The Role of Stanniocalcin-1 in Vascular Cell Function

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2013

A thesis submitted for the degree of Doctor of Philosophy

Declaration

I, Rudina Tahiri, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Stanniocalcin-1 (STC-1) is a 56kDa disulphide-linked homodimeric glycoprotein in vertebrates, with the capacity to regulate the extracellular ionised calcium (Ca^{2+}) concentration. Specifically, in fish, Stanniocalcin (STC) is released into bloodstream in response to rising serum calcium levels by acting on calcium sensitive receptors on both gill and intestine epithelial cells to reduce the calcium uptake. Initially, STC was identified as a secreted hormone having an inhibitory effect on whole body Ca^{2+} influx that prevents hypercalcemia. However the function of this protein in mammals is still unclear.

During angiogenesis, essential angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) and Platelet-derived Growth Factor (PDGF), initiate cellular responses, intracellular signaling events that drive proliferation, migration and survival of vascular endothelial and smooth muscle cells (SMCs), respectively. Thus, it is important to understand the physiology of the molecular mechanism through which VEGF and/or PDGF exerts its biological activities as well as its role in malignancies in order to develop anti-angiogenic strategies for therapeutic intervention.

In the first part of this thesis, the role of STC-1 in endothelial cell function was characteristed using varirous approaches. Analysis of VEGF-stimulated migration showed that STC-1 can modulate endothelial cell motility but has no effect on endothelial cell signalling.

Here, I identified for the first time that PDGF-BB but not PDGF-AA, strongly upregulates expression of STC-1 in vascular smooth muscle cells. PDGF-BB-induced STC-1 expression is at least partially mediated via PDGFRα and possibly PDGFRα/β heterodimers, but there appears to be compensation between the two receptors. However, the partial inhibition of STC-1 expression by siRNA-mediated knockdown of both PDGFRs raises the possibility that an unconventional pathway is also involved not mediated by known PDGFRs, or by a non-Receptor Tyrosine Kinase (RTK) pathway.

Furthermore, a novel synergistic effect of STC-1 and PDGF-BB in SMCs on phosphorylation of Akt/PBK protein that leads to GSK3 inhibition was observed. As

a PDGF-induced protein, STC-1 is implicated in modulating PDGF-mediated cell motility, and is a potential modulator of PI3K-Akt pathway activation. Akt enhanced phosphorylation might indicate a role for STC-1 in regulating SMC survival.

Acknowledgements

Praise and Gratitude belongs to Our Lord, Lord of Universe.

My sincere gratitude for my supervisors Professor Ian Zachary, Dr. Ian Evans and my past supervisor Dr. David Homes is so great that I hardly could find any excellent way of describing it.
Their guidance, patience, continuous support, and above all their inspiring motivation during my PhD research in this laboratory is endlessly appreciated. I could not have imagined a better supervision for my PhD study.
I would like to thank you the British Heart Foundation for funding the work in this project.
Thank you also to all members of our laboratory and Ark Therapeutics Ltd., each and every one repeatedly helping me and kindly providing support and reagents for this work; Dr. Paul Frankel, Dr. Lily Cheng, Dr. Hayian Jia and Dr. Berger Herzog for their encouragement, comments and continuous support and guidance. I am genuinely grateful to them for helping with setting up my experiments and sharing their molecular biology knowledge with me and for useful discussions.
I would like to thank Dr. Caroline Pellet-Many for her invaluable teaching of smooth muscle cells characteristics and constant help and guidance with technical support. I am grateful to my fellow labmate Dr. Maiko Yamaji for her generous help with PCR techniques and constant availability for an open scientific discussion.
I also I would like to thank Dr. Vedanta Mehta and Miss Angela Barrett for being so kind to me and creating a helpful and friendly environment for me in the lab.
Many thanks to all members of the Centre for Cardiovascular Biology and Medicine for helping in many areas of my research.
Finally, to my family, thank you so greatly for being so loving and compassionate to me and for bearing my stress, my struggle, my passion. I realise that without your

tender love, and as my youngest son would describe, your *gigantic patience* and understanding I could not be here today, fulfilling a dream.

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Abbreviations

ABC	ATP-binding cassette
Ad	Adenovirus
Akt	Protein kinase B (PKB)
Asn	Asparagine
ATP	Adenosine 5’ -triphosphate
bp	Base pair(s)
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
Ca^{2+} SC	Calcium sensitive channel
CAG	Cytosine Adenine Guanine (nucleotide codes)
cAMP	Adenosine 3’, 5’-cyclin monophosphate
cDNA	Complementary DNA
Crk	CT10 Regulator of Kinase where CT10 is the avian virus
CS	Corpuscles of Stannius
Da	Daltons
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulphoxide
DNA	2’- Deoxyribonucleic acid
DNase	2’- Deoxyribonuclease
Ds	Double stranded
dT	2’- Deoxythimidine phosphate
DTT	Dithiothreitol
E	Embryonic day, time after fertilisation
EBM	Endothelial basal medium
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra acetic acid
eNOS	Endothelial nitric oxyde synthase
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal- regulated kinase
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FGF	Fibroblast growth factor

Flt	Fms-like tyrosine kinase
Fms	Fibroblast motility stimulating factor
GAG	Glycoaminoglycan
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GFP	Green fluoresent protein
GTP	Guanosine triphosphate
H_2O2	Hydrogen peroxide
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
IKB	Inhibitor of NF-kB
Ig	Immunoglobulin
IL-1	Interleukin-1
IP_3	Inositol -1, 4, 5-triphosphate
Kb	Kilobases
kDa	KiloDalton
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney epithelial cells
MMP	Mitochondrial membrane potential
MMPs	Matrix degrading metalloproteinase
MOPS	3-(N-morpholino) propanesulfonic acid
MEF	Murine embryonal fibroblast
MOI	Multiple of infection, number of viral particles per cell
NF-kB	Nuclear factor-kappa B
NO	Nitric acid
NRP1	Neuropilin 1
Nt	Nucleotide
ORF	Open reading frame
p38- MAPK	p38 mitogen-activated protein kinase
p53	Tumor protein 53
p130 Cas	Protein 130 Cas (Crk-associated substrate)
P-	Phospho-/phosphorylated
PAGE	Poliacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline tween
PCR	Polimerase chain reaction
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptors
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PI3K	Phosphatidylinositide 3 kinase

PIP3	Phosphatidylinositol 3, 4, 5-triphosphate
PIGF	Placental growth factor
pH	Measure of the activity of the hydrogen ion
PTCs	Proximal tubule cells
PVDF	Polyvilylidene fluoride
Ras	Rat sarcoma
RNA	Ribonucleid acid
RNase	Ribonuclease
RT	Reverse transcriptase/reverse transcription
RTK	Receptor Tyrosine Kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacryl amide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SH2	Src homology domain 2
SH3	Src homology domain 3
SHP2	Src homology domain 2 containing tyrosine phosphatase
sHsp	Small heat shock protein
siRNA	Small interfering RNA
SMC	Smooth muscle cell
Src	Sarcoma
STC	Stanniocalcin
TBS	Tris buffer saline
TBST	Tris buffer saline tween
Thr	Threonine
TNF	Tumor necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
Tyr	Tyrosine
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WT	Wild-type

Chapter 1

1 Introduction

Stanniocalcin (STC) is a glycosylated homodimeric protein so called because it is produced by the Corpuscles of Stannius (CS) in teleostean and holostean fish (bony fish). The CS are fish-specific endocrine glands associated with the kidney, which were first described by Professor H. Stannius (assuming that they were fish adrenals (Stannius, 1839; Wagner, Hampong, Park, & Copp, 1986). Originally, STC was described as a regulatory hormone involved in calcium and phosphate regulation and homeostasis, positively regulated by extracellular ionised calcium (Ca^{2+}) from the aquatic environment (Hanssen, Aarden, van der Venne, Pang, & Wendelaar Bonga, 1991). In response to a rise in serum calcium levels, CS cells undergo a stepwise increase in STC secretion with each successive rise in calcium concentration. Upon release, STC acts on calcium sensitive receptors on both gill and gut epithelial cells to reduce calcium uptake. Subsequently, STC has an inhibitory effect on whole body calcium influx that prevents hypercalcemia and also stimulates phosphate (Pi) reabsorption by the kidneys (Chang, Jellinek, & Reddel, 2003). Inhibition of the uptake of calcium through the gills and intestines by STC, together with stimulation of inorganic phosphate reabsorption has been widely confirmed (Filvaroff et al., 2002; Sterba, Wagner, Schroedter, & Friesen, 1993; Wagner, Fargher, Milliken, McKeown, & Copp, 1993; Wagner, Jaworski, & Haddad, 1998) and its function in bony fish has been established as a calcium and phosphate regulatory hormone.

1.1 Stanniocalcin

As mentioned previously, Stanniocalcin or as initially called teleocalcin, was first identified as a secreted homodimeric protein from CS. It took more than a century, however for the functional importance of these specialised glands for gill calcium transport to be established by the demonstration that surgical removal of CS glands caused hypercalcemia (Fontaine, 1964). Removal of the CS also reduces plasma Na^+ and Cl^- concentrations and decreases aortic blood pressure (Butler & Alia Cadinouche, 1995; B. H. Yeung, Law, & Wong, 2012). Further studies showed that CS extracts inhibited gill calcium transport (Fenwick & So, 1974). Subsequently, it was found that CS glands produced a 56 kDa glycoprotein that inhibited Ca^{2+} influx from the water without affecting Ca^{2+} efflux, thus promoting hypocalcemia (Flik et al., 1990; Lafeber, Flik, Wendelaar Bonga, & Perry, 1988; Wagner, De Niu, Jaworski, Radman, & Chiarot, 1997; Wagner et al., 1993; Wagner et al., 1986). It was suggested that the inhibition of Ca^{2+} influx could be the result of a specific modulation of the Ca^{2+} uptake mechanism in the gill by reducing whole body Ca^{2+} uptake (Lafeber et al., 1988). Furthermore, consistent with a role of STC in fish calcium homeostasis in response to hypercalcemic stimuli, increased serum Ca^{2+} stimulates STC release by the CS, an effect mediated via calcium-sensing receptors (CaSR) identified on the surface of cultured CS cells. STC mRNA stability is also effected from elevated plasma Ca^{2+} concentration, indicating that Ca^{2+} levels as the primary stimulus, control storage and synthesis rate of STC in CS cells (Ellis & Wagner, 1995; Greenwood, Flik, Wagner, & Balment, 2009; Radman, McCudden, James, Nemeth, & Wagner, 2002). It is now well established that STC functions in fish to regulate Ca^{2+} levels primarily by inhibiting Ca^{2+} movement through the gills (Fig. 1.1).

It seems likely that evolutionary conservation of a role for the CS and STC in regulating Ca^{2+} homeostasis is closely linked to the aquatic environment of fish and the oviparous nature of fish development in which embryos are surrounded by water with widely differing calcium concentrations. It is generally accepted that STC plays a major role in mineral homeostasis in both marine and freshwater fish, with, for example, little difference between freshwater and marine salmon in the blood level and sensitivity of STC to calcium levels. Indeed, in seawater, where the Ca^{2+} concentrations are 10 times higher than that of extracellular fluid, in the absence of STC, Ca^{2+} ions will penetrate by

diffusion across the gills, skin and intestines as calcium moves along an electrochemical gradient, causing hypercalcemia in these organs.

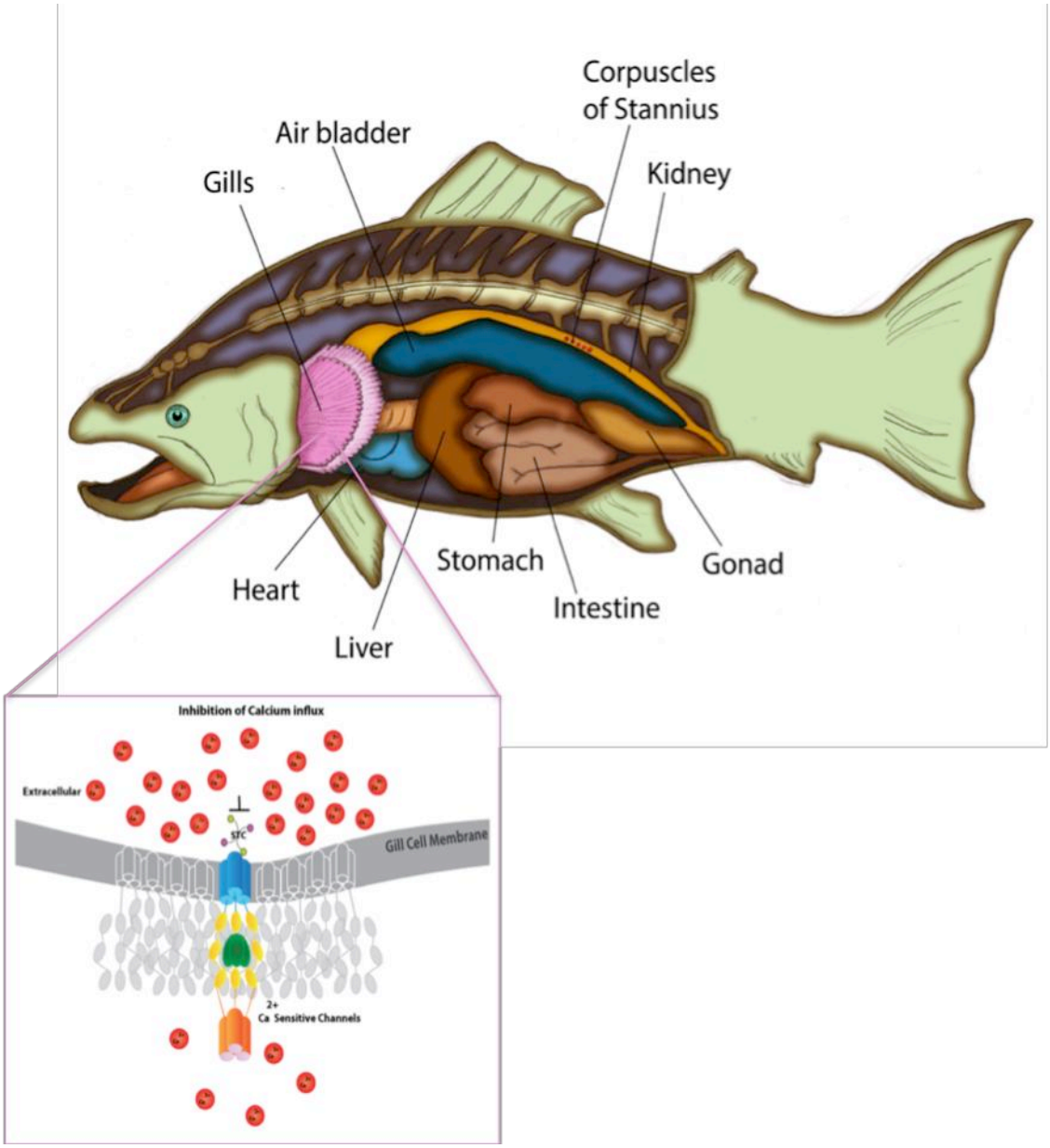


Figure 1.1: Physiological action of STC in fish

This diagram represents the possible mechanism by which CS gland-derived STC acts on epithelial Ca^{2+} channels to inhibit calcium movement by preventing hypercalcemia in the fish. STC is thought to bind to CaSCs and potentially promotes complex formation that leads to inhibition of CaSCs resulting in Ca^{2+} influx homeostasis.

However the precise mechanism by which STC prevents hypercalcemia in fish remains unclear, and one of the main obstacles to defining the mechanism has been the inability to identify the STC receptor on its target cells. The effect of STC may partly be mediated via reduced mRNA expression of epithelial Ca^{2+} channels, as has been shown in Zebrafish (Tseng et al., 2009). STC-mediated Ca^{2+} channel inhibition in gill epithelial cells may also involve the activation of the cyclicAMP- Protein kinase A (PKC) pathway (Lu, Wagner, & Renfro, 1994). Salmon-derived STC has a concentration-dependent stimulatory effect on renal inorganic phosphate (Pi) reabsorption by flounder renal proximal tubule cells (PTCs) whereas Ca^{2+} transport is unaffected (Lu et al., 1994). Furthermore, STC increased cAMP levels, and transeptithelial STC-induced luminal-to-peritubular Pi flux was prevented by pre-incubation with H-89, a protein kinase A inhibitor. Thus, the authors concluded that the effect of STC on Pi transport by flounder PTCs is mediated through the cAMP-PKA pathway. The increased renal Pi retention might also elevate plasma Pi, thereby promoting Ca^{2+} and Pi deposition into bone and scales. Previous work also supported these findings by suggesting that STC not only reduces Ca^{2+} entry but actively promotes the efflux of intracellular Ca^{2+} load, an action that is consistent with the antihypercalcemic role of STC (Fenwick & Brasseur, 1991).

STC production in fish is not confined to the CS glands and, in fact, the *stc* gene is variably expressed at lower levels in most organs. For example, in the rainbow trout, the highest levels of expression outside the CS glands are in the male and female gonads and the anterior region of the kidney (Richards, Fenton, Syed, & Wagner, 2012), whereas in flounder, *Platichthys flesus*, STC is highly expressed in the caudal neurosecretory system. (Greenwood et al., 2009; Tseng et al., 2009).

Apparently, the gene product in these other tissues does not necessarily perform the same biological functions as that in CS gland-derived STC. For instance a more heavily glycosylated variant is produced by the ovaries in female rainbow trout that may have a role in reproduction (McCudden, Kogon, DiMattia, & Wagner, 2001). It has further been proposed that *stc* expression in different tissues may have variable physiological effects on development and/or homeostasis as either autocrine or paracrine factors (Richards et al., 2012).

1.1.1 Stanniocalcin in mammals

Normal physiological processes such as muscle contraction, nerve signal transmission and cellular metabolism constantly require regulatory homeostatic mechanisms that ensure an effective control of extracellular calcium levels. Aquatic vertebrates are surrounded by water, which acts as a continuous and abundant source of calcium; whereas terrestrial vertebrates are dependent on a dietary supply of calcium. Therefore, these two different environments, land and water, might be expected to have profound functional and evolutionary implications for calcium-regulating homeostatic mechanisms.

In terrestrial vertebrates there are two main hormones important for calcium homeostasis: the hypercalcemic factor, parathyroid hormone (PTH), produced by the parathyroid glands and the hypocalcemic factor, calcitonin (CT). Both hormones act via specific receptors localized in the gut, bone and kidney. A third important factor is vitamin D, in particular its metabolite 1, 25-dihydroxyvitamin D_3 which acts as a total body calcium-regulating hormone. Therefore, the presence in terrestrial vertebrates of STC homologs as antihypercalcemic hormones was originally doubted, as they were considered functionally redundant, particularly since terrestrial animals do not face the threat of hypercalcemia that fish are exposed to. Moreover, no homologous organs for CS have been identified in higher vertebrates. However, several studies showed that fish STC had PTH-like biological activity in mammalian cells (Lafeber, Herrmann-Erlee, Flik, & Wendelaar Bonga, 1989; Yoshiko, Kosugi, & Koide, 1996), and subsequently two independent laboratories discovered a mammalian ortholog of fish STC, renamed STC-1. STC-1 was identified by mRNA differential display of genes involved in the control of cellular proliferation and by a random sequencing screen of a human fetal lung cDNA library (Chang et al., 1995; Olsen, Cepeda, Zhang, Rosen, & Vozzolo, 1996). Further immunological and biological assays revealed that a similar protein product to fish STC was expressed in human and rat kidney.

A second human and mouse STC gene and protein, STC-2 was subsequently identified during a search in expressed sequence tag (EST) databases (Chang & Reddel, 1998), and more recently, a second fish STC has also been identified (Luo, Pisarska, & Hsueh,

2005).

The STC-1 protein sequence has approximately 80% amino acid similarity with fish STC. This similarity between the two proteins indicates that the role of STC-1 as an anti-hypercalcemic factor may be conserved from fish to mammals (Chang, Dunham, Jeffrey, & Reddel, 1996). STC-2 protein seems quite different from STC-1 sharing only 34% identity. Indeed, mammalian STC-1 is more closely related to fish STC-1 than to mammalian STC-2. Like fish STC, mammalian STC-1 and STC-2 are secreted as disulfide-linked homodimeric proteins with a conserved N-linked glycosylation site [Asn-X-Thr (Ser)] and conserved cysteine residues (see Figure 1.2). There are 11 cysteine residues present in STC-1 monomers, forming five intramonomeric disulphide bonds and one intermonomeric disulphide bond. In contrast, STC-2 has 15 cysteine residues, albeit with the same spacing as fish STC and STC-1. In addition, mammalian STC-2 has 15 histidine residues clustered towards the C-terminus that are not present in either STC-1 or the fish STC-2 sequence (Luo et al., 2005). This might indicate a possible role of mammalian STC-2 in interaction with transition metals (DiMattia, Varghese, & Wagner, 1998; Moore et al., 1999).

1.1.2 Structure of STC-1 gene

1.1.2.1 Fish STC

The fish *stc* gene from *F. rubripes* contains five exons spanning about 3 kb. Based on the comparison of *stc1* gene structures, exons 3 and 4 of *F. rubripes stc1* could be derived from a single exon with an intron insertion during evolution. Overall, the conservation of exon-intron boundaries among all *stc1* and *stc2* genes suggests that they originated from a common ancestral gene before the divergence of fish and mammals (Luo et al., 2005; McCudden, Kogon, et al., 2001).

Four stanniocalcin genes have been identified and their products were isolated and characterized in *T. nigroviridis* and other teleost fishes. The isolated Tetraodon *stc* transcripts contain the pro-peptide and the entire mature peptide-coding region and they share high sequence similarity and conserved sequence motifs such as the N-terminal glycosylation domain and cysteine residues with the teleost and tetrapod homologues. A duplication of the *stc1* and *stc2* genes in the teleost lineage presumably gave rise to the

four Tetraodon *stcs* and their presence in a variety of fish tissues is in agreement with their proposed paracrine, autocrine and intracrine roles in fish species (Schein et al., 2012).

In teleosts, the physiological role of STC-1-A is well established and a rise in extracellular Ca^{2+} levels above the physiological level activates its expression and promotes STC secretion by CS. Unlike *stc1*-a, the other *stc* genes exhibited low expression levels and no significant changes in response to increased environmental calcium in any of the tissues (CS, gills, and kidney) analysed (Schein et al., 2012).

1.1.2.2 Mammalian STC-1 gene

The gene encoding human *stc-1* is located on the short arm of chromosome 8 (8p11.2-p21). The *Stc1* gene contains four exons spanning about 13kb with 91% nucleotide sequence identity with mouse *stc-1* gene in coding regions (GenBank accession number AF512563) with a highly conserved long 3'- untranslated region (UTR) sequence (Chang et al., 1995; Varghese, Wong, Deol, Wagner, & DiMattia, 1998; Yoshiko & Aubin, 2004). The 5'- UTR of *Stc1 gene* but not of *Stc2*, is rich in the noncontiguous trinucleotide repeat, CAG, with 19 such repeats clustered within 102 nucleotides of the proximal transcription start site. In *stc-1*, four blocks of trinucleotide repeats consist of three to six CAG repeats, which are separated by 6-15 nucleotides (Chang et al., 1998). These repeats are found in the promoters of many genes and constitute regions that can affect DNA structure and modulate gene expression. Interestingly, length polymorphisms such as CAG repeats or other trinucleotide repeats (CGG and GAA) are sometimes associated with the pathogenesis of diseases, as documented in Huntington's disease. However, the significance of CAG repeats in the *stc-1* gene is presently still unknown (Ishibashi & Imai, 2002).

1.2 Mammalian STC-1

The mammalian *stc-1* gene is expressed as a predominant 4 kb transcript and an additional 2 kb transcript with significant expression of STC-1 seen in the heart, kidney, prostate, brain, bone, and thyroid gland, and the highest expression of STC-1 mRNA and protein in the ovary (McCudden, Tam, & Wagner, 2001; Paciga, Watson, DiMattia,

& Wagner, 2002). Indeed, STC-1 appears to circulate in the bloodstream during pregnancy and lactation, implying a possible role in reproductive biology (Chakraborty et al., 2007; Deol, Varghese, Wagner, & Dimattia, 2000; Li, Luo, & Wang, 2003; Tremblay et al., 2009).

1.2.1 Human STC-1 protein

With the exception of pregnancy and lactation, measurements of mammalian STC-1 in serum have shown low, even undetectable hormone levels. This is in contrast to fish in which STC circulates continuously for the maintenance of serum Ca^{2+} levels. The wide tissue distribution of endogenous STC-1 in mammals suggests that STC-1 might function as an autocrine/paracrine factor, intended for local-cellular signalling and regulation of cell function rather than acting as a classic endocrine hormone (Chang et al., 2003; Madsen et al., 1998; Wagner, Guiraudon, Milliken, & Copp, 1995).

This notion is now, however, in dispute, since (James, Seitelbach, McCudden, & Wagner, 2005) have shown that mammalian red blood cells have significant carrying capacity for the hormone STC-1. They also described an additional STC-1 binding activity in glomerular filtrate suggestive of the existence of a soluble STC-binding protein. Together, these findings suggest that red blood cells and/or STC-1 binding protein may have specific roles in the transport of STC-1 to tissues that would otherwise have no access to the hormone, or a possible role in sequestering the hormone from the circulation (James et al., 2005).

The mammalian STC-1 cDNA sequence predicts a protein of 247 amino acid residues with a single N-linked glycosylation consensus site. Immunoblotting analysis of human tissue-derived and recombinant STC-1 has revealed that STC-1 is a disulphide-linked homodimer of 25-kDa monomeric subunits and has a molecular mass of approximately 50 kDa under nonreducing conditions (J. Zhang et al., 1998). However, western blot analysis under non-reducing conditions demonstrates that STC-1 secreted from bovine thecal/interstial cells consisted of three higher molecular weight proteins of 84, 112, and 135 kDa. These STC-1 variant forms appear to not be warranted to different glycosylated forms, could be due to the combination of oligomerisation (Chang et al., 2003; Paciga et al., 2002).

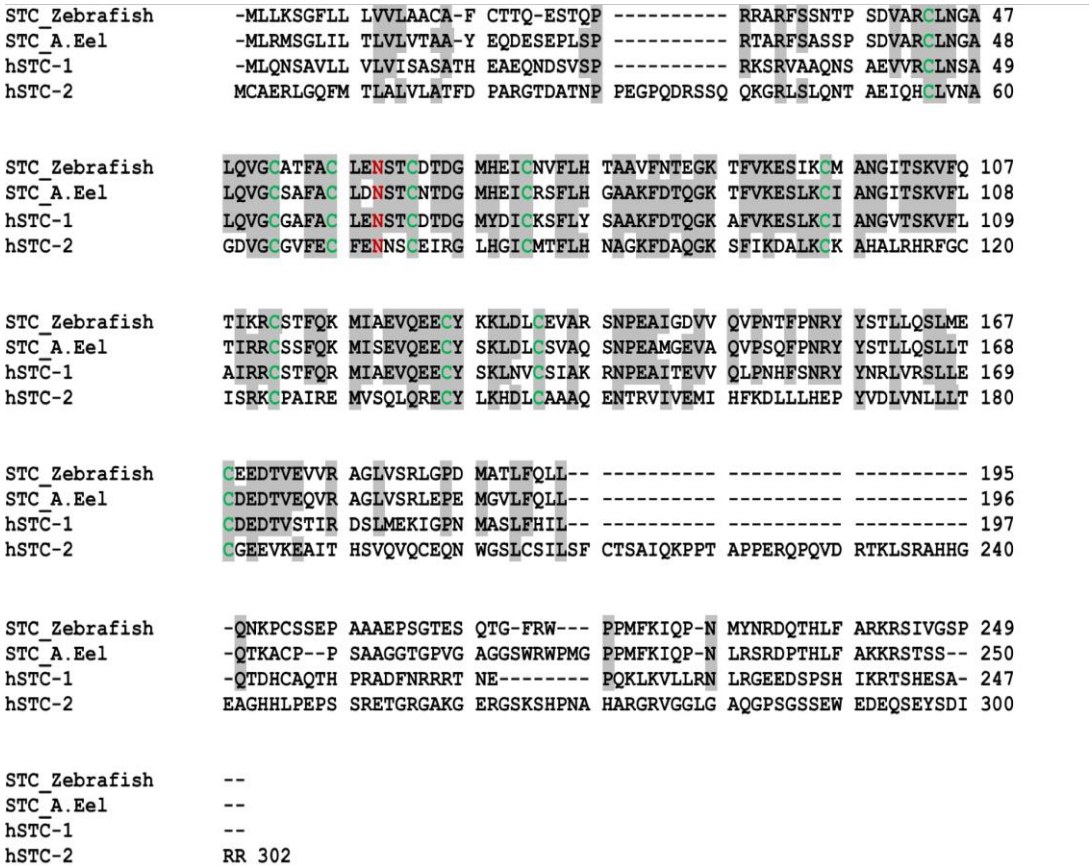


Figure 1.2: A comparison of fish STCs with human STC-1 and STC-2

The sequence alignment of amino acid sequences of STC-1s from zebrafish *(Danio rerio(Brachydanio rerio)*; GenBank accession #Q6PHV3), and Australian eel (*Anguilla australis*; accession #P18301) with human STC-1(accession #P52823) and STC-2 (accession #O76061) was performed by the clustalw program. The shaded amino acids indicate 100% identity. The putative N-linked glycosylation site is highlighted in red and the ten conserved cysteine residues are highlighted in green.

All three proteins were preferentially associated with cholesterol lipid droplets, suggesting a regulatory effect on the steroidogenic pathway (Paciga et al., 2002). There is very little knowledge on the structure of STC-1 variants so far.

An analysis of the human STC-1 amino acid sequence using online Prosite software revealed the presence of a consensus protein kinase ATP-binding domain near the N-terminus of the protein (see Figure 1.2), a domain found in kinases, heat shock proteins and ATP-binding transporters. The possibility that STC-1 might have a kinase activity seems unlikely as it lacks a catalytic domain. It has been demonstrated however, that ATP is able to bind to STC-1 thereby antagonising STC-1 binding to its mitochondrial receptors (see section 1.2.2) (Ellard et al., 2007). Jellinek et al., (2000) showed that the human fibrosarcoma cell line, HT1080, secretes both STC-1 and STC-2 as phosphorylated proteins and indicated that STC-1 is phosphorylated exclusively on serine residue(s). However, the mechanism of STC-1 phosphorylation in HT1080 cells was not clearly demonstrated (Jellinek et al., 2000).

Based on structural similarities between fish STC and STC-1, it is possible that STC-1 is conserved through evolution from fish to mammals. Some important insights into the evolutionary conservation between these proteins have been yielded by analysis of the essential role of human STC-1 in calcium regulation in mammals. Olsen et al (1996) demonstrated the ability of human recombinant STC-1 (hrSTC-1) to inhibit the transport of calcium through fish gills (Olsen et al., 1996) and injection of hrSTC-1 into rats reduced renal phosphate excretion (Wagner et al., 1997). Furthermore, hrSTC-1 decreases intestinal calcium uptake and simultaneously increases phosphate reabsorption in *in vitro* studies with pig and rat cells (Madsen et al., 1998). Published data also suggest a role for STC-1 in the control of intracellular Ca^{2+} in rat cardiomyocytes (Sheikh-Hamad et al., 2003) and neurons (Serlachius, Zhang, & Andersson, 2004). These findings suggest that mammalian STC-1 might contribute as a regulator of mineral homeostasis, with an effect on Ca^{2+} metabolism (reviewed in (Yoshiko & Aubin, 2004).

1.2.2 STC-1 receptors

As mentioned above, elucidation of the mechanisms of action of STC in fish has been hampered by the inability so far to identify STC receptors on its target cells. Similarly, no receptors for mammalian STC have been molecularly cloned, identified or characterized. However, a number of studies have suggested the existence of various putative STC-1 receptors or high affinity binding sites. Using an STC-alkaline phosphatase (AP) fusion protein, receptor-binding assays on mammalian liver and kidney revealed the presence of STC-1 receptors on mitochondria and plasma membranes (McCudden, James, Hasilo, & Wagner, 2002). These receptors had saturable, high-affinity (0.25–0.8 nM), and displaceable binding sites for STC-1 in cells of the kidney, liver, breast, and ovaries (McCudden et al., 2002; McCudden, Majewski, Chakrabarti, & Wagner, 2004; Sheikh-Hamad, 2010). Analysis of membrane and mitochondrial receptors revealed that they have similar binding affinities, indicating that they are similar but regulated at different cell-proportions, with 90% at mitochondrial binding site, while 10% are located at the plasma membrane. The identity of the mitochondrial STC receptor(s) is yet to be established.

Secreted STC-1 is thought to be an autocrine/paracrine hormone, that targets neighbouring cells, where it binds to cell surface receptors, and is subsequently sequestered into mitochondria. Sazonova et al (2008) proposed two different models, which involve the trafficking of STC-1 from the cell surface to the mitochondria (Sazonova et al., 2008).

The first model proposes that the membrane receptor acts as a facilitator of STC-1 entry only, as opposed to a chaperone that accompanies STC-1 to the mitochondria, and consistent with model it was shown that no apparent receptor downregulation occurred after either short or long term exposure of Madin-Darby Canine Kidney Epithelial (MDCK) cells to high ligand concentrations. The second model implies that the membrane receptors do in fact serve as a chaperone to facilitate translocation of STC-1 to the mitochondria and consistent with this model, Wang, et al. (2009) also reported a rapid internalization of recombinant STC-1 by freshly isolated murine peritoneal macrophages and localization to the mitochondria within 10 min (Y. Wang et al., 2009). Although STC-1 receptor levels do not seem to be reduced by excessive STC-1 concentrations, when calcium-rich medium is used to inhibit STC-1 secretion, a

significant upregulation of STC-1 binding sites is observed, indicating a calcium-dependent STC-1 effect on receptor recycling and/or expression (Sazonova et al., 2008; Sheikh-Hamad, 2010).

As in mammals, (Richards et al., 2012) reported the localization of fish STC-1 receptors, using freshwater rainbow trout as a model system. The fish receptor was found to be present on both microsomal membranes and mitochondria. The receptors had high affinity binding sites (3.2–3.7 nM), similar to those reported in mammals, and in line with the concentrations of circulating STC-1 (0.1–5.0 nM) in fish serum. However, in fish as well as in mammals, ligand sequestration occurs in a mitochondrial compartment. *In situ* ligand binding studies revealed the presence of specific, STC-1 binding sites in most fish tissues and/or organs, including brain, cartilage, adipocytes, skeletal, cardiac and visceral smooth muscle, liver, kidney, gastrointestinal tract, the eye, and the teeth (Richards et al., 2012). Red blood cells exhibited the strongest binding activity, followed by cardiac myocytes, and to a lesser extent in liver hepatocytes (Richards et al., 2012). Nephron epithelial cells were generally receptor-positive in both the anterior and posterior portions of the kidney, whereas newly developing nephron segments were receptor-negative. Thus it appears that STC-1 receptor expression might be developmentally regulated. Unexpectedly, there was low binding activity in STC-1 producing cells from the CS of both salmon and trout, whereas a sub-set of CS cells displayed a higher level of binding, indicating that secreted STC-1 may feed back on its cells of origin.

Despite the accumulating evidence of STC-1 binding sites, the STC-1 receptor has not yet been purified or cloned or molecularly characterised. Furthermore, its downstream signalling pathways are essentially unknown. One study, in flounder proximal tubules, has suggested that the receptor may be G-protein coupled (Lu et al 1994), the evidence for this being that the effects of STC-1 on apical phosphate transport are PKA-dependent (blocked by H-89 and mimicked by forskolin) and accompanied by increased cAMP production (Lu et al., 1994; Richards et al., 2012). Nevertheless, the definitive characterization of STC receptors in fish and mammals remains an important but elusive goal essential for determining STC function.

1.3 Role of STC-1 in development

Transgenic mice over-expressing hSTC-1 have been generated by two independent laboratories using different promoters (Filvaroff et al., 2002; Varghese et al., 2002). Filvaroff et al (2002) inserted the gene for human STC-1 (hSTC-1) downstream of the rat muscle specific myosin light chain 2 promoter sequence to achieve efficient expression of hSTC-1 in muscle (mainly skeletal) but no other tissues. Overexpression of STC-1 in these mice resulted in growth retardation with distinct changes in the musculoskeletal system and osteoclast activity (see Table 1). Additionally, transgenic lines revealed significantly higher levels of serum ionized calcium but normal serum phosphate levels compared with wild-type mice. Furthermore, measurements of baseline vascular density strongly suggested that STC-1 transgenic mice had significantly higher capillary density in organs like heart and kidney and tissues like muscle and bone, compared with age-matched littermates. These changes could be due to direct effects of STC-1 on endothelial cells and their environment, consistent with previous studies showing up-regulation of STC-1 upon endothelial cell differentiation (Kahn et al., 2000). STC-1 could also affect muscle mass, function, bone size, and structure (Filvaroff et al., 2002). Varghese et al analysed transgenic mice overexpressing STC-1 using the metallothionein I minimal promoter. These mice had a growth retardation and reproduction phenotype. The highest levels of expression of STC-1 protein were detected in liver, heart, mammary glands and brain. Interestingly transgenic mRNA levels in heart and liver were consistently 2- and 5-fold higher in females, respectively, than those in males (Filvaroff et al., 2002; Varghese et al., 2002). In both studies, transgenic expression of hSTC-1 affected growth and reproduction, as manifested by dwarfism and reduced litter size, with elevated calcium levels in at least some lines of transgenic mice, in spite of their different hSTC-1 tissue expression profiles. In addition to dwarfism, muscle-specific transgenic mice had an energy wasting phenotype indicated by higher food and oxygen consumption rates and mitochondrial hypertrophy at the sites of transgene expression, suggesting the involvement of STC-1 in cellular metabolism and possibly a developmental phenotype (Ellard et al., 2007; Filvaroff et al., 2002).

In contrast, *Stc1*$^{-/-}$ knockout mice did not exhibit gross morphological or behavioural abnormalities compared to wild-type mice (Chang, Cha, Koentgen, & Reddel, 2005). Histological

examination of the lung, heart, liver, kidney, brain, spleen, stomach, small intestine, colon, ovary, uterus, and testis did not reveal any overt differences (Chang et al., 2005). Parallel findings from a double knock out study suggested that the absence of mouse *Stc1* and/or *Stc2* genes has no effect on serum calcium and phosphate levels (Chang et al., 2008). It is therefore unlikely that mammalian STC-1 or STC-2 play a critical role in the prevention of systemic hypercalcaemia under normal physiological conditions. This conclusion is also largely consistent with results obtained with overexpression of STC-1 in transgenic mice. Therefore, it seems reasonable to assume that the role of mammalian STC-1 is not restricted to the anti-hypercalcemic effects attributed to fish STC-1; its role in mineral homeostasis under normal conditions requires more investigation.

1.4 Physiological roles of STC-1

The physiological role of mammalian STC-1 is only beginning to be elucidated. Although the study of the biological activity of STC-1 has increased in the past decade, many questions regarding its role in the systems where it is expressed and various signals that trigger STC-1 secretion remained unanswered. In contrast to fish STC-1 which is largely regulated by elevated Ca^{2+} levels, mammalian STC-1 appears to be regulated in numerous physiological and pathophysiological processes such as angiogenesis (Bell et al., 2001; Holmes & Zachary, 2008; D. Liu, Jia, Holmes, Stannard, & Zachary, 2003; S. Yang et al., 2002) bone and muscle development (Filvaroff et al., 2002; Yoshiko, Aubin, & Maeda, 2002), inflammation (Huang et al., 2009; Y. Wang et al., 2009), hypoxia (H. Y. Yeung et al., 2005; Zhang et al., 2000), differentiation (K. Z. Zhang et al., 1998), neuroprotection, cellular stress response (Nguyen, Chang, & Reddel, 2009) and carcinogenesis (Chang et al., 2003; Neri & Bicknell, 2005).

Table 1.1 Murine phenotypes of STC-1 and STC-2

Protein	+/+++	-/-	Phenotype	References
Transgenic overexpression STC-1	Viable		Dwarfism and skeletal abnormalities High capillary density	(Filvaroff et al., 2002; Varghese et al., 2002)
Transgenic overexpression STC-2	Viable		Substantial postnatal growth retardation. Intrauterine growth-restrictive phenotype.	(Gagliardi, Kuo, Raulic, Wagner, & DiMattia, 2005)
STC-1		Viable	No observed phenotype	(Chang et al., 2005)
STC-1/STC-2		Viable	Increased body weight	(Chang et al., 2008)
STC-2		Viable	Increased body weight	(Chang et al., 2008)

Several studies have suggested that based on expression profiles, STC-1 may have a potential role in angiogenesis (Kahn et al., 2000), wound healing (Iyer et al., 1999) and capillary morphogenesis (Bell et al 2001), because it is highly induced by factors that may directly or indirectly be linked to angiogenic pathways (Zlot et al., 2003). For example, the observation that STC-1 is highly upregulated in endothelial cells treated with VEGF suggested a distinctive role for STC-1 in angiogenesis or the tumour vasculature *in vivo* (Fujiwara et al., 2000; Gerritsen et al., 2002; Holmes & Zachary, 2008; Neri & Bicknell, 2005). Additionally, it has been reported that HGF alone, and synergistically in combination with VEGF, induced vascular morphogenesis *in vitro* and angiogenesis *in vivo*. The analysis of the gene expression profile of endothelial cells undergoing HGF- and VEGF-stimulated morphogenesis identified STC-1, as one of the most highly upregulated genes in the *in vitro* model (Zlot et al., 2003). Hypoxia can also increase STC-1 gene expression in several cancerous tissues and cell-lines (H. Y. Yeung et al., 2005). In addition, there is evidence that hSTC-1 is a Hypoxia Inducible Factor-1 (HIF-1) target gene, and strongly supports the involvement of STC-1 in hypoxic tumor angiogenesis (Law, Ching, Lai, & Wong, 2010).

In situ hybridization has shown a clear upregulation of STC-1 expression in tumour blood vessels, which suggests that STC-1 could be a tumour vascular marker (Gerritsen et al., 2002).

1.4.1 Protection from oxidative stress

STC-1 has been implicated in the regulation of a key pathway involved in the response to oxidative stress, energy metabolism, governing cellular survival and proliferation. The involvement of STC-1 in cellular energy metabolism was initially investigated by McCudden et al. (2002). Analysis of MDCK cells transfected with a mouse soluble STC-AP fusion protein indicated that STC-1 localizes to the inner mitochondrial membrane and enhances NADH disappearance. The receptor binding assays also revealed the presence of a class of high affinity STC receptors in nephron epithelial cells and liver hepatocytes which were present on both the plasma membrane and mitochondria. More significantly perhaps, this study identified STC as a putative regulator of mitochondrial metabolism, as STC-1 had dose-dependent stimulatory

effects on electron transport in bovine sub-mitochondrial particles within the nanomolar range (Ellard et al., 2007; McCudden et al., 2002).

Muscle-specific transgenic mice studies (Filvaroff et al., 2002; Varghese et al., 2002) as previously mentioned, showed an association between mitochondrial hypertrophy and energy wasting phenotypes with excessive STC-1 mitochondrial expression. These findings pointed to energy-related functions of STC-1 and also suggested that STC-1 might be a stimulator of electron transport chain activity (ETC). Using intact functional mitochondria as well as whole cells, STC-1 was found to substantially increase state III oxygen consumption rate and uncouple oxidative phosphorylation as indicated by reduced ADP to oxygen ratios (Ellard et al., 2007).

In addition, Wang et al (2009) further examined the respiratory effects of STC-1 on macrophage function and survival. In freshly isolated peritoneal macrophages and cultured murine macrophages, addition of rhSTC1 to the medium had a dose and time–dependent effect on ATP reduction. In contrast, using total cell lysates, no differences were observed in the activities of complex I, complex II, complex I + III, complex II + III, and complex IV in rhSTC1-treated cells compared with vehicle-treated controls. It is unlikely therefore that STC-1 directly inhibiting mitochondrial respiratory chain complexes is the cause for the decline in intracellular ATP levels (Y. Wang et al., 2009).

As a result of the uncoupling of mitochondrial oxidative phosphorylation, STC-1 was also expected to decrease the generation of reactive oxygen species (ROS). The first direct evidence of a protective role of STC-1 against ROS was demonstrated using dihydroethidium (DHE) staining as an indicator of superoxide ($O_2^{\cdot -}$) generation. Incubation with rhSTC-1 for 24 hours led to a 40% reduction of superoxide generation in wild-type macrophages, whereas no effect was observed in macrophages from uncoupling protein 2 (UCP_2)-/- mice. In addition, there was a cumulative reduction in mitochondrial membrane potential after a 24 hour treatment, sufficient for an attenuation of superoxide generation (Y. Wang et al., 2009)

In contrast to these findings, STC-null mouse embryo fibroblasts (MEFs) appear to be resistant to oxidative stress induced by H_2O_2 treatment due to increased MEK and

ERK1/2 kinase activation. These data suggest that STC-1 expression could act to decrease survival of MEFs in response to oxidative stress by downregulating MEK and ERK1/2 activation (Nguyen et al., 2009).

1.4.1.1 Induction of UCP_2

UCPs are important mitochondrial anion carriers that localize at the inner mitochondrial membrane and facilitate H^+ leakage from the mitochondrial intermembrane space to the matrix (Huang et al., 2009; Sheikh-Hamad, 2010; Y. Wang et al., 2009). UCPs regulate the mitochondrial membrane potential through dissipation of the proton gradient across the inner mitochondrial membrane, without ATP generation. Whereas UCP_1 expression is restricted to brown adipose tissue, UCP_2 is more ubiquitously expressed, with expression also occurring in several phagocytic cell types (D. Park et al., 2011).

The mechanism by which STC-1 reduces superoxide generation and enhances cell survival in macrophages is unknown, but appears to be mitochondrial uncoupling dependent, through increased UCP_2 expression, and attenuation of ROS generation, resulting in a modest depolarisation of the mitochondrial membrane potential (MMP) (Huang et al., 2009; Y. Wang et al., 2009). Wang et al (2009) examined the effect of hrSTC-1 on UCP_2 expression in cultured murine magrophages. An increase in UCP_2 was observed within the first hour of rhSTC-1 treatment. Given the suppression of superoxide generation by STC-1 in macrophages, the authors suggested that STC-1-mediated superoxide reduction is not NADPH oxidase dependent (Sheikh-Hamad, 2010; Y. Wang et al., 2009).

1.4.2 Cell survival

A possible role of STC-1 as an anti-apoptotic factor is supported by the finding that STC-1 is able to promote tolerance of mouse brains and hearts against ischemia (Westberg, Serlachius, Lankila, & Andersson, 2007). Their data provide an insight into the possible role of STC-1 in cell survival under hypoxic conditions. In agreement with these findings, Block et al (2008) also demonstrated that STC-1 reduced the number of apoptotic lung cancer epithelial cells following hypoxia. A number of recent studies

have implicated STC-1 in a possible role in tumor progression, showing that STC-1 expression is regulated by p53 and NFκB in apoptotic nasopharyngeal and colon cancer cells. In addition STC-1 expression is induced in hypoxic tumor microenvironments (Lai et al., 2007; Law et al., 2010).

Similarly, serum-starved STC-1-treated macrophages exhibit lower cellular ATP levels in a dose and time-dependent manner, which might be followed by apoptosis. However, despite the decline in ATP levels, STC-1 actually appears to enhance cell viability, suggesting a role for STC-1 in cellular protection. This may be due to cell cycle arrest in the G1 phase (Y. Wang et al., 2009). Ohkouchi et al. (2012) reported that H_2O_2-treated A549 lung carcinoma cells exhibited improved cell survival when co-cultured with mesenchymal stromal cells (MSCs) than when grown alone. This increased cell survival was attributed to STC-1 since STC-1 blocking antibodies prevented this effect. Furthermore, it was found that the STC-1 secreted by MSCs upregulated UCP_2 expression in A549 cells, thus making them resistant to ROS and enhancing anaerobic glycolysis, the latter phenomenon being a feature of many cancer cells known as the Warburg effect. Similarly, Ohkouchi et al (2012) showed that rSTC1 decreased ROS levels and the MMP of A549 cells, as well as increasing lactate production. All of these effects were shown to be dependent on UCP_2 upregulation. This study raises the possibility of using antibodies or antagonists to STC-1 as an anti-cancer therapy (Ohkouchi et al., 2012).

Data regarding the regulation of STC-1 by hypoxia and its role in reduction in ROS generation in macrophages further suggests a possible important role of STC-1 in promoting cell survival, although there is controversy as to whether STC-1 is pro-survival or pro-apoptotic. For example, in contrast to the previous studies, Law et al (2008) demonstrated that trichostatin A (TSA) induced apoptotic cell death in human colon adenoma cells (HT29), accompanied by a time and dose-dependent induction of STC-1 levels. TSA is a histone deacetylase (HDAC) inhibitor that is known to promote histone and non-histone protein acetylation, an essential post-translational process in regulating chromatin topology, gene transcription and intracellular signalling. Treatment of tumor cells with HDAC inhibitors has been shown to induce transcriptional activation of genes that are essential for the inhibition of cell growth, metastasis, and angiogenesis but also induce apoptosis. The treatment of HT29 cells with 500 nM of TSA significantly stimulated STC-1 gene expression at 4 h of treatment, and this was shown to be

mediated by NFκB acetylation. The induction of STC-1 expression by TSA suggests a role for STC-1 in the cellular apoptotic process in human carcinogenesis (Law et al., 2010; Law, Lai, Lui, Wan, & Wong, 2008).

1.5 STC-1, cancer and angiogenesis

1.5.1 STC in cancer

STC-1 was originally cloned as part of a search for cancer-related genes (Chang et al., 1995) and since then STC-1 has been shown to be highly expressed in many different human tumours suggesting that the elevation of this protein might be involved in remodulation of tumorigenic microenvironment (Chang et al 2003;Yeung et al 2012). Increased STC-1 expression has been reported in the following human neoplasms: non-small cell lung cancer (Du, Gu, Li, & Gao, 2011), colorectal cancers and hepatocelluar carcinomas (Fujiwara et al., 2000; Tamura et al., 2011), breast carcinoma (McCudden et al., 2004; Wascher et al., 2003), ovarian cancer (G. Liu et al., 2010), and leukemia (Tohmiya et al., 2004). In breast cancer, the levels of both STC-1 and STC-2 were reported to correlate with the expression of estrogen receptor (ER). The importance of STCs for cancer progression or tumour growth is at present unclear, but some studies report that STC-1 may promote tumour cell growth by enhancing the expression of an important vascular growth factor, VEGF in gastric cancer cells. Thus, the role of STC-1 is thought to be mediated through the alteration of microenvironment, which in turn induces neovascularisation of tumors without direct effect on proliferation of cancer cells (He et al., 2011; G. Liu et al., 2010).

1.5.2 STC and angiogenesis

One way in which STCs could affect the tumour microenvironment is through regulation of angiogenesis. Since mice deficient in either STC-1 or STC-2 or both STCs, are viable and exhibit no gross or overt defects in development (see Table 1 above), it is unlikely that STCs play essential or important roles in vasculogenesis or embryonic angiogenesis or early development of the cardiovascular system. However, this does not preclude an important role of STCs in angiogenesis in adult organisms, particularly that associated with disease or disease-related processes. In this context, it

is relevant to note, as mentioned above, that mice transgenically over-expressing STC-1 had significantly higher capillary density in several tissues including heart, kidney, muscle and bone (Table 1). It is therefore of interest that in several genome array profiling studies of gene regulation in models of angiogenesis (blood vessel formation; discussed below) and in response to the essential angiogenic factor, VEGF, upregulation of STC-1 has been a common observation array profiling studies of both VEGF-A_{165}-regulated gene expression and gene expression in *in vitro* models of capillary angiogenesis (Bell et al., 2001; Gerritsen et al., 2002; Glienke et al., 2000; Holmes & Zachary, 2008; Kahn et al., 2000; D. Liu et al., 2003; S. Yang et al., 2002). It is clear from these studies that STC-1 mRNA expression is upregulated in angiogenesis in *in vitro* models, and is upregulated in human endothelial cells in response to VEGF. At the time that I began work on this thesis, it was unknown how STC-1 regulated angiogenesis, or whether it might play a significant role in this process. Zlot et al (2003) reported that recombinant STC-1 inhibited Hepatocyte Growth Factor (HGF)-induced endothelial cell migration and morphogenesis in vitro, but had no detectable effect on responses to Vascular Endothelial Growth Factor (VEGF or VEGF-A) or Fibroblast Growth Factor-2 (FGF-2). Nevertheless, given that VEGF-A strongly upregulated STC-1 expression at the level of mRNA and protein expression (Holmes & Zachary, 2008) it remained possible that STC-1 might regulate VEGF responses in endothelial cells, and one of the aims of this thesis was to explore that possibility.

1.6 Vascular development

In large multicellular organisms in all vertebrates, the vasculature is an internal transport system that delivers oxygen and nutrients to peripheral tissues, carries metabolites and macromolecules generated by one tissue to other tissues, and removes waste products. This network initially starts to form during embryonic development in a process termed vasculogenesis.

1.6.1 Vasculogenesis

Vasculogenesis involves the differentiation of endothelial precursors or angioblasts and their assembly into a primitive vascular network, comprising the primary capillary plexuses and the major primordial vessels, principally (in mice, for example) the dorsal aorta and cardinal vein. The haemangioblasts are multipotent precursor cells that form blood islands in the yolk sac, where they differentiate into haematopoietic and endothelial cells. Vascular growth factor receptor-2 (VEGFR-2, also called Flk-1 and KDR in mice and humans, respectively) is known to be a haemangioblast cell surface marker, since embryos lacking VEGFR-2 show defects in both haematopoietic and endothelial lineages (Risau, 1997). During their differentiation, haematopoietic progenitor cells lose VEGFR-2 expression whereas the transcription factor Scl/tal-1 expression is induced. Endothelial progenitors maintain VEGFR-2 expression, which plays a key role in delineating their fate as endothelial cells. Subsequently this newly formed, primitive vessel structure, termed the primary vascular plexus, successively expands, while other angioblasts aggregate directly to form the dorsal aorta and cardinal vein; further growth and remodelling of these embryonic structures, involving angiogenesis, vessel morphogenesis and pruning, and recruitment of mural cells or pericytes and vascular smooth muscle cells, results in the formation of a more organised complex mature vascular network (Carmeliet & Jain, 2011).

1.6.2 Angiogenesis

Angiogenesis is the predominant mechanism of blood vessel formation in later stages of embryonic development and in adulthood physiologically and in disease. During angiogenesis, endothelial cells undergo proliferation, differentiation, migration and morphogenesis in a series of events tightly coordinated by the actions of a variety of growth factors and cell-adhesion molecules. Under the influence of the key angiogenic factor, VEGF (specifically VEGF-A), trans-endothelial permeability of parental vessels increases plasma proteins (like fibronectin, fibrinogen etc) extravasate and provide a provisional structure for ECs to migrate on. VEGF-A is also essential for selection of the endothelial tip cells, which form at the tips of new advancing angiogenic sprouts (see Figure 1.3). Signalling via the Delta-like ligand 4 (Dll4)/Notch receptor pathway also plays a key role in regulating VEGF-mediated tip cell selection by suppressing the

tip cell phenotype in neighbouring cells and determining formation of stalk cells, in order to ensure organised rather than chaotic vessel sprouting (Phng & Gerhardt, 2009). Subsequent migration of endothelial tip cells from their resident site followed by endothelial stalk cells, is also facilitated by increased expression and secretion of matrix proteases such as matrix metalloproteinases (MMPs) disrupting and remodelling surrounding extracellular matrix (ECM) and loosening inter-endothelial contacts. Matrix-bound angiogenic factors such as VEGF, TGF-beta and FGF are released and proteolytically activated. Thus, the activities of MMPs inhibitors such as Tissue Inhibitors of Metalloproteinases (TIMPs) play a critical role in vessel growth and maintenance. TIMPs tightly control the basement membrane deposition during vessel maturation and the generation of anti-angiogenic fragments such as tumstatin and angiostatin1. Chemotactic migration of endothelial tip cells, exhibiting many extended filopodia, is then followed by the assembly of endothelial tip and stalk cells into solid cords which later leads to formation of vessels with a central lumen (Fig. 1.3).

VEGF guides in a co-ordinated manner the tip cell migration, regulation of lumen formation and vessel size, by production of a VEGF gradient. Once the endothelial channels, protected by the surrounding pro-survival factors, are formed into lumens they require a more stable, durable and functional structural support. This stabilisation of nude nascent vessels is established with the recruitment of mural cells and leads to endothelium quiescence, differentiation, survival and haemostatic regulation. Mural cells such as vascular smooth muscle cells (VSMC) and pericytes play an important role in providing vessel stability and integrity through interstitial matrix deposition and direct interaction with the endothelium. VSMC contribute more in larger vessels like arteries and veins, whereas pericytes directly interact with the endothelial layer in smaller distal vessels such as capillaries, venules and arterioles.

In contrast to sprouting vessel formation, non-sprouting angiogenesis, as initially described in lung development (Short, Bennett, & et al., 1950), involves the splitting of pre-existing vessels by transcapillary pillars or posts of extracellular matrix (Risau, 1997).

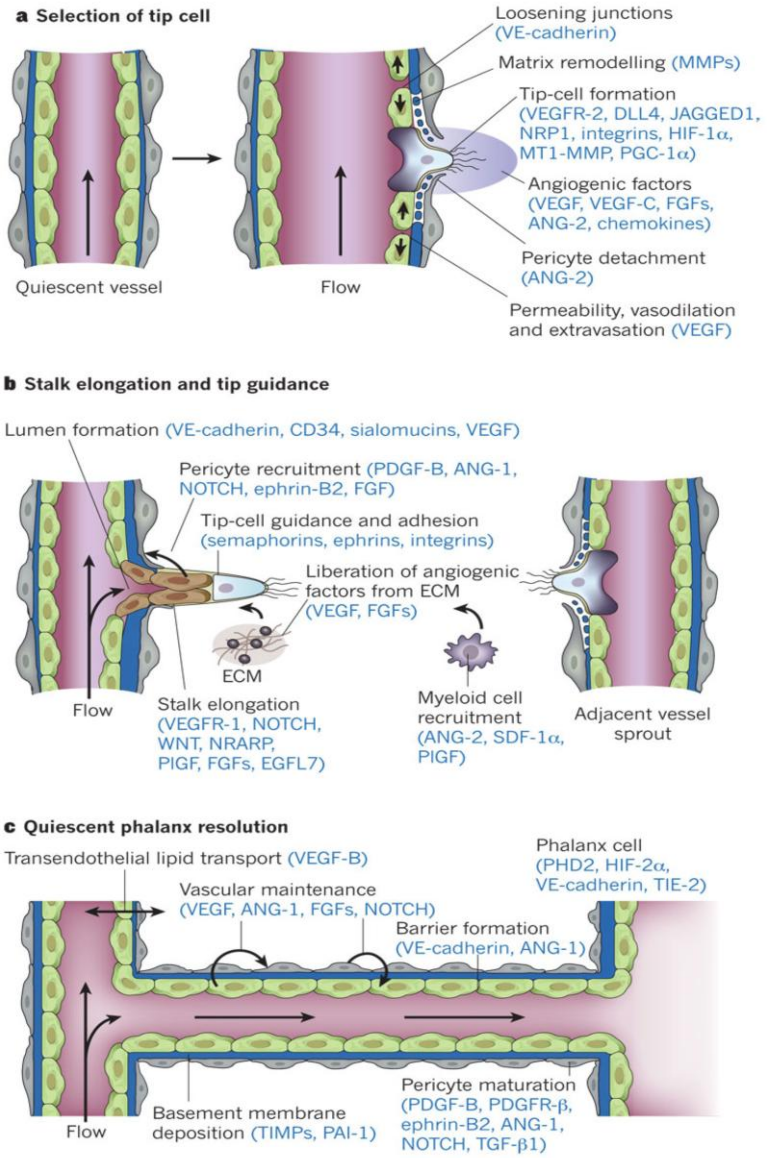


Figure 1.3: Main stages and processes in angiogenic sprouting (from Carmeliet & Jain 2011)

Schematic diagrams illustrating the main stages, cellular processes and angiogenic factors and their receptors involved in sprouting angiogenesis. See text and Carmeliet & Jain (2011) for further details.

During this process, there is no requirement for immediate endothelial cell proliferation, but rather the rearrangement and remodelling of the existing endothelium. Moreover, critical processes such as proteolytic degradation of basement membrane or invasion of surrounding tissues may not occur. Despite strong morphological evidence that supports a role for this process in various tissues, little is known about the physiological role and molecular regulation of non-sprouting angiogenesis (intussusception) (reviewed in Carmeliet & Jain 2011). Vessels, particularly in diseases such as cancer, may also form via other mechanisms such as vasculogenesis from tissue or bone marrow-derived progenitors, vessel co-option, vascular mimicry and direct tumour to endothelial cell differentiation (reviewed in Carmeliet & Jain 2011).

1.6.2.1 The endothelium

The endothelium was originally defined by the Swiss anatomist Wilhelm His in 1865, as the cell lining of blood vessels, lymphatics, and mesothelial-lined cavities. This definition would be restricted later on, to include only the inner cell layer of blood and lymphatics vessels.

This inner cellular flat layer of endothelial cells, one cell thick, forms the tunica intima layer of blood vessels. In many physiological functions, endothelial cells play an important role as a microenvironment sensor, vasomotor toner, blood cell trafficker, hemostatic balancer, and regulator of permeability and survival, and control of innate and adaptive immunity.

1.6.3 Vascular-derived angiogenic factors and signalling

Vascular cells secrete and produce a variety of growth factors that are capable, either alone or in concert with other growth factors, of promoting angiogenesis. The most extensively studied factors that have been implicated in the regulation of angiogenesis include VEGF, bFGF, PDGF, angiopoietins (Ang)-1 and -2 and their receptors, the Ties, Ephs/ephrins, as well as Notch and its ligands (eg. Dll4). Indeed their potential effects on facilitation of any stage of angiogenesis are critical in determining the fate of the mature vasculature.

1.6.3.1 VEGF

VEGF, so named due to its apparent specificity for endothelial cells, is a potent mitogen that was first isolated from medium conditioned by bovine pituitary follicular cells (Ferrara & Henzel, 1989) and subsequently purified and cloned (Keck et al., 1989; Leung, Cachianes, Kuang, Goeddel, & Ferrara, 1989).

VEGF was actually initially described as vascular permeability factor (VPF) due to its ability to induce vascular fluid leakage in the guinea-pig skin (Keck et al., 1989; Senger et al., 1983). Since these early reports it has been shown that VEGF promotes angiogenesis in three-dimensional in vitro models, as well as in vivo models. A critical role for VEGF in blood vessel development has been shown by many investigators highlighting the pro- angiogenic effects like the ability to induce survival, migration and vascular permeability in endothelial cells (Connolly et al., 1989). The angiogenic properties of VEGF make it an attractive area of interest for possible therapeutic usage and/or modulation in various pathological conditions. In addition there are several indications that point to VEGF as a crucial mediator of neovascularization of aggressive tumours (Aiello et al., 1994; Kim et al., 1993).

1.6.3.2 The VEGF family

The VEGF family of growth factors consists of five heparin-binding homodimeric glycoproteins of 45 kDa, VEGF-A, -B, -C, -D and placental growth factor (PlGF); in addition there is a virus-encoded factor, VEGF-E, and VEGF-F which is found in viperinae snake venom (reviewed in (Holmes & Zachary, 2005) .

The VEGF-A gene is located on chromosome 6p21.9 in the human genome and is arranged into eight exons separated by seven introns (Houck et al., 1991). In humans, cDNA sequence analysis has revealed four different isoforms of VEGF-A, that are generated by alternate splicing of exons 6 and 7 consisting of 121, 165, 189 and 206 amino acids long, (29). The presence or the absence of exons 6 or 7 that encode for the heparin-binding domain is the main characteristic feature that determines the relative diffusibility of these isoforms. Thus the smaller acidic isoform, VEGF-A121, does not bind heparin and is freely diffusible. In contrast, VEGF-189 and VEGF-206 have

regions rich in basic residues due to the inclusion of exon 6, and are almost completely sequestered in the ECM having a high affinity for heparin (Robinson & Stringer, 2001). It has been reported that a lack of heparin binding affinity results in a reduction in the mitogenic activity of VEGF suggesting that the heparin (or ECM) binding domain is an essential stimulatory cue for initiation of correct vascular formation (Ruhrberg et al., 2002). ECM sequestered VEGF can be released from the bound form by heparinase or by plasmin cleavage at the C-terminus generating a soluble bioactive fragment (J. E. Park, Keller, & Ferrara, 1993). In addition exons 7 and 8 also encode the domains responsible for binding to the VEGF co-receptor, Neuropilin-1 (NRP-1) (Guo, Jia, Song, Warren, & Donner, 1995; Jia et al., 2004; Soker, Takashima, Miao, Neufeld, & Klagsbrun, 1998). Together, differential binding to heparin and NRP-1 are thought to largely explain the varying angiogenic potential of the splice variants.

VEGF-A165 is regarded as the most biologically active isoform in terms of endothelial biology and angiogenesis. As a result it has been the most intensively studied and best characterized isoform. As may be expected, VEGF-A165 has intermediate properties whereby a significant fraction, but not all, remains bound to the cell surface and ECM.

VEGF-A is able to induce proliferation, migration and survival of endothelial cells in vitro and blood vessel permeabilisation in vivo (Neufeld, Cohen, Gengrinovitch, & Poltorak, 1999). Moreover, VEGF-A165 gene expression is regulated by oxygen availability (Shweiki, Itin, Soffer, & Keshet, 1992), and its production in response to hypoxia is an important regulator of abnormal angiogenesis in several diseases such as diabetic retinopathy (Adamis et al., 1994), psoriasis (Detmar et al., 1994) and cancer (Shweiki, Neeman, Itin, & Keshet, 1995). Gene targetting in mice has further confirmed a pivotal role for VEGF-A in angiogeneic processes, with the absence of only one VEGF-A allele causing severe and lethal disruption to the developing vasculature (Carmeliet et al., 1996; Ferrara, 1996).

VEGF-B is a pro-angiogenic growth factor, which can form heterodimers with VEGF-A (Silvestre et al., 2003). Gene disruption studies observed that VEGF- B knockout mice are viable and fertile (Aase et al., 2001; Bellomo et al., 2000; Silvestre et al., 2003). Bellomo et al (2000) reported that VEGF-B-/- mice have smaller hearts than wild type mice, suggesting a role of VEGF-B in cardiac development, while Aase et al (2001)

found that atrial conduction was impaired, indicating that VEGF-B could also be important in adult heart function (Aase et al., 2001). Recent studies have also suggested that VEGF-B can promote neuronal survival and induce endothelial fatty acid uptake (Fischer et al., 2008; Hagberg et al., 2010).

Both VEGF-C and VEGF-D bind to VEGFR2 and VEGFR3, though proteolytic cleavage of the N- and C-terminal domains of the full-length proteins enhances their affinity for VEGFR2 (Koch, Tugues, Li, Gualandi, & Claesson-Welsh, 2011); Figure 1.4). However, whereas VEGF-C knockout mice die at E15.5-17.5 with severe oedema and abnormal lymphatic vessel formation (M. J. Karkkainen et al., 2004), VEGF-D -/- mice are viable and healthy, showing no vascular or lymphatic abnormalities (Baldwin et al., 2005). Thus VEGF-C is required for lymphatic angiogenesis whereas VEGF-D does not appear to have a developmental role. Nevertheless, studies have shown that VEGF-D does not stimulate endothelial cell proliferation in vitro, but can promote angiogenesis in adult animal models (Anisimov et al., 2009; Jia et al., 2004; A. M. Karkkainen et al., 2009).

Originally discovered as a VEGF homologue, Placenta growth factor (PlGF) primarily binds to VEGFR1, has little or no direct mitogenic activity and does not appear to have a particular role in development (Carmeliet et al., 2001; J. E. Park, Chen, Winer, Houck, & Ferrara, 1994). Studies have indicated that PlGF may regulate angiogenesis by direct or indirect mechanisms. For example PlGF can form heterodimers with VEGF-A and these are poor inducers of angiogenic pathways compared with VEGF-A homodimers. Conversely, PlGF homodimers out compete VEGF-A for binding to VEGFR1, thereby enhancing VEGF-A binding to VEGFR2 in endothelial cells (Cao et al 2009). Given its potential effect on enhancement of VEGF responses, anti-PlGF antibodies have been used to inhibit the growth of tumours in transplantable tumour models with mixed results (Carmeliet & Jain, 2011). In preclinical models, PlGF protein or gene delivery increases the revascularization of ischaemic tissues (Carmeliet & Jain, 2011). However, the effectiveness of PlGF as either a therapeutic agent or target remains unclear.

1.6.3.3 Regulation of VEGF gene expression

The regulation of VEGF gene expression plays a major role in blood vessel growth. VEGF mRNA expression is rapidly upregulated by exposure to low cellular oxygen

concentrations (hypoxia) in a variety of cultured cells. Hypoxia-induced VEGF expression is a result of a heterodimer transcription factor complex, Hypoxia inducible factor-1 (HIF-1) α and β binding to hypoxia response element (HRE) in the VEGF promoter. Under normoxic conditions HIF-1α hydroxylated on key proline residues by the oxygen-dependent enzyme, prolyl hydroxylase (PH); hydroxylated HIF-1α is ubiquitinylated and is recognized by the von Hippel-Lindau gene product protein, which is essential for HIF-1α proteasomal degradation. During hypoxia, however, PH is inhibited, non-hydroxylated HIF-1α accumulates and forms a complex with HIF-1β, which then translocates to the nucleus. This translocation leads to the HIF-1 complex and other transcriptional co-activators inducing VEGF transcription (Ferrara & Kerbel, 2005). In addition, VEGF mRNA expression has been shown to be induced by several cytokines or growth factors, such as PDGF-BB, TGF-b and FGF-2 in cells and during neovascularization and/or tumorigenesis (Ferrara & Kerbel, 2005; Stavri, Hong, et al., 1995; Stavri, Zachary, Baskerville, Martin, & Erusalimsky, 1995).

1.6.4 VEGF receptors

Three cell surface tyrosine kinases (RTK) have been identified as VEGF receptors (VEGFRs): VEGFR1 / Flt-1, VEGFR2 / KDR (mouse fetal liver kinase-1, Flk-1) and VEGFR3 / Flt-4 (Neufeld et al., 1999). In addition some VEGF isoforms bind to the co-receptors, NRP1 and 2 (Pellet-Many et al., 2011; Pellet-Many, Frankel, Jia, & Zachary, 2008; Soker et al., 1998). The three VEGFRs share a similar structural composition with an N-terminal extracellular domain of seven immunoglobulin (Ig)-like domains (VEGFR3 has six, domain five is replaced by a disulphide bond), a single transmembrane region and a consensus cytoplasmic tyrosine kinase catalytic region, which is divided into two parts by an insert sequence. Figure 1.4 shows the VEGF receptors and summarises their interaction with different VEGF isoforms.
VEGFR1 has a 10-fold higher affinity for the main VEGF isoform, VEGF- A165, than VEGFR2 (Petrova, Makinen, & Alitalo, 1999). Nevertheless, the VEGFR1 undergoes little detectable phosphorylation when bound to VEGF-A165, whereas, VEGF-A165 binding to VEGFR2 results in autophosphorylation on several major sites and is followed by the activation of downstream signalling (Zachary, 2003).

1.6.4.1 VEGFR1 & VEGFR2

VEGFR1 has a molecular weight of about 210 kDa, but can also be secreted as a soluble form, lacking the seventh Ig-like domain (transmembrane sequence). Soluble VEGFR1 acts as a physiological negative regulator of VEGF action by sequestering VEGF-A and preventing VEGFR-2 signalling (Kendall & Thomas, 1993; Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006).

In situ hybridization studies have shown that VEGFR1 is highly expressed in endothelial cells during embryonic development, then downregulated in adult vascular endothelium (Millauer et al., 1993). VEGFR1-knockout mice are embryonic lethal with apparent vascular defects. Endothelial cell differentiation was unaffected but their assembly into functional channels was severely impaired, implying an essential role for

VEGFR1 in vascular organisation (Fong, Rossant, Gertsenstein, & Breitman, 1995). In comparison to VEGFR2, VEGFR1 has weak tyrosine kinase autophosphorylation activity following stimulation by VEGF with no direct mitogenic signalling observed (Seetharam et al., 1995). In response to VEGF-A, six residues in the C-terminal tail of VEGFR1 including tyrosines 1169, 1213, 1242, 1309, 1327, and 1333 have been identified as phosphorylation sites, although the extent of phosphorylation of some of these residues appears to be minimal.

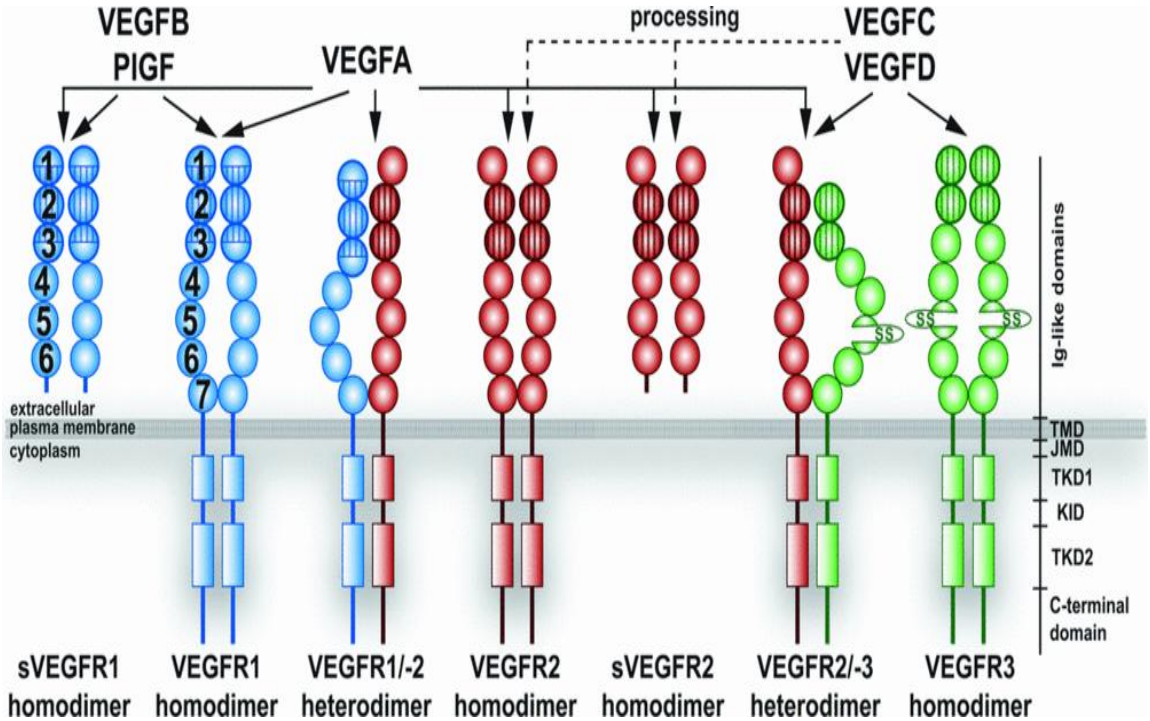


Figure 1.4: Binding specificities of five VEGFs (VEGFA, VEGFB, VEGFC, VEGFD and PlGF) to three VEGFRs.

Proteolytic processing of VEGFC and VEGFD enhances affinity for VEGFR2. VEGFR Ig-like domains involved in VEGF binding are hatched circles. VEGFRs 1 and 2 are also expressed as soluble forms (sVEGFR1 and sVEGFR2) comprising the extracellular domain lacking the seventh Ig-like domain. Key: JMD, juxtamembrane domain; KID, kinase insert domain; TMD, transmembrane domain; TKD1, ATP-binding domain; TKD2, phosphotransferase domain. Figure from Koch et al 2011.

PlGF has also been reported to play a role in crosstalk between Flt1 and Flk1 (Autiero, Luttun, Tjwa, & Carmeliet, 2003). Furthermore VEGFR1 may be differentially activated by different ligands like VEGF-A and PIGF, possibly due to ligand-specific phosphorylation, which results in distinct biological effects (Autiero et al., 2003; Roskoski, 2007). In addition, genetic deletion of the intracellular tyrosine kinase domain of VEGFR1 in mice showed that this domain is not required for vascular development, indicating that VEGF-A-induced stimulation through VEGFR1 tyrosine kinase may not be required for early vessel development (Hiratsuka et al 1998). Nevertheless further studies with these mice have shown that the tyrosine kinase activity of this receptor does appear to play a role in pathogenic angiogenesis, particularly in tumours where high levels of VEGFR1-specific ligands, such as PlGF, are produced (Hiratsuka et al., 2001; Hiratsuka, Minowa, Kuno, Noda, & Shibuya, 1998). In addition, activation of VEGFR1 is implicated in the increased expression of MMPs and in the paracrine release of growth factors from endothelial cells (Shibuya, 2006).

VEGFR2 binds to the different isoforms of VEGF-A (110–165 amino acid residues), and the fully processed forms of VEGF-C, VEGF-D and VEGF-E (Fig. 1.4). VEGFR2 is the key mediator of the mitogenic, angiogenic, chemotactic and vascular permeability-enhancing effect of VEGF-A. Although VEGFR2 has lower affinity for VEGF than VEGFR1, VEGFR2 is responsible for most of VEGF signal transduction in ECs (Zachary, 2003).

VEGFR2 was thought to be specific for endothelial cells and bone marrow progenitor cells, but some studies have also demonstrated the existence of VEGFR2 expression in neuronal progenitor cells (Quinn, Peters, De Vries, Ferrara, & Williams, 1993; X. Yang & Cepko, 1996). VEGFR2-deficient mice die in utero between E8.5-9.5 due to lack of vasculogenesis and blood island formation (Shalaby et al., 1995). In knockout embryos, severe early defects in the development of haematopoietic and endothelial cells as well as disorganized blood vessels were observed and indicate that VEGFR2 is essential for blood vessel formation and vasculogenesis.

Multiple tyrosine phosphorylation sites in VEGFR2 have been shown to be phosphorylated in response to VEGF and are potentially involved in essential signalling pathways stimulating endothelial cell proliferation and migration, indicating that VEGFR2 is the key signalling receptor for mediating VEGF activity in endothelial cells

(Waltenberger, Claesson-Welsh, Siegbahn, Shibuya, & Heldin, 1994). There are at least six tyrosine residues in the cytoplasmic domain of VEGFR2 that become phosphorylated after VEGF binding: Y951 and Y996 (in the kinase domain), Y1054 and Y1059 (in the catalytic domain) and Y1175 and Y1214 (in the c-terminus). Residue Y1175 has been shown to be a critical mediator of VEGFR2 signalling, with several studies indicating that phosphorylated Y1175 acts as a docking site for various signalling proteins. For example, Y1175 phosphorylation results in binding and activation of phospholipase Cγ1 (PLCγ1), which then activates the PKC signalling pathway (Takahashi & Shibuya, 2001). Y1175 also recruits the Shb adaptor protein, which subsequently activates the phosphoinositide-3' kinase (PI3K) pathway (Holmqvist et al., 2004) and another adaptor protein, Sck (Igarashi, Shigeta, Isohara, Yamano, & Uno, 1998; Ratcliffe et al., 2002; Warner, Lopez-Dee, Knight, Feramisco, & Prigent, 2000). Knock-in mice that have Y1173 (which is the murine equivalent to Y1175) mutated to phenylalanine die during early embyogenesis with similar vascular defects to VEGFR2 knock-out mice (Sakurai, Ohgimoto, Kataoka, Yoshida, & Shibuya, 2005). Mutation of Y951 to phenylalanine inhibits VEGF-induced stress fibre formation and endothelial migration. This residue has also been found to associate with VRAP (VEGFR2 associated protein), which interacts with src (Matsumoto et al., 2004; Wu et al., 2000). However, src activation is primarily thought to occur via Y1057 (the murine equivalent of Y1059) and association with c-Cbl (Meyer, Sacks, & Rahimi, 2008). Furthermore, activated src has a regulatory role in Y1175 phsophorylation since phosphorylation of this residue was inhibited when Y1057 was mutated (Meyer et al., 2008). Y1054 and Y1059 may also regulate VEGFR2 internalization since mutation of theses sites reduced VEGF-mediated endocytosis of the receptor (Dougher et al 1999). Y1214 is reported to be involved in NCK binding leading to activation of the small GTPase, cdc42, and further downstream, p38MAPK (Lamalice, Houle, & Huot, 2006; Lamalice, Houle, Jourdan, & Huot, 2004). However, mice with a mutation of the murine residue equivalent to Y1214 (Y1212F) were viable and fertile, without any obvious phenotype (Sakurai et al., 2005).

1.6.4.2 VEGFR3

VEGFR3 plays a key role in remodelling the primary capillary plexus in the embryo and contributes to angiogenesis and lymphatic blood vessel development in the adult (Dumont et al., 1998). VEGFR3 is expressed during embryonic vascular development after which its expression declines and is subsequently restricted to lymphatic vessels (Kaipainen et al., 1995). VEGFR3 binds VEGF-C and VEGF-D but not VEGF-A. VEGFR3 is the only VEGF receptor that has a distinct extracellular rearrangement of its Ig-like domains. Proteolytic cleavage in the sixth Ig domain of the receptor causes a disulphide linkage between the two components of the original chain (Pajusola et al., 1992).

1.6.4.3 Neuropilin-1 & -2

Based upon chemical cross-linking studies, Soker et al identified a VEGF receptor in HUVEC samples that differed from VEGFR1 and VEGFR2 (Soker, Fidder, Neufeld, & Klagsbrun, 1996). These investigators purified this receptor from human MDA-MB-231 breast cancer cells and showed that it is identical to neuropilin-1 (NRP1), a surprising result at the time owing to its initial characterization as a neuronal recognition molecule and a neuronal cell adhesion molecule (Soker et al., 1998). Subsequent work by Chen et al (2000) led to the discovery of the related neuropilin-2 (NRP2). NRPs are non-protein tyrosine kinase co-receptors for various members of VEGF family (reviewed in (Pellet-Many et al., 2008). In neurons, they also act as co-receptors for the class 3 semaphorins, which are secreted glycoproteins that serve as chemorepulsive axon guidance molecules capable of collapsing axonal growth cones and repelling axons of ganglia during neurogenesis. NRP1 binds to some isoforms of VEGF-A, VEGF-B and PlGF2, whereas NRP2 binds to VEGF-A145, VEGF-A165, VEGF-C and -D (Gluzman-Poltorak, Cohen, Herzog, & Neufeld, 2000; Mamluk et al., 2002) (see 1.8.4.1 and Fig. 1.4). In addition, soluble forms of NRPs composed of most of the extracellular portion are generated by alternative splicing, and are secreted by various tissues. sNRPs maintain their ability to bind semaphorins and VEGF and may therefore act as decoy receptors and preventing ligand binding to cell surface receptors (Cackowski, Xu, Hu, & Cheng, 2004; Gagnon et al., 2000).

NRPs are transmembrane receptors containing a large extracellular region, a transmembrane segment, and a short (≈40 amino acid residue) intracellular portion that contains a PDZ domain-binding motif (Fig. 1.5). The b1 and b2 domains in the extracellular domain are essential for VEGF binding. NRP-1 and NRP-2, act as co-receptors with VEGFR1, VEGFR2, and VEGFR3 that transduce VEGF signalling, though their precise role in VEGF signalling is unclear (Pellet-Many et al., 2008).

The critical role of NRP1 in vascular development is shown in gene-targeting studies, in which NRP1 knockout mice died at embryonic age (E)10-14 with multiple vascular and neuronal defects (Kawasaki et al., 1999). Moreover generation of endothelial-specific NRP1-null mice showed that NRP1 deficient mice also died at E12.5 with major cardiac defects and abnormal arterial branching (Gu et al., 2003). In contrast a knock-in mutant NRP1 that binds VEGF but not semaphorin did not result in death until the neonatal stage, with the mice showing impaired sensory neuronal pathfinding. Although NRP2 shares 44% homology with NRP1 and is expressed in many of the same cells as NRP1 it cannot compensate for the loss of NRP1 in knockout models. Indeed, NRP2 knockout studies indicate NRP2 has a restricted role in lymphangiogenesis and sensory guidance (H. Chen et al., 2000; Yuan et al., 2002).

Complex formation between NRP1 and VEGFR2 in endothelial cells have been shown to increase VEGF-stimulated migration possibly by enhancing VEGFR2 cell signalling by stabilising and prolonging ligand binding. Indeed there is evidence that the cytoplasmic domain of NRP1 participates in complex formation, since VEGFR2-NRP1 association is decreased if the PDZ binding domain is deleted (Prahst et al., 2008). Nevertheless, overexpression of a mutant NRP1 lacking the cytoplasmic domain (Nrp1ΔC) does not impact on ligand induced VEGFR2 phosphorylation (at tyrosine residue 1175) as opposed to NRP1 knockdown by siRNA which significantly reduces phosphorylation at this residue (Evans et al., 2011). Although the cytoplasmic tail of NRPs has no known transductional signalling role, some studies have indicated that it serves as a docking site for downstream signaling molecules, alone or in conjunction with co-receptors. Indeed, the PDZ binding domain of NRP1 can interact with synectin (also known as GIPC1) in a yeast two-hybrid screen (Cai & Reed, 1999). Further studies demonstrated that NRP1 associated with synectin in endothelial cells and synectin knockdown reduced VEGF-induced migration (L. Wang, Mukhopadhyay, &

Xu, 2006). Synectin regulates G-protein-coupled receptor signalling and interacts with proteins involved in cytoskeletal scaffolds (Abramow-Newerly, Roy, Nunn, & Chidiac, 2006; M. Liu & Horowitz, 2006). Synectin may also be involved in NRP1-mediated cell attachment since Synectin-NRP1 binding induces α5β1 integrin internalisation (Valdembri et al., 2009).

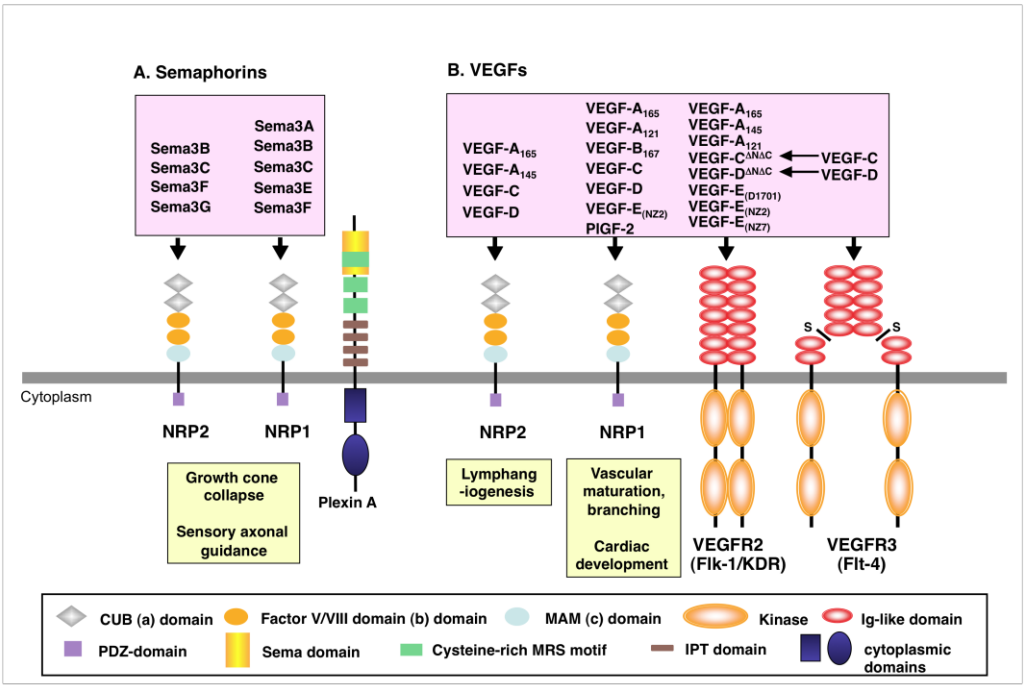


Figure 1.5: Neuropilins, their ligands and co-receptors.

A. Class 3 Semaphorins bind NRP1 and NRP2 with differing specificities. Plexins A1 and A2 are the major signalling receptors for Semaphorins, and complex with NRPs and Semaphorins to form a complex essential for growth cone collapse and axonal guidance in neurogenesis. The plexin cytoplasmic domain contains two regions of homology found in GTPase activating proteins. Key: IPT, immunoglobulin-like domains shared by plexins and transcription factors; MRS, Met-related sequences. **B.** Some isoforms of members of the VEGF family bind to NRP1 and NRP2 as indicated. The VEGF-A_{121} isoform binds to NRP1 but does not promote NRP1/VEGFR2 complexation. NRP1 may also associate with VEGFR1/Flt1, and NRP2 is reported to complex with the receptor for VEGF-C and VEGF-D, VEGFR3. Key: Ig-like, immunoglobulin-like. Figure adapted from Pellet-Many et al 2008.

Although synectin knockout in zebrafish phenocopies zebrafish NRP1 knockouts (Chittenden et al., 2006; L. Wang et al., 2006), this is not the case with mice (Chittenden et al., 2006). Synectin knockout mice are viable, although they are smaller than wild type mice and exhibit microvascular arterial defects (Chittenden et al., 2006). It is clear therefore that synectin alone does not mediate intracellular NRP1 function.

1.7 Vascular Smooth Muscle Cell physiology

Larger blood vessels (arterioles, arteries, venules and veins) consist of a single layer of endothelial cells surrounding the vessel lumen, and the vessel wall, primarily composed of a continuous layer of vascular smooth muscle cells (SMC), and an outer adventitial layer containing fibroblasts, connective tissue, nerves and in large vessels a microvasculture called the vasa vasorum (see Figure 1.6). During development, smooth muscle cells and pericytes play major roles in the formation, remodelling, stabilisation, maturation and function of the vascular network. The primary roles of SMC in the mature vasculature are contraction, regulation of blood vessel tone, blood pressure, and blood flow distribution (Owens, 1995).

Fully mature SMC are multifunctional mesenchymal cells which display a marked phenotypic plasticity. Thus, changes in the local environment can result in profound and reversible changes in their differentiated phenotype. Depending on specific local conditions, which could be a result of cell interactions, alteration of extracellular matrix, or in response to cytokines, SMC will undergo adaptive changes often resulting in remodelling of the vessel wall. Thus, SMC within adult animals retain remarkable phenotypic diversity and plasticity during normal development, during repair of vascular injury, and in disease states including arteriosclerosis and tumour angiogenesis. These phenotypic alterations commonly termed “dedifferentiation”, are modulations of the differentiated state involving reversible interconversions between phenotypes with an impact on the normal structure or function of the differentiated SMC. The spectrum of phenotypes expressed by SMC emphasizes their transition between and within two polar distinct well-described states: contractile and synthetic (reviewed in (Gomez & Owens, 2012; Owens, Kumar, & Wamhoff, 2004).

The synthetic phenotype is predominant during vascular development or regeneration, where SMC play a key role in morphogenesis of the blood vessels, simultaneously

participating in vessel growth and remodelling. At the early stages of vasculogenesis, SMC are highly proliferative, migratory and synthetic, producing extracellular matrix components such as collagen, elastin, cadherens, integrins and proteoglycans that constitute a major portion of the blood vessel wall while at the same time acquiring contractile capacities. In this synthetic state, SMC possess abundant gap junctions with endothelial cells and communicate directly through connexon-containing gap junctions (Owens et al., 2004). The process of recruitment of SMC or pericytes is critical for the establishment of a physiologically remodelled and mature vessel.

In contrast, contractile SMC exist mainly within established adult blood vessels, proliferate at an extremely low rate, are largely non-migratory, and exhibit very low biosynthetic activity. In this state 80-90% of the cell volume is occupied by contractile apparatus, whereas organelles such as endoplasmic reticulum, Golgi and free ribosomes are few in number and located in the perinuclear region. Mature fully differentiated SMC perform their contractile function by expressing a unique repertoire of contractile proteins, such as myosin, tropomyosin, actin, ion channels, and signalling molecules. These proteins are required for contractile properties that are distinct from terminally differentiated skeletal or cardiac muscles cells. *In vivo* observations have shown that the SMC responsible for the neointimal thickening in the rat carotid artery balloon injury model appear to switch into a reversible phenotypic modulation in response to the insult. Under these complicated local conditions, SMC undergo a change from their normal function of contraction and maintenance of vessel wall tone to one of repair of the wound by migration, synthesis of matrix and/or cell replication (Gomez & Owens, 2012). These SMC exhibit an ability to proliferate, and express PDGF-BB mRNA but low levels of PDGFRα, similar to that seen as in SMC in neonatal rat (Majesky, Benditt, & Schwartz, 1988; Majesky, Giachelli, Reidy, & Schwartz, 1992).

However, the origin of the SMCs, which form the neointimal thickening, remains controversial. Once wound healing is complete, the cells appear to return to their previous phenotype and function (Campbell, Campbell, Manderson, Horrigan, & Rennick, 1988).

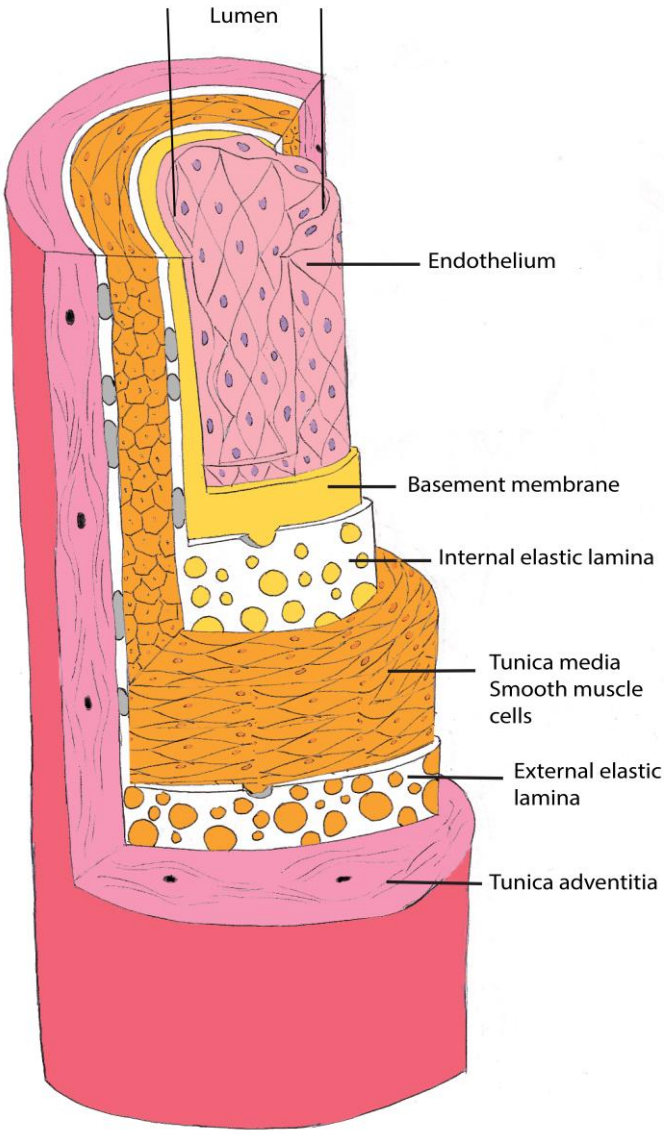


Figure 1.6: Anatomic view of a multilayered arterial vessel wall

This is a schematic diagram showing the cross-sectional view of an arterial wall and its wall components in the mature cardiovascular system.

There is strong evidence that phenotypic modulation of SMC plays a major role in a number of major diseases in humans including atherosclerosis, cancer, and hypertension (Owens et al., 2004). Indeed, the complexity of different phenotypes that may be manifested by SMC is evident within atherosclerotic lesions. These are characterised by an increase in troponin, myosin, β-actin and the vimentin to desmin protein ratio (Skalli, Bloom, Ropraz, Azzarone, & Gabbiani, 1986; Valente et al., 1988).

1.7.1 SMC in disease

Atherosclerosis is an inflammatory pathology, which is thought to result from damage involving oxidative stress to the vascular endothelium lining the artery. Endothelial injury results in the migration of inflammatory cells such as macrophages and T-lymphocytes into the arterial wall. The macrophages develop into foam cells resulting in the accumulation of lipid, followed by proliferation and migration of SMC from the media into the intima. The occurrence of proliferation and subsequent accumulation of SMC results in further intimal expansion. Accumulation of SMC in the tunica intima (neointima formation) plays a crucial role in the pathogenesis of atherosclerosis, and is also implicated in restenosis following angioplasty or stenting (Ross, 1995, 1999). In the neointima, growth factors and proteolytic agents induce SMC proliferation and migration. During this process, SMC undergo phenotypic modulation, from a contractile to a synthetic phenotype, resulting in reduced expression of typical SMC contractile proteins and an enhanced response to growth and chemotactic factors (Orlandi & Bennett, 2010).

There is very clear evidence that the morphological, biochemical, physiological and molecular properties of SMC vary at different stages of atherosclerosis, and also within different lesion types and between different regions within a given lesion. For example, in early lesion development SMC phenotypic change plays a maladaptive role, whereas it may have a beneficial adaptive role in stabilization of plaques in mature lesions. However, at the later stages of atherosclerosis, it contributes to plaque destabilization through VSMC apoptosis and /or activation of various protease cascades. It is not yet

known whether atherosclerosis plays a role in initiating SMC phenotypic switching or if it is the SMC modulation that participates in the development and progression of atherosclerotic lesions (Ross, 1999).

1.7.2 Platelet Derived Growth Factors

Platelet-derived growth factor (PDGF) was initially characterised as a serum growth factor for fibroblast, smooth muscle cells and glia cells (Ross, Glomset, Kariya, & Harker, 1974). PDGFs are a family of closely related growth factors with an approximate molecular weight of 30 kDa. There are five disulphide-linked homo- and heterodimers of homologous isoforms (Fig. 6): the A and the B polypeptide chains, which give rise to three dimeric bioactive ligands, AA, BB and AB, and the recently identified PDGF-C and PDGF-D chains (Fredriksson, Li, & Eriksson, 2004; Stroobant & Waterfield, 1984). The heterodimer PDGF-AB is currently believed to be the only heterodimer form in human platelets with somewhat different signaling properties from the homodimers (Ekman, Thuresson, Heldin, & Ronnstrand, 1999), and its physiological role still remains to be established. Since these findings substantial experimental evidence has shown that PDGF through its receptors plays an important role in embryonic development. Increased PDGF activity has also been linked with several pathological conditions and diseases such as cancer. This has led to increased interest in PDGFs as a therapeutic target, and PDGF/PDGFRs inhibitors are currently being tested in both pre-clinical models as well as human clinical trials.

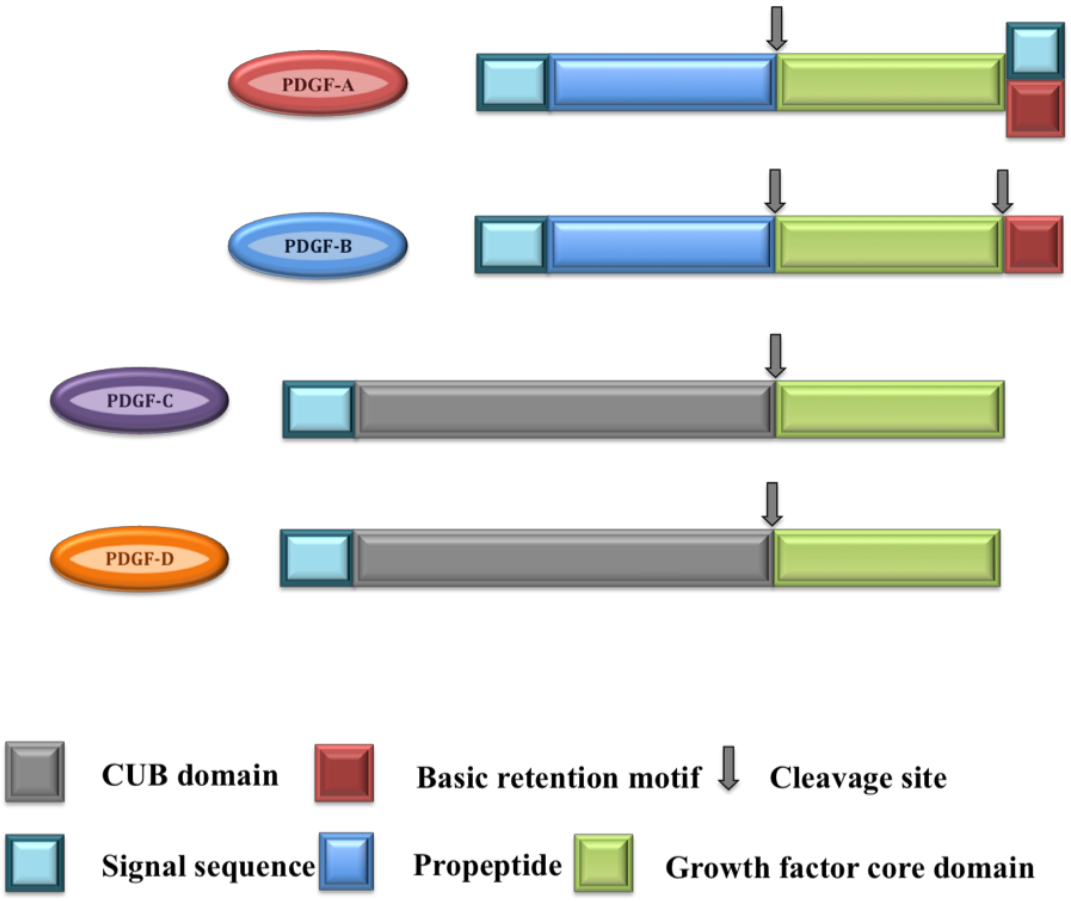


Figure 1.7: Proteolytic processing of PDGF family

This picture is a representation of the proteolytic processing of PDGF precursor polypeptides and their domains. PDGF isoforms are close structurally related growth factors that are produced as precursor molecules and undergo proteolytic processing in their N terminal pro-peptides and in C terminal as in the case of B-chain. Soluble inactive forms of PDGF- C and PDGF-D include their characteristic CUB domain, which is proteolytic removed extracellulally ready to bind to the PDGF receptors.

1.7.2.1 PDGF family

Mammalian PDGF family members carry a growth factor core domain containing a conserved set of cysteine residues, the so-called cysteine knot motif (Fredriksson et al., 2004). The cysteine knot is an eight-cysteine residue ring with three intramolecular and one intermolecular disulphide bond per monomer. The core domain is necessary and sufficient for the stability of the ligand dimeric structure to bind and functionally activate its receptors (Andrae, Gallini, & Betsholtz, 2008). Other related growth factors such as VEGFs share a similar structure.

The crystal structure of PDGF-BB (Oefner, D'Arcy, Winkler, Eggimann, & Hosang, 1992) revealed that each monomeric molecule has two long highly twisted antiparrallel β-strands and the two subunits are associated in a head-tail conformation, which is also found in VEGF .

The A and B chains of PDGF are 60% similar in their amino acid sequences, while the C and D chains display approximately 50% identity (Fredriksson et al., 2004; Heldin & Westermark, 1990). PDGFs are all expressed and assembled as inactive dimeric precursors and terminal proteolytic processing is necessary for their biological activity (Fig. 1.6). PDGF-A and PDGF-B have short N-terminal extensions upstream of the growth factor domain, that undergoes intracellular proteolytic processing by furin or related proprotein convertases. PDGF-B also requires C-terminal processing, which is suggested to be essential to acquire receptor-binding ability (Fredriksson et al., 2004; Siegfried, Khatib, Benjannet, Chretien, & Seidah, 2003). In contrast, PDGF-C and PDGF-D chains display a distinct protein domain, the so-called CUB domain, as part of their N-terminal extensions. Both PDGF-C and PDGF-D are secreted as latent ligands, requiring activation by limited extracellular proteolysis to release the growth factor domains from the CUB domains by plasmin and/or other serum proteases. The proteolytic processing of PDGF polypeptides is shown in figure 1.7.

Additionally, in their C-termini, both PDGF-C and PDGF-D lack amino acid sequence extensions referred to as the retention motif, while PDGF-A and B have mainly basic amino acid sequences involved in binding to extracellular matrix. However, CUB

domains are implicated in protein–protein and protein-carbohydrate interactions that might regulate the extracellular distribution of latent C and D chains.

The genes encoding for the A and B chains are located on human chromosomes 7 and 22, while C and D genes are located on chromosomes 4 and 11, respectively (Fredriksson et al., 2004). The genes are structurally related with similar exon/intron organizations, and they have apparently arisen by gene duplication and evolutionary divergence. In addition, alternative splicing of exon 6 in the PDGFA gene leads to the formation of multiple PDGF-A secreted polypeptides, with or without the retention motif, (Bonthron, Collins, Grzeschik, van Roy, & Speleman, 1992; Rorsman & Betsholtz, 1992).

PDGFs are primarily characterised as paracrine growth factors which are released from cells and bind to their extracellular receptors, and may also be engaged in autocrine loops in tumours, similar to those recently described for VEGF-A required for endothelial survival and vascular maintenance (Lee et al., 2007).

In general, PDGFs are expressed in discrete populations of cells and act locally as paracrine growth factors to drive different cellular responses (for review, see (Betsholtz, 2004; Hoch & Soriano, 2003). PDGF expression is highly regulated in vivo during development and in certain physiological hypertrophic responses, whereas in cells, expression is responsive to a variety of stimuli, including hypoxia, thrombin, cytokines, and growth factors, including PDGF itself (for review, see (Heldin & Westermark, 1999).

The PDGF-BB homodimer is the predominant and most intensively studied isoform. It is produced by activated platelets and lesion macrophages and stimulates SMC proliferation, migration and survival. It also induces rapid downregulation of selective contractile markers in cultured SMC (Dandre & Owens, 2004). It is physiologically important in intercellular crosstalk between endothelial cells secreting PDGF-BB and SMC expressing PDGF-BB receptors. Furthermore, both VSMC and endothelial cells secrete PDGF-BB (Jacot & Wong, 2008).

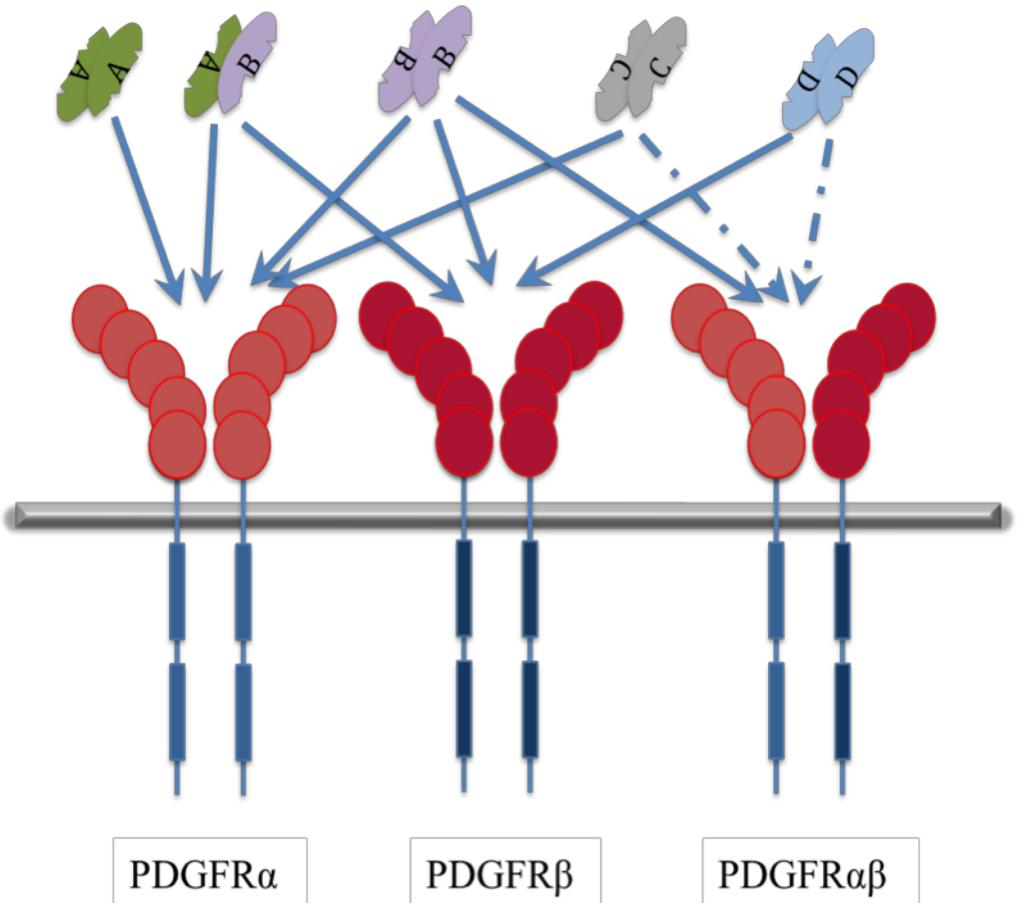


Figure 1.8: PDGF–PDGFR interactions in cultured cells.

Each chain of the PDGF family member binds and activates homo- and heterodimeric complexes of PDGFR-α and PDGFR-β, indicated by solid arrows, whereas hached arrows indicate weak bindings. Upon ligand activication, receptor dimerisation and autophosphorylation determine its mitogenic responses.

1.7.3 PDGF Receptors

PDGF family members bind to two different cell surface receptors, PDGFR-α and PDGFR-β (Fig. 1.8). PDGFR-α binds all three isoforms (PDGF-AA, -AB, -BB) with high and similar affinity. In contrast, PDGFR-β was found to bind to PDGF-BB with high affinity, but with approximately 10-fold lower affinity to PDGF-AB, and does not bind at all to PDGF-AA.

In humans and other mammals, two structurally and functionally related PDGFRs (PDGF receptor tyrosine kinases) have been identified, namely PDGFRα and PDGFRβ (Fig. 1.8). The PDGFRs are plasma membrane spanning receptors with a similar organization with an extracellular ligand-binding domain composed of five immunoglobulin-like loops, a single transmembrane domain, a regulatory juxtamembrane domain, a split tyrosine kinase domain and a C-terminal tail. The gene encoding PDGFRα is located in chromosome 4q11-4q12 and is transcribed as a 6.5 kb messenger RNA, while PDGFRβ gene is positioned in chromosome 5q33 producing a 5.5kb mRNA (Claesson-Welsh, Eriksson, Westermark, & Heldin, 1989). After proteolytic processing of their signal peptides each precursor protein is modified into N-linked and O-linked glycosylated mature form of 170 kDa PDGFRα and 190 kDa PDGFRβ and the size of their glycosylation varies among different cell types (Claesson-Welsh, 1994).

There is an overall pattern of PDGFR expression in fibroblasts and SMC of different species, though there are reports of exclusive expression of PDGFRα in platelets (Vassbotn, Havnen, Heldin, & Holmsen, 1994) and PDGFRβ in pericytes and myoblasts (Sundberg, Ljungstrom, Lindmark, Gerdin, & Rubin, 1993) However, in development PDGFRs specific functions totally depend on cell type and context-specific expression.

Binding of PDGF to the N-terminal part of the extracellular domain of the receptor causes dimerization of receptors to form homo- or heterodimers, which is an essential and key event for receptor activation. Dimeric PDGF isoforms bind simultaneously to the receptor, such that the ligand forms a bridge between two receptors, resulting in

receptor dimerisation. Receptor dimerization occurs in a dose-dependent manner and is accompanied by changes in the intracellular domain conformation. The conformational changes in turn enable autophosphorylation of multiple tyrosine residues on the receptor dimeric intracellular domains by ATP binding. Specific downstream signal molecules bind to these phosphorylated tyrosines via SH2 (Src homology 2) or PTB (Phospho Tyrosine binding) motifs involving protein-protein interactions, which initiate a cascade of signal transduction.

According to a common model of how a tyrosine kinase receptor autophosphorylates itself, a basal kinase activity or "unlocking" trigger to phosphorylate a particular tyrosine residue serves as a substrate for full enzymatic activity directed toward other tyrosine residues in the receptor molecules as well as other substrates for the kinase. However, analysis of site-directed mutations in PDGFRs has begun to shed light on *in vivo* functions of specific phosphorylation sites.

PDGFR-α and -β homodimers as well as heterodimeric receptors, transduce potent overlapping, but not identical, mitogenic signals (Heldin & Westermark, 1999). It is notable that no heterodimers involving PDGF-C and PDGF-D chains have been described so far (Fredriksson et al., 2004).

VSMC, fibroblasts and many other cells types express both receptor isoforms, PDGFR-β is the major isoform expressed by pericytes (Sundberg et al., 1993) whereas PDGFR-α is expressed by other cell types such as platelets (Vassbotn et al., 1994).

1.7.4 PDGFRs signal transduction

As mentioned previously, PDGF-ligand binding and receptor dimerisation results in conformational changes that lead to autophosphorylation of specific tyrosine residues in the intracellular domain. This initiates a cascade of downstream mitogenic signals involving multiple SH2-domain-containing proteins that bind directly to the phosphorylated tyrosine residues of the activated receptors.

Receptor tyrosine phosphorylation sites induced by ligand binding serve two purposes: firstly, to regulate the state of activity within kinase domain and phosphatase disactivation. Secondly, to create docking sites for downstream signal transduction molecules, which leads in kinase-substrates interaction through SH2 domains.

Mutation of Tyr 857 into phenylalanine in second part of the tyrosine kinase domain of PDGFRβ and Tyr 849 for PDGFRα showed that phosphorylation of this tyrosine residue has an important role in positive regulation of kinase activity (Kazlauskas and Kovalenko, 2004). Binding to tyrosine phosphorylation sites involves recruitment of many signal transduction molecules containing also SH3 domains that activate other downstream molecules, via binding to proline-rich stretches and "pleckstrin homology" domains such as Sos 1, or other GTP-binding proteins. A range of signalling molecules associate with PDGFR phosphorylation sites *in vitro*.

1.7.4.1 Downstream signal transduction molecules

Src family tyrosine kinases: Two tyrosine residues in the juxtamembrane domain, Tyr 579 and Tyr 581 are involved in binding and activation of members of the Src family of cytoplasmic kinases, Src, Fyn, and Yes, resulting in increase of kinase activity (Gould & Hunter, 1988). Despite the fact that Src is known to be involved in stimulation of cell cycle progression, binding of Src to PDGFRα was shown to not be so important for its mitogenic response (DeMali & Kazlauskas, 1998). Although PDGFRα phosphorylation results in an initial increase in Src kinase activity, PDGFRα mutated on two tyrosine residues Tyr 572 and Tyr 574 responsible for Src binding was not be able to induce PDGF-AA-mediated Src activation but did not prevent DNA synthesis when the receptors were expressed in pig aortic endothelial cells, indicating that initial burst of Src activity might be only required for efficient tyrosine phosphorylation of selected signaling molecules in an activated cell but not in a later event as cell proliferation (Gelderloos, Rosenkranz, Bazenet, & Kazlauskas, 1998; Hooshmand-Rad, Yokote, Heldin, & Claesson-Welsh, 1998).

Phosphatidylinositol 3-kinase (PI3K): is a family of proteins, which generates intracellular pool of phosphoatidylinositol 3,4,5 –triphosphoate (PIP3). PDGFRs interact and tyrosine phosphorylate p85 subunit of PI3K but not of p110 by association of its two SH2 domains and one SH3 domain with two phosphorylated tyrosine residues, Tyr 740 and 751, in the PDGFRβ kinase insert (Fantl et al., 1992; Kashishian, Kazlauskas, & Cooper, 1992; Klippel, Escobedo, Fantl, & Williams, 1992). The PDGFRβ contains two optimized binding sites for SH2 domains of p85, PI3K for binding to their respective phospho-tyrosine residues, whereas PLCγ binding site Tyr

1021 could be a competitive site for PI3K as well (Songyang et al., 1993). The p85 phosphorylation by PDGFRs promotes directed cell migration, actin reorganization and cell survival through activation of downstream effectors, most importantly the serine/threonine kinase Akt/PKB.

PLC (phospholipase C) γ: This enzyme hydrolyses PIP2 to generate the second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), in turn leading, respectively to mobilization of Ca^{2+} from intracellular stores, and activation of certain members of the PKC family. In vivo studies have shown that Tyr 1009 and Tyr 1021 in the C-terminal region of PDGFRs bind and activate PLCγ possibly by interactions with both of two SH2-containing domains in PLCγ (Ronnstrand et al., 1992). PDGFR-mediated PLCγ activation is important for cell mitogenesis and motility (Kundra, Soker, & Zetter, 1994).

The ERK cascade: Furthermore, PDGF receptors also recruit and associate with several additional signalling molecules, adaptors such as **Grb2** and **Shc** that bind through its SH3 domains to the Ras guanine nucleotide exchange factors mSos1 and mSos2 which accelerate Ras GTPase activity that is particular important in coupling PDGF receptors to downstream MAPK signalling pathways involved in the control of cell proliferation and differentiation. Ras protein in turn undergoes a conformational change that serves as a switch for its activation cycle between an inactive GDP-bound form and a biologically active GTP-bound state. Consequently, active Ras GTP binds and activates other downstream targets like Raf-1 protein-serine/threonine kinase which leads to the activation of mitogenic ERK cascade (Andrae et al., 2008; van der Geer, Henkemeyer, Jacks, & Pawson, 1997). PDGFs are also able to activate other MAPK pathways, such as p38 kinase.

Excessive or uncontrolled PDGFR signaling may be dangerous, so that a stringent feedback control mechanism exits by which PDGFR tyrosine phosphorylation is tightly regulated by endocytotic internalization and degradation of ligand-receptor complex, and/or by dephosphorylation of PDGFRs and its substrates through SH2-domain **PTPs** (protein tyrosine phosphatases). Ligand bound PDGF receptors are dephosphorylated by tyrosine phosphatase SHP-2 that is also able to dephosphorylate Src protein (Stokoe and McCormick 1997).

1.7.4.2 The differences in PDGFRα and PDGFRβ cellular signalling

After PDGFRs activation a number of signaling cascades are triggered that leads to multiple cellular and developmental responses. Although there are many similarities between PDGFRα and PDGFRβ signal transduction, comparison of downstream cellular regulation and developmental signal demonstrates that there are also a number of differences. For instance, in contrast to PDGFRβ signalling, disruption of PDGFRα-mediated PI3K activation is important in normal skeletal development.

As mention above, even though both receptors recruit and interact with PI3K, PLCγ, SHP-2, only PDGFRβ stably associates with GTPase-activating protein, instead different binding site exist for SHP2 phosphatase, SHP-2 binds to the kinase insert of the PDGFRα, but to the tail of the PDGFRβ (Heidaran, Pierce, Jensen, Matsui, & Aaronson, 1990).

Differences in signalling mechanisms of the α and β receptors are also observed in disease state SMC, for example in the genetically hypertensive rat strain used as a model for human hypertension. In this study, PDGF-AA and PDGF-BB had different effects on DNA and protein synthesis. Therefore, in this cell type, it was suggested that PDGFRα, which was abnormally expressed, could cause cellular hypertrophy during hypertension whereas PDGFRβ elicits a mitogenic response (Inui, Kitami, Tani, Kondo, & Inagami, 1994).

In addition, while PDGF-AA is known to stimulate DNA synthesis and proliferation in normal SMC from a variety of species, PDGF-BB is both a potent mitogen for SMC and also a potent chemoattractant, whereas several studies indicate that PDGF-AA has either no chemotactic activity in SMC or negatively regulates the chemotactic response to PDGF-BB (Koyama, Hart, & Clowes, 1994; Koyama, Morisaki, Saito, & Yoshida, 1992; Siegbahn, Hammacher, Westermark, & Heldin, 1990).

The restricted expression patterns of PDGF receptors as well as their cell interaction sites are considered to be important and specific for their functional and developmental effects. Gene targeting studies have identified some specific characteristic receptor regulation by showing that PDGFRβ signaling can fully recompense for loss of

PDGFRα-specific signaling and can direct certain cellular functions that α cannot (Klinghoffer, Mueting-Nelsen, Faerman, Shani, & Soriano, 2001). Distinct PDGFRβ signals are required to promote optimal function of specific cell types such as SMCs whereas PDGFRα signals are important for tissue derived from nonneuronal cranial neural crest cells (Klinghoffer et al., 2001; Soriano, 1997).

1.7.5 Role of PDGF in development

Distinct biological functions of the PDGF isoforms during development are largely studied by targeted disruption of gene encoding the PDGF-AA and PDGF-BB or PDGFRs in mice. PDGF-A deficient embryos survived only around three weeks of age with severe lung alveoli malfunctions, leading to alveolar myofibroblast undifferentiated phenotype (Bostrom et al., 1996).

Loss of function of PDGF-BB chain resulted in abnormally developed kidney glomeruli, a dilated heart and vasculature hemorrhages, causing perinatal death. The disruption of PDGF-BB reflects its primary importance in angiogenesis as a major mediator of pericyte recruitment to newly sprouting capillaries, and as a major mitogen and chemoattractant for SMC. Targeted disruption of the PDGF β receptor gene is lethal at or just before birth, and the mutants mice are anemic, suffer from haemorrhage and have reduced platelet counts (thrombocytopenic); these mice also exhibit defects in kidney glomerulus maturation probably secondary to a loss of kidney mesangial cells (Soriano 1994).

No obvious abnormalities were observed in central nerve system of the β receptor mutant. Both PDGF-BB chain and β receptor mutants show a similar phenotype important in kidney and vascular wall development, supporting the idea that PDGF-BB and β receptor signalling are crucial of these tissues during development (Soriano, 1994).

Mutant α receptor mice die during early embryonic stages and exhibit incomplete cephalic closure, severe dermal defects and significant defects in skull and skeleton. Disruption of PDGFRα signalling seems to be mainly important for non-neuronal cardiac crest development similar with defects observed in PDGF-A/C double knockouts (Morrison-Graham, Schatteman, Bork, Bowen-Pope, & Weston, 1992; Soriano, 1997).

1.8 Aims of this thesis

At the outset of this thesis it was known that STC-1 expression was upregulated in models of angiogenesis and in cultured endothelial cells in response to VEGF. However, very little was known regarding the role of STCs in either endothelial cell function or in angiogenesis. Various lines of evidence did indicate that STC-1 might regulate different aspects of mammalian cell function, though neither the mechanisms mediating these cellular effects, nor their physiological or pathological significance were understood. It was also largely unknown at this time whether STCs were expressed or were regulated in SMC. Therefore the overall goals of this thesis is to increase our understanding of STC-1 function in vasculature, specifically to:

- Examine the role of STC-1 in endothelial cell function, and in angiogenesis in cell culture models using a variety of approaches, including treatment with recombinant STC-1 protein (which had recently become commercially available), inhibition of endogenous STC-1 expression using specifically targeted small interfering RNA (siRNA), and over-expression of STC-1 using an adenovirus encoding STC-1 to allow for efficient transduction of primary endothelial cells. Some pilot work had been performed in this laboratory immediately prior to the commencement of my PhD work, which had generated STC-1-encoding adenoviruses, and suggested that STC-1 did not regulate endothelial cell proliferation in response to VEGF, that STC-1 over-expression could negatively regulate VEGF-induced endothelial cell migration. However, these data were preliminary and I therefore aimed to reproduce these data, and to further examine the role of STC-1 in VEGF functions.

Since VSMC are an important component of the vasculature, I also hypothesised that STC upregulated in endothelial cells might have implications for regulation of VSMC function. VSMC in culture are also highly synthetic cells, and I therefore also hypothesised that STCs might be expressed in VSMC, and that their expression might be regulated in VSMC.

- Examine STC-1 expression in VSMC, and to assess whether STC-1 expression was regulated by growth factors for VSMC, particularly PDGFs.
- Analyse the role of STC-1 in the regulatory functions of VSMC that might be relevant for their role in development or disease, such as cell migration, survival or proliferation.
- Investigate possible mechanisms through which STC could act in either endothelial cells or VSMC.

Chapter 2

2 Material and Methods

Each experimental technique used in this thesis is described below. Supporting information, which explains the key principle for each method was extracted from Wilson and Walker (2005, 6th edition) and/or product literature as appropriate.

2.1 General materials

Endothelial cell basal medium (EBM) and endothelial growth supplements (Singlequots) were purchased from Lonza and fetal bovine serum (FBS) from Life Technologies Ltd. Smooth muscle cells (SMC), SMC basal media and SMC growth supplements were obtained from TCS CellWorks Ltd.

Recombinant human PDGF-BB (disulfide-linked homodimer of two B chains) and PDGF-AA (homodimer of A chain) were purchased from Peprotech EC Ltd. Human recombinant VEGF-A_{165} (mature secreted 165 amino acid (aa) form) was from R&D Systems. Primary antibody suppliers and dilutions are given in Table 2.1.

siRNAs were from either Life Technologies Ltd, ThermoFisher Scientific or Qiagen as indicated in Table 2.2. Lipofectamine® RNAiMAX siRNA Transfection Reagent was provided from Life Technologies Ltd. DuoSet ELISA Development kit for the detection of STC-1 was obtained from R&D Systems. Complete™ Protease inhibitor cocktail was from Roche Diagnostics Ltd and Phosphatase inhibitors were from Sigma. All other chemicals or reagents were from standard suppliers or as listed in the text.

2.2 Human Cell culture

All cells were grown in cell cultureware (BD Biosciences) under sterile conditions in a 37°C, 95% air/5% CO_2 incubator.

2.2.1 Smooth Muscle Cells

Human Coronary Artery Smooth Muscle Cells (HCASMCs, TCS CellWorks) or Human Aortic Smooth Muscle Cells (HAoSMCs, TCS CellWorks) were grown in Smooth muscle cell (SMC) growth medium comprising SMC basal medium plus SMC growth supplement (containing gentamycin (25μg/ml) amphotericin B (50 μg/ml) and 2.5 % v/v FBS.

Experiments used confluent SMCs at passages 3-7 and those used for signalling studies were starved overnight in serum free SMC medium. Cells were then washed in serum-free medium and incubated in serum-free medium with or without the addition of growth factors for the indicated times. For some studies, cells were pre-incubated with pharmacological inhibitors or 100 ng/ml recombinant human Stanniocalcin-1 protein (rhSTC-1, MyBioSource) for 30 mins prior to the addition of other growth factors, for example, 30ng/ml recombinant human PDGF-BB for a further 10 mins.

2.2.2 Endothelial Cells

Human Umbilical Vein Endothelial Cells (HUVEC, TCS CellWorks) were grown on gelatin-coated cultureware in endothelial growth medium (EGM; consisting of EBM supplemented with 10% v/v FBS, and Singlequots; containing 10 ng/ml recombinant human epidermal growth factor, 12 μg/ml bovine brain extract and 50 μg/ml

gentamicin-ampicillin). Experiments utilized confluent HUVECs at passage 3 or 4 that were rendered quiescent by overnight incubation in EBM containing 0.3% FBS prepared as a dilution. Prior to the addition of factors and other treatments, the quiescent cells were washed twice in serum-free EBM and then incubated with or without the addition of factors for 24 hours.

2.2.3 Human dermal fibroblasts

Human dermal fibroblasts (HDF) were grown in M106 medium (Life Technologies Ltd) supplemented with Low Serum Growth Supplement (Life Technologies Ltd; containing 2% v/v FBS, 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor and 10 µg/ml heparin).

2.3 SDS-PAGE and Western blotting

2.3.1 Principle

Eletrophoresis is a separation technique based on the movement of charged macromolecules under the influence of an electrical field. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) is the most widely used protein separation technique. SDS-PAGE separates denatured proteins migrating through a gel according to their polypeptide size.

The protein samples are diluted and denatured in buffer containing the ionic detergent SDS, forming an imposed negatively charged complex and DTT which cleaves any disulphide bonds. Using a precast bis-tris gel of appropriate pore size, proteins migrate through a cross-linked polyacrylamide gel in response to an applied electrical field. The eletrophoretic mobility of SDS bound polypeptides will strictly depend on their molecular size, resulting in an effective separation with smaller molecules running faster than larger molecules. After separation by SDS-PAGE electrophoresis the migrated proteins are transferred from the gel matrix to a polyvinylidene fluoride (PVDF) membrane. During transfer, the proteins bind to this membrane, and are immobilised. For immuno-detection, protein-binding sites on the surface of the membrane are blocked by incubating with a protein-rich solution, in this case, fat-free

milk and the immobilised proteins are then readily accessible to a specific primary antibody. Primary antibody is then detected by a second antibody conjugated to horseradish peroxidase. A chemiluminescent substrate is then added, which allows the chemiluminescent reaction between the enzyme and the substrate to occur by emitting light. The emitted light is then detected using photographic film, followed by densitometric analysis.

Western-blotting is often described as a semi-quantitative technique, with the amount of protein originally present in the samples proportional to the amount of peroxidase enzyme bound to the protein on the membrane, and so the amount of light generated and area together with intensity of the band present on the film. Routinely, western blotting samples were not protein assayed before loading, instead equivalent amounts of cells (based on equal coverage of tissue culture plastic) were lysed in equal amounts of buffer, and equal volumes were loaded. Usually, blotting with an antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a house-keeping enzyme that catalyses the sixth step of glycolysis, was used to visually confirm comparable protein loading.

2.3.2 Preparation of cell supernatants and lysates for western blotting

Serum-deprived cells were washed twice with warm basal medium and incubated in a 37°C, 5% CO_2 incubator for the required time with and without growth factors (for SMC, these were 30ng/ml PDGF/BB, 30 ng/ml PDGF/AA and 30 ng/ml PDGF/BB and 25 ng/ml basic FGF; for HUVECs 25 ng/ml VEGF was used). In some experiments, cells were preincubated for 30 mins with kinase inhibitors or STC-1 prior to growth factor treatment.

After treatments, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and immediately lysed in Cell Lysis Buffer (Cell Signaling Technology, Inc.) supplemented with 0.5 μl/ml Protease Inhibitor Cocktail and Phosphatase Inhibitor cocktails 1 & 2. Lysates were clarified by passage through a QIAshredder (Qiagen

Ltd.), and subsequently centrifuged (16 000 ×*g*, 4 °C, 10 min). Pellets were discarded and supernatants were stored at –80 °C until required for SDS PAGE.

Cell culture supernatants were collected, and clarified by centrifugation (2860 ×*g*, 4 °C, 10 min) to pellet the cell debris. Clarified supernatants were concentrated through an Amicon Ultra-4 Centrifugal Filter Device (Millipore Corp.).

2.3.3 Deglycosylation

STC-1 is *N*-glycosylated to varying degrees depending on the site of secretion. In certain experiments deglycosylation of STC-1 was performed to simplify the western blotting data. In these instances STC-1 was deglycosylated using 25500unit/µL Peptide-*N*-Glycosidase F (PNGaseF) (New England Biolabs, Inc.) as previously described (Homes & Zachary 2008). 36 µl of concentrated supernatants were denatured with 1X Glycoprotein Denaturing Buffer at 100°C for 10 minutes. After the addition of 1% NP-40 and G7 Reaction Buffer (50 mM sodium phosphate pH 7.5), two-fold dilutions of PNGase F were added and the reaction mix was incubated for 1 hour at 37°C.
In other experiments, PNGase F was used in combination with chondoitinase ABC and heparinase to characterise in more detail the glycosylated forms produced by SMCs. Bacterially produced chondroitinase ABC *(*from *Proteus vulgaris*) and heparinase (*Flavobacterium heparinum)* are endolyases that catalyse the cleavage of glycosaminoglycan side chains from the proteoglycan core protein. Their primary substrates are chondroitin sulphate and heparan sulphate proteoglycan, respectively.

Chondroitinase ABC (Sigma-Aldrich) was reconstituted in a buffer containing 0.01% w/v BSA and subsequently diluted into a buffer containing 50mM Tris, pH 8.0, with 60 mM sodium acetate and 0.02% w/v BSA, and Heparinase I and III (heparitinase; Sigma) was prepared in a buffer containing 20 mM Tris-HCl, pH 7.5, containing 0.1mg/ml BSA and 4 mM $CaCl_2$. The concentrated cell culture supernantants of the HCASMCs were treated with the enzymes by adding a final concentration of 1 unit per ml and then samples were incubated for 2 hours at 37°C. Samples were then analysed by SDS-PAGE and immunoblotting.

Unit definition for chondroitinase: One unit will liberate 1.0 mmole of 2-acetamido-2-deoxy-3-O-(b-D-gluc-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose from chondroitin sulfate A or 1.0 mmole of 2-acetamido- 2-deoxy-3-O-(b-D-gluc-4-ene-pyranosyluronic acid)-6-O-sulfo-D-galactose from chondroitin sulfate C per minute at pH 8.0 at 37 °C.

2.3.4 SDS PAGE and Western blotting

Proteins were separated by SDS–polyacrylamide gel electrophoresis in NuPAGE 4-12% Bis–Tris gels using MOPS running buffer systems (Life Technologies Ltd.). After electrophoresis, proteins were then transferred to 0.45µm Invitrolon PVDF membrane (Life Technologies Ltd.). Non-specific binding to the membrane was blocked by incubation in 5% w/v nonfat dried milk and 0.1% v/v Tween 20 in PBS with shaking at room temperature for one hour. Membranes were then incubated for either two hours at room temperature or overnight incubation at 4 °C with primary antibodies (see Table 2.1) diluted in blocking solution. The membranes were washed three times each for 15 mins with PBS-T and then incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (at 1:10000 dilution). Immunodetection was visualised using chemiluminescent detection according to the manufacturers protocol (ECL Plus, GE Healthcare Plc). Blots were scanned and protein bands quantified by scanning densitometry of autoradiograms along with a calibration strip and analysed using ImageJ (National Institute of Health; http://rsbweb.nih.gov/ij/). Equal protein loading was assessed, with parallel samples with control antibodies to reference proteins, such as GAPDH and alpha Tubulin where appropriate. In addition, where protein phosphorylation was determined, the results are expressed as a ratio to the total (ie phosphorylated and unphosphorylated) protein for each sample.

Table 2.1 Antibodies used in this study

Antibodies-Target	Host	Dilutions	Company
STC-1	Goat	1:1000	R&D Systems
STC-1	Chicken	1:800	Abcam Plc.
Phospho-Akt (Ser 473)	Rabbit	1:1000	Cell Signalling Technologies Inc.
Akt	Rabbit	1:1000	Cell Signalling Technologies Inc.
Phospho-GSK-3α/β (Ser 21/9)	Rabbit	1:1000	Cell Signalling Technologies Inc.
p38	Mouse	1:1000	BD Transduction Labs.
Phospho-p38 (T180-Y182)	Rabbit	1:1000	Cell Signalling Technologies Inc..
GAPDH V-18	Goat	1:1000	Santa Cruz Biotechnology
PDGFαR C-20	Rabbit	1:1000	Santa Cruz Biotechnology
Phospho-PDGFαR Tyr 745	Goat	1:1000	Santa Cruz Biotechnology
PDGFβR P-20	Rabbit	1:1000	Santa Cruz Biotechnology

Phospho-PDGFβR Tyr 1021	Rabbit	1:1000	Santa Cruz Biotechnology
Phospho-PDGFαR (T849)/PDGFβR (T857)	Rabbit	1:1000	Cell Signalling Technologies Inc.
Alpha Tubulin	Mouse	1:2000	Santa Cruz Biotechnology
FAK	Rabbit	1:500	Cell Signalling Technologies Inc.
Phospho-FAK Tyr 407	Rabbit	1:1000	Cell Signalling Technologies Inc.
p130 Cas	Mouse	1:1000	BD Transduction Labs.
Phospho-p130 Cas (Tyr 410)	Rabbit	1:1000	Cell Signalling Technologies Inc.

2.4 Small interfering RNA (siRNA) Transfection

2.4.1 Principle of siRNA gene silencing

RNA interference (RNAi) is a highly used technique applied to inhibit the expression or replication of specific mRNAs in a cell, therefore reducing the abundance of the protein, potentially blocking protein function. RNAi is a natural occurring cellular regulatory mechanism of controlling gene expression such as inhibiting the expression or replication of pathogenic double-stranded, dsRNA from certain viruses.

In the RNAi response, an mRNA-targeted mechanism degrades mRNA using ds RNAs, which are processed into short, 21-23 nucleotide fragments, called small interfering RNAs (siRNAs) (Aagaard et al 2008). siRNAs are snipped from longer precursor dsRNA chains by an endonuclease called Dicer. The anti-sense strand of these siRNAs is then used by an RNA-induced silencing complex (RISC) through a base-pairing

interaction, to guide mRNA cleavage, and promote mRNA degradation. For experimental purposes, this defence mechanism is deployed for the exogenous administration of synthetic dsRNAs, similar to Dicer products, which are engineered to precisely match the protein-encoding nucleotide sequence of the target mRNA.

siRNAs do not readily cross the cellular membrane as result of their negative charge and size. Cellular delivery of siRNAs is usually achieved via complex formation with cationic liposome-like reagents such as oligofectamine or RNAiMAX, which are then released into the cytoplasm where they associate with RISC complex.

2.4.2 Experimental details

2.4.2.1SMCs

To explore what functional role STC-1 plays in SMC cells it was important to be able to effectively knock down STC-1 using targeted siRNAs. However, SMCs are relatively resistant to high efficiency transfection with siRNAs. Whilst nucleofection by eletroporation has been previously demonstrated to be an effective approach, which was previously used in this lab to investigate the role of critical genes such as Neuropilin 1(NRP1) in SMCs (C. Pellet-Many, PhD Thesis 2010), obtaining a good STC-1 knockdown in SMCs using this approach presented a difficult challenge.

Initial experiments were performed using two different siRNAs as described below. Confluent cultures of HCASMCs were transfected using the Amaxa basic nuclefector kit for primary smooth muscle cells (Lonza). Briefly, the cells were plated at approximately 80% confluence and transfected on the following day using 100 nM final concentration of siRNA, non-target siRNA (# D-001810-01-05) and targeted STC-1-specific siRNA (# J-006477-07) from Dharmacon, Silencer Pre-designed Neuropilin-1 (NRP-1) siRNA (#4914), Silencer Negative Control #1 siRNA (Ambion Inc.) in nucleofection reagent solution provided with the kit. The cells were then incubated in SMC medium with 10% smooth muscle cell growth supplements for 24 h. Forty eight hours after transfection, cells were either harvested or rendered quiescent overnight prior to growth factor treatments. The major obstacle to using this approach was that for

STC-1 this method was able to achieve a reduction in STC-1 expression in some experiments, but other experiments produced inconsistent results. In addition, nucleofection also resulted in substantial levels of SMC cell death.

Therefore, a new transfection reagent, siRNA duplex-Lipofectamine™ RNAiMAX (Life Technologies Ltd.) was tested and optimised for delivering STC-1 siRNA and the non-targetting control siRNA into SMCs. Optimisation consisted of using transfecting cells at low and high confluency and using two different RNAiMAX concentrations. Briefly, SMC cells were seeded in two 6-well plates and transfected when the cells reached either 30% or 80% confluence, according to the manufacturer's protocol. siRNAs (20 μM) were diluted in 250 μl Opti-MEM I reduced serum medium (Opti-MEM, Life Technologies Ltd) and mixed with either 2.5 or 5 μl of transfection reagent pre-diluted in 250 μl Opti-MEM. After 20 min incubation at room temperature, the complexes were added to the cells in a final volume of 2 ml medium. Cells were harvested after 48 hours.

After 48 hours post transfection, the results showed that STC-1 expression was down-regulated compared to the non-targeting control. Furthermore, the highest degree of STC-1 knockdown was achieved at the lower confluency (approx. 30%) and highest concentration of RNAiMAX. Thus, this optimized protocol, detailed below, was used in all subsequent experiments.

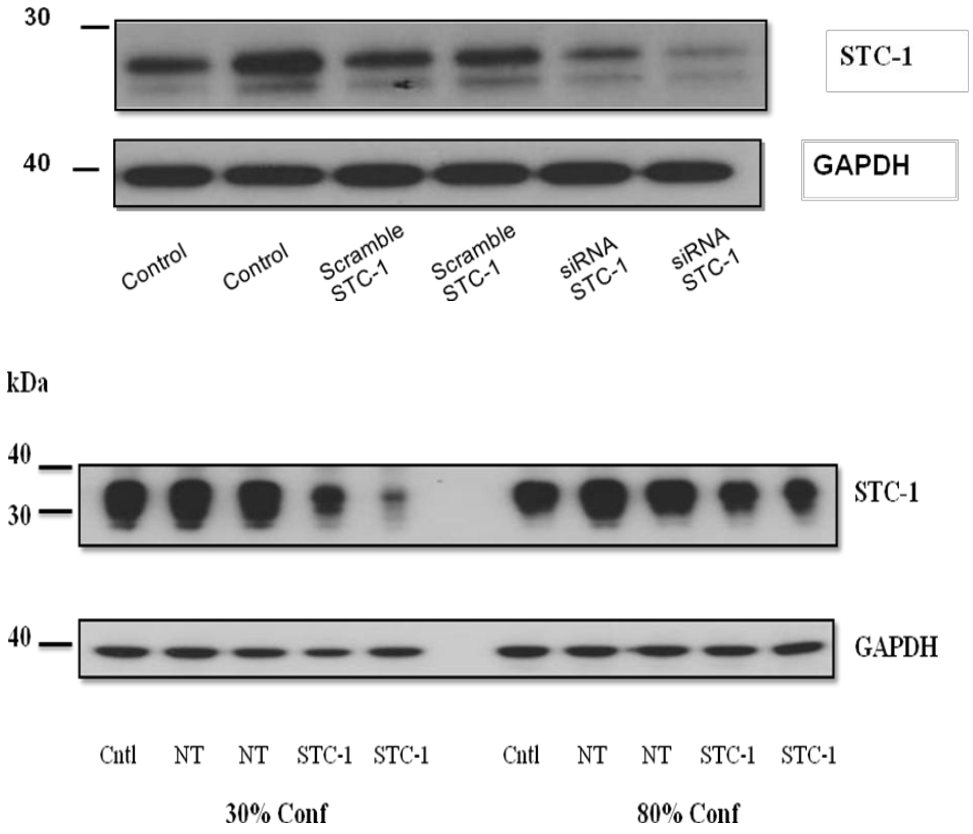


Figure 2.1: STC-1 gene knockdown mediated by two different applications

A. HCASMC were transfected with STC-1 siRNA using nucleofection. Deglycosylated supernantant samples were analyzed by SDS-PAGE gels and western blots using chicken anti-human STC-1 antibody. **B**. HCASMC at high and low confluences were transfected with siRNA using 2.5 and 5 μL Lipofectamine for up to 48 hr. Conditioned media from transfected cells were immunoblotted with goat anti-STC-1 antibody.

2.4.2.1.1 Procedure

Target genes of interest were downregulated using pre-designed duplex siRNAs from Dharmacon or Ambion as detailed in Table 2.2. Non-targeting, negative control #1 siRNA (Ambion) was used to control for the non-specific effects of siRNA transfection.

siRNA transfection was performed using siRNA duplex-Lipofectamine™ RNAiMAXand the cells were transfected at approximately 30% confluency. siRNA (20 μM) were diluted in 250 μl Opti-MEM I reduced serum medium and mixed with 5 μl of RNAiMAX pre-diluted in 250 μl Opti-MEM. After 20 min incubation at room temperature, the complexes were added to the cells in a final volume of 2 ml medium. Cells were harvested after 48 hr and the knockdowns were confirmed by western blotting, using appropriate antibodies.

2.4.2.2 HUVECs

Confluent HUVECs in 6-well plate were transfected using oligofectamine (Life Technologies Ltd). Briefly, siRNAs were diluted in warm Opti-MEM I, then incubated with 5% v/v oligofectamine for 25 mins to allow the siRNA/oligofectamine complex formation. Cells were washed with Opti-MEM and incubated for 4 hours with siRNA/oligofectamine/Opti-MEM solution (final siRNA concentration 200μM, 1ml/well). After 4 hr, the medium was adjusted to 10% v/v FBS and the following day changed to EGM. For functional studies, knockdown was assessed by lysing the fraction of unused parallel cultures of transfected cells in that particular experiment and analyzing these lysates via western blotting.

Table 2.2: Nucleotide catalogue number of siRNAs used in this study

Target	Catalogue Number	Company
Non-Target (NT)	1027281	Qiagen
STC-1	J-006477-07	Dharmacon
PDGFRα	4392420	Ambion
PDGFRβ	4390824	Ambion
PKC	AM51323	Ambion
NRP1	4914	Ambion

2.5 Polymerase Chain Reaction

2.5.1 Principle of PCR

Polymerase Chain Reaction (PCR) is an accurate, reliable molecular biology technique for the amplification of a single or a few copies of a piece of DNA across several orders of magnitude, generating many copies of an identical sequence of DNA. The starting material may be genomic DNA, RNA, cloned DNA or forensic samples.

The technique uses a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*, to enzymatically synthesize a complementary DNA sequence from nucleotides, by using single-stranded DNA as a template, and primers. Primers are DNA oligonucleotides, which are required for initiation of DNA synthesis because they prime the DNA sample ready for the polymerase to bind and begin to copy. This enables the polymerase to selectively amplify the DNA sequence of interest.

During PCR, thermal cycling is employed with alternate and rapid heating and cooling of the PCR sample to a defined series of temperatures. These changes in temperature are used to control the activity of the polymerase and the binding of primers. Initiation of the PCR reaction occurs at high temperature such as 95°C where all double helix stranded DNAs are “melted” into single strands. At a lower temperature, each strand is then allowed to hybridise with the complementary primers, followed by DNA enzymatic extension using DNA polymerase to selectively amplify the target DNA (called reverse transcription where RNA is used as the starting material). The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions. Successive cycles will thus generate an exponentially increased number of DNA fragments, which can be visualized directly using agorose gel eletrophoresis followed by ethidium bromide staining.

2.5.2 Principle of RT-PCR

Quantitative real-time polymerase chain reaction (QRT-PCR or Q-PCR) is a modified form of the standard PCR technique that allows the monitoring of the accumulated PCR products in real time. Providing that the PCR reaction is proceeding exponentially (ie. neither the primers, nucleotides or Taq is limiting the rate of reaction) there is a quantitative relationship between amount of starting target sequence and amount of PCR product. The accumulation of PCR products is quantified by use of an integral fluorescent reporter molecule. The fluorescence emitted by the reporter molecule increases as the enzyme-catalysed DNA synthesis accumulates with each cycle of amplification.

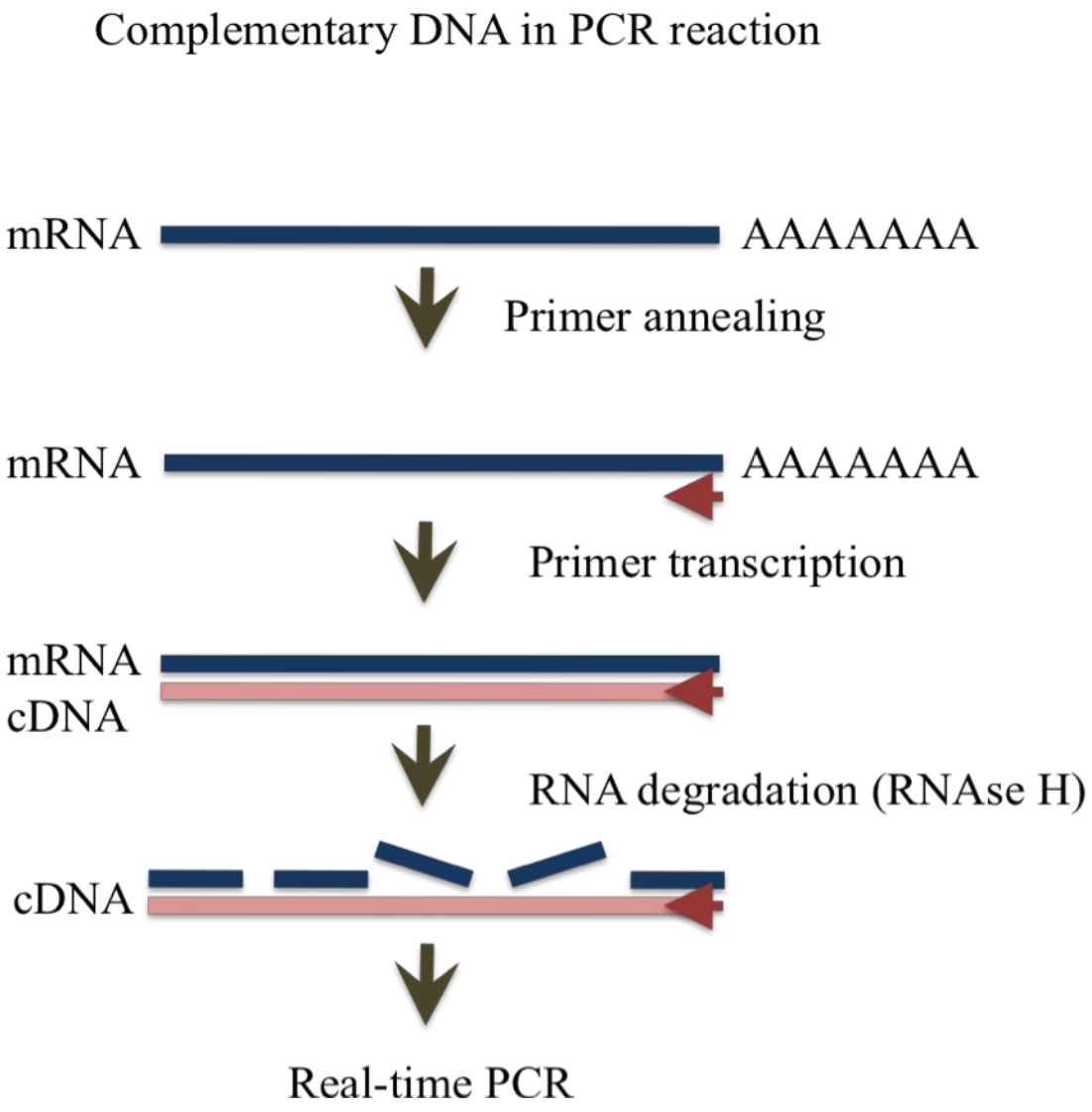


Figure 2.2: Schematic presentation of the reverse transcription reaction

A commonly used reporter molecule is SYBR-Green, a dye that binds to the minor groove of double-stranded DNA but not to single-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence also increases. SYBR Green has the advantage of being relatively inexpensive, easy to use, and sensitive.

2.5.3 Experimental details

Total RNA was purified from SMCs treated with 30ng/ml PDGF-BB or vehicle for indicated times and purified using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. During purification, RNA was digested with RNase-free DNase I (Qiagen) eradicating any residual genomic DNA. The DNase I was removed in a subsequent wash step, then, single-stranded complementary DNA (cDNA) was selectively synthesised from 2 µg of total mRNA with oligo$(dT)_{12–18}$ primer using the Superscript II first-strand synthesis system for RT-PCR (Life Technologies Ltd) according to manufacturer's instructions.

The relative quantity of STC-1 and control gene GAPDH mRNA in each sample was determined using cDNA template with the Light Cycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics) and the following conditions: 2 min incubation at 50 °C, pre-denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C, 15 s; and 60 °C, 1 min. The primers used in this application are detailed in the Table 2.3. RT-PCR analyses were performed in duplicate for each sample using RNA preparations from at least three independent experiments.

2.5.4 Agarose gel electrophoresis

To confirm that the primers used in the QRT-PCR for *stc* and *gapdh* genes were specific and produced the PCR products of the correct size, the amplified PCR products were run on an agarose gel. PCR products were mixed with 6x loading buffer (Fermentas; final concentration 1x) and run at 120V on a 1% agarose gel containing 200ng/ml ethidium bromide, using TAE running buffer (40mM tris-acetate, pH 8.3, 1mM EDTA).

Table 2.3 Nucleotide sequences of STC-1 and GAPDH entry primers

Gene	Template Product length (PL)	Oligo Name (length)	Sequence (5'→3')
NM_003155.2	2008.....2028	Forward STC-1 (21)	GAGACAGGAAAAACC AACAGG
	PL=236		
	2243.....2223	Reverce STC-1 (21)	GATGAAAGGGGACGA GTAAAG
NM_002046.3	822.....841	Forward GAPDH (20)	GTCAGTGGTGGACCTG ACCT
	PL=251		
	1072.....1053	Reverce GAPDH (20)	CCCTGTTGCTGTAGCC AAAT

The sizes of PCR products were determined by comparison with a DNA molecular weight marker (100 bp ladder; Fermentas), which contains DNA fragments of known size, run on the gel alongside the PCR products. Following electrophoresis the gel was viewed and photographed under ultraviolet light. Figure 2.3 shows the size of the primers products obtained for the QRT-PCR reaction. Analysis of the gel and showed that both primers produced a single product corresponding to to their expected molecular weights, with the STC-1 cDNA product being around 236 bp and GAPDH at around 251 bp. No other non-specific bands were observed in the negative control lanes (lacking template cDNA).

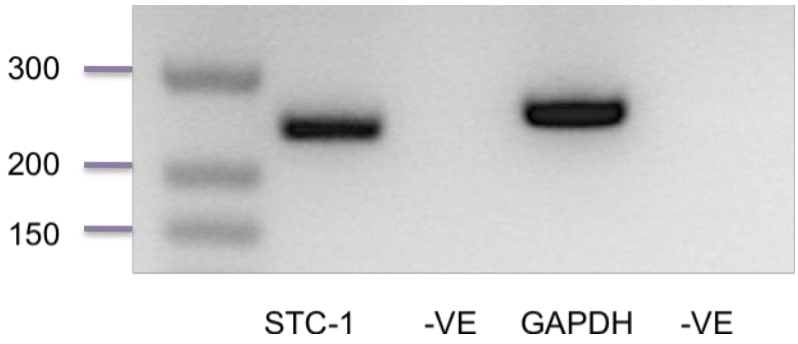


Figure 2.3 Ethidium bromide-stained PCR products after agarose gel electrophoresis of the DNA fragments amplified from the cDNA of the samples used in RT-PCR reactions, using STC-1 and GAPDH primers. PCR products were analysed in 2% agarose TBE electrophoresis gels with negative control samples representing the PCR reaction carried out without cDNA template (–VE;). The gel also shows a DNA ladder and the DNA bands in the STC-1 and GAPDH lanes indicate successful amplification of the target DNA fragments of expected length. The gel shown is representative of the results obtained from two independent experiments.

2.6 Enzyme-linked immunosorbent assay (ELISA) for STC-1

2.6.1 Principle of ELISA

Enzyme-linked immunosorbent assay (ELISA) is a plate-based assay designed for detecting and quantitatively evaluating the presence of substances such as peptides, proteins, antibodies and hormones. In an ELISA, an antigen is immobilized to a solid surface or a primary antibody coated on the surface and then complexed with an antibody that is linked to a reporter-enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

2.6.2 Experimental details

Quiescent HCASMCs were treated with or without 30ng/ml PDGF-BB, and cell culture supernatants were concentrated by filter centrifugation (as detailed in 2.3.2) prior to use of the duoset Elisa (R&D, Systems). For quantification of hSTC-1 in serum-free medium, 96-well microplates (C8 Maxisorp 445101, Nunc) were coated overnight at room temperature (RT) with 1.6 µg/ml capture antibody, goat anti-human STC-1 in PBS without carrier protein (R&D, Systems). The following day, each well was washed three times with washing buffer (0.05% v/v Tween 20, pH 7.2-7.4) and blocked with 300 µl 1% v/v BSA in PBS (reagent diluent, pH 7.2-7.4) for 1 hour at RT. After washing cells three times, 100 µl diluted supernantants were applied to duplicate wells after being diluted with reagent diluent. A standard curve was set up using 100 µl of the purified recombinant human STC-1 (0–4 ng/ml) in reagent diluent. The plates were incubated for 2 h at RT followed by washing three times and application of 100 µl of 200ng/ml biotinylated detection goat anti-human STC-1 antibody (R&D, Systems) for 2 h at RT. After washing three times, 100 µl of 1:200 diluted streptavidin conjugated to horseradish peroxidase was added, and further incubated for 20 minutes at RT. The Substrate solution (a 1:1 mixture of color reagent A, H_2O_2 and reagent B, containing tetramethylbenzidine; R&D Systems) was applied and plates were incubated at RT in the dark. The reaction was stopped after 15–20 min with 50 µl of 2 M H_2SO_4 and absorbance was immediately measured in a Tecan Magellan microplate reader at 450 nm against a reference wavelength of 560 nm.

2.7 Adenovirus Infection

To examine the effect of STC-1 over-expression on smooth muscle and endothelial cell function, an adenovirus (Ad) expressing wild type STC-1 (Ad.STC-1) was used together with a control adenovirus, expressing lac Z (Ad.lacZ). The wild type STC-1 and lac Z expressing adenoviruses were kindly provided from Dr David Holmes and some details of adenovirus production are given below.

Adenoviruses are highly useful vehicles for delivering genes or DNA constructs to cells, permitting analysis of the signaling pathways and/or cell function involved in the

overproduction of protein of choice on these cells. Adenoviruses are non-enveloped double-stranded DNA viruses whose capsid is mainly composed of pentons (penton base and fibre monomers) and hexons. The viral infection begins with the attachment of the fibre proteins to a cellular receptor named CAR (for coxsackievirus and adenovirus receptor) (Benihoud, Yeh, & Perricaudet, 1999), followed by the interaction of the penton base with αvβ3 and αvβ5 proteins belonging to the Integrin family. After adenoviral internalisation through receptor-mediated endocytosis, the virus escapes from the early endosomal compartment and is transported into the nucleus where viral transcription and replication initiates. The adenovirus genome is extra-chromosomally expressed without integration into the host cell genome.

Once inside the nucleus, adenoviral genes are expressed in two distinct phases, early and late, by viral DNA replication. The early phase is responsible for early (E1) genes expression coding for regulatory proteins required for replication of viral genome and later gene expression. Assembly of viral components followed by completion of the virus cycle subsequently triggers host cell death and the release of virion progeny (Russell, 2000). In this thesis, replication-defective adenoviral vectors are used, which are unable to produce additional viral particles to infect other cells since the E1 and E3 genes have been deleted. The expression level of the DNA sequence of interest in the adenovirus is under the control of host transcription machinery and initially increases over time before decreasing as the viral genome is diluted by cell division.

2.7.1 Adenoviral Gateway® cloning systems and vectors

Adenoviral systems are now popular platforms for reliable gene delivery and high-level transient expression in any mammalian cell type. Adenoviral Gateway® Technology is a fast, easy, and accurate cloning strategy allowing a DNA fragment to be inserted into a universal entry vector (pENTR/D-TOPO) and then rapidly transferred into pre-designed destination vectors (Fig. 2.4).

Linear pENTR/D-TOPO vector has a four nucleotide overhang conjugated to a topoisomerase I from Vaccinia virus which allows the ligation and insertion of 5' CACC blunt-end PCR products (desired gene insert) into the plasmid. The vector also contains a kanamycin resistance gene for selection of positive transformants. Use of this

system allows the relocation of a sequence of interest at any destination vector via recombinational cloning between attL1/L2 sites in entry vector and the attLR1/R2 sites in the destination vector which are ampicillin selected.

pAd/CMV/V5-DESTTM (36.7 kb) is a destination vector that can express high levels of the gene of interest in essentially any mammalian cell type, producing replication-incompetent E1- and E3- deleted Ad5 adenoviral particles when transfected into the 293A Cell Line. The 293A Cell Line facilitates the initial production, amplification, and titering of replication-incompetent adenoviruses. The 293A cells contain a stably integrated copy of the E1 gene that supplies the E1 proteins (E1a and E1b) required to generate recombinant adenovirus where the gene of interest is driven by immediate early human cytomegalovirus (CMV) promoter for high-level constitutive expression. The control plasmid, pAd/CMV/V5-GW/lacZ, is included for use as a positive expression control in the mammalian cell line of choice.

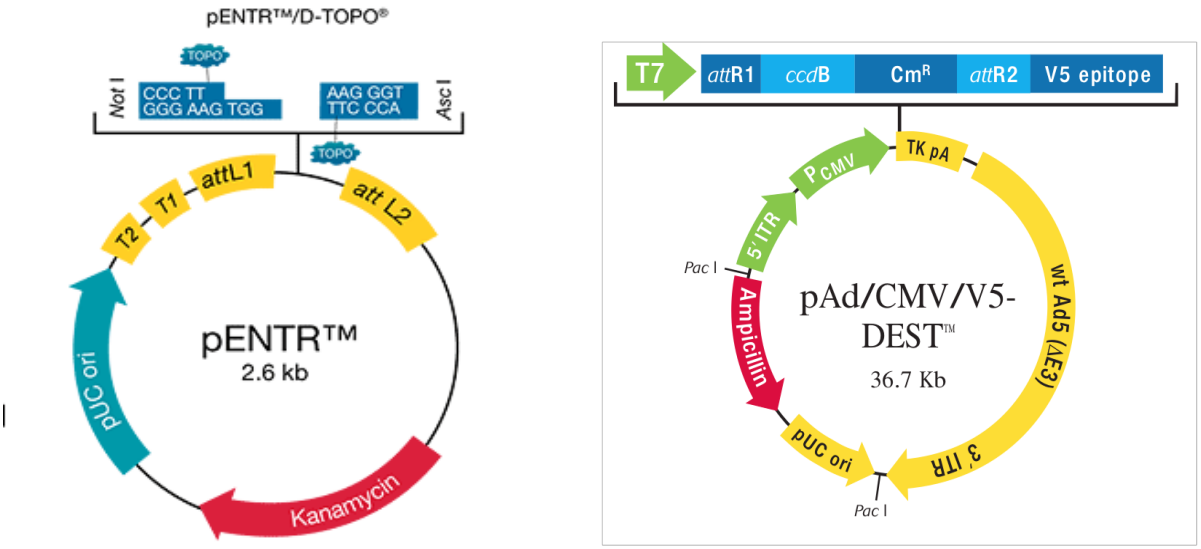


Figure 2.4: Adenoviral Gateway® cloning vectors

Schematic illustration of entry and destination vectors used in STC-1 adenoviral production from the Invitrogen Gateway® Technology manuals.

2.8 Cell migration assay

2.8.1 Assay principle

Transwell migration is commonly used to study migration of cells towards a chemoattractant. It was originally introduced for the analysis of leukocyte chemotaxis (Boyden 1962). The Transwell consists of two medium filled chambers separated by a microporous membrane. The upper chamber, a cylindrical cell culture insert, contains a polycarbonate membrane at the bottom with a defined pore size (in these experiments a pore size of 8 μm was used). Cells are seeded in the upper compartment in the serum-free media, while chemoattractant are placed in the chamber below. Migratory cells therefore move through the pores toward the chemoattractant in the other chamber. The number of cells that have migrated to the lower side of the membrane is determined after removing non-migrated cells (on the upper surface of the membrane) by staining the membrane and then counting migrated cells under a microscope.

2.8.2 Procedure

Confluent HUVECs/HCASMCs were trypsinised and resuspended in serum-free media at 3x 10^5/3x 10^4 cells/ml respectively. In some experiments, prior to seeding, cells were incubated at this stage for 30 mins in the presence of 100ng/ml hrSTC-1 with agitation every 5-10 mins.

Cells culture inserts (8μm membrane pore size, 6.4mm diameter, Falcon Becton Dickinson) were inserted into a 24-well plate and in the lower chamber, 750 μl serum-free media with or without 25ng/ml VEGF/ 30ng/ml PDGF (for HUVECs/HCASMCs respectively) was added. Cell suspensions (500 μl) were added to the upper chamber and the Transwell was incubated at 37°C for 4 hr. Cells that have not migrated were scrapped from the upper side of the membrane using a cotton bud, and the cells on the lower side of the membrane were fixed and stained with the Reastain Quick Diff kit (Reagena). Nuclei of migrated cells were counted in four fields using an eyepiece indexed graticule at 100x magnification. The mean of four counts per insert was used as the count for each sample or insert.

2.9 Tubulogenesis co-culture assay

In vitro angiogenesis was determined by using a co-culture tubulogenesis assay (Friis et al., 2003). Briefly, human dermal fibroblasts (HDF) were plated out in 24-well plates and grown until confluent in M106 medium. HUVECs were incubated for 48 h with adenovirus expressing either STC-1 of LacZ at a multiplicity of infection (MOI) of 20. HUVECs were then trypsinised and resuspended at 2 x 10^5 cells/ml in EBM plus 1% FBS. The M106 medium on the HDFs was replaced with 0.5 ml EBM plus 0.5% FBS and ± 25 ng/ml VEGF. Finally, 1x10^4 HUVECs (50 µl cell suspension) were added to each well. HUVECs and fibroblasts were propagated in co-culture for 7 days at 37°C and 5% CO_2 incubator with the medium being changed after 3 days. After 1 week incubation, the medium was removed, clarified by centrifugation and stored at –20 °C for later analysis of STC-1 levels by Western blotting. Tube formation was visualised by staining for von Willebrand factor using the following method. All incubation steps were performed at room temperature and with gentle agitation. Cells were fixed in absolute ethanol for 2 hours, followed by washing twice with PBS-T and blocking with 5% milk in PBS-T for 30 min. Cells were then incubated with an antibody to von Willebrand factor (Abcam), diluted 1 in 1000 in PBS-Tween 20 with 5% milk for 1 h. After washing with PBS-T, bound antibody was detected with a biotinylated secondary antibody (Chemicon,) diluted 1 in 2000 in PBS-T and incubated for 30 min. Avidin-Biotin complexes were formed using the ABC reagent (Vector Laboratories), following manufacturer's protocol and staining was finally visualized with diaminobenzidine (Sigma). Tube formation was imaged using a Leica stereomicroscope and four representative fields were captured for each well. Images were analyzed using the Angiosys software package (TCS Cellworks; http://www.tcscellworks.co.uk/pricing_ordering/product_detail.php?CI_ID=13) that measures average tube length, total tube length, number of tubes and the number of branch points.

2.10 Cell viability (XTT) assay

2.10.1 Assay principle

The XTT cell proliferation assay is an effective method to measure cell growth, viability and cytotoxicity. Tetrazolium salt, (XTT) a colourless or slightly yellow compound is reduced in metabolically active cells via mitochondrial dehydrogenase, to produce the bright orange coloured formazan dye. The formation of formazan can be quantified spectrophotometrically and the amount of dye produced directly correlates to the number of metabolically active cells present. Actively proliferating cells increase their metabolic activity while cells exposed to toxins such as hydrogen peroxide (H_2O_2) will have decreased activity. H_2O_2 is formed in the eukaryotic cell as a by-product of various oxidases and superoxide dismutases. Hydrogen peroxide accumulation in cells causes oxidative damage like oxidation of cellular targets such as DNA, proteins, and lipids leading to mutagenesis and cell death.

2.10.2 Procedure

HCASMCs (1.5 x10^3 cells/100 µl) were seeded in a 96-well plate and transfected with negative control, STC-1 siRNA or mock (no siRNA) using the Lipofectamine RNAiMax protocol (Section 2.4.2.1.1) for 48 hr and incubated 24hr in basal medium. The following day, cells were washed and treated with and without 30ng/ml PDGF-BB for 1hr and hydrogen peroxide for 24hr. Medium used for 24hr incubation were saved to assess STC-1 knockdown. The following day, 50µl of XTT reagent (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulphonic acid hydrate) was added to each well/medium. The absorbance of the formazan product, reflecting cell viability, was measured at 490 nm. Each assay was performed in duplicate. A similar procedure was followed for the treatment of cells with recombinant STC-1 using the same reagents.

2.11 Statistical analysis

All results are reported as the means ± SEM of at least three independent experiments, unless otherwise indicated. Statistical analysis was performedusing Prism (GraphPad)

either by one or two-way ANOVA as appropriate, with Bonferroni's test for multiple comparison of the differences between groups and the value of $p < 0.05$ was considered to be statistically significant.

Each graph is presented data mean with error bars representing the standard error of the mean (SEM) unless otherwise stated on the text.

Chapter 3

3 Results: Effect of STC-1 on Endothelial Cell function

Genome-wide analysis of VEGF-induced gene expression in HUVECs led to identification of STC-1 as a VEGF-responsive gene (D. Liu et al., 2003). Further work in this laboratory explored in more detail STC-1 induction in endothelial cells and showed that VEGF and FGF synergistically upregulated STC-1 mRNA and protein expression. This was found to be mediated by the PKC and ERK pathways (Holmes & Zachary, 2008). This and other findings discussed in the introduction, led to the suggestion that regulation of this factor may be a key feature of the angiogenic response to VEGF and possibly other angiogenic cytokines. However the role of VEGF-induced STC-1 gene expression in the endothelium specifically or the vasculature in general is still not fully known. Before I began my PhD, limited pilot work had been performed examining the effects of recombinant STC-1 on endothelial cell proliferation and migration, and had not revealed any significant effects (unpublished results). However, since these were pilot experiments, at the start of my PhD, I examined effects of STC-1 in endothelial cells in more detail using recombinant human STC-1 (rhSTC-1), and by

adenoviral overexpression of STC, and by targeted knockdown of STC-1.

3.1 Induction of STC-1 by VEGF-A

As previously identified, the angiogenic cytokine, VEGF, is able to induce STC-1 expression. To confirm the STC-1 upregulation in response to VEGF treatment in HUVECs, concentrated media from HUVECs incubated with or without 25 ng/ml VEGF for 24 hours were prepared for immunoblotting. Analysis of western blots showed, as expected, a strong increase in expression of STC-1 protein migrating as a major band of ~35 kDa in HUVECs, in agreement with previously published data from this laboratory (Holmes and Zachary 2008). A representative blot obtained from these experiments is shown in Figure 3.1.

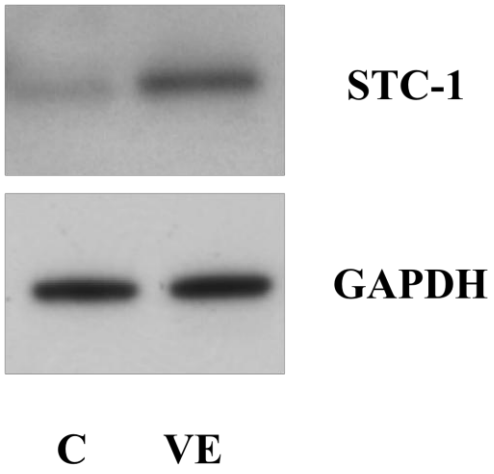


Figure 3.1: STC-1 upregulation by VEGF

Confluent, serum-deprived HUVECs were treated with 25ng/ml VEGF (VE) or no addition (C) for 24 hours. The conditioned medium was clarified by centrifugation, concentrated and analysed by western blotting using antibodies to STC-1 and GAPDH. The blot shown is representative of three independent experiments yielding similar results.

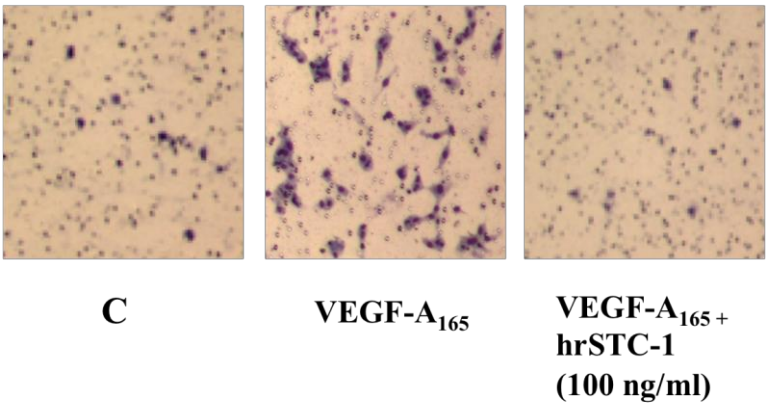
3.2 Role of STC-1 in VEGF-stimulated cell migration

Given the evidence suggesting a role for STC-1 in angiogenesis, I explored whether STC-1 could affect VEGF-mediated migration. The role of STC-1 in the migratory response of HUVECs towards VEGF was examined by the pre-treatment of cells for 30 min with different concentrations of recombinant STC-1. Treatment with STC-1 alone for 4 hours at concentrations of 1 to 100 ng/ml showed no effect on migration of HUVECs (Fig.3.2). Stimulation of HUVECs with VEGF alone resulted in a near 6-fold increase in migration compared to the control (no VEGF) (Fig. 3.2). Pre-treatment of HUVECs with up to 50 ng/ml hrSTC-1 had no effect on VEGF-induced migration. In contrast however, pre-treatment with 100 ng/ml hrSTC-1 significantly decreased endothelial migration by approximately 40% compared to the effect of VEGF alone (Fig 3.2).

3.3 Analysis of recombinant STC-1 on VEGF-induced signalling

The inhibitory effect of 100ng/ml hrSTC-1 on VEGF-dependent migration, led me to examine the role of STC-1 on VEGF-stimulated signalling pathways. HUVECS were pre-treated with two different concentrations of hrSTC-1 (50 and 100ng/ml) followed by stimulation for 10 minutes with VEGF (25 ng/ml) or no addition. Whole-cell lysates were prepared and immunoblotted to determine the activation states of several important signalling molecules involved in VEGF downstream signalling such as Akt, p38 kinase, FAK, p130 Cas and ERK1/2 (Fig 3.3).

In several initial experiments, no apparent effect was observed in response to hrSTC-1 treatment on different VEGF-induced downstream signalling pathways. It was noted that while VEGF increased activity of ERK1/2, Akt and p38kinase, I was unable to observe increases in p130Cas or FAK Y407 phosphorylation (Fig. 3.3).



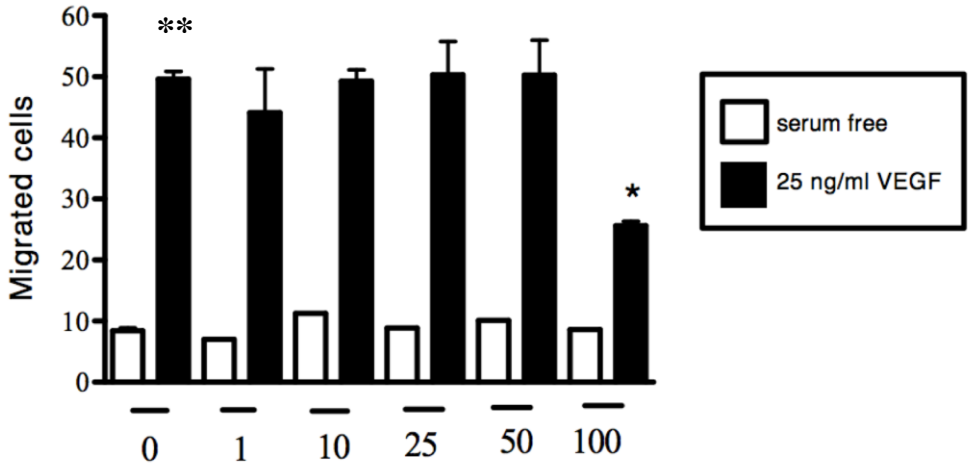


Figure 3.2: Effect of hrSTC-1 on HUVEC migration in response to VEGF-A_{165}

HUVEC cells were pre-incubated in suspension in serum free medium with hrSTC-1 for 30 minutes at the indicated concentrations and then transferred into transwell migration assay chambers. Migration of the cells towards 25ng/ml VEGF (V) or no addition (serum free) was assessed after incubation at 37°C for 4 hours. The cells that had migrated were fixed, stained and counted. Photographs of representative transwell membranes are presented above. Results are shown as means ± SEM from three independent experiments (below), analysed by two-way ANOVA with Bonferroni's test. ** P=0.001 compared to control (0), and * P=0.01 versus 100ng/ml hrSTC-1 treatment.

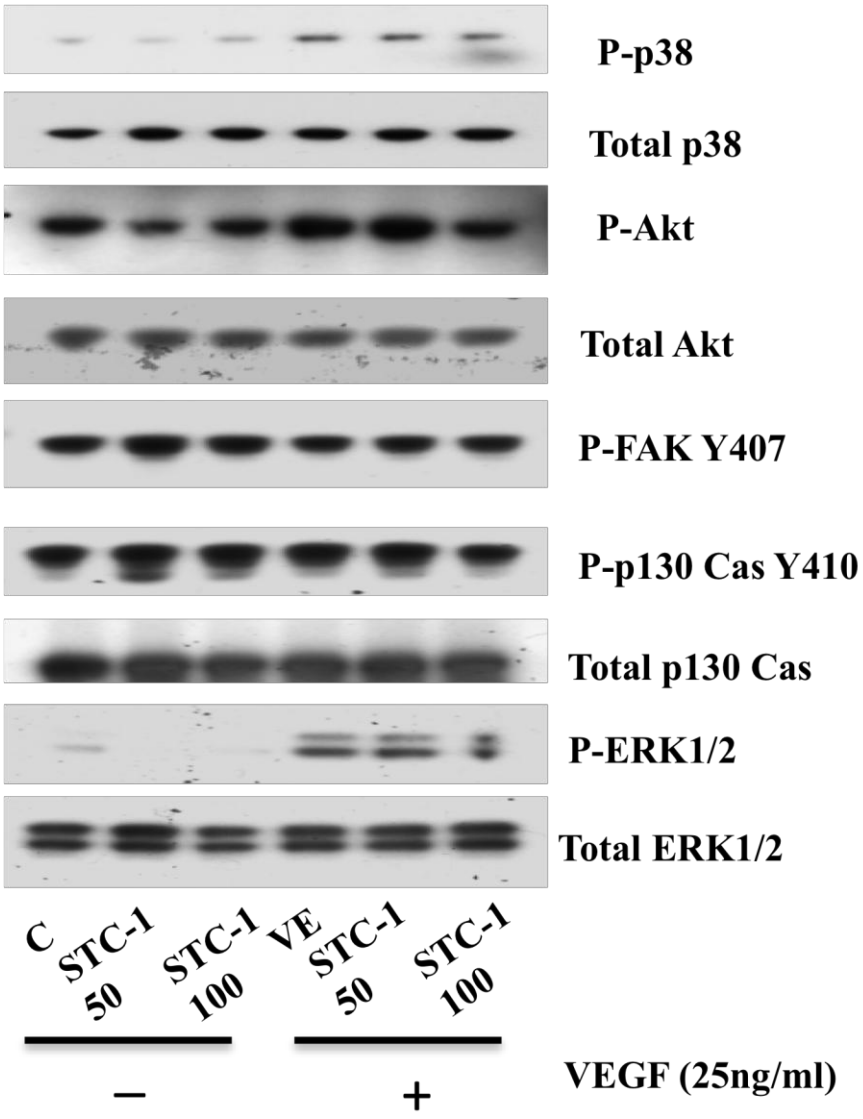


Figure 3.3 Effect of STC-1 on VEGF stimulation of signalling pathways.

Confluent, serum-deprived HUVECs were pretreated for 30 mins with the indicated concentrations (ng/ml) of hrSTC-1. Cells were then treated with 25 ng/ml VEGF (VE) or received no addition (C) for 10 mins followed by lysis and immunoblotting with the indicated antibodies. Results are representative of at least two independent experiments yielding similar results.

3.4 Adenovirus mediated overexpression of STC-1 in HUVECs

Incubating HUVECs with hrSTC-1 show how these cells respond to exogenous STC-1. However endothelial cells express significant amounts of STC-1 and I hypothesised that STC-1 may therefore have an autocrine effect, which cannot be mimicked by addition of exogenous STC-1. It is also possible that the hrSTC-1 used in my initial studies was not processed or post-translationally modified in ways that are important for its biological function. In order to investigate these possibilities, adenoviral infection of HUVECs was used to overexpress STC-1. To look at the levels of STC-1 in the cells and its secretion into the medium, two different multiplicities of infection (MOI) were used based on the viral titres. Confluent HUVECs were infected with adenoviruses expressing either STC-1 (Ad.STC-1) or the control gene, Lac Z (Ad.Lac Z), at MOIs of 5 and 10. 48 hours after infection, the medium and cell lysates were immunoblotted for STC-1. The results, shown in Figure 3.4, show that STC-1 immunoreactive bands were strongly expressed in both the cells and the medium from Ad.STC-1-infected cells as compared with Ad.Lac Z-infected cells. The major bands sprecifically increased in medium from Ad.STC-1-infected cells were ~35, ~60 and ~80 kDa. The major 35kDa band is similar to that previously reported in this laboratory for endogenous STC-1 (Holmes and Zachary 2008). It was decided that MOI of 10 would be used for further experiments.

3.4.1 Effect of STC-1 overexpression on VEGF-stimulated migration and signalling

I initially investigated whether STC-1 adenoviral overexpression could have any effect on endothelial cell migration. HUVECS were infected with either Ad.STC-1 or Ad.Lac Z at a MOI of 10 for 48 hours, and their migration towards VEGF was assessed in a transwell assay. Infection of endothelial cells with Ad.STC-1 adenovirus at MOI of 10 significantly increased the STC-1 levels secreted in media a compared to control cells infected with Ad.Lac Z (data not shown) but did not significantly alter VEGF-induced HUVEC migration (Fig 3.5). It was also noted that adenoviral infection in itself had a slight effect on the basal level of HUVECs migration in the absence of VEGF stimulation (Fig 3.5).

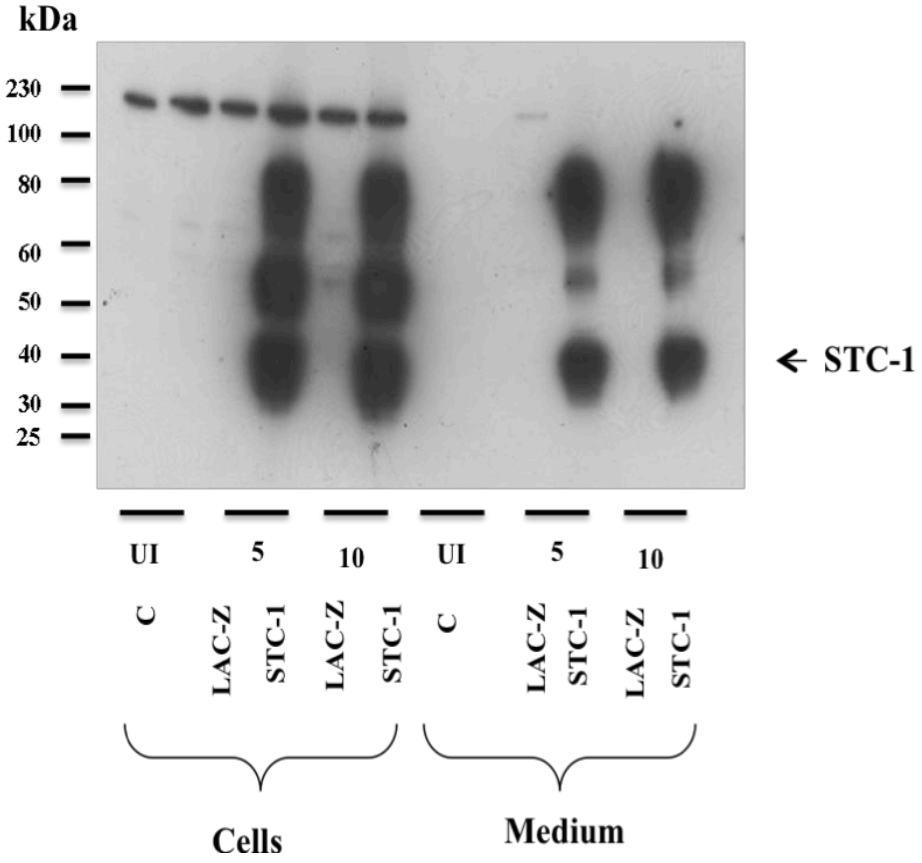


Figure 3.4 Effect of adenovirus-mediated overexpression of STC-1 in HUVEC

HUVECs were infected with the indicated adenovirus for 48hr at MOI of 5 and 10. Serum-starved cells were then incubated for 4hr. Concentrated supernantant medium and lysed cells samples of uninfected (UI) and infected cells with indicated adenoviruses were immunoblotted with STC-1 antibodies.

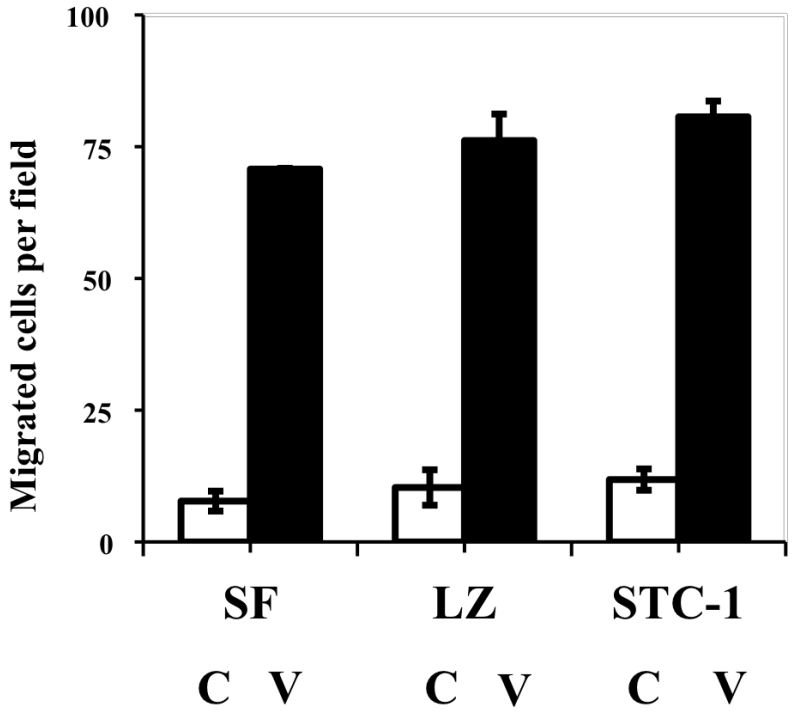


Figure 3.5: Effect of adenovirus-mediated overexpression of STC-1 on migration

HUVECs were either uninfected (SF), or infected with Ad.Lac Z (LZ) or Ad.STC-1 for 48 hr at an MOI of 10. Migration was then assessed in a transwell assay towards 25ng/mL VEGF (V) or no addition (C) after 4 hr treatment, after which the migrated cells were fixed, stained and counted. Results are means ± SEM from three independent experiments and were analysed by two-way ANOVA, with Bonferroni's test for multiple pair-wise comparisons. No significant differences were observed between Ad.Lac Z and Ad.STC-1 infected cells in response to VEGF treatment.

Despite the lack of effect on migration, I next investigated the role of adenovirus-mediated STC-1 overexpression on VEGF stimulated signalling. HUVECs were infected with Ad.STC-1 or Ad.Lac Z for 48 hrs. Cells were then serum starved overnight and treated with and without VEGF for 10 mins. Cells were lysed and western blot analysis was performed. As expected, VEGF treatment resulted in increased phosphorylation of P38 MAP kinase and ERK1/2 (Fig. 3.6). Akt phosphorylation was increased by VEGF in the absence of adenovirus, but it was noted that both Ad.LacZ and Ad.STC-1 both enhanced Akt phsophorylation regardless of VEGF stimulation. This is likely to represent a non-specific effect of viral infection on HUVECs and similar results have been obtained by other colleagues in the lab. Curiously, p130Cas and FAK phosphorylation levels were largely unaffected by VEGF stimulation even in the absence of viral infection, although basal levels of phsophorylation were relatively high. Most importantly, there was no indication that STC-1 overexpression had any effect on either the VEGF-stimulated or basal levels of phosphorylation of any of the proteins examined (Fig. 3.6).

3.4.2 Effect of STC-1 overexpression in VEGF-stimulated tubulogenesis

The co-culture tubulogenesis assay is used as an in vitro model of angiogenesis. A monolayer of fibroblasts together with stimulation by VEGF provides a model of interactions between endothelial cells and stromal cells that at least partially mimicks the microenvironment in which sprouting angiogenesis occurs in vivo. This assay was used to determine whether overexpression of STC-1, using Ad.STC-1 infection, had any effect on the abilty of HUVECs to form tube like structures. Both uninfected HUVECs, and cells infected with Ad.STC-1 virus showed an extended network of endothelial cell tubules after 7- to 10-days culture in the presence of VEGF, whereas cells cultured without VEGF showed little or no tubulogenesis (Fig. 3.7A, B). It was confirmed that infection with Ad.STC-1 resulted in a sustained increase in secreted STC-1 throughout the assay period from 1 to 7 days, and that VEGF also increased STC-1 protein expression after 7 days adenoviral infection in ininfected, Ad.LacZ-infected and Ad.STC-1-infected cells (Fig 3.7C). It was difficult to see VEGF-stimulated STC-1 expression in day 1 cultures, because of the difficulty in exposing the gel long enough

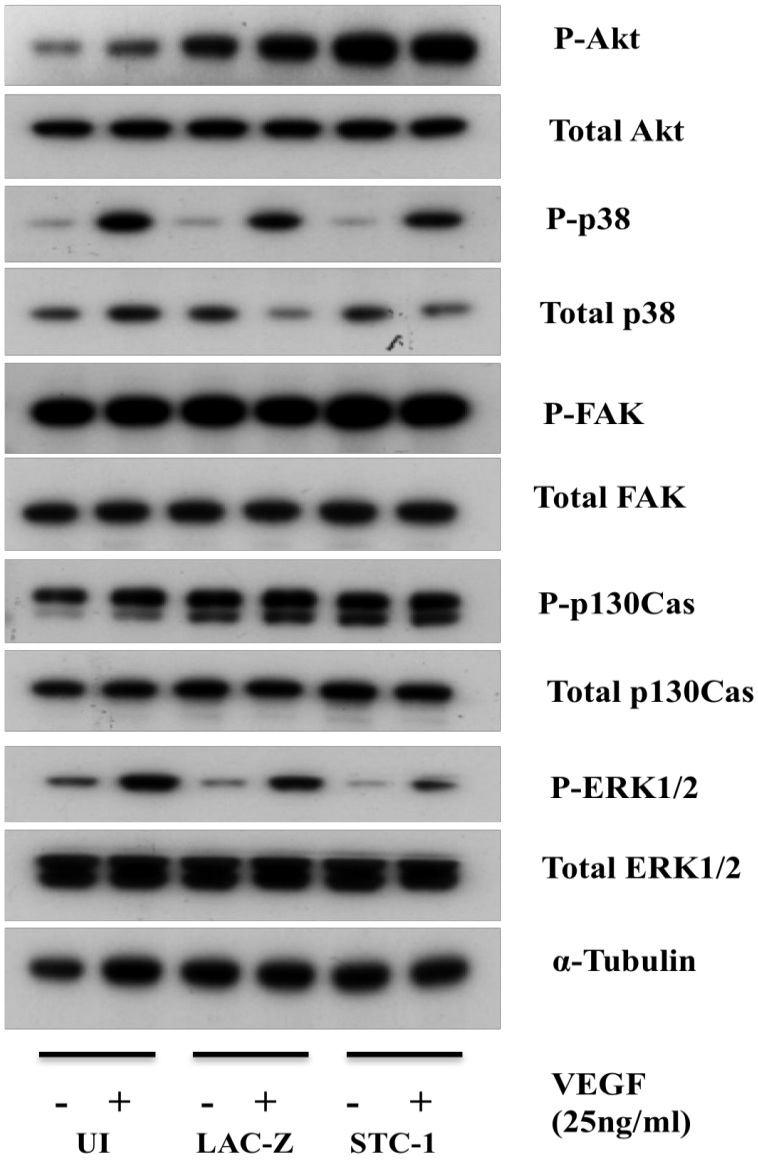


Figure 3.6: Effect of adenovirus-mediated overexpression of STC-1 on phosphorylation of VEGF- induced signalling

HUVECs were infected with the indicated adenovirus for 48hr at MOI of 10. Serum-starved cells were then treated with (+) or without (-) 25 ng/ml VEGF for 10 mins followed by lysis and immunoblotting with the indicated antibodies. Results are representative of at least two independent experiments.

due to very high STC-1 expression in Ad.STC-1-infected cells (Fig. 3.7C). There was a trend for VEGF treatment of Ad.STC-1-infected cells to have increased numbers of branch points and total tube length compared to uninfected and Ad.Lac Z infected cells, however this was not statistically significant. Furthermore, cells infected with Ad.Lac Z exhibited generally elevated and variable basal levels of tube formation in the absence of VEGF. Thus the mean number of branch points and total tube length in Ad.LacZ-infected cells was similar with or without VEGF treatment and similar to that found in non-infected cells after VEGF stimulation. This made it difficult to interpret the effect of Ad.STC-1 overexpression in this assay. The reason for the high level of tube formation in the Ad.Lac Z treated cells in the absence of growth factor is unknown, but it is unlikely to be an effect of virus infection since Ad.STC-1 treated cells behaved in a similar manner to uninfected cells without VEGF.

3.5 STC-1 knockdown and endothelial migration

The results from experiments using Ad.STC-1 overexpression indicated that adenovirus mediated STC-1 expression had no significant effect on VEGF-stimulated migration when compared to that observed with Ad.Lac Z control. This may indicate that endogenous levels of STC-1 are sufficient to maximally drive endothelial migration. We therefore determined whether reducing endogenous STC-1 production by siRNA mediated knockdown could have any effect on endothelial cell migration. Representative blots demonstrating effective knockdown of STC-1 are shown in Figure 3.8A. A trend was observed for knockdown of STC-1 to modestly reduce VEGF-stimulated HUVEC migration, but this effect did not reach statistical significance (Fig 3.8B).

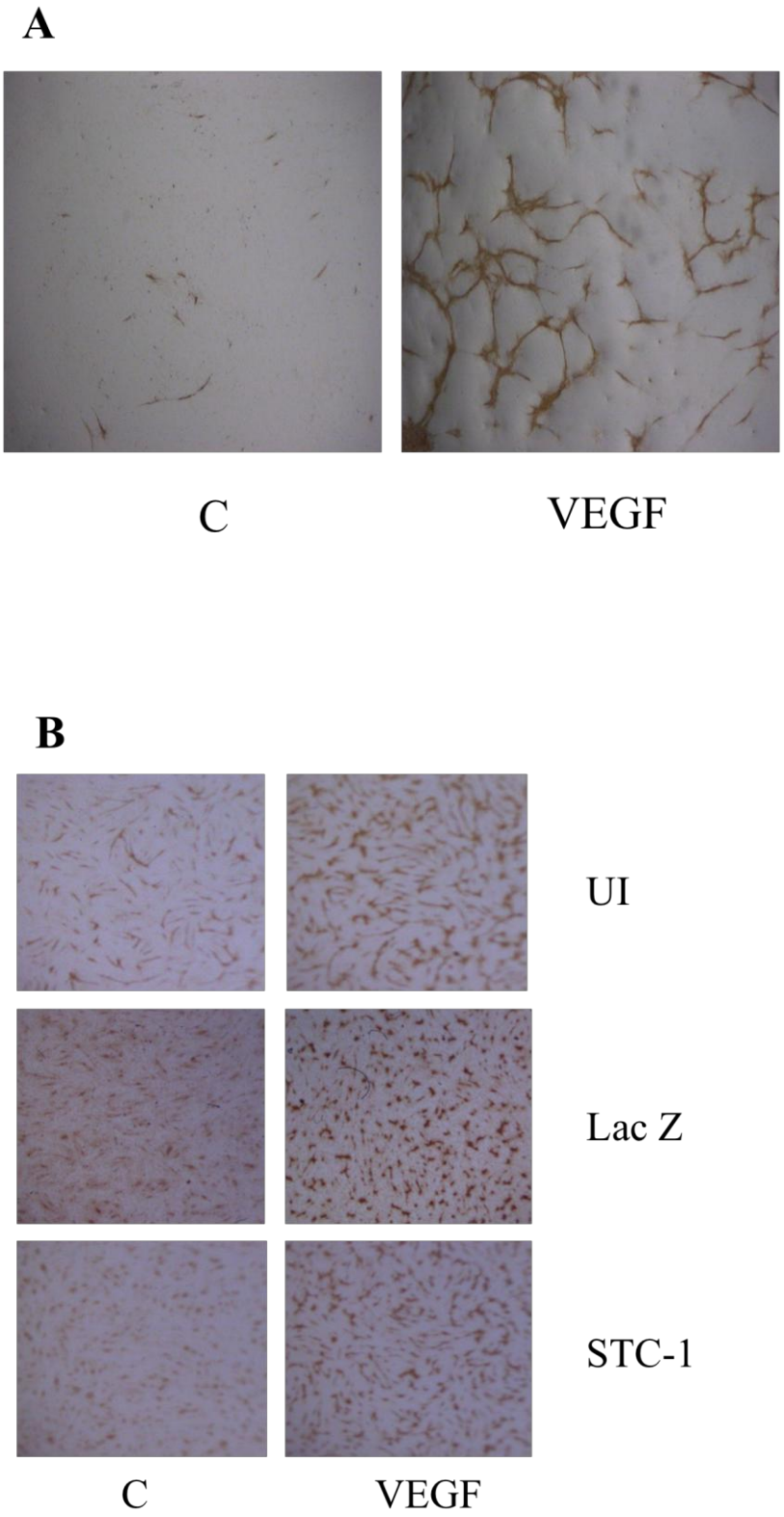


Figure 3.7: Effect of adenovirus overexpression of STC-1 in a VEGF-mediated angiogenic coculture assay

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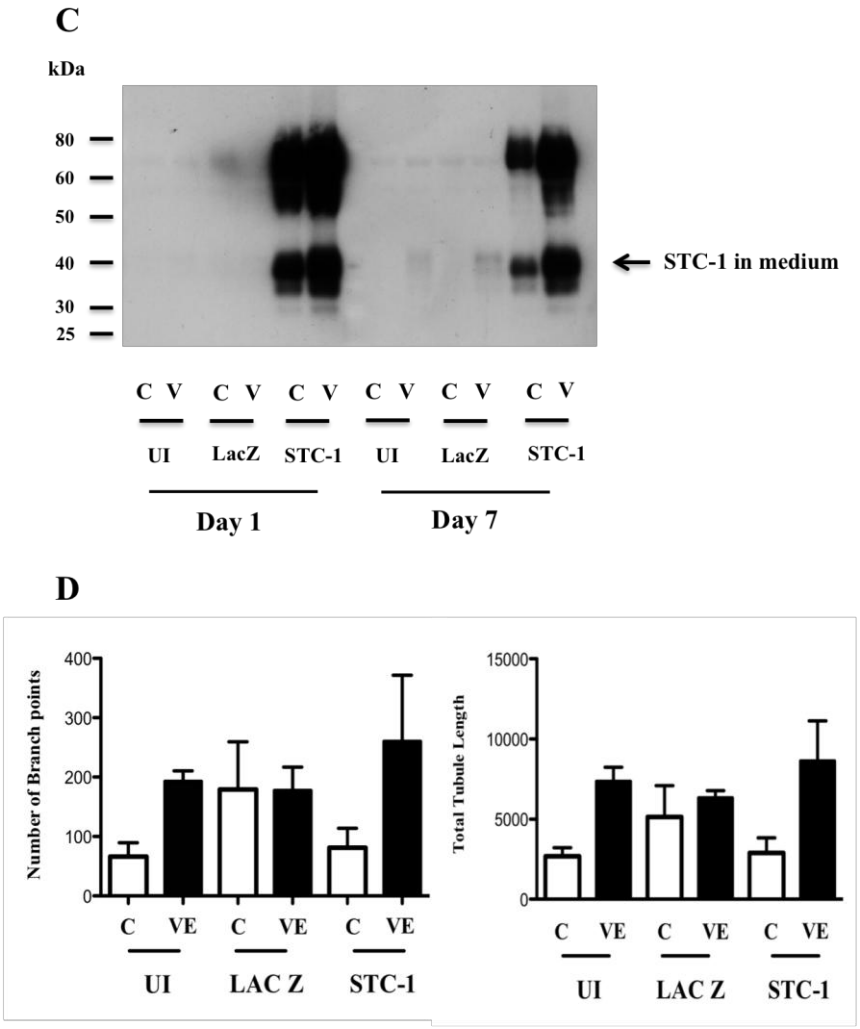

Figure 3.7: Effect of adenovirus overexpression of STC-1 in a VEGF-mediated angiogenic coculture assay

Human dermal fibroblasts were grown in 24-well plates to confluence. HUVECs were infected with either Ad.Lac Z or Ad.STC-1 at MOIs of 10 or no virus (UI) for 48 hr. Trypsinised HUVECs were then seeded (10,000 cells per well) on top of the fibroblast cell layer. Cell co-cultures were then maintained for 7 days in the presence of EBM plus 0.5% serum alone (C) or EBM plus 0.5% serum and 25ng/ ml VEGF (VE). During the period of the assay, the medium was removed every 3 days and replaced with fresh EBM plus 0.5% FBS ± VEGF. The removed medium was clarified by centrifugation and used to assess secreted STC-1 levels by immunoblotting. Endothelial cells were then visualized by immunostaining for von Willebrand

factor (vWF). (**A**) VEGF induces tube formation in co-culture. Representative results are presented showing that VEGF (25 ng/ml) induces extensive tube formation in the co-culture model using non-infected endothelial cells (right hand panel) compared to cells cultured in the absence of VEGF (left hand panel). (**B**) Representative pictures of vWF-stained HUVECS after 7 days in co-culture. The top two panels show non-infected HUVECs in the absence (left hand panel) or presence (right hand panel) of 25 ng/ml VEGF. The middle two panels show Ad.Lac Z infected HUVECs in the absence (left hand panel) or presence (right hand panel) of 25 ng/ml VEGF. The lower two panels show Ad.STC-1 infected HUVECs in the absence (left hand panel) or presence (right hand panel) of 25 ng/ml VEGF. (**C**) Ad.STC-1 results in increased STC-1 expression in the medium throughout the period of the assay. Media was collected during the periodic media changes, clarified by centrifugation and immunoblotted for STC-1. The figure is representative of media from three different assays. (**D**) Quantitation of coculture assays. After immunostaining with vWF antibody, tube formation was digitised and quantified using Angiosys software. The bar graph represents the mean ± SEM number of branch points (left hand graph) or total tubule length (right hand graph) counted in three independent experiments. Statistical analysis by two-way ANOVA, with Bonferroni's test for multiple pair-wise comparisons indicated *$P < 0.05$ for C *vs* VE in UI and STC-1 groups for both branch points and total tube length.

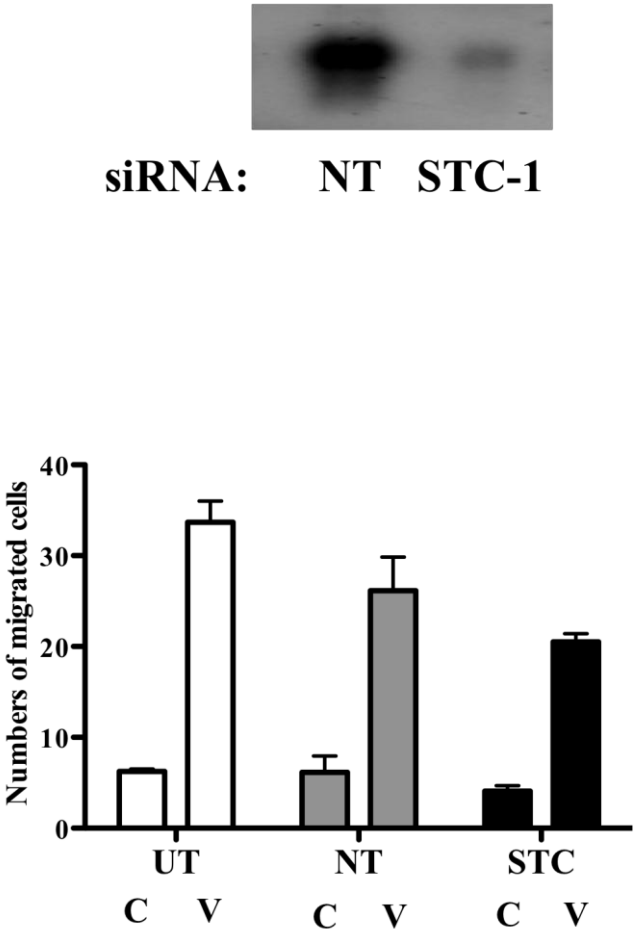

Figure 3.8: Effect of siRNA-mediated STC-1 knockdown on VEGF-induced migration

A. Representative blot indicating the degree of knockdown caused by siRNA-mediated transfection. Supernantant media from siRNA-transfected cells were collected and prepared for immunoblotting and probed with an antibody to STC-1. **B.** HUVECs were pre-treated for 48 hr with STC-1 siRNA and a non-targeting control siRNA (NT) or no treatment, untreated (UT). Cells were trypsinised and migration assay was assessed in a transwell assay using 25ng/ml VEGF (V) or no addition (C) as the chemoatttractant. Data are from three independent experiments. Analysis of VEGF- stimulated migration by two-way ANOVA indicated no significant effect achieved by STC-1 siRNA treated cells compared to the control NT siRNA, ($p>0.05$).

3.6 Discussion

In cardiovascular biology, VEGF is a critical cytokine exhibiting multiple diverse biological functions in endothelial cells. For example, VEGF signalling induces the migration and survival of endothelial cells. Gene expression profiling studies in endothelial cells and in models of sprouting angiogenesis or capillary formation have suggested a possible role of STC-1 in angiogenesis and/or its potential importance in the modulation of neovascular diseases (Gerritsen et al., 2002; Holmes & Zachary, 2008; Kahn et al., 2000; D. Liu et al., 2003; S. Yang et al., 2002). Currently however, relatively little is known about how expression of STC-1 affects endothelial angiogenic responses. In this chapter I aimed to identify possible functional roles of STC-1 in VEGF- mediated endothelial cell responses.

Since VEGF plays an important role in a number of processes required for angiogenesis in endothelial cells, including migration, I initially confirmed that VEGF does upregulate STC-1 expression (Fig. 3.1). I next investigated the role of STC-1 in VEGF-stimulated cell migration, using recombinant STC-1. In these studies, 100 ng/ml STC-1 resulted in an approximately 40% reduction in VEGF-induced migration whereas STC-1 at up to 50 ng/ml had no effect on VEGF-mediated migration (Fig. 3.2). This was somewhat unexpected since the finding that STC-1 was upregulated in response to VEGF might suggest a pro-angiogenic role for this factor. However, it also possible that STC-1 might be required for modulating responses to VEGF by limiting angiogenic processes at higher concentrations of STC-1. Indeed, (Zlot et al., 2003) showed that STC-1 acted as a selective autocrine negative modulator of HGF-induced endothelial migration and morphogenesis (cord formation) on Matrigel. Although the expression of both HGF and its receptor c-Met are known to be up-regulated in both physiological and pathological angiogenesis, HGF is not considered to be an endothelial-selective mitogen and morphogen. In contrast to the data presented in this thesis however, (Zlot et al., 2003) did not see an effect of STC-1 on VEGF-induced migration, even when 1 µg/ml STC-1 was used. In addition they also failed to show VEGF-induced upregulation of STC-1 expression in a HUVEC monolayer, although an increase was noted in a 3D culture. The reasons for these discrepant results are unknown; however, it

should be noted that (Zlot et al., 2003) used a supraphysiological dose of VEGF (400 ng/ml) to induce STC-1 expression which may result in endothelial cells becoming non responsive due to VEGF receptor downregulation. In addition, the migration assay used by (Zlot et al., 2003) lasted 18 h compared to 4 h in my migration assays; therefore it is possible that the inhibitory effect of STC-1 on VEGF-induced endothelial migration observed in this thesis may be transient. In addition the recombinant STC-1 used by (Zlot et al., 2003) was from a different source to the one used in this thesis and this may also partly account for the discrepancies.

Analysis of VEGF-stimulated signalling revealed that STC-1 had no effect on any of the VEGF-stimulated signalling pathways investigated (Fig. 3.3). An important pathway involved in VEGF-induced signalling is phosphorylation of the nonreceptor tyrosine kinase FAK, which act as a mediator between extracellular matrix and cytoskeleton (Abu-Ghazaleh, Kabir, Jia, Lobo, & Zachary, 2001). Recent findings suggested that STC-1 reduced HGF-induced FAK phosphorylation (Zlot et al., 2003). In contrast, STC-1 pre-incubation had no effect on VEGF-induced FAK phosphorylation in the studies presented here. I was also not able to observe any change in VEGF-induced FAK phosphorylation. In addition, VEGF mediated phosphorylation of another protein important in cell migration p130Cas (Crk-associated substrate) was also not significantly increased. This was unexpected since other colleagues in this and other laboratories have shown that VEGF inducesphosphorylation of Y407 FAK and Y410 p130Cas in HUVECs (Evans et al., 2011; Herzog, Pellet-Many, Britton, Hartzoulakis, & Zachary, 2011). It is difficult to explain this difference, but it is possible that the batches or passages of HUVECs used for this set of experiments were less responsive or possibly less quiescent than usual, resulting in weaker signalling responses. Overall, I was unable to attribute the inhibitory effect of 100 ng/ml STC-1 on VEGF–induced migration to changes in any of the signalling pathways I investigated. Given that both FAK and p130Cas are phosphorylated at multiple sites, in further work it would be desirable to have examined effects of STC-1 on VEGF-induced phosphorylation of FAK and p130Cas at different tyrosine residues, eg FAKY397 or FAKY861, which are also known to be regulated by VEGF. In addition, VEGF also regulates cell migration via multiple other pathways, eg the small GTPase, Rac. Further work should undertake a more comprehensive study of effects of STC-1 on VEGF-induced chemotactic

signalling.

Additionally, it should be noted, that the inhibitory effect of hrSTC-1 on VEGF-induced migration was observed at only a single concentration of STC-1 (100ng/ml), and it is unclear that this concentration is physiologically relevant. There is limited information available on in vivo levels of circulating STC-1 in mammals, making it difficult to evaluate the physiological relevance of 100ng/ml STC-1. However, it is also possible that in pathophysiological situations, STC-1 may be strongly over-expressed locally, similar to the strong over-expression of VEGF observed in many cancers or neovascular eye diseases. It should also be noted that since endothelial cells produce and secrete endogenous STC-1, they therefore may not necessarily be particularly responsive to exogenously administered STC-1. To address this, further experiments could be performed in which exogenous STC-1 is used in cells in which endogenous STC-1 expression is inhibited using targeted siRNA-mediated knockdown.

Other concerns about experiments involving exogenously added hrSTC-1 are that STC-1 may function in an autocrine fashion and that its post-translational processing may be important for its function, aspects that may not be mimicked by treatment with hrSTC-1. To address this, experiments were performed using an adenovirus to overexpress STC-1 in endothelial cells. Adenoviral overexpression of STC-1 had no effect on HUVEC migration towards VEGF, in contrast to the inhibitory effect seen with recombinant STC-1 (Fig. 3.5); Ad.STC-1 also caused no apparent changes in the phosphorylation of any of the VEGF-induced signalling proteins examined (Fig. 3.6). These negative findings may indicate that endogenously produced STC-1 in Ad.STC-1-infected endothelial cells does not accumulate sufficiently to reproduce the inhibitory effect caused by 100 ng/ml hrSTC-1. It was observed that there was a slight alteration in Akt phosphorylation in response to VEGF treatment in the presence of adenovirus overexpressed STC-1 in comparison to Lac Z infected cells. I also noticed that basal levels of Akt activity was altered in infected Lac Z cells as well, although VEGF-stimulated Akt phosphorylation was greater in the presence of STC-1. This made the interpretation of these results difficult since it was difficult to know whether enhanced Akt phosphorylation was due simply to adenoviral infection or to STC-1 over-expression. This question could be partly addressed using a different control vector, eg Ad.GFP, or by lowering the MOI of infection to reduce the viral load.

Overall, I was unable to find any evidence by which either endogenous or exogenously administered STC-1 could influence VEGF signalling. It may be that STC-1 acts by influencing the activity of other growth factors/cytokines, HGF for example. In addition, Chen et al. 2008 described a critical role for STC-1 in regulating endothelial cell function under normal or inflammatory conditions by inhibiting TNF-α-induced monolayer permeability in endothelial cells. TNF-α-stimulated expression of the junction molecules, ZO-1 and claudin-1, was downregulated by STC-1 at both the mRNA and protein levels, suggesting a new role for STC-1. However, this study found that STC-1 failed to block the VEGF-induced increase in cell permeability, indicating that the negative effect of STC-1 could be specific to TNF-α.

In a fibroblast/endothelial cell coculture model of angiogenesis, the formation of new capillary-like structures composed of endothelial cells, adenoviral STC-1 overexpression also did not significantly either positively or negatively modulate tubulogenesis, though (Figs. 3.7). However, the interpretation of these results was complicated by a stimulatory effect on tubulogenesis of Ad.LacZ infection alone. The reason for this is unknown but may be related to the increased basal Akt phosphorylation also observed after viral infection (Fig. 3.6), although this is unlikely since Ad.STC-1 produced no similar increase in tubulogenesis in the absence of VEGF.
Knockdown of STC-1 in HUVECS resulted in a trend for lower VEGF-stimulated migration in comparison of non-target siRNA, but this was not significant (Fig. 3.8) . It is possible that a more substantial knockdown of STC-1 expression may be required to significantly affect VEGF-induced migration and this needs further investigation. Taken together with the other migration assay data, it could be hypothesised that endogenous and exogenously administered STC-1 have different effects. Endogenous STC-1 may be required, at a low level, for optimal endothelial motility and increasing STC-1 somewhat above this level using adenoviral over-expression has no additional effect. In contrast, high levels of exogenous STC-1 may be inhibitory. Obviously much more data is required to support this hypothesis, nonetheless it does fit with the idea put forward by other groups that STC-1 may act as a stabilising signal during angiogenesis, promoting the maturation of new blood vessels rather than further growth (Zlot et al 2003).

Given the largely negative data presented here it could be concluded that STC-1 has little or no effect on endothelial cells. Nevertheless, there is considerable evidence that STC-1 does influence endothelial function. For example STC-1 increases intracellular Ca^{2+} levels in quiescent HUVECs (Chakraborty et al., 2007). The mechanism involved was not explored, however a similar effect was observed in epithelial tumour cell lines and STC-1 in these cells was found to act via modulation of purinergic signaling (Block et al 2010).

As already discussed, Chen et al 2008 reported that STC-1 inhibited TNF-α induced endothelial permeability and this is in broad agreement with the findings by Chakraborty et al who showed that STC-1 inhibited monocyte transmigration through a HUVEC monolayer (Chakraborty et al., 2007). Indeed it seems that STC-1 may have a more general role in inhibiting macrophage and leucocyte migration (C. Chen, Jamaluddin, Yan, Sheikh-Hamad, & Yao, 2008; Kanellis et al., 2004).

Further evidence of a role for STC-1 in endothelial biology comes from microarray data which demonstrates that STC-1 exerts both short-term (2 h) and long-term (24 h) effects on gene expression in cultured HUVECs, regulating a number of genes that are important to cell growth and differentiation such as growth factors and regulators of cell cycle and apoptosis in endothelial cells (Chakraborty et al., 2007). Once again, however, the mechanism behind these transcriptional effects was not explored.

Overall therefore it can be concluded that although VEGF regulates endothelial STC-1 expression, STC-1 regulates endothelial function largely through non-VEGF mediated pathways, or else regulates other VEGF functions not examined in this chapter. However, the data presented here indicates that STC-1 is not a pro-angiogenic signal but may instead act to maintain newly formed vessels or in the functions of mature vascular endothelium, or endothelial functions under conditions of stress. More work is required to test these hypotheses and investigate in more detail the mechanisms by which STC-1 exerts its effects on endothelial cell biology.

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Chapter 4

4 PDGF regulation of STC-1 expression

Various studies have suggested that STC-1 may have a role in wound healing, angiogenesis and cancer (Law et al., 2010). In the previous Chapter, though I confirmed that VEGF-A_{165} upregulates STC-1 protein expression (Holmes & Zachary, 2008; D. Liu et al., 2003{Holmes, 2008 #1048), I was however, unable to find definitive evidence for a role for STC-1 in VEGF-mediated endothelial function. Given that STC-1 is a secreted protein, it is possible that the main effects of endothelial derived STC-1 may actually be paracrine in nature, possibly acting on vascular smooth muscle cells (SMC). Indeed by secretion of various factors, such as endothelial derived-PDGF, endothelial cells can modulate the cellular responses of SMC and pericytes (mural cells involved in stabilizing capillaries) including proliferation, migration and vessel stability (Hellstrom, Kalen, Lindahl, Abramsson, & Betsholtz, 1999). In addition, it was also of interest to examine whether VSMC could themselves express STC-1. At the beginning of this thesis, STC-1 was not known to be expressed in SMC or to regulate SMC function. Firstly, therefore, I investigated whether STC-1 is expressed in SMC and whether PDGF signaling could affect STC-1 expression. Several approaches are described below which aim to generate an overall picture of STC-1 upregulation in response to the PDGF-BB isoform in SMCs.

4.1 PDGF-BB stimulated STC-1 gene expression

The possibility that PDGF might regulate the effect of STC-1 expression was initially investigated in two different human primary SMCs, Aortic (Ao) SMCs and Coronary artery (CA) SMCs. Analysis of PDGF-BB-induced STC-1 expression was performed by real-time, quantitative RT-PCR using gene-specific STC-1 primers and GAPDH as a reference gene. Treatment of a confluent monolayer of human CASMCs with 30ng/ml PDGF-BB for different times, showed that PDGF-BB treatment induced expression of STC-1 mRNA significantly after 45 minutes, with a maximum increase of approximately 14-fold at 90 minutes (p<0.001), thereafter expression declined to just above basal level after 24 hours (Fig. 4.1).

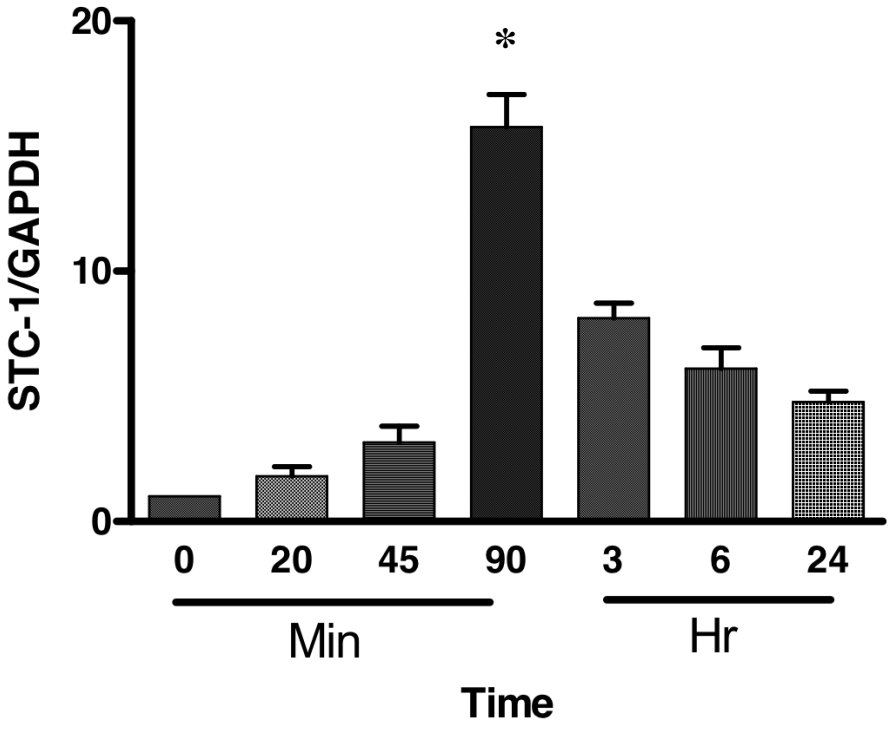

Figure 4.1: Regulation of STC-1 gene expression by PDGF-BB

Confluent, serum–starved CASMCs were treated with 30 ng/ml PDGF for the indicated times before mRNA extraction. STC-1 mRNA levels were determined by real-time quantitative RT-PCR and normalised to GAPDH mRNA levels. The results are expressed as mean fold increases ± SEM from three independent experiments, * p<0.001 versus 0 hr time point.

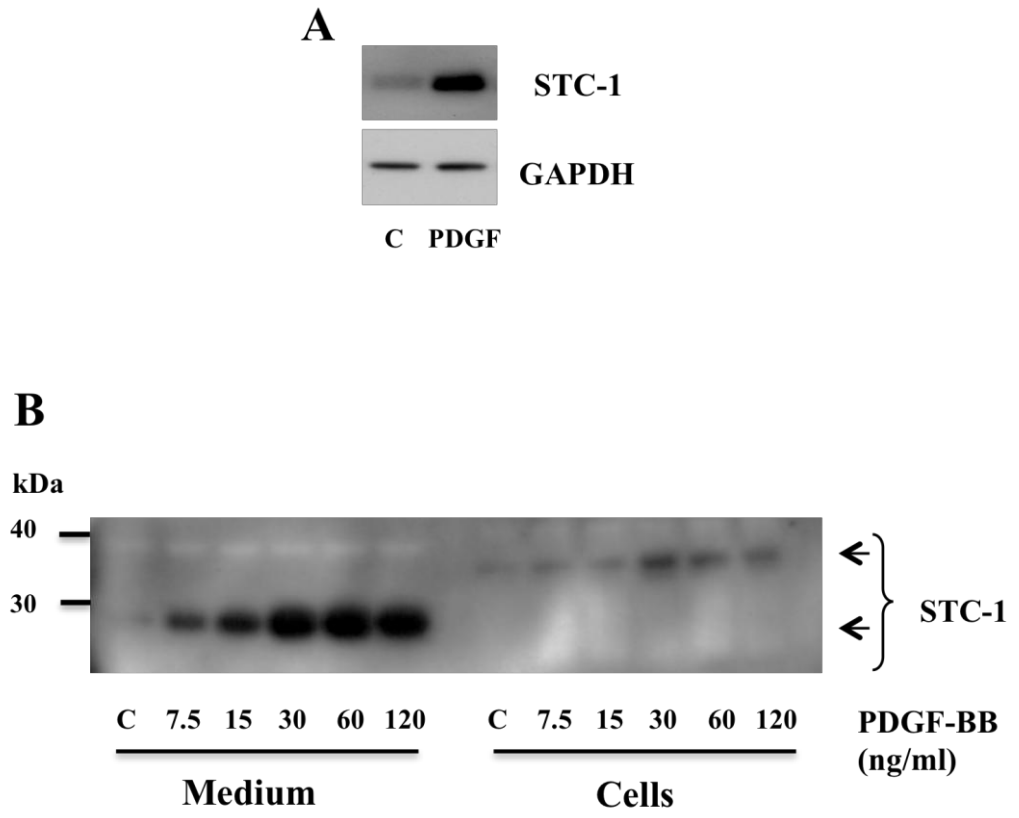

Figure 4.2: Analysis of intracellular and secreted STC-1 protein induction

A. Confluent, serum-starved HAoSMCs were treated with or without 30ng/ml PDGF-BB for 24 hours. Conditioned media were concentrated then deglycosylated with PNGase F enzyme and immunoblotted and probed with indicated antibodies. **B**. HCASMCs were treated with either different concentrations of PDGF-BB as indicated or no addition (C) for 24 hours. Conditioned media and whole cell extracts were prepared for SDS-PAGE, immunoblotted and probed with chicken anti-human STC-1 antibody from Abcam Plc.

4.2 Analysis of PDGF regulation of STC-1 protein secretion

Having established that PDGF BB upregulates STC-1 mRNA expression I used Western blot analysis to confirm that STC-1 protein expression is also increased. Because STC-1 is generally secreted from cells imunoblotting was performed on concentrated cell media supernatants as well as whole cell lysates. Supernantants and whole-cell lysates were prepared from serum-starved cells after treatment with different PDGF-BB concentrations for 24 hours.

In an initial experiment when the concentrated supernantants were not deglycosylated, the antibody used could not detect the secreted STC-1 in the supernatant. However, as previously reported, STC-1 is a glycosylated protein in different systems (Holmes & Zachary, 2008). Therefore, secreted proteins in the cell supernantants were concentrated and deglycosylated and run in parallel with whole-cell lysates. The western blot analysis showed a clear detection of secreted protein after PDGF treatment. However, a consistent problem was that STC-1 protein levels in the cell lysates were very faint compared to the supernantants (Fig. 4.2). This was exacerbated by the use of a relatively ineffective antibody (raised in chicken to human STC-1), which was the best one available at the time. It was then decided to perform western blots only use concentrated supernantant from cell cultures in all subsequent experiments. Better antibodies with greater sensitivity were also identified. In particular, blots were performed using different antibodies from R&D Systems.

4.2.1 STC-1 is N-glycosylated in SMCs

Western blotting of supernantants prepared from SMCs revealed that STC-1 was expressed as a single band in SMCs approximately 35-kDa molecular size (Fig. 4.3). A similar molecular weight band of STC-1 is also expressed in endothelial cells and several cancers (see chapter 3 and (Holmes & Zachary, 2008). As previously shown, STC derived from corpuscles of Stannius (CS) has a carbohydrate moiety attached at a

single consensus N-linked glycosylation site at the consensus site, Asn_{29}-Ser_{30}-Thr_{31} of the mature protein core. It has been shown that the glycosylation patterns of CS and ovarian STC differ in the size of this posttranslational modification. Fish ovarian STC is substantially more highly glycosylated than the CS-derived hormone. To assess if STC-1 secreted protein in SMC contains a similar N-linked glycosylation as in fish species, PNGase F digestion was performed on SMCs concentrated supernantants. PNGase F is an amidase, which cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins.

Treatment of supernatants from SMCs with PNGase F enzyme resulted in a shift in STC-1 molecular weight by approximately 5-8 kDa as compared to untreated samples, to a band with a molecular weight of 28 kDa (Fig. 4.3). In contrast, treatment with enzymes, which specifically remove O-linked glycosylated moieties, namely either chondroitinase (which removes chondroitin sulphate chains), or with heparitinase (which removes heparan sulphate-rich chains) had little or no effect on STC-1 molecular weight. It was noted that chondroitinase and heparatinase particularly in combination, did reduce the level of immunoreactive STC-1, but conversion of glycosylated STC-1 to other species by these enzymes was not detected. Furthermore, combinations of PNGase with chondroitinase and heparatinase made no further difference to the level of STC-1 compared with PNGase alone. This result indicates that secreted STC-1 in SMCs is an N-linked glycosylated protein.

4.2.2 PDGF-induced STC-1 expression is restricted to the BB isoform

Having shown that PDGF-BB upregulates STC-1 expression in SMCs, I determined whether other growth factors, including the PDGF isoform AA could also induce STC-1 expression. The results showed, as before, that treatment with 30ng/ml PDGF-BB significantly increased STC-1 protein release in conditioned culture medium, whereas PDGF-AA had no effect (Fig. 4.4). This suggests that STC-1 stimulation could be regulated via PDGFRβ as PDGF-BB binds to β and α receptors with high affinity while PDGF-AA acts only through α receptors.

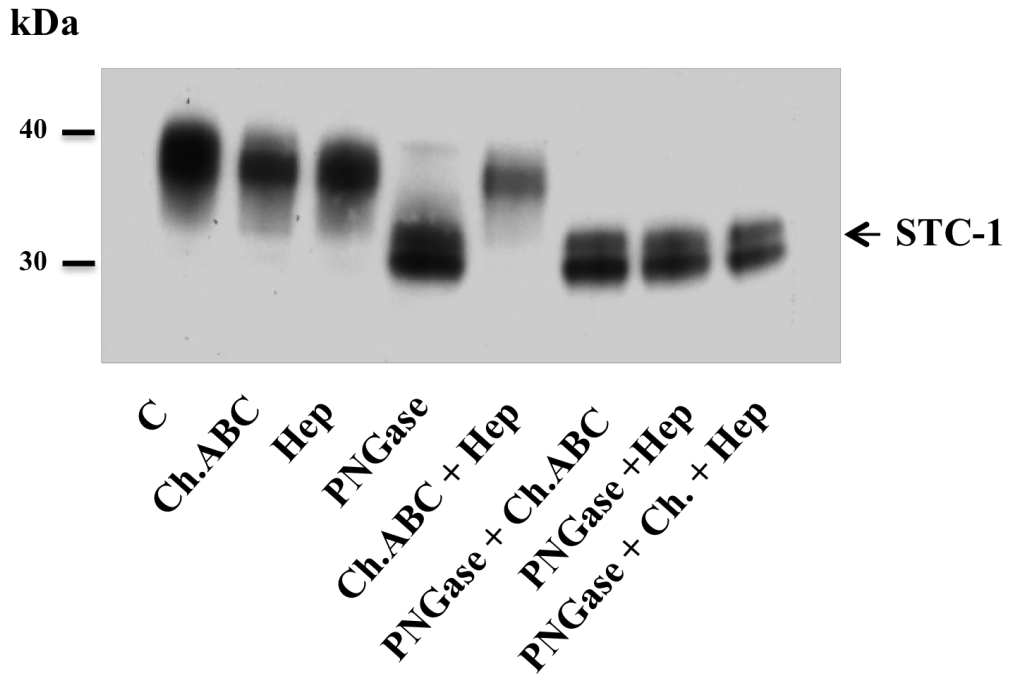

Figure 4.3: Glycosylation of STC-1 in SMCs

Conditioned medium from CASMCs was concentrated then treated with and without PNGase F, chondroitinase (Ch.ABC) and heparitinase III (Hep) or in combination as indicated. Supernantants were immunoblotted and probed with goat STC-1 antibody from R&D Systems.

As expected, treatment with VEGF-A_{165} had no effect in SMCs and additionally, FGF-2, a known mitogen for SMCs, also had no effect on STC-1. It should also be noted that a combination of PDGF-BB and FGF did not have a synergistic effect on STC-1 expression, in contrast to the effect of VEGF and FGF combined in HUVECs (Holmes & Zachary, 2008). Similar data showing STC-1 upregulation specifically by PDGF-BB was also observed in AoSMCs (Fig. 4.2A).

4.2.3 Time and dose response of PDGF-stimulated STC-1 secretion

Following the initial analysis of upregulation of STC-1 by PDGF-BB in SMCs I performed further characterization of this response. A time course experiment was carried out over a period of 24 hours. Cell culture supernatants from PDGF-BB-treated cells were collected at 0, 1.5, 3, 6 and 24 h and assayed for secreted STC-1. Western blot data (Fig. 4.5) showed that PDGF-BB caused a significant time–dependent increase in the stimulation of STC-1, which was detectable as early as three hours and significantly different from basal conditions at 24 h. Nevertheless, there was also a time-dependent increase in STC-1 secretion over 24 h in the absence of PDGF-BB stimulation and this may reflect a response of the cells to prolonged serum starvation. Since the 24 hr time point resulted in the strongest STC-1 upregulation, this was used in subsequent PDGF-BB experiments, unless otherwise stated.

PDGF-BB-stimulated STC-1 expression was additionally examined using ELISA to quantify time-dependent STC-1 expression in PDGF-treated and untreated cells (Fig. 4.6). Concentrated supernatants from three independent experiments were subsequently used in a sandwich ELISA kit designed to measure STC-1 protein in cell culture supernatants. In agreement to the Western blot data, increasing STC-1 expression in conditioned culture medium was observed in PDGF-stimulated samples. However, there was also a time-dependent stimulation of STC-1 in SMCs in basal conditions over a time of 24 h, such that PDGF-induced STC-1 expression was not statistically significant compared with untreated SMCs using this method, despite PDGF-BB-stimulated STC-1 being considerably greater than STC-1 expression after 24 h incubation in serum-free medium. This may be due to the increased variability seen with this assay.

The influence of different concentrations of PDGF-BB on STC-1 stimulation was also further examined using the goat anti-STC-1 antibody. In response to different PDGF-BB concentrations, STC-1 protein levels were increased after 24 hours treatment as seen in Figure. 4.7A. Western blotting of supernantants indicated a significant increase in STC-1 in cells was obtained with 60 and 120ng/ml PDGF-BB treatments compared to unstimulated cells ($p<0.05$).

Together, these results indicated that the expression of STC-1 was upregulated in a time and concentration-dependent manner by PDGF-BB, whereas PDGF-AA and FGF had no effect in SMCs.

4.3 PDGF-stimulated upregulation of STC-1 is mediated via PDGFRs

PDGF-BB is a major initiator of angiogenesis and its biological effects are mediated via two major protein tyrosine kinase receptors, PDGFRβ and PDGFRα, acting both as a chemoattractant and a proliferating reagent, and is responsible for the migration of the SMC and the formation of the neointima (Levitzki, 2004). The involvement of PDGF receptors in mediation of PDGF mitogenic activation of SMCs was examined using time and dose-dependent activation.

4.3.1 Effect of PDGF-BB on PDGF receptors

Initial experiments were performed to characterise PDGF-induced α and β receptor phosphorylation. Immunoblotting of lysates prepared from CASMCs treated with 30 ng/mL PDGF-BB for different times with specific antibodies to PDGFRα and PDGFRß, indicated that PDGF-BB was able to induce rapid phosphorylation of both β and α-receptors in SMCs detectable after 2.5 mins, reaching a maximum at 2.5-10 and 2.5-5 minutes for β and α-receptors respectively, and thereafter declining (Fig. 4.8).

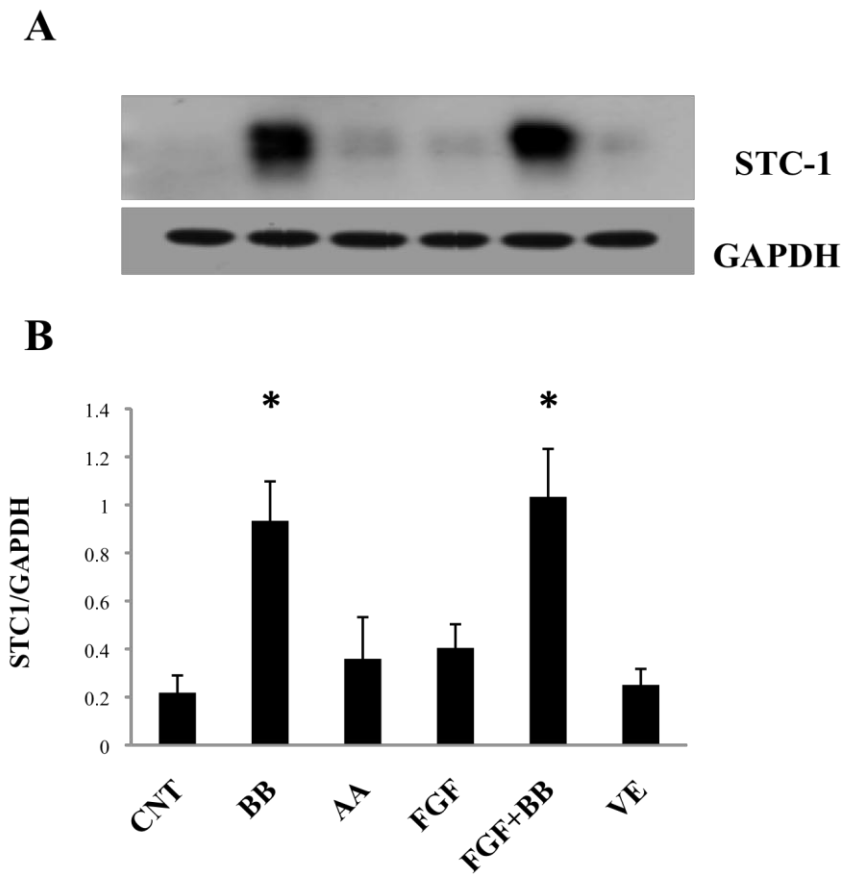

Figure 4.4: Specificity of PDGF-BB in STC-1 upregulation

A. Conditioned medium from quiescent cultures of HCASMCs treated for 24 hours with 30ng/ml PDGF-BB, 30ng/ml PDGF-AA, 25ng/ml VEGF-$A_{165,}$ 25ng/ml FGF-2, or no treatment (CNT) were concentrated and then analyzed by SDS-PAGE gels and Western blotting with goat antibodies to human STC-1. Cell lysates were separately prepared and blotted with anti-GAPDH antibody. **B**. Quantified data of densitometry of immunoblots for the PDGF-BB-induced STC-1 protein levels. Results are representative of three independent experiments n=3,with error bars ± SEM, * P<0.01.

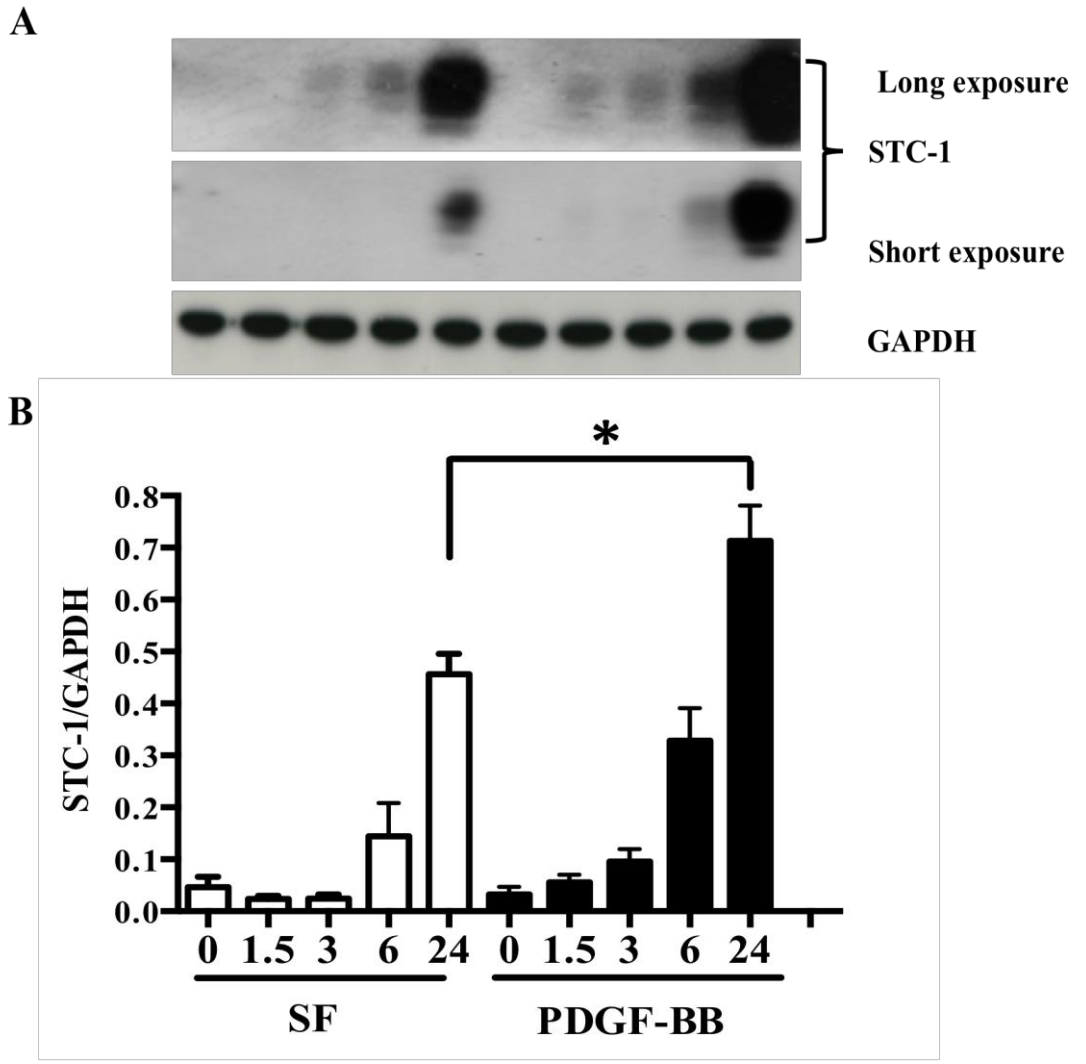

Figure 4.5: Analysis of time course of PDGF-BB simulation of STC-1 secretion in SMCs by Western blotting

A, Quiescent SMCs were stimulated with and without 30ng/ml PDGF-BB for the indicated times (0, 1.5, 3, 6, 24 hours) and concentrated media samples were separated by SDS-PAGE electrophoresis. Western blot analysis was performed with a goat polyclonal STC-1 antibody. The cell lysates were run in a separate gel and blotted for GAPDH as controls. Representative Western blotting results are shown. **B**, Bar graph showing the densitometric quantification of STC-1 expression normalized to GAPDH levels from three independent experiments. Data are means +/-SEM, * $P<0.05$ for 24 hours PDGF-BB versus 24 hours without PDGF-BB (SF).

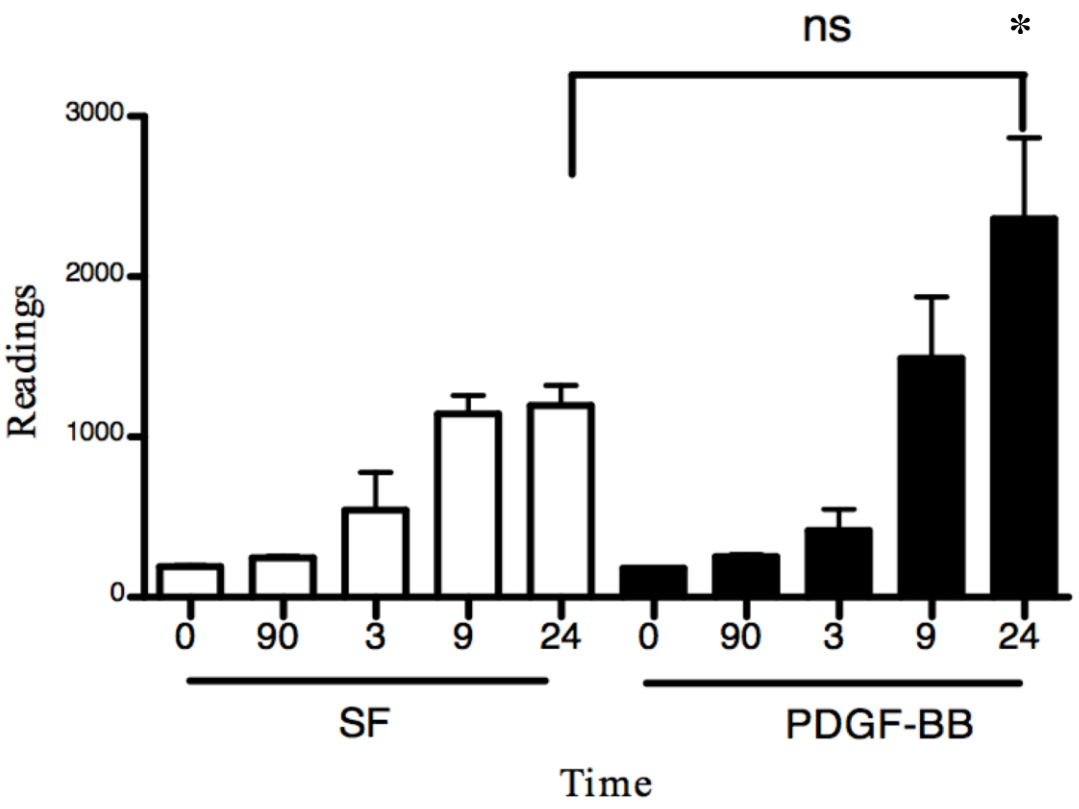

Figure 4.6: Time course of PDGF-BB induced change of STC-1 protein level in HCASMC analysed by ELISA

Cells were incubated with and without 30ng/ml PDFG-BB for indicated times and STC-1 protein released into cell culture media was measured by ELISA in three independent experiments (means +/- SEM), ∗ $P < 0.01$ PDGF-BB (24) hr versus PDGF-BB (0).

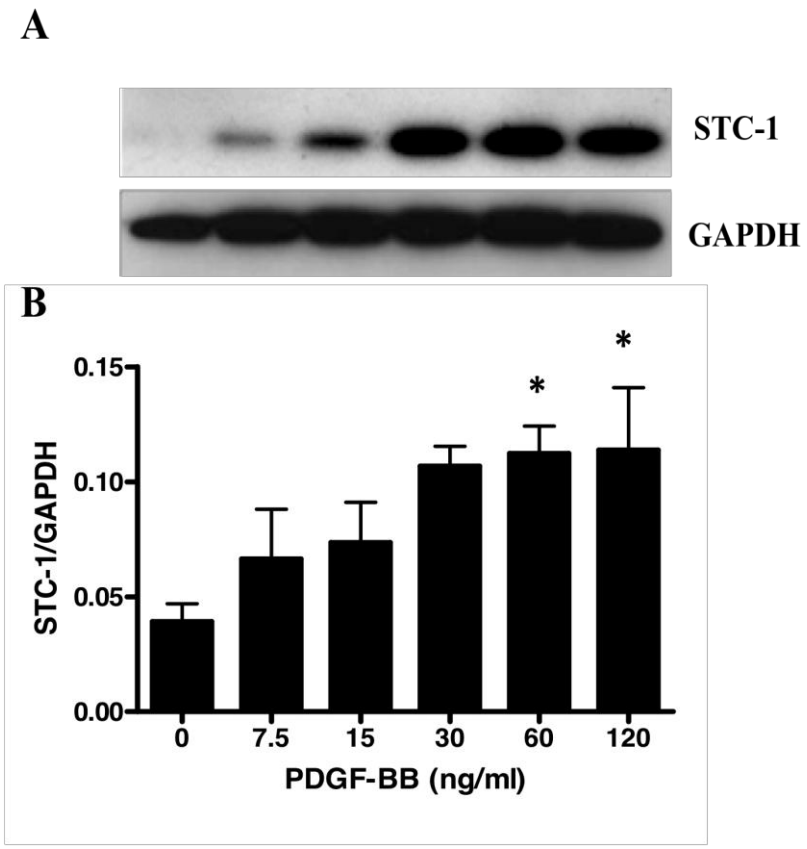

Figure 4.7: Effects of increasing PDGF-BB concentration on STC-1 induction

A. Concentration-dependent response of STC-1 expression to PDGF-BB. Confluent HCASMCs were cultured in serum-free conditions for 24 hours and then incubated with no addition (0) or with various concentrations of PDGF-BB ranging from 7.5 ng/ml^{-1} to 120 ng/ml^{-1} for a further 24 hours. The level of deglycosylated STC-1 protein was detected by immunoblot analysis using a chicken anti-STC-1 antibody. **B**. Quantification of the effect of various PDGF-BB concentrations on upregulation of STC-1. Data from three independent experiments are represented by densitometric normalized values to GAPDH using ImageJ. Results were assessed by one-way Anova with differences located by Borferroni's test for multiple comparison tests. Results are means +/-SEM, * P<0.05 compared versus control (0).

The phosphorylation of PDGFR isotypes in SMCs was directly evaluated by immunoblot analysis of cell extracts in comparison to untreated controls. Western blot analysis showed that activation of the PDGFRβ persists for longer than PDGFRα, since β receptor phosphorylation level does not decrease until 30 min whereas α-receptor phosphorylation becomes attenuated by 10 mins (Fig. 4.8). This may reflect a difference in the rates of endocytotic receptor internalization, or possibly receptor-specific regulation of phosphorylation by, for example, tyrosine phosphatases.

4.3.2 PDGF-stimulated downstream signalling

PDGF, via activation of its receptor tyrosine kinases (RTK) is known to increase tyrosine phosphorylation and activation of a number of cellular components such as MAPKs, PI3K, and phospholipase C-γ, which are known to be involved in multiple cellular and developmental responses. Initial experiments were performed to characterize PDGF-BB downstream signaling pathways.

Using antibodies directed specifically to the various phosphorylation sites of different signaling proteins involved in different PDGF signaling pathways, it was determined that PDGF-BB induces a marked phosphorylation of p38 MAPK, ERK1/2 and Akt kinases. Time and dose dependent treatments of confluent serum-starved SMC cultures with PDGF-BB, indicated that maximal kinase activation was obtained at 10-20 mins after PDGF-BB addition within a range of 10-50 ng/ml PDGF-BB (Fig. 4.9).

PDGF treatment reproducibly induced downstream signaling as determined by immunoblots. However there were differences observed in the duration of activation. For example, the Akt activation persisted for up to 60 mins. In contrast, p38 kinase seemed to be more transiently phsophorylated with a return to basal levels by 20 mins, while ERk1/2 kinase remained phosphorylated for up to 20 mins.

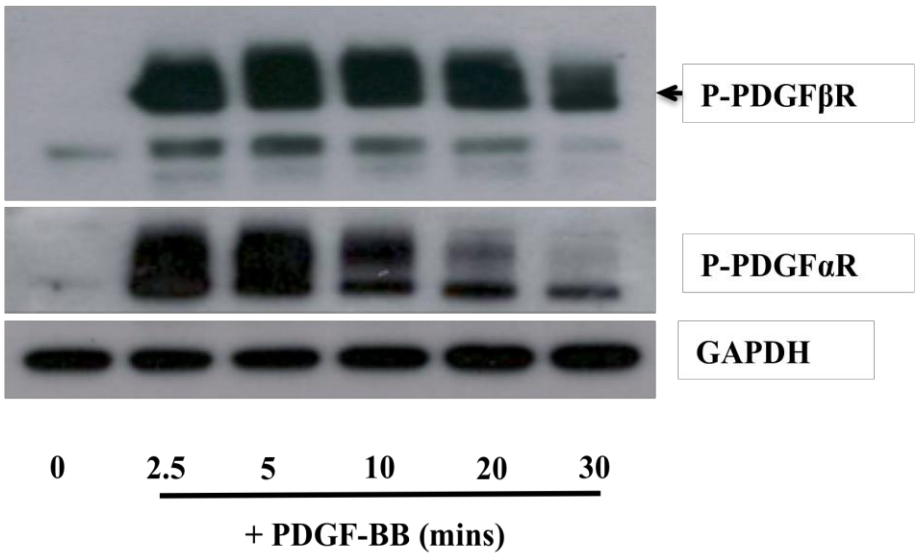

Figure 4.8: PDGF-B stimulates phosphorylation of PDGFRα and PDGFRβ in CASMC

Confluent, serum deprived CASMC were stimulated with 30ng/ml PDGF-BB for the indicated times before lysis. Samples were then Western blotted with phospho-specific antibodies to both PDGFRs or the reference protein GAPDH. The results show the rapid autophosphorylation of PDGF Receptor β (Tyr1021) and PDGF Receptor α (Tyr754). Results are representative of three independent experiments yielding similar results.

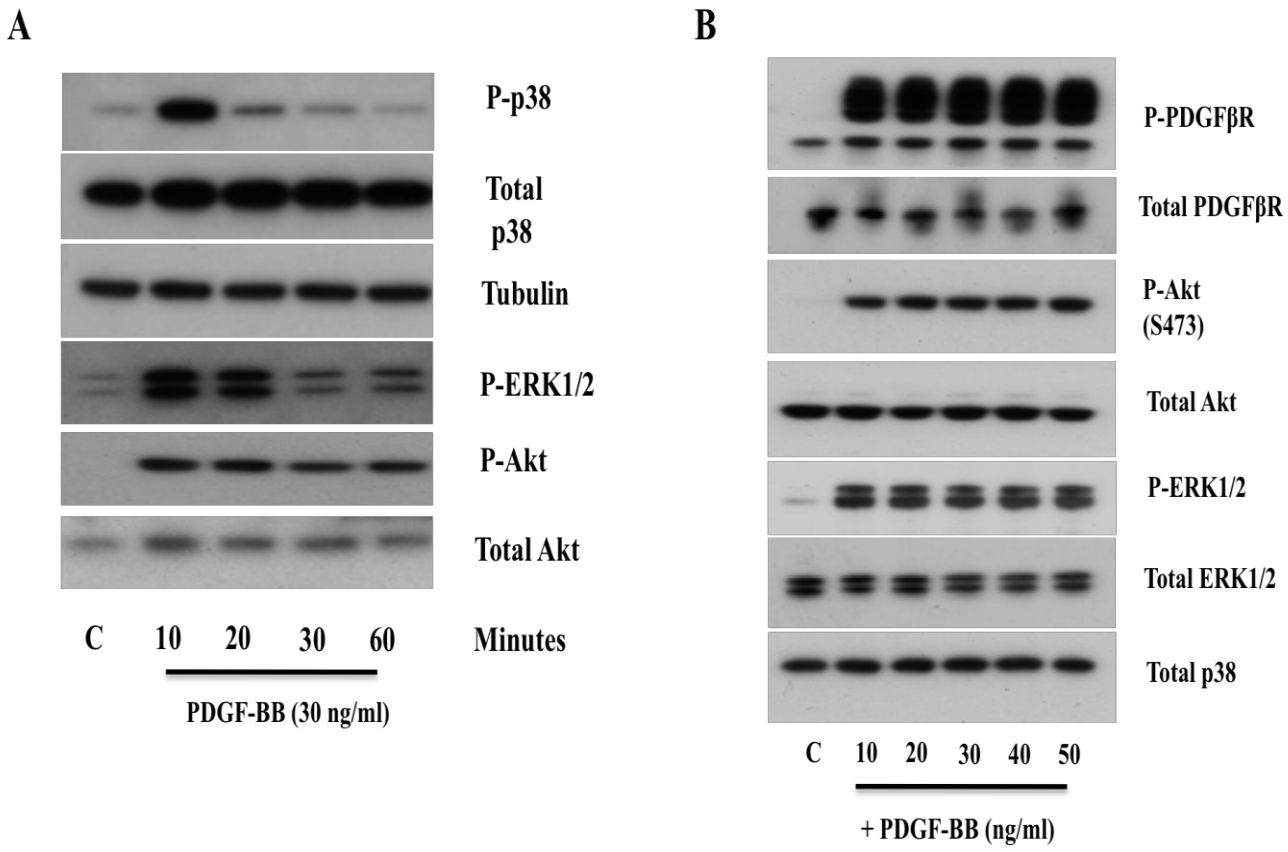

Figure. 4.9: Effect of time and dose responses of PDGF-BB for phosphorylation of p38 MAPK, ERK1/2, and Akt

A. Confluent, serum-deprived CASMCs were treated with 30ng/ml PDGF-BB for the indicated times before harvesting the cells for immunoblot analysis. Samples were then probed with the indicated antibodies. Control (C) means sample in SFM with no PDGF-BB treatment.

B. Serum-deprived, CASMCs were stimulated for 10 min with either different concentrations of PDGF-BB or no addition (C). Cell lysates were then prepared and immunoblotted with the indicated antibodies. Data in both A & B are representative of at least three independent experiments.

4.3.3 Effect of Knockdown of PDGFRs on STC-1 upregulation

In order to establish that the PDGF-BB upregulation of STC-1 was mediated via its receptors in SMCs two approaches were used: pharmacological inhibition of PDGFRs, and siRNA-mediated knockdown of both receptors.

The role of PDGFRs was initially examined using siRNA knockdown targeting PDGF receptors. The effective knockdown of both PDGF receptors is shown in figure 4.10. These data show that siRNA targeted to PDGFRα specifically and markedly reduced expression of PDGFRα with little effect on PDGFRβ expression while PDGFRβ–specific siRNA reduced PDGFRβ expression with little effect on PDGFRα level.

The involvement of PDGFRα and PDGFRβ on STC-1 expression was initially examined using siRNA knockdown of each receptor individually (Fig. 4.11). In this same experiment STC-1 siRNA was used to confirm the identity of the major STC-1 protein band. Unexpectedly, siRNA directed against PDGFRβ appeared to increase STC-1 protein secretion, whereas PDGFRα knockdown showed a trend for reduced PDGF-BB-stimulated STC-1 upregulation compared to either non-targeted control siRNA or the effect of PDGFRβ–targeted siRNA, although this effect was not statistically significant (Fig.4.11A, B). Thus, these data indicate that homodimeric PDGFRβ does not appear to be responsible for PDGF-BB induced STC-1 upregulation, but suggest a role in part for PDGFRα. Since PDGF-BB binds to and activates both receptors and also binds to heterodimers, it was possible that PDGF-BB-induced STC-1 expression might be mediated through homodimeric PDGFRα activation and/or by PDGFRαβ- heterodimers. Nevertheless, the fact that PDGFRα knockdown alone was not sufficient to significantly reduce PDGF-BB induced STC-1 expression suggests either there is some compensatory role played by PDGFRβ, or it may simply be that the level of knockdown was not enough to completely prevent signalling through this receptor. It is also important to note that due to the need to treat with PDGF-BB for 24 h to obtain robust STC-1 protein upregulation, PDGFR immunoreactivity is markedly decreased due mainly to ligand-induced receptor-mediated endocytosis, receptor degradation and down-regulation following activation.

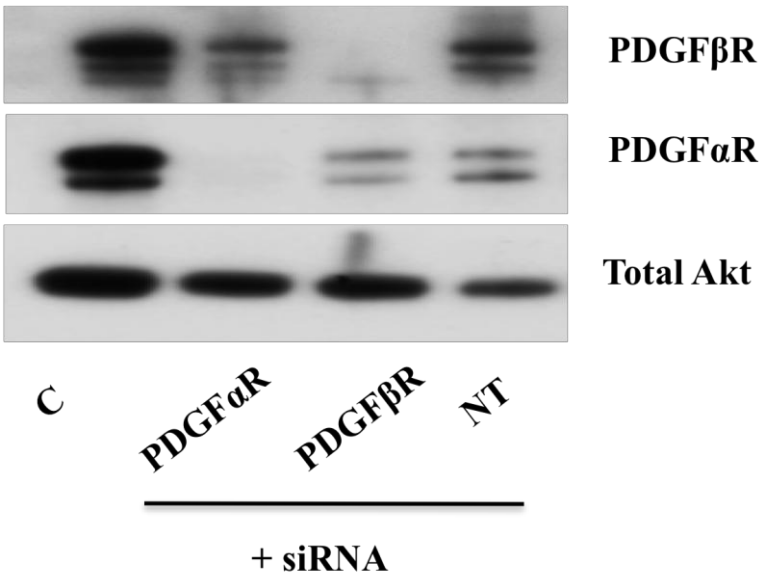

Figure 4.10: Effects of siRNAs directed against PDGRαR and PDGFβR on PDGR expression

CASMC were transfected with 25nM siRNA targeting PDGRαR, PDGFβR, STC-1 and NT (non-targeting siRNA) using lipofectamine RNAiMAX, or untransfected and untreated sample Control (C) and transfected and PDGF-BB treated sample, (+PDGF-BB). After 72 hours, serum-starved cells were treated for 10 mins treatment with 30ng/ml PDGF-BB. Cell extracts were immunoblotted and probed with the indicated antibodies. In this experiment total Akt was used as a loading control.

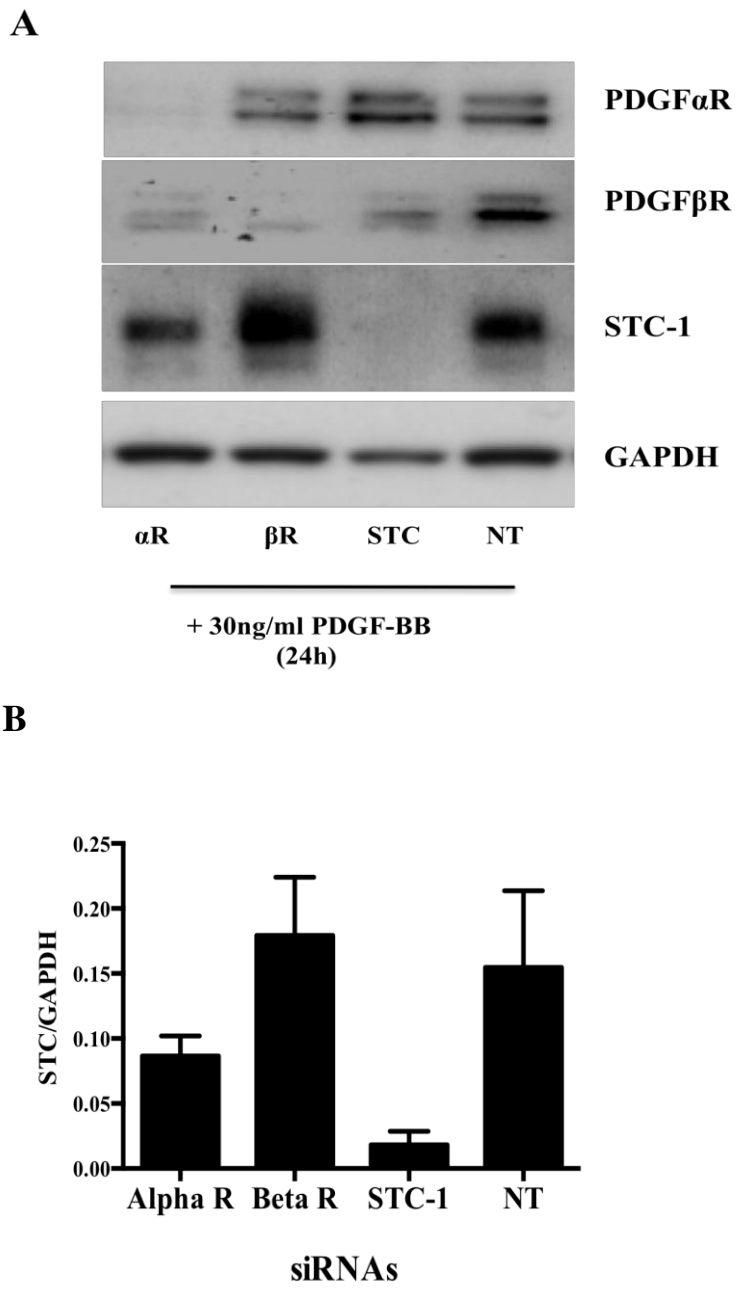

Figure 4.11: Effect of PDGFαR and PDGFβR siRNAs on STC-1 stimulation

A. Serum- deprived HCASMCs were transfected with 25nM siRNA targeting PDGFαR, PDGFβR, STC-1 and NT (non-targeting siRNA) using lipofectamine RNAiMAX reagent. After 72 hr serum-starved cells were treated for further 24 hr with 30ng/ml PDGF-BB. Lysates and supernantants were immunoblotted and probed with indicated antibodies. Results are representative of three independent experiments. **B**. Quantification of results from part A. Results were analysed by two-way ANOVA using Bonferroni's test for multiple pair-wise comparisons.

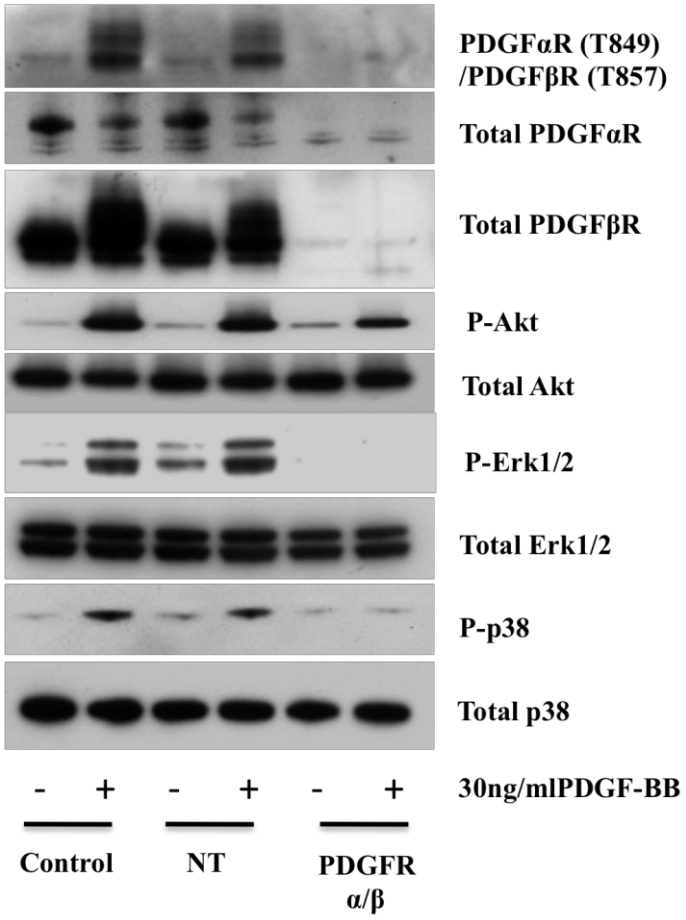

Figure 4.12: Effect of double PDGFR knockdown on downstream signalling

SMCs were incubated with or without (Control) 25nM siRNA of PDGF receptors and non-specific siRNA (NT). Cells were then treated with for 10 mins with 30ng/ml PDGF-BB (+) or no addition (-) and harvested. Cell extracts were immunoblotted and probed with the indicated antibodies.

4.3.3.1 Double knockdown of PDGFαR and PDGFβR

Taken together, the results from experiments using siRNA targeting either homodimeric PDGFαR or PDGFβR indicated that knockdown of either receptor alone failed to have a dramatic effect on STC-1 induction. It seemed likely from these results that there was some functional redundancy involved in PDGF-stimulated STC-1 by these receptors. To address this, double siRNA knockdowns targeting both PDGFRs in SMCs were performed.

Initially, I determined whether double PDGFβR and PDGFαR knockdown was effective in inhibiting PDGF-BB stimulation of receptor activation and downstream signaling. As shown in Fig. 4.12, double knockdown of PDGFβR and PDGFαR strongly inhibited activation of both receptors and activation of ERK and p38 kinase in response to PDGF-BB. In addition, PDGF-BB-induced Akt activation was also inhibited, though these siRNAs were insufficient for complete inhibition of Akt activation, despite strong inhibition of PDGFβR and PDGFαR phosphorylation and of other signaling pathways examined (Fig. 4.12).

To assess whether PDGFRβ and PDGFRα play redundant and compensatory roles in STC-1 upregulation, expression of both PDGF receptors was reduced in SMCs using double siRNA knockdown as before, and cells were treated for 24 hours with or without PDGF-BB to induce STC-1 expression. Results from experiments using double siRNA targeting both PDGFRα and PDGFRβ indicated that receptors levels were substantially reduced. As expected 24 hours treatment with PDGF-BB caused a marked decrease in expression of both receptors, more striking for than PDGFRβ than PDGFRα, and a marked increase in STC-1 protein expression. However, although there was a noticeable trend for reduced PDGF-stimulated STC-1 protein levels, this was not statistically significant in three independent experiments (Fig. 4.13). It was also noted in these experiments that non-targeted control siRNA also decreased PDGFR expression compared with untransfected control cells, most likely reflecting an effect of transfection conditions on receptor expression.

4.3.3.2 NRP1 and PKCα knockdown

It has previously been reported that STC-1 expression is mediated via NRP1 and PKC-dependent pathways in response to VEGF treatment in endothelial cells (Holmes & Zachary, 2008; Sazonova et al., 2008). Initially, the role of NRP1, a co-receptor for PDGFRα and β (Pellet-Many et al., 2011), was examined using a specific siRNA. A representative blot of the effect of NRP1 knockdown on PDGF-induced STC-1 protein expression is shown in Fig. 4.14A. NRP1 knockdown did not significantly affect PDGF-BB-stimulated STC-1 expression compared to the non-targeted control siRNA.

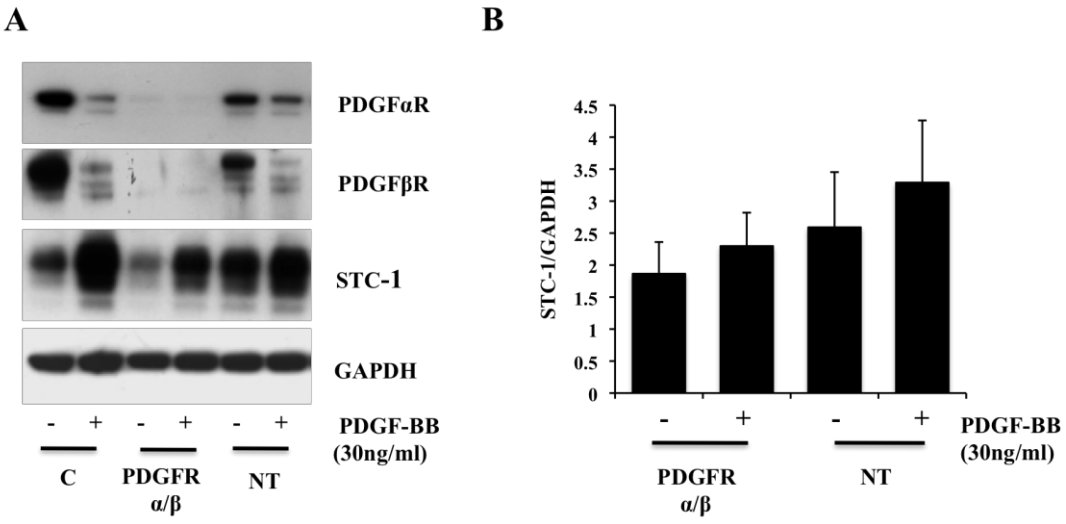

Figure 4.13: Effect of double PDGF receptor knockdown on PDGF-stimulated STC-1 expression

A. SMCs were treated with 25nM of the indicated siRNA or without any siRNA (C). After 72 hours, cells were then treated for 24hr with 30ng/ml PDGF-BB (+) or with no addition (-) and then lysed. Cell extracts were immunoblotted and probed with the indicated antibodies. **B**. Quantification of the effect of PDGFαR and PDGFβR siRNAs on STC-1 induction. Results are mean ± SEM STC-1 levels normalised to GAPDH, from three independent experiments. Differences between siRNAs were analysed by one-way ANOVA using Bonferroni's test for multiple pair-wise comparisons.

Initial attempts were made to examine the role of specific signaling pathways in PDGF-BB-induced STC-1 expression using inhibitors of signaling pathways, eg of PKC, MAPK and PI3K pathways. However, due to the need to treat for 24 hours with PDGF-BB to induce robust STC-1 expression, it was found that such a longer-term treatment with pharmacological inhibitors, eg GF109203X or wortmannin, had severe effects on cell viability, making interpretation of these data very difficult. Since PKC was implicated in mediating STC-1 expression (Holmes & Zachary, 2008; Sazonova et al., 2008), and PKCα is a major PKC isoform in cultured human CASMC (Yasunari, Maeda, Minami, & Yoshikawa, 2001), I instead examined the effect of PKCα-targeted siRNA. A representative blot of the effect of PKCα knockdown on PDGF-induced STC-1 protein expression is shown in Fig. 4.14B. PKCα knockdown did not significantly reduce, and in fact somewhat enhanced, PDGF-BB-stimulated STC-1 expression compared to the non-targeted control siRNA (Fig. 4.14B).

4.3.4 Pharmacological inhibition of PDGF Receptors

Two problems encountered using siRNA are incomplete knockdown and off-target effects. An alternative approach, which may address at least the first of these drawbacks is to use pharmacological membrane-permeable inhibitors. Therefore, the role of PDGFR signalling was examined using the PDGFR kinase inhibitor AG 1296. Quinoxaline AG 1296, an ATP-competitive blocker, is a highly potent and selective inhibitor of PDGFR and its family members Kit and FIt3.

To examine the effect of AG1296 on the PDGF receptors, SMCs were pretreated with 10μM AG1296 followed by PDGF-BB treatment. As expected, after 10 min PDGF treatment, AG 1296 at 10μM completely blocked PDGF- stimulated phosphorylation of both α- and β-receptors at Tyrosine 849 and 857 respectively (Fig. 4.15), in agreement with previously published data (Kovalenko et al., 1997). This region of the α- and β-receptors is 90% structurally similar and is located in the bi-partite receptor kinase domain. β-Receptor phosphorylation of tyrosine 1021, a PLCγ binding site (Morrison, Kaplan, Rhee, & Williams, 1990), was also reduced, albeit not completely inhibited. It should also be noted that the decrease in PDGFRα immunoreactivity usually seen with PDGF-BB stimulation was completely absent in the AG1296 treated cells.

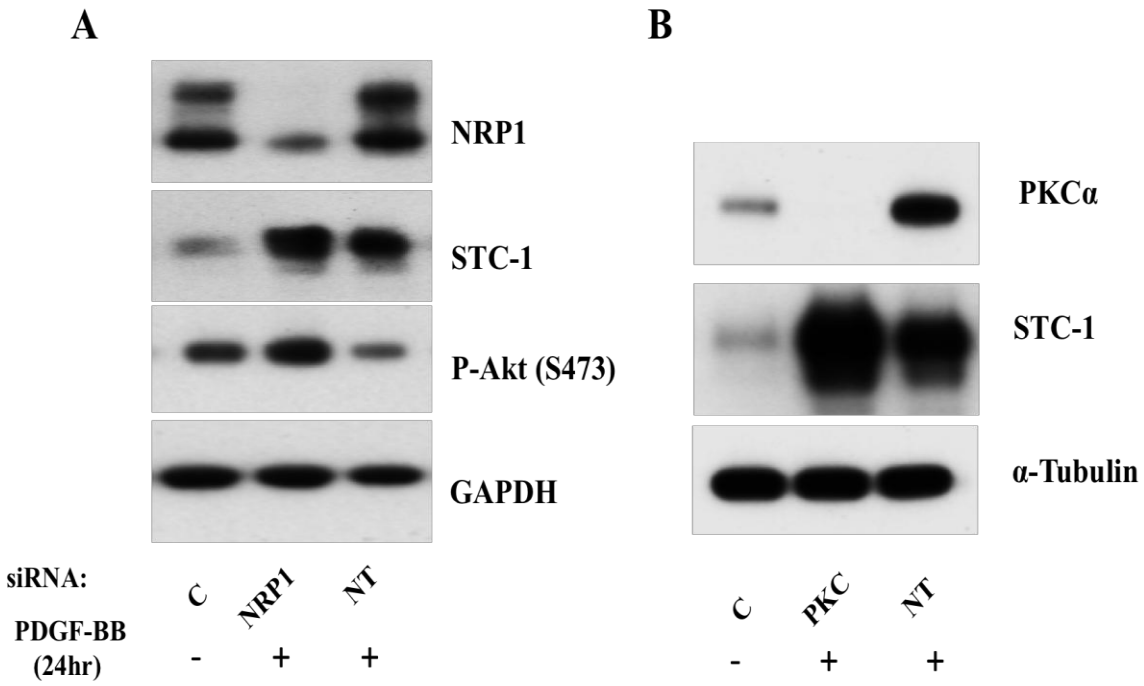

Figure 4.14: Effect of NRP1 knockdown on PDGF-stimulated STC-1

A. CASMC were transfected with 25nM siRNA targeting NRP1 and NT (non-targeting siRNA) using lipofectamine RNAiMAX, or untransfected (C). After 72 hr, serum-starved cells were treated for further 24 hr with 30ng/ml PDGF-BB. Cell lysates were immunoblotted and probed with the indicated antibodies. Supernantants were concentrated and immunoblotted and probed for STC-1. **B.** CASMC were transfected with 25nM siRNA targeting PKCα and NT using lipofectamine RNAiMAX, or untransfected (C). After 72 hr, serum-starved cells were treated for further 24 hr with 30ng/ml PDGF-BB. Cell lysates were immunoblotted and probed with the indicated antibodies. Supernantants were concentrated and immunoblotted and probed for STC-1.

PDGF- induced phosphorylation of the downstream signaling molecules, p38 MAPK and ERK1/2, was fully inhibited, and Akt phosphorylation was markedly reduced in AG1296-treated cells (Fig. 4.15). The effect of AG1296 on PDGF-stimulated STC-1 regulation and phosphorylation of Akt was also examined after 24 hr PDGF-BB treatment. AG1296 strongly reduced the activation of Akt, and also prevented the attenuation of PDGFRα expression as indicated by the lack of decrease in its immunoreactivity following PDGF-BB stimulation. In contrast the reduction in PDGFRβ level was unaffected by AG1296, suggesting that the inhibitor may prevent PDGFRα internalization but has little effect on PDGFRβ internalization and degradation. Surprisingly, STC-1 induction was not affected by the inhibitor, perhaps suggesting that residual PDGFRs signalling, resistant to the inhibitor, may be sufficient to drive STC-1 upregulation. Other possible explanations for the lack of effect of the PDGFRs inhibitor are discussed below.

4.4 Discussion

STC-1 gene expression is widely seen in tissues like heart, liver, lung, adrenal glands, kidney, prostate and ovary. The expression of STC-1 is induced by various stimuli, such as VEGF, hypoxia/oxidative stress and inflammation. However, at the start of this thesis, there were no reports regarding PDGF-mediated STC-1 expression in SMCs, or effects of PDGFs on STC expression.

The possible effect of PDGF in STC-1 expression was examined in human CASMCs and AoSMCs. A major finding of this chapter is that PDGF induced expression of STC-1 mRNAs indicated by quantitative PCR. Further experiments indicated that PDGF-BB stimulated STC-1 protein expression in a time and dose-dependent manner in CASMCs. Interestingly, while STC-1 expression was strongly upregulated by PDGF-BB, PDGF-AA, another potent SMC mitogen that acts through the activation of PDGFRα only, had no effect on PDGF-stimulated STC-1 expression.

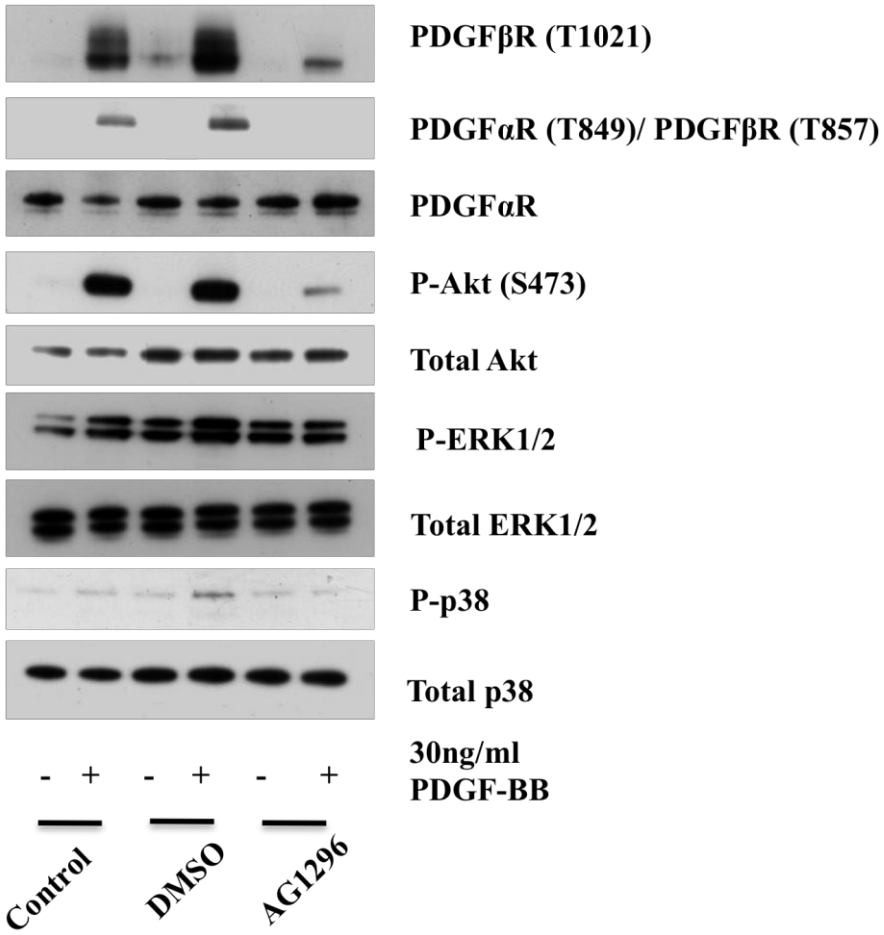

Figure 4.15: Tyrphostin AG1296 does not completely inhibit PDGF-stimulated downstream signaling

Confluent, serum-starved HCASMCs were pre-incubated with or without (Control) 10μM of AG1296 or solvent alone (0.1% DMSO) for 30 mins followed by 10 mins treatment with 30ng/ml PDGF-BB (+) or no addition (-). Cells were then lysed and immunoblotted using the indicated antibodies. These data are representative of at least two independent experiments.

Since PDGF-BB binds and specifically activates both PDGF transmembrane receptors, α and β, whereas PDGF-AA binds specifically to PDGFRα, the selectivity of PDGF-BB for STC-1 upregulation strongly suggested that STC-1 expression would be mediated via PDGFRβ. However siRNA-mediated receptor knockdowns of either PDGFRα or PDGFRβ suggested that PDGFRα or possibly PDGFRα/β heterodimers might be the primary receptor(s) responsible for STC-1 upregulation. In contrast, PDGFRβ siRNA knockdown had no effect on PDGF-stimulated STC-1 expression. However, it was also evident that knockdown of either PDGFRα or PDGFRβ, or of both PDGFRα and PDGFRβ only partially reduced PDGF-BB-induced STC-1 expression. The most likely explanation is that STC-1 induction by PDGF-BB only requires a low level of receptor activation and the knockdown was insufficient to completely prevent this. This would also explain the reduced but significant Akt activation seen after double receptor knockdown (Fig. 4.12). This explanation implies that very low levels of PDGFR activity or only a fraction of the total cellular pool is required for downstream PDGF functional signalling necessary for STC-1 upregulation, while a larger PDGFR pool is required for activation of some signaling pathways, eg ERK1/2 and p38 MAPK.

It is unclear why PDGF-BB might induce STC-1 upregulation via PDGFRα, while PDGF-AA had no effect, but this might reflect distinct conformational changes in PDGFRα upon binding of these two ligands that may elicit divergent intracellular signalling through the same receptor. There is at least one reported example of differential regulation of PDGF-BB–induced activation as opposed to PDGF-BB-induced activity (Rolny, Spillmann, Lindahl, & Claesson-Welsh, 2002). The results presented here appear to represent another instance of differential PDGFRα signalling by PDGF-AA and BB. Further work is required to confirm this hypothesis, for example investigation of STC-1 expression in cells specifically expressing PDGFRα or PDGFRβ and mutant PDGFRs. As an alternative to PDGF receptor knockdowns, I also used the PDGF receptor inhibitor AG1296. This compound should, in theory, completely block all PDGFR mediated signalling. However despite blocking PDGF-BB-induced PDGFR tyrosine phosphorylation, p38 and ERK1/2 phosphorylation and strongly reducing Akt activation, this inhibitor had little effect on STC-1 induction.

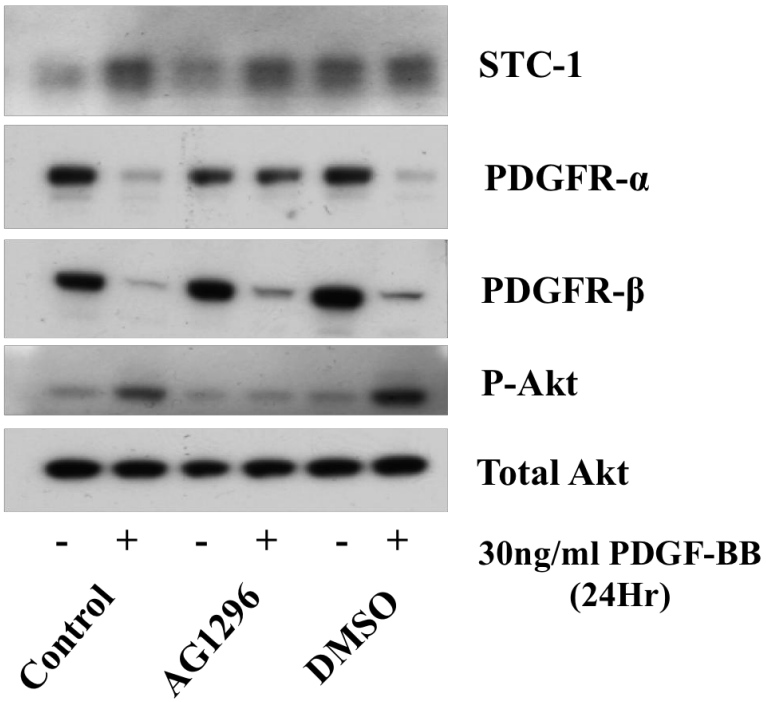

Figure 4.16: Tyrphostin AG1296 has little effect on PDGF-stimulated STC-1 expression

Confluent, serum-starved HCASMCs were pre-incubated with or without (Control) 10μM of inhibitor or solvent alone (0.1% DMSO) for 30 mins followed by 24 hours treatment with 30ng/ml PDGF-BB (+) and no addition (-). Cells were then lysed and immunoblotted using the indicated antibodies. These data are representative of at least two independent experiments.

This was a surprising result, which is difficult to explain. It is possible that metabolism of the drug over a 24 h treatment might be sufficient to reduce effective concentrations of the drug to allow some PDGFR signaling sufficient to allow STC-1 expression to recover. I also noted that while AG1296 prevented downregulation of PDGFRα it had no effect on downregulation of PDGFRβ. Since receptor downregulation is a consequence of receptor activation and subsequent ligand-induced endocytosis and degradation, one interpretation of my results is that, though AG1296 transiently inhibited both PDGFRs, it had a more sustained inhibitory effect on PDGFRα, allowing longer-term PDGFRβ activity to compensate for inhibition of PDGFRα. This possibility warrants further study, perhaps by examination of effects of AG1296 on PDGFRα and PDGFRβ kinase activity at different times.

VEGF- stimulated STC-1 upregulation in endothelial cells had been reported to occur via PKC and NRP1-dependent pathways (Holmes & Zachary, 2008). NRP1 is thought to be important for VEGF-stimulated STC-1 expression through the ability of NRP1 to potentially stabilise VEGFR2/KDR activity, suggesting that STC-1 expression is regulated by NRP1/KDR signalling. However, NRP1 knockdown was not able to reduce PDGF-stimulated STC-1 expression in SMCs, in contrast to VEGF-stimulated STC-1 expression in endothelial cells (Holmes & Zachary 2008), suggesting that NRP1 may be important in VEGF-stimulated STC-1 expression in HUVECs, but not in SMCs. It was also found that VEGF-induced STC-1 mRNA expression was blocked by a broad spectrum inhibitor of PKC (GF109203X), suggesting that in endothelial cells STC-1 expression is mediated through PKC activation (Holmes & Zachary, 2008). I did not use PKC inhibitors in this chapter, because the 24 hour treatment required to strongly upregulate STC-1 protein, would result in toxicity and a large loss of cell viability (unpublished observations). However, in the current study, siRNA-mediated dowregulation of PKCα appeared to increase rather than decrease PDGF-stimulated STC-1 expression levels. This finding is in agreement with a recent study that showed PKCα knockdown strongly upregulated STC-1 levels in a breast cancer cell line (Cornmark, Lonne, Jogi, & Larsson, 2011). In further work I would need to examine the signaling pathways mediating PDGF-BB-induced STC-1 expression more fully, by investigating effects of shorter-term treatments with signaling pathway inhibitors on STC-1 mRNA expression, and using knockdowns to other PKC isoforms expressed in

SMC.

Given the partial effects of PDGFR knockdown and the lack of effect of a non-selective PDGFR inhibitor on STC-1 expression, other possible mechanisms for PDGF-BB-induced STC-1 expression need to be considered. Another possible explanation for my data is that a novel mechanism not mediated solely or partly by known PDGFRs is responsible for PDGF-BB-induced STC-1 expression. There is however no strong evidence in the literature for such a mechanism. A non-PDGFR receptor, LRP1 (low density lipoprotein receptor-related protein 1) has been shown to modulate PDGF signalling but this occurs via interaction with PDGFRβ (Muratoglu, Mikhailenko, Newton, Migliorini, & Strickland, 2010). However, it is possible that STC-1 expression may be enhanced by interaction of PDGF-BB with other cell-surface molecules such as heparan sulphate or chondroitin sulphate glycosaminoglycans (HSPGs, CSPGs), such as perlecan or syndecans, which are strongly expressed in SMC. PDGF-B chains bind to HSPG and CSPG, and these interactions regulate biological functions of PDGF-B in development (Garcia-Olivas, Vilaro, Reina, & Castel, 2007; Lindblom et al., 2003); (Abramsson et al., 2007). This is a possibility, which could be tested initially by examining effects of targeted knockdown of known proteoglycans in SMC on PDGF-BB-induced STC-1 expression. I also found that STC-1 was constitutively expressed in SMC in the absence of serum or PDGF treatment, and that this constitutive expression was considerable. It is therefore possible that PDGF-B may sequester some extracellular or cell surface component that negatively regulates basal STC-1 expression, and can thereby enhance constitutive STC-1 expression.

In summary, the results in this chapter show that PDGF-BB stimulates STC-1 mRNA and protein expression in arterial SMC, and also that STC-1 is constitutively expressed in these cells. PDGF-BB-induced expressed is at least partially mediated via PDGFRα and possibly PDGFRα/β heterodimers, but there appears to be compensation between the two receptors. However, the partial inhibition of STC-1 expression by knockdown of both PDGFRs raises the possibility that an unconventional pathway is also involved not mediated by known PDGFRs, or by a non-RTK pathway.

Chapter 5

5 Effects of STC-1 on vascular smooth muscle cell function

In the cardiovascular system, PDGF has been implicated in a variety of physiological and pathophysiological processes, including recruitment and proliferation of vascular mural cells during development, and pathophysiological SMC accumulation in vascular pathologies such as atherosclerosis and restenosis. PDGF-BB-induced chemotactic migration of SMC is thought to play a particularly important role in these processes. Having demonstrated that PDGF-BB induces STC-1 expression in primary cultures of SMC in Chapter 4, I decided to investigate the role of STC-1 protein expression in PDGF-BB mediated SMC functions, focusing particularly on PDGF-BB functional signaling and on PDGF-BB-induced cell migration.

5.1 Effects of rhSTC-1 on PDGF-BB signalling

PDGF-BB, via PDGFRs, activates several intracellular signaling pathways, including the ERK1/2 cascade, PLC-γ leading to activation of PKC, and the PI3K pathway leading to activation of Akt. One of the major downstream targets of PDGF-BB signalling is the serine/threonine kinase, Akt. There is strong evidence in the literature that Akt is a crucial downstream target of PI3K and it is likely to be responsible for

many of the biological consequences of PI3K activation in SMC. For example, SMC survival is dependent on Akt signalling (Allard, Figg, Bennett, & Littlewood, 2008).

To assess whether SMC signal transduction could be altered by PDGF-BB induced STC-1 expression, CASMCs were pre-treated with recombinant human STC-1 (hrSTC-1) protein for 30 min, followed by a further 10 min PDGF-BB treatment (Fig. 5.1A). As expected, immunoblotting using phosphorylation-specific antibodies demonstrated a robust increase in Akt phosphorylation (Ser 473) 10 min after PDGF-BB stimulation when compared with untreated cells. Interestingly, the immunoblotting analysis also revealed a synergistic effect of STC-1 on PDGF-induced Akt activation compared with the effect of PDGF alone. Quantification of Akt phosphorylation in several experiments showed that synergistic enhancement of PDGF-BB-induced Akt activity by STC-1 was consistent and statistically significant, while STC-1 alone had no detectable effect on Akt activity (Fig. 5.1B).

The involvement of STC-1 in PDGF-induced Akt phosphorylation was further examined after different times of PDGF-BB treatment. SMCs were pre-incubated with 100ng/ml hrSTC-1 for 30 min followed by stimulation with PDGF-BB or vehicle over a period of two hours. The levels of Akt phosphorylation were examined as well as other PDGF-induced downstream pathways in SMCs. The synergistic augmentation of Akt phosphorylation was found to occur for up to 2 hours treatment with STC-1 and PDGF, whereas ERK1/2 phosphorylation was not affected (Fig. 5.2). In contrast, PDGF-BB induced p38 phosphorylation appeared to be decreased at 10 min treatment after STC-1 preincubation compared to PDGF-BB alone.

The influence of different concentrations of hrSTC-1 on PDGF-stimulated Akt phosphorylation was also examined. Pre-treatment of SMCs with various concentrations of STC-1 for 30 mins resulted in the enhancement of PDGF-induced Akt phosphorylation, an effect that was evident at all the STC-1 concentrations used from 25-600 ng/ml (Fig. 5.3). It was noted that the same STC-1 concentrations alone in the absence of PDGF-BB, had no effect on Akt activity. Both the time and concentration experiments results indicated that STC-1 significantly enhances PDGF-stimulated Akt phosphorylation.

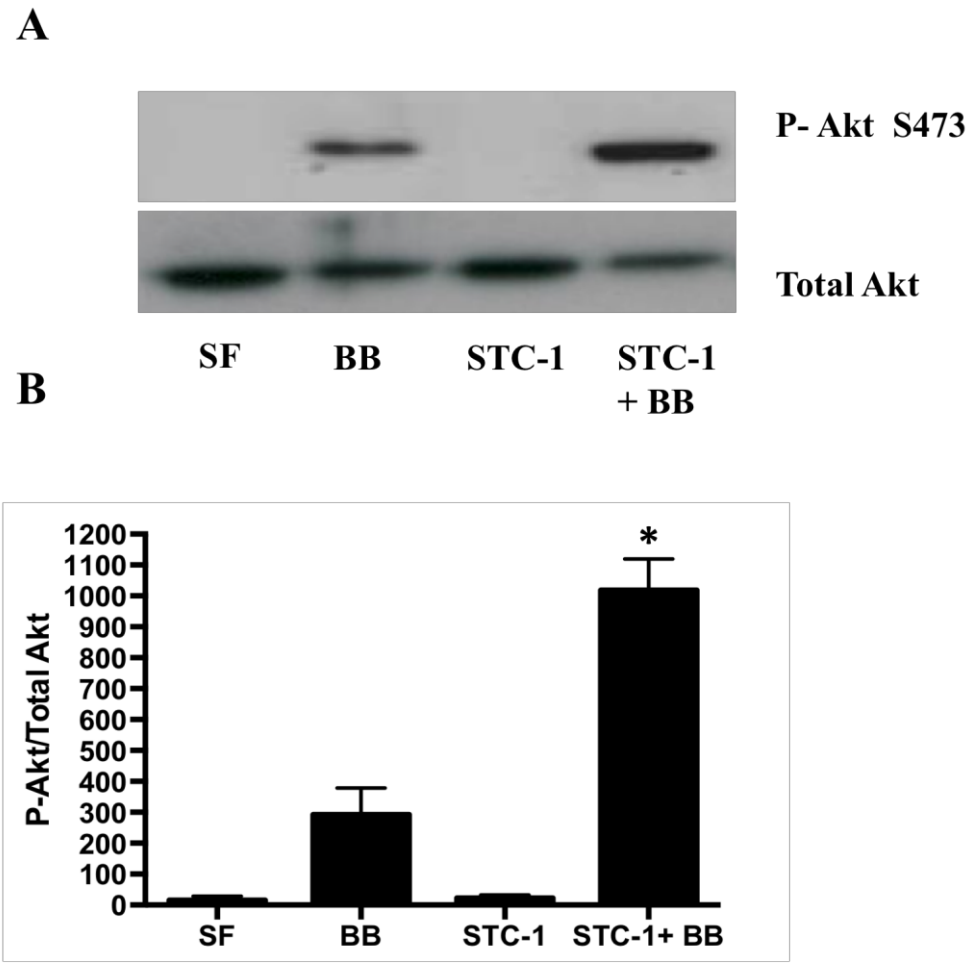

Figure 5.1: Synergistic effect of hr STC-1 and PDGF-BB on Akt activation

A. HCASMCs were pre-treated with or without human recombinant STC-1 for 30 mins at 100ng/ml, and then treated for 10 minutes with or without PDGF-BB at 30 ng/ml. Cell lysates were then prepared and immunoblotted with antibodies to Akt phosphorylated at Ser473, or total Akt. **B**. Western blot data for phosphorylated Akt was quantified from three independent experiments and are presented as means +/- SEM normalized to total Akt levels. PDGF-BB+STC-1 treatment resulted in a significant increase in Akt activity after 10 min of treatment compared with PDGF-BB alone (*$p < 0.001$).

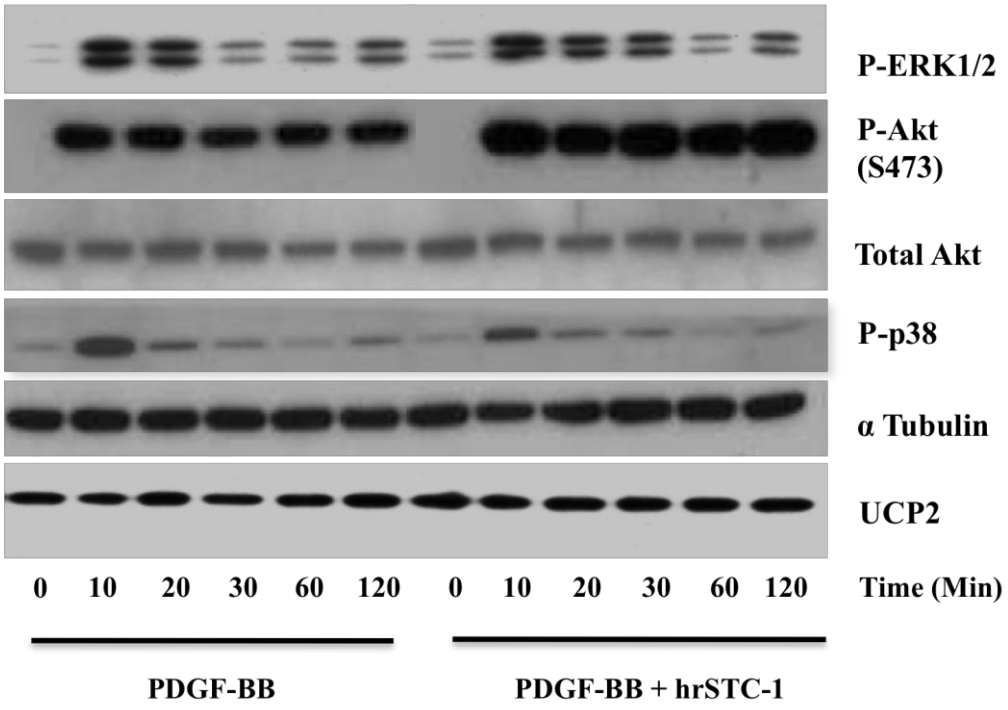

Figure 5.2: STC-1 is involved in enhancement of Akt activation but attenuates p38 phosphorylation

Confluent serum-starved HCASMC were treated with or without addition of 100ng/ml hrSTC-1, and subsequently treated with or either no addition (0) or with 30 ng/ml PDGF-BB for the indicated times. Cells were then lysed and immunoblotted with the indicated antibodies. Results are representative of two independent experiments.

5.2 The effect of STC-1 on downstream targets of the Akt pathway

Activation of Akt has been reported to phosphorylate multiple downstream effectors, such as glycogen synthase kinase 3 (GSK3). GSK3 is a key regulator of many cell functions, including the control of cell division, apoptosis, microtubule organization and the specification of cell fates during embryonic development. GSK3 is a highly conserved serine/threonine protein kinase that is negatively regulated by Akt. GSK3 activity is inhibited by phosphorylation of two known isoforms, GSK3α at ser 21, and GSK3β at ser 9. To further investigate the effect of STC-1 on SMCs, Akt downstream signaling was examined.

5.2.1 Effect of STC-1 on PDGF-BB-induced GSK3 phosphorylation

To determine whether PDGF-stimulated Akt enhanced activation by STC-1 could also enhance downstream target proteins of the Akt pathway, the effect of STC-1 on GSK3α/β phosphorylation at Ser 21 was examined using recombinant STC-1 protein. Representative blots demonstrated that STC-1 strongly enhanced PDGF-stimulated GSK3α/β phosphorylation at Ser 21 for the α- isoform and Ser 9 for the β-isoform, whereas p38 kinase phosphorylation was attenuated (Fig. 5.4). As expected, phosphorylation of Akt was enhanced.

5.2.2 Effect of STC-1 knockdown on PDGF-BB-induced Akt signalling

To further examine the importance of STC-1 in regulating PDGF-induced Akt phosphorylation in SMCs, STC-1 was knocked down using targeted siRNAs. Representative blots of the effect of STC-1 knockdown on PDGF-stimulated Akt phosphorylation are shown in Figure 5.5A, with the quantified effect on Akt phosphorylation present in Figure 5.5B.

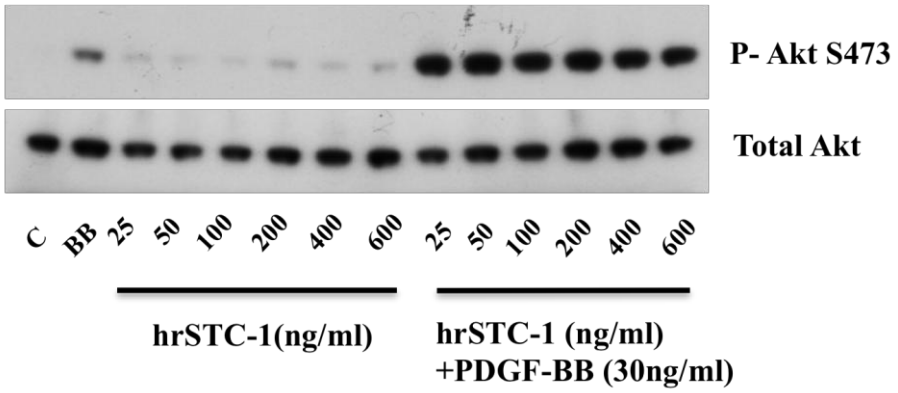

Figure. 5.3: Effects of different hrSTC-1concentrations on Akt phosphorylation

Serum–starved, confluent HCASMCs were pre-treated with various concentrations of hrSTC-1 as indicated for 30mins and then treated for 10 minutes with or without PDGF-BB at 30 ng/ml. Cell lysates were then prepared and immunoblotted for the indicated antibodies. This result is representative of two independent experiments.

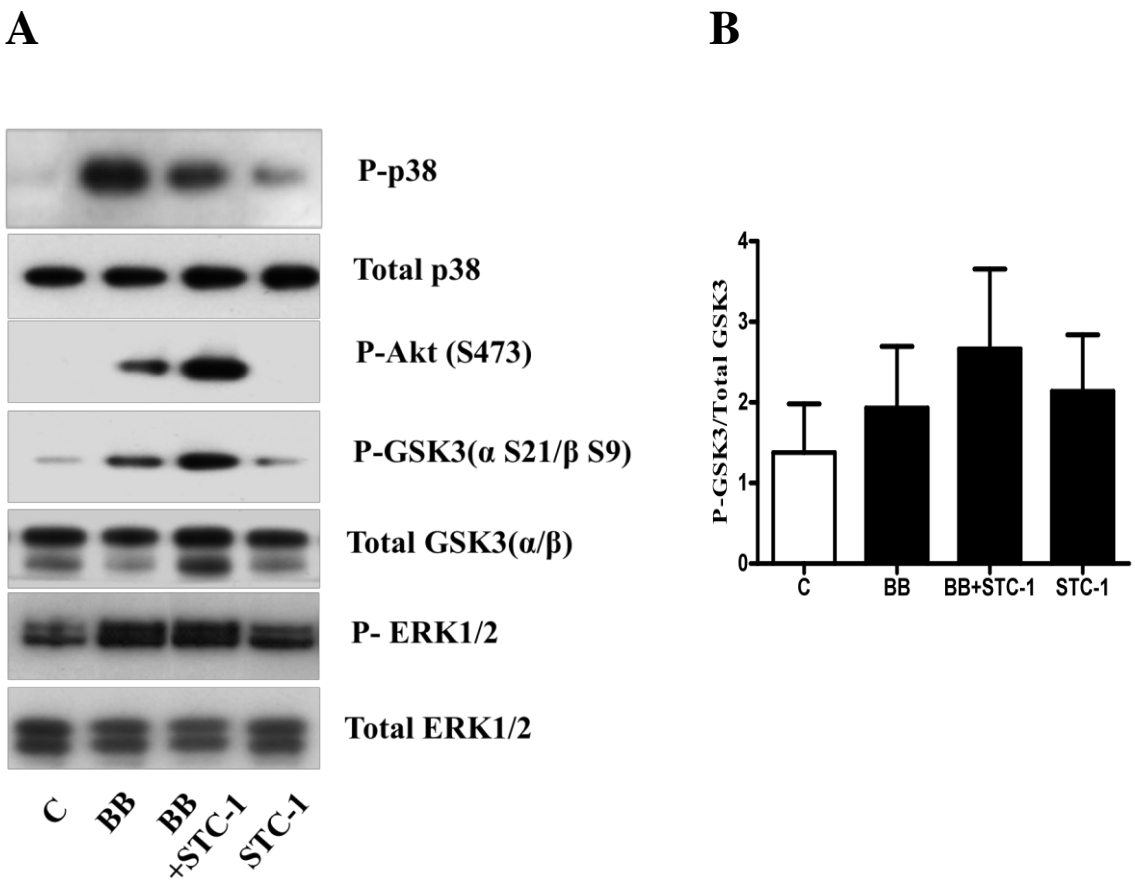

Figure 5.4: Effects of STC-1 on PDGF-BB-induced GSK3 phosphorylation

A. Confluent, serum-starved CASMCs were pre-incubated for 30 mins with 100ng/ml hrSTC-1, followed by 10 mins treatment with or either no addition (C) or 30ng/ml PDGF-BB (BB). Cells were then lysed, blotted and probed with the indicated antibodies. These blots are representative of 3 independent experiments yielding similar results. **B**. Quantification of the effect of STC-1 on PDGF-mediated GSK3 phosphorylation. Results are from three independent experiments and are presented as means +/- SEM normalized to total GSK3 levels without reaching any statistical significance ($p>0.05$).

STC-1 knockdown appeared to reduce PDGF-induced Akt phosphorylation, although this was not statistically significant. There was no effect of STC-1 knockdown on ERK1/2 phosphorylation. It was also observed that the levels of Akt and ERK1/2 phosphorylation in transfected samples (both NT and STC-1 siRNAs) were increased in the absence of ligand, suggesting a non-specific effect of transfection and further complicates interpretation of data with these siRNAs.

5.3 Adenovirus overexpression of wild-type STC-1 protein in SMCs

To complement the results using hrSTC-1 and to further validate the synergistic effect of STC-1 in PDGF-stimulated Akt phosphorylation, adenovirus overexpression of STC-1 in SMCs was used. SMCs were infected with adenoviruses overexpressing STC-1 (Ad.STC-1) or β-galactosidase (Ad.Lac Z) as a control.

I first assessed the effect of viral infection on STC-1 protein overexpression in CASMCs in a time and MOI-dependent manner compared to the control infection with Ad.Lac Z. A range of MOIs were used to infect cells for 48 hours, from 5 to 100 infectious units per cell. Infection with Ad.STC-1 caused a MOI-dependent increase in STC-1 expression in contrast to Ad.Lac Z-infected cells which showed no increase in basal STC-1 expression. Immunoblots revealed a gradual and stable increase of STC-1 with a readily detectable level of overexpression that lies between MOI of 10 and 100 (Fig. 5.6).

Similarly, a time course experiment using an MOI of 10 showed that STC-1 expression in the medium of Ad.STC-1 infected cells increased with time over a period of up to 4 h after cells had been transferred to fresh medium, indicating that adenovirally-expressed STC-1 is secreted rapidly from SMC. There was no increase in STC-1 expression in Ad.Lac Z infected cells over this time period (Fig. 5.7).

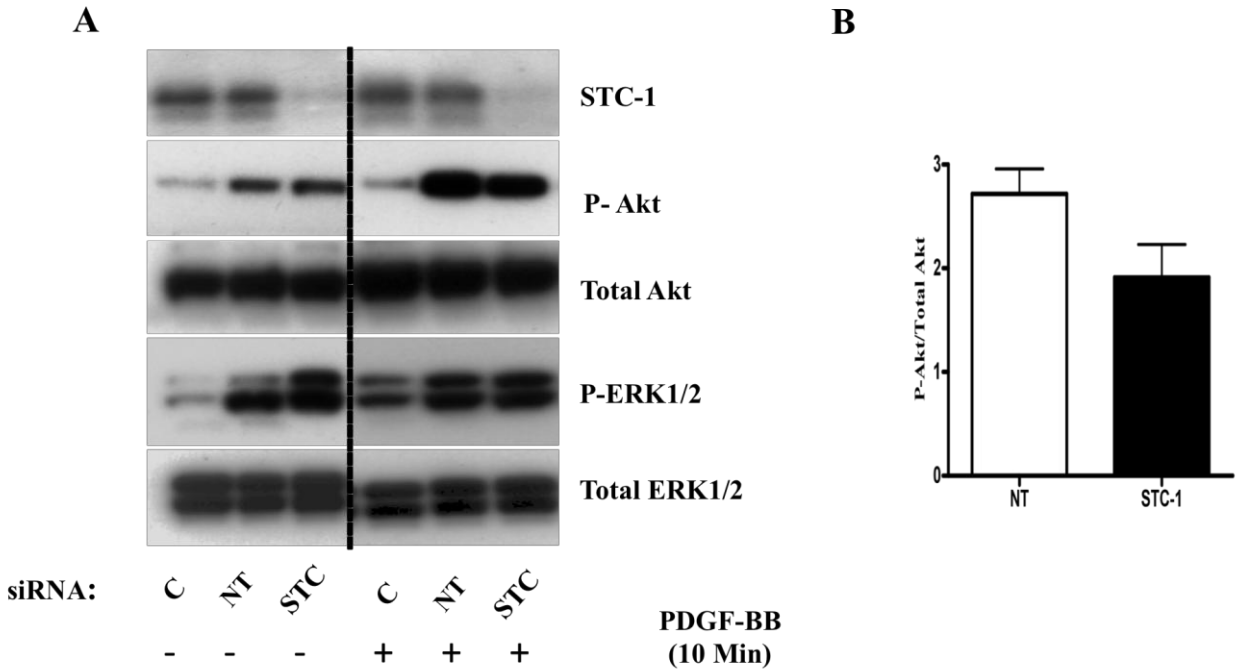

Figure 5.5: Effect of STC-1 knockdown on PDGF-stimulated signalling

A. HCASMC were transfected with 25nM targeting STC-1 or NT (non-targeting) siRNA using lipofectamine RNAiMAX, or untransfected (C). After 72 hr, serum-starved cells were treated for 10 mins with 30ng/ml PDGF-BB or without addition (-). Cell lysates were immunoblotted and probed with the indicated antibodies. Supernantants were concentrated and immunoblotted and probed for STC-1. Data presented are from four independent experiments. The dashed line indicates that the blots are derived from different regions of the same blot. **B.** Quantification of results for Akt phosphorylation in panel A, (n = 4) are presented normalised to total Akt levels. Analysis by two-way ANOVA with Bonferroni's test for multiple comparisons showed no significant difference.

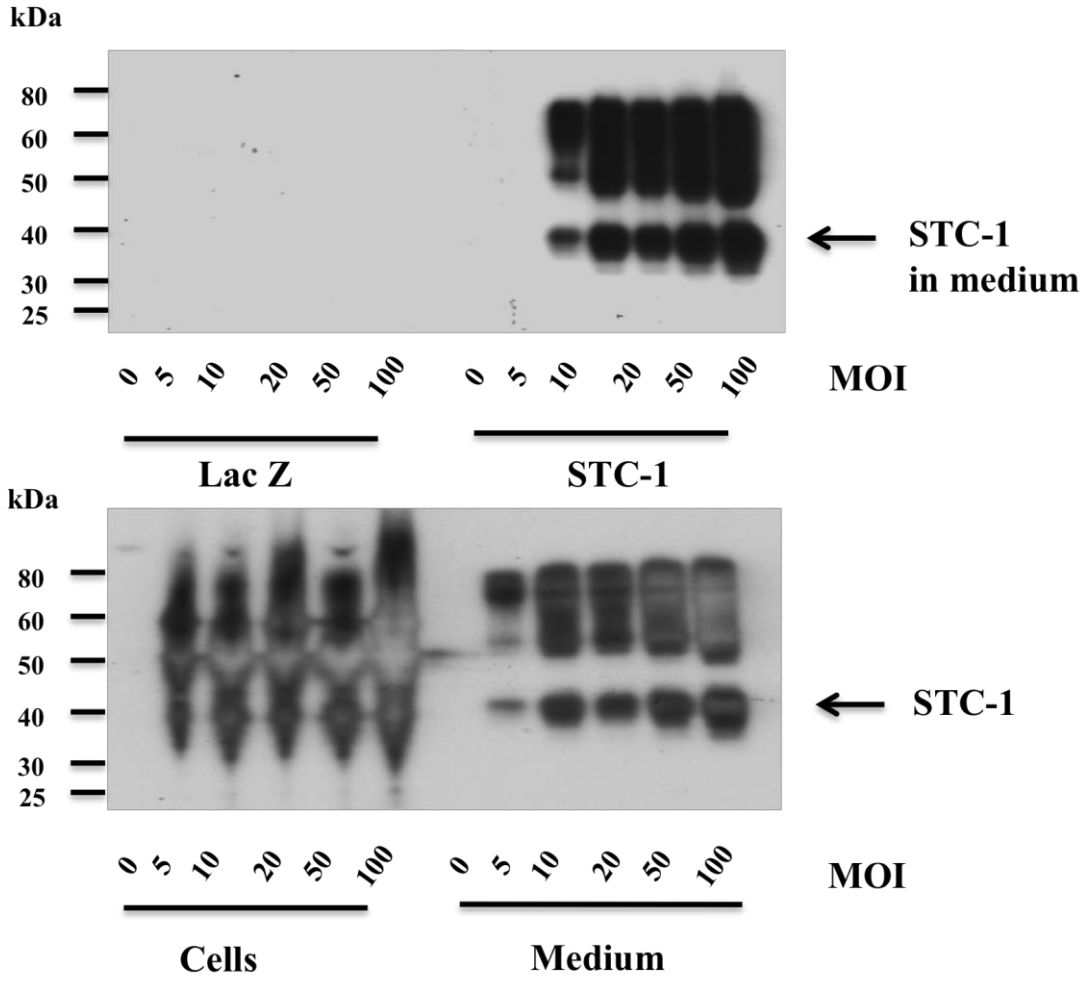

Figure 5.6: Overexpression of STC-1 using adenoviruses

HCASMCs were incubated with adenovirus encoding wild-type STC-1 and contol Lac Z at the indicated multiplicity of infection (MOI) for 48h. Cells were then lysed and supernantant were collected and prepared for immunoblotting. Position of STC-1 is indicated by the arrow based on it molecular weight around 40 kDa, whereas the upper band could be unspecific interaction due to the viral expression. Data are from a single experiment.

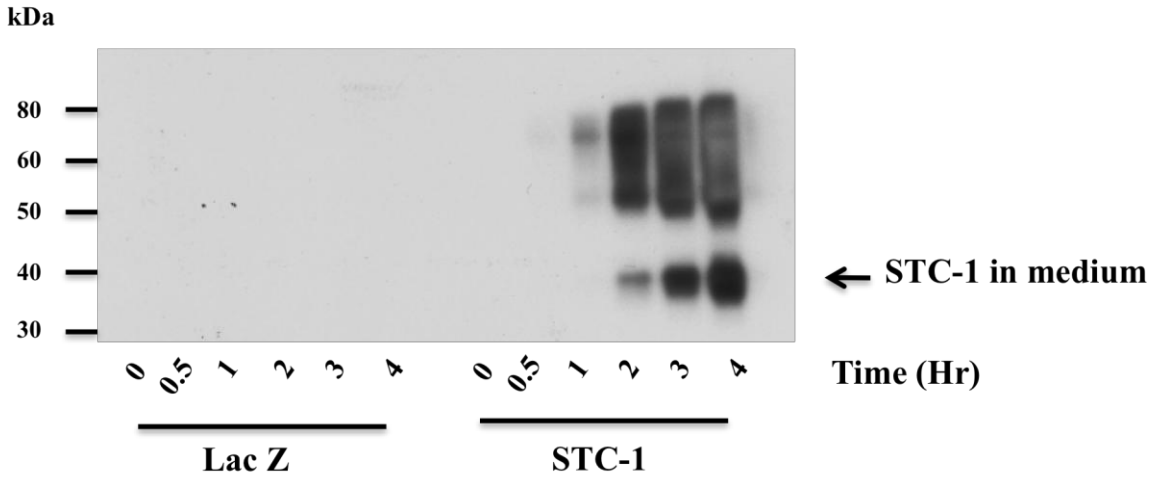

Figure 5.7: Time dependent overexpression of STC-1

CASMCs were incubated with adenovirus encoding STC-1 or Lac Z at an MOI of 10 for 48h. Serum- deprived cells were washed two times with serum free medium and supernantant samples were collected at the indicated times and immunoblotted and probed with anti-STC-1 antibody. Data are representative from two independent experiments.

To determine the involvement of overexpression of STC-1 in PDGF-induced Akt pathway, I initially investigated whether adenovirus infection would have a non-specific effect on Akt phosphorylation in SMCs. This preliminary experiment was performed because I had seen that adenoviral infection had increased basal Akt phosphorylation in endothelial cells (Chapter 3, Fig. 3.5). I observed that both Ad.Lac Z and Ad.STC-1 infection resulted in a MOI-dependent effect on Akt phosphorylation in the absence of ligand stimulation. It could be argued that Ad.STC-1 had a slightly greater effect than Ad.LacZ at lower MOIs, perhaps indicating that STC-1 does enhance Akt signalling.

However the increase in basal Akt phosphorylation upon viral infection made it difficult to assess the effect of STC-1 overexpression on PDGF-stimulated Akt phosphorylation. Therefore adenovirus-mediated expression of STC-1 was not further used as an approach to examine the role of STC-1 in PDGF-stimulated Akt phosphorylation enhancement. It should be noted that this particular observation has been observed previously in this laboratory in work with other adenoviruses generated by the same methodology as the ones used in this study.

5.4 Role of STC-1 in PDGF-stimulated cell migration

After observing the synergistic effect of STC-1 in PDGF-mediated Akt pathway activation, I decided to study the effect of STC-1 in PDGF-stimulated SMC migration which is considered an important process in the abnormal accumulation of SMC associated with atherosclerosis, restenosis, graft stenosis and other vessel wall pathologies.

5.4.1 Effect of hrSTC-1 on PDGF-induced cell migration

Initially, the involvement of STC-1 in SMC migration was examined with different concentrations of hrSTC-1. Since STC-1 was consitituitvely produced by SMC, I hypothesized that STC-1 was unlikely to act as a chemotactic factor by itself, but may have a regulatory role in SMC chemotaxis. Therefore I examined initially whether addition of STC-1 alone to SMC could affect SMC migration. These initial studies

showed that STC-1 by itself was not able to cause any effect on SMC migration (Fig 5.9). In contrast, PDGF-BB stimulation resulted in a significant activation of SMC migration by nearly 6 fold (Fig. 5.9).

To assess the role of STC-1 in PDGF-stimulated migration, CASMCs were pre-treated with different concentrations of hrSTC-1 and then allowed to migrate towards PDGF-BB or vehicle in transwell assays (Fig. 5.10).

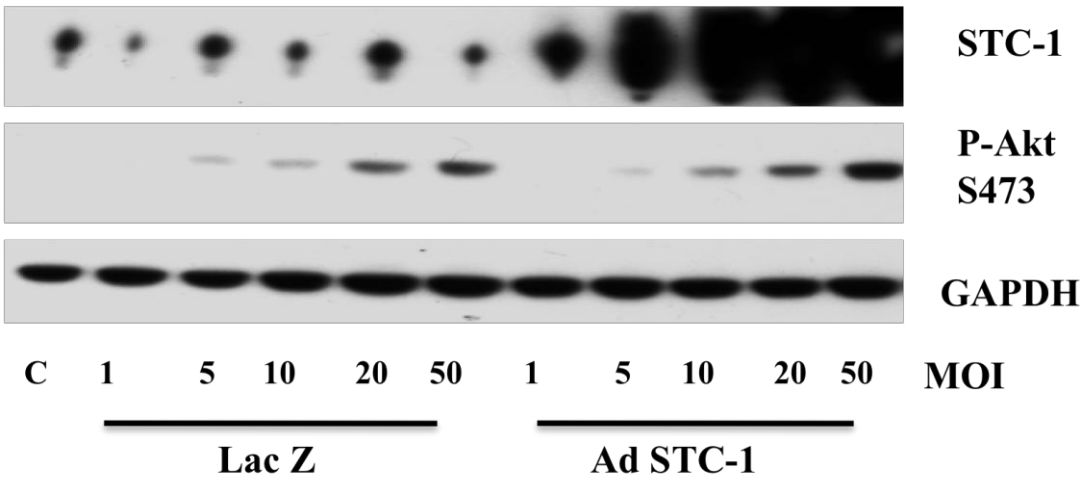

Figure 5.8: Adenoviral overexpression of STC-1 and lac Z increases the phosphorylation of Akt kinase in CASMCs

CASMC were infected with Ad.STC-1 or Ad.Lac Z using different MOI as indicated. 48 hours after infection, cells were harvested and immunoblotted and probed with the indicated antibodies. Supernantants were concentrated and blotted for STC-1. The results are representative of at least 3 independent experiments yielding similar results.

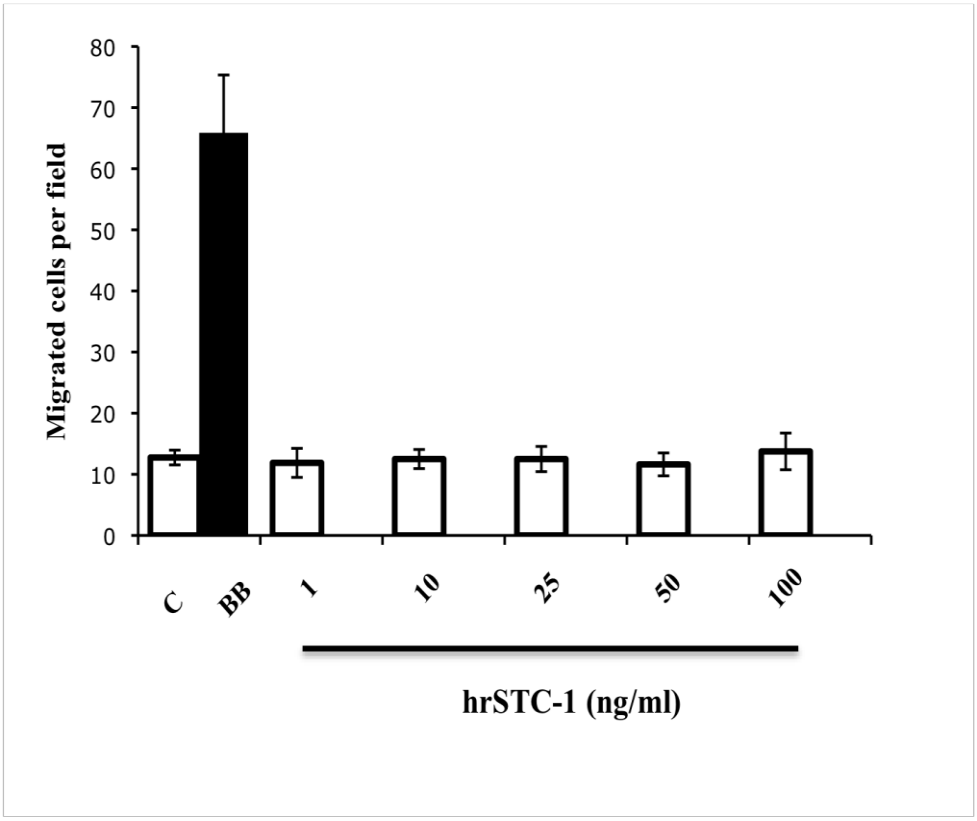

Figure 5.9: CASMC migration in response to PDGF-BB and STC-1

CASMC cells were trypsinised and treated for 30 minutes with hrSTC-1 at the indicated concentrations and then seeded into a transwell migration assay chamber and allowed to migrate at 37°C for 4 hours. In parallel, other cells were allowed to migrate towards 30ng/ml PDGF-BB or no addition (serum free) in the lower chamber and the cells were allowed to migrate at 37°C for 4 hours. Then the cells that migrated were fixed, stained and assessed by counting. The results are means +/- SEM obtained from three independent experiments.

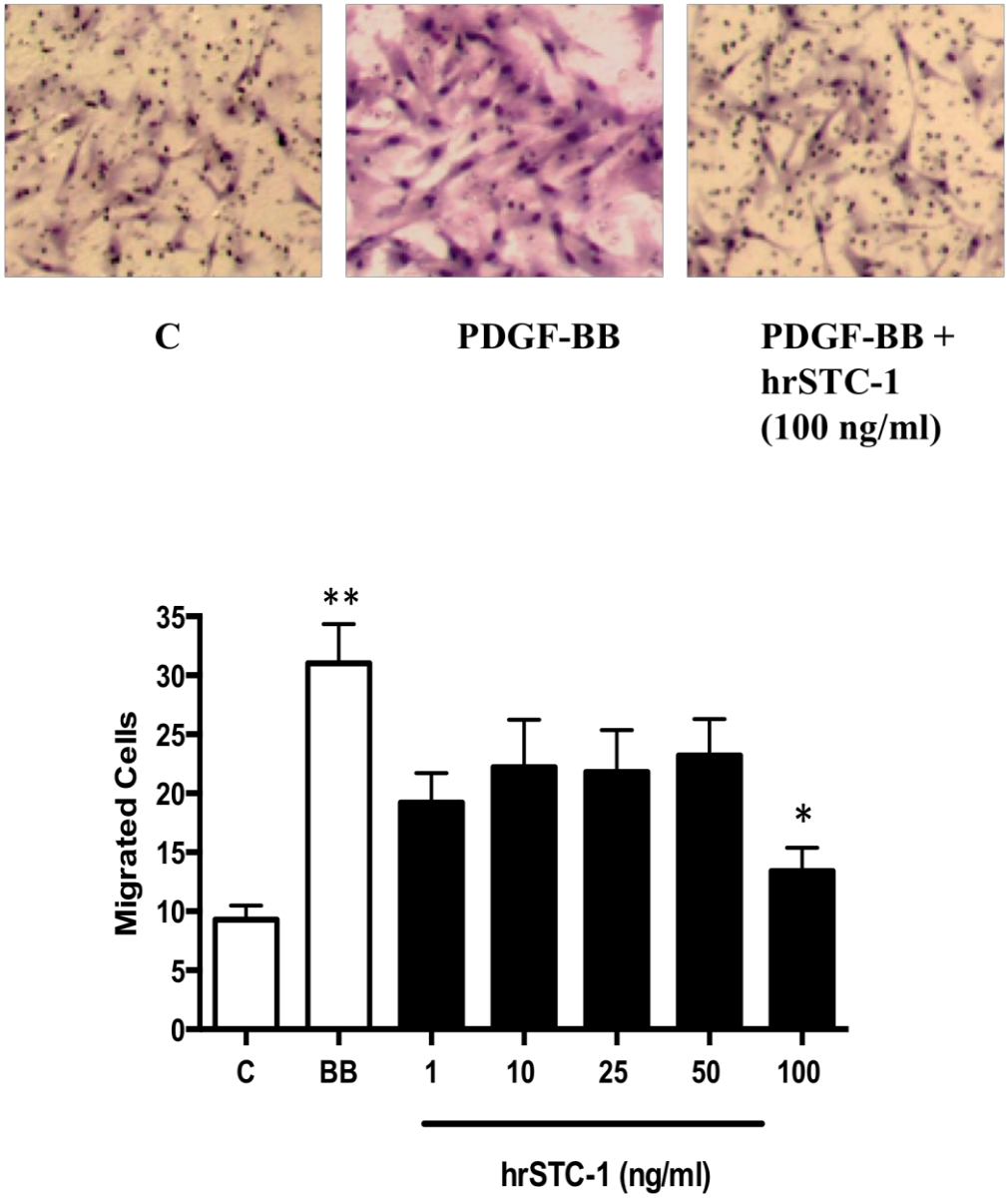

Figure 5.10: hrSTC-1 reduces CASMC migration in response to PDGF-BB

CASMC cells were pre-incubated in suspension with hrSTC-1 at the indicated concentrations and then transferred into transwell migration assay chamber. Migration of the cells was assessed towards 30ng/ml PDGF-BB or no addition (SF) at 37°C for 4 hours. Then the cells that migrated were fixed, stained and counted. Data are from four independent experiments. Analysis of PDGF-BB-stimulated migration by two-way ANOVA indicated a significant reduction by 100ng/ml hrSTC-1 treated cells compare to the PDGF-BB only treated cells, PDGF-BB vs 100ng/ml hrSTC-1, * P=0.01 and SF vs PDGF-BB, ** P=0.001.

The data showed that hrSTC-1 concentrations ranging between 1-50ng/ml had little effect on PDGF-induced CASMCs migration, but the higher dose of 100ng/ml resulted in a significant reduction in the number of cells that migrated towards a 30ng/ml PDGF gradient compared to cells that were not pretreated with STC-1 (Fig. 5.10). It should also be noted that this is strikingly similar to the effect of 100ng/ml STC-1 on VEGF-mediated migration in endothelial cells (Chapter 3; Fig. 3.2).

5.4.2 Effect of STC-1 knockdown on PDGF-stimulated SMC migration

A role for STC-1 in PDGF-stimulated cell migration was also examined by depleting STC-1 in CASMCs with siRNA and assessing the migratory response towards the chemoattractant, PDGF-BB. Transfection of cells with siRNAs targeted to STC-1 caused a large reduction in the amount of STC-1 secretion by these cells (Fig. 5.11A). STC-1 knockdown also caused a trend towards reduced migration of SMCs to PDGF but this was not statistically significant (Fig. 5.11 B).

To check the consistency of the results of siRNA transfection experiments on PDGF-mediated migration, a second siRNA was used. STC-1 knockdown using the second STC-1 siRNA showed a similar effect to the first STC-1 siRNA, with a small trend towards decreased migration, but no significant effect on PDGF-stimulated migration of STC-1 compared with non-targeted siRNA (Fig. 5.12).

5.4.3 Effects of adenoviral overexpression of STC-1 on migration

The role of STC-1 in PDGF-stimulated cell migration was further examined by infection of CASMCs with adenoviruses to overexpress STC-1 or the control, Ad.Lac Z. Adenovirus-mediated overexpression of STC-1 had no effect on the migratory response of SMCs in the presence or absence of PDGF chemoattractant, compared to Lac Z virus-infected cells (Fig. 5.13).

5.5 Involvement of STC-1 in SMC survival

Another key biological role for Akt is in pro-survival or anti-apoptitic cell signaling. The role of STC-1 in SMC survival was assessed by pre-treatment with hrSTC-1 followed by PDGF-BB or no treattment.

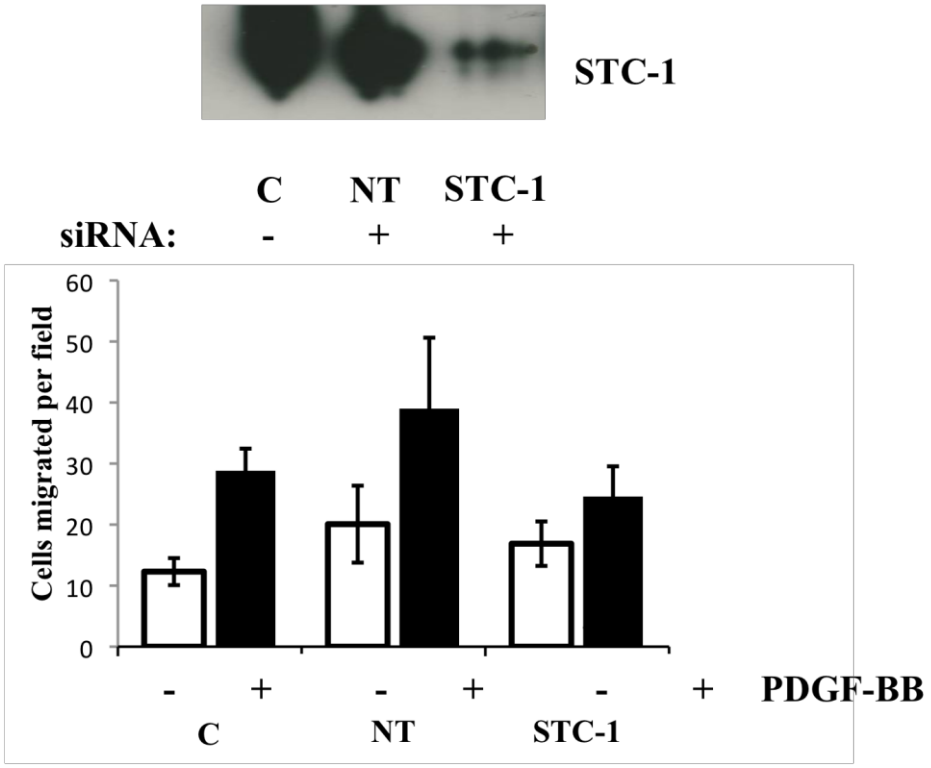

Figure 5.11: Effect of STC-1 knockdown on PDGF-BB-mediated cell migration

A. CASMC cells were transfected with STC-1 siRNA or a non-targeting control siRNA (NT) or no treatment (Control). After 48 h, cells were trypsinised and SMC migration was assessed in a transwell assay using 30ng/ml PDGF-BB or no addition (-) as the chemoatttractant. Data are from three independent experiments. Analysis of PDGF-BB- stimulated migration by one-way ANOVA indicated no significant effect achieved by STC-1 siRNA treated cells compared to the control siRNA. **B.** Representative blot showing STC-1 knockdown achieved by STC-1 siRNA transfection. Concentrated supernantants from the media were immunoblotted with antibodies against STC-1.

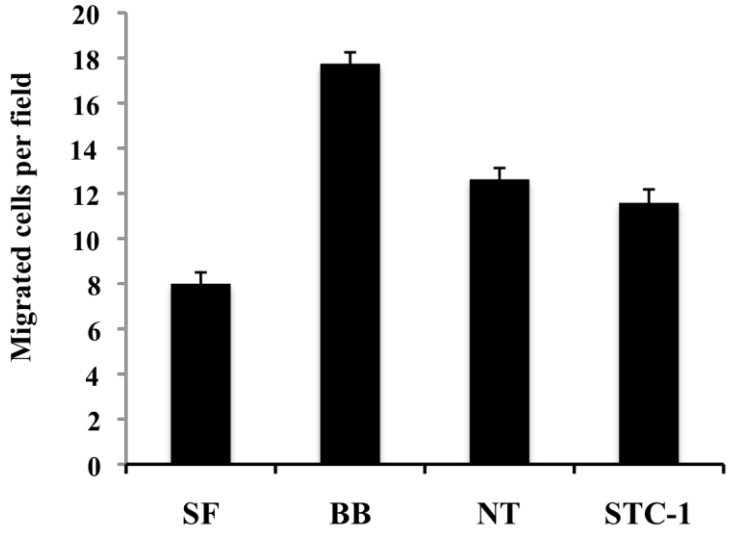

Figure 5.12: Effect of STC-1 knockdown by using a second siRNA on PDGF-BB-mediated cell migration

CASMC cells were transfected with a second STC-1 siRNA or a non-targeting control (NT) or no treatment (SF & BB). After 48 h, cells were trypsinised and migration was assessed in a transwell assay using 30ng/ml PDGF-BB or no addition (SF) as the chemoatttractant. Data are from three independent experiments. Analysis of PDGF-BB-stimulated migration by one-way ANOVA indicated no significant effect achieved by STC-1 siRNA treated cells compared to the control siRNA.

Apoptosis was induced by treatment with 1 mM hydrogen peroxide (H_2O_2) and the number of viable cells was assessed using the XTT (sodium 3′- [1-(phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy- 6-nitro) benzene sulfonic acid hydrate) viability assay.

H_2O_2 decreased the number of viable SMC, an effect that was not altered in the presence of PDGF-BB (Fig. 5.14). Treatment with STC-1 produced a modest trend towards a reduction in cell viability, while a combination of STC-1 and PDGF-BB, although this was not statistically significant. In addition treatment with a combination of STC and PDGF-BB produced a more prominent increase in cell viability following H_2O_2 treatment.

5.6 Discussion

The data reported in Chapter 4 indicated that PDGF stimulates STC-1 expression in SMCs via a possible PDGFRα-mediated pathway. In this Chapter, the role of STC-1 in PDGF- stimulated downstream signalling cascades and associated biological functions were examined, to further understand the biological relevance of PDGF-stimulated STC-1 upregulation in SMC.

Human recombinant (hr) STC-1 protein was used to explore whether STC-1, as a secreted factor following PDGF stimulation in SMC, has an impact on PDGF-induced downstream signaling. The major finding of this chapter is that treatment of SMCs with hrSTC-1 significantly increased PDGF-BB stimulated Akt phosphorylation in a time and concentration-dependent manner. Since STC-1 had no detectable effect on the level of Akt activity, these findings indicate a synergistic activation of Akt in the presence of STC-1 and PDGF in SMCs.

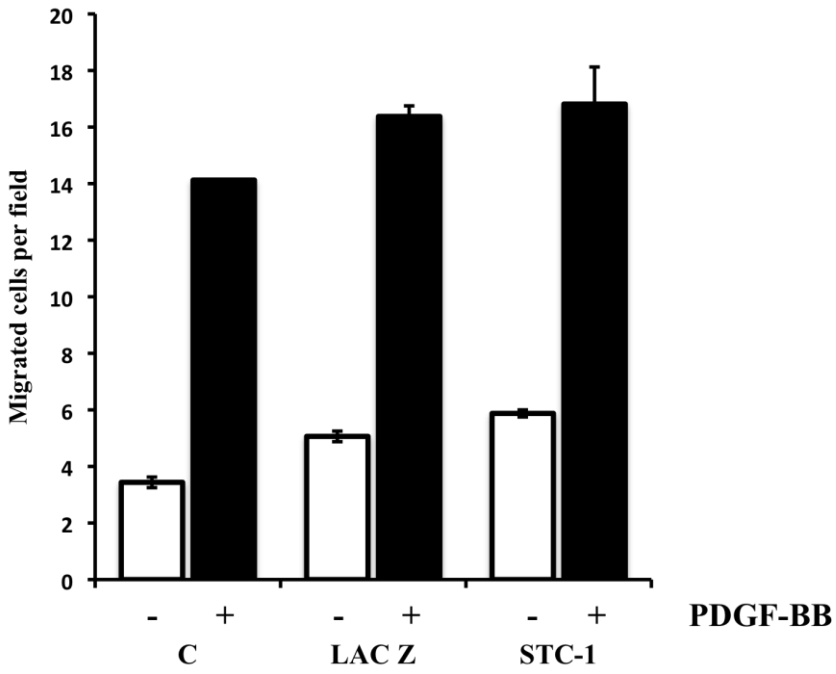

Figure 5.13: Effect of adenovirus-mediated overexpression of STC-1 on cell migration

CASMC were infected with either Ad.STC-1 or Ad.lac Z for 48 hr at MOI of 10. Uninfected, serum starved cells were trypsinised and added to the upper chamber of a transwell migration assay chamber. The supernantants of infected cells were collected and were added to the lower chamber with or without the addition of 30ng/ml PDGF-BB as the chemoattractant. After 4 hours, cells that migrated were fixed, stained and the number of migrated cells was assessed by counting. Data presented are mean +/- SEM numbers of migrated cells from three independent experiments.

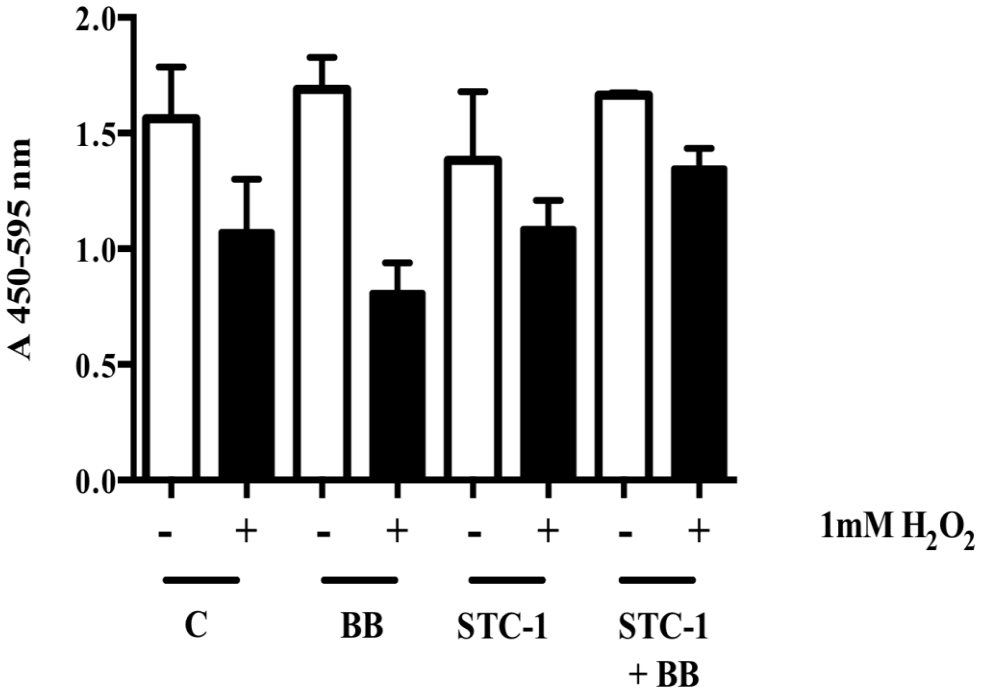

Figure 5.14: Effects of STC-1 on PDGF-induced survival

HCASMC were pre-treated with or without addition of 100ng/ml STC-1 for 30 mins followed by stimulation with or without 30ng/ml PDGF-BB for a further 60 mins. Cells were then incubated overnight in the presence (+) or absence (-) of 1mM H_2O_2 to induce apoptosis. Cells were then stained with XTT reagent. The absorbance of the formazan product, reflecting cell viability, was measured at 595 nm. Results are mean +/- SEM obtained from three independent experiments.

Akt, a serine-threonine protein kinase, is the most crucial downstream mediator of the RTK-PI3K complex. The family of Akts has been found to have key regulatory roles in many cellular processes in SMC including normal cell growth, cell cycle progression, and cell survival (Allard et al., 2008; Shiojima & Walsh, 2002). PDGF-dependent activation of PI3K/Akt pathway has also been reported to regulate cell motility (Millette, Rauch, Kenagy, Daum, & Clowes, 2006). Constitutive activation of the PI3K/Akt pathway has been shown to inhibit apoptosis in various cell types and blocks programmed cell death induced by a variety of apoptotic stimuli (Shiojima & Walsh, 2002). The mechanism by which Akt protects cells from apoptosis is complex, since Akt once activated, directly phosphorylates several components of the cell-death machinery such as Bad, caspase 9, GSK3 and members of the FoxO family (Allard et al., 2008). For example, one consequence of Akt activity is phosphorylation of Bad, a pro-apoptotic member of the Bcl-2 family of proteins, which dissociates the heterodimeric complex formed with the survival factor Bcl-xl thereby restoring the anti-apoptotic function of Bcl-xl. Allard et al. (2008) demonstrated that vascular SMC survival in response to a physiological death stimulus is dependent on Akt signalling. Akt-mediated phosphorylation of GSK-3 at serines 21 or 9 causes inhibition of GSK3, and this can therefore prevent the promotion of apoptosis by GSK3-mediated activation of p53 or inactivation of survival factors. However, it is important to recognise that GSK3 may both promote and inhibit apoptosis, and its role in this process is controversial (Beurel & Jope, 2006). The effect of STC-1 on PDGF-BB-induced Akt activation prompted me to further investigate whether this effect extended to downstream effectors such as GSK3. Results presented in this chapter show that STC-1 also enhanced the stimulation of GSK3 phosphorylation by PDGF. These results suggest that the synergistic effect of STC-1 on PDGF-stimulated Akt phosphorylation may also enhance GSK3 inhibitory loop activation by Akt. In contrast to effects on the Akt pathway, there was some indication in my data that the phosphorylation levels of p38 in response to PDGF-BB were attenuated as result of STC-1 pre-treatment. However, this observation requires more work to verify that this was a statistically significant effect. Nevertheless, it is of interest that there are several examples in the literature of crosstalk between the p38 and Akt pathways (Berra, Diaz-Meco, & Moscat, 1998; Gratton et al., 2001).

Knockdown of STC-1 via siRNA had no significant effect on PDGF-stimulated Akt phosphorylation, although a noticeable trend towards inhibition was seen compared to the control non-targeted siRNA. The lack of a significant effect after STC-1 knockdown may be due to a number of reasons. Firstly, if endogenous STC-1 production is to some degree involved in mediating PDGF-bb-induced Akt activation, the degree of knockdown may not have been sufficient to affect PDGF-stimulated Akt activation. Alternatively, STC-1 may simply not be required at all for activation of Akt by PDGF-BB alone, but can only enhance activity.

High STC-1 expression in the tumor vasculature and under hypoxic conditions may have protective and regulatory roles in cell survival (Lal et al., 2001; H. Y. Yeung et al., 2005). Overexpression of STC-1 protein has also been associated with several cancers including ovarian cancer, but no essential function has been pinpointed (G. Liu et al., 2010). In addition, the effects of STC-1 on metabolic rate and uncoupling of oxidative stress suggest that STC-1 may be involved in delaying the onset of hypoxia-induced apoptosis in human neuronal cells (Zhang et al., 2000). Greater cellular phosphate uptake by neuronal cells could reduce the rise in intracellular calcium caused by hypoxia, with an anti-apoptotic effect. In light of these data, it is possible that effects of STC-1 on respiration and mitochondrial calcium accumulation could be part of a pro-survival pathway in metabolically active, mitochondria-rich cells (Ellard et al., 2007; Zhang et al., 2000). Recent data shows that overexpression of STC-1 is associated with increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl, which leads to the inhibition of cleavage of procaspase-3, -8 and -9 (G. Liu et al., 2010). However, my results suggest that in SMCs, STC-1 is involved in the enhancement of phosphorylation of Akt and the Akt effector, GSK3, by PDGF. However, more work is required to validate this conclusion. It would be interesting, for example to examine effects of STC-1 on PDGF-BB-induced Akt activation in other cell types. I would also like to examine effects of STC-1 on a wider range of Akt substrates. Furthermore, the mechanism through which STC-1 regulates PDGF-BB-induced Akt activation is unclear. My working hypothesis is that STC-1 binds to a specific cell surface receptor in SMC, which is able to mediate a synergistic interaction with PDGF-BB to enhance Akt signaling at some point proximal to Akt in the signaling cascade. One possibility is that STC-1 might enhance PDK-mediated phosphorylation of ser 473. Another possibility is

that STC-1 acts synergistically at the level of PI3K activation. It would be important to test these possibilities. The PDK responsible for phosphorylation of Akt ser 473 is unknown, but it would be possible to examine whether STC-1 can enhance phosphorylation of the p85 subunit of PI3K. At present a receptor for STC-1 has not been molecularly identified in any organism and this will be discussed in Chapter 6. However, a possible alternative to a mechanism mediated via a specific STC-1 receptor is that STC-1 might interact with PDGF-BB and/or with PDGFRs to enhance PDGF-BB interaction with its receptor in a way that can selectively enhance Akt activation. It is not clear how this might occur, however.

Synergistic enhancement of Akt activity by STC-1 could shift the balance between antiapoptotic and proapoptotic signalling in favour of decreased cell death. Though I was unable to show a statistically significant effect of STC-1 on SMC viability, a trend towards a synergistic effect of STC-1 in combination with PDGF-BB was observed. These data would need to be confirmed using different assays of apoptosis, eg using other markers of apoptosis such as caspase activation, or FAK cleavage, TUNEL staining, or FACS analysis of annexin V staining. One problem I encountered in this aspect of the work is that cultured SMC are quite resistant to apoptosis. For example, while serum withdrawal is a strong stimulus for apoptosis in HUVECs, it has little effect on SMC death. On the other hand, pharmacological inducers of cell death using, for example, staurosporine, etoposide, or anisomycin, would have such powerful effects that it might be difficult to show a survival effect of STC-1.

Broadly in support of my data, several reports in the literature have shown a significant role for STC-1 in survival or protection against supeoxide generation through induction of mitochondrial uncoupling proteins (Huang et al., 2012; Y. Wang et al., 2009). Liu et al. (2010) examined the association of STC-1 expression in ovarian cancer cells and apoptosis using annexin V fluorescence apoptosis assay. Retroviral-mediated overexpression of STC-1 appeared to lower the percentage of apoptotic cells rather than in corresponding control cells, suggesting that STC-1 may have anti-apoptotic activity. Whilst, in ovarian xenograft model in nude mice, siRNA-linked STC-1vectors caused nearly twice the percentage increase in apoptosis than in tumors derived from corresponding control cells (G. Liu et al., 2010). Other cell types such as mesenchymal

stem cells, secrete STC-1, and studies have demonstrated that the secretion of STC-1 from these cells reduces ROS- or UV irradiation - stimulated cell death in a co-culture system (Block et al., 2008; Ohkouchi et al., 2012). Furthermore, the addition of STC-1 recombinant protein into A549 cells exposed to 100 μmol/l H_2O_2 resulted in the restoration of ROS to control levels by increasing anaerobic glycolysis together with UCP_2 expression, while siRNA-mediated STC-1 knockdown inhibited cytoprotection of H_2O_2-injured cells (Ohkouchi et al., 2012). However, the underlying mechanism by which STC-1 enhances excessive anaerobic glycolysis and protection during the oxidative response it is still unclear (Ohkouchi et al., 2012). Other studies suggest that the molecular mechanism of ROS protection provided by STC-1 is a result of induced UCP_2 expression and decreased protein carbonyl and nitrotyrosine levels which are generated by ROS (Roddy et al., 2012; Y. Wang et al., 2009). Additionally, STC-1 has been reported to be neuro- and renal-protective (Huang et al., 2009; Zhang et al., 2000), and upregulated in ischemic preconditioning (Westberg et al., 2007), observations that may or may not be explained by its multiple effects in lowering ROS generation. Recently, UCP_2 has been implicated as a regulator of phagocytic engulfment of apoptotic cells, and data have shown an essential role for UCP_2 in apoptotic cells clearance *in vivo* (D. Park et al., 2011). However, it is not yet known if STC-1 is involved in phagocytosis of apoptotic cells. It is also unknown whether STC-1 regulates UCPs in SMC. I examined this question during the course of my thesis, but did not see any effect of STC-1 on UCP_2 expression in SMC cultures (Fig. 5.2). I also tried to examine the effect of UCP_2-targeted siRNA on the response of SMC to STC-1, but was unable to obtain effective UCP_2 knockdown (results not shown). Therefore, I did not pursue the role of UCP_2 further.

In contrast to the idea that STC-1 might have an anti-apoptotic effect, there are data indicating that STC-1 has a negative effect on pro-survival signalling pathways and/or has a positive effect on cell death signalling. For example, (Nguyen et al., 2009) demonstrated that treatment of wild type mouse embryo fibroblasts with H_2O_2 induced STC-1 expression 40-fold and reduced their survival much more than did a similar treatment of *STC-1*$^{-/-}$cells. Moreover, their data showed a role for STC-1 in the oxidative stress response suggesting that STC-1 functions in a negative feedback loop to regulate MEK and ERK1/2. However, the precise mechanism by which STC-1 inhibits

MEK activation and mediates apoptosis is not known and requires further investigation (Nguyen et al., 2009). In addition, V5-tagged pLenti6.3/ V5-DEST overexpression of exogenous STC-1 was found to sensitize histone deacetylase inhibitor, (Dox)-induced cellular apoptosis induced by doxorubicin, an important class of anticancer agent, also suggesting a pro-apoptotic role of STC-1 in human nasopharyngeal carcinoma cell line CNE2 (Ching, Yeung, & Wong, 2012).

I would like to have complemented studies of effects of recombinant STC-1 on Akt activity with results from adenovirus-mediated STC-1 overexpression. However, this was not practical because both Ad.Lac Z and Ad.STC-1 consistently increased Akt phosphorylation, indicating that increased Akt activity is a possible consequence of adenovirus transduction in these cells. The basis of increased Akt phosphorylation in adenovirally infected cells is unclear, but might involve the triggering of intracellular protective machinery, which consequently alters cellular behaviour.

Results in this chapter also showed that treatment of SMCs with 100ng/ml hrSTC-1 significantly decreased PDGF-stimulated SMCs migration. As previously observed in Chapter 3, 100 ng/ml of STC-1 also partially inhibited HUVEC migration in the presence of VEGF, suggesting a common mechanism of action for STC-1 regulation of migration in these different cell types. However, adenovirus-mediated overexpression of wild type STC-1 did not significantly alter PDGF-induced SMCs migration when compared to control Lac Z adenovirus. Again, this is similar to the results observed in HUVECs and may indicate that the cells are not responsive to intracellular STC-1, or that adenoviral ATC-1 overexpression does not produce sufficient concentrations of STC-1 to have an effect. Studies with two different siRNAs showed that STC-1 knockdown had no significant effect on PDGF-stimulated migration, although a detectable trend towards inhibition was seen with both siRNAs examined.

The importance of STC-1 in PDGF-stimulated migration is not clear, but other results also suggest a regulatory role of STC-1 in cell migration. Using the human keratinocyte model HaCa T cells, (B. Yeung & Wong, 2011) showed that STC-1 knockdown was found to significantly inhibit cell migration, while V5-tagged STC-1 overexpression significantly enhanced migration towards staurosporine, a non-specific kinase inhibitor. However, it is widely know that staurosporine is used as an apoptotic agent, and its

effect on dephosphorylation of a key migratory kinase, FAK, could contribute to the overall results (Kabir, Lobo, & Zachary, 2002). Furthermore, STC-1 overexpression in the unstimulated cells failed to induce cell migration.

The reasons behind the lack of statistically significant results, obtained with STC-1 knockdown and STC-1 overexpression in PDGF-induced migration, could be the result of a low number of repeated experiments. A higher number of experiments might have produced more convincing results that could have confirmed a functional distinction between control cells and cells treated with recombinant STC-1 or with STC-1 knockdown or over-expression. Other data reported in this chapter, such as increased GSK3 phosphorylation as result of a synergistic effect of STC-1 and PDGF-BB treatment, showed a trend for an increase in phoshorylation at Ser 21 for alpha and Ser 9 for beta isoforms. A greater statistically significant effect could have been obtained if more experiments had been performed in order to address variation between experiments due to other factors like differences between cell batches and culture conditions.

Overall, the results obtained in this chapter indicate that STC-1 can enhance PDGF-BB-stimulated Akt activity via an unknown mechanism, an effect which other data produced in this chapter suggests but does not prove, may mediate cytoprotection after a H_2O_2-induced insult. Other data in this chapter shows that STC-1 at 100 ng/ml also partially inhibits PDGF-BB-induced chemotaxis.

Chapter 6

6 Discussion

After its initial discovery as a major hormone produced by the corpuscles of Stannius (CS) in bony fish, STC-1 was identified in humans by mRNA differential display of genes involved in the control of cellular proliferation and by a random sequencing screen of a human fetal lung cDNA library (Chang et al., 1995; Olsen et al., 1996). It is well-established that STC-1 acts as an anti-hypercalcaemic hormone in teleost fish and it was anticipated that the mammalian homologue might similarly play a role in calcium and/or phosphate homeostasis. Indeed, STC-1 can regulate Ca2+ fluxes in mammalian cells. However, there appears to be no physiological role of STC-1 in mammalian growth or calcium or phosphate homeostasis because STC-1 deficient mice have no overt phenotype and display no alteration in serum calcium and phosphate (Chang et al 2005). STC-2-deficient mice display a 10-15% increase in body weight compared with littermate controls, but this was independent of pituitary function or effects on the growth hormone/IGF-I axis, and was not associated with changes in serum calcium or

phosphate, or changes in bone development (Chang et al., 2008). Therefore the function of STCs in mammals remains an enigma. Furthermore, human STC-1 is implicated in several physiological and pathological processes in mammals including angiogenesis, and responses to ischemia and stress (Chang et al., 2003). The precise involvement of STC-1 in these systems is still unclear and remains to be further studied. However, work by our group and others have indentified STC-1 as an immediate-early VEGF-regulated gene in endothelial cells (Bell et al., 2001; Holmes & Zachary, 2008; D. Liu et al., 2003; Zlot et al., 2003). It was also demonstrated that FGF-2 synergistically enhanced STC-1 mRNA and protein expression induced by VEGF-A_{165} (Holmes & Zachary, 2008). These findings highlighted the possible role of STC-1 upregulation in pathophysiological neovasculation and/or as an important regulatory factor in the angiogenic milieu.

The studies presented in Chapter 3 were designed to examine the possibility that STC-1 could regulate endothelial functions of the key angiogenic cytokine, VEGF, centrally involved in angiogenic responses such as cell migration and tubulogenesis, and major VEGF signaling pathways. The results obtained indicated little effect of modulating STC-1 levels in assays of migration, cell signaling and tubulogenesis using recombinant STC-1, adenoviral over-expression or targeted siRNA-mediated knockdown. The one exception was an inhibitory effect on VEGF-induced migration at 100ng/ml (discussed in Chapter 3.6). Given the largely negative data presented here it could be concluded that STC-1 has little or no effect on endothelial cells. Nevertheless, there is considerable evidence that STC-1 does influence endothelial function. For example STC-1 increases intracellular Ca^{2+} levels in quiescent HUVECs (Chakraborty et al., 2007). The mechanism involved was not explored, however a similar effect was observed in epithelial tumour cell lines and STC-1 in these cells was found to act via modulation of purinergic signaling (Block, DiMattia, & Prockop, 2010). As already discussed, Chen et al 2008 reported that STC-1 inhibited TNF-α induced endothelial permeability, suggesting a new role for STC-1 in maintaining an anti-inflammatory endothelial barrier, and this finding is in broad agreement with the findings of Chakraborty et al., 2007 who showed that STC-1 inhibited monocyte transmigration through a HUVEC monolayer (Chakraborty et al., 2007). Indeed it seems that STC-1 may have a more general role in inhibiting macrophage and leucocyte migration (C. Chen et al., 2008; Kanellis et al., 2004). Furthermore, despite the redundancy of STC-1 in mouse

development, surprisingly little work has been performed on the role of STC-1 in zebrafish development. One study tried to investigate the effect of STC-1 in zebrafish using STC-1 morpholino-mediated knockdown, and demonstrated that zebrafish STC-1 regulates Ca^{2+} transport via negatively effecting the expression of gill epithelial Ca channels to reduce Ca^{2+} uptake in zebrafish embryos (Tseng et al., 2009). However, it would also be interesting to examine the effects of STC-1 and/or STC-2 morpholino knockdown on zebrafish embryonic vascular development using the fli-GFP fish line, in which jellyfish green fluorescent protein (GFP) expression is under the control of the endothelial cell-specific *fli* promoter. It is also possible that STC-1 may have a more specific role in mammalian angiogenesis in disease-related situations such as cancer. For example, high expression of STC-1 in the tumour vasculature and under hypoxic conditions has been suggested to have protective and regulatory roles in cell survival (Lal et al., 2001; H. Y. Yeung et al., 2005).

Overall however, my data suggest that although VEGF regulates endothelial STC-1 expression, STC-1 regulates endothelial function largely through non-VEGF mediated pathways, or else regulates other VEGF functions not examined in this chapter. However, the data presented here indicates that STC-1 is not a pro-angiogenic signal but may instead act to maintain newly formed vessels or in the functions of mature vascular endothelium, or endothelial functions under conditions of stress. More work is required to test these hypotheses and investigate in more detail the mechanisms by which STC-1 exerts its effects on endothelial cell biology.

A major novel finding of this thesis is that STC-1 mRNA and protein expression is also induced by PDGF-BB in human arterial SMC, an effect mediated at least partly via PDGFRα. STC-1 protein is also expressed constitutively in SMC at a significant level. I also showed that STC-1 secreted by SMC is post-translationally modified by N-linked glyosylation. These findings add significantly to existing knowledge about the regulation of STC-1 expression, and have potential novel implications for the regulation of SMC function. However, a weakness of this part of the thesis is that I was unable to show strong inhibition of the effect of PDGF-BB on STC-1 protein expression by knockdown or pharmacological inhibition of PDGFRs, possible reasons for which were

discussed in Chapter 4. An alternative approach to examining the role of PDGFRs in STC-1 regulation would be to use mouse SMC from mice deficient in either PDGFRα or PDGFRβ, such as those generated by (French, Creemers, & Tallquist, 2008).

One possibility that I explored is that STC-1 could regulate functions mediated by PDGF-BB in SMC. PDGF-BB is a potent chemoattractant for SMC, and PDGF-BB expression is increased in endothelial tip cells. This chemotactic activity is thought to play an important role in biological functions of PDGF-BB such as recruitment of pericytes to newly sprouting capillaries, or of SMC in arteriogenesis, and in pathological processes such as atherosclerosis and restenosis after angioplasty which are charcaterised by abnormal accumulation of SMC in the neointima of affected arteries. The finding that STC-1 could significantly inhibit PDGF-BB-induced SMC migration is therefore potentially of interest as a regulatory mechanism in biological functions of PDGF-BB. It is unlikely that STC-1 plays a critical role in SMC recruitment to new blood vessels in development, given that STC-1 deficient mice have no noticeable phenotype. However, it was found that mice overexpressing STC-1 exhibit a 30-50% reduction in post-natal growth as well as reduced litter sizes that appear to be independent of any effect on pituitary function or production of Growth Hormone or IGF-I (Varghese et al., 2002). The cause of this effect of STC-1 over-expression in mice is unknown, but it is interesting to speculate whether it could be due to a negative regulatory effect of high levels of STC-1 on angiogenesis consistent with my observation. This idea is also supported by a previous study showing that STC-1 negatively regulates HGF-induced endothelial cell migration (Zlot et al., 2003). However, another recent study examined metastatic growth of colorectal carcinoma cell lines co-injected with either wild-type of STC-1-deficient fibroblasts derived from STC-1-/- mice. This paper showed that tumours grown together with STC-1-deficient fibroblasts exhibited fewer metastases compared with tumours grown with wild-type fibroblasts (Pena et al., 2013). Furthermore, these fibroblasts are responsive to PDGF-BB, and PDGF-BB induced STC-1 mRNA expression in them. These findings are more suggestive of a role of fibroblast-derived STC-1 in promoting tumour cell migration involved in metastatic spread, than a negative regulatory role. The role of STC-1 in regulating chemotactic effects of PDGF-BB in cells of mesenchymal origin (eg SMC, pericytes, fibroblasts) is a question that would need to be examined in further work

using animal models of angiogenesis and SMC migration, or of stromal cell interactions with tumour cells, and investigating the effects of STC-1 overexpression locally (using adenoviral over-expression) or in transgenically over-expressing mice. The functional role of STC-1 glycosylation is also unknown, but may be important for its secretion. This could be examined by identifying the main sites of N-linked glycosylation, and analysing their importance by over-expressing mutated forms of STC-1.

A key unanswered question is whether STC-1 acts via a cell-surface or other cellular receptor. There is strong evidence through analysis of ligand-receptor binding assays revealing the presence of STC-1 receptors on mitochondria and plasma membranes with ligand sequestration appearing to occur in a mitochondrial compartment in fish as well as in mammals (McCudden et al., 2002; Richards et al., 2012). It has not been explained, however, how extracellular STC-1 is able to enter the cell in order to bind mitochondrial receptors. Presumably some mechanism would need to exist to allow for STC-1 to enter the cell and cross the plasma membrane, but at present this is unclear. Furthermore, despite the accumulating evidence of a possible STC-1 receptor, which could serve as the transducer by which STC-1 could influence intracellular signalling and thus exert physiological responses, the STC-1 receptor has not yet been purified or molecularly characterized. Furthermore, the downstream signalling pathways regulated by STC-1 are essentially unclear. It remains attractive to suggest that STC-1 effects are mediated by specific cellular receptors. Technologies have been developed which could be useful in identifying STC-1 receptors, such as the ligand-based, receptor-capture (LRC) technology employing a trio-functionality reagent TRICEPTS for the subsequent capture of glycoprotein receptors on living cells and tissues (Frei et al., 2012). A simpler approach to identification of STC-1 receptors might be covalent cross-linking of radiolabelled STC-1 to binding components available at the cell surface. Such an approach was successful in initially identifying a novel cell surface binding component for VEGF, later found to be Neuropilin-1 using a combination of Con A Sepharose and VEGF-Sepharose affinity chromatography (Soker et al., 1998). It was not within the scope of this thesis to molecularly define the STC-1 receptor in endothelial cells or SMC, but this should be a major goal of future STC-1 research. It is of course possible that STC-1 acts in an entirely different way and lacks a specific high-affinity cell surface receptor. For example STC-1 might function in mammals as a circulating

protein, which might bind to plasma proteins, and is involved in protein transport in the circulation, or possibly lacks a defined function.

Another unanswered question is how STC-1 is able to alter specific intracellular signalling pathways. There is very little known about regulation of intracellular signalling by STC-1. Since PDGF-BB induced STC-1 expression in SMC, I asked whether this could regulate PDGF-BB-dependent signalling in SMC. My results showed that though STC-1 was not able to promote signalling through several major signalling cascades, it did significantly enhance PDGF-BB-induced Akt activation as determined by Akt phosphorylation at serine 473, and also enhanced PDGF-BB-induced phosphorylation of the Akt substrate, GSK3. My data also suggested that STC-1 might inhibit PDGF-BB-induced p38 kinase activity, though these data are ore preliminary. As discussed in chapter 5, how STC-1 might enhance PDGF-BB signalling through the Akt pathway is unclear, though I speculate that this is most likely due to some receptor-mediated effect of STC-1 which is able to synergise with PDGF-BB signalling upstream of Akt as depicted in Figure 6.1. The fact that STC-1 did not cause a general enhancement of PDGF-BB signalling, argues against this being a non-specific effect of STC-1, though this cannot be ruled out.

Positive regulation of Akt signalling by STC-1 suggests that STC-1 should also enhance cellular functions regulated by Akt, such as cell survival. In support of this possibility I was able show evidence for a synergistic survival effect of STC-1 and PDGF-BB in the increased SMC cell death induced by hydrogen peroxide, though this effect was not statistically significant. However, Akt is also implicated in cell migration, and STC-1 did not enhance PDGF-BB-induced cell migration. Further work should examine more fully the effect of STC-1 on the apoptosis/survival of SMC using a variety of assays and in response to a variety of apoptotic stimuli. In support of a role of STC-1 in promoting cell survival, and as mentioned above, high expression of STC-1 in some tumours, eg ovarian cancer, and under hypoxic conditions has been suggested to have protective and regulatory roles in cell survival (Lal et al., 2001; H. Y. Yeung et al., 2005).

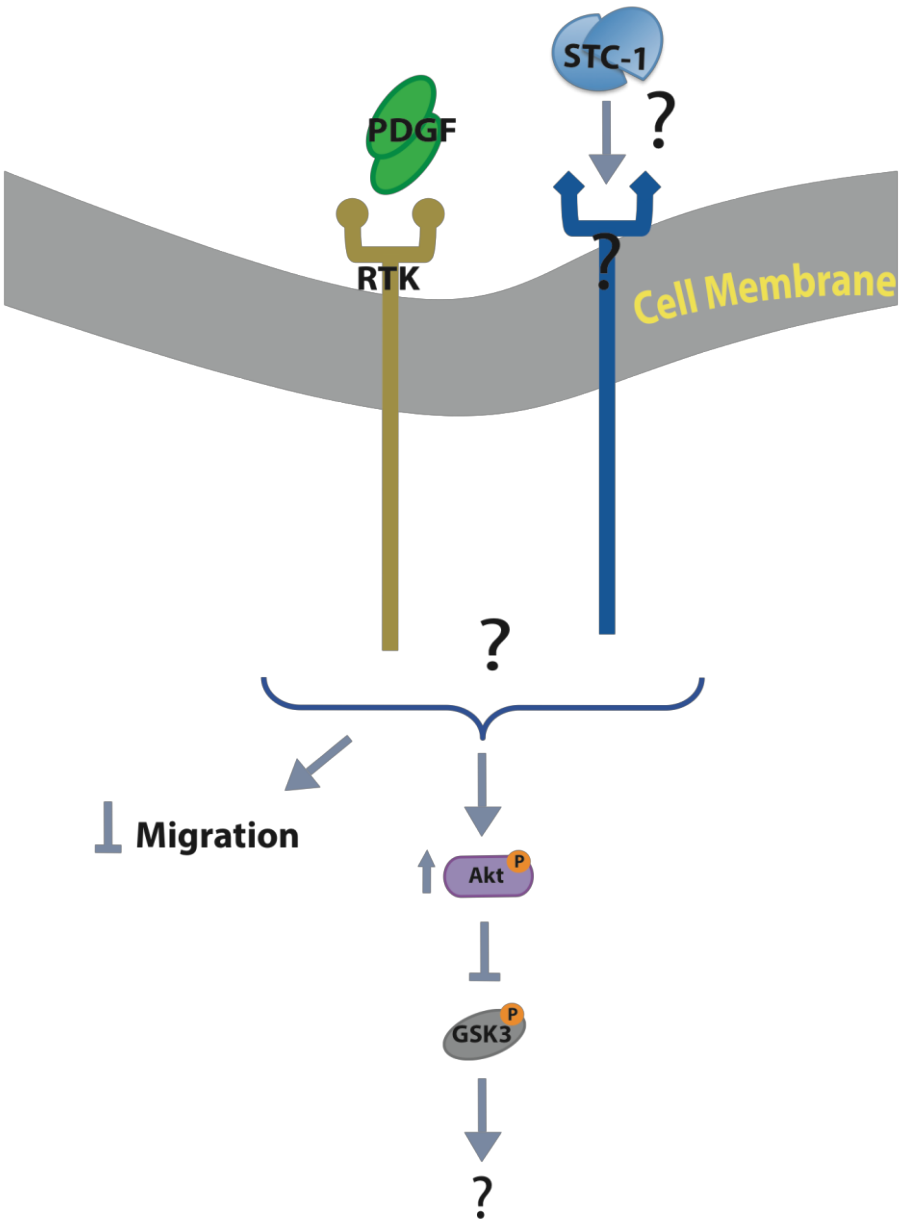

Figure 6.1: Proposed model for STC-1 function in smooth muscle cells

Schematic diagram of STC-1 signalling pathway highlights some aspects of extracellular secreted STC-1 role in SMCs. We hypothesis that STC-1 might bind to a specific cell surface receptors initiating intracellular signaling cascades that might lead to various cellular responses, and the unknown effects are indicated with a question mark. Embedded in this model are the STC-1 functional effects observed in the present study.

In addition, the effects of STC-1 on metabolic rate and uncoupling of oxidative stress suggest STC-1 involvement in delaying the onset of hypoxia-induced apoptosis in human neuronal cells (Zhang et al., 2000). Enhanced cellular phosphate uptake by neuronal cells (caused by STC-1), might diminish the hypoxia-induced rise in intracellular calcium, thereby delaying apoptosis. In light of these data, it is possible that cyto-protective effects of STC-1 on respiration and mitochondrial calcium accumulation could be part of a novel anti-apoptotic pathway, operative in metabolically active, mitochondria-rich cells (Ellard et al., 2007; Zhang et al., 2000). Recent data shows that overexpression of STC-1 is associated with increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl, which leads to the inhibition of cleavage of procaspase-3, -8 and -9 (G. Liu et al., 2010). Another future challenge will be the identification of how the STC-1 and PDGF-mediated PI3K pathways converge to mediate Akt phosphorylation in SMC, and to address whether this effect can also be identified in other PDGF-responsive cell types.

The observed accumulation of secreted STC-1 expressed from both vascular endothelial cells and SMCs might indicate that STC-1 could be a critical modulator for cell-cell regulatory interactions and/or exchange of signals between these cell types. However the cellular consequences of such modulation as well as the primary stimulatory cells responsible for excessive secretion of STC-1 could vary depending on numerous physiological parameters regulated by proteins under the control of different primary and/or secondary messengers molecules. In terms of the role of STC-1 in endothelium-SMCs interaction, co-culture *ex vivo* angiogenesis-related assays such as polymer matrices assay, Matrigel or aortic ring assays may be useful.

There is strong evidence from studies in fish and in some mammalian cells that STC-1 has a role in regulating Ca^{2+} fluxes and homeostasis. Furthermore, numerous studies have indicated that the intracellular Ca^{2+}-associated signaling in vascular cells, particularly in SMCs, is primarily controlled by the influx of extracellular Ca^{2+} and IP3-dependent Ca^{2+} release from intracellular stores such as endoplasmic/sarcoplasmic reticulum (ER/SR) through Ca^{2+}-induced Ca^{2+} release (CICR). Moreover, this

extracellular Ca^{2+} ions mobility is so tightly regulated that innumerable cellular processes such as motility, adhesion, metabolism, proliferation and apoptosis are initiated as results of it. Consequently, vascular calcium modulation of vasodilator responses play a critical role in the peripheral vascular resistance and establishment of vascular tone, thereby serve to maintain the homeostasis of blood circulation. However it is unclear how STC-1 secreted in subintimal region between endothelium and SMCs could possibly modulate or inhibit Ca^{2+} sparks and consequently induce membrane depolarization that might be importantly involved in the control of vascular tone and vasomotor responses.

In comparison to STC-1, its homologue STC-2 has a direct role in Ca^{2+} cellular regulation acting as a negative regulator of Ca^{2+} influx through plasma membrane-localized store-operated Ca^{2+} channels, (SOCs) (Zeiger et al., 2011). This study identified a potential role for STC-2 in the cellular stress responses by limiting the ER Ca^{2+} sensor STIM1-mediated activation of Ca^{2+} influx, thus increasing stress- induced cellular viability. Although in this study, I was unable to determine whether STC-1 plays a role in cell survival, and I did not examine STC-1 regulation of Ca^{2+} signalling in SMC due to lack of access to facilities for these studies, and to lack of time, it would important to examine if STC-1 acts as a regulator of intracellular SMC Ca^{2+} homeostasis, using the fluorescent imaging of STC-$1^{-/-}$ mouse embryonic fibroblasts loaded with the ratiometric dye Fura-2 as in Zieger et al (2011).

Given that STC-1 is implicated in regulating mitochondrial functions, it is possible that STC-1 complexation with its putative membrane-spanning receptor might target mitochondrial functions, possibly involving enhanced oxygen consumption rate and uncoupling of oxidative phosphorylation (Ellard et al., 2007). However, the exact mechanism involved needs to be elucidated. Whether STC-1/receptor complex is able to associate with intracellular mitochondrial proteins or recruit other intracellular binding partners remains unclear.

Another clue to a possible function of STC-1 has come from sequence analysis studies, which identified a consensus protein kinase ATP-binding domain in human STC-1 near the N-terminus of the protein, and studies showing that ATP is able to bind to STC-1 thereby antagonising STC-1 binding to its mitochondrial receptors (Ellard et al., 2007).

It is also believed that secreted STC-1 contains one or more unidentified serine residues that may be substrates for extracellular phosphorylation by ecto-PKC during or soon after secretion from the cell (Jellinek et al., 2000). While the addition of phosphates to STC-1 may cause conformational changes, it may subsequently help regulate protein processing, structure and its biological activity. Apart from these findings, STC-1 so far lacks a clear domain structure that might provide insights into its function.

In conclusion, this investigation is the first description of regulation of STC-1 expression in SMC, its induction by PDGF-BB, and of a role for STC-1 in regulating PDGF-BB-dependent Akt signalling and SMC migration. However, investigation of STC-1 presents a number of significant challenges, particularly the lack of unambiguous identification of its receptor, and mechanism of action or biological function in mammals. Understanding and determining the underlying mechanisms of STC-1-mediated effects on SMCs and in the vasculature more generally will require much further work.

7 Reference

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