The transcriptional regulation of VEGF production from pulmonary artery smooth muscle cells by TGFβ_1

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ABSTRACT

Pulmonary Hypertension (PH) is a rare and fatal disease of the pulmonary vasculature. Hyperplasia and hypertrophy of smooth muscle cells in muscular arteries and arrival of smooth muscle cell in non-muscular pulmonary arteries are key pathological features of PH. VEGF has a strong role in embryonic vasculo/angiogenesis and adult vascular protection. VEGF and its main signalling receptor, VEGFR2, are expressed on endothelial and smooth muscle cells within remodelling arteries and VEGFR inhibition leads to the development of PH in rats. Furthermore over expression of VEGF in monocrotaline models of PH prevents the progression of remodelling. Here we tested the effect of mediators relevant to PH on VEGF release from pulmonary artery smooth muscle cells (PASMCs). TGFβ_1 exerted the greatest effect and we went on to characterise the signal transduction and transcriptional mechanisms involved. TGFβ_1 acted via two T cell factor (TCF) binding sites within the VEGF promoter and increased basal levels of TCF4 association with the VEGF promoter. TGFβ_1 also induced Smad2, 3 and 4 association with the VEGF promoter within the same region as TCF4 and inhibition of Smads abolished the TGFβ_1 effect. We found that a GSK3β/β-catenin/Smad2/3 nuclear complex was present under unstimulated conditions and that TGFβ_1 caused inhibition of glycogen synthase kinase 3β (GSK3β) and decreased β-catenin phosphorylation. Unphosphorylated β-catenin associated with the VEGF promoter in response to TGFβ_1, while phosphorylated β-catenin did not. Collectively these studies suggest that dephosphorylation of β-catenin, in response to TGFβ_1, allowed association of a preformed protein complex with TCF4 at the VEGF promoter, which in turn increased VEGF transcription.

Finally we showed that murine PASMCs heterozygous for *BMPR II*, the most commonly mutated gene in PH, produced increased VEGF protein and mRNA in response to TGFβ_1 than their wild type controls.

PUBLICATIONS

Full papers.

Clifford R.L., Deacon K., Knox A.J. Novel regulation of vascular endothelial growth factor-A (VEGF-A) by TGFbeta 1: Requirement for Smads, beta -catenin and GSK3beta. Journal of Biological Chemistry. 2008 Oct 23 [E-pub ahead of print]

Abstracts

Clifford R.L., Deacon K., Knox A.J. Transforming Growth Factor β1 (TGFβ1) Regulation of Vascular Endothelial Growth Factor (VEGF) in Pulmonary Artery Smooth Muscle Cells (PASMCs). (Abstract presented at British Thoracic Society Winter Meeting 2006 and the American Thoracic Society meeting 2007 San Francisco)

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5-HT	5-hydroxytryptamine
5-HTT	5-HT transporter
Act D	Actinomycin D
Akt/PKB	Akt/Protein Kinase B
ALK	Activin receptor-like kinase
AML	Acute myelogenous leukaemia
Ang2	angiopoietin 2
AP-1	Activator Protein-1
APC	Adenomatous polyposis coli
ARE	AU rich element
ATF	Activating transcription factor
ATP	Adenosine triphosphate
AUF	AU rich element binding factor
β2M	β-2 microglobulin
BAMBI	BMP and activin membrane-bound inhibitor
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
BK	Bradykinin
BMP	Bone Morphogenetic Protein
BMPR II	BMP Receptor II
β-TrCP	β-transducin repeat-containing protein
CBP	CREB binding protein
CEBP	CCAAT/enhancer binding protein
ChIP	Chromation Immunoprecipitation
CK	casein kinase
Co-IP	Co-Immunoprecipitation
COX	cyclooxygenase
CRD	Cysteine rich domain
CREB	cAMP response element binding
CTBP	C-terminal binding protein
DV1	Dishevelled
EGF	Epidermal Growth Factor
ERK	Extracellular-signal-regulated kinase
ET-1	Endothelin-1
FEVR	Familial Exudative Vitreoretinopathy
FGF	Fibroblast growth factor
Fz	Frizzled
GAIT	IFN-γ activated inhibitor of translation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF	Growth Differentiation Fcator
GSK3β	Glycogen synthase kinase 3 β
GTP	Guanosine-5'-triphosphate
HAT	Histone acetylase
HBP1	HMG-box transcription factor 1
HDAC	Histone deacetylase
HGF	Hepatocyte Growth Factor
HHT	Hereditary Haemorrhagic telangiectasia
HIF	Hypoxia Inducible Factor
HMG	Hugh mobility group
HRE	Hypoxia response element
HSPG	heparin surface proteoglycans
HUVEC	Human umbilical vein endothelial cell
IFNγ	Interferon γ
IGF-1	Insulin like growth factor
IL-x	Interleukin-x
IRES	Internal ribosome entry site
JNK	Jun N terminal kinase
KGF	Keratinocyte Growth factor
LAP	Latency Associated Peptide
LEF	Lymphoid enhancer factor-1
LF2000	Lipofectamine 2000
LRP	LDL receptor related protein
LTBP	Latent TGFβ binding protein

LUC	Luciferase
MAPK	Mitogen Activated Protein Kinase
MEF	Mouse embryonic fibroblast
MH1/2	Mad-homology
MMP	Matrix metalloproteinase
MTR	Mithromycin
NLK	Nemo-like kinase
NO	Nitric oxide
NOS	NO synthase
NRP	Neuropilins
PAP	Pulmonary artery pressure
PASMCs	Pulmonary artery smooth muscle cells
PCR	Polymerase Chain reaction
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PDGFR	PDGF receptor
PGE2	prostaglandin E2
PGI2	Prostacyclin
PGI-R	Prostacyclin Receptor
PH	Pulmonary hypertension
PI3K	Phosphoinositide 3-kinase
PIC	Protease Inhbitor Cocktail
PKA	Protein kinase A
PKC	Protein Kinase C
PLCγ	Phospholipase C γ
PlGF	Placental Growth Factor
POEMS	polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy and skin change)
PP2A	Protein Phosphatase 2A
PPHN	Persistant pulmonary hypertension of the newborn
PTB	phosphotyrosine binding domain
RTK	Receptor tyrosine kinas
RVSP	right ventricular systolic pressure
SAD	Smad Activation Domain
SARA	Smad anchor for receptor activation
SBE	Smad Binding Element
SEM	Standard Error of Mean
SH2	Src Homology 2
SIS3	Specific Inhibitor of Smad3
Sp-1	Specificity protein-1
TAK	TGFβ activated kinas
TCF	T Cell Factor
TGFβ1	Transforming Growth Factor beta
TGFβR	TGFβ Receptor
TMB	
TNFα	Tumour Necrosis Factor α
uPA	urokinase-type plasminogen activator
uPAR	uPA recpeptor
UTR	untranslated region
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VIP	Vasoactive intestinal peptide
VRAP	VEGF receptor associated protein
WRE	Wnt response element

1 INTRODUCTION

1.1 PULMONARY CIRCULATION

“and after the blood has been refined in the right ventricle of the heart, it must reach the left ventricle where it is impregnated with the vital spirit (pneuma) but there is no opening between these two ventricles…..And thus this blood after it is refined must flow via the vena arteriosa (pulmonary artery) to the lungs where it must spread and be mingled with air…” [1]. The first recorded description of the pulmonary circulation was written by Ala ad-Din Abu al-Hassan Ali Ibn Abi-Hazm al-Qarshi (known as Ibn Nafis Damashqi) in 1242 A.D. It was the first indication that the whole circulation was separated into the systemic circulation (that which is pumped by the heart into the arterial system and fine capillaries before returning to the right ventricle via the venous system) and the pulmonary circulation (that in which the blood of the right ventricle is pumped, via the pulmonary artery, into the lungs for oxygenation and returns via the pulmonary vein to the left atrium).

It is now known that the pulmonary circulation is a complex and intricate system that can withstand and adapt to an extreme blood flow experienced by no other part of the circulation. The pulmonary vascular bed increases its cross sectional area and recruits previously unperfused vessels to maintain a healthy pulmonary artery pressure of between 12-16 mmHg [2]. However constant barrage of the pulmonary vasculature by the entire cardiac output makes it vulnerable to injury and disease, including pulmonary hypertension (PH).

1.1.1 Organisation and Structure of the Pulmonary Arteries

The main pulmonary artery, with a diameter of ~30mm, splits and branches to form subsequent ‘generations’ of arteries, each of smaller cross sectional area

and reduced vascular volume. Throughout the vessel generations the composition of the vessel wall structure changes in relation to its function and position.

The larger vessels (generations 17-13), including the main pulmonary artery are termed elastic arteries, due to the presence of three or more layers of elastic laminae within the media, in addition to the internal and external elastic laminae which surround the media. The elastic vessels consists of adventitial, muscular (media) and intimal layers [3]. The adventitia consists of thin collagenous connective tissue containing elastic fibres and few smooth muscle cells. The media is characterised by numerous fenestrated elastic membranes, enmeshed in which are smooth muscle cells and collagenous fibres. The intima is lined by endothelial cells and the subendothelial tissue contains myofibroblast type cells [4].

Generations 13-3 consist of the muscular arteries which contain a significantly greater amount of muscle in relation to their diameter compared to the elastic vessels.

The percentage of muscle in relation to diameter decreases throughout the generations, with generations 5-3 being referred to as only partially muscular. Within the smaller vessels of this subset, the internal laminae disappear. Finally, generations 5-1 consist of the non-muscular vessels, in which no elastic laminae are present and the smooth muscle cells are replaced by pericytes, which fuse to the endothelial lining and are able to differentiate into smooth muscle cells [3, 4].

1.2 PATHOBIOLOGY OF PULMONARY HYPERTENSION

Pulmonary hypertension (PH) is clinically defined as a mean pulmonary artery pressure of greater than 25mmHg at rest or 30mmHg on exertion [5]. PH is an umbrella term for a group of diseases all characterised by elevated pulmonary artery pressure leading to right ventricular heart failure. PH patients, without treatment, have an estimated life expectancy of less than 3 years from diagnosis; less than that of many neoplastic diseases [6]. Multifactoral pathobiologic mechanisms, initiated idiopathically or due to association with an underlying disease or genetic predisposition [7], result in a loss of compliance of pulmonary vessels, reduced vessel vasodilatory function, thrombosis and increased vascular cell proliferation and remodelling [8]. All result in increased vascular resistance and it is becoming clear that remodelling is pivotal to the progression of PH.

1.2.1 Cellular Changes in Pulmonary Hypertension

1.2.1.1 Endothelial Cells

In the lungs of 80% [3] of idiopathic PH patients and severe cases of secondary PH the normal endothelial cell monolayer is replaced by dysregulated, intraluminal endothelial cell growth known as plexiform lesions [9]. Plexiform arteriopathy is thought to be associated with all forms of PH [10]. While historically it was believed these lesions were an end stage product in PH, it is now clear that theses lesions are dynamic with continuing endothelial cell proliferation. The endothelial cells of plexiform lesions have different gene expression patterns to those outside the lesions, for example they express high levels of smooth muscle cell mitogens and vasoconstrictants, for example

endothelin-1 and thromboxane A_2 [11]. Further, Voelkel *et al.*, hypothesise that a triggering injury or stimulus results in initial endothelial cell apoptosis which acts as a selection barrier. Subsequently phenotypically altered endothelial cells emerge which proliferate abnormally and are apoptosis resistant [12].

In addition to increased cell growth signalling in pulmonary hypertensive vessels, there is also a reduction in growth suppressant signalling. For example prostacyclin synthase [13] and nitric oxide synthase [14] expression is reduced in the endothelial cells of plexiform lesions, both of which are involved in vessel relaxation as well as inhibition of cell proliferation. Furthermore, the transforming growth factor beta receptor II (TGFβRII), a growth suppressor in a number of contexts, and Bax, a pro-apoptotic signalling protein are reduced in plexiform lesions. Also the endothelial cells appear visually different. Rat endothelial cells are swollen, with increased lamellar structures and organelles, in response to hypoxia [3, 15]

1.2.1.2 Smooth Muscle Cells

A common feature of remodelling in all classifications of PH are increases in the amount of muscle in the partially-muscular arteries and the appearance and expansion of smooth muscle into the small, peripheral, normally non-muscular pulmonary arteries [8]. In the partially muscular arteries the increased smooth muscle content is postulated to be due to hyperplasia and hypertrophy of pre-existing smooth muscle cells, probably as a compensatory strengthening mechanism in response to chronic increases in pulmonary artery pressure [3]. This is in contrast to the smooth muscle cells of the normal adult pulmonary artery which display minimal cell division. In the originally non-muscular

arteries the precursor cells are thought to be fibroblasts, recruited to the vessel from the interstitium, and pericytes [16] which both take on a smooth muscle like phenotype.

Further, there is evidence to suggest that the media of the pulmonary artery does not consist, of a single homogenous, differentiated, contractile population of smooth muscle cells, as historically believed, but of numerous sub-populations of cells. Each population is phenotypically different and reacts differently to stress and hypertensive stimuli. It is believed that in PH, stimulation of a sub-set of these cells to proliferate also stimulates them to produce mitogens and matrix proteins (collagen elastin and proteoglycans) which stimulate an alternative sub-population to contribute to the disease process [17], making the final vascular lesion the result of numerous cell types and cellular processes.

In addition, in severe PH a layer of 'smooth muscle cell-like' cells is seen beneath the endothelium forming a compartment called the neointima. This layer of cells is thought to originate from smooth muscle cells of the media and fibroblast migration from the adventitia [3].

1.2.1.3 Fibroblasts

The adventitial layer of the pulmonary artery has been implicated as a modulator of pulmonary artery remodelling through its interaction with the media and intima [18]. The adventitia is highly populated with fibroblasts and, especially that of the small, muscular arteries, becomes thickened in PH. Adventitial fibroblasts, in response to PH, begin to express smooth muscle cell contractile proteins, especially α-smooth muscle actin. These 'myofibroblasts'

therefore have contractile properties. Further, these cells have enhanced proliferative and synthetic properties.

The thickening of the adventitia may be due to the rate of fibroblast proliferation greatly exceeding the rate of fibroblast apoptosis [18].

1.2.2 Other Features of Vascular Remodelling

1.2.2.1 Extracellular Matrix Deposition

Increased deposition of matrix in the pulmonary artery reduces compliance of the vessel. Both elastin and collagen deposition are increased in PH, as a result of production by endothelial and smooth muscle cells [3].

1.2.3 Genetic Basis of Pulmonary Hypertension

Familial PH is a subclass of PH in which patients have an underlying genetic predisposition. Most prevalent of these mutations are heterozygous mutations of the bone morphogenetic protein (BMP) receptor type II gene (*BMPR II*). BMPR II is a receptor of the TGFβ superfamily (refer to page 48 for more detail). BMPR II is a constitutively active serine/threonine kinase receptor and requires association with BMP type I receptors to form a heterocomplex for active signalling. Its main ligands are BMP2, BMP4, BMP7, GDF6 (growth differentiation factor 6) and GDF7. Ligand binding results in receptor activation and phosphorylation of the downstream signalling proteins, Smads 1, 5 and 8. Smads 1/5/8 translocate to the nucleus and determine BMP signalling via interaction with BMP responsive gene promoters and regulation of gene transcription. Numerous mutations of *BMPR II* have been identified within the ligand binding domain, cysteine kinase domain, non-cysteine kinase domain and cytoplasmic tail of BMPR II. Mutation results in failure of the

receptors to traffic correctly to the cell membrane and/or failure of downstream receptor signalling [8]. Consequences of reduced receptor signalling include increased pulmonary artery smooth muscle mass and endothelial cell apoptosis, processes important in vascular remodelling. Mutations in Activin-like kinase type-1 (ALK1) and endoglin, receptors in the TGFβ family, are present in PH patients with associated hereditary hemorrhagic telangiectasia [19]. The mutations have reduced penetrance and are age and sex dependent, conferring increased risk but not definite development of PH.

1.2.4 Mediators in Pulmonary Hypertension

Dysregulation of numerous mediators contributes to the development of idiopathic PH and may be the 'second hit' required for individuals with a genetic predisposition to develop PH. Endothelin-1 (ET-1) is produced by endothelial cells and is a potent vasoconstrictor and smooth muscle cell mitogen [2]. It is over-expressed in PH animal models and patients [20]. ET-1 expression correlates with pulmonary vascular resistance in idiopathic PH patients and ET-1 receptor expression is greater in PH arteries [19]. In addition, the endothelin converting enzyme (ECE), which converts the inactive 38 amino acid big-ET-1 to the 21 amino acid active ET-1 , is abundantly expressed in pulmonary artery endothelial cells from idiopathic PH patients [2]. The serum levels of other inflammatory cytokines including interleukin-1 (IL-1β) and tumour necrosis factor α (TNFα) are increased in primary PH patients and patients with PH secondary to POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy and skin change) disorder [21-23].

Prostacylin is a product of arachidonate metabolism and dilates the pulmonary circulation via activation of the cyclic adenosine monophosphate (cAMP)-dependent pathways. Prostacyclin also inhibits vascular smooth muscle cell proliferation and decreases platelet aggregation [8]. Prostacyclin production and prostacyclin synthase expression are reduced in PH patients. Furthermore, prostacyclin receptor expression is reduced in severe PH [19, 20]. In contrast, thromboxane, also a product of arachidonate metabolism, but a vasoconstrictant, is present in increased levels in PH patients, suggesting the vasodilation/vasoconstriction balance of arachidonate metabolism is defective [19]. Prostacylin analogues were the first effective therapy for PH. A further vasodilator and inhibitor of smooth muscle cell proliferation, nitric oxide (NO), is also reduced in PH due to reduced NO synthase expression (specifically NOS III or eNOS[2]) [8, 19].

Serotonin is produced by pulmonary neuroepithelial bodies and stored in platelets [8]. It causes pulmonary vasoconstriction and smooth muscle cell proliferation [20] and circulating plasma levels of serotonin are elevated in PH patients [8]. Smooth muscle cells from PH patients have a heightened sensitivity to the proliferation inducing effects of serotonin than control cells and mutations in the serotonin transporter (5-HTT) are linked to PH development [19].

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen and its expression is increased in the PH pulmonary vasculature. It is postulated to mediate a protective role in PH as VEGF gene transfer in PH animal models

prevents the progression of PH. Expression of other growth factors including PDGF, bFGF, IGF-1 and EGF is also increased in PH and enhanced growth factor production is implicated in PH remodelling [8]. PDGF is a potent mitogen and chemoattractant for pulmonary vascular smooth muscle cells and its receptors are upregulated in lamb models of PH. Furthermore PDGFR antagonists reverse PH in animal models [19].

Vasoactive intestinal peptide (VIP) in also implicated in PH pathobiology. It is a potent vasodilator, an inhibitor of smooth muscle cell proliferation, an anti-inflammatory and an antiapoptotic. VIP reduces pulmonary vascular resistance in monocrotaline induced PH rabbits and inhibits proliferation of PASMCs from idiopathic PH patients [19].

1.2.5 Treatment of Pulmonary Hypertension

Current therapy strategies for PH are diverse and complicated due to the degree of diversity in the underlying pathology of the disease; different categories of PH may require and respond very differently to different therapies. Most therapies have focused on inducing arterial dilation as opposed to preventing and reversing pulmonary remodelling. The non-specific symptoms with which PH presents means patients, by the time of diagnosis, have developed remodelling. This makes the modulation of vessel dilation alone inadequate and has brought remodelling to the forefront of therapy research.

In brief, all patients are placed on anticoagulant therapy (providing there is no contraindication) despite any definite evidence of the benefit. Diuretics and continuous oxygen therapy are often prescribed to prevent or delay right heart

failure [24]. Calcium channel blockers are now known to be effective in less than 10% of patients with PH (those with a positive vasodilator challenge) [7]. PH patients produce reduced levels prostacyclin, a product of the arachidonic acid cascade known to promote vasodilation and inhibit vascular proliferation [7]. Inhaled iloprost, a long acting prostacyclin analogue, is now a first-line choice of therapy by clinicians for patients with moderate to severe PH [24]. A potential for reduced efficacy compared to earlier, less stable prostacyclin analogues, can result in iloprost being replaced by epoprostnol or treprostinil, however their requirement for intravenous or subcutaneous administration makes them unattractive to patients and often results in catheter related infections [24].

Endothelin-1 is a vasoconstrictor and smooth muscle cell mitogen known to be elevated in the plasma and lung tissue of patients with certain forms of PH, with increased concentration correlating with disease severity [7]. Endothelin signals through two receptors, ET_A and ET_B. Bosentan is an ET_A and ET_B antagonist and benefits patients with moderate to severe PH [24]. In addition, two ET_A receptor specific inhibitors, sitaxsentan and ambrisentan are also approved for PH treatment [7]. However hepatotoxicity is a side effect of this group of drugs and results in withdrawal of treatment from approximately 6% of patients [6].

Phosphodiesterase inhibition results in vasodilation by preventing the degradation of the vasodilator cyclic guanosine monophosphate. As a result, sildenfil, a potent and specific inhibitor of PDE-5 (the primary isoform

expressed in the pulmonary vasculature) has been approved for the treatment of PH [7, 24].

Despite the improvement of pharmacological agents in the treatment of PH, surgery and lung transplant still remains an important strategy for end stage disease and is the only curative treatment for idiopathic PH.

1.3 VASCULAR ENDOTHELIAL GROWTH FACTOR

1.3.1 General Overview

Vascular Endothelial Growth Factor (VEGF) and vascular permeability factor (VPF) were initially identified as two independent proteins; VPF, capable of permeabilising the endothelium of numerous vascular beds to plasma protein and VEGF, an endothelial cell specific mitogen. However molecular cloning of the two proteins revealed them be the same molecule [25], and a distant relative (20% homology) of the platelet derived growth factor (PDGF) A and B chains. [26, 27].

Since its identification in the 1980s, 6 VEGF related genes have been identified, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E (a form found in papapoxvirus [28]), VEGF-F (a form found in snake venom [28]) and placental growth factor (PlGF). Further, six mammalian VEGF-A isoforms have been characterised and are named by there amino acid number (VEGF-A 121, 145,165, 183, 189 and 206) and created by alternative splicing of VEGF-A mRNA [25, 29] (Figure 1-1). Mouse isoforms are a single amino acid shorter [28]. All isoforms are secreted as covalently linked homodimers (VEGF-A_{165}

being a 46-kDa homodimeric glycoprotein of two 23-kDa monomers [26]), linked initially by hydrophobic interaction, and terminally by disulphide stabilisation [30].

The *VEGFA* gene is organised as 8 exons separated by 7 introns and is localised to chromosome 6p21.3. The coding region spans 14kb [31]. The most abundant and biologically active VEGF-A isoform, VEGF-A_{165}, lacks residues encoded by exon 6, while the 121 amino acid isoform lacks residues encoded by exons 6 and 7 [32]. These differences in splicing result in proteins with altered affinity for heparin and a resultant differential in diffusion ability. The weakly acidic $VEGF_{121}$ diffuses freely through tissue due to exon 6 and 7 splicing resulting in loss of 15 basic amino acids which have affinity for heparin [30], while 50-75% of $VEGF_{165}$, upon secretion, binds to cell surface heparin sulphate proteoglycans (HSPGs) and ~100% of the more basic $VEGF_{189}$ and $VEGF_{206}$ binds HSPGs providing a sequestered pool of VEGF in the extracellular matrix. The heparin bound isoforms can be released to a soluble form via heparinase and plasmin cleavage. Thus VEGF can become available to cells via at least two different mechanisms: as a freely diffusible protein or following protease activation and cleavage to smaller isoforms [29]. Loss of the heparin binding domain of VEGF-A_{165} results in loss of its mitogenic activity. As such VEGF-A_{165} is believed to possess optimal characteristics of bioavailability and biological potency [32]. $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{183}$ have a wide tissue distribution while $VEGF_{145}$ and $VEGF_{206}$ are comparatively rare [30].

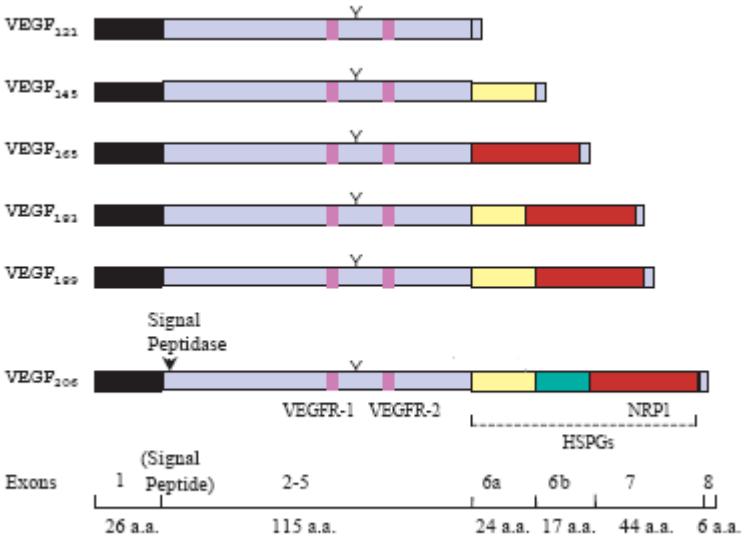


Figure 1-1 The Splice Variants of human VEGF-A.

Adapted from [30]. Alternative splicing of the human VEGF gene produces six isoforms, which differ by the presence or absence of sequences encoded by exons 6 and 7. The difference in resultant protein affects their ability to bind heparin. Amino acid residues and the exons from which the variants are derived are shown at the bottom of the figure. Sites of interaction with VEGFRs and NRPs are indicated. 'Y' represents possible glycosylation sites.

VEGF-B, isolated and characterised in 1996, has structural similarities to VEGF-A and is abundantly expressed in the heart and skeletal muscle [33]. It exists as two isoforms of 167 and 186 residues. Both have identical 115 amino acid N terminals but differ in their C-termini [34]. VEGF-B_{167} is nonglycosyltaed, binds heparin and is mostly sequestered to the extracellular matrix, while VEGF-B_{186} is O-glycosylated and freely diffusible [35]. VEGF-B can form heterodimers with VEGF-A [35].

VEGF-C was identified in 1996 as the ligand for VEGFR-3 [36], while VEGF-D was identified in the late 1990s by computer based homology searching. VEGF-D has closest homology to VEGF C and is expressed abundantly in heart, skeletal muscle, lung, colon and small intestine [37]. Both VEGF-C and D are produced as long precursor proteins which are proteolytically processed to generate a number of isoforms which have differential receptor affinity [34]. VEGF-C expression is predominantly limited to regions where lymphatic vessels develop and is implicated in lymph angiogenesis [26, 35].

PlGF was isolated from a cDNA library in 1991. It is a 149-amino acid protein with 53% homology to VEGF-A [27, 38] and is abundantly expressed in the placenta, heart and lungs [35]. It has three isoforms of 131 (PlGF-1), 152 (PlGF-2) and 203 (PlGF-3) amino acids [34] and can form VEGF:PlGF heterodimers with potent mitogenic activity, in addition to PlGF homodimers with relatively weak mitogenic activity [27, 30]. Only the 152 amino acid form binds heparin [35].

1.3.2 Regulation of VEGF

1.3.2.1 Transcriptional Regulation

Transcriptional regulation of the *VEGF* gene is under tight control. As studies show lack of single VEGF allele is sufficient to cause lethality it is clear that the amount of VEGF present is vital to whether its role is executed correctly.

The VEGF promoter contains binding sites for numerous transcription factors including HIF-1, Sp-1, CREB, p53 and TCF. VEGF mRNA is regulated by

exposure to low oxygen tension via the hypoxia sensitive transcription factor hypoxia inducible factor-1 (HIF-1) in a variety of normal and transformed cells [31]. HIF-1 is a basic, heterotrimeric, helix-loop-helix protein consisting of two subunits, HIF-1α and HIF-1β [31]. HIF-1α is constitutively degraded by prolyl hydroxylation. Under hypoxia prolyl hydroxylases are inhibited and HIF-1α degradation prevented. HIF-1α and HIF-1β are stabilised and bind to hypoxia response elements (HRE) in the promoter or enhancer region of the *VEGF* gene [28, 39]. HIF-1α can also act in combination with other transcriptional regulators to affect VEGF mRNA levels, and in some cases maximal VEGF expression requires association of HIF-1 and further factors. For example Jeon *et al.,* showed TGFβ induced VEGF transcription in mouse macrophages required HIF-1α association with the HRE on the VEGF promoter in addition to Smad3 and Smad4 association with Smad binding elements (SBE) further downstream the VEGF promoter [40]. HIF-1α can also interact with Mdm2, a protein that can bind and inhibit the tumour suppressor p53, and induce VEGF via a complex requiring both proteins [41]. Interestingly Schmidt *et al.,* have shown that hypoxic regulation of VEGF transcription can be independent of HIF, and in mouse embryonic fibroblasts, hypoxia induces NF-κB translocation which stimulates binding of the Ap-1 subunit junB to the VEGF promoter and increases VEGF expression [42]. Furthermore, Arany *et al*., show that oxygen depletion in skeletal muscle cells causes an increase in peroxisome-proliferator-activated-receptor-γ coactivator 1α (PGC-1α) which subsequently increases binding of the orphan nuclear receptor ERR-α to consensus ERR-A-binding sequences (AAGGTCA) within the VEGF promoter resulting in increases VEGF transcription [43].

The Specificity Protein 1 (Sp-1) transcription factor also transcriptionally controls VEGF expression, both basally and in response to stimuli. Sp1 was one of the first transcription factors to be cloned in 1987. It belongs to a multigene family which also includes Sp2, Sp3 and Sp4 [44]. Sp1 can act independently or in combination with other proteins from the same family. For example, a combination of Sp1, Sp3 and Sp4 is required for VEGF transcription in pancreatic cancer cells via proximal GC-rich sites in the VEGF promoter [45]. Phosphorylation of Sp1 is required for transcriptional activity and a number of signalling pathways increase VEGF transcription via regulation of Sp1 phosphorylation, for example, stimulation of cells with Hepatocyte Growth Factor (HGF) [46], Epidermal Growth Factor (EGF) and TGFβ results in Sp1 phosphorylation as does activation of numerous kinases including casein kinase II, ERK, protein kinase A (PKA) [44] and Akt [47]. An alternative mechanism of Sp-1 regulation is via modulation of Sp protein levels, for example the down regulation of VEGF by cyclooxygenase-2 inhibitors in pancreatic cancer cells occurs via activation of proteasome-dependent degradation of both Sp1 and Sp4 [48].

With a known role for VEGF in the female menstrual cycle it is not surprising that VEGF expression is regulated by 17β estradiol via an estrogen response element (ERE) within the VEGF promoter. This ERE also binds the estrogen receptors α and β. Progesterone is also capable up-regulating VEGF transcription via progesterone receptors A and B and three progesterone response elements (PRE1,2 and 3). However mutation of these three sites does

not completely abrogate the response to progesterone suggesting they also act via alternative regulatory sites within the VEGF promoter [44].

Jeon *et al.*, have shown that VEGF transcription can be induced by activation of the PKA pathway, resulting in association of cyclic AMP-responsive element binding protein (CREB) to one of three CREB responsive elements (CREs) in the mouse VEGF promoter [49]. Further, the von Hippel-Lindau (VHL) or p53 protein, a key tumour suppressor, can increase VEGF mRNA levels and oxidised phospholipids can induce binding of activating transcription factor-4 (ATF4) to the VEGF promoter [50].

A number of cytokines and growth factors increase VEGF mRNA in specific cell types, for example EGF in cultured glioblastoma cells, TGFβ in cultures of quiescent fibroblast and epithelial cells, keratinocyte growth factor 1 (KGF1) in keratinocytes, interleukin 1α (IL-1α) and prostaglandin E_2 (PGE_2) in synovial fibroblasts [31], insulin like growth factor-1 (IGF-1) in colorectal carcinoma cells, angiotensin II in human mesangial cells, PDGF-BB in human smooth muscle cells and IL-6 in a number of cells [31, 51].

The necessity for tight regulation of VEGF expression results in complex regulatory mechanisms that differ depending on cell type and environment. The examples covered here do not come close to covering the full range of transcriptional mechanisms described in the vast published literature.

1.3.2.2 Post-transcriptional Regulation

Control of mRNA stability is an important control point in VEGF regulation. For example hypoxia is known to transcriptionally regulate VEGF but can also control VEGF mRNA stability. Destabilising elements are present in the 3' untranslated region (UTR), the coding region of the mRNA and also within the 5' UTR. VEGF has an unusually long and GC rich 5' UTR which may provide the distinction between it and other mRNAs where no 5'UTR determined stabilisation is observed. Stabilisation of VEGF mRNA by hypoxia requires action via all three of these regions in combination, with no single region individually conferring stabilisation [52]. A transacting factor which mediates stabilising functions within the 3' UTR of VEGF under hypoxic conditions is HuR, a member of the RNA-binding protein, Elav-like, family which binds AU-rich elements (AREs) of which there are eight in the 3' UTR of VEGF [53]. How HuR then modifies stability is unclear but it may inhibit endonuclease activity preventing mRNA cleavage and exonuclease mediated degradation. Alternatively it may inhibit deadenylase activity preventing deadenylation resulting in mRNA rapid exonuclease degradation [30, 54]. Proteins that destabilise mRNA can also bind AREs, for example, AUF1 and tristetraprolin [53].

AREs have also been implicated in the translational regulation of gene expression and Vell *et al.,* have identified this as a level of VEGF regulation. They found that the presence of the zinc finger-containing mRNA binding protein *Zfp36l1* results in reduced VEGF mRNA loading onto polysomes and therefore reduced VEGF translation [55]. Regulation of VEGF translation has

also been reported in response to interferon γ (IFN-γ) in monocytic cells. IFN-γ induces VEGF mRNA and protein up to 8 hours, while at 24 hours there is no induction of protein despite an abundance of VEGF mRNA. This was due to a blocking of VEGF translation by association of the IFN-γ-activated inhibitor of translation (GAIT) with GAIT element in the 3'UTR of VEGF mRNA [56].

1.3.3 VEGF Receptors and Signalling

1.3.3.1 The VEGF Receptors

Initially receptors for VEGF were identified on vascular endothelial cells [32] and subsequently on bone marrow-derived cells [32], haematopoietic stem cells, macrophages, megakaryocytes, monocytes [57], trophoblasts, renal mesangial cells, neurons and platelets [34, 35]. The VEGFs interact differentially with three VEGF receptor tyrosine kinases (RTK), VEGFR-1/Flt-2 (*fms*-like tyrosine kinase), VEGFR-2/Flk-1/KDR (kinase-insert-domain containing receptor) and VEGFR-3/Flt4 [25]. VEGFR-1 and VEGFR-2 are 45% identical in amino acid structure [27]. Alternative splicing of VEGFR-1 mRNA results in a soluble form of the receptor, sFlt-1, which is truncated on the C-terminal side of the sixth Ig domain [27]. In addition some VEGF family members can bind co-receptor Neuropilins.

The VEGFRs belong to the class III receptor tyrosine kinases of the platelet derived growth factor (PDGF) receptor subfamily [34]. Receptor tyrosine kinases catalyse the transfer of the γ phosphate of adenosine triphosphate (ATP) to hydroxyl groups of tyrosine's on target proteins [58]. VEGFR-1 and VEGFR-2 have seven immunoglobulin-like domains in the extracellular domain, a single transmembrane domain and a consensus tyrosine kinase

domain, interrupted by a 70-amino-acid kinase-insert domain, within the cytoplasmic domain [26, 28, 29, 32]. Immunoglobulin domains 2 and 3 are necessary and sufficient for ligand binding [59]. Ig domain 2 is the primary ligand binding domain and Ig domain 3 confers ligand specificity [28]. Ig domain 4 mediates receptor dimerisation, Ig domain 5 and 6 are required for VEGF retention following binding while Ig domain 1 regulates ligand binding [30].

VEGFR-3 differs slightly in structure to VEGFR-1 and VEGFR-2 due to cleavage of the fifth extracellular immunoglobulin loop resulting in the separated polypeptides being linked by a disulfide bridge [57]. In humans alternative splicing of the VEGFR-3 generates two isoforms that differ in their C-termini [35]. VEGFR3 is expressed on all endothelial cells during development but is restricted to lymphatic endothelial cells in the adult [35].

VEGF receptors are monomers at the cell membrane and ligand binding induces dimerisation of the receptors. VEGFRs form either homo- or heterodimers depending on the binding properties of the ligand [28]. Dimerisation results in activation of kinase activity and trans-autophosphorylation of residues in the cytoplasmic domain of the receptors. These autophosphorylated sites function as binding sites for SH2 (src homology 2) or PTB (phosphotyrosine binding domains) domains of numerous signalling proteins and result in the assembly of a signalling complex containing proteins with a wide array of intrinsic abilities including enzymatic activity and the ability to interact with phospholipids, nucleic acids and other proteins [58]. VEGFR-1 has seven major autophosphorylation sites within its

C-terminal tail, Y794, Y1169, Y1213, Y1242, Y1309, Y1327 and Y1333 [34]. These act as docking sites for proteins including Phospholipase C-γ (PLC-γ), adaptor protein Nck, phosphatase SHP-2 and the p85 subunit of Phosphoinositide-3 kinase (PI3K) [34]. Six autophosphorylation sites have been identified in VEGFR-2, Y951 and Y996 in the kinase insert domain of the protein, Y1054 and Y1059 in the kinase domain (phosphorylation of which is required for maximal kinase activity) and Y1175 and Y1214 in the C-terminal tail [26, 57]. Phosphorylation of these sites recruits the adaptor proteins Shc, Grb2 and Nck, the protein tyrosine phosphatases SHP-1 and 2, and the kinases PLCγ and PI3K [34]. Phosphatases such as SHP-1 and 2 can negatively regulate the receptors by counteracting the receptors activating phosphorylation [28]. Seven autophosphorylation sites have been identified in VEGFR-3. Y1063 and Y1068 are within the kinase domain and can regulate kinase activity, while Y1230, Y1231, Y1265, Y1337 and Y1363 are located in the C-terminal tail and known to recruit proteins including Shc protein phosphatase.

Termination of VEGFR signalling can be achieved by dephosphorylation, rapid degradation of the receptors via the proteasome pathway and internalisation of the receptors and degradation in the lysosomes [28].

1.3.3.2 VEGF Ligand:Receptor Specificity

VEGFR-1 binds VEGF-A with a K_d of 10-20pM [29], having a ten fold higher affinity for VEGF-A than VEGFR-2 (K_d of 75-125pM), while VEGFR-3 does not bind VEGF-A. Upon dimerisation VEGFR-2 increases its affinity for VEGF 100 fold while dimerised VEGFR-1 has increased affinity of only 2 fold

[59]. PlGF and VEGF-B bind with high affinity only to VEGFR-1, however VEGF/PlGF heterodimers are known to have biological activity and bind VEGFR-2 [26]. VEGF-C and VEGF-D bind VEGFR-3 in their propeptide form while as processed mature ligands can bind VEGFR-2 in addition to having increased affinity for VEGFR-3 [36, 37, 57]. VEGF-E binds exclusively to VEGFR-2 and in some forms to neuropilin 1[34]. VEGF-F interacts with both VEGFR-1 and 2 [28]. An illustration of VEGF specificity for its receptors is shown in Figure 1-2.

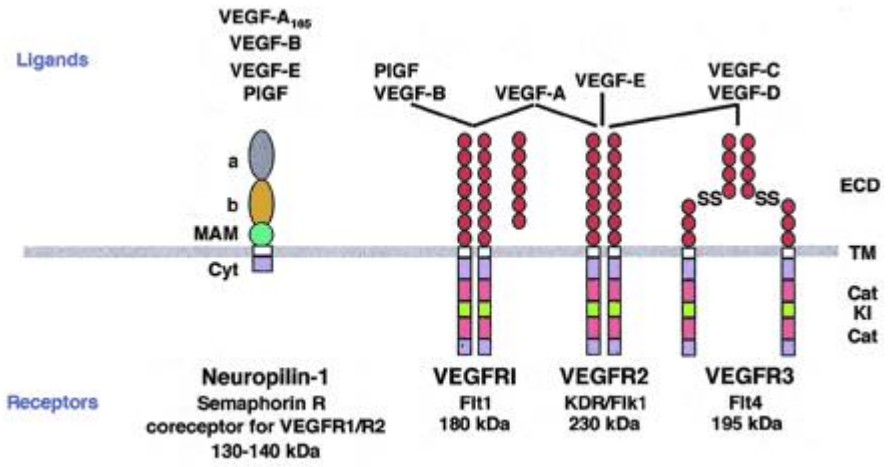


Figure 1-2 Vascular endothelial growth factor ligands and receptors.

Adapted from [26]. ECD = Extracellular Domain; TM = transmembrane domain; Cat = catalytic domain; KI = kinase insert domain;

VEGF-A's receptor binding domain (residues 8-109) includes two binding 'hot spots' which cross the VEGF dimer interface. Five of the seven high importance residues on VEGF-A for VEGFR-2 binding are also of high

importance for VEGFR-1 binding [59]. Further, PlGF and VEGF-B compete with VEGF-A for VEGFR-1 binding, suggesting the sites of interaction on VEGFR-1 for all three ligands are highly similar [34].

1.3.3.3 VEGF Receptor Downstream Signalling

VEGFR-1 is an 180kDa transmembrane glycoprotein and the first of the VEGF receptors to be identified. Its precise role is still unclear, partly due to temporal and spatial regulation of its signalling [32, 34, 57]. VEGFR-1 is the first of the VEGF receptors to be expressed during development but at a lower level than VEGFR-2 [35]. Mice expressing only VEGFR-1 lacking the kinase domain are able to develop normally but mice lacking VEGFR-1 altogether are not viable. This suggests a crucial role for VEGFR-1 that is not dependent on its kinase activity. It may be that the intracellular domain of VEGFR-1 is positioned such that it represses the kinase activity of its kinase domain by preventing the exposure of regulatory sequences in the kinase domain [28]. Current opinion suggests VEGFR-1 to be a negative regulator of VEGFR-2, or a 'decoy' receptor, acting as a 'sink' for VEGF-A and preventing its association with VEGFR-2 [26, 31]. Mice lacking both the transmembrane domain and the kinase domain have only a 50% survival rate suggesting that anchoring of the protein to the membrane is important for its signalling role and supports a role as co-effector of VEGFR-2 signalling. A soluble form of VEGFR-1 (sVEGFR-1) is expressed *in vivo* as a splice variant of the full VEGFR-1. sVEGFR-1 has high affinity for VEGF-A and sequesters in away from VEGFR-2 and further supports the 'decoy' hypothesis [57]. Further, VEGFR-1 kinase activity is only seen to increase marginally in response to ligand binding and its phosphorylation is only detectable upon overexpresson

of the protein suggesting the receptor possesses little signalling ability [57]. Additional complexity is introduced by evidence for crosstalk between VEGFR-1 and VEGFR-2, for example, VEGFR-2 mediated endothelial cell proliferation can be suppressed by VEGFR-1 [28].

VEGFR-2 is a 200-230 kDa protein and the major mediator of the mitogenic, angiogenic, migratory and permeability inducing effects of VEGF [32, 57]. Its autophosphorylation in response to VEGF-A is easily detectable and delineation of its downstream signalling a priority to researchers [28]. VEGFR-2 activates PLCγ resulting in activation of the mitogen-activated protein kinase (MAPK)/ extracellular-signal-regulated kinase 1/2 (ERK1/2) signalling cascade and protein kinase C (PKC) via Tyr1173 [28, 31]. This same tyrosine residue and Tyr1175 are also coupled to PI3K activation resulting in an increase in the lipid phosphatidylinositol $(3,4,5)P_3$ and subsequent AKT/PKB activation [57]. These pathways promote endothelial cell proliferation and survival. VEGFR-2 signalling also results in endothelial cell migration via formation of a complex containing VRAP (VEGF receptor-associated protein) at phosphorylated Tyr951 and actin reorganisation via Tyr1214 phosphorylation and p38 MAPK activation. VEGFR-2 expression is upregulated by VEGF, VEGF-C and VEGF-D [35].

VEGFR-3 is a 195 kDa protein and its adult expression is limited to lymphatic endothelial cells. Blockade of VEGFR-3 in adult mice results in lymphatic abnormalities with no effect on the blood vasculature, consistent with its adult role being limited to lymph angiogenesis [57]. VEGFR3-/- mice die at day 9.5

due to deficient vessel remodelling. This is postulated to be due to an increased availability of VEGF-C and VEGF-D to signal through VEGFR-2. Little specificity of VEGFR-3 signalling has been investigated, however phosphorylation of Tyr 1337 creates an association site for the Shc-Grb2 complex, two adaptor proteins which can result in Ras activation [28]. Other signal transducers implicated in VEGFR-3 signalling include ERK1/2, PKC, PI3K and PKB/AKT. VEGFR-3:VEGFR-2 heterodimers can form and directly influence the signalling capacity of VEGFR-3 as VEGFR-2 does not phosphorylate Tyr1337 and thus affects the ability of the downstream substrates to interact with VEGFR-3 [57].

1.3.3.4 The Neuropilins

Distinct to the tyrosine kinase activity containing receptors for VEGF are the Neuropilins (NRP1 and NRP2). Neuropilins are transmembrane proteins with a short cytoplasmic tail and no known signalling function. They were originally identified at the growing tips of axons in specific neurons [34]. Neuropilins can bind all isoforms of VEGF, except VEGF-A_{121} due to the lack of the exon 7 encoded basic residues, VEGF-B, VEGF-E and a splice variant of placental growth factor (PlGF-2) [26, 32]. In some contexts NRP1 enhances binding of VEGF-A_{165} to VEGFR-2 and may explain the increased mitogenic activity of the 165 isoform over the 121 isoform [26, 29, 32, 34]. Also, inhibition of $VEGF_{165}$ binding to NRP-1 can prevent VEGF binding to the VEGFR-2 and downstream mitogenic effects, suggesting NRP-1 presents VEGF to VEGFR-2 in a way that enhances the effectiveness of VEGFR-2 signalling [31].

1.3.4 Physiological role of VEGF in the Vasculature

Initial evidence for the requirement for VEGF in embryonic vasculogenesis (endothelial cell differentiation) and angiogenesis (sprouting of new capillaries from preexisting vessels [26]) came in 1996 when Carmeliet *et al.*, and Ferrara *et al.*, showed loss of a single VEGF allele in mice was sufficient to cause embryonic lethality between days 11 and 12, with the mice showing growth retardation, defective vascularisation of numerous organs, rudimentary aortas, decreased ventricular wall thickness and a reduction of red blood cells in the blood islands [29, 32]. Subsequently, Carmeliet *et al.* targeted exons 6 and 7 of VEGF in a Cre/loxP system and effectively generated the non-heparin binding $VEGF_{120}$ isoform. Of the homozygous $VEGF_{120}$ knockouts generated 50% died within hours of birth. However, those which survived this initial phase exhibited enlarged hearts, irregular heart beats, weak heart contractions and defective angiogenesis [60]. In addition mice lacking $VEGF_{188}$ display impaired arteriolar development and ~50% die at birth [35]. Collectively these studies indicate the importance of VEGF expression and also show that isoforms other than $VEGF_{165}$ cannot compensate for loss of $VEGF_{165}$ and illustrates the discrete roles the different isoforms play.

Mice specific for VEGF receptor deficiency also show vascular abnormalities. VEGFR-1 -/- mice die *in utero* between days 8.5 and 9.5 with mature, differentiated endothelial cells but an inability of endothelial cells to form organised vascular channels due to excessive proliferation of endothelial progenitor cells, again suggesting VEGFR-1's negative role in VEGF signalling [26, 29, 32]. VEGFR-2 -/- mice also die *in utero* between days 8.5

and 9.5 but in contrast to VEGFR-1 -/- mice this is due to a lack of vasculogenesis (i.e. differentiated endothelial cells) and a failure to develop blood islands (masses of angioblasts and hemoblasts which form blood vessels in the embryonic yolk sac) and organised blood vessels [26, 32], supporting a positive role for VEGFR-2 in VEGF signalling. Further, mutation of Tyr1173 to phenylalanine results in vascular defects similar to those seen in VEGFR-2-/- mice. VEGFR-3 -/- mice die at day 9.5 due to defects in the remodelling of the primary vascular network and cardiovascular failure, while endothelial cell differentiation and the initial formation of primary vascular networks are not disturbed, suggesting it plays a general role in early cardiovascular development and it is only after generation of the lymphatic system that VEGFR-3 becomes restricted to the lymphatic system [34].

Neuropilin 1 (NRP-1) overexpression in mice results in vascular abnormalities including excess capillaries, excess dilated blood vessels and heart malformation. NRP-1 -/- mice die at day 10.5-12.5 with cardiovascular failure, defects in the aorta and other large blood vessels, and aberrant yolc sac vascularisation [26, 34].

Collectively these studies show a critical role for VEGF and its signalling in embryonic vasculogenesis and angiogenesis, however the biological role of VEGF in the adult vasculature is harder to identify, as VEGF deficiency is lethal. Recent experimental techniques have however suggested a role for VEGF in adult vascular protection [26]. VEGF induces mediators with vascular protective effects, for example, the vasodilators, nitric oxide (NO) and

prostacyclin (PGI_2). Additional vaso-protective roles of these mediators include inhibition of vascular smooth muscle proliferation and anti-platelet/anti-thrombotic actions [26]. In agreement with a protective role, mice exposed to inducible targeting of the VEGF gene (which only partially inhibits VEGF) have increased mortality, reduced growth, impaired organ growth, decreased proliferation of numerous cell types and an increased apoptotic index of endothelial cells, however the effects of targeted VEGF loss were less significant as the post-natal age of the mice increased [29].

VEGF can promote vascular endothelial cell survival, a process necessary for vascular protection and maintenance. A least part of this effect is because VEGF can induce anti-apoptotic proteins (Bcl-2 and A1 [29, 32]) and phosphorylation of focal adhesion kinase, whose dephosphorylation is an early response to apoptotic stimuli [26]. Furthermore, VEGF can activate the anti-apoptotic kinase Akt/PKB via PI3K and VEGFR-2 [26]. VEGF also induces the expression of proteases by endothelial cells, such as interstitial collagenase and the urokinase-type and tissue-type plasminogen activators. These proteases allow cell migration required for angiogenesis [30].

Adult female reproductive tract angiogenesis requires VEGF. VEGF mRNA in mice, rats and primates, is temporally and spatially related to the proliferation of blood vessels suggesting VEGF is a mediator of the cyclical growth of blood vessels that occurs in the female reproductive tract [29]. More recently the development of an inhibitor of rodent VEGF has shown inhibition to result in complete suppression of corpus luteum angiogenesis and failure of the

endometrium to develop in a rat model of hormonally induced ovulation [60]. Finally, adult wound healing also requires VEGF. In rodents, VEGF mRNA is maximally expressed by surface epidermal keratinocytes soon after dermal injury [27].

In addition a number of diseases including proliferative retinopathies, age-related macular degeneration, tumours, rheumatoid arthritis and psoriasis, have angiogenic pathology [31].

1.3.5 VEGF in Pulmonary Hypertension

Abnormal VEGF and angiogenic processes are associated with a wide array of diseases. This can be due to either an enhanced angiogenic response, for example, in rheumatoid arthritis, diabetic retinopathy and tumour development, or loss or destruction of angiogenic response, for example, following a myocardial infarction [34]. Whether induction or inhibition of VEGF and other angiogenic signals is of benefit to patients is disease specific and requires careful targeting of any such treatment to specific regions.

The role of VEGF in PH is not well defined and there are contradictory reports in the literature as to whether it performs a beneficial or detrimental role.

VEGF and VEGFR-2 expression on endothelial cells and smooth muscle cells has been reported in areas of remodelling in both primary PH patients and secondary PH patients [61, 62], and suggested to several researchers that the remodelling and formation of plexiform lesions that occurs in PH might be akin to a process of disordered angiogenesis, similar to that seen in tumour

formation [61]. Expression of both VEGF and its primary signalling receptor, VEGFR-2, on the same cells suggested autocrine or paracrine signalling of VEGF back onto the endothelial cells. It was further shown that inhibition of VEGFR-1 and VEGFR-2 by the synthetic inhibitor SU5416, in rats, in combination with hypoxia or shear stress, was sufficient to cause endothelial cell death. Endothelial cell death was followed by selection of an apoptosis resistant population of endothelial cells which were highly proliferative and caused the obliteration of the pulmonary artery lumen. Initial apoptosis of endothelial cells was an absolute requirement for development of the proliferative endothelial cells and the author postulated that factors released from the apoptotic cells caused the proliferation [63, 64]. The conditioned media from the initial apoptotic endothelial cells increased vascular smooth muscle growth, a key pathological symptom observed in PH patients. Factors present in the conditioned media included TGFβ_1 and VEGF. TGFβ_1 conferred smooth muscle cell proliferation while VEGF conferred protection of smooth muscle cells from apoptosis, the combination of which resulted in an overall increase in smooth muscle. Further, Voelkel *et al*., have shown that VEGFR inhibition can cause transition between an endothelial cell phenotype and a smooth muscle cell phenotype suggesting a reduction in VEGF signalling may result in increased muscularisation in the pulmonary artery [65].

The precise role of VEGF is difficult to define. In models, loss of VEGF signalling results in endothelial cell death and a subsequent increase in endothelial cell proliferation and differentiation of endothelial cells into smooth muscle like cells, suggesting a lack of VEGF to be detrimental and

therefore a protective role for VEGF. In contrast however there is evidence that the presence of VEGF production (following endothelial cell apoptosis) increases smooth muscle cell content within the remodelled arteries, suggesting that VEGF is detrimental.

In animal based models in which the levels of VEGF are experimentally increased, VEGF appears to confer a protective role. In rats exposed to monocrotaline (MCT), a phytotoxin derived from the seeds of *Crotalaria spectabilis*, development of PH is evident from increased right ventricular systolic pressure (RVSP) and increased right ventricular to left ventricular plus septal (LV) weight ratios (RV/LV). Cell based gene transfer of VEGF delivered at the time of MCT exposure or 14-28 days after MCT exposure, significantly reduces both RSVP and RV/LV [66]. A similar experiment performed by Zhao *et al*., had a further group in which endothelial nitric oxide synthase (eNOS) was transfected into the rats. They showed that VEGF could prevent progression of PH (i.e. it prevented further increases in RSVP) but that it could not cause regression of PH (i.e. a reduction of RSVP to the control levels seen in those rats not exposed to MCT), however eNOS could, to an extent, reverse the PH [67].

In a rabbit bleomycin model of PH, VEGF gene transfer prevented the development of PH as signified by a reduced pulmonary artery pressure (PAP), reduced thickening of the artery wall, and reduced narrowing of the cavity of mid- and small-sized pulmonary arteries. Also, endothelial cell VEGF mRNA in the bleomycin induced PH rabbits was lower than in control animals [68, 69]. Le Cras *et al*., showed in newborn rats that inhibition of VEGFR

signalling using SU5416, was sufficient to increase right ventricular weight and pulmonary arterial wall thickness, markers of PH that persisted into adulthood.

Persistent PH of the newborn (PPHN) is a disease characterised by failure of pulmonary vascular resistance to fall at birth and results in vascular changes similar to other forms of PH, for example, endothelial cell dysfunction, elevated pulmonary vascular resistance and impaired endothelium-dependent vasoconstriction. In a fetal sheep model of PPHN VEGF protein expression was reduced by 78% and inhibition of VEGF in normal fetal sheep resulted in impaired endothelium dependent vasodilation, right ventricular hypertrophy and muscularisation of the small pulmonary arteries[70]. Recovery of VEGF levels in PH sheep by administration of intrapulmonary recombinant VEGF reduced PAP, restored vasodilation and attenuated structural remodelling of the pulmonary arteries [71].

In contrast to the studies in which VEGF is decreased in response to the induction of PH, in studies in which PH is induced in lambs and rats by high pulmonary blood flow, increased VEGF protein and mRNA levels are present in the smooth muscle cells of the pulmonary artery. Further, VEGFR levels are increased in the adjacent endothelium [72, 73]. Also studies utilising hypoxia induced PH also see an increase in VEGF and VEGF-1 expression, however a decrease in VEGFR-2 expression is observed [74, 75].

While the precise role for VEGF in PH is still unclear a greater understanding of its roles and regulatory mechanisms and how these relate to the progression of PH may shed new insight on the pathophysiology of this disorder. The study of VEGF regulation in pulmonary vasculature cells is important as it may provide ways of manipulating VEGF and thus regulating PH in the future.

1.4 TRANSFORMING GROWTH FACTOR B

1.4.1 General Overview

Transforming growth factor β (TGFβ) is the prototype of the TGFβ family which consists of a large number of structurally related, secreted, polypeptide growth factors, each regulating a wide range of cellular processes, from cell proliferation, motility, adhesion and differentiation to cell death, in organisms from fruitfly to human. TGFβ is expressed in complex temporal and tissue specific patterns, allowing it to play a role of some sort in all tissues [76, 77]. The family members and their percent homology to bone morphogenetic protein (BMP) 2 are shown in Table 1

Table 1 The transforming growth factor β family.

Adapted from [76]. GDF, growth differentiation factor. CDMP, cartilage-derived morphogenetic protein. MIS/AMH, Müllerian inhibiting substance/anti-Müllerian hormone. GDNF, glial cell-derived neurotrophic factor.

Names [Homologues]	% Homology to BMP2
BMP2 subfamily	
BMP2 [Dpp^D]	100
BMP4	92
BMP5 subfamily	
BMP5 [60 A^D]	61

BMP6/Vgr1	61
BMP7/OP1	60
BMP8/OP2	55
GDF5 subfamily	
GDF5/CDMP1	57
GDF6/CDMP2	54
GDF7	57
Vg1 subfamily	
GDF1 [$Vg1^X$]	42
GDF3/Vgr2	53
BMP3 subfamily	
BMP3/osteogenin	48
GDF10	46
Intermediate members	
Nodal [Xnr 1 to 3^X	42
Dorsalin	40
GDF8	41
GDF9	34
Activin subfamily	
Activin βA	42
Activin βB	42
Activin βC	37
Activin βE	40
TGF-β subfamily	
TGF-β1	35
TGF-β2	34
TGFβ3	36
Distant members	
MIS/AMH	27
Inhibin α	22
GDNF	23

Mature TGFβs are 25kDa homodimers (β1/β2 and β2/β3 heterodimers are reported). All are synthesised as larger precursor monomers, of which the mature TGFβ monomers represent the C-terminal 112 amino acids. Differences between the TGFβs are mainly seen in the N-terminal of the proteins and include three N-glycosylation sites in TGFβ1 and β2 but four in TGFβ3, and the presence of the RGDL (arg-gly-asp-leu) motif, used to confer interactions between TGFβ and cell surface and matrix proteins, in TGFβ1 and

β3 but not TGFβ2. The genes for the human TGFβs are situated on three separate chromosomes: TGFβ1 on chromosome 19q13, β2 on 1q41 and β3 on 14q24 [77]. The genes are controlled by differentially regulated promoters and are known to be under the control of posttranscriptional regulation [78].

TGFβs are secreted as latent, biologically inactive protein dimers due to non-covalent association of the N-terminal section of the TGFβ precursor, called the latency associated peptide (LAP), with the C-terminal of the TGFβ precursor (active TGFβ molecule). Further, a protein called the latent TGFβ binding protein (LTBP) binds the LAP by disulphide bonds to create a large latent complex. This large latent complex may facilitate TGFβ secretion and its ability to bind components of the extracellular matrix controls cellular localisation of the latent complex [77] and bioavailability of active TGFβ. Activation can occur via proteolytic or non-proteolytic mechanisms and is a tightly regulated process. Non-proteolytic mechanisms include heat, acid and reactive oxygen species. Proteases implicated in TGFβ activation include plasmin, thrombin, elastase, matrix metalloproteinase (MMP)-2 and MMP-9 [79]. Further the integrin αvβ6 may be critical in TGFβ activation in the lung [79].

In contrast, BMPs are secreted in an active form and regulated through reversible interactions with extracellular antagonists, including noggin, chordin, and DAN [80].

1.4.2 TGFβ Receptors and Signalling

TGFβ family members signal through a family of transmembrane serine/threonine kinases. The family is split into two subfamilies based on their structural and functional similarities; type I receptors and type II receptors (betaglycan is sometimes referred to as a type III receptor, however it confers no signalling role but is involved in increasing the affinity of TGFβ for its type II receptor [81]). The receptors are glycoproteins of approximately 55kDa (Type I) and 70kDa (Type II), and are present as homodimers in the plasma membrane [82]. Their extracellular domains are short and N-glycosylated. The transmembrane and cytoplasmic juxtamembrane regions have no specific features but contain phosphorylation sites which confer signalling specificity. Type I receptors possess a highly conserved 30 amino acid region preceding the kinase domain, termed a GS domain due to a characteristic TTSGSGSG sequence within it. Phosphorylation of the threonine and serine residues within this domain, by the type II receptor is required for activation of signalling in response to ligand. The kinase domain of the two types of receptor is a canonical serine/threonine kinase domain. Type I receptors phosphorylate their substrates, Smad proteins, on serine residues, while type II receptors phosphorylate themselves and type I receptors on serine and threonine residues. Type II receptors have a short cytoplasmic tail which can be phosphorylated but confers no signalling ability, in contrast to tyrosine kinase receptors. Type I receptors have virtually no C-terminal tail [76]. The various receptors by their subtype are given in Figure 1-3 A and by type I:type II interaction and ligand specificity in Figure 1-3 B.

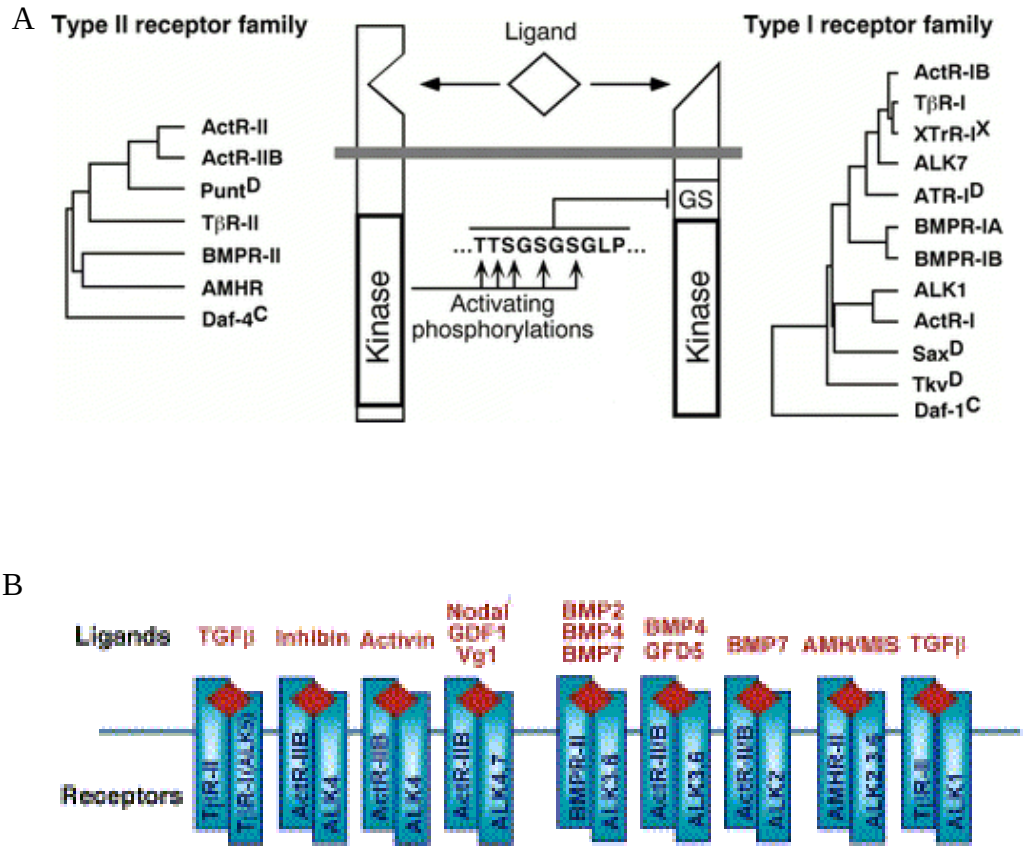


Figure 1-3 TGFβ family of receptors.

A) Shown by receptor sub type. Adapted from [76] B) Shown as Type I and Type 1 complexes with an indication of ligand specificity. Adapted from [83]

The structure of the TGFβ dimers confers their binding to receptors. Binding of a single TGFβ dimer brings together pairs of type I and type II receptors to form a heterotetramer of two type I and two type II receptors. Two modes of binding have been reported and are dependent on the ligand and receptor type. TGFβ and activin receptors are activated by binding of the ligand first to the type II receptor followed by recruitment of the type I receptor. The type I receptor in this scenario can only bind ligand when the ligand is in complex with the type II receptor and not when it is free in solution. The binding

typical of BMP receptors is termed cooperative as it involves high affinity binding of ligand to receptor when both types of receptor are expressed together but low affinity when either type is expressed alone [76]. Upon ligand binding the type II receptor phosphorylates the type I receptor on serine and threonine residues within the GS domain [78]. The type I receptor contains a region called the L45 loop which confers downstream signalling specificity [78]. This loop differs by four amino acids between TGFβ and BMP type I receptors and can select between the separate TGFβ and BMP downstream signalling pathways [84]. Whether phosphorylation of the type I receptor results in increased kinase activity or provides a docking sites for its substrate, the Smads, is still unclear, however the end result is phosphorylation of one of five 'receptor-regulated' Smad proteins [76, 78].

Smad proteins were identified by genetic studies in *Drosophila* and *Caenorhabditis elegans.* The original member was called *Mothers against dpp (Mad),* while further *Mad* homologues were called *Sma-2, Smad-3 and Sma-4.* Eight related Smad proteins have been identified in mammals. Smads are a conserved family of intracellular signal transducers for the TGFβ superfamily [78]. The Smads are divided into three subclasses: receptor-activated Smads (R-Smads), common-partner Smads (Co-Smads) and inhibitory Smads (I-Smads or anti-Smad). Smads 1,5,8,2 and 3 are R-Smads, Smad4 is the only Co-Smad and Smads 6 and 7 are I-Smads. R-Smads and Co-Smads have homologous N and C termini, called MH1 (Mad-homology 1) and MH2 domains respectively [78]. R-Smads have a SSXS phosphorylation motif in their C termini. R-Smads are the only group of Smads that can be directly

phosphorylated by the type I receptor kinase and can be further divided into those commonly downstream of BMP family signalling, Smads1, 5 and 8, and those commonly downstream of TGFβ and Activin, Smads 2 and 3. Ligand induced activation of the type I receptor allows interaction between the L3 loop within the MH2 domain of the specific R-Smad and the L45 loop of the specific type I receptor, placing the R-Smad in a perfect position for phosphorylation within the SSXS motif. Accessory proteins, for example, Smad anchor for receptor activation (SARA), interact with non-activated Smad2 and the receptor to assist phosphorylation of the Smad [85]. Upon phosphorylation, R-Smads have reduced affinity for the type I receptor and increased affinity for the L3 loop of Co-Smad, Smad4 [78]. Hence a R-Smad:Co-Smad complex if formed which translocates and accumulates in the nucleus, regulating transcription via association with specific gene promoters (Figure 1-4). Smad4 is consistent irrespective of whether TGFβ or BMP signalling is initiated. Smad6 inhibits BMP signalling while Smad7 inhibits TGFβ and Activin signalling [76]. It is possible this inhibition occurs via interruption of receptor phosphorylation of R-Smads, via competition of the I-Smads for Smad4 [76], or via recruitment of a phosphatase to the type I receptor, thus dephosphorylating it and preventing its signalling [82]. Further the localisation of Smad7 alters in response to TGFβ. In unstimulated cells Smad7 is found in the nucleus, while in stimulated cells it shuttles to the cytoplasm allowing interaction with R-Smads and receptors [86]. Interestingly I-Smad expression is upregulated in response to TGFβ family signalling providing an autoinhibitory mechanism in TGFβ signalling [78].

The MH1 and MH2 domains of R-Smads and Co-Smads are highly conserved and confer specific functions. Between the MH1 and MH2 domains is a linker region of variable length and sequence. In a basal state the MH1 domain inhibits the transcriptional and biological actions of the MH2 domain due to an intrinsic affinity the domains have for each other [76]. Phosphorylation of the R-Smad MH2 domain SSXS motif domain causes a conformational change in the Smad proteins which relieves the inhibitory effect of the MH1 domain [78]. In the activated state the MH1 domain of both R-Smads and Co-Smads has DNA binding activity [76]. The MH2 domain has a number of roles; in R-Smads it determines association with the type I receptor and other R-Smads, while in R-Smads and Co-Smads it is involved in Co-Smad:R-Smad interaction, interaction with DNA binding proteins and the activation of transcription. The linker region contains MAP-kinase phosphorylation sites [76].

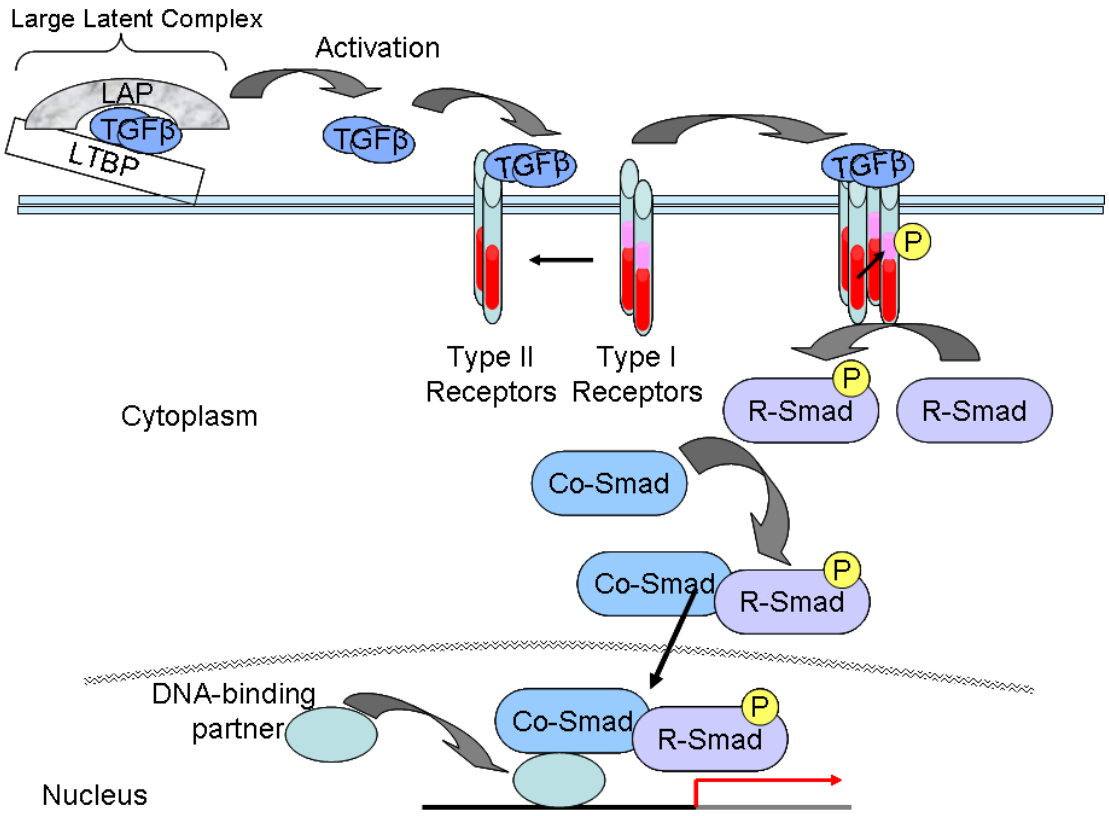


Figure 1-4 The TGFβ Signalling Pathway

Secreted TGFβ homodimers are maintained inactive by association with the latency associated peptide (LAP) and the latent TGFβ binding protein (LTBP), in the large latent complex. Activation of TGFβ by one of a number of tightly regulated processes allows its association with Type II and Type I receptors and induces the formation of a receptor heterotetramer. The Type II receptor phosphorylates the Type I receptor on serine and threonine residues in its GS domain (pink). In response to Type I receptor phosphorylation the R-Smad is recruited and phosphorylated within its SSXS motif. Phosphorylation of the R-Smad reduces its affinity for the receptor and increases its affinity for the Co-Smad. The R-Smad/Co-Smad complex translocates to the nucleus where they associate with promoters of TGFβ regulated genes, in combination with DNA binding partners.

Termination of Smad signalling may be conferred by phosphatases as well as ubiquitination and proteosome-mediated degradation of activated R-Smads. Smad 1 and 5 are targeted for destruction in the cytoplasm of unstimulated

cells by Smad ubiquitinaton regulatory factor 1 (Smurf 1 - a WW domain containing E3 ubiquitin ligase), while Smad2 is ubiquitinated in the nucleus and undergoes proteosome-mediated degradation [83]. Phosphorylated Smad3 is also ubiquitinated in the nucleus but is exported to the cytoplasm prior to proteosomal degradation [85]. Smurf1 and 2 also mediate ubiquitination of active TGFβ receptors by a process in which Smurfs interact with nuclear Smad7 following TGFβ stimulation and cause nuclear exportation of the Smurf:Smad7 complex. Once in the cytoplasm the Smad7:Smurf complex ubiquitinates the receptors and targets them for degradation in proteosomes or lysosomes. [83-85]. TGFβ receptors can also be internalised by clathrin-dependent mechanisms, however this promotes signalling and recycling of receptors as apposed to degradation [87]. TGFβ receptor signalling can also be inactivated by prevention of a type I:type II complex formation. This was first demonstrated by the decoy receptor BAMBI (BMP and activin membrane-bound inhibitor) which directly interacts with the type II receptor thus preventing association with type I receptors [87]. Also TGFβ signalling can induce the transcription of genes whose resultant protein interacts with Smads to inhibit further signalling. For example, transcription of the transcriptional repressor ATF3 by TGFβ represses the transcription of Id1. This is called a 'self enabling' TGFβ transcriptional response and allows regulation of extended TGFβ signalling [82].

1.4.3 Transcriptional Regulation by TGFβ

Stimulation of a cell with TGFβ results in the regulation of hundreds or genes, both negatively and positively. Smad4 and R-Smads (except for Smad2) can bind DNA via the Smad binding element (SBE) within promoters which

contains only 5 base pairs, 5'-CAGAC-3'. This occurs via a β hairpin in the MH1 domain of Smads interacting with bases in the SBE. Smad2 has a 30 residue insertion close to the β hairpin that results in its poor ability to bind DNA [83]. Promoters of genes known to contain an SBE include *type VII collagen*, *JunB* and *type 1 plasminogen activator inhibitor* (*PAI-1*) [78]. Genes can often contain multiple SBEs. Association of Smads with the SBE alone is of low affinity and mediates relatively low specificity of DNA binding due to the high occurrence of NGNC motifs in promoter DNA (on average once in the regulatory region of any average sized gene! [81]) and similar affinity of Smad1, 3 and 4 for the SBE. [86]. It is believed that the interaction of Smads with other transcription factors, transcriptional co-activators and co-repressors is required for specificity of TGFβ mediated transcription. Perhaps the first of these to be identified was the interaction between the transcription factor Fast-1, Smad2 and Smad4 that is required for transcription of the *Mix.2* gene. Smad2 directly interacted with Fast-1 via its MH2 domain, while Smad4 interacted with the DNA via its MH1 domain and activated transcription via its MH2 domain [76]. Fast can also bind the *goosecoid* promoter and activate its transcription in response to TGFβ. In this scenario Fast binds constitutively to an 'activin-response element' but is only able to activate transcription upon association of Smad2 with Fast and of Smad2:Smad4 with an adjacent SBE. Interestingly if Smad2 is replaced with Smad3 the transcription of *goosecoid* is inhibited [86], a good example of how Smads determine specificity of transcription.

Smads can also activate transcription via sites other than the canonical SBEs, for example, the GC rich Sp-1 [78, 83] and AP-1 binding sites [86]. The transcriptional response via AP-1 sites in response to Smad binding alone is weak and made stronger by activated forms of the proteins canonically associated with the AP-1 sites, c-jun and c-fos [86]. Further DNA-binding partners of the Smads include; ATF2, a basic helix-loop-helix leucine zipper protein that binds cAMP response elements (CREs); TFE3, which binds DNA, including the *PAI-1* promoter, via an E-box element and requires specific spacing between the E-box and the two adjacent SBEs to transduce TGFβ signals [81, 86]; the acute myelogenous leukaemia (AML) family of proteins interact with the MH2 domains of Smad3 and Smad4, apparently constitutively within the cytoplasm, and form an active transcriptional complex upon recruitment of further factors when entering the nucleus, to regulate the *IgA* gene [81].

Activation of transcription by Smads requires, in some instances, the recruitment of the co-factors p300 and CREB binding protein (CBP) to promoters. These proteins can act as bridges between other transcriptional complex proteins and the transcriptional machinery but also have intrinsic histone acetyltransferase (HAT) activity. The acetylation of histones reduces their overall positive charge and therefore their attraction to negatively charged DNA, resulting in an 'unfolding' of chromatin structure that is required for the interaction of transcriptional machinery and proteins with the DNA, and thus for transcription. Smad4 contains a Smad-activation domain (SAD) which allows stronger association with p300/CBP co-activators and may confer a

crucial role for Smad4 in regulating the efficiency of transactivation by these proteins [85].

TGFβ can also recruit histone deacetylases (HDACs). HDAC recruitment has the opposing effect to HAT recruitment and represses transcription. Smads can interact with a number of proteins which have the ability to recruit HDACs, for example the homeodomain protein TGIF and the corepressor proteins Ski and SnoN. [86]. Interestingly Ski and SnoN are direct target genes of TGFβ and represent a negative feedback loop [87].

Finally Smad proteins can regulate transcription by removing transcriptional repressors. For example, BMP-activated Smad complexes cause *osteopontin* expression by interacting with, and dislodging, the homeodomain protein Hoxc-8 which represses transcription when bound to its cognate DNA.

1.4.4 Smad Independent TGFβ Signalling

TGFβ signalling can occur independently of Smad proteins. TGFβ activates other signalling cascades including the Mitogen Activated Protein Kinase (MAPK) pathway of ERK, JNK and p38MAPK. However these pathways are poorly characterised and a direct interaction between the type I receptors and kinases upstream of the MAPKs has not yet been demonstrated [88]. TGFβ can activate Rho-like GTPases such as RhoA, Rac and Cdc14, and this may allow further downstream activation of kinase cascades.

1.4.5 The TGFβ Family and the Vasculature

The role of TGFβ in vascular development was first recognised when mutations in TGFβ related genes were identified in familial vascular

pathologies, for example, hereditary haemorrhagic telangiectasia (HHT) in which mutations of the *ENG* gene which encodes endoglin, a type III TGFβ receptor, result in vascular lesions and arteriovenous malformation and Loeys-Dietz syndrome which is associated with mutations in *TGFBR1* and *TGFBR2* and symptoms include aortic aneurysms [89].

A key role for TGFβ signalling in angiogenesis is the promotion of vessel muscularisation. $TGF\beta_1$ signalling to adjacent mesenchymal cells promotes smooth muscle cell and pericyte differentiation [89]. 50% of $TGF\beta_1$ -/-mice die at day E10.5 due to defects in the development of the yolk sac vasculature. The differentiation of endothelial cells into tubes is also lacking and results in fragile vessel walls. Failure of endothelial tight contacts and smooth muscle cell differentiation causes leakage of blood cells into the yolk sac [90]. Further assessment is limited as mice with different genetic backgrounds display different effects in response to $TGF\beta_1$ removal, possibly due to compensation by other TGFβ isoforms [90, 91].

TGFβR-II deficient mice have also been engineered. These share numerous characteristics with the $TGF\beta_1$ -/- mice, for example, deficiency is lethal at day E10.5 and results from defects in yolk sac vasculogenesis and haematopoiesis. Blood vessels are formed but they are dilated and incompletely attached to surrounding endodermal layers [90]. A reduction of cellular adhesiveness may be due to a reduction in the of amount of fibronectin, (an extracellular matrix protein normally deposited between two layers of the yolk sac), produced by the poorly differentiated endothelial cells [91]. Embryos deficient in the type

III TGFβR endoglin die at day E11.5 from defective vascular development. Vascular organisation and mature blood vessels are lacking from the yolk sac. Both endothelial cell organisation and vascular smooth muscle cell development are poor in the yolk sac by day E8.5-10.5. Furthermore, evidence for a failure of endothelial remodelling is present in the embryo as well as the yolk sac. Endoglin -/- embryos have no smooth muscle cells surrounding the major blood vessels. Abnormal cardiac development is also evident [91]. ALK-1 (a type I TGFβ receptor) deficient mice die at day E11.5. They exhibit a lack of mature vessels in the yolk sac (those that are present are dilated), excessive growth of endothelial cells resulting in the fusion of capillary networks, delayed differentiation of smooth muscle cells and a failure of smooth muscle cells to localise to the endothelium. These mice also display ateriovenous malformation in the vascular bed of the embryo due to fusion of major arteries and veins. This is thought to be due to a lack of Ephrin-B2 (involved in boundary formation between arteries and veins) expression [92]. ALK-1 -/- mice also have high expression of the vasculogenesis/angiogenesis related genes, VEGF, Angiopoietin- 2 (Ang2) and urokinase-type plasminogen activator (uPA) [91]. Mice deficient in ALK5 (TGFβIR) display severe defects in vascular development of both the embryo and yolk sac. They also have enhanced endothelial cell proliferation and improper endothelial cell migratory function. As with TGFβR-II deficient mice they display impaired fibronectin production [93]. Smad5 deficient mice also show defects linked to vascular development. They die between E10.5-11.5 due to circulatory system defects. The yolk sacs lack an organised vasculature and show loose attachment between the two yolk sac layers. Furthermore, the embryos have large blood

vessels surrounded by insufficient smooth muscle and lack a capillary network in the developing brain [91]. Recently mice deficient in the TGFβ activated kinase TAK1 have been engineered. TAK1 is a member of the MAPK pathway whose activity is rapidly induced by TGFβ. TAK1 deficient embryos have defects in the developing embryo and yolk sac, including dilation and misbranching of vessels and a lack of smooth muscle cells [94].

Collectively these studies show TGFβ family signalling plays a crucial and incompletely defined role in vascular development. TGFβ signalling contributes to a number of processes involved in angiogenesis including the maintenance of the integrity of the vessel wall, the recruitment of smooth muscle cells, the deposition of extracellular matrix and the differentiation of endothelial cells into arteries and veins [92].

1.4.6 TGFβ and Pulmonary Hypertension

Interest in the TGFβ family with regard to PH was initiated in 1994 when Botney *et al*., found increased expression of TGFβ2 and TGFβ3 in the pulmonary arteries of patients with primary PH [95]. Subsequently in 2000, mutations of the *BMPR-II* gene were found to be associated with the familial form of the disease [96] and over expression of a dominant negative BMPR II mutation, selectively in the smooth muscle cells of mice, was sufficient to cause PH [97]. 80% of familial PH families show a form of mutation in *BMPR II* [98], however familial PH accounts for only approximately 6% of total PH patients. Further of the people with the *BMPR II* mutation only 10-20% will develop the disease due to poor penetrance of the mutation. Thus this mutation is not the only requirement for the development of the disease and a ‘second

hit' is required [99]. Numerous mutations (>50 in 2005 [100]) have been identified, including nonsense and frame shift mutations in the extracellular domain which lead to premature truncation of the transcripts and no production of transmembrane BMPR-II protein, missense mutations in conserved cysteine residues of the extracellular domain which prevent trafficking of the receptors to the membrane, missense and frame shift mutations in the kinase domain that lead to improper downstream Smad1 and MAPK signalling and frame shift or nonsense mutations in the cytoplasmic tail which affect downstream signalling of the receptor [80].

Shortly after the identification of the *BMPR II* mutation in familial PH 25% of cases in a study of sporadic or idiopathic PH were also found to hold *BMPR II* mutations [101], and underlined the importance of this signalling pathway in PH. Furthermore, mutations in the TGFβ family members, ALK-1 and endoglin have also been identified in PH presenting in childhood [100] and reduced levels of BMPR II have been reported in the absence of a detectable genetic mutation [102].

Functional effects of the mutation include a change in growth response of pulmonary artery smooth muscle cells to both BMP2, 4 and 7 and $TGF\beta_1$. Morrell *et al.,* showed PASMC proliferation from control (healthy) cells to be inhibited by $TGF\beta_1$ and the BMPs, while PASMCs from primary PH patients with a *BMPR II* mutation showed loss of inhibition in response to the BMPs and increased proliferation in response to $TGF\beta_1$. Smooth muscle proliferation is a key component of PH pathology [103]. $TGF\beta_1$ is not a known ligand of

BMPR II and suggests more widespread defects in TGFβ family signalling than just those immediately downstream of BMPR II. Contrary to the inhibitory response of $TGF\beta_1$ on normal PASMCs stated above, Sturrock *et al*., showed $TGF\beta_1$ to induce proliferation of human PAMSC. This may be a temporal effect as Morrell *et al*., measured proliferation at 14 hours while Sturrock *et al*., observed at 72 hours [104]. Further evidence for a role of TGFβ is provided by Mata-Greenwood *et al*., who have shown, in an increased pulmonary blood flow model of PH in sheep, that $TGF\beta_1$ levels are increased in hypertensive sheep compared to control animals and that the increased levels temporally coincide with increased medial thickness [105]. The effect of *BMPR II* mutation on BMP4 signalling and loss of inhibition of proliferation as observed by Morrell *et al*., was due to an imbalance in Smad1/MAPK signalling downstream of the receptor. P38 MAPK and ERK1/2 mediated proliferation and inhibition of apoptosis in PASMCs while Smad1 signalling inhibited proliferation. In PH PASMCs Smad1 signalling was reduced, resulting in loss of the anti-proliferative response [106]. Further Zhang *et al*., have shown BMP protection of PASMCs from apoptosis to be greater in primary PH cells than in normal controls, an effect which would further increase the level of smooth muscle in the PH artery [102].

In a monocrotaline model of PH in rats, the development of PH coincided with an increased level of phosphorylated Smad2, suggesting increased TGFβ signalling was present. Increased phosphorylated Smad2 correlated with enhanced pulmonary vascular endothelial cell apoptosis and initiation of vascular smooth muscle cell proliferation. Inhibition of TGFβ signalling with

an inhibitor of ALK-5 reduced Smad2 phosphorylation and attenuated the development of PH in a dose dependent manner. Inhibition of TGFβ signalling also had beneficial effects on established monocrotaline induced PH [107]. Interestingly the presence of the *BMPR II* mutation can reduce the BMP2 induced protection from apoptosis in endothelial cells suggesting increased endothelial cell apoptosis could be a early response in a number of models of PH [108]. However the level of phosphorylated Smad2 in monocrotaline rats is contradicted by Zakrzewicz *et al*., who show levels of phospho-Smad2 to be reduced in response to monocrotaline. They also show ALK-1, TGFβR-2, endoglin, Smad3, Smad4 and TGFβ induced apoptosis of PASMCs to be reduced [109]. Whereas Richter *et al*., detected very little phospho-Smad2 in PAMSCs from normal or idiopathic PH patients but saw an increase in endothelial phospho-Smad2 in idiopathic PH [110]. In addition Mata-Greenwood *et al*., [105] have shown an increase in ALK-1 expression and a decrease in ALK-5 expression in an increased blood flow model of PH which contradicts the observation of Zakrzewicz *et al*.

In a screen of genes differentially affected by BMP2 in normal and idiopathic PH patients, *TGFβ, TGFβR type I, TGFβR type II* and *Smad2* genes negatively correlated with pulmonary artery pressure, suggesting, in this context that they may be protective [111].

It is clear that the TGFβ family affect the pulmonary vasculature and are modulated in various models of PH and also in tissue from patients. However it is also clear that this is model and patient dependent and may depend on

variants such as stage of the disease. Further study of the regulation of this pathway may provide insight into the pathogenesis of PH.

1.5 WNT/B-CATENIN/TCF SIGNALLING

1.5.1 General Overview

The Wnts comprise a large family (around 20 in mammals [112]) of highly conserved cysteine-rich, 39-46Kda, secreted growth factors [113, 114]. Their signals are pleiotropic with effects that are important for development and homeostatic processes throughout the species, from worms to mammals, including cell proliferation, differentiation, apoptosis and migration [113, 115]. Some variation may be due to the Wnt family being defined by amino acid sequence rather than functional properties of the proteins [116, 117]. Furthermore, more than 50 component proteins have been identified to transduce Wnt signals [114]. Wnts are split into two classes by their function, the wnt1 class signals preferentially through canonical β-catenin dependent signalling while the Wnt5a class stimulate intracellular calcium release [118]. Research into Wnt signalling has focused mainly on the canonical, β catenin dependent signalling pathway (see Figure 1-5 for overview), however other pathways and interactions are accumulating. Canonical signalling involves Wnt proteins being released or presented by signalling cells to target cells, where they bind the Frizzled (Fz)/low density lipoprotein (LDL) receptor-related protein (LRP) complex on the cells surface. Frizzled receptors are seven-transmembrane receptors with a long N-terminal extension called a cysteine-rich domain (CRD), to which Wnt proteins bind. The LRP family are a group of single-pass transmembrane molecules that are vital for Wnt

signalling but by an unknown mechanism. The receptors transduce the signal to a number of intracellular proteins including, Dishevelled (Dvl), glycogen synthase kinase-3β (GSK3β), axin, Adenomatous Polyposis Coli (APC), and the transcriptional regulator, β-catenin [117]. How signal is transduced to or via these proteins is not clear. Dvl can directly interact with Fz and be phosphorylated by PAR-1 and CKII upon interaction [116]. Also, Axin can interact with LRP, and Axin and Dvl are thought to heterodimerise, possibly disrupting a GSK/APC/Axin/β-catenin complex [112]. The GSK/APC/Axin complex maintains cytoplasmic β-catenin levels low by targeting it for continuous proteasome-mediated degradation. This process involves phosphorylation of the N-terminal of β-catenin by the serine/threonine kinases, casein kinase 1α (CKIα; phosphorylates Ser45 [113]) and GSK-3β (phosphorylates Thr 41, Ser37, Ser33, Asp32 and Gly34 [113]). Axin and APC act as scaffold proteins to maintain β-catenin and the kinases in the correct conformation for phosphorylation. Phosphorylated β-catenin (ser33/37 [119]) is recognised by β-TrCP (beta-transducin repeats containing protein), a protein which functions as a substrate recognition subunit for the E3 ubiquitin ligase complex [112]. Subsequently β-catenin is ubiquitinated and degraded by the proteasome. Upon Wnt signalling β-catenin is no longer phosphorylated and the degradation pathway is inhibited, allowing hypophosphorylated β-catenin levels to accumulate. β-catenin also moves into the nucleus. Inhibition of β-catenin degradation may be due to recruitment of Axin to the receptor and therefore physical breaking up of the degradation complex, dephosphorylation of β-catenin by phosphatases such as PP2A, or inhibition of or removal of GSK3β from the complex (possibly via interaction with a protein called Frta

which binds Dvl [113]) thus preventing β-catenin phosphorylation [117]. Nuclear β-catenin interacts with a group of transcription factors called the lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) to regulate transcription [117].

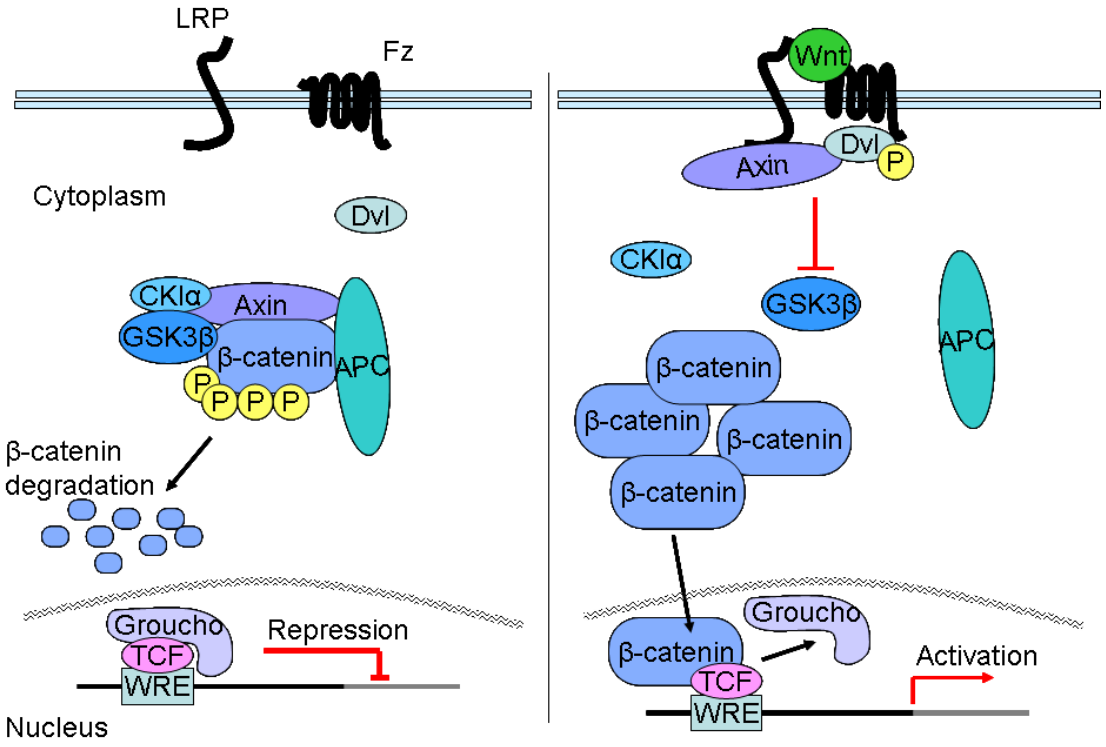


Figure 1-5 The Wnt/β-Catenin Signalling Pathway.

In the absence of Wnt, β-catenin binds to the "destruction complex" containing axin, APC (adenomatous polyposis coli), GSK3β (glycogen synthase kinase) and CK1α (Casein kinase), and is phosphorylated by GSK3β and CK1α, resulting in its degradation by the proteosome (left). Wnt binds the Frizzled (Fz)/LRP (low-density lipoprotein receptor-related protein) receptor complex which mediates signalling via Axin and Dvl (dishevelled) to inhibit GSK3β. β-catenin escapes the destruction complex, becomes hypophosphorylated and translocates to the nucleus. In the nucleus β-catenin displaces the repressive Groucho and activates Wnt responsive genes in combination with TCF.

1.5.2 Glycogen Synthase Kinase 3

Glycogen synthase kinase 3 has two isoforms, GSK3β and GSK3α. GSK3β and α have >98% homology within their kinase domains but differ substantially outside this domain. GSK3β has a mass of 47kDa, while GSK3α is 51kDa due to a glycine rich N-terminal extension [120]. Despite being structurally similar the two isoforms are not functionally identical. GSK3β ablation in mice is lethal due to liver degeneration and this cannot be rescued by GSK3α [120]. Over 40 proteins can be phosphorylated by GSK3 including 18 transcription factors [121]. Their range of targets suggests involvement of these kinases in a broad range of cellular functions. In fact, GSK3 is involved in the regulation of cell fate, Wnt and Hedgehog signal transduction, protein synthesis, glycogen metabolism, mitosis and apoptosis [122]. As a consequence the activity of the GSK3s requires tight regulation.

GSK3 recognises the substrate consensus sequence S/T-X-X-X-Phospho-S/T, where X represents any amino acid and GSK3 phosphorylates the first S/T. Thus, GSK3 phosphorylates substrates that are pre-phosphorylated in preference to those that are not [122]. Structurally this phenomenon is due to three residues, R96, R180, K205, which are in close proximity to each other on the surface of GSK3β and interact with the negative charge of the pre-phosphorylated target protein, aligning the substrate in the optimal position for GSK3s kinase activation loop [122].

GSK3 kinase activity can be activated and inhibited by phosphorylation. Phosphorylation of Ser9 and Ser21 in GSK3β and GSK3α respectively,

significantly reduces kinase activity due to creation of a pseudosubstrate which associates with the positive R96, R180, K205 groove and occupies the catalytic site preventing phosphorylation of real substrates [120]. Several kinases can phosphorylate the inhibitory serine residues in GSK3 including PKB/Akt, protein kinase A (PKA), protein kinase C (PKC) and p90Rsk [121]. Phosphorylation of Tyr216 and Tyr 279 in GSK3β and GSK3α respectively, by an unknown kinase, facilitates GSK3 kinase activity.

Further regulation of GSK3 occurs by modulation of intracellular localisation and binding proteins. While traditionally considered a cytoplasmic protein, GSK is present in the nucleus and mitochondria. Different cellular locations provide access to different substrates, for example in the nucleus GSK3 can phosphorylate transcription factors including p53, TCF and CREB among others. GSK3 cellular location is not static and translocation in and out of the nucleus is regulated by stimuli including PKB/Akt [121]. Binding of GSK3 to proteins including Frat-1 may also regulate its cellular localisation as Frat-1 facilitates nuclear export of GSK3 [121]

GSK3 was originally identified and named for its role in glycogen synthesis. In the absence of insulin glycogen synthase is primed for phosphorylation by GSK3, by phosphorylation at ser657 by casein kinase II (CK2). Subsequently glycogen synthase is phosphorylated at ser653, 649, 645 and 641 by GSK3 and inhibited. Elevated blood sugar levels increase insulin levels and initiate signalling that results in Akt/PKB activation. Akt/PKB phosphorylates GSK3 and inhibits it, preventing glycogen synthase phosphorylation. Thus glycogen

synthase is active and glycogen stores are created [122]. GSK3 also plays a well defined inhibitory role in Wnt/β-catenin signalling by constitutively phosphorylating N-terminal serine's in β-catenin and targeting it for proteosomal degradation. Only upon GSK3 inhibition by Ser9/21 phosphorylation can β-catenin become hypophosphorylated, accumulate and signal.

1.5.3 Transcriptional Regulation by β-Catenin

β-catenin is a member of the armadillo family of proteins, which are characterised by a central domain consisting of a repeating 42 amino acid motif called the 'arm repeat', thought to be a versatile protein binding interface [123]. β-catenin has 12 such arms which form a groove within which the binding of Axin, APC and TCF is mutually exclusive [112]. The groove preferentially binds phosphorylated proteins. These two facts in combination partially explain β-catenin localisation and signalling [123].

Canonical regulation of transcription by β-catenin involves its interaction with one of four members of the TCF/LEF family of transcription factors, TCF-1, LEF-1, TCF-3 and TCF-4, via their N-terminus [112, 124, 125]. Wnt target genes can be differentially regulated in a tissue and developmentally controlled manner such that in a certain cell at a given time both wnt-responsive and non-responsive genes exist [126]. The fact that all TCFs bind β-catenin and interact with the same sequence of DNA does not immediately agree with the diverse and tightly regulated gene regulation that occurs in response to wnt signalling. Thus it is not perhaps surprising that the control of transcription by TCFs and

β-catenin is not straight forward and requires the interaction of numerous co-proteins.

In an unstimulated cell TCFs have high affinity for the DNA sequence (A/T)(A/T)CAA(A/T)GG, known as the Wnt-response element (WRE) and bind DNA directly through their high mobility group (HMG) domain [113, 125]. In unstimulated cells TCFs act as repressors of Wnt target genes by associating with the transcriptional repressors, Groucho, CtBP (C terminal Binding Protein) and HBP1 (HMG-box transcription factor 1) [113]. Groucho recruits histone deacetylases (HDACs) which regulate chromatin structure to repress transcription [116]. Upon cell stimulation the movement of β-catenin into the nucleus and its association with TCFs converts the TCF complex into a transcriptional activator complex. The manner in which this occurs is cell type and stimulus dependent but can include displacement of Groucho and replacement with histone acetylases such as CBP/p300 which alter chromatin structure to allow transcription. Trimethylation of histone H3 has recently been found to be important in the regulation of Wnt mediated transcription and may occur via β-catenin mediated recruitment of the SET1 histone methyltranferase complex to Wnt regulated promoters. The SET1 complex associates with the C-terminal of β-catenin [115]. Wöhrle *et al*., have recently shown that an overall 'Wnt responsive' state of genes is characterised by the presence of acetylated histone H3, methylated H3K4 and DNA hypomethylation of promoters [126]. Thus epigenetic mechanisms are being seen as increasingly important in the regulation of Wnt signalling.

β-catenin can also interact with the Wnt co-activators Bcl9/Lgs and Pygopus [115]. Numerous other co-regulators have been identified, for example the protein Chibby binds the C-terminus of β-catenin and prevents its association with DNA, ICAT (inhibitor of β-catenin and TCF4 [119]) blocks TCF/β-catenin interaction and causes dissociation of β-catenin from CBP/p300 [117]. Specificity can also be introduced into TCF/β-catenin transcriptional regulation via the involvement of other transcription factors or transcriptional modulators at distinct binding sites on DNA, for example Smad4 can bind Smad binding elements (SBEs) in concert with the binding of TCF:β-catenin to WREs [124].

Members of the β-catenin destruction complex can also act as transcriptional regulators. For example, APC can be recruited to the enhancer region of the *c-myc* promoter, in complex with β-TrCP and CtBP and may mediate the exchange of co-activator (i.e. p300/CBP) and co-repressor (i.e. Groucho) complexes at gene promoters during transcriptional cycles [115].

Further regulation can occur at the level of TCF itself which can be phosphorylated by the MAP kinase NEMO-like kinase (NLK) resulting in a loss of DNA affinity of the TCF/β-catenin complex [116, 117]. Conversely phosphorylation of TCF3 by CK1 enhances TCF3 binding to β-catenin [123]. Phosphorylation of β-catenin at Ser552 and Ser675 by Akt and PKA can enhance TCF/β-catenin reporter activation, possibly via the recruitment of HATs [123]. Lef-1 and TCF-4 can also be sumolated, however the effects of sumolation on transcriptional activity can be either positive or negative

depending on context and requires further investigation to understand its full role [113].

A large number of Wnt target genes have been identified including members of the Wnt pathway (e.g. *Fz, Axin2, β-TcRP, TCF1* and *LEF1*), providing feedback control during Wnt signalling [117]. Further genes known to be regulated by TCF include *c-myc, c-jun, fra-1* and *cyclin D1* [113] .

1.5.4 Wnt/β-catenin/TCF and the Vasculature

In vitro experiments suggest a role for Wnt signalling in endothelial cell proliferation as over expression of Wnt1 in primary endothelial cells causes β-catenin stabilisation dependent TCF/LEF mediated gene transcription and proliferation of endothelial cells [118, 127], as does expression of stable β-catenin in human umbilical vein endothelial cells [128]. This suggests that Wnt signalling may play a role in the formation and differentiation of the vasculature as endothelial cell proliferation is key to these processes. In agreement, the activation of Wnt/β-catenin signalling induces formation of a capillary like network of primary endothelial cells in vitro [129]. Microvascular endothelial cells in culture express Wnt-5a, Wnt-7a, and Wnt-10b and human umbilical vein endothelial cells (HUVECs) express Fz-4, Fz-5 and Fz-6 proteins and *Fz-1,3,5* and *7* genes [130]. Wnt-2 is expressed in the fetal vessels of the placenta and Wnt-2 deficient mouse embryos fail to establish a proper fetal capillary network in the placenta due to either reduced proliferation or increased apoptosis of endothelial cells [129, 130]. Thus Wnt-2 is implicated in proper placental vascularisation. Knockout of Wnt-4 in mice prevents formation of the male-specific coelomic blood vessels [129, 130].

Wnt-7b may be critical for maintenance of smooth muscle cells in the major pulmonary vessels as mutant mice exhibit increased smooth muscle cell hypertrophy and death [129, 131]. Mutant embryos die of respiratory failure and have enlarged, branched vessels, with a thickened smooth muscle layer and significant haemorrhage around the large pulmonary vessels of the lungs due to lack of smooth muscle integrity. Consistently, over expression of β-catenin increases vascular smooth muscle cell proliferation and inhibits apoptosis while dominant negative TCF4 induced apoptosis [131]. Furthermore, Wnt-5a and Frizzled 3 are known to be expressed by pulmonary artery smooth muscle cells [129]. Frizzled-5 deficient mice die at day E11 due to defective yolk sac angiogenesis as evident by reduced endothelial cell proliferation and disrupted placental vasculogenesis [118, 129]. These defects are thought to occur during angiogenic remodelling as the primary vessel structure forms normally [130].

Disruption of cell:cell adhesions to allow cell migration is critical to the process of angiogenesis. β-catenin binds to the plasma membrane in cell adherens junctions and VEGF stimulated phosphorylation of β-catenin disrupts β-catenin:cadherin complexes in these junctions resulting in weaker cell adhesion and progression of new vessel formation in response to myocardial infarction [118]. Furthermore, β-catenin regulates VEGF expression in colon cancer cells and human endothelial cells [127, 132, 133], in addition to numerous other angiogenic proteins including Ephrins, FGF18, FGF20, IL-8, endothelin-1, CX43, uPAR, MMP7 and MMP3 [129, 130].

Activation of the Wnt/β-catenin signalling pathway is also implicated in early cardiac myogenesis as indicated by an increase in *Wnt-3A* and *Wnt-8A* expression, hypophosphorylation of β-catenin, accumulation of β-catenin and induction of TCF/LEF dependent transcription in differentiating pluripotent cardiac P19CL6 cells [134].

LRP5 mutations are associated with vascular defects of the eye (osteoperosis-pseudoglioma syndrome or OPPG) [117]. Also mutations in both LRP5 and Frizzled 4 are associated with familial exudative vitrepathy (FEVR), a hereditary disease characterised by defective vasculogenesis in the peripheral retina [117]. The disease is inherited in an autosomal dominant manner and the dominance is thought to be due to the mutant, non-signalling, frizzled acting in a dominant negative fashion to prevent signalling of the wild type protein [130]. Norrin, a cysteine knot protein, with no structural similarity to Wnts, but that binds Frizzled 4 and activates canonical β-catenin signalling is mutated in Norrie disease which is characterised by vascular abnormalities in the eye and blindness [112]. Mutated forms of Norrin are also associated with Coats disease and retinopathy of prematurity (ROP), all of which exhibit defects of retinal vascular development [130].

1.5.5 Wnt/β-catenin/TCF and Pulmonary Hypertension

The role of Wnt signalling, β-catenin and TCF/LEF in PH is poorly reported in the current available published literature. β-catenin is highly expressed and Wnt7a expression is lost within plexiform lesions, compared to surrounding cells [135]. Wnt and β-catenin may be involved in the putative transition of endothelial cells to mesenchymal, smooth muscle like cells in pulmonary

vascular remodelling [136]. A number of posters at recent meetings have shown a growing interest in the Wnt pathway. For example, Sklepkiewicz *et al*. showed that β-catenin and GSK3β protein levels are increased in rats exposed to the monocroaline model of PH compared to control rats. They also showed increased GSK3β phosphorylation at the inhibitory Ser9 position in response to PH development. Inhibition of GSK3β by phosphorylation results in reduced phosphorylation of β-catenin and its cytoplasmic and nuclear accumulation due to lack of β-catenin destruction by the proteosome. The same group also showed that expression of a dominant negative GSK3β construct causes a decrease in vascular smooth muscle cell proliferation [137], providing functional evidence for a potential role of GSK3β in PH. However this contradicts with the previously mentioned report of β-catenin overexpression resulting in the proliferation of vascular smooth muscle cells [132].

Interestingly, Deng *et al*., have shown BMP4, $TGF\beta_1$, Endothelin-1 and Serotonin to induce GSK3β phosphorylation at Ser9 in pulmonary artery smooth muscle cells and for pharmacological inhibition of GSK3β to cause pulmonary artery smooth muscle cell hypertrophy [138].

Additionally de Jesus Perez *et al*., have shown BMP2 mediated motility of PASMCs to require β-catenin expression. Thus, PASMC hyperplasia, hypertrophy and motility, three functions that are inappropriately regulated in PH can be linked to β-catenin expression. Further, β-catenin expression is linked to stimuli thought to contribute to PH, including BMPs, TGFβ, ET-1 and serotonin. This evidence in combination with the growing interest in

angio- and vasculogenesis regulation by Wnt/β-catenin signalling provides convincing, if preliminary, evidence for a role of the pathway in PH.

1.6 SUMMARY

In summary, PH is a diverse disease with numerous factors implicated as the initiating trigger for the arterial dysfunction which results in almost complete occlusion of the pulmonary artery and fatality in patients. These triggers include genetic predisposition, environmental factors such as exposure to hypoxia, and defects in molecular cell signalling.

To date, evidence has suggested a role for the angiogenic molecule, VEGF, in the pathogenesis of PH. However, definition of either a protective or a damaging role for VEGF is so far elusive. Smooth muscle cells play a pivotal role in the progression of PH as they contribute to both the arterial remodelling and the reduced vasodilatory function of the artery. The secretion of VEGF by human PASMCs has not been studied before. We are therefore interested in determining whether PASMCs secrete VEGF. We have also introduced the intricacies of VEGF regulation in different cellular contexts and aim to study the mechanisms by which VEGF production from PASMCs is regulated by mediators relevant to PH.

2 HYPOTHESIS AND AIMS OF THE THESIS

2.1 HYPOTHESIS AND AIMS OF THE THESIS

As summarised in the introduction, VEGF is implicated in the pathogenesis of PH and PASMCs are involved in the pulmonary artery remodelling that occurs in all forms of PH. Despite these observations the secretion of VEGF from human PASMCs has not previously been studied. We have also discussed the levels at which VEGF expression is regulated and the range of stimuli known to mediate VEGF expression. The current studies aim to address the hypothesis that modulators important in PH progression and known to induce VEGF in other cell types, including prostaglandins, TGFβ family members and inflammatory cytokines, regulate the secretion of VEGF from PASMCs.

The specific aims we sought to address were to:

- confirm that normal human PASMCs produce VEGF protein
- determine if TGFβ family members, prostaglandins and inflammatory cytokines regulate VEGF production
- select the greatest inducer of VEGF and determine whether VEGF production is regulated transcriptionally or post transcriptionally
- define specific signal transduction and transcriptional complexes required for VEGF regulation
- determine if *BMPR II* mutation affects the regulation of VEGF in wild type and heterozygous *BMPR II* knock out murine PASMCs

3 MATERIALS AND METHODS

3.1 METHODS

3.1.1 Cell Culture

3.1.1.1 Human Pulmonary Artery Smooth Muscle Cells

Proximal Human Pulmonary Artery Smooth Muscle Cells (HPASMCs) from a 22 year old male were purchased, at passage three and cultured to passage six, in Smooth Muscle Cell Growth Medium supplemented with 2µM L-glutamine, 5µg/ml insulin, 0.5ng/ml recombinant human epidermal growth factor, 2ng/ml recombinant human basic fibroblast growth factor, foetal bovine serum (5% v/v final concentration), 25µg/ml gentamicin and 50ng/ml amphotericin B, under 5%CO2/95% air in a humidified incubator at 37°C, for experimental use. All experiments were performed at passage six.

Once at the required confluence cells were growth arrested for the required period of time in serum free Dulbecco's Modified Eagle Medium (DMEM) containing 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine, and 2.5 µg/ml amphotericin B but excluding fetal calf serum. For transfection experiments only, penicillin, streptomycin and amphotericin B were excluded from the media as per the transfection reagent manufacturer's protocol.

Medium was replaced with serum free with or without stimulant.

3.1.1.2 Mouse Embryonic Fibroblasts

3.1.1.2.1 Smad Knockout MEFs

Wild type, homozygous Smad 2 knock out and homozygous Smad 3 knock out mouse embryonic fibroblast cells were a kind gift from Erwin Böttinger [139]. MEFs were grown to confluence in DMEM, containing 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine and 2.5 µg/ml amphotericin B under 5%CO2/95% air in a humidified incubator at 37°C. Once confluent the MEFs were detached with trypsin-EDTA and seeded to 24 well plates at a density of 5 x 10^4 cells/well and cultured to the density required for the specific experimental procedure. Cells at the required confluence were growth arrested by serum withdrawal in serum free DMEM, containing 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine, and 2.5 µg/ml amphotericin B. For transfection experiments only, penicillin, streptomycin and amphotericin B were excluded from serum free media as per the transfection reagent manufacturer's protocol.

Medium was replaced with serum free with or without TGFβ_1.

3.1.1.2.2 GSK3-β Knockout MEFs

Wild type and homozygous GSK3β MEFs were a kind gift from James R. Woodgett [140]. MEFs were grown to confluence in DMEM containing 10% fetal calf serum, 0.11g/L sodium pyruvate and 3.7g/L sodium bicarbonate under 5%CO2/95% air in a humidified incubator at 37°C. Once confluent MEFs were detached with trypsin-EDTA and split into tissue culture plates depending on the experimental requirements, and cultured to the density required for the specific experimental procedure. Cells at the required confluence were growth arrested by serum withdrawal in serum free DMEM, containing 100 units/ml penicillin, 100 µg/ml streptomycin, and 4 mM L-glutamine. For transfection experiments only, penicillin and streptomycin were

excluded from serum free media as per the transfection reagent manufacturer's protocol.

Medium was replaced with serum free with or without TGFβ_1.

3.1.1.3 Mouse Pulmonary Artery Smooth Muscle Cells: *BMPR II* +/+ and -/+

Pulmonary artery smooth muscle cells from wild type mice and mice heterozygous for *Bone Morphogenetic Protein Receptor II* (*BMPR2*) were a kind gift from Nicholas Morrell [106]. Mouse PASMCs were cultured to passage 7 in DMEM, containing 20% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 4 mM L-glutamine under 5%CO2/95% air in a humidified incubator at 37°C. All experiments were performed at passage 7.

Once at the required confluence cells were growth arrested for the required period of time in serum free DMEM containing 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine, and 2.5 µg/ml amphotericin B but excluding fetal calf serum.

Medium was replaced with serum free with or without TGFβ_1.

3.1.2 VEGF Assay

3.1.2.1 Human VEGF ELISA

The concentration of human VEGF-A in culture medium supernatants was determined by Enzyme-Linked Immunosorbent Assay (ELISA). Confluent PASMCs were serum starved for 24 hours and treated for the required time before supernatants were collected. Supernatants were stored at -20°C until assayed according to manufacturer's instructions. Briefly, a monoclonal antibody (mouse anti-human VEGF) specific for VEGF was precoated onto a

96 well plate, over night at room temperature. The plate was washed 3 times (0.05% Tween 20 in phosphate buffered saline) and blocked for a minimum of 1 hour with reagent diluent (1% bovine serum albumin in phosphate buffered saline (PBS)). Standards were diluted using 2 fold serial dilutions in reagent diluent to provide a seven point standard curve from 2000ng/ml to 0ng/ml. Plates were washed and standard and sample supernatants (100 µl) were pipetted into the wells and incubated for 2 hours at room temperature. Any VEGF present was bound by the immobilized antibody. Plates were washed three times to remove any unbound substances and 100 µl of an enzyme-linked polyclonal antibody (biotinylated goat anti-human VEGF) specific for VEGF was added to the wells and incubated for an additional 2 hours at room temperature. Plates were washed again to remove any unbound antibody-enzyme reagent, and 50µl of Streptavidin-HRP solution added and incubated for 20 minutes. 100 µl of a substrate solution (50:50 v/v H2O2 and tetramethylbenzidine (TMB substrate reagent set)) was then added for 20 minutes and colour developed in proportion to the amount of VEGF bound in the initial step. The reaction was stopped by adding 50 µl of stop solution (2 N sulfuric acid); the degree of colour generated was determined by measuring the optical density at 450 nm (reference Filter 570 nm) within 30 minutes in a Dynatech MR500 microplate reader (Billinghurst, West Sussex, UK). The VEGF concentrations of unknown samples were calculated using the standard curve. The results were expressed as picogram/millilitre. This kit shows no cross reactivity with human placental growth factor, VEGF-C or VEGF-D.

3.1.2.2 Mouse VEGF ELISA

The concentration of mouse VEGF164 and mouse VEGF120 in culture medium supernatants was determined by Enzyme-Linked Immunosorbent Assay (ELISA). Confluent MEFs were serum starved for 24 hours and treated for the required time before supernatants were collected. Supernatants were stored at -20°C until assayed according to manufacturer's instructions. Briefly, a monoclonal antibody (goat anti-mouse VEGF) specific for VEGF was precoated onto a 96 well plate, over night at room temperature. The plate was washed 3 times (0.05% Tween 20 in phosphate buffered saline) and blocked for a minimum of 1 hour with reagent diluent (1% bovine serum albumin in phosphate buffered saline (PBS)). Standards were diluted using 2 fold serial dilutions in reagent diluent to provide a seven point standard curve from 1000ng/ml to 0ng/ml. Plates were washed and standard and sample supernatants (100 µl) were pipetted into the wells and incubated for 2 hours at room temperature. Any VEGF present was bound by the immobilized antibody. Plates were washed three times to remove any unbound substances and 100 µl of an enzyme-linked polyclonal antibody (biotinylated goat anti-mouse VEGF) specific for VEGF was added to the wells and incubated for an additional 2 hours at room temperature. Plates were washed again to remove any unbound antibody-enzyme reagent, and 50µl of Streptavidin-HRP solution added and incubated for 20 minutes. 100 µl of a substrate solution (50:50 v/v H2O2 and tetramethylbenzidine) was then added for 20 minutes and colour developed in proportion to the amount of VEGF bound in the initial step. The reaction was stopped by adding 50 µl of stop solution (2 N sulphuric acid); the degree of colour generated was determined by measuring the optical density at

450 nm (reference Filter 570 nm) within 30 minutes in a Dynatech MR500 microplate reader. The VEGF concentrations of unknown samples were calculated using the standard curve. The results were expressed as picogram/millilitre. This kit shows no cross reactivity with human VEGF121 or human VEGF165.

3.1.3 RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cells were grown to confluence in 6-well plates and growth arrested for 24 hours. They were then treated as required, collected and stored at -80°C until required. Total RNA was isolated by using the RNeasy Plus mini kit following the manufacturer's protocol with gDNA Eliminator spin columns. RNA concentrations were determined by dual wavelength spectrophotometry (260 and 280nm) and stored at -80°C until required.

3.1.3.1 Reverse transcription

1µg of total RNA was heated to 72°C for 5 minutes with 0.6 µg of oligo(dT)15 primer and 2µM dNTPs. Following heating RNA was reverse transcribed in a total volume of 25 µl including 132 units of Moloney murine leukemia virus reverse transcriptase, 26.4 units of RNase inhibitor, and 1x M-MLV RT buffer. The reaction was incubated at 42 °C for 90 min. RT products were stored at -20°C until required.

3.1.3.2 PCR of cDNA

Aliquots of the RT products were subsequently used for PCR amplification. 2 µl of RT products was brought to a volume of 50 µl containing 1.5mM $MgCl_2$, 0.25 mM of each dTNPs, 1.25 units of GoTaq® DNA polymerase, 0.5 µM of

both the upstream and downstream PCR primers, and 1x Green GoTaq® Reaction Buffer.

Amplification was carried out with a PTC-100 programmable thermal controller (Bio-Rad Laboratories Ltd, Hertfordshire, UK) after an initial denaturation at 94 °C for 3 min. This was followed by 30 cycles of PCR using the following temperature and time profile: denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min, primer extension at 72 °C for 1 min, and a final extension of 72 °C for 10 min. The following primers were used: VEGF sense 5'- ATCTGCATGGTGATGTTGGA-3' and VEGF antisense 5'-GGGCAGAATCATCACGAAGT-3' and GAPDH sense 5'-CGGAGTCAACGGATTTGGTCGTATTGG-3', GAPDH antisense 5'-GCTCCTGGAAGATGGTGATGGGATTTCC-3'.

The PCR products were visualized by electrophoresis on a 2% agarose gel in 0.5x TBE buffer after staining with 0.5 µg/ml ethidium bromide. The ultraviolet (UV)-illuminated gels were photographed.

3.1.3.3 Quantitative Real-time RT-PCR

Human VEGF-A expression was determined using primer sequences: sense 5'-ATCTGCATGGTGATGTTGGA -3' and antisense 5'-GGGCAGAATCATCACGAAGT -3'. Human VEGFR2 expression was determined using primer sequences: sense 5'-GGCTAATACAACTCTTCAAATTAC

-3' and antisense 5'-CCGAGGCCAAGTCAGTTTCCCGG-3' Human β_2-Microglobulin was used as the housekeeping gene using the primer sequences: Sense 5'-AATCCAAATGCGGCATCT-3' and antisense 5'-GAGTATGCCTGCCGTGTG-3'. Murine VEGF-A expression was

determined using the following primer sequences: Sense 5'-CTGTGCAGGCTGCTGTAACG-3' and antisense 5'-GTTCCCGAAACCCTGAGGAG-3'. Murine VEGFR2 expression was determined using the following primer sequences: Sense 5'-GGCGGTGGTGACAGTATCTT -3' and antisense 5'-GTCACTGACAGAGGCGATGA -3'. Murine β-actin was used as a housekeeping gene using the following primer sequence: Sense 5'-AAATCGTGCGTGACATCAAA-3' and antisense 5'-AAGGAAGGCTGGAAAAGAGC-3'. 1.6µl of reverse-transcribed cDNA was subjected to real-time PCR using Excite Real-time Mastermix with SYBR green and the Mx3000P® QPCR System (Stratagene, California, US). Each reaction consisted of 1x Excite mastermix, SYBR green (1:60000 final concentration), 40 nM of both sense and antisense primers, 1.6 µl of DNA (or dH_2O), and H_2O to a final volume of 20µl. Thermal cycler conditions included incubation at 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s and 59 °C for 1 min. Integration of the fluorescent SYBR green into the PCR product was monitored after each annealing step. Amplification of one specific product was confirmed by melting curve analysis, where a single melting peak eliminated the possibility of primer-dimer association. For melting curve analysis to be performed the products were heated from 55 to 95 °C after the 50 cycles. Negative controls consisting of no template were always included, and all samples were assayed in duplicate. Changes in VEGF-A expression were normalised to changes in house keeping gene and expressed as relative expression compared to the control sample.

3.1.4 Transfections

The required cell type was grown to the confluence stated in the transfection reagents protocol in 24 well plates and serum starved in DMEM without antibiotics and serum for the specified time. The transfection reagent varied depending on cell type and the construct transfected. Those used were Lipofectamine 2000, Fugene 6 and Fugene HD. Complexes containing the concentration of luciferase linked DNA and the ratio of transfection reagent determined by optimisation experiments were formed in antibiotic and serum free media and incubated at room temperature for a minimum of 20 minutes. Cells were always co-transfected with the internal control plasmid pRL-SV40 containing the Renilla Luciferase gene. 100µl of media containing the DNA:reagent complex was added directly to each well (without removal of the media already contained in the wells). Following incubation of the cells with the complex for the required time the media was removed and replaced with 500µl control media or media containing the required stimulus. Following stimulation the cells were rinsed once in PBS and then harvested using 1 x passive lysis buffer and stored at -20°C for short term storage or -80°C for longer term storage. Firefly and renilla luciferase activities were measured using the Dual Luciferase Assay System Kit and a Microlumat Plus LB 96V luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbag, Germany).

The VEGF promoter-driven luciferase constructs were a kind gift from Professor Dieter Marmé, Institute of Molecular Oncology, Tumor Biology Center, Freiburg, Germany[141] . The Sp-1 reporter construct containing 6 Sp-

1 binding sites was a kind gift from Professor Jeffrey E. Kudlow, School of Medicine, The University of Alabama at Birmingham [142]. The TCF reporter, TOPglow and negative control, FOPglow were purchased from Millipore. The AP2-reporter construct was a kind gift from Professor Helen Hurst, CRUK, London, UK [143]. The p53 *cis*-Reporting System was purchased from Stratgene, California, US. Dominant negative Smad2 and Smad3 constructs were a kind gift from Dr Anne Sturrock Div. of Respiratory, Critical Care and Occupational Pulmonary Medicine, University of Utah Medical Center [104]. Dominant negative TCF4 was a kind gift from Dr Thilo Hagen, Wolfson Digestive Diseases Centre, University of Nottingham, UK [144]. Smad2 and Smad3 expression constructs were a kind gift from Elizabeth J. Robertson Department of Molecular and Cellular Biology, Harvard University, USA [145]

3.1.5 Site Directed Mutagenesis of VEGF-318-Luc TCF binding sites

All site directed mutagenesis was conducted by Dr Karl Deacon with the Stratagene QuikChange® II Site-Directed Mutagenesis Kit according to the kit protocol. Briefly 50µl control reactions were created containing 1 x reaction buffer, 10ng pWhitescript 4.5-kb control plasmid, 125ng control primer #1 and control primer#2, 1µl dNTP mix, 38.5µl water and 1µl *PfuUltra*HF DNA polymerase. Sample reactions were prepared containing 1x reaction buffer, 5ng, 10ng, 20ng or 50ng dsDNA template (VEGF-318-Luc),125ng sense and antisense primer (as listed below), 1µl dNTP mix,, 1µl *PfuUltra*HF DNA polymerase and water to a total volume of 50ul for each reaction. The cycling conditions used are as follows: 95^{o}C for 30 seconds for 1 cycle and 18 cycles of 95^{o}C for 30 seconds, 55^{o}C for 1 minute, 68^{o}C for 6 minutes (1 minute per

kilobase of plasmid template). Following amplification, 1µl *Dpn* I restriction enzyme was added to the reaction and incubated for 1 hour at 37°C to digest the parental DNA. The remaining mutated DNA was transformed into XL1-blue supercompetent cells and grown on LB-Ampicillin plates for >16hrs. Colonies were selected, grown in NZY+ broth and isolated using the QIAprep Spin Miniprep Kit.

TCF binding sites were mutated individually at residue 110 base pairs from the transcription start site and 142 base pairs from the transcription start site. The 110 sense and antisense primer sequences were as follows, CGC GTGTGG AAG GGC C GAG GCT CGC CTG TCC, GGACAG GCGAGC CTC G GCC CTT CCA CAC GCG. The 142 sense and antisense primers were as follows, GCT TCA CTG AGC GTC CGC C GAG CCC GGG CCC GAGC, GCTC GGG CCC GGG CTC G GCG GAC GCT CAG TGA AGC. The underlined residues represent the mutant residues. The double 110/142 mutation was created by putting the 110 mutant construct through another round of site directed mutagenesis with the 142 primers. All mutations were confirmed by DNA sequencing at the University of Nottingham's Biopolymer Synthesis and Analysis Unit on a 3130 ABI PRISM.

3.1.6 Western Blots

Cells were cultured to confluence in 6 well plates, serum starved and treated using the drug or reagent at the concentrations and time courses stated. At the end of treatment cells were washed with PBS and treated with an extraction buffer [50mM TRIS-HCl pH7.4, 1% Nonidet P40, 0.25% sodium deoxycholate, 150mM NaCl and 1mM ethylenediaminetetraacetic acid

(EDTA) supplemented with 1mM phenylmethanesulphonylfluoride (PMSF), 1mM Sodium Orthovanadate (Na_3VO_4), 0.1mM Dithiothreitol (DTT), 0.4ug/ml leupeptin and pepstatin] with cell scraping. The cell extract was stored at -20°C until required. Prior to use samples were centrifuged at 14000 RPM at 4°C to remove cell debris and protein concentrations were determined using the Pierce bicinchoninic acid (BCA) assay. Protein samples were mixed with 4x Lamellae Buffer (25% 0.5M Tris HCl pH 6.8, 20% 100% glycerol, 20% 10% SDS, 40mg bromophenol blue, 35% dH_2O) and boiled for 5mins to denature the proteins. Protein samples were subject to electrophoresis in 10% SDS-polyacrylamide gel. Separated proteins were electroblotted to PDVF membranes and the blot was blocked for 1 h at room temperature with blocking buffer (0.1% TBST with 5% fat-free dried milk powder). The blot was then incubated with primary antibody (1/1000 dilution), at 4°C overnight. The blot was washed with 0.1% TBST, and incubated with HRP-conjugated secondary antibodies (1/2000 dilution with 5% fat-free dried milk in 0.1% TBST) for 1 h. The blot was washed again, incubated with ECL Western blotting detection reagent and developed on hyperfilm.

The antibodies used were; β-catenin (H-102, sc-7199, Santa Cruz Biotechnology, inc., California, US), GAPDH (AbD Serotec, Oxford, UK) Total Smad2/3 (#3102 Cell signalling Technology, Massachusetts, USA), phospho (serine 465/467) Smad2 (#3101 Cell Signalling Technology), dephosphorylated β-catenin (ab19451 Abcam, Cambridge, UK), phospho-β-catenin (Ser33/37/Thr41) (Cell Signalling Technology #9561), Lamin A/C (sc-

7292 Santa Cruz Biotechnology), total GSK3β (#9315 Cell Signalling Technology) and phospho-Ser9- GSK3β (#9336 Cell Signalling Technology).

3.1.7 Nuclear and Cytoplasmic compartment separation

Cells were grown to confluence in 100mm dishes, serum starved for 24 hours and incubated for the specified times with or without 1ng/ml TGFβ_1 or 10μM SB216763. Cytoplasmic and nuclear fractions were separated using Sigma's CelLytic™ NuCLEAR™ Extraction Kit. Briefly, following stimulation cells were washed twice with PBS, and scraped into conical centrifuge tubes. The sample was centrifuged for 5mins at 450xg and 4°C. The supernatant was discarded to leave approximately 50μl packed cell volume (PVC). 250μl 1x lysis buffer supplemented with 1% 0.1M DTT and 1% protease inhibitor cocktail (PIC) was added to the PCV and gently resuspended, avoiding foam. The samples were incubated on ice for 15 minutes to allow swelling. A final concentration of 0.6% IGEPAL CA-360 detergent was added to the sample and the samples were vortexed vigorously for 10 seconds to allow cell lysis. Samples were centrifuged immediately at 11,000 x g and 4°C for 30 seconds. The cytoplasmic fraction supernatant was transferred to a fresh tube. A subsequent wash in DTT and PIC supplemented lysis buffer was performed at this stage to minimise contamination of the nuclear fraction with cytoplasmic proteins. Extraction buffer was prepared by adding 1% 0.1M DTT and 1% PIC to the required volume of Extraction buffer (0.42M salt concentration). The nuclear pellet was resuspended in 33.3μl (2/3 PCV) supplemented Extraction buffer and vortexed for a minimum of 15 minutes. The sample was centrifuged for 5 minutes at 20,000 x g at 4°C. The nuclear fraction supernatant was

transferred to a fresh tube and the nuclear and cytoplasmic fractions were quickly stored at -80.

3.1.8 Chromatin Immunoprecipitation

PASMCs were cultured to confluence in 75-cm^2 flasks (2 per condition), growth arrested for 24 hours, and incubated with 1ng/ml TGFβ_1 for 0 and 2.5 hours. The ChIP Assay was performed using the ChIP-IT Express kit following the manufacturer's protocol. Briefly, cells were fixed to preserve protein and DNA interactions using 37% formaldehyde in minimal cell culture medium, removed from the flask by scraping, lysed and sheared by sonication. Chromatin was split into 50µl aliquots. 10µl of chromatin from each condition was removed for use as 'Input control DNA'. The remainder of the chromatin aliquot was incubated overnight at 4° in a 100µl solution containing Protein G magnetic beads, PIC and 4µg of target antibody or associated IgG control. The magnetic beads were washed prior to elution of immunoprecipitated DNA. The crosslinks were then reversed and all samples including inputs were then incubated at 65°C for 2.5 hours and then for 1 hour at 37°C in the presence of proteinase K to remove the proteins. Immunoprecipitated DNA was ready for use in PCR following the addition of a solution to stop proteinase K activity. Input DNA underwent a further phenol/chloroform extraction before being used in PCR.

The antibodies used were; TCF4 (N-20) (sc-8631 Santa Cruz Biotechnology), Smad2 (phospho S465 + S467) (ab16509, Abcam), Smad3 (ab28379), total β-Catenin (06-734 Millipore, Watford, UK) dephosphorylated β-catenin (ab19451 Abcam, Cambridge, UK) Smad 4 (06-693, Millipore), total GSK3β

(#9315 Cell Signalling Technology), phospho-β-catenin (Ser33/37/Thr41) (Cell Signalling Technology #9561), normal rabbit IgG (AB-105-C, R and D Systems), normal mouse IgG2a (M5409, Sigma) and normal mouse IgG1 (MAB002, R and D Systems) and normal goat IgG control (ab-108-C abcam).

The VEGF primers yielded a 161-bp pair product corresponding to -262 to -101 of the VEGF gene promoter and were: sense 5'-GCGTGTCTCTGGACAGAGTTT-3', and antisense 5'-AGCCTCAGCCCTTCCACA-3'. The 'upstream VEGF' primers yielded a 232-bp product corresponding to -1589 to -1357 of the VEGF gene promoter and were: sense 5'-GAGGCTATGCCAGCTGTAGG-3' and antisense 5'-CCCTTTTCCTCCAACTCTCC-3'. Amplification was carried out with a PTC-100 programmable thermal controller (Bio-Rad Laboratories Ltd, Hertfordshire, UK) after an initial denaturation at 94 °C for 5 min. This was followed by 30-40 cycles of PCR using the following temperature and time profile: denaturation at 94 °C for 0.5 min, primer annealing at 59 °C for 0.5 min, primer extension at 72 °C for 0.5 min, and a final extension of 72 °C for 1 min. The PCR products were visualised by electrophoresis on 2% agarose gel in 0.5xTBE buffer after staining with 0.5ug/ml ethidium bromide. The ultraviolet-illuminated gels were photographed.

3.1.9 Nuclear Co-immunoprecipitation

PASMCs were cultured to confluence in 225 cm^2 flasks, growth arrested, and incubated for 2 hours with or without 1ng/ml TGFβ_1. The Co-IP assay was performed using the Universal Magnetic Co-IP Kit according to the manufacturer's protocol. Briefly, the cells were washed with PBS

supplemented with phosphatase and deacetylases inhibitors and then scraped from the flask in the PBS/inhibitors solution. Nuclei were isolated by resuspending the cell pellet in hypotonic buffer supplemented with phosphatase inhibitors, deacetylases inhibitors, PIC, and PMSF and incubating the sample on ice for 15 minutes, followed by the addition of detergent and centrifugation at 14,000 x g for 30 seconds at 4°C. The nuclear fraction was then digested to release all protein complexes from DNA. Samples were centrifuged a final time to remove debris and transferred to fresh centrifuge tubes. Phosphatase, protease and deacetylases inhibitors are included at all stages to minimise protein modifications. Nuclear extracts were incubated on rolling shakers at 4°C for 4 hours with target antibody (at the dilution stated on the datasheet) or relevant IgG control at the corresponding concentration. A 'buffer only' sample (± antibody) was also performed on each occasion to account for non-specific binding of buffer components to the antibody. After the 4 hour incubation, Protein G magnetic beads were added to the complex and the sample incubated for a further hour. Samples were centrifuged briefly to remove sample from the lid and then washed 4 times with wash buffer supplemented with phosphatase, protease and deacetylases inhibitors and PMSF. Following the final wash, the bead pellets were resuspending in 2x Reducing loading buffer (130mM Tris pH 6.8, 4% SDS, 0.02% bromophenol blue, 20% glycerol and 100nM DTT) and stored at -20°C. The samples were split in half and run on two gels to allow blotting for an increased number of associated proteins. The antibodies used for co-immunoprecipitation were Smad2 (#3122 Cell Signalling Technology) and GSK3β (#9315 Cell Signalling Technology). In addition light chain specific secondary antibodies were used.

3.1.10 Cell Viability

The toxicity of all the chemicals and vehicles used in this study was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) assay [146]. At the end of the experiment culture media was removed and replaced with 250 µl of serum free media containing 1mg/ml thiazolyl blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (Sigma), then incubated for 20 minutes at 37 °C. This medium was removed and the plates dried overnight. 250 µl of dimethyl sulfoxide (DMSO) was then added to dissolve the blue-coloured tetrazolium. The optical density was read at 550 nm in a TECAN GENios (Tecan UK Limited, Theale, Reading, UK) microplate reader. Viability was set as 100% in control cells.

3.1.11 Statistical Analysis

Data are expressed as means and SEM from n determinants. Statistical analysis was performed with the software program GraphPad Prism 4. For experiments with only two data sets, unpaired two-tailed Students t-test was used to determine the significance of differences between the two means. On all other occasions a one way ANOVA was performed with either a Bonferri's post test, to compare two specific columns or Dunnet post test, to compare all conditions to a single control lane. For Western blot and standard gel PCR experimtents representative experiment results are shown.

3.2 MATERIALS

PASMCs (Product Code CC-2581 Lot Number 6F0511) were purchased from Lonza Group Ltd, Basel, Switzerland. Smooth Muscle Cell Growth Medium was purchased from TCS Cellworks, Buckingham, UK. Dulbecco's Modified

Eagle Medium (DMEM), penicillin, streptomycin, L-Glutamine, amphotericin B, trypsin-EDTA, PBS, Tween 20, BSA, phenol:choloform, sodium pyruvate, sodium bicarbonate, TBE buffer, Ethidium Bromide, Trizma base, sodium deoxycholate, EDTA, PMSF, NA_3VO_4, DTT, leupeptin, pepstatin, SDS, Nonidet p40 and bromophenol blue were purchased from Sigma, Poole, Dorset, UK. Fetal Calf Serum was purchased from Harlan UK Ltd, Bicester, Oxon, England. Recombinant human TGFβ_1, recombinant murine Wnt3a, human and mouse VEGF ELISAs were purchased from R and D systems, Abingdon, Oxon, UK. DMEM (cat# 12100-061) for GSK3β +/+ and -/- MEFs,.

Lipofectamine 2000 and agarose were purchased from Invitrogen, Paisley, UK. RNeasy Plus mini kit and QIAprep Spin Miniprep Kit were purchased from Qiagen, West Sussex, UK. Moloney murine leukemia virus reverse transcriptase, RNase inhibitor, 1x M-MLV RT buffer, $MgCl_2$, GoTaq® DNA polymerase, 1x Green GoTaq® Reaction Buffer, internal control plasmid pRL-SV40, 1 x passive lysis transfection buffer, Dual Luciferase Assay System Kit, oligo dT, dNTPs, were purchased from Promega, Madison, WI. Excite Real-time Mastermix with SYBR green was purchased from Biogene, Cambridge, UK. Fugene 6, Fugene HD were purchased from Roche Diagnostics, Indianapolis, US. QuikChange® II Site-Directed Mutagenesis Kit was purchased from Stratagene, La Jolla, California, US. The BCA Assay was purchased from Thermo Scientific, Rockford, Illinois, US. HRP-conjugated secondary antibodies were purchased from Dako, Cambridge, UK. ECL Western blotting detection reagent was purchased from GE Healthcare UK Ltd, Buckinghamshire, UK. SB216763 GSK3β inhibitor was purchased from

Tocris Bioscience, Bristol, UK. The ChIP-IT Express kit and the Universal Magnetic Co-IP Kit were purchased from Active Motif, Rixensart, Belgium. Light chain specific secondary antibodies were purchased from Jackson ImmunoResearch, via Stratech Scientifc Ltd., Suffolk UK. Sulphuric acid, hydrochloric acid and sodium chloride were purchased from Fisher Scientific, Loughborough, Leicestershire, UK. SIS3 and MTT were purchased from Merck Chemicals Ltd, Darmstadt, Germany. TMB substrate set was purchased from Becton Dickinson, Franklin Lakes, New Jersey, US. Primers were purchased from Eurofins, London, UK. PDVF membrane was purchased from BioRad Laboratories, Hurcules, California. Blotto, non-fat dry milk was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, California, US

4 THE EFFECT OF TGFB FAMILY MEMBERS, INFLAMMATORY CYTOKINES AND PROSTAGLANDINS ON VEGF PRODUCTION FROM HUMAN PULMONARY ARTERY SMOOTH MUSCLE CELLS

4.1 INTRODUCTION

During the development of pulmonary hypertension massive remodelling of the arteries occurs resulting in narrowing of the artery lumen and increased pulmonary vascular resistance. Several cells types are involved in this process including pulmonary artery endothelial cells and pulmonary artery smooth muscle cells.

The angiogenic molecule VEGF is implicated in the progression of pulmonary hypertension, due to its ability to protect endothelial cells and smooth muscle cells from apoptosis. Moreover immunohistochemical studies have shown VEGF staining in the smooth muscle layer of pulmonary arteries from pulmonary hypertensive patients [61]. The regulation of VEGF production from human PASMCs in culture has not been investigated. Furthermore the primary angiogenic signalling receptor for VEGF, VEGFR-2 is expressed on pulmonary artery endothelial cells adjacent to pulmonary artery smooth muscle cells expressing VEGF [62], thus providing a possible paracrine target for VEGF produced from smooth muscle cells.

Current paradigms regarding the pathobiology of pulmonary hypertension strongly implicate the TGFβ family. TGFβ molecules have been shown to be expressed in higher levels in the arteries of PH patients [95], the BMPR II mutations underlie 80% of familial PH cases [99] and 25% of idiopathic PH cases [101] and result in altered growth responses of pulmonary artery smooth muscle cells to TGFβ and the BMPs [103]. More recently TGFβ$_1$ has been

shown to be released from dysfunctional endothelial cells in models of PH and confers a proliferative response on adjacent smooth muscle cells [12].

Inflammatory cytokines have also been a focus of research into the underlying mechanism of pulmonary hypertension. For example the serum concentrations of interleukin-1β (IL-1β) and endothelin-1 are markedly increased in primary pulmonary hypertension compared to control patients [21, 23]. In COPD patients with pulmonary hypertension, exhaled breath and arterial levels of endothelin-1 were increased and correlated with increased pulmonary artery pressure [in press]. Endothelin-1 is known to induce vascular smooth muscle cell proliferation [23] and endothelin receptor antagonists have reasonable efficacy in the treatment of pulmonary hypertension [147]. In addition Lesprit *et al*., found that Il-1β, tumour necrosis factor α (TNFα) and VEGF levels were increased in the serum of patients with PH secondary to the rare multisystem disorder POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy and skin change) [22].

Bradykinin, a potent vasodilator, may also be involved in regulating pulmonary hypertension as Taraseviciene *et al*., have shown that a bradykinin antagonist prevented the development of pulmonary hypertension in a hypoxia model [148].

Prostaglandins are the products of arachidonic acid conversion by cyclooxygenase (COX) enzymes. In hypoxia models of pulmonary hypertension, the inducible isoform of the COX enzymes, COX-2 is upregulated and results in an increase in prostaglandins including PGE_2 and a

hypoxia mediated decrease in pulmonary artery smooth muscle cell proliferation [149]. Thus prostaglandins may be protective in pulmonary hypertension. In agreement with this the current 'Gold Standard' treatment for pulmonary hypertension is intravenous infusion of stable prostacyclin analogues, for example Iloprost and Carbaprostacyclin. Furthermore, Iloprost has been shown to inhibit pulmonary artery smooth muscle cell proliferation [150, 151]. Perhaps contradictory to this however, Iloprost and Carbaprostacyclin have been show to cause VEGF dependent increases in angiogenesis [152].

In summary VEGF appears an important modulator of PH but its role is poorly defined and its secretion from PASMCs unreported. A diverse range of mediators are implicated in PH pathogenesis and some can regulate VEGF production in alternative contexts. Here we tested the hypothesis that normal human pulmonary artery smooth muscle cells produce VEGF and that the TGFβ family members, inflammatory mediators and prostaglandins would regulate VEGF secretion.

4.2 AIMS

The aims of this chapter were to determine:

- if human PASMCs produce VEGF
- if members of the TGFβ family regulate VEGF expression from PASMCs
- if a selection of inflammatory cytokines regulate VEGF expression from PASMCs

- if PGE_2 and prostacyclin (PGI_2) analogues regulate VEGF production from PASMCs

4.3 METHODS

PAMSCs were cultured to confluence in 24 well plates. Confluent PASMCs were serum starved for 24 hours. The medium was replaced with 500µl serum free medium containing the stimuli stated at the specified concentration. Following 24 hour incubation the supernatants were removed and VEGF was either measured immediately by ELISA as described in the Materials and Methods chapter, or stored at -20°C until the ELISA could be performed.

4.4 RESULTS

4.4.1 PASMCs produce VEGF protein under basal conditions and TGFβs increases the level of VEGF produced while BMPs do not

We initially performed experiments to determine if PASMCs were able to secrete VEGF and the effect, if any, that members of the TGFβ superfamily would have on the amount of VEGF produced. The TGFβ superfamily can be separated into those which canonically signal via Smads 2 and 3, the TGFβs, and those which utilise Smads 1,3 and 5, the BMPs. A level of basal VEGF expression was present in all experiments. Stimulation of PAMSCs for 24 hours with 1ng/ml $TGF\beta_1$, 1ng/ml $TGF\beta_2$ and 25ng/ml Activin A caused an increase in VEGF production by 2.11, 2.06 and 1.69 fold above control respectively (Figure 4-1). In contrast 10ng/ml BMP2 and 10ng/ml BMP4 did not increase VEGF production above control levels.

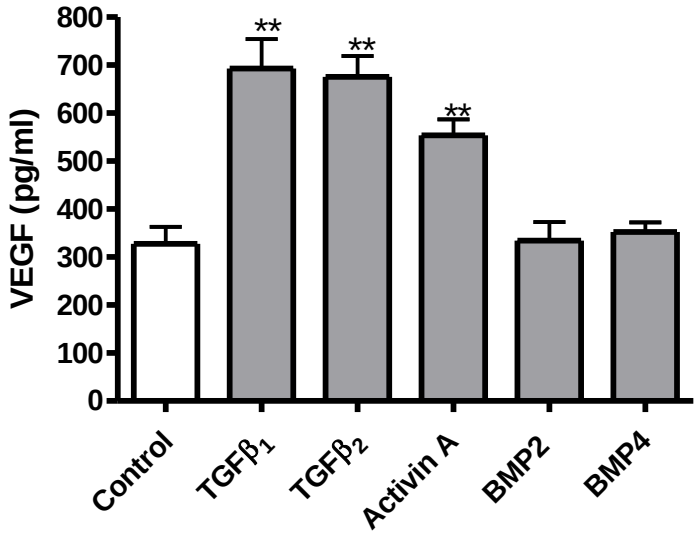


Figure 4-1 The effect of TGFβ family members on VEGF production from PASMCs

VEGF protein accumulation in the supernatants from PASMCs. Confluent PASMCs were serum starved for 24 hours and incubated for a further 24 hours in medium containing either 1ng/ml TGFβ_1, 1ng/ml TGFβ_2, 25ng/ml Activin A, 10ng/ml BMP2 or 10ng/ml BMP4. Bars represent the mean ± s.e.m from triplicate determinants in a minimum of three independent experiments. (**, $p < 0.01$ by one way ANOVA with Dunnet post test)

4.4.2 Inflammatory cytokines, prostaglandin E_2 and prostacyclin analogues do not increase VEGF production

Having established that members of the TGFβ family induced VEGF we were interested to know if other mediators implicated in pulmonary hypertension pathobiology were able to modulate VEGF production. First PASMCs were incubated for 24 hours with a selection of inflammatory cytokines. While TNFα and endothelin-1 did not significantly affect VEGF levels, both Bradykinin and IL-1β significantly reduced VEGF production compared to controls (Figure 4-2).

Subsequently PASMCs were incubated for 24 hours with PGE_2 or the prostacyclin analogues, iloprost and carbaprostacyclin. 10µm iloprost and 10µM carbaprostacyclin did not alter VEGF production whereas10µM PGE_2 significantly reduced VEGF production (Figure 4-3).

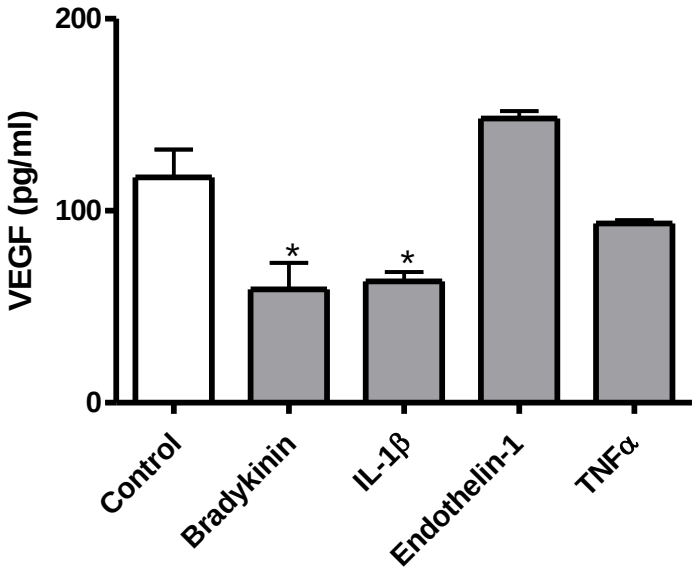


Figure 4-2 The effect of inflammatory cytokines on VEGF production by PAMSCs

VEGF protein accumulation in the supernatants from PASMCs. Confluent PASMCs were serum starved for 24 hours and incubated for a further 24 hours in medium containing either 10µM Bradykinin, 10ng/ml Interleukin-1β (IL-1β), 10ng/ml Endothelin-1 or 10ng/ml Tumour Necrosis Factor α (TNFα). Bars represent the mean ± s.e.m from triplicate determinants in a minimum of three independent experiments. (*, $p < 0.05$ by one way ANOVA with Dunnet post test)

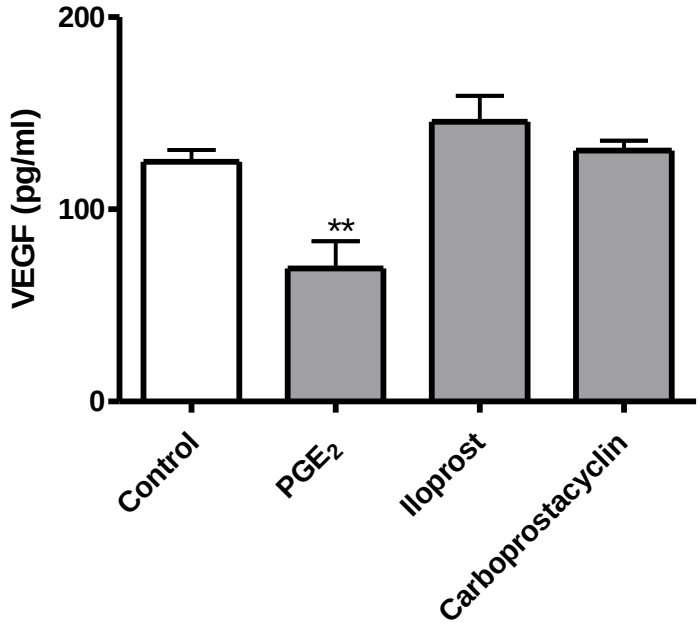


Figure 4-3 The effect of prostanoids on VEGF production by PASMCs

VEGF protein accumulation in the supernatants from PASMCs. Confluent PASMCs were serum starved for 24 hours and incubated for a further 24 hours in medium containing either 10µM PGE_2, 10µM Iloprost or 10µM Carbaprostacyclin.. Bars represent the mean ± s.e.m from triplicate determinants in a minimum of three independent experiments. (**, $p < 0.01$ by one way ANOVA with Dunnet post test)

4.4.3 TGFβ$_1$ increases VEGF production from PASMCs in a concentration and time dependent manner

Following the observation that human PASMCs were able to produce VEGF protein and analysis of the initial screen for relevant molecules that can regulate the VEGF production we decided to focus on TGFβ$_1$ as a stimulant as it produced the greatest increase in VEGF production and has greater implications in pulmonary hypertension than the other TGFβ related stimuli. Initially we performed a concentration response to TGFβ$_1$ (Figure 4-4).

PASMCs were incubated for 24 hours with the stated concentration of TGFβ$_1$. A VEGF induction above control levels was seen at concentrations of 1ng/ml and greater. Subsequently a time course was performed to establish the time scale for VEGF induction by TGFβ$_1$. A significant increase in VEGF production in response to 1ng/ml TGFβ$_1$ was seen from 8 hours (Figure 4-5).

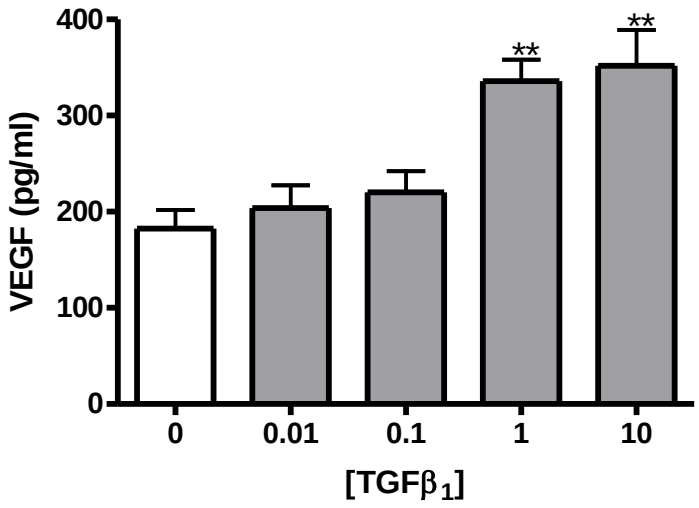


Figure 4-4 Concentration response of TGFβ1 on VEGF production from PASMCs

VEGF protein accumulation in the supernatants from PASMCs. Confluent PASMCs were serum starved for 24 hours and incubated for a further 24 hours in medium containing the stated concentration of TGFβ$_1$. Bars represent the mean ± s.e.m from triplicate determinants in a minimum of three independent experiments. (**, $p < 0.01$ by one way ANOVA with Dunnet post test)

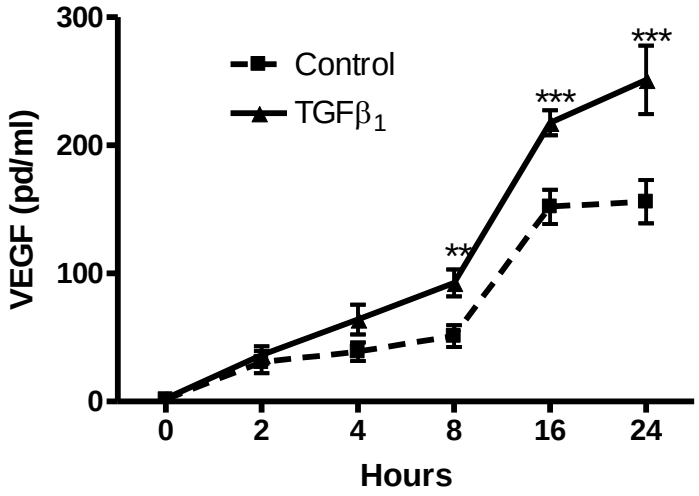


Figure 4-5 Time course of basal and TGFβ induced VEGF production from PASMCs

VEGF protein accumulation in the supernatant from PASMCs. Confluent PASMCs were serum starved for 24 hours in the presence and absence of $TGF\beta_1$ for the times stated. Points represent the mean ± s.e.m from triplicate determinants in a minimum of three independent experiments. (**, $p < 0.01$; and ***, $p < 0.001$ by one way ANOVA with Bonferroni's post test).

4.5 DISCUSSION

The major findings of the studies in this chapter are that cultured confluent normal human PASMCs secrete VEGF protein under basal conditions and that differential regulation of VEGF occurs in response to a number of stimuli relevant to pulmonary hypertension. The main stimuli which increased VEGF production were the TGFβ family of signalling molecules. In contrast Bradykinin, IL-1β and PGE_2 decrease VEGF secreted from PASMCs. All assays were performed using DuoSet ELISA kits developed to detect only $VEGF_{165}$ and $VEGF_{121}$. Standard curves were run on all plates to calculate the concentrations in the unknown samples and to confirm the assay was working.

There are similarities and differences between the effects seen in our studies in PASM cells with those reported in other biological systems. Bradykinin induces VEGF secretion from cultured stromal rat fibroblasts [153] and bradykinin and VEGF co-localise in mice with developing tumours [154]. Similarly IL-1β has been reported to increase VEGF release in several other cell types [155-157] and in a number of cases its regulation has been characterised,. For example, in the lung epithelial cell line A549, VEGF is upregulated by IL-1β via a pathway involving PI-3kinase/AKT/mTOR, NF-κB, COX-2 and HIF-1α [155]. Upregulation of VEGF by PGE_2 has been reported in human airway smooth muscle cells [158], human synovial fibroblasts [159] and the mink embryo [160]. This occurs via EP_2 and EP_4 receptors, an increase in cyclic AMP and binding of Sp-1 and AP2 transcription factors to the VEGF promoter. In rat gastric microvascular endothelial cells PGE_2 increases VEGF via ERK2 and JNK MAP kinases [161]. All of these studies contrast with our findings as we found that bradykinin, IL-1beta and PGE_2 all reduced VEGF release. There are no other reports of these stimuli reducing VEGF release in any cell system although VEGF-D has recently been shown to be down regulated by IL-1β [162]. It would seem unlikely that differences in the concentrations of stimuli used or incubation time can account for these contradictory effects of Il-1β, bradykinin and PGE_2 on VEGF production as the concentrations and incubation times in our study are not dissimilar to those in studies where upregulation was seen with the same stimuli. A more likely explanation is that there is a difference in the regulatory pathways between pulmonary vascular smooth muscle cells and these other structural cells. This could be due to an altered complement of receptors or components of the

intracellular signalling cascade such as kinases or transcription factors. However in hindsight, it may have been valuable to perform more detailed time course and concentration response studies. PGE_2 is produced from conversion of arachidonic acid by the cyclooxygenase enzymes, COX-1 and COX-2. Bradykinin and IL-1β have been shown to increase PGE_2 release from PASMCs via upregulation of COX-2 protein [163]. As the effect of bradykinin and IL-1β mirror the effect of PGE_2 on VEGF release from PASMCs it is likely that IL-1β and bradykinin are acting via induction of PGE_2. It would be interesting to investigate this possibility using the non-specific COX inhibitor, indomethacin and/or the COX-2 specific inhibitor NS398.

We also found that VEGF production from PASMCs was unresponsive to the prostacyclin analogues, iloprost and carbaprotacyclin, TNFα, endothelin-1 and BMP2 and BMP4. Iloprost has been shown to increase VEGF mRNA in rat aortic smooth muscle cells [164] which probably reflects a difference between cell types. Interestingly a separate group recently showed both iloprost and carbaprostacyclin to induce VEGF protein production at 48 hours in human lung fibroblasts [165] suggesting different regulatory systems exist in different cell types and may explain the lack of induction in PASMCs. The regulation of VEGF by TNFα also appears to be cell specific and both up and down-regulation have been reported. For example TNFα transgenic mice (i.e. mice overexpressing TNFα) have reduced levels of lung VEGF mRNA, suggesting TNFα down regulates VEGF [166]. However, in both human first trimester trophoblast cells [167] and human retinal epithelium cells [168] VEGF protein and mRNA levels are increased by TNFα stimulation, albeit at different points

in time. Equally, depending on the study investigated, the effect of endothelin-1 on VEGF can be positive or negative. In human ovarian carcinoma cells [169] and airway smooth muscle cells [170] endothelin-1 has been seen to increase VEGF mRNA and protein, while in rat osteoblastic cells endothelin-1 decreases VEGF mRNA [171]. Furthermore an endothelin type A receptor antagonist causes a recovery of VEGF mRNA in diabetic rat heart [172]. The BMPs however seem to consistently increase VEGF expression in the literature available [173-175].

The most important aspect of our studies was that we found that members of the TGFβ signalling family, TGFβ_1, TGFβ_2 and Activin A significantly increased VEGF secretion from PASMCs. Our observations in PASMCs agree with previous reports that Activin A can induce VEGF. This has been shown in bovine aortic endothelial cells [176], human hepatoma cell lines [177] and corneal epithelial cells [178]. In human hepatoma cell lines the regulation of VEGF by Activin A was shown to be transcriptional and rely upon association of Smad2 with Sp-1 and association of the complex with the VEGF promoter [177]. Similarly TGFβ_2 has been shown to induce VEGF via a mechanism requiring NF-κB, a transcription factor implicated in multiple cellular processes [168]. Transcriptional regulation of VEGF by TGFβ_1 has been described previously although the regulatory mechanisms appear to be cell and species specific. For example in mouse macrophages, TGFβ_1 induces Smad3/4 binding to Smad binding elements (SBE) and HIF1α binding to hypoxia response elements (HRE) in the promoter of VEGF [40], while in human

cholangiocellular carcinoma cell lines TGFβ_1 induces Sp-1 and Sp-3 binding to the VEGF promoter [179].

In conclusion, the studies in this chapter show VEGF protein production from PAMSCs can be regulated by a number of stimuli relevant to pulmonary hypertension, of which TGFβ_1 caused the most significant change. Due to the evidence implicating transcriptional control in the regulation of VEGF by TGFβ_1 Chapter 5 investigates whether transcriptional regulation of VEGF occurs in PASMCs in response to TGFβ_1.

5 TRANSCRIPTIONAL REGULATION OF VEGF BY TGFB REQUIRES INCREASED TCF4 BINDING TO THE VEGF PROMOTER

5.1 INTRODUCTION

In Chapter 4 we found that members of the TGFβ family increased VEGF production from PASMCs. $TGF\beta_1$ was more potent than $TGF\beta_2$ and Activin A, in increasing VEGF release and caused a time and concentration dependent increase in VEGF secretion. In this chapter we turned our attention to the mechanisms involved in this effect.

VEGF regulation can occur at a number of levels including transcription, post-transcription, translation, and release of extracellular matrix binding isoforms from the cell surface [180]. Posttranscriptional regulation involves a number of processes including stabilisation of VEGF mRNA by binding of the RNA binding protein HuR to AU rich elements (AREs) in the 3'untranslated region (UTR) of VEGF mRNA [180], while translational regulation depends on the presence of internal ribosome entry sites (IRES) in the 5' UTRs [181]. Transcriptional regulation of VEGF involves a plethora of external factors. The VEGF promoter contains binding sites for several transcription factors including, Sp1, AP-2, Egr-1, p53, TCF and HIF-1α [44].

As mentioned in the discussion of Chapter 4 $TGF\beta_1$ can regulate VEGF transcriptionally via interaction of its signalling proteins Smad2, Smad3 and Smad4 with various transcriptional co-factors, including HIF1α and Sp-1 [40, 179], at the VEGF promoter. These interactions are cell specific however and the mechanisms regulating VEGF production in response to TGFβ in PASMC are unknown.

Here we tested the hypothesis that TGFβ_1 can regulate PASMC VEGF production at the transcriptional level via a specific region (s) of the VEGF promoter and specific transcription factor(s).

5.2 AIMS

The aims of this chapter were to determine;

- if TGFβ_1 regulated VEGF mRNA levels
- if any changes in VEGF mRNA were due to transcriptional as apposed to post-transcriptional mechanisms
- if one or more specific promoter binding sites and/or transcription factors were required for TGFβ_1 induction of VEGF transcription

5.3 METHODS

5.3.1 Inhibitor Studies

PASMCs were cultured to confluence and serum starved for 24 hours. Subsequently the media was replaced with serum free medium containing inhibitor (Actinomycin D (5μg/ml) or Mithramycin concentration response) or DMSO vehicle control. TGFβ_1 (1ng/ml final concentration) was added after 30 mins and samples were taken at the stated times and stored appropriately. ELISA and PCR were performed as per the Materials and Methods chapter.

5.3.2 Transfections

5.3.2.1 Luciferase Reporter Construct Transfections

Confluent PASMCs were serum starved for 14-16 hours and transfected with a 1:2 ratio of DNA: LF2000 for 2 hours. The medium was then removed and

replaced with medium containing 10ng/ml TGFβ_1 or 75ng/ml Wnt3a for 3.5 hours.

5.3.2.2 Dominant Negative Co-transfections

Confluent cells were serum starved for 7-9 hours and transfected with 1:2 ratio of DNA: Fugene HD for 14-16 hours. Medium was then removed and replaced with medium containing 10mg/ml TGFβ_1 for 24 hours.

5.3.3 Chromatin Immunoprecipitation

The antibodies used in this chapter were TCF4(N20) (sc-8631 X) and normal Goat IgG control (ab-108-C).

5.4 RESULTS

5.4.1 TGFβ_1 induces VEGF mRNA production by PASMCs

It has been shown previously, in some cell types, that TGFβ_1 can regulate VEGF levels by increasing mRNA levels [40, 179]. To determine if TGFβ_1 induced VEGF mRNA in human PASMCs we performed both standard PCR (Figure 5-1 B) and quantitative real time PCR (Figure 5-1 A) on cDNA reverse transcribed from RNA samples taken across a time course of 8 hours in response to 1ng/ml TGFβ_1. TGFβ_1 induced VEGF mRNA levels across the time course, by both RT-PCR and real time PCR. A representative real time PCR graph is shown as differences in relative expression levels across the individual experiments gave large errors when combined.

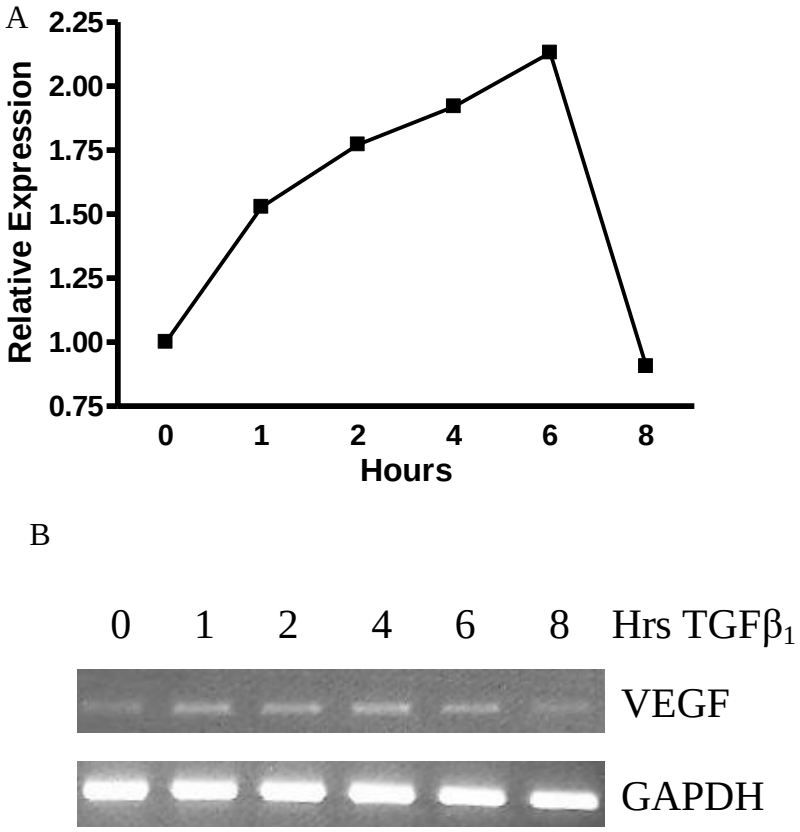


Figure 5-1 The effect of TGFβ1 on VEGF mRNA from PAMSCs

$TGF\beta_1$ mediated VEGF mRNA. Confluent PASMCs were serum starved for 24 hours and incubated for the times indicated in medium containing 1ng/ml $TGF\beta_1$. A) The housekeeping gene β_2-microglobulin and VEGF mRNA were measured by quantitative real-time PCR. B) the housekeeping gene GAPDH and VEGF mRNA were measured by RT-PCR. Real time and RT-PCR were each performed three times. A representative graph and gel are shown.

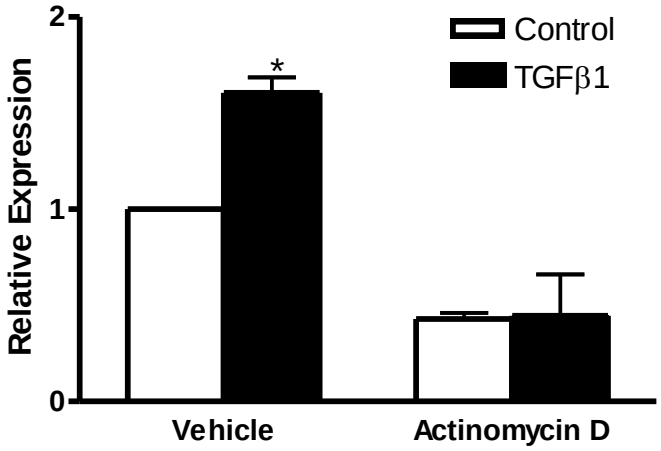
5.4.2 $TGF\beta_1$ induced VEGF mRNA and protein production are abolished by the inhibitor of transcription, Actinomycin D

To determine whether the increase in VEGF mRNA in response to $TGF\beta_1$ in PASMCs was due to increased transcription we used the inhibitor of transcription, Actinomycin D. This compound can be toxic to cells and MTT assays were performed prior to experiments to minimise toxicity. Data in

Appendix-1 shows Actinomycin D did not cause cell death at 2 hours, however significant cell death was seen at 4.5 and 6 hours. Toxicity was not dependent on the concentration of Actinomycin D or TGFβ_1. As a result Actinomycin D experiments were performed with the highest concentration of Actinomycin D to ensure an effect, but experiments were kept under 2 hours when investigating its effects on mRNA, as induction is seen after 1 hour. However, when investigating the effects on protein production a longer incubation was necessary and as such the VEGF concentrations obtained were always normalised to MTT data.

A significant increase in TGFβ_1 induced VEGF mRNA level was reproduced by both real time PCR (Figure 5-2 A) and RT-PCR (Figure 5-2 B) and Actinomycin D prevented TGFβ_1 dependent increases in VEGF mRNA.

A)



B)

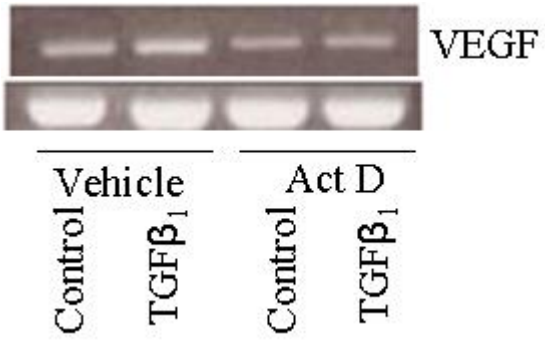


Figure 5-2 The effect of Actinomycin D on TGFβ1 induced VEGF mRNA in PASMCs

$TGF\beta_1$ mediated VEGF mRNA induction with and without 30 minute preincubation with 5μg/ml Actinomycin D, an inhibitor of transcripton, following 24 hour serum starvation. A) The housekeeping gene β_2-microglobulin and VEGF mRNA were measured by quantitative real-time PCR. B) the housekeeping gene GAPDH and VEGF mRNA were measured by RT-PCR. Each point represents the mean ± s.e.m from three individual experiments (*, $p < 0.05$ by one way ANOVA with Bonferroni's post test)

Furthermore, as shown previously in Chapter 4, $TGF\beta_1$ significantly increased VEGF protein secretion after 6 and 8 hours in comparison to control levels (grey lines Figure 5-3), but this induction was prevented by Actinomycin D (black lines Figure 5-3). Actinomycin D also reduced the basal levels of VEGF production, suggesting there was an element of transcriptional regulation to basal VEGF levels. Both mRNA and protein data suggest the $TGF\beta_1$ mediated effect on VEGF in PASMCs occurred via a transcriptional mechanism.

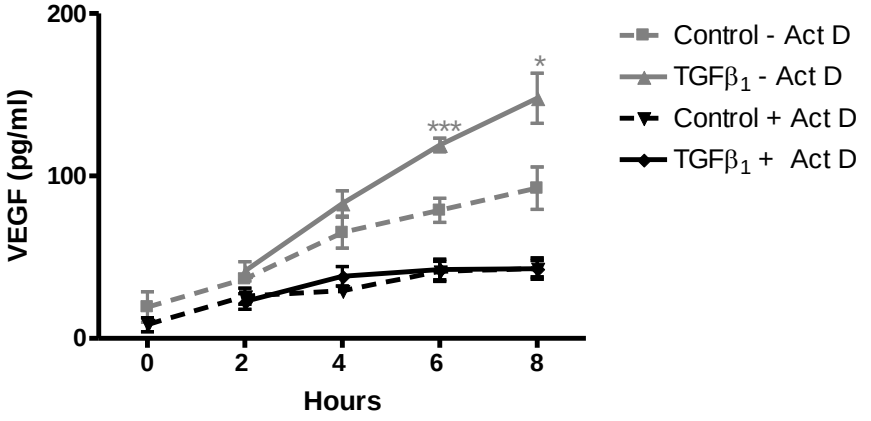


Figure 5-3 The effect of Actinomycin D on TGFβ1 induced VEGF protein production by PASMCs

VEGF accumulation in the supernatants from PASMCs. Confluent PASMCs were serum starved for 24 hours followed by a 30 minute pre-incubation with 5µg/ml Actinomycin D or DMSO vehicle control and subsequent time course to $TGF\beta_1$. Points represent the mean ± s.e.m from three individual experiments (*, $p < 0.05$; and ***, $p < 0.001$ by one way ANOVA with Bonferroni's post test)

5.4.3 $TGF\beta_1$ can activate a VEGF promoter luciferase reporter construct

Subsequently luciferase reporters were used to confirm transcriptional activity in response to $TGF\beta_1$. Luciferase reporters consist of fragments of promoter sequences, or repeats of specific transcription factor binding sites, ligated to the firefly luciferase gene. When a stimulus induces transcriptional activity of the DNA sequence the firefly luciferase gene is transcribed and the resulting protein measured as described in the Materials and Methods chapter. $TGF\beta_1$ caused a 1.65 fold increase in VEGF promoter reporter luciferase over control (Figure 5-4). The promoter reporter available contains of a section of the VEGF promoter from -2018bp upstream of the transcription start site to +50bp

downstream of the transcription start site and its activation further confirmed transcriptional regulation of VEGF by TGFβ_1 in PASMCs.

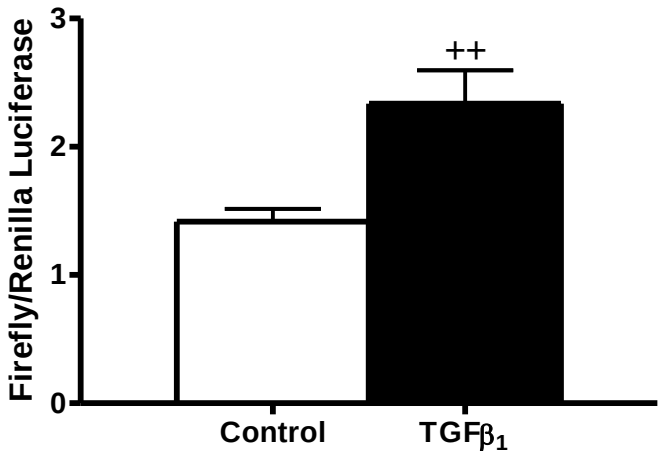


Figure 5-4 The effect of TGFβ1 on a VEGF promoter luciferase reporter

Luciferase activity in PASMCs transiently transfected for 2 hr with a 2068-bp fragment of the VEGF promoter (-2018/+50) ligated to a luciferase reporter construct. Cells were cultured to confluence, growth arrested, and transfected with 1μg of DNA and 2μl of LF2000 per well. There was an increase in promoter activity in cells stimulated for 3.5hrs with 10ng/ml TGFβ_1 compared to unstimulated controls. (++, $p < 0.01$ by unpaired two-tailed student t-test)

5.4.4 TGFβ1 induction of VEGF requires a 182-bp region of the VEGF promoter between -239 and -85bp upstream of the transcription start site

Having determined that TGFβ_1 was signalling via the promoter region of VEGF we used a VEGF promoter deletion series to determine the area of the promoter required. A representation of the series if given on the left hand side of Figure 5-5. Each different symbol represents a different transcription factor binding site known to be present within that region of the VEGF promoter.

The 2068 bp construct is the largest of the series and contains the maximum amount of regulatory sequence and transcription factor binding sites. Each of the other constructs are created by restriction enzyme cleavage at a specific site within the 2068 bp construct such that the constructs gradually get shorter, losing specific regulatory sequence and transcription factor binding sites each time. Consequently the region of the promoter required for a specific response can be narrowed down to within the last responding deletion series construct. In Figure 5-4 the VEGF-2068-Luc was used and responded well to TGFβ_1 stimulation. In Figure 5-5 data is shown for transfection of the whole deletion series in human PASMCs. A significant response to TGFβ_1 was seen with all constructs up to and including the VEGF-318-Luc. No response was seen with the VEGF-135-Luc. This suggests that the region of the VEGF promoter required for a response to TGFβ_1 is a 182bp section between -239 and -85 upstream of the transcription start site.

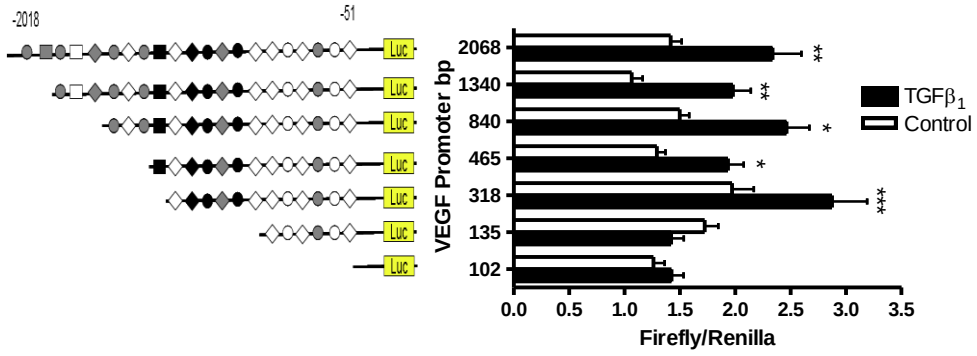


Figure 5-5 Effect of TGFβ1 on a transiently transfected VEGF promoter deletion series in PASMCs

Left) a representation of the VEGF promoter-driven luciferase constructs used in transfection studies. Each different symbol represents a different transcription factor binding site. Not all binding sites are illustrated. Pairs of bars on the right hand side graph correspond to the

adjacent reporter representation. Right) Increases in luciferase expression in PASMC transiently transfected with the deletion series of the VEGF promoter luciferase constructs after 3.5hr incubation with TGFβ1. Cells were cultured to confluence, growth arrested, and transfected for 2 hours with 1μg of DNA and 2μl of LF2000 per well. Cells were then stimulated for 3.5 hrs with 10ng/ml TGFβ1 (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ by ANOVA with Bonferroni's post test)

5.4.5 TGFβ$_1$ induction of VEGF in PASMCs does not involve Sp-1, AP-2 or p53 binding sites but may involve TCF sites

The 182bp section of the VEGF promoter identified as crucial to the TGFβ$_1$ response in deletion series studies, contains at least 60 putative transcription factor binding sites, including GR (glucocorticoid receptor) sites, NF-E (nuclear factor ets-like) sites, Gal4 binding sites, Sp-1 binding sites, GCF (GC factor) sites, AP2 binding sites, T cell factor (TCF) binding sites and p53 binding sites. We began to investigate which of these binding sites could be activated by first determining if any of the transcription factors which bind to these sites were activated by TGFβ$_1$. To do this we used luciferase reporters containing multiple repeats of the specific transcription factor binding site ligated to the firefly luciferase gene. A representation of the alignment of the sites we investigated in this way is given in Figure 5-6.

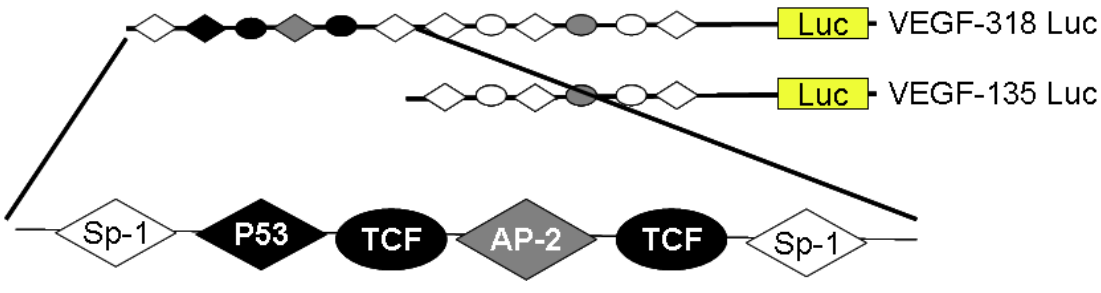


Figure 5-6 A selection the transcription factor binding sites present in the VEGF promoter between the 318bp and 315 bp constructs

Representation of the section of the VEGF promoter between the 318bp VEGF promoter reporter and the 135bp VEGF promoter reporter. The expanded section highlights a selection of the transcription factor binding sites within the region of the promoter.

The first transcription factor we investigated was Sp-1. There are at least 6 putative Sp-1 binding sites within the relevant section of the VEGF promoter. The Sp-1 reporter and the control vector were transfected into PASMCs and simulated for 3.5 hours with 10ng/ml $TGF\beta_1$. As PGE_2 has been shown previously to activate this reporter in PASMCs [158] it was used as a positive control. PGE_2 increased luciferase activity twofold but $TGF\beta_1$ had no effect (Figure 5-7). Activation of the control vector was negligible in response to $TGF\beta_1$ or PGE_2.

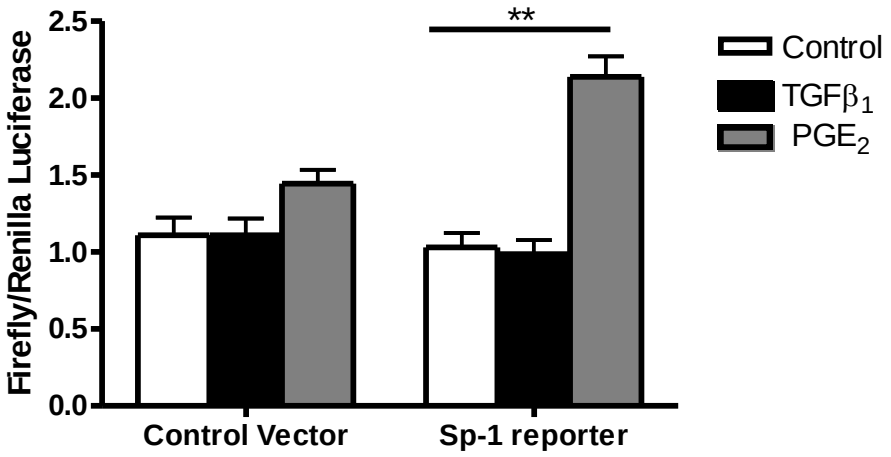


Figure 5-7 Effect of TGFβ1 and PGE2 on a transiently transfected Sp-1 luciferase reporter in PASMCs

Levels of luciferase expression in PASMC. Confluent serum starved PASMCs were transiently transfected with 0.5µg/well Sp-1 luciferase reporter or 0.5µg/well control plasmid with 1µl/well LF2000, for two hours. Subsequently the medium was replaced with medium with or without 10ng/ml $TGF\beta_1$ or 10µM PGE_2 and incubated for a further 3.5 hours. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. (**, $p < 0.01$ by one way ANOVA with Dunnet post test).

Mithramycin is a chemotherapeutic which selectively binds GC- rich regions of DNA to inhibit transcription. The Sp-1 binding site is GC-rich and as a consequence, the binding of Mithramycin prevents Sp-1 binding [182]. We used Mithramycin to confirm that Sp-1 was not involved in $TGF\beta_1$ regulation of VEGF in PAMSCs. 30 minute pre-incubation with increasing concentrations of Mithramycin, followed by 24 hour incubation with $TGF\beta_1$ did not reduce the fold increase in $TGF\beta_1$ stimulated VEGF over basal levels, but caused an increase (Figure 5-8). The apparent increase was due to an inhibitory effect of Mithramycin on basal levels of VEGF. These data suggest that Sp-1 is not involved in $TGF\beta_1$ induced VEGF production but may play a role in regulating basal VEGF production.

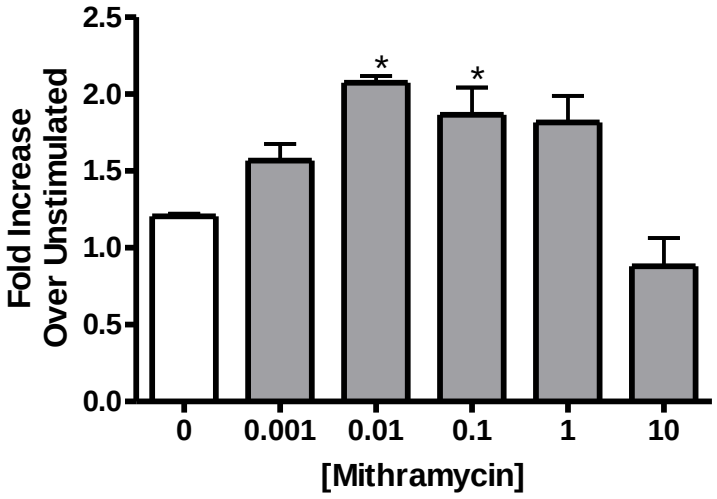


Figure 5-8 The effect of the Sp-1 inhibitor, Mithramycin, on TGFβ1 induced VEGF production from PASMCs

VEGF protein accumulation in the supernatants from PASMCs. Confluent PASMCs were serum starved for 24 hours and pre-incubated for 30 minutes with the stated concentration of Mithramycin, followed by 24 hour incubation with and without 1ng/ml $TGF\beta_1$. Bars represent the fold increase in $TGF\beta_1$ stimulated VEGF release over basal VEGF release for each condition. Bar represent the mean ± s.e.m from duplicate samples in two individual experiments. (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ by one way ANOVA with Dunnet post test

Having ruled out a role for Sp-1 in $TGF\beta_1$ induced VEGF in PASMCs we next investigated a role of AP-2. As with Sp-1 we used an AP2 luciferase reporter to establish if $TGF\beta_1$ could stimulate AP2 mediated transcription. $TGF\beta_1$ did not stimulate the AP2 reporter (Figure 5-9). Retinoic acid stimulates the AP-2 reporter in a human teratocarcinoma NT2 cell line [183] and was used as a positive control. Retinoic acid did stimulate the reporter on one occasion (Figure 5-9), confirming the reporter was working, but we were not able to repeat the effect and significance was never reached.

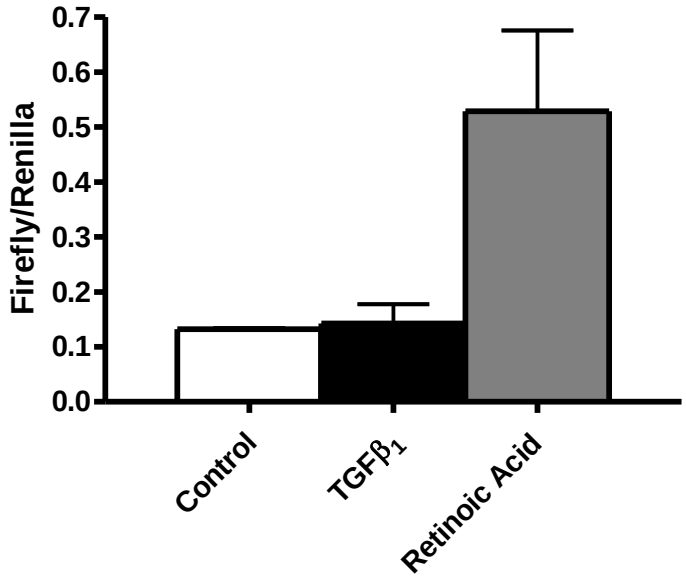


Figure 5-9 Effect of Retinoic Acid on a transiently transfected AP-2 luciferase reporter

Levels of luciferase expression in PASMC. Confluent serum starved PASMCs were transiently transfected with 0.5µg/well AP-2 luciferase reporter with 1µl/well LF2000, for two hours. Subsequently the medium was replaced with medium with or without 10ng/ml TGFβ_1 or 1µm Retinoic Acid and incubated for a further 3.5 hours. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from individual experiments. No significance was reached.

The subsequent transcription factor we investigated was the tumour suppressor transcription factor p53. The p53-luciferase reporter was transfected into PASMCs and incubated for 3.5 hours with 10ng/ml TGFβ_1. Alternatively, as a positive control, the p53-luciferase construct was co-transfected with the p53 expression construct, pFC-p53. The p53 expression construct induced a 1.7 fold increase in p53-Luc activity while TGFβ_1 had no effect (Figure 5-10). Thus, p53 is unlikely to be involved in TGFβ_1 induction of VEGF in PASMCs.

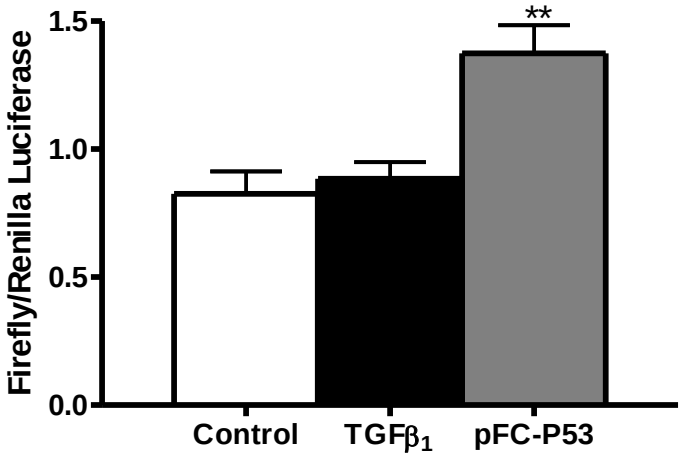


Figure 5-10 The effect of TGFβ1 and a p53 expression construct (pFC-P53) on a transiently transfected p53 reporter

Levels of luciferase expression in PASMC. Confluent serum starved PASMCs were transiently transfected with 0.5µg/well p53 luciferase reporter with 1µl/well LF2000 for two hours, or 0.5µg/well p53 luciferase and 0.5µg/well p53 expression plasmid, pFC-p53, with 2µl LF2000/well for 3 hours. Subsequently the medium was replaced with medium with or without 10ng/ml $TGF\beta_1$ and incubated for a further 3.5 hours. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. (**, $p < 0.01$ by ANOVA with Dunnet post test).

Finally we investigated whether TCF dependent induction of transcription could be activated by $TGF\beta_1$. There are two TCF binding sites within the region of interest in the VEGF promoter. The TCF reporter, Topglow and the negative control reporter, Fopglow, which contains multiple repeats of a mutated TCF binding site, were transfected into PASMCs. $TGF\beta_1$ caused a 2 fold increase in TCF-luciferase reporter, Topglow, activity with no induction of the negative control Fopglow construct (Figure 5-11). This data showed that $TGF\beta_1$ can induce TCF dependent transcription in PASMCs and the fact that

TCF sites exist within the VEGF promoter suggests TCF may be involved in TGFβ_1 induction of VEGF in these cells.

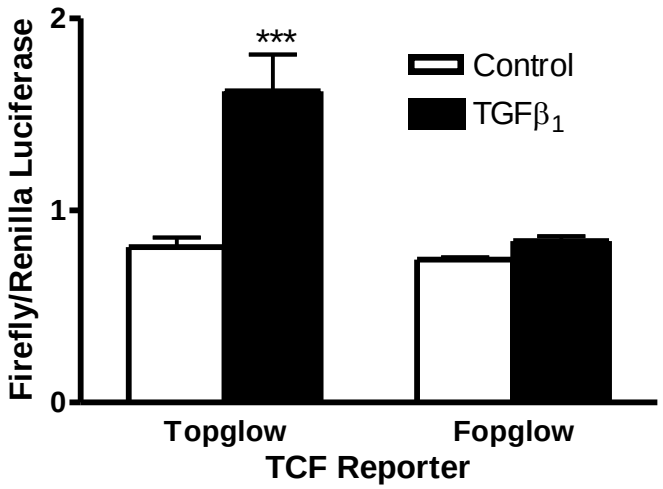


Figure 5-11 The effect of TGFβ1 on the transiently transfected TCF luciferase reporter, Topglow, and negative control reporter, Fopglow

Levels of luciferase expression in PASMC. Confluent serum starved PASMCs were transiently transfected with 0.5µg/well TCF luciferase reporter, Topglow, or 0.5µg/well control plasmid, Fopglow, with 1µl/well LF2000, for two hours. Subsequently the medium was replaced with medium with or without 10ng/ml TGFβ_1 and incubated for a further 3.5 hours. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. (***, $p < 0.001$ by ANOVA with Bonferroni's post test).

The transcription factor reporters used in the above experiments contain only the sequences required for the specific transcription factor to bind and therefore cannot account for any interaction that might occur between different transcription factors at the native promoter. As the TCF, Sp-1, p53 and AP2 binding sites are in close proximity on the VEGF promoter it is feasible that interactions could occur between the different transcription factors and their binding sites. The TCF sites were the only individual site that responded to TGFβ_1, and as a result we used a canonical TCF stimulus, Wnt3a, to establish if

Sp-1, AP2 and p53 dependent transcription in PASMCs could be induced by interaction with TCF. Thus, the TCF, Sp-1, AP2, p53 and relevant control vectors were transfected into PASMCs and stimulated for 3.5 hours with 75ng/ml Wnt3a. Only the TCF reporter, Topglow responded to Wnt3a (Figure 5-12), suggesting that in PASMCs TCF cannot induce Sp-1, AP2 or p53 dependent transcription and it is unlikely an interaction between TCF, AP2, Sp-1 or p53 occurs at the native VEGF promoter in response to TGFβ_1.

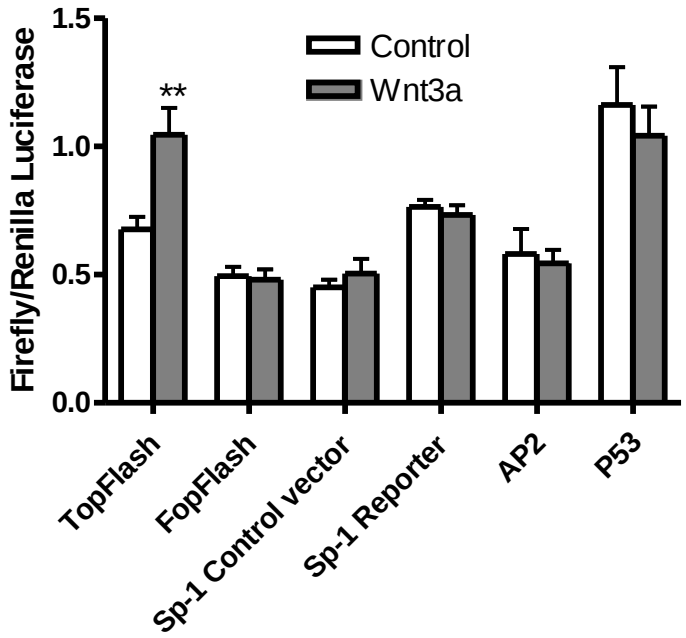


Figure 5-12 The effect of the canonical TCF stimulant, Wnt3a, on the Sp-1, AP-2, p53 and TCF reporters

Levels of luciferase expression in PASMCs. Confluent serum starved PASMCs were transiently transfected with 0.5µg/well TCF, Sp-2, AP-2 and p53 luciferase reporters with 1µl/well LF2000 for 2 hours. Subsequently the medium was removed and replaced with medium containing 75ng/ml Wnt3a and incubated for a further 3.5 hours. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from two individual experiments (**, $p < 0.01$ by ANOVA with Bonferroni's post test).

5.4.6 TGFβ$_1$ induction of VEGF in PASMCs depends on TCF4 binding to TCF binding sites within the VEGF promoter

Having shown TGFβ$_1$s ability to activate a TCF luciferase reporter it was important to determine that the TCF sites in the VEGF promoter were responsible for the activation by TGFβ$_1$. Site directed mutagenesis was therefore carried out on the VEGF-318 luciferase reporter (the shortest VEGF-promoter luciferase reporter to respond to TGFβ$_1$) to mutate the two TCF binding sites individually. A double mutant was also created. A representation of the mutations introduced is shown under the graph in Figure 5-13.

Wild type (wt) VEGF-318 Luc, VEGF-318Δ110 Luc (Δ110), VEGF-318Δ142 Luc (Δ142) and VEGF-318Δ110/142 Luc (Δ110/142) were transfected into PASMCs and stimulated for 3.5 hours with 10ng/ml TGFβ$_1$. Wt VEGF-318 Luc was significantly induced by TGFβ$_1$ as previously seen in Figure 5-5, while mutation of the individual TCF sites or both sites together prevented a response to TGFβ$_1$ (Figure 5-13) suggesting a critical requirement for both TCF sites in the induction of VEGF by TGFβ$_1$ in PASMCs. Interestingly mutation of both TCF sites also induced basal VEGF-318 luciferase activity.

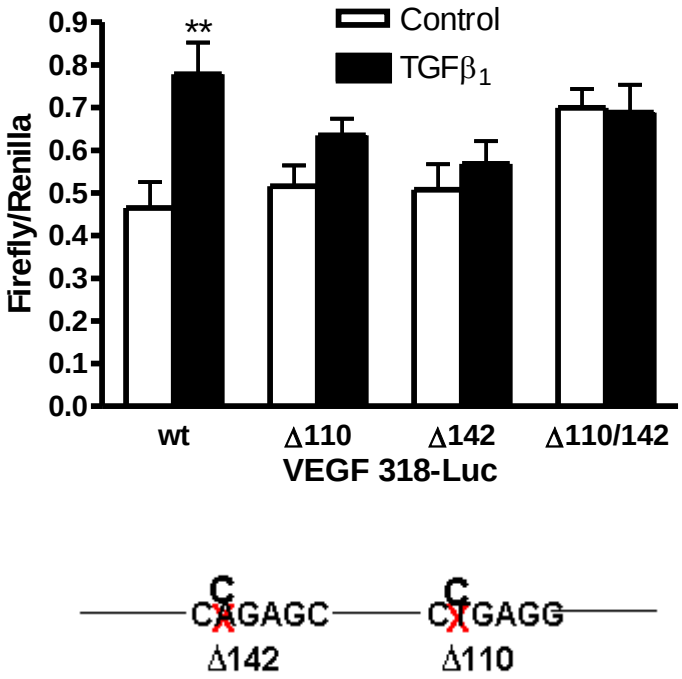


Figure 5-13 The effect of site directed mutation of individual TCF binding sites within the VEGF-318 luciferase reporter on TGFβ1 luciferase induction

Levels of luciferase expression in PASMCs. Confluent serum starved PASMCs were transiently transfected with 1µg/well of a wild type 318-bp fragment of the VEGF promoter (-267/+50) ligated to a luciferase reporter construct, or site directed mutants of the TCF binding site at the -110bp position, the -142bp position, or both sites of the VEGF 318-luciferase construct, with 2µl/well LF2000 for 2 hours. Medium was then removed and cells were stimulated for 3.5 hrs with 10ng/ml TGFβ$_1$. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. (**, $p < 0.01$ by ANOVA with Bonferroni's post test).

To confirm the role of TCFs in the induction of VEGF in response to TGFβ$_1$ by an alternative method and also to establish which TCF isoform was involved we transiently transfected a dominant negative TCF4 construct into our cells. TCF4 was initially selected as it can be produced in the longest form of all the TCFs and thus contains the maximum number of interaction domains [125], it

has been shown to regulate VEGF expression in colon cancer [133] and, from a technical point of view, it is the only TCF with a published specific antibody suitable for ChIP [126].

The dominant negative TCF4 construct or related control vector, were co-transfected with the VEGF-318 luciferase reporter. In the presence of the empty control vector TGFβ_1 increased VEGF-318 luciferase activity (Figure 5-14). However in the presence of dominant negative TCF4 the TGFβ_1 response was abolished. This data further confirms a role for TCFs in TGFβ_1 induced VEGF production and provides initial evidence that TCF4 is the isoform involved.

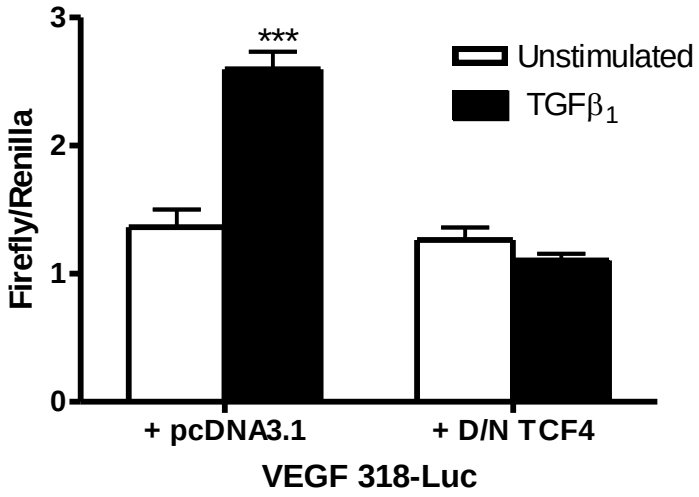


Figure 5-14 The effect of dominant negative TCF4 co-transfection on TGFβ1 induction of the VEGF-318 luciferase reporter

Levels of luciferase expression in PASMCs. Confluent serum starved PASMCs were transiently transfected with 0.4µg/well VEGF-318 luciferase and co-transfected with either 0.2µg/well control plasmid, pcDNA3.1, or 0.2µg/well dominant negative TCF4 plasmid and 1.2µl Fugene HD/well for 16-20 hrs. The medium was then removed and replaced with medium containing 10ng/ml TGFβ_1. Each bar represents the mean ± s.e.m from a minimum of

triplicate samples from three individual experiments. (***, $p < 0.001$ by ANOVA with Bonferroni's post test).

We went on to determine whether TCF could interact with the native VEGF promoter using ChIP. ChIP involves fixing whole cells such that protein:DNA interactions are maintained. The DNA is then sheared into uniform sized sections by sonication and incubated with an antibody against the transcriptional modulator of interest. The protein is immunoprecipitated, bringing with it any interacting portions of DNA. The protein:DNA crosslinks are then reversed, the protein removed by proteinase K incubation, and the DNA recovered. The DNA present is analysed by PCR. We performed ChIP using a TCF4 antibody and relevant IgG control to ensure data generated was not due to non-specific association of DNA with the antibody. Unstimulated control cells and cells stimulated for 2.5 hours with 1ng/ml $TGF\beta_1$ were subject to immunoprecipitation. Two sets of PCR primers were designed and used. The first set (VEGF -262 to -161) was designed to cover the two TCF binding sites shown to be important in the previous studies. The second set (VEGF upstream) was designed to cover an irrelevant region of the VEGF promoter, further upstream than the TCF sites of interest and in a region of the promoter determined as not critical for $TGF\beta_1$ induction of VEGF in the deletion series study, notably -1589 to -1357 upstream of the VEGF transcription start site (within the VEGF-2068 luciferase reporter). This second set of primers controls for non specific binding of the transcription factors to irrelevant regions of DNA. TCF4 binds the relevant region of the VEGF promoter under basal conditions and this binding is increased in response to $TGF\beta_1$ stimulation

(Figure 5-15). Furthermore no band is visible in the IgG and upstream primer negative controls, ensuring the result is specific to TCF4 and the TCF binding site section of the promoter. Inputs show the presence of DNA prior to immunoprecipitation.

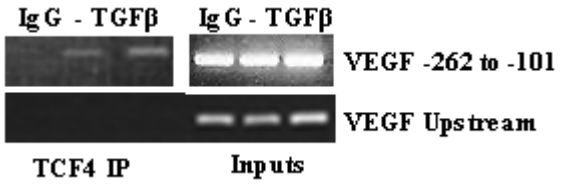


Figure 5-15 The effect of TGFβ1 on TCF4 binding to the relevant 161-bp region of the VEGF promoter

Representative PCR gel of a TCF4 ChIP assay. Confluent PASMCs were serum starved for 24 hours followed by 2.5 hours incubation with and without $TGF\beta_1$. Immunoprecipitation was carried out using an antibody to TCF4 or relevant control IgG. The PCR primers were amplified in the -262 to -101 region of the VEGF promoter. VEGF Upstream primers were used as a negative control for non specific binding of the immunoprecipitated proteins to irrelevant regions of the promoter.

Finally we wished to determine whether the increase in TCF4 association with the VEGF promoter was due to $TGF\beta_1$ increasing TCF4 protein. TCF4 protein was present in unstimulated samples at all time points and $TGF\beta_1$ had no effect on the amount of TCF4 present (

Figure 5-16).

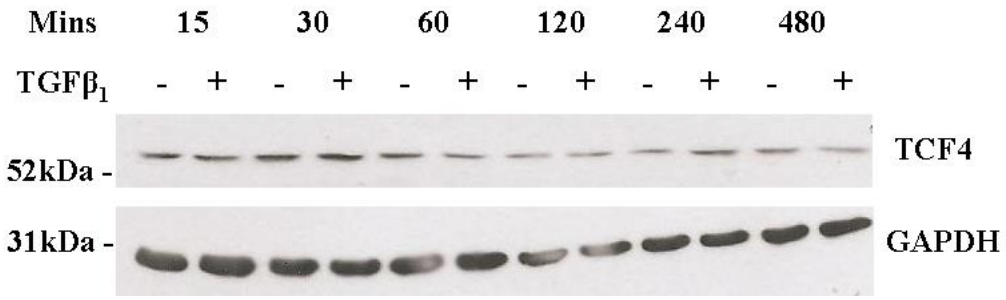


Figure 5-16 The effect of TGFβ1 on total TCF4 protein levels

$TGF\beta_1$ induced TCF4 protein. GAPDH was used as a house keeping control. Confluent, serum starved PASMCs were incubated with 1ng/ml $TGF\beta_1$ across an 8 hour time course. Protein was extracted for western blot. The blots shown are representative of similar results achieved in 2 independent experiments.

5.5 DISCUSSION

In Chapter 4, we found that $TGF\beta_1$ concentration and time dependently increased the concentration of VEGF protein secreted from PASMCs. The major findings from the studies in the current chapter are that $TGF\beta_1$ can induce VEGF mRNA levels via a transcriptional mechanism requiring increased binding of the T Cell Factor 4 (TCF4) transcription factor to one of two TCF binding sites within the VEGF promoter. $TGF\beta_1$ induction of the VEGF gene with a requirement for TCF has not previously been reported.

$TGF\beta_1$ can increase VEGF mRNA in a number of cell types including AKR-2B mouse embryonic fibroblasts, A549 human lung adnenocarcinoma cells [184], human keratinocytes (HaCaT) [185], vascular smooth muscle cells [51], human endothelial cells (HMECs), human hepatoma cells (Hep3B) [186], rat tubular epithelial cells (NRK52E) [187], mouse macrophage cells (WEHI3)

[40] and human cholangiocellular carcinoma cells (TFK-1) [179]. To determine if TGFβ$_1$ increased VEGF mRNA in PASMCs we measured VEGF mRNA by standard RT-PCR and quantitative real time PCR and showed that TGFβ$_1$ did induce VEGF mRNA. The data generated by real time PCR was variable and only a representative graph is shown. We believe this variability is due to a combination of a relatively small signal window, i.e. TGFβ$_1$ induced VEGF over basal VEGF levels, and an incredibly sensitive technique. The small signal window meant that even small differences in both VEGF Ct values and housekeeping gene β2-microglobulin Ct values created large differences in relative expression values. Furthermore, VEGF mRNA has been found to be unstable and rapidly degraded under normal conditions [52], a characteristic that is understandable for a gene that needs to be tightly regulated, and slight differences in stability between experiments may have contributed to the high error at later time points. We were unable to create VEGF mRNA stability data in our PASMCs due to the toxicity levels of the transcriptional inhibitor Actinomycin D which is shown in Appendix 1. While we were able to generate protein secretion data following long incubation with Actinomycin D, by normalising the concentrations generated to MTT assay results, the toxic effect of the compound on cells resulted in an insufficient amount of cell material to extract sufficient RNA for PCR analysis. Despite these issue TGFβ$_1$ induced VEGF mRNA was consistently increased after 1 hour incubation with 1ng/ml TGFβ$_1$.

Frequent accounts of TGFβ$_1$ induced VEGF mRNA requiring increased transcription exist [40, 179, 186] however TGFβ$_1$ can also regulate VEGF

mRNA levels post-transcriptionally by stabilising VEGF mRNA [188]. We incubated PASMCs for 30 minutes prior to TGFβ$_1$ stimulation with the inhibitor of transcription Actinomycin D, and showed at both the mRNA and protein level that inhibition of transcription abolished the TGFβ$_1$ mediated effect, suggesting TGFβ$_1$ was acting transcriptionally. This was substantiated by VEGF-promoter reporter gene experiments in which TGFβ$_1$ increased luciferase activity. Transient transfection studies of promoter reporter genes have their limitations. The DNA sequence ligated to the firefly gene is not the full native promoter sequence of the gene and possible important upstream regulatory sequences may be lost. Furthermore, the transfected DNA is not incorporated into the cells native chromatin structure and thus any regulation that depends on epigenetic mechanisms will not be observed. Indeed in some cases transfected constructs become sequestered in the cytoplasm, not even entering the nucleus. Due to this transfection studies are always used as a supportive method or as initial data confirmed by other techniques.

Next we showed, using a VEGF promoter reporter deletion series, that TGFβ$_1$ induced luciferase required a specific region of the reporter to be present, namely the 182bp region between the 318bp construct and the 135bp construct, which corresponds to -239 to -85bp upstream of the transcription start site on the native VEGF promoter. This section of the VEGF promoter contains at least 60 putative transcription factor binding sites (as established using the transcription element search system (TESS) http://www.cbil.upenn.edu/cgi-bin/tess/tess). To begin identifying which of these sites can be activated by TGFβ$_1$ signalling we used reporter constructs containing multiple repeats of

each individual transcription factor binding site. These have a further caveat to there use over the promoter reporters in that the high number of binding sites can cause the factor of interest to be sequestered to the reporter and away from its normal cellular role resulting in signalling defects and possible cell death. Thus it was important to optimise the amount of DNA transfected into cells, and, as previously, confirm results with alternative methods. We discounted Sp-1, AP2 and p53 from our investigations using this method, and determined a possible role for TCFs.

The TCFs form a subfamily of the high-mobility group (HMG)-box-containing superfamily of transcription factors and are grouped along with LEF1 in the TCF/LEF family [125]. Four members exist in the vertebrate family, TCF1, LEF1, TCF3 and TCF4. They all have slight variations in their structure but generally consist of four domains; an N-terminal β-catenin binding domain; a central domain; a well-conserved HMG DNA-binding domain, including a nuclear localisation signal; and a long C-terminal tail [125]. They canonically exist at the base of Wnt/β-catenin signalling. A more in depth discussion of this signalling will be necessary in future chapters, but for now, to determine if TCF signalling required association with Sp-1, AP2 or p53, we stimulated the reporter constructs with Wnt3a, a specific inducer of TCF signalling, to see if TCF could act in complex with one of the factors, to induce activity of these reporters. No such activity was observed and provided final closure on the possible role of Sp-1, AP2 and p53.

To provide an initial link between TGFβ_1 activation of TCF dependent transcription and a) the VEGF promoter and b) the specific 182bp region identified in deletion series studies, constructs were created by site directed mutagenesis that contained mutations of the TCF sites within the VEGF-318 Luc construct. Three constructs were created, the first with an A/G mutation at the 142 position, the second with a T/C mutation at the 110 position and the third contained both mutations. Mutation of either TCF site was sufficient to prevent TGFβ_1 induction of the reporter, confirming that the TCF sites specific to the VEGF promoter are required for TGFβ_1 induced VEGF production, and inferring that a single TCF site is sufficient for TGFβ_1 induction but that either site can be utilised. Interestingly when both TCF sites are mutated an increase in basal luciferase activity is seen. This suggests that the TCF sites play a role in the regulation basal VEGF levels. In agreement with this is ChIP data presented at the end of the chapter showing TCF4 association with the VEGF promoter under basal conditions. It is well reported that TCFs associate with promoters under basal conditions and exist in complex with transcriptional repressors, for example Groucho, which is known to recruit HDACs and repress transcription via deacetylation of histones within the chromatin structure [113, 116]. Thus it is possible that (Figure 5-17) in the absence of TGFβ_1 stimulation, TCF4, in association with co-repressors, occupies one of the TCF binding sites (wnt response elements (WRE)) and keeps basal VEGF levels restricted. Upon TGFβ_1 stimulation other factors are recruited to the TCF sites, in addition to further TCF, which allows the response to TGFβ_1. The TGFβ_1 mediated recruitment of transcriptional activators occurs without displacement of the co-factors which maintain restricted basal VEGF levels. It

is only when both TCF sites are removed in a false system (i.e. mutation of the sites) that TCF, in association with its co-factors, can no longer associate with the VEGF promoter and as such the repression of basal VEGF expression is lifted and basal luciferase levels increase. Furthermore, TGFβ$_1$ induction above the increased basal levels is not possible due an absence of TCF binding sites available for the TCF4 binding required for a TGFβ$_1$ mediated response. It would have been interesting to perform ChIP experiments on unstimulated PASMCs to establish whether co-repressors, such as Groucho and HDACs, are present at the VEGF promoter and whether TGFβ$_1$ stimulation regulated any such complex.

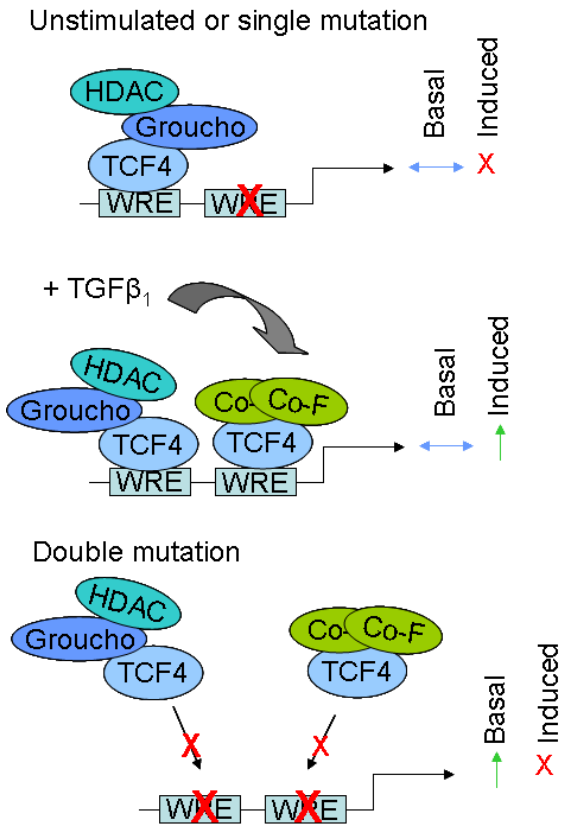


Figure 5-17 Schematic of the possible role of TCF4 in basal and TGFβ1 induced VEGF expression

In unstimulated cells or upon mutation of a single WRE, TCF4 in association with published co-repressors associates with the VEGF promoter to maintain a repressed level of VEGF expression (↔). Upon TGFβ_1 stimulation, the repression of basal VEGF transcription remains, while TGFβ_1 induces binding of increased TCF4 and possible other Co-factors (Co-F) to increase (↑) VEGF transcription. Mutation of both WREs prevents association of the repressor complex, causing increased basal expression, and prevents association of the TGFβ_1 induced complex, preventing an increase in induced transcription.

As mentioned above, there are 4 members of the TCF/LEF family which all bind equally well to the wnt response element (WRE). Here we used expression of a dominant negative TCF construct and ChIP to provide evidence for the specific role of TCF4 in the induction of VEGF by TGF$\beta_1$in PAMSCs. The dominant negative construct used was specific for TCF4, however it is possible that it interacted with and had dominant negative effects on different members of the TCF/LEF family and as such ChIP was performed with an antibody specific for TCF4 to ensure that it was TCF4 that associated with the native VEGF promoter. Use of primers designed specifically around the two TCF binding sites (WREs) of interest and also irrelevant 'upstream' primers went some way to ensuring that the binding of TCF4 was specific to the region we were interested in and was not just an intrinsic, non-specific, 'stickiness' of TCF4 to any DNA sequence.

Regulation of VEGF transcription requiring TCF4 has been described previously. For example two TCF binding sites at -805bp and -629bp upstream of the VEGF transcription start site were found to be necessary for K-ras activation of VEGF in Caco-2 human colonic adenocarcinoma cells [189],

while increased TCF4 expression is required for PGE_2 induction of VEGF in the human colon cell line, LS-174T [190]. In contrast our studies show a requirement for two TCF binding sites at -143bp and -111bp upstream of the VEGF transcription start site and increased binding of TCF4 to these sites independently of TCF4 protein levels. Further, $TGF\beta_1$ regulation of VEGF has not previously been shown involve TCF4 in any cell type.

In conclusion, in this chapter we have shown $TGF\beta_1$ to transcriptionally regulate VEGF via two TCF binding sites, or wnt response elements (WREs), and an increase in TCF4 binding to this site. We have also discussed the possibility of TCF/LEF family member's contribution to basal VEGF levels.

It is unlikely that binding of TCF4 alone to the VEGF promoter is sufficient to result in the VEGF response, especially as it is not a 'typical' transcription factor downstream of $TGF\beta_1$, but is better known for its role at the transcriptional end of Wnt/β-catenin signalling. As such the following two chapters will first investigate the role of TGFβ signalling in the process, followed by the role of Wnt/β-catenin signalling.

6 THE REQUIREMENT FOR SMADS IN TGFB INDUCED VEGF EXPRESSION

6.1 INTRODUCTION

In the previous chapter we showed that TGFβ_1 increased binding of TCF4 to the VEGF promoter and that this was necessary for TGFβ_1 induction of VEGF production. TCF/LEFs are however poor transcription factors by themselves, and although they are able to bind the consensus Wnt response element ((A/T)(A/T)CAA(A/T)GG) they require other co-factors to influence transcription [125].

Canonical TGFβ_1 signalling involves activation of Type I and Type II receptor serine/threonine kinases and subsequent phosphorylation of the intracellular signalling proteins Smad2 and/or Smad3 [76]. This phosphorylation causes Smad2/3 to lose affinity for the Type I receptor and associate with the Co-Smad, Smad4. The Smad2/3/4 complex then translocates to the nucleus [78]. In the nucleus Smads bind Smad response elements (SBEs) (CAGAC) to regulate transcription [83]. However due to the high occurrence of the SBE sequence in promoter sequences Smad binding to SBEs is of low specificity and affinity [81]. Further more, Smads can also modulate transcription without the presence of an SBE, by associating with other transcription factors at their binding site [86].

It therefore seemed likely that Smads might be co-factors required for TCF4 mediated regulation of VEGF in response to TGFβ_1 in PASMCs, with an association between TCF4 and Smads providing the point of interaction for Smads at the VEGF promoter.

In this chapter we tested the hypothesis that Smads are required for TGFβ_1 induced VEGF production from PASMCs.

6.2 AIMS

The aims of this chapter were to determine;

- if canonical TGFβ_1/Smad signalling is active in PASMCs
- if Smads have a signalling role in TGFβ_1 induction of VEGF
- if Smads associate with the native VEGF promoter at the region shown to bind TCF4 in the previous chapter
- if studies in wild type, Smad2 and Smad3 null mouse embryonic fibroblasts (MEFs) could corroborate the data generated in PASMCs

6.3 METHODS

6.3.1 Western Blot

Cells were stimulated with 1ng/ml TGFβ_1 for 0, 15, 30, 60, 120, 240 and 480 minutes.

Two wells of a six well plate were used per condition and samples were kept on ice. The antibodies used in this chapter were phospho (serine 165/167) Smad2 (#3101 CST) and total Smad2/3 (#3101 CST).

6.3.2 Specific Inhibitor of Smad3 studies

Cells were cultured to confluence in 24 well plates and serum starved for 24 hours in 500µl media. A 30 minute pre-incubation with the stated concentration of SIS3 or DMSO vehicle control was carried out prior to addition of TGFβ_1 at a final concentration of 1ng/ml. Concentrations of

inhibitor were made up such that the final concentration of DMSO in the media was the same for all SIS3 concentrations. With the studies in MEFs concentrations were normalised to cell counts due to difference in cell number required to achieve confluence in each cell line.

6.3.3 Chromatin Immunoprecipitation

The antibodies used in this chapter are Smad2 (phospho s465/467) (ab16509. Abcam), Smad3 (ab28379), Smad4 (06-693, Millipore) and normal rabbit IgG (AB-105-C, R and D Systems)

6.3.4 Transfections

6.3.4.1 PASMC Co-transfections

Confluent cells were serum starved for 7-9 hours and transfected with 1:2 ratio of DNA:Fugene HD for 14-16 hours. The medium was then removed and replaced with medium containing 10ng/ml TGFβ_1 for 24 hours.

6.3.4.2 MEF Standard transfection

60-80% confluent cells were serum starved for 7-9 hours and transfected using a 1:3 ratio of DNA:Fugene6 for between 14 and 16 hours. The medium was then replaced with medium containing 10ng/ml TGFβ_1 for 24 hours.

6.3.4.3 MEF Co-transfection for Smad recovery

60-80% confluent cells were serum starved for 7-9 hours and transfected using a 1:3 ratio of DNA:Fugene6 for between 14 and 16 hours. The medium was then replaced with serum free medium and the cells were left to express the Smad protein for 24 hours. Subsequently the medium was replaced with medium containing 10ng/ml TGFβ_1 for 24 hours.

6.4 RESULTS

6.4.1 Transcriptional regulation of VEGF by TGFβ$_1$ in PASMCs requires Smad2, Smad3 and Smad4

Canonical TGFβ$_1$ signalling involves activation of two types of receptor serine/threonine kinases which results in phosphorylation of the intracellular signal transducer Smad proteins. Smad2 and Smad3 are the Smads most commonly activated by phosphorylation in response to TGFβ$_1$. To confirm that canonical TGFβ$_1$ signalling is active in PASMCs we performed a western blot for phosphorylated Smad2 in whole cell lysates. There was minimal basal Smad2 phosphorylation in serum starved PASMCs, whilst 15 minutes stimulation with 1ng/ml TGFβ$_1$ increased Smad2 phosphorylation (Figure 6-1). Total Smad2/3 was used as a loading control. This result confirms canonical TGFβ$_1$ signalling is present and active in PASMCs.

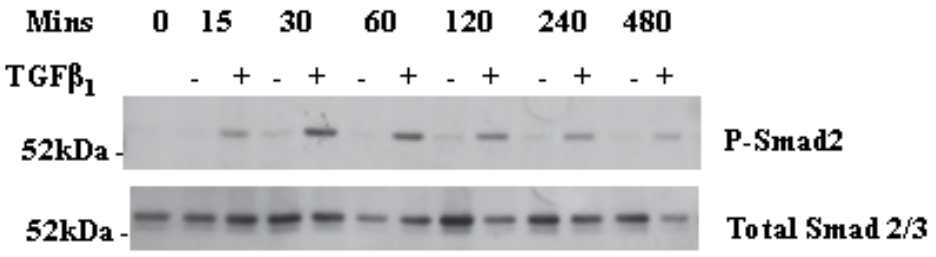


Figure 6-1 The effect of TGFβ1 on Smad2 phosphorylation

TGFβ$_1$ induced phosphorylated Smad2 protein. Total Smad2/3 was used as a house keeping control. Confluent, serum starved PASMCs were incubated with 1ng/ml TGFβ$_1$ across an 8 hour time course. Protein was extracted for western blot. The blots shown are representative of similar results achieved in 3 independent experiments.

These experiments show TGFβ$_1$ signalling to be active but do not infer a role for the Smad proteins in the regulation of VEGF in PASMCs. To determine whether at least one of the Smad proteins were involved in the TGFβ$_1$ regulation of VEGF in PASMCs we performed experiments using a Smad3 inhibitor (6,7-Dimethoxy-2-((2E)-3-(1-methyl-2-phenyl-1H-pyrrolo[2,3-b]pyridin-3-yl-prop-2-enoyl))-1,2,3,4-tetrahydroisoquinoline) (SIS3). This inhibitor abrogates Smad3 phosphorylation, prevents its association with Smad4 and reduces its DNA binding ability while having no effect on Smad2 phosphorylation, Smad4 and 7 protein levels or MAPK, ERK and PI3K phosphorylation [191]. TGFβ$_1$ induced a significant increase in VEGF production from PASMCs which was abolished by all concentrations of the SIS3 (Figure 6-2), suggesting Smad3 is critical to the signalling mechanism utilised by TGFβ$_1$. Little effect was seen on basal VEGF production.

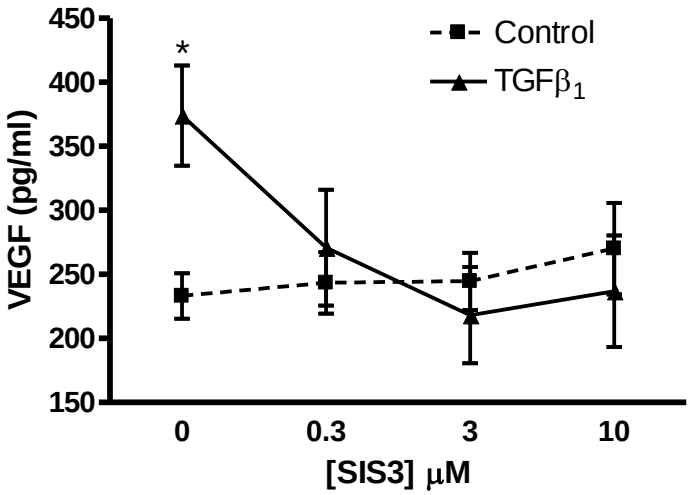


Figure 6-2 The effect of the Specific Inhibitor of Smad3 (SIS3) on VEGF production from PASMCs in response to TGFβ1

VEGF protein accumulation in the supernatants from PASMCs. Confluent PASMCs were serum starved for 24 hours and pre-incubated for 30 minutes with the stated concentration of SIS3, followed by 24 hour incubation with 1ng/ml TGFβ$_1$. Points represent the mean ± s.e.m

from triplicate samples in 2 individual experiments. (*, $p < 0.05$ by one way ANOVA with Bonferroni's post test).

Smad2 and Smad3, upon activation, associate with Smad4 and translocate to the nucleus. Phosphorylation of Smad3 and its association with Smad4 causes a conformational change in Smad3 and Smad4 protein structure which reveals their DNA binding domains allowing them to act as transcriptional regulators at the promoters of $TGF\beta_1$ responsive genes. Smad2 has a 30 base insertion within the region close to the DNA binding which prevents its direct association with DNA however it can be associated in a transcriptional complex [76, 78, 83].

Having shown that, at a minimum, Smad3 is critical to the induction of VEGF from PASMCs by $TGF\beta_1$, we next determined whether Smad3 or Smad2 were required for transcriptional regulation of VEGF. Consequently we co-transfected dominant negative Smad2 and dominant negative Smad3 constructs with the VEGF reporter construct, VEGF-318 Luc. When the control vector, pCMV5, was over expressed $TGF\beta_1$ increased luciferase activity, while dominant negative Smad2 or Smad3 abolished the $TGF\beta_1$ response (Figure 6-3). This suggests that both Smad2 and Smad3 play a role in induction of VEGF by $TGF\beta_1$ in PASMCs.

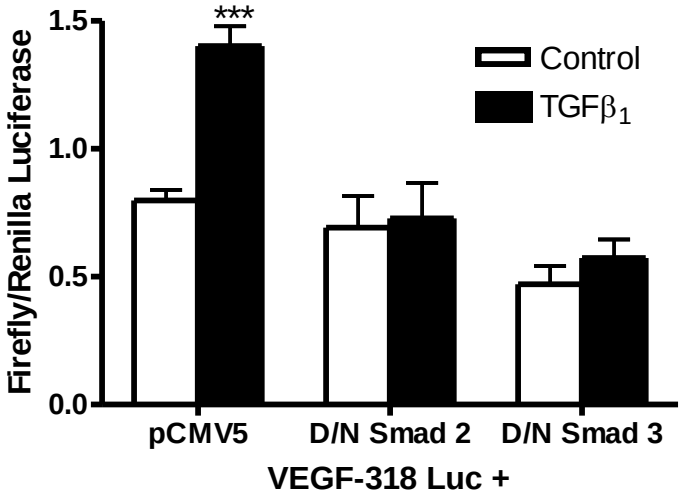


Figure 6-3 The effect of dominant negative Smad transfection on the VEGF-318 luciferase reporter

Levels of luciferase expression in PASMCs. Confluent serum starved PASMCs were transiently transfected with 0.2μg/well VEGF-318 Luc and 0.3μg/well of pCMV5, dominant negative Smad2 or dominant negative Smad3 and 1μl/well of Fugene HD for 16-20 hours. The medium was removed and replaced with medium containing 10ng/ml TGFβ$_1$. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. (***, $p < 0.001$ by one way ANOVA with Bonferroni's post test)

Subsequently we determined whether Smads were having a direct effect on TCF mediated transcription. We co-transfected the dominant negative Smad2 and Smad3 constructs with the TCF luciferase, Topglow, or its negative control, Fopglow. When the control vector pCMV5 was over expressed the Topglow, but not the Fopglow reporter, was able to respond to TGFβ$_1$ (Figure 6-4) as was shown in Chapter 5. However transfection of either dominant negative Smad2 or Smad3 abolished TGFβ$_1$ increased luciferase activity. This suggests that Smad2 and Smad3 are able to directly interfere with TCF induced transcription.

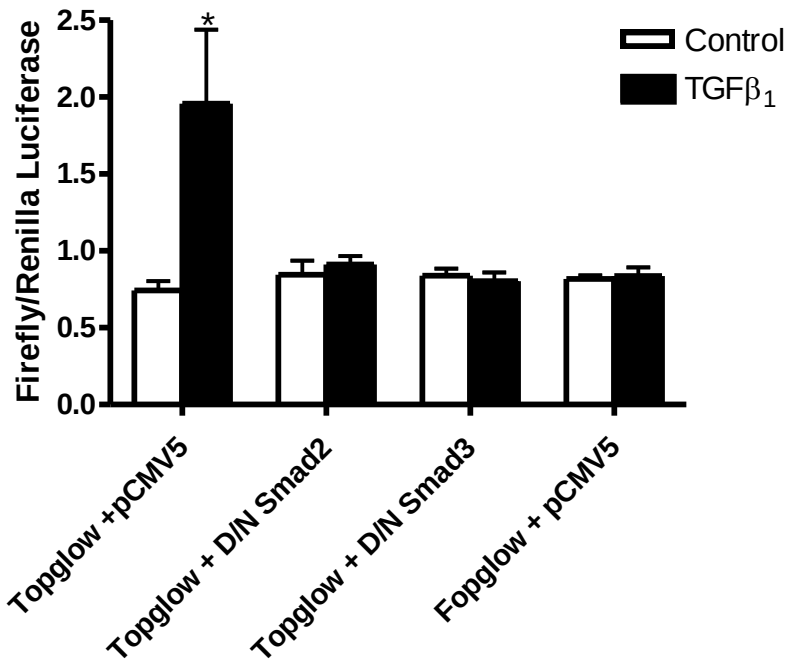


Figure 6-4 The effect of dominant negative Smad transfection on the TCF luciferase reporter, Topglow and the negative control reporter Fopglow.

Levels of luciferase expression in PASMCs. Confluent serum starved PASMCs were transiently transfected with 0.2μg/well Topglow or Fopglow and 0.3μg/well of pCMV5, dominant negative Smad 2 or dominant negative Smad 3 and 1μl/well of Fugene HD for 16-20 hours. The medium was removed and replaced with medium containing 10ng/ml TGFβ1. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. (*, $p < 0.05$ by one way ANOVA with Bonferroni's post test)

This data shows that Smad signalling is required for TGFβ_1 induction of VEGF in PASMCs. We subsequently determined the effect of Smads on the native VEGF promoter. To determine whether Smad2, Smad3 and Smad4 could associate with the 182bp region of the VEGF promoter shown to bind TCF4, ChIP was performed with antibodies for Smad2, Smad3, Smad4 and their corresponding control IgG. TGFβ_1 increased association of Smad2, Smad3 and Smad4 at the VEGF promoter (Figure 6-5). Some basal association of

Smad2 was also evident. VEGF upstream primers did not amplify any DNA suggesting Smad binding to the VEGF promoter is specific to the region of the VEGF promoter shown to bind TCF4 in Chapter (-1). Inputs show the presence of DNA prior to immunoprecipitation.

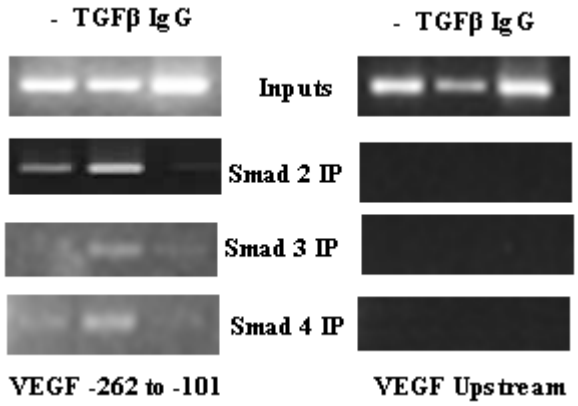


Figure 6-5 The effect of TGFβ1 on the binding of Smad2, Smad3 and Smad4 to the relevant 161bp section of the VEGF promoter

Representative PCR gels of Smad ChIP assays. Confluent PASMCs were serum starved for 24 hours followed by 2.5 hours incubation with or without 1ng/ml $TGF\beta_1$. Immunoprecipitation was carried out using antibodies to Smad2, Smad3, Smad4 and corresponding control IgG. The PCR primers were amplified in the -262 to -101 region of the VEGF promoter. VEGF upstream primers are a negative control for non-specific binding of the immunoprecipitated proteins to irrelevant regions of the promoter.

6.4.2 Studies in Smad2 and Smad3 knock-out mouse embryonic fibroblasts substantiate a role for Smad2 and Smad3 in $TGF\beta_1$ regulation of VEGF

Embryonic fibroblasts isolated from mice with knockout of a specific gene are a useful tool for establishing the role of a specific protein in a system or the cellular and molecular mechanism of a specific genes regulation. We obtained

Smad2 and Smad3 deficient mouse embryonic fibroblasts (MEFs) along with the corresponding wild type cells from Professor Erwin Böttinger. Initially we determined if VEGF was produced by these cells and if TGFβ$_1$ increased VEGF production in the wild type cells and if this induction was different in Smad2 and Smad3 deficient MEFs. A concentration response to TGFβ$_1$ was performed at 48 hours. Wild type MEFs released VEGF basally and VEGF was significantly increased by TGFβ$_1$, with a peak concentration observed at 5ng/ml TGFβ$_1$ (Figure 6-6). Smad2-/- MEFs had reduced basal levels of VEGF production but responded to TGFβ$_1$, again with a peak VEGF concentration in response to 5ng/ml TGFβ$_1$. However the peak level of VEGF produced by Smad2-/- was reduced in comparison to the wild type MEFs (Figure 6-6). Smad 3-/- MEFs produced very little basal VEGF and responded minimally to TGFβ$_1$ (Figure 6-6).

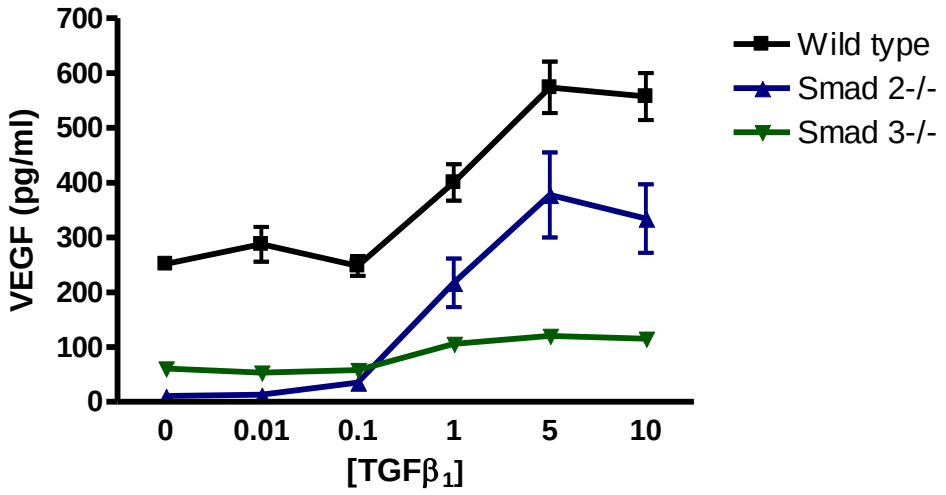


Figure 6-6 Concentration response of TGFβ1 on VEGF production from wild type, Smad2 deficient and Smad3 deficient mouse embryonic fibroblasts

VEGF protein accumulation in the supernatants from wild type, Smad2 deficient and Smad3 deficient MEFs. Confluent MEFs were serum starved for 24 hours and incubated for 48 hours with the stated concentration of TGFβ$_1$. Points represent the mean ± s.e.m from a minimum of

triplicate samples from three individual experiments. Concentrations are normalised to cell counts.

We next performed a time course to 1ng/ml TGFβ$_1$ (Figure 6-7). Wild type MEFs had the highest basal and TGFβ$_1$ induced VEGF expression at all time points, while Smad2-/- MEFs had a reduced basal level of VEGF production but responded to TGFβ$_1$ at all time points, and Smad3-/- MEFs had the lowest basal VEGF expression and showed minimal response to TGFβ$_1$ at any time point. This data agrees with the data in PASMCs using the Smad3 inhibitor which shows a necessity for Smad3 in TGFβ$_1$ mediated induction of VEGF.

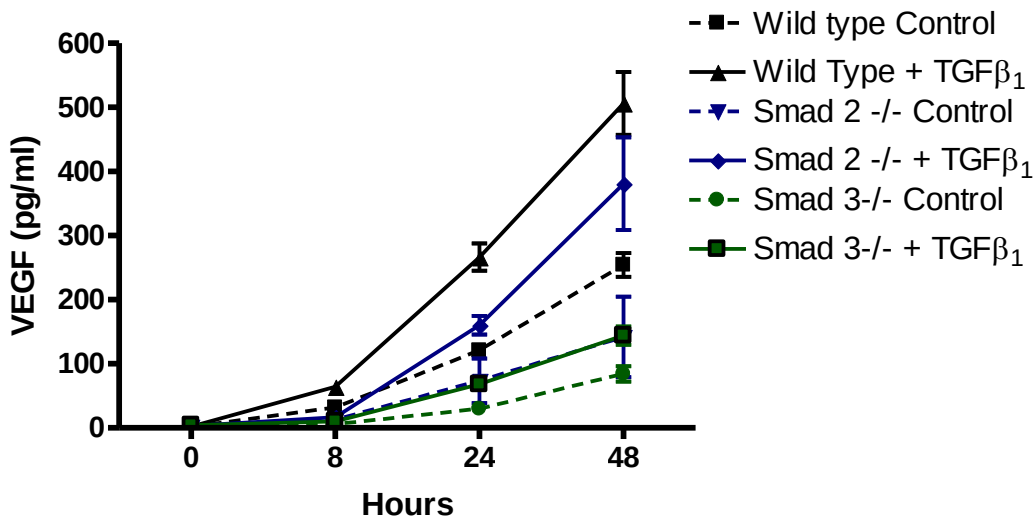


Figure 6-7 Time course of basal and TGFβ1 induced VEGF release from wild type, Smad2 deficient and Smad3 deficient mouse embryonic fibroblasts

VEGF protein accumulation in the supernatants from wild type, Smad2 deficient and Smad3 deficient MEFs. Confluent MEFs were serum starved for 24 hours and incubated for the stated time with 1ng/ml TGFβ1. Points represent the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. Concentrations are normalised to cell counts.

As the data generated in the MEFs showed similarities to the data generated using the Smad3 inhibitor in PASMCs we went on to study the effects of SIS3 in the MEF cell lines. As in PASMCs, SIS3 abolished the effect of TGFβ_1 on VEGF production from wild type MEFs, with little effect on basal VEGF production (Figure 6-8). Interestingly the SIS3 also prevented the increase in VEGF production seen in Smad2-/- cells (Figure 6-9) suggesting that the reduced level of VEGF production seen in these cells may be due to a level of compensation for loss of Smad2 by Smad3. As expected TGFβ_1 had no significant effect on VEGF production from Smad3-/- MEFs and the SIS3 had no effect on VEGF concentrations (Figure 6-10).

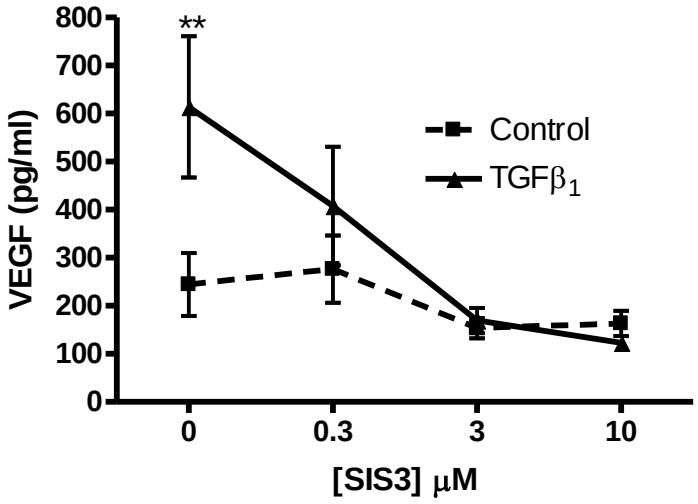


Figure 6-8 The effect of a Specific Inhibitor of Smad 3 (SIS3) on VEGF production from wild type mouse embryonic fibroblasts in response to TGFβ1

VEGF protein accumulation in the supernatants from wild type MEFs. Confluent MEFs were serum starved for 24 hours and pre-incubated for 30 minutes with the stated concentration of SIS3, followed by 24 hour incubation with 1ng/ml TGFβ_1. Points represent the mean ± s.e.m from triplicate samples in 2 individual experiments. Concentrations are normalised to cell counts. (**, $p<0.01$ by one way ANOVA with Bonferroni's post test).

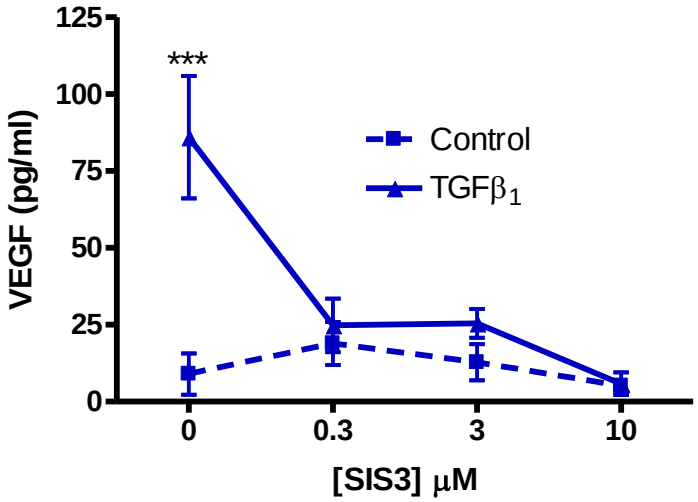


Figure 6-9 The effect of a Specific Inhibitor of Smad 3 (SIS3) on VEGF production from Smad2 deficient mouse embryonic fibroblasts in response to TGFβ1

VEGF protein accumulation in the supernatants from Smad2 deficient MEFs. Confluent Smad 2-/- MEFs were serum starved for 24 hours and pre-incubated for 30 minutes with the stated concentration of SIS3, followed by 24 hour incubation with 1ng/ml TGFβ$_1$. Points represent the mean ± s.e.m from triplicate samples in 2 individual experiments. Concentrations are normalised to cell counts. (***, $p < 0.001$ by one way ANOVA with Bonferroni's post test).

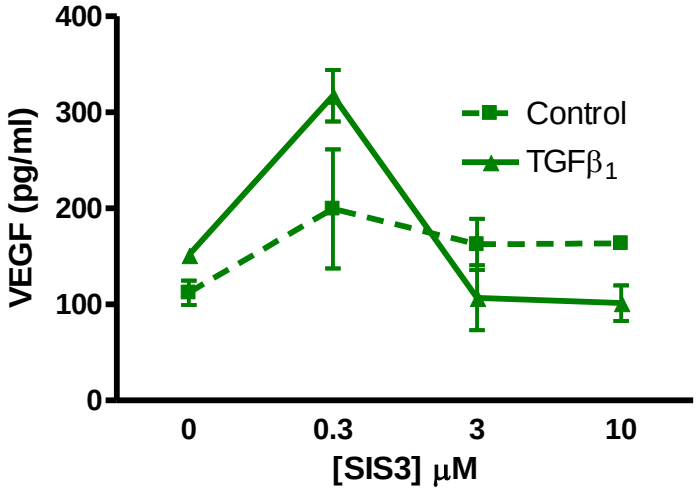


Figure 6-10 The effect of a Specific Inhibitor of Smad 3 (SIS3) on VEGF production from Smad3 deficient mouse embryonic fibroblasts in response to TGFβ1

VEGF protein accumulation in the supernatants from Smad3 deficient MEFs. Confluent Smad 3-/- MEFs were serum starved for 24 hours and pre-incubated for 30 minutes with the stated concentration of SIS3, followed by 24 hour incubation with 1ng/ml TGFβ_1. Points represent the mean ± s.e.m from triplicate samples in 2 individual experiments. Concentrations are normalised to cell counts. No significance was reached.

To determine if loss of Smad proteins would effect the ability of the VEGF promoter luciferase reporter to respond to TGFβ_1 and to test whether the TCF binding sites are as critical for responses in MEFs as in PASMCs, the VEGF-318 luciferase reporter and the TCF site mutant constructs were transfected initially into wild type MEFs. TGFβ_1 increased luciferase activity of the VEGF-318 reporter and this induction was abolished when one or both of the TCF binding sites were mutated (Figure 6-11). This agrees with the PASMC data.

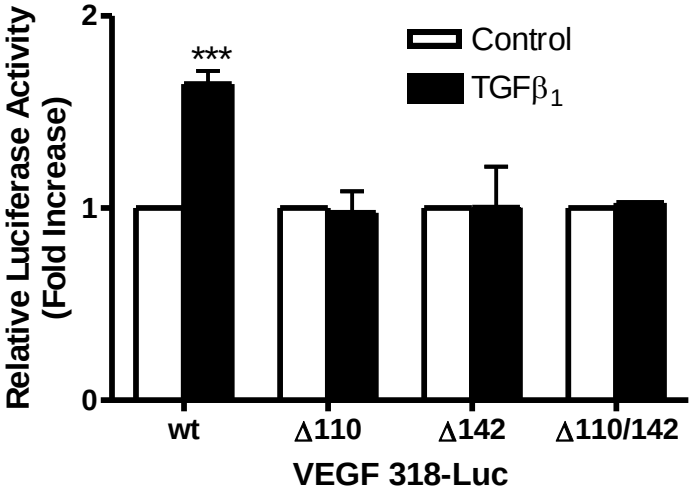


Figure 6-11 The effect of TGFβ1 on the wild type VEGF-318 luciferase reporter and the TCF site mutant VEGF-318 constructs in wild type mouse embryonic fibroblasts

Levels of luciferase expression in wild type MEFs. 60-80% confluent serum starved wild type MEFs were transiently transfected with either 0.5µg/well wild type (wt) VEGF-318 Luc,

0.5µg/well TCF binding site mutant at the 110 position (Δ110), 0.5µg/well TCF binding site mutant at the 142 position (Δ142) or 0.5µg/well TCF binding site mutant at both the 110 and 1422 position (Δ110/142) and 1.5µl/well of Fugene 6 for 16-20 hours. The medium was removed and replaced with medium containing 10ng/ml TGFβ_1. Each bar represents the fold increase in TGFβ_1 stimulated luciferase activity over basal luciferase activity. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. (*, $p < 0.05$; **, $p<0.01$; and ***, $p< 0.001$ by one way ANOVA with Bonferroni's post test)

Having determined that the VEGF-318 luciferase construct was activated in the wild type MEFs and was apparently under the same control with respect to the TCF sites as the PASMCs, we studied the effect of loss of Smad2 and Smad3 on the TGFβ_1 effect. TGFβ_1 did not activate any of the luciferase constructs in either the Smad2-/- or Smad3-/- MEFs (Figure 6-12) suggesting that both Smad2 and Smad3 are required for TGFβ_1 increased VEGF promoter luciferase activity. Further, a decrease in luciferase activity was seen in response to TGFβ_1 in the Smad3-/- MEFs with all but the double mutant construct.

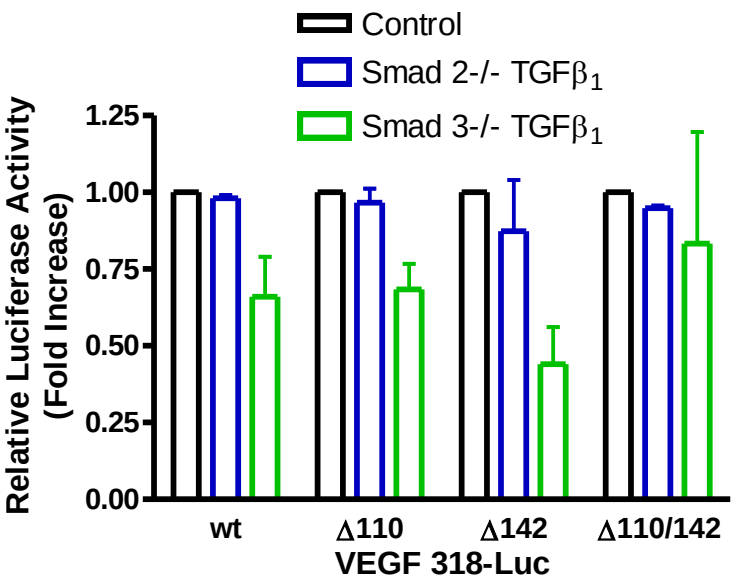


Figure 6-12 The effect of TGFβ1 on the wild type VEGF-318 luciferase reporter and the TCF site mutant VEGF-318 constructs in Smad2 and Smad3 deficient mouse embryonic fibroblasts

Levels of luciferase expression in Smad2 and Smad3 deficient MEFs. 60-80% confluent serum starved Smad 2-/- MEFs were transiently transfected with either 0.2µg/well wild type (wt) VEGF-318 Luc, 0.2µg/well TCF binding site mutant at the 110 position (Δ110), 0.2µg/well TCF binding site mutant at the 142 position (Δ142) or 0.2µg/well TCF binding site mutant at both the 110 and 142 position (Δ110/142) and co-transfected with 0.3µg/well control vector pCAAG and 1.5µl/well of Fugene 6 for 16-20 hours. Smad 3-/- MEFs were transiently transfected with either 0.2µg/well wild type (wt) VEGF-318 Luc, 0.2µg/well TCF binding site mutant at the 110 position (Δ110), 0.2µg/well TCF binding site mutant at the 142 position (Δ142) or 0.2µg/well TCF binding site mutant at both the 110 and 142 position (Δ110/142) and co-transfected with 0.4µg/well control vector pCAAG and 1.8µl/well of Fugene 6 for 16-20 hours. The medium was removed and replaced with medium containing 10ng/ml $TGF\beta_1$. Each bar represents the fold increase in $TGF\beta_1$ stimulated luciferase activity over basal luciferase activity. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. No significance was reached.

To determine if recovery of the 'missing' Smad could recover the TGFβ$_1$ effect and to determine if the TCF binding sites perform a similar function in the Smad2 and Smad3 deficient cells we performed co-transfection of the VEGF luciferase reporters and the 'missing' Smad. Recovery of the 'missing' Smad increased TGFβ$_1$ induced luciferase activity from the wild type VEGF-318 luciferase construct, while the TCF site mutants were still unable to respond (Figure 6-13). This agrees with data in the PASMCs using the TCF mutant transfections and dominant negative Smads to prevent activation of the Topglow TCF reporter. It also shows that the TCF binding sites are required for Smads to have a transcriptional effect in MEFs and suggests that Smads and TCF4 form a complex at the TCF binding site as apposed to Smads acting at a distinct site within the promoter.

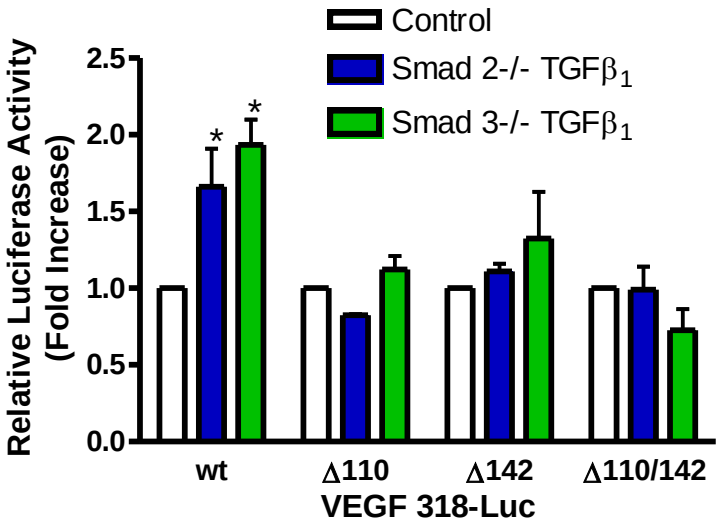


Figure 6-13 The effect of TGFβ1 on the wild type VEGF-318 luciferase reporter and the TCF site mutant VEGF-318 constructs in Smad2 and Smad3 deficient mouse embryonic fibroblasts following recovery of Smad expression

Levels of luciferase expression in Smad2 and Smad3 deficient MEFs. 60-80% confluent, serum starved Smad 2-/- MEFs were transiently transfected with either 0.2µg/well wild type

(wt) VEGF-318 Luc, 0.2µg/well TCF binding site mutant at the 110 position (Δ110), 0.2µg/well TCF binding site mutant at the 142 position (Δ142) or 0.2µg/well TCF binding site mutant at both the 110 and 142 position (Δ110/142) and co-transfected with 0.3µg/well Smad2 expression vector and 1.5µl/well of Fugene 6 for 16-20 hours. Smad 3-/- MEFs were transiently transfected with either 0.2µg/well wild type (wt) VEGF-318 Luc, 0.2µg/well TCF binding site mutant at the 110 position (Δ110), 0.2µg/well TCF binding site mutant at the 142 position (Δ142) or 0.2µg/well TCF binding site mutant at both the 110 and 142 position (Δ110/142) and co-transfected with 0.4µg/well Smad3 expression vector and 1.8µl/well of Fugene 6 for 16-20 hours. The medium was removed and replaced with medium containing 10ng/ml TGFβ_1. Each bar represents the fold increase in TGFβ_1 stimulated luciferase activity over basal luciferase activity. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. (*, $p < 0.05$ by one way ANOVA with Bonferroni's post test)

6.5 DISCUSSION

In Chapter 5 we found that TGFβ_1 transcriptionally regulated VEGF in PASMCs via a mechanism requiring increased binding of TCF4 to one of two TCF binding sites (or wnt response elements WREs) within a 182bp region of the human VEGF promoter. The major findings from the studies in the current chapter are that Smad2, Smad3 and Smad4 are also involved in the transcriptional regulation of VEGF by TGFβ_1 in PASMCs and also play a role in TGFβ_1 induction of VEGF in mouse embryonic fibroblasts (MEFs). We also show that the TCF binding sites are required in MEFs.

TCFs are poor transcription factors on their own; in fact as previously discussed they often act as transcriptional repressors in unstimulated cells [125]. Canonical TGFβ_1 signalling activates Smad proteins to act as

transcription factors or transcriptional regulators. Consequently we hypothesised that Smads may play a role, in association with TCF4, at the VEGF promoter in response to TGFβ_1 in PASMCs.

To confirm that canonical Smad signalling is present in PASMCs Smad2 phosphorylation was investigated by western blot. Phosphorylation of Smad2 was seen after 15 minutes and confirmed active signalling. Subsequently to provide evidence that TGFβ_1 activation of Smads was required for VEGF production from PASMCs an inhibitor of Smad3 was used. This inhibitor prevents the phosphorylation of Smad3 in response to TGFβ_1, its consequent association with co-Smad, Smad4, and also prevents its ability to bind consensus Smad binding elements (SBEs). It has been shown to have no effect on Smad2 phosphorylation or the expression levels of Smad4 and Smad7 (inhibitory Smad). Furthermore it does not effect phosphorylation by p38 MAP kinase, ERK or PI-3-kinase. As a result it is thought to be a 'specific inhibitor of Smad3' (SIS3) [191]. We used the Smad3 inhibitor in PASMCs and showed that even at low concentrations (0.3µM) it was able to abolish the TGFβ_1 mediated increase in VEGF. As the inhibitor has been shown to have no effect on Smad2 this data suggests that Smad3 is absolutely critical to the induction of VEGF by TGFβ_1 in PASMCs. However, when we used dominant negative Smads to help substantiate a role for Smads, both dominant negative Smad3 and Smad2 abolished TGFβ_1 induced increases in VEGF-318 luciferase reporter activity, suggesting both Smads are involved. As with all dominant negative constructs however, they can be unspecific and a dominant negative Smad can interact with and prevent signalling via other Smad proteins. As

such we performed ChIP using antibodies against Smad2, Smad3 and Smad4 to establish whether one or more of them were associated with the native VEGF promoter in PASMCs in response to TGFβ_1. We showed that binding of all three Smads increases upon TGFβ_1 stimulation, confirming that all three Smads play a role in VEGF transcription. A dominant negative Smad4 construct is also available [192] and it may have been beneficial to perform the dominant negative transfection experiments with this construct also. However such experiments would have been open to the same specificity issue as the dominant negative Smad2 and Smad3 experiments and as such we do not feel lack of these experiments detracts significantly from the data. Unfortunately we were not able to perform Re-ChIP, a technique in which following immunoprecipitation with one antibody the DNA is immunoprecipitated again with a second antibody to determine whether both proteins are present at the same time. As such it is possible that the proteins may be mutually exclusive and do not, in reality exist at the VEGF promoter in the same cell at the same time. However the Smad3 inhibitor data suggests that this is not the case and that at least Smad3 must be present for TGFβ_1 induction of VEGF transcription to occur. It would have been interesting to see what effect an inhibitor of Smad2 has on VEGF expression however such a compound does not exist and the dominant negative Smad2 was the best alternative. Furthermore Smad2 was associated with the VEGF promoter under basal conditions suggesting it may play a role in the regulation of basal VEGF production.

To assist in confirming a role for Smads in TGFβ_1 induced VEGF production we obtained Smad2 deficient and Smad3 deficient mouse embryonic

fibroblasts, along with the corresponding wild type cells from Erwin Böttinger's group. The wild type MEFs were able to produce VEGF basally and this was increased approximately 2.2 fold in response to TGFβ_1, similar to the 2.11 fold seen in PASMCs under the same conditions. Interestingly the Smad2 deficient cells produced a much lower basal level of VEGF but were still able to respond to TGFβ_1. This reduction in basal levels of VEGF production by Smad2 deficient MEFs could agree with the basal association of Smad2 with the VEGF promoter in PASMCs and the suggestion that Smad2 regulates basal VEGF promoter activity. However basal production of VEGF from Smad3 deficient MEFs is also reduced and no Smad3 is seen to be basally associated with the VEGF promoter, preventing a definitive conclusion for the role of Smads in basal VEGF production being reached. Smad3 deficient MEFs also responded very little to TGFβ_1 substantiating the critical role for Smad3 shown with the Smad3 inhibitor in PASMCs. Furthermore, the Smad3 inhibitor abolished responses to TGFβ_1 in both wild type and Smad2 deficient MEFs. The effect of the Smad3 inhibitor in Smad2 deficient MEFs suggests that the response to TGFβ_1 seen in these cells is due to compensation of Smad2 loss by Smad3. The fact that Smad3 deficient MEFs are unable to respond to TGFβ_1 suggests the same level of compensation is not achieved by Smad2 in Smad3 deficient MEFs. These studies in MEFs confirm a crucial role for Smad3 in TGFβ_1 induced production of VEGF and suggest that while Smad2 is involved it may not be as critical as Smad3 and can be, to some extent, compensated for by Smad3.

In addition to the protein data generated in the MEFs we determined if TGFβ_1 was able to induce the VEGF-318 luciferase reporter in the MEFs and whether the TCF sites were critical in this cell type also. The wild type, Δ110, Δ142 and Δ110/142 TCF site mutants of the VEGF-318 luciferase were transfected into the 3 cells lines. TGFβ_1 induced a significant increase in luciferase activity from the wild type 318 construct in the wild type MEFs and interestingly this was abolished when the TCF binding sites were mutated, suggesting the transcriptional regulation of VEGF in MEFs, by TGFβ_1 is also dependent on the TCF binding sites. It would have been interesting to perform chromatin immunoprecipitation in these cells to establish if increased binding of TCF4 to the VEGF promoter was also required, however time restraints prevented this. In both the Smad2 and Smad3 deficient cells an induction of the wild type or the mutated constructs was not seen. However upon recovery of the necessary Smad by co-transfection the response of the wild type 318 reporter was restored while the mutated constructs remained unstimulated. With respect to the data from these experiments generated in the Smad3 deficient cells the lack of induction in the absence of Smad3 is in agreement with previous experiments showing a critical role for Smad3 for example, the lack of protein secretion in response to TGFβ_1 in Smad3 deficient MEFs and the data generated with the Smad3 inhibitor. However, we expected to see a small induction of the VEGF-318 reporter in the Smad2 deficient MEFs as they were able to secrete increased amounts of protein in response to TGFβ_1. We believe the ability of Smad2 deficient cells to respond to TGFβ_1 at the protein level is due to compensation by Smad3 as the TGFβ_1 response is inhibited by the Smad3 inhibitor. We suggest that the inability of the VEGF-318 reporter to

respond to TGFβ_1 in Smad2 deficient cells is due to the inability of these false reporters to integrate into the native transcriptional machinery of the cell. It is likely that the compensatory mechanism of Smad3 requires regions of the promoter not present within the VEGF-318 promoter and/or regulation of transcriptional modulators only present in the native transcriptional complex, for example, modulation of histones, histone acetyltransferases or deacteylases. This can be supported by the frequent reports of Smad3 interaction with the transcriptional co-activators CREB binding protein (CBP) and p300 [193-196]. The determination of VEGF mRNA levels in response to TGFβ_1, by real time PCR in the MEFs would have further contributed to an explanation for the apparent differences between VEGF protein production and VEGF promoter activity in response to TGFβ_1 in the absence of Smad2. For example the increase in VEGF protein in response to TGFβ_1 may be independent of transcription and be due to translational effects.

The requirement for Smads in the regulation of VEGF in response to TGFβ_1 has been shown previously. Sànchez-Elsner *et al.*, showed Smad3 association with a Smad binding element (SBE) within a region of the VEGF promoter between -1006 and -954bp upstream of the transcription start site is required for VEGF transcription induced by TGFβ_1 in human epithelioid carcinoma HeLa cells and monkey kidney COS cells [186]. While Jeon *et al.*, also showed Smad3 association with one of two SBEs was required for TGFβ_1 induced VEGF transcription in mouse macrophages via a similar region of the mouse VEGF promoter (-935 to -916bp) [40]. These previous studies agree with our observation that Smad3 appears to the more critical Smad protein

involved, however we see association at a region of the promoter that is previously unreported and a region that does not contain a SBE. The data generated from the TCF site mutants in the Smad deficient MEFs suggests that TCF4 association with TCF binding sites is a prerequisite for Smad association and the fact that no SBE is present suggests that Smad association with the VEGF promoter in PASMCs is indirect, via TCF. In addition the ability of dominant negative Smads to prevent TGFβ_1 activation of TCF dependent transcription (i.e. the Topglow reporters) suggests that TCF and the Smads are able to interact with other. This is also substantiated by our ChIP data in PASMCs in which the primers were specifically designed to cover a small region of the promoter and suggests that the Smads and TCF4 associate within close proximity of each other. It would have been ideal if Re-ChIP could have been used to confirm definite association between the proteins at the VEGF promoter but the sensitivity of this technique was not optimised in time for the experiments to be performed.

In conclusion the studies in this chapter have shown the transcriptional regulation of VEGF by TGFβ_1 in PASMCs to require Smad2, Smad3 and Smad4 association with the VEGF promoter. In addition the ability of the Smad protein to transduce their effects depends on the presence of TCF binding sites and probably a direct interaction with TCF4. There are no previous reports of TGFβ_1 regulation of VEGF transcription via an interaction with TCFs.

7 THE ROLE OF B-CATENIN AND GSK3B IN TGFB INDUCED VEGF EXPRESSION

7.1 INTRODUCTION

In Chapter 5 we showed that TGFβ_1 transcriptional regulation of VEGF in PASMCs required one of two TCF binding sites and increased binding of TCF4 to those sites. In Chapter 6 we explained that TCF/LEFs alone are rarely able to initiate transcription themselves and require interaction with other transcriptional modulators. We focused on establishing a role for the signalling elements downstream of TGFβ_1 and showed involvement of Smads 2, 3 and 4 in the regulation of VEGF by TGFβ_1 in PASMCs.

In the current chapter we investigate the signalling components canonically upstream of TCF/LEFs, namely the Wnt/β-catenin signalling pathway. The Wnt family of proteins are the most well known stimulators of this pathway, and β-catenin is the signalling protein which transduces the signal between the cytoplasm and nucleus. Once in the nucleus β-catenin can associate with TCF/LEFs to initiate transcription of several genes including *c-myc, c-jun* and *cyclin D1* [113]. In the absence of Wnt, β-catenin is contained in a 'destruction complex' consisting of β-catenin, Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α). While in this complex β-catenin is phosphorylated at Ser45 by CK1α and Thr41, Ser37, Ser33, Asp32 and Gly34 by GSK3β [113]. β-catenin phosphorylated at Ser33/37 is recognised by the substrate recognition subunit (β-TrCP) for the E3 ubiquitin ligase complex [112]. Subsequently β-catenin is ubiquitinated and degraded by the proteosome. Destruction of β-catenin prevents its translocation into the nucleus and removes its ability to regulate transcription. Upon Wnt stimulation a poorly characterised series of signalling events results

in the prevention of β-catenin phosphorylation. This is thought to be due to either removal of phosphate groups by phosphatases such as PP2A, or inhibition of the constitutively active kinase GSK3β [117]. Once dephosphorylated, β-catenin is no longer degraded and can accumulate in the cytoplasm. Cytoplasmic accumulation allows β-catenin translocation to the nucleus where it associates with TCF/LEF to regulate transcription.

Regulation of VEGF transcription via accumulation and nuclear translocation of β-catenin due to inhibition of GSK3β has been seen previously in human umbilical vein endothelial cells in response to Ginsenoside-Rg1, the most prevalent active constituent of ginseng [197]. Furthermore, TGFβ_1 has been shown to induce β-catenin accumulation in desmoid cells [198] and to induce β-catenin nuclear translocation in a Smad3 dependent manner in bone marrow-derived adult human mesenchymal stem cells [199]. Thus we postulated that the TGFβ_1 and β-catenin signalling pathways might interact, upstream of TCF, to regulate VEGF expression.

In this chapter we investigate the hypothesis that the β-catenin and GSK3β pathway is modulated by TGFβ_1 in PASMCs and that this contributes to VEGF expression.

7.2 AIMS

The aims of this chapter were to determine:

- if β-catenin can associate with the VEGF promoter
- if TGFβ_1 can regulate β-catenin function

- if TGFβ_1 regulation of β-catenin affects the ability of β-catenin to associate with the VEGF promoter.
- if TGFβ_1 mediated regulation of β-catenin involves changes in GSK3β activity
- if regulation of GSK3β if required for VEGF expression in response to TGFβ_1

7.3 METHODS

7.3.1 Western Blot

7.3.1.1 Whole cell lysates

Cells were stimulated for 0, 15, 30, 60, 120, 240 and 480 minutes with 1ng/ml TGFβ_1. Two wells of a six well plate were used per condition and samples were kept on ice. The antibodies used in this chapter were total β-catenin (clone H-102, sc-7199), GAPDH (OBT 1636), and total GSK3β (CST 9315).

7.3.1.2 Nuclear/Cytoplasm extracts

Confluent, serum starved cells were stimulated for 4 hours or 0, 1 and 2 hours with either 1ng/ml TGFβ_1 or 10μM SB216763. The antibodies used in this chapter were Lamin A/C (sc-7292), unphosphorylated β-catenin (ab19451), phosphor-ser33/37/thr41-β-catenin (CST 9561), total β-catenin (clone H-102, sc-7199), total GSK3β (CST 9315) and phosphor-ser9-GSK3β (CST 9561).

7.3.2 Co-immunoprecipitations

Two T225cm^2 flasks were used per condition. The antibodies used for immunopreciption were Smad2 (CST 3122) and total GSK3β (CST 9315).

The antibodies used for the immunoblots were total β-catenin (clone H-102, sc-7199) and total GSK3β (CST 9315).

7.3.3 Chromatin Immunoprecipitation

The antibodies used in this chapter were total β-catenin (Millipore, 06-734), unphosphorylated β-catenin (ab19451), phosphor-ser33/37/thr41-β-catenin (CST 9561) and total GSK3β (CST 9315).

7.3.4 GSK3β Inhibitor Studies

Cells were cultured to confluence in 24 well plates and serum starved for 24 hours in 500µl media. A 30 minute pre-incubation with the stated concentration of SB-216763 or DMSO vehicle control was carried out prior to addition of $TGF\beta_1$ into the required wells at a final concentration of 1ng/ml. Concentrations of inhibitor were made up so that the final concentration of DMSO in the media was the same for all SB-216763 concentrations. An MTT assay was performed on all occasions.

7.3.5 Transfection

7.3.5.1 Standard Transfection

Confluent PASMCs were serum starved for 14-16 hours and transfected with a 1:2 ratio of DNA: LF2000 for 2 hours. The medium was then removed and replaced with medium containing 10µM SB-216763 for 3.5 hours.

7.3.5.2 Co-transfection

Confluent cells were serum starved for 7-9 hours and transfected with 1:2 ratio of DNA: Fugene HD for 14-16 hours. Medium was then removed and replaced with medium containing 10mg/ml $TGF\beta_1$ for 24 hours.

7.4 RESULTS

7.4.1 β-Catenin Associates with the VEGF promoter in response to TGFβ1

Due to the requirement for β-catenin in TCF mediated regulation of transcription in various cell and system contexts [112, 117, 124, 125], we investigated whether β-catenin associated with the VEGF promoter in PASMCs and if TGFβ1 was able to modulate β-catenin association. In contrast to TCF4, β-catenin was not basally associated with the VEGF promoter suggesting there is no basal β-catenin/TCF signalling at the VEGF promoter (Figure 7-1). β-catenin association with the VEGF promoter was induced by 2.5 hour stimulation with 1ng/ml TGFβ1. No amplification was seen when using the 'upstream' primers suggesting β-catenin association is specific and within the same region of the promoter as TCF4 and Smad2, Smad3 and Smad4.

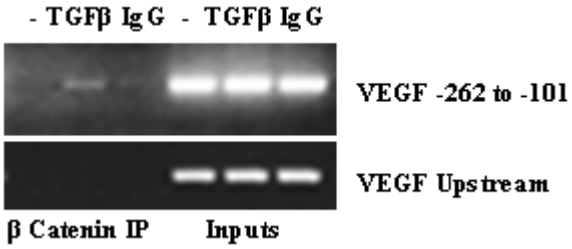


Figure 7-1 The effects of TGFβ1 on β-catenin binding to the relevant 161-bp region of the VEGF promoter

Representative PCR gel of a total β-catenin ChIP assay. Confluent PASMCs were serum starved for 24 hours followed by 2.5 hours incubation with and without TGFβ1. Immunoprecipitation was carried out using an antibody to β-catenin or relevant control IgG. The PCR primers were amplified in the -262 to -101 region of the VEGF promoter. VEGF Upstream primers were used as a negative control for non specific binding of the immunoprecipitated proteins to irrelevant regions of the promoter.

7.4.2 TGFβ$_1$ does not regulate β-catenin stability or cellular location

Canonically, regulation of transcription by the TCF/β-catenin complex requires inhibition of β-catenin degradation and accumulation of β-catenin in the cytoplasm, followed by its nuclear translocation. Once in the nucleus β-catenin can associate with TCF to regulate transcription [112, 117]. We investigated whether TGFβ$_1$ was able to prevent β-catenin degradation and allow its accumulation. Whole cell lysates were taken from a time course to TGFβ$_1$ and separated by SDS-PAGE. The resulting membrane was probed with a total β-catenin antibody and a loading control GAPDH antibody. TGFβ$_1$ did not increase whole cell β-catenin levels (Figure 7-2). In addition, the GSK3β inhibitor did not induce β-catenin protein levels.

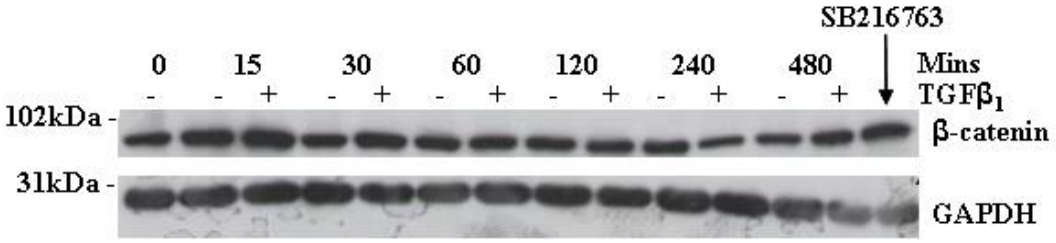


Figure 7-2 The effect of TGFβ1 on β-catenin protein levels over time

TGFβ$_1$ induced β-catenin protein levels. GAPDH was used as a house keeping control. Confluent, serum starved PASMCs were incubated with 1ng/ml TGFβ$_1$ across an 8 hour time course. GSK3β inhibitor, SB216763, was used a β-catenin inducing positive control. Protein was taken for western blot. The blots shown are representative of similar results achieved in 3 independent experiments.

Having shown that TGFβ$_1$ was not regulating whole cell levels of β-catenin in PASMCs we next determined if TGFβ$_1$ was contributing to the nuclear translocation of β-catenin. Nuclear and cytoplasmic fractions were isolated from confluent PASMCs that were either unstimulated or stimulated for 4 hours with 1ng/ml TGFβ$_1$ or 10μM SB216763 GSK3β inhibitor. The fractions were separated by SDS-PAGE and the membrane probed with a total β-catenin antibody and the nuclear marker Lamin A/C. TGFβ$_1$ did not increase the nuclear levels of β-catenin (Figure 7-3). There was a high basal level of nuclear β-catenin in PASMCs and the GSK3β inhibitor did not induce further nuclear translocation.

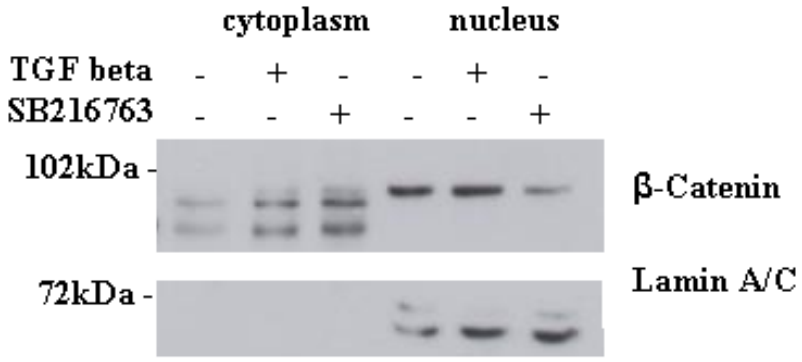


Figure 7-3 The effect of TGFβ1 on the cellular location of β-catenin

TGFβ1 induced β-catenin in cytoplasm and nuclear extracts from PASMCs. Lamin A/C was used as a nuclear marker and nuclear loading control. Confluent serum starved PASMCs were incubated with 1ng/ml TGFβ$_1$, 10μM SB216763 or left unstimulated for 4 hours. Protein was extracted for western blot. Probing shown is with Santa Cruz antibody sc-7199 against amino acids 680-781 of the human β-catenin C terminus. The blots shown are representative of similar results achieved in 3 independent experiments.

7.4.3 TGFβ1 regulates β-catenin phosphorylation status

Guger and Gumbiner [200] showed modulation of β-catenin phosphorylation can regulate its signalling activity without a coinciding accumulation of β-catenin. β-catenin that is not phosphorylated at N-terminal serines 33/37/45 was shown to be more active. We therefore probed our nuclear/cytoplasm extracts with an antibody against unphosphorylated ser33/37 of β-catenin. TGFβ1 significantly increased the level of unphosphorylated β-catenin (Figure 7-4). The extent of regulation shown by TGFβ1 was similar to the effect of the GSK3β inhibitor, SB216763, suggesting TGFβ1 may be modulating its effects via GSK3β.

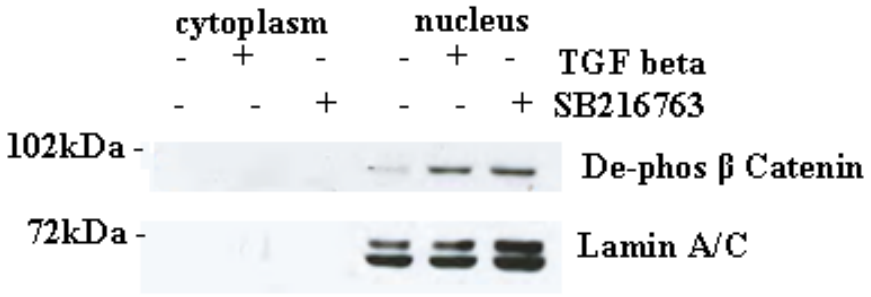


Figure 7-4 The effect of TGFβ1 on dephosphorylated, active, β-catenin

TGFβ1 induced dephosphorlyated β-catenin in cytoplasm and nuclear extracts from PASMCs. Lamin A/C was used as a nuclear marker and nuclear loading control. Confluent serum starved PASMCs were incubated with 1ng/ml TGFβ1, 10μM SB216763 or left unstimulated for 4 hours. Protein was extracted for western blot. Probing shown is with Abcam antibody ab19451 against dephosphorylated amino acids 27-37 of β-catenin. The blots shown are representative of similar results achieved in 3 independent experiments.

To confirm the change in phosphorylation detected by the unphosphorylated β-catenin antibody was real and not an antibody effect we probed the nuclear/cytoplasm extracts with an antibody against phosphorylated

ser33/37/Thr41. There was basal β-catenin phosphorylation in unstimulated cells and the level of phosphorylation was decreased in both TGFβ$_1$ and GSK3β inhibitor stimulated cells (Figure 7-5). This is consistent with Figure 7-4 in which little unphosphorylated β-catenin was present under basal conditions but unphosphorylated β-catenin was induced by TGFβ$_1$ and SB216763 stimulation.

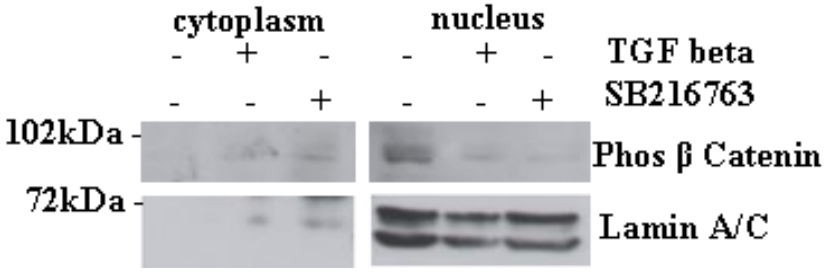


Figure 7-5 The effect of TGFβ1 on phosphorylated, inactive, β-catenin

TGFβ1 induced phosphorylated β-catenin in cytoplasm and nuclear extracts from PASMCs. Lamin A/C was used as a nuclear marker and nuclear loading control. Confluent serum starved PASMCs were incubated with 1ng/ml TGFβ$_1$, 10μM SB216763 or left unstimulated for 4 hours. Protein was extracted for western blot. Probing shown is with Cell Signalling Technology antibody #9561 against phosphorylated amino acids ser33/37/thr41 of β-catenin. The blots shown are representative of similar results achieved in 3 independent experiments.

7.4.4 Dephosphorylated but not phosphorylated β-catenin associates with the VEGF promoter in response to TGFβ$_1$

Having shown that TGFβ$_1$ regulated β-catenin phosphorylation status we next determined if this was relevant to VEGF transcription. Having showed that total β-catenin associates with the VEGF promoter (**Error! Reference source not found.**) we next determined whether the β-catenin associated with the

VEGF promoter was phosphorylated or not. Chromatin immunoprecipitation showed binding of unphosphorylated β-catenin (Figure 7-6) but not phosphorylated β-catenin (Figure 7-7) to VEGF promoter after TGFβ_1 stimulation suggesting that the β-catenin associated with the VEGF promoter is dephosphorylated.

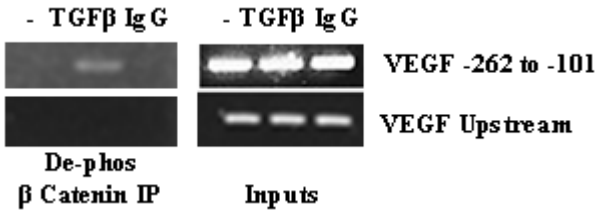


Figure 7-6 The effect of TGFβ1 on dephosphorylated β-catenin association with the relevant 161-bp region of the VEGF promoter

Representative PCR gel of a dephosphorylated β-catenin ChIP assay. Confluent PASMCs were serum starved for 24 hours followed by 2.5 hours incubation with and without TGFβ_1. Immunoprecipitation was carried out using an antibody to dephosphorylated β-catenin or relevant control IgG. The PCR primers were amplified in the -262 to -101 region of the VEGF promoter. VEGF Upstream primers were used as a negative control for non specific binding of the immunoprecipitated proteins to irrelevant regions of the promoter.

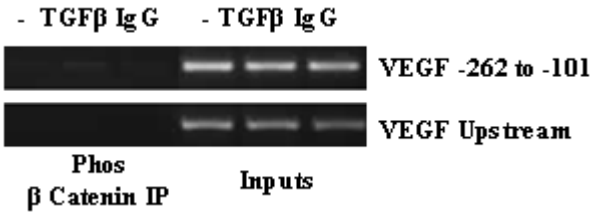


Figure 7-7 The effect of TGFβ1 on phosphorylated β-catenin association with the relevant 161-bp region of the VEGF promoter

Representative PCR gel of a phosphorylated β-catenin ChIP assay. Confluent PASMCs were serum starved for 24 hours followed by 2.5 hours incubation with and without TGFβ_1. Immunoprecipitation was carried out using an antibody to phosphorylated β-catenin or relevant control IgG. The PCR primers were amplified in the -262 to -101 region of the VEGF

promoter. VEGF Upstream primers were used as a negative control for non specific binding of the immunoprecipitated proteins to irrelevant regions of the promoter.

7.4.5 TGFβ$_1$ inhibits GSK3β activity independently of total GSK3β levels or cellular location

Glycogen synthase kinase (GSK) 3β is present within the β-catenin destruction complex and mediates β-catenin degradation by phosphorylating β-catenin on serine-33, serine-37 and threonine-41. We have shown regulation of phosphorylation of these sites on β-catenin by TGFβ$_1$ and were interested to determine if TGFβ$_1$ was able to negatively regulate GSK3β in PASMCs. Initially we investigated whether TGFβ$_1$ altered total GSK3β levels in the PASMCs to result in β-catenin dephosphorylation. Confluent PASMCs were incubated with 1ng/ml TGFβ$_1$, lysed, and whole cell lysates separated by SDS-PAGE. The resulting membrane was probed with total GSK3β antibody and a GAPDH loading control. TGFβ$_1$ had no effect on the levels of GSK3β expressed in PASMCs (Figure 7-8).

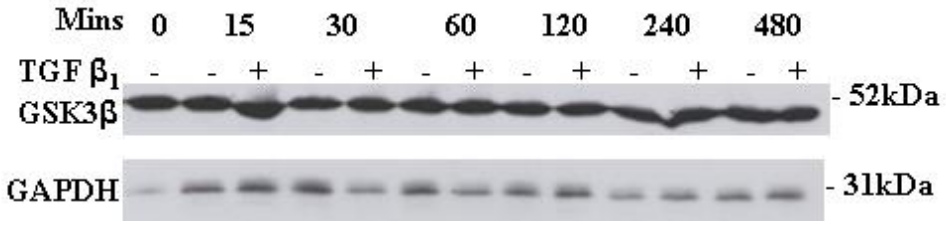


Figure 7-8 The effect of TGFβ1 on GSK3β protein levels over time

TGFβ$_1$ induced GSK3β protein levels. GAPDH was used as a house keeping control. Confluent, serum starved PASMCs were incubated with 1ng/ml TGFβ$_1$ across an 8 hour time course. Protein was taken for western blot. The blots shown are representative of similar results achieved in 3 independent experiments.

Having shown no effect of TGFβ$_1$ on whole cell GSK3β levels we investigated TGFβ$_1$ effects on GSK3β cellular location. A TGFβ$_1$ time course (0, 1 and 2 hours) was performed on serum starved confluent PASMCs and nuclear and cytoplasmic fractions were extracted, separated by SDS-PAGE and probed for total GSK3β and the nuclear marker and loading control Lamin A/C. GSK3β was constitutively present in the nucleus of PASMCs and this was not altered by TGFβ$_1$ (Figure 7-9 top blot). Finally we determined if TGFβ$_1$ decreased the intrinsic and constitutive kinase activity of GSK3β. Phosphorylation of serine 9 of GSK3β results in its inhibition. Nuclear/cytoplasmic extracts were reprobed with an antibody against phospho-serine 9-GSK3β. TGFβ$_1$ increased the level of phosphorylated serine9 on GSK3β (Figure 7-9 middle blot). This data suggests that while TGFβ$_1$ does not regulate GSK3β whole cell levels or cellular location it can inhibit its kinase activity causing a resultant increase in unphosphorylated β-catenin.

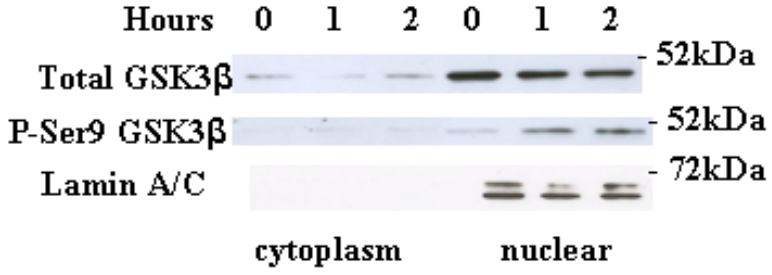


Figure 7-9 The effect of TGFβ1 on GSK3β cellular location and phosphorylation of GSK3β-ser9

TGFβ$_1$ induced GSK3β in cytoplasm and nuclear extracts from PASMCs. Lamin A/C was used as a nuclear marker and nuclear loading control. Confluent serum starved PASMCs were incubated with 1ng/ml TGFβ$_1$ for 1 and 2 hours or left unstimulated. Protein was extracted for

western blot. The blots were probed for total GSK3β and phosphor ser9 GSK3β. The blots shown are representative of similar results achieved in 3 independent experiments.

7.4.6 GSK3β inhibition is required for TGFβ$_1$ mediated VEGF expression

The previous data shows that TGFβ$_1$ regulates GSK3β activity but does not necessarily implicate GSK3β in the specific regulation of VEGF by TGFβ$_1$. We therefore investigated the effects of GSK3β modulation on VEGF expression.

SB-216763 is a well characterised selective inhibitor of GSK3β. We examined whether SB-216763 could regulate VEGF protein secretion. As the constitutively active GSK3β acts to phosphorylate β-catenin, we expected it to prevent β-catenin association with the VEGF promoter and inhibit TGFβ$_1$ induced VEGF production. We hypothesised that GSK3β inhibition would increase VEGF protein production. Confluent, serum starved PASMCs were pre-incubated with increasing concentrations of SB-216763 for 30 minutes prior to 24 hours with or without TGFβ$_1$. SB-216763 had no effect on TGFβ$_1$ induced VEGF production but increased control VEGF production, to the level of TGFβ$_1$ induced VEGF at the highest SB216763 concentrations (Figure 7-10). This suggests that GSK3β is involved in the production of VEGF by PASMCs and that TGFβ$_1$ acts upstream of GSK3β as the inhibitor did not modulate the TGFβ$_1$ induced effect.

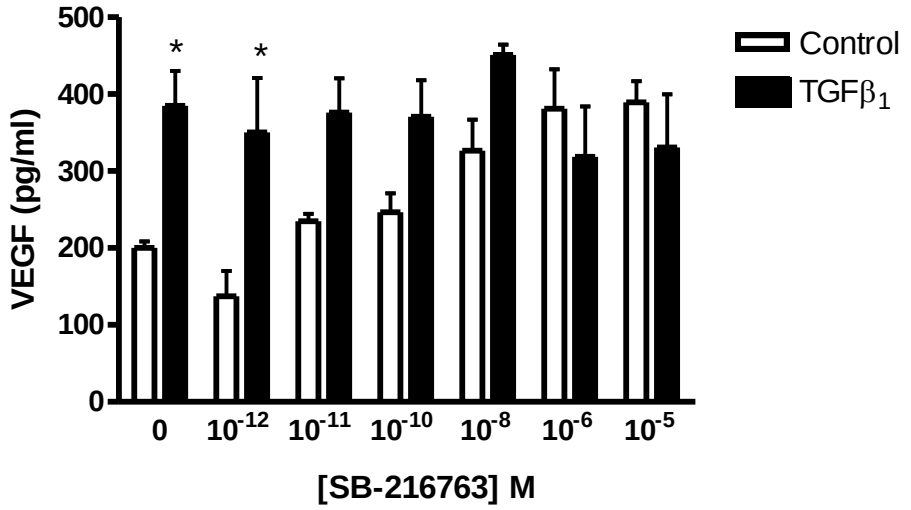


Figure 7-10 The effect of the GSK3β inhibitor, SB216763, on VEGF production from PASMCs in response to TGFβ1

VEGF protein accumulation in the supernatants from PASMCs. Confluent PASMCs were serum starved for 24 hours and preincubated for 30 minutes with the stated concentration of the GSK3β inhibitor, SB216763, followed by 24 hour incubation with 1ng/ml TGFβ1. Bars represent the mean ± s.e.m from triplicate samples in 2 individual experiments. (*, $p < 0.05$ by one way ANOVA with Bonferri's post test)

To further substantiate a role for GSK3β in the regulation of VEGF in PAMSCs we investigated the effect GSK3β inhibition on VEGF promoter luciferase reporter activity. The 318 bp reporter was used to provide evidence for a role of GSK3β at the site of transcriptional regulation. Confluent, serum starved PASMCs were transfected with the 318bp-VEGF luciferase reporter and incubated for 3.5 hours with 10μM SB216763. An increase in luciferase activity in response to the GSK3β inhibitor was seen (Figure 7-11) suggesting that GSK3β influences the transcriptional regulation of VEGF.

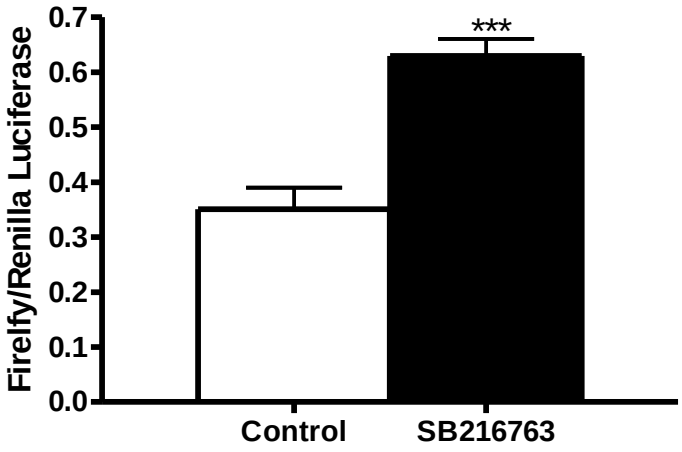


Figure 7-11 The effect of the GSK3β inhibitor, SB216763, on the transiently transfected VEGF-318 promoter luciferase reporter

Levels of luciferase expression in PASMC. Confluent serum starved PASMCs were transiently transfected with 1µg/well VEGF-318 luciferase and 2µl/well LF2000 for 2 hours. The medium was removed and replaced with medium containing 10µM SB216763 for 3.5 hours. Bars represent the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. (***, $p < 0.001$ by one way ANOVA with Bonferri's post test)

As in Chapter 6 we used mouse embryonic fibroblasts generated from mice with Smad proteins removed as a tool for investigating the role of Smads. Similarly wild type and GSK3β deficient (GSK3β -/-) MEFs were kindly provided by Dr James Woodgett of the Samuel Lunenfeld Research Institute, Toronto. As wild type MEFs had been shown in previous experiments to respond well to 1ng/ml TGFβ_1 (Chapter 6 Figure 6-6) we performed a time course in the wild type and GSK3β-/- MEFs and collected the resulting supernatants for ELISA. VEGF concentrations were normalised to cell number. Wild type MEFs produced VEGF basally and VEGF concentrations were significantly increased by TGFβ_1 at 4 hours and later, slightly earlier than the TGFβ_1 response in PASMCs. GSK3β-/- MEFs had a dramatically

increased basal level of VEGF production, over and above the TGFβ$_1$ induced VEGF levels in the wild type MEFs. Furthermore, TGFβ$_1$ did not significantly increase VEGF production over the high basal levels. These observations agree with the data generated using the GSK3β inhibitor in PASMCs.

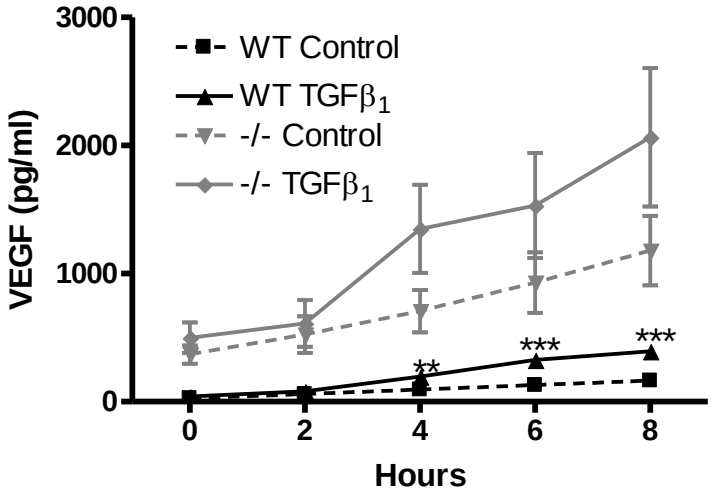


Figure 7-12 The effect of TGFβ1 on VEGF production from wild type and GSK3β deficient (-/-) mouse embryonic fibroblasts

Murine VEGF protein accumulation in the supernatants from wild type (WT – black bars) and GSK3β deficient (-/- - grey bars) mouse embryonic fibroblasts (MEFs). Confluent, serum starved MEFs were stimulated with 1ng/ml TGFβ$_1$ across an 8 hour time course. Points represent the mean ± s.e.m from triplicate samples from 3 independent experiments. (**, $p < 0.01$; and ***, $p < 0.001$ by one way ANOVA with Bonferri's post test)

The effect of TGFβ$_1$ on VEGF mRNA was also determined in wild type and GSK3β-/- MEFs. In wild type MEFs, VEGF mRNA levels increased by 1 hour, with a peak response at 1 hour which decreased over the 8 hours (Figure 7-13 – black lines). Consistent with the ELISA data generated in these cells the GSK3β deficient cells had an increased basal level of expression in

comparison to the wild type MEFs and TGFβ$_1$ did not induce further increases in VEGF mRNA above basal levels (Figure 7-13 – grey lines).

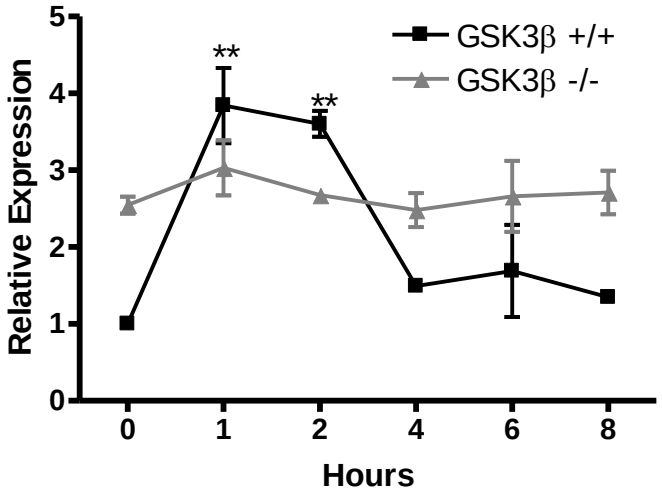


Figure 7-13 The effect of TGFβ1 on VEGF mRNA production by wild type (GSK3β+/+) and GSK3β deficient (GSK3β-/-) mouse embryonic fibroblasts

TGFβ1 mediated murine VEGF mRNA induction. Confluent wild type (+/+ black lines) and GSK3β deficient (-/- grey lines) MEFs were serum starved of 24 hours and further incubated for the times stated in medium containing 1ng/ml TGFβ1. The housekeeping gene murine β-actin and murine VEGF mRNA were measured by quantitative real-time PCR. Each point represents the mean ± s.e.m from three individual experiments. (**, $p < 0.01$ by one way ANOVA with Dunnet post test)

This data shows the effect of inhibiting GSK3β within the PASMC system or removing GSK3β from the MEF model system. In order to provide a more complete approach we investigated the effect of increased GSK3β by over expression of wild type GSK3β in PASMCs. We over expressed GSK3β in combination with the 318bp-VEGF luciferase reporter to gain insight into the region of the promoter at which GSK3β was mediating its effects, and also the largest of the promoter reporters, the 2068bp-VEGF reporter to ensure that any effect of GSK3β expression was detected. Confluent PASMCs were co-

transfected with the VEGF-promoter reporter and either the wild type GSK3β expression plasmid or the empty vector control, pFlag CMV2. When the empty control vector, pFlag CMV2, was over expressed TGFβ$_1$ did increase luciferase activity (Figure 7-14). However when wild type GSK3β was over expressed the TGFβ$_1$ response was abolished. Wild type GSK3β over expression also inhibited the 318bp VEGF-luciferase reporter (Figure 7-15). These results provide further evidence for role for GSK3β in the regulation of VEGF by TGFβ$_1$ in PASMCs.

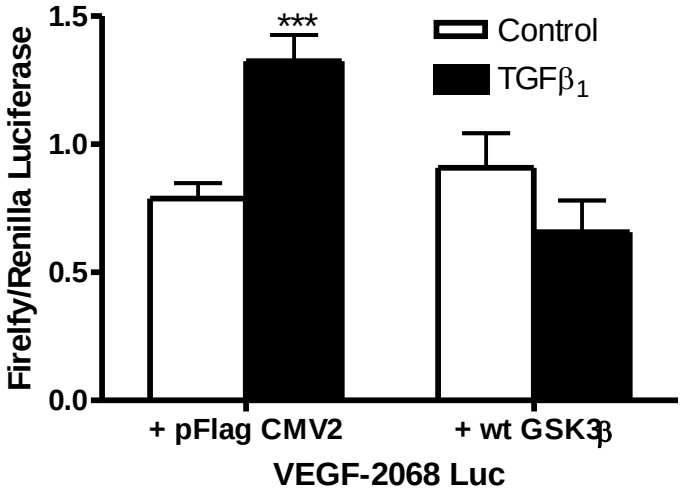


Figure 7-14 The effect of wild type GSK3β over expression on the TGFβ1 induced luciferase activity of the VEGF-2068 promoter luciferase reporter

Levels of luciferase expression in PAMSCs. Confluent serum starved PASMCs were transiently transfected for 15-16 hours with 0.4µg/well VEGF-2068-Luc and 0.2µg/well control vector, pFlag CMV2, or wild type GSK3β. Fugene HD was the transfection reagent used at a ratio of 1:2 DNA:Fugene HD. The medium was then removed and replaced with medium containing 10ng/ml TGFβ$_1$. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. (***, $p < 0.001$ by one way ANOVA with Bonferri's post test)

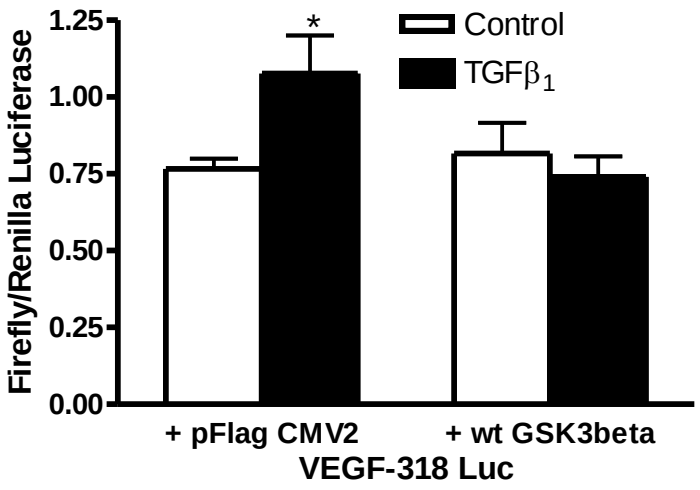


Figure 7-15 The effect of wild type GSK3β over expression on the TGFβ1 induced luciferase activity of the VEGF-318 promoter luciferase reporter

Levels of luciferase expression in PAMSCs. Confluent serum starved PASMCs were transiently transfected for 15-16 hours 0.2μg/well VEGF-318-Luc and 0.3μg/well control vector, pFlag CMV2, or wild type GSK3β. Fugene HD was the transfection reagent used at a ratio of 1:2 DNA:Fugene HD. The medium was then removed and replaced with medium containing 10ng/ml $TGF\beta_1$. Each bar represents the mean ± s.e.m from a minimum of triplicate samples from three individual experiments. (*, $p < 0.05$ by one way ANOVA with Bonferri's post test)

Finally we investigated whether GSK3β could associate with the native VEGF promoter in PASMCs by performing ChIP using an anti total GSK3β antibody. There was no GSK3β association under basal conditions but 2.5 hour $TGF\beta_1$ induction caused GSK3β association with the native VEGF promoter, confirming GSK3βs role in the $TGF\beta_1$ induced production of VEGF in PASMCs (Figure 7-16). Furthermore the primers used suggest GSK3β associates in the same promoter region as TCF4, β-catenin and the Smads.

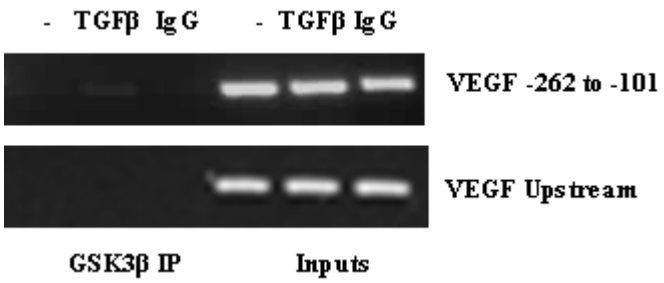


Figure 7-16 The effect of TGFβ1 on GSK3β association with the relevant 161-bp region of the VEGF promoter

Representative PCR gel of a GSK3β ChIP assay. Confluent PASMCs were serum starved for 24 hours followed by 2.5 hours incubation with and without $TGF\beta_1$. Immunoprecipitation was carried out using an antibody to GSK3β or relevant control IgG. The PCR primers were amplified in the -262 to -101 region of the VEGF promoter. VEGF Upstream primers were used as a negative control for non specific binding of the immunoprecipitated proteins to irrelevant regions of the promoter.

7.4.7 Smad2 directly binds both β-catenin and GSK3β under basal conditions and in the presence of $TGF\beta_1$

So far the data to suggest that a complex between members of the $TGF\beta_1$ canonical signalling pathway and members of the β-catenin/TCF signalling pathway form a complex is limited to ChIP data using primers which amplify a specific, limited region of the VEGF promoter. In order to convincingly suggest a protein complex is formed at the VEGF promoter we performed co-immunoprecipitation assays.

Initially immunoprecipitation with an anti Smad2 antibody was performed. Our co-immunoprecipitations were performed on nuclear extracts so all associations shown represent nuclear complexes. Under basal and $TGF\beta_1$ induced conditions Smad2 complexed with β-catenin (Figure 7-17 i).

Furthermore, Smad2 complexed with GSK3β (Figure 7-17 ii). This suggests that a Smad2/β-catenin/GSK3β complex is present in unstimulated cells and is not regulated by TGFβ$_1$. In agreement with this, immunoprecipitation with an anti GSK3β antibody followed by immunoblot with an anti-β-catenin antibody showed β-catenin and GSK3β to complex under basal conditions with no regulation of the complex by TGFβ$_1$ (Figure 7-18). This suggests that TGFβ$_1$ does not regulate the formation of the protein complex but regulates its association with the VEGF promoter and its transcriptional activity.

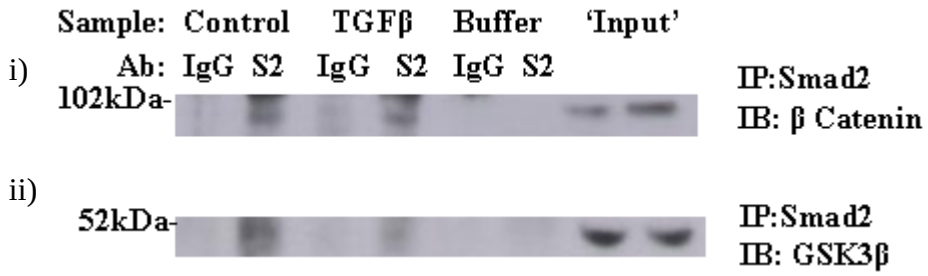


Figure 7-17 The effect of TGFβ1 on the co-immunoprecipitation of Smad2 with β-catenin and GSK3β

Native physical interactions between Smad2, β-catenin and GSK3β *in vivo*. Immunoprecipitation of endogenous Smad2 with β-catenin in PASMCs. Nuclear extract was co-immunoprecipitated with magnetic IgG beads in the presence of a Smad2 antibody or relevant IgG control antibody. The immunoprecipitates were then separated by SDS-PAGE and probed with i) an anti β-catenin antibody and ii) an anti-GSK3β antibody. The blots shown are representative of similar results achieved in 3 independent experiments.

Figure 7-18 The effect of TGFβ1 on the co-immunoprecipitation of GSK3β with β-catenin

Native physical interactions between β-catenin and GSK3β *in vivo*. Immunoprecipitation of endogenous GSK3β with β-catenin in PASMCs. Nuclear extract was co-immunoprecipitated with magnetic IgG beads in the presence of a GSK3β antibody or relevant IgG control antibody. The immunoprecipitates were then separated by SDS-PAGE and probed with an anti β-catenin antibody. The blots shown are representative of similar results achieved in 3 independent experiments.

7.5 DISCUSSION

In previous chapters we found that TGFβ$_1$ regulation of VEGF transcription requires one of two TCF binding sites within the VEGF promoter, increased binding of TCF4 and association of Smads 2,3 and 4 with the VEGF promoter upon TGFβ$_1$ stimulation. The major findings from the current chapter are that β-catenin and GSK3β, proteins canonically upstream of TCF/LEF, also associate with the VEGF promoter and that β-catenin must be dephosphorylated, via TGFβ$_1$ inhibition of GSK3β, for the association with the VEGF promoter to occur. We also show that a protein complex exists between β-catenin, GSK3β and Smad2 and that this complex is formed in the absence of TGFβ$_1$ stimulation and is not altered by TGFβ$_1$ simulation. We suggest that TGFβ$_1$ mediated dephosphorylation of β-catenin allows association of the preformed complex with TCF4 at the VEGF promoter resulting in increased VEGF transcription.

Initially we determined whether β-catenin was involved in VEGF transcriptional regulation and whether TGFβ$_1$ was able to regulate β-catenin. ChIP studies showed that β-catenin did not associate with the VEGF promoter under basal conditions but that TGFβ$_1$ induced its association. To our

knowledge this is the first time TGFβ$_1$ has been shown to cause β-catenin promoter association. It is certainly the first time that TGFβ$_1$ been shown to cause β-catenin association specifically to the VEGF promoter. However, β-catenin dependent regulation of VEGF has been described previously. For example, Leung *et al*., and Skurk *et al*., found cytoplasmic β-catenin accumulation and subsequent β-catenin nuclear translocation is required for Ginsenoside-Rg1 induction of VEGF in human endothelial cells [128, 197]. While Zhang *et al*., showed over expression of a mutated, stable form of β-catenin increased VEGF reporter activity in HeLa cells (cervical cancer cell line) [133] and Easwaran *et al*., described β-catenin dependent up-regulation of VEGF in human colon cancer cells [132].

While previous studies have shown TGFβ$_1$ can increase β-catenin expression levels [198] and stimulate β-catenin nuclear translocation [199] TGFβ$_1$ did not regulate whole cell β-catenin or β-catenin nuclear translocation in our studies in PASMCs. Furthermore, the level of β-catenin expressed in unstimulated cells was high across the 8 hour time course, β-catenin was constitutively present in the nucleus and β-catenin expression and nuclear translocation could not be induced by the positive control GSK3β inhibitor, SB216763. This seemed unusual as canonical β-catenin signalling involves β-catenin degradation in unstimulated cells, due to phosphorylation of serine and threonine residues within its N terminal, by the constitutively active kinase GSK3β, causing it to be recognised by the proteasome apparatus, resulting in low levels of basal whole cell β-catenin. We therefore, expected to see a low level of whole cell β-catenin in unstimulated PASMCs and, at a minimum, an

accumulation in response to SB21676. In contrast we observed a high, GSK3β inhibition insensitive, level of basal whole cell β-catenin. Also it is 'normally' only upon GSK3β inhibition and resultant hypophosphorylation of β-catenin that β-catenin accumulates in the cytoplasm and translocates to the nucleus. We therefore expected nuclear levels of β-catenin to be low in unstimulated cells and induced by GSK3β inhibition. However we observed constitutive nuclear β-catenin that was not increased further in response to SB216763. Consistent with our observations in PASMCs a number of studies have shown that β-catenin's signalling activity does not always correlate with accumulated levels and nuclear translocation. For example Young *et al*., [201] showed over expression of a stable β-catenin mutant (β-catenin S37A) could increase TCF/LEF transcriptional activity without any cytosolic accumulation of the mutant β-catenin. Furthermore Nelson *et al*., [202] showed an increase in active β-catenin can occur in a protein synthesis independent manner and suggest that a pre-existing pool of inactive β-catenin protein can be activated to signal without the requirement for ongoing protein synthesis. Korinek *et al*., [203] also showed that inhibition of β-catenin signalling can occur without a loss of β-catenin protein levels via interaction of β-catenin with the adenomatous polyposis coli (APC) tumour suppressor protein. We were interested in investigating the loss of β-catenin using conditional knock-out MEFs and obtained β-catenin Flox/Flox MEF cells from Kun-Liang Guans group in Michigan, USA [204]. Unfortunately the cells did not resuscitate well from liquid nitrogen storage in either our hands or the original groups and we were not able to use them.

Most previous reports of basal nuclear β-catenin expression show that it exists in an active transcriptional complex with TCF/LEF [205, 206]. This is contradictory to our data in PASMCs as, while we found that β-catenin was constitutively present within the nucleus, there was no basal level of β-catenin association at the VEGF promoter suggesting no constitutive β-catenin:TCF association. Unfortunately we were unable to confirm a direct protein:protein interaction by co-immunoprecipitation due to the poor quality of TCF4 antibodies and low expression level of TCF4. Had time allowed we would have tried to achieve a β-catenin:TCF4 immunoprecipitation by overexpression of 'tagged' versions of the proteins thus removing the issues of low protein levels and poor specific TCF4 antibodies. This method has been used previously by other groups to successfully co-immunoprecipitate the two proteins [207, 208].

It is not clear why nuclear β-catenin is prevented from forming an interaction with TCF/LEF in unstimulated PASMCs but one possibility is involvement of the APC protein. It has been shown previously that APC can prevent transcription of TCF/LEF dependent genes by interaction with β-catenin and effective sequestration of β-catenin from TCF/LEF [209]. The β-catenin binding sites for TCF/LEF and APC have been shown to overlap and therefore binding of β-catenin to either protein is mutually exclusive [210]. It would have been interesting to conduct immunoprecipitation of β-catenin with APC to establish if APC is present within the basal protein complex, preventing association of the complex with TCF4 and the VEGF promoter, and whether the association altered upon TGFβ_1 stimulation. Alternatively, Prieve *et al.*,

[211] have shown that the presence of β-catenin and TCF/LEF within the nucleus is not, in a cell specific manner, sufficient to allow activation of gene expression and that a third component is required. The lack of basal β-catenin association at the VEGF promoter, despite the presence of nuclear β-catenin, may simply be due to lack of a co-factor required for β-catenin DNA association and that association of this cofactor with β-catenin occurs in response to TGFβ_1. We have not identified such a component in the PASMCs as we have shown the interaction of β-catenin with TGFβ signalling Smad proteins was constitutive. However we identified a modification of β-catenin itself in response to TGFβ_1 - an increase in the level of unphosphorylated amino acids 27-37 of β-catenin (and also a decrease in phospho Ser33/Ser37/Thr41 β-catenin). We have also shown by ChIP that the phosphorylated version of β-catenin did not associate with the VEGF promoter, while the dephosphorylated version did. Thus we suggest that in unstimulated PASMCs nuclear β-catenin is prevented from associating with TCF4 at the VEGF promoter by phosphorylation, and only upon TGFβ_1 stimulation and a resultant hypophosphorylation of β-catenin does β-catenin associate at the VEGF promoter. Consistent with this, it has been shown that regulation of β-catenin phosphorylation controls its ability to activate transcription [200] and Sadot *et al*., showed by mobility gel shift assay that phospho-β-catenin formed a complex with LEF-1 but did not form a ternary complex with LEF/TCF binding sites on 293T bovine epithelial cell DNA in unstimulated cells [212]. How dephosphorylation of β-catenin affects its ability to associate with DNA is unclear. It is possible that phosphorylation of β-catenin prevents it from associating with TCF4 and thus its association with DNA. TCFs are negatively

charged and interact with the positively charged groove within β-catenin created by the 'arm repeat'. Phosphorylation of β-catenin may create sufficient negative charge on the protein to repel an interaction between it and TCF4 [123, 213]. A similar explanation could account for the inability of phospho-β-catenin to associate with negatively charged DNA. Alternatively dephosphorylation may cause an as yet unidentified inhibitory co-factor to dissociate or a stimulatory factor to associate but this would require further investigation to confirm. One point to note from the western blot experiments performed, and a possible limitation, is that the antibody that detects unphosphorylated β-catenin does not 'reach' as far as Thr41, which is covered by the anti-phospho-β-catenin antibody. A number of antibodies against phospho-β-catenin-ser33/37 were sampled, however the resultant blots had a large number of non specific bands present within the 90kDa region making it impossible to identify which band corresponded to phospho-β-catenin. As a consequence the phospho Ser33/ser37/thr42 β-catenin antibody was used as the best compromise.

We were subsequently interested in the mechanism by which TGFβ_1 was able to reduce β-catenin phosphorylation. The most obvious target for investigation was glycogen synthase kinase 3β (GSK3β). This kinase can phosphorylate β-catenin at the serine and threonine residues detected by the phospho-β-catenin antibodies we used. Also TGFβ_1 regulates GSK3β activity in desmoids tumour cells [198]. The initial evidence that TGFβ_1 regulation of β-catenin phosphorylation involved GSK3β was the observation that the GSK3β inhibitor, SB216763 caused a similar level of β-catenin dephosphorylation to

TGFβ$_1$ stimulation. Furthermore, incubation of PASMCs with both TGFβ$_1$ and SB216713 did not cause additive dephosphorylation of β-catenin. We went on to show that TGFβ$_1$ did not alter whole cell GSK3β expression, or affect the amount of GSK3β present in the nucleus of PASMCs. GSK3β expression levels have been shown previously to be regulated by Isoflavone in prostate cancer cells [214], however this is the only example of GSK3β regulation by expression level that we have been able to identify suggesting it is uncommon and cell type specific. GSK3β nuclear localisation can occur in response to stimuli other than TGFβ$_1$, for example, apoptotic stimuli (heat shock and staurosprine) [215] and hepatectomy in rats [216] however there is no evidence to suggest nuclear shuttling can occur in response to TGFβ$_1$. Interestingly, GSK3β was constitutively present within the nucleus of the PASMCs and was later found to co-immunoprecipitate with nuclear β-catenin and Smad2. This places GSK3β in an ideal position to regulate β-catenin phosphorylation in response to TGFβ$_1$ in PASMCs. Co-immunoprecipitation of β-catenin and GSK3β has been shown previously [214, 217]. However ours is the first report of GSK3β association with Smad2. Having found no regulation of GSK3β expression or localisation by TGFβ$_1$ we hypothesised that TGFβ$_1$ would be able to affect the kinase activity of GSK3β. GSK3β is a constitutively active kinase that is inhibited by phosphorylation at the ser9 position. We showed that TGFβ$_1$ could increase GSK3β-Ser9 phosphorylation suggesting that TGFβ$_1$ acts to inhibit GSK3β kinase activity. This is consistent with previous studies showing that TGFβ$_1$ increased phosphorylation of GSK3β [198]. If time had allowed it would have been ideal to confirm this observation with a GSK3β kinase assay. TGFβ$_1$ mediated increases in GSK3β phosphorylation

have been shown previously to require activation of PKB/Akt kinase activity [218]. PKB/Akt phosphorylates both GSK3β and GSK3α at Ser9 and Ser21 respectively, to inhibit their kinase activity [120]. Bakin *et al*., showed TGFβ$_1$ mediated activation of Rho GTPase and phosphtidylinositol-3-kinase (PI3K) resulted in PKB/Akt phosphorylation and activation and subsequent GSK3β phosphorylation in NMuMG (mouse, NAMRU, mammary gland) cells [218]. While Gingery *et al*., show TGFβ$_1$ mediated activation of TGFβ Activated kinase-1 (TAK1) results in phosphorylation and activation of PKB/Akt [219]. Furthermore Krymskaya *et al*., have shown TGFβ type I and type II receptors to interact with PI 3-kinase in airway smooth muscle cells [220]. Unfortunately time limitations prevented full investigation of the signalling upstream of GSK3β phosphorylation in PASMCs however we were able to obtain preliminary data in PKB/Akt deficient MEFs that one or more PKB/Akt isoforms may be involved in TGFβ$_1$ regulation of VEGF.

Having shown the ability of TGFβ$_1$ to regulate GSK3β we went on to show that GSK3β inhibition was required for a) increased VEGF protein production from PASMCs, by ELISA, and b) increased transcriptional activity of the VEGF promoter, by reporter assay, via pharmacological inhibition of GSK3β using SB-216763. Investigation of the effect of the inhibitor on PASMC VEGF mRNA levels would have further strengthened this data. The effect of increased GSK3β expression was investigated via over expression of wild type GSK3β in PASMCs and resulted in loss of TGFβ$_1$ induced VEGF reporter activity. Furthermore, GSK3β deficient MEFs had increased levels of basal VEGF production in comparison to wild type MEFs, to the extent that while

TGFβ$_1$ stimulation of VEGF production was possible in the wild type MEFs it was not in the GSK3β deficient MEFs, at either the protein or RNA levels. Finally, to incorporate the effects of TGFβ$_1$ on GSK3β activity and the effects on GSK3β modulation of VEGF we showed GSK3β was able to associate with the native PASMC VEGF promoter in response to TGFβ$_1$. As with previous ChIP experiments, GSK3β association at the VEGF promoter was detected using primers designed around the two TCF binding sites and strongly suggests a TCF4:β-catenin:Smad2,3,4:GSK3β complex exists at the VEGF promoter in response to TGFβ$_1$ stimulation. We believe this to be the first description of GSK3β ChIP. It is unlikely that GSK3β binds directly to the VEGF promoter as structural analysis of GSK3β has not described a DNA binding domain. However the protein interaction shown to occur between GSK3β, β-catenin and Smad2 in PASMCs provides a mechanism for its interaction with the DNA associated complex.

Finally we investigated, by co-immunoprecipitation, the protein complex that exists under basal and TGFβ$_1$ stimulated conditions in PASMCs. We found that GSK3β associated with both β-catenin and Smad2. We also showed an association between Smad2 and β-catenin. This association was constitutively present in unstimulated cells and was not altered by TGFβ$_1$ stimulation. This contrasts to Jian *et al'*s., observations that Smad3 association with β-catenin increases in response to TGFβ$_1$ stimulation. Furthermore, the same group show Smad3:GSK3β association to decrease in response to TGFβ$_1$ [199]. We were unable to optimise our immunoprecipitation method to allow successful Smad3 immunoprecipitation, however data in previous chapters suggests

Smad3 plays a similar if not compensatory role to Smad2 in PASMCs and as such we would have expected a similar result from Smad3 immunoprecipitation. We feel the differences between our observation and those seen by Jian *et al.*, are likely due to cell specific effects as Jian *et al*'s., studies were performed in bone marrow-derived adult human mesenchymal stem cells [199]. ChIP using antibodies against phosphorylated and unphosphorylated β-catenin suggests that modulation of β-catenin phosphorylation determines association of the protein complex with DNA, however it would have been interesting to obtain immunoprecipitations and resultant immunoblots with antibodies against phospho-Smad2, phospho-GSK3β and phospho-β-catenin to confirm that the proteins present in the immuno-complex were being regulated by TGFβ_1. Attempts at this proved futil however. We believe this is due to the low level of immunoprecipitated protein as phospho-GSK3β and unphosphorylated β-catenin were detected in the 'input' samples on a number of occasions but proved undetectable in the immunoprecipitated samples. Detection of phospho-Smad2 or any of the total Smads (all ~60kDa molecular weight) was prevented by a non-specific band present at approximately 60kDa in all co-immunoprecipitations, despite utilising a light chain specific secondary antibody.

A summary diagram of the data obtained in this and the previous three chapters is shown in Figure 7-19. Canonical TGFβ signalling proteins and β-catenin signalling proteins combine into a transcriptional complex that, upon TGFβ_1 stimulation, associates with TCF4 at Wnt response elements (WREs) within the VEGF promoter, to increase VEGF expression. Cooperation of TGFβ

signalling with Wnt signalling is previously described. In COS cells, LEF1 binds both Smad2 and Smad3 in response to TGFβ$_1$ and causes synergistic activation of the *Xtwn* promoter via LEF1 binding to a WRE and Smads binding to Smad binding elements (SBEs) [221]. In contrast to our findings this was shown to be independent of β-catenin. Interestingly in *Xenopus* embryos Wnt stimulation of the *Xtwn* promoter depends on the formation of a Smad4/LEF1/β-catenin complex. The murine *gastrin* promoter is also synergistically regulated by Smad3/4 and TCF4/β-catenin and also requires the presence of WREs and SBEs for maximal TGFβ$_1$ induced increases in murine gastrin expression. However they also provide evidence for the presence of TCF4/β-catenin/Smad complexes at the WREs alone and the SBEs alone. This ability of a TCF4/β-catenin/Smad complex to form at the WRE is consistent with our data as we were unable to identify a SBE with the region of the VEGF promoter we had determined as necessary for TGFβ$_1$ induction.

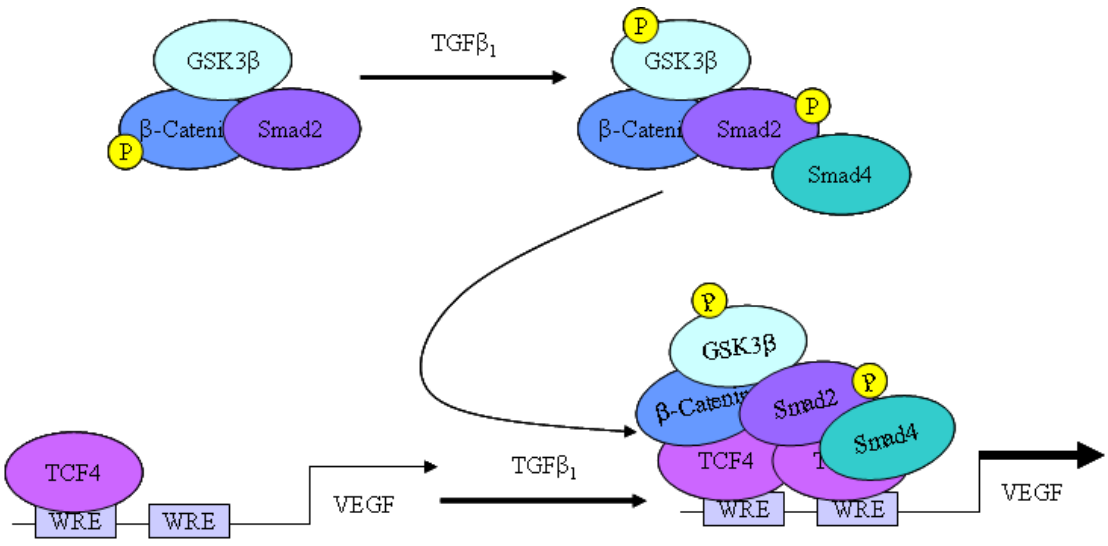


Figure 7-19 Schematic representation of the signalling events implicated by the current data

Phosphorylated β-catenin, Smad2 and GSK3β are constitutively associated in the nucleus of PAMSCs. TCF4 is associated with Wnt reponse elements (WREs) under basal conditions and play a role in basal VEGF production. TGFβ1 stimulation results in the phosphorylation of Smad2 and its association with Smad4. TGFβ1 causes an increase in GSK3β-ser9 phosphorylation and resultant decrease in β-catenin phosphorylation. These events result in a transcriptional complex containing Smad2, 3, and 4, dephosphorylated β-catenin and phosphorylated GSK3β which associate with the VEGF promoter, along with further TCF4, at two TCF binding sites in response to TGFβ1. This results in increased VEGF expression.

In conclusion, in this chapter we have identified the requirement for β-catenin and GSK3β in TGFβ_1 regulation of VEGF in PASMCs and discussed the presence of a transcriptional complex containing Smad2/3/4, β-catenin and GSK3β which is present under basal conditions and associates with TCF4 at WREs within the VEGF promoter upon TGFβ_1 mediated dephosphorylation of β-catenin.

8 THE EFFECT OF BMPR II MUTATION ON TGFB INDUCED MURINE VEGF AND VEGFR2

8.1 INTRODUCTION

Mutation of the *BMPR-II* gene is present in more than 80% of familial PH cases and up to 25% of idiopathic pulmonary PH cases [96, 98, 99, 101]. The *BMPR II* gene encodes the Bone Morphogenetic Protein Receptor II protein. Reduction of BMPR II protein has been reported in PH cases with no detectable mutation of the *BMPR II* gene [102]. BMPR II is required for signalling of the bone morphogenetic proteins (BMPs) and mutation of the gene causes several defects in the protein including loss of the protein trafficking to the membrane [222], altered kinase activity and cytoplasmic domain changes which affect downstream signalling [80]. BMPs are members of the TGFβ super family of signalling proteins. *BMPR II* mutation causes defects in the growth response of pulmonary artery smooth muscle cells to BMP2, 4 ,7 and $TGF\beta_1$ resulting in a loss of $TGF\beta_1$ mediated inhibition of proliferation and increased smooth muscle [103]. Increased smooth muscle within the pulmonary artery is a common feature of remodelling in all classifications of PH [8].

In previous chapters we characterised the signalling and transcriptional mechanisms involved in $TGF\beta_1$ regulation of VEGF in normal human PASMCs. Here we investigate the effect of heterozygous *BMPR II* knock out in mouse PASMCs on $TGF\beta_1$ induced VEGF, as a model of PH.

8.2 AIMS

The aims of this chapter were to determine;

- if wild type murine PASMCs produce VEGF in response to TGFβ_1
- if TGFβ_1 induced VEGF production from murine PASMCs is affected by BMPR II mutation
- if human and murine PASMCs express the VEGF receptors and whether expression levels are regulated by TGFβ_1
- if BMPR II mutation affects VEGFR expression in murine PASMCs

8.3 METHODS

Cells were cultured to confluence, serum starved for 24 hours and incubated for the stated times with the stated concentrations of TGFβ_1.

8.4 RESULTS

8.4.1 The TGFβ1 induced VEGF response is enhanced in BMPR II +/- mouse PASMCs compared to wild type mouse PASMCs

To investigate the effect of heterozygous *BMPR II* knockout on TGFβ_1 induced VEGF production we stimulated confluent, serum starved, wild type and BMPR2 +/- mouse PASMCs (mPASMCs) for 24 hours with a concentration range of human TGFβ_1. Stimulation above basal levels was seen in both cell types at concentrations of 1ng/ml and greater (Figure 8-1). Further, a significant increase in TGFβ_1 induced VEGF secretion occurred in heterozygous BMPR II knock out mPASMCs compared to the TGFβ_1 induced VEGF secretion from wild type mPASMCs, at concentrations of 1ng/ml and greater.

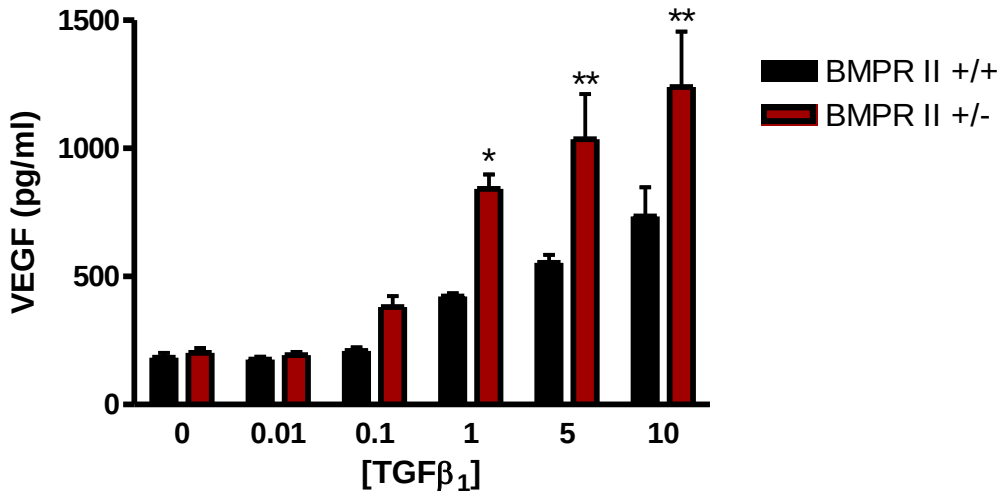


Figure 8-1 Concentration response of TGFβ1 on VEGF production from BMPR II +/+ and BMPR II +/- mouse pulmonary artery smooth muscle cells

VEGF protein accumulation in the supernatants from BMPR II +/+ and BMPR II +/- mouse pulmonary artery smooth muscle cells. Confluent mPASMCs were serum starved for 24 hours and incubated for 24 hours with the stated concentration of $TGF\beta_1$. Points represent the mean ± s.e.m from a minimum of triplicate samples from two individual experiments. Concentrations are normalised to cell counts. (*, $p < 0.05$; and **, $p<0.01$ by one way ANOVA with Bonferroni's post test)

Subsequently we performed a time course to 1ng/ml $TGF\beta_1$ in wild type and BMPR II heterozygous knock out mice. At all time points basal VEGF production was greater in BMPR II knock out mPASMCs and $TGF\beta_1$ induced VEGF concentrations in BMPR II +/- mPASMCs reached levels 3.5 fold greater than $TGF\beta_1$ induced VEGF concentrations in wild type mPASMCs (Figure 8-2).

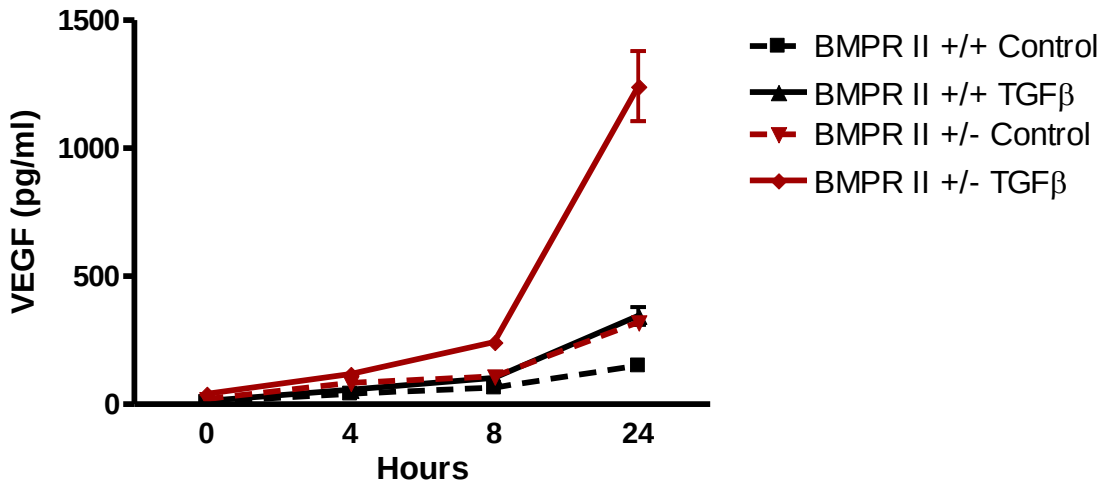


Figure 8-2 Time course of basal and TGFβ1 induced VEGF release from BMPR II +/+ and BMPR II +/- mouse pulmonary artery smooth muscle cells

VEGF protein accumulation in the supernatants from BMPR II +/+ and BMPR II +/- mouse PASMCs. Confluent mPASMCs were serum starved for 24 hours and incubated for the stated time with 1ng/ml $TGF\beta_1$. Points represent the mean ± s.e.m from a minimum of triplicate samples from two individual experiments. Concentrations are normalised to cell counts.

We then investigated the effect of heterozygous BMPR II knock out on $TGF\beta_1$ induced VEGF mRNA. Real time PCR was performed on cDNA from an 8 hour, 1ng/ml $TGF\beta_1$ time course in mPASMCs. In both wild type and BMPR II +/- mPASMCs murine VEGF mRNA was induced by 1 hour (Figure 8-3). There was also an increase in $TGF\beta_1$ induced VEGF mRNA levels in the BMPR II +/- mPASMCs when compared to the wild type cells (Figure 8-3). This data suggests $TGF\beta_1$ induced VEGF production is augmented by heterozygous knock out of the BMPR II gene.

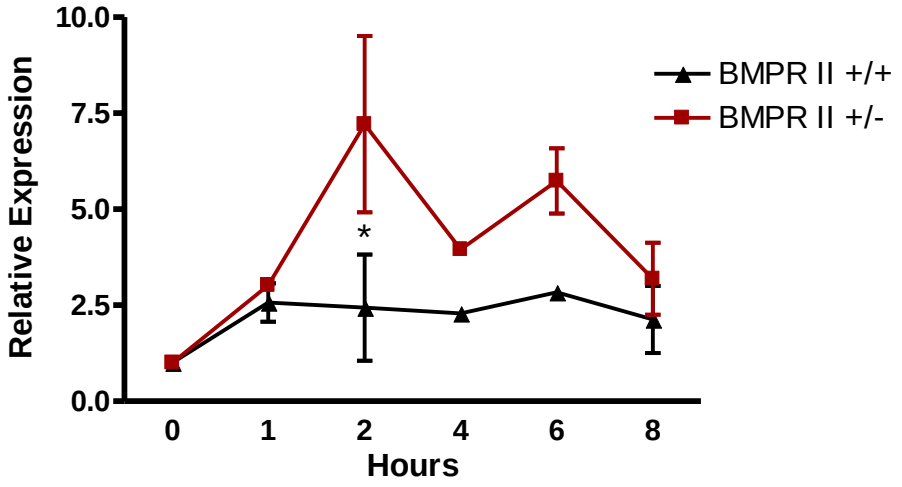


Figure 8-3 The effect of TGFβ1 on VEGF mRNA production by BMPR II +/+ and BMPR II +/- mouse pulmonary artery smooth muscle cells

$TGF\beta_1$ mediated murine VEGF mRNA induction. Confluent BMPR II +/+ and BMPR II +/- mouse pulmonary artery smooth muscle cells were serum starved of 24 hours and further incubated for the times stated in medium containing 1ng/ml $TGF\beta_1$. The housekeeping gene murine β-actin and murine VEGF mRNA were measured by quantitative real-time PCR. Each point represents the mean ± s.e.m from two individual experiments. (*, $p < 0.05$ by one way ANOVA with Bonferroni's post test)

8.4.2 TGFβ1 increases VEGFR2 expression in human PASMCs and this may be enhanced in BMPR II +/- mouse PASMCs compared to wild type mouse PASMCs

We went on to study the expression of the VEGF receptors by PASMCs. Real time PCR was performed on cDNA from an 8 hour, 1ng/ml $TGF\beta_1$ time course in human PASMCs. Amplification of VEGFR1 was not seen despite the use of different primer combinations suggesting that VEGFR1 is not expressed on human PASMCs, however the primers should be used against a positive

control to confirm this. In contrast amplification of VEGFR2 cDNA was successful and TGFβ_1 increased VEGFR2 expression (Figure 8-4), although this did not reach statistical significance.

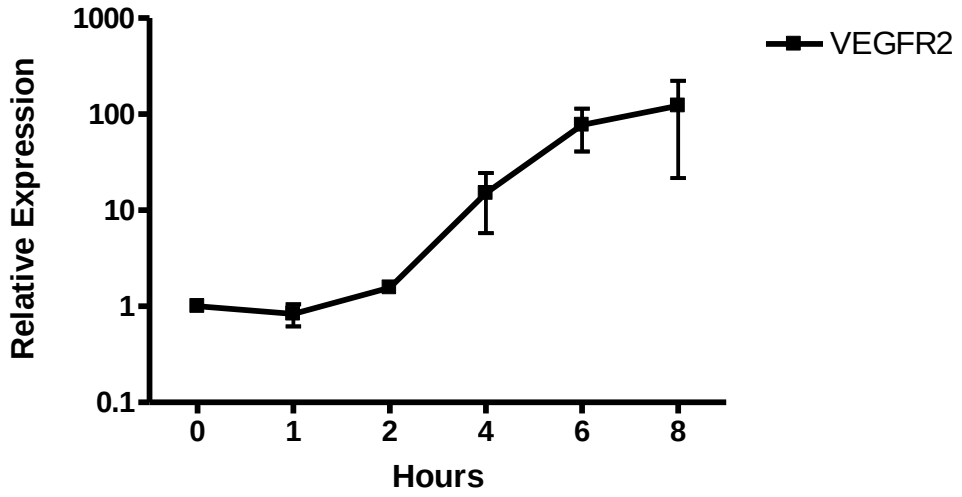


Figure 8-4 The effect of TGFβ1 on VEGFR2 mRNA production by PASMCs

TGFβ_1 mediated VEGFR2 mRNA. Confluent human PASMCs were serum starved for 24 hours and incubated for the times indicated in medium containing 1ng/ml TGFβ_1. The house keeping gene human β_2-microglobulin and VEGFR2 were measured by quantitative real-time PCR.

Having shown regulation of VEGFR2 expression by TGFβ_1 in human PASMCs we investigated the expression on VEGFR2 in wild type and BMPR II heterozygous knock out mouse PASMCs. We also looked for VEGFR1 expression, but did not get amplification of the cDNA, strengthening the hypothesis that VEGFR1 is not expressed by PASMCs. Amplification of VEGFR2 was successful in both wild type and BMPR II +/- cells and expression was increased by TGFβ_1 in both cell lines at 2 hours (Figure 8-5). Furthermore, a trend towards increased TGFβ_1 induced VEGFR2 expression in

the BMPR II +/- cells in comparison to wild type cells was seen although this did not reach statistical significance.

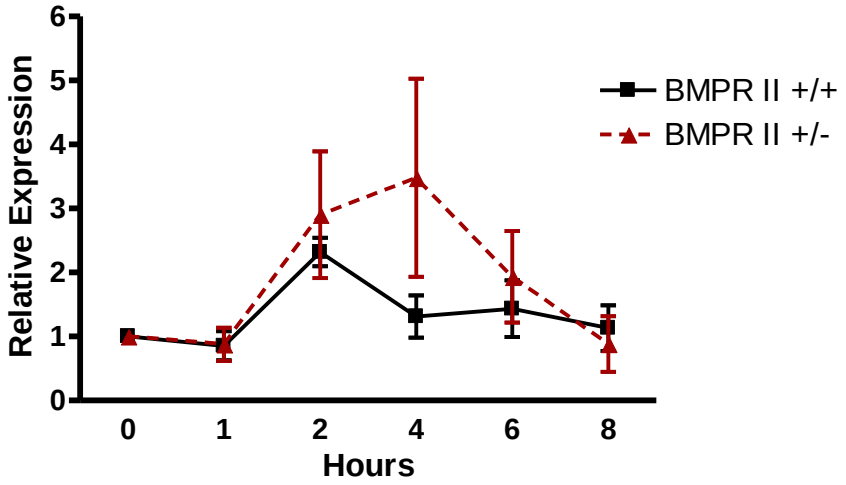


Figure 8-5 The effect of TGFβ1 on VEGFR2 mRNA production from BMPR II +/+ and BMPR II +/- mouse pulmonary artery smooth muscle cells

$TGF\beta_1$ mediated murine VEGFR2 mRNA. Confluent mPASMCs were serum starved for 24 hours and incubated for the times indicated in medium containing 1ng/ml $TGF\beta_1$. The house keeping gene murine β-actin and VEGFR2 were measured by quantitative real-time PCR.

8.5 DISCUSSION

The major findings of the studies in this chapter are that cultured murine PASMCs secreted VEGF protein and produced VEGF mRNA under basal conditions and that $TGF\beta_1$ increased VEGF production. Also $TGF\beta_1$ induced murine VEGF was augmented by *BMPR II* mutation. Furthermore, we showed that VEGFR2 was expressed by human and mouse PASMCs and that VEGFR2 expression may be regulated by $TGF\beta_1$ and *BMPR II* mutation.

No previous studies have looked at the effect of *BMPR II* gene mutation on $TGF\beta_1$ induced gene expression. While $TGF\beta_1$ is not a recognised ligand for

BMPR2 its cellular effects in the presence on *BMPR II* mutation have been shown to be altered. For example, TGFβs inhibitory effect on PASMC proliferation is converted to a stimulatory effect in cells from patients with *BMPR II* mutation positive PAH [103]. BMP and TGFβ signalling converges at the level of the co-Smad, Smad4 and shared signalling through this protein potentially accounts for the ability of TGFβ_1 signalling to be modulated by *BMPR II* mutation. While transcriptional responses to BMP4 are reduced in *BMPR II* mutation positive human PASMCs [98], we show an increased responsiveness of VEGF to TGFβ_1 in *BMPR II* +/- mPASMCs. Clearly an important further step would be to determine if this effect is also true in human *BMPR II* mutated PASMCs or, alternatively, to use siRNA to knock down *BMPR II* levels in normal human PASMCs. It would also be interesting to ascertain if the enhanced TGFβ_1 effect is gene specific or a general transcriptional response. This could be ascertained using a luciferase reporter sensitive to TGFβ signalling, for example the Cignal SMAD Reporter (luc) Kit from SABioscinces. An increased response from this reporter in *BMPR II* mutated cells would suggest intrinsic increased transcriptional ability of Smads. However this method has its disadvantages and does not mean that every gene in a *BMPR II* mutant cell would display increased responsiveness to TGFβ_1. For example, the reporter does not allow for regulation of gene transcription by co-factors or interacting transcription factors, which could act positively or negatively, at adjacent binding sequences or for regulation of genes by TGFβ_1 via non-Smad binding sites, for example, the regulation of VEGF shown in the previous chapters which occurs without a Smad binding element being present.

The increased production of VEGF by PASMCs as a consequence of *BMPR II* mutation may contribute to the pathogenesis of pulmonary hypertension. VEGF has mitogenic, angiogenic, migratory and permeability inducing effects making it a target of intense research in the cancer field [32, 57]. The remodelling process that occurs in PH has been likened to that of tumour formation suggesting VEGF may contribute to PH remodelling [61]. Consistent with this, VEGF secreted from damaged endothelial cells protects vascular smooth muscle cells from apoptosis causing increased smooth muscle mass [64] and in a blood flow induced model of PH in sheep VEGF protein and mRNA levels are increased in PASMCs [72, 73]. In contradiction, inhibition of VEGF causes apoptosis and subsequent proliferation of endothelial cells [63] and gene transfer of VEGF in to animal models of PH can prevent or reduce progression of PH [66-69].

Identification of VEGFR2 expression by PASMCs was unexpected as VEGFR2 expression was originally thought to be confined to endothelial cells. The TGFβ_1 and *BMPR II* mutation effect on VEGFR2 mRNA did not reach significance due to the high variability between individual real-time PCR experiments. It is undoubtedly necessary to show that VEGFR2 protein is expressed and also utilise flow cytometry data to confirm VEGFR2 expression at the cell surface. This could be followed by investigation of TGFβ_1 and BMPR II effects on protein/cell surface levels to confirm the possible effects seen on mRNA. However VEGFR2 expression in PASMCs creates the possibility of autocrine VEGF signalling. Further the increased levels of

VEGFR2 and VEGF in *BMPR II* mutant cells provides two points of amplification for autocrine VEGF signalling. If VEGF and VEGF signalling are detrimental to PH this amounts to a devastating signalling loop, however if VEGF is protective an autocrine loop could represent a powerful compensatory mechanism.

Damaged endothelial cells that produce VEGF also secrete TGFβ_1 and the TGFβ_1 produced causes smooth muscle cell proliferation [63]. Furthermore, TGFβ_1 levels are increased in sheep models of PH and positively correlate with increased media thickness [105]. The potential combination of excess TGFβ_1 expression and increased intrinsic transcriptional activity of Smads could be detrimental to PH patients. However it is expected that TGFβ_1 regulates the expression of genes that are protective against PH development and it would require a screen of a number of genes implicated as 'good' or 'bad' in PH to determine any overall TGFβ_1 effects when *BMPR II* is mutated. Nevertheless, TGFβ_1 regulation of gene expression represents an important and thus far poorly explored target in understanding the molecular cell biology behind PH.

9 CONCLUSION AND SUGGESTIONS FOR FUTURE STUDIES

Recent advances in the therapy for pulmonary hypertension (PH) have improved the outlook for patients and the discovery of *BMPR II* mutations in familial and sporadic forms of the disease have provided an element of focus to the study of PH pathogenesis. However, PH remains a fatal disease with heart and lung transplant providing the only reprieve. The study of the molecular mechanisms involved in the regulation of putative PH targets represents a vital area of research. In the current study we show a novel mechanism by which $TGF\beta_1$ regulates VEGF expression in pulmonary artery smooth muscle cells (PASMCs). PASMCs are a key cell type in the development of PH. A feature common to all forms of PH is an increase in muscle content of muscular arteries and the appearance of smooth muscle in non-muscular arteries. $TGF\beta_1$ inhibits the proliferation of healthy PASMCs but increases proliferation of primary PH PASMCs [103]. VEGF is secreted from ovine PASMCs in a $TGF\beta_1$ dependent manner in response to cyclic stretch and is associated with the remodelling the occurs upon the development of PH in the ovine PH model [223]. In the current study we showed $TGF\beta_1$ increased VEGF secretion from PASMCs and went on to evaluate the transcriptional mechanisms required for the $TGF\beta_1$ effect. Our initial studies identified a specific 182bp region of the VEGF promoter to be critical and also determined increased association of the transcription factor TCF4 to be required. TCF4 is required for VEGF transcription in human colon cancer cell lines [189, 190], however TCF4 dependent VEGF transcription in response to $TGF\beta_1$ is previously unreported. We also identified a potential role for TCF4 in the regulation of basal VEGF transcription. This may be relevant to PH pathogenesis as, in our final chapter,

we show *BMPR II* mutation in murine PASMCs results in increased secretion of basal VEGF. An interesting area of further investigation would be to characterise the potential transcriptional complex associated with TCF4 at the VEGF promoter under basal conditions and whether this alters in response to $TGF\beta_1$ or in PASMCs from PH patients. In particular Groucho is known to bind TCF/LEFs and repress transcription by binding histone deacetylases (HDACs) [224]. HDACs modify the N-terminal domains of histones by deacetylating lysine residues resulting in chromatin rearrangement into a secondary structure not conducive to gene transcription. β-catenin is thought to compete for TCF4 binding with Groucho and β-catenin translocation to the nucleus is postulated to be sufficient to displace Groucho resulting in activated transcription [116]. Association of Groucho and HDACs at the basal VEGF promoter could be investigated using ChIP.

We went on to investigate the role of TGFβ signalling proteins in TCF4 dependent VEGF transcription. Cooperation between Wnt (TCF4 upstream signalling) and TGFβ signalling components is present at the *Xtwn* promoter and the murine *gastrin* promoter [208, 221] but not previously described at the VEGF promoter. We showed Smad2, Smad3 and Smad4 associated with the VEGF promoter, within the same region as TCF4, in response to $TGF\beta_1$. Further, we suggested that Smad3 was critical to the $TGF\beta_1$ effect and was capable of compensating for Smad2. One of the main areas of future interest, which would strengthen the data in this section of the study, would be to develop a sensitive Re-ChIP method. This allows the DNA from an initial immunoprecipitation (e.g. TCF4) to be 'put through' a second

immunoprecipitation (e.g. Smad3) to determine whether both proteins are associated within a complex at the same region of DNA at the same time. Once optimised this technique would have extensive potential for characterising transcriptional complexes. It may also be of interest to determine the method by which Smad3 can compensate for Smad2 and whether, in its compensatory role, it associates with the same or a distinct region of the promoter. Its inability to fully recover TGFβ_1 dependent VEGF secretion to wild type levels in Smad2 -/- MEFs and to stimulate the 318-bp VEGF luciferase reporter in Smad2-/- MEFs suggests that it acts via a different mechanism to when it is present in concert with Smad2.

Subsequently we investigated signalling components upstream of TCF4 and their role in the TGFβ_1 effect. We showed β-catenin association with the VEGF promoter that was independent of β-catenin whole cell protein levels and β-catenin nuclear translocation. β-catenin association with the VEGF promoter required TGFβ_1 mediated dephosphorylation of β-catenin-Ser33/37. Dephosphorylation was a consequence of TGFβ_1 inhibition of GSK3β. Furthermore GSK3β associated with the VEGF promoter in response to TGFβ_1. This data suggested a transcriptional complex at the VEGF promoter consisting of TCF4, Smad2, Smad3, Smad4, dephosphorylated β-catenin and phosphorylated GSK3β. Co-immunoprecipitations (IP) confirmed interactions between GSK3β, β-catenin and Smad2. Future studies would be beneficial to optimise the co-immunoprecipitation technique to allow IP of native TCF4 and Smad3. Alternatively, if expression levels are too low or if antibodies are too poor we have tagged over expression constructs for Smads2/3 and 4, TCF4 and

β-catenin that could be expressed and immunoprecipitated. A further area of interest is the mechanism by which the protein complex is prevented from associating with the VEGF promoter until β-catenin is dephosphorylated. It is possible that the Adenomatosis Polyposis Coli (APC) protein is bound to β-catenin in its phosphorylated state, preventing its association with TCF4 at the promoter, and dephosphorylation displaces APC. Alternatively it is possible that TGFβ_1 stimulation allows association of further transcriptional regulator proteins whose association is limited by β-catenin phosphorylation. These proteins may be members of the histone acetyl transferase (HAT) family. β-catenin binds p300 via its C-terminus Armadillo repeat and this enhances TCF/β-catenin responsive gene expression via P300 acetylation of β-catenin lysine345 which causes increased affinity of β-catenin for TCF4 [207, 224] . Whether HAT association is dependent on β-catenin phosphorylation status is not known but could be investigated using co-immunoprecipitation of β-catenin associated proteins prior to and following GSK3β inhibition. Further investigation using over expressed β-catenin mutated at phosphorylation sites is also possible. CBP/P300 association with a dephosphorylated protein in preference to a phosphorylated protein is however contrary to previous reports of CBP/p300 interaction with specifically phosphorylated CREB [225] and Smad3 [226].

Studies to investigate the signalling responsible for TGFβ_1 mediated phosphorylation and inhibition of GSK3β could also be performed. GSK3β is commonly a target of phosphorylation by PKB/Akt and TGFβ_1 can activate PKB/Akt via Rho GTPase and PI3K [218] and TGFβ Activated Kinase (TAK)-

1 [219]. Preliminary experiments in PKB/Akt null MEFs suggest a possible role of PKB/Akt in the TGFβ$_1$ regulation of VEGF but this could be expanded at a number of levels. Activation of PKB/Akt requires its phosphorylation at ser473 and thr308 [227]. Determination of the effect of TGFβ$_1$ on PKB phosphorylation, and therefore activity, could be determined by western blot. An allosteric (interacts at a site other than the active site) inhibitor of PKB/Akt is available that regulates downstream GSK3β activity [228]. This could be used on cultured cells to determine if it can inhibit TGFβ$_1$ induced VEGF secretion, VEGF mRNA and VEGF promoter reporter activity. Expression plasmids of dominant negative Akt [229] and constitutively active PKB2 [230] are available and could be used to investigate the role of PKB/Akt by molecular transfection techniques. Further, as the TGFβ receptor TGFβR II binds PI3K (a possible kinase upstream of GSK3β and PKB) in human airway smooth muscle cells [220], if a PI3K dependent mechanism is shown, it would be interesting to show whether PI3K can bind the TGFβR II in PASMCs.

The role of β-catenin and associated signalling in pulmonary hypertension is poorly characterised. Rai *et al*., [135] have shown high expression of β-catenin and loss of Wnt7a within plexiform lesions. While Arciniegas *et al*., [136] believe Wnt and β-catenin to be involved in the putative transition of endothelial cells to mesenchymal, smooth muscle like cells in pulmonary vascular remodelling. Further Arciniegas *et al*., believe interaction between TGFβ and Wnt signalling to be important in endothelial-mesenchymal transition. Recent meeting abstracts have shown increased β-catenin and GSK3β expression in the monocrotaline model of PH and a decrease in

vascular smooth muscle cell proliferation upon expression of dominant negative GSK3β [137]. Further BMP4, TGFβ$_{1,}$ Endothelin-1 and serotonin increase GSK3β Ser9 phosphorylation and GSK3β inhibition causes PASMC hypertrophy, while BMP2 increases PASMC motility [231]. These preliminary reports suggest a role for β-catenin and also coordinated TGFβ and Wnt signalling in PH. Future studies to identify other targets of β-catenin mediated transcription in normal PASMCs would be of great interest and may identify the genes responsible for the functional effects of β-catenin on PASMCs. This could be achieved by utilising siRNA technology to 'knock out' β-catenin in normal PAMSCs and determine differential gene and protein expression levels in response to β-catenin loss. Further, differential regulation of VEGF by β-catenin and TGFβ$_{1,}$ and other β-catenin target genes identified, could be investigated in *BMPR II* knock out murine PASMCs and PASMCs from both *BMPR II* mutation positive and negative PH patient PASMCs. Dysfunctional β-catenin signalling may represent a novel target of PH research.

The final section of the current studies started to explore the effect of *BMPR II* mutation on TGFβ$_1$ transcriptional regulation of VEGF and its receptor VEGFR2. The first opportunity for future investigation identified from these studies is to determine why TGFβ$_1$ mediated VEGF expression is augmented upon heterozygous loss of *BMPR II*. Potential possibilities include differential:

- expression of TGFβ and Wnt signalling components, for example Smads, β-catenin, TCF4 and GSK3β
- activation of Smads, β-catenin and GSK3β
- transcriptional activity of Smads and TCF4/β-catenin

- expression of signalling inhibitors, for example APC and Smad7
- expression of TGFβ receptors or TGFβ receptor binding
- expression/recruitment/activity of transcriptional co-activators/repressors.

We would also need to strengthen and confirm the data on VEGFR2 expression.

In conclusion, in the current study we have shown that $TGF\beta_1$ transcriptionally regulates VEGF secretion from normal PASMCs via a novel pathway requiring the association of a TCF4/β-catenin/Smad2/3/4/GSK3β complex with one of two WREs within a 182bp region of the VEGF promoter. This association was dependent on $TGF\beta_1$ mediated inhibition of the constitutively active kinase GSK3β and resultant dephosphorylation of β-catenin N-teminal serine residues. Preliminary experiments have suggested that this mechanism maybe disrupted in PH associated with reduced BMPR II expression.

Opportunities for further investigation lie in the identification and study of additional target PH genes that are regulated by $TGF\beta_1$ and the differences in $TGF\beta_1$ induced gene expression in, initially murine *BMPR II* heterozygous knock out mice, and moving into human PH PASMCs. A number of potential genes have been identified. Platelet derived growth factor (PDGF) in a potent mitogen and chemoattractant for vascular smooth muscle cells and both PDGFR α and β are upregulated in a lamb model of chronic uterine PH. Further the PDGFR antagonist, imatinib or Gleevec, has shown promising

results in PH animal models [19]. Also TGFβ$_1$ can increase PDGFRβ and PDGF B expression and decrease PDGFRα expression [232, 233].

Endothelin-1 (ET-1) is a smooth muscle cell mitogen and vasoconstrictant. ET-1 expression is abundant in remodelled pulmonary arteries, its expression correlates with vascular resistance and its receptor density is greater in PH arteries [19]. Bosentan, a dual ET-A/ET-B receptor antagonist is a current therapy for PH [24]. Also TGFβ$_1$ increases ET-1 protein secretion from human and rat PASMCs [234].

Cox-2 expression in response to hypoxia reduces PASMC proliferation [149] and analogues of the Cox-2 product, prostacyclin, are used as the 'Gold Standard' treatment for PH [24]. Further, prostacyclin receptor (PGI-R) expression is reduced on smooth muscle cells from remodelled PH arteries and PGI-R knock out mice develop a greater degree of PH in response to hypoxia than wild type controls [235]. TGFβ$_1$ can increase Cox-2 expression and release of the Cox-2 products, prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) [163, 236, 237], while BMP4 can decrease Cox-2 expression [236] making Cox-2 a prime target for altered regulation in the presence of *BMPR II* mutation.

The LL (long) variant of the serotonin (5HT) transporter (5HTT) is more frequently carried by PH patients than control patients and PH patients have increased lung tissue 5-HTT mRNA than control patients [238]. Further, PASMCs from primary PH patients have greater uptake of 5-HT, increased expression of 5-HTT and proliferate faster in response to 5-HT than control cells [238]. While a direct link between TGFβ$_1$ and 5-HT expression is lacking, BMPR2 II +/- mice do not develop PH spontaneously but infusion of

5-HT is sufficient to initiate PH development in association with an inhibition of Smad1/3/8 phosphorylation [239].

Above are a limited number of example molecules that have relevance to PH and the potential to be regulated by TGFβ_1 and numerous more are possible.

10 REFERENCES

1. Azizi, M.H., T. Nayernouri, and F. Azizi, *A brief history of the discovery of the circulation of blood in the human body.* Arch Iran Med, 2008. **11**(3): p. 345-50.
2. Mandegar, M., et al., *Cellular and molecular mechanisms of pulmonary vascular remodeling: role in the development of pulmonary hypertension.* Microvasc Res, 2004. **68**(2): p. 75-103.
3. J. M. B. Hughes, N.W.M., *Pulmonary Circulation: From Basic Mechanisms to Clinical Practice* 2001: Imperial College Press.
4. Hiatt, L.P.G.a.J.L., *Colour Atlas of Histololgy*. Third ed. 2000: Lippincott Williams and Wilkins.
5. Manes, A. and C. Campana, *Pulmonary hypertension: classification and diagnostic algorithm.* Ital Heart J, 2005. **6**(10): p. 834-9.
6. Baloira, A., *[Pulmonary hypertension treatment: future prospects]*. Arch Bronconeumol, 2007. **43**(3): p. 131-5.
7. Chin, K.M. and L.J. Rubin, *Pulmonary arterial hypertension.* J Am Coll Cardiol, 2008. **51**(16): p. 1527-38.
8. Humbert, M., et al., *Cellular and molecular pathobiology of pulmonary arterial hypertension.* J Am Coll Cardiol, 2004. **43**(12 Suppl S): p. 13S-24S.
9. Yeager, M.E., et al., *Microsatellite instability of endothelial cell growth and apoptosis genes within plexiform lesions in primary pulmonary hypertension.* Circ Res, 2001. **88**(1): p. E2-E11.
10. Cool, C.D., et al., *Three-dimensional reconstruction of pulmonary arteries in plexiform pulmonary hypertension using cell-specific markers. Evidence for a dynamic and heterogeneous process of pulmonary endothelial cell growth.* Am J Pathol, 1999. **155**(2): p. 411-9.
11. Giaid, A., et al., *Expression of endothelin-1 in the lungs of patients with pulmonary hypertension.* N Engl J Med, 1993. **328**(24): p. 1732-9.
12. Sakao, S., et al., *Apoptosis of pulmonary microvascular endothelial cells stimulates vascular smooth muscle cell growth.* Am J Physiol Lung Cell Mol Physiol, 2006. **291**(3): p. L362-8.
13. Tuder, R.M., et al., *Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension.* Am J Respir Crit Care Med, 1999. **159**(6): p. 1925-32.
14. Giaid, A. and D. Saleh, *Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension.* N Engl J Med, 1995. **333**(4): p. 214-21.
15. Meyrick, B. and L. Reid, *The effect of continued hypoxia on rat pulmonary arterial circulation. An ultrastructural study.* Lab Invest, 1978. **38**(2): p. 188-200.
16. Jones, R., M. Jacobson, and W. Steudel, *alpha-smooth-muscle actin and microvascular precursor smooth-muscle cells in pulmonary hypertension.* Am J Respir Cell Mol Biol, 1999. **20**(4): p. 582-94.
17. Stenmark, K.R. and M.G. Frid, *Smooth muscle cell heterogeneity: role of specific smooth muscle cell subpopulations in pulmonary vascular disease.* Chest, 1998. **114**(1 Suppl): p. 82S-90S.

18. Strauss, B.H. and M. Rabinovitch, *Adventitial fibroblasts: defining a role in vessel wall remodeling.* Am J Respir Cell Mol Biol, 2000. **22**(1): p. 1-3.
19. Said, S.I., *Mediators and modulators of pulmonary arterial hypertension.* Am J Physiol Lung Cell Mol Physiol, 2006. **291**(4): p. L547-58.
20. Zaiman, A., et al., *One hundred years of research in the pathogenesis of pulmonary hypertension.* Am J Respir Cell Mol Biol, 2005. **33**(5): p. 425-31.
21. Humbert, M., et al., *Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension.* Am J Respir Crit Care Med, 1995. **151**(5): p. 1628-31.
22. Lesprit, P., et al., *Pulmonary hypertension in POEMS syndrome: a new feature mediated by cytokines.* Am J Respir Crit Care Med, 1998. **157**(3 Pt 1): p. 907-11.
23. Yoshibayashi, M., et al., *Plasma endothelin concentrations in patients with pulmonary hypertension associated with congenital heart defects. Evidence for increased production of endothelin in pulmonary circulation.* Circulation, 1991. **84**(6): p. 2280-5.
24. Ulrich, S., M. Fischler, and R. Speich, *Update on therapies for pulmonary hypertension.* Swiss Med Wkly, 2007. **137**(5-6): p. 73-82.
25. Ribatti, D., A. Vacca, and M. Presta, *The discovery of angiogenic factors: a historical review.* Gen Pharmacol, 2000. **35**(5): p. 227-31.
26. Zachary, I., *Signaling mechanisms mediating vascular protective actions of vascular endothelial growth factor.* Am J Physiol Cell Physiol, 2001. **280**(6): p. C1375-86.
27. Thomas, K.A., *Vascular endothelial growth factor, a potent and selective angiogenic agent.* J Biol Chem, 1996. **271**(2): p. 603-6.
28. Olsson, A.K., et al., *VEGF receptor signalling - in control of vascular function.* Nat Rev Mol Cell Biol, 2006. **7**(5): p. 359-71.
29. Ferrara, N., *Role of vascular endothelial growth factor in regulation of physiological angiogenesis.* Am J Physiol Cell Physiol, 2001. **280**(6): p. C1358-66.
30. Robinson, C.J. and S.E. Stringer, *The splice variants of vascular endothelial growth factor (VEGF) and their receptors.* J Cell Sci, 2001. **114**(Pt 5): p. 853-65.
31. Ferrara, N., *Role of vascular endothelial growth factor in the regulation of angiogenesis.* Kidney Int, 1999. **56**(3): p. 794-814.
32. Ferrara, N., H.P. Gerber, and J. LeCouter, *The biology of VEGF and its receptors.* Nat Med, 2003. **9**(6): p. 669-76.
33. Olofsson, B., et al., *Vascular endothelial growth factor B, a novel growth factor for endothelial cells.* Proc Natl Acad Sci U S A, 1996. **93**(6): p. 2576-81.
34. Petrova, T.V., T. Makinen, and K. Alitalo, *Signaling via vascular endothelial growth factor receptors.* Exp Cell Res, 1999. **253**(1): p. 117-30.
35. Tammela, T., et al., *The biology of vascular endothelial growth factors.* Cardiovasc Res, 2005. **65**(3): p. 550-63.

36. Joukov, V., et al., *A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases.* Embo J, 1996. **15**(2): p. 290-98.
37. Achen, M.G., et al., *Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4).* Proc Natl Acad Sci U S A, 1998. **95**(2): p. 548-53.
38. Maglione, D., et al., *Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor.* Proc Natl Acad Sci U S A, 1991. **88**(20): p. 9267-71.
39. Josko, J. and M. Mazurek, *Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis.* Med Sci Monit, 2004. **10**(4): p. RA89-98.
40. Jeon, S.H., et al., *Mechanisms underlying TGF-beta1-induced expression of VEGF and Flk-1 in mouse macrophages and their implications for angiogenesis.* J Leukoc Biol, 2007. **81**(2): p. 557-66.
41. LaRusch, G.A., et al., *Nutlin3 blocks vascular endothelial growth factor induction by preventing the interaction between hypoxia inducible factor 1alpha and Hdm2.* Cancer Res, 2007. **67**(2): p. 450-4.
42. Schmidt, D., et al., *Critical role for NF-kappaB-induced JunB in VEGF regulation and tumor angiogenesis.* Embo J, 2007. **26**(3): p. 710-9.
43. Arany, Z., et al., *HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha.* Nature, 2008. **451**(7181): p. 1008-12.
44. Pages, G. and J. Pouyssegur, *Transcriptional regulation of the Vascular Endothelial Growth Factor gene--a concert of activating factors.* Cardiovasc Res, 2005. **65**(3): p. 564-73.
45. Abdelrahim, M., et al., *Role of Sp proteins in regulation of vascular endothelial growth factor expression and proliferation of pancreatic cancer cells.* Cancer Res, 2004. **64**(18): p. 6740-9.
46. Reisinger, K., R. Kaufmann, and J. Gille, *Increased Sp1 phosphorylation as a mechanism of hepatocyte growth factor (HGF/SF)-induced vascular endothelial growth factor (VEGF/VPF) transcription.* J Cell Sci, 2003. **116**(Pt 2): p. 225-38.
47. Pore, N., et al., *Sp1 is involved in Akt-mediated induction of VEGF expression through an HIF-1-independent mechanism.* Mol Biol Cell, 2004. **15**(11): p. 4841-53.
48. Abdelrahim, M. and S. Safe, *Cyclooxygenase-2 inhibitors decrease vascular endothelial growth factor expression in colon cancer cells by enhanced degradation of Sp1 and Sp4 proteins.* Mol Pharmacol, 2005. **68**(2): p. 317-29.
49. Jeon, S.H., et al., *The PKA/CREB pathway is closely involved in VEGF expression in mouse macrophages.* Mol Cells, 2007. **23**(1): p. 23-9.
50. Oskolkova, O.V., et al., *ATF4-dependent transcription is a key mechanism in VEGF up-regulation by oxidized phospholipids: critical role of oxidized sn-2 residues in activation of unfolded protein response.* Blood, 2008. **112**(2): p. 330-9.
51. Brogi, E., et al., *Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only.* Circulation, 1994. **90**(2): p. 649-52.

52. Dibbens, J.A., et al., *Hypoxic regulation of vascular endothelial growth factor mRNA stability requires the cooperation of multiple RNA elements.* Mol Biol Cell, 1999. **10**(4): p. 907-19.
53. Vumbaca, F., et al., *Double-stranded RNA-binding protein regulates vascular endothelial growth factor mRNA stability, translation, and breast cancer angiogenesis.* Mol Cell Biol, 2008. **28**(2): p. 772-83.
54. Levy, N.S., et al., *Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR.* J Biol Chem, 1998. **273**(11): p. 6417-23.
55. Bell, S.E., et al., *The RNA binding protein Zfp36l1 is required for normal vascularisation and post-transcriptionally regulates VEGF expression.* Dev Dyn, 2006. **235**(11): p. 3144-55.
56. Ray, P.S. and P.L. Fox, *A post-transcriptional pathway represses monocyte VEGF-A expression and angiogenic activity.* Embo J, 2007. **26**(14): p. 3360-72.
57. Cross, M.J., et al., *VEGF-receptor signal transduction.* Trends Biochem Sci, 2003. **28**(9): p. 488-94.
58. Schlessinger, J., *Cell signaling by receptor tyrosine kinases.* Cell, 2000. **103**(2): p. 211-25.
59. Wiesmann, C., et al., *Crystal structure at 1.7 A resolution of VEGF in complex with domain 2 of the Flt-1 receptor.* Cell, 1997. **91**(5): p. 695-704.
60. Carmeliet, P., et al., *Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188.* Nat Med, 1999. **5**(5): p. 495-502.
61. Tuder, R.M., et al., *Expression of angiogenesis-related molecules in plexiform lesions in severe pulmonary hypertension: evidence for a process of disordered angiogenesis.* J Pathol, 2001. **195**(3): p. 367-74.
62. Hirose, S., et al., *Expression of vascular endothelial growth factor and its receptors correlates closely with formation of the plexiform lesion in human pulmonary hypertension.* Pathol Int, 2000. **50**(6): p. 472-9.
63. Taraseviciene-Stewart, L., et al., *Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension.* Faseb J, 2001. **15**(2): p. 427-38.
64. Sakao, S., et al., *Initial apoptosis is followed by increased proliferation of apoptosis-resistant endothelial cells.* Faseb J, 2005. **19**(9): p. 1178-80.
65. Sakao, S., et al., *VEGF-R blockade causes endothelial cell apoptosis, expansion of surviving CD34+ precursor cells and transdifferentiation to smooth muscle-like and neuronal-like cells.* Faseb J, 2007. **21**(13): p. 3640-52.
66. Campbell, A.I., et al., *Cell-based gene transfer of vascular endothelial growth factor attenuates monocrotaline-induced pulmonary hypertension.* Circulation, 2001. **104**(18): p. 2242-8.
67. Zhao, Y.D., et al., *Microvascular regeneration in established pulmonary hypertension by angiogenic gene transfer.* Am J Respir Cell Mol Biol, 2006. **35**(2): p. 182-9.

68. Gong, F.Q., et al., *[Effect of VEGF gene transfer on the bleomycin-induced pulmonary hypertension in immature rabbits].* Zhejiang Da Xue Xue Bao Yi Xue Ban, 2005. **34**(6): p. 551-6.
69. Gong, F.Q., et al., *VEGFmRNA and eNOSmRNA expression in immature rabbits with bleomycin-induced pulmonary hypertension.* J Zhejiang Univ Sci, 2004. **5**(8): p. 995-1000.
70. Grover, T.R., et al., *Intrauterine hypertension decreases lung VEGF expression and VEGF inhibition causes pulmonary hypertension in the ovine fetus.* Am J Physiol Lung Cell Mol Physiol, 2003. **284**(3): p. L508-17.
71. Grover, T.R., et al., *rhVEGF treatment preserves pulmonary vascular reactivity and structure in an experimental model of pulmonary hypertension in fetal sheep.* Am J Physiol Lung Cell Mol Physiol, 2005. **289**(2): p. L315-21.
72. Lam, C.F., et al., *Functional adaptation and remodeling of pulmonary artery in flow-induced pulmonary hypertension.* Am J Physiol Heart Circ Physiol, 2005. **289**(6): p. H2334-41.
73. Mata-Greenwood, E., et al., *Expression of VEGF and its receptors Flt-1 and Flk-1/KDR is altered in lambs with increased pulmonary blood flow and pulmonary hypertension.* Am J Physiol Lung Cell Mol Physiol, 2003. **285**(1): p. L222-31.
74. Nadeau, S., et al., *Changes in expression of vascular endothelial growth factor and its receptors in neonatal hypoxia-induced pulmonary hypertension.* Pediatr Res, 2005. **58**(2): p. 199-205.
75. Partovian, C., et al., *Heart and lung VEGF mRNA expression in rats with monocrotaline- or hypoxia-induced pulmonary hypertension.* Am J Physiol, 1998. **275**(6 Pt 2): p. H1948-56.
76. Massague, J., *TGF-beta signal transduction.* Annu Rev Biochem, 1998. **67**: p. 753-91.
77. Lawrence, D.A., *Transforming growth factor-beta: an overview.* Kidney Int Suppl, 1995. **49**: p. S19-23.
78. Piek, E., C.H. Heldin, and P. Ten Dijke, *Specificity, diversity, and regulation in TGF-beta superfamily signaling.* Faseb J, 1999. **13**(15): p. 2105-24.
79. Jenkins, G., *The role of proteases in transforming growth factor-beta activation.* Int J Biochem Cell Biol, 2008. **40**(6-7): p. 1068-78.
80. Bobik, A., *Transforming growth factor-betas and vascular disorders.* Arterioscler Thromb Vasc Biol, 2006. **26**(8): p. 1712-20.
81. Massague, J. and D. Wotton, *Transcriptional control by the TGF-beta/Smad signaling system.* Embo J, 2000. **19**(8): p. 1745-54.
82. ten Dijke, P. and C.S. Hill, *New insights into TGF-beta-Smad signalling.* Trends Biochem Sci, 2004. **29**(5): p. 265-73.
83. Shi, Y. and J. Massague, *Mechanisms of TGF-beta signaling from cell membrane to the nucleus.* Cell, 2003. **113**(6): p. 685-700.
84. Lutz, M. and P. Knaus, *Integration of the TGF-beta pathway into the cellular signalling network.* Cell Signal, 2002. **14**(12): p. 977-88.
85. 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.
86. Attisano, L. and J.L. Wrana, *Smads as transcriptional co-modulators.* Curr Opin Cell Biol, 2000. **12**(2): p. 235-43.

87. Itoh, S. and P. ten Dijke, *Negative regulation of TGF-beta receptor/Smad signal transduction.* Curr Opin Cell Biol, 2007. **19**(2): p. 176-84.
88. Derynck, R. and Y.E. Zhang, *Smad-dependent and Smad-independent pathways in TGF-beta family signalling.* Nature, 2003. **425**(6958): p. 577-84.
89. ten Dijke, P. and H.M. Arthur, *Extracellular control of TGFbeta signalling in vascular development and disease.* Nat Rev Mol Cell Biol, 2007. **8**(11): p. 857-69.
90. Pepper, M.S., *Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity.* Cytokine Growth Factor Rev, 1997. **8**(1): p. 21-43.
91. Goumans, M.J. and C. Mummery, *Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice.* Int J Dev Biol, 2000. **44**(3): p. 253-65.
92. Bertolino, P., et al., *Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders.* Chest, 2005. **128**(6 Suppl): p. 585S-590S.
93. Larsson, J., et al., *Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice.* Embo J, 2001. **20**(7): p. 1663-73.
94. Jadrich, J.L., M.B. O'Connor, and E. Coucouvanis, *The TGF beta activated kinase TAK1 regulates vascular development in vivo.* Development, 2006. **133**(8): p. 1529-41.
95. Botney, M.D., L. Bahadori, and L.I. Gold, *Vascular remodeling in primary pulmonary hypertension. Potential role for transforming growth factor-beta.* Am J Pathol, 1994. **144**(2): p. 286-95.
96. Lane, K.B., et al., *Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. The International PPH Consortium.* Nat Genet, 2000. **26**(1): p. 81-4.
97. West, J., et al., *Suppression of type II bone morphogenic protein receptor in vascular smooth muscle induces pulmonary arterial hypertension in transgenic mice.* Chest, 2005. **128**(6 Suppl): p. 553S.
98. Yang, J., et al., *Mutations in bone morphogenetic protein type II receptor cause dysregulation of Id gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension.* Circ Res, 2008. **102**(10): p. 1212-21.
99. De Caestecker, M. and B. Meyrick, *Bone morphogenetic proteins, genetics and the pathophysiology of primary pulmonary hypertension.* Respir Res, 2001. **2**(4): p. 193-7.
100. Harrison, R.E., et al., *Transforming growth factor-beta receptor mutations and pulmonary arterial hypertension in childhood.* Circulation, 2005. **111**(4): p. 435-41.
101. Thomson, J.R., et al., *Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-beta family.* J Med Genet, 2000. **37**(10): p. 741-5.

102. Zhang, S., et al., *Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells.* Am J Physiol Lung Cell Mol Physiol, 2003. **285**(3): p. L740-54.
103. Morrell, N.W., et al., *Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins.* Circulation, 2001. **104**(7): p. 790-5.
104. Sturrock, A., et al., *Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells.* Am J Physiol Lung Cell Mol Physiol, 2006. **290**(4): p. L661-L673.
105. Mata-Greenwood, E., et al., *Alterations in TGF-beta1 expression in lambs with increased pulmonary blood flow and pulmonary hypertension.* Am J Physiol Lung Cell Mol Physiol, 2003. **285**(1): p. L209-21.
106. Yang, X., et al., *Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension.* Circ Res, 2005. **96**(10): p. 1053-63.
107. Zaiman, A.L., et al., *Role of the TGF-beta/Alk5 signaling pathway in monocrotaline-induced pulmonary hypertension.* Am J Respir Crit Care Med, 2008. **177**(8): p. 896-905.
108. Teichert-Kuliszewska, K., et al., *Bone morphogenetic protein receptor-2 signaling promotes pulmonary arterial endothelial cell survival: implications for loss-of-function mutations in the pathogenesis of pulmonary hypertension.* Circ Res, 2006. **98**(2): p. 209-17.
109. Zakrzewicz, A., et al., *The transforming growth factor-beta/Smad2,3 signalling axis is impaired in experimental pulmonary hypertension.* Eur Respir J, 2007. **29**(6): p. 1094-104.
110. Richter, A., et al., *Impaired transforming growth factor-beta signaling in idiopathic pulmonary arterial hypertension.* Am J Respir Crit Care Med, 2004. **170**(12): p. 1340-8.
111. Fantozzi, I., et al., *Divergent effects of BMP-2 on gene expression in pulmonary artery smooth muscle cells from normal subjects and patients with idiopathic pulmonary arterial hypertension.* Exp Lung Res, 2005. **31**(8): p. 783-806.
112. Clevers, H., *Wnt/beta-catenin signaling in development and disease.* Cell, 2006. **127**(3): p. 469-80.
113. Kikuchi, A., S. Kishida, and H. Yamamoto, *Regulation of Wnt signaling by protein-protein interaction and post-translational modifications.* Exp Mol Med, 2006. **38**(1): p. 1-10.
114. Takahashi-Yanaga, F. and T. Sasaguri, *[Wnt/beta-catenin signaling pathway and drug discovery].* Fukuoka Igaku Zasshi, 2007. **98**(4): p. 93-9.
115. Willert, K. and K.A. Jones, *Wnt signaling: is the party in the nucleus?* Genes Dev, 2006. **20**(11): p. 1394-404.
116. Gordon, M.D. and R. Nusse, *Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors.* J Biol Chem, 2006. **281**(32): p. 22429-33.
117. Logan, C.Y. and R. Nusse, *The Wnt signaling pathway in development and disease.* Annu Rev Cell Dev Biol, 2004. **20**: p. 781-810.

118. van Gijn, M.E., et al., *The wnt-frizzled cascade in cardiovascular disease.* Cardiovasc Res, 2002. **55**(1): p. 16-24.
119. Xu, W. and D. Kimelman, *Mechanistic insights from structural studies of beta-catenin and its binding partners.* J Cell Sci, 2007. **120**(Pt 19): p. 3337-44.
120. Doble, B.W. and J.R. Woodgett, *GSK-3: tricks of the trade for a multi-tasking kinase.* J Cell Sci, 2003. **116**(Pt 7): p. 1175-86.
121. Jope, R.S. and G.V. Johnson, *The glamour and gloom of glycogen synthase kinase-3.* Trends Biochem Sci, 2004. **29**(2): p. 95-102.
122. Forde, J.E. and T.C. Dale, *Glycogen synthase kinase 3: a key regulator of cellular fate.* Cell Mol Life Sci, 2007. **64**(15): p. 1930-44.
123. Daugherty, R.L. and C.J. Gottardi, *Phospho-regulation of Beta-catenin adhesion and signaling functions.* Physiology (Bethesda), 2007. **22**: p. 303-9.
124. Hecht, A. and R. Kemler, *Curbing the nuclear activities of beta-catenin. Control over Wnt target gene expression.* EMBO Rep, 2000. **1**(1): p. 24-8.
125. Hoppler, S. and C.L. Kavanagh, *Wnt signalling: variety at the core.* J Cell Sci, 2007. **120**(Pt 3): p. 385-93.
126. Wohrle, S., B. Wallmen, and A. Hecht, *Differential control of Wnt target genes involves epigenetic mechanisms and selective promoter occupancy by T-cell factors.* Mol Cell Biol, 2007. **27**(23): p. 8164-77.
127. Wright, M., et al., *Identification of a Wnt-responsive signal transduction pathway in primary endothelial cells.* Biochem Biophys Res Commun, 1999. **263**(2): p. 384-8.
128. Skurk, C., et al., *Glycogen-Synthase Kinase3beta/beta-catenin axis promotes angiogenesis through activation of vascular endothelial growth factor signaling in endothelial cells.* Circ Res, 2005. **96**(3): p. 308-18.
129. Masckauchan, T.N. and J. Kitajewski, *Wnt/Frizzled signaling in the vasculature: new angiogenic factors in sight.* Physiology (Bethesda), 2006. **21**: p. 181-8.
130. Zerlin, M., M.A. Julius, and J. Kitajewski, *Wnt/Frizzled signaling in angiogenesis.* Angiogenesis, 2008. **11**(1): p. 63-9.
131. Shu, W., et al., *Wnt7b regulates mesenchymal proliferation and vascular development in the lung.* Development, 2002. **129**(20): p. 4831-42.
132. Easwaran, V., et al., *beta-Catenin regulates vascular endothelial growth factor expression in colon cancer.* Cancer Res, 2003. **63**(12): p. 3145-53.
133. Zhang, X., J.P. Gaspard, and D.C. Chung, *Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia.* Cancer Res, 2001. **61**(16): p. 6050-4.
134. Nakamura, T., et al., *A Wnt- and beta -catenin-dependent pathway for mammalian cardiac myogenesis.* Proc Natl Acad Sci U S A, 2003. **100**(10): p. 5834-9.
135. Rai, P.R., et al., *The cancer paradigm of severe pulmonary arterial hypertension.* Am J Respir Crit Care Med, 2008. **178**(6): p. 558-64.
136. Arciniegas, E., et al., *Perspectives on endothelial-to-mesenchymal transition: potential contribution to vascular remodeling in chronic*

pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol, 2007. **293**(1): p. L1-8.
137. P. Sklepkiewicz, S.S.P., X. Tian, H.A. Ghofrani, N. Weissmann, W. Seeger, F. Grimminger, R.T. Schermuly, *Glycogen Synthase Kinase 3 beta/beta Catenin System in Pulmonary Arterial Hypertension*, in *American Thoracic Society International Conference*. 2008: Toronto.
138. H. Dneg, J.K.B., J. Lei, D.J. Pinsky, M.B. Hershenson, *Bone Morphogenic Protein (BMP#0-4, Transforming Growth Factor (TGF)-beta, Serotonin (5-HT) and Endothelin (ET)-1 Each Induce Pulmonary Vasculaar Smooth Muscle Hypertrophy*, in *American Thoracic Society International Conference*. 2008: Toronto.
139. Piek, E., et al., *Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts.* J Biol Chem, 2001. **276**(23): p. 19945-53.
140. Hoeflich, K.P., et al., *Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation.* Nature, 2000. **406**(6791): p. 86-90.
141. Finkenzeller, G., A. Technau, and D. Marme, *Hypoxia-induced transcription of the vascular endothelial growth factor gene is independent of functional AP-1 transcription factor.* Biochem Biophys Res Commun, 1995. **208**(1): p. 432-9.
142. Biggs, J.R., J.E. Kudlow, and A.S. Kraft, *The role of the transcription factor Sp1 in regulating the expression of the WAF1/CIP1 gene in U937 leukemic cells.* J Biol Chem, 1996. **271**(2): p. 901-6.
143. Bamforth, S.D., et al., *Cardiac malformations, adrenal agenesis, neural crest defects and exencephaly in mice lacking Cited2, a new Tfap2 co-activator.* Nat Genet, 2001. **29**(4): p. 469-74.
144. Hagen, T., et al., *Signalling activity of beta-catenin targeted to different subcellular compartments.* Biochem J, 2004. **379**(Pt 2): p. 471-7.
145. Dunn, N.R., et al., *Mice exclusively expressing the short isoform of Smad2 develop normally and are viable and fertile.* Genes Dev, 2005. **19**(1): p. 152-63.
146. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.* J Immunol Methods, 1983. **65**(1-2): p. 55-63.
147. Dupuis, J. and M.M. Hoeper, *Endothelin receptor antagonists in pulmonary arterial hypertension.* Eur Respir J, 2008. **31**(2): p. 407-15.
148. Taraseviciene-Stewart, L., et al., *A bradykinin antagonist and a caspase inhibitor prevent severe pulmonary hypertension in a rat model.* Can J Physiol Pharmacol, 2002. **80**(4): p. 269-74.
149. Yang, X., et al., *Hypoxic induction of cox-2 regulates proliferation of human pulmonary artery smooth muscle cells.* Am J Respir Cell Mol Biol, 2002. **27**(6): p. 688-96.
150. Clapp, L.H., et al., *Differential effects of stable prostacyclin analogs on smooth muscle proliferation and cyclic AMP generation in human pulmonary artery.* Am J Respir Cell Mol Biol, 2002. **26**(2): p. 194-201.
151. Wharton, J., et al., *Prostacyclin analogues differentially inhibit growth of distal and proximal human pulmonary artery smooth muscle cells.* Circulation, 2000. **102**(25): p. 3130-6.

152. Pola, R., et al., *Comparative analysis of the in vivo angiogenic properties of stable prostacyclin analogs: a possible role for peroxisome proliferator-activated receptors.* J Mol Cell Cardiol, 2004. **36**(3): p. 363-70.
153. Ikeda, Y., et al., *Host stromal bradykinin B2 receptor signaling facilitates tumor-associated angiogenesis and tumor growth.* Cancer Res, 2004. **64**(15): p. 5178-85.
154. Ishihara, K., et al., *Roles of bradykinin in vascular permeability and angiogenesis in solid tumor.* Int Immunopharmacol, 2002. **2**(4): p. 499-509.
155. Jung, Y.J., et al., *IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis.* Faseb J, 2003. **17**(14): p. 2115-7.
156. Maruyama, K., et al., *Interleukin-1 beta upregulates cardiac expression of vascular endothelial growth factor and its receptor KDR/flk-1 via activation of protein tyrosine kinases.* J Mol Cell Cardiol, 1999. **31**(3): p. 607-17.
157. Sola-Villa, D., et al., *IL-1beta induces VEGF, independently of PGE2 induction, mainly through the PI3-K/mTOR pathway in renal mesangial cells.* Kidney Int, 2006. **70**(11): p. 1935-41.
158. Bradbury, D., et al., *Vascular endothelial growth factor induction by prostaglandin E2 in human airway smooth muscle cells is mediated by E prostanoid EP2/EP4 receptors and SP-1 transcription factor binding sites.* J Biol Chem, 2005. **280**(34): p. 29993-30000.
159. Inoue, H., et al., *Regulation by PGE2 of the production of interleukin-6, macrophage colony stimulating factor, and vascular endothelial growth factor in human synovial fibroblasts.* Br J Pharmacol, 2002. **136**(2): p. 287-95.
160. Lopes, F.L., et al., *Transcriptional regulation of uterine vascular endothelial growth factor during early gestation in a carnivore model, Mustela vison.* J Biol Chem, 2006. **281**(34): p. 24602-11.
161. Pai, R., et al., *PGE(2) stimulates VEGF expression in endothelial cells via ERK2/JNK1 signaling pathways.* Biochem Biophys Res Commun, 2001. **286**(5): p. 923-8.
162. Mountain, D.J., M. Singh, and K. Singh, *Downregulation of VEGF-D expression by interleukin-1beta in cardiac microvascular endothelial cells is mediated by MAPKs and PKCalpha/beta1*. J Cell Physiol, 2008. **215**(2): p. 337-43.
163. Bradbury, D.A., et al., *Effect of bradykinin, TGF-beta1, IL-1beta, and hypoxia on COX-2 expression in pulmonary artery smooth muscle cells.* Am J Physiol Lung Cell Mol Physiol, 2002. **283**(4): p. L717-25.
164. Pueyo, M.E., et al., *Regulation of vascular endothelial growth factor expression by cAMP in rat aortic smooth muscle cells.* Exp Cell Res, 1998. **238**(2): p. 354-8.
165. Kamio, K., et al., *Prostacyclin analogs stimulate VEGF production from human lung fibroblasts in culture.* Am J Physiol Lung Cell Mol Physiol, 2008. **294**(6): p. L1226-32.

166. Fujita, M., et al., *Pulmonary hypertension in TNF-alpha-overexpressing mice is associated with decreased VEGF gene expression.* J Appl Physiol, 2002. **93**(6): p. 2162-70.
167. Choi, S.J., et al., *Effects of cytokines on VEGF expression and secretion by human first trimester trophoblast cell line.* Am J Reprod Immunol, 2002. **48**(2): p. 70-6.
168. Bian, Z.M., S.G. Elner, and V.M. Elner, *Regulation of VEGF mRNA expression and protein secretion by TGF-beta2 in human retinal pigment epithelial cells.* Exp Eye Res, 2007. **84**(5): p. 812-22.
169. Spinella, F., et al., *Endothelin-1-induced prostaglandin E2-EP2, EP4 signaling regulates vascular endothelial growth factor production and ovarian carcinoma cell invasion.* J Biol Chem, 2004. **279**(45): p. 46700-5.
170. Alagappan, V.K., et al., *Vasoactive peptides upregulate mRNA expression and secretion of vascular endothelial growth factor in human airway smooth muscle cells.* Cell Biochem Biophys, 2007. **47**(1): p. 109-18.
171. Qu, G. and H.P. von Schroeder, *Role of osterix in endothelin-1-induced downregulation of vascular endothelial growth factor in osteoblastic cells.* Bone, 2006. **38**(1): p. 21-9.
172. Jesmin, S., et al., *Differential effects of selective endothelin type a receptor antagonist on the gene expression of vascular endothelial growth factor and its receptors in streptozotocin-induced diabetic heart.* Exp Biol Med (Maywood), 2006. **231**(6): p. 902-6.
173. Bluteau, G., et al., *VEGF and VEGF receptors are differentially expressed in chondrocytes.* Bone, 2007. **40**(3): p. 568-76.
174. Tokuda, H., et al., *p38 MAP kinase regulates BMP-4-stimulated VEGF synthesis via p70 S6 kinase in osteoblasts.* Am J Physiol Endocrinol Metab, 2003. **284**(6): p. E1202-9.
175. Yao, Y., et al., *Regulation of bone morphogenetic protein-4 by matrix GLA protein in vascular endothelial cells involves activin-like kinase receptor 1.* J Biol Chem, 2006. **281**(45): p. 33921-30.
176. Maeshima, K., et al., *Crucial role of activin a in tubulogenesis of endothelial cells induced by vascular endothelial growth factor.* Endocrinology, 2004. **145**(8): p. 3739-45.
177. Wagner, K., et al., *Activin A stimulates vascular endothelial growth factor gene transcription in human hepatocellular carcinoma cells.* Gastroenterology, 2004. **126**(7): p. 1828-43.
178. Poulaki, V., et al., *Activin a in the regulation of corneal neovascularization and vascular endothelial growth factor expression.* Am J Pathol, 2004. **164**(4): p. 1293-302.
179. Benckert, C., et al., *Transforming growth factor beta 1 stimulates vascular endothelial growth factor gene transcription in human cholangiocellular carcinoma cells.* Cancer Res, 2003. **63**(5): p. 1083-92.
180. Yoo, P.S., A.L. Mulkeen, and C.H. Cha, *Post-transcriptional regulation of vascular endothelial growth factor: implications for tumor angiogenesis.* World J Gastroenterol, 2006. **12**(31): p. 4937-42.
181. Liu, L. and M.C. Simon, *Regulation of transcription and translation by hypoxia.* Cancer Biol Ther, 2004. **3**(6): p. 492-7.

182. Blume, S.W., et al., *Mithramycin inhibits SP1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo.* J Clin Invest, 1991. **88**(5): p. 1613-21.
183. Luscher, B., et al., *Regulation of transcription factor AP-2 by the morphogen retinoic acid and by second messengers.* Genes Dev, 1989. **3**(10): p. 1507-17.
184. Pertovaara, L., et al., *Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells.* J Biol Chem, 1994. **269**(9): p. 6271-4.
185. Frank, S., et al., *Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing.* J Biol Chem, 1995. **270**(21): p. 12607-13.
186. Sanchez-Elsner, T., et al., *Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression.* J Biol Chem, 2001. **276**(42): p. 38527-35.
187. Nakagawa, T., et al., *TGF-beta induces proangiogenic and antiangiogenic factors via parallel but distinct Smad pathways.* Kidney Int, 2004. **66**(2): p. 605-13.
188. Kanies, C.L., *Enrichment of AU-Rich element containing mRNAs during intestinal cell epithelial-mesenchymal transition: roles, mechanisms and significance*, in *Cancer Biology*. 2008, Vanderbilt University: Nashville. p. 144.
189. Li, J., et al., *Oncogenic K-ras stimulates Wnt signaling in colon cancer through inhibition of GSK-3beta.* Gastroenterology, 2005. **128**(7): p. 1907-18.
190. Shao, J., et al., *Prostaglandin E2 Stimulates the beta-catenin/T cell factor-dependent transcription in colon cancer.* J Biol Chem, 2005. **280**(28): p. 26565-72.
191. Jinnin, M., H. Ihn, and K. Tamaki, *Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-beta1-induced extracellular matrix expression.* Mol Pharmacol, 2006. **69**(2): p. 597-607.
192. Medici, D., E.D. Hay, and D.A. Goodenough, *Cooperation between snail and LEF-1 transcription factors is essential for TGF-beta1-induced epithelial-mesenchymal transition.* Mol Biol Cell, 2006. **17**(4): p. 1871-9.
193. Feng, X.H., et al., *The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation.* Genes Dev, 1998. **12**(14): p. 2153-63.
194. Janknecht, R., N.J. Wells, and T. Hunter, *TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300.* Genes Dev, 1998. **12**(14): p. 2114-9.
195. Pouponnot, C., L. Jayaraman, and J. Massague, *Physical and functional interaction of SMADs and p300/CBP.* J Biol Chem, 1998. **273**(36): p. 22865-8.
196. Topper, J.N., et al., *CREB binding protein is a required coactivator for Smad-dependent, transforming growth factor beta transcriptional*

responses in endothelial cells. Proc Natl Acad Sci U S A, 1998. **95**(16): p. 9506-11.
197. Leung, K.W., et al., *Ginsenoside-Rg1 induces vascular endothelial growth factor expression through the glucocorticoid receptor-related phosphatidylinositol 3-kinase/Akt and beta-catenin/T-cell factor-dependent pathway in human endothelial cells.* J Biol Chem, 2006. **281**(47): p. 36280-8.
198. Amini Nik, S., et al., *TGF-beta modulates beta-Catenin stability and signaling in mesenchymal proliferations.* Exp Cell Res, 2007. **313**(13): p. 2887-95.
199. Jian, H., et al., *Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells.* Genes Dev, 2006. **20**(6): p. 666-74.
200. Guger, K.A. and B.M. Gumbiner, *A mode of regulation of beta-catenin signaling activity in Xenopus embryos independent of its levels.* Dev Biol, 2000. **223**(2): p. 441-8.
201. Young, C.S., et al., *Wnt-1 induces growth, cytosolic beta-catenin, and Tcf/Lef transcriptional activation in Rat-1 fibroblasts.* Mol Cell Biol, 1998. **18**(5): p. 2474-85.
202. Nelson, R.W. and B.M. Gumbiner, *A cell-free assay system for beta-catenin signaling that recapitulates direct inductive events in the early xenopus laevis embryo.* J Cell Biol, 1999. **147**(2): p. 367-74.
203. Korinek, V., et al., *Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma.* Science, 1997. **275**(5307): p. 1784-7.
204. Inoki, K., et al., *TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth.* Cell, 2006. **126**(5): p. 955-68.
205. Barker, N., P.J. Morin, and H. Clevers, *The Yin-Yang of TCF/beta-catenin signaling.* Adv Cancer Res, 2000. **77**: p. 1-24.
206. Persad, S., et al., *Tumor suppressor PTEN inhibits nuclear accumulation of beta-catenin and T cell/lymphoid enhancer factor 1-mediated transcriptional activation.* J Cell Biol, 2001. **153**(6): p. 1161-74.
207. Levy, L., et al., *Acetylation of beta-catenin by p300 regulates beta-catenin-Tcf4 interaction.* Mol Cell Biol, 2004. **24**(8): p. 3404-14.
208. Lei, S., et al., *The murine gastrin promoter is synergistically activated by transforming growth factor-beta/Smad and Wnt signaling pathways.* J Biol Chem, 2004. **279**(41): p. 42492-502.
209. Neufeld, K.L., et al., *APC-mediated downregulation of beta-catenin activity involves nuclear sequestration and nuclear export.* EMBO Rep, 2000. **1**(6): p. 519-23.
210. Orsulic, S., et al., *E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation.* J Cell Sci, 1999. **112 (Pt 8)**: p. 1237-45.
211. Prieve, M.G. and M.L. Waterman, *Nuclear localization and formation of beta-catenin-lymphoid enhancer factor 1 complexes are not sufficient for activation of gene expression.* Mol Cell Biol, 1999. **19**(6): p. 4503-15.

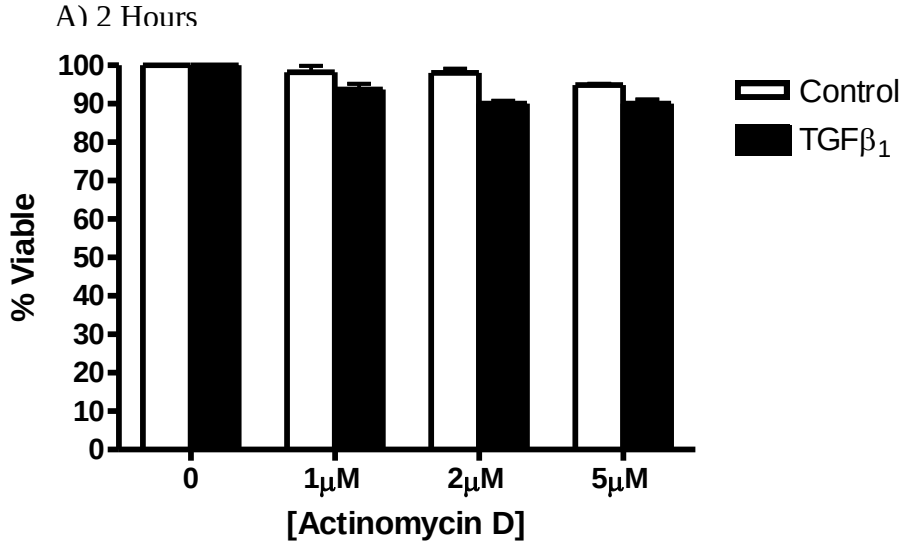
212. Sadot, E., et al., *Regulation of S33/S37 phosphorylated beta-catenin in normal and transformed cells.* J Cell Sci, 2002. **115**(Pt 13): p. 2771-80.
213. Whitmarsh, A.J. and R.J. Davis, *Regulation of transcription factor function by phosphorylation.* Cell Mol Life Sci, 2000. **57**(8-9): p. 1172-83.
214. Li, Y., et al., *Regulation of Akt/FOXO3a/GSK-3beta/AR signaling network by isoflavone in prostate cancer cells.* J Biol Chem, 2008. **283**(41): p. 27707-16.
215. Bijur, G.N. and R.S. Jope, *Proapoptotic stimuli induce nuclear accumulation of glycogen synthase kinase-3 beta.* J Biol Chem, 2001. **276**(40): p. 37436-42.
216. Chen, H., et al., *Inhibition of GSK-3beta decreases NF-kappaB-dependent gene expression and impairs the rat liver regeneration.* J Cell Biochem, 2007. **102**(5): p. 1281-9.
217. Kang, D.E., et al., *Presenilin 1 facilitates the constitutive turnover of beta-catenin: differential activity of Alzheimer's disease-linked PS1 mutants in the beta-catenin-signaling pathway.* J Neurosci, 1999. **19**(11): p. 4229-37.
218. Bakin, A.V., et al., *Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration.* J Biol Chem, 2000. **275**(47): p. 36803-10.
219. Gingery, A., et al., *TGF-beta coordinately activates TAK1/MEK/AKT/NFkB and SMAD pathways to promote osteoclast survival.* Exp Cell Res, 2008. **314**(15): p. 2725-38.
220. Krymskaya, V.P., et al., *TGF-beta 1 modulates EGF-stimulated phosphatidylinositol 3-kinase activity in human airway smooth muscle cells.* Am J Physiol, 1997. **273**(6 Pt 1): p. L1220-7.
221. Letamendia, A., E. Labbe, and L. Attisano, *Transcriptional regulation by Smads: crosstalk between the TGF-beta and Wnt pathways.* J Bone Joint Surg Am, 2001. **83-A Suppl 1**(Pt 1): p. S31-9.
222. Sobolewski, A., et al., *Failure of bone morphogenetic protein receptor trafficking in pulmonary arterial hypertension: potential for rescue.* Hum Mol Genet, 2008. **17**(20): p. 3180-90.
223. Black, S.M., et al., *Cyclic stretch increases VEGF expression in pulmonary arterial smooth muscle cells via TGF-1 and reactive oxygen species: a requirement for NAD(P)H oxidase.* Conf Proc IEEE Eng Med Biol Soc, 2004. **7**: p. 5053-6.
224. Hurlstone, A. and H. Clevers, *T-cell factors: turn-ons and turn-offs.* Embo J, 2002. **21**(10): p. 2303-11.
225. Lundblad, J.R., et al., *Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP.* Nature, 1995. **374**(6517): p. 85-8.
226. Shen, X., et al., *TGF-beta-induced phosphorylation of Smad3 regulates its interaction with coactivator p300/CREB-binding protein.* Mol Biol Cell, 1998. **9**(12): p. 3309-19.
227. Chen, R., et al., *Regulation of Akt/PKB activation by tyrosine phosphorylation.* J Biol Chem, 2001. **276**(34): p. 31858-62.
228. Green, C.J., et al., *Use of Akt inhibitor and a drug-resistant mutant validates a critical role for protein kinase B/Akt in the insulin-*

dependent regulation of glucose and system A amino acid uptake. J Biol Chem, 2008. **283**(41): p. 27653-67.
229. Franke, T.F., et al., *The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase.* Cell, 1995. **81**(5): p. 727-36.
230. Zhu, X., et al., *Constitutively active PKB/Akt inhibited apoptosis and down-regulated beta1,4-galactosyltransferase 1 in hepatocarcinoma cells.* Biochem Biophys Res Commun, 2003. **309**(2): p. 279-85.
231. H. Deng, J.K.B., J. Lei, D.J. Pinsky, M.B. Hershenson, *Bone Morphogenic Protein (BMP#0-4, Transforming Growth Factor (TGF)-beta, Serotonin (5-HT) and Endothelin (ET)-1 Each Induce Pulmonary Vasculaar Smooth Muscle Hypertrophy*, in *American Thoracic Society International Conference*. 2008: Toronto.
232. Gronwald, R.G., R.A. Seifert, and D.F. Bowen-Pope, *Differential regulation of expression of two platelet-derived growth factor receptor subunits by transforming growth factor-beta.* J Biol Chem, 1989. **264**(14): p. 8120-5.
233. Haberstroh, U., et al., *TGF-beta stimulates rat mesangial cell proliferation in culture: role of PDGF beta-receptor expression.* Am J Physiol, 1993. **264**(2 Pt 2): p. F199-205.
234. Markewitz, B.A., et al., *Regulation of endothelin-1 synthesis in human pulmonary arterial smooth muscle cells. Effects of transforming growth factor-beta and hypoxia.* Cardiovasc Res, 2001. **49**(1): p. 200-6.
235. Hoshikawa, Y., et al., *Prostacyclin receptor-dependent modulation of pulmonary vascular remodeling.* Am J Respir Crit Care Med, 2001. **164**(2): p. 314-8.
236. Sheares, K.K., et al., *Differential effects of TGF-beta1 and BMP-4 on the hypoxic induction of cyclooxygenase-2 in human pulmonary artery smooth muscle cells.* Am J Physiol Lung Cell Mol Physiol, 2004. **287**(5): p. L919-27.
237. Ristimaki, A., O. Ylikorkala, and L. Viinikka, *Effect of growth factors on human vascular endothelial cell prostacyclin production.* Arteriosclerosis, 1990. **10**(4): p. 653-7.
238. Rabinovitch, M., *Linking a serotonin transporter polymorphism to vascular smooth muscle proliferation in patients with primary pulmonary hypertension.* J Clin Invest, 2001. **108**(8): p. 1109-11.
239. Dempsie, Y. and M.R. MacLean, *Pulmonary hypertension: therapeutic targets within the serotonin system.* Br J Pharmacol, 2008. **155**(4): p. 455-62.

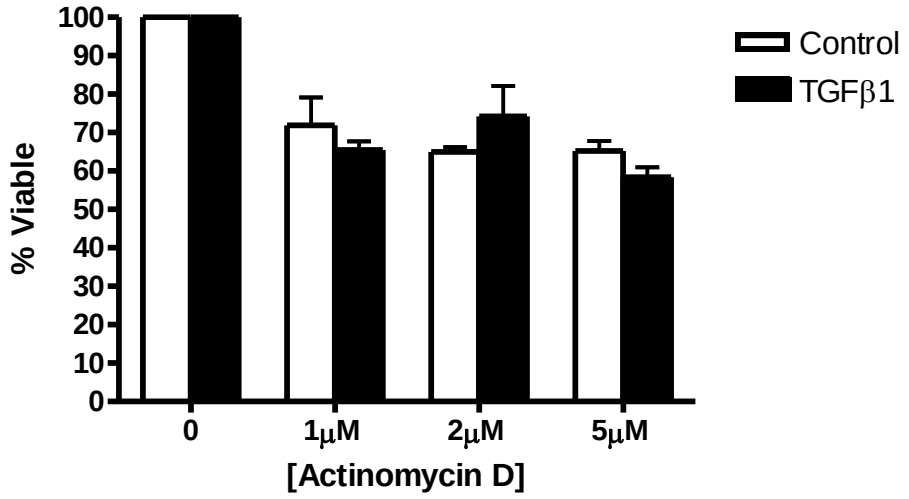
11 APPENDIX 1 – TOXICITY OF ACTINOMYCIN D

11.1 TOXICITY OF ACTINOMYCIN D

Actinomycin can be toxic to cells and as such were performed a number of MTT assays to establish if toxicity could be minimised. As can be seen from Figure 11-1 Actinomycin D did not cause cell death at 2 hours, however significant cell death was seen at 4.5 and 6 hours. Toxicity was not dependent on the concentration of Actinomycin D or TGFβ_1. As a result Actinomycin D experiments were performed with the highest concentration of Actinomycin D to ensure an effect, but experiments were kept under 2 hours when investigating its effects on mRNA, as significant induction is seen after 1 hour and at later time points cell death reduces cell number to the extent that sufficient RNA cannot be extracted to reverse transcribe and amplify. However, when investigating the effects on protein production a longer incubation was necessary and as such the VEGF concentrations obtained were always normalised to MTT data.



B) 4.5 Hours



C) 6 Hours

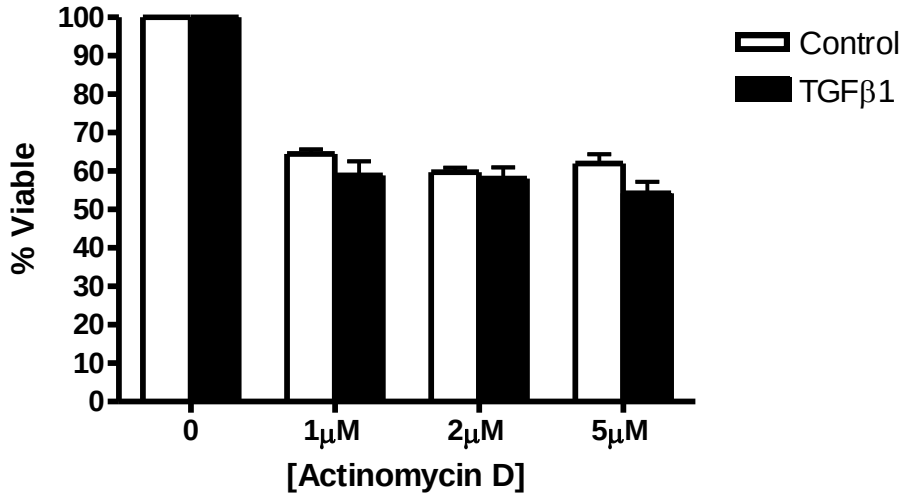


Figure 11-1 The toxicity of the inhibitor of transcription, Actinomycin D

MTT assay following incubation of PASMCs with Actinomycin D. Confluent PASMCs were serum starved for 24 hours and incubated for A) 2 hours, B) 4.5 hours and C) 6 Hours, with various concentrations of Actinomycin D. Bars represent the mean ± s.e.m of triplicate sample from a single experiment.