREFACE

The identification of TGFβ1, almost twenty-five years ago, sparked a wave of important discoveries in the field of signal transduction. These findings revealed a complex and intricate network of signaling and effector molecules involved in the regulation of the TGFβ pathway. Countless studies have shown that deregulation of TGFβ signaling components is implicated in the pathogenesis of numerous human diseases, illustrating the importance of the TGFβ signaling pathway.

Although the TGFβ signaling pathway has been well studied and many of the key players have been identified, new effector proteins continue to be discovered and their functions need to be elucidated. In light of our recent discovery, the identification of a novel negative feedback loop mechanism involving GRK2, this chapter focuses on deciphering the mechanism of action of GRK2, downstream of TGFβ signaling. This chapter identifies and postulates the role of potential GRK2-interacting partners, paving the way for future studies that will eventually result in the development of targeted therapies.

3.2 INTRODUCTION

The TGFβ signaling pathway regulates a broad range of cellular processes, including cell growth, differentiation and apoptosis, in nearly all cell types [2, 3, 10]. TGFβ and its receptors are widely expressed in all tissues and the regulatory role they play is of central importance to human diseases. In the early stages of disease, TGFβ is initially anti-proliferative and is often considered a "protective cytokine" [3]. For instance, in the early stages of cancer, TGFβ contributes to tumor suppression by efficiently inhibiting cell proliferation and inducing apoptosis. However, in the later stages of the disease, the TGFβ growth inhibitory effects are replaced by invasive and pro-metastatic signals. At this point, the role of TGFβ signaling switches to promote the invasion of epithelial pre-malignant lesions by inducing EMT, thereby mediating cellular metastasis. Prevention and control of this switch in regulation is critical for patient survival, since metastasis represents the last stage in tumorigenesis and is the leading cause of cancer patient mortality. As such, the tumor-permissive effects of TGFβ present a therapeutic opportunity, since by blocking this signaling network one can interrupt the mechanisms essential for disease progression. Evidence of this therapy is clearly illustrated by the recent plethora of TGFβ antagonists that are currently in development and their apparent lack of adverse effects [112].

Previous work in our lab identified GRK2 as a downstream target of TGFβ signaling. Although primarily studied in liver carcinomas, the effect of TGFβ-induced GRK2 upregulation was found not to be tissue specific when extended to other cells lines, and could thus be an important regulatory mechanism of TGFβ signaling. In particular, GRK2 was shown to act as a negative feedback loop and to physically interact with and phosphorylate the R-Smads on a single serine/threonine residue within the linker domain (T197 for Smad2 and S157 for Smad3) [42]. This GRK2-induced Smad phosphorylation blocks TGFβ-induced Smad activation by preventing the phosphorylation of the C-terminal serine residues (SSXS motif) of the Smads, by TβRI. Inability to phosphorylate the C-terminal residues prevents nuclear translocation of the Smads, thereby inhibiting the biological effects of TGFβ. This GRK2 function defines a novel antagonistic pathway to TGFβ signaling, inviting further investigation and characterization of its bearing.

The phosphorylation of a residue within the Smad2/3 linker region provides a platform for the assembly of a regulatory complex at this site. Phosphorylation is a very important regulatory event, often acting like an "on-off switch" for cellular or enzymatic activity. In fact, orchestration of complex cellular events such as cell cycle progression and apoptosis often involve the assembly of multi-molecular complexes at precise subcellular locations within the cell. Much of this temporal and spatial control is thought to be achieved through phosphorylation-dependent protein-protein interactions, giving rise to the formation of signaling complexes that can be tightly regulated by the action of various kinases and phosphatases. This phospho-dependent signaling is best exemplified by phosphorylation of tyrosine residues residing within short sequence motifs, which generates sites for various src homology domain 2 (SH2) and phosphotyrosine-binding domain (PTB)-containing proteins [113]. It is conceivable that the phosphoresidue identified within the Smad linker region could serve as a recruitment site for adaptor/effector molecules, which will relay the antagonistic effect of GRK2 on TGFβ signaling.

Recent studies have identified the 14-3-3 proteins, the modular signaling domain (WW domain) and the Forkhead-associated (FHA) domain as phosphoserine/phosphothreonine (pS/pT) binding domains. Through direct binding to short pS/pT sequence motifs contained within their phosphoprotein targets, these domains are capable of forming signaling complexes [114, 115]. Of particular interest are the FHA domains that were originally identified as having a sequence profile of ~75 aa found in a variety of proteins, including a number of Forkhead-type transcription factors [116]. The binding of FHA

domains to their target phosphopeptides is mediated primarily by the three positions immediately C-terminal to the pT, in particular at the pT+3 position [117]. Screening of a synthetic oriented phosphopeptide library revealed two optimal FHA binding sequences. FHA1 strongly selected for Asp in the pT+3 position with weaker selection for Ile and Leu (pTxxD/I/L), while FHA2 showed a strong preference for Ile and a weaker selection for Leu (pTxxI/L) [118]. Interestingly, the optimal binding sequence of FHA2 match the sequence surrounding T197 in Smad2 (pTHSI) and surrounding S157 in Smad3 (pSHSI).

In order to identify any specific adaptor/effector molecules that may interact with the GRK2 phosphorylation site within the Smad2/3 linker domain, we generated four 10 aa peptides representing the sequence surrounding T197 of Smad2 and S157 of Smad3, in both their phosphorylated and non-phosphorylated forms.

3.3 HYPOTHESIS & OBJECTIVES

The TGFβ signaling pathway has become a candidate for site-directed therapy in disease, particularly in cancer. Discovery of the novel negative feedback loop involving the downstream target of TGFβ, GRK2, suggests an important regulatory role for this kinase.

Screening of a phosphopeptide library revealed two optimal FHA binding sequences matching the sequence within the Smad2/3 linker domain known to interact with GRK2. It is possible that this region may represent a binding motif for FHA-containing effector molecules. As such, we propose to identify phospho-T197- and phospho-S157-interacting proteins from total cell lysates, using affinity chromatography purification.

Identification of adaptor/effector molecule(s) that relay the GRK2 inhibitory signal is(are) critical to further our understanding of this novel antagonistic pathway in TGFβ signaling and for the development of new targeted therapies.

3.4 MATERIALS & METHODS

Cell Culture: Human liver cancer (HUH7) and human breast cancer (MCF7) cells were cultured in DMEM supplemented with 10% FBS, 100units/ml penicillin, 10mg/ml streptomycin, and 2mM glutamine (Invitrogen). Cells were grown to ~80% confluence and then growth arrested overnight in serum-free DMEM supplemented with 100units/ml penicillin, 10mg/ml streptomycin, and 2mM glutamine. Similarly, rat VSMCs were cultured in low glucose DMEM supplemented with 10% heat-inactivated FBS, 100units/ml penicillin, 10mg/ml streptomycin, and 2mM glutamine. Cells were grown to ~80%

confluence and then growth arrested overnight in serum-free low glucose DMEM supplemented with 100units/ml penicillin, 10mg/ml streptomycin, and 2mM glutamine.

Column Preparation and Ligand Coupling: Specific 10 aa phosphopeptides representing the sequence surrounding T197 (KLDDY(T-p)HSIP) of Smad2 or the non-phosphorylated version of the peptide (KLDDYTHSIP), in addition to the phosphopeptides representing the sequence surrounding S157 (KLDDY(S-p)HSIP) or the non-phosphorylated version of the peptide (KLDDYSHSIP), were synthesized and purified by high performance liquid chromatography. An extra N-terminal lysine residue was added to each peptide, allowing for covalent coupling to the HiTrap NHS-activated Sepharose High Performance affinity column matrix (Amersham Biosciences).

Using a peristaltic pump, the HiTrap columns were washed with ice-cold acidification buffer [1mM HCl] in preparation for peptide coupling. The lyophilized peptides were dissolved in coupling buffer [0.2M $NaHCO_3$, 0.5M NaCl, pH8.3] to a concentration of 10mg/ml and for a final volume of 1ml. The peptides were subsequently passed through to the acid-washed columns and left for 4hrs at 4°C to allow maximal coupling to the matrix. Following incubation, excess unbound active groups were deactivated and non-specifically bound peptides were washed out using a series of alternating high and low pH washes. Neutralization buffer was then injected into the peptide-bound columns for storage at 4°C.

Preparation of Lysates and Experimental Treatment:

Preparation of lysates: To identify proteins that specifically interact with the phosphopeptide and not with the unphosphorylated peptide, total cell lysates from HUH7s, MCF7s and VSMCs were collected in sterile ice-cold 1X PBS and lysed with lysis buffer [50mM Hepes, pH7.5; 150mM sodium chloride; 100mM sodium fluoride; 10mM sodium pyrophosphate; 5mM EDTA, pH 8.0; 10% glycerol; 0.5% Nonidet P40; 0.5% sodium deoxycholate] supplemented with protease inhibitors 1mM PMSF, 10μg/ml aprotinin, 10μg/ml leupeptin, 2μg/ml pepstatin, (BioShop). Lysates were rotated at high speed for 1h at 4°C. This was followed by centrifugation at 14,000rpm for 15min at 4°C. Supernatants were passed through a 45μm filter and divided equally for passage through each column.

Binding and Eluting: Before passing the lysates through the peptide-coupled columns, the columns were washed with 2mL of 1X PBS, followed by 3mL of elution buffer [100mM glycine, 0.5M NaCl, pH 2.7], and were finally equilibrated with 10mL of 1X PBS. Cell lysates were then slowly circulated through the column for 2hrs at 4°C. Once complete, the columns were washed with 10mL 1X PBS and bound proteins were eluted

with 5mL of elution buffer, whereby 15 fractions of 0.5mL (13 drops) each were collected in eppendorfs containing 45µL of neutralization buffer [1M TrisHCl, pH 9.0]. Collected fractions were mixed thoroughly to prevent degradation. The columns were de-equilibrated with 10mL of 1X PBS and prepared for storage at 4°C by circulating 5mL of storage buffer [0.05M Na_2HPO_4, 0.1% NaN_3]. Fractions were stored at -80°C until further use.

Acetone Precipitation: Acetone, cooled to -20°C, was added to the eluted fractions in a volume four times that of the sample being precipitated (800µL of acetone to 200µL of sample). The tubes were vortexed gently before overnight incubation at -20°C. The following day, the samples were centrifuged at 14,000rpm for 10min. The supernatant was carefully decanted, so as not to dislodge the protein pellet from the bottom of the tube, and the tubes left open for ~30min to allow any remaining acetone to evaporate. The remaining pellet was resuspended and dissolved in 2X SDS Loading Buffer.

SDS-PAGE and Gel staining: Glass plates, spacers and combs were washed with 10% acetic acid, rinsed with sterile dH_2O, and air dried. Lab coat and acetic acid-washed gloves were worn at all times in order to prevent keratin contamination.

The collected fractions were resolved on a 7.5% polyacrylamide gel, to allow optimal protein separation, and analyzed by silver stain as per the manufacturer's instructions (Invitrogen SilverQuest Silver Staining Kit, For Mass Spectrometry-Compatible Silver Staining of Proteins in Polyacrylamide Gels).

Gels were stored in a hermetically sealed bag containing 5mL 1% acetic acid.

Gel Extraction, Sample Destaining and Sample Analysis: Comparative analysis of protein bands in the unphosphorylated peptide-bound column and in the phosphopeptide-bound column revealed several interesting targets. Protein bands were extracted using sterile scalpel blades and were transferred to labeled sterile eppendorfs using sterile tweezers. Extracted bands were destained as per the manufacturer's directions (Invitrogen SilverQuest, Destainer) and sent to Applied Biosystems, MDS Sciex, for trypsin digest and subsequent analysis by mass spectrometry (4000 QTRAP LC/MS/MS System).

3.5 PRELIMINARY RESULTS

Smad2- and Smad3-Specific Peptides and Phosphopeptides Demonstrate Specific Binding to Potential Target Proteins from Cell Lysates: In order to identify specific adaptor/effector molecules that interact with the GRK2 phosphorylation site within the Smad2/3 linker domain, peptides corresponding to the phosphorylated and to the non-

phosphorylated sequence in the linker region, were synthesized. Whole cell lysates from HUH7, MCF7 and VSMCs were circulated through the four peptide-bound affinity columns and bound proteins were eluted. Eluted fractions were concentrated using acetone, resolved on SDS-PAGE and visualized by silver stain.

Both the Smad2 (Fig.15) and the Smad3 (Fig.16) gels showed multiple protein interactions, some specific to the phosphopeptide-bound column, whereas some only interacted with the non-phosphorylated peptide-bound column. These results support our hypothesis that a single phosphoresidue with a specific amino acid environment can trigger recognition and binding of an interacting partner.

Proteins Identified from the Peptide Analysis: When choosing which protein bands to send for sequence analysis, it was preferential to select the darkest bands, as they suggested a higher protein concentration from which to extract information. Although one band is cut, it may in fact contain several different peptides of similar molecular weight, making it difficult to identify a single protein. Moreover, trypsin digestion and protein fragmentation greatly reduces the amount of protein available for sequence analysis.

The 4000 QTRAP LC/MS/MS System was used to analyze our samples. It is ideal for metabolite identification and proteomic applications, including protein identification. Collisional focusing and linear ion trap technologies are combined to maximize MS/MS sensitivity. A triple quadrupole multiple reaction monitoring system serves to increase sensitivity for improved quantification and Information Dependent Acquisition permits focused analysis of specific ions of interest. Identified sequences were screened using ProteinPilot™, which combines the Mascot search engine with new Algorithms, simultaneously searching over 150 biological modifications, genetic variants, unexpected cleavages, and user-defined modifications [119]. Identified proteins, accession number, percent coverage and other related information was provided.

Five phosphoresidue-binding protein bands and one non-phosphoresidue-binding protein band were extracted from the gel and analyzed by mass spectrometry (Table 1). Included in the table is the Percent Sequence Homology, which refers to the percentage of fragment sequences of the unknown peptide that can align against the sequence of a known protein, i.e. the total percentage of amino acids that are identical between the two sequences after aligning them. The number of fragments and the fragment length are useful criteria for judging the relevance of the resulting protein match, as it is easier to match up several smaller fragments than to have homology over a larger area.

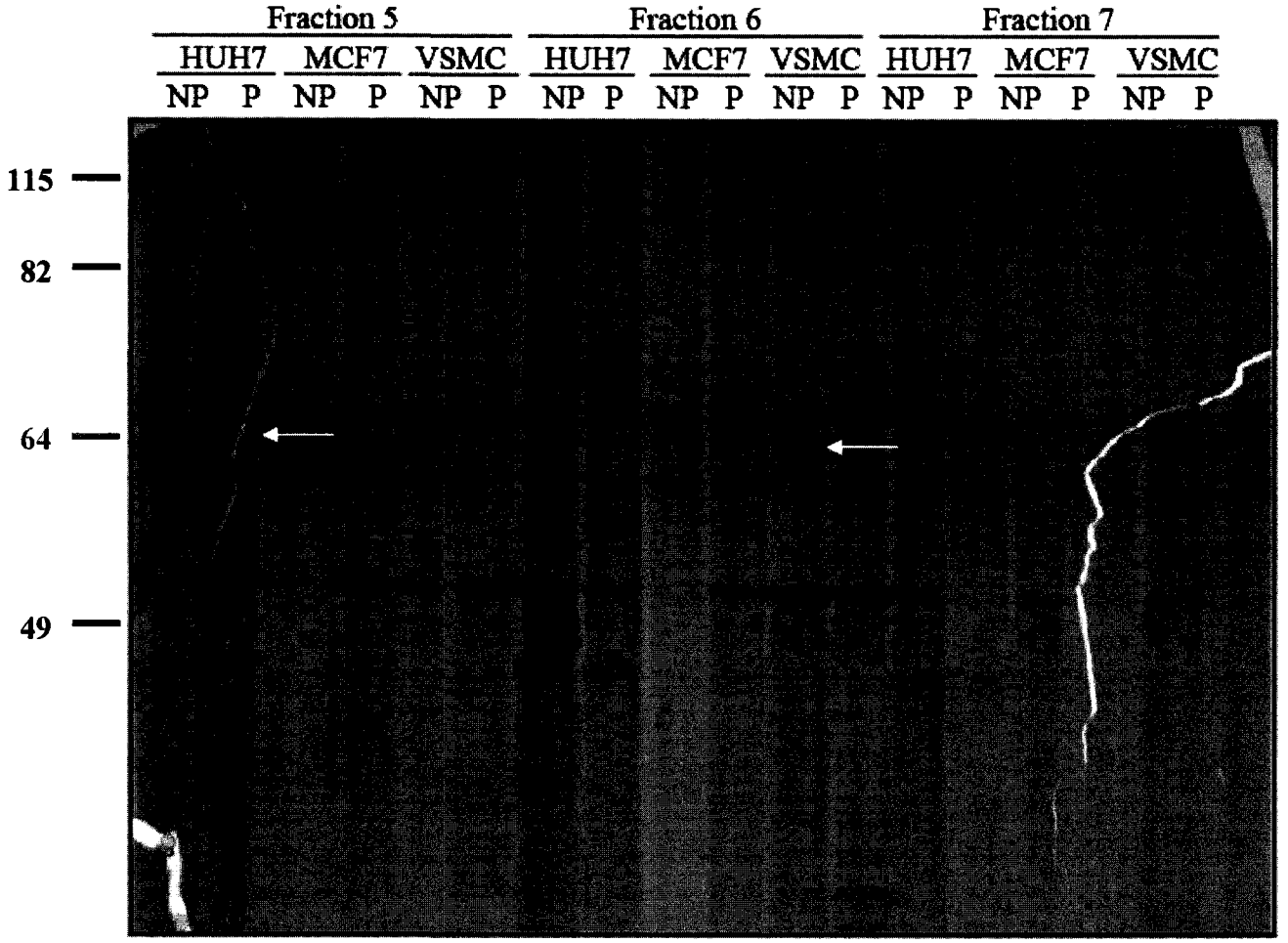


Fig.15: Smad2-specific peptides and phoshphopeptides demonstrate specific binding to potential target proteins from cell lysates

A silver stained SDS-PAGE from affinity purified proteins from Fractions 5, 6 and 7 of human hepatocarcinoma cells (HUH7), human breast cancer cells (MCF7) and vascular smooth muscle cells (VSMC), showing specific binding to either a non-phosphorylated or a phosphorylated peptide-bound column representing the GRK2 mediated phosphorylated residues on Smad2. The arrows indicate the bands that were sent for analysis.

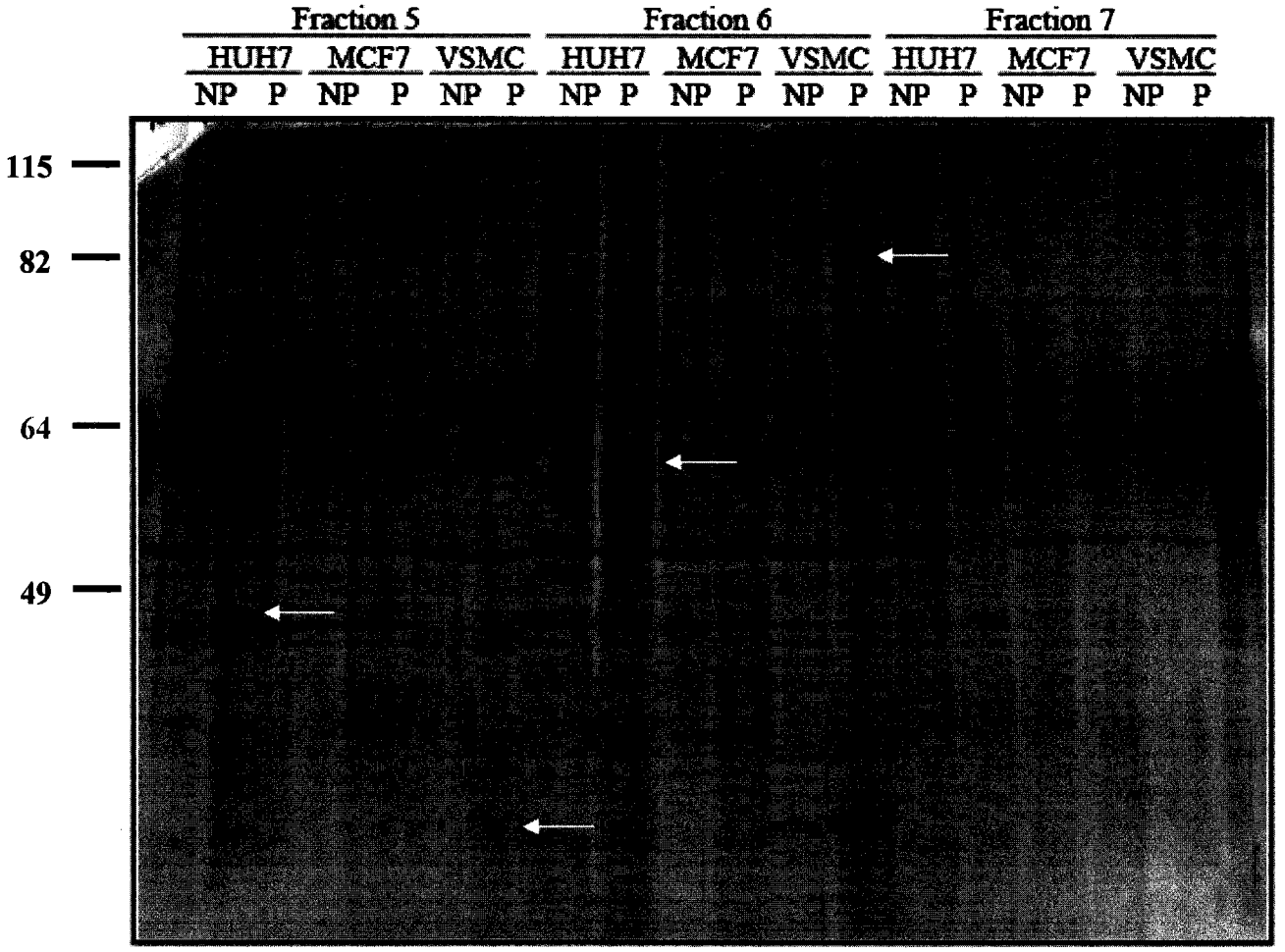


Fig.16: Smad3-specific peptides and phoshphopeptides demonstrate specific binding to potential target proteins from cell lysates

A silver stained SDS-PAGE from affinity purified proteins from Fractions 5, 6 and 7 of human hepatocarcinoma cells (HUH7), human breast cancer cells (MCF7) and vascular smooth muscle cells (VSMC), showing specific binding to either a non-phosphorylated or a phosphorylated peptide-bound column representing the GRK2 mediated phosphorylated residues on Smad3. The arrows indicate the bands that were sent for analysis.

Smad	Sample Name	mw (kDa)	Protein Identified from Peptide	Sequence Homology (%)
2	F5_B2_P_HUH7	~ 64	Caspase-12 Carboxypeptidase D precursor Coronin-1C (hCRNN4)	10.7 7.9 5.9
2	F6_B2_NP_VSMC	~ 64	Dermcidin MAGUK p55 subfamily 5	54.5 11.6
3	F5_B1_P_HUH7	< 49	Caspase-12	16.8
3	F5_B2_P_VSMC	< 49	Predicted protein Hypothetical protein	N/A
3	F6_B5_P_HUH7	< 49	Eukaryotic Elongation Factor 1 α 1	19.3
3	F6_B1_P_VSMC	> 64	Dermcidin DiGeorge Syndrome critical region 6 PP3856	20.9 18.2 3.3

Table 1: Specific peptide-binding proteins submitted for identification – Summary of the bands extracted from the Smad2 and the Smad3 gels sent for partial sequence identification using the 4000 QTRAP LC/MS/MS from Applied Biosystems, MDS Sciex. Samples were named as follows: Fraction#_Band#_Non-phosphorylated/Phosphorylated Peptide_Cell Type. Percent Sequence Homology refers to the total percentage of amino acids that are identical between the unknown peptide sequence and a known protein sequence, after aligning them.

Listed below are the proteins identified and their respective roles in cellular processes. Although some of the proteins identified displayed low sequence homology with the peptides sent for analysis, the aim of this preliminary screen was to familiarizing ourselves with the genre of proteins we could expect to find interacting with the Smad linker region.

Caspase-12:

Smad2, Fraction 5, Band 2 of the Phosphoresidue column, HUH7, ~64kDa

Smad3, Fraction 5, Band 1 of the Phosphoresidue column, HUH7, ~64kDa

The phosphoresidue-binding protein isolated from the Smad2 gel, HUH7 column showed 10.7% sequence homology with Caspase-12. In the Smad3 gel, it showed 16.8%.

Caspases are cysteinyl aspartate-specific proteinases known for their role in apoptosis, inflammation and cytokine maturation. They are classified into two subfamilies based on structure, function and substrate specificity: (i) the inflammatory caspases, composed of caspases-1, -4, -5, and -12 or (ii) the apoptotic caspases, composed of caspases-2, -3, -6, -7, -8, -9, and -10 [84, 120],. There is no human caspase-11.

Caspase-12 expression can be found in almost all tissues, with the highest levels being observed in the lung, stomach and small intestine. However, constitutive protein expression is restricted to skeletal muscle, heart, brain, liver, eye, testis and to a much lesser extent in the lymph nodes, thymus and spleen [84]. Importantly, caspase-12 has been implicated in neurodegenerative disorders including Alzheimer's disease.

A controversial role for caspase-12 is as key mediator of ER-stress-induced cell death [120, 121]. Caspase-12 exists in a proform on the cytoplasmic side of the ER. Two hypotheses for its activation, without mitochondrial involvement, have been proposed. One involves translocation of cytosolic caspase-7, during ER stress, to the ER surface, whereas the other involves increased Ca^{2+}, leading to the movement of calpain to the ER surface [121]. However, multiple groups failed to reproduce results demonstrating that caspase-12 is dispensable for ER stress-initiated apoptosis [84], which is probably due to the fact that they did not discriminate between caspase-12 autocleavage and the calpain cleavage event.

More recently, caspase-12 has been shown to associate with caspase-1 and inhibit its activity. The inhibitory function of caspase-12 is a dominant effect and is detrimental to the individual, as it leads to increased susceptibility to infection and severe sepsis [84]. A single nucleotide polymorphism in caspase-12, occurring in exon 4, changes the codon from a stop to an arginine residue, which results in the synthesis of a full length protein. Most individuals express the truncated form of caspase-12. The full length variant is only

expressed in about 20% of individuals, mostly of African descent, dampening their inflammatory response to endotoxins, and increasing their risk of developing sepsis [84].

Interestingly, the enzymatic function of caspase-12 is not required for its inhibitory effect on caspase-1 [84, 122]. In this regard, caspase-12 seems to be the cellular FLICE-inhibitory protein, which blocks death receptor-induced apoptosis (cFLIP) counterpart for regulating the inflammatory branch of the caspase cascade. These results suggest that caspase-12 functions not only in the haematopoietic system, but may also modulate the inflammatory response in other tissues [122].

Carboxypeptidase D precursor (Metallocarboxypeptidase D, gp180):

Smad2, Fraction 5, Band 2 of the Phosphoresidue column, HUH7, ~64kDa

Type I membrane protein, Carboxypeptidase D (CPD) had 7.9% sequence homology with the HUH7 column of Smad2 gel phosphoresidue-binding peptide.

Carboxypeptidases are a family of enzymes that process peptides and hormones by removing basic amino acids from their C-terminus, thereby modulating activity [123]. Originally discovered as a 180-kDa glycoprotein-receptor for duck hepatitis B virus, eventually the bovine, murine and human forms were cloned and characterized, and found to play an important role in the secretory pathway [123, 124].

CPD, is a type I membrane protein with a single transmembrane sequence near the C-terminus. It contains three domains of which only domains I and II are enzymatically active. The inactive domain III has retained some residues potentially involved in substrate binding, suggesting a role in the binding and presenting of peptide substrates [125].

Expression of CPD is ubiquitous, as it is present in numerous cell types where it may play an important constitutive function in the processing of proteins, polypeptides or pro-hormones that transit the secretory pathway [125]. Consequently, it is no surprise that CPD is primarily found in the *trans*-Golgi network of the cell. Also present in the *trans*-Golgi network and immature secretory vesicles is furin, a protease involved in releasing the C-terminal end of TGFβ proproteins. Thus, CPD has the right cellular and subcellular distribution to participate in the processing of neuroendocrine peptides, as well as to play a major role in the processing of proteins initially cleaved by furin [126]. Like furin, the CPD active site uses a glutamic residue and a tightly bound penta-co-ordinated Zn^{2+} cofactor to catalyze the cleavage of C-terminal basic amino acid residues [125].

Nevertheless, significant amounts of CPD can also be found on the plasma membrane, indicating that this protein can also function as a cell surface protein [127].

Coronin-1C (Coronin-3, hCRNN4):

Smad2, Fraction 5, Band 2 of the Phosphoresidue column, HUH7, ~64kDa

Extracted from the HUH7 column of Smad2, the phosphoresidue-binding protein was shown to have 5.9% sequence homology with Coronin-1C.

Coronin-1C/Coronin-3 belongs to a family of seven mammalian proteins that play a central role in various cellular processes including wound healing, cell proliferation, signal transduction, transcriptional regulation, remodeling of the cytoskeleton, and regulation of vesicular trafficking [83]. They are characterized by the presence of at least four WD repeats in the core of the protein (Fig.17), a primary structure unlike that of any other actin-binding protein known at the time of discovery. Moreover, they have a sequence similar to that of the β subunits of trimeric G proteins, which have seven WD repeats [128]. Such proteins are often part of multi-subunit complexes formed through WD40 domain interactions [129], thus serving as a platform for protein-protein interaction.

Interestingly, the interaction of coronin proteins with the actin cytoskeleton is regulated by phosphorylation. Consensus phosphorylation sites are widespread among the coronins, and it is likely that phosphorylation is a common modification in this protein family and that it plays an important role in its regulation [128]. In addition to maintaining cellular structural integrity through its three-dimensional network of filamentous polymers, the cytoskeleton may participate in agonist-stimulated signal transduction [106]. In fact, several WD-repeat proteins have been linked to human disease [83].

Dermcidin precursor (preproteolysin):

Smad2, Fraction 6, Band 2 of the Non-Phosphoresidue column, VSMC (~64kDa)

Smad3, Fraction 6, Band 1 of the Phosphoresidue column, VSMC (>64kDa)

The Smad2 peptide-binding and the Smad3 phosphoresidue-binding peptides extracted from VSMC columns showed 54.5% and 20.9% homology with Dermcidin, respectively.

Dermcidin is a recently discovered antimicrobial peptide from the innate immune defense system of the human skin. Found in sweat, it prevents local and systemic invasion of pathogens by modulating surface colonization [130]. Recently, it was found to be expressed in tubular structures of cutaneous mixed tumours and has been proposed as a survival factor in neuronal cells, hepatoma cells and breast carcinomas [130-132].

Dermcidin is a 110 aa protein composed of a signal peptide, proteolysis-inducing factor-core peptide (PIF-CP), a propeptide, and a skin antimicrobial called DCD-1. In 2006, Lowrie *et al.* showed that Dermcidin protected against oxidative stress induced by

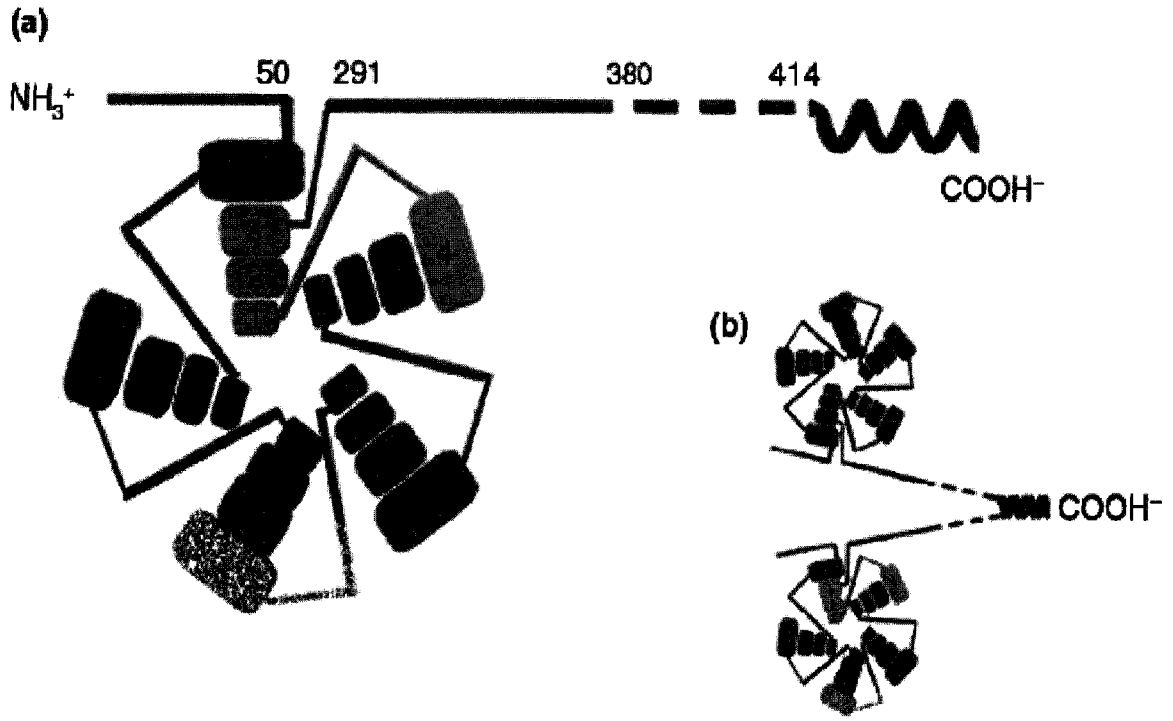


Fig.17: Basic core structure of Coronin proteins

Model of coronin structure based on the β-propeller structure of G protein β subunits. (a) Monomer. Numbers represent the approximate amino acid positions in *Dictyostelium* coronin. An individual WD repeat (in green) is shown to fold into four β sheets (numbered) that span two 'propeller blades'. β Sheet 4 of the third repeat (mottled) is more variable than others among mammalian coronins. The conserved region (N-terminus to ~380 aa) is followed by a unique region of variable length. The C-terminal (~32 aa) is thought to form an α-helical coiled coil. (b) Dimer with subunits joined at the coiled coils (de Hostos 1999)

glucose oxidase, by protecting from both apoptosis and necrosis. However, this protection was abrogated by the mutation of the N32 asparagine residue alone or in combination with the mutation of both asparagine residues of the PIF-CP sequence. Furthermore, they attribute the survival advantage of Dermcidin to the PIF-CP fragment. Screening of tissues for PIF binding demonstrated substantial binding only to skeletal muscle and liver [133].

Y-P30, another peptide arising from the same gene as PIF, has also been proposed as a survival protein. These peptides share the came core sequence, demonstrating evolutionary conservation, thus it is highly probable that this gene may have important cellular function.

MAGUK p55 subfamily member 5:

Smad2, Fraction 6, Band 2 of the Non-Phosphoresidue column, VSMC, ~64kDa

Membrane-associated guanylate kinase (MAGUK) scaffolding protein shared 11.6% homology with the non-phosphopeptide-binding protein from the Smad2 HUH7 column.

Diverse extracellular signals are coupled to intracellular signal transduction pathways and to the cytoskeleton at the plasma membrane [43] by MAGUKs, members of a ubiquitous multidomain protein family. Consequently, they have been extensively studied for their role in targeting proteins in polarized cells, such as neurons and epithelia [134]. Establishment and maintenance of polarity relies on polarized vesicle trafficking and precise targeting of proteins to discrete membrane subdomains in response to extracellular and spatial cues [134]. It is through the use of scaffolding proteins that these specific locations are kept organized and distinct, allowing for precise and timely expression. Disruption of these processes, altering the polarized epithelial phenotype, can result in severe developmental defects [135].

All MAGUK proteins are defined by a basic core structure of three domains (Fig.18): (i) a Src homology 3 (SH3) domain, (ii) a guanylate kinase (GUK) domain, and (iii) a PDZ domain [43, 136]. Based on their role in other proteins, SH3 domains are expected to bind the actin cytoskeleton, proteins involved in signal transduction or both [43] via proline-rich motifs. The GUK domain shares some homology with the enzyme that catalyzes the ATP-dependent conversion of GMP to GDP. Sequence divergence has allowed for the variation of nucleotide-binding capabilities. Consequently, it shows little or no kinase activity. However, the GUK domain has been found to engage in inter and intramolecular interactions with the SH3 domain, via mechanisms not involving the proline-rich recognition site of SH3. Intermolecular interaction could lead to the formation of large scaffolding complexes, containing different combinations of various domain-specific

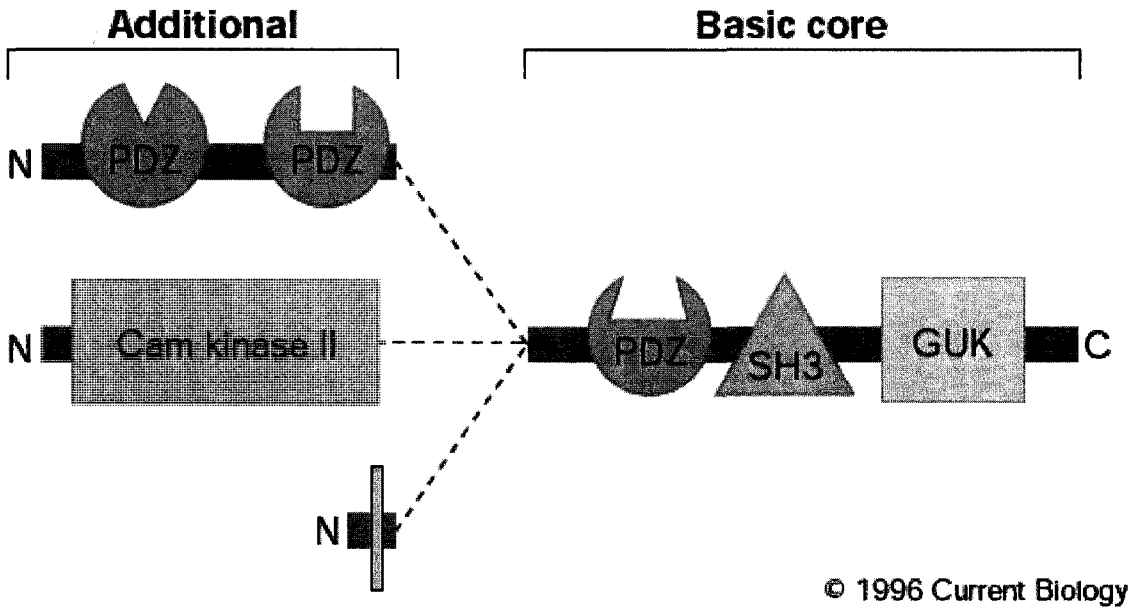


Fig.18: Basic core structure of MAGUK proteins

The basic core of a MAGUK protein consists of a PDZ, an SH3 and a GUK domain. Some MAGUKs have additional domains at the N-terminus, such as two PDZ domains or a domain with homology to CaMKII (Anderson 1996)

binding proteins, bringing together a variety of transmembrane receptors at the basolateral surface of their associated cytoplasmic proteins [135]. Finally, the PDZ domains are found in various proteins, in single or in multiple copies. They can dimerize or bind the C-terminal regions of integral membrane and intracellular proteins. Functionally, PDZ-containing proteins are involved in the targeting of their interacting partners to specific subcellular domains, in the formation of PDZ-based scaffolds to stabilize interacting proteins within macromolecular complexes, in the trafficking of PDZ interacting proteins along microtubules, synaptic vesicle exocytosis and in signal transduction [137]. The MAGUK p55 subfamily contains a single PDZ domain, which binds the C-terminal EYYV motif of glycophorin C, an integral erythrocyte membrane protein [135]. In fact, in the red blood cell, p55 links the cytoplasmic tail of glycophorin C to the actin network [43].

Many cellular complexes are assembled on scaffold proteins. Multi-domain scaffolding proteins can assemble a combination of cell adhesion molecules, cytoskeletal proteins, receptors, ion channels and their signaling components at specific membrane sites [135]. Thus all MAGUK proteins are likely to share some basic integrative function in coordinating signals at the cell cortex, perhaps the ability to link transmembrane proteins to cytosolic proteins, while interacting with G protein cascades [43].

Eukaryotic translation elongation factor 1 α 1 (eEF1α1):

Smad3, Fraction 6, Band 5 of the Phosphoresidue column, HUH7 (<49)

The phosphoresidue-binding peptide cut from the Smad3 HUH7 column showed 19.3% homology with the known protein eEF1α1.

In early 1990, eEF1α2 was identified as a tissue-specific variant of eEF1α1 (formerly EF-1α). The two forms of eEF1α are encoded by separate loci, but the resulting proteins are 92% identical and 98% similar, having essentially the same function during protein translation [138, 139]. Each stage of protein translation is controlled by multimeric protein factors, eukaryotic Initiation Factor, eukaryotic Elongation Factor and eukaryotic Release Factor, that respectively regulate initiation, elongation and termination [138]. Both eEF1α1 and 2 directly bind amino acylated tRNA and direct its association with the ribosome and mRNA codon during polypeptide elongation.

Despite this similarity, the expression patterns of the two isoforms are markedly different. In humans, eEF1α1 is ubiquitously expressed [138] and thus is involved in protein synthesis, stress-sensing, apoptosis and cellular proliferation [140]. eEF1α2, on the other hand, is restricted to the heart, brain, and skeletal muscle.

In 2000, Izawa, Fukata *et al.* showed that, in addition to being a cofactor of polypeptide elongation, eEF1α1 has filamentous actin (F-actin)-binding and -bundling activities, as well as microtubule-severing activity. By extension, eEF1α1 was also proposed to act as a signaling molecule in proliferating cells, after nuclear translocation, upon binding to other nucleic-acid-binding proteins. Inside the nucleus, eEF1α can bind DNA, RNA and RNA polymerase, potentially playing a role in transcriptional regulation [140]. It has been suggested by Lee (2003) that increased eEF1α1 and 2 expression may not specifically increase protein production, but rather lead to an overall increase in protein translation. Increased protein synthesis may enhance cell replication, as sufficient protein material would be available to fulfill the metabolic requirements of cell division.

Interestingly, eEF1α was also identified as a novel substrate of Rhokinase. Rho is a small GTPase involved in signaling pathways that regulate actin-cytoskeletal structure, cell morphology, cell aggregation, cell-cell adhesion, cell motility, cytokinesis, and smooth muscle contraction [141]. Phosphorylation of eEF1α by Rhokinase decreases the F-actin-binding and -bundling activities of eEF1α. Because F-actin and aminoacyl-tRNA compete with each other for eEF1α binding, Rho-kinase phosphorylates eEF1α to weaken its F-actin-binding and to increase its binding to aminoacyl-tRNA, promoting localized protein synthesis associated with the actin cytoskeleton [141]. These results suggest that the Rho/Rho-kinase pathway regulates cytoskeletal organization via eEF1α phosphorylation.

DiGeorge Syndrome critical region gene 6 (DGCR6):

Smad3, Fraction 6, Band 1 of the Phosphoresidue column, VSMC, >64kDa

The Smad3 VSMC phosphopeptide-bound protein sequence matched 18.2% with DGCR6.

DiGeorge syndrome is a rare congenital disease associated with microdeletions of the 22q11.2 chromosomal region, often resulting in recurrent infection, heart defects, and characteristic facial features. DGCR6 was first isolated in 1995 and found to share homology with the *Drosophila melanogaster* gonadal protein and with the laminin γ-1 (LAMC1) chain. Expression of DGCR6 was found in all tissues, except the placenta, and was highest in adult heart and skeletal muscle [142], which is partly composed of VSMCs.

Increasing evidence has linked DGCR6 homologue, LAMC1, to possible defects in the development of neural crest cells, in the DiGeorge syndrome. LAMC1 is a highly conserved and well-studied protein with functions in tissue assembly, cell migration and attachment and differentiation [142]. It binds to cells via a receptor and is thought to

mediate the attachment, migration and organization of cells into tissues, during embryonic development, by interacting with other ECM components.

PP3856:

Smad3, Fraction 6, Band 1 of the Phosphoresidue column, VSMC, >64kDa

The Smad3 gel VSMC column-extracted phosphoresidue-binding protein showed 3.3% homology with Nicotinate Phosphoribosyltransferase (NPRTase), also known as, PP3856.

NPRTase catalyzes the first step in the biosynthesis of NAD from nicotinate [143], in what is described as the Preiss-Handler pathway [144]. This enzyme occurs in most mammalian tissues, as it is part of nucleotide metabolism, which is essential for transcription. It transfers a phosphoribosyl group to a base from 5-phosphoribosyl-1-pyrophosphate, forming nicotinic acid mononucleotide from nicotinic acid. Although NPRTase can be stimulated by ATP, it is not absolutely dependent on it for activity.

3.6 DISCUSSION

This chapter describes the preliminary screening of potential GRK2 phosphoresidue-binding adaptor/effector proteins.

Despite the extensive studies that have contributed to deciphering the TGFβ signaling pathway, many of its components are still unknown and have yet to be characterized. Our lab discovered a novel negative feedback loop mechanism involving GRK2, a downstream target of TGFβ. GRK2 prevents Smad signaling by physically interacting with and phosphorylating a specific serine/threonine residue within the Smad2/3 linker region. Similarity between two FHA domain binding sequences and the GRK2 phosphorylation site suggest that these residues may act as recruitment sites for adaptor/effector molecules.

Peptides representing the target residues on both Smad2 and 3, in both their phosphorylated and non-phosphorylated forms, were coupled to affinity columns through which whole cell lysates from HUH7, MCF7 and VSMCs were circulated. Bound proteins were eluted, resolved on SDS-PAGE and silver stained.

A range of cell types were sampled in order to screen for cell-specific binding patterns. We anticipated to see a change in the amount of protein binding as the fraction number increased, particularly in the phosphopeptide-bound column, suggesting the recruitment and/or release of an adaptor/effecor molecule from the specific region. Both the Smad2 and 3 gels showed some specific phosphopeptide-binding proteins. Others were even found to

interact with only the non-phosphorylated peptide-bound column. Select samples were sent for trypsin digest, partial sequence analysis and identification by mass spectrometry.

From the 6 samples analyzed, 10 proteins were identified, some of which perform specific functions that might be relevant to the role of an adaptor/effector molecule that may interact with GRK2 phosphorylation site within the Smad2/3 linker region.

Dermcidin has been shown to be protective against oxidative stress and apoptosis. Despite its interesting role as a regulator of apoptosis and stress-activated factors, Dermcidin was eluted from both columns, suggesting a more general role in cell processes.

The DGCR6, which also shares homology with the LAMC1 protein that functions in tissue assembly, migration, attachment and differentiation, could potentially be regulated by the Smad2/3 linker region phosphorylation events, as it shares functions with TGFβ.

CPD, primarily found in the *trans*-Golgi membrane, is a ubiquitous enzyme involved in propeptide processing. Regulated proteolysis could represent a powerful mechanism for local and temporal control over protein activation [27]. Studies have also described CPD as part of an autoregulatory feedback loop, where it is both "upstream" and "downstream" of TGFβ signaling [123].

Caspase-12 was identified from two extracted HUH7 samples, present at approximately the same molecular weight. Interestingly, activation of caspase-12 has been attributed to ER stress, which plays a critical role in liver injury, as high levels of caspase-12 are expressed there [121]. Recently, however, caspase-12 has been shown to inhibit the activity of caspase-1, which processes actin [84]. Various cellular processes including embryogenesis, migration and adhesion involve the dynamic remodeling of the actin cytoskeleton. Thus, it conceivable that caspase-12 may manipulate the actin cytoskeleton to facilitate signaling.

Another actin-binding protein identified was Coronin-1C/Coronin-3. Coronins play a central role in various cellular processes, particularly signal transduction, transcriptional regulation, remodeling of the cytoskeleton, and regulation of vesicular trafficking. In addition to having at least four WD repeats, which have been linked to human disease[83], Coronins have a sequence similar to that of G protein β subunits [128]. Interestingly, GRK2 is activated by free Gβγ subunits [96]. Gβγ subunits translate the intensity of a G protein-stimulated signal into GRK2 activity to switch off the signal-generating receptor [96]. It is conceivable that Coronin could be involved in mediating GRK2 activity within the linker region or in attenuating any surrounding GPCR signal that may be interfering.

Because of the role Smads play in signal transduction, MAGUK scaffolding proteins fit the profile of potential adaptor/effector molecule, as their role is to organize protein complexes in order to control the precise targeting of proteins to discrete membrane subdomains in response to extracellular and spatial cues [134]. The PDZ domain acts as a scaffold for the recruitment of several proteins [43] and the identified MAGUK p55 subfamily member 5 contains one PDZ domain. GITs, ubiquitous multifunctional binding proteins for GRKs, interact with a variety of signaling molecules involved in cellular processes such as cytoskeletal dynamics, membrane trafficking, cell adhesion and signal scaffolding. Given the role of both MAGUK and GIT proteins, as well as their diverse implications in cellular signaling, it is possible that recruitment of GRK to such scaffolding complexes might engage GRKs in functional and/or regulatory interactions [95].

Among the regulatory signals that may be emanating from the GRK2 phospho-region, it is possible to promote gene expression. In fact, the main role of the widely expressed eEF1α1 is to direct amino acylated tRNA binding to the ribosome, during elongation. And, it has also been shown to translocate to the nucleus [140], where it can bind DNA, RNA or RNA polymerase and regulate transcription. eEF1α1 also interacts with the cytoskeleton, such that phosphorylation of eEF1α1 regulates the organization of the actin cytoskeleton through the small G-protein kinase pathway. Thus, it is possible that recruitment to the linker region and phosphorylation of eEF1α1 might lead to cytoskeletal reorganization, assisted cell signaling, nuclear translocation and possibly, activation of gene expression.

Interestingly, an enzyme involved in the biosynthesis of NAD, PP3856, was identified. PP3856 is an essential component of nucleotide metabolism, required for transcription. Identification of this protein could be indicative of the recruitment of transcriptional factors to this GRK2-phosphorylated binding site.

Taken together, these results suggest that the actin skeleton plays an integral role in arranging scaffolds, in regulating cell signaling in both a location- and time-dependent manner, and that it also plays a potential role in mediating transcription and translation. Such regulatory abilities, in conjunction with phosphorylated activation or regulation, makes some of these identified proteins interesting targets of future studies regarding potential interaction with GRK2. Elucidation of this regulatory negative feedback loop is critical to further our understanding of the regulatory mechanisms involved in the complex and essential TGFβ signaling pathway. Moreover, it will contribute greatly to the development of novel targeted therapies.

CHAPTER 4: FINAL DISCUSSION

This thesis investigated the importance of the role that GRK2 plays in both TGFβ and GPCR signaling and crosstalk mechanisms. In chapter two, our results define a novel regulatory and antagonistic mechanism between the two distinct growth factor superfamilies (TGFβ and GPCR), and identify GRK2 as the key mediator of the crosstalk between these two signaling pathways. Moreover, chapter three discusses the role of the GRK2 phosphorylation site within the Smad2/3 linker region as a potential platform for the assembly of a regulatory complex. Analysis by mass-spectrometry identified several potential phosphoresidue-binding proteins, which could be involved in potentiating the antagonistic effects of GRK2 on TGFβ signaling.

The TGFβ signaling cascade regulates a plethora of essential physiological functions, as is made evident by the various human diseases in which TGFβ deregulation has been observed [3]. While other signaling pathways have been shown to regulate Smad-mediated TGFβ signaling [53-55, 145], relatively less is known about the impact of TGFβ signal transduction on signaling from other receptor superfamilies.

When first identified by our laboratory as a downstream target of the TGFβ signaling cascade, GRK2 had not been implicated in the downstream signaling of any other single-transmembrane serine/threonine kinase growth factor receptor [42]. In fact, this was the first time GRK2 was found to act in a negative feedback loop, terminating TGFβ-induced signaling. This inhibitory effect was not tissue specific, suggesting an important regulatory role for GRK2. Moreover, as GRK2 is a key regulatory kinase involved in the early initiating steps towards desensitization of GPCRs, it also suggested a potential role for GRK2 in mediating crosstalk with other signaling pathways, particularly the GPCRs.

Interestingly, AngII, the predominant peptide hormone of the renin-angiotensin system (RAS), exerts opposite effects to those of TGFβ, particularly in the regulation of cell growth, proliferation and migration in VSMCs [146, 147]. Although the TGFβ and the AngII signaling pathways have been shown to be intricately intertwined [2, 23, 103], only limited information is currently known regarding the mechanism of their actions [103]. As such, we sought to examine the crosstalk mechanisms between two receptor families, and to investigate whether TGFβ-induced increase in GRK2 would affect GPCR signaling.

Our results showed that, in VSMCs, activation of the TGFβ signaling cascade resulted in a time-dependent increase of GRK2 expression, leading to the inhibition of AngII-induced ERK phosphorylation, as well as to the inhibition of AngII-mediated VSMC

proliferation and migration. Use of an antisense oligonucleotide blocking GRK2 expression restored normal cellular behaviour, attributing the inhibitory effects to GRK2. Moreover, we found that TGFβ-mediated increase of GRK2 expression blocked AngII-mediated ERK phosphorylation and activation of the downstream target HIF-1α, without affecting MEK phosphorylation. Interestingly, induction of HIF-1α closely follows ERK phosphorylation [148] and, it has been shown that AngII exerts its proliferative and anti-apoptotic functions through the ERK pathway [149]. Our results suggest that the GRK2-mediated inhibitory effects are independent of receptor phosphorylation and internalization, and directly target ERK activation. Altered levels of GRK2 have been described in various diseases [87], often acting at the level of receptor phosphorylation and internalization. However, increased levels of GRK2 have been associated with cardiovascular disease, in which both TGFβ [3] and AngII also play central regulatory roles [23, 97]. Based on our results, it is conceivable that upon TGFβ-induced GRK2 upregulation, excess GRK2 can interact with MEK and prevent AngII-induced ERK phosphorylation. In fact, increasing evidence has emphasized the interdependence between Smad signaling and the activation of specific kinase pathways in diseased states [8]. These results, in combination with the results of the antisense oligo experiments, highlight the central role of GRK2 in mediating TGFβ and GPCR crosstalk signaling. It would be interesting to investigate the state of TGFβ signaling in clinical patients with hypertension or in patients with cardiovascular disease. TGFβ is a highly immunosuppressive cytokine [1, 40], thus it is possible that disruption of the TGFβ signaling pathway can result in susceptibility to autoimmune disease. Such findings could present new avenues to which TGFβ- and novel GRK2-targeted therapies could be applied. As such, chapter three investigated the possible role of the GRK2-mediated phosphorylation site within the Smad2/3 linker region as recruitment site for regulatory complex assembly.

As previously described, our laboratory found that GRK2 specifically interacts with and phosphorylates a serine/threonine residue within the Smad2/3 linker region, thus terminating TGFβ signaling [42]. By identifying proteins that specifically interact with the phosphopeptide, or in some cases with the non-phosphorylated peptide, we sought to elucidate the mechanism by which GRK2 terminates TGFβ signaling.

Although some of the extracted peptides did not show significant homology with the identified proteins, this was an initial screen with the purpose of identifying any potential interacting protein. Thus, all identified proteins were researched for a potential role in

signal transduction or cellular regulation. Interestingly, identified peptides ranged from scaffolding proteins that mediate signal-specificity, to transcription factors that may be involved in target gene regulation, and to caspases that may activate apoptosis or other stress-activated events. In fact, all the proteins identified could be working in concert to regulate various cellular functions. Coronins, whose interaction with the actin cytoskeleton is regulated by phosphorylation, can be recruited to the site of GRK2 phosphorylation within the linker region and serve as a platform for protein-protein interaction and signal transduction. At the same time, CPD could be processing proteins and influencing signal transduction within the cell. Caspase-12, Dermcidin and DGCR6 could exert their effects on cell survival, stress-response and migration, while PP3856 and eEF1α1 regulate gene transcription and protein translation, respectively. Interestingly, MAGUKs were extracted from the non-phosphorylated peptide-bound column. It is possible that loss of MAGUK binding to this site, after GRK2 phosphorylation, could account for the loss of cell polarity or for the induction of EMT, which are often observed in tumors or in diseased cells. Further studies, particularly comparative mass spectrometry studies between the peptides eluted from the phophopeptide-bound column and from the non-phosphorylated peptide-bound column, are warranted in this area. Such studies would be better at isolating proteins that specifically bind to either column.

Taken together, our results define GRK2 as a central regulator in the crosstalk between TGFβ and AngII signaling. GRK2 is a ubiquitously expressed kinase with an important regulatory role in signaling and in disease. Although it is involved in receptor desensitization and internalization, we propose that GRK2 acts as the key mediator of crosstalk between TGFβ and GPCR superfamilies, by blocking AngII-induced ERK phosphorylation. Moreover, we identify potential GRK2-interacting proteins that may be involved in antagonizing the TGFβ signaling pathway. Better understanding and characterization of this novel antagonistic pathway can lead to the development of targeted therapies in a wide range of human diseases.

REFERENCES

1. Wahl, S.M., *Transforming growth factor-beta: innately bipolar.* Curr Opin Immunol, 2007. **19**(1): p. 55-62.
2. Wang, W., V. Koka, and H.Y. Lan, *Transforming growth factor-beta and Smad signalling in kidney diseases.* Nephrology (Carlton), 2005. **10**(1): p. 48-56.
3. Ruiz-Ortega, M., et al., *TGF-beta signaling in vascular fibrosis.* Cardiovasc Res, 2007. **74**(2): p. 196-206.
4. Wang, W., et al., *Essential role of Smad3 in angiotensin II-induced vascular fibrosis.* Circ Res, 2006. **98**(8): p. 1032-9.
5. Massague, J., *TGF-beta signal transduction.* Annu Rev Biochem, 1998. **67**: p. 753-91.
6. Wakefield, L.M. and A.B. Roberts, *TGF-beta signaling: positive and negative effects on tumorigenesis.* Curr Opin Genet Dev, 2002. **12**(1): p. 22-9.
7. Massague, J., S.W. Blain, and R.S. Lo, *TGFbeta signaling in growth control, cancer, and heritable disorders.* Cell, 2000. **103**(2): p. 295-309.
8. Martin, M.M., et al., *TGF-beta1 stimulates human AT1 receptor expression in lung fibroblasts by cross talk between the Smad, p38 MAPK, JNK, and PI3K signaling pathways.* Am J Physiol Lung Cell Mol Physiol, 2007. **293**(3): p. L790-9.
9. Massague, J., *How cells read TGF-beta signals.* Nat Rev Mol Cell Biol, 2000. **1**(3): p. 169-78.
10. Singh, N.N. and D.P. Ramji, *The role of transforming growth factor-beta in atherosclerosis.* Cytokine Growth Factor Rev, 2006. **17**(6): p. 487-99.
11. Itoh, S., et al., *Signaling of transforming growth factor-beta family members through Smad proteins.* Eur J Biochem, 2000. **267**(24): p. 6954-67.
12. Lin, S.J., et al., *The structural basis of TGF-beta, bone morphogenetic protein, and activin ligand binding.* Reproduction, 2006. **132**(2): p. 179-90.
13. Ho, J.W.Y., *Cell Growth Regulation by TGF-beta Family Members - Identification of Novel Antagonists*, in *Department of Medicine*. 2006, McGill University: Montréal.
14. Itman, C., et al., *All in the family: TGF-beta family action in testis development.* Reproduction, 2006. **132**(2): p. 233-46.
15. Knight, P.G. and C. Glister, *TGF-beta superfamily members and ovarian follicle development.* Reproduction, 2006. **132**(2): p. 191-206.
16. Thompson, T.B., et al., *Beta A versus beta B: is it merely a matter of expression?* Mol Cell Endocrinol, 2004. **225**(1-2): p. 9-17.

17. Herpin, A., C. Lelong, and P. Favrel, *Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans.* Dev Comp Immunol, 2004. **28**(5): p. 461-85.
18. Massague, J., J. Seoane, and D. Wotton, *Smad transcription factors.* Genes Dev, 2005. **19**(23): p. 2783-810.
19. Elliott, R.L. and G.C. Blobe, *Role of transforming growth factor Beta in human cancer.* J Clin Oncol, 2005. **23**(9): p. 2078-93.
20. Hinck, A.P., et al., *Transforming growth factor beta 1: three-dimensional structure in solution and comparison with the X-ray structure of transforming growth factor beta 2.* Biochemistry, 1996. **35**(26): p. 8517-34.
21. Annes, J.P., J.S. Munger, and D.B. Rifkin, *Making sense of latent TGFbeta activation.* J Cell Sci, 2003. **116**(Pt 2): p. 217-24.
22. Massague, J. and Y.G. Chen, *Controlling TGF-beta signaling.* Genes Dev, 2000. **14**(6): p. 627-44.
23. Rodriguez-Vita, J., et al., *Angiotensin II activates the Smad pathway in vascular smooth muscle cells by a transforming growth factor-beta-independent mechanism.* Circulation, 2005. **111**(19): p. 2509-17.
24. Bilezikjian, L.M., et al., *Pituitary actions of ligands of the TGF-beta family: activins and inhibins.* Reproduction, 2006. **132**(2): p. 207-15.
25. Mellor, S.L., et al., *Localization of activin beta(A)-, beta(B)-, and beta(C)-subunits in humanprostate and evidence for formation of new activin heterodimers of beta(C)-subunit.* J Clin Endocrinol Metab, 2000. **85**(12): p. 4851-8.
26. Wang, H. and B.K. Tsang, *Nodal signalling and apoptosis.* Reproduction, 2007. **133**(5): p. 847-53.
27. Hogan, B.L., *Bone morphogenetic proteins: multifunctional regulators of vertebrate development.* Genes Dev, 1996. **10**(13): p. 1580-94.
28. Granjeiro, J.M., et al., *Bone morphogenetic proteins: from structure to clinical use.* Braz J Med Biol Res, 2005. **38**(10): p. 1463-73.
29. Saarma, M., *GDNF - a stranger in the TGF-beta superfamily?* Eur J Biochem, 2000. **267**(24): p. 6968-71.
30. Cate, R.L., et al., *Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells.* Cell, 1986. **45**(5): p. 685-98.
31. Yamamoto, M., et al., *The transcription factor FoxH1 (FAST) mediates Nodal signaling during anterior-posterior patterning and node formation in the mouse.* Genes Dev, 2001. **15**(10): p. 1242-56.

32. Mathews, L.S. and W.W. Vale, *Expression cloning of an activin receptor, a predicted transmembrane serine kinase.* Cell, 1991. **65**(6): p. 973-82.
33. Massague, J. and D. Wotton, *Transcriptional control by the TGF-beta/Smad signaling system.* Embo J, 2000. **19**(8): p. 1745-54.
34. Le Roy, C. and J.L. Wrana, *Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling.* Nat Rev Mol Cell Biol, 2005. **6**(2): p. 112-26.
35. Lefkowitz, R.J. and S.K. Shenoy, *Transduction of receptor signals by beta-arrestins.* Science, 2005. **308**(5721): p. 512-7.
36. Moustakas, A., S. Souchelnytskyi, and C.H. Heldin, *Smad regulation in TGF-beta signal transduction.* J Cell Sci, 2001. **114**(Pt 24): p. 4359-69.
37. Miyazono, K., P. ten Dijke, and C.H. Heldin, *TGF-beta signaling by Smad proteins.* Adv Immunol, 2000. **75**: p. 115-57.
38. Euler-Taimor, G. and J. Heger, *The complex pattern of SMAD signaling in the cardiovascular system.* Cardiovasc Res, 2006. **69**(1): p. 15-25.
39. ten Dijke, P. and C.S. Hill, *New insights into TGF-beta-Smad signalling.* Trends Biochem Sci, 2004. **29**(5): p. 265-73.
40. Kim, I.Y., M.M. Kim, and S.J. Kim, *Transforming growth factor-beta : biology and clinical relevance.* J Biochem Mol Biol, 2005. **38**(1): p. 1-8.
41. Wu, G., et al., *Structural basis of Smad2 recognition by the Smad anchor for receptor activation.* Science, 2000. **287**(5450): p. 92-7.
42. Ho, J., et al., *The G protein-coupled receptor kinase-2 is a TGFbeta-inducible antagonist of TGFbeta signal transduction.* Embo J, 2005. **24**(18): p. 3247-58.
43. Anderson, J.M., *Cell signalling: MAGUK magic.* Curr Biol, 1996. **6**(4): p. 382-4.
44. Dong, C., et al., *Microtubule binding to Smads may regulate TGF beta activity.* Mol Cell, 2000. **5**(1): p. 27-34.
45. Sasaki, A., et al., *Filamin associates with Smads and regulates transforming growth factor-beta signaling.* J Biol Chem, 2001. **276**(21): p. 17871-7.
46. Halayko, A.J. and J. Solway, *Molecular mechanisms of phenotypic plasticity in smooth muscle cells.* J Appl Physiol, 2001. **90**(1): p. 358-68.
47. Kang, Y., et al., *Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway.* Proc Natl Acad Sci U S A, 2005. **102**(39): p. 13909-14.
48. Derynck, R. and Y.E. Zhang, *Smad-dependent and Smad-independent pathways in TGF-beta family signalling.* Nature, 2003. **425**(6958): p. 577-84.
49. Unsicker, K., *Transforming growth factor-beta.* Eur J Biochem, 2000. **267**(24): p. 6953.
50. Shi, Y., et al., *A structural basis for mutational inactivation of the tumour suppressor Smad4.* Nature, 1997. **388**(6637): p. 87-93.

51. Liu, X., et al., *Ski/Sno and TGF-beta signaling.* Cytokine Growth Factor Rev, 2001. **12**(1): p. 1-8.
52. Inman, G.J., F.J. Nicolas, and C.S. Hill, *Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity.* Mol Cell, 2002. **10**(2): p. 283-94.
53. Kretzschmar, M., et al., *A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras.* Genes Dev, 1999. **13**(7): p. 804-16.
54. Matsuura, I., et al., *Cyclin-dependent kinases regulate the antiproliferative function of Smads.* Nature, 2004. **430**(6996): p. 226-31.
55. Wicks, S.J., et al., *Inactivation of smad-transforming growth factor beta signaling by Ca(2+)-calmodulin-dependent protein kinase II.* Mol Cell Biol, 2000. **20**(21): p. 8103-11.
56. Engel, M.E., et al., *Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription.* J Biol Chem, 1999. **274**(52): p. 37413-20.
57. Yakymovych, I., et al., *Inhibition of transforming growth factor-beta signaling by low molecular weight compounds interfering with ATP- or substrate-binding sites of the TGF beta type I receptor kinase.* Biochemistry, 2002. **41**(36): p. 11000-7.
58. Conery, A.R., et al., *Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis.* Nat Cell Biol, 2004. **6**(4): p. 366-72.
59. Chang, H., A.L. Lau, and M.M. Matzuk, *Studying TGF-beta superfamily signaling by knockouts and knockins.* Mol Cell Endocrinol, 2001. **180**(1-2): p. 39-46.
60. Thiery, J.P., *Epithelial-mesenchymal transitions in tumour progression.* Nat Rev Cancer, 2002. **2**(6): p. 442-54.
61. Yingling, J.M., K.L. Blanchard, and J.S. Sawyer, *Development of TGF-beta signalling inhibitors for cancer therapy.* Nat Rev Drug Discov, 2004. **3**(12): p. 1011-22.
62. Siegel, P.M. and J. Massague, *Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer.* Nat Rev Cancer, 2003. **3**(11): p. 807-21.
63. Dumont, N. and C.L. Arteaga, *Targeting the TGF beta signaling network in human neoplasia.* Cancer Cell, 2003. **3**(6): p. 531-6.
64. Massague, J., *G1 cell-cycle control and cancer.* Nature, 2004. **432**(7015): p. 298-306.
65. Uchida, K., et al., *Involvement of MAP kinase cascades in Smad7 transcriptional regulation.* Biochem Biophys Res Commun, 2001. **289**(2): p. 376-81.
66. Rich, J., A. Borton, and X. Wang, *Transforming growth factor-beta signaling in cancer.* Microsc Res Tech, 2001. **52**(4): p. 363-73.
67. Moustakas, A., et al., *Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation.* Immunol Lett, 2002. **82**(1-2): p. 85-91.

68. Christofori, G., *New signals from the invasive front.* Nature, 2006. **441**(7092): p. 444-50.
69. Norton, L. and J. Massague, *Is cancer a disease of self-seeding?* Nat Med, 2006. **12**(8): p. 875-8.
70. Liu, X., H. Hu, and J.Q. Yin, *Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis.* Liver Int, 2006. **26**(1): p. 8-22.
71. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.
72. Libby, P., P.M. Ridker, and A. Maseri, *Inflammation and atherosclerosis.* Circulation, 2002. **105**(9): p. 1135-43.
73. Yue, J. and K.M. Mulder, *Requirement of Ras/MAPK pathway activation by transforming growth factor beta for transforming growth factor beta 1 production in a smad-dependent pathway.* J Biol Chem, 2000. **275**(45): p. 35656.
74. Funaba, M., C.M. Zimmerman, and L.S. Mathews, *Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase.* J Biol Chem, 2002. **277**(44): p. 41361-8.
75. Edlund, S., et al., *Transforming growth factor-beta1 (TGF-beta)-induced apoptosis of prostate cancer cells involves Smad7-dependent activation of p38 by TGF-beta-activated kinase 1 and mitogen-activated protein kinase kinase 3.* Mol Biol Cell, 2003. **14**(2): p. 529-44.
76. Sanchez-Capelo, A., *Dual role for TGF-beta1 in apoptosis.* Cytokine Growth Factor Rev, 2005. **16**(1): p. 15-34.
77. Pessah, M., et al., *c-Jun interacts with the corepressor TG-interacting factor (TGIF) to suppress Smad2 transcriptional activity.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6198-203.
78. Abecassis, L., et al., *Evidence for a role of MSK1 in transforming growth factor-beta-mediated responses through p38alpha and Smad signaling pathways.* J Biol Chem, 2004. **279**(29): p. 30474-9.
79. Ohshima, T. and K. Shimotohno, *Transforming growth factor-beta-mediated signaling via the p38 MAP kinase pathway activates Smad-dependent transcription through SUMO-1 modification of Smad4.* J Biol Chem, 2003. **278**(51): p. 50833-42.
80. Dowdy, S.C., A. Mariani, and R. Janknecht, *HER2/Neu- and TAK1-mediated up-regulation of the transforming growth factor beta inhibitor Smad7 via the ETS protein ER81.* J Biol Chem, 2003. **278**(45): p. 44377-84.
81. Chen, Y.J., et al., *Interplay of PI3K and cAMP/PKA signaling, and rapamycin-hypersensitivity in TGFbeta1 enhancement of FSH-stimulated steroidogenesis in rat ovarian granulosa cells.* J Endocrinol, 2007. **192**(2): p. 405-19.

82. Barrios-Rodiles, M., et al., *High-throughput mapping of a dynamic signaling network in mammalian cells.* Science, 2005. **307**(5715): p. 1621-5.
83. Rosentreter, A., et al., *Coronin 3 involvement in F-actin-dependent processes at the cell cortex.* Exp Cell Res, 2007. **313**(5): p. 878-95.
84. Scott, A.M. and M. Saleh, *The inflammatory caspases: guardians against infections and sepsis.* Cell Death Differ, 2007. **14**(1): p. 23-31.
85. Reddy, K.B., et al., *Maspin expression inversely correlates with breast tumor progression in MMTV/TGF-alpha transgenic mouse model.* Oncogene, 2001. **20**(45): p. 6538-43.
86. Bakin, A.V., et al., *A critical role of tropomyosins in TGF-beta regulation of the actin cytoskeleton and cell motility in epithelial cells.* Mol Biol Cell, 2004. **15**(10): p. 4682-94.
87. Premont, R.T. and R.R. Gainetdinov, *Physiological roles of G protein-coupled receptor kinases and arrestins.* Annu Rev Physiol, 2007. **69**: p. 511-34.
88. Yang, W. and S.H. Xia, *Mechanisms of regulation and function of G-protein-coupled receptor kinases.* World J Gastroenterol, 2006. **12**(48): p. 7753-7.
89. Penela, P., C. Ribas, and F. Mayor, Jr., *Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases.* Cell Signal, 2003. **15**(11): p. 973-81.
90. Laporte, S.A., et al., *The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3712-7.
91. Claing, A., et al., *Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins.* Prog Neurobiol, 2002. **66**(2): p. 61-79.
92. Beaulieu, J.M. and M.G. Caron, *Beta-arrestin goes nuclear.* Cell, 2005. **123**(5): p. 755-7.
93. Hata, J.A. and W.J. Koch, *Phosphorylation of G protein-coupled receptors: GPCR kinases in heart disease.* Mol Interv, 2003. **3**(5): p. 264-72.
94. Penn, R.B., A.N. Pronin, and J.L. Benovic, *Regulation of G protein-coupled receptor kinases.* Trends Cardiovasc Med, 2000. **10**(2): p. 81-9.
95. Ribas, C., et al., *The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling.* Biochim Biophys Acta, 2007. **1768**(4): p. 913-22.
96. Eichmann, T., et al., *The amino-terminal domain of G-protein-coupled receptor kinase 2 is a regulatory Gbeta gamma binding site.* J Biol Chem, 2003. **278**(10): p. 8052-7.

97. Higuchi, S., et al., *Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology.* Clin Sci (Lond), 2007. **112**(8): p. 417-28.
98. Kohout, T.A. and R.J. Lefkowitz, *Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization.* Mol Pharmacol, 2003. **63**(1): p. 9-18.
99. DeWire, S.M., et al., *Beta-arrestins and cell signaling.* Annu Rev Physiol, 2007. **69**: p. 483-510.
100. Roux, P.P. and J. Blenis, *ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions.* Microbiol Mol Biol Rev, 2004. **68**(2): p. 320-44.
101. Mulder, K.M., *Role of Ras and Mapks in TGFbeta signaling.* Cytokine Growth Factor Rev, 2000. **11**(1-2): p. 23-35.
102. Wetzker, R. and F.D. Bohmer, *Transactivation joins multiple tracks to the ERK/MAPK cascade.* Nat Rev Mol Cell Biol, 2003. **4**(8): p. 651-7.
103. Rosenkranz, S., *TGF-beta1 and angiotensin networking in cardiac remodeling.* Cardiovasc Res, 2004. **63**(3): p. 423-32.
104. Chen, Z., et al., *MAP kinases.* Chem Rev, 2001. **101**(8): p. 2449-76.
105. Liu, H.W., et al., *Angiotensin II regulates phosphoinositide 3 kinase/Akt cascade via a negative crosstalk between AT1 and AT2 receptors in skin fibroblasts of human hypertrophic scars.* Life Sci, 2006. **79**(5): p. 475-83.
106. Touyz, R.M., G. Yao, and E.L. Schiffrin, *Role of the actin cytoskeleton in angiotensin II signaling in human vascular smooth muscle cells.* Can J Physiol Pharmacol, 2005. **83**(1): p. 91-7.
107. Inagami, T. and S. Eguchi, *Angiotensin II-mediated vascular smooth muscle cell growth signaling.* Braz J Med Biol Res, 2000. **33**(6): p. 619-24.
108. Schmidt, M., et al., *Ras-independent activation of the Raf/MEK/ERK pathway upon calcium-induced differentiation of keratinocytes.* J Biol Chem, 2000. **275**(52): p. 41011-7.
109. Hunyady, L., et al., *Mechanisms and functions of AT(1) angiotensin receptor internalization.* Regul Pept, 2000. **91**(1-3): p. 29-44.
110. Santos, R.A., M.J. Campagnole-Santos, and S.P. Andrade, *Angiotensin-(1-7): an update.* Regul Pept, 2000. **91**(1-3): p. 45-62.
111. Sorescu, D., *Smad3 mediates angiotensin II- and TGF-beta1-induced vascular fibrosis: Smad3 thickens the plot.* Circ Res, 2006. **98**(8): p. 988-9.
112. Yang, Y.A., et al., *Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects.* J Clin Invest, 2002. **109**(12): p. 1607-15.

113. Pawson, T. and P. Nash, *Protein-protein interactions define specificity in signal transduction.* Genes Dev, 2000. **14**(9): p. 1027-47.
114. Durocher, D., et al., *The FHA domain is a modular phosphopeptide recognition motif.* Mol Cell, 1999. **4**(3): p. 387-94.
115. Yaffe, M.B. and L.C. Cantley, *Signal transduction. Grabbing phosphoproteins.* Nature, 1999. **402**(6757): p. 30-1.
116. Hofmann, K. and P. Bucher, *The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors.* Trends Biochem Sci, 1995. **20**(9): p. 347-9.
117. Durocher, D., et al., *The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms.* Mol Cell, 2000. **6**(5): p. 1169-82.
118. Durocher, D., et al., *The molecular basis of FHA Domain:Phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms.* Mol Cell, 2000. **6**(5): p. 1169-82.
119. Guo, X., *4000 QTRAP™ LC/MS/MS System.* 2007, Applied Biosystems/MDS Sciex.
120. Saleh, M., et al., *Differential modulation of endotoxin responsiveness by human caspase-12 polymorphisms.* Nature, 2004. **429**(6987): p. 75-9.
121. Xie, Q., et al., *Effect of tauroursodeoxycholic acid on endoplasmic reticulum stress-induced caspase-12 activation.* Hepatology, 2002. **36**(3): p. 592-601.
122. Saleh, M., et al., *Enhanced bacterial clearance and sepsis resistance in caspase-12-deficient mice.* Nature, 2006. **440**(7087): p. 1064-8.
123. Hoff, N.P., et al., *Carboxypeptidase D: A Novel TGF-beta Target Gene Dysregulated in Patients with Lupus Erythematosus.* J Clin Immunol, 2007.
124. Song, L. and L.D. Fricker, *Tissue distribution and characterization of soluble and membrane-bound forms of metallocarboxypeptidase D.* J Biol Chem, 1996. **271**(46): p. 28884-9.
125. O'Malley, P.G., et al., *Characterization of a novel, cytokine-inducible carboxypeptidase D isoform in haematopoietic tumour cells.* Biochem J, 2005. **390**(Pt 3): p. 665-73.
126. Novikova, E.G., et al., *Characterization of the enzymatic properties of the first and second domains of metallocarboxypeptidase D.* J Biol Chem, 1999. **274**(41): p. 28887-92.
127. Timblin, B., M. Rehli, and R.A. Skidgel, *Structural characterization of the human carboxypeptidase D gene and its promoter.* Int Immunopharmacol, 2002. **2**(13-14): p. 1907-17.

128. de Hostos, E.L., *The coronin family of actin-associated proteins.* Trends Cell Biol, 1999. **9**(9): p. 345-50.
129. Clapham, D.E. and E.J. Neer, *New roles for G-protein beta gamma-dimers in transmembrane signalling.* Nature, 1993. **365**(6445): p. 403-6.
130. Lai, Y.P., et al., *Functional and structural characterization of recombinant dermcidin-1L, a human antimicrobial peptide.* Biochem Biophys Res Commun, 2005. **328**(1): p. 243-50.
131. Helms, M.W., et al., *First evidence supporting a potential role for the BMP/SMAD pathway in the progression of oestrogen receptor-positive breast cancer.* J Pathol, 2005. **206**(3): p. 366-76.
132. Stewart, G.D., et al., *Dermcidin expression confers a survival advantage in prostate cancer cells subjected to oxidative stress or hypoxia.* Prostate, 2007. **67**(12): p. 1308-17.
133. Lowrie, A.G., et al., *Dermcidin expression in hepatic cells improves survival without N-glycosylation, but requires asparagine residues.* Br J Cancer, 2006. **94**(11): p. 1663-71.
134. Roh, M.H., et al., *The Maguk protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost.* J Cell Biol, 2002. **157**(1): p. 161-72.
135. Caruana, G., *Genetic studies define MAGUK proteins as regulators of epithelial cell polarity.* Int J Dev Biol, 2002. **46**(4): p. 511-8.
136. Stucke, V.M., et al., *The MAGUK protein MPP7 binds to the polarity protein hDlg1 and facilitates epithelial tight junction formation.* Mol Biol Cell, 2007. **18**(5): p. 1744-55.
137. Ho, J.W.Y., *Indentification and Characterization of a Novel Interaction Between CASK and the Transforming Growth Factor Beta Receptor Interacting Protein*, in *Department of Physiology*. 2002, McGill University: Montréal. p. 80.
138. Lee, J.M., *The role of protein elongation factor eEF1A2 in ovarian cancer.* Reprod Biol Endocrinol, 2003. **1**: p. 69.
139. Tomlinson, V.A., et al., *Translation elongation factor eEF1A2 is a potential oncoprotein that is overexpressed in two-thirds of breast tumours.* BMC Cancer, 2005. **5**: p. 113.
140. Goodchild, A., et al., *Cytotoxic G-rich oligodeoxynucleotides: putative protein targets and required sequence motif.* Nucleic Acids Res, 2007. **35**(13): p. 4562-72.
141. Izawa, T., et al., *Elongation factor-1 alpha is a novel substrate of rho-associated kinase.* Biochem Biophys Res Commun, 2000. **278**(1): p. 72-8.

142. Demczuk, S., G. Thomas, and A. Aurias, *Isolation of a novel gene from the DiGeorge syndrome critical region with homology to Drosophila gdl and to human LAMC1 genes.* Hum Mol Genet, 1996. **5**(5): p. 633-8.
143. Niedel, J. and L.S. Dietrich, *Nicotinate phosphoribosyltransferase of human erythrocytes. Purification and properties.* J Biol Chem, 1973. **248**(10): p. 3500-5.
144. Brenner, C., *Evolution of NAD biosynthetic enzymes.* Structure, 2005. **13**(9): p. 1239-40.
145. Kretzschmar, M., J. Doody, and J. Massague, *Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1.* Nature, 1997. **389**(6651): p. 618-22.
146. Burnier, M. and H.R. Brunner, *Angiotensin II receptor antagonists.* Lancet, 2000. **355**(9204): p. 637-45.
147. Schmidt-Ott, K.M., S. Kagiyama, and M.I. Phillips, *The multiple actions of angiotensin II in atherosclerosis.* Regul Pept, 2000. **93**(1-3): p. 65-77.
148. Richard, D.E., et al., *p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1.* J Biol Chem, 1999. **274**(46): p. 32631-7.
149. Fischer, O.M., et al., *EGFR signal transactivation in cancer cells.* Biochem Soc Trans, 2003. **31**(Pt 6): p. 1203-8.

APPENDICES