# Stanniocalcin-1 and vascular remodelling at the maternal-fetal interface

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# Declaration of Thesis

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. The work presented is entirely my own, except where stated otherwise by reference or acknowledgment.

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28th February 2023

# Abstract

Maternal vascular remodelling is an essential process which takes place during the early stages of pregnancy to enable increased nutrients and respiratory gases to the developing fetus. During this process, endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are lost from maternal spiral arteries and are replaced with fetal trophoblast cells. Defects in this process are associated with severe pregnancy complications, such as pre-eclampsia. Trophoblast cells have previously been shown to stimulate both ECs and VSMCs to express stanniocalcin-1 (STC-1). This protein plays a diverse role within female reproductive tissues and is implicated in physiological and pathophysiological cardiovascular function. This study aimed to understand the regulation of STC-1 in vascular cells and elucidate its role in the vascular remodelling process. STC-1 was found to be expressed within first trimester maternal decidual tissue in the presence of trophoblast cells. Trophoblast cell secreted factors stimulated secretion of STC-1 from VSMCs and ECs but did not affect intracellular STC-1 expression. It was demonstrated that trophoblast conditioned media (TCM) contains a wide range of cytokines but the specific factor(s) which induced vascular cell STC-1 secretion was not identified. TCM stimulation of vascular cells induced activation of proteins in major cell signalling pathways including Akt, glycogen synthase kinase 3β, mitogen-activated protein kinase, and serum/glucocorticoid regulated kinase 1. Akt was found to be implicated in the regulation of TCM-induced STC-1 secretion from ECs. Activation of protein kinase C (PKC) induced secretion of STC-1 from vascular cells, but TCM-induced secretion of STC-1 is not regulated through PKC. To determine the role of STC-1 in this system, tools to overexpress and knockdown STC-1 expression in VSMCs and ECs were developed. Overexpression of STC-1 reduced the rate of EC migration but did not affect VSMC migration.

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# List of Abbreviations:

AC - adenylyl cyclase

aPL - antiphospholipid antibodies

ATP - adenosine triphosphate

BFA - brefeldin A

BSA - bovine serum albumin

cAMP - cyclic adenosine monophosphate

CCL5 - C-C motif chemokine ligand 5

CCR1 - C-C motif chemokine receptor 1

CDK2 - cyclin-dependent kinase 2

CHX - cycloheximide

dNK - decidual natural killer

EC – endothelial cell

ECM – extracellular matrix

eGFP - enhanced green fluorescent protein

ELISA - enzyme-linked immunosorbent assay

ERK - extracellular signal-regulated kinases

eSC - endometrial stromal cell

ESE - early secretory phase

EVT - extravillous trophoblast

FasL - Fas ligand

FCS - fetal calf serum

FGF-2 - fibroblast growth factor 2

FGR - fetal growth restriction

GFP - green fluorescent protein

GPCR - G protein-coupled receptor

GSK3β - glycogen synthase kinase 3 beta

hCG- human chorionic gonadotropin

HGF - hepatocyte growth factor

HIF - hypoxia-inducible factor

IFNγ - interferon gamma

IGF - insulin-like growth factor

IKK- IκB kinase

IL – interleukin

IRES - internal ribosome entry site

JAK - janus kinase

LH - luteinising hormone

MAPK - mitogen-activated protein kinase

MAPKAPK-2 - MAP kinase-activated protein kinase 2

MEK - mitogen-activated protein kinase kinase

MMP - matrix metalloproteinase

MSE - mid-secretory phase

mTORC-2 - mammalian target of rapamycin complex 2

NDRG1 - N-myc downstream regulated 1

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

PAPP-A - pregnancy-associated plasma protein-A

PBS - phosphate buffered saline

PE - proliferative phase

PI3K - phosphoinositide 3-kinase

PKA – protein kinase A

PKB – protein kinase B

PKC – protein kinase C

PlGF - placental growth factor

PMA - phorbol 12-myristate 13-acetate

RI - resistance index

ROS - reactive oxygen species

RTK - receptor tyrosine kinase

RUPP - reduced uterine perfusion pressure

SDS-PAGE - sodium dodecyl-sulfate polyacrylamide gel electrophoresis

sFlt-1 - soluble fms-like tyrosine kinase 1

SGK1 - serum/glucocorticoid regulated kinase 1.

STAT3 - signal transducer and activator of transcription 3

STC-1 – stanniocalcin-1

TBS - tris buffered saline

TCM – trophoblast conditioned media

TGFβ - transforming growth factor beta

TNBC - triple-negative breast cancer

TNF - tumour necrosis factor

TRAIL - TNF-related apoptosis inducing ligand

VEGF - vascular endothelial growth factor

VSMC – vascular smooth muscle cell

# Chapter 1: General Introduction

In human pregnancy, sufficient uteroplacental blood flow is essential to ensure a normal pregnancy outcome and is achieved by extensive growth and remodelling of the maternal uterine circulation, as well as the creation of a new fetal vascular organ, the placenta (Osol and Mandala, 2009).

## 1.1 Uterine vascular anatomy

The blood supply to the uterus consists of a branched structure with successive decreases in vessel diameter as they progress through the myometrium and endometrium. In the non-pregnant state, blood flow to the human uterus is supplied by the left and right uterine arteries. At intervals along their length, the uterine arteries give rise to arcuate arteries that supply the myometrium. These then branch off into radial arteries which are directed towards the lumen of the uterus (Burton, Woods, *et al.*, 2009). As these reach the myometrial-endometrial boundary, each radial artery gives off lateral branches, forming basal arteries which supply the myometrium and the deeper basalis parts of the endometrium, and also continues as spiral arteries (Pijnenborg, Vercruysse and Hanssens, 2006) (Figure 1.1).

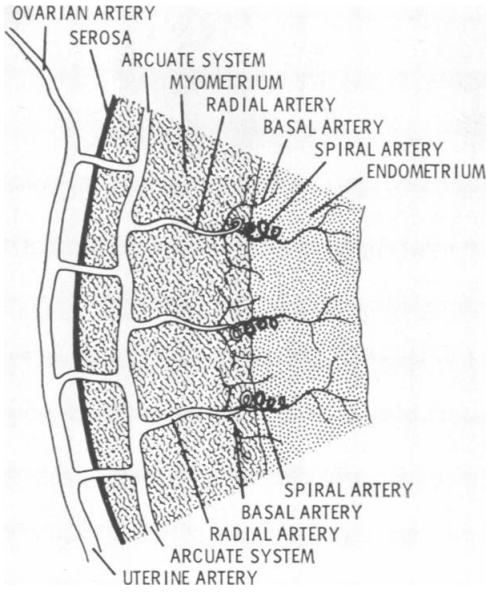



**Figure 1.1 A diagram of blood supply to the non-pregnant uterus.**

*(From: Robertson, 1976).*

Spiral arteries are highly coiled arteries which supply blood to the endometrial layer and, in the pregnant uterus, these arteries span the inner myometrium and decidua (Burton, Woods, *et al.*, 2009). Spiral arteries develop in the second half of the menstrual cycle under the influence of progesterone (Ferenczy, Bertrand and Gelfand, 1979). In the non-pregnant uterus, they have muscular walls and a highly elastic lamina (Robertson and Warner, 1974) and are composed of vascular smooth muscle cells (VSMCs) coiled around a central lumen formed by a single layer of endothelial cells (ECs). In the absence of pregnancy, spiral arteries are lost during menstrual shedding (Espinoza *et al.*, 2006). During pregnancy spiral arteries are highly remodelled from a low flow, high resistance vessel to a high flow, low resistance vessel (Burton, Woods, *et al.*, 2009). This remodelling occurs as a result of a loss of VSMCs and elastic lamina from the vessel wall. In addition, the endothelium is temporarily replaced with a trophoblast layer but this is restored later in pregnancy (Brosens, Robertson and Dixon, 1967).

Unmodified myometrial spiral arteries have a luminal diameter of approximately 200 µm (Boyd, 1970), whilst the diameter of a remodelled vessel can increase to up to 2 mm (Benirschke and Driscoll, 1967). This increase in size enables a significant increase in blood supply to the fetoplacental unit. Early studies showed that in singleton pregnancies, the total uteroplacental blood flow increases from a baseline value of 20-50 ml/minute to 450-800 ml/minute (Assali *et al.*, 1953; Metcalfe *et al.*, 1955; Assali, Rauramo and Peltonen, 1960). It is thought that almost all 100-150 arteries in the placental bed are remodelled (Lyall, 2005), with spiral arteries at the centre of the placenta undergoing more extensive remodelling than those at the periphery (Brosens, Robertson and Dixon, 1967). Spiral artery remodelling is mediated by two distinct mechanisms: trophoblast-independent and trophoblast-dependent.

## 1.2 Trophoblast-independent spiral artery remodelling

Although the source of controversy in the field, it is thought that spiral artery remodelling begins prior to trophoblast invasion and is thought to prime the vessels for trophoblast invasion. Studies by Craven *et al.* 1998 and Kam *et al.* 1999 compared spiral arteries from normal and ectopic pregnancies using histological and immunochemical approaches (Craven, Morgan and Ward, 1998; Kam *et al.*, 1999). Both studies showed distinct changes in spiral arteries in the absence of trophoblasts, such as EC activation and alterations in VSMC layer organisation. Craven *et al.* 1998 suggested these changes to be indicative of the first stages of physiological change within the spiral arteries. However, the term "physiological change" was originally used to describe the "disappearance of the normal muscular and elastic structures of arteries and their replacement by fibrinoid material in which trophoblast cells are embedded" (Brosens, Robertson and Dixon, 1967), indicating a requirement for the presence of trophoblasts. These early trophoblast-independent

changes within spiral arteries which precede trophoblast-dependent remodelling are, therefore, now referred to as decidua-associated remodelling (Pijnenborg, Vercruysse and Hanssens, 2006).

Non-trophoblast-induced changes also occur in other arteries including radial, arcuate, and uterine arteries where trophoblast invasion does not take place. All of these arteries undergo profound dilation during pregnancy (Arts, 1961; Burchell, 1967). For example, the diameter of the uterine artery has been shown to double by 6.5 weeks of pregnancy and the other vessels of the uterine circulation successively increase. By mid-pregnancy, the diameter of the arcuate arteries exceeds that of the uterine vessels (Burchell, 1967).

Vascular remodelling is not limited to arteries as uterine veins have also been shown to expand during pregnancy, but this is not as well-studied. In rats, the diameter of the main uterine vein has been shown to increase 65% during pregnancy and also double in length. These changes are also associated with a reduction in elastin content and an increase in venous distensibility (Page *et al.*, 2002).

The molecular mechanisms underlying this decidua-associated transformation preceding trophoblast invasion has not been well-studied. It is thought that endocrine stimulation involving oestrogen and progesterone may play a role in this process by regulating endometrial blood vessel growth and regression (Rogers and Abberton, 2003). Oestrogen is known to influence vascular reactivity by stimulating nitric oxide synthesis (Huang *et al.*, 2000) and increase vessel permeability and EC proliferation through increased vascular endothelial growth factor (VEGF) release (Aberdeen *et al.*, 2008). The effects of progesterone are thought to be indirect through its effects on the recruitment of immune cells to the endometrium (Sentman *et al.*, 2004). Moreover, both EC and VSMC possess

numerous human chorionic gonadotropin (hCG) receptors, particularly within the smaller resistance branches of the artery (Toth *et al.*, 1994). hCG stimulation has been shown to cause vasodilation through enhancing eicosanoid production (Toth *et al.*, 2001). Placental growth factor (PlGF) has also been shown to induce vasodilation of endometrial arteries through increasing nitric oxide production (Osol *et al.*, 2008), consistent with the effects of oestrogen in this system.

## 1.3 Trophoblast-dependent spiral artery remodelling

The human placenta consists of a branching villous structure composed of blood vessels in a core of mesenchymal connective tissue. This structure is surrounded by two layers of specialised placental cells termed trophoblast. The inner layer consists of cytotrophoblasts, and the outer layer consists of multinucleated syncytiotrophoblasts which covers the entire surface of the placenta acting as a barrier to the maternal circulation and is the site of gas and nutrient exchange (James, Whitley and Cartwright, 2010). Cytotrophoblast in villous tips differentiate into extravillous trophoblast (EVT) and form the stratified structure called the cell column. Here, EVTs lose their proliferative activity and acquire invasive properties enabling invasion into the decidua and subsequent remodelling of the spiral arteries (Sato, Fujiwara and Konishi, 2012).

EVT invasion occurs through two routes: interstitial through the decidua or endovascular via the distal ends of the spiral arteries. Interstitial EVTs invade the uterine wall whereas endovascular EVTs migrate along the lumen of the spiral artery as far as the inner one-third of the myometrium and continue until the middle of the second trimester of pregnancy (Pijnenborg *et al.*, 1981). After this point, the VSMCs and the elastic extracellular matrix (ECM) from the spiral arteries is lost and replaced with a fibrin-based deposit, fibrinoid (Robertson, Brosens and Dixon, 1967).

Interstitial EVT invasion is thought to influence the cells of the vessel wall by preparing them for subsequent endovascular EVT invasion (Pijnenborg *et al.*, 1983; Kam *et al.*, 1999). Prior to 8 weeks of gestation, endovascular trophoblast cells flow into the spiral artery lumen forming a plug blocking the flow of maternal blood into the intervillous space (Burton, Jauniaux and Watson, 1999), this helps to create a low oxygen environment to protect the fetus against potentially harmful reactive oxygen species (Sato, Fujiwara and Konishi, 2012). After 8 weeks, the plugs begin to disassociate, and the endovascular trophoblasts migrate retrograde along the spiral artery lumen, replacing maternal ECs (Burton, Jauniaux and Watson, 1999). At this point, maternal arteries are connected with the intervillous space, reaching a fully established uteroplacental circulation by 12 weeks of gestation (Coppens *et al.*, 1996).

Together these two invasive processes, completed by 20-22 weeks of gestation, transform spiral arteries from small, resistance vessels to large, low resistance vessels enabling adequate placental perfusion which is vital for a successful pregnancy (Ramsey, 1981).

## 1.4 Regulation of trophoblast differentiation

Cytotrophoblast differentiation into EVT requires stringent regulation to ensure adequate spiral artery remodelling. Any defects in this process can lead to a reduction in the number of spiral arteries remodelled and a reduced depth of remodelling. The precise mechanisms controlling the differentiation of these cells have not yet been fully elucidated, however, it is apparent that this process is tightly regulated through the actions of adhesion molecules such as integrins and E-cadherins, transcription factors such as activator protein-1 family members (Janatpour *et al.*, 1999; Bamberger *et al.*, 2004), and cytokines and growth factors including the transforming growth factor beta (TGFβ) and interleukin (IL) families (Bischoff, Meisser and Campana, 2000).

## 1.5 Mechanisms underlying EVT invasion of spiral arteries

The factors which preferentially direct interstitial EVTs towards the maternal spiral artery for invasion have not been fully characterised. It is thought, however, that high oxygen tension in maternal arteries promotes trophoblast differentiation towards an invasive phenotype, which could act to facilitate interstitial EVT invasion (Genbacev *et al.*, 1997). It has also been proposed that the presence of maternal platelets in maternal spiral arteries promotes interstitial EVT invasion (Sato *et al.*, 2005). Histological examination of the human placental bed has shown that activated maternal platelets are trapped by endovascular trophoblast aggregates that are formed inside the spiral artery lumen and, *in vitro,* co-culture of platelets with EVTs has been shown to induce Matrigel invasion of EVTs (Sato *et al.*, 2005). The effect of activated maternal platelets on EVT invasion is indirect as it has been demonstrated that soluble factors derived from platelets direct interstitial EVT invasion towards the spiral artery. For example, the chemokine receptor, C-C motif chemokine receptor 1 (CCR1), has been found to be expressed on isolated EVT cells (Sato *et al.*, 2003). Its ligand, C-C motif chemokine ligand 5 (CCL5), is secreted by activated platelets and is one of the soluble factors which directs EVT invasion. These findings suggest a model in which the presence of maternal platelets provides a positive feedback mechanism to promote interstitial EVT invasion (Sato, Fujiwara and Konishi, 2012).

## 1.6 Mechanisms underlying spiral artery remodelling

The precise mechanisms underlying the process of spiral artery remodelling have yet to be fully determined, however, it is evident from research efforts in the field that several distinct changes take place within the vascular cells and their environment. This includes extracellular matrix (ECM) restructuring, vascular cell de-differentiation, migration, alterations in cell adhesion and increased sensitivity to apoptotic stimuli (Osol and

Mandala, 2009; Harris, 2011) (Figure 1.2). It is clear that these events are not mutually exclusive and may be interdependent. For example, loss of EC adhesion could result in apoptosis, and changes in ECM composition could result in alterations in the differentiation state of VSMCs (Whitley and Cartwright, 2009).

These mechanisms appear to be regulated by both endovascular and interstitial EVTs. Interstitial EVTs are more likely to influence VSMC behaviour, whilst endovascular EVTs primarily target ECs (Whitley and Cartwright, 2009). It is also thought that spiral artery remodelling is further influenced by the cells of the maternal immune system and by changes in blood flow and pressure that occur during the early stages of pregnancy (Whitley and Cartwright, 2010).

The ability to fully characterise the spiral artery remodelling process has been hindered by a lack of availability of material at all stages of gestation and a lack of appropriate animal models. However, a combination of cell lines and *ex vivo* tissue studies have been employed over recent years to help elucidate the mechanisms underlying the spiral artery remodelling process.

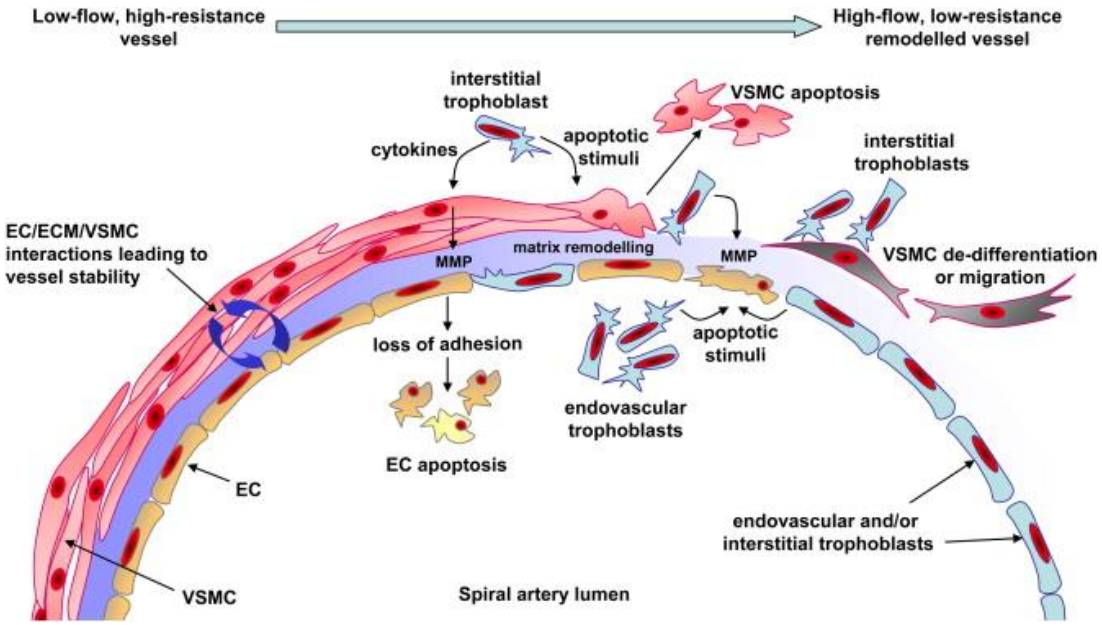



**Figure 1.2 A diagram of the potential mechanisms involved in trophoblast-dependent spiral artery remodelling.**

*(From: Whitley and Cartwright, 2010)*

## 1.6.1 Apoptosis

It is well established that apoptosis, the process of programmed cell death, plays a key role in both normal and pathological vascular remodelling (Korshunov and Berk, 2008). It has become clear, however, in recent years that the balance between pro- and anti-apoptotic stimuli is vital in preventing excessive remodelling. In a quiescent vessel, cell death is matched with cell proliferation and without this regulation, excessive cell death can occur resulting in vascular wall instability and the onset of pathological conditions such as aortic aneurism (Whitley and Cartwright, 2010). In the spiral arteries, it is known that there are many factors which promote EC survival including a number of cytokines and growth factors such as VEGF, angiopoietin-1 and fibroblast growth factor 2 (FGF-2) (Mallat and Tedgui, 2000). The invasion of trophoblasts, however, seems to alter the balance in the vessel towards cell death, perhaps through the release of pro-apoptotic factors or

promotion of a loss of cell adhesion. The loss of ECs combined with the presence of trophoblasts might then act to stimulate the induction of VSMC death in the vessel. The mechanisms underlying the regulation of apoptosis in this system are not completely clear, however, several cytokines produced by trophoblasts have been implicated in this process. Three members of the tumour necrosis factor (TNF) family produced by trophoblasts; TNF-α, TNF-related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL) appear to be involved in regulating vascular cell apoptosis (King *et al.*, 1995; Pijnenborg *et al.*, 1998; Hammer *et al.*, 1999; Hammer and Dohr, 2000; Ashton *et al.*, 2005; Keogh *et al.*, 2007). TNF-α has been shown to bind to and activate two receptors; TNF-receptors 1 and 2 on ECs and VSMCs (Goetze *et al.*, 1999). Depending on the recruitment of downstream molecules, this pathway can promote either cell survival or apoptosis based on the overall balance between pro- and anti-apoptotic factors within the local environment (Whitley and Cartwright, 2010). In addition, both VSMCs and ECs also express receptors for TRAIL on their cell surface; TRAIL-receptor 1 and TRAIL-receptor 2. TRAIL is synthesised by first trimester cytotrophoblasts and the binding of this molecule to these receptors is known to induce apoptosis (Secchiero *et al.*, 2003; Keogh *et al.*, 2007). FasL has been detected at the maternal-fetal interface where it is expressed by villous, extravillous, and syncytiotrophoblasts and it binds to and activates the cell surface receptor Fas (Runic *et al.*, 1996). Fas is expressed on both ECs and VSMCs and studies have suggested a role for this ligand in the induction of apoptosis (Ashton *et al.*, 2005).

### 1.6.2 ECM restructuring

The structure of the spiral artery is maintained by the ECM. The wall of the un-remodelled spiral arteries consists of three ECM layers with the cells of the vessel on top of or embedded in the ECM. The intima consists of a single layer of ECs on a basement

membrane consisting of collagen type-IV and laminins. The lamina forms a tightly woven layer of elastin and collagen type-IV fibres with fibronectin to separate the ECs and VSMCs and provide strength and support to the vessel wall. Small pores within the ECM allow ECs and VSMCs to form direct cell to cell contact via myo-endothelial junctions. The second layer of the vessel consists of elastic fibres surrounding the medial VSMCs which forms an external elastic lamina. This is surrounded by the adventitial layer consisting of collagen fibres and fibroblasts (McGrath *et al.*, 2005; Arribas, Hinek and González, 2006). The adventitia and the intima form barriers against invading interstitial and endovascular trophoblasts, respectively (Harris and Aplin, 2007).

During the process of spiral artery remodelling, the integrity of the ECM is compromised. This begins during decidua-associated remodelling and correlates with the presence of maternal leukocytes (Craven, Morgan and Ward, 1998; Kam *et al.*, 1999; Smith *et al.*, 2009). Following interstitial trophoblast invasion, the elastic lamina in the myometrial segment of the spiral artery is disrupted causing dilation of the artery lumen, intimal oedema, and widening of the intercellular spaces of the media (De Wolf *et al.*, 1980; Pijnenborg *et al.*, 1983). Following endovascular trophoblast invasion, further disorganisation of the intima and media occurs disrupting EC interactions and degrading elastin (Khong *et al.*, 1986; Khong, Adema and Erwich, 2003). Enzymes are released by both the vascular cells and trophoblasts at this stage (Lim *et al.*, 1997; Bischof, Meisser and Campana, 1998; Smith *et al.*, 2009) which further aid in the disruption of the ECM. These include the family of matrix metalloproteinases (MMPs) which are zinc-dependent endopeptidases that play a role in the degradation of all ECM components. MMPs are produced by ECs (MMP-1 and -9), VSMCs (MMP-2,-9 and -12), decidual natural killer cells, macrophages and invasive EVT (MMP-1, -2, -9, and - 12) (Lim *et al.*, 1997; Bischof, Meisser and Campana, 1998; Smith *et*

*al.*, 2009). Decidual natural killer cells also secrete granzyme, a serine-protease that degrades a number of substrates including collagen IV and fibronectin and this may play an important role in the vascular remodelling process (Podack *et al.*, 1988; Simon *et al.*, 1991). The effect of MMPs on the ECM induces the release of a number of biologically active molecules. For example, the activity of MMP-2 can release TGFβ bound to the ECM (Mott and Werb, 2004) and MMP-9 can induce the release of VEGF. TGFβ is known to regulate trophoblast invasion and motility (Tse, Whitley and Cartwright, 2002) and VEGF acts as a EC survival factor which may counter the apoptotic effects of trophoblasts. In addition, MMP-2 and -9 are known to induce cleavage of a component of the spiral artery basement membrane, collagen XVIII, which is produced by ECs and VSMCs (Saarela *et al.*, 1998). Cleavage of collagen XVIII releases endostatin which inhibits EC proliferation (Shichiri and Hirata, 2001) and stimulates apoptosis (Dhanabal *et al.*, 1999; Hanai *et al.*, 2002).

The process of ECM degradation is tightly regulated and the survival of ECs and VSMCs depends on their interactions with the ECM and various intracellular signals. This process ultimately results in apoptosis or migration of the vascular cells, and it is likely that this depends on the extent and the duration of the process but the specific factors influencing this are not clear (Whitley and Cartwright, 2010).

### 1.6.3 Fibrinoid deposition

Another characteristic of "physiological change" in the spiral arteries as described by Brosens *et al* is the secretion of fibrinoid material by EVTs (Brosens, Robertson and Dixon, 1967). This fibrinoid material is composed of fibronectin, collagen type IV and laminin (Brosens, Robertson and Dixon, 1967; Frank *et al.*, 1994). Secretion of fibrinoid material in this way results in trophoblasts becoming embedded within the matrix. It is thought that these fibrinoid deposits act to retain the structure of the newly remodelled vessel and can

also form a basement membrane upon which re-endothelialisation can occur (Brosens, Robertson and Dixon, 1967).

### 1.6.4 VSMC de-differentiation

The behaviour and phenotype of VSMCs are affected by the extent to which they are differentiated. VSMCs can switch between a 'functional' (contractile) and 'synthetic' (proliferative) phenotype following changes in gene expression (Kaplan-Albuquerque *et al.*, 2005). In a healthy adult artery, the majority of VSMCs have a contractile phenotype and express contractile proteins such as smooth muscle α-actin, smooth muscle myosin heavy chain, calponin and smooth muscle 22α. VSMCs with a contractile phenotype do not generally proliferate, migrate or secrete ECM (Owens, Kumar and Wamhoff, 2004). In response to changes in extracellular cues, such as those present during the process of vascular remodelling, VSMCs can adopt a more synthetic phenotype (Wilcox, 1992). VSMCs with a synthetic phenotype typically display migratory and proliferative behaviour accompanied with a loss or reduction in the expression of VSMC-specific contractile proteins (Wilcox, 1992). In the context of spiral artery remodelling, it is thought that a switch to a synthetic phenotype results in an increase in sensitivity to apoptotic stimuli (Su *et al.*, 2006; Halka *et al.*, 2008) and increased migration away from the vessel (Newby, 2006). Within the remodelling vessel, the differentiation state of VSMCs may be affected by a number of external factors including the presence of other cell types and the ECM (Owens, 2007; Rzucidlo, Martin and Powell, 2007). For example, ECs promote VSMC differentiation by stimulating the expression of contractile proteins (Fillinger *et al.*, 1997), and also inhibit VSMC matrix deposition and proliferation (Powell *et al.*, 1997). Trophoblasts release key inflammatory cytokines including TNFα, which in combination

with interleukin-1 beta (IL-1β), acts to stimulate expression of the inducible form of nitric oxide synthase in VSMCs, which could induce VSMC apoptosis (Geng *et al.*, 1996).

### 1.6.5 The effect of haemodynamics on vessel structure and vascular remodelling

Interactions between ECs and VSMCs in the remodelling spiral artery can be affected by a range of factors including shear stress and pressure, as well as autocrine and paracrine signals. These signals can be derived either from within the vessel wall itself or the surrounding tissue, and can affect cellular properties such as differentiation state or cell survival (Pijnenborg, 2000).

There are a number of haemodynamic changes that take place in the remodelling spiral arteries. For example, the formation of the endovascular trophoblast plug that occurs in the first few weeks of gestation dramatically reduces blood flow in the vessels and increases the internal pressure of the vessel (Hamilton and Boyd, 1960). As pregnancy progresses, the pressure within the vessel falls as the VSMCs in the myometrial segment lose their structured organisation following interstitial trophoblast invasion (Pijnenborg *et al.*, 1983). When the trophoblast plug begins to dissociate, blood flow into the intervillous space increases as well as the shear stress experienced by the vessel wall. It is not clear how the haemodynamic changes influence spiral artery remodelling. It is likely, however, that the response of vascular cells to invading trophoblasts is modulated by the haemodynamic stresses faced within the vessel (Pijnenborg, 2000).

In other vascular systems, the effect that mechanical forces can have on vessel structure has been better described. It has been demonstrated that mechanical forces can affect EC and VSMC differentiation, growth, and response to external stimuli (Riha *et al.*, 2005). As physiological levels of shear stress have been shown to inhibit EC apoptosis induced by

stimuli including TNFα and reactive oxygen species (ROS) (Dimmeler *et al.*, 1997; Li, Haga and Chien, 2005), it is possible that the loss of laminar blood flow in the spiral arteries caused by trophoblast plugging could result in increased susceptibility of ECs to trophoblast-mediated apoptotic stimuli. It is also possible that changes in haemodynamics in the remodelling vessel could causes changes in vascular cell interactions. For example, ECs can alter the phenotype of VSMCs which affects matrix deposition (Hastings *et al.*, 2007), and VSMCs can influence the response of ECs to shear stress (Hoerstrup *et al.*, 2001; Imberti *et al.*, 2002).

### 1.6.6 The role of maternal immune cells in vascular remodelling

Maternal immune cells have been implicated in the process of spiral artery remodelling. Specific maternal natural killer cells, known as decidual natural killer (dNK) cells form the major component of immune cells within the decidua basalis, followed by macrophages and T cells (Jabrane-Ferrat and Siewiera, 2014). dNK cells are present at the time of spiral artery remodelling and their numbers decrease from mid-gestation (Moffett-King, 2002).

The precise role of dNK cells in spiral artery remodelling has yet to be fully elucidated, however, animal studies have suggested a role for dNK cells in this process. Mice depleted of dNK cells display inadequate spiral artery remodelling in a process regulated by the production of interferon gamma (IFNγ) (Ashkar, Di Santo and Croy, 2000). In humans, dNK cells have been implicated in influencing trophoblast function and have been reported to display both pro-invasive (Hanna *et al.*, 2006; De Oliveira *et al.*, 2010; Lash *et al.*, 2010) and anti-invasive (Hu *et al.*, 2006) effects on trophoblasts. Differences in the effect on trophoblast function between dNK cells isolated from normal pregnancies and from those at risk of developing pre-eclampsia have also been reported (Fraser *et al.*, 2012). dNK cells

from normal pregnancies promote migration, whereas those from high-risk pregnancies have no effect on trophoblast motility. Moreover, dNK cells from normal pregnancies induce apoptotic changes in VSMCs and ECs, whereas dNK cells from high-risk pregnancies fail to induce vascular apoptosis and secrete fewer pro-apoptotic factors (Fraser *et al.*, 2012). dNK cells can also produce MMPs which could aid in the degradation of extracellular matrix proteins in the spiral artery (Naruse *et al.*, 2009).

## 1.7 Consequences of impaired spiral artery remodelling

It is clear that spiral artery remodelling plays a key role in the establishment and maintenance of a normal pregnancy. The various mechanisms involved in this process have been shown to be interdependent and require tight spatial and temporal regulation to ensure adequate arterial conversion. Inadequate spiral artery remodelling has been associated with the onset of pregnancy disorders including recurrent miscarriage, pre-eclampsia, and fetal growth restriction (Whitley and Cartwright, 2009).

### 1.7.1 Pre-eclampsia

Pre-eclampsia is a multifactorial syndrome of pregnancy which is thought to be caused by a combination of genetic, environmental, immunological, and nutritional factors. It affects up to 3-8% of all pregnancies and is an important cause of maternal and perinatal morbidity and mortality. In developing countries, the number of pregnancies affected by pre-eclampsia rises to up to 15% (Ghulmiyyah and Sibai, 2012) resulting in 60,000 maternal deaths annually worldwide (Mongraw-Chaffin, Cirillo and Cohn, 2010; Young, Levine and Karumanchi, 2010).

Pre-eclampsia is the prodromal syndrome of eclampsia. Eclampsia affects approximately 1 in 2000 pregnancies in the United Kingdom and is associated with high maternal morbidity

and mortality (Douglas and Redman, 1994). Eclampsia is defined as the occurrence of convulsions during pregnancy or in the first 10 days postpartum in association with at least two of the following features: hypertension, proteinuria, thrombocytopenia or an increased plasma aspartate transaminase concentration (Douglas and Redman, 1994).

Pre-eclampsia is characterised by new onset hypertension (blood pressure ≥ 140/90 mmHg) combined with one or more other features such as proteinuria, maternal organ dysfunction (including liver, kidney or neurological), or uteroplacental dysfunction, such as fetal growth restriction (FGR) and/or abnormal Doppler ultrasound findings of uteroplacental blood flow (Brown *et al.*, 2018). This classification differentiates pre-eclampsia from the more common and less dangerous condition of gestational hypertension which is defined as new onset hypertension in pregnancy without proteinuria (Davey and MacGillivray, 1988).

In terms of the pathophysiology of pre-eclampsia, it is believed that there are at least two sub-types: early onset and late onset (Tranquilli *et al.*, 2013). Early onset pre-eclampsia is widely acknowledged to be caused primarily by placental defects, whilst the pathogenesis of late onset pre-eclampsia is thought to be underpinned by interactions between senescence of the placenta and a maternal genetic predisposition to cardiovascular and metabolic disease (Burton *et al.*, 2019).

There are several risk factors that can contribute to the onset of pre-eclampsia. The risk of developing pre-eclampsia is higher in a first pregnancy (~4%), and it is thought that there is a protective effect of a normal first pregnancy as there is a lower risk in subsequent pregnancies (~2%). The risk of recurrence is high (~15%) following one pre-eclamptic pregnancy and even higher (~32%) following two pre-eclamptic pregnancies (Hernández-Díaz, Toh and Cnattingius, 2009). Other risk factors for pre-eclampsia include: pre-existing

chronic hypertension, pre-gestational diabetes, chronic renal disease, multifetal pregnancy, pre-pregnancy body mass index (BMI) >30, previous stillbirth, nulliparity, maternal age >40, long inter-pregnancy interval (>5 years), assisted reproduction, previous FGR, and previous placental abruption (Duckitt and Harrington, 2005; Abalos *et al.*, 2014; Bartsch *et al.*, 2016).

The onset of pre-eclampsia in pregnancy can have profound effects on the health of the mother causing hypertension, kidney damage, liver failure, central nervous system damage, cardiomyopathy, and could eventually result in mortality (Armaly *et al.*, 2018). An even higher mortality rate is observed when pre-eclampsia is associated with HELLP (haemolysis, elevated liver enzymes, low platelets) syndrome, liver haemorrhage, or pulmonary oedema (Ghulmiyyah and Sibai, 2012). Furthermore, a pre-eclamptic pregnancy can have lifelong health implications for both the mother and the baby. For the mother, the risk of cardiovascular disease and death increases two-fold following a pre-eclamptic pregnancy (Bellamy *et al.*, 2007) and for the baby, there is a significant increase in the risk of cardiovascular disease and stroke in later life (Kajantie *et al.*, 2009), as well as high blood pressure in young adulthood (Davis *et al.*, 2012).

The specific pathophysiology underlying the condition of pre-eclampsia has not yet been fully elucidated and is the source of much controversy in the field. It is apparent that the processes of placentation and vascular remodelling may potentially have defects which result in the onset of the condition. In addition, various risk factors and genetic variations may act to predispose women to developing the disorder (Burton *et al.*, 2019).

Though the symptoms of pre-eclampsia do not present until the second trimester, it is evident that the pathogenesis is established in the first trimester at the time of trophoblast invasion and spiral artery remodelling. Defective vascular remodelling in the first trimester

may contribute to the pathogenesis of the condition. It has been found that spiral arteries derived from pre-eclamptic pregnancies have a reduced mean external diameter compared to those from normal pregnancies (200 µm compared to 500 µm) (Brosens, Robertson and Dixon, 1972) (Figure 1.3). This defect in vascular remodelling dramatically reduces blood supply to the placenta resulting in poor placental perfusion (Zhou *et al.*, 1993; Redman and Sargent, 2001). Several factors have been implicated in the poor remodelling of spiral arteries resulting in the onset of pre-eclampsia and other pregnancy complications. These include reduced differentiation of EVT into an invasive phenotype, increased trophoblast apoptosis, an imbalance in the functions of migratory and invasive EVT, and the inability of cytotrophoblasts to adopt an endovascular phenotype, resulting in a reduced pool capable of remodelling the spiral arteries (Zhou, Damsky and Fisher, 1997; Allaire *et al.*, 2000; Lala and Chakraborty, 2003). In addition, it has been found that cytotrophoblasts in pre-eclamptic pregnancies show a reduction in their ability to produce MMPs (Reister *et al.*, 2006) yet elevated production of E-cadherin and the anti-invasive factor TGF-β (Moffett-King, 2002). It is unclear exactly what causes this dysregulation in EVT differentiation, proliferation and function, however, it has been found that levels of several cytokines involved in the control of these processes are altered in the blood of women with pre-eclampsia (Kharfi *et al.*, 2003).

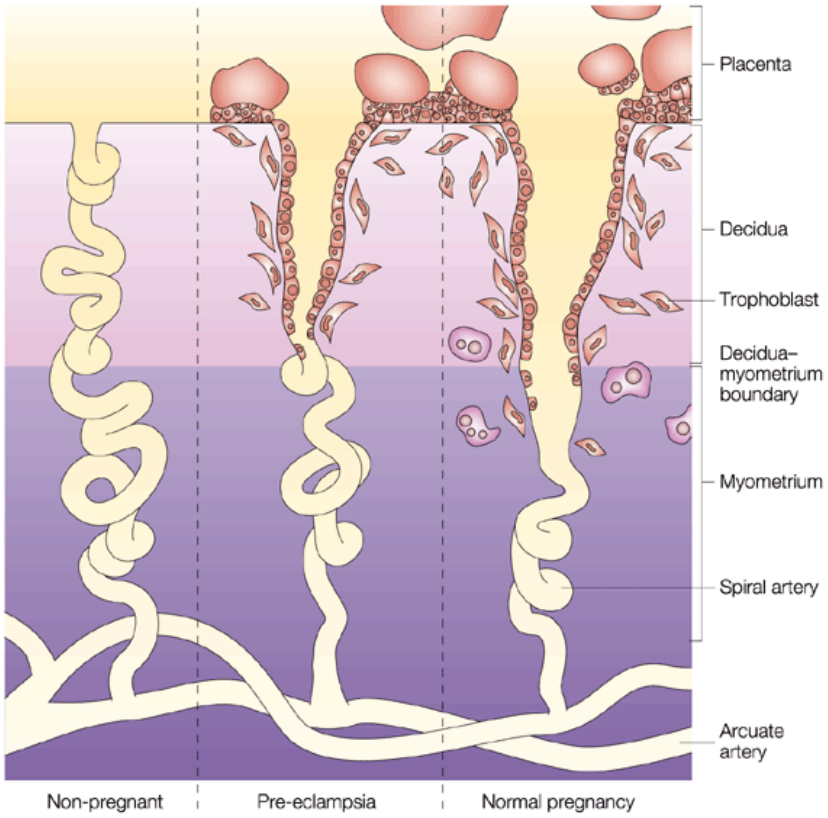



**Figure 1.3 A diagram to show the difference in spiral artery remodelling between normal and pre-eclamptic pregnancies.**

*(From: Bell, 2004)*

It is evident that there are many different mechanisms underlying the pathogenesis of pre-eclampsia and it is impossible to define a single cause. Our knowledge of the condition is hampered by the problems we face in studying human pregnancy *in vivo*, thus, the only known cure for pre-eclampsia at present is delivery of the placenta (Powe, Levine and Karumanchi, 2011).

### 1.7.2 Recurrent miscarriage

Another pregnancy disorder associated with impaired placentation and vascular remodelling is recurrent miscarriage which is defined as three or more consecutive

pregnancy losses before the 20th week of pregnancy (Garrido-Gimenez and Alijotas-Reig, 2015). It affects 1-3% of pregnancies and its occurrence rises steeply with maternal age (Rai and Regan, 2006). There are a number of genetic, structural, infective, endocrine, and immune causes that have been attributed to the onset of recurrent miscarriage (Rai and Regan, 2006). Herein, the causes relating to placentation and the remodelling process will be discussed, these include the involvement of uterine natural killer cells, oxidative stress, and antiphospholipid antibodies (aPL).

It has been proposed that impaired decidualisation may be attributed to the onset of recurrent miscarriage. In endometrial cells derived from a woman with recurrent miscarriage, increased expression of the marker of decidualisation, prokineticin-1, has been reported. This is indicative of an increased period of endometrial receptivity which is thought to disrupt the normal process of embryo selection and the maternal responses to embryonic signals (Salker *et al.*, 2010). In addition, aberrations in first trimester uteroplacental blood flow has been linked to the aetiology of recurrent miscarriage. Premature disassociation of trophoblast plugs resulting in aberrant blood flow into the intervillous space has been reported in cases of recurrent miscarriage. This can result in increased placental oxidative stress and trophoblast degeneration (Hempstock *et al.*, 2003). In the mid-luteal phase endometrium of women suffering from idiopathic recurrent miscarriage, an increase in the density of uterine natural killer (NK) cells has been reported. It is thought that this could also contribute to the increased peri-implantation blood flow (Quenby *et al.*, 2009).

Women with idiopathic recurrent miscarriage have increased aPL in comparison to normal fertile women (Buckingham and Chamley, 2009). aPL are a heterogeneous group of autoantibodies which are reactive with negatively charged phospholipids and their

associated plasma protein co-factors and are associated with a number of pregnancy complications. The relevance of increased aPL in cases of recurrent miscarriage is unclear, but aPL have been shown to have direct placental effects such as inhibiting trophoblast differentiation and invasion, as well as inhibiting endometrial decidualisation (Buckingham and Chamley, 2009).

### 1.7.3 Fetal growth restriction (FGR)

FGR is a pregnancy complication in which the fetus fails to reach its full growth potential due to impaired placental function, which may result from a variety of factors (Martins, Biggio and Abuhamad, 2020).

FGR occurs in up to 10% of pregnancies and is one of the leading causes of infant morbidity and mortality (Bernstein *et al.*, 2000; Unterscheider *et al.*, 2014; Swanson and David, 2015). The aetiology of FGR is complex and is thought to be caused by a number of maternal, fetal, and placental causes (Swanson and David, 2015). Maternal factors implicated in the aetiology of FGR include hypertension, diabetes, cardiopulmonary disease, anaemia, malnutrition, and smoking. Fetal causes include genetic factors, congenital malformations, fetal infections, and multifetal pregnancy. Placental causes include: placental insufficiency, placental infarction, and placental mosaicism (Sławek-Szmyt *et al.*, 2022). FGR has been classified into two categories, early onset which is diagnosed before 32 weeks gestation, and late onset which is diagnosed at or after 32 weeks gestation (Figueras *et al.*, 2018; Martins, Biggio and Abuhamad, 2020). Early onset FGR is frequently associated with pre-eclampsia in 60-70% of cases and is thought to be caused by abnormal placentation (Mecacci *et al.*, 2021). Late onset FGR is thought to be less related to pre-eclampsia and does not seem to be determined by abnormal placentation in the first trimester (Figueras *et al.*, 2018). It should be noted that the diagnosis of FGR is distinct from the small-for-

gestational-age diagnosis which describes fetuses which are small but healthy and at lower risk of abnormal perinatal outcomes (Unterscheider *et al.*, 2013).

Although pre-eclampsia and FGR are distinct conditions, they are known to share a similar pathogenesis of inadequate placentation, inflammation, and maternal vascular dysfunction (Lane-Cordova *et al.*, 2019). It is thought that early onset FGR is the result of poor trophoblastic invasion, leading to insufficient spiral artery remodelling and, therefore, increased maternal peripheral vascular resistance (Bamfo *et al.*, 2007; Staff, Dechend and Redman, 2013; Burton and Jauniaux, 2018). It is also hypothesised that pre-existing defects in maternal cardiovascular function characterised by low cardiac output and high systemic vascular resistance may cause insufficient placental perfusion and trophoblast impairment (Foo *et al.*, 2018; Tay *et al.*, 2019; Mecacci *et al.*, 2021). Vascular dysfunction in early onset FGR results in a small placenta, compromised feto-placental angiogenesis and villous development, and reduced uteroplacental blood flow resulting in insufficient maternal to fetal transfer of oxygen and nutrients (Mecacci *et al.*, 2021). In comparison to pre-eclampsia, however, FGR alone does not present with the same inflammatory and cardiovascular responses that are characteristic of pre-eclampsia. For example, the anti-angiogenic factors such as soluble endoglin, soluble fms-like tyrosine kinase 1 (sFlt-1), and endostatin are all known to be increased in cases of pre-eclampsia, but not in cases of normotensive FGR (Shibata *et al.*, 2005).

## 1.8 Animal models of pre-eclampsia

Our understanding of pre-eclampsia and other pregnancy complications whose aetiology occurs in the first trimester has been hampered by a lack of appropriate models. As pre-eclampsia only occurs in humans and certain ape species of the gorilla genus (Thornton and

Onwude, 1992) and does not occur naturally in laboratory animal models, the characteristics and symptoms need to be induced in these models (Marshall *et al.*, 2018).

Several animal models of pre-eclampsia have been proposed based on the perceived causes and mechanisms of the condition. One such model is a uteroplacental ischemia/reduced uterine perfusion pressure (RUPP) model based on the observation that decreasing uterine blood flow increases maternal blood pressure (Sunderland, Hennessy and Makris, 2011). Creation of this model generally involves partial or complete occlusion of the abdominal aorta and/or occlusion of one or both uterine arteries and have been created in a number of animals including rabbits (Losonczy, Brown and Venuto, 1992), dogs (Abitbol, 1981; Woods and Brooks, 1989), rhesus monkeys (Andrew Combs, Katz and Kitzmiller, 1993), baboons (Cavanagh *et al.*, 1977), and sheep (Clark, Durnwald and Austin, 1982). The most widely used RUPP model is the rat RUPP model which has been well-characterised and reproduced in numerous studies (Granger *et al.*, 2006). In this model, uteroplacental perfusion is reduced by approximately 40% resulting in pregnancy specific increases in mean arterial blood pressure, as well as proteinuria, endothelial dysfunction, and FGR (Llinás *et al.*, 2002; Walsh *et al.*, 2009). This RUPP model is known to most closely represent severe pre-term pre-eclampsia and is associated with an imbalance in angiogenic factors, such as increased sFlt-1 and soluble endoglin, and decreased VEGF and PlGF.

In addition to RUPP models, several genetically manipulated mouse models have also been used to model the pathophysiology of pre-eclampsia. Nitric oxide synthase knockout mouse models have been created which are hypertensive both before and during pregnancy, and display proteinuria, reduced cardiac output, uterine artery dysfunction, FGR, and placental hypoxia, without elevated levels of sFlt-1 (Kulandavelu *et al.*, 2012; Kusinski *et al.*, 2012). Furthermore, several other mouse models have been created which model pre-eclampsia

including catechol-O-methyltransferase (COMT) deficient mouse models and the borderline hypertensive mouse strain (BPH/5) which demonstrate a pre-eclamptic-like phenotype when pregnant (Davisson *et al.*, 2002; Kanasaki *et al.*, 2008).

Several pharmacological animal models of pre-eclampsia have also been developed to induce some of the clinical features of the condition. These include nitric oxide inhibition models which display symptoms including hypertension, proteinuria, and FGR (Molnär, *et al.*, 1994; Salas *et al.*, 1995). In addition, angiogenic based models of pre-eclampsia have been developed in which exogenous administration of sFlt-1 or soluble endoglin results the onset of disease-specific symptoms (Maynard *et al.*, 2003; Venkatesha *et al.*, 2006).

Although a number of animal models of pre-eclampsia exist, they vary in their reproducibility and characterisation. None, so far, are capable of exactly representing the human condition (McCarthy *et al.*, 2011). Due to the heterogeneity of pre-eclampsia, it is difficult to create a standard model which would reflect the range of clinical symptoms presented in humans, and differences in the aetiology between early and late onset pre-eclampsia complicate this further (Marshall *et al.*, 2018). The existing models have contributed to increasing our understanding of the pathogenesis of this disorder, but extrapolation of these data to the human condition should be with caution (McCarthy *et al.*, 2011).

## 1.9 Spiral artery remodelling and stanniocalcin-1 (STC-1)

Stanniocalcin-1 (STC-1) is a glycoprotein that has been implicated in the spiral artery remodelling process in early pregnancy. A 2013 study by Wallace and colleagues investigated the effect of fetal trophoblast cell secreted factors on vascular cell gene expression to further understand the trophoblast/vascular cell interactions which occur

during the spiral artery remodelling process in early pregnancy (Wallace *et al.*, 2013). A three-dimensional spheroid co-culture of ECs and VSMCs was used to model an inverted vessel lumen. Following treatment of the spheroid co-culture with conditioned media from an EVT cell line, 101 vascular cell genes showed significant up/downregulation. Of these, STC-1 gene expression was upregulated two-fold (Wallace *et al.*, 2013).

STC-1 is known to be implicated in roles in both normal female reproductive physiology, and also in the pathophysiology of several reproductive disorders (Bishop, Cartwright and Whitley, 2021). STC-1 is widely expressed in female reproductive tissues including the ovaries (Varghese *et al.*, 1998; Deol *et al.*, 2000), uterus (Stasko, DiMattia and Wagner, 2001; Xiao *et al.*, 2006; Allegra *et al.*, 2009), and the placenta (Uusküla *et al.*, 2012; Juhanson *et al.*, 2016; Abid *et al.*, 2020). In addition, it has been shown that although STC-1 is usually undetectable in the circulation, during pregnancy, detectable levels have been reported (Deol *et al.*, 2000). Moreover, in complicated pregnancies, third trimester and post-partum serum levels of STC-1 are even further elevated (Uusküla *et al.*, 2012; Abid *et al.*, 2020). The current evidence is suggestive of a key role for STC-1 within female reproduction and within both normal and complicated pregnancies. The relevance of STC-1 upregulation within this system and the mechanisms underlying this, however, have yet to be elucidated.

## 1.10 STC-1

Stanniocalcin (STC) is a homodimeric glycoprotein first isolated in bony fish where it is secreted by the corpuscles of Stannius, small endocrine glands located on the ventral surface of the kidneys (Stannius, 1839). Surgical removal of these glands in the 1960s was found to result in hypercalcaemia, implicating STC in calcium homeostasis in fish (Fontaine, 1964).

In the late 1980s, the anti-hypercalcaemic factor secreted by the corpuscles of Stannius was purified and identified as STC (Wagner *et al.*, 1986; Lafeber *et al.*, 1988). No comparable structure to the corpuscles of Stannius had been identified in mammals, thus it was assumed that the STC gene had been lost during evolution (Yeung, Law and Wong, 2012). Early studies investigating the role of fish STC in mammalian cells, however, reported physiological roles for STC in the mammalian system (Lafeber *et al.*, 1989; Yoshiko, Kosugi and Koide, 1996). Moreover, evidence of a STC-like protein was found in human serum and kidney extracts following observed immunoreactivity with salmon STC antiserum (Wagner *et al.*, 1995). These findings led to the initial hypothesis of the existence of a mammalian STC orthologue.

The mammalian orthologue of STC was discovered in the 1990s following the successful cloning of mouse and human cDNAs by two independent groups (Chang *et al.*, 1995; Olsen *et al.*, 1996). The amino acid sequence of this orthologue shows ~61% identity and ~73% similarity with various fish STCs (Chang *et al.*, 1995). In 1998, a second member of the mammalian STC family was identified by several research groups (Chang and Reddel, 1998; DiMattia, Varghese and Wagner, 1998; Ishibashi *et al.*, 1998), resulting in the renaming of mammalian STC as STC-1, and the newly identified protein as STC-2 (Chang and Reddel, 1998).

### 1.10.1 Structure of STC-1

Human STC-1 cDNA encodes a protein of 247 amino acids (Chang *et al.*, 1995) and is composed of four exons (Chang *et al.*, 1998). STC-1 has a predicted molecular weight of 27 kDa (Trindade *et al.*, 2009), however, several studies have pointed to the existence of di- or multimers (Zhang *et al.*, 1998; Paciga *et al.*, 2002, 2005). The dimeric 56 kDa form of STC-1 is known as STC50 and a number of higher molecular weight variants have also been

identified in ovarian cells, adrenocortical cells and adipocytes, and are collectively referred to as 'big STC'. Within this category, at least three molecular weights: 84, 112, and 135 kDa have been described (Paciga *et al.*, 2002, 2005).

The human STC-1 protein is analogous to fish STC. The first 204 amino acids of human STC-1 show 92% similarity to that of salmon, however, the last 43 residues at the C-terminus differ immensely (Chang, Jellinek and Reddel, 2003). In contrast, human STC-2 encodes a protein of 302 amino acids which shows just 34% identity to both human STC-1 and eel STC, with the greatest sequence similarity present at the N-terminus (Chang, Jellinek and Reddel, 2003). At this terminus, a conserved cysteine motif is found in both fish and human STCs. In STC-1, there are 11 cysteine residues with the same spacing as those in fish STCs (Butkus *et al.*, 1987; Wagner *et al.*, 1992). STC-2 possesses 15 cysteine residues, but only 10 of which have the same spacing as fish STC and STC-1 (Moore *et al.*, 1999). During translation of the protein, 10 out of the 11 cysteines form intrachain disulphide linkages and the 11th cysteine residue functions in forming the interchain dimer (Trindade *et al.*, 2009), allowing STC-1 to exist as a homodimer in its native state (Lafeber *et al.*, 1988). The lack of spatial conservation of the 11th cysteine residue in STC-2 compared to fish STC/STC-1, combined with the presence of four additional cysteine residues in this protein, leads to the prediction that STC-2 has a different tertiary structure when compared with other STCs. Despite some structural similarities between both STC-1 and STC-2, there is no evidence, thus far, to suggest that these proteins are capable of heterodimerisation (Joshi, 2020).

### 1.10.2 STC-1 secretion and protein secretion pathways

Another defining feature of STC-1, which is also conserved amongst the STCs, is the presence of an N-linked glycosylation consensus sequence. In STC-1, this is present at residues 62–64; consistent with the position of this sequence in fish STC (Butkus *et al.*,

1987; Wagner *et al.*, 1992). Protein glycosylation is important for protein functions such as stability, folding, and secretion (Dwek *et al.*, 2002). N-glycosylation is a type of glycosylation in which N-glycans are directly attached to asparagine residue in proteins, Asn-X-Thr/Ser (where X represents any amino acid except proline) (Lennarz, 1987; Ruddock and Molinari, 2006).

STC-1 is secreted from most cells and contains a signal peptide sequence of ~24 amino acids and a pro-sequence of ~15 amino acids, both of which are processed to yield the mature forms of the protein (Moore *et al.*, 1999). In most cell types, STC-1 is produced in large amounts intracellularly in lightly glycosylated forms, however, when secreted, STC-1 is highly glycosylated and phosphorylated by protein kinase C (Jellinek *et al.*, 2000).

Eukaryotic cells possess an elaborate endomembrane system consisting of a number of independent organelles that function sequentially to enable protein secretion. The ER is the entry point into the secretory pathway for newly synthesised proteins. Ribosomes dock onto a protein pore in the ER membrane, releasing the new protein into the lumen of the ER. The primary role of the ER is to provide an environment for proper protein folding, enabled by chaperone proteins. Post-translational modification of proteins, such as glycosylation, also occurs in the ER (Lee *et al.*, 2004). Folded, partially glycosylated proteins are then transported to the Golgi apparatus and move through the Golgi sub-compartments (cis, medial, trans) before entering the trans-Golgi network which sorts proteins into distinct transport pathways aided by clathrin and adaptor proteins (Ponnambalam and Baldwin, 2009).

Proteins can be secreted from cells by either a constitutive or regulated secretory pathway and these two pathways diverge in the trans-Golgi network (Kelly, 1985; Moore, 1987). The

constitutive pathway operates in all cells and in this pathway, proteins are secreted at the same rate at which they are synthesised. Many soluble proteins are continually secreted from the cell by this pathway, and this also supplies the plasma membrane with newly synthesised lipids and proteins. Constitutively secreted proteins are packaged into small vesicles which fuse directly with the plasma membrane without prior storage. In regulated secretion, newly synthesised proteins destined for secretion are stored at high concentrations in secretory vesicles until an appropriate stimulus is received by the cell (Brion, Miller and Moore, 1992). The pathway by which STC-1 is secreted from cells has not yet been characterised.

### 1.10.3 Expression pattern of STC-1 in mammals

STC-1 has a wide expression pattern in mammals, with reported expression in many tissues including the heart, lungs, liver, adrenal gland, kidney, ovary, prostate, colon, bone, and spleen (Chang *et al.*, 1995; Olsen *et al.*, 1996; Varghese *et al.*, 1998; Yoshiko and Aubin, 2004; Liu *et al.*, 2010; Law *et al.*, 2011) and the highest expression levels are found in the ovary, kidney, prostate, and thyroid (Moore *et al.*, 1999). STC-1 is not normally found in the circulation (De Niu *et al.*, 2000; Deol *et al.*, 2000) suggesting that STC-1 generally acts as a paracrine/autocrine factor (Yeung, Law and Wong, 2012).

STC-1 is targeted to specific tissues through a receptor-mediated process. Several studies have reported the existence of a STC-1 receptor. Through the use of an STC-1 alkaline phosphatase fusion protein, composed of mouse STC-1 cDNA fused to the human placental alkaline phosphatase gene, high affinity (0.25–0.8 nm), saturable and displaceable binding sites for STC-1 were identified in cells of the kidney, liver, lung, brain, skeletal muscle, spleen, breast, and ovaries (McCudden *et al.*, 2002; Paciga *et al.*, 2003; Hasilo *et al.*, 2005). Within the kidney and liver, 90% of the binding sites are mitochondrial and the remaining

are located at the plasma membrane (McCudden *et al.*, 2002). Similarly, in mammary gland alveolar cells in non-pregnant and pregnant rats, STC-1 receptors are mitochondrial and plasma membrane-associated, however, during lactation STC-1 is targeted instead to specific nuclear receptors (Hasilo *et al.*, 2005). In the ovaries, STC-1 and its receptor are preferentially targeted to cholesterol/lipid storage droplets (Paciga *et al.*, 2003).

### 1.10.4 Regulation of mammalian STC-1 expression

In mammals, regulation of STC-1 expression is tissue-specific and is mediated by a variety of stimuli and through a number of signalling pathways. Consistent with its role as an anti-hypercalcaemic hormone, STC-1 is regulated by calcium. For example, in human fibroblast cells, extracellular calcium induced a 10-fold increase in steady-state STC-1 mRNA levels (Chang *et al.*, 1995). Similarly, in the canine renal cell line, MDCK, STC-1 expression was induced 8-fold under conditions of osmotic stress, but only in the presence of high extracellular calcium, confirming a role for extracellular calcium in STC-1 regulation (Sheikh-Hamad, Rouse and Yang, 2000). In addition, the active metabolite of vitamin $D_3$, calcitriol, has been shown to affect STC-1 expression in a tissue-specific manner (Honda *et al.*, 1999). In female rats treated with calcitriol, STC-1 mRNA levels were increased 3-fold in the kidney, but had no effect on ovarian STC-1 expression (Honda *et al.*, 1999).

In a number of mammalian cell lines, hypoxia has also been shown to be a key regulator of STC-1 expression (Lal *et al.*, 2001; Yeung *et al.*, 2005; Ito *et al.*, 2014). Hypoxia induces expression of hypoxia-inducible factor (HIF)-1 alpha, which in turn upregulates STC-1 expression (Ito *et al.*, 2014). This upregulation is mediated through the hypoxia response element found in the promoter of STC-1 (Law *et al.*, 2010). Additionally, glucocorticoids have been shown to influence mammalian STC-1 expression. Hydrocortisone and dexamethasone significantly reduce steady-state STC-1 mRNA levels in a mouse

corticotrope and a human fibrosarcoma cell line, but this effect is antagonised by activation of the cyclic adenosine monophosphate (cAMP) signalling pathway (Groves, Wagner and DiMattia, 2001). cAMP signalling has also been implicated in the regulation of STC-1 gene expression in ovarian cells (Paciga *et al.*, 2002; Paciga, DiMattia and Wagner, 2004). In ovarian thecal interstitial cells, stimulation of STC-1 expression by hCG is mediated through activation of the cAMP/protein kinase A (PKA) pathway (Paciga *et al.*, 2002). Similarly, in luteal cells of the ovary, STC-1 expression is regulated by activation of the cAMP/PKA pathway (Paciga, DiMattia and Wagner, 2004).

Within the vascular system, STC-1 expression in vascular ECs is upregulated by lysophosphatidylcholine (lysoPC), a component of oxidised lipoproteins which is present in atherosclerotic lesions and has pro-atherogenic properties (Sato *et al.*, 1998). Moreover, STC-1 expression was found to be significantly upregulated in vascular ECs in a three-dimensional model of angiogenesis where phorbol 12-myristate 13-acetate (PMA), FGF-2 and VEGF were used to stimulate tube formation (Kahn *et al.*, 2000).

## 1.11 The role of STC-1 in mammals

### 1.11.1 STC-1 in angiogenesis

Several studies have implicated STC-1 in angiogenesis. STC-1 has been shown to promote expression of VEGF in human umbilical vein endothelial cells (HUVECs) (Law and Wong, 2013) and gastric cancer cells (He *et al.*, 2011), and induce angiogenic sprouting regulated through the VEGF/VEGF receptor 2 (VEGFR2) pathway (Law and Wong, 2013). Moreover, STC-1 has been found to be upregulated in an *in vitro* model of angiogenesis and specifically expressed in the vasculature of colon carcinomas (Gerritsen, Peale Jr. and Wu, 2002). Additionally, it has been proposed that STC-1 may play a regulatory role in angiogenesis, acting as a stabilising factor contributing to the maturation of newly formed blood vessels

through selective modulation of hepatocyte growth factor (HGF)-induced EC migration and morphogenesis (Zlot *et al.*, 2003).

STC-1 has also been implicated in pathological angiogenesis in a rat model of laser-induced choroidal neovascularisation. In this model, STC-1 stimulates the pathological growth of new blood vessels in choroidal tissue through upregulation of VEGF and VEGFR2 expression (Zhao *et al.*, 2018).

### 1.11.2 STC-1 in cell proliferation

STC-1 plays a key role in the process of cell proliferation, predominantly in the pathology of cancers. Overexpression of STC-1 has been shown to induce cell proliferation in prostate cancer cells via cyclin E1/cyclin-dependent kinase 2 (CDK2) (Bai *et al.*, 2017; Costa *et al.*, 2020). Similarly, in clear cell renal cell carcinoma cell lines, silencing of STC-1 expression resulted in inhibition of cell proliferation (Ma *et al.*, 2015). In gastric cancer cells, overexpression of STC-1 has been demonstrated to induce cell proliferation, but only under conditions of hypoxia (Wang *et al.*, 2019). In contrast, in cervical cancer cells, overexpression of STC-1 has been shown to inhibit cell proliferation (Guo *et al.*, 2013).

In breast cancer, the effect of STC-1 on cell proliferation depends on the subtype. In triple-negative breast cancer (TNBC) cell lines, it has been reported that STC-1 has no effect on cell proliferation (Chang *et al.*, 2015). However, in a luminal breast cancer cell line which expresses the oestrogen and progesterone receptors, knock-down of STC-1 was shown to inhibit cell proliferation (Daniel and Lange, 2009). Similarly, in another oestrogen-receptor positive cell line, overexpression of STC-1 was shown to promote cell proliferation (Hou *et al.*, 2021). The difference in the effect of STC-1 in these two subtypes can be explained by the finding that the STC-1 gene is co-expressed with the oestrogen receptor and is an

oestrogen responsive gene (Bouras *et al.*, 2002; McCudden *et al.*, 2004). Therefore, it is likely that its effect on cell proliferation will be specific to oestrogen receptor-expressing breast cancer cells.

### 1.11.3 STC-1 in apoptosis

In addition to playing a key role in cell proliferation, STC-1 has also been implicated in the regulation of apoptosis. Depending on cell type and environment, STC-1 is involved in either pro- or anti-apoptotic roles.

In some cancer cells, STC-1 has been associated with pro-apoptotic roles. For example, in cervical cancer cells, overexpression of STC-1 induces apoptosis via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) phospho-P65 (Ser536) by phosphoinositide 3-kinase (PI3K)/Akt, IκBα and IκB kinase (IKK) signalling (Pan *et al.*, 2017). Similarly, in hepatocellular carcinoma cell lines, STC-1 up-regulates pro-apoptotic genes such as interleukin-12 (IL-12) and NOD-like receptor family pyrin domain-containing 3. STC-1 has also been shown to be regulated by the tumour suppressor protein p53 in nasopharyngeal cancer cells. In these cells, oxidative stress or trichostatin-A-induced apoptosis stimulates overexpression of STC-1 through activation of p53 (Lai *et al.*, 2007; Ching, Yeung and Wong, 2012).

STC-1 also exerts pro-apoptotic effects in normal physiology and in response to cellular stress. Treatment of fetal rat metatarsal bones with recombinant STC-1 has been shown to increase the number of apoptotic chondrocytes in the growth plate (Wu, Yoshiko and De Luca, 2006). In response to hydrogen-peroxide induced stress in mouse embryo fibroblasts, loss of function of STC-1 results in increased resistance to apoptosis. STC-1 has been shown to exert these pro-apoptotic effects through downregulation of mitogen-activated protein

kinase kinase (MEK) and consequent downregulation of pro-survival signalling pathway extracellular signal-regulated kinases (ERK)1/2 (Nguyen, Chang and Reddel, 2009).

STC-1 also plays an anti-apoptotic role in some cell types, and this has been shown to be regulated by hypoxia. STC-1 expression is upregulated under hypoxic conditions in a number of human cancer cells and in human lung epithelial cells and this is mediated by HIF-1 (Yeung *et al.*, 2005; Ito *et al.*, 2014). In hypoxic gastric cancer cells, STC-1 expression is elevated and is implicated in anti-apoptotic, pro-proliferative roles (Wang *et al.*, 2019).

In addition to hypoxia-induced stress, STC-1 is also implicated in anti-apoptotic roles in response to a range of different cellular stresses. For example, STC-1 reduces reactive oxygen species-induced apoptosis in lung cancer cells by uncoupling oxidative phosphorylation (Ohkouchi *et al.*, 2012). Furthermore, in breast cancer cells, overexpression of STC-1 has been demonstrated to reduce apoptosis following X-ray irradiation treatment (Hou *et al.*, 2021). Recombinant STC-1 treatment of mice lungs decreases the extent of apoptosis following lipopolysaccharide-induced injury (Tang *et al.*, 2014). In addition, multipotent stem cells have been shown to upregulate and secrete STC-1 to reduce apoptosis of lung cancer epithelial cells (Block *et al.*, 2009).

### 1.11.4 STC-1 in cell migration

In cancer cells, STC-1 has been implicated in both pro- and anti-migratory roles. In cervical cancer cells, knock down of STC-1 resulted in enhanced cell migration and overexpression of STC-1 inhibited invasion of cervical cancer cells (Guo *et al.*, 2013). In contrast, STC-1 has been shown to act as a pro-migratory factor in cancer cells which likely relates to function in metastasis. For example, STC-1 promotes migration of breast cancer cells (Hou *et al.*, 2021), renal carcinoma cells (Ma *et al.*, 2015), hypoxic gastric cancer cells (Wang *et al.*,

2019), ovarian cancer cells (Liu *et al.*, 2010; Lin *et al.*, 2022), and colorectal cancer cells (Pena *et al.*, 2013). In line with this, greater levels of STC-1 expression have been observed in metastatic cell lines and tissues (Ma *et al.*, 2015; Lin *et al.*, 2022).

STC-1 has also been demonstrated to play an inhibitory role in immune cell migration in the cancerous tumour cell environment. Tumour mass consists of heterogeneous populations of cancer cells and infiltrating immune cells and tumour progression is strongly influenced by the interactions between these cells (Leung and Wong, 2020). In a co-culture model of human metastatic hepatocellular carcinoma cells overexpressing STC-1 with a macrophage cell line, STC-1 demonstrated an inhibitory effect on macrophage migration/infiltration (Leung and Wong, 2020). It is thought that the effect of STC-1 on macrophage migration in this way may negatively impact tumour development (Leung and Wong, 2020). Similarly, STC-1 has been found to inhibit the transmigration of macrophages and T lymphocytes through ECs, but not affect the transmigration of neutrophils and B lymphocytes (Chakraborty *et al.*, 2007). It is thought that this may relate STC-1 to a role in transendothelial migration of inflammatory cells to attenuate the influx of these cells to sites of tissue injury (Chakraborty *et al.*, 2007).

In angiogenesis, STC-1 also displays contrasting effects on cell migration. Directly, STC-1 overexpression in ECs promotes migration (Law and Wong, 2013), however, indirectly, STC-1 selectively modulates EC migration in response to growth factors. Recombinant STC-1 specifically inhibits the migratory response of ECs following HGF-stimulation but not following stimulation with VEGF or FGF-2 (Zlot *et al.*, 2003).

## 1.12 STC-1 in the female reproductive system

Numerous studies have highlighted the specific role of STC-1 in the physiology and pathophysiology of the female reproductive system. STC-1 is widely expressed in female reproductive tissues including the ovaries (Deol *et al.*, 2000; Paciga *et al.*, 2002; Luo *et al.*, 2004; Basini *et al.*, 2010; Jepsen *et al.*, 2016), uterus (Stasko, DiMattia and Wagner, 2001; Xiao *et al.*, 2006; Allegra *et al.*, 2009), and placenta (Uusküla *et al.*, 2012; Juhanson *et al.*, 2016; Abid *et al.*, 2020). Amongst all female reproductive tissues, the highest expression of STC-1 is found in the ovaries where its expression pattern and roles are best characterised (Varghese *et al.*, 1998; Deol *et al.*, 2000). STC-1 expression is virtually undetectable in the male testis (Varghese *et al.*, 1998; Deol *et al.*, 2000), suggesting that its role is specific to female reproduction (Bishop, Cartwright and Whitley, 2021).

### 1.12.1 The expression pattern of STC-1 in the mammalian ovary

STC-1 expression has been reported in the ovaries of mice (Deol *et al.*, 2000), rats (Paciga *et al.*, 2002; Luo *et al.*, 2004), pigs (Basini *et al.*, 2010; Baioni *et al.*, 2011), bovines (Paciga *et al.*, 2002, 2003; Paciga, DiMattia and Wagner, 2004), and humans (Jepsen *et al.*, 2016).

The localisation of STC-1 expression within the ovary varies between species. In mice, STC-1 gene and protein expression have been reported in the secondary interstitial and theca interna cells whilst STC-1 protein expression is only found in oocytes and corpus luteal cells (Varghese *et al.*, 1998). In pigs, STC-1 protein is expressed in the ovarian follicular fluid and is produced by granulosa cells (Basini *et al.*, 2010), while STC-1 gene expression is detectable in the thecal layer of antral follicles, consistent with observations in mice, rats, and bovines (Varghese *et al.*, 1998; Paciga *et al.*, 2002). The discordance between STC-1 gene and protein expression observed in the mammalian ovary is thought to be indicative

of a mechanism in which certain ovarian cells do not express the STC-1 gene but function as targets for STC-1 and therefore sequester the protein (Varghese *et al.*, 1998).

#### 1.12.1.1 Regulation of ovarian STC-1 expression

In mice, expression of ovarian STC-1 is regulated by luteinising hormone (LH) (Deol *et al.*, 2000), whereas in rat and bovine ovarian cells, hCG has been shown to stimulate secretion of STC-1 (Paciga *et al.*, 2002). The effect of hCG on ovarian cells is mimicked by treatment with forskolin, suggesting that STC-1 regulation is mediated through the cAMP/PKA pathway (Paciga *et al.*, 2002).

#### 1.12.1.2 Structure and sub-cellular targeting of ovarian STC-1

The structure of ovarian STC-1 is physically distinct from STC-1 identified in other mammalian tissues and has been referred to as 'big STC-1' (Paciga *et al.*, 2002). STC-1 derived from thecal interstitial cells is larger than STC50 found in other mammalian tissues. In the native state, STC-1 derived from thecal interstitial cells comprises three molecular mass species of 84, 112, and 135 kDa (Paciga *et al.*, 2002). Chemical reduction of big STC-1 resulted in a 45 kDa species, implying that the three molecular masses are polymers of two or more subunits which are stabilised by disulphide linkages (Paciga *et al.*, 2002).

STC-1 receptors in reproductive tissues have been little studied, however, big STC-1 receptors have been described (Paciga *et al.*, 2003). In bovine luteal cells, populations of big STC-1 receptors have been identified in cholesterol/lipid storage droplets and some targeting to the luteal cell plasma membrane is also evident, however, the affinity of the plasma membrane receptor is 10-fold lower than that of the cholesterol/lipid storage droplets receptor (Paciga *et al.*, 2003). This receptor-mediated targeting in luteal cells provides an explanation for the observed discordances in the patterns of STC-1 mRNA and

protein distribution in ovarian cells. For example, although both STC-1 gene and protein expression are detectable in the thecal interstitial compartment in mice, rats and bovines (Varghese *et al.*, 1998; Deol *et al.*, 2000; Paciga *et al.*, 2002), only STC-1 protein expression is present in oocytes and corpus luteal cells (Varghese *et al.*, 1998; Deol *et al.*, 2000), thus, it is postulated that oocytes and luteal cells may be targets for STC-1 in this case and sequester the protein (Varghese *et al.*, 1998).

#### 1.12.1.3 The role of STC-1 in the mammalian ovary

Within ovarian cells, STC-1 has been implicated in roles in steroidogenesis and folliculogenesis. In bovine luteal cells and rat granulosa cells, STC-1 has been reported to decrease gonadotropin-stimulated progesterone synthesis (Paciga *et al.*, 2003; Luo *et al.*, 2004). It is thought that STC-1 binds to high specificity and affinity receptors on the cell plasma membrane and acts downstream of adenylyl cyclase in the cAMP/PKA pathway to exert these effects on progesterone biosynthesis (Luo *et al.*, 2004).

STC-1 has also been reported to regulate pig granulosa cell redox status which is important in folliculogenesis. In granulosa cells, STC-1 stimulates the production of superoxide anion (Baioni *et al.*, 2011), a ROS implicated in modulating ovarian steroidogenesis (Sawada and Carlson, 1996; Jain *et al.*, 2000; Behrman *et al.*, 2001) and in inducing ovulation (Behrman *et al.*, 2001; Fujii, Iuchi and Okada, 2005). It is likely that STC-1 exerts this effect through its stimulatory effects on the mitochondrial electron transport chain (Ellard *et al.*, 2007) as mitochondria are the main sites of superoxide anion generation (Murphy, 2009) but the specific mechanisms are not yet understood.

In addition, STC-1 has been shown to inhibit the proteolytic activity of the metzincin metalloproteinase, pregnancy-associated plasma protein-A (PAPP-A), *in vitro* through non-

covalent, high affinity binding (Kløverpris *et al.*, 2015). PAPP-A is secreted from granulosa cells (Conover *et al.*, 2001) and is known to regulate signalling of insulin-like growth factor (IGF) which is involved in several physiological ovarian functions such as follicular growth, steroidogenesis and ovulation (Kwintkiewicz and Giudice, 2009). In human ovaries, inhibitory complexes between PAPP-A and STC-1 have been detected in follicular fluid leading to a decrease in IGF release (Jepsen *et al.*, 2016). It is postulated that STC-1 may be involved in the decline in IGF-mediated follicular growth through its inhibitory actions on PAPP-A (Jepsen *et al.*, 2016).

### 1.12.2 The expression pattern of STC-1 in mammalian endometrium

Endometrial expression of STC-1 has been described in several mammals, including humans, rats, pigs, sheep, and horses (Song *et al.*, 2006, 2009; Xiao *et al.*, 2006; Kikuchi *et al.*, 2011; Aghajanova *et al.*, 2016; Khatun *et al.*, 2020). Several high-throughput gene expression profiling studies have implicated STC-1 in human endometrial functions and report fluctuating tissue expression levels throughout the menstrual cycle. Endometrial expression of STC-1 is high during the mid-secretory phase (MSE) (Talbi *et al.*, 2006; Aghajanova *et al.*, 2016; Boggavarapu *et al.*, 2016; Suhorutshenko *et al.*, 2018) which correlates with the expression of receptivity markers during the window of implantation (Allegra *et al.*, 2009). The specific expression pattern and cellular localisation of STC-1 in the mammalian endometrium has been poorly studied and the existing literature suffers from restricted sample sizes. More recent studies have also reported dysregulation of STC-1 in human endometrial pathologies and, although clinically relevant, these studies highlight the need for further research in this area (Bishop, Cartwright and Whitley, 2021).

#### 1.12.2.1 Dysregulation of STC-1 in endometriosis

STC-1 has been implicated in the pathogenesis of female reproductive disorders including polycystic ovary syndrome (Khatun *et al.*, 2020) and endometriosis (Aghajanova *et al.*, 2016). Endometriosis is an oestrogen-dependent gynaecological condition characterised by chronic inflammation and the presence and growth of ectopic endometrial tissue (Hickey, Ballard and Farquhar, 2014). Altered endometrial expression of STC-1 in endometriosis has been described (Aghajanova *et al.*, 2016). In healthy women, endometrial STC-1 gene expression fluctuates throughout the menstrual cycle (Aghajanova *et al.*, 2016). In the endometrium of women with endometriosis, however, STC-1 gene expression is significantly increased in MSE compared to the proliferative phase (PE) or early secretory phase (ESE). Endometrial STC-1 gene expression in PE and MSE is also 2-fold higher in women with endometriosis compared to healthy women (Aghajanova *et al.*, 2016). Although not reported for women with endometriosis, up-regulation of STC-1 protein in endometrial fluid in MSE compared to ESE is observed in healthy women (Aghajanova *et al.*, 2016). Abundance of STC-1 in the secretome of receptive MSE endometrium is highly indicative of a role in the process of implantation (Aghajanova *et al.*, 2016), but further studies are required to confirm this role.

On a cellular level, STC-1 protein is expressed in the cytoplasm of epithelial and stromal cells from both women with and without endometriosis (Aghajanova *et al.*, 2016). Within the endometrium of healthy women, STC-1 protein expression is higher in the luminal epithelium and stroma compared to the glandular epithelium. In the endometrium of women with endometriosis, no difference in STC-1 protein expression was found between epithelial cells, however, endometrial stromal cell (eSC) expression of STC-1 was lower in endometriosis compared to healthy cells (Aghajanova *et al.*, 2016).

In endometrial stromal fibroblasts decidualised with cAMP, STC-1 gene expression was up-regulated over 230-fold in healthy women, however, in women with endometriosis, only a 45-fold increase was observed (Aghajanova *et al.*, 2016). This indicates that STC-1 expression in endometrial stromal fibroblasts is induced by cAMP and therefore may be mediated though the PKA pathway, but this will require further investigation. The observed reduction in the up-regulation of STC-1 in cAMP-decidualised endometrial stromal fibroblasts from women with endometriosis may be indicative of a role in the pathogenesis of decidualisation defects (Aghajanova *et al.*, 2016).

## 1.12.3 The expression and role of STC-1 in early gestation

### 1.12.3.1 The expression and role of STC-1 in the uterus during decidualisation and blastocyst implantation

Blastocyst implantation is a tightly controlled physiological process which requires complex interactions between the developing embryo and the uterus (Paria *et al.*, 2002). Dramatic changes in gene expression accompany this process to aid decidualisation and remodelling of the uterine architecture (Stasko, DiMattia and Wagner, 2001). Several studies have implicated STC-1 in this process in numerous species through the observation of changes in its gene and protein expression throughout the decidualisation and implantation processes. The strategies underlying these processes vary significantly across species, thus STC-1 expression and localisation also appear to be species-specific.

Early studies in mice first suggested a role for STC-1 in decidualisation and blastocyst implantation. Following blastocyst implantation, STC-1 gene expression in the mouse uterus shifts from the uterine epithelium to the mesometrial stromal cells. Between days 6.5-8.5 of pregnancy, STC-1 gene expression then moves to the cells of the mesometrial lateral sinusoids and declines thereafter (Stasko, DiMattia and Wagner, 2001). The localisation of

STC-1 protein varies from its gene expression in this context. STC-1 protein is reported to accumulate in epithelial, stromal, and decidual cells, as well as in fetal giant trophoblast cells throughout the implantation process. The mRNA-protein disparities observed here indicate that STC-1 is transcribed in one cell type and then sequestered by target cells for action, suggestive of a paracrine/autocrine signalling role during these processes in the uterus (Stasko, DiMattia and Wagner, 2001).

In rats, STC-1 mRNA is present in the stromal cells surrounding the implanting blastocyst (Xiao *et al.*, 2006). It is notable, however, that in pseudopregnant rats, corresponding STC-1 gene expression is not observed suggesting that STC-1 expression in the uterus is specifically induced by developing embryos and implanting blastocysts. This hypothesis is further evidenced by the report that STC-1 mRNA is maintained at a basal level during an induced delay in implantation but is elevated following termination of the delay. In addition, STC-1 gene and protein expression are concentrated at sites with the implanting blastocyst compared to other uterine segments (Xiao *et al.*, 2006).

Uterine expression of STC-1 during implantation has also been investigated in other mammals including sheep, pigs, and horses. In sheep, STC-1 gene expression is confined to the endometrial glands at the start of blastocyst implantation and is induced by progesterone. Later in pregnancy, STC-1 expression is then stimulated by placental hormones (Song *et al.*, 2006). STC-1 protein is also present in the endometrial glands but is secreted into the uterine lumen where it is detectable in placental areolae which transport uterine gland secretions across the placenta and into the fetal-placental circulation. Secretion of STC-1 from uterine glands into the fetal circulation and allantoic fluid in this manner may suggest an exocrine role in this system (Song *et al.*, 2006).

In pigs and horses, uterine STC-1 expression is detectable earlier than in sheep, from the point of conceptus attachment, consistent with findings from rodent studies (Stasko, DiMattia and Wagner, 2001; Xiao *et al.*, 2006; Song *et al.*, 2009; Kikuchi *et al.*, 2011). In pigs, STC-1 expression is limited to the peri-implantation period of pregnancy and is stimulated by progesterone and oestrogen (Song *et al.*, 2009). Similarly, oestrogen stimulates STC-1 expression in the equine uterus (Kikuchi *et al.*, 2011). In both pigs and horses, STC-1 protein is detectable in the uterine luminal fluids, indicating secretion of STC-1 from the endometrial glands towards the conceptus (Song *et al.*, 2009; Kikuchi *et al.*, 2011). The observed temporal and spatial expression of STC-1 in pigs and horses suggests that STC-1 may play an important role in conceptus attachment during implantation (Song *et al.*, 2009; Kikuchi *et al.*, 2011), whereas in rodents and sheep, STC-1 may be involved in later stages of implantation and decidualisation.

In humans, the expression of STC-1 in the uterus during blastocyst implantation has been little studied due to the challenges in investigating human pregnancy *in vivo.* However, through investigation of endometrial gene expression in women who underwent successful IVF treatment, it was found that STC-1 is one of only six genes which retained homogenous expression during the window of implantation (Allegra *et al.*, 2009). The localisation, expression pattern, and role of STC-1 in the human uterus during implantation is unclear and will require further research.

The present research strongly suggests that STC-1 plays a key role in the uterus during early pregnancy, particularly during the implantation/peri-implantation period. Therefore, data obtained from studies using genetically engineered mouse models are surprising. In STC-1 null mice, no important change in fertility was detectable (Chang *et al.*, 2005). It is thought that other gene products are capable of fully compensating for STC-1 in this context, such

as STC-2. No change in the expression of STC-2 in organs in the STC-1 null mice was detected suggesting that this is not the compensatory factor (Chang *et al.*, 2005). In contrast, transgenic overexpression of human STC-1 in mice resulted in reduced female reproductive capacity (Varghese *et al.*, 2002). It is unclear what causes this, but from the data on uterine STC-1 expression in mice (Stasko, DiMattia and Wagner, 2001), it is apparent that dramatic and precise shifts in expression accompany blastocyst implantation, thus, it is postulated that transgenic overexpression may interfere with this tightly controlled process (Varghese *et al.*, 2002).

#### 1.12.3.2 STC-1 in placental development

The expression of placental genes varies significantly during pregnancy, with genes regulating the cell cycle, differentiation, motility, and angiogenesis being up-regulated in early pregnancy, whereas genes involved in lipid metabolism, stress response, and signal transduction are highly expressed at term (Winn *et al.*, 2007). Transcriptome profiling of human placental gene expression dynamics from early to mid-gestation has revealed significant STC-1 gene expression during this period with a peak of expression during mid-gestation. Interestingly, placental STC-1 expression also remains high at term, but only in complicated pregnancies (Uusküla *et al.*, 2012). Several single-nucleotide polymorphisms (SNPs) in the STC-1 gene have been identified which appear to directly modulate placental STC-1 transcript levels (Juhanson *et al.*, 2016). It is possible that these SNPs explain the observed variation in placental STC-1 gene expression levels between normal and complicated pregnancies, but this requires further study (Juhanson *et al.*, 2016). Although the precise role of placental STC-1 in pregnancy has yet to be elucidated, current findings in the field suggest that it plays a role in maintaining a healthy pregnancy, but may also be implicated in the pathology of pregnancy complications (Abid *et al.*, 2020).

Within the first trimester placenta, STC-1 has been shown to be predominantly expressed in syncytiotrophoblast and cytotrophoblast cells. STC-1 protein expression is also reported in placental endothelial and stromal cells but at lower expression levels (Abid *et al.*, 2020). Moreover, placental cells have been demonstrated to secrete STC-1, and in the choriocarcinoma-derived cytotrophoblast cell line (BeWo), this is stimulated by low oxygen conditions (1% $O_2$) combined with induction of intracellular cAMP (Abid *et al.*, 2020). Pharmacological modulation of PKA showed that secretion of STC-1 from BeWo cells is mediated through this pathway (Abid *et al.*, 2020). Interestingly, elevation of intracellular cAMP in normoxia had no effect on the secretion of STC-1 by BeWo cells. Similar data from first trimester chorionic villus tissue shows that STC-1 secretion was greater under conditions of low oxygen (Abid *et al.*, 2020). Further investigation of the pathways underlying STC-1 secretion under these conditions has shown that this process is mediated through activation of the PI3K/Akt/serum/glucocorticoid regulated kinase 1 (SGK-1) pathway, primarily through activation of mammalian target of rapamycin complex 2 (mTORC-2). In addition, inhibition of the transcription factor HIF-2α resulted in complete inhibition of STC-1 secretion in this system, suggesting a key role for this factor (Abid *et al.*, 2020). This contrasts previous findings investigating cells from other mammalian tissues, such as nasopharyngeal cancer cells, where HIF-1 has been implicated as a key regulator of STC-1 expression (Yeung *et al.*, 2005; Law *et al.*, 2010).

The observed increase in STC-1 secretion exclusively under hypoxic conditions is significant in relation to the *in vivo* situation. In early gestation, the oxygen concentration at the maternal-fetal interface is thought to be around 1-2% (Rodesch *et al.*, 1992). Around the $10^{th}$ week of pregnancy, however, blood flow to the developing placenta changes significantly due to remodelling of the maternal spiral arteries and the loss of trophoblast

plugs (Burton, Yung, *et al.*, 2009). In complicated pregnancies, such as those with pre-eclampsia or FGR where vascular remodelling and placentation are known to be insufficient, placental STC-1 expression and secretion have been reported to be increased (Uusküla *et al.*, 2012; Abid *et al.*, 2020). This finding suggests that low oxygen may be a stimulus for STC-1 expression in the developing placenta.

#### 1.12.3.3 STC-1 in the maternal circulation during pregnancy

In healthy, non-pregnant individuals, STC-1 is absent or undetectable in the circulation. During pregnancy, detectable levels of STC-1 have been reported, and are suggestive of an endocrine role in pregnancy (Deol *et al.*, 2000). The source of circulating STC-1 in pregnancy is currently unclear. It is postulated that circulating STC-1 may originate from the ovary, due to the coincident rise in ovarian STC-1 gene expression with increased serum STC-1 levels (Deol *et al.*, 2000). Although it is suggested, that the placenta may be the source of circulating STC-1 due to observed STC-1 secretion from placental cells (Abid *et al.*, 2020) the continued presence of STC-1 post-partum indicates both the placenta and the ovaries may be the source, or that the tissue origin changes post-partum, possibly to support the physiology of lactation. In pregnancies complicated by conditions whose aetiology is thought to occur in the first trimester, such as pre-eclampsia, FGR, and gestational diabetes, third trimester and post-partum serum levels of STC-1 are even further elevated (Uusküla *et al.*, 2012; Abid *et al.*, 2020). This is consistent with increased secretion of STC-1 seen in first trimester placentas derived from pregnancies at risk of developing pre-eclampsia (Abid *et al.*, 2020). The presence of STC-1 in the maternal circulation during pregnancy has only been investigated in the third trimester and post-partum (Uusküla *et al.*, 2012; Juhanson *et al.*, 2016; Abid *et al.*, 2020), therefore, it is unclear at which stage of

pregnancy STC-1 is released into the bloodstream. To further understand this, longitudinal studies on maternal serum are required (Bishop, Cartwright and Whitley, 2021).

#### 1.12.3.4 STC-1 in maternal spiral artery remodelling in pregnancy

Collectively, the present research on STC-1 in the uterus, placenta, and maternal circulation indicate its involvement in the establishment and maintenance of pregnancy. A role for STC-1 in the remodelling of maternal vasculature that occurs in early pregnancy has also been postulated (Wallace *et al.*, 2013). Investigation of trophoblast-induced changes in human decidual spiral artery remodelling using a three-dimensional spheroid culture system to model the interactions that occur between the different cell types *in vivo* revealed two-fold up-regulation of STC-1 gene expression in vascular cells following incubation with trophoblast-secreted factors (Wallace *et al.*, 2013). Despite its upregulation in this system, its specific function has yet to be described. As previous studies have implicated STC-1 in physiological and pathophysiological cardiovascular function, and it is postulated to play a key role in the uterus and placenta in early pregnancy, it was hypothesised that STC-1 may be involved in the process of spiral artery remodelling.

It is evident from the current research that a number of tightly controlled cellular events take place during the vascular remodelling process (Whitley and Cartwright, 2010). The existing literature on the functional roles of STC-1 suggests its potential mechanisms of action within the remodelling spiral artery. For example, STC-1 has been shown to inhibit the action of inflammatory cytokines, including TNF-α (Chen *et al.*, 2008; Sheikh-Hamad, 2010), an important factor in the regulation of vascular cell apoptosis in artery remodelling (Rastogi *et al.*, 2012). In addition, STC-1 antagonises the activity of vascular reactive factors, including angiotensin II and HGF (Zlot *et al.*, 2003; Liu *et al.*, 2012; Moreau *et al.*, 2012). Both factors are known to be present within the environment of the remodelling spiral

artery and could influence the behaviour of vascular cells. Furthermore, overexpression of STC-1 has been shown to promote angiogenesis *in vivo and in vitro* (Bell et al., 2001; Gerritsen, Peale Jr. and Wu, 2002; Liu et al., 2003; He et al., 2011), suggesting that it may have different roles depending on concentration or the local environment. It is crucial to maintain a balance of signals within the remodelling spiral artery to allow vessel change but also apply limits. Therefore, it is possible that altering levels of STC-1 acts as a local control mechanism to prevent excessive remodelling, but this is not yet understood. In addition, how trophoblast-induced STC-1 expression from vascular cells is regulated has yet to be investigated.

## 1.13 Aims and objectives of this thesis

This research aimed to improve our understanding of STC-1 in vascular remodelling at the maternal-fetal interface in early pregnancy. An EC cell line and a VSMC cell line were used as a model of the maternal spiral artery. The vascular cell lines were treated with conditioned media collected from an EVT cell line to model the fetal trophoblast/vascular cell interactions that occur in the remodelling spiral artery *in vivo.* It was hypothesised that STC-1 expression in vascular cells of the remodelling spiral artery is stimulated by trophoblast cell secreted factors and STC-1 expression in this context relates to a functional role in the spiral artery remodelling process.

To examine this hypothesis, the aims and objectives of this thesis were as follows:

1. To characterise STC-1 in the maternal decidua.
2. To understand how STC-1 expression is regulated in a cell line model of the remodelling spiral artery in early pregnancy.

3. To optimise tools to assess the functional role of STC-1 in the remodelling spiral artery.
4. To understand the functional role played by STC-1 in vascular cells of the remodelling spiral artery.

# Chapter 2: Materials and methods

## 2.1 Cell culture

All cell lines used in this study were cultured in a humidified 5% $CO_2$ incubator at 37°C. All cell culture reagents were obtained from Sigma-Aldrich (Dorset, UK) unless otherwise stated. When required, all cell lines were passaged by removing the medium, washing once with phosphate buffered saline (PBS) and incubating with 2.5-5 ml trypsin for 1 minute to induce detachment of cells from the plate. Trypsin was then neutralised by adding an equal volume of complete cell culture medium. Cells were harvested in a 50 ml centrifuge tube (Corning, NY, USA) and centrifuged at 400 *g* for 5 minutes before resuspending the resulting cell pellet in fresh media.

### 2.1.1 Cell lines used in this study

#### 2.1.1.1 SGHPL-4

The SGHPL-4 cell line was derived from first trimester human EVT cells transfected with pSV3neo, a plasmid containing the large T-antigen of SV40 (Choy and Manyonda, 1998). These cells have been shown to maintain the characteristics of EVT cells including the expression of markers such as human placental lactogen and human chorionic gonadotrophin (Choy and Manyonda, 1998; Cartwright, Holden and Whitley, 1999).

SGHPL-4 cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal calf serum (FCS), 2 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were used until passage number 25 and split 1:3 every 3-4 days.

#### 2.1.1.2 SGHEC-7

The SGHEC-7 cell line was derived from HUVECs transfected with the pSV3neo plasmid. These cells divide rapidly and retain a number of differentiated EC functions throughout

their lifespan, including expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin and binding properties (Fickling, Tooze and Whitley, 1992; Cartwright, Whitley and Johnstone, 1995).

SGHEC-7 cells were cultured in a 1:1 ratio of Medium 199 supplemented with Earle's modified salts (M199) and RPMI 1640 medium with 5% (v/v) FCS, 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml endothelial cell growth supplement (ECGS) and 0.09 mg/ml heparin. The cells were used until passage number 25 and split 1:3 every 3-4 days.

#### 2.1.1.3 SGHVSM-9

SGHVSM-9 cells were obtained from human aorta and transfected with the pSV3neo plasmid (Harris *et al.*, 2006). SGHVSM-9 cells were cultured in Ham's F10 medium containing 10% (v/v) FCS, 2 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were used until passage number 95 and split 1:3 every 5-7 days.

## 2.2 Immunocytochemistry staining of SGHEC-7 and SGHVSM-9 cells

SGHEC-7 and SGHVSM-9 cells were seeded onto gelatin-coated (1% (w/v porcine gelatin (Sigma-Aldrich, Dorset, UK) in deionised water) 13 mm glass coverslips in a 24-well plate at a density of 2 x $10^4$ and 3 x $10^4$ cells per well respectively. The cells were incubated overnight at 37°C with 5% $CO_2$ in complete culture medium.

The cells were then stained with MitoTracker™ Orange CMTMRos (Thermo Fisher Scientific, Waltham, MA, USA) using the manufacturer's protocol. Briefly, the culture medium was removed, and cells were washed once in PBS. MitoTracker™ Orange CMTMRos was diluted in phenol red-free RPMI 1640 medium without FCS to a final concentration of 100 nM. 500

µl of diluted MitoTracker™ Orange CMTMRos was added to each well and the cells were incubated at 37°C with 5% $CO_2$ for 30 minutes. The MitoTracker™ Orange CMTMRos/media solution was then removed, and the cells were washed twice with PBS. The cells were fixed using ice-cold methanol for 10 minutes before permeabilising in PBS containing 0.2% (v/v) Tween® 20 for 10 minutes. The cells were then washed twice for 5 minutes with PBS and blocked with 10% (v/v) goat serum in PBS for 20 minutes at room temperature. The fixed and blocked cells were then incubated in primary antibody; mouse anti-human STC-1 (5 µg/ml in PBS containing 0.2% (v/v) Tween® 20 and 1.5% (v/v) goat serum) (R&D Systems, MN, USA) or isotype-matched immunoglobulin control (Mouse IgG2b kappa Isotype Control) (5 µg/ml in PBS containing 0.2% (v/v) Tween® 20 and 1.5% (v/v) goat serum) (Invitrogen, Waltham, Massachusetts, USA) for 1 hour at room temperature. The cells were then washed 3 times with PBS and incubated in secondary antibody; goat anti-mouse biotinylated immunoglobulin G (5 µg/ml in PBS containing 0.2% (v/v) Tween® 20 and 1.5% (v/v) goat serum) (Vector Laboratories, Newark, CA, USA) for 45 minutes at room temperature. The cells were washed with PBS again and incubated in Streptavidin Alexa Fluor™ 488 Conjugate (4 µg/ml in PBS containing 0.2% (v/v) Tween® 20 and 1.5% (v/v) goat serum) (Thermo Fisher Scientific, Waltham, MA, USA) in the dark for 30 minutes at room temperature. The cells were washed 3 times with PBS before storing in PBS with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Newark, CA, USA) until imaging. The cells were imaged using a Nikon A1R Confocal microscope (Nikon, Tokyo, Japan).

**Table 2.1 Primary antibodies used in immunocytochemistry experiments in this study.**

| Primary Antibody | Type | Working concentration | Source |
|---|---|---|---|
| STC-1 | Monoclonal mouse | 5 µg/ml | R&D Systems |
| IgG2b kappa Isotype Control | Mouse | 5 µg/ml | Invitrogen |

## 2.3 Generation of trophoblast conditioned media (TCM)

SGHPL-4 cells were grown in 175cm$^2$ flasks (Corning, NY, USA) until confluent. Once confluent, the cells were harvested into a 50 ml centrifuge tube (Corning, NY, USA) and the resulting cell suspension was centrifuged at 400 *g* for 5 minutes. The supernatant was removed and replaced with 50 ml PBS to thoroughly remove any trypsin and cell culture medium from the cells. Cells were centrifuged again, and the resulting pellet was resuspended in 20 ml cell culture medium before counting using a haemocytometer. Cells were then incubated with gelatin-coated Cytodex® microcarrier beads (Sigma-Aldrich, Dorset, UK) (50µl Cytodex® microcarrier beads suspended in PBS per 1 x $10^6$ cells) and 25 mM HEPES buffer in phenol red-free RPMI 1640 medium containing 10% (v/v) FCS on a roller set to the lowest speed and incubated at 37°C. Prior to use, the Cytodex® microcarrier beads were suspended in PBS (400 mg in 20 ml PBS) and autoclaved at 120°C.

After 24 hours, the cell suspension was centrifuged at 200 *g* for 2.5 minutes and the supernatant was removed. The pellet was gently washed twice with phenol red-free RPMI 1640 medium without FCS before incubating in 20 ml phenol red-free RPMI 1640 medium without FCS and 25 mM HEPES buffer on a roller at 37°C.

After 48 hours, the conditioned media was collected by centrifugation at 200 *g* for 2.5 minutes and the resulting supernatant was harvested in a 50 ml centrifuge tube. The

supernatant was centrifuged again at 3000 *g* before filtering through a sterile 1 µm glass fibre Acrodisc® syringe filter (Pall Corporation, NY, USA) to remove any cell debris. The conditioned media was then filtered through a 0.2 µm filter (Pall Corporation, NY, USA) to sterilise. The conditioned media was concentrated 20-fold using VivaSpin columns (3000 molecular weight cut-off) to remove any molecules less than 3 kDa in size (Sartorius Stedium, Surrey, UK) before the VivaSpin column was washed with 10 ml phenol red-free RPMI 1640 medium without FCS. The resulting conditioned media was aliquoted, and the protein concentration determined by a Bradford assay before storing at -20°C.

## 2.4 Cellular stimulation with cytokines and inhibitors

SGHEC-7 or SGHVSM-9 cells were seeded into 6-well plates (Corning, NY, USA) at a density of 5 x $10^5$ cells and 6 x $10^5$ cells respectively. Cells were incubated for 5 hours before media was changed to phenol red-free RPMI 1640 medium without FCS. Cells were incubated at 37°C, 5% $CO_2$ overnight.

Cells were treated with various cytokines or inhibitors. In the cases where the effect of inhibitors was assessed, these were added 20 minutes before the stimulus. Cells were incubated at 37°C, 5% $CO_2$, 21% $O_2$ during this time period. Where inhibitors were prepared in dimethyl sulfoxide (DMSO), an equivalent volume of DMSO was added as a vehicle control.

TCM was added to cells at a concentration of 50 µg/ml for 2 hours before removing the media from the cells and washing twice with PBS. The media was replaced with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS. TCM contains many factors that may interfere with subsequent analysis, so the treatment media was removed at this stage prior to incubation. Cells were then incubated at 37°C, 5% $CO_2$, and 21% $O_2$.

After 24 hours, the media from each well was collected and centrifuged at 18,000 *g* for 5 minutes to remove any suspended cells. The suspended cells were retained and lysed with the cell monolayer. The supernatant was retained for analysis via ELISA.

## 2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of STC-1 in cell culture supernatants was determined by ELISA, using the Human Stanniocalcin 1 DuoSet ELISA kit (R&D Systems, MN, USA). The final concentration of STC-1 in the cell culture supernatants was expressed per mg of total cellular protein as determined by Bradford assay. The ELISA was performed using the manufacturer's protocol as follows:

Plate preparation: The Capture Antibody was diluted to the working concentration in PBS (Sigma-Aldrich, Dorset, UK) and was used to coat a 96-well microplate with 100 µl per well. The plate was then incubated overnight at room temperature. The plate was then washed by filling each well with 400 µl wash buffer (0.05% Tween® 20 in PBS) using a squirt bottle and removing the excess liquid by inverting the plate against a clean paper towel. Washing was repeated twice more. The plates were then blocked by adding 300 µl Reagent Diluent (included in the ELISA kit) to each well and incubating at room temperature for one hour.

Assay procedure: After incubation, the wash step was repeated and 100 µl of standards and samples were added to each well and incubated for 2 hours at room temperature. When required, samples were diluted in phenol red-free RPMI 1640 medium containing 5% (v/v) FCS.

Following incubation, the plate was washed three times with wash buffer and 100 µl of Detection Antibody, diluted in Reagent Diluent containing 2% (v/v) Normal Goat Serum

(NGS) (Vector Laboratories, Burlingame, CA, United States) was added. The plate was incubated for 2 hours at room temperature.

The plate was washed three times with wash buffer again and 100 µl of the working dilution of Streptavidin-HRP was added to each well. The plate was incubated for 20 minutes at room temperature away from direct light.

Following washing, 100 µl of TMB One Component HRP Microwell Substrate Solution (Surmodics, Minnesota, USA) was added to each well and incubated for 20 minutes at room temperature away from direct light. Stop Solution (50 µl) (2 N $H_2SO_4$) was added to each well and the optical density of each well at 450 nm was determined immediately using a SpectraMax® 340 Microplate Spectrophotometer (Molecular Devices, San Jose, CA, United States).

## 2.6 Cell lysis and Bradford assay

To determine the total cellular protein concentration, the cells were harvested by scraping and lysed in 200 µl RIPA buffer (1x PBS, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS containing 0.1 mM EDTA with added inhibitors, 1mM phenylmethylsulfonyl fluoride (PMSF), 0.3 µM aprotinin, 1 mM sodium orthovanadate and PhosSTOP™ (one tablet per 10 ml RIPA buffer) (Roche, Basel, Switzerland) for 25 minutes on ice. The lysate was sonicated at 60% amplification three times for 5 seconds each before centrifugation at 4°C, 18,000 *g* for 10 minutes. The protein concentration of the lysate was determined by Bradford assay.

## 2.7 Western blot analysis

Cells were collected and lysed as described previously. 30-50 µg of protein per well was resolved by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on

7.5%, 10%, or 12% polyacrylamide gels prior to transfer to Immobilon®-FL PVDF membranes (Merck Millipore, Burlington, MA, USA). To allow normalisation of the western blot, the total protein in each sample was determined using REVERT™ Total Protein Stain (LI-COR Biosciences, NE, USA). Non-specific reactivity was blocked using a 1:1 ratio of Tris Buffered Saline (TBS) and Odyssey® Blocking Buffer (LI-COR Biosciences, NE, USA) for 1 hour at room temperature. Membranes were incubated in primary antibody in either a 1:1 ratio of 0.2% (v/v) Tween®20 in TBS (0.2% TBS-T) and Odyssey® Blocking Buffer or 5% (w/v) BSA in 0.1% (v/v) Tween®20 in TBS (0.1% TBS-T) or 5% (w/v) low fat milk powder in 0.1% TBS-T overnight at 4°C. The membranes were washed in 0.1% TBS-T and incubated in secondary antibody (1:10,000 in a 1:1 ratio of 0.2% TBS-T and Odyssey® Blocking Buffer with 0.1% (v/v) 10% SDS or 5% (w/v) low fat milk powder in 0.1% TBS-T) for 1 hour at room temperature. Detection was performed using the Odyssey® CLx Imaging system (LI-COR Biosciences, Nebraska, USA) or using enhanced chemiluminescence with the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) with Signal Accumulation Mode to enable optimal membrane exposure. The resulting images were analysed using LI-COR Image Studio™ Lite Quantification Software (LI-COR Biosciences, Nebraska, USA) where the intensity of each band was determined and expressed as a ratio to the total protein intensity of the same sample.

**Table 2.2 Primary antibodies used in western blot experiments in this study.**

| Primary Antibody | Type | Dilution | Buffer | Source |
|---|---|---|---|---|
| STC-1 | Monoclonal mouse | 1:500/1:1000 | 1:1 of 0.2% TBS-T and Odyssey® Blocking Buffer | R&D Systems |
| Phospho-44/42-MAPK (Thr202/Tyr204) | Monoclonal rabbit | 1:1000 | 5% (w/v) BSA in 0.1% TBS-T | Cell Signalling Technology |
| Phospho-NDRG1 (Thr346) | Monoclonal rabbit | 1:1000 | 5% (w/v) BSA in 0.1% TBS-T | Cell Signalling Technology |
| Phospho-Akt (Ser473) | Monoclonal rabbit | 1:2000 | 5% (w/v) BSA in 0.1% TBS-T | Cell Signalling Technology |
| Phospho-EGFR (Tyr1068) | Polyclonal rabbit | 1:500 | 5% (w/v) low fat milk powder in 0.1% TBS-T | Invitrogen |
| Phospho-STAT3 (Tyr705) | Monoclonal rabbit | 1:1000 | 5% (w/v) BSA in 0.1% TBS-T | Cell Signalling Technology |
| Phospho-STAT3 (Ser727) | Monoclonal mouse | 1:1000 | 5% (w/v) BSA in 0.1% TBS-T | Cell Signalling Technology |

| | | | | |
|---|---|---|---|---|
| Phospho-MAPKAPK-2 (Thr334) | Monoclonal rabbit | 1:1000 | 5% (w/v) BSA in 0.1% TBS-T | Cell Signalling Technology |
| Recombinant Anti-Calponin 1 | Monoclonal rabbit | 1:1000 | 5% (w/v) low fat milk powder in 0.1% TBS-T | Abcam |
| Smooth muscle actin | Monoclonal mouse | 1:1000 | 5% (w/v) low fat milk powder in 0.1% TBS-T | Dako |
| KLF4 | Monoclonal rabbit | 1:1000 | 5% (w/v) BSA in 0.1% TBS-T | Abcam |
| PKC epsilon | Monoclonal mouse | 1:250 | 5% (w/v) low fat milk powder in 0.1% TBS-T | R&D Systems |
| Phospho-GSK-3β (Ser9) | Polyclonal rabbit | 1:1000 | 5% (w/v) BSA in 0.1% TBS-T | Cell Signalling Technology |

**Table 2.3 Secondary antibodies used in western blot experiments in this study.**

| Secondary Antibody | Type | Dilution | Buffer | Source |
|---|---|---|---|---|
| Alexa Fluor™ 750 IgG | Goat anti-Mouse | 1:10,000 | 0.2% TBS-T and Odyssey® Blocking Buffer with 0.1% (v/v) 10% SDS | Thermo Fisher Scientific |
| Alexa Fluor™ 700 IgG | Goat anti-Rabbit | 1:10,000 | 0.2% TBS-T and Odyssey® Blocking Buffer with 0.1% (v/v) 10% SDS | Thermo Fisher Scientific |
| IRDye® 800CW IgG | Donkey anti-Rabbit | 1:10,000 | 5% (w/v) low fat milk powder in 0.1% TBS-T | LI-COR Biosciences |
| HRP-conjugate IgG | Goat anti-Rabbit | 1:10,000 | 5% (w/v) low fat milk powder in 0.1% TBS-T | Sigma-Aldrich |

## 2.8 Peptide blocking to confirm antibody specificity

The binding specificity of the mouse anti-human STC-1 monoclonal antibody (R&D Systems, MN, USA) for the detection of STC-1 in SGHEC-7 and SGHVSM-9 cell lysates was confirmed by pre-incubating the antibody with 8 times excess recombinant human STC-1 (R&D Systems, MN, USA). The strength of the signal between samples stained with control or blocked antibody in western blot experiments was compared to determine antibody specificity.

Mouse anti-human STC-1 antibody (1:750) and recombinant protein were added to a 1:1 ratio of 0.2% TBS-T and Odyssey® Blocking Buffer. Antibody alone (1:750 in a 1:1 ratio of 0.2% TBS-T and Odyssey® Blocking Buffer) was used as a control. The control and antibody/recombinant protein mixtures were incubated overnight at 4°C on a tube roller. Meanwhile, 25 µg of SGHEC-7 and SGHVSM-9 lysates were resolved by SDS-PAGE on 12% polyacrylamide gels and transferred to Immobilon®-FL PVDF membranes (Merck Millipore, Burlington, MA, USA). The membranes were incubated in the control or antibody/recombinant protein mixtures for 2 hours at room temperature on a tube roller before washing in 0.1% TBS-T and incubating in secondary antibody Alexa Fluor™ 750 goat anti-mouse IgG (1:10,000 in a 1:1 ratio of 0.2% TBS-T and Odyssey® Blocking Buffer with 1:10,000 10% SDS (w/v)) (Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour at room temperature.

Detection was performed using the Odyssey® CLx Imaging system (LI-COR Biosciences, Nebraska, USA) and the resulting images were analysed using LI-COR Image Studio™ Lite Quantification Software (LI-COR Biosciences, Nebraska, USA) where the intensity of each band was determined and expressed as a ratio to the total protein intensity of the same sample.

## 2.9 Western blot analysis of maternal decidual tissue

Products of conception were obtained from women undergoing elective termination of pregnancy between 8-14 weeks gestation at St George's University Hospital, NHS Foundation Trust. Wandsworth Local Research Ethics committee approval was in place and all patients provided written informed consent (ref: 01.96.8). Inclusion criteria for this study included singleton pregnancy, gestational age 8-14 weeks, normal fetal anatomy and nuchal translucency thickness, and no known maternal medical condition or history of

recurrent miscarriage. Prior to termination of pregnancy, Doppler ultrasound screening of uterine arteries was performed on the women by a trained sonographer to establish the resistance indices (RI) of the arteries. In this study, a high-RI is defined as a pregnancy with bilateral uterine artery notches and a mean RI≥95th centile, while a normal-RI presents no uterine artery notches and a mean RI<95$^{th}$ centile. Pregnancies with a high-RI have poorly remodelled spiral arteries and are five-times more likely to develop pre-eclampsia than those with a normal-RI (Prefumo, Sebire and Thilaganathan, 2004; Leslie *et al.*, 2015).

Maternal decidual tissue was isolated from the first trimester termination of pregnancy samples and washed in 1 x Hanks' Balanced Salt Solution (HBSS) with 2.5 µg/ml amphotericin B and snap frozen in liquid nitrogen. The tissue was stored at -80°C until use.

To lyse the decidual tissue, a 2 cm$^2$ section was cut using a scalpel and placed in a 2 ml Lysing Matrix D tube (MP Biomedicals, Irvine, CA, USA) containing 500 µl RIPA buffer with 1mM PMSF, 0.3 µM aprotinin, 1 mM sodium orthovanadate and PhosSTOP™ (one tablet per 10 ml RIPA buffer) (Roche, Basel, Switzerland). The Lysing Matrix D tubes containing decidual tissue were placed in a FastPrep-24™ Classic bead beating grinder and lysis system (MP Biomedicals, Irvine, CA, USA) and homogenised at 3 times at 4 m/s for 20 seconds each. The tubes were then centrifuged at 4°C, 18,000 *g* for 5 minutes. If the tissue was not completed homogenised at this stage, the homogenisation and centrifugation steps were repeated. The supernatant was transferred to a clean 1.5 ml Eppendorf tube and the samples were sonicated at 60% amplification for 30 seconds before centrifugation at 4°C, 18,000 *g* for 10 minutes. The supernatants were transferred to new 1.5 ml Eppendorf tubes and the protein concentration of the lysate was determined by Bradford assay.

30 µg of protein per well was resolved by SDS-PAGE on 12% polyacrylamide gels prior to transfer to Immobilon®-FL PVDF membranes (Merck Millipore, Burlington, MA, USA). To allow normalisation of the western blot, the total protein in each sample was determined using REVERT™ Total Protein Stain (LI-COR Biosciences, NE, USA). Non-specific reactivity was blocked using a 1:1 ratio of TBS and Odyssey® Blocking Buffer (LI-COR Biosciences, NE, USA) for 1 hour at room temperature. Membranes were incubated in primary antibody; mouse anti-human STC-1 (1:1000 in a 1:1 ratio of 0.2% TBS-T and Odyssey® Blocking Buffer) (R&D Systems, MN, USA) overnight at 4°C. The membranes were washed in 0.1% TBS-T and incubated in secondary antibody Alexa Fluor™ 750 goat anti-mouse IgG (1:10,000 in a 1:1 ratio of 0.2% TBS-T and Odyssey® Blocking Buffer with 1:10,000 10% (w/v) SDS) (Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour at room temperature. Detection was performed using the Odyssey® CLx Imaging system (LI-COR Biosciences, Nebraska, USA). The resulting images were analysed using LI-COR Image Studio™ Lite Quantification Software (LI-COR Biosciences, Nebraska, USA) where the intensity of each band was determined and expressed as a ratio to the total protein intensity of the same sample.

## 2.10 Proteome Profiler Human XL Cytokine Array

The relative levels of cytokines and chemokines within TCM were determined using a Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, MN, USA) as per the manufacturer's instructions. Briefly, the nitrocellulose membrane spotted with 105 capture antibodies was blocked using Array Buffer 6 which serves as a block buffer (included in the kit) for 1 hour at room temperature. TCM (77 µg) diluted in Array Buffer 6 was added to the membrane and incubated overnight at 4°C on a rocking platform shaker. The membrane was then washed three times for 10 minutes in Wash Buffer (included in the kit) prior to incubation in Detection Antibody Cocktail for 1 hour at room temperature on a rocking

platform shaker. The wash steps were repeated, and the membrane was incubated in IRDye 800CW Streptavidin (1:2000 in Array Buffer) (LI-COR Biosciences, NE, USA) for 30 minutes at room temperature on a rocking platform shaker. Following further washing, the membrane was analysed using the Odyssey® CLx Imaging system (LI-COR Biosciences, NE, USA) with the following parameters: Resolution: 84 µm, Quality: Medium, Focus offset: 0.0 mm, Intensity: 5.

For analysis of the membrane, the pixel density of each capture antibody spot was determined using ImageJ® Software (Schneider, Rasband and Eliceiri, 2012) and the average pixel density of the duplicated spots was calculated using Microsoft Excel (Microsoft Corporation, Washington, USA). The pixel densities were then normalised against the reference spots on the membrane.

## 2.11 Phospho-receptor tyrosine kinase (RTK) array

The relative phosphorylation of human RTKs in SGHEC-7 and SGHVSM-9 cells following stimulation with TCM was determined using a Proteome Profiler Human Phospho-RTK Array Kit (R&D Systems, MN, USA) as per the manufacturer's instructions. Briefly, SGHEC-7 and SGHVSM-9 cells were seeded into 6 cm dishes (Corning, NY, USA) at a density of 2 x $10^6$ cells per dish. Cells were incubated for 5 hours before media was changed to phenol red-free RPMI 1640 medium without FCS. Cells were incubated at 37°C, 5% $CO_2$ overnight.

Cells were treated with TCM (50 µg/ml) in phenol red-free RPMI 1640 medium without FCS, or an equivalent volume of media without TCM as a control, for 5 or 15 minutes. The media was removed, and the cells were washed twice with ice-cold PBS. 500 µl of Lysis Buffer 17 prepared with 10 µg/mL Aprotinin, 10 µg/mL Leupeptin, and 10 µg/mL Pepstatin A was added, and the cells were harvested by scraping before transferring to 1.5 ml Eppendorf

tubes. The lysates were rocked for 30 minutes at 4°C on a tube roller before centrifuging at 14,000 *g* for 5 minutes at 4°C. The supernatant was transferred to a clean Eppendorf tube and the protein concentration of the lysate was determined by Bradford assay. Lysates were kept on ice prior to use.

All kit reagents were brought to room temperature prior to use. The nitrocellulose membranes spotted with 49 different anti-RTK antibodies were blocked using Array Buffer 1 (included in the kit) for 1 hour at room temperature on a rocking platform shaker. Following blocking, 200-300 µg of SGHEC-7 and SGHVSM-9 cell lysates diluted in Array Buffer 1 were added to the membranes and incubated overnight on a rocking platform shaker at 4°C. The membranes were then washed three times in Wash Buffer (included in the kit) and Anti-Phospho-Tyrosine-HRP Detection Antibody (diluted 1:5000 in Array Buffer 2 (included in the kit)) was added to the membranes and incubated for 2 hours at room temperature on a rocking platform shaker. The membranes were washed three times in Wash Buffer and placed on the bottom sheet of a plastic sheet protector. 1 ml of Chemi Reagent Mix (included in the kit) was added to each membrane and the membranes were covered with the top sheet of the plastic sheet protector and incubated for 1 minute. Excess Chemi Reagent Mix was removed, and the membranes were analysed using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) with Signal Accumulation Mode to enable optimal membrane exposure. In this case, 3 minutes was the optimal exposure time.

## 2.12 Construction of STC-1$_{mutant}$ and STC-1$_{overexpression}$ plasmids

The STC-1$_{overexpression}$ plasmid is a bicistronic plasmid consisting of human STC-1 mRNA and enhanced green fluorescent protein (eGFP) created for the purpose of overexpression

studies. The STC-1 cDNA clone was obtained from Source Bioscience in a pCMV-Sport6 vector (Source Bioscience, Nottingham, UK). The cDNA clone was generated from RNA sourced from an anonymous pool of tissue samples by the National Institute of Health Mammalian Gene Collection Library (IMAGE ID: 5191420) (GenBank Accession Number: BC029044).

The STC-1 cDNA was cloned into a pCAGGS bicistronic vector containing the eGFP gene (Hitoshi, Ken-ichi and Jun-ichi, 1991). This vector was modified with an internal ribosome entry site (IRES) which allows the co-expression of two proteins from a single mRNA transcript. The eGFP gene is downstream and under the control of the IRES. The SV40 origin in the original pCAGGS vector has been inactivated and several restriction enzyme sites have been modified to increase the number of unique cloning sites.

The STC-1 cDNA was cloned into the pCAGGS vector using restriction sites SphI and EcoRI. As STC-1 contains an internal SphI site, this was first altered (GCATGC to GCCTGC) using high fidelity polymerase chain reaction (PCR) in two separate reactions using the following primers: Reaction 1: STC-1 Forward (ggggcatgCTCCAAAACTCAGCAGTG) and STC-1ΔLow (GGAGTTTTCCAGGCAGGCAAAAGCCCC). Reaction 2: STC-1 Reverse (ggggaattcTTATGCACTCTCATGGGATGTGCGT) and STC-1ΔTop (GGGGCTTTTGCCTGCCTGGAAAACTCC). The PCR products from each reaction were then combined and SphI and EcoRI sites were added to the 5' and 3' ends respectively using the following primers: STC-1 Forward (ggggcatgCTCCAAAACTCAGCAGTG) and STC-1 Reverse (ggggaattcTTATGCACTCTCATGGGATGTGCGT). The resulting PCR product was verified by agarose gel electrophoresis and the correct gel slice was excised. DNA was purified from the gel slice using guanidinium thiocyanate. The resulting DNA was ligated into the pCAGGS vector and transformed into competent DH5α *Escherichia coli* cells. The resulting plasmid

DNA was isolated through alkaline lysis mini-preparation and the DNA sequence was verified through sequencing (Source Bioscience, Nottingham, UK). Correct plasmid DNA was then prepared through midi-preparation using the PureYield™ Plasmid Midiprep System (Promega, WI, USA).

The STC-$1_{mutant}$ plasmid was created as a control to the STC-$1_{overexpression}$ plasmid. This plasmid is an amended version of the STC-$1_{overexpression}$ plasmid which contains a single cytosine to thymine base pair change in amino acid residue 23 of STC-1 mRNA to introduce pre-mature stop codon (CAG to TAG). This results in the formation of a 22 amino acid truncated product. Using the STC-$1_{overexpression}$ plasmid as a template, recombinant PCR was used to create a single base pair change through two separate reactions using the following primers: Reaction 1: STC-1 Forward (ggggcatgCTCCAAAACTCAGCAGTG) and STC-1 Mutant Low (CAGAGTCATTCTACTCCGCCTCATGGG). Reaction 2: STC-1 Reverse (ggggaattcTTATGCACTCTCATGGGATGTGCGT) and STC-1 Mutant Top (CCCATGAGGCGGAGTAGAATGACTCTG). The resulting PCR products were verified by agarose gel electrophoresis and the correct gel slices were excised. DNA was purified from the gel slices using guanidinium thiocyanate and purified DNA from both reactions was combined. High fidelity PCR with the following primers: STC-1 Forward (ggggcatgCTCCAAAACTCAGCAGTG) and STC-1 Reverse (ggggaattcTTATGCACTCTCATGGGATGTGCGT) was used to combine and amplify the recombinant PCR products. The resulting PCR product was verified by agarose gel electrophoresis and the correct gel slice was excised. DNA was purified using guanidinium thiocyanate. The purified DNA was digested with restriction enzymes SphI and EcoRI. At this stage, the STC-$1_{overexpression}$ plasmid was also digested with the same enzymes to allow introduction of the new mutant insert. The resulting digested insert was ligated into the

STC-$1_{overexpression}$ plasmid and transformed into competent DH5α *Escherichia coli* cells. The resulting plasmid DNA was isolated through alkaline lysis mini-preparation and the DNA sequence was verified through sequencing (Source Bioscience, Nottingham, UK). Correct plasmid DNA was then prepared through midi-preparation using the PureYield™ Plasmid Midiprep System (Promega, WI, USA).

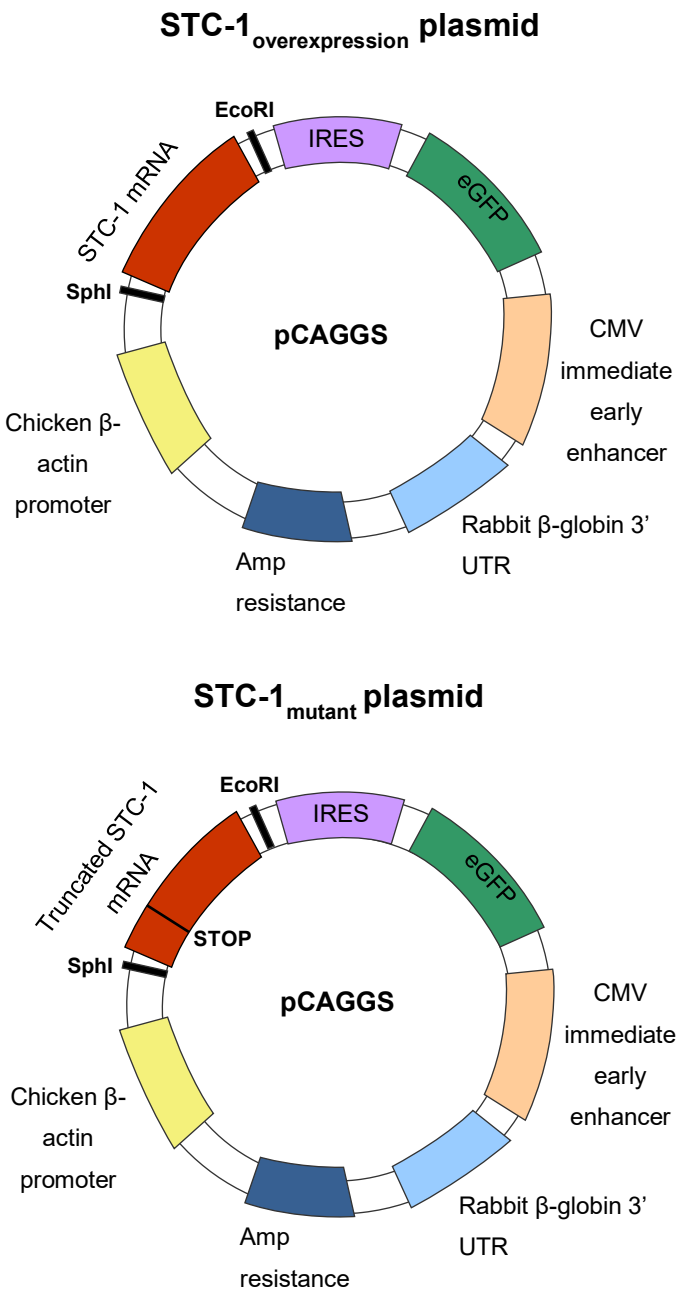



**Figure 2.1 Schematic representation of the STC-1$_{overexpression}$ and STC-1$_{mutant}$ plasmids.**

*IRES, internal ribosome entry site; eGFP, enhanced green fluorescent protein; CMV, cytomegalovirus; UTR, untranslated region; Amp, ampicillin.*

**Table 2.4 Plasmids generated in this study.**

| Plasmid | Enzymes | Vector |
|---|---|---|
| $STC\text{-}1_{mutant}$ | SphI/EcoRI | pCAGGS |
| $STC\text{-}1_{overexpression}$ | SphI/EcoRI | pCAGGS |

**Table 2.5 Commercial plasmids used in this study.**

| Plasmid | Source |
|---|---|
| pBABE-puro | Addgene, MA, USA |
| $VB_{control}$ | VectorBuilder, IL, USA |
| $VB_{STC\text{-}1}$ | VectorBuilder, IL, USA |
| Control siRNA | Santa Cruz Biotechnology, TX, USA |
| STC-1 siRNA | Santa Cruz Biotechnology, TX, USA |
| Control shRNA | Santa Cruz Biotechnology, TX, USA |
| STC-1 shRNA | Santa Cruz Biotechnology, TX, USA |

## 2.13 Puromycin kill curve

SGHEC-7 and SGHVSM-9 cells were seeded in triplicate in 48-well plates (Corning, NY, USA) at a density of $3 \times 10^4$ and $4 \times 10^4$ per well respectively. The cells were incubated in complete cell culture medium overnight at 37°C with 5% $CO_2$. Cells were treated with a range of concentrations of puromycin (InvivoGen, San Diego, CA, USA) in complete cell culture medium. For SGHEC-7 cells, the following concentrations of puromycin were tested: 0.4 µg/ml, 0.3 µg/ml, 0.2 µg/ml, 0.1 µg/ml, 0.05 µg/ml, 0.025 µg/ml, 0.01 µg/ml, and 0

µg/ml. For SGHVSM-9 cells, the following concentrations of puromycin were tested: 0.8 µg/ml, 0.6 µg/ml, 0.4 µg/ml, 0.2 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 0.01 µg/ml, and 0 µg/ml. Cells were incubated at 37°C with 5% $CO_2$ for 7 days and percentage cell survival was empirically determined every 24 hours. The resulting data was plotted onto survival curves (Appendix Figure 1). It was determined that 0.4 µg/ml was the optimal concentration for both cell lines, however, following transfection, there was little cell death using this concentration so the puromycin concentration was increased to 1 µg/ml.

## 2.14 Transfection of SGHEC-7 and SGHVSM-9 cells using the Lonza Nucleofector® system

SGHEC-7 and SGHVSM-9 cells were transfected using the Lonza Nucleofector® system (Lonza, Basel, Switzerland) following the manufacturer's protocol. For SGHEC-7 cells, the HUVEC Nucleofector® Kit was used and for SGHVSM-9 cells, the human aortic smooth muscle cell (AoSMC) Nucleofector® Kit was used.

Prior to starting the transfection protocol, 1.5 ml of complete SGHEC-7 and SGHVSM-9 cell media was added to wells of a 6-well plate (Corning, NY, USA) and placed in a 37°C, 5% $CO_2$, humidified incubator to pre-warm and equilibrate the plates. Additionally, the entire supplement (included in the kit) was added to the Nucleofector® solution.

SGHEC-7 and SGHVSM-9 cells were grown and maintained in 175$cm^2$ flasks (Corning, NY, USA) until 90% confluent. The cell culture medium was then removed, and cells were harvested by trypsinisation into a 50 ml centrifuge tube (Corning, NY, USA). The resulting cell suspension was centrifuged at 400 *g* for 5 minutes. The supernatant was then removed, and the cells were resuspended in a known volume of complete culture medium. The cells were counted using a haemocytometer. For SGHEC-7 cells, 5 x $10^5$ cells were used per well and for SGHVSM-9 cells, 7.5 x $10^5$ cells were used per well. The desired quantity of cells was

then centrifuged at 400 *g* for 5 minutes before resuspending in 100 µl Nucleofector® solution containing supplement. 1.5 µg of plasmid DNA or 300 nM siRNA was added and the cell/Nucleofector® solution/DNA suspension was transferred into a cuvette (included in the kit). The cuvettes were individually placed in the Nucleofector® II Device (Lonza, Basel, Switzerland) and SGHEC-7 cells were transfected using programme U-001 and SGHVSM-9 cells were transfected using programme U-025. Following transfection, ~500 µl of pre-warmed culture media was added to each cuvette and the sample was transferred into the 6-well plate. The transfected cells were allowed to attach to the plate for 5 hours before removing media and replacing with fresh complete culture media. For experiments investigating secretion or intracellular expression of STC-1 following transfection of plasmid DNA or siRNA, cells were incubated at 37°C with 5% $CO_2$ for 24 or 48 hours respectively, before harvesting cell media and cell monolayer for ELISA and western blot analysis. For wound healing assays following transfection of plasmid DNA, cells were incubated for 48 hours before scratch formation and analysis by time lapse microscopy.

## 2.15 shRNA lentiviral transduction of SGHVSM-9 cells

SGHVSM-9 cells were seeded in 6-well plates (Corning, NY, USA) at a density of 4 x $10^5$ cells per well and grown in complete culture medium until 50% confluent. On the day of infection, culture medium was removed replaced with 5 µg/ml of Polybrene® (Santa Cruz Biotechnology, TX, USA) diluted in complete culture medium. The optimal concentration of Polybrene® for this cell line was empirically determined prior to lentiviral transduction, and 5 µg/ml was identified as the optimal concentration. The lentiviral particles were thawed at room temperature and mixed gently just before use. Cells were infected with the lentiviral particles, either control shRNA Lentiviral Particles-A (containing a shRNA construct encoding a scrambled sequence that will not lead to the specific degradation of any known

cellular mRNA) or STC-1 shRNA Lentiviral Particles (a pool of concentrated viral particles containing 3 target-specific constructs that encode 19-25 nucleotide shRNA designed to knock down STC-1 gene expression) (both Santa Cruz Biotechnology, TX, USA). A range of infectious units of lentivirus (IFU) was added different wells; 1 x $10^5$ IFU, 5 x $10^4$ IFU, 2.5 x $10^4$ IFU and 1.25 x $10^4$ IFU. The plates were swirled gently to mix, and the infected cells were incubated overnight at 37°C with 5% $CO_2$. The culture medium was removed and replaced with complete medium without Polybrene®. The cells were maintained and split into 6 cm plates (Corning, NY, USA) once confluent. Cells were cultured for a week before adding complete culture medium containing 1 µg/ml puromycin to allow for selection of cells stably expressing shRNA. The cells were maintained in complete culture medium containing 1 µg/ml puromycin.

## 2.16 Wound healing assay

SGHEC-7 and SGHVSM-9 cells were transfected with 1.5 µg of either STC-$1_{mutant}$ or STC-$1_{overexpression}$ plasmid, as described in section 2.14, and seeded into 12-well plates (Corning, NY, USA) at a density of 5 x $10^5$ cells per well in triplicate. Cells were incubated for 5 hours before removing the media and replacing with fresh complete media. Cells were then incubated at 37°C, 5% $CO_2$ for 48 hours.

Using a 10 µl pipette tip attached to a vacuum aspirator, a vertical scratch was made through the middle of each well. Remaining cell media and detached cells were removed, and the cells were washed twice with PBS. Complete SGHVSM-9 media was added to SGHVSM-9 cells, and 1:1 ratio of M199 and RPMI 1640 medium with 5% (v/v) FCS, 2 mM (v/v) L-Glutamine, 100 U/ml (v/v) penicillin, 100 µg/ml (v/v) streptomycin (no added growth factors) was added to SGHEC-7 cells. Time-lapse microscopy was used to image the cells every 15 minutes over a 24-hour period using an Olympus IX81 inverted microscope

(Olympus, Tokyo, Japan) with a motorised stage (Prior Scientific, Cambridge, UK). Images were captured using a C4742-95 digital camera (Hamamatsu, Tokyo, Japan) and Volocity 6.1 software (Perkin Elmer, MA, USA). The stage was enclosed in a heated, humidified chamber (Solent Scientific, Cambridge, UK) at 37°C with 5% $CO_2$ in air.

The resulting images were analysed using the Wound Healing Size plugin for ImageJ® from Suarez-Arnedo and colleagues (Suarez-Arnedo *et al.*, 2020) to determine the rate of cell migration. This plugin allows quantification of wound area, wound coverage of total area, average wound width, and width standard deviation in images obtained from a wound healing assay. Rate of cell migration ($R_M$) was calculated using the formula: $R_M = W_i – W_f/t$ where $W_i$ is the average of the initial wound width (μm), $W_f$ is the average of the final wound width (μm) and t is the time span of the assay in hours.

## 2.17 Statistical analysis

Data were plotted onto graphs and statistical analysis was carried out using GraphPad Prism Version 9 for Windows (GraphPad Software, La Jolla, CA, USA). Normal distribution of data was assumed and a two-tailed, unpaired *t* test or a one-way ANOVA were used to determine statistical differences against the indicated group (ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

# Chapter 3: Characterisation of STC-1 in the maternal decidua

Following the discovery of STC-1 in the 1990s, research in the field has focussed on characterisation of this protein within the mammalian system. It is known that STC-1 can exist in different states depending on tissue type. For example, dimeric and multimeric forms of STC-1 have been identified as well higher molecular weight variants in some cell types (Zhang *et al.*, 1998; Paciga *et al.*, 2002, 2005). STC-1 characterisation is further complicated as it is expressed intracellularly but also secreted (Moore *et al.*, 1999; Jellinek *et al.*, 2000).

STC-1 expression has been investigated within many female reproductive tissues, including the ovaries, placenta, and the uterus. Uterine expression of STC-1 during the establishment of pregnancy in the processes of decidualisation and implantation in several animal models, including humans, have been described (Stasko, DiMattia and Wagner, 2001; Song *et al.*, 2006, 2009; Xiao *et al.*, 2006; Allegra *et al.*, 2009; Kikuchi *et al.*, 2011). The decidua is a transient but important platform in the uterine tissue comprised of differentiated endometrial stromal cells, newly generated maternal vascular cells, and maternal blood cells (Mori *et al.*, 2016). Decidualisation of the endometrium can occur even in the absence of a fertilised conceptus and commences in the mid-secretory phase of the menstrual cycle (Dockery *et al.*, 1988). It is a vital process for human pregnancy, and it functions to provide maternal immune tolerance, protect the fetus, and regulate placentation (Salker *et al.*, 2010).

Prior to the commencement of this study, the expression of STC-1 in spiral arteries in human maternal decidual tissue was assessed by immunohistochemical staining of consecutive sections of decidual tissue isolated from first trimester termination of

pregnancy samples. This showed that STC-1 is expressed in ECs in the remodelled vessels of the decidua basalis, where trophoblasts are present, which is demonstrated by co-incident staining of STC-1, von Willebrand factor (vWF) (EC marker) and cytokeratin 7 (CK-7) (trophoblast marker) (Figure 3.1). This aligns with findings from previous three-dimensional cell line model studies which showed that placental EVT cells can induce expression of STC-1 from vascular cells (Wallace *et al.*, 2013).

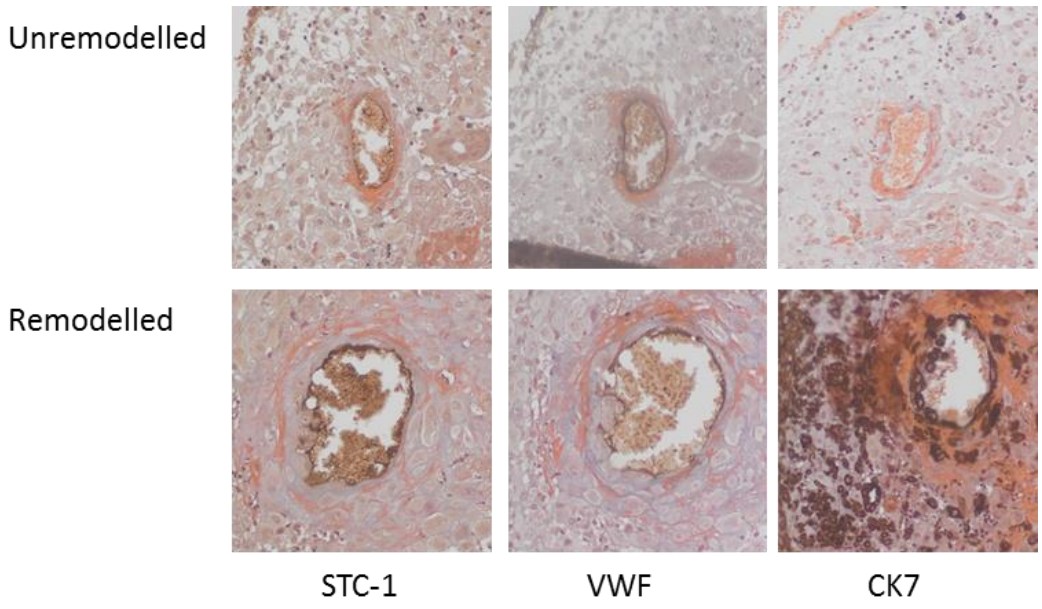



**Figure 3.1 Immunohistochemical staining of spiral arteries in maternal decidual tissue isolated from first trimester termination of pregnancy samples.**

*The top panel shows three serial sections of unremodelled vessels stained for STC-1, vWF (EC marker), and CK7 (trophoblast marker). The bottom panel shows three serial sections of a remodelled vessel stained for the same markers. (Experiment performed by Sandra Ashton, St George's University of London).*

The effect of pregnancy complications on the expression of STC-1 in human maternal decidual tissue was assessed in this study. In pregnancies complicated by conditions whose aetiology is thought to occur in the first trimester, it is known that third trimester and post-partum serum levels of STC-1 are elevated (Uusküla *et al.*, 2012; Abid *et al.*, 2020).

Furthermore, in complicated pregnancies, or those at increased risk of developing complications, STC-1 expression is increased in both first trimester and term placentas (Uusküla *et al.*, 2012; Abid *et al.*, 2020). It is, therefore, postulated that STC-1 expression may be increased in maternal decidual tissue from pregnancies at risk of developing complications.

Due to the considerable ethical and practical issues of studying first trimester human pregnancy *in vivo*, and a lack of appropriate animal models, two human vascular cell lines were selected for use in this study as a model of the maternal spiral artery. An EC cell line, SGHEC-7, and a VSMC cell line, SGHVSM-9. These cell lines have previously been used to model the maternal spiral artery (Wallace *et al.*, 2013) but STC-1 expression has not yet been characterised in these cells. This study aimed to investigate both the intracellular expression pattern of STC-1 in these cell lines, as well as the basal expression levels of STC-1.

## 3.1 Results

### 3.1.1 The specificity of the mouse anti-human STC-1 monoclonal antibody used in western blot experiments in this study

Preliminary western blot experiments in this study using a STC-1 mouse anti-human monoclonal antibody (R&D Systems, MN, USA) resulted in the detection of two molecular weights: 56 kDa and 35 kDa. Since STC-1 has several reported molecular weights (Zhang *et al.*, 1998; Paciga *et al.*, 2002, 2005), it was essential to confirm the specificity of this antibody before continuing with its use for the detection of STC-1 in subsequent experiments in this study. The 56 kDa band of STC-1 was not observed consistently between samples and experimental repeats, therefore, the 35 kDa band was used for the detection of STC-1 in this study. This was also consistent with the molecular weight of STC-1 reported by the manufacturer of the antibody used herein.

To achieve this, western blot analysis was performed on SGHEC-7 and SGHVSM-9 lysates with STC-1 antibody pre-blocked with recombinant human STC-1 (R&D Systems, MN, USA) and control un-blocked antibody. Detection of STC-1 using pre-blocked antibody resulted in a ~110-fold and ~22-fold reduction in STC-1 band density compared to control un-blocked antibody in SGHEC-7 and SGHVSM-9 cells, respectively, confirming that the STC-1 antibody used in this study is specific for STC-1 in these cell lines (Figure 3.2).

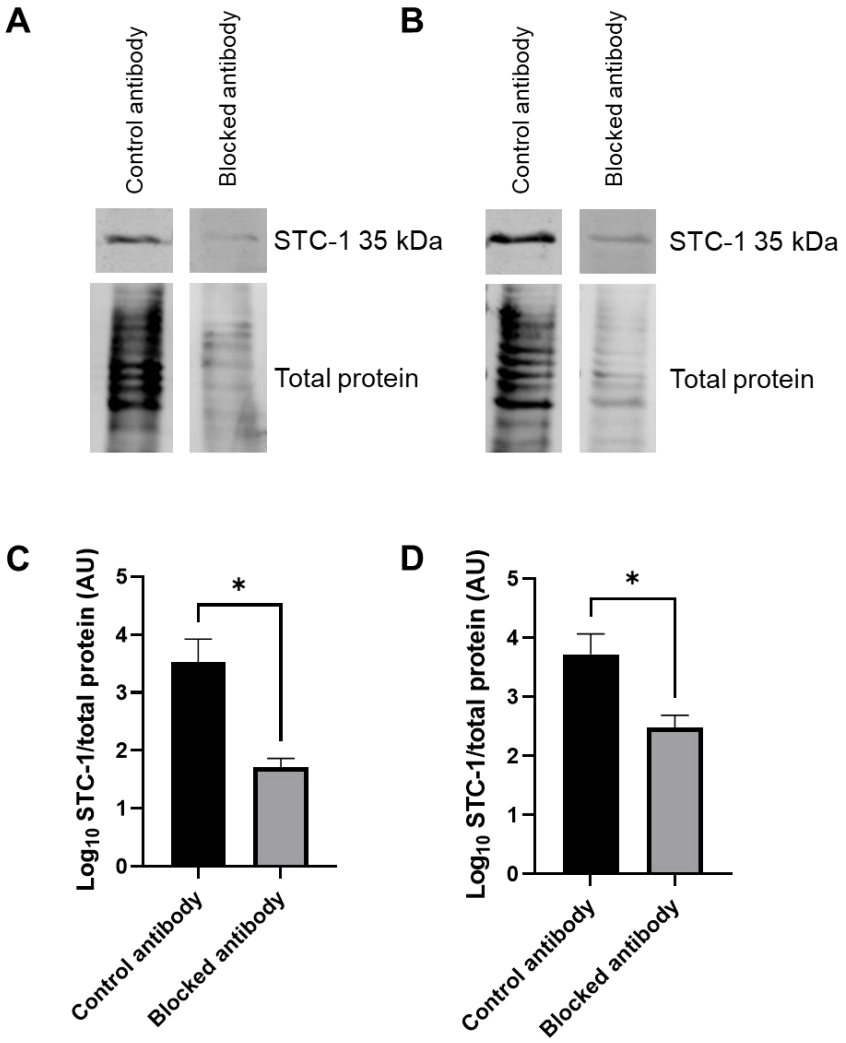



**Figure 3.2 Analysis of the binding specificity of the mouse anti-human STC-1 monoclonal antibody for the detection of STC-1 in SGHEC-7 and SGHVSM-9 cell lysates.**

*Expression of STC-1 determined by western blot analysis of SGHEC-7 (A) and SGHVSM-9 (B) cell lysates with control un-blocked antibody or STC-1 antibody pre-blocked with recombinant human STC-1 (R&D Systems, MN, USA). Quantification of western blot analysis for the detection of STC-1 in (C) SGHEC-7 and (D) SGHVSM-9 cells (n = 3). Data are expressed as band density normalised to total protein of the corresponding lane. $Log_{10}$-transformed data of STC-1/total protein (AU). Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (* = P ≤ 0.05).*

### 3.1.2 Expression of STC-1 in first trimester maternal decidual tissue from normal pregnancies and from those at risk of developing complications

Maternal decidual tissue was obtained from first trimester termination of pregnancy samples and the samples were first divided into groups based on gestational age; 16 samples were pre-11 weeks gestational age, and 16 samples were post-11 weeks. 2 $cm^2$ sections of snap frozen decidual tissue were lysed and analysed by SDS-PAGE. The expression of STC-1 in the lysates was compared between groups. No significant difference was found in the expression of STC-1 in decidual lysates pre- and post-11 weeks gestational age (Figure 3.3A).

Expression of STC-1 in maternal decidual tissue was further analysed by assessing the difference in expression between samples from pregnancies with a normal RI, and from pregnancies with a high RI which were at a 5-fold higher risk of developing pre-eclampsia if the pregnancy had progressed to term (Prefumo, Sebire and Thilaganathan, 2004; Leslie *et al.*, 2015). As STC-1 expression is known to be upregulated in the maternal circulation and placenta in complicated pregnancies (Uusküla *et al.*, 2012; Abid *et al.*, 2020), it was hypothesised that STC-1 expression may be elevated in first trimester decidual tissue from pregnancies at risk of developing complications. 16 samples were obtained from pregnancies with a normal RI and 16 samples were obtained from pregnancies with a high RI. No significant difference was found in the expression of STC-1 between the normal and high-risk pregnancy groups (Figure 3.3B).

The analysis was further refined and the expression of STC-1 in maternal decidual tissue from normal and high-risk pregnancy groups, pre- and post-11 weeks gestational age was assessed. In the pre-11 weeks gestational age group, no significant difference in expression

of STC-1 was found between the normal and high-risk pregnancy groups (Figure 3.3C). Similarly, in the post-11 weeks gestational age group, no significant difference in expression of STC-1 was found between the normal and high-risk pregnancy groups (Figure 3.3D).

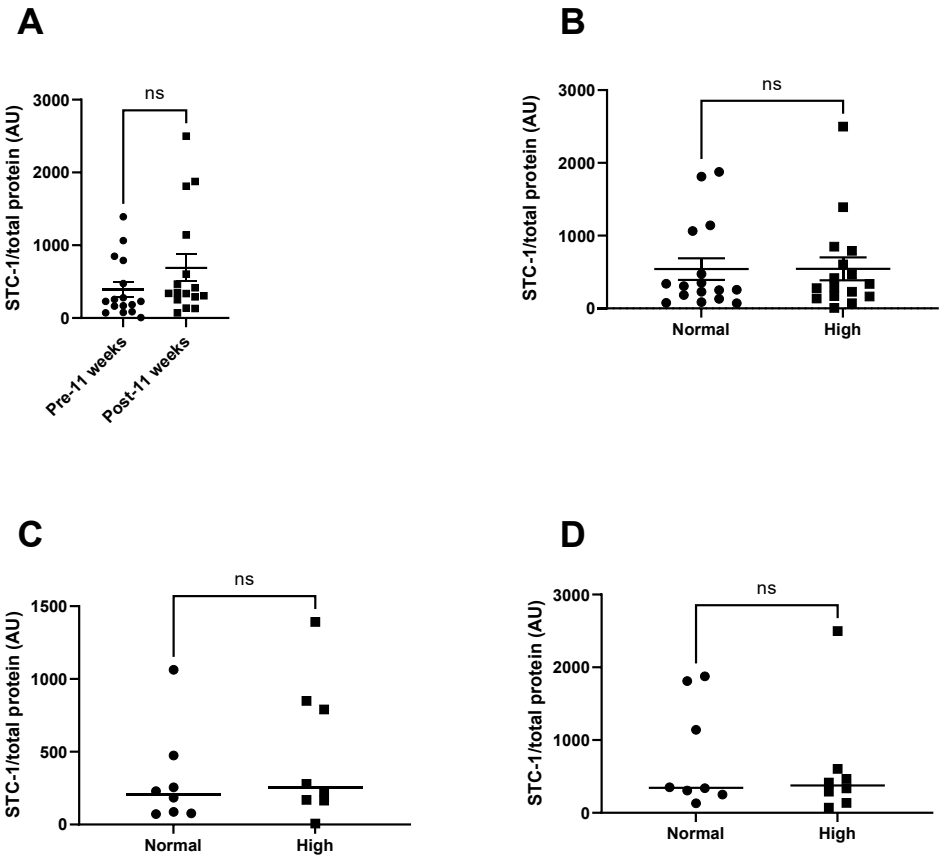



**Figure 3.3 Expression of STC-1 in maternal decidual tissue from normal pregnancies and from those at increased risk of developing complications.**

*(A) STC-1 expression in maternal decidual tissue pre- (n = 16) and post-11 weeks gestational age (n = 16), (B) STC-1 expression in maternal decidual tissue from pregnancies with a normal RI (n = 16) and from those with a high RI (n = 16), (C) STC-1 expression in pre-11 week gestational age maternal decidual tissue from pregnancies with a normal RI (n = 8) and from those with a high RI (n = 8), (D) STC-1 expression in post-11 week gestational age maternal decidual tissue from pregnancies with a normal RI (n = 8) and from those with a high RI (n = 8). Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (ns = not significant).*

### 3.1.3 Characterisation of intracellular STC-1 expression in SGHEC-7 and SGHVSM-9 cells

Two human vascular cell lines were used in this study as a model of the maternal spiral artery: SGHEC-7 EC line and SGHVSM-9 VSMC line. Immunocytochemical analysis of the cell lines was performed to characterise the intracellular expression of STC-1. SGHEC-7 and SGHVSM-9 cells were grown on gelatin-coated slides and fixed before staining mitochondria with MitoTracker™ Orange CMTMRos. STC-1 labelled in green shows cytoplasmic expression in both cell lines (Figure 3.4).

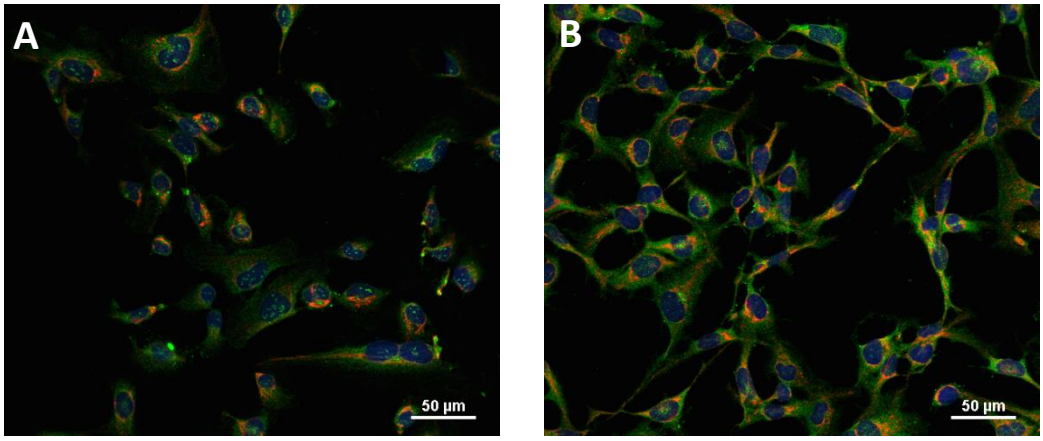



**Figure 3.4 Immunocytochemical staining of SGHEC-7 cells and SGHVSM-9 cells.**

*Confocal z-stack image of SGHEC-7 cells (A) and SGHVSM-9 cells (B). STC-1 is labelled in green, mitochondria are labelled in red using MitoTracker™ Orange CMTMRos, nuclei are labelled with DAPI (blue). 20x objective lens, scale bar 50 µm.*

### 3.1.4 Unstimulated SGHVSM-9 cells display higher levels of STC-1 secretion compared to unstimulated SGHEC-7 cells, whilst unstimulated intracellular expression of STC-1 is consistent between cell lines

To assess the basal level of intracellular STC-1 expression from SGHEC-7 and SGHVSM-9 cell lines, both cell lines were cultured for 48 hours before harvesting the cell monolayer for analysis by western blot. To assess the basal level of STC-1 secretion from both cell lines, the cells were cultured for 24 hours before collecting cell culture supernatant for analysis using an ELISA specific for the detection of STC-1. Unstimulated SGHVSM-9 cells demonstrate >10-fold higher secretion of STC-1 than unstimulated SGHEC-7 cells (Figure 3.5A, $P \leq 0.0001$). In contrast, there is no significant difference in intracellular expression of STC-1 between both cell lines (Figure 3.5B and C).

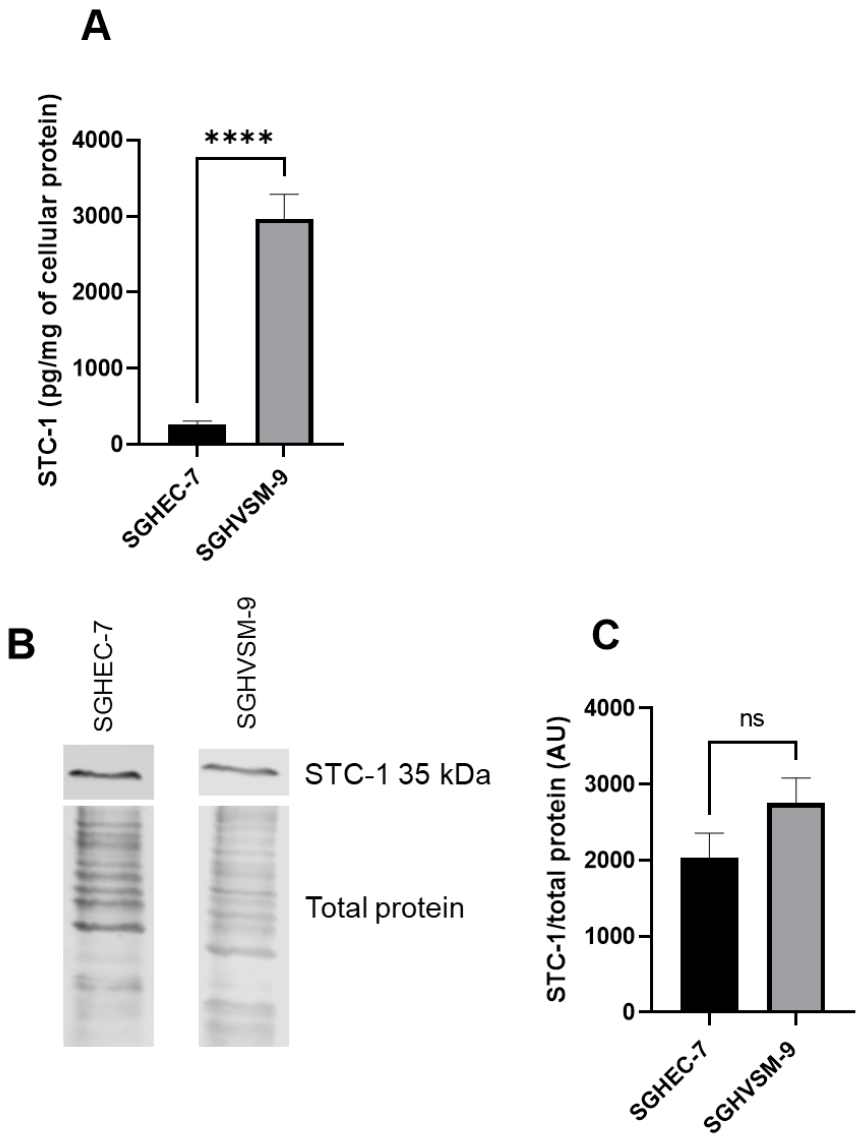



**Figure 3.5 Secretion and intracellular expression of STC-1 by unstimulated SGHEC-7 and SGHVSM-9 cells.**

*(A) Secretion of STC-1 from SGHEC-7 and SGHVSM-9 cells into the culture medium over a 24-hour period was determined by ELISA (SGHEC-7 - n = 13, SGHVSM-9 – n = 22). (B) Intracellular expression of STC-1 in SGHEC-7 and SGHVSM-9 cells cultured for 48 hours was determined by western blot analysis. (C) Quantification of western blot analysis for the detection of STC-1 in SGHEC-7 and SGHVSM-9 cells (n = 3). Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (**** = P ≤ 0.0001, ns = not significant).*

## 3.2 Discussion

A primary aim of this study was to characterise the expression of STC-1 in the first trimester maternal decidua and in the vascular cell line models used in this study. STC-1 expression in the uterus during the processes of decidualisation and blastocyst implantation has previously been characterised in several non-human mammalian species. Dynamic changes in its gene and protein expression throughout the processes of both decidualisation and implantation have been reported and its expression and localisation in the uterus appears to vary between species (Stasko, DiMattia and Wagner, 2001; Song *et al.*, 2006, 2009; Xiao *et al.*, 2006; Kikuchi *et al.*, 2011). In the human decidua, expression of STC-1 during the window of implantation in the mid-secretory phase of the menstrual cycle in non-pregnant women has previously been described (Allegra *et al.*, 2009), however, the expression pattern and subcellular localisation of STC-1 in this tissue, as well as the effect of potential pregnancy complications on the expression of STC-1 has not yet been reported.

Previous work conducted prior to this study demonstrated for the first time that STC-1 is clearly expressed in ECs in human maternal vessels undergoing physiological remodelling in first trimester decidual tissue. In the remodelling vessels of the decidua basalis, where trophoblasts are present, STC-1 is expressed and co-localises with the EC marker, vWF, and the trophoblast marker, CK-7 (Figure 3.1). This correlates with previous studies which demonstrated that trophoblast cell-secreted factors induce STC-1 gene expression in a vascular cell model of the maternal spiral artery (Wallace *et al.*, 2013).

The expression of STC-1 in whole first trimester decidual tissue from a range of gestational ages was also assessed. It was first investigated whether gestational age influenced STC-1 expression in the maternal decidua. The tissue samples were divided into two gestational age groups, pre- and post-11 weeks (Figure 3.3A). It is known that trophoblast plugs block

the spiral arteries until the 11th week of pregnancy preventing maternal blood flow into the intervillous space and inducing a relatively low oxygen environment (Burton, Woods, *et al.*, 2009; Weiss *et al.*, 2016). Separation of groups by this gestational age enabled the effects of changes in maternal blood flow and oxygen concentration on the expression of decidual STC-1 to be investigated. STC-1 expression was found to remain consistent between gestational age groups (Figure 3.3A). The effect of increased risk of pre-eclampsia on STC-1 expression in the maternal decidua was also assessed. The tissue samples were divided into a normal RI group and a high RI group, which were at greater risk of developing pre-eclampsia had the pregnancy progressed to term. STC-1 expression was also found to be consistent between the two RI groups, suggesting that in the first trimester, risk of pregnancy complication does not influence decidual STC-1 expression (Figure 3.3B). Further refinement of the analysis investigating the effect of both variables, gestational age and risk of pregnancy complication, on the expression of STC-1 in the maternal decidua also showed no significant difference in STC-1 expression (Figure 3.3C and D). It is possible that differences in STC-1 expression in normal and complicated pregnancies may only be observable later in pregnancy or at term, as the majority of the current literature has only shown increased STC-1 expression at this stage. It is also plausible that a greater number of maternal decidual tissue samples are needed to fully investigate whether differences in STC-1 expression are present between normal and complicated pregnancies.

Complete characterisation of STC-1 across mammalian cell types is difficult as it is known that in the mammalian system, STC-1 can exist in several multimeric forms as well as higher molecular weight variants (Zhang *et al.*, 1998; Paciga *et al.*, 2002, 2005). Moreover, STC-1 is known to target specific cellular compartments through a receptor-mediated process and

this targeting appears to vary depending on cell type (McCudden *et al.*, 2002; Paciga *et al.*, 2003; Hasilo *et al.*, 2005).

Prior to this study, expression of STC-1 in vascular cells had not been characterised. To investigate this, two vascular cell line models were used in this study: SGHEC-7 EC line and SGHVSM-9 VSMC line. This study aimed to characterise the intracellular expression pattern of STC-1 in these cells, as well as quantify the basal levels of intracellular expression and secretion.

Immunocytochemical staining of both cell line models employed in this study revealed widespread cytoplasmic expression of STC-1 (Figure 3.4). Subcellularly, STC-1 has previously been reported to co-localise with the mitochondria, plasma membrane, nucleus and cholesterol/lipid storage droplets depending on cell type (McCudden *et al.*, 2002; Paciga *et al.*, 2003; Hasilo *et al.*, 2005). To further characterise the sub-cellular localisation of STC-1 in vascular cells and investigate whether STC-1 is targeted to specific organelles, further co-staining and co-localisation analysis would be required.

Western blot analysis of both SGHEC-7 and SGHVSM-9 cell lines used in this study for the detection of STC-1 revealed consistent levels of expression between cell lines (Figure 3.5B and C). In contrast, basal levels of STC-1 secretion varied significantly between cell lines, with 10-fold higher secretion demonstrated in SGHVSM-9 cells (Figure 3.5A). It has previously been demonstrated that levels of STC-1 secretion vary between cell types, for example, both intracellular and secreted levels of STC-1 have been characterised in a range of ovarian cells, including RAS-transformed ovarian epithelial cell lines and human ovarian epithelial cancer cell lines (Liu *et al.*, 2010). In RAS-transformed cell lines, intracellular STC-1 expression was higher compared to their corresponding parental cells and intracellular STC-

1 expression varies between ovarian cancer cell lines depending on their mutation pattern. Similarly, secreted levels of STC-1 were found to vary amongst human ovarian epithelial cell lines, RAS-transformed cell lines displayed higher levels of STC-1 secretion compared to their parental cells and levels of secretion varied amongst ovarian epithelial cancer cell lines (Liu *et al.*, 2010). The levels of STC-1 secretion from SGHVSM-9 cells and SGHEC-7 cells reported in this study were in a similar range to those reported from ovarian cell lines described previously (Liu *et al.*, 2010).

As discussed in section 1.10.2 of this thesis, STC-1 is known to be N-glycosylated at amino acid residues 62–64 (Butkus *et al.*, 1987; Wagner *et al.*, 1992). It is important to consider the implication of STC-1 glycosylation when interpreting the ELISA results obtained in this study. The immunogen sequence of the capture antibody included in the STC-1 ELISA kit (R&D Systems, MN, USA) used in this study consists of amino acids Thr18-Ala247 of the STC-1 protein. This antibody will, therefore, detect the glycosylation sequence of STC-1. Intracellular and secreted forms of STC-1 have been reported to be differentially glycosylated (Jellinek *et al.*, 2000), but there is no evidence to suggest that the capture antibody in the ELISA can differentiate between lightly or highly glycosylated forms of STC-1. Future work characterising secreted STC-1 by ELISA would benefit from investigation of whether differential glycosylation affects detection in this way. A comparison between ELSIA results from cells treated with PNGase F to remove N-linked glycosylation as described previously (Luo *et al.*, 2004), with untreated cells would indicate whether glycosylation affects the results obtained by the ELISA used in this study.

# Chapter 4: Regulation of STC-1 expression in VSMCs and ECs

During the process of spiral artery remodelling in the first trimester of pregnancy, there is considerable interplay between placental cells and the vascular cells of the maternal vessels. It is known that trophoblast cells synthesise and secrete numerous factors which may influence the structure of vessels at the maternal-fetal interface in both a positive and negative manner, and that it is the balance of factors in the local environment that will determine the extent of remodelling (Wallace *et al.*, 2013). It has previously been demonstrated that EVT stimulate both ECs and VSMCs to express the STC-1 gene (Wallace *et al.*, 2013), but the effect of trophoblast secreted factors on STC-1 protein expression and secretion has not yet been established. Moreover, although a number of factors secreted by trophoblast cells have been identified (Aldo *et al.*, 2007; Hering *et al.*, 2008; Harris *et al.*, 2010), the complete profile of factors secreted by trophoblast cells has not yet been described.

Investigation of STC-1 expression in a number of mammalian systems has revealed that regulation of its expression is underpinned by a range of different cell signalling pathways and stimuli, and this appears to be tissue- and/or cell-type dependent (Chang *et al.*, 1995; Honda *et al.*, 1999; Sheikh-Hamad, Rouse and Yang, 2000; Paciga *et al.*, 2002; Paciga, DiMattia and Wagner, 2004). The cell signalling pathways underlying trophoblast-induced STC-1 expression and secretion within the vascular cells of the remodelling maternal spiral artery have not yet been described. It is likely that there are a number of trophoblast-derived factors that will act either individually or in synchrony to influence expression of STC-1 and it is possible that this is mediated through a number of cell signalling pathways (Figure 4.1)

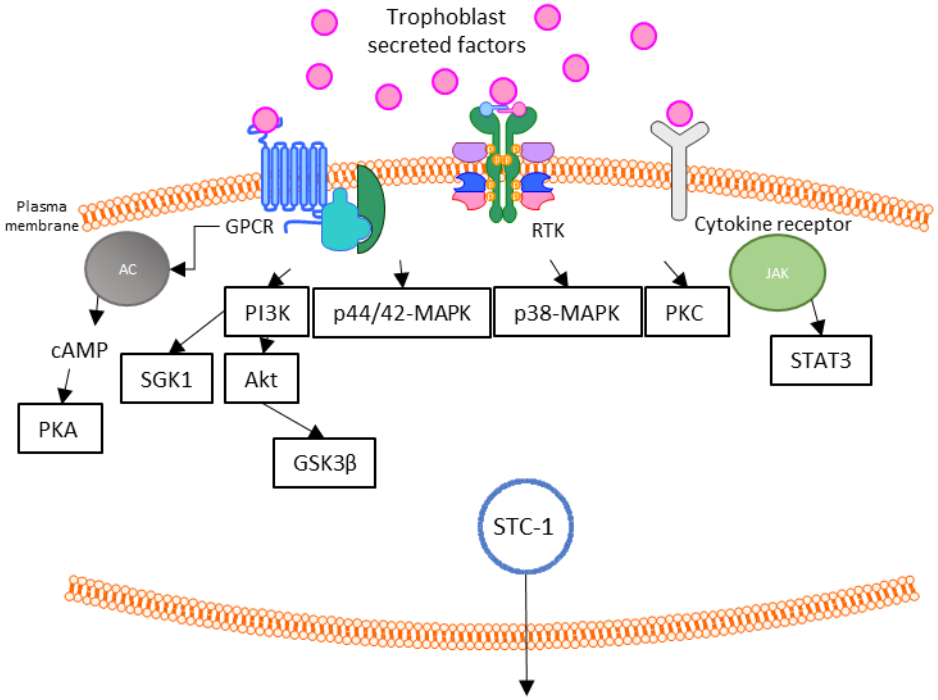



**Figure 4.1 A schematic illustration of the cell signalling pathways which could underly TCM-induced STC-1 secretion from vascular cells which will be investigated in this study.**

*GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PI3K, phosphoinositide 3-kinase; SGK-1, serum/glucocorticoid regulated kinase 1; GSK3β, glycogen synthase kinase 3 beta; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; JAK, janus kinase; STAT3, signal transducer and activator of transcription 3; STC-1, stanniocalcin-1. Figure was prepared using the Motifolio drawing toolkit.*

## 4.1 Results

### 4.1.1 Trophoblast conditioned media (TCM) stimulates secretion of STC-1 from SGHEC-7 and SGHVSM-9 cells.

To determine whether interaction of EVTs with ECs and VSMCs stimulates STC-1 protein expression and secretion, SGHEC-7 and SGHVSM-9 cells were stimulated with TCM. TCM contains all the factors secreted by EVT cells and, when used to stimulate vascular cells, it can be used to model the EVT-vascular cell interactions which occur *in vivo*.

SGHVSM-9 and SGHEC-7 cells were cultured with TCM (50 µg/ml) in phenol red-free RPMI 1640 medium without FCS for 2 hours. The treatment media was then removed, the cells were washed twice with PBS and incubated in phenol red-free RPMI 1640 medium containing 5% (v/v) FCS. Following 24-hour incubation, the cell culture medium was collected and analysed by ELISA. Preliminary work conducted prior to the commencement of this study demonstrated that 2 hours treatment with TCM was sufficient to induce a significant effect on STC-1 secretion from SGHEC-7 cells (Appendix Figure 2). TCM contains many factors, including STC-1, which may interfere with the subsequent analysis, therefore, the treatment media was removed at this stage prior to incubation.

In SGHEC-7 cells and SGHVSM-9 cells, TCM treatment induced a ~15-fold and ~3-fold increase in STC-1 secretion in comparison to untreated cells, respectively (Figure 4.2, $P \leq 0.0001$).

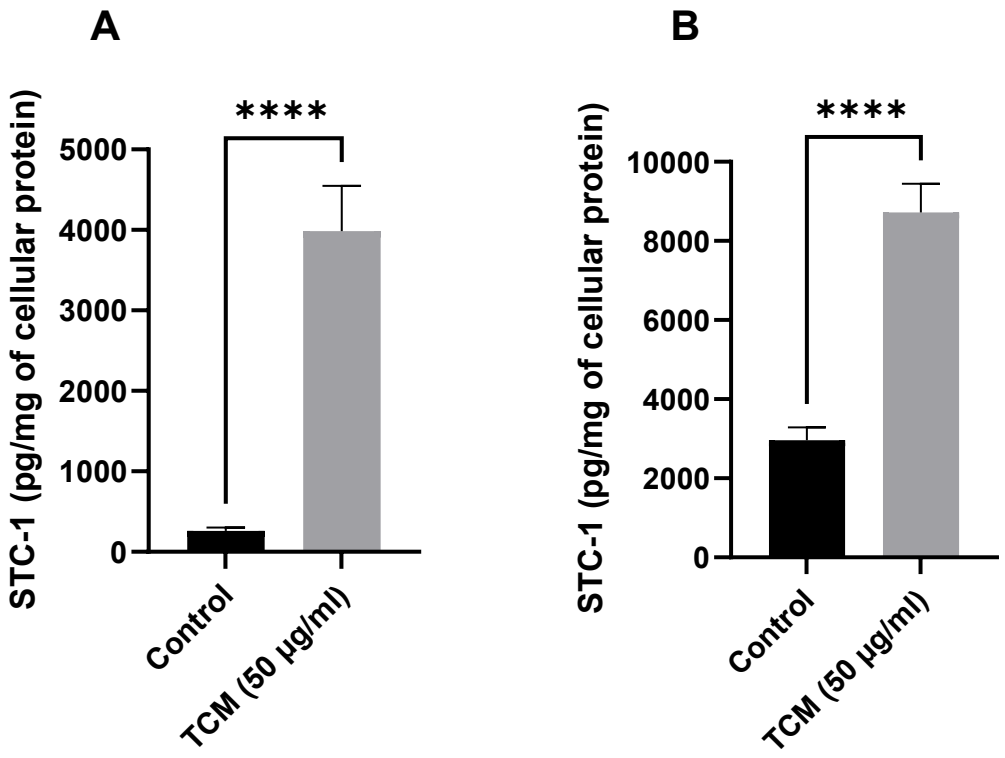



**Figure 4.2 The effect of TCM on STC-1 secretion from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 24-hour period following 2 hours treatment with TCM (50 µg/ml) was determined by ELISA (SGHEC-7 - n = 13, SGHVSM-9 – n = 22). Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (**** = P ≤ 0.0001).*

### 4.1.2 TCM treatment of SGHEC-7 and SGHVSM-9 cells has no effect on intracellular expression of STC-1

The effect of TCM treatment on intracellular expression of STC-1 in SGHEC-7 and SGHVSM-9 cells was also investigated. SGHEC-7 and SGHVSM-9 cells were cultured in the presence of TCM (50 μg/ml) in phenol red-free RPMI 1640 medium without FCS for 2 hours. The treatment media was then removed and replaced with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS for 24 hours. The cell monolayer was then harvested and lysed for western blot analysis. TCM treatment had no significant effect on intracellular expression of STC-1 in either cell type (Figure 4.3).

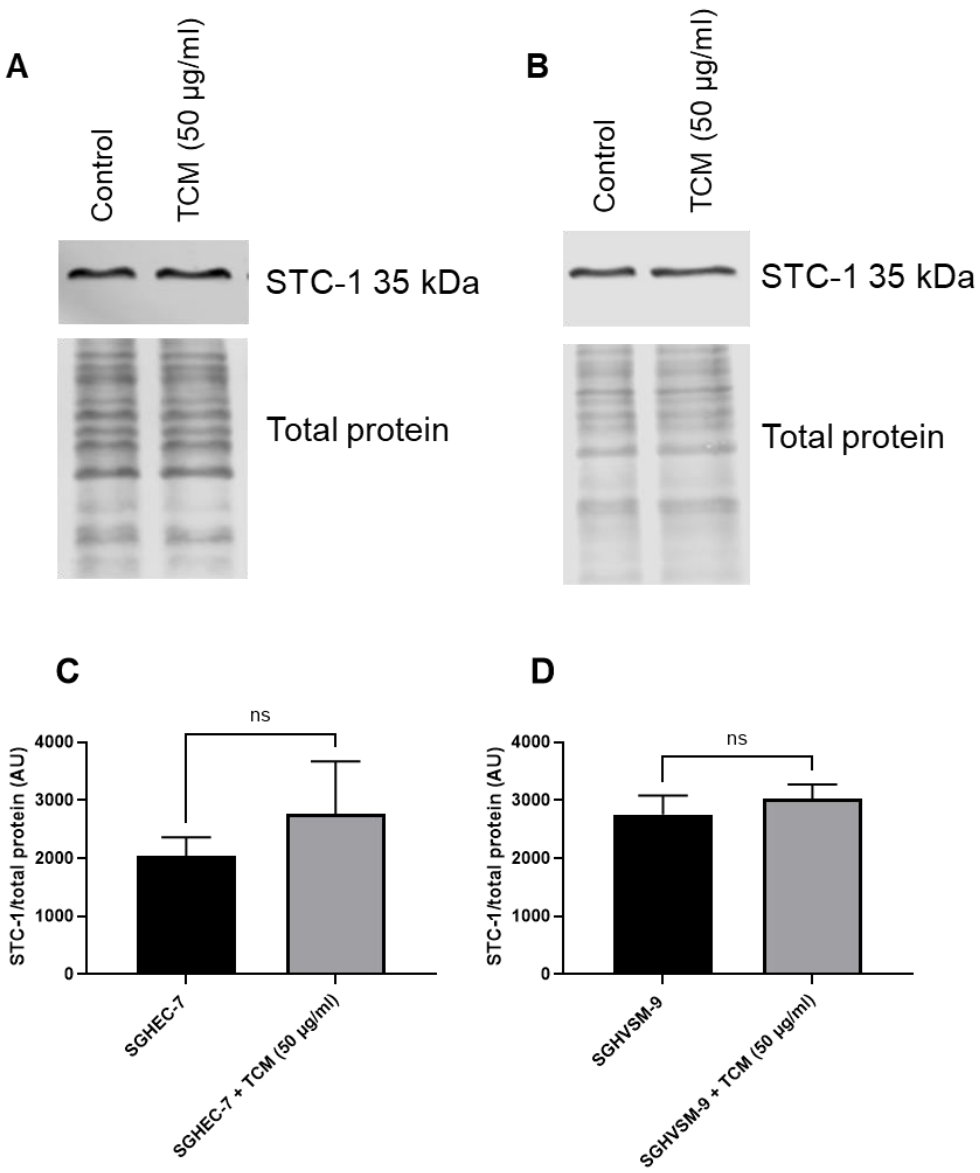



**Figure 4.3 The effect of TCM on intracellular expression of STC-1 from SGHEC-7 and SGHVSM-9 cells.**

*Expression of STC-1 determined by western blot analysis of SGHEC-7 (A) and SGHVSM-9 (B) cells cultured for 24 hours following 2 hours treatment with TCM (50 µg/ml). Quantification of western blot analysis for the detection of STC-1 in (C) SGHEC-7 and (D) SGHVSM-9 cells (n = 3). Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (ns = not significant)*

### 4.1.3 Inhibition of protein synthesis had no effect on TCM-induced STC-1 secretion from SGHEC-7 or SGHVSM-9 cells, but protein transport inhibition reduced TCM-induced STC-1 secretion from SGHVSM-9 cells.

The observation that TCM induces STC-1 secretion from SGHEC-7 and SGHVSM-9 cells but does not affect intracellular STC-1 protein expression led to the hypothesis that TCM may only act to stimulate secretion of pre-formed protein with no effect on *de novo* protein synthesis. To investigate this, cells were treated with cycloheximide (CHX) (Sigma-Aldrich, Dorset, UK), a protein synthesis inhibitor, and brefeldin A (BFA) (Sigma-Aldrich, Dorset, UK), a protein transport inhibitor which blocks transport of proteins from the endoplasmic reticulum to the Golgi complex.

Initially, a range of concentrations of CHX and BFA were added to SGHEC-7 and SGHVSM-9 cells to determine the optimal concentration to induce an effect with minimal toxicity. The range of concentrations used were previously reported to induce an effect on STC-1 expression or had previously been used in these cell types (Li and Wong, 2008; Moeller *et al.*, 2011; Seye *et al.*, 2012). For SGHEC-7 cells, the highest concentrations of CHX and BFA which could be used with minimal cell toxicity were 1 µg/ml CHX and 2.5 µg/ml BFA. For SGHVSM-9 cells, it was possible to use higher concentrations of each reagent without toxicity, therefore, 10 µg/ml CHX and 5 µg/ml BFA were used.

In addition, two methods were tested. The methods were based on previous reports in which cells were pre-treated with CHX or BFA for 2 hours prior to stimulation (Deng, 2022). In the first method, TCM (50 µg/ml) was added to the CHX/BFA media following the 2-hour pre-incubation and the cells were incubated for a further 2 hours. The media was then removed and replaced with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS for 24 hours. The second method was identical, except CHX and BFA were added back into the

phenol red-free RPMI 1640 medium containing 5% (v/v) FCS for the final 24-hour incubation. The first method did not result in any effect in either cell line (data not shown), therefore, the second method was used.

SGHEC-7 cells were treated with 1 µg/ml CHX or 2.5 µg/ml BFA for 2 hours before stimulation with TCM for an additional 2 hours. The cell culture medium was then replaced with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS and either 1 µg/ml CHX or 2.5 µg/ml BFA and the cells were incubated for 24 hours. SGHVSM-9 cells were treated in the same way but with higher concentrations of CHX and BFA, 10 µg/ml and 5 µg/ml BFA, respectively.

Treatment of TCM-stimulated SGHVSM-9 cells with BFA resulted in a ~2-fold reduction in STC-1 secretion (Figure 4.4D, $P \leq 0.05$), whereas treatment with CHX had no significant effect on STC-1 secretion (Figure 4.4C). In SGHEC-7 cells, however, treatment with CHX and BFA had no significant effect on TCM-induced STC-1 secretion (Figure 4.4A and B).

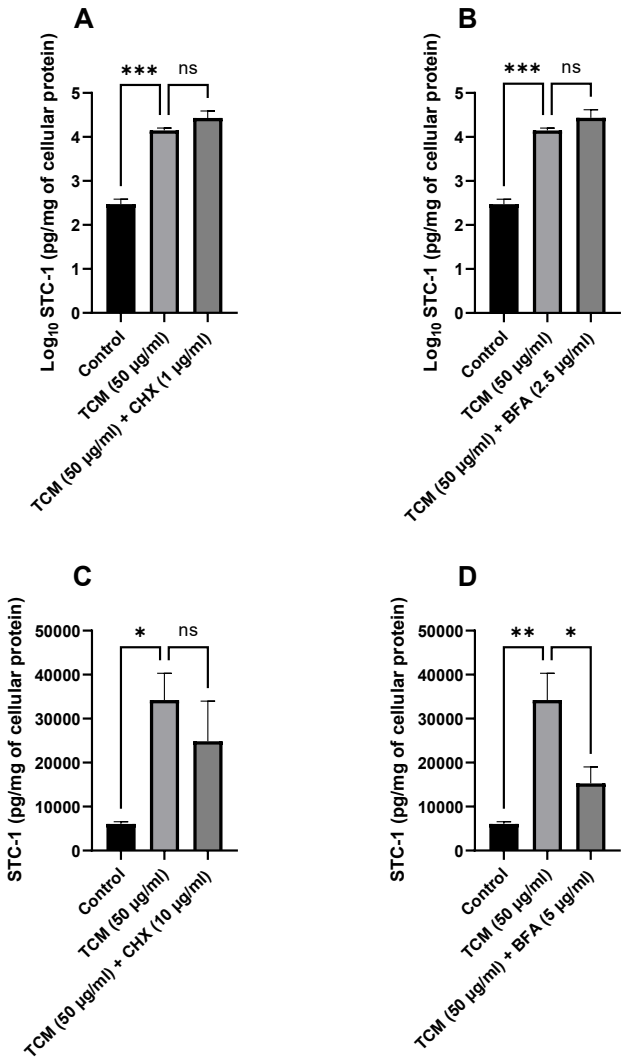



**Figure 4.4 The effect of inhibition of protein synthesis or protein transport on TCM-induced secretion of STC-1 from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 cells (A, B) into the culture medium over a 24-hour period following 2 hours pre-treatment with CHX (1 µg/ml) (A) or BFA (2.5 µg/ml) (B) and 2 hours treatment with TCM (50 µg/ml) was determined by ELISA (n =3). $Log_{10}$-transformed data of STC-1 (pg/mg of cellular protein). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, *** = P ≤ 0.001). Secretion of STC-1 from SGHVSM-9 cells (C, D) into the culture medium over a 24-hour period following 2 hours pre-treatment with CHX (10 µg/ml) (C) or BFA (5 µg/ml) (D) and 2 hours treatment with TCM (50 µg/ml) was determined by ELISA (n =3). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05, ** = P ≤ 0.01).*

### 4.1.4 Proteome Profiler Human XL Cytokine Array analysis of cytokines and growth factors in TCM

The observation that TCM stimulates the secretion of STC-1 from SGHEC-7 and SGHVSM-9 cells led to two main questions; 1) What factor(s) in TCM is/are responsible for inducing the secretion of STC-1 from these cells? 2) Which cell signalling pathway is responsible for regulating STC-1 secretion in these cells?

To address the first question, TCM was screened using a cytokine array (Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, MN, USA)) to determine the relative levels of 105 human cytokines and growth factors (as listed in Appendix Table 1). Analysis of this array showed that each of the 105 cytokines and growth factors on the array were present in TCM (Appendix Figure 3).

Eleven cytokines and growth factors that were identified to be present in TCM were tested individually in SGHEC-7 cells to assess their effect on STC-1 secretion. The cytokines/growth factors tested are listed in Table 4.1. The range of concentrations of cytokines/growth factors used here were chosen as they were previously established to induce an effect in this cell line on expression and secretion of other target proteins. None of the cytokines/growth factors tested had any effect on STC-1 secretion from SGHEC-7 cells and there was no obvious change in cell morphology or survival following treatment (data not shown).

**Table 4.1 Cytokines/growth factors tested in SGHEC-7 cells for their effect on STC-1 secretion.**

| Cytokine/growth factor | Concentrations tested | Company |
|---|---|---|
| Vascular endothelial growth factor A (VEGF-A) | 100 ng/ml, 200 ng/ml | PeproTech, NJ, USA |
| Hepatocyte growth factor (HGF) | 10 ng/ml, 100 ng/ml | PeproTech, NJ, USA |
| Platelet-derived growth factor-BB (PDGF-BB) | 50 ng/ml, 100 ng/ml | PeproTech, NJ, USA |
| Transforming growth factor-beta (TGF-β) | 20 ng/ml, 40 ng/ml | R&D Systems, MN, USA |
| Interleukin-1 beta (IL-1β) | 5 ng/ml, 10 ng/ml | PeproTech, NJ, USA |
| Fibroblast growth factor 2 (FGF-2) | 50 ng/ml, 100 ng/ml | PeproTech, NJ, USA |
| Epidermal growth factor (EGF) | 50 ng/ml, 100 ng/ml | PeproTech, NJ, USA |
| C-X-C motif chemokine ligand 10 (CXCL10) | 100 ng/ml | PeproTech, NJ, USA |
| Interferon gamma (IFN-γ) | 500 U/ml | PeproTech, NJ, USA |
| Leptin | 100 ng/ml | PeproTech, NJ, USA |
| Tumour necrosis factor alpha (TNF-α) | 30 ng/ml | PeproTech, NJ, USA |

### 4.1.5 TCM treatment of SGHVSM-9 cells induces phosphorylation of epidermal growth factor receptor

After conducting the cytokine array, it was evident that TCM contains a wide range of cytokines and growth factors and one or more of these must be involved in stimulating the secretion of STC-1 from SGHEC-7 and SGHVSM-9 cells. Assessing the effect of each individual factors or combination of factors on the STC-1 secretion would be extremely time-consuming and expensive. Therefore, it was decided to investigate the effect of TCM on the activation of receptor tyrosine kinases (RTKs) to aid identification of which factors might be underlying TCM-induced secretion of STC-1 from vascular cells. To achieve this, a Human Phospho-Receptor Tyrosine Kinase (Phospho-RTK) Array (R&D Systems, MN, USA) was used to screen for phosphorylated RTKs following TCM treatment in SGHEC-7 and SGHVSM-9 cells (Figure 4.5).

The Phospho-RTK Array contains antibodies against 49 different RTKs. It is likely that activation of the different RTKs by TCM will require different time periods of stimulation. Therefore, SGHEC-7 and SGHVSM-9 cells were treated with TCM for two different time periods: 5 and 15 minutes.

SGHEC-7 and SGHVSM-9 cells were cultured for 5 hours in complete media before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. The cells were treated with TCM (50 μg/ml) for 5 and 15 minutes. The culture medium was removed, and the cell monolayer was lysed for analysis using the Human Phospho-RTK Array kit. Following 5 minutes treatment with TCM, a weak signal for phosphorylated epidermal growth factor receptor (EGFR) in SGHVSM-9 cells was detected (Figure 4.5B). In SGHEC-7 cells, 5 minutes treatment with TCM did not result in phosphorylation of any RTK

on this array (Figure 4.5C and D). Similarly, 15 minutes treatment of SGHEC-7 and SGHVSM-9 cells with TCM did not result in phosphorylation of any RTK on the array (data not shown).

To confirm activation of EGFR in SGHVSM-9 cells following TCM stimulation and to ensure that the result obtained using TCM-treated SGHEC-7 lysates was accurate and not caused by technical issues associated with the use of antibody-based arrays such as variations in antibody binding specificity, the expression of phospho-EGFR (Tyr1068) in cells stimulated with TCM was assessed by western blot (Figure 4.6).

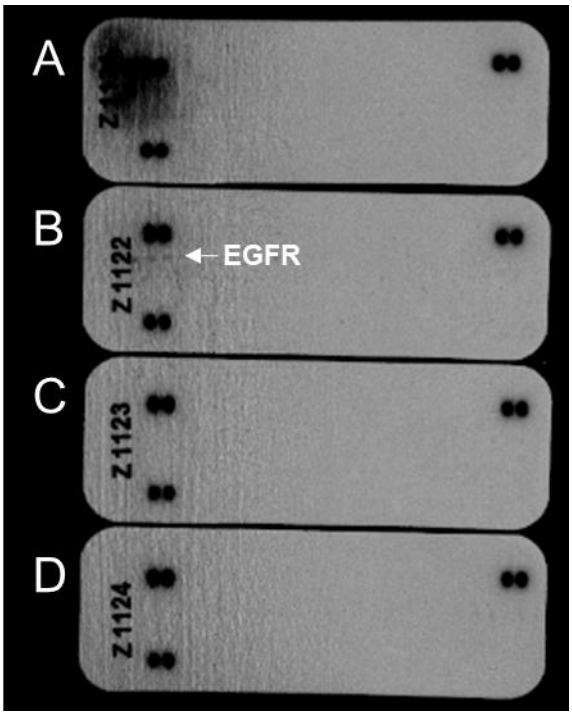



**Figure 4.5 A Phospho-RTK array to screen the relative levels of phosphorylated RTKs in SGHVSM-9 cells and SGHEC-7 cells following 5 minutes treatment with TCM.**

*(A) Untreated SGHVSM-9 cells, (B) SGHVSM-9 cells treated with TCM (50 µg/ml), (C) Untreated SGHEC-7 cells, (D) SGHEC-7 cells treated with TCM (50 µg/ml).*

Both cell lines were cultured for 5 hours in complete media before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. The cells were treated with TCM (50 µg/ml) for 10 minutes prior to removing the culture medium and harvesting the cell monolayer for analysis through western blot. The cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane. These membranes were probed for the detection of phospho-EGFR (Tyr1068). Expression of phospho-EGFR (Tyr1068) increased ~3-fold in SGHVSM-9 cells following 10 minutes stimulation with TCM (Figure 4.6B, D $P \leq 0.05$).

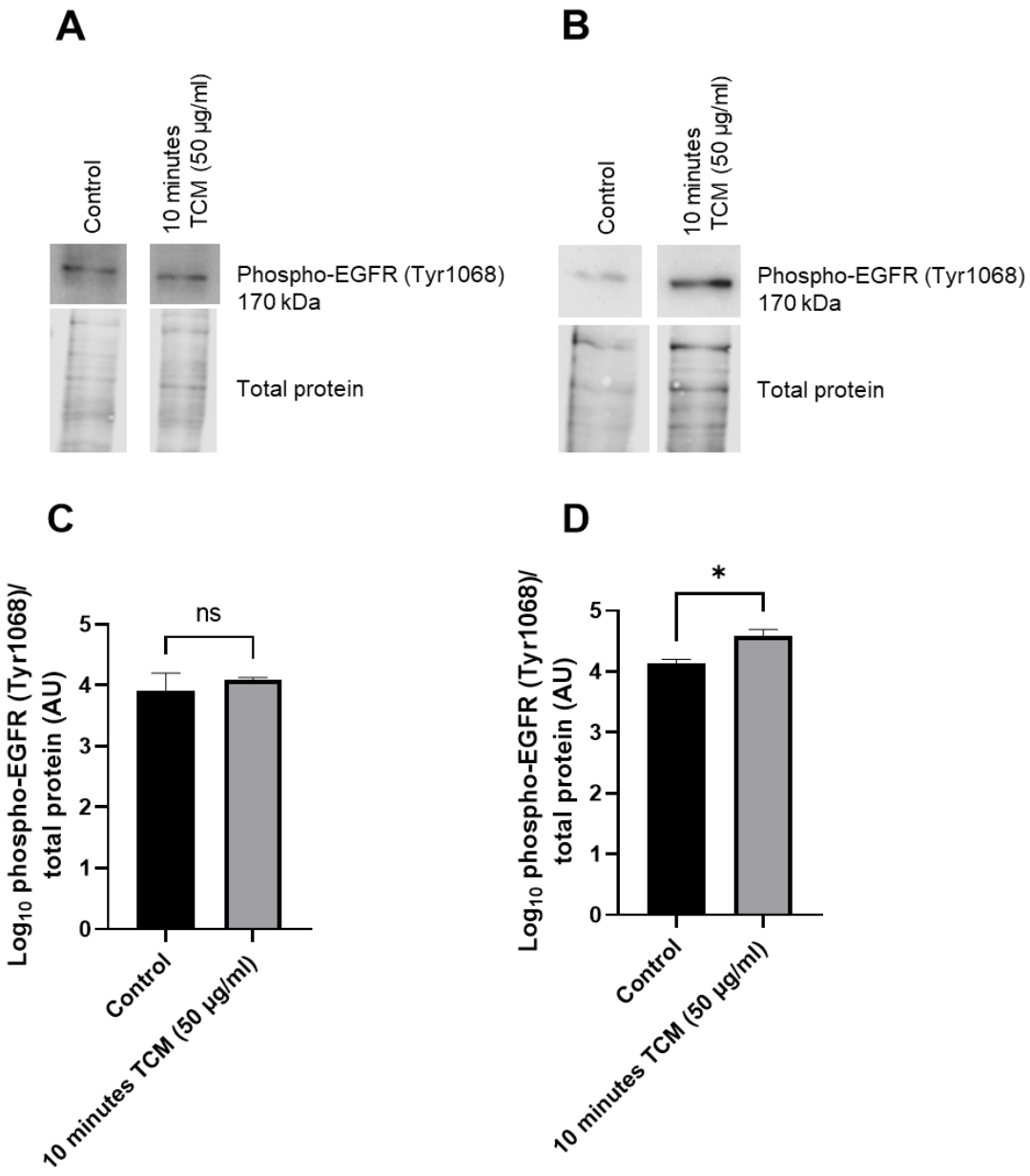



**Figure 4.6 The effect of TCM treatment on the expression of phospho-EGFR (Tyr1068).**

*Expression of phospho-EGFR (Tyr1068) in SGHEC-7 (A) and SGHVSM-9 (B) cells following 10 minutes treatment with TCM (50 µg/ml) was determined by western blot. Quantification of western blot analysis for the detection of phospho-EGFR (Tyr1068) in (C) SGHEC-7 and (D) SGHVSM-9 cells (n = 3). Data are expressed as band density normalised to total protein of the corresponding lane. $Log_{10}$-transformed data of phospho-EGFR (Tyr1068)/total protein (AU). Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (ns = not significant, * = P ≤ 0.05).*

### 4.1.6 Broad-spectrum receptor tyrosine kinase (RTK) inhibition did not affect TCM-induced STC-1 secretion from SGHEC-7 or SGHVSM-9 cells

As an additional approach to investigate the possible role of RTK signalling in TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells, a broad-spectrum RTK inhibitor, sunitinib malate (SU11248) (Tocris Bioscience, Bristol, UK) was used. SU11248 is an ATP-competitive, multitargeted tyrosine kinase inhibitor which inhibits cellular signalling by targeting multiple RTKs including EGFR, PDGFR-α,-β, VEGFR-1, -2, KIT, FLT3, RET and CSF-1R (Shukla *et al.*, 2009).

SGHEC-7 and SGHVSM-9 cells were cultured for 5 hours in complete media before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. Cells were then treated with 2.5 µM and 5 µM SU11248 for 20 minutes. These concentrations used were chosen as the half maximal inhibitory concentration (IC50) of SU11248 in HUVECs was previously established to be 1.6 µM (Zlacká *et al.*, 2022) and SU11248 has previously been used in the range of 1-5 µM in these cells (Lai *et al.*, 2017; Juengel *et al.*, 2021). TCM (50 µg/ml) was added to cells for 2 hours before removing the treatment media and replacing with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS. Following 24-hour incubation, the cell culture medium was removed and analysed by ELISA. Treatment of SGHEC-7 and SGHVSM-9 cells with SU11248 had no significant effect on TCM-induced STC-1 secretion at the inhibitor concentrations tested (Figure 4.7).

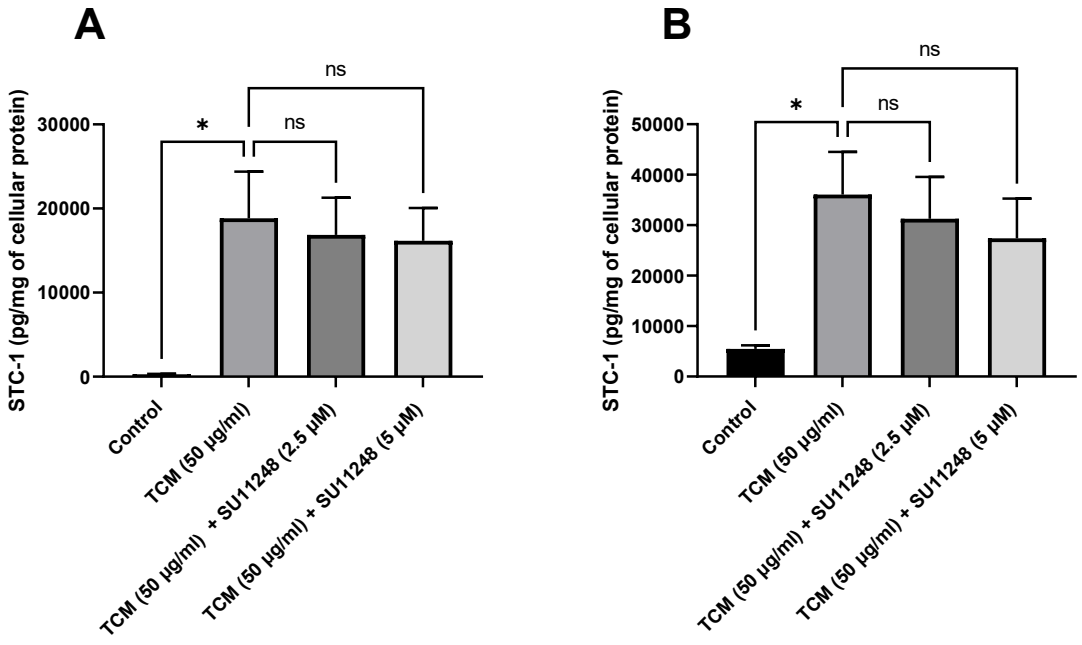



**Figure 4.7 The effect of broad-spectrum RTK inhibition using sunitinib malate (SU11248) on TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 24-hour period following 2 hours treatment with SU11248 (2.5 µM or 5 µM) and TCM (50 µg/ml) was determined by ELISA (n =3). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05).*

### 4.1.7 TCM treatment of SGHEC-7 and SGHVSM-9 cells induced activation of proteins in major cell signalling pathways

To further investigate the mechanisms underlying TCM-induced secretion of STC-1 from vascular cells, the effect of TCM treatment on the activation of proteins in major cell signalling pathways in SGHEC-7 and SGHVSM-9 cells was assessed.

SGHEC-7 and SGHVSM-9 cells were cultured for 5 hours in complete media before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. The cells were treated with TCM (50 μg/ml) for 5, 10, and 30 minutes prior to removing the culture medium and harvesting the cell monolayer for analysis through western blot. The cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane. These membranes were probed for the detection of phospho-GSK-3β (Ser9), phospho-AKT (Ser473), phospho-p44/42 MAPK (Thr202/Tyr204), and phospho-NDRG1 (Thr346).

In SGHEC-7 cells, 10 minutes TCM treatment induced a significant increase in the expression of phospho-AKT (Ser473) (~10-fold compared to control) and phospho-p44/42 MAPK (Thr202/Tyr204) (~2.5-fold compared to control), and 30 minutes TCM treatment significantly increased expression of phospho-NDRG1 (Thr346) (~2.6-fold compared to control) (Figure 4.8B, C and D,$P \leq 0.05$). The expression of phospho-GSK-3β (Ser9) increased ~3-fold following 10 minutes TCM treatment, but this change did not reach statistical significance (Figure 4.8A).

In SGHVSM-9 cells, 5 minutes TCM treatment induced a significant increase in the expression of phospho-AKT (Ser473) (~5.5-fold compared to control) (Figure 4.9B, $P \leq 0.05$). Expression of phospho-GSK-3β (Ser9) was significantly increased after 5 and 10 minutes TCM treatment (~2.2-fold compared to control) (Figure 4.9A, $P \leq 0.05$). Expression of

phospho-p44/42 MAPK (Thr202/Tyr204) was significantly increased after 10 minutes TCM treatment (~2.6-fold compared to control) (Figure 4.9C, $P \leq 0.01$) and phospho-NDRG1 (Thr346) expression was significantly increased after 10 and 30 minutes (~2.3-fold and ~2.1-fold compared to control, respectively) ($P \leq 0.01$, $P \leq 0.05$, respectively) TCM treatment (Figure 4.9D).

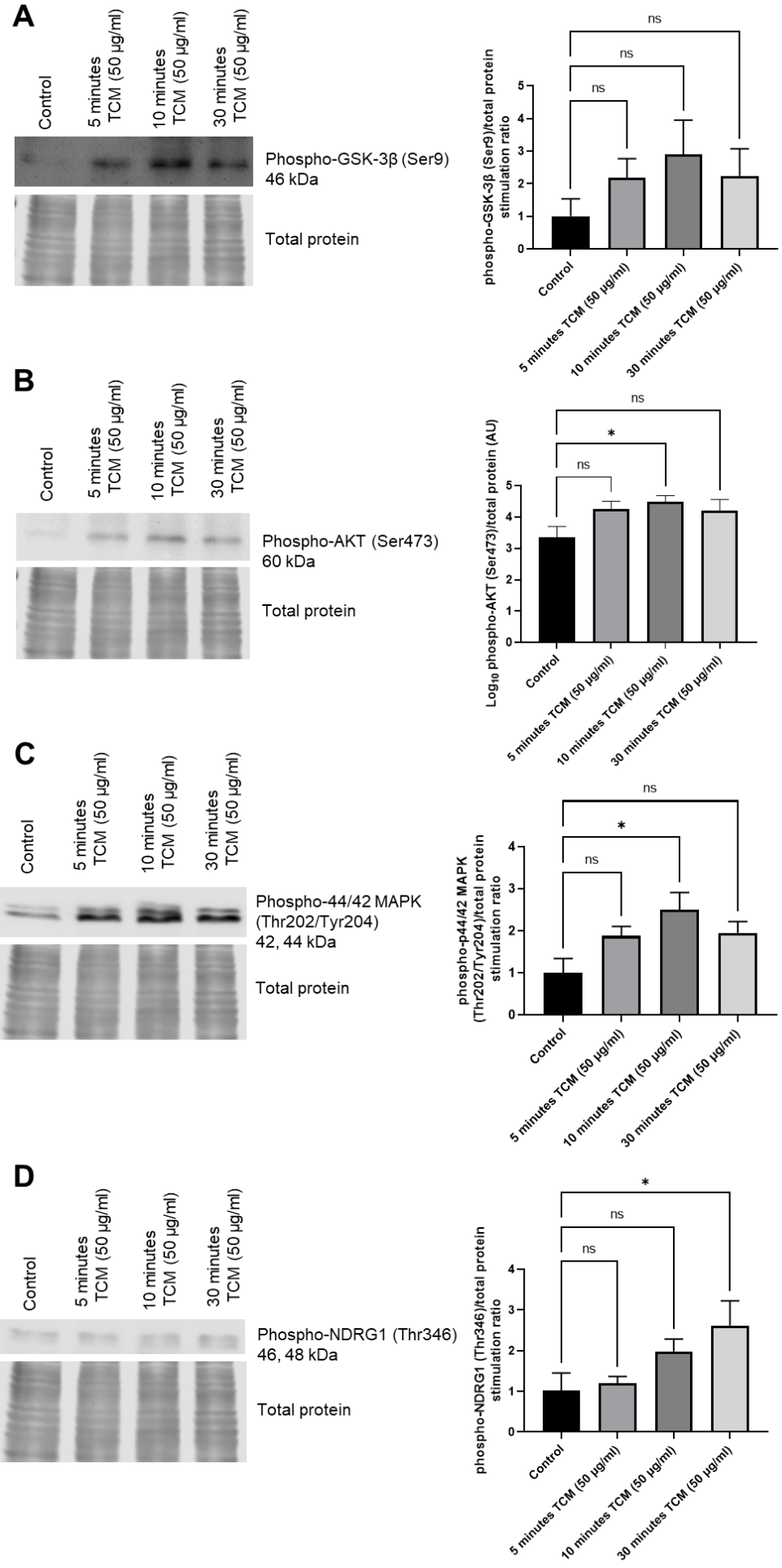

A
Control
5 minutes TCM (50 µg/ml)
10 minutes TCM (50 µg/ml)
30 minutes TCM (50 µg/ml)
Phospho-GSK-3β (Ser9) 46 kDa
Total protein
phospho-GSK-3β (Ser9)/total protein stimulation ratio
ns
B
Phospho-AKT (Ser473) 60 kDa
Log10 phospho-AKT (Ser473)/total protein (AU)
*
C
Phospho-44/42 MAPK (Thr202/Tyr204) 42, 44 kDa
phospho-p44/42 MAPK (Thr202/Tyr204)/total protein stimulation ratio
D
Phospho-NDRG1 (Thr346) 46, 48 kDa
phospho-NDRG1 (Thr346)/total protein stimulation ratio

**Figure 4.8 The effect of TCM treatment on the activation of proteins in major cell signalling pathways in SGHEC-7 cells.**

*Expression of (A) phospho-GSK-3β (Ser9) (n = 4), (B) phospho-AKT (Ser473) (n = 5), (C) phospho-p44/42 MAPK (Thr202/Tyr204) (n = 5), (D) phospho-NDRG1 (Thr346) (n = 5) in SGHEC-7 cells following 5, 10, and 30 minutes treatment with TCM (50 µg/ml) was determined by western blot. Data are expressed as band density normalised to total protein of the corresponding lane. (A, C, and D) are presented as stimulation ratios where each dataset is expressed as a ratio to the average of the control dataset. (B) is $Log_{10}$-transformed data of phospho-AKT (Ser473)/total protein (AU). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05).*

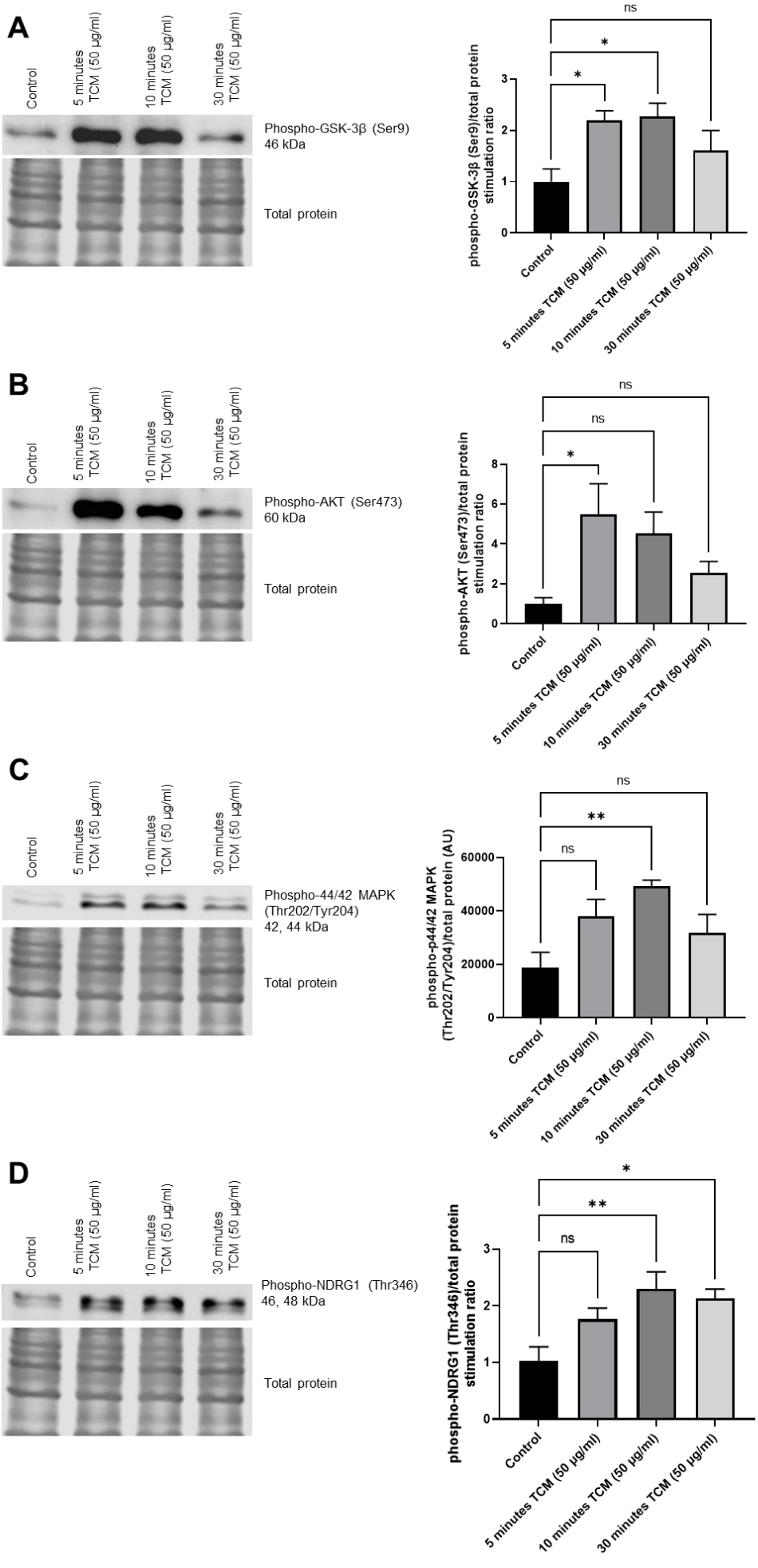

A
Control
5 minutes TCM (50 µg/ml)
10 minutes TCM (50 µg/ml)
30 minutes TCM (50 µg/ml)
Phospho-GSK-3β (Ser9)
46 kDa
Total protein
phospho-GSK-3β (Ser9)/total protein stimulation ratio
ns
*
*
B
Phospho-AKT (Ser473)
60 kDa
Total protein
phospho-AKT (Ser473)/total protein stimulation ratio
ns
ns
*
C
Phospho-44/42 MAPK
(Thr202/Tyr204)
42, 44 kDa
Total protein
phospho-p44/42 MAPK
(Thr202/Tyr204)/total protein (AU)
ns
**
ns
D
Phospho-NDRG1 (Thr346)
46, 48 kDa
Total protein
phospho-NDRG1 (Thr346)/total protein stimulation ratio
*
**
ns

**Figure 4.9 The effect of TCM treatment on the activation of proteins in major cell signalling pathways in SGHVSM-9 cells.**

*Expression of (A) phospho-GSK-3β (Ser9) (n = 4), (B) phospho-AKT (Ser473) (n = 5), (C) phospho-p44/42 MAPK (Thr202/Tyr204) (n = 5), (D) phospho-NDRG1 (Thr346) (n = 5) in SGHVSM-9 cells following 5, 10, and 30 minutes treatment with TCM (50 µg/ml) was determined by western blot. Data are expressed as band density normalised to total protein of the corresponding lane. (A, B, and D) are presented as stimulation ratios where each dataset is expressed as a ratio to the average of the control dataset. Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05, ** = P ≤ 0.01).*

### 4.1.8 Inhibition of phosphoinositide 3-kinase (PI3K) in SGHEC-7 and SGHVSM-9 cells did not affect TCM-induced STC-1 secretion, but PI3K activation reduced TCM-induced STC-1 secretion from SGHEC-7 cells

PI3K signalling is a commonly implicated intracellular pathway in the regulation of cell growth, motility, survival, metabolism, and angiogenesis (Katso *et al.*, 2003; Engelman, Luo and Cantley, 2006). PI3K is activated by many extracellular signals including growth factors, cytokines, and hormones. These factors bind to transmembrane receptors such as RTKs and G protein-coupled receptors (GPCRs) (Guo *et al.*, 2015; Fruman *et al.*, 2017). The PI3K pathway has previously been implicated in the regulation of STC-1 expression in human skin fibroblasts and trophoblast cells (Moeller *et al.*, 2011; Abid *et al.*, 2020). Therefore, the role of the PI3K pathway in the regulation of TCM-induced STC-1 secretion from vascular cells was investigated.

To assess the role of the PI3K pathway in the regulation of STC-1 secretion, a PI3K inhibitor, LY294002 (Calbiochem®, San Diego, California, USA) and a PI3K activator 740-YP (Tocris Bioscience, Bristol, UK) were used to treat SGHEC-7 and SGHVSM-9 cells.

Prior to using LY294002 to assess the effect of PI3K inhibition on STC-1 secretion, it was necessary to determine its efficacy in inhibiting the target pathway in these cells. To achieve this, the effect of the highest concentration of this inhibitor used in this study (50 µM) on the expression of phospho-AKT (Ser473) in SGHEC-7 and SGHVSM-9 cells was assessed. As Akt is a downstream target of PI3K a reduction in phosphorylation of this kinase would indicate effective inhibition of the PI3K pathway by LY294002.

Cells were seeded in 3.5 cm plates and cultured in complete media for 5 hours before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating

overnight. The cells were then pre-treated with LY294002 (50 μM) for 20 minutes before incubating with TCM (50 μg/ml) for 10 minutes. The media was removed, and the cell monolayer was harvested for analysis through western blot. The cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane for the detection of phospho-AKT (Ser473).

Treatment of TCM-stimulated SGHEC-7 and SGHVSM-9 cells with LY294002 (50 μM) resulted in a significant reduction in the expression of phospho-AKT (Ser473) (Figure 4.10A, B SGHEC-7 – $P \leq 0.01$, Figure 4.10C, D SGHVSM-9 – $P \leq 0.05$) indicating that the PI3K pathway was inhibited by LY294002 (50 μM) in this system.

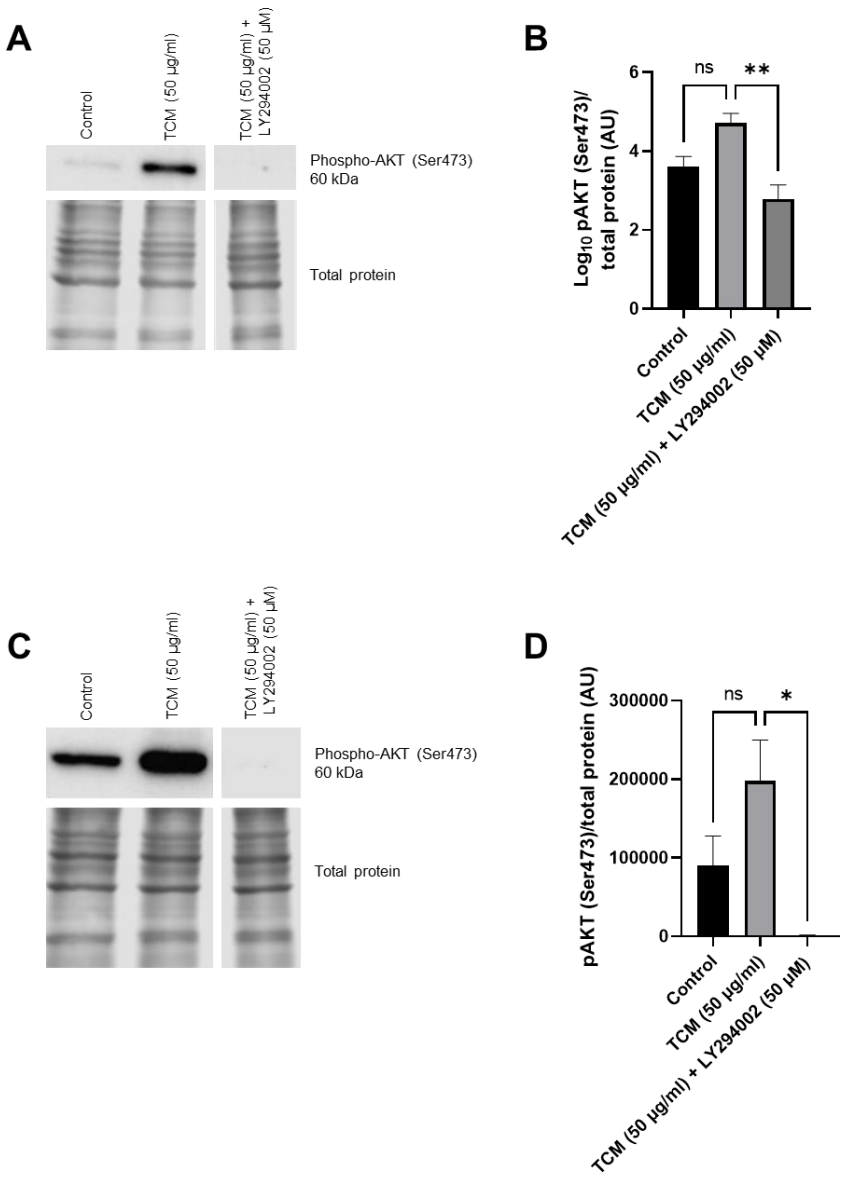



**Figure 4.10 LY294002 treatment of TCM-stimulated SGHEC-7 and SGHVSM-9 cells reduced expression of phospho-AKT (Ser473).**

*Expression of phospho-AKT (Ser473) in (A) SGHEC-7 cells and (C) SGHVSM-9 cells following 20 minutes pre-treatment with LY294002 (50 µM) and 10 minutes treatment with TCM (50 µg/ml) was determined by western blot. Quantification of western blot analysis for the detection of phospho-AKT (Ser473) in (B) SGHEC-7 and (D) SGHVSM-9 cells (n = 3). Data are expressed as band density normalised to total protein of the corresponding lane. (B) is $Log_{10}$-transformed data of pAKT (Ser473)/total protein (AU). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05, ** = P ≤ 0.01).*

In SGHEC-7 and SGHVSM-9 cells, the effect of LY294002 on both unstimulated and TCM-induced STC-1 secretion was investigated. Three concentrations of LY294002 were tested: 10 µM, 25 µM, and 50 µM. This range was selected as LY294002 used at these concentrations has previously been demonstrated to induce an effect on STC-1 expression (Moeller *et al.*, 2011; Abid *et al.*, 2020).

SGHEC-7 and SGHVSM-9 cells were cultured for 5 hours in complete media before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. Cells were then treated with 10 µM, 25 µM, and 50 µM LY294002 or 50 µg/ml 740-YP in phenol red-free RPMI 1640 medium without FCS for 20 minutes. TCM (50 µg/ml) was added to cells for 2 hours before removing the treatment media and replacing with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS. Following 24-hour incubation, the cell culture medium was removed and analysed by ELISA.

LY294002 treatment of unstimulated and TCM-stimulated SGHEC-7 and SGHVSM-9 cells had no significant effect on STC-1 secretion at any of the concentrations tested (Figure 4.11A and B). Similarly, in SGHVSM-9 cells, treatment with the PI3K activator 740-YP (50 µg/ml) had no effect on unstimulated or TCM-induced STC-1 secretion (Figure 4.11D). In SGHEC-7 cells, activation of PI3K with 740-YP had no effect on unstimulated STC-1 secretion, however, PI3K activation resulted in a significant reduction in TCM-induced STC-1 secretion (Figure 4.11C, $P \leq 0.05$).

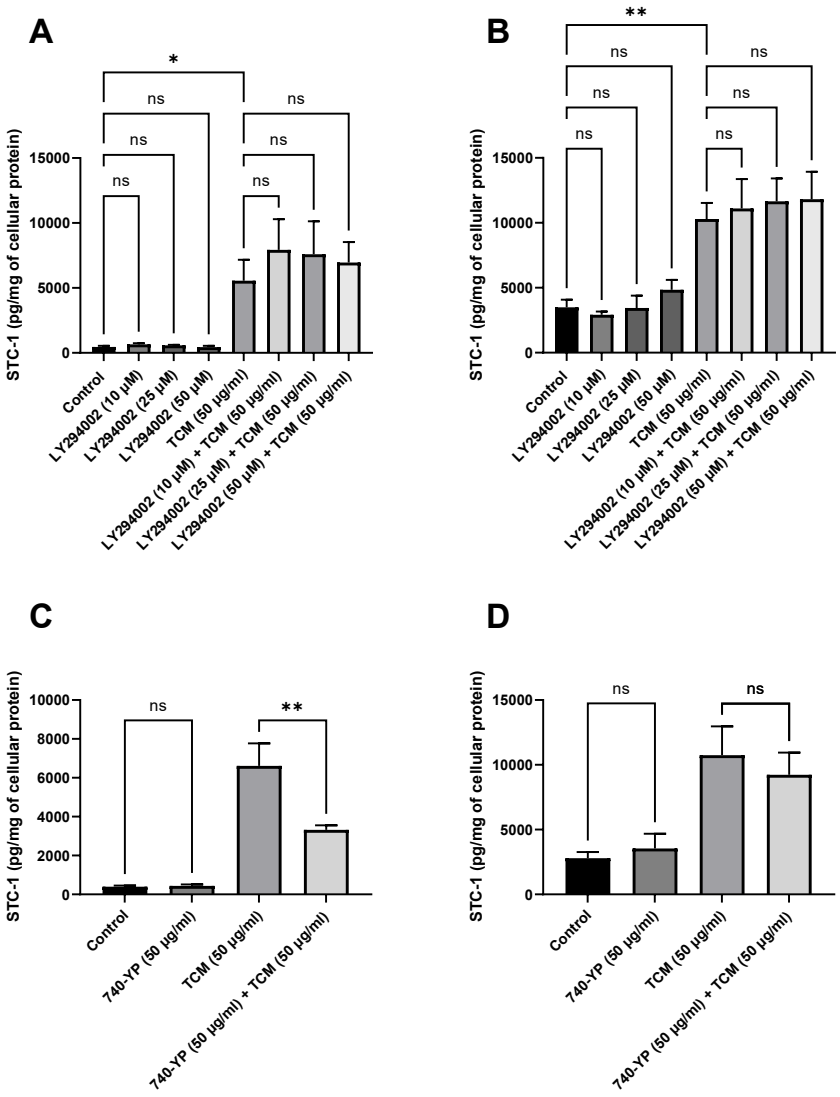



**Figure 4.11 The effect of inhibition and activation of phosphoinositide 3-kinase (PI3K) on TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 24-hour period following 2 hours treatment with LY294002 10 µM (n = 3), 25 µM (n = 3), and 50 µM (n = 7) +/- TCM (50 µg/ml) was determined by ELISA. Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05, ** = P ≤ 0.01). Secretion of STC-1 from SGHEC-7 cells (C) and SGHVSM-9 cells (D) into the culture medium over a 24-hour period following 2 hours treatment with 740-YP (50 µg/ml) +/- TCM (50 µg/ml) was determined by ELISA (n = 3). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, ** = P ≤ 0.01).*

### 4.1.9 Inhibition of Akt increased TCM-induced STC-1 secretion from SGHEC-7 cells, but not from SGHVSM-9 cells

The effect of inhibition of the serine/threonine kinase, Akt (also known as protein kinase B), on STC-1 secretion from SGHEC-7 and SGHVMS-9 cells was also investigated. Akt is a downstream effector in the PI3K signalling pathway (Liu *et al.*, 2009), and previous experimentation demonstrated its activation in SGHEC-7 and SGHVSM-9 cells following TCM treatment (section 4.1.7).

SGHEC-7 and SGHVSM-9 cells were treated with three concentrations of AKT Inhibitor IV (Calbiochem®, San Diego, California, USA): 2.5 µM, 5 µM, and 10 µM. This inhibitor is a cell-permeable compound which is known to suppress phosphorylation of Akt on residues Ser473 and Thr308 (Meinig and Peterson, 2015).

Prior to using AKT Inhibitor IV to assess the effect of Akt inhibition on STC-1 secretion, it was necessary to determine its efficacy in inhibiting the target pathway in these cells. To achieve this, the effect of the highest concentration of this inhibitor used in this study (10 µM) on the expression of phospho-AKT (Ser473) in SGHEC-7 and SGHVSM-9 cells was assessed.

Cells were seeded in 3.5 cm plates and cultured in complete media for 5 hours before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. The cells were then pre-treated with AKT Inhibitor IV (10 µM) for 20 minutes before incubating with TCM (50 µg/ml) for 10 minutes. The media was removed, and the cell monolayer was harvested for analysis through western blot. The cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane for the detection of phospho-AKT (Ser473).

Treatment of TCM-stimulated SGHEC-7 cells with AKT Inhibitor IV (10 μM) resulted in a significant reduction in the expression of phospho-AKT (Ser473) (Figure 4.12A, B $P \leq 0.05$) indicating that this kinase is inhibited in this system. In TCM-stimulated SGHVSM-9 cells, treatment with AKT Inhibitor IV (10 μM) resulted in a 2.5-fold decrease in phospho-AKT (Ser473) expression but this did not reach statistical significance (Figure 4.12C, D).

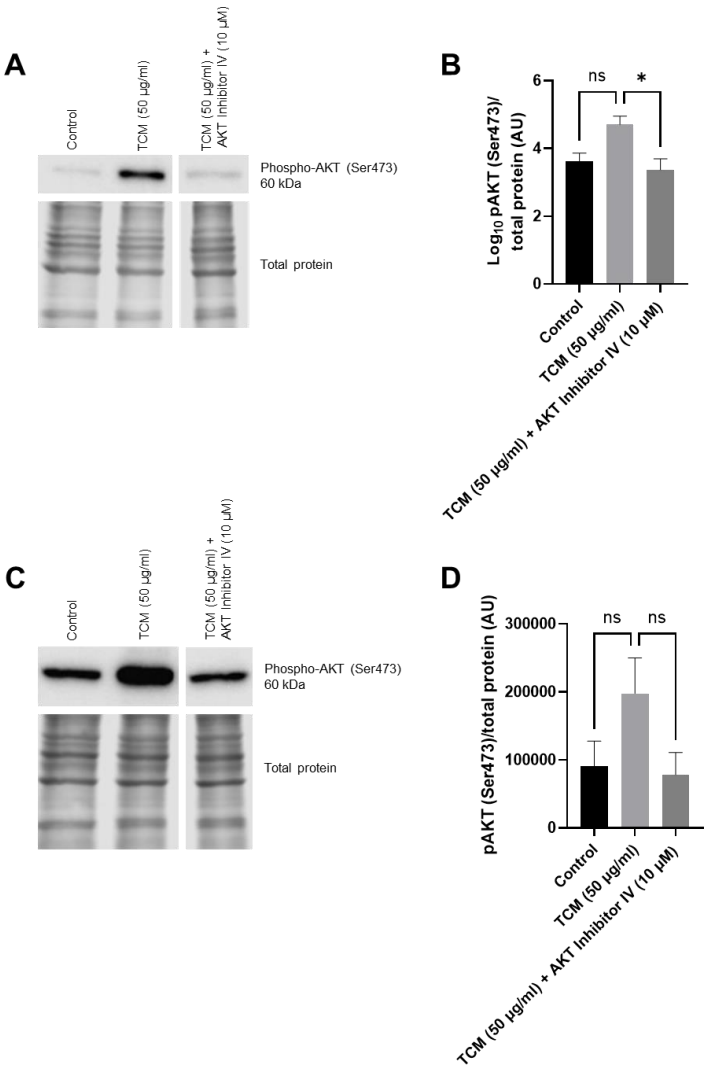



**Figure 4.12 AKT Inhibitor IV treatment of TCM-stimulated SGHEC-7 and SGHVSM-9 cells reduced expression of phospho-AKT (Ser473).**

*Expression of phospho-AKT (Ser473) in (A) SGHEC-7 cells and (C) SGHVSM-9 cells following 20 minutes pre-treatment with AKT Inhibitor IV (10 µM) and 10 minutes treatment with TCM (50 µg/ml) was determined by western blot. Quantification of western blot analysis for the detection of phospho-AKT (Ser473) in (B) SGHEC-7 and (D) SGHVSM-9 cells (n = 3). Data are expressed as band density normalised to total protein of the corresponding lane. (B) is $Log_{10}$-transformed data of pAKT (Ser473)/total protein (AU). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05).*

The effect of AKT Inhibitor IV was assessed on unstimulated and TCM-treated cells. SGHEC-7 and SGHVSM-9 cells were cultured for 5 hours in complete media before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. Cells were then treated with 2.5 µM, 5 µM, and 10 µM AKT Inhibitor IV in phenol red-free RPMI 1640 medium without FCS for 20 minutes. TCM (50 µg/ml) was added to cells where the effect of TCM-stimulation was assessed for 2 hours before removing the treatment media and replacing with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS. Following 24-hour incubation, the cell culture medium was removed and analysed by ELISA.

In SGHVSM-9 cells, AKT Inhibitor IV had no significant effect on STC-1 secretion in unstimulated or TCM-treated cells at any of the concentrations tested (Figure 4.13B).

In SGHEC-7 cells, AKT Inhibitor IV had no significant effect on unstimulated STC-1 secretion at any of the concentrations tested, however, treatment with 10 µM AKT Inhibitor IV resulted in a ~1.9-fold increase in STC-1 secretion in TCM-treated cells (Figure 4.13A, $P \leq 0.05$).

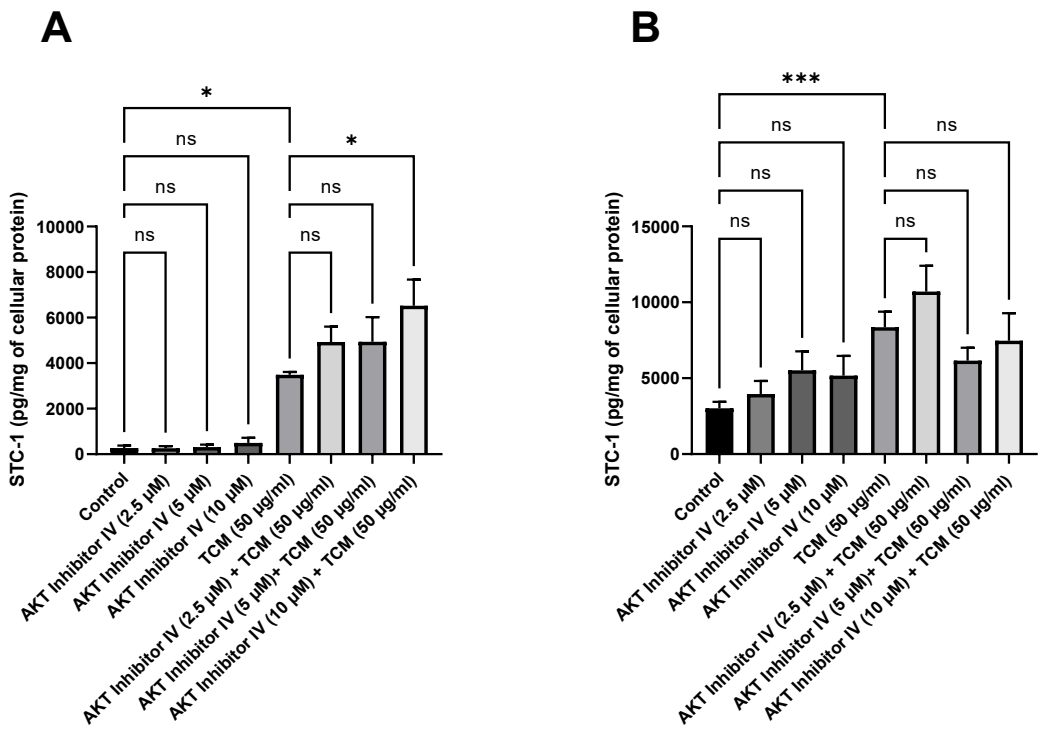



**Figure 4.13 The effect of inhibition of Akt on TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 24-hour period following 2 hours treatment with AKT Inhibitor IV 2.5 µM (SGHEC-7 – n = 3, SGHVSM-9 – n = 4), 5 µM (SGHEC-7 – n = 3, SGHVSM-9 – n = 12), and 10 µM (SGHEC-7 – n = 3, SGHVSM-9 – n = 4) +/- TCM (50 µg/ml) was determined by ELISA. Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05, *** = P ≤ 0.001).*

### 4.1.10 Inhibition of serum/glucocorticoid regulated kinase 1 (SGK1) increased TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells

The effect of inhibiting SGK1 on STC-1 secretion from SGHEC-7 and SGHVSM-9 cells was investigated. SGK1 is a member of the AGC family of serine-threonine kinases and shares the greatest sequence homology with the AKT family (Di Cristofano, 2017). Phosphorylation and activation of SGK1 is controlled by the PI3K signalling pathway (Kobayashi and Cohen, 1999; Park *et al.*, 1999) and previous experimentation demonstrated activation of its downstream kinase, N-myc downstream regulated 1 (NDRG1), in SGHEC-7 and SGHVSM-9 cells following TCM treatment (section 4.1.7). Therefore, to further investigate the role of the PI3K signalling pathway in TCM-induced secretion of STC-1 from vascular cells, the role of SGK1 in this process was assessed.

SGHEC-7 and SGHVSM-9 cells were treated with three concentrations of a specific SGK1 inhibitor, GSK650394 (Tocris Bioscience, Bristol, UK): 25 µM, 50 µM, and 100 µM. Prior to using GSK650394 to assess the effect of SGK1 inhibition on STC-1 secretion, it was necessary to determine its efficacy in inhibiting the target pathway in these cells. To achieve this, the effect of the highest concentration of this inhibitor used in this study (100 µM) on the expression of phospho-NDRG1 (Thr346) in SGHEC-7 and SGHVSM-9 cells was assessed. As NDRG1 is a downstream target of SGK1, a reduction in phosphorylation of this kinase would indicate effective inhibition of SGK1 by GSK650394.

Cells were seeded in 3.5 cm plates and cultured in complete media for 5 hours before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. The cells were then pre-treated with GSK650394 (100 µM) for 20 minutes before incubating with TCM (50 µg/ml) for 10 minutes. The media was removed, and the cell

monolayer was harvested for analysis through western blot. The cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane for the detection of phospho-NDRG1 (Thr346).

Treatment of TCM-stimulated SGHEC-7 and SGHVSM-9 cells with GSK650394 (100 μM) resulted in a significant reduction in the expression in the phosphorylation of NDRG1 (Thr346) (Figure 4.14A, B SGHEC-7 - $P \leq 0.0001$, Figure 4.14C, D SGHVSM-9 -$P \leq 0.001$) indicating that this kinase is inhibited in this system.

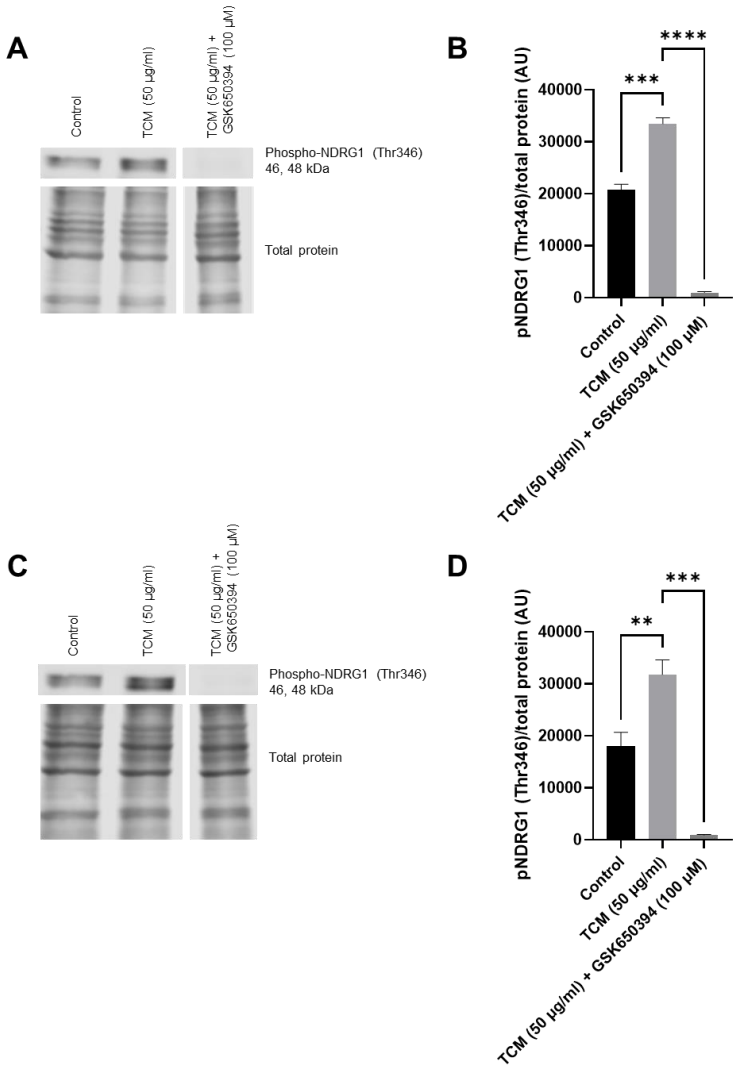



**Figure 4.14 GSK650394 treatment of TCM-stimulated SGHEC-7 and SGHVSM-9 cells reduced expression of phospho-NDRG1 (Thr346).**

*Expression of phospho-NDRG1 (Thr346) in (A) SGHEC-7 cells and (C) SGHVSM-9 cells following 20 minutes pre-treatment with GSK650394 (100 µM) and 10 minutes treatment with TCM (50 µg/ml) was determined by western blot. Quantification of western blot analysis for the detection of phospho-NDRG1 (Thr346) in (B) SGHEC-7 and (D) SGHVSM-9 cells (n = 3). Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM. Data were analysed with a one-way ANOVA (** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001).*

The effect of GSK650394 treatment was assessed on both unstimulated and TCM-treated cells. SGHEC-7 and SGHVSM-9 cells were cultured for 5 hours in complete media before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. Cells were then treated with 25 µM, 50 µM, and 100 µM GSK650394 in phenol red-free RPMI 1640 medium without FCS for 20 minutes. TCM (50 µg/ml) was added to cells where the effect of TCM-stimulation was assessed for 2 hours before removing the treatment media and replacing with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS. Following 24-hour incubation, the cell culture medium was removed and analysed by ELISA.

In unstimulated SGHEC-7 and SGHVSM-9 cells, GSK650394 treatment had no significant effect on STC-1 secretion at any concentration tested (Figure 4.15). In TCM-treated cells, however, treatment with 100 µM GSK650394 resulted in a significant increase in STC-1 secretion in both cell lines (Figure 4.15, $P \leq 0.0001$).

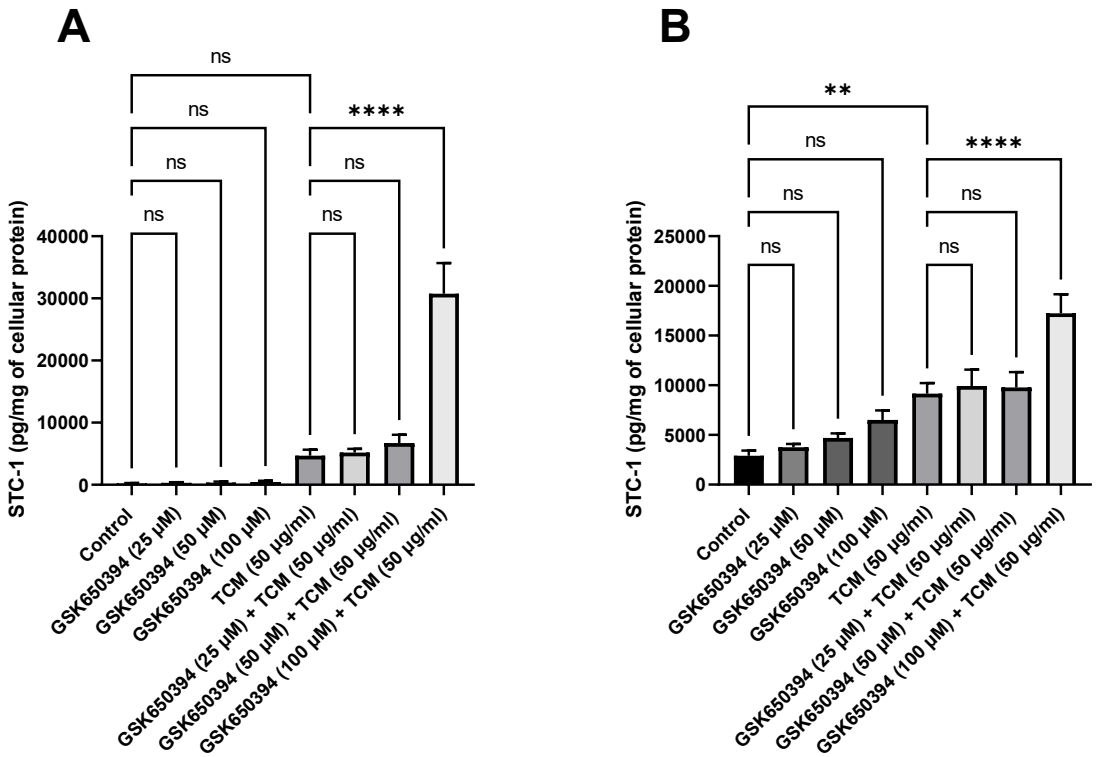



**Figure 4.15 The effect of inhibiting serum/glucocorticoid regulated kinase 1 (SGK1) on TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 24-hour period following 2 hours treatment with GSK650394 25 µM (SGHEC-7 – n = 3, SGHVSM-9 – n = 6), 50 µM (SGHEC-7 – n = 3, SGHVSM-9 – n = 6), and 100 µM (SGHEC-7 – n = 7, SGHVSM-9 – n = 10) +/- TCM (50 µg/ml) was determined by ELISA. Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, ** = P ≤ 0.01, **** = P ≤ 0.0001).*

### 4.1.11 Inhibition of glycogen synthase kinase 3β (GSK3β) in SGHEC-7 and SGHVSM-9 cells did not affect TCM-induced STC-1 secretion

The role of another serine/threonine kinase, GSK3β, in TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells was also investigated. GSK3β is a multifunctional kinase implicated in the Wnt/β-catenin pathway. When active, it enables the formation of a fully functional destruction complex for β-catenin to maintain low levels of this protein (Logan and Nusse, 2004) and previous experimentation demonstrated its activation in SGHVSM-9 cells following TCM treatment (section 4.1.7).

SGHEC-7 and SGHVSM-9 cells were cultured for 5 hours in complete media before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. Cells were then treated with three concentrations of the GSK3β inhibitor, Kenpaullone (Tocris Bioscience, Bristol, UK): 1 μM, 5 μM, and 10 μM, or the GSK3β inhibitor with enhanced selectivity, 1-Azakenpaullone (Cayman Chemical, Ann Arbor, Michigan, USA): 50 nM and 100 nM in phenol red-free RPMI 1640 medium without FCS for 20 minutes. TCM (50 μg/ml) was added to the cells for 2 hours before removing the treatment media and replacing with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS. Following 24-hour incubation, the cell culture medium was removed and analysed by ELISA.

Kenpaullone and 1-Azakenpaullone treatment of TCM-stimulated cells had no significant effect on STC-1 secretion at any concentration tested (Figure 4.16).

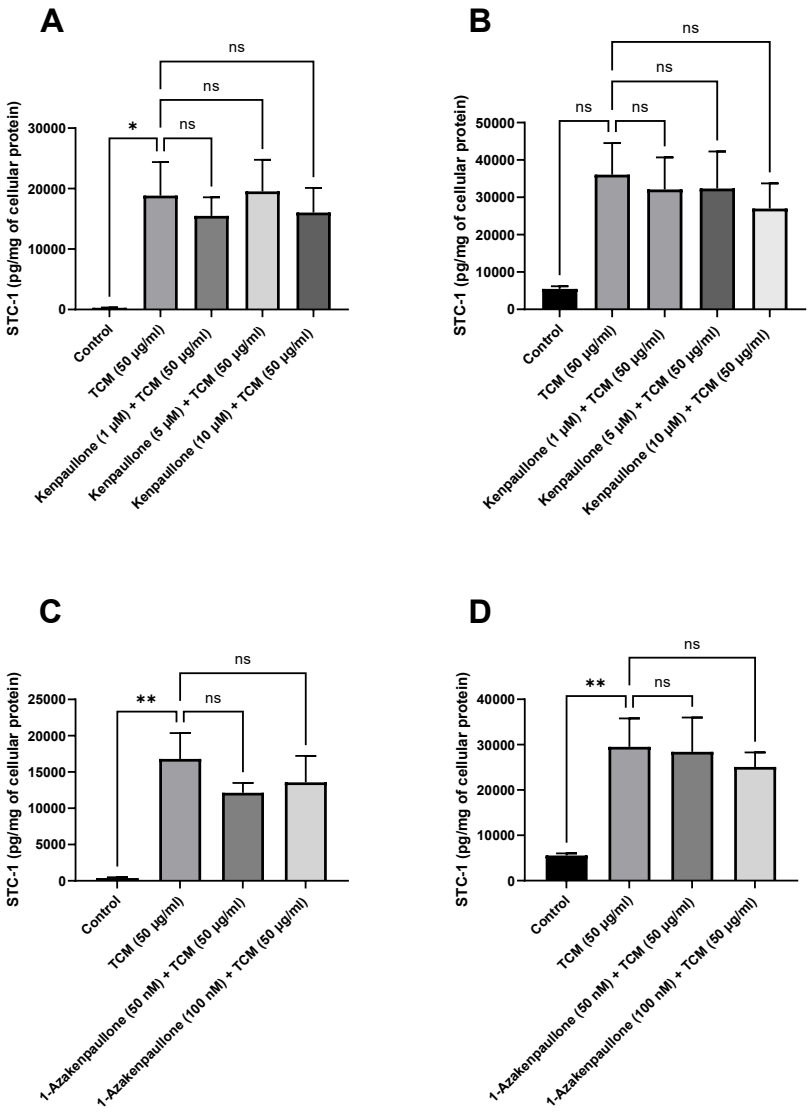



**Figure 4.16 The effect of inhibiting glycogen synthase kinase 3β (GSK3β) on TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 24-hour period following 2 hours treatment with Kenpaullone (1 µM, 5 µM, and 10 µM) with TCM (50 µg/ml) was determined by ELISA (n = 3). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05). Secretion of STC-1 from SGHEC-7 cells (C) and SGHVSM-9 cells (D) into the culture medium over a 24-hour period following 2 hours treatment with 1-Azakenpaullone 50 nM and 100 nM (n = 3) with TCM (50 µg/ml) (n = 5) was determined by ELISA. Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, ** = P ≤ 0.01).*

### 4.1.12 Inhibiting mitogen-activated protein kinases (MAPK) in SGHEC-7 and SGHVSM-9 cells did not affect TCM-induced STC-1 secretion

The role of p44/42-MAPK and p38-MAPK in the regulation of TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells was investigated. Previous experimentation demonstrated activation of p44/42-MAPK in SGHEC-7 and SGHVSM-9 cells following TCM treatment (section 4.1.7).

To determine the role of p44/42-MAPK, three concentrations of PD 98059 (Tocris Bioscience, Bristol, UK) were used to treat cells: 10 µM, 25µM, and 50 µM. PD 98059 is a highly selective inhibitor of MEK1 activation and the MAP kinase cascade (Di Paola *et al.*, 2010). The role of p38-MAPK was investigated by treating cells with three concentrations of SB203580 (Calbiochem®, San Diego, California, USA): 5 µM, 10 µM, and 25 µM. SB203580 is a selective competitive inhibitor of ATP binding to p38-MAPK (Shi *et al.*, 2015).

Prior to using PD 98059 and SB203580 to assess the effect of MAPK inhibition on STC-1 secretion, it was necessary to determine efficacy of these inhibitors in these cells. To achieve this, the effect of the highest inhibitor concentrations used in this study, PD 98059 50 µM and SB203580 25 µM, on the expression of p44/42-MAPK (Thr202/Tyr204) and pMAPKAPK-2 (Thr334), respectively, in SGHEC-7 and SGHVSM-9 cells was assessed. p38-MAPK is known to phosphorylate MAP kinase-activated protein kinase 2 (MAPKAPK-2) (Sreekanth *et al.*, 2016), therefore, a reduction in phosphorylation of this protein would indicate effective inhibition of p38-MAPK by SB203580.

Cells were seeded in 3.5 cm plates and cultured in complete media for 5 hours before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. The cells were then pre-treated with PD 98059 (50 µM) or SB203580 (25 µM) for 20 minutes before incubating with TCM (50 µg/ml) for 10 minutes. The media was

removed, and the cell monolayer was harvested for analysis through western blot. The cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane for the detection of p44/42-MAPK (Thr202/Tyr204) and pMAPKAPK-2 (Thr334).

Treatment of TCM-stimulated SGHEC-7 and SGHVSM-9 cells with PD 98059 (50 μM) resulted in a significant reduction in the expression of p44/42-MAPK (Thr202/Tyr204) (Figure 4.17A SGHEC-7 - $P \leq 0.0001$, Figure 4.17B SGHVSM-9 - $P \leq 0.01$) indicating that this kinase is inhibited in this system.

Treatment of TCM-stimulated SGHEC-7 and SGHVSM-9 cells with SB203580 (25 μM) resulted in a ~11-fold and ~4-fold reduction in the expression of pMAPKAPK-2 (Thr334), respectively, but this did not reach statistical significance (Figure 4.17C and D).

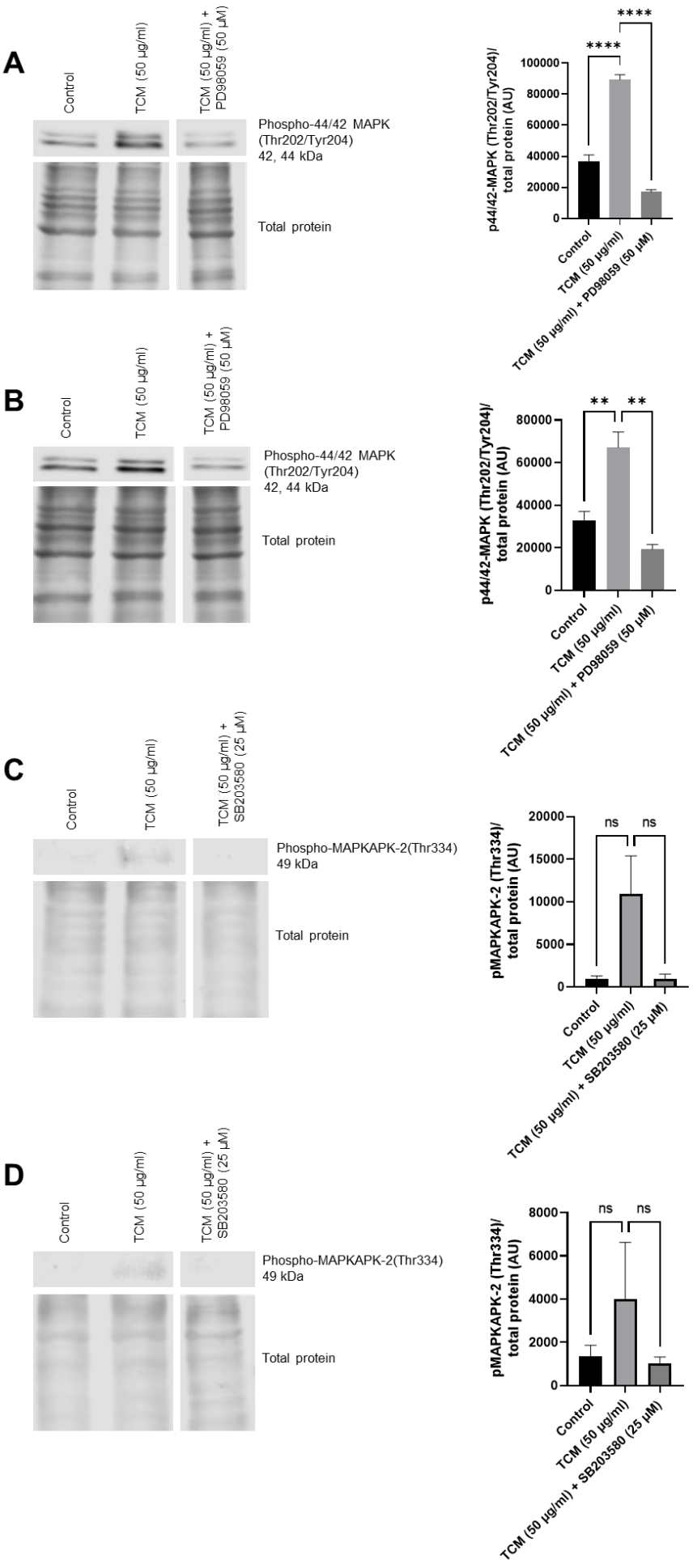

A
Control
TCM (50 µg/ml)
TCM (50 µg/ml) + PD98059 (50 µM)
Phospho-44/42 MAPK (Thr202/Tyr204) 42, 44 kDa
Total protein
p44/42-MAPK (Thr202/Tyr204)/ total protein (AU)
****
****
B
Control
TCM (50 µg/ml)
TCM (50 µg/ml) + PD98059 (50 µM)
Phospho-44/42 MAPK (Thr202/Tyr204) 42, 44 kDa
Total protein
p44/42-MAPK (Thr202/Tyr204)/ total protein (AU)
**
**
C
Control
TCM (50 µg/ml)
TCM (50 µg/ml) + SB203580 (25 µM)
Phospho-MAPKAPK-2(Thr334) 49 kDa
Total protein
pMAPKAPK-2 (Thr334)/ total protein (AU)
ns
ns
D
Control
TCM (50 µg/ml)
TCM (50 µg/ml) + SB203580 (25 µM)
Phospho-MAPKAPK-2(Thr334) 49 kDa
Total protein
pMAPKAPK-2 (Thr334)/ total protein (AU)
ns
ns

**Figure 4.17 PD 98059 and SB203580 treatment of TCM-stimulated SGHEC-7 and SGHVSM-9 cells reduced expression of p44/42-MAPK (Thr202/Tyr204) and pMAPKAPK-2 (Thr334), respectively.**

*Expression of p44/42-MAPK (Thr202/Tyr204) in (A) SGHEC-7 cells and (B) SGHVSM-9 cells following 20 minutes pre-treatment with PD 98059 (50 µM) and 10 minutes treatment with TCM (50 µg/ml) was determined by western blot (n = 3). Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM. Data were analysed with a one-way ANOVA (** = P ≤ 0.01, **** = P ≤ 0.0001). Expression of pMAPKAPK-2 (Thr334) in (C) SGHEC-7 cells and (D) SGHVSM-9 cells following 20 minutes pre-treatment with SB203580 (25 µM) and 10 minutes treatment with TCM (50 µg/ml) was determined by western blot (n = 3). Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant).*

The effect of p44/42-MAPK and p38-MAPK inhibition was assessed on TCM-treated cells. SGHEC-7 and SGHVSM-9 cells were cultured for 5 hours in complete media before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. Cells were then treated with 10 µM, 25µM, and 50 µM PD 98059 or 5 µM, 10 µM, and 25 µM SB203580 in phenol red-free RPMI 1640 medium without FCS for 20 minutes. TCM (50 µg/ml) was added to cells for 2 hours before removing the treatment media and replacing with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS. Following 24-hour incubation, the cell culture medium was removed and analysed by ELISA. Inhibition of p44/42-MAPK and p38-MAPK did not result in a significant effect on TCM-induced STC-1 secretion from either cell line at any of the inhibitor concentrations tested (Figure 4.18).

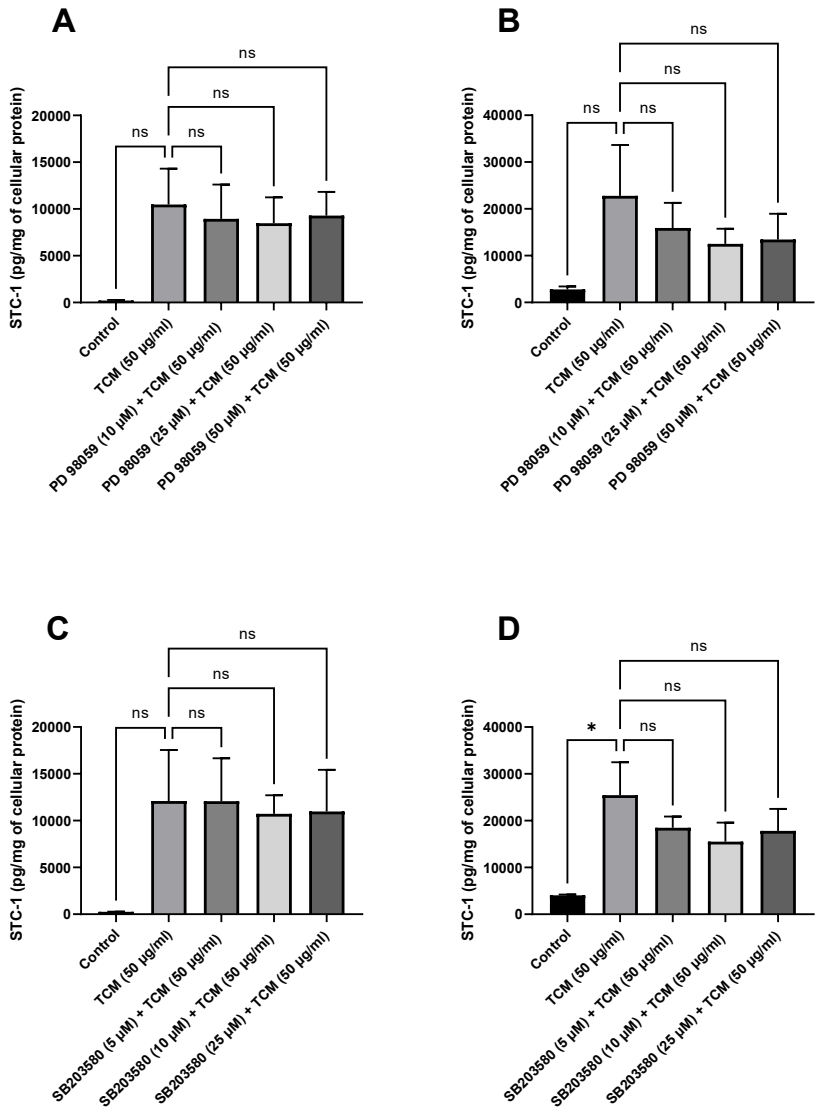



**Figure 4.18 The effect inhibiting mitogen-activated protein kinases (MAPK) on TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 24-hour period following 2 hours treatment with PD 98059 (10 µM, 25µM, and 50 µM) with TCM (50 µg/ml) was determined by ELISA (n = 3). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant). Secretion of STC-1 from SGHEC-7 cells (C) and SGHVSM-9 cells (D) into the culture medium over a 24-hour period following 2 hours treatment with SB203580 (5 µM, 10 µM, and 25 µM) with TCM (50 µg/ml) was determined by ELISA (n = 3). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05).*

### 4.1.13 Inhibition of signal transducer and activator of transcription 3 (STAT3) in SGHEC-7 and SGHVSM-9 cells did not affect TCM-induced STC-1 secretion

The effect of STAT3 inhibition on TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells was assessed. STAT3 is a transcription factor which is known to be activated by a number of factors including IL-6, IL-10, IL-11, IL-15, IL-22, leukaemia inhibitory factor (LIF), HGF, leptin, and growth hormone (GH) (Mizoguchi, 2012), all of which have been shown to be present in TCM (section 4.1.4). It was hypothesised that activation of STAT3 by trophoblast-secreted factors may be implicated in the regulation of STC-1 expression in vascular cells.

To determine the role of STAT3 in regulation of STC-1 secretion, SGHEC-7 and SGHVSM-9 cells were treated with three concentrations of 5,15-diphenylporphyrin (5,15-DPP) (Calbiochem®, San Diego, California, USA): 1 μM, 5 μM, and 10 μM. 5,15-DPP is a selective inhibitor of STAT3 which prevents dimerization through interaction with the Src homology 2 domain, reducing nuclear translocation and DNA binding (Uehara *et al.*, 2009). Prior to using 5,15-DPP to assess the effect of STAT3 inhibition on STC-1 secretion, it was necessary to determine its efficacy in inhibiting the target pathway in these cells. To achieve this, the effect of the highest concentration of this inhibitor used in this study (10 μM) on the expression of phospho-STAT3 (Tyr705) in SGHEC-7 and SGHVSM-9 cells was assessed. Phosphorylation of STAT3 on Tyr705 promotes dimerisation, nuclear translocation, and DNA binding and thus is an indicator of STAT3 activity (Guadagnin *et al.*, 2015). A reduction in phosphorylation of STAT3 at this site following 5,15-DPP treatment would therefore indicate effective inhibition of STAT3.

Cells were seeded in 3.5 cm plates and cultured in complete media for 5 hours before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. The cells were then pre-treated with 5,15-DPP (10 μM) for 20 minutes before incubating with TCM (50 μg/ml) for 10 minutes. The media was removed, and the cell monolayer was harvested for analysis through western blot. The cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane for the detection of phospho-STAT3 (Tyr705).

Treatment of TCM-stimulated SGHEC-7 cells with 5,15-DPP (10 μM) resulted in a significant reduction in expression of phospho-STAT3 (Tyr705) (Figure 4.19A, B $P \leq 0.01$). Expression of phospho-STAT3 (Tyr705) remained unchanged, however, in TCM-stimulated SGHVSM-9 cells following 5,15-DPP (10 μM) treatment (Figure 4.19C, D).

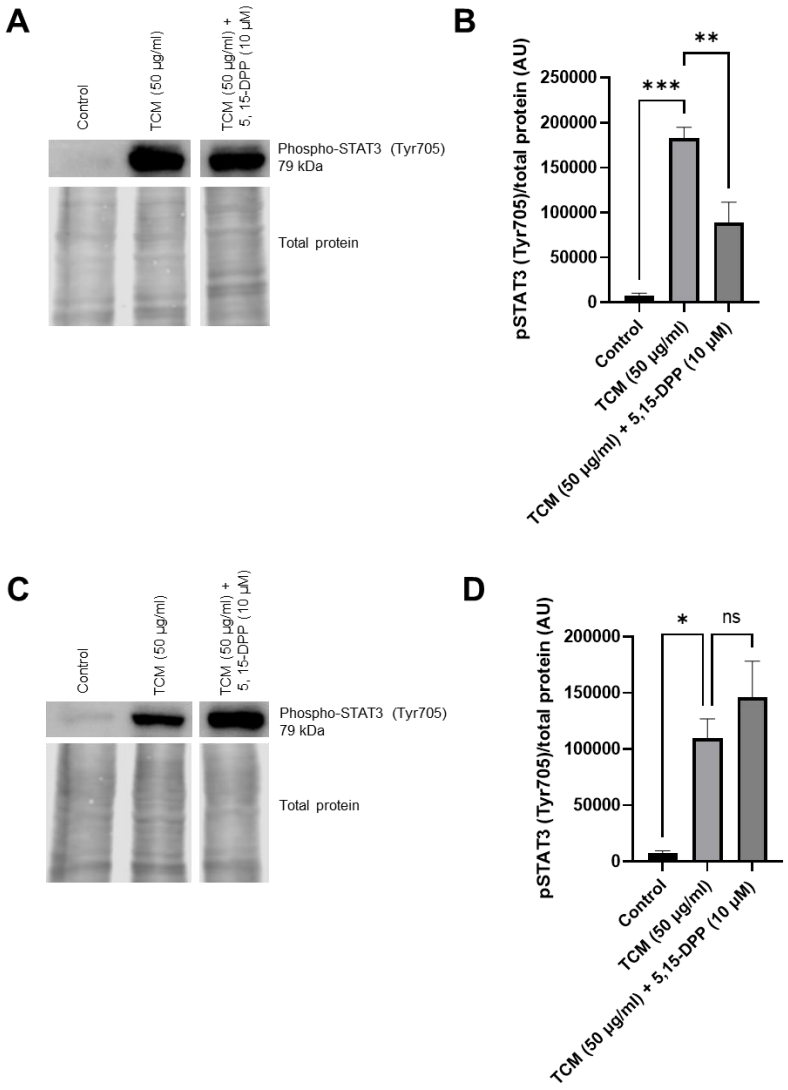



**Figure 4.19 The effect 5,15-DPP treatment of TCM-stimulated SGHEC-7 and SGHVSM-9 cells on the expression of phospho-STAT3 (Tyr705).**

*Expression of phospho-STAT3 (Tyr705) in (A) SGHEC-7 cells and (C) SGHVSM-9 cells following 20 minutes pre-treatment with 5,15-DPP (10 µM) and 10 minutes treatment with TCM (50 µg/ml) was determined by western blot. Quantification of western blot analysis for the detection of phospho-STAT3 (Tyr705) in (B) SGHEC-7 and (D) SGHVSM-9 cells (n = 3). Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).*

The effect of STAT3 inhibition was assessed on TCM-treated cells. SGHEC-7 and SGHVSM-9 cells were cultured for 5 hours in complete media before changing the media to phenol red-free RPMI 1640 medium without FCS and incubating overnight. Cells were then treated with 1 μM, 5 μM, and 10 μM 5,15-DPP in phenol red-free RPMI 1640 medium without FCS for 20 minutes. TCM (50 μg/ml) was added to cells for 2 hours before removing the treatment media and replacing with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS. Following 24-hour incubation, the cell culture medium was removed and analysed by ELISA. Treatment of SGHEC-7 and SGHVSM-9 cells with 5,15-DPP did not result in a significant change in TCM-induced STC-1 secretion at any of the inhibitor concentrations tested (Figure 4.20).

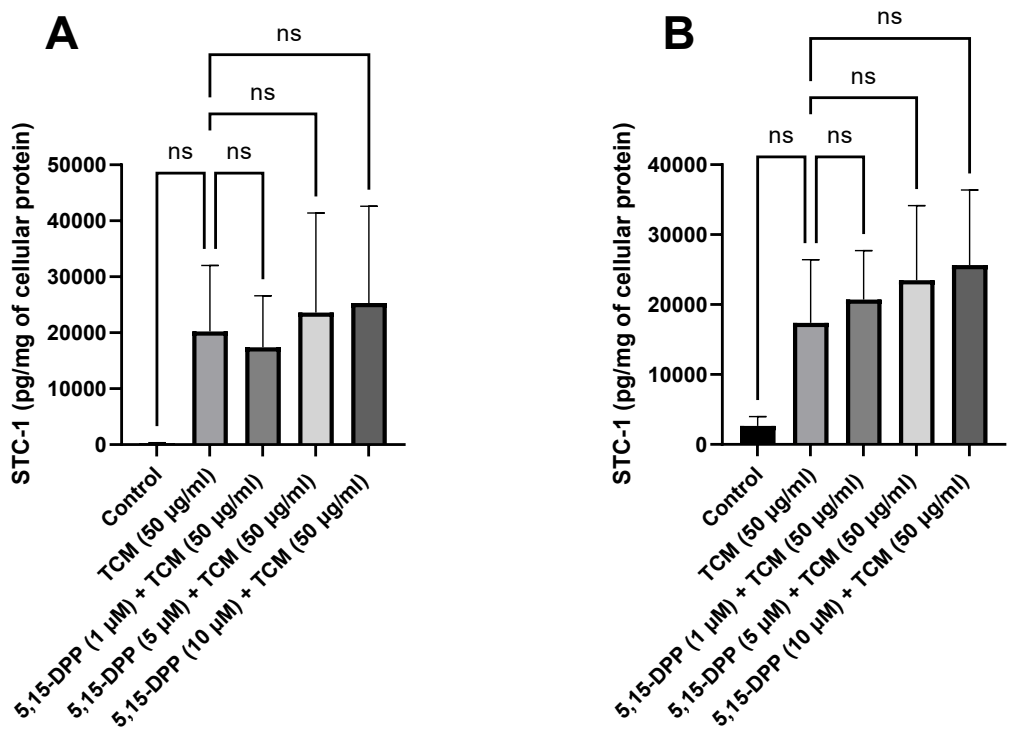



**Figure 4.20 The effect of inhibiting signal transducer and activator of transcription 3 (STAT3) on TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 24-hour period following 2 hours treatment with 5,15-diphenylporphyrin (5,15-DPP) (1 µM, 5 µM, and 10 µM) with TCM (50 µg/ml) was determined by ELISA (n = 3). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant).*

### 4.1.14 Cyclic adenosine monophosphate (cAMP) stimulation and inhibition of protein kinase A (PKA) had no effect on STC-1 secretion from SGHEC-7 and SGHVSM-9 cells

The cAMP/PKA pathway has been shown to regulate the expression of STC-1 in rat and bovine thecal interstitial cells (Paciga *et al.*, 2002; Paciga, DiMattia and Wagner, 2004), human endometrial stromal cells, (Aghajanova *et al.*, 2016; Khatun *et al.*, 2020), and human trophoblast cells under conditions of hypoxia (Abid *et al.*, 2020). For this reason, it was decided to investigate the effect of cAMP stimulation on STC-1 expression in vascular cells.

SGHVSM-9 cells were treated with three concentrations of the Adenosine-3', 5'-cyclic monophosphate (cAMP) analogue, 8-Bromo adenosine-3', 5'-cyclic monophosphate cAMP (8 Br-cAMP) (Biolog Life Science Institute, Bremen, Germany): 25 µM, 50 µM, and 100 µM. SGHEC-7 cells were treated with only 100 µM 8 Br-cAMP. None of the concentrations of 8 Br-cAMP tested had any significant effect on STC-1 secretion in either cell line (Figure 4.21A and B).

The role of the downstream target of cAMP, PKA, in the regulation of STC-1 secretion in SGHVSM-9 cells was also investigated. The PKA inhibitor N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinoline sulfonamide (H89) (Tocris Bioscience, Bristol, UK) was used at a concentration of 20 µM to investigate the effect of PKA inhibition on TCM-stimulated SGHVSM-9 cells. Treatment of TCM-stimulated cells with H89 had no significant effect on STC-1 secretion (Figure 4.21C).

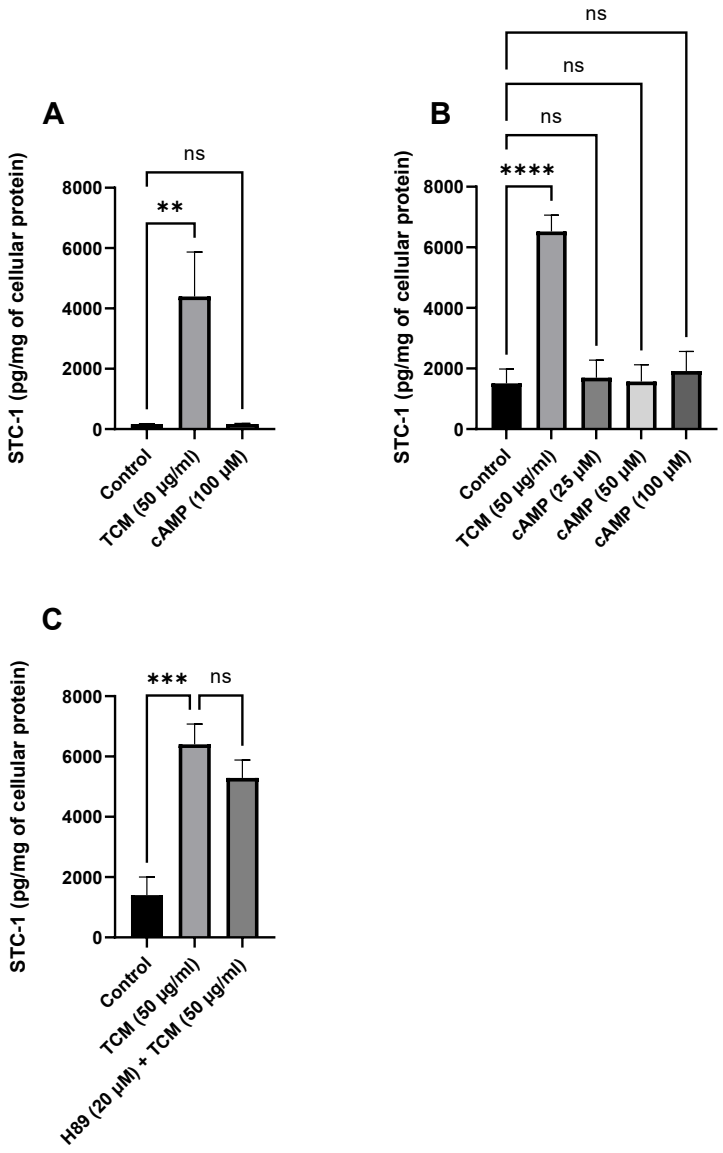



**Figure 4.21 The effect of cyclic adenosine monophosphate (cAMP) stimulation and protein kinase A (PKA) inhibition on STC-1 secretion from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 24-hour period following 2 hours treatment with 8 Br-cAMP (SGHEC-7 - 100 µM, SGHVSM-9 - 25 µM, 50 µM, and 100 µM) or TCM (50 µg/ml) was determined by ELISA (n = 5). Secretion of STC-1 from SGHVSM-9 cells (C) into the culture medium over a 24-hour period following 2 hours treatment with H89 (20 µM) and TCM (50 µg/ml) was determined by ELISA (n = 4). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, ** = P ≤ 0.01, *** = P ≤ 0.001, ****, P ≤ 0.0001).*

### 4.1.15 Activation of protein kinase C (PKC) induces secretion of STC-1 from SGHEC-7 and SGHVSM-9 cells, but TCM-induced secretion of STC-1 is not regulated through PKC

The role of PKC activation in the regulation of STC-1 secretion from vascular cells was assessed. PMA (Sigma-Aldrich, Dorset, UK) is a phorbol ester commonly used to activate PKC isoforms belonging to the conventional (PKC-α, -β1, -β2, and -γ) and novel (PKC-δ, -ε, -θ, and -η) PKC families which both require diacylglycerol (DAG) activation (Geraldes and King, 2010; Neeli and Radic, 2013). PMA elicits its effect on PKC activation through penetrating the cell membrane and mimicking DAG (Neeli and Radic, 2013).

Treatment of SGHEC-7 and SGHVSM-9 cells with PMA (20 nM) led to a significant increase in STC-1 secretion from both cell lines, far greater than that induced by TCM-treatment (Figure 4.22, $P \leq 0.0001$). PMA-induced STC-1 secretion was significantly reduced when the cells were treated with a broad-spectrum PKC inhibitor, Go 6983 (1 μM) (inhibits PKCα, PKCβ, PKCγ, PKCδ and PKCζ) (Sigma-Aldrich, Dorset, UK), in combination with PMA (Figure 4.22A - $P \leq 0.001$, Figure 4.22B - $P \leq 0.05$). Conversely, TCM-induced STC-1 secretion was not significantly reduced following broad-spectrum PKC inhibition in either cell line (Figure 4.22).

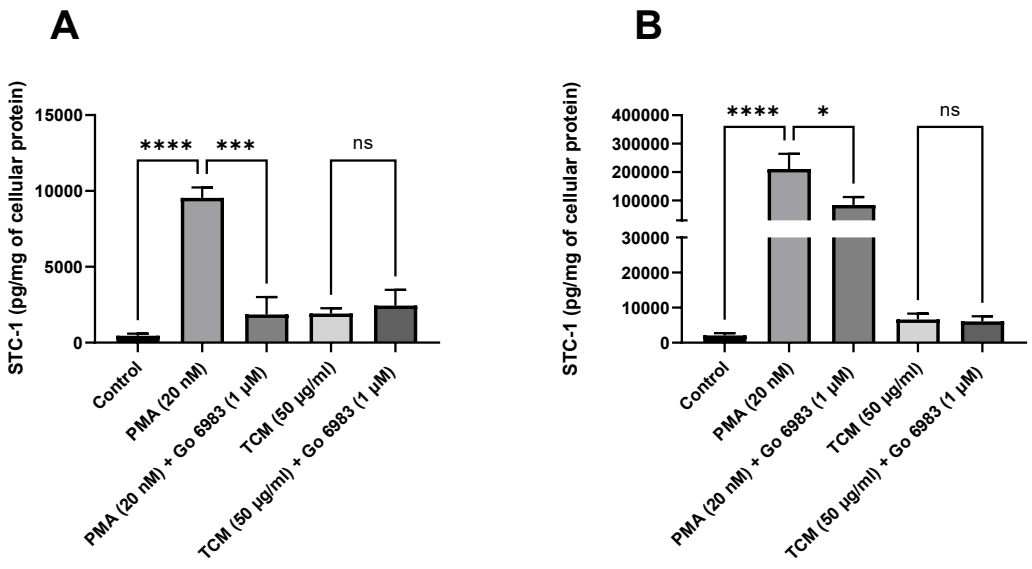



**Figure 4.22 The effect of protein kinase C (PKC) activation and broad-spectrum PKC inhibition on STC-1 secretion from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 (A) and SGHVSM-9 (B) into the culture medium over a 24-hour period following 2 hours treatment with PMA (20 nM) or TCM (50 µg/ml) +/- Go 6983 (1 µM) was determined by ELISA (SGHEC-7 – n = 3, SGHVSM-9 = n = 5). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, * = P ≤ 0.05, *** = P ≤ 0.001, **** = P ≤ 0.0001).*

### 4.1.16 Inhibition of PKC epsilon in SGHEC-7 and SGHVSM-9 cells did not affect TCM-induced STC-1 secretion

Go 6983 does not inhibit the PKC isoform epsilon (PKCε) which is activated by PMA, therefore, the role of this isoform was assessed independently. Prior to assessing the role of PKCε in TCM-induced STC-1 secretion, it was first established that PKCε is expressed in both SGHEC-7 and SGHVSM-9 cells. Both cell lines were grown under normal culture conditions in complete media prior to trypsinisation and centrifugation. The resulting cell pellet was washed twice in PBS and frozen at -20°C. The cell pellet was lysed as described previously for analysis through western blot. The cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane. These membranes were probed for the detection of PKCε which was found to be expressed by both cell lines (Figure 4.23).

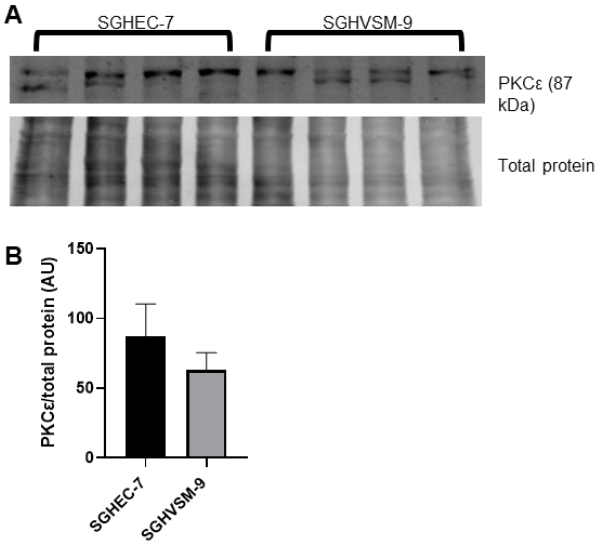



**Figure 4.23 PKCε is expressed by both vascular cell lines.**

*(A) PKCε expression determined by western blot analysis of SGHEC-7 and SGHVSM-9 cells for PKCε (87 kDa – top band) and total protein staining as a loading control. (B) Quantification of western blot analysis for the detection of PKCε. Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM.*

A specific peptide inhibitor of PKC epsilon (Santa Cruz Biotechnology, TX, USA) was then used to assess the effect of this isoform on TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells. This PKCε inhibitor peptide is an octapeptide that selectively and reversibly inhibits the translocation of PKCε. It does not contain any sequence to facilitate cellular uptake, therefore passage across the plasma membrane may be limited. For this reason, two methods were tested: 1) transfection of cells with the PKCε inhibitor peptide using the Lonza Nucleofector® system, 2) adding the PKCε inhibitor peptide directly to the cell treatment media. Both methods have previously been shown to induce an effect in other systems (Gayen *et al.*, 2009; Kuan *et al.*, 2014; Li *et al.*, 2015; Benoist *et al.*, 2019). For the transfection method, 1 µM PKCε inhibitor peptide was transfected into SGHEC-7 and SGHVSM-9 cells and the cells were incubated for 4 hours before treating with TCM for 2 hours. This concentration of peptide was used for transfection as it was previously reported to induce an effect when introduced to cells via this method (Li *et al.*, 2015). After 2 hours incubation, the treatment media was removed and replaced with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS. The cell culture media was harvested after 24 hours for analysis by ELISA. Transfection of SGHEC-7 and SGHVSM-9 cells with the PKCε inhibitor peptide had no significant effect on TCM-induced STC-1 secretion (Figure 4.24A and B).

In the second method, the PKCε inhibitor peptide was added directly to the cell treatment media for 20 minutes prior to 2-hour stimulation with TCM. For this method, a range of concentrations of the PKCε inhibitor peptide was used: 5 µM, 10 µM, and 20 µM which was based on previous reports using this peptide for the treatment of cell lines using this method (Kuan *et al.*, 2014; Benoist *et al.*, 2019; Jang, Lee and Park, 2020; Owari *et al.*, 2020). After 2 hours incubation, the treatment media was removed and replaced with phenol red-free RPMI 1640 medium containing 5% (v/v) FCS. The cell culture media was

harvested after 24 hours for analysis by ELISA. Treatment of SGHEC-7 and SGHVSM-9 cells with the PKCε inhibitor peptide in this way also did not result in any significant effect on TCM-induced STC-1 secretion at any of the concentrations tested (Figure 4.24C and D).

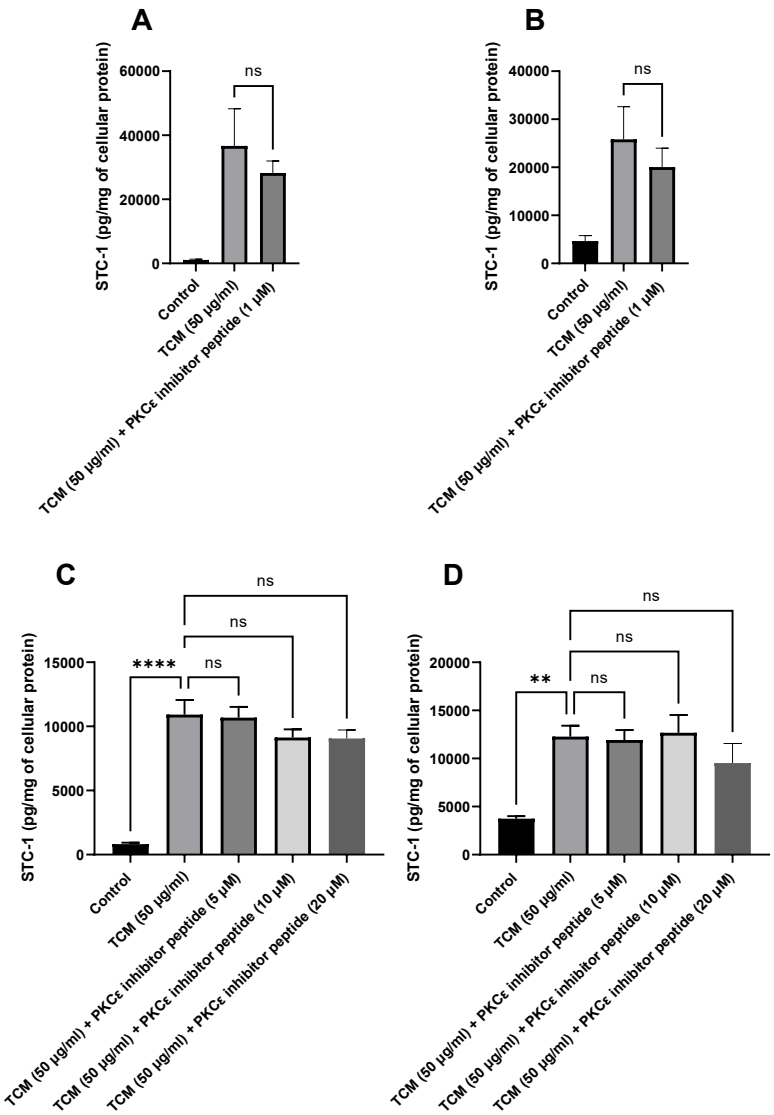



**Figure 4.24 The effect of inhibition of PKCε on TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) transfected with PKCε inhibitor peptide (1µM) and treated with TCM (50 µg/ml) for two hours was detected by ELISA (n = 3). Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (ns = not significant). Secretion of STC-1 from SGHEC-7 cells (C) and SGHVSM-9 cells (D) into the culture medium over a 24-hour period following 2 hours treatment with PKCε inhibitor peptide (5 µM, 10 µM, and 20 µM) and TCM (50 µg/ml) was determined by ELISA (n = 3). Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, ** = P ≤ 0.01, **** = P ≤ 0.0001).*

## 4.2 Discussion

Previous work has shown that the presence of trophoblast cells *in vivo* results in increased expression of STC-1 protein in ECs of the first trimester decidua (Figure 3.1) and that trophoblast-secreted factors upregulate STC-1 gene expression in an *in vitro* three-dimensional vascular cell model (Wallace *et al.*, 2013). However, the effect of trophoblast-secreted factors on intracellular and secreted STC-1 protein expression from vascular cells had not been investigated. A key aim of this study was, therefore, to understand the effect of trophoblast-secreted factors on both intracellular and secreted expression of STC-1 protein from SGHEC-7 and SGHVSM-9 cells and characterise the profile of factors secreted by trophoblast cells which may induce this effect. It was also important to elucidate the mechanisms underlying regulation of trophoblast-induced effects on STC-1 protein expression.

STC-1 protein is known to be retained by the cell as well as being secreted from these cell lines, therefore, the effect of trophoblast-secreted factors on both intracellular and secreted protein was investigated. TCM was generated from the EVT cell line SGHPL-4 and contains all the factors secreted by these cells. The results from this study showed that TCM treatment of SGHEC-7 and SGHVSM-9 cells resulted in a significant increase in STC-1 secretion from both cell lines (Figure 4.2). The effect of TCM on intracellular STC-1 expression was not consistent with its effect on the secreted protein as TCM treatment of both SGHEC-7 and SGHVSM-9 cells had no effect on intracellular protein levels (Figure 4.3).

It is clear from this data and previous findings that, following stimulation of vascular cells with TCM, levels of both STC-1 mRNA (Wallace *et al.*, 2013) and secreted protein increase, whilst intracellular protein levels remain constant. From this, the following hypothesis can be proposed. Vascular cells maintain steady intracellular stores of STC-1, which is supported

by immunocytochemical findings in this study that showed significant cytoplasmic expression of STC-1 (Figure 3.4), as well as western blot analysis that showed consistent intracellular STC-1 expression in both cell lines (Figure 3.5). Upon stimulation, STC-1 gene expression is increased, and this is translated into protein. To explain why there is an observable increase in protein secretion and not intracellular expression at this stage, it could be postulated that upon stimulation, pre-formed STC-1 is released from the cells, and the intracellular stores are replenished following translation of new mRNA (Figure 4.25). Although the pathway by which STC-1 is secreted from cells has not yet been characterised, these results suggest that secretion of STC-1 from these cells is mediated through the regulatory secretory pathway as newly synthesised STC-1 appears to be stored within the cell until the stimulus is received.

To investigate this, protein synthesis and protein transport were inhibited in TCM-treated cells using CHX and BFA, respectively, to determine whether TCM-induced STC-1 secretion is dependent on prior protein synthesis. In SGHVSM-9 cells, protein transport inhibition with BFA resulted in a significant decrease in STC-1 secretion, whilst CHX treatment had no effect. In SGHEC-7 cells, no change in secretion was observed following treatment with CHX or BFA (Figure 4.4).

This data aligns with the hypothesis proposed above. It is likely that TCM stimulation of SGHVSM-9 induces secretion of pre-formed STC-1 mediated through the regulatory secretory pathway as this is significantly reduced following treatment with BFA. In contrast, the same result was not observed following treatment of SGHEC-7 cells with BFA (Figure 4.4). It may be possible that there are differences in the way that STC-1 is stored in these two cell lines. SGHEC-7 cells may have greater stores of STC-1 in the Golgi complex compared to the ER, thus, blocking ER to Golgi protein transport with BFA would have no

effect on secretion. It has previously been shown that BFA is more effective at blocking secretion of certain cytokines such as TNFα compared to other protein transport inhibitors such as monensin which blocks trans-Golgi protein transport (O'Neil-Andersen and Lawrence, 2002). Therefore, it is plausible that a protein transport inhibitor such as monensin may be required to block secretion of STC-1 from SGHEC-7 cells. The effect of BFA or monensin treatment on expression of STC-1 has not previously been reported, therefore, it is difficult to ascertain whether STC-1 may be differentially stored amongst cell types, and this should be addressed in future studies.

Following this hypothesis, it was thought that CHX treatment of the vascular cells would likely result in a reduction in STC-1 protein expression as it is known that TCM-treatment of these cells increases STC-1 mRNA (Wallace *et al.*, 2013), thus inhibiting translation of this mRNA would reduce protein levels. In this study, the effect of CHX on STC-1 secretion was used as an end point and no change in secretion was observed in either cell line (Figure 4.4). It is possible that a change in STC-1 secretion would not immediately be observed following CHX treatment as these cells likely contain large stores of pre-formed STC-1, so secretion would not be affected until these stores were sufficiently depleted. Long-term treatment with CHX may, therefore, result in a reduction in STC-1 secretion, but it would be difficult to assess this with minimal cell toxicity. Assessment of intracellular protein expression by western blot analysis following CHX treatment may be an effective approach to demonstrate the effect of CHX on intracellular stores of STC-1. It should also be noted that reduced doses of CHX and BFA were used to treat SGHEC-7 cells compared to SGHVSM-9 cells (10-fold lower dose of CHX and 2-fold lower dose of BFA) to reduce toxicity in this cell line. It is possible that, although these doses avoided excess cell death, they may not have been sufficient to exert an effect on these cells. Future work would benefit from

treating SGHEC-7 cells with the same doses of CHX and BFA used in this study followed by assessment of expression of proteins previously shown to be affected by these inhibitors. This would help to confirm whether the doses used in this study are sufficient to exert an effect on protein synthesis and transport. The effect of cellular stimulation on the *de novo* synthesis of STC-1 using CHX has been described previously. In trichostatin A-treated colon adenocarcinoma cells, CHX treatment did not affect STC-1 mRNA expression, however, in dexamethasone-treated rat Sertoli cells and triiodothyronine-treated skin fibroblasts, CHX treatment significantly reduced STC-1 mRNA expression, suggesting that stimulated STC-1 expression is dependent on *de novo* protein synthesis (Law *et al.*, 2008; Li and Wong, 2008; Moeller *et al.*, 2011). It appears from these findings that the mechanisms underlying STC-1 protein expression vary between cell types and the specific stimulus.

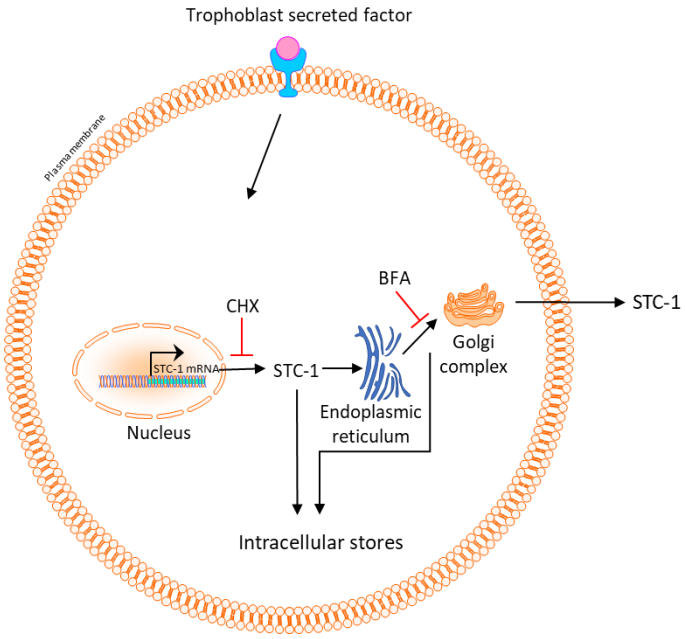



**Figure 4.25 A schematic diagram illustrating the potential model of STC-1 protein synthesis, transport, and storage in vascular cells following TCM stimulation.**

*CHX, cycloheximide; BFA, brefeldin A; STC-1, stanniocalcin-1. Figure was prepared using the Motifolio drawing toolkit.*

A wide array of factors are secreted by the human placenta, and these are thought to contribute to the maternal metabolic and cardiovascular adaptation to pregnancy (Rosario *et al.*, 2021). For example, secreted placental lactogen has been shown to promote β-cell proliferation and increase glucose-stimulated insulin secretion (Brelje, Parsons and Sorenson, 1994; Sorenson and Brelje, 1997; Kim *et al.*, 2010; Baeyens *et al.*, 2016), and placental growth hormone induces skeletal muscle insulin resistance in the mother (Barbour *et al.*, 2004). The secretome of primary human syncytiotrophoblast cells has previously been characterised using Stable Isotope Labelling with Amino Acids in Cell Culture (SILAC) followed by mass spectrometry. This identified over 1000 proteins in the secretome, 50% of which are associated with extracellular vesicles (Rosario *et al.*, 2021). Trophoblast-derived extracellular vesicles including exosomes, microvesicles, and apoptotic bodies have previously been shown to play a key role in placental orchestration of pregnancy and maternal immune sensing of the fetus (Stefanski *et al.*, 2019). Alongside extracellular vesicles, a large proportion of the secreted proteins were associated with the cytoplasm, nucleus, and lysosome (Rosario *et al.*, 2021). Gene ontology analysis of the trophoblast secretome revealed that many of these proteins are associated with catalytic and transporter activity and were linked to processes such as metabolism, energy pathways, and cell communication. Furthermore, pathway analysis demonstrated that the trophoblast secretome was enriched for cardiovascular system development and function, consistent with the hypothesis that trophoblast secreted proteins are involved in maternal cardiovascular adaptation to pregnancy (Rosario *et al.*, 2021).

To date, the complete profile of factors secreted by human EVT cells has not been characterised, however, it has been previously shown that factors secreted by EVT cells act on vascular cells in the local environment to influence expression of a number of genes,

including STC-1 (Wallace *et al.*, 2013) This study aimed to characterise the profile of factors secreted by EVTs cells through the use of a cytokine array. This showed that EVT cells secrete all 105 cytokines present on the array (section 4.1.4), including a number of major pro-inflammatory cytokines (IL-6, IL-8, MCP-1, IP-10, RANTES, ICAM-1, and VCAM-1) and anti-inflammatory cytokines (IL-1RA, IL-4, IL-10, IL-11, and IL-13) (Zhang and An, 2007) as well as a number of factors shown to play important roles in EC biology and at the maternal-fetal interface such as VEGF, TGF-β, HGF, and IL1-β (Librach *et al.*, 1994; Tse, Whitley and Cartwright, 2002; Li, Zhao and Ren, 2016). It is noteworthy that these arrays are only semi-quantitative, and the pixel density of the spots will partially depend on the affinity of the antibodies on the array. Future studies would benefit from the use of quantitative assays such as a Luminex® Assay (R&D Systems, MN, USA) which would allow for the relative levels of different cytokines to be determined. In addition, more comprehensive analysis of the EVT secretome using a method such as SILAC followed by mass spectrometry as used previously to characterise the human syncytiotrophoblast secretome (Rosario *et al.*, 2021), would help to elucidate the full profile of factors present in the secretome, and identify those which may regulate maternal physiology and fetal development.

Assessing the effect of each cytokine identified on the array individually on STC-1 secretion from vascular cells was beyond the scope of this study, especially as a range of concentrations of each cytokine would be required to fully determine their effects on STC-1 secretion. For this reason, cytokines and growth factors previously shown to play roles in EC biology and at the maternal-fetal interface were selected to determine their effects individually on STC-1 secretion in SGHEC-7 cells. Treatment of SGHEC-7 cells with these cytokines and growth factors did not result in any significant effect on STC-1 secretion at

any of the concentrations tested (section 4.1.4). The effects of various cytokines on STC-1 secretion by ECs has previously been assessed (Zlot *et al.*, 2003). These included six of the cytokines tested in this study: VEGF-A, HGF, TGF-β, IFNγ, FGF-2, and TNFα. Consistent with the results obtained herein, previous work treating HUVECs with VEGF-A, TGF-β, and IFNγ at concentrations equal to or greater than those used in the present study did not induce a significant increase in STC-1 secretion. Similarly, TNFα, used at a concentration three-times lower than that used in this study, did not induce STC-1 secretion (Zlot *et al.*, 2003). It is of note that the results obtained previously on the effect of VEGF-A on expression of STC-1 from ECs are conflicting. The study by Zlot *et al.* reported no change in secretion of STC-1 from HUVECs following treatment with 400 ng/ml VEGF-A after 24-72 hours (Zlot *et al.*, 2003), consistent with the results obtained herein following 24-hour stimulation of SGHEC-7 cells with 100 ng/ml and 200 ng/ml VEGF-A. In contrast, a study by Holmes *et al.* reported a significant increase in STC-1 secretion from HUVECs following 24-hour stimulation with 25 ng/ml VEGF-A (Holmes and Zachary, 2008). It is possible that this discrepancy in results can be explained by differences in methodology for the detection of secreted STC-1. The present study and that by Zlot *et al.* used ELISA for the detection of secreted STC-1, however the study by Holmes *et al.* used western blot analysis on concentrated cell culture media. It is possible that the sensitivity of the ELISA assays used was not sufficient to detect the increase in VEGF-stimulated STC-1 and that this could only be detected following concentration of the culture media. It is evident that role of VEGF in the expression of STC-1 from vascular cells is not clear and further study with a wider range of concentrations and time points for stimulation would be required to clarify its role.

In contrast to the results from the present study, Zlot *et al.* also demonstrated that treatment of HUVECs with HGF and FGF-2 resulted in a significant increase in STC-1

secretion (Zlot *et al.*, 2003). In this study, however, much higher concentrations of both HGF and FGF-2 (400 ng/ml) were used compared to those used herein (10 ng/ml, 100 ng/ml and 50 ng/ml, 100 ng/ml, respectively). The time period for stimulation also varied between these studies. In the referenced study, an increase in STC-1 secretion was only detectable following 72 hours cytokine stimulation whilst no change was detected after 24 hours stimulation (Zlot *et al.*, 2003), the time point used in the present study. It is possible that a longer period of stimulation and higher concentration of growth factors would have been required to induce secretion of STC- 1 from SGHEC-7 cells. It should also be considered that EVT-secreted factors likely work synergistically in the local environment to induce an effect on STC-1 secretion thus the action of each factor individually would not be sufficient to exert an effect. This is evidenced by a previous finding which demonstrated that the stimulatory effect of VEGF-A on STC-1 secretion from HUVECs was synergistically enhanced by co-treatment with FGF-2 (Holmes and Zachary, 2008). Future work to elucidate the EVT-secreted factors which induce STC-1 secretion would benefit from co-treatment with a range of growth factors and cytokines.

As the initial screen of several factors present in TCM did not aid identification of the factors involved in mediating STC-1 secretion, a further screening approach was taken to identify activation of cell surface RTKs following TCM stimulation. Identifying activated RTKs using this approach was a time and cost-effective way to narrow down which factors in TCM may elicit a response in the vascular cells. The results from the Phospho-RTK Array revealed a weak signal for phosphorylation of EGFR in SGHVSM-9 cells following 5 minutes TCM treatment (Figure 4.5). The minimal response observed with this array was unexpected. It is possible that shorter time points for TCM stimulation of the vascular cells, such as 30 seconds to 1 minute, would have been required for activation of all of the RTKs

on the array as their activation is known to be time sensitive and will vary for each RTK. Due to the high cost of the array kit, testing multiple time points was not within the scope of this study. Future work would also benefit from cellular stimulation with a positive control to confirm the efficacy of the array. For example, treatment of cells with recombinant epidermal growth factor (EGF) or HGF should result in phosphorylation of EGFR and hepatocyte growth factor receptor (HGFR) which would be visible on the array. The results obtained using the array kit will also be limited by the affinity of the antibodies, therefore, activation of EGFR was confirmed by western blot analysis for the detection of pEGFR. Expression of pEGFR was significantly increased in SGHVSM-9 cells following 10 minutes TCM treatment (Figure 4.6). TCM treatment of SGHEC-7 cells did not result in phosphorylation of any RTK on the array after either 5- or 15-minutes stimulation with TCM and no change in pEGFR expression following TCM-treatment was detectable by western blot analysis (Figure 4.5, 4.6).

After establishing that TCM treatment activates EGFR in SGHVSM-9 cells, a broad-spectrum RTK inhibitor, SU11248 which inhibits several RTKs including EGFR, was used to assess the effect of inhibition of cell signalling by a number of RTKs on STC-1 secretion from both vascular cell lines (Figure 4.7). Although the Phospho-RTK array did not demonstrate activation of any RTKs in TCM-treated SGHEC-7 cells, the effect of broad-spectrum RTK inhibition was still assessed in this cell line. This approach was taken because the Phospho-RTK array is known to have technical limitations such as the aforementioned variations in antibody affinity as well as the difficulty in selecting a time point for TCM stimulation in which would enable activation of all of the RTKs present on the array. Pharmacological inhibition using SU11248 therefore enabled an additional approach to study the involvement of RTKs in this cell line. RTK inhibition in this way did not result in any

significant effect on STC-1 secretion from either vascular cell line (Figure 4.7). It may be possible that the factors present in TCM which induce STC-1 expression do not signal through RTKs, or SU11248 does not inhibit the RTK through which this is mediated.

To further investigate TCM stimulation on SGHEC-7 and SGHVSM-9 cells, the effect of TCM on the activation of a number of intermediates in common cell signalling pathways was assessed (Figure 4.8, 4.9). This provided another means of screening the pathways underlying TCM-induced effects on these cells. TCM-stimulation of both SGHEC-7 and SGHVSM-9 cells resulted in phosphorylation of Akt, p44/42-MAPK, and NDRG1. Phosphorylation of GSK3β occurred in SGHVSM-9 cells following TCM treatment but not in SGHEC-7 cells (Figure 4.8, 4.9). Phosphorylation of these proteins following TCM-stimulation provided an indication that the factors present in TCM activated these downstream signalling cascades.

To understand which cell signalling pathways might be involved in regulating TCM-induced STC-1 secretion from SGHEC-7 and SGHVSM-9 cells, the approach taken was to pharmacologically inhibit key proteins in common cell signalling pathways. If a reduction of TCM-induced STC-1 secretion was observed following inhibition of these proteins, it would suggest that the pathway could be underlying the regulation of TCM-induced STC-1 secretion.

The role of PI3K signalling in TCM-induced secretion of STC-1 from SGHEC-7 and SGHVSM-9 cells was first investigated. PI3K is a major signalling pathway downstream of RTKs and GPCRs and since its downstream effector, Akt, was found to be activated in these cells following TCM stimulation, it was hypothesised that this pathway may be involved in regulating TCM-induced STC-1 secretion. Expression of STC-1 has previously been shown to

be regulated through the PI3K pathway in TNBC cells, human trophoblast cells, and human skin fibroblast cells (Moeller *et al.*, 2011; Jeon *et al.*, 2016; Abid *et al.*, 2020). In this study, inhibition of PI3K had no effect STC-1 secretion from the vascular cell lines, however, activation of PI3K with 740-YP resulted in a significant reduction in TCM-induced STC-1 secretion in SGHEC-7 cells (Figure 4.11).

To confirm this result, the effect of inhibition of Akt, which is downstream of PI3K, was investigated. Overexpression of constitutively active Akt has previously been shown to increase expression of STC-1 in TNBC cells (Jeon *et al.*, 2016), yet inhibition of Akt also increased STC-1 gene expression in human epidermal keratinocyte cells (Yeung and Wong, 2011), Inhibition of Akt resulted in a significant increase in secretion of STC-1 from SGHEC-7 cells at the highest tested dose of 10 μM (Figure 4.13). This supports the finding in this cell line where activation of PI3K with 740-YP resulted in a significant decrease in STC-1 secretion (Figure 4.11). As an increase in expression of STC-1 was observed following pharmacological inhibition and a decrease was found following activation, it could be suggested that treatment of the cells in this way removed a negative regulator of STC-1. It has previously been proposed that downstream targets of Akt may be implicated in the regulation of STC-1 expression such as mTOR, IKK, and Mdm2 (Yeung and Wong, 2011). For example, activation of Akt could increase NF-κB activity via phosphorylation of IKK (Kane *et al.*, 1999), resulting in the negative effect of NF-κB on STC-1 expression reported in the mouse neuroblastoma cell model (Yeung *et al.*, 2003). Furthermore, Akt could inhibit p53 via phosphorylation of Mdm2 (Mayo and Donner, 2001), wherein p53 could stimulate STC-1 expression in human nasopharyngeal cancer cells (Lai *et al.*, 2007).

SGK-1 is a serine/threonine kinase which is activated through phosphorylation by phosphoinositide-dependent protein kinase-1 (PDK-1) and mTORC2, signalling

intermediates downstream of PI3K (Kobayashi and Cohen, 1999; Park *et al.*, 1999; García-Martínez and Alessi, 2008). SGK-1 has increasingly been implicated as a protein kinase with important roles within the vasculature (Zhong *et al.*, 2014) and it has also previously been established that TCM stimulates both SGHEC-7 and SGHVSM-9 cells to express the SGK-1 gene (Wallace *et al.*, 2013), potentially indicating a role for this kinase at the maternal-fetal interface. Inhibition of SGK-1 with GSK650394 resulted in a significant increase in TCM-induced STC-1 secretion from both vascular cell lines when used at 100 µM (Figure 4.15). It is known that GSK650394 may display off target effects on Akt and other related kinases when used at this concentration, therefore, it is possible that inhibition of Akt may occur which would explain this result. Investigating the effect of GSK650394 on Akt activity when used at 100 µM by western blot analysis would help to clarify whether these off-target effects occur and would aid interpretation of this result. Future work would also benefit from additional approaches to confirm if the result obtained with pharmacological inhibition of SGK-1 with GSK650394 was caused by off-target effects, for example, the use of inhibitors which have different mechanisms of action, or the use of an SGK-1 siRNA to enable specific knockdown of this protein.

The effect of inhibition of GSK3β on STC-1 secretion was also assessed. GSK3β is known to be regulated by Akt through phosphorylation on Ser9 which inhibits its activity (Buttrick and Wakefield, 2008; Hermida, Dinesh Kumar and Leslie, 2017). GSK3β has previously been implicated in the regulation of STC-1 expression in human epidermal keratinocyte cells where treatment with GSK3β inhibition by lithium chloride inhibited STC-1 gene expression (Yeung and Wong, 2011). Treatment of TCM-treated vascular cells with both Kenpaullone and 1-Azakenpaullone had no effect on STC-1 secretion from either cell line (Figure 4.16),

suggesting that GSK3β is not involved in the regulation of TCM-induced STC-1 expression in these cells.

The data presented thus far was not suggestive of a direct role for the PI3K/Akt pathway in regulation of TCM-induced STC-1 expression, therefore, the role of other common cell signalling pathways, the MAPK pathways was investigated. The subfamilies of MAPKs, p44/42-MAPK and p38-MAPK, are known to be activated by both RTKs and GPCRs through which the factors present in TCM are likely act (Robinson and Dickenson, 2001). For this reason, it was thought that these pathways may be involved in regulating TCM-induced STC-1 expression. In addition, the MAPK pathway has previously been implicated in the regulation of STC-1 in human neural crest-derived tumour cells where IL6 signalling through the p44/42-MAPK pathway induces expression of STC-1 (Westberg *et al.*, 2007). p44/p42-MAPK was also found to be activated in both SGHEC-7 and SGHVSM-9 cells following TCM treatment so it was thought that this pathway may play a role in this process. Inhibition of this kinase did not, however, result in any significant change in TCM-induced STC-1 secretion in this study (Figure 4.18).

The role of the other MAPK subfamily, p38-MAPK, in this process was also assessed. p38-MAPK is known to be activated by a number of pro-inflammatory cytokines such as IL1 and TNFα (Wei and Liu, 2002; Zhao *et al.*, 2016), both of which have been shown to be present in TCM in this study. This pathway had not previously been implicated in the regulation of STC-1 expression. Consistent with p44/42-MAPK inhibition, inhibition of p38-MAPK did not affect TCM-induced STC-1 secretion from the vascular cell lines (Figure 4.18). From these results, it can be suggested that, although the p44/42-MAPK pathway is activated in these cells following TCM treatment, it is unlikely that any of the MAPK signalling pathways underly TCM-induced STC-1 secretion from vascular cells.

Since the MAPK signalling pathways were not found to underly TCM-induced STC-1 secretion, the role of another common signalling pathway, the JAK-STAT pathway, in this process was investigated. A number of cytokines signal via the JAK-STAT pathway, many of which are present in TCM (Morris, Kershaw and Babon, 2018), thus it was thought that this pathway may underpin TCM-induced effects on STC-1 expression. Inhibition of STAT3 did not result in any change in STC-1 secretion suggesting that this pathway is also not involved in this process (Figure 4.20). It should be noted, however, that inhibition of STAT3 with 5,15-DPP in SGHVSM-9 cells did not result in a significant reduction in expression of phospho-STAT3 (Tyr705) (Figure 4.19C, D), indicating that 5,15-DPP was not effective in inhibiting STAT3 in SGHVSM-9 cells at the concentrations used in this study. It is possible that a higher concentration of 5,15-DPP would be required to inhibit STAT3 in this cell line and this should be assessed in future studies.

The role of cAMP and PKA in secretion of STC-1 from vascular cells was investigated in this study. cAMP signalling is essential in initiation of decidualisation and the cAMP/PKA pathway is known to be activated in early pregnancy in the presence of hCG (Brar *et al.*, 1997; Weedon-Fekjær and Taskén, 2012). A number of previous studies have implicated the cAMP/PKA pathway in the regulation of STC-1 expression in a range of mammalian cells including rat and bovine ovarian thecal interstitial cells, human endometrial stromal fibroblasts, and human trophoblast cells (Paciga *et al.*, 2002; Paciga, DiMattia and Wagner, 2004; Aghajanova *et al.*, 2016; Abid *et al.*, 2020; Khatun *et al.*, 2020). Therefore, it was thought that STC-1 may be similarly regulated in vascular cells. In this study, stimulation of the vascular cell lines with cAMP did not induce any change in STC-1 secretion and inhibition of PKA had no effect on TCM-induced STC-1 secretion from SGHVSM-9 cells, suggesting that this pathway is not involved (Figure 4.21).

The role of PKC in the regulation of STC-1 expression in vascular cells was also investigated in this study. STC-1 is known to be phosphorylated by PKC in human fibrosarcoma cells (Jellinek *et al.*, 2000) and the PKC isoform, PKCα, has previously been implicated in the regulation of STC-1 expression in breast cancer cells where PKCα suppresses the expression of STC-1 (Cornmark *et al.*, 2011). PKCs are members of the AGC family and play diverse roles in a variety of cellular functions (Dempsey *et al.*, 2000; Newton, 2001). The isoforms of the PKC family are divided into three groups based on their lipid and cofactor requirements: the diacylglycerol (DAG) and calcium-sensitive conventional isoforms (α, βI, βII and γ), the DAG-sensitive and calcium-insensitive novel isoforms (δ, η, θ, and ε), and the phosphatidylinositol trisphosphate-sensitive atypical isoforms (ζ, ι, μ and γ) (Violin and Newton, 2003).

PKC was activated in SGHEC-7 and SGHVSM-9 cells using PMA which resulted in a significant increase in STC-1 secretion from both cell lines (Figure 4.22). STC-1 secretion from SGHEC-7 and SGHVSM-9 cells was ~5-fold or ~30-fold higher following PMA treatment compared to TCM treatment, respectively. As anticipated, PMA-induced STC-1 secretion was significantly reduced following broad-spectrum PKC inhibition, however, this inhibition had no effect on TCM-induced STC-1 secretion (Figure 4.22). This suggests that TCM-induced stimulation of STC-1 is not regulated by the PKC isoforms inhibited by Go 6983 (PKCα, PKCβ, PKCγ, PKCδ and PKCζ). As PKCε is activated by PMA but not inhibited by Go 6983, the effect of inhibition of this isoform on TCM-induced STC-1 secretion was assessed independently. No effect on TCM-induced STC-1 secretion was observed following inhibition of PKCε using either method tested in this study (Figure 4.24). The result obtained here indicates that PKC is a key regulator of STC-1 expression in SGHEC-7 and SGHVSM-9 cells but suggests that the PKC family does not underpin TCM-induced STC-1 expression in these cells (Figure 4.26). It

is not known from the results presented herein whether PKCε inhibition using the PKCε inhibitor peptide effectively inhibited PKCε in these cells at the concentrations used. It would be beneficial to assess the expression of PKCε in these cells following treatment with the PKCε inhibitor peptide to confirm its inhibition.

The findings in this study suggest that the cell signalling pathways previously shown to be involved in the regulation of STC-1 expression in a number of mammalian cell types are not similarly implicated in TCM-induced STC-1 expression in vascular cells. It is likely that STC-1 secretion will be differentially regulated in different cells, therefore, the mechanisms underlying its regulation will vary which would provide an explanation for many of the results obtained herein. It should also be noted that differences in methodologies between studies will influence the results obtained. In addition, since TCM contains many factors, it is likely a combination of these factors will act to induce an effect on STC-1 expression from vascular cells. These factors will signal through a number of different signalling cascades to influence STC-1 expression and it is possible that inhibition of each pathway individually may not be sufficient to significantly reduce STC-1 secretion. Future work would benefit from simultaneous inhibition of signalling pathways which are known to be activated in these cells following TCM-stimulation.

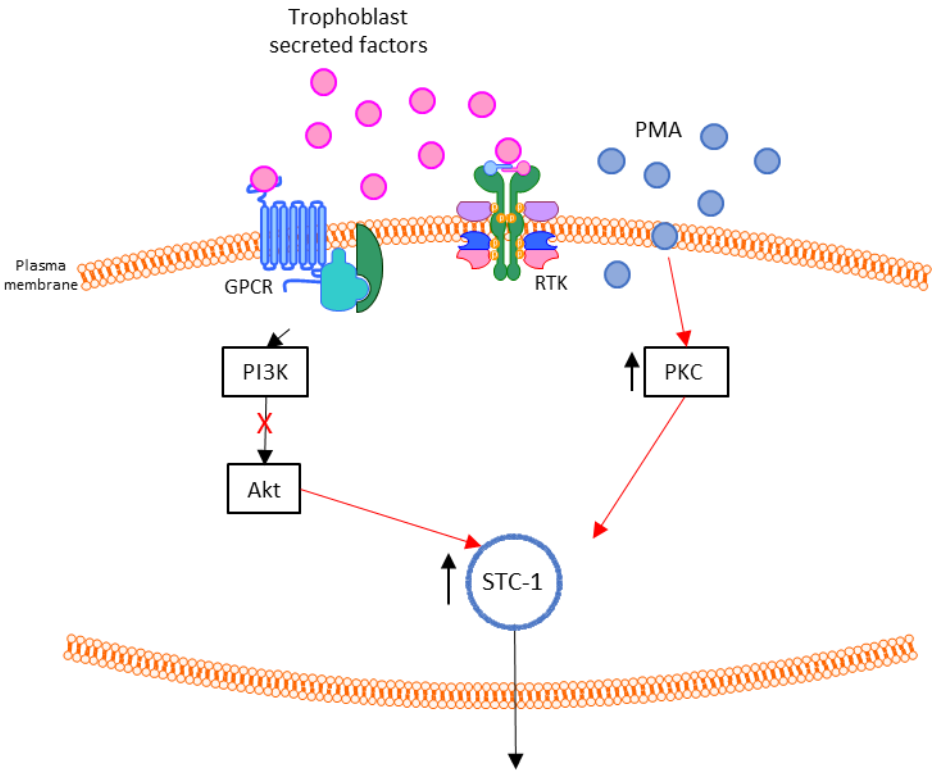



**Figure 4.26 A schematic illustration summarising the cell signalling pathways which underly STC-1 secretion from vascular cells based on findings in this study.**

*GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; PMA, phorbol 12-myristate 13-acetate; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; STC-1, stanniocalcin-1. Figure was prepared using the Motifolio drawing toolkit.*

# Chapter 5: Developing tools to assess the functional role of STC-1 at the maternal-fetal interface

STC-1 was first implicated in the field of female reproduction over 20 years ago, but despite this, we are only now beginning to understand the mechanisms underlying its regulation in female reproduction and its role within this system. Our understanding of STC-1 within female reproduction, and specifically within the remodelling of first trimester spiral arteries, has been hindered by a lack of suitable models and tools. To further understand the role played by STC-1 within the vascular cell model of the maternal spiral artery, it was desirable to develop and optimise robust and simple genetic manipulation tools which could be used in a variety of mammalian cell types.

The first approach to this aim involved the creation of STC-1 overexpression plasmids. One plasmid employed in this study, $STC\text{-}1_{overexpression}$, consisted of human STC-1 mRNA and enhanced green fluorescent protein (eGFP) in a bicistronic vector to enable simultaneous expression of both genes. The second plasmid used in this study was a commercially available STC-1 overexpression plasmid, $VB_{STC\text{-}1}$, which also co-expresses STC-1 with an eGFP/puromycin resistance fusion protein.

The second approach to this aim involved the development of two methods to knock down STC-1 gene expression. One of which was a commercially available siRNA and the other was a commercially available shRNA, both specific to STC-1. The aim of developing and optimising these tools for use in this cell line model system was to enable further investigation of the role played by STC-1 in the key cellular functions involved in the spiral artery remodelling process.

## 5.1 Results

### 5.1.1 Transient overexpression of STC-1 using the STC-$1_{overexpression}$ plasmid resulted in a significant increase in STC-1 secretion in SGHEC-7 and SGHVSM-9 cells but did not affect intracellular STC-1 expression

To assess the efficacy of the STC-$1_{overexpression}$ plasmid for use in overexpression studies, SGHEC-7 and SGHVSM-9 cells were transfected with the STC-$1_{overexpression}$ plasmid, and the STC-$1_{mutant}$ plasmid as a control, using the Lonza Nucleofector® system. The cells were then cultured for 24 hours before collecting the culture medium for analysis by ELISA and harvesting the cell monolayer for western blot analysis. Transfection of both SGHEC-7 and SGHVSM-9 cells with the STC-$1_{overexpression}$ plasmid resulted in a ~140-fold increase in STC-1 secretion compared to transfection with the STC-$1_{mutant}$ control plasmid (Figure 5.1A and B, $P \leq 0.0001$).

Transfection of both SGHEC-7 and SGHVSM-9 cells with the STC-$1_{overexpression}$ plasmid did not, however, result in a significant change in intracellular STC-1 expression compared to transfection with the STC-$1_{mutant}$ control plasmid (Figure 5.1C, D, E, and F).

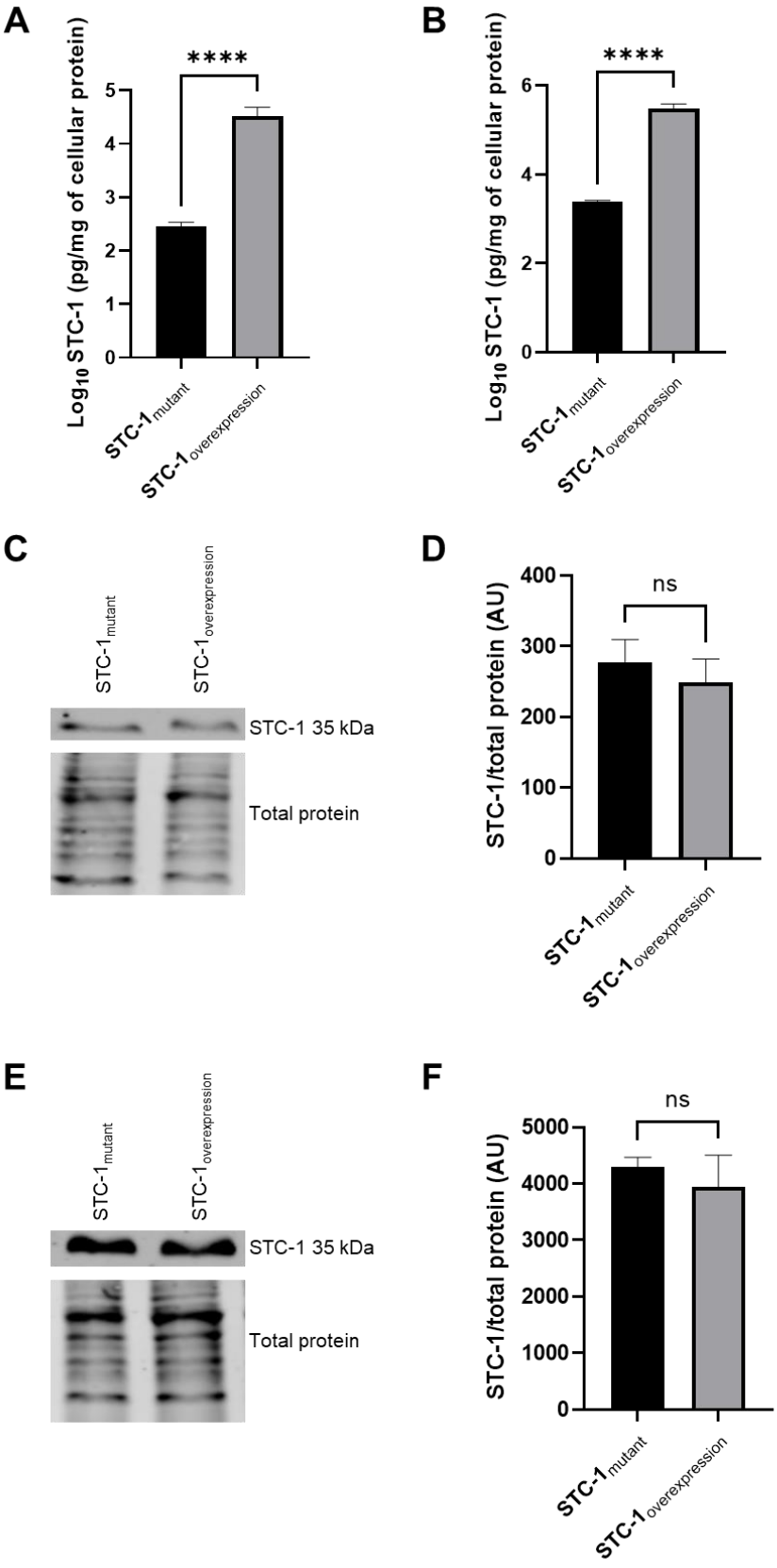

A
****
Log10 STC-1 (pg/mg of cellular protein)
0
1
2
3
4
5
STC-1mutant
STC-1overexpression
B
****
Log10 STC-1 (pg/mg of cellular protein)
0
2
4
6
STC-1mutant
STC-1overexpression
C
STC-1mutant
STC-1overexpression
STC-1 35 kDa
Total protein
D
ns
STC-1/total protein (AU)
0
100
200
300
400
STC-1mutant
STC-1overexpression
E
STC-1mutant
STC-1overexpression
STC-1 35 kDa
Total protein
F
ns
STC-1/total protein (AU)
0
1000
2000
3000
4000
5000
STC-1mutant
STC-1overexpression

**Figure 5.1 Secretion and intracellular expression of STC-1 in SGHEC-7 and SGHVSM-9 cells following transfection with STC-$1_{overexpression}$ and STC-$1_{mutant}$ plasmids.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 24-hour period following transfection with STC-$1_{overexpression}$ and STC-$1_{mutant}$ plasmids was determined by ELISA (n = 4). $Log_{10}$-transformed data of STC-1 (pg/mg of cellular protein). Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (**** = P ≤ 0.0001). Intracellular expression of STC-1 in SGHEC-7 cells (C) and SGHVSM-9 cells (E) transfected with STC-$1_{overexpression}$ and STC-$1_{mutant}$ plasmids and cultured for 48 hours was determined by western blot. Quantification of western blot analysis for the detection of STC-1 in (D) SGHEC-7 and (F) SGHVSM-9 cells (n = 4). Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (ns = not significant).*

### 5.1.2 Development of a stable STC-1 overexpression cell line using the STC-$1_{overexpression}$ plasmid

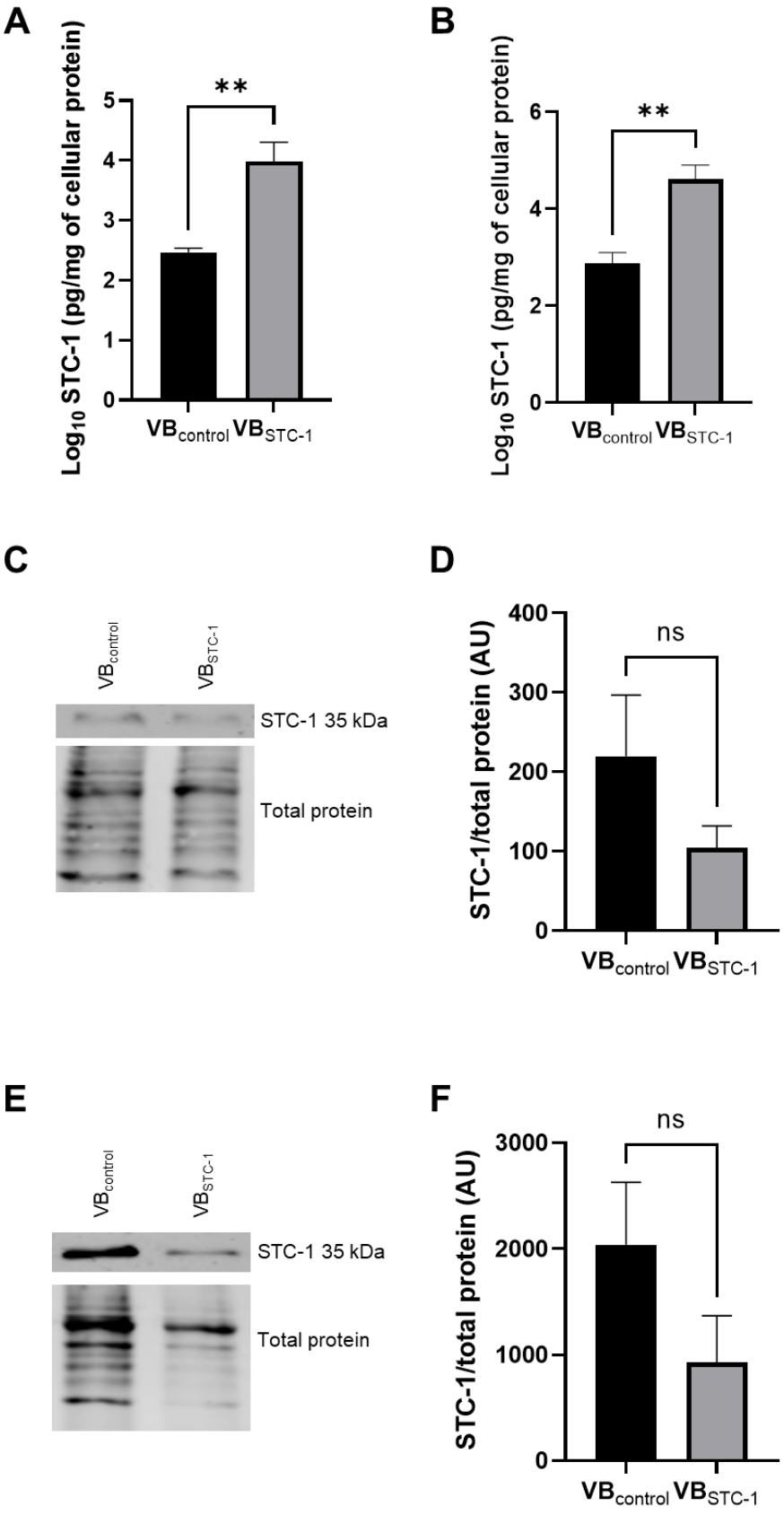
Following successful validation of the STC-$1_{overexpression}$ plasmid and the STC-$1_{mutant}$ plasmid for use in overexpression studies (Figure 5.1), the creation of SGHEC-7 and SGHVSM-9 cell lines which stably overexpress each plasmid was attempted to reduce the requirement for transient transfections. SGHEC-7 and SGHVSM-9 cells were co-transfected using the Lonza Nucleofector® system with STC-$1_{overexpression}$ plasmid or STC-$1_{mutant}$ plasmid and pBABE-puro plasmid (Morgenstern and Land, 1990) in a 9:1 ratio respectively to introduce puromycin resistance and allow for selection of cells. Cells were maintained in 1 µg/ml puromycin to allow for selection of positively expressing cells. 20 surviving colonies were selected and expanded before assessing STC-1 secretion by ELISA to confirm successful integration and expression of the plasmids. This process was repeated numerous times, and on each occasion STC-1 secretion was not found to be increased in the selected colonies transfected with the STC-$1_{overexpression}$ plasmid compared to cells expressing the STC-$1_{mutant}$ plasmid (data not shown). In addition, upon visualisation of cells at this stage using fluorescent microscopy, it was not possible to detect GFP fluorescence indicating that stable transfection was not successful.

### 5.1.3 Transient overexpression of STC-1 using the $VB_{STC-1}$ plasmid resulted in a significant increase in STC-1 secretion in SGHEC-7 and SGHVSM-9 cells, but did not alter intracellular STC-1 expression

As the previous approach to creating a stable cell line using co-transfection was unsuccessful, it was decided to employ the use of a commercially available plasmid $VB_{STC-1}$ (VectorBuilder, Chicago, Illinois, USA) and the corresponding control plasmid, $VB_{control}$ (VectorBuilder, Chicago, Illinois, USA) containing a non-coding sequence. These plasmids contain an integrated puromycin resistance gene, and it was anticipated that this could help overcome issues with co-transfection.

To assess the efficacy of the $VB_{STC-1}$ plasmid for use in overexpression studies, SGHEC-7 and SGHVSM-9 cells were transfected with the $VB_{STC-1}$ plasmid and the $VB_{control}$ plasmid as a control using the Lonza Nucleofector® system. The cells were then cultured for 24 hours before collecting the culture medium for analysis by ELISA and harvesting the cell monolayer for western blot analysis. Transfection of both SGHEC-7 and SGHVSM-9 cells with the $VB_{STC-1}$ plasmid resulted in an over 60-fold increase in STC-1 secretion compared to transfection with the $VB_{control}$ control plasmid (Figure 5.2A and B, $P \leq 0.01$).

Consistent with the result observed with the $STC\text{-}1_{overexpression}$ plasmid, transfection of both SGHEC-7 and SGHVSM-9 cells with the $VB_{STC-1}$ plasmid did not result in a significant change in intracellular STC-1 expression compared to transfection with the $VB_{control}$ plasmid (Figure 5.2C, D, E, and F).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Log10 STC-1 (pg/mg of cellular protein)
**
VBcontrol
VBSTC-1
B
Log10 STC-1 (pg/mg of cellular protein)
**
VBcontrol
VBSTC-1
C
VBcontrol
VBSTC-1
STC-1 35 kDa
Total protein
D
STC-1/total protein (AU)
ns
VBcontrol
VBSTC-1
E
VBcontrol
VBSTC-1
STC-1 35 kDa
Total protein
F
STC-1/total protein (AU)
ns
VBcontrol
VBSTC-1

**Figure 5.2 Secretion and intracellular expression of STC-1 in SGHEC-7 and SGHVSM-9 cells following transfection with $VB_{STC-1}$ and $VB_{control}$ plasmids.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 24-hour period following transfection with $VB_{STC-1}$ and $VB_{control}$ plasmids was determined by ELISA (n = 4). $Log_{10}$-transformed data of STC-1 (pg/mg of cellular protein). Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (** = P ≤ 0.01). Intracellular expression of STC-1 in SGHEC-7 cells (C) and SGHVSM-9 cells (E) transfected with $VB_{STC-1}$ and $VB_{control}$ plasmids and cultured for 48 hours was determined by western blot. Quantification of western blot analysis for the detection of STC-1 in (D) SGHEC-7 and (F) SGHVSM-9 cells (n = 4). Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (ns = not significant).*

### 5.1.4 Development of a stable STC-1 overexpression cell line using the $VB_{STC-1}$ plasmid

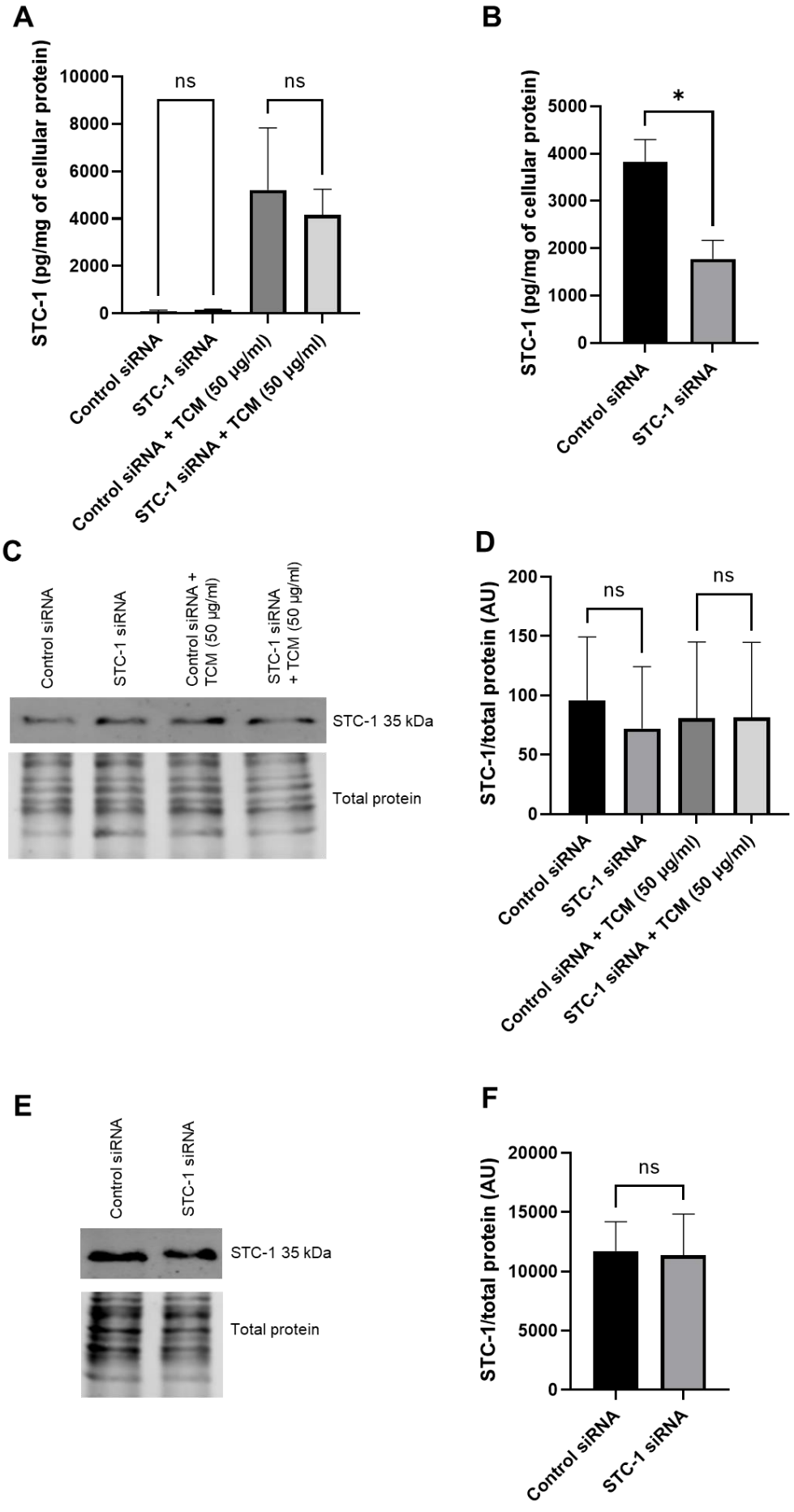
To create cell lines which stably express the $VB_{STC-1}$ plasmid or the $VB_{control}$ plasmid, SGHEC-7 and SGHVSM-9 cells were transfected using the Lonza Nucleofector® system with either plasmid. Cells were maintained in 1 µg/ml puromycin to allow for selection of positively expressing cells. 16 surviving colonies were selected and expanded before assessing STC-1 secretion by ELISA to confirm successful integration and expression of the plasmids. None of the surviving colonies transfected with the $VB_{STC-1}$ plasmid displayed increased secretion of STC-1 compared to colonies which were transfected with the $VB_{control}$ plasmid (data not shown). Consistent with the result obtained when attempting creation of stable cell lines with the $STC\text{-}1_{overexpression}$ plasmid, GFP fluorescence was not visible at this stage, indicating that stable transfection was unsuccessful.

### 5.1.5 Transient knockdown of STC-1 using a specific siRNA reduced secretion of STC-1 from SGHVSM-9 cells, but did not alter intracellular STC-1 expression

As another method to assess the functional role of STC-1 in the vasculature, a specific siRNA was optimised to knock down expression of STC-1 in SGHEC-7 and SGHVSM-9 cells. The STC-1 siRNA used in this study consisted of a pool of three STC-1-specific 19-25 nucleotide siRNAs designed to knockdown STC-1 gene expression. As a control, a siRNA consisting of a scrambled sequence which does not lead to the degradation of any known mRNA was used (both Santa Cruz Biotechnology, TX, USA).

To assess the efficacy of STC-1 siRNA for use in knockdown studies, SGHEC-7 and SGHVSM-9 cells were transfected with STC-1 siRNA and control siRNA using the Lonza Nucleofector® system. The cells were then cultured for 48 hours before collecting the culture medium for analysis by ELISA and harvesting the cell monolayer for western blot analysis. Transfection of SGHVSM-9 cells with STC-1 siRNA resulted in a ~2-fold reduction in STC-1 secretion compared to transfection with control siRNA (Figure 5.3B, $P \leq 0.05$). As basal secretion of STC-1 from SGHEC-7 cells is very low (Figure 3.5), cells were treated with TCM for 2 hours 24 hours post-transfection with siRNA. No significant change in STC-1 secretion was observed in TCM-treated SGHEC-7 cells following transfection with STC-1 siRNA (Figure 5.3A).

Consistent with the result observed following overexpression of STC-1, transfection of both SGHEC-7 and SGHVSM-9 cells with STC-1 siRNA did not result in a significant change in intracellular STC-1 expression compared to transfection with control siRNA (Figure 5.3C, D, E and F).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8000
6000
4000
2000
0
ns
ns
Control siRNA
STC-1 siRNA
Control siRNA + TCM (50 µg/ml)
STC-1 siRNA + TCM (50 µg/ml)
B
STC-1 (pg/mg of cellular protein)
5000
4000
3000
2000
1000
0
*
Control siRNA
STC-1 siRNA
C
Control siRNA
STC-1 siRNA
Control siRNA + TCM (50 µg/ml)
STC-1 siRNA + TCM (50 µg/ml)
STC-1 35 kDa
Total protein
D
STC-1/total protein (AU)
200
150
100
50
0
ns
ns
Control siRNA
STC-1 siRNA
Control siRNA + TCM (50 µg/ml)
STC-1 siRNA + TCM (50 µg/ml)
E
Control siRNA
STC-1 siRNA
STC-1 35 kDa
Total protein
F
STC-1/total protein (AU)
20000
15000
10000
5000
0
ns
Control siRNA
STC-1 siRNA

**Figure 5.3 Secretion and intracellular expression of STC-1 in SGHEC-7 and SGHVSM-9 cells following transfection with STC-1 siRNA and control siRNA plasmids.**

*Secretion of STC-1 from SGHEC-7 cells (A) and SGHVSM-9 cells (B) into the culture medium over a 48-hour period following transfection with control siRNA and STC-1 siRNA plasmids was determined by ELISA. SGHEC-7 cells were treated with TCM (50 µg/ml) for 2 hours prior to 48-hour incubation (SGHEC-7 - n = 3, SGHVSM-9 - n = 4). Results are mean ± SEM. In (A) Data were analysed with a one-way ANOVA (ns = not significant). In (B) Data were analysed with a two-tailed, unpaired t test (* = P ≤ 0.05). Intracellular expression of STC-1 in SGHEC-7 cells (C) and SGHVSM-9 cells (E) transfected with control siRNA and STC-1 siRNA plasmids and cultured for 48 hours was determined by western blot. SGHEC-7 cells were treated with TCM (50 µg/ml) for 2 hours prior to 48-hour incubation. Quantification of western blot analysis for the detection of STC-1 in (D) SGHEC-7 and (F) SGHVSM-9 cells (n = 3). Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM. In (D) Data were analysed with a one-way ANOVA (ns = not significant). In (F) Data were analysed with a two-tailed, unpaired t test (ns = not significant).*

### 5.1.6 Development and characterisation of a SGHVSM-9 STC-1 shRNA stable cell line

An additional approach to investigate the effect of STC-1 knockdown in SGHVSM-9 cells was the creation of a stable cell line which expresses STC-1 shRNA through lentiviral transduction. This was established to reduce the require for transient transfections using STC-1 siRNA. The STC-1 shRNA Lentiviral Particles used in this study contain three STC-1-specific constructs encoding 19-25 nucleotide shRNA designed to knock down STC-1 gene expression and contained a puromycin resistance gene to enable selection of positively expressing cells (Santa Cruz Biotechnology, TX, USA). Lentiviral transduction was used here to overcome the previous issues of low transfection efficiency using the Lonza Nucleofector® system.

SGHVSM-9 cells were infected with either STC-1 shRNA Lentiviral Particles or Control shRNA Lentiviral Particles (Santa Cruz Biotechnology, TX, USA) and maintained in culture prior to puromycin selection. Surviving colonies were selected and expanded before assessing STC-1 expression to confirm successful infection with the shRNA constructs. SGHVSM-9 cells stably expressing the STC-1 shRNA construct displayed 5-fold less secretion of STC-1 compared to control shRNA (Figure 5.4A, $P \leq 0.001$). In contrast, following treatment with TCM (50 µg/ml), there was no significant difference in STC-1 secretion between STC-1 or control shRNA stably expressing cells (Figure 5.4B). Intracellular expression of STC-1 also remained consistent between STC-1 or control shRNA stably expressing cells (Figure 5.4C, D, E and F).

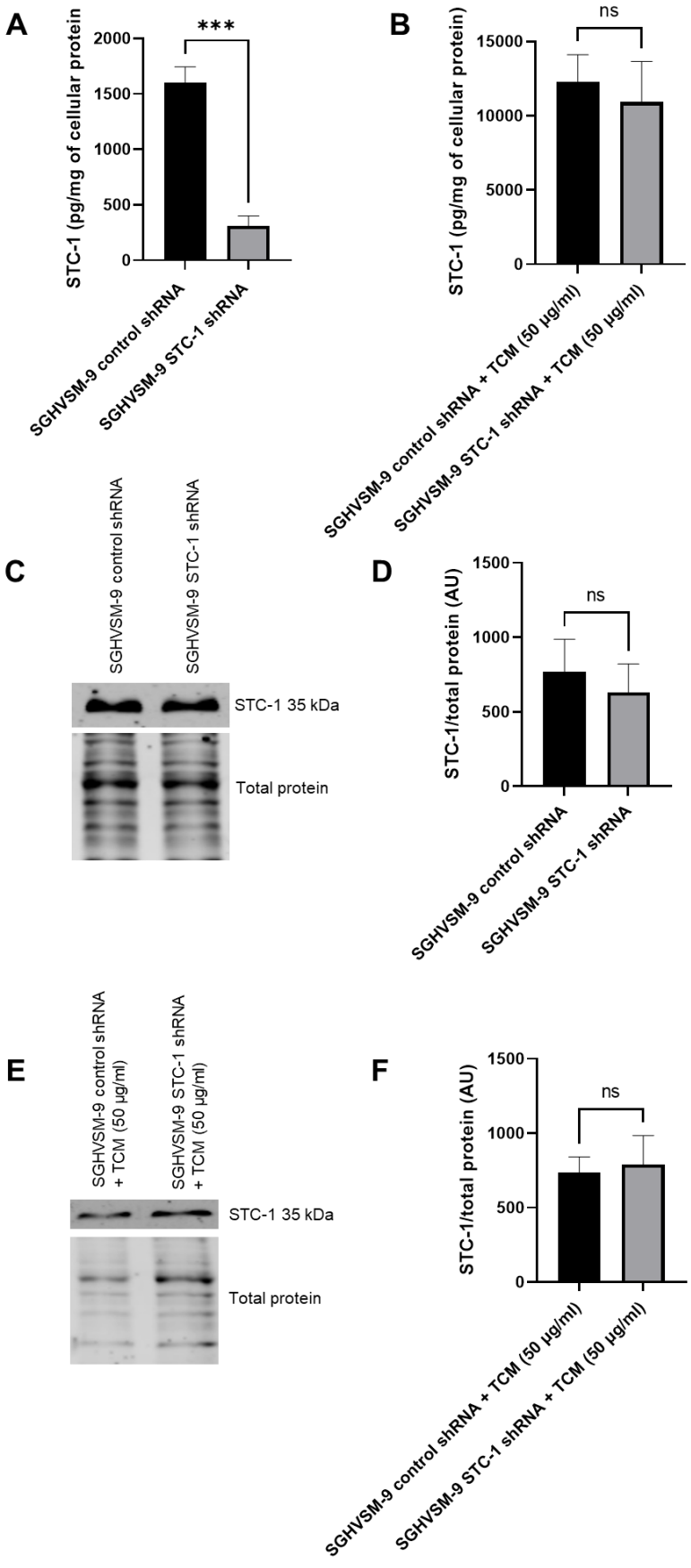

A
STC-1 (pg/mg of cellular protein
2000
1500
1000
500
0
***
SGHVSM-9 control shRNA
SGHVSM-9 STC-1 shRNA
B
STC-1 (pg/mg of cellular protein
15000
10000
5000
0
ns
SGHVSM-9 control shRNA + TCM (50 µg/ml)
SGHVSM-9 STC-1 shRNA + TCM (50 µg/ml)
C
SGHVSM-9 control shRNA
SGHVSM-9 STC-1 shRNA
STC-1 35 kDa
Total protein
D
STC-1/total protein (AU)
1500
1000
500
0
ns
SGHVSM-9 control shRNA
SGHVSM-9 STC-1 shRNA
E
SGHVSM-9 control shRNA + TCM (50 µg/ml)
SGHVSM-9 STC-1 shRNA + TCM (50 µg/ml)
STC-1 35 kDa
Total protein
F
STC-1/total protein (AU)
1500
1000
500
0
ns
SGHVSM-9 control shRNA + TCM (50 µg/ml)
SGHVSM-9 STC-1 shRNA + TCM (50 µg/ml)

**Figure 5.4 Secretion and intracellular expression of STC-1 in SGHVSM-9 cells stably overexpressing STC-1 shRNA and control shRNA.**

*Secretion of STC-1 from SGHVSM-9 cells stably overexpressing control shRNA and STC-1 shRNA unstimulated (A) (n = 4) and (B) stimulated with TCM (50 µg/ml) for 2 hours (n = 8) into the culture medium over a 24-hour period was determined by ELISA. Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant, *** = P ≤ 0.001). (C) Intracellular expression of STC-1 in SGHVSM-9 cells stably overexpressing control shRNA and STC-1 shRNA unstimulated and (E) stimulated with TCM (50 µg/ml) for 2 hours and then cultured for 48 hours was determined by western blot. Quantification of western blot analysis for the detection of STC-1 in (D) SGHVSM-9 cells stably overexpressing control shRNA and STC-1 shRNA unstimulated (n = 4) and (F) stimulated with TCM (50 µg/ml) (n = 8). Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM. Data were analysed with a one-way ANOVA (ns = not significant).*

## 5.2 Discussion

This study aimed to create and optimise a range of tools to genetically manipulate STC-1 expression and secretion which could be used in a variety of mammalian cell lines. Several previous studies have employed the use of STC-1 overexpression and knockdown plasmids to assess the role of STC-1 in a number of mammalian systems (Wang *et al.*, 2011; Wu *et al.*, 2014; Huang *et al.*, 2015; Chao *et al.*, 2021; Liu *et al.*, 2022). To date, however, none of these plasmids had integrated GFP to aid detection through fluorescent microscopy.

The first tool created in this study was a STC-1 overexpression plasmid, STC-$1_{overexpression}$. This plasmid enabled co-expression of STC-1 mRNA and eGFP in a bicistronic vector. The bicistronic design of this plasmid hosted many benefits. Unlike in vectors which express selectable markers from a unique promoter, in bicistronic vectors both proteins are under the control of the same promoter, so detection of the protein encoded by the second cistron is evidence that the first cistron is also being expressed (Martin *et al.*, 2006). Bicistronic expression in this way enables screening and selection for cells that are expressing the gene of interest at high levels.

The vascular cell lines used in this study, SGHEC-7 and SGHVSM-9 were found to be very difficult to transfect. Several chemical transfection methods were trialled which yielded varying results (data not shown). These included the cationic polymer polyethylenimine (PEI), and the commercially available cationic reagents ViaFect™ (Promega, WI, USA) and ScreenFect™A (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). All of these chemical methods resulted in very low transfection efficiency and/or high cell toxicity. To overcome these issues, the physical transfection method of Nucleofection® was used (Lonza, Basel, Switzerland). Nucleofection® is a transfection method based on electroporation and works by using a transient electric field to induce pores in the cell

membrane as well as using cell-type specific solutions to enable transfer of genetic material directly into the cells' nucleus (Ren *et al.*, 2022). This method produced the highest transfection efficiency and lowest levels of cell toxicity in both vascular cell lines of all of transfection methods tested and therefore was used for subsequent transfection experiments in this study.

Following creation of the STC-$1_{overexpression}$ plasmid and optimisation of transfection protocol, it was demonstrated that transfection of both vascular cell lines with this plasmid resulted in a significant increase in STC-1 secretion, yet intracellular protein levels remained consistent (Figure 5.1). This aligns with previous findings in this study where TCM-stimulation of vascular cells increased STC-1 secretion without altering intracellular STC-1 protein levels (Figure 4.2 and 4.3). In line with the hypothesis proposed in Chapter 4, it is likely that overexpression of STC-1 in this way increases gene expression and that this is translated into protein which is immediately secreted, whilst the stable intracellular stores of STC-1 are maintained, indicative of a switch to constitutive secretion. To confirm this, future studies could investigate the effect of BFA treatment on STC-1 overexpressing cells to give insight into the mechanisms underlying STC-1 protein transport following overexpression.

To date, this is the first study to assess secretion of STC-1 from mammalian cell lines following transient overexpression. Previous studies in the field have examined intracellular expression of STC-1 following transient overexpression in ovarian cancer cell lines and hippocampal neurons and have reported significant increases in intracellular STC-1 expression as determined by western blot (Liu *et al.*, 2010; Chao *et al.*, 2021). It is possible that the mechanisms underlying storage and secretion of STC-1 vary between cell types which could explain the differences between this study and others. In addition, differences

in the overexpression constructs used in each study may influence targeting of the overexpressed protein.

Following validation of the STC-$1_{overexpression}$ plasmid and the STC-$1_{mutant}$ plasmid for use in overexpression studies in SGHEC-7 and SGHVSM-9 cells, it was decided to try and create stable overexpression cell lines to reduce the need for transient transfections and which could be used for functional analysis of STC-1. This approach was taken as both of these cell lines proved to be difficult to transfect. Despite Nucleofection® resulting in the highest transfection efficiency of all the methods tested, this efficiency was not as high as anticipated. Therefore, it was decided that the generation of stable overexpression cell lines would provide a more robust and consistent approach to overexpression. The first approach to stable cell line generation in this study involved co-transfection of the STC-$1_{overexpression}$ plasmid or STC-$1_{mutant}$ plasmid with a puromycin-resistance plasmid. The method was repeated a number of times and on each occasion, puromycin resistant colonies were selected and expanded but following characterisation, the cells were found not to overexpress STC-1 as determined by ELISA (data not shown).To try and overcome these technical challenges, a commercially available STC-1 overexpression plasmid, $VB_{STC-1}$, was used which co-expressed STC-1 with a puromycin/eGFP fusion protein. This should have offered a number of advantages. Firstly, the fused nature of GFP and the puromycin resistance gene in this plasmid meant that successfully transfected cells could be tracked by fluorescence and selected by puromycin resistance. Secondly, as the puromycin resistance gene was integrated into this plasmid, it helped to avoid low transfection efficiency associated with co-transfection of antibiotic resistance plasmids.

Transfection of both SGHEC-7 and SGHVSM-9 cells with the $VB_{STC-1}$ plasmid resulted in a significant increase in STC-1 secretion compared to the control but, consistent with

transfection with the STC-$1_{overexpression}$ plasmid, no change in intracellular STC-1 expression was found (Figure 5.2). Following validation of these plasmids for use in overexpression studies, stable cell line generation using the $VB_{STC-1}$ plasmid and $VB_{control}$ plasmid was attempted. Consistent with the result obtained following attempted stable cell line generation with the STC-$1_{overexpression}$ plasmid, expanded puromycin resistant colonies were not found to overexpress STC-1 (data not shown). The reasons for this are unclear as it was anticipated that the integrated puromycin resistance gene in this plasmid would ensure that colonies which survived selective pressure would contain the plasmid and therefore express STC-1. It is possible that the selective pressure induced by puromycin was not high enough to eliminate all non-expressing cells and, therefore, these cells ultimately outgrew the expressing cells. A higher concentration of puromycin in future studies may help to overcome this issue. Future work may also benefit from the use a different method of transfection, such as lentiviral transduction to create stable cell lines for STC-1 overexpression as this method has previously been used to create stable HUVEC cell lines which overexpress STC-1 (Law and Wong, 2013). However, considering the high levels of cell death observed during the attempts to create these cell lines, it is possible that overexpression of STC-1 at this level may be toxic to cells. High concentrations of some proteins are known to harm cells in several ways, for example, by activating or overloading specific biological pathways, disrupting regulation, or by aggregating together resulting in the production of artifacts (Vavouri *et al.*, 2009; Makanae *et al.*, 2013; Tang and Amon, 2013). It is plausible that STC-1 expression requires tight regulation to prevent cell toxicity so overexpression may not be possible in these cell lines. Future work to study the role of STC-1 in these cell lines should, therefore, focus on the optimisation of STC-1 knockdown techniques which are less likely to result in significant cell damage.

To this end, a commercially available specific STC-1 siRNA was optimised for use in knockdown studies. As SGHVSM-9 cells were previously shown to secrete a higher basal level of STC-1 compared to SGHEC-7 cells, it is likely that a knockdown approach would be better suited for functional analysis in this cell line. In SGHEC-7 cells, prior stimulation with TCM was required as basal secretion is very low, therefore, the effect of siRNA knockdown on TCM-stimulated SGHEC-7 cells was assessed. The results from this study revealed that siRNA-mediated knockdown of STC-1 is an effective tool to reduce secretion of STC-1 from SGHVSM-9 cells, but not from unstimulated or TCM-stimulated SGHEC-7 cells (Figure 5.3). The reasons for differences in efficacy of STC-1 knockdown between the cell lines are not clear. This may be explained by variations in transfection efficiency between the two cell lines or it has previously been suggested that highly expressed genes are more susceptible to siRNA-mediated gene knockdown (Hong *et al.*, 2014). Therefore, higher basal STC-1 expression in SGHVSM-9 cells may explain the observed result that siRNA gene knockdown is more efficient in this cell line compared to SGHEC-7 cells. The results from this study also showed that siRNA-mediated knockdown of STC-1 did not affect intracellular protein levels from either vascular cell line. The reasons underlying this are unclear. It is possible that the effect of STC-1 siRNA on intracellular protein was time-sensitive, and that an earlier time point for analysis would have been required to see an effect. Future studies using a time-course to assess the effect of STC-1 siRNA on intracellular and secreted protein would clarify if a shorter time point, such as 24 hours, may have been optimal to observe an effect of STC-1 knockdown. Analysis of cells treated with control or STC-1 siRNA by quantitative PCR to assess changes in STC-1 gene expression would also be beneficial to confirm the efficacy of siRNA treatment and clarify whether the observed reduction in STC-1 secretion from SGHVSM-9 cells following transfection with STC-1 siRNA was genuine.

To again reduce the requirement for transient transfections, generation of a stable STC-1 knockdown SGHVSM-9 cell line was attempted using a specific STC-1 shRNA and lentiviral transduction. Following the results obtained when optimising STC-1 siRNA for use in the vascular cell lines, it was decided attempt stable STC-1 knockdown only in SGHVSM-9 cells. It was thought that higher basal STC-1 secretion would render this method more effective, and knockdown of STC-1 would be more relevant in functional studies. Following lentiviral transduction of STC-1 shRNA in SGHVSM-9 cells and selection and expansion of positively-expressing colonies, cells were found to secrete significantly less STC-1 compared to cells stably expressing control shRNA (Figure 5.4). Interestingly, the reduction in STC-1 secretion between STC-1 shRNA and control shRNA cells following TCM stimulation did not reach statistical significance (Figure 5.4). It is possible that the increased expression in STC-1 induced by TCM treatment is so great that changes in knockdown could not be detected.

In summary, two approaches for STC-1 overexpression were generated and optimised in this study, the STC-$1_{overexpression}$ plasmid and $VB_{STC-1}$ plasmid, as well as two approaches for STC-1 knockdown, siRNA and shRNA. These represent useful tools which can be employed in functional analysis studies of STC-1 in vascular cells. The issues with transfection efficiency faced using the vascular cell lines in this study were unforeseen. Despite optimising Nucleofection® as a method to transfect the vascular cell lines, the efficiency of this method was not as high as expected, and creation of stable cell lines was not possible within the timeframe of this study. It is clear that these cell lines are very difficult to transfect, and a different approach may be required for future functional studies. Infection of SGHVSM-9 cells with lentiviral shRNA plasmids appeared promising, and future work to optimise the efficiency of this method so that changes in STC-1 expression post-stimulation can be observed would be beneficial. In addition, the best approach to assess STC-1

overexpression may be to amend the current STC-$1_{overexpression}$ plasmid to create a lentiviral plasmid. This may help to overcome transfection efficiency issues and enable more extensive functional analysis.

# Chapter 6: The functional role of STC-1 in VSMCs and ECs at the maternal-fetal interface

STC-1 acts in a pleiotropic manner in mammalian systems. It has been implicated in a diverse range of processes including mineral homeostasis (Olsen *et al.*, 1996), angiogenesis (He *et al.*, 2011; Law and Wong, 2013), organogenesis (Jiang *et al.*, 2000; Stasko and Wagner, 2001), cell proliferation (Bai *et al.*, 2017), apoptosis (Kim *et al.*, 2013), retinal degeneration (Roddy *et al.*, 2012), cerebral ischemia (Zhang *et al.*, 2000), inflammation (Mohammadipoor *et al.*, 2016), tumourigenesis (Liu *et al.*, 2010), and anti-oxidative activity (Kim *et al.*, 2013; Wu *et al.*, 2014; Bonfante *et al.*, 2020). In the female reproductive system specifically, STC-1 has been linked to roles including steroidogenesis (Paciga *et al.*, 2002, 2003; Luo *et al.*, 2004), ROS homeostasis (Basini *et al.*, 2010; Baioni *et al.*, 2011), and decidualisation and blastocyst implantation in early pregnancy (Stasko and Wagner, 2001; Song *et al.*, 2006, 2009; Xiao *et al.*, 2006; Allegra *et al.*, 2009). The functional role of STC-1 at the maternal-fetal interface in the first trimester of pregnancy, however, has yet to be uncovered. As STC-1 has previously been implicated in a diverse range of mammalian processes, it is likely that it will play varied roles within this context. In the present study, the role played by STC-1 in the vascular cell model of the maternal spiral artery was assessed.

## 6.1 Results

### 6.1.1 Overexpression of STC-1 resulted in suppression of SGHEC-7 cell migration

The role of STC-1 in vascular cell migration was assessed using a wound healing assay. SGHEC-7 and SGHVSM-9 cells were transfected with $STC\text{-}1_{overexpression}$ or $STC\text{-}1_{mutant}$ plasmids before creating a vertical scratch in the middle of each well and assessing cell migration through time-lapse microscopy. Overexpression of STC-1 in SGHEC-7 cells resulted in a ~1.6-fold reduction in the rate of cell migration (Figure 6.1A, $P \leq 0.001$) Overexpression of STC-1 in SGHVSM-9 cells had no significant effect on the rate of cell migration (Figure 6.1B).

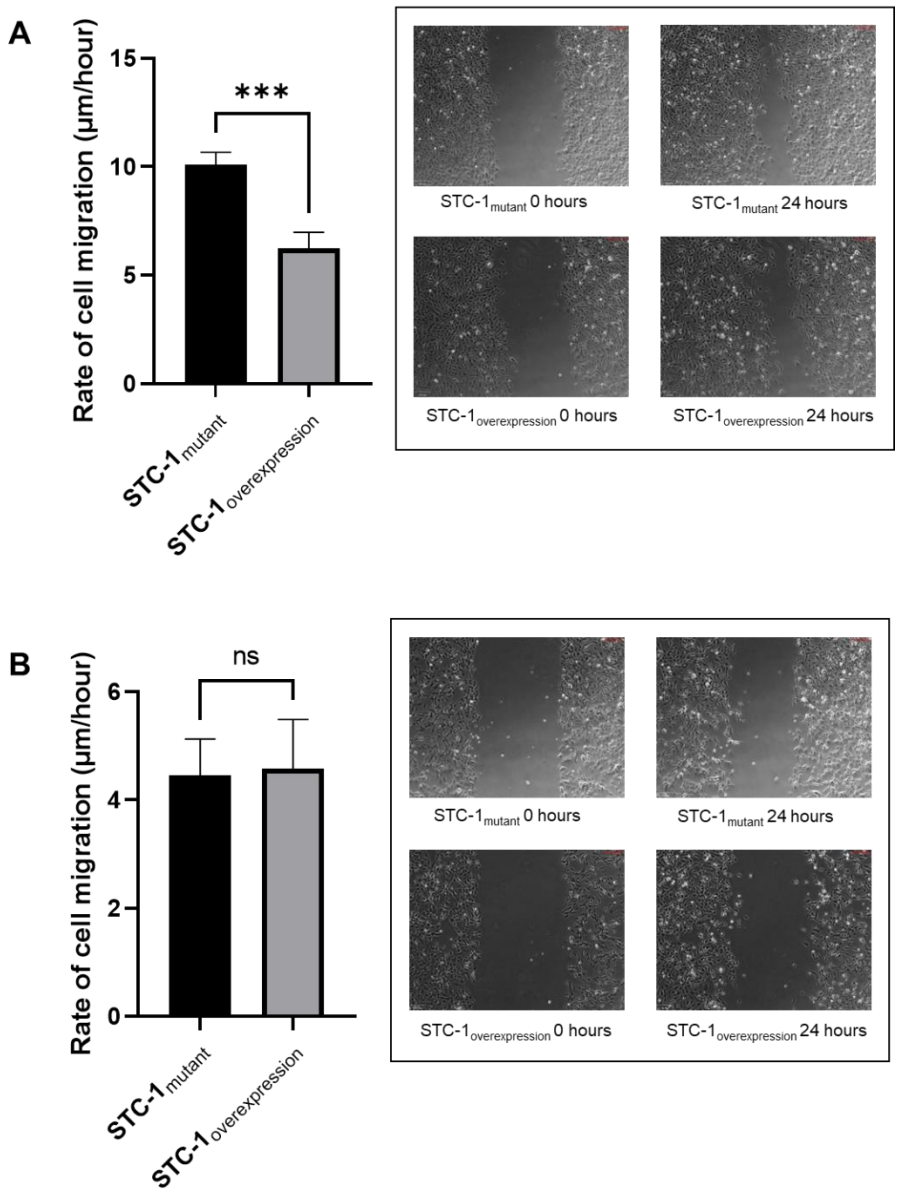



**Figure 6.1 The effect of overexpression of STC-1 on migration of SGHEC-7 and SGHVSM-9 cells.**

*SGHEC-7 (A) cells and SGHVSM-9 cells (B) were transfected with STC-1$_{mutant\ or}$ STC-1$_{overexpression}$ plasmids and the rate of cell migration (µm/hour) was assessed by a wound healing assay. Time-lapse microscopy was used to image the cells every 15 minutes over a 24-hour period (SGHEC-7 – n= 4, SGHVSM-9 – n = 3). The included representative images were taken at the first and last experimental time point. Results are mean ± SEM. Data were analysed with a two-tailed, unpaired t test (ns = not significant, *** = P ≤ 0.001).*

### 6.1.2 Overexpression and knockdown of STC-1 in SGHVSM-9 cells did not affect expression of VSMC differentiation markers

It was hypothesised that STC-1 may play a role in modulating the differentiated state of VSMCs in remodelling spiral arteries. To investigate this, the expression of markers of VSMC differentiation including Krüppel-like factor 4 (KLF4), calponin, and smooth muscle actin was assessed through western blot in SGHVSM-9 cells either overexpressing STC-1 or expressing STC-1 siRNA.

SGHVSM-9 cells were transfected using the Lonza Nucleofector® system with either the $STC\text{-}1_{overexpression}$ plasmid, the $VB_{STC\text{-}1}$ plasmid, or STC-1 siRNA, and corresponding control for each construct and cultured for 24 hours for overexpression or 48 hours for knockdown. The cell monolayer was then harvested for analysis by western blot. The cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane. These membranes were probed for the detection of KLF4, calponin, and smooth muscle actin. Overexpression of STC-1 in SGHVSM-9 cells did not result in a significant change in expression of KLF4, calponin, or smooth muscle actin (Figure 6.2A, C, and E). Similarly, knock down of STC-1 did not result in a significant change in expression of any of the VSMC differentiation markers (Figure 6.2 B, D, and F).

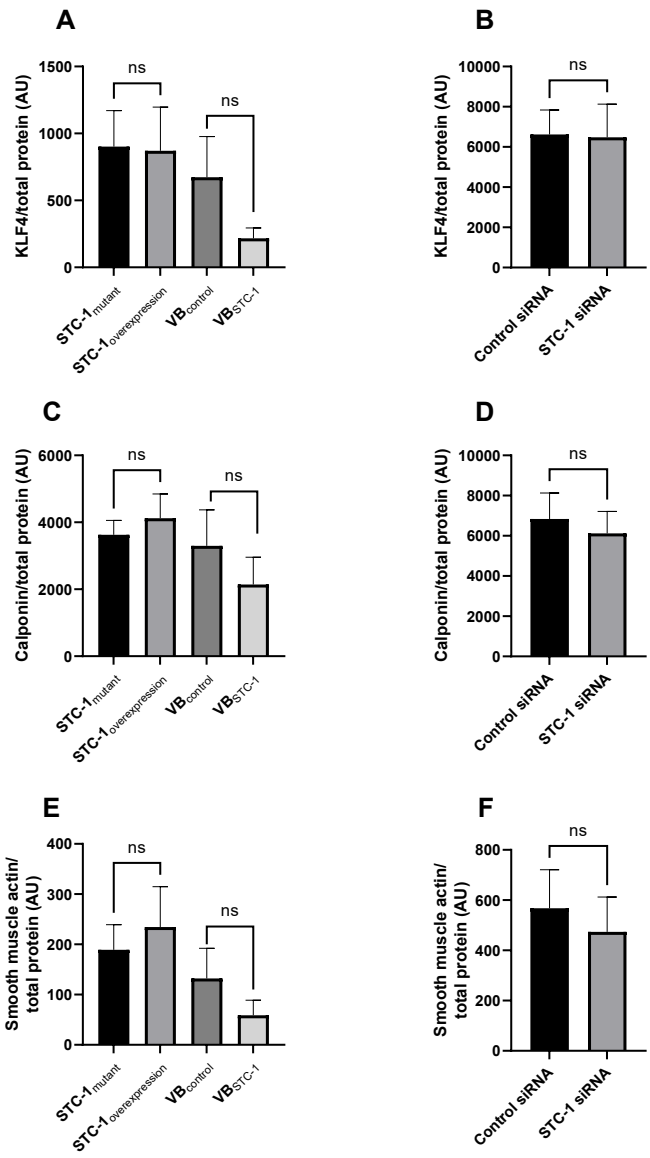



**Figure 6.2 The effect of overexpression and knockdown of STC-1 on the expression of differentiation markers in SGHVSM-9 cells.**

*Intracellular expression of KLF4 (A, B), calponin (C, D), and smooth muscle actin (E, F) in SGHVSM-9 cells following transfection with STC-1$_{overexpression}$ and STC-1$_{mutant}$ plasmids, VB$_{STC-1}$ and VB$_{control}$ plasmids (A, C, E) or STC-1 siRNA and control siRNA plasmids (B, D, F) and cultured for 24 hours (overexpression) or 48 hours (knockdown) was determined by western blot (n = 4). Data are expressed as band density normalised to total protein of the corresponding lane. Results are mean ± SEM. Data were analysed with independent two-tailed, unpaired t tests (ns = not significant).*

## 6.2 Discussion

STC-1 has been implicated in a number of processes in the mammalian system, but its role in vascular remodelling in the first trimester of human pregnancy has not been established. This study aimed to characterise the role of STC-1 in this process. A number of mechanisms are involved in trophoblast-dependent spiral artery remodelling including VSMC de-differentiation, cell migration, extracellular matrix restructuring, and changes in sensitivity to death-inducing ligands (Whitley and Cartwright, 2010).

The role of STC-1 in migration of SGHEC-7 and SGHVSM-9 cells was assessed in this study. The current literature suggests a complex picture is emerging regarding the effect of STC-1 on cell migration. STC-1 has been implicated in both pro- and anti-migratory roles in a number of cancer cells including cervical, breast, renal, gastric, ovarian, and colorectal cancer cells (Liu *et al.*, 2010; Guo *et al.*, 2013; Pena *et al.*, 2013; Ma *et al.*, 2015; Wang *et al.*, 2019; Hou *et al.*, 2021; Lin *et al.*, 2022).

The role of STC-1 in vascular cell migration has been investigated in previous studies. In VSMCs, STC-1 has been shown to suppress angiotensin II-induced migration in a model of atherosclerosis (Yamamoto *et al.*, 2016). In ECs, STC-1 has been shown to play both pro- and anti-migratory roles. During angiogenesis, overexpression of STC-1 has been shown promote migration, but recombinant STC-1 treatment of ECs inhibits HGF-induced migration (Zlot *et al.*, 2003; Law and Wong, 2013). In this study, SGHEC-7 cells overexpressing STC-1 displayed a reduced rate of cell migration compared to the control, however, overexpression of STC-1 in SGHVSM-9 cells had no effect on cell migration (Figure 6.1). The present study and the study by Law and Wong 2013 both investigated the effect of STC-1 overexpression on EC migration yet reported conflicting results. There were, however, differences in the methodology used in these studies. The study by Law and

Wong investigated migration of ECs which stably overexpressed STC-1 through a transwell migration assay (Law and Wong, 2013), whilst, in the present study, migration of ECs which transiently expressed STC-1 was assessed by a wound healing assay. These differences in methodology may explain the conflicting results. It should be noted that the inhibitory effect of STC-1 on EC migration observed in this study was also reported in the study by Zlot *et al.* following treatment of ECs with recombinant STC-1 (Zlot *et al.*, 2003). It is clear from the results obtained in this study and previously that the role of STC-1 on EC migration is complex and STC-1 may play both pro- and anti-migratory roles based on concentration or the presence of local stimuli and elucidating its role is further complicated by the differences in methodology in the current literature.

In a healthy vessel, ECs and VSMCs are largely non-motile and non-proliferative. During physiological remodelling, however, ECs and VSMCs are lost from the spiral artery and replaced by trophoblast cells. Vascular cell migration is known to be an important mechanism in this process (Bulmer *et al.*, 2012). As STC-1 expression is upregulated in vascular cells following contact with trophoblast cells and is further elevated in the local environment in complicated pregnancies (Uusküla *et al.*, 2012; Wallace *et al.*, 2013; Abid *et al.*, 2020), it is plausible that STC-1 overexpression induced in this study could appropriately model the *in vivo* situation. For this reason, the finding that STC-1 overexpression results in suppression of EC migration is interesting. Extrapolation of this data could suggest that high expression of STC-1 as reported in complicated pregnancies may contribute to reduced EC migration which could impact on spiral artery remodelling. It has previously been demonstrated that STC-1 overexpression promotes arterial re-endothelialisation through enhancing mice arterial EC migration capacity (Liu *et al.*, 2022), yet the opposite was reported herein. It is possible that the effect of STC-1 may vary depending on the local

environment and, in the context of pregnancy, it could be that STC-1 plays a role in preventing re-endothelialisation, but this would require further study.

It is important to note that the efficiency of transient transfection of the cells used in this study was not as high as anticipated. Although it was demonstrated that STC-1 secretion was significantly increased following transfection and, as a paracrine factor, secreted STC-1 will act on neighbouring cells, it is likely that a low percentage of cells in each well were successfully transfected. It is possible that low transfection efficiency could influence the results obtained in this study. This could, therefore, be improved by the use of stable overexpression cell lines to overcome transfection efficiency issues associated with transient transfection. To further investigate STC-1 in vascular cell migration, the effect of STC-1 knockdown could be assessed to confirm a role for STC-1 in this process. It should also be noted that during the course of the wound healing assay, the cells will proliferate as well as migrate into the gap. Cell proliferation in this way may interfere with the measurement of cell migration. Further work to elucidate the role of STC-1 in vascular cell migration would benefit from the use of drugs such as actinomycin D to inhibit cell proliferation and enable the effect of cell migration alone to be determined (Jonkman *et al.*, 2014).

The role of STC-1 in VSMC de-differentiation during spiral artery remodelling was also assessed in this study. VSMCs are capable of switching between a 'functional' (contractile) and 'synthetic' (proliferative) phenotype following changes in gene expression (Kaplan-Albuquerque *et al.*, 2005). In a healthy artery, the majority of VSMCs exhibit a contractile phenotype and are largely non-proliferative (Owens, Kumar and Wamhoff, 2004). In response to changes in extracellular cues, VSMCs can adopt a more synthetic phenotype (Wilcox, 1992). The cellular mechanisms underlying loss of VSMCs from remodelling spiral

arteries is not fully understood, however, VSMC de-differentiation has been shown to be an important feature of early remodelling (Robson *et al.*, 2019). It was hypothesised that STC-1 may play a role in the modulation of VSMC differentiation state, however, STC-1 overexpression and knockdown were not shown to have any significant effect on the expression KLF4, calponin, or smooth muscle actin in SGHVSM-9 cells in this study (Figure 6.2), suggesting that STC-1 does not play a role in the process.

# Chapter 7: Final discussion and future work

Research in this field clearly demonstrates that establishment and maintenance of a successful pregnancy requires complex interplay between multiple maternal and placental factors. Sufficient spiral artery remodelling is essential to enable adequate uteroplacental blood flow to support development of the fetus. If this remodelling is insufficient, a number of severe pregnancy complications can develop, compromising the health of both the mother and fetus during the perinatal period but also later in life (Burton *et al.*, 2019). Numerous research efforts in the field have attempted to unravel the complex molecular and cellular interactions that occur at the maternal-fetal interface to aid our understanding of both the physiology and pathophysiology underlying the vascular remodelling process. These studies have highlighted the involvement of a range of factors which may contribute to normal or insufficient vascular remodelling. Due to the complexity of the aetiology of pre-eclampsia and differences in the pathophysiology between early and late onset forms of the disorder, it is unlikely that any single biomarker or pairing of biomarkers will be able to predict all forms of pre-eclampsia. Therefore, it is vital that research in this area continues to uncover additional factors that may contribute to the development of this pregnancy disorder.

STC-1 is a factor which had been implicated in the process of spiral artery remodelling through previous gene array studies where trophoblast secreted factors induced STC-1 gene expression in a maternal spiral artery cell line model (Wallace *et al.*, 2013). Prior to this study, however, the regulation and role of STC-1 in this system had not been investigated. Moreover, despite clear upregulation of STC-1 in the placenta and maternal circulation in complicated pregnancies (Uusküla *et al.*, 2012; Abid *et al.*, 2020), the relevance of this was not understood.

It is clear from previous research that the field of STC-1 biology is emerging and our current of understanding of this protein is limited. The present literature points to specific and important roles for STC-1 within the mammalian system and, in particular, in female reproductive tissues. However, the majority of the literature in the field of STC-1 biology focusses on its spatial and temporal expression patterns in a range of tissues with fewer studies focussed on determining its role. STC-1 has been implicated in the pathology of a range of mammalian diseases (Gerritsen, Peale Jr. and Wu, 2002; Uusküla *et al.*, 2012; Aghajanova *et al.*, 2016; Zhao *et al.*, 2018; Abid *et al.*, 2020; Khatun *et al.*, 2020), but the mechanisms underlying STC-1 expression and its role in many of these pathologies are not fully understood. In this way, much of the literature is speculative and therefore renders extrapolation of this data to other mammalian tissues difficult. Since the expression and role of STC-1 in vascular cells in early pregnancy had not been investigated, a major challenge of this study was understanding which data from previous studies would be relevant to this system and could direct research in this area.

To understand STC-1 in early pregnancy, this study first aimed to characterise STC-1 expression within first trimester maternal decidual tissue and in cell lines modelling the spiral artery to elucidate its regulation and role in this system. Data collected prior to the commencement of this study demonstrated that STC-1 is expressed in endothelial cells of remodelling spiral arteries in first trimester human decidual tissue (Figure 3.1). Expression of STC-1 in this context supported previous studies which reported trophoblast-induced STC-1 gene expression in a three-dimensional vascular cell line model (Wallace *et al.*, 2013). In this way, it is likely that STC-1 expression would also be detected in VSMCs of the remodelling spiral arteries, but this was not assessed and should be a topic of future research. Since STC-1 is known to be upregulated in the placenta and maternal circulation

in complicated pregnancies (Uusküla *et al.*, 2012; Abid *et al.*, 2020), it was hypothesised that upregulation of STC-1 may also occur in first trimester decidual tissue from complicated pregnancies. STC-1 expression was not found to differ between normal pregnancies and those at risk of pre-eclampsia, nor was its expression affected by gestational age (Figure 3.3). There are challenges in obtaining high numbers of first trimester decidual tissue samples which restricted large scale analysis of STC-1 in this tissue. Future research expanding on these findings using a larger pool of samples would clarify whether changes in STC-1 expression occur in this context.

Data obtained using vascular cell lines in this study are indicative of a system in which trophoblast invasion of spiral arteries in early pregnancy stimulates secretion of STC-1 from vascular cells. Careful consideration was taken to model the impact of biological variation whilst still using cell lines. TCM was made in batches and a different batch was used for each experimental repeat which enabled better modelling of biological variation. This study was the first to screen the extensive profile of secreted factors from trophoblasts. As demonstrated, TCM contains a wide range of cytokines and growth factors (section 4.1.4), and each of these will act on vascular cells in different ways to induce an effect on STC-1 expression. This comprehensive screen was a useful starting point for this research, but as previously discussed, the array used in this study was semi-quantitative and therefore did not indicate the relative levels of these factors which might be important. In the present study, it was not possible to identify the specific factor(s) which underpins this process, and it is likely that these factors will act in a synergistic manner. Future work would benefit from a more comprehensive analysis of these factors and how they may act both individually and collectively on vascular cells to influence STC-1 expression.

A major aim of this study was to elucidate the cell signalling pathways underlying the regulation of STC-1 expression. A key finding was that Akt is implicated in the regulation of TCM-induced STC-1 secretion (Figure 4.13), in line with previous findings (Yeung and Wong, 2011). Future work would benefit from pharmacological inhibition of proteins downstream of Akt to further elucidate this mechanism. In addition, PKC appears to be implicated in regulating STC-1 secretion from vascular cells, but not that induced by TCM (Figure 4.22). This finding is relevant in the context of vascular biology and could provide insight into STC-1 regulation in cells from different vascular beds. It is interesting that mechanisms underlying the regulation of TCM-induced STC-1 expression in vascular cells were not consistent with those implicated in other reproductive tissues. In ovarian cells, endometrial stromal cells, and trophoblast cells (Paciga *et al.*, 2002; Paciga, DiMattia and Wagner, 2004; Aghajanova *et al.*, 2016; Abid *et al.*, 2020; Khatun *et al.*, 2020) expression of STC-1 is mediated through the PKA pathway and is induced by cAMP, but this was not demonstrated in the model in this study (Figure 4.21). It is apparent that the mechanisms underlying STC-1 expression are complex and vary depending on cell type and stimuli present in the local environment. Although the specific signalling mechanisms underlying TCM-induced STC-1 secretion were not fully elucidated in this study, importantly, the present data provided confirmation of common signalling pathways that are not involved in this process and will help direct future work in this area.

Another important aim of this study was to understand the role that STC-1 plays in the remodelling spiral artery. In pursuit of this, two approaches for STC-1 overexpression and knockdown were optimised. Despite difficulties faced with the generation of stable overexpression cell lines, the two overexpression plasmids employed in this study, STC-$1_{overexpression}$ and $VB_{STC\text{-}1}$, as well as STC-1 siRNA, were found to be effective for use in initial

functional analysis of STC-1 on vascular cells (Figure 6.1 and 6.2). STC-1 has been implicated in several roles depending on tissue origin but its role in vascular cells of the remodelling spiral artery had not been assessed. A key finding from this study was that overexpression of STC-1 in SGHEC-7 cells using the $STC\text{-}1_{overexpression}$ plasmid generated herein resulted in suppression of cell migration (Figure 6.1). STC-1 appears to act in both pro- and anti-migratory roles in vascular cells and this seems to be dependent on local stimuli (Zlot *et al.*, 2003; Law and Wong, 2013; Yamamoto *et al.*, 2016). Vascular cell migration is important within the remodelling spiral artery and since STC-1 is upregulated in the presence of trophoblast cells and is further elevated in the local environment in complicated pregnancies (Uusküla *et al.*, 2012; Wallace *et al.*, 2013; Abid *et al.*, 2020), it is possible that suppression of EC migration by STC-1 is a key role for this protein in this system. This finding is also interesting from the perspective of general vascular biology and may provide insight into the role of STC-1 in ECs from different vascular beds.

The use of transfected cell lines in this study offered a number of advantages over the use of primary cells. For example, cell lines are cost effective, easy to maintain, and retain a stable phenotype from passage to passage. Using transfected cell lines also eliminates patient-to-patient variation which allows for multiple replicates of the same experiment to be performed (Balon and Wiatrak, 2021). In addition, transfected cell lines require less growth factors for survival in culture which reduces the potential that these factors will interfere with subsequent analysis. Several caveats are, however, associated with the use of cell lines, for example, although a lack of patient-to-patient variation can be advantageous, it also reduces the ability of cell lines to reflect biological variation which can be achieved with the use of patient-derived primary cells. In addition, since transfected cell lines are genetically manipulated, it is difficult to ensure that cell lines display and maintain

functional features consistent with those seen in primary cells and they may exhibit changes to their phenotype and responsiveness to stimuli (Kaur and Dufour, 2012). It is also noteworthy that in the *in vivo* environment, cells are surrounded by other cells and ECM in a three-dimensional manner, therefore, two-dimensional cell culture may not adequately represent the three-dimensional environment of cells. Cells grown in two-dimensional culture display morphological changes which can influence many cellular processes including cell proliferation, differentiation, apoptosis, and gene and protein expression (Tibbitt and Anseth, 2009). In addition, changes in gene expression have been reported in cells grown in two-dimensional culture including up-regulation of genes involved in cell cycling, metabolism, and turnover of macromolecules (Birgersdotter, Sandberg and Ernberg, 2005; LaMarca *et al.*, 2005). The use of a three-dimensional co-culture system of SGHEC-7 and SGHVSM-9 cells as described previously (Wallace *et al.*, 2013) could help to overcome this limitation and better model the complex mechanical and biological interplay experienced by the vascular cells *in vivo*, but this approach is difficult to scale up.

It should be noted that the SGHEC-7 cell line used in this study was derived from a human umbilical vein and the SGHVSM-9 cell line was derived from adult human aorta, thus, neither of these cell lines have been derived directly from a human spiral artery. There may be cell-specific differences between the cells used in this study and spiral artery vascular cells. For example, as they originate from a different vascular bed, they will not have been subject to same pregnancy-specific endocrine stimuli as decidual vascular cells such as oestrogen and progesterone. These hormones are known to induce changes in gene and protein expression of key enzymes and mediators involved in modulating uterine artery contractility (Chang and Zhang, 2008) and these changes may not be consistent in cells isolated from a different vascular bed. It is important to consider this when interpreting the

results obtained in this study. Despite this, it is of note that the origin of these cell lines allows the findings from this study to be applicable to vascular biology in general.

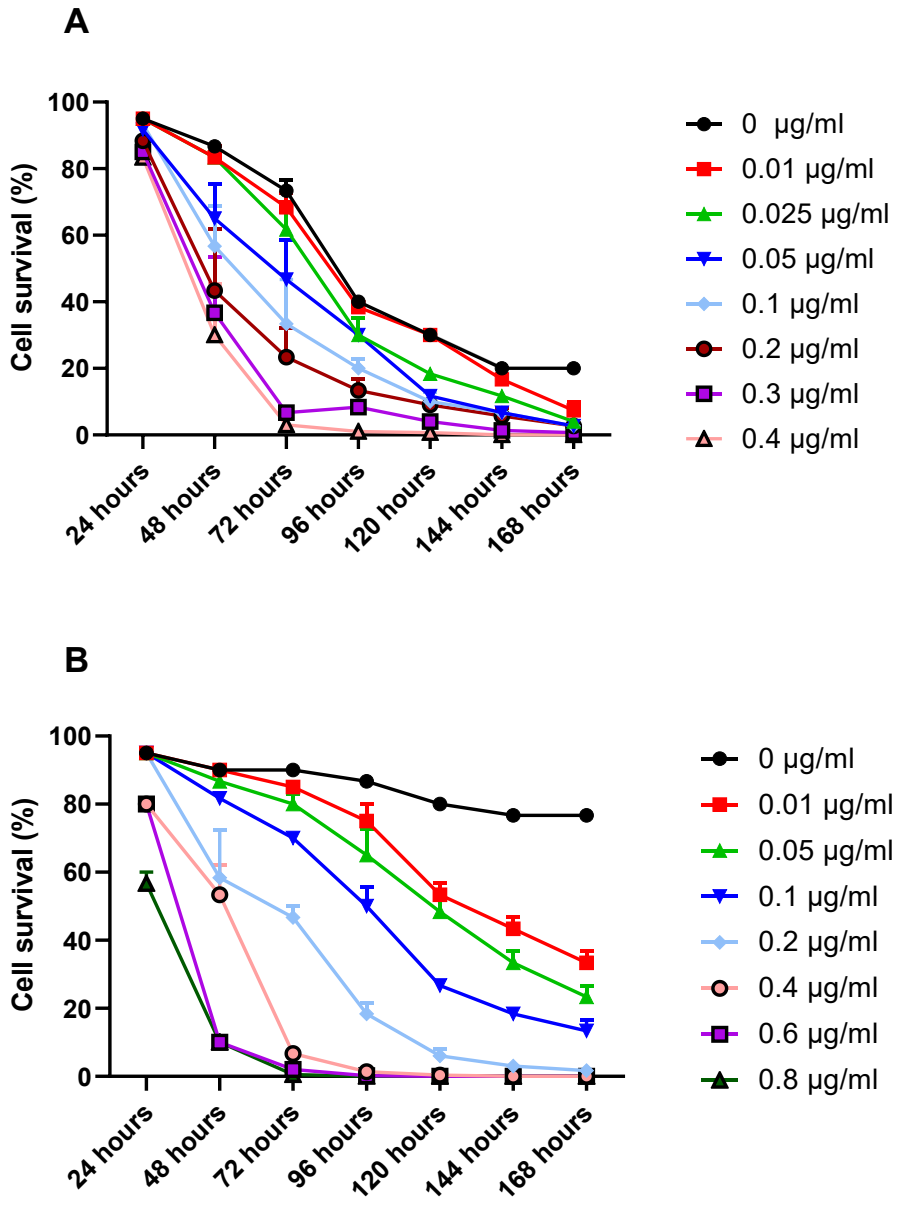
To date, no isolations of spiral artery vascular cells have been carried out due to the size of the vessels and the limitations in obtaining first-trimester tissue, therefore, the use of transfected cell line models is currently the most appropriate approach to conduct this research. Future research would benefit from the optimisation of methods to isolate vascular smooth muscle cells and endothelial cells from the human first trimester spiral artery. At present, isolation of these cells in sufficient numbers has proved challenging, and it has been difficult to isolate these cells without significant contamination with other decidual cells. In addition, although the use of primary cells enables better modelling of the *in vivo* system, as well as allowing patient-to-patient variation to be reflected, they also harbour several limitations. For example, primary cells grow at a slower rate and have a shorter lifespan compared to transfected cell lines which limits their use in numerous experimental repeats. Moreover, consistent with the use of transfected cell lines, primary cells will also require two-dimensional cell culture and therefore will face the same morphological and genetic changes as transfected cell lines when cultured in this way.

Given the extensive ethical implications associated with the study of human pregnancy *in vivo*, and the lack of suitable animal models to study the first trimester spiral artery remodelling process, the use of vascular cell lines to model this process *in vitro* was the most appropriate choice for this study. Despite the potential limitations associated with the use of the vascular cell lines employed in this study, it is important to note that these cells have previously been used to model the maternal spiral artery and the effect of interactions with trophoblast cells on the expression of STC-1 (Wallace *et al.*, 2013). Furthermore, in terms of STC-1 protein expression, these cells lines appropriately reflected

the findings from maternal tissue. For example, previous immunohistochemical analysis of maternal decidual tissue revealed an increase in expression of STC-1 in ECs in the presence of trophoblasts (Figure 3.1), and in SGHEC-7 cells, increased secretion of STC-1 was observed following culture with TCM (Figure 4.2).

In summary, the present research demonstrates that STC-1 is clearly expressed in first trimester maternal decidual tissue and reports a model in which trophoblast secreted factors act to induce STC-1 expression in this context. The extensive range of factors present in TCM has been identified, and this research presents two cell signalling pathways that may be involved in both TCM-stimulated and unstimulated STC-1 expression from vascular cells. Genetic manipulation tools optimised in this study will aid future research to uncover the role of STC-1 in vascular remodelling. The present data combined with previous research strongly implicate a role for STC-1 in spiral artery remodelling. This study has progressed our knowledge in this area and postulated avenues for future research which could help shed light on the role of STC-1 in early pregnancy and further understand its dysregulation in complicated pregnancies.

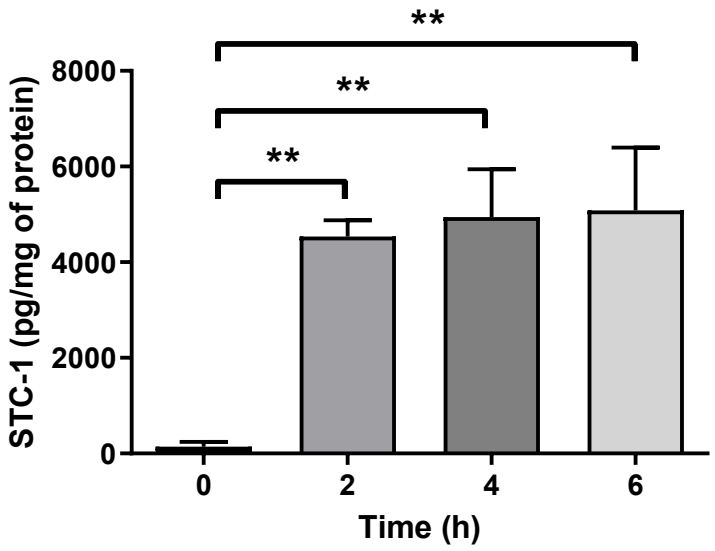
# Appendix




**Appendix Figure 1. Puromycin kill curves.**

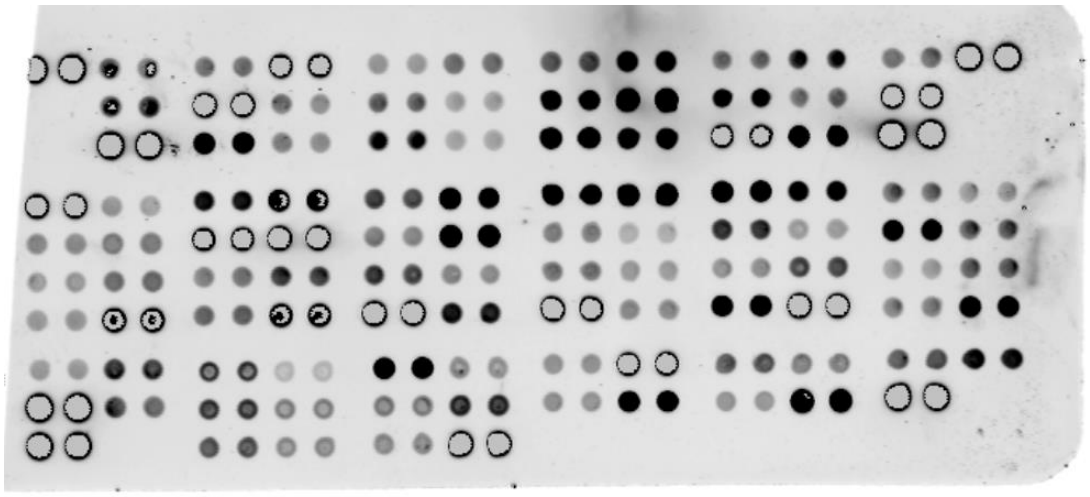
*Percentage survival of SGHEC-7 (A) and SGHVSM-9 cells (B) following treatment with increasing concentrations of puromycin (µg/ml) was empirically determined every 24 hours for 7 days. Cells were seeded in triplicate and the results are an average percentage of the three wells at each puromycin concentration. Results are mean ± SEM.*

24h
150μg/ml
n=5




**Appendix Figure 2. Time course experiment to assess the effect of TCM on STC-1 secretion from SGHEC-7 cells.**

*Secretion of STC-1 from SGHEC-7 cells into the culture medium over a 24-hour period following 2 hours treatment with TCM (150 μg/ml) was determined by ELISA (n = 5). Results are mean ± SEM. Data were analysed with a one-way ANOVA (** = P ≤ 0.01).*



**Appendix Figure 3. Proteome Profiler Human XL Cytokine Array.**

*The relative levels of cytokines and chemokines within TCM were determined using a Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, MN, USA). TCM (77 µg) was diluted in Array Buffer 6 (included in the array kit) and added to the membrane before incubating overnight. The array was performed following the manufacturer's instructions and the membrane was analysed using the Odyssey® CLx Imaging system (LI-COR Biosciences, Nebraska, USA) with the following parameters: Resolution: 84 µm, Quality: Medium, Focus offset: 0.0 mm, Intensity: 5.*

**Appendix Table 1 Cytokine Panel in Proteome Profiler Human XL Cytokine Array Kit.**

| | | |
|---|---|---|
| Adiponectin/Acrp30 | Interferon (IFN)-gamma | C–C motif chemokine ligand 2 (CCL2)/ Monocyte chemoattractant protein-1 (MCP-1) |
| Angiogenin | Insulin-like growth factor binding protein 2 (IGFBP-2) | C–C motif chemokine ligand 7 (CCL7)/ Monocyte chemoattractant protein-3 (MCP-3) |
| Angiopoietin-1 | Insulin-like growth factor binding protein 2 (IGFBP-3) | Macrophage colony-stimulating factor (M-CSF) |
| Angiopoietin-2 | Interleukin-1 alpha (IL-1 alpha)/ Interleukin-1F1 (IL-1F1) | Macrophage migration inhibitory factor (MIF) |
| Apolipoprotein A1 | Interleukin-1 beta (IL-1 beta)/ Interleukin-1F2 (IL-1F2) | C-X-C motif ligand 9 (CXCL9)/ Monokine induced by gamma interferon (MIG) |
| B-cell activating factor (BAFF)/ B Lymphocyte Stimulator (BLyS)/ Tumour Necrosis Factor Superfamily Member 13B (TNFSF13B) | Interleukin 1 receptor antagonist (IL-1RA)/ Interleukin-1F3 (IL-1F3) | C–C motif chemokine ligand 3 (CCL3)/ C–C motif chemokine ligand 4 (CCL4)/ Macrophage inflammatory protein (MIP)-1 alpha/beta |
| Brain-derived neurotrophic factor (BDNF) | Interleukin-2 (IL-2) | C–C motif chemokine ligand 20 (CCL20)/ Macrophage inflammatory protein (MIP)-3 alpha |
| Cluster of differentiation 14 (CD14) | Interleukin-3 (IL-3) | C–C motif chemokine ligand 19 (CCL19)/ Macrophage |

| | | |
|---|---|---|
| | | inflammatory protein (MIP)-3 beta |
| Cluster of differentiation 30 (CD30) | Interleukin-4 (IL-4) | Matrix metalloproteinase 9 (MMP-9) |
| Cluster of differentiation 31 (CD31)/ Platelet endothelial cell adhesion molecule-1 (PECAM-1) | Interleukin-5 (IL-5) | Myeloperoxidase |
| Cluster of differentiation 40 (CD40) Ligand/ Tumour Necrosis Factor (Ligand) Superfamily Member 5 (TNFSF5) | Interleukin-6 (IL-6) | Osteopontin (OPN)/ Secreted Phosphoprotein 1 (SPP1) |
| Chitinase 3-like | Interleukin-8 (IL-8) | Platelet-derived growth factor (PDGF)-AA |
| Complement Component C5/C5a | Interleukin-10 (IL-10) | Platelet-derived growth factor (PDGF)-AB/BB |
| Complement Factor D | Interleukin-11 (IL-11) | Pentraxin 3/ Tumour Necrosis Factor-stimulated gene 14 (TSG-14) |
| C-Reactive Protein (CRP) | Interleukin-12 (IL-12) p70 | C-X-C Motif Chemokine Ligand 4 (CXCL4)/ Platelet factor 4 (PF4) |
| Cripto-1 | Interleukin-13 (IL-13) | Receptor for advanced glycation end products (RAGE) |
| Cystatin C | Interleukin-15 (IL-15) | C–C motif chemokine ligand 5 (CCL5)/ Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES) |

| | | |
|---|---|---|
| Dickkopf WNT signalling pathway inhibitor 1 (DKK-1) | Interleukin-16 (IL-16) | Retinol binding protein 4 (RBP4) |
| Dipeptidyl peptidase IV (DPPIV)/ Cluster of differentiation 26 (CD26) | Interleukin-17A (IL-17A) | Relaxin-2 |
| Epidermal growth factor (EGF) | Interleukin-18 Binding Protein a (IL-18 BPa) | Resistin |
| C-X-C Motif Chemokine Ligand 5 (CXCL5)/ Epithelial-derived neutrophil-activating peptide 78 (ENA-78) | Interleukin-19 (IL-19) | C-X-C Motif Chemokine Ligand 12 (CXCL12)/ Stromal cell-derived factor (SDF)-1 alpha |
| Endoglin/ Cluster of differentiation 105 (CD105) | Interleukin-22 (IL-22) | Serpin E1/ Plasminogen activator inhibitor-1 (PAI-1) |
| Extracellular matrix metalloproteinase inducer (EMMPRIN)/ Cluster of differentiation 147 (CD147) | Interleukin-23 (IL-23) | Sex hormone-binding globulin (SHBG) |
| Fas Ligand | Interleukin-24 (IL-24) | Suppression Of Tumorigenicity 2 (ST2)/ Interleukin-1 Receptor 4 (IL1 R4) |
| Fibroblast growth factor (FGF) basic | Interleukin-27 (IL-27) | C–C motif chemokine ligand 17 (CCL17)/ Thymus- and activation-regulated chemokine (TARC) |
| Keratinocyte growth factor (KGF)/ Fibroblast growth factor 7 (FGF-7) | Interleukin-31 (IL-31) | Trefoil Factor 3 (TFF3) |
| Fibroblast growth factor 19 (FGF-19) | Interleukin-32 (IL-32) alpha/beta/gamma | Transferrin receptor (TfR) |

| | | |
|---|---|---|
| Fms-Like Tyrosine Kinase 3 (FLT-3) Ligand | Interleukin-33 (IL-33) | Transforming growth factor (TGF)-alpha |
| Granulocyte colony stimulating factor (G-CSF) | Interleukin-34 (IL-34) | Thrombospondin-1 |
| Growth/Differentiation Factor-15 (GDF-15) | C-X-C motif chemokine ligand 10 (CXCL10)/ Interferon gamma-induced protein 10 (IP-10) | T-cell immunoglobulin 1 (TIM-1) |
| Granulocyte-macrophage colony-stimulating factor (GM-CSF) | C-X-C motif chemokine 11 (CXCL11)/ Interferon-inducible T-cell alpha chemoattractant (I-TAC) | Tumour Necrosis Factor (TNF)-alpha |
| C-X-C Motif Chemokine Ligand 1 (CXCL1)/ Growth-regulated oncogene (GRO) alpha | Kallikrein 3/ Prostate Specific Antigen (PSA) | Urokinase-type plasminogen activator receptor (uPAR) |
| Growth Hormone (GH) | Leptin | Vascular cell adhesion molecule 1 (VCAM-1) |
| Hepatocyte Growth Factor (HGF) | Leukaemia inhibitory factor (LIF) | Vascular endothelial growth factor (VEGF) |
| Intercellular Adhesion Molecule 1 (ICAM-1)/ Cluster of differentiation 54 (CD54) | Lipocalin-2/ Neutrophil gelatinase-associated lipocalin (NGAL) | Vitamin D binding protein |

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