Regulation of vascular calcification by syndecan 4

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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Abstract
A thesis submitted to The University of Manchester by Samantha Jayne Borland for the degree of Doctor of Philosophy entitled ‘Regulation of vascular calcification by syndecan 4’, September 2015.

Vascular calcification is the formation of mineralised tissue, bone and/or cartilage within blood vessels. It involves the osteogenic differentiation of vascular smooth muscle cells (VSMCs), VSMC apoptosis and extracellular matrix mineralisation. This pathology has the potential to be regulated by proteoglycans (PGs), which comprise a protein core and glycosaminoglycan (GAG) chains. This study tests the hypothesis that PGs regulate vascular calcification.

VSMCs were cultured with β-glycerophosphate (β-GP) to induce mineralisation. Osteogenic differentiation was confirmed by the up-regulation of Runx2 and Msx2, and down-regulation of αSMA and SM22α mRNA. During VSMC osteogenic differentiation and mineralisation, the mRNA expression levels of syndecan 1, biglycan, decorin, versican, osteoglycin and lumican mRNAs were down-regulated. In contrast, syndecan 4 mRNA expression was increased during the late stages of VSMC mineralisation. Syndecan 4 is a transmembrane PG, and knocking-down syndecan 4 expression using siRNA increased VSMC mineralisation. Previous studies have shown that syndecan 4 regulates fibroblast growth factor-2 (FGF-2) signalling, and FGF-2 mRNA and protein expression were increased during the late stages of VSMC mineralisation. Exogenous FGF-2 inhibited VSMC mineralisation, and this ability was lost in the absence of syndecan 4, suggesting that syndecan 4 is required for the inhibitory effect of FGF-2 on VSMC mineralisation. However, over-expressing syndecan 4 also increased VSMC mineralisation, suggesting that syndecan 4 levels must be finely regulated in VSMCs.

Syndecan 4 displays heparan sulphate (HS) and/or chondroitin/dermatan sulphate (CS/DS) GAG chains on its extracellular domain, and these GAGs can regulate FGF-2 signalling. The mRNA levels of specific CS/DS (C4ST1, C4ST2, DS epimerase-1, -2) biosynthetic enzymes were up-regulated in mineralising VSMCs. To correlate the glycomic transcription profile of mineralising VSMCs with the GAGs synthesised by these cells, 3H-GAGs were isolated from confluent and mineralising VSMCs and characterised using specific scission agents. The ratios of DS to CS and CS/DS 4-O-sulphation were increased in mineralising VSMCs. In contrast, there was no change in HS disaccharide composition. Knocking-down EXT1 (a crucial HS biosynthetic enzyme) expression using siRNA increased VSMC mineralisation and reduced FGF-2-induced Akt activation. The cytoplasmic domain of syndecan 4 also regulates FGF-2 signalling through its interaction with protein kinase Cα (PKCα). Inhibiting PKCα activity with G66976, or knocking-down PKCα expression using siRNA, increased VSMC mineralisation. The HS chains displayed on syndecan 4 (and possibly other PGs) and syndecan 4’s interaction with PKCα may therefore be required for the inhibitory effect of syndecan 4 on VSMC mineralisation.

Overall these studies suggest that syndecan 4 expression is increased in mineralising VSMC to maintain FGF-2 signalling, and in turn, slow down the mineralisation process.
Declaration

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Samantha Jayne Borland
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School of Medicine
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<th>Description</th>
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<tbody>
<tr>
<td>2-OST</td>
<td>2-O-sulphotransferase</td>
</tr>
<tr>
<td>3-OST</td>
<td>3-O-sulphotransferase</td>
</tr>
<tr>
<td>6-OST</td>
<td>6-O-sulphotransferase</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AMAC</td>
<td>2-aminoacridone</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C4ST</td>
<td>Chondroitin 4-O-sulfotransferase</td>
</tr>
<tr>
<td>C6ST</td>
<td>Chondroitin 6-O-sulfotransferase</td>
</tr>
<tr>
<td>CASK</td>
<td>Calcium/calmodulin-associated serine/threonine kinase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>ChSy-1</td>
<td>Chondroitin synthase-1</td>
</tr>
<tr>
<td>ChSy-2</td>
<td>Chondroitin synthase-2</td>
</tr>
<tr>
<td>ChSy-3</td>
<td>Chondroitin synthase-3</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
</tr>
<tr>
<td>cv</td>
<td>Column volume</td>
</tr>
<tr>
<td>CVC</td>
<td>Calcifying vascular cell</td>
</tr>
<tr>
<td>D4ST1</td>
<td>Dermatan 4-O-sulfotransferase 1</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylethanolamine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>dp</td>
<td>Degree of polymerisation</td>
</tr>
<tr>
<td>dp2</td>
<td>Disaccharide</td>
</tr>
<tr>
<td>dp4</td>
<td>Tetrasaccharide</td>
</tr>
<tr>
<td>dp6</td>
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<tr>
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<td>Dermatan sulphate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric acid synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin-radixin-moesin</td>
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<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>EXT</td>
<td>Exostosin</td>
</tr>
<tr>
<td>EXTL</td>
<td>Exostosin-like glycosyltranferase</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glucoronic acid</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylgulosamine</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
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HEK293T  Human embryonic kidney 293T
HexA  Hexuronic acid
HMO  Hereditary multiple osteochondroma
HRP  Horseradish peroxidase
HS  Heparan sulphate
HSPG  Heparan sulphate proteoglycan
HUVEC  Human umbilical cord endothelial cell
IdoA  Iduronic acid
IL-1β  Interleukin-1β
LB  Lysogeny broth
LDLR  Low-density lipoprotein receptor
MGP  Matrix Gla protein
MMP  Matrix metalloproteinase
MSC  Mesenchymal stem cell
Msx  Muscle segment homeobox
NaCl  Sodium chloride
NaP  Sodium phosphate
NDST  N-deacetylase N-sulphotransferase
NPP1  Nucleotide pyrophosphatase 1
Osx  Osterix
Ox-LDL  Oxidised low-density lipoprotein
PAPS  3’ phosphoadenosine-5’ phosphosulphate
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PDK1  3-phosphoinositide dependent protein kinase 1
PG  Proteoglycan
PI3K  Phosphoinositide 3-kinase
PIP₂  Phosphotidylinositol (4,5)-bisphosphate
PIP₃  Phosphatidylinositol (3,4,5)-trisphosphate
PKC  Protein kinase C
PLCγ  Phospholipase Cγ
PMA  Phorbol-12-myristate-13-acetate
qPCR  Quantitative polymerase chain reaction
R-Smads  Regulatory-Smads
RT  Room temperature
Runx2  Runt-related transcription factor 2
SAX-HPLC  Strong anion exchange-high performance liquid chromatography
SD  Standard deviation
SEM  Standard error of mean
siRNA  Small interfering RNA
SM22α  Smooth muscle 22α
SMC  Smooth muscle cell
SOC  Super optimal broth with catabolite repression
Sulf  Sulfatase
T2DM  Type 2 diabetes mellitus
TBST  Tris-buffered saline tween
TGF-β1  Transforming growth factor-β1
TNF  Tumour necrosis factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>TRPC6</td>
<td>Transient receptor potential cation channel 6</td>
</tr>
<tr>
<td>UA</td>
<td>Uronic acid</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine 5′diphosphate</td>
</tr>
<tr>
<td>UST</td>
<td>Uronyl 2-O-sulfotransferase</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>XT</td>
<td>Xylosyltransferase</td>
</tr>
<tr>
<td>αSMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>β-GP</td>
<td>β-glycerophosphate</td>
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1 Introduction

Vascular calcification is the formation of mineralised tissue, bone and/or cartilage within the vessel wall. Most patients with cardiovascular disease have some degree of calcification, although it is most prevalent in patients with chronic kidney disease (CKD), type 2 diabetes mellitus (T2DM) and atherosclerosis (Lehto et al., 1996; Nakayama et al., 2011). Vascular calcification is also associated with ageing and ~60% of 60-year olds will have some degree of calcification (Rennenberg et al., 2009). Calcification is not only highly prevalent in these diseases, but there is now substantial evidence that it contributes to the morbidity and mortality associated with these common conditions (Rennenberg et al., 2009; London et al., 2011).

The arterial wall is composed of three major layers: the tunica intima, tunica media and tunica adventitia (Figure 1.1A). Intimal calcification occurs within atherosclerotic plaques (Figure 1.1B). In this instance, calcification can increase the risk of plaque rupture, leading to stroke and myocardial infarction; however, this is highly dependent on the distribution of calcification within the plaque (Ehara et al., 2004). Medial calcification is associated with ageing, CKD and T2DM, and occurs along the elastic lamellae of the arterial wall media (Figure 1.1C). Calcification within the medial layer of the vessel wall can reduce aortic and arterial elasticity, which impairs cardiovascular haemodynamics and results in a significantly elevated risk of morbidity and mortality in the form of hypertension, lower limb amputation, cardiac hypertrophy and sudden cardiac death (Rennenberg et al., 2009; London et al., 2011). Intimal and medial calcification can occur independently of each other, but in patients with atherosclerosis and CKD, both intimal and medial calcification has been observed in the same arterial segment (Nakamura et al., 2009).

Calcific uremic arteriolopathy, also known as calciphylaxis, is a further clinical manifestation of medial calcification. This condition is a rare, but serious, complication of end-stage CKD and is characterised by medial calcification in the microvessels of the dermal, pulmonary and mesenteric vascular beds (Hayden et al., 2008). Calcification may also develop in the cardiac valve leaflets leading to calcific aortic stenosis (Yutzey et al., 2014).

Existing approaches for the prevention of vascular calcification are limited as the small number of drugs (e.g. bisphosphonates, EDTA chelation therapies) to enter clinical trials have had limited beneficial impact and/or their potentially harmful effects to bone health have outweighed their benefits (Wu et al., 2013). For this reason, attention has now turned to therapies that target the molecular mechanisms of calcification itself. One little explored potential target is the proteoglycan (PG) family of proteins and their associated glycosaminoglycan (GAG) chains, which is the focus of this thesis. GAGs are a family of long, unbranched polysaccharide chains that are made by almost all animal cells. Different disaccharide compositions characterise the 3 major $O$-linked sulphated GAGs (Figure 1.2): (1) hexuronic acid (HexA) (Glucuronic Acid (GlcA) or Iduronic Acid (IdoA)) and $N$-acetylglucosamine (GlcNAc) in heparan sulphate (HS); (2) GlcA
Figure 1.1. Calcification within the vessel wall. (A) Cross-section through a blood vessel demonstrates that the arterial wall is composed of three layers: the tunica intima, tunica media and tunica adventitia. The intima surrounds the vessel lumen, and consists of a single layer of endothelial cells that line the lumen of the vessel wall and the internal elastic lamina. The medial layer comprises the majority of the arterial wall; it is composed of smooth muscle cells and concentric regions of matrix consisting mainly of elastin (external elastic lamina). The adventitia is the outermost layer of the arterial wall, containing connective tissue and microvessels (vaso vasorum). Image not drawn to scale. (B-C) Histological sections of human arteries reveal calcification occurs within two sites of the vessel wall. (B) Non-atherosclerotic human artery stained with von kossa shows calcification in the medial layer (mineral stained dark purple, arrows). (C) Human artery stained with von Kossa shows calcification within the neointima of an atherosclerotic plaque (mineral stained black, arrows). Images in (B-C) were reprinted from (Sage et al., 2010) with permissions from Macmillan Publishers Ltd: Nat Rev Cardiol. (License no. 3699480516417).
Figure 1.2. Repeating disaccharide units in HS, CS and DS. The sugar backbone of HS is composed of repeating disaccharide units of GlcA and GlcNAc residues with varying proportions of IdoA. CS is composed repeating disaccharide units of GlcA and GalNAc. DS is closely related to CS, and its repeating disaccharide units contain IdoA instead of or in addition to GlcA. These sugar residues can be modified by the addition of a sulphate group at various carbon (C) positions as indicated by ‘S’. GalNAc, N-acetylgalactosamine; GlcA, GlcNAc, N-acetylglucosamine; Glucuronic Acid; IdoA, Iduronic Acid.

and N-acetylgalactosamine (GalNAc) in chondroitin sulphate (CS); (3) IdoA and GalNAc in dermatan sulphate (DS). In addition, 3 other classes of GAGs have been identified. Heparin is a highly sulphated form of HS secreted by mast cells on the PG core protein, serglycin, and is a commonly used anti-coagulant drug in the clinic. Keratan sulphate is a complex GAG that is composed of alternating galactose and GlcNAc units. Finally, hyaluronan is a non-sulphated GAG, synthesised at the plasma membrane, which is formed by a linear chain of repeating, unmodified disaccharides.

With the exception of hyaluronan, all GAGs exist as components of PGs, and PGs can be secreted into the ECM, inserted into the plasma membrane or stored within secretory granules. Several PGs have been identified which can be classified based on their GAG composition: (i) HS containing PGs (e.g. glypicans 1-6); (ii) CS/DS containing PGs (e.g. versican, decorin, biglycan); (iii) CS/DS and/or HS containing PGs (e.g. betaglycan; perlecan, syndecans 1-4); (iv) keratan sulphate containing PGs (e.g. osteoglycin, lumican). In PGs, anywhere between one (e.g. decorin) to over one hundred GAG chains (e.g. aggrecan) can be covalently linked to the core protein.

Several PGs and their GAG chains have previously been implicated in physiological bone formation and/or the regulation of signalling pathways that are important in this process (reviewed by (Rodgers et al., 2008)). Interestingly, several key findings suggest that vascular
calcification may involve many of the same processes as physiological bone formation. First, bone-like tissue has been identified in diseased vessels (Qiao et al., 2003), with 10-20% of human atherosclerotic vessels containing fully formed trabecular bone (Hunt et al., 2002). Second, detailed analyses of calcified lesions have identified the presence of hydroxyapatite, the principal mineral in bone (Schmid et al., 1980; Duer et al., 2008). Third, transcription factors that regulate osteoblast differentiation and mineralisation have been detected in human calcified blood vessels, including runt-related transcription factor 2 (Runx2, also referred to as Cbfa1), muscle segment homeobox-2 (Msx2) and osterix (Osx), suggesting vascular calcification is an active and cell-regulated process, and not a mere precipitation of mineral (Tyson et al., 2003; Moe et al., 2003; Shroff et al., 2008b; Shimizu et al., 2011). Indeed, non-mineral components of bone are also detected in calcified vessels, including osteoblast-like cells (Speer et al., 2009; Naik et al., 2012), as well as regulators of bone formation such as bone morphogenetic proteins (BMPs) and alkaline phosphatase (ALP) (Bostrom et al., 1993; Shanahan et al., 2000).

Given the similarities between bone formation and vascular calcification, it is intuitive to consider that GAGs and their PG cores may also play a role in regulating vascular calcification. Indeed, the PGs decorin (Fischer et al., 2004; Yan et al., 2011) and biglycan (Song et al., 2012) have been previously implicated in the regulation of vascular calcification (see section 1.2.5), but whether other PGs are involved in this process has not been explored in detail. In addition, GAGs have been identified as a major component of calcified atherosclerotic plaques (Duer et al., 2008), but the roles of GAGs in vascular calcification are also largely unexplored. Therefore, this Introduction will initially review vascular calcification and how it is regulated, and will then introduce PGs and their GAG chains, and how they may modulate this pathology.

1.1 Mechanisms of vascular calcification

Vascular calcification can be characterised by several key events including the differentiation of vascular smooth muscle cells (VSMCs), VSMC apoptosis, calcifying matrix vesicle release and extracellular matrix (ECM) degradation, which will be discussed in detail below.

1.1.1 Osteogenic differentiation of VSMCs

One of the major features of vascular calcification is the differentiation of cells within the vessel wall to an osteo/chondrocyte-like phenotype and the deposition of a matrix that becomes calcified; however, the order in which these two events occur has recently become an area of controversy (discussed further below). The origin of osteo/chondrocyte-like cells in atherosclerotic and medial calcification has been determined using lineage tracing in vivo. For this, the LacZ transgene was inserted into mouse VSMCs using the smooth muscle 22α (SM22α)-Cre promoter; calcified aortic sections were then stained with X-gal to demonstrate which osteo/chondrocyte-like cells had once expressed LacZ (i.e. osteoblasts/chondrocytes which originated as VSMCs). In calcified aortic sections from matrix Gla protein (MGP)-null mice (a model of medial calcification), 97% of the osteoblast/chondrocyte-like cells present within the lesions had once exhibited a SMC phenotype (Speer et al., 2009). In contrast, only 75-88% of osteo/chondrocyte-like cells were derived from SMCs in calcified atherosclerotic sections from apolipoprotein E (ApoE)-null mice fed a high-fat diet (Naik et al., 2012). The greater contribution
of other cell types in osteo/chondrogenesis in atherosclerotic, compared to medial, calcification may account for this discrepancy. Indeed, a variety of cell types in the vessel wall are capable of differentiating into an osteoblast-like phenotype, including vascular pericytes (Collett et al., 2003), multi-potent vascular stem cells (Tang et al., 2012), endothelial cells (Cheng et al., 2013; Yao et al., 2013), adventitial myofibroblasts (Shao et al., 2005; Al-Aly et al., 2007) and circulating progenitors from bone marrow (Naik et al., 2012). However, as demonstrated in the lineage tracing studies described above, the majority of osteo/chondrocyte-like cells in calcified blood vessels originate from VSMCs; this cell type will therefore be the focus of this thesis.

VSMCs reside in the medial layer of blood vessels and exhibit a contractile phenotype that is characterised by the expression of high levels of contractile proteins, including α-smooth muscle actin (αSMA) and SM22α (Shanahan et al., 1993). In response to injury, these contractile VSMCs differentiate into a proliferative, synthetic state which is characterised by the expression of lower levels of contractile proteins (Rzucidlo et al., 2007). Therefore, in the adult, VSMCs retain phenotypic plasticity rather than being terminally differentiated. Indeed, when cultured in the presence of raised phosphate levels (>2 mM) in vitro, VSMCs differentiate into osteoblast-like cells and deposit a matrix that becomes calcified (Shioi et al., 1995; Steitz et al., 2001; Son et al., 2006; Collett et al., 2007; Son et al., 2007; Alam et al., 2009), similar to what is observed in vivo (see above). Recent studies have suggested that although VSMCs express osteoblast-related genes when cultured in osteogenic-inducing medium, the gene expression profile of these cells is distinguishable from true osteoblasts (Alves et al., 2014); hence they are referred to as osteoblast-like cells. VSMCs can also undergo chondrogenic differentiation (Neven et al., 2010; Tyson et al., 2003); however, this aspect of calcification is not the focus of this thesis will not be further discussed in this Introduction.

The osteoblast-like cells located in calcified blood vessels may also arise from a sub-population of VSMCs, termed by Watson et al. (1994) as CVCs. These cells are thought to represent approximately 30% of total VSMCs (Watson et al., 1994), and can be distinguished by the expression of the pericyte marker 3G5 (Bostrom et al., 1993). In vitro, CVCs spontaneously undergo osteogenic differentiation and form mineralised nodules (Bostrom et al., 1993), in contrast to the ‘hill and valley’ morphology exhibited by mineralising VSMCs (Collett et al., 2007; Alam et al., 2009). Therefore, CVCs may be more primitive than normal VSMCs. Indeed, further studies have shown that CVCs express similar markers to mesenchymal stem cells (MSCs), and they can be induced to differentiate along the chondrogenic, leiomyogenic and stromogenic lineages in vitro (Tintut et al., 2003), suggesting that CVCs may be more closely related to MSCs than VSMCs.

It has been reported that only 20-40% of cloned CVC populations form nodules that become mineralised (Watson et al., 1994). It is not known why some SMC preparations deposit a mineralised matrix, while others do not, although it has been reported that only 204 out of 398 bovine VSMC preparations stained positive for ALP (Jono et al., 1997). ALP modulates calcification by catalysing the breakdown of pyrophosphate, which potently inhibits vascular calcification by preventing the formation of hydroxyapatite (Lomashvili et al., 2004). ALP also increases the levels of free phosphate, thereby promoting VSMC osteogenic differentiation and
mineralisation (Shanahan et al., 2011). In addition to ALP, it has also been reported that different levels of BMP-2 and MGP can differentially regulate human CVC calcification. In this regard, CVC calcification is inhibited by MGP in preparations of cells with high expression of BMP-2 relative to MGP, but increased in preparations of cells with low expression of BMP-2 relative to MGP (Zebboudj et al., 2003). Together these studies suggest that different levels of MGP, BMP-2 and ALP (or other unknown proteins) in VSMCs/CVCs may regulate their ability to undergo osteogenic differentiation and mineralisation.

It was originally proposed that the deposition of a mineralised matrix by VSMCs is preceded, or occurs simultaneously, with the differentiation of these cells. However, evidence is now accumulating to suggest that this may not always be the case. For example, MGP-null mice develop extensive and rapid calcification within 9 days of age, but only express chondrogenic markers (aggrecan, Sox9) 5 days after the onset of calcification (Khavandgar et al., 2014). Moreover, there is no change in the mRNA expression levels of osteogenic markers (Runx2, Osx) in the aortas before, or after, the onset of calcification in MGP-null mice (Khavandgar et al., 2014). It has also been reported that there is no change in osteogenic and SMC marker expression during human VSMC mineralisation *in vitro* (Borland & Canfield, unpublished findings) (Alves et al., 2014), and there is no evidence for VSMC osteogenic differentiation in calcified breast arteries from uraemic women (O’Neill and Adams, 2014). Together these studies suggest that VSMC osteogenic differentiation may in fact occur as a consequence of mineralisation, and in some instances, VSMC osteogenic differentiation may not even be required for the deposition of a mineralised matrix in the vessel wall. Consistent with the former hypothesis, exposure to hydroxyapatite induces the osteogenic differentiation of mouse VSMCs (Sage et al., 2011).

### 1.1.2 Markers of VSMC osteogenic differentiation

In normal bone development, the co-ordinated actions of developmental programs mediate the osteogenic differentiation of MSCs, which is characterised by the up-regulation of Runx2, Msx2 and Osx. When cultured in high phosphate levels or β-GP, VSMCs lose their characteristic SMC markers (αSMA, SM22α) and develop osteoblastic features by up-regulating Runx2, Msx2 and Osx expression (Steitz et al., 2001; Dhore et al., 2001; Tyson et al., 2003; Speer et al., 2009; Osako et al., 2010), similar to what is observed in human calcified blood vessels (Tyson et al., 2003; Moe et al., 2003; Shroff et al., 2008b; Shimizu et al., 2011). These osteogenic transcription factors are therefore used to indicate the differentiation of VSMCs into osteoblast-like cells, as will be discussed further below.

#### (i) Runx2

Runx2 is the master regulator of bone development and is an early marker of osteogenic differentiation (Otto et al., 1997). Runx2 is barely detectable in healthy blood vessels, but its expression is markedly increased in areas of calcification within the vessel wall (Tyson et al., 2003; Moe et al., 2003). The expression of Runx2 is also very low in quiescent VSMCs, but its expression is up-regulated prior to the onset of matrix mineralisation (Speer et al., 2009), further suggesting Runx2 may be an important regulator of vascular calcification. Indeed, it has been
shown using small interfering RNA (siRNA) that Runx2 is necessary for phosphate-induced mineralisation of VSMCs in vitro (Speer et al., 2009; Zhou et al., 2014), and SMC-specific deletion of Runx2 in ApoE-null mice inhibits high-fat diet-induced vascular calcification (Sun et al., 2012). Runx2 exerts its effects on mineralisation by controlling the expression of several osteogenic transcription factors and markers including ALP and osteocalcin (Figure 1.3).

**Figure 1.3. Schematic overview of the transcription factors involved in VSMC osteogenic differentiation.** This figure is limited to interactions related to the studies presented in this thesis. Wnt signals may originate from Msx2-expressing cells of the tunica adventitia (e.g. myofibroblasts). Figure based on information from (Nakashima et al., 2002; Shao et al., 2005; Al-Aly et al., 2007; Matsubara et al., 2008; Shanahan et al., 2011; Taylor et al., 2011).

Whilst Runx2 is required for vascular calcification in vitro and in vivo, smooth muscle-specific over-expression of Runx2 does not induce spontaneous vascular calcification in mice (Raaz et al., 2015). These data could importantly suggest that whilst Runx2 mediates the osteogenic differentiation and mineralisation of VSMCs, other factors must also be present (e.g. high phosphate) for this event to proceed.

**(ii) Osx**

Osx is a zinc finger-containing transcriptional regulator of osteoblast differentiation that is expressed in developing bones and is essential for embryonic bone development (Nakashima et al., 2002). It has been suggested that Osx functions downstream of Runx2, as Runx2-null mice do not express Osx (Nakashima et al., 2002) and re-introduction of Runx2 into Runx2-deficient MSCs induces Osx expression (Matsubara et al., 2008). Osx regulates the expression and/or activity of several osteoblast-related genes including osteocalcin and ALP; however Osx can regulate the expression of these genes in Runx2-null MSCs suggesting a Runx2-independent pathway may also exist in osteogenesis (Matsubara et al., 2008) (discussed further in section 1.1.2ii). Osx may also regulate the osteogenic differentiation of VSMCs as Osx expression is increased in human calcified blood vessels (Shroff et al., 2008b), and it has been
detected during the osteogenic differentiation of VSMCs *in vitro* (Taylor et al., 2011). However, the direct role of Osx in VSMC osteogenic differentiation is currently unknown.

(iii) **Msx2**

The pro-calcific factor, BMP-2, is capable of inducing Osx expression in the absence of Runx2 (Matsubara et al., 2008), suggesting the presence of a Runx2-independent pathway in osteogenesis. It is thought that this Runx2-independent pathway is mediated by Msx2 (Matsubara et al., 2008) (Figure 1.3), an osteogenic transcription factor that is essential for normal bone development (Satokata et al., 2000). Msx2 expression is up-regulated in human calcified blood vessels (Tyson et al., 2003; Shimizu et al., 2011) and mineralising VSMCs (Osako et al., 2010), suggesting Msx2 may also have a role in vascular calcification. Indeed, oxidised low-density lipoproteins (Ox-LDLs) enhance the osteogenic differentiation of VSMCs in an Msx2/Osx-dependent manner (Taylor et al., 2011), and SMC-specific over-expression of Msx2 significantly increases the number of calcified coronary artery lesions in low-density lipoprotein receptor (LDLR)$^{-/-}$ mice, which develop diabetes and vascular calcification when fed a high-fat diet (Shao et al., 2005).

Msx2 may regulate VSMC osteogenic differentiation and mineralisation via several different mechanisms, although this may be dependent on the stimulatory factors present. For example, Msx2 induces ALP activity in bovine VSMCs, which may occur through stimulation of Osx expression (Taylor et al., 2011). It has also been demonstrated that Wnt/β-catenin signalling is a downstream target of BMP-2-induced Msx2 activation. Specifically, Msx2 up-regulates the expression of Wnt7a, Wnt3a, Wnt5a and down-regulates the LRP5/6 inhibitor, Dkk1 in adventitial myofibroblasts. Paracrine Wnt signals elaborated by Msx2-expressing cells of the tunica adventitia then program the osteogenic differentiation and mineralisation of VSMCs in the tunica media via nuclear localisation of β-catenin and consequent activation of type I collagen expression and ALP activity (Shao et al., 2005; Al-Aly et al., 2007).

In contrast to the effects of over-expressing Msx2 in vascular calcification (Shao et al., 2005), SMC-specific knock-down of Msx2 expression in LDLR-null mice has no effect on the development of high-fat diet-induced vascular calcification when compared to controls (Cheng et al., 2014). Together these data suggest that increases in Msx2 expression mediate the osteogenic differentiation and mineralisation of VSMCs, but this process cannot be inhibited by the removal of Msx2. It is possible that knocking-down Msx2 expression *in vivo* may be compensated for by a second muscle segment homeobox gene, Msx1. Consistent with this hypothesis, SMC-specific knock-down of both Msx1 and Msx2 expression in LDLR-null mice inhibited high-fat diet-induced vascular calcification (Cheng et al., 2014). Strategies that block both Msx1 and Msx2 function may, therefore, be effective in the treatment of vascular calcification, although this must be approached with caution as Msx1/Msx2 double knock-out mice die during embryogenesis due to profound deficiencies in the development of structures derived from the cranial and cardiac neural crest (Ishii et al., 2005).

Overall these studies demonstrate that Runx2, Osx and Msx2 mediate the osteogenic differentiation and mineralisation of VSMCs (Figure 1.3), although over-expression of Runx2 or
Msx2 does not induce spontaneous calcification on their own (Shao et al., 2005; Raaz et al., 2015). Other factors (e.g. high phosphate, VSMC apoptosis, matrix vesicle release) must therefore be present for calcification to occur.

1.1.3 VSMC apoptosis and Akt

VSMC apoptosis is another key event in the calcification cascade (Proudfoot et al., 2000; Son et al., 2006; Collett et al., 2007; Clarke et al., 2008). Apoptotic bodies are small (~0.3 µm to 1 µm in diameter), membrane-bound vesicles that bud off from the membranes of apoptotic cells, and when released from VSMCs, they act as a source of calcium and phosphate. These apoptotic bodies thereby provide a microenvironment suitable to initiate crystal formation, and apoptotic bodies have been detected in the areas surrounding calcifying VSMCs in vitro (Proudfoot et al., 2000) and in human calcified blood vessels (Shroff et al., 2008b). VSMC apoptosis appears to play a pro-calcific role in the calcification cascade as stimulation of apoptosis with anti-Fas IgM increases VSMC mineralisation in vitro (Proudfoot et al., 2000), whereas inhibition of VSMC apoptosis with the general caspase inhibitor, zVAD.fmk, reduces calcification in vitro (Proudfoot et al., 2000; Collett et al., 2007; Son et al., 2006) and ex vivo (Mune et al., 2009). Chronic stimulation of VSMC apoptosis in ApoE-null mice also increases the number of calcified vascular lesions when compared to controls (Clarke et al., 2008).

Several studies have suggested that growth arrest-specific gene 6 (Gas6) may be an important regulator of VSMC apoptosis in vascular calcification. During high phosphate-induced VSMC mineralisation in vitro, the expression of Gas6 and its receptor Axl are decreased (Collett et al., 2007; Son et al., 2006). The Gas6-Axl pathway inhibits VSMC apoptosis through phosphoinositide 3-kinase (PI3K)-Akt signalling, and over-expressing Axl in VSMCs inhibits matrix mineralisation in vitro (Son et al., 2006; Son et al., 2007; Collett et al., 2007). Akt regulates a variety of cellular processes including apoptosis and proliferation (reviewed in (Manning and Cantley, 2007)). Akt contains a pleckstrin homology (PH) domain which binds to PI3K-generated phospha tidylinositol (3,4,5)-trisphosphate (PIP3) in the plasma membrane with high affinity (Franke et al., 1997). Akt can then be phosphorylated at threonine 308 (Thr308) and serine 473 (Ser473) by 3-phosphoinositide dependent protein kinase 1 (PDK1) and mTOR complex 2 (mTORC2), respectively (reviewed in (Manning and Cantley, 2007)).

Akt regulates apoptosis via several possible mechanisms. For example, activation of Akt results in the phosphorylation and activation of the anti-apoptotic protein, Bcl-2 (Ruvolo et al., 2001). In contrast, Akt-induced phosphorylation of the pro-apoptotic protein, Bad, can result in its inactivation and prevents it from binding to Bcl-2 (Zha et al., 1996; del Peso et al., 1997). In vascular calcification, phosphate down-regulates Gas6-Axl, leading to reduced PI3K-mediated Akt phosphorylation, and increased VSMC apoptosis (Son et al., 2007). Statins restore Gas6-Axl signalling and Akt phosphorylation, and can therefore inhibit high phosphate-induced VSMC apoptosis and mineralisation in vitro (Son et al., 2006; Son et al., 2007). Overall these studies suggest that PI3K/Akt signalling plays an inhibitory role in vascular calcification, and approaches to restore this signalling pathway may be an effective treatment for this condition.
1.1.4 Matrix vesicles

Matrix vesicles are small (30-300 nM in diameter), membrane-bound structures that pinch-off from the plasma membrane of VSMCs, and have been detected in calcified blood vessels (Kapustin et al., 2011; New et al., 2013; Kapustin et al., 2015). It was originally believed that matrix vesicle release was as an adaptive response by VSMCs to mineral imbalance, which aimed to remove excess intracellular calcium and phosphate from the cell and prevent cell death (Shroff et al., 2010). However, the composition of these matrix vesicles is altered in calcifying VSMCs and they can provide a microenvironment in which hydroxyapatite formation can occur (Reynolds et al., 2004; Reynolds et al., 2005; Kapustin et al., 2011; Shroff et al., 2010; Kapustin et al., 2015).

Under normal conditions, matrix vesicles are prevented from inducing mineralisation due to the presence of fetuin-A (Reynolds et al., 2004; Reynolds et al., 2005) and MGP (Kapustin et al., 2011), both of which are potent inhibitors of vascular calcification (Table 1.1). These mineralisation inhibitors are down-regulated in matrix vesicles released from calcifying VSMCs, leading to calcification (Reynolds et al., 2004; Reynolds et al., 2005; Kapustin et al., 2011). Mineralisation-competent matrix vesicles also contain elevated levels of ALP (Chen et al., 2008) and annexin VI (Chen et al., 2008; Kapustin et al., 2011). Annexin VI has been shown to mediate calcium uptake into matrix vesicles, and knocking-down annexin VI expression reduces VSMC mineralisation in vitro (Chen et al., 2008; Kapustin et al., 2011). Together these studies suggest that matrix vesicles play an essential role in vascular calcification, and as their production can occur prior to matrix mineralisation (Reynolds et al., 2004), these vesicles may initiate this process.

1.1.5 ECM degradation

ECM degradation is another key event in vascular calcification (reviewed in (Pai and Giachelli, 2010)), and is largely mediated by specific matrix metalloproteinases (MMPs) (Chen et al., 2011; Aoshima et al., 2012). MMP-2 and MMP-9 are gelatinases that degrade elastin (as well as other proteins such as PGs, see section 1.3.1ii), and MMP-2 and MMP-9 expression and/or activity is increased during vascular calcification (Chen et al., 2011; Kapustin et al., 2011; Pai et al., 2011). These MMPs are thought to accelerate vascular calcification by increasing the degradation of elastin (Chen et al., 2011) and by releasing the pro-calcific protein, TGF-β1, from the ECM (see section 1.2.2ii). Indeed, the soluble elastin-derived peptide α-elastin has been shown to accelerate high phosphate-induced mineralisation in human VSMCs in vitro (Hosaka et al., 2009), and deficiency of cathepsin S (an elastolytic enzyme) abolished atherosclerotic plaque calcification in uraemic ApoE-null mice (Aikawa et al., 2009). In humans, disruption of the internal elastic lamina in calcified elastic arteries from uremic patients has also been observed (Ibels et al., 1979).

Whilst elastin degradation appears to play a pro-calcific role in vascular calcification, α-elastin did not stimulate the deposition of a mineralised matrix by human VSMCs under normal phosphate load (1.4 mM) in vitro (Hosaka et al., 2009). Therefore, a phosphate stimulus may be required in conjunction with elastin degradation to exert pro-calcific effects.
<table>
<thead>
<tr>
<th>Regulatory factor</th>
<th>Role(s) in vascular calcification</th>
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<tbody>
<tr>
<td>BMP-2, BMP-4</td>
<td>BMP-2 (Li et al., 2008; Liberman et al., 2011) and BMP-4 (Yao et al., 2009) promote osteogenic differentiation and/or mineralisation of VSMCs. Inhibiting vascular BMP-2/-4 activity with MGP over-expression reduces aortic mineral deposition in ApoE-null mice fed a high fat diet (Yao et al., 2010a).</td>
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<tr>
<td>TGF-β1</td>
<td>Promotes the osteogenic differentiation and mineralisation of VSMCs (Kanno et al., 2008; Wang et al., 2010); the mechanism by which TGF-β1 exerts this effect is currently unknown.</td>
</tr>
<tr>
<td>TNF</td>
<td>TNF accelerates VSMC mineralisation (Tintut et al., 2000; Son et al., 2008; Aoshima et al., 2012; Masuda et al., 2013). The anti-TNF monoclonal antibody, infliximab, reduces high fat diet-induced aortic calcification in LDLR-null mice (Al-Aly et al., 2007), and ApoE-null mice with CKD (Masuda et al., 2013).</td>
</tr>
<tr>
<td>ALP</td>
<td>ALP activity is an early marker of osteogenic differentiation and is an inducer of calcification. ALP catalyses the breakdown of pyrophosphate (Lomashvili et al., 2004), a potent inhibitor of calcium-phosphate deposition. ALP also increases the levels of free phosphate, thereby promoting VSMC osteogenic differentiation and mineralisation (Shanahan et al., 2011).</td>
</tr>
<tr>
<td>Calcium</td>
<td>Alterations in calcium homeostasis trigger the generation of mineralisation-competent matrix vesicles by VSMCs (Kapustin and Shanahan, 2012). Elevated calcium levels also promote vascular calcification by stimulating VSMC apoptosis and ER stress (reviewed in (Shanahan et al., 2011)).</td>
</tr>
<tr>
<td>Phosphate</td>
<td>CKD patients typically present with hyperphosphataemia (&gt;2 mM), and serum levels of phosphate correlate with the extent and severity of vascular calcification (Goodman et al., 2000; Shigematsu et al., 2003). Raised phosphate levels promote VSMC osteogenic differentiation and mineralisation (Steitz et al., 2001; Dhore et al., 2001; Tyson et al., 2003; Speer et al., 2009), ECM degradation (Kapustin et al., 2011; Chen et al., 2011) and VSMC apoptosis (Son et al., 2006; Son et al., 2007).</td>
</tr>
<tr>
<td>BMP-7</td>
<td>Inhibits osteogenic differentiation of VSMCs in vitro (Kang et al., 2010) and in vivo (Davies et al., 2003; Davies et al., 2005), likely by promoting the smooth muscle phenotype of these cells (Dorai et al., 2000).</td>
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<tr>
<td>MGP</td>
<td>MGP-null mice develop spontaneous vascular calcification (Luo et al., 1997). MGP inhibits calcification by preventing the growth of hydroxyapatite crystals (Lomashvili et al., 2004), and through the inhibition of BMP-2 (Zebboudj et al., 2003).</td>
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<tr>
<td>Fetuin-A</td>
<td>Fetuin-A null mice develop spontaneous soft tissue calcification (Jahnen-Dechent et al., 1997). Fetuin-A inhibits calcification by binding calcium phosphate crystals and promoting their removal (Heiss et al., 2008).</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>Pyrophosphate treatment of rats with CKD and vascular calcification significantly reduces calcified lesions (O’Neill et al., 2011). Mice lacking expression of the pyrophosphate synthase, NPP1, spontaneously develop vascular calcification by two months of age (Lomashvili et al., 2014), and mutations in ENPP1 (gene encoding NPP1) cause generalised arterial calcification of infancy (Rutsch et al., 2008). Pyrophosphate inhibits hydroxyapatite crystal formation (Fleisch et al., 1966).</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Osteoblast marker and an inducible inhibitor of calcification in its phosphorylated form (Steitz et al., 2002; Speer et al., 2005). The mechanism by which osteopontin exerts its effect has not yet been fully elucidated, although it has been reported that phosphorylated osteopontin directly associates with apatite deposits and blocks crystal growth (Steitz et al., 2002).</td>
</tr>
</tbody>
</table>

Promoters are shown in red, and inhibitors of vascular calcification are shown in blue. ALP, alkaline phosphatase; BMP, bone morphogenetic protein; CKD, chronic kidney disease; ER,
1.2 Regulation of vascular calcification

The initiation and development of vascular calcification is regulated by a wide range of factors, including metabolic imbalances, inflammation and genetic mutations (Sage et al., 2010; Shanahan et al., 2011; Demer and Tintut, 2014). The balance between stimulatory and inhibitory factors normally prevents the development of vascular calcification, but this balance can be lost in disease, resulting in the deposition of a mineralised matrix in the vessel wall. In this section, the role of phosphate, inflammation, BMPs, and fibroblast growth factors (FGFs) in the pathogenesis of vascular calcification will be discussed, as these factors are the most relevant to this thesis. PGs that have been previously implicated in vascular calcification will also be reviewed. Some of the other factors that regulate vascular calcification are summarised in Table 1.1.

1.2.1 Phosphate

Elevated phosphate is known to be a crucial risk factor and mediator of vascular calcification (Shanahan et al., 2011). CKD patients typically present with hyperphosphataemia (>2 mM), and serum levels of phosphate have been shown to correlate with the extent and severity of vascular calcification (Goodman et al., 2000; Shigematsu et al., 2003). Culturing isolated bovine VSMCs in the presence of the phosphate donor, \( \beta \)-glycerophosphate (\( \beta \)-GP), is a widely used \textit{in vitro} model of vascular calcification (Shioi et al., 1995; Collett et al., 2007; Alam et al., 2009). \( \beta \)-GP is broken down by ALP to release inorganic phosphate, a major promoter of vascular calcification (Shanahan et al., 2011), leading to the formation of calcified, multi-cellular areas within the culture dish. Human VSMCs can also be induced to differentiate along the osteogenic lineage when cultured in the presence of elevated phosphate levels (2.6 mM) (Son et al., 2006; Son et al., 2007), or elevated calcium levels (2.6 mM) and \( \beta \)-GP (Yan et al., 2011). With time in culture, calcifying human VSMCs aggregate locally to form bone-like nodules, in a manner similar to that observed \textit{in vivo}.

High phosphate conditions enhance the uptake of phosphate by VSMCs through the type III sodium-dependent phosphate co-transporters, Pit-1 and Pit-2 (Jono et al., 2000; Villa-Bellosta et al., 2007). Pit-1 appears to play a crucial role in the development of vascular calcification, as knocking-down its expression in VSMCs inhibited phosphate-induced mineralisation and up-regulation of Runx2 (Li et al., 2006). However, SMC-specific knock-down of Pit-1 had no effect on the development of vascular calcification in a mouse model of CKD (Crouthamel et al., 2013). It is possible that Pit-2 may compensate for reductions in VSMC Pit-1 as high phosphate-induced mineralisation was restored in Pit-1-null VSMCs when Pit-2 was over-expressed, and knocking-down Pit-2 expression in Pit-1-null VSMCs decreased high phosphate-induced mineralisation \textit{in vitro} (Crouthamel et al., 2013). Further work is required to determine if SMC-specific deletion of both Pit-1 and Pit-2 reduces vascular calcification \textit{in vivo}.
Elevated phosphate can regulate VSMC osteogenic differentiation and mineralisation via several different mechanisms (Shanahan et al., 2011). For example, high phosphate (3.3 mM) increases the methylation of the SM22α promoter increases in human VSMCs and rat aortic rings. Methylation results in the loss of SM22α expression, which can be reversed by the inhibition of DNA methylation using the anti-arrhythmic drug, procaine (Montes de Oca et al., 2010). Elevated phosphate also promotes ECM degradation through increased expression and activity of MMP-2 (Kapustin et al., 2011; Chen et al., 2011), and it can induce VSMC apoptosis through the down-regulation of the anti-apoptotic protein, Gas6 (Son et al., 2006; Son et al., 2007); both of these events lead to calcification of the vessel wall.

Although elevated phosphate levels clearly play a role in the development of vascular calcification, it has been suggested that phosphate levels alone cannot explain the accelerated mineralisation observed in CKD patients. In this regard, treatment of cultured VSMCs with uraemic serum isolated from CKD patients increases osteogenic differentiation and mineralisation of VSMCs, but this effect was independent of changes in phosphate concentration (Chen et al., 2002; Moe et al., 2003). Together these studies suggest that elevated phosphate levels, in addition to other modulatory factors, are involved in the development of vascular calcification.

1.2.2 Inflammation

Inflammation is commonly associated with vascular calcification in atherosclerosis, although it also has a role in T2DM and CKD (Shao et al., 2010). Recent studies have demonstrated that inflammation co-localises with atherosclerotic plaque calcification (Aikawa et al., 2007; Abdelbaky et al., 2013), and a variety of inflammatory cells including macrophages localise to sites of calcification in the vessel wall (Kirsch et al., 2015). These inflammatory cells release a range of inflammatory proteins that are capable of accelerating vascular calcification, including tumour necrosis factor (TNF) (Tintut et al., 2000; Son et al., 2008; Aoshima et al., 2012; Masuda et al., 2013), interleukin-1β (IL-1β) (Parhami et al., 2002) and TGF-β1 (Watson et al., 1994; Kanno et al., 2008; Wang et al., 2010). Macrophages also release ECM-degrading MMPs and mineralisation-inducing matrix vesicles (New et al., 2013), both of which lead to calcification of the vessel wall.

Whilst inflammation clearly plays a role in vascular calcification, it is currently unknown whether inflammation is a cause or consequence of this process. Macrophage infiltration precedes the formation of osteoblast-like cells in a mouse model of atherosclerosis (Aikawa et al., 2007), although the inflammatory proteins released by these macrophages also promote calcification in the vessel wall (see above). It is therefore possible that a positive feedback loop may occur; in this regard, inflammation stimulates mineral deposition, and in turn, mineral deposition enhances the infiltration of inflammatory cells and production of inflammatory proteins within the vessel wall. Indeed, calcium phosphate crystals dose-dependently stimulate the release of TNF and IL-1β from human macrophages in vitro (Nadra et al., 2005).
(i) **TNF**

The inflammatory protein, TNF, has been long regarded as an important regulator of vascular calcification. *In vitro*, TNF accelerates VSMC mineralisation (Tintut et al., 2000; Son et al., 2008; Aoshima et al., 2012; Masuda et al., 2013), and it is also drives the osteo/chondrogenic differentiation programme in precursor cells located in the vessel wall (Al-Aly et al., 2007). Given the role of TNF in vascular calcification, it is intuitive to consider that blocking the activity of TNF could inhibit this process. Consistent with this suggestion, the anti-TNF monoclonal antibody, infliximab, reduced high-fat diet-induced aortic calcification in LDLR-null mice (Al-Aly et al., 2007), and it also reduced calcification in ApoE-null mice with CKD (Masuda et al., 2013).

TNF may regulate VSMC mineralisation via several possible mechanisms. For example, TNF has been shown to activate the Msx2-Wnt/β-catenin signalling pathway, which stimulates osteo/chondrogenic differentiation of VSMCs (Al-Aly et al., 2007). TNF can also reduce the expression of the anti-apoptotic protein, Gas6 (Son et al., 2008), thereby promoting VSMC apoptosis and mineralisation. Together these studies suggest that TNF plays a pro-calcific role in the development of vascular calcification.

(ii) **TGF-β1**

Three TGF-β isoforms (TGF-β1, 2 and 3) are present in mammals; however the TGF-β1 isoform is the most prevalent (Guo and Chen, 2012). A role for TGF-β1 in the pathogenesis of vascular calcification was first suggested by Watson et al. who reported a dramatic increase in nodule formation following treatment of bovine CVCs with this growth factor (Watson et al., 1994). This result was recently verified in rat VSMCs (Wang et al., 2010). In light of this evidence, it is intuitive to consider that blocking TGF-β1 signalling could inhibit vascular calcification. Consistent with this suggestion, a neutralising monoclonal antibody against the TGF-β1 type I receptor attenuated the expression of osteogenic differentiation markers in VSMCs cultured in osteogenic medium (Kanno et al., 2008; Wang et al., 2010) and chemical inhibition of the TGF-β type 1 receptor attenuated high phosphate and Ox-LDL-induced mineralisation of human VSMCs (Yan et al., 2011). The mechanism by which TGF-β1 modulates this process, however, remains unclear.

1.2.3 **BMPs**

Long known to induce ectopic bone formation when injected subcutaneously in mice (Wozney et al., 1988), BMPs (specifically BMP-2, -4, -7) are now also recognised as critical mediators of vascular calcification. The BMPs modulate gene transcription via Smad-dependent (canonical) and Smad-independent (non-canonical) signalling pathways (reviewed in (Cai et al., 2012)). In canonical BMP signalling, BMPs bind a heterodimeric complex consisting of two types of receptor serine/threonine (Ser/Thr) protein kinases, known as the BMP type I and type II receptors. BMP binding to the type II receptor results in the recruitment and phosphorylation of the type I receptor, which in turn initiates intracellular signalling by phosphorylating the regulatory-Smad proteins (R-Smads), Smad1, Smad5 and Smad8 (Smads1/5/8). Once activated, Smad1/5/8 form heteromeric complexes with the common mediator (co)-Smad4 and translocate to the nucleus where they modulate target gene expression by directly binding to
Smad-binding elements, or indirectly through interactions with DNA-binding co-factors, co-activators and co-repressors (Figure 1.4).

BMP signalling is regulated at several points throughout the pathway (Figure 1.4). Several diffusible antagonists, including noggin (Zimmerman et al., 1996) and MGP (Table 1.1), bind BMPs and block their interaction with signalling receptors. BMP signalling is further negatively regulated by the inhibitory Smads (Smad6, Smad7), which recruit the Smad ubiquitin ligase also regulate BMP signalling, although this is highly dependent on the cellular context and sulphation patterning of GAG chains (discussed further in section 1.5.1).

Figure 1.4. Schematic overview of the BMP signalling pathway. BMP ligands bind to BMP type II receptors, leading to the recruitment and phosphorylation of the BMP type I receptor. After being activated by the BMP type II receptor, the BMP type I receptor phosphorylates (P) and activates Smad1/Smad5/Smad8 (R-Smads) which form a heteromeric complex with Smad4 to propagate the signal into the cell. BMP signalling is regulated by Smad6/Smad7 (I-Smads) and extracellular regulators such as matrix Gla protein (MGP) and noggin. Figure adapted from (Cai et al., 2012).
**BMP-2/-4**

BMP-2 is a potent osteoinductive factor and its expression was first reported in human calcified atherosclerotic lesions in 1993 (Bostrom et al., 1993). Expression levels of BMP-2 increase during the formation of calcified CVC nodules (Bostrom et al., 1993), and its expression in VSMCs can be stimulated by a wide range of factors associated with the condition such as inflammation (Al-Aly et al., 2007; Ikeda et al., 2012). It is now recognised that BMP-2 plays an important role in promoting vascular calcification as recombinant BMP-2 induces the osteogenic differentiation and mineralisation of VSMCs in vitro (Li et al., 2008; Liberman et al., 2011). It has been suggested that BMP-2 participates in this process by inducing the up-regulation of Runx2 and Msx2, and stimulating the loss of SMC marker genes such as SM22α in mineralising VSMCs (Hayashi et al., 2006; Liberman et al., 2011).

A second BMP family member, BMP-4, has also been shown to play a role in vascular calcification. BMP-4 is a potent osteoinductive factor in normal bone development and its expression has been localised to areas of calcification in human atherosclerotic lesions (Dhore et al., 2001). In vitro, BMP-4 accelerates the osteogenic differentiation of CVCs (Yao et al., 2008; Yao et al., 2009), and BMP-4 is responsible for receptor activator of nuclear factor kappa B ligand (RANKL)-induced VSMC mineralisation in vitro (Panizo et al., 2009). Given the role of BMP-2/-4 in the development of vascular calcification, it is intuitive to consider that blocking the activity of these BMPs could inhibit this process. Consistent with this, blockade of the BMP type I receptor function with the small molecule inhibitor, LDN-193189, has been reported to reduce high-fat diet-induced vascular osteogenic activity and calcification in LDLR-null mice (Derwall et al., 2012). Inhibition of vascular BMP-2/-4 activity by MGP over-expression also led to a reduction in high-fat diet-induced aortic mineral deposition in ApoE-null mice (Yao et al., 2010b). Therefore, BMP-2/-4 inhibition could provide a novel therapeutic target in the treatment of vascular calcification.

**BMP-7**

BMP-7 promotes the osteogenic differentiation of osteoblastic precursors (Cheng et al., 2003), but inhibits VSMC osteogenic differentiation and mineralisation (Davies et al., 2005; Kang et al., 2010). In vivo, recombinant BMP-7 treatment also abolishes the development of calcified vascular lesions in a mouse model of atherosclerosis and CKD (Davies et al., 2003). BMP-7 may exert these inhibitory effects in vascular calcification via several possible mechanisms. For example, BMP-7 up-regulates αSMA expression in human VSMCs in vitro, suggesting that BMP-7 may inhibit VSMC osteogenic differentiation and mineralisation by promoting the SMC phenotype of these cells (Dorai et al., 2000). A second possibility is that BMP-7 inhibits vascular calcification in vivo by stimulating bone formation and thereby reducing serum phosphate levels (Davies et al., 2005). Together these studies suggest that BMP-7 plays a stimulatory role in bone formation but an inhibitory role in vascular calcification. Inhibition of BMP-7 signalling when targeting BMP-2/-4 in vascular calcification must therefore be carefully considered.
1.2.4 FGFs

The FGFs are a large family of secreted glycoproteins that can be classified as either paracrine- or endocrine-acting. Paracrine FGFs, such as FGF-2, are readily sequestered to the ECM by PGs displaying HS chains (HSPGs) to limit their diffusion within the extracellular space. For signal propagation, the paracrine FGFs bind to a cell surface FGF-receptor (FGFR1-4) in a ternary complex consisting of FGF, FGFR and HPSGs. The formation of these paracrine FGF-FGFR complexes is critically dependent on the sulphation patterning within HS chains (discussed further in section 1.5.2). Dimerisation of the ternary paracrine FGF-FGFR-HPSG complex leads to a conformational shift in the FGFR structure, resulting in the transphosphorylation of the intracellular tyrosine kinase domain. Subsequent downstream signalling events occurs through: (i) phospholipase C\(\gamma\) (PLC\(\gamma\)) and protein kinase C (PKC); (ii) Ras-Erk1/2 (iii) PI3K-Akt (Turner and Grose, 2010). The paracrine FGF signalling pathway is summarised in Figure 1.5.

Figure 1.5. Schematic overview of the paracrine FGF signalling pathway. Paracrine FGF ligands bind to a cell surface FGFR in a ternary complex consisting of FGF-FGFR-HSPG. HSPGs act both as co-receptors and modulators of FGF bioavailability in the extracellular space. Ligand binding to FGFR induces the dimerisation of FGFR, resulting in the cross-phosphorylation of tyrosine residues in the intracellular domain of the receptor tyrosine kinase, and activation of downstream signalling events. DAG, diacylglycerol; Erk1/2, extracellular related kinase 1/2; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FRS2, fibroblast growth factor receptor substrate 2; Gab1, Grb2-associated protein 1; Grb2, growth factor receptor-bound protein 2; HSPGs, heparan sulphate proteoglycans; PI3K, Akt, PKC, Raf. 
phosphoinositide 3-kinase; PLCγ, phospholipase Cγ; PKC, protein kinase C; SOS, son of sevenless. Figure based on information from (Turner and Grose, 2010; Ornitz and Marie, 2015).

Unlike the paracrine FGFs, the endocrine FGFs (FGF-19, FGF-21 and FGF-23) have poor affinity for HS (Goetz et al., 2007). Importantly, this enables these ligands to diffuse away from the site of their secretion and enter the bloodstream to reach their target organs (Goetz et al., 2007; Asada et al., 2009). As the endocrine FGFs have poor binding affinity for HS, these ligands require Klotho co-receptors to bind their FGFR (Urakawa et al., 2006; Kurosu et al., 2007). Consistent with this, HS is not a component of the signal transduction unit of FGF-19 and FGF-23 (Goetz et al., 2012). Interestingly, Goetz et al. (2012) demonstrated that a paracrine FGF could be converted into an endocrine ligand by substituting its C-terminal tail for that of an endocrine FGF, which contains the Klotho-binding site. Therefore, the endocrine FGFs activate downstream signalling through a FGF-FGFR-Klotho complex and do not appear to rely on HS.

(i) FGF-2
The paracrine FGF ligand, FGF-2, is a critical regulator of osteogenesis and bone development (Marie et al., 2012; Ornitz and Marie, 2015), although its role in this process is complex. Bone formation and mineralisation is reduced in FGF-2-null mice, and also in cells isolated from these mice (Montero et al., 2000; Naganawa et al., 2006). However, whilst short-term FGF-2 treatment for the first 3-8 days of an osteogenic-inducing protocol (which takes up to 9-14 days) stimulates matrix mineralisation in calvarial osteoblasts (Fakhry et al., 2005; Ling et al., 2006) and MSCs (Dombrowski et al., 2009), continuous FGF-2 treatment inhibits mineralisation in these cells (Debiais et al., 1998; Fakhry et al., 2005; Ling et al., 2006; Dombrowski et al., 2009; Biver et al., 2012). Together these studies could suggest that FGF-2 is required initially to promote mineralisation, but then must be down-regulated so mineralisation can proceed.

To date, only one study has examined the effect of exogenous FGF-2 on osteogenic marker expression in VSMCs (Nakahara et al., 2010). Short-term (up to 12 hours) incubation with FGF-2 stimulated the expression of osteopontin and osteocalcin mRNA by rat VSMCs in vitro (Nakahara et al., 2010). However, it was also stated that FGF-2 did not induce mineralisation in these cells, although how these studies were performed was not clear (Nakahara et al., 2010). Further work is therefore required to determine the role of FGF-2 in VSMC osteogenic differentiation, and also its role in regulating the deposition of a mineralised matrix by these cells.

(ii) FGF-23
FGF-23 is released by skeletal osteocytes in response to increased serum phosphate levels, and it acts on the kidney to reduce phosphate resorption (Shimada et al., 2004). FGF-23 was originally considered as a biomarker of vascular calcification as high FGF-23 levels were associated with calcification in the vessel wall (Nasrallah et al., 2010). However, more recent studies have suggested that there is no association between FGF-23 and calcification in the coronary arteries and aortas of patients with CKD (Scialla et al., 2013); much controversy also surrounds whether FGF-23 plays a regulatory role in vascular calcification. For example,
exogenous FGF-23 had no effect on phosphate-induced mineralisation in bovine (Lindberg et al., 2013) and human VSMCs (Scialla et al., 2013), although the FGF-23 co-receptor Klotho could not be detected/was very lowly expressed at the mRNA level in these cells. In contrast to these studies, exogenous FGF-23 increased high phosphate-induced rat aortic ring mineralisation ex vivo, and rat VSMC mineralisation in vitro (Jimbo et al., 2014). The reasons for these discrepancies are currently unknown, although it may reflect species-specific roles of FGF-23 in VSMC mineralisation. Further work is required to determine if FGF-23 is a biomarker and/or causative agent of vascular calcification.

### 1.2.5 Decorin & biglycan

Decorin and biglycan are small leucine-rich PGs that display CS/DS chains. Both decorin and biglycan have been implicated in the regulation of osteogenesis (summarised in Table 1.2) and vascular calcification, but much discord exists over the expression levels of these PGs in vascular calcification (Table 1.2, and discussed in more detail below). Decorin co-localises with mineral deposits in human atherosclerotic plaques (Fischer et al., 2004), and decorin mRNA and/or protein expression is increased in mineralising VSMCs (Fischer et al., 2004; Alves et al., 2014), calcified rat vessels (Shibata et al., 2010) and human calcified thoracic aortic aneurysms (Matsumoto et al., 2012a). In contrast, decorin protein expression is decreased in human calcified aortic valves (Matsumoto et al., 2012b) and unchanged in mineralising human VSMCs in vitro (Yan et al., 2011). This disparity in results may be due to use of different cell systems, species, and/or the techniques used to measure the mRNA and/or protein levels of specific PG core proteins during vascular calcification.

Previous studies have shown that decorin may promote vascular calcification as viral-mediated over-expression of decorin accelerates VSMC mineralisation (Fischer et al., 2004; Yan et al., 2011), likely through increased TGF-β1 signalling in these cells (Yan et al., 2011). Importantly, the GAG chains displayed on decorin are vital to this PG’s role in accelerating human VSMC mineralisation in vitro (Yan et al., 2011), suggesting that GAGs may also be an important regulator of vascular calcification. Indeed, GAGs have been identified as a major component of human calcified atherosclerotic plaques (Duer et al., 2008). In contrast to its role in vascular calcification, over-expressing decorin inhibits matrix mineralisation in MC3T3-E1 osteoblasts (Mochida et al., 2009). Therefore, decorin may modulate calcification in a cell-type dependent manner.

Biglycan protein expression is also increased in human calcified aortic aneurysms (Matsumoto et al., 2012a), but biglycan mRNA expression is either decreased or unchanged in in vitro models of vascular calcification (Fischer et al., 2004; Shibata et al., 2010). It has been suggested that biglycan plays a stimulatory role in vascular calcification as exogenous addition of soluble biglycan promotes the formation of calcified nodules in human aortic valve interstitial cells (Song et al., 2012). The signalling pathways regulated by biglycan in vascular calcification specifically are currently unknown; however, previous studies have reported that biglycan is required for BMP-4-induced mouse calvarial osteoblast mineralisation (Chen et al., 2004). As
BMP-4 also plays a pro-calcific role in vascular calcification, one could hypothesise that biglycan promotes vascular calcification by enhancing this signalling pathway.

The expression levels of other PGs have also been documented in in vitro and/or in vivo models of vascular calcification, but again, much discord exists over the expression levels of these PGs in this condition (summarised in Table 1.2). In light of these findings, there is a pressing need to characterise the expression profile of several PG core proteins simultaneously during the osteogenic differentiation and mineralisation of VSMCs in order to highlight other PGs with a novel role in this process; this aim will be addressed in this thesis.

In summary, vascular calcification is not just a passive precipitation of calcium phosphate in the vessel wall, but like physiological bone formation, it is an active and cell-regulated process. Vascular calcification is regulated by a wide range of activators and inhibitors, the balance of which is disrupted in disease (e.g. CKD, T2DM) leading to the formation of mineralised tissue, bone and/or cartilage-like structures within the vessel wall (Figure 1.6). A single factor does not regulate vascular calcification, and the factors driving calcification can be different in different patients. Moreover, these factors may also act differently on different arterial beds (Schlieper, 2014). Multiple interventions may therefore need to be used in combination to treat this disease, and in the age of personalised medicine, this may need to be tailored for on a patient-by-patient basis (Wanner, 2015).

1.3 PGs: role of syndecans in vascular disease and osteogenesis
Several PGs have previously been implicated in the regulation of bone development and/or signalling pathways that are important in this process (summarised in Table 1.2) (Rodgers et al., 2008). Of these PGs, decorin (Fischer et al., 2004; Yan et al., 2011) and biglycan (Song et al., 2012) have also been implicated in the regulation of vascular calcification (see section 1.2.5 and Table 1.2), but whether other PGs are involved in this process has not been explored in detail. In this section, the role of the syndecan family (syndecan 4 specifically) of PGs in the vasculature and osteogenic signalling pathways will be discussed, as this PG family will form a major part of this thesis; their role in vascular calcification is currently unknown.

1.3.1 Syndecans
The syndecans are a family of membrane-spanning PGs, each comprising a core protein ranging from 20-40 kDa that is modified with GAG chains. Four syndecan genes are present in higher vertebrates: syndecan-1 is the major syndecan of epithelial cells including vascular endothelium, syndecan-2 is present mostly in mesenchymal, neuronal and VSMC cells, and syndecan-3 is the major syndecan of the nervous system (Alexopoulou et al., 2007). In contrast, syndecan 4 is ubiquitously expressed, albeit at a lower level than the other syndecan genes in the same cell (Alexopoulou et al., 2007). Each syndecan has a short C-terminal cytoplasmic domain, a single-span transmembrane domain, and an extracellular N-terminal domain with attachment sites for 3 to 5 HS or CS/DS chains. The extracellular domain shows low sequence homology between the syndecan genes, whilst the transmembrane and cytoplasmic domains are highly conserved (Alexopoulou et al., 2007; Multhaupt et al., 2009).
Table 1.2. Summary of PGs and their potentiation involvement in vascular calcification and/or bone formation.

<table>
<thead>
<tr>
<th>PG</th>
<th>GAG chains</th>
<th>Location</th>
<th>Expression and role in vascular calcification</th>
<th>Expression and role in osteogenic signalling/bone development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glypican 4</td>
<td>HS</td>
<td>Cell surface</td>
<td>Unknown</td>
<td>Glypican 4 mRNA is increased in the MG-63 osteosarcoma cell line during osteogenic differentiation and mineralisation (Kumarasuriyar et al., 2009). Glypican 4 deficiency causes craniofacial defects in zebrafish, including a smaller head and shorter jawbones (LeClair et al., 2009); defects speculated to be due to decreased Wnt signaling.</td>
</tr>
<tr>
<td>Perlecan</td>
<td>HS or CS/DS</td>
<td>Basement membrane</td>
<td>Perlecan mRNA expression is decreased in calcified rat vessels, but there is no significant change in mineralising rat VSMCs in vitro (Shibata et al., 2010). Role in vascular calcification is unknown.</td>
<td>Perlecan-null mice develop lethal chondrodysplasia, characterised by shortened long bones and craniofacial abnormalities; these animals die shortly after birth (Arikawa-Hirasawa et al., 1999). HS and CS/DS on growth plate perlecan mediates the binding and delivery of FGF-2 to the FGFRs (Smith et al., 2007), suggesting that perlecan may act as a regulator of FGF activity in bone.</td>
</tr>
<tr>
<td>Betaglycan</td>
<td>HS or CS/DS</td>
<td>Cell surface</td>
<td>Unknown</td>
<td>Betaglycan regulates BMP signaling, although this appears to be in a ligand-dependent manner. For example, betaglycan inhibits BMP-7 signalling in a hepatocyte HepG2 cell line (Wlater and Vale, 2003), but potentiates BMP-2 signalling by promoting ligand binding to the type I BMP receptor (Kirkbride et al., 2008). Role in bone development is unknown.</td>
</tr>
<tr>
<td>Biglycan</td>
<td>CS/DS</td>
<td>ECM</td>
<td>Biglycan protein expression is increased in human calcified aortic aneurysms (Matsumoto et al., 2012a), but biglycan mRNA expression is either decreased or unchanged in in vitro models of vascular calcification (Fischer et al., 2004; Shibata et al., 2010). Exogenous biglycan promotes calcified nodule formation in human aortic valve interstitial cells (Song et al., 2012). Elastin degradation promotes vascular calcification (Hosaka et al., 2009; Aikawa et al., 2009), and over-expressing biglycan inhibits elastin assembly in VSMCs (Hwang et al., 2008).</td>
<td>Biglycan is abundantly expressed in bone (Fisher et al., 1983). Biglycan-null mice have a reduced growth rate and bone mass due to lower levels of bone formation in these mice (Xu et al., 1998). Biglycan also regulates BMP-4-induced mouse calvarial osteoblast mineralisation in vitro (Chen et al., 2004), suggesting that biglycan plays a pro-calcific role in osteogenesis.</td>
</tr>
<tr>
<td>Protein</td>
<td>CS/DS ECM</td>
<td>Function and Expression in Vascular Calcification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-----------</td>
<td>--------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Decorin</strong></td>
<td>CS/DS ECM</td>
<td>Decorin co-localises with calcification in human atherosclerotic plaques (Fischer et al., 2004), and decorin mRNA or protein expression is increased in both <em>in vitro</em> (Fischer et al., 2004; Alves et al., 2014) and <em>in vivo</em> (Shibata et al., 2010) models of vascular calcification. In contrast, decorin protein expression is decreased in human calcified aortic valves (Matsumoto et al., 2012b) and unchanged in mineralising human VSMCs <em>in vitro</em> (Yan et al., 2011). Viral-mediated over-expression of decorin accelerates the mineralisation of VSMCs (Fischer et al., 2004; Yan et al., 2011), likely through the up-regulation of TGF-β1 signalling in these cells (Yan et al., 2011). Decorin mRNA and protein expression is decreased during the deposition of a mineralised matrix by MC3T3-E1 osteoblasts (Mochida et al., 2009) and MSCs (Hoshiba et al., 2009). Over-expressing decorin inhibits matrix mineralisation in MC3T3-E1 osteoblasts (Mochida et al., 2009). Bone defects have not been reported in decorin-null mice, although biglycan may compensate for a loss of decorin in bone development <em>in vivo</em> (Corsi et al., 2002).</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Versican</strong></td>
<td>CS/DS ECM</td>
<td>Versican protein is decreased in human calcified thoracic aorta aneurysms (Matsumoto et al., 2012a) and human calcified aortic valves (Matsumoto et al., 2012b), but versican mRNA expression is unchanged in calcified rat vessels (Shibata et al., 2010) and mineralising VSMCs <em>in vitro</em> (Fischer et al., 2004; Shibata et al., 2010). Role in vascular calcification is unknown; however, elastin degradation promotes vascular calcification (Hosaka et al., 2009; Aikawa et al., 2009) and over-expressing versican inhibits elastin assembly in VSMCs (Huang et al., 2006). Versican protein is expressed during the early stages of MSC osteogenic differentiation, but is down-regulated upon mineralisation of the ECM (Hoshiba et al., 2009). Versican follows the same pattern of expression during intramembranous ossification <em>in vivo</em> (Nakamura et al., 2005a). Role in bone development is unknown.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lumican</strong></td>
<td>KS ECM</td>
<td>Lumican protein expression is down-regulated in human calcified aortic valves (Matsumoto et al., 2012b). In contrast lumican expression is increased in human calcified thoracic aorta aneurysms (Matsumoto et al., 2012a) and calcified rat vessels (Shibata et al., 2010), but unchanged in mineralising rat VSMCs <em>in vitro</em> (Shibata et al., 2010). Role in vascular calcification is unknown. Lumican mRNA is expressed by differentiating and mature osteoblasts, but not by proliferating pre-osteoblasts. Lumican can be considered as a marker of osteogenic differentiation (Raouf et al., 2002). Role in bone development is unknown.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Osteoglycin</strong></td>
<td>KS ECM</td>
<td>Osteoglycin is a potential marker of differentiated VSMCs, and its expression is not associated with sites of calcification within human atherosclerotic plaques (Shanahan et al., 1997). Osteoglycin protein expression is down-regulated in human calcified aortic valves (Matsumoto et al., 2012b), but osteoglycin mRNA expression is increased in calcified rat vessels (Shibata et al., 2010). Knocking-down osteoglycin expression has no effect on aortic calcification in ApoE-null mice (Moncayo-Arlandi et al., 2014). Osteoglycin mRNA expression is increased during MC3T3-E1 osteoblast mineralisation (Tanaka et al., 2012). <em>In vitro</em>, over-expressing osteoglycin expression in MC3T3-E1 osteoblasts inhibits matrix mineralisation, however, the level of osteoglycin over-expression at the protein level was not confirmed in these cells (Tanaka et al., 2012). Role in bone development is unknown.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.6. Regulation of vascular calcification. Promoters are shown in red, and inhibitors of vascular calcification are shown in blue. Figure based on information/data from (Shanahan et al., 2011; Sage et al., 2011; Schlieper, 2014).
Single knock-outs of syndecan 1 (Stepp et al., 2002), syndecan 3 (Kaksonen et al., 2002) or syndecan 4 (Echtermeyer et al., 2001) yield viable and fertile mice, with no gross abnormalities; a syndecan 2-null mouse is yet to be generated. These results suggest some degree of redundancy between the syndecan genes during embryonic development. However, such redundancy does not seem to exist in post-natal life as the phenotypes of the single syndecan knock-out mice diverge in post-natal tissue injury or disease (discussed further below).

(i) **Structure**

The cytoplasmic domain of the syndecans mediates their interaction with the cytoskeleton. This domain can be divided into a pair of conserved regions (C1 and C2) that is present in all isoforms, and a variable (V) region that is unique to each syndecan (Figure 1.7). The C1 region of the cytoplasmic domain facilitates receptor interactions with the cytoskeleton, and in the case of syndecan 2, the C1 region has been shown to interact with actin-associated ezrin-radixin-moesin (ERM) proteins (Granés et al., 2003). The functional role for this interaction is currently unclear, although ERM phosphorylation is increased in glomeruli isolated from syndecan 4-null mice when compared to wild type controls (Liu et al., 2012). Whilst syndecan 4 has not been shown to directly interact with ERM, it is possible that syndecan 4 can also interact with these proteins as the C1 region is conserved between the syndecans. The C2 region of the syndecans interact with a number of PDZ-domain-containing proteins, including syntenin, synectin (also known as GIPC1) and Ca\(^{2+}\)/calmodulin-associated serine/threonine kinase (CASK), which have been reviewed in detail elsewhere (Multhaupt et al., 2009).

The V region of the syndecan core protein is unique to each syndecan, and to date, most studies have investigated the role of the V region in syndecan 4. Notably, the V region of syndecan 4 binds phosphatidylinositol (4,5)-bisphosphate (PIP\(_2\)) promoting dimerisation (Oh et al., 1998; Whiteford et al., 2008), thereby providing a platform for the binding and activation of PKC\(\alpha\). This interaction is important for syndecan 4 signaling, as it promotes the assembly of stress fibers and focal adhesions in combination with integrins of the \(\beta_1\) class (Whiteford et al., 2007; Bass et al., 2007). Since the small GTPases Rac and Rho are downstream of PKC\(\alpha\), syndecan 4 also regulates the activity of guanine nucleotide exchange factors (GEF), GTPase activators (GAPs), and guanine dissociation inhibitors (GDIs), thereby influencing cell adhesion and migration via RhoA and Rac (reviewed in (Multhaupt et al., 2009)). Regulation of PKC\(\alpha\) activity by syndecan 4 will be considered in more detail in Chapter 7.

The transmembrane domain of all syndecans contains a GXXXG motif that promotes the formation of SDS-resistant dimers (Choi et al., 2005). All syndecans have a very similar transmembrane domain sequence, and on the basis of synthetic peptide experiments, it has been suggested that the syndecans could form heterodimers (Dews and Mackenzie, 2007). However, this suggestion is yet to be verified *in vitro* or *in vivo*. 

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Figure 1.7. Syndecan structure. Schematic overview of the syndecan core protein; image is not to scale. Potential interactions between the different domains of the syndecan proteins and other ligands are shown. Figure adapted from (Alexopoulou et al., 2007; Multhaupt et al., 2009).

The GAG chains displayed on the extracellular domain (also referred to as the ‘ectodomain’) of the syndecan core proteins can mediate their interaction with a wide range of extracellular ligands, including FGF-2, TGF-β1, and ECM molecules such as fibrillin-1 (Alexopoulou et al., 2007; Xian et al., 2010). Syndecans usually possess 3-5 HS chains, although additional chondroitin/dermatan sulfate chains may be present on syndecans 1, 3 and 4 (Shworak et al., 1994; Ueno et al., 2001; Deepa et al., 2004; Gopal et al., 2010). The HS chains are close to the N-terminus and are attached to serine/glycine sites on the core protein, whilst the CS/DS chains are more membrane-proximal (Multhaupt et al., 2009). The necessity of these GAG chains for syndecan function have been revealed, with a loss of HS chains on syndecan 4 preventing the correct formation of focal adhesions, reducing focal adhesion kinase activity, and affecting the cells ability to exert tension on ECM molecules (Gopal et al., 2010). The biosynthesis of HS and CS/DS and the role of these GAGs in regulating growth factor signalling will be discussed in sections 1.4 and 1.5, respectively.

Evidence is now emerging that the ectodomain of the syndecans do not just display their associated GAG chains, but they also possess biological function. For example, a bacterially-expressed syndecan 4 ectodomain fused to a GST fusion protein can support MSC attachment
and spreading (Whiteford et al., 2007), and site-directed mutagenesis of conserved sequences within the syndecan 4 ectodomain have revealed an NXIP motif is important for its cell adhesion activity (Whiteford and Couchman, 2006). In the case of syndecans 2 and 4, the integrins promoting cell adhesion belong to the β1 class (Whiteford et al., 2007). However, whilst syndecan 4 plays a clear role in cell adhesion, this appears to be dependent on the cell-type used. In this regard, mesenchymal cells adhere to the syndecan 4 ectodomain, while several epithelial cell lines failed to respond even though they possess β1 integrin (Whiteford et al., 2007). This reason for such cell type-specific behaviour is currently unclear.

(ii) Shedding
Syndecans can be shed from the cell surface; this is a naturally occurring process and is facilitated via MMPs (including MMP-2 and MMP-9), which cleave the syndecan core protein (Fitzgerald et al., 2000; Manon-Jensen et al., 2010; Manon-Jensen et al., 2013; Ramnath et al., 2014). Cell surface shedding of syndecans by proteolytic cleavage at a site proximal to the plasma membrane generates soluble proteoglycan fragments that may diffuse away and stimulate effects on other cells, or serve as a competitive inhibitor of endogenous syndecan signalling (reviewed in (Manon-Jensen et al., 2010)). In this regard, cardiac rupture and mortality was increased in syndecan 4-null mice following myocardial infarction, but over-expressing the shed form of syndecan 4 in the hearts of wild-type mice also resulted in increased mortality (Matsui et al., 2011). Furthermore, lipopolysaccharide-induced shedding of syndecan 4 from the cell surface of cardiac fibroblasts reduced focal adhesion formation when compared to controls, resembling genetic ablation of syndecan 4 in the same cells (Strand et al., 2013). As increases in syndecan 4 mRNA expression correlate with increased syndecan 4 shedding from the cell surface (Strand et al., 2013), care must therefore be taken when modulating syndecan expression in disease. This will be particularly important in conditions where MMP expression/activity is increased, such as vascular calcification (see section 1.1.5).

(iii) Roles of syndecans in vascular disease
Syndecans 1-4 are expressed by VSMCs (Cizmeci-Smith et al., 1997; Rauch et al., 2005; Shibata et al., 2010), and there is increasing evidence to suggest that this family of PGs may play an important role in the vasculature, particularly in disease (reviewed in (Alexopoulou et al., 2007)). The expression levels of syndecan 1 (Nikkari et al., 1994) and syndecan 4 (Nikkari et al., 1994; Cizmeci-Smith et al., 1997; Ikesue et al., 2011) are both increased following vascular injury, although these PGs appear to have opposing roles in this process. In this regard, ligation-induced vascular injury is reduced in syndecan 4-null mice (Ikesue et al., 2011), but increased in syndecan 1-null mice (Fukai et al., 2009), when compared to wild-type controls. It has been reported recently that syndecan 1 is a regulator of mature VSMC phenotype and that the expression of the VSMC markers, αSMA and calponin, are reduced in syndecan 1-null mouse VSMCs (Chaterji et al., 2014). Under normal, healthy conditions VSMCs reside in a quiescent, contractile state, however, in response to vascular injury or disease these cells are able to re-enter the cell cycle converting to a synthetic, proliferative phenotype (see review by
Therefore, the de-differentiation of syndecan 1-deficient VSMCs may be responsible for the enhanced response to ligation-induced vascular injury in syndecan 1-null mice.

It has been proposed that knocking-down syndecan 4 expression in mice plays a protective role in ligation-induced vascular injury by reducing VSMC proliferation, but it has no effect on VSMC apoptosis (Ikesue et al., 2011). However, in other tissue injury settings such as myocardial infarction, syndecan 4 is believed to play a protective role through the inhibition of cardiac myocyte apoptosis (Echtermeyer et al., 2011; Xie et al., 2012). In this regard, cardiac myocyte apoptosis is increased in syndecan 4-null mice following myocardial infarction (Echtermeyer et al., 2011), whilst cardiac myocyte apoptosis is reduced when syndecan 4 is over-expressed in the hearts of rats 1 day following the induction of myocardial infarction in these animals (Xie et al., 2012). It is therefore possible that the effects of syndecan 4 on apoptosis are tissue-specific.

Whilst loss of syndecan 4 expression appears to play a protective role in ligation-induced vascular injury (Ikesue et al., 2011), atherosclerotic plaque susceptibility is increased in syndecan 4/LDLR double knock-out mice fed a high-fat diet (Baeyens et al., 2014). Atherosclerosis typically develops in regions of blood vessels where fluid flow patterns are disturbed, and where endothelial cells fail to align in the direction of flow. Endothelial cells from the thoracic arteries of syndecan 4/LDLR double knock-out mice were poorly aligned in vivo, and depletion of syndecan 4 in human umbilical cord endothelial cells (HUVECs) also inhibited flow-induced alignment in vitro (Baeyens et al., 2014). The potential beneficial role of syndecan 4 in the vasculature is further supported by the finding that deletion of syndecan 4 in mice increases basal blood pressure (~10 mmHg) when compared to wild-type controls (Partovian et al., 2008). Conversely, over-expression of syndecan 4 enhances coronary microvessel vasodilation in response to FGF-2 (Li et al., 2002). Syndecan 4 regulates FGF-2-induced Akt phosphorylation via its interactions with PKCα (Partovian et al., 2008; Ju and Simons, 2013), which in turn modulates endothelial nitric oxide synthase (eNOS) phosphorylation and the production of nitric oxide, a potent vasodilator (Partovian et al., 2008) (Figure 1.8). Together these studies suggest that the role of syndecan 4 in the vasculature is highly dependent on the disease state.
Figure 1.8. Syndecan 4 regulates FGF-2-induced Akt activation through PKCα. Syndecan 4-dependent activation of PKCα has a crucial role in the assembly of mTORC2 (also called PDK2) and activation of PDK1 (Partovian et al., 2008; Ju and Simons, 2013). Both mTORC2 and PDK1, in turn, control the activation of Akt by two phosphorylation events: Thr308 phosphorylation is accomplished by PDK1, and Ser473 phosphorylation by mTORC2. In the absence of syndecan 4, PKCα activation is reduced, leading to impaired assembly of mTORC2 (Partovian et al., 2008) and diminished activation of PDK1, which it controls through Pak1/2 (Ju and Simons, 2013). Figure is not to scale. Figure adapted from Elfenbein and Simons (2014).

The role of syndecan 2 in vascular disease is currently unknown, however like syndecan 1 (Chaterji et al., 2014), syndecan 2 may regulate SMC marker expression in VSMCs. In this regard, knocking-down syndecan 2 expression in human aortic VSMCs using siRNA abrogates the increase in αSMA and SM22α expression in response to Notch signalling in these cells (Zhao et al., 2012). However, lentivirus-induced over-expression of syndecan 2 in human aortic SMCs was not sufficient to induce αSMA expression, nor did it further increase αSMA expression in response to Notch signalling in these cells (Zhao et al., 2012). These data suggest that syndecan 2 cooperates with Notch to enhance SMC marker expression in VSMCs, but syndecan 2 alone does not regulate the expression of αSMA and SM22α.
Syndecan 4 mRNA expression is increased in calcified rat vessels (Shibata et al., 2010); however, the expression profiles and roles of syndecans 1, 2 and 3 in vascular calcification are currently unknown. Interestingly, κ-carrageenan (which causes obstructive nephropathy via its deposition in the collecting ducts)-induced nephropathy is enhanced in syndecan 4-null mice (Ishiguro et al., 2001b), suggesting that syndecan 4 has a protective role in this kidney disease model. As vascular calcification is highly prevalent in patients with CKD (Shanahan et al., 2011), one could hypothesise that syndecan 4 may prevent kidney disease, and in turn, vascular calcification in this disease setting. Indeed, it has been reported that calcification was increased in the placental vessel labyrinth of syndecan 4-null embryos when compared to wild type controls (Ishiguro et al., 2000), although the role of syndecan 4 in disease-induced vascular calcification is currently unknown.

(iv) Roles of syndecans in osteogenesis

All four syndecan genes are expressed in calvarial osteoblasts (Song et al., 2007; Teplyuk et al., 2009); however, of the four syndecan genes, the majority of studies have focused on the involvement of syndecan 2 and 4 in osteogenesis, which will be discussed in more detail below. Whilst syndecan 1 expression is decreased during osteoblast differentiation (Birch and Skerry, 1999), the expression levels of syndecan 3 during this process have not been documented. The roles of syndecan 1 and syndecan 3 in osteogenesis are also unknown.

Syndecan 4 mRNA expression is increased during the osteogenic differentiation of the mouse osteoblastic cell line, MC3T3-E1 (Jackson et al., 2007), and its expression is up-regulated by the osteogenic factor FGF-2 (see section 1.2.4i) in VSMCs (Cizmeci-Smith et al., 1997) and calvarial osteoblasts (Song et al., 2007; Teplyuk et al., 2009). Syndecan 4 may play a regulatory role in osteoblast mineralisation as syndecan 4 antibody treatment reduced mineralised nodule formation in rat calvarial osteoblasts cultured under osteogenic conditions (Song et al., 2007); although, there were several deficiencies regarding the experimental design of this study to consider. For example, cells were treated with the syndecan 4 antibody for 2 hours, followed by treatment with 10 ng/ml FGF-2 in osteogenic culture medium for three days. Cells were then cultured with for a further 14 days in osteogenic culture medium alone. It is unlikely that the effects of the syndecan 4 blocking antibody lasted the duration of the experiment, so it is therefore difficult to interpret the validity of their results.

The role of syndecan 4 in osteogenesis in vivo is also unclear. Bone formation is not altered in syndecan 4 null-nice, although this result may be confounded by a compensatory increase in syndecan 2 in these mice (Bertrand et al., 2013). Indeed, syndecan 2 may play an important role in osteoblast differentiation, independent of the other syndecan genes. For example, syndecan 2 mRNA is expressed during osteoblast differentiation (Birch and Skerry, 1999; Molteni et al., 1999) and its expression is up-regulated by the pro-calcific factor BMP-2 (Gutierrez et al., 2006). The specific regulation of syndecan 2 expression during osteoblast differentiation, and in response to osteogenic factors, suggests that this PG may have a role in osteogenesis. Consistent with this hypothesis, silencing syndecan 2 expression in human
osteoblasts using siRNA impairs cell adhesion and actin skeleton formation (Wang et al., 2011b); however the role of syndecan 2 in osteoblast mineralisation was not investigated in this study.

Whilst bone formation is not altered in syndecan 4-null mice, fracture healing is impaired when compared to wild type controls (Bertrand et al., 2013). Fracture healing could be restored in syndecan 4 null-mice using the anti-TNF monoclonal antibody, certolizumab (Bertrand et al., 2013), suggesting the inflammatory response is impaired in the absence of syndecan 4. The induction of syndecan 4 expression in response to TNF may therefore represent a negative feedback response to help to dampen the inflammation present in different disease settings (Zhang et al., 1999; Wang et al., 2011a; Tan et al., 2012; Bertrand et al., 2013; Strand et al., 2013; Ramnath et al., 2014). Indeed, syndecan 4 is capable of exerting anti-inflammatory effects in models of pulmonary inflammation (Tanino et al., 2012), myocardial infarction (Xie et al., 2012) and pressure-overload (Strand et al., 2013).

In summary, no single syndecan deletion in mice leads to an embryonic phenotype, possibly due to some degree of redundancy between the syndecan genes (Bertrand et al., 2013). However, phenotypes in single syndecan gene knock-out mice can appear in postnatal tissue injury, including the vasculature (Fukai et al., 2009; Ikesue et al., 2011; Baeyens et al., 2014), kidneys (Ishiguro et al., 2001b) and bones (Bertrand et al., 2013), suggesting that compensation may not occur under stress conditions. Whilst the syndecans can positively or negatively regulate tissue injury, it is becoming increasingly apparent that the expression levels of the syndecan genes must be finely balanced in such settings. For example, wound healing is delayed in syndecan 1-null mice (Stepp et al., 2002), and over-expressing syndecan 1 in mice also leads to delayed wound healing with defective granulation tissue angiogenesis (Elenius et al., 2004). Similar observations have also been made for syndecan 4 in wound closure (Longley et al., 1999; Echtermeyer et al., 2001) and myocardial infarction (Matsui et al., 2011). Further work is therefore required to determine the effects of manipulating syndecan expression in disease.

1.4 HS and CS/DS biosynthesis

As mentioned previously, the ability of PGs (including syndecans) to bind a wide variety of ligands is primarily (although not entirely) a function of the GAG chains displayed on their core proteins. The sugar backbone of GAGs has been conserved throughout evolution (Esko and Lindahl, 2001), and heterogeneity in HS and CS/DS polysaccharide chains is instead exerted through the number of repeating disaccharide units (usually between 20 and 400 disaccharides) and the sulphonation motifs present within individual chains. The level and distribution of sulphonation within these chains is tightly regulated leading to tissue-specific patterning (Maccarana et al., 1996; ten Dam et al., 2004; Johnson et al., 2007; Baldwin et al., 2008). Indeed, GAG biosynthesis is non-template driven and is mediated by the co-ordinated action of polymerases, epimerases, sulphotransferases and sulfatases (Sulfs), as well as enzymes involved in precursor formation. The following section will focus on the biosynthesis of HS and
CS/DS, as these GAGs are displayed on syndecan core proteins and are most relevant to this thesis.

1.4.1 Tetrasaccharide linkage region

Biosynthesis of the sulphated GAGs is initiated in the Golgi apparatus and requires the formation of a tetrasaccharide link region composed of Glucuronic Acid-Galactose-Galactose-Xylose (GlcA-Gal-Gal-Xyl). A Xyl residue is first transferred from the corresponding UDP-sugar to the hydroxyl group (-OH) of a serine residue on the core protein by a xylosyltransferase (XT). Two isoforms of XT exist, XTI and XTII, with tissue-specific expression patterns present in mammals (Gotting et al., 2007). The remaining linkage region is synthesised by the sequential transfer of two Gal residues (by galactosyltransferase I/II (GaLT I/II)) and GlcA (by glucuronosyltransferase I (GlcATI)), from their corresponding UDP-sugars. After formation of this linkage region, the pathways for HS and CS/DS biosynthesis diverge.

1.4.2 HS

Members of the Exostosin-like (EXTL) gene family (EXTL1-3) transfer N-acetylglucosamine (GlcNAc) to the linkage region and commit the intermediate PG molecule to the assembly of HS (Carlsson and Kjellen, 2012). This addition initiates the polymerisation of the growing HS chain by the Exostosin1 (EXT1)/EXT2 polymerase complex, which adds alternating units of GlcA and GlcNAc from their corresponding UDP-sugars. While EXT1 alone can catalyse the polymerisation of the HS backbone, EXT2 does not seem to have the capacity to polymerise HS chains by itself (Busse and Kusche-Gullberg, 2003). Instead, it has been suggested that the role of EXT2 in HS biosynthesis is to act as chaperone for EXT1 (Wei et al., 2000; McCormick et al., 2000). Therefore, both EXT1 and EXT2 are required for the polymerisation of HS.

As the HS chain grows, it undergoes a series of modifications involving the N-deacetylase N-sulphotransferases (NDSTs), epimerases, sulphotransferases, and Sulfs (Figure 1.9). With the exception of C5-epimerase and 2-O-sulphotransferase (2-OST), the HS modification enzymes all belong to multi-isomer families. The first modification enzyme, NDST, removes N-acetyl groups from selected GlcNAc residues and replaces them with sulphate groups to form N-sulphoglucosamine (GlcNS). These NDST enzymes, of which 4 isoenzymes exist, are key in determining the sulphation patterning in HS, with the initial positioning of N-sulphate groups directing subsequent sulphation events in nascent HS chain (Kjellén, 2003). This event is followed by the epimerisation of GlcA to IdoA via the action of C5 epimerase. In most cases, GlcA residues must be linked to N-sulphated GlcNAc residues at the non-reducing end of the HS chain for epimerisation to occur (Jacobsson et al., 1984). Therefore, IdoA residues are typically (but not always) confined to N-sulphated domains.

Nascent HS chains are further modified by O-sulphation. 2-OST adds a sulphate group to the 2-OH position of GlcA or IdoA, although 2-O-sulphation is more closely associated with sites of epimerisation i.e. IdoA (Rong et al., 2001). Subsequently, four 6-O-sulphotransferase (6-OST1, 2, 3a, 3b) can add a sulphate group to the 6-OH position of GlcNAc or GlcNS, and in certain cases, the 3-OH position of GlcNAc or GlcNS may be sulphated by 3-O-sulphotransferase (3-
OST1-7). HS may also be modified by the activity of the cell membrane-displayed Sulfs (Sulf1 or Sulf2) that remove sulphate groups from the 6-OH position in GlcNS. The incomplete, yet interdependent, action of the NDSTs, epimerases and sulphotransferases (which have been suggested to form a GAGosome (Esko and Selleck, 2002)) leads to the organised domain structure in the mature HS chain with highly sulphated regions (NS domains) dispersed with low/non-sulphated domains (NA domains). Intermediately sulphated regions (NA/NS domains) form boundaries between these two domains (Figure 1.10) (Gallagher, 2001).

The biosynthesis of HS may be regulated at multiple points. First, the organisation of the domain pattern in HS varies widely between tissues (Ledin et al., 2004) and is likely to be determined by the cell- and tissue-specific expression of different enzyme isoforms of NDSTs, 6-OST, 3-OST and Sulfs (Lindahl and Li, 2009). Second, the expression levels of these individual enzymes can influence the HS domain pattern. For example, mouse ESCs undergoing neural differentiation show increased expression of NDST4, 3-OST, 6-OST2 and 6-OST3, correlating with increases in N-, 6-O and 3-O sulphation in HS chains derived from these cells (Johnson et al., 2007).

Importantly, changes in the mRNA levels of HS biosynthetic enzymes do not necessarily correlate with changes in HS composition. Indeed, NDST activity and HS chain N-sulphation are increased in the brains of α-l-iduronidase-null mice (a model of mucopolysaccharide I Hurler), but this increase did not correlate with increased mRNA expression levels of the four NDST genes in the brains of these mice (Holley et al., 2011a). HS chain modifications are highly dependent on the interactions between specific HS biosynthetic enzymes, and NDST1 activity can be regulated by the expression levels of the HS chain polymerising enzyme EXT2 (Presto et al., 2008) and the sulphate donor 3' phosphoadenosine-5' phosphosulphate (PAPS) (Carlsson et al., 2008). These findings highlight the need to correlate changes in HS biosynthetic enzyme expression and activity with HS chain composition.
Figure 1.9. Schematic overview of HS biosynthesis. Following synthesis of the tetrasaccharide link region (orange), the polymerase complex composed of EXT1 and EXT2 adds alternating units of GlcA and GlcNac to the non-reducing end of the chain. As the chain grows, NDST removes N-acetyl groups from selected GlcNAc residues and replaces them with a sulphate group to form GlcNS (purple). N-deacetylation/N-sulphation is followed by epimerisation of GlcA to IdoA (red) and stepwise addition of sulphate groups to the 2-OH position of IdoA (blue), and the 6-OH position of GlcNAc/GlcNS (green). In certain cases, GlcNAc/GlcNS will be sulphated at the 3-OH position (dark pink), and selected sulphate groups will be removed from the 6-OH position of GlcNAc/GlcNS (light pink). 2-OST, 2-O-sulphotransferase; 3-OST, 3-O-sulphotransferase; 6-OST, 6-O-sulphotransferase; EXT, exostosin; NDST, N-deacetylase N-sulphotransferase; Sulf, sulfatases. Figure based on information from (Kreuger and Kjellén, 2012).
1.4.3 CS/DS

Transfer of GalNAc to the linkage region by CS N-acetylgalactosaminyltransferase-I (CHGN1, also referred to as CS GalNAcT-I) commits the intermediate PG molecule to the assembly of CS/DS. This addition initiates the polymerisation of the growing CS/DS chain by the glycosyltransferases, of which four have been identified to date: chondroitin synthase-1 (ChSy-1) (Kitagawa et al., 2001), chondroitin synthase-2 (ChSy-2) (Izumikawa et al., 2007), chondroitin synthase-3 (ChSy-3) (Izumikawa et al., 2008), chondroitin polymerising factor 2 (ChPF2) (Kitagawa et al., 2003).

During assembly, some GlcA residues are converted to IdoA by DS epimerase-1 or DS epimerase-2, thereby converting CS to DS; these chains are subsequently modified by specific sulphotransferases (Figure 1.11). Dermatan 4-O-sulphotransferase-1 (D4ST1) adds a sulphate group to the 4-OH position of GalNAc, which can then be modified by the addition of a sulphate group to the 6-OH position of GalNAc(4S) by GalNAc(4S)-6ST. In some cases, GalNAc(4S) will also be modified by uronyl 2-O-sulphotransferase (UST, also referred to as CS/DS 2-OST), which catalyses the transfer of a sulphate group to the 2-OH position of IdoA. D4ST1 must be present to generate IdoA-containing disaccharides in DS (Pacheco et al., 2009a), which may explain the observation that IdoA blocks typically contain GalNAc(4S) instead of GalNAc(6S) (Thelin et al., 2013). The 6-O-sulphation pathway in DS remains to be clarified, but may be mediated by chondroitin 6-O-sulphotransferase-1 or -2 (C6ST1/C6ST2) (Figure 1.11). In di-sulphated structures, 2-O-sulphation of IdoA (or GlcA) always follows 4-O- or 6-O-sulphation of GalNAc.

In the absence of GlcA epimerisation, the biosynthesis of CS proceeds (Figure 1.11). CS chains are modified by the chondroitin 4-O-sulphotransferases (C4ST1/C4ST2/C4ST3) and C6ST1/C6ST2, which add a sulphate group to the 4-OH and 6-OH position of GalNAc, respectively. Subsequently, UST can add a sulphate group to the 2-OH position of GlcA following 4-O- or 6-O-sulphation of GalNAc, and GalNAc(4S)-6ST adds a sulphate group to the 6-OH position of GalNAc(4S).
Figure 1.11. Schematic overview of CS/DS biosynthesis. In IdoA containing units, epimerisation always occurs before sulphation. In disulphated structures, 2-O-sulphation follows both 4-O and 6-O sulphation, while 4-O sulphation precedes 6-O sulphation when a 4S, 6-di-O- sulphated unit is generated. iO, iA, iB, iC, iD units contain IdoA and can be classified as DS disaccharides. A, B, C, D and E units do not contain IdoA and can be classified as CS disaccharides. Dotted lines indicate putative pathways because iC and iD units formation has not been clarified yet. C4ST, chondroitin 4-O-sulphotransferase; C6ST, chondroitin 6-O-sulphotransferase; D4ST1, dermatan 4-O-sulphotransferase 1; GalNAc(4S)-6ST, GalNAc(4S)-6-O-sulphotransferase; UST, uronyl 2-O-sulphotransferase. Figure adapted from (Malmström et al., 2012).
The term CS/DS, which will be used throughout this thesis, refers to the hybrid nature of these chains. In this regard, the conversion of GlcA to IdoA is variable, and can range from one IdoA residue per chain to almost 100% IdoA. Typically, IdoA residues can be found either in small blocks (≥6 IdoA residues in succession), as alternating GlcA/IdoA blocks, or as isolated IdoA residues in unmodified GlcA blocks (Theelin et al., 2013). This modification of CS/DS chains is similar to the pattern of N-sulphation observed in HS (Figure 1.10), and is thought to be cell/tissue-specific (Cheng et al., 1994; Ohtake-Niimi et al., 2010; Zhao et al., 2013). However, a cell can also produce different CS/DS chains depending on the core protein. For example, the decorin/biglycan isolated from human skin fibroblasts contained 60% IdoA, whilst versican only contained 7% IdoA (Pacheco et al., 2009b). The factors regulating DS biosynthesis on different PG core proteins, however, remain unclear.

Due to the negative charge of the attached GAG chains, PGs can interact with a number of cell surface and matrix proteins, and cytokines, thereby regulating both physiological and pathophysiological processes, including bone formation (Rodgers et al., 2008). GAGs are abundantly expressed in bone (Prince and Navia, 1983; Waddington et al., 1989; Waddington and Embery, 1991; Duer et al., 2008), and they have also been identified as a major component of human calcified atherosclerotic plaques (Duer et al., 2008). However, the composition of GAGs in calcified vessels/mineralising VSMCs, and their role in this process has not been examined; the sulphation patterning of HS and CS/DS during VSMC mineralisation will therefore be investigated in this thesis.

1.5 Regulation of osteogenic signalling pathways by HS and CS/DS

As mentioned previously, the ability of PGs (including the syndecans) to bind a wide variety of ligands is primarily (although not entirely) a function of their GAG chains. In this section, the role of HS and CS/DS in regulating BMP and FGF signalling will be discussed as these factors play an important role in the regulation of vascular calcification, and are therefore of most relevance to this thesis. The role of HS and CS/DS in regulating osteoblast differentiation and/or mineralisation will also be discussed.

1.5.1 BMPs

BMP-2 and BMP-4 are potent pro-calcific factors in vascular calcification, whereas BMP-7 plays an inhibitory role (discussed in section 1.2.3). BMP signalling can be regulated at several points throughout the pathway (Figure 1.4), including by the GAG chains, HS and CS/DS. The role of HS in regulating BMP signalling has been examined by targeting various HS biosynthetic enzymes, such as EXT1, using gene knock-out approaches (Johnson et al., 2007; Holley et al., 2011b; Kraushaar et al., 2012). Depleting endogenous HS in mouse embryonic stem cells (ESCs) results in impaired/reduced BMP signalling (Kraushaar et al., 2012), suggesting that HS positively regulates BMP signalling in these cells. In contrast, when bacterial heparinases were used to degrade cell surface HS in human MSCs, BMP signalling was enhanced (Manton et al., 2007). However, these results must be treated with caution because the bacterial heparinases can remove most of the HS chain bound to a core protein, although a small HS ‘stub’ often
remains. It is possible that the resulting short HS chains may possess altered biological activity, and it is therefore unclear if the findings of the latter study reflect changes in ligand signalling activity and/or depletion of HS.

In a complementary approach, exogenous HS can be applied to HS-deficient and/or HS-competent cells to determine the effects of specific HS motifs in regulating BMP signalling. Using this approach, the response to BMP-4 is reduced in EXT1-null mouse ESCs, but this can be recovered by the addition of exogenous heparin, indicating that HS is required for successful BMP signalling (Holley et al., 2011b). Consistent with this suggestion, BMP-4 is more prone to degradation in the culture medium taken from EXT1-null mouse ESCs, and this could be prevented by the addition of heparin (Kraushaar et al., 2012). Sulphation patterning in HS can further influence its regulation of BMP signalling. Previous studies have shown that noggin is most efficiently sequestered at the cell surface to inhibit BMP signalling by binding HS sequences composed of 10 or more monosaccharides which contain N-, 6-O- and 2-O-sulphation (Viviano et al., 2004). This study went on to show that Sulf1-induced removal of sulphate groups from the C6 position in GlcNS is necessary for the release of noggin from the cell surface and a restoration of BMP-4 responsiveness in chinese hamster ovary (CHO) cells. Together these studies demonstrate that carefully modulating the sulphation patterning in HS can be used to activate or inhibit the BMP responsiveness of a particular cell.

In contrast to HS, the role of CS/DS in regulating BMP signalling is, with few exceptions, unknown. CS containing GlcA-GalNAc(4S,6S) as its major disaccharide species (referred to as CS-E) increases the deposition of a mineralised matrix by the mouse osteoblastic MC3T3-E1 cell line; however this could be blocked using a neutralising antibody against BMP-4 (Miyazaki et al., 2008). Whilst CS-E increased MC3T3-E1 mineralisation, monosulphated CS (GlcA-GalNAc(4S) and GlcA-GalNAc(6S)) had no effect (Miyazaki et al., 2008). Together these data suggest that CS modulates BMP-4 signalling and osteoblast mineralisation in a sulphation-dependent manner. A role for CS/DS in regulating BMP signalling has also been demonstrated in vivo. Knocking-down C4ST1 expression in mice results in severe dwarfism due to defects in endochondral ossification (Klüppel et al., 2005). This phenotype was associated with reduced BMP-induced Smad1 phosphorylation in the growth plate of C4ST1-null mice, although exogenous BMP-2-induced Smad1 phosphorylation was similar in explants from wild-type and C4ST1-null mice. Therefore, the loss of BMP signaling in C4ST1-null mice is not due to an intrinsic inability of the cells to respond to BMP, but instead, the ability of BMPs to diffuse or access all surface signaling receptors may be compromised in the absence of 4-O-sulphated CS.

1.5.2 FGFs

The FGFs are emerging as important regulators of vascular calcification (discussed in section 1.2.4), and it is well known that HS and CS/DS regulate FGF signalling. In this regard, the FGFs are able to bind to FGFRs with high affinity, but this interaction and subsequent signaling events are amplified in the presence of HS, which is thought to act as a co-receptor for FGFFGFR
binding (Yayon et al., 1991; Rapraeger et al., 1991; Kan et al., 1999). Early studies in primary neural cells and differentiated teratocarcinoma cells demonstrated that alterations in HS sulphation patterns and core protein expression occurred concomitantly with the temporal expression of FGFs (Jiang et al., 1995; Brickman et al., 1998). In a more recent report, Pickford et al demonstrated that heparin/HS is an absolute requirement for the transduction of FGF-mediated signals across the cell membrane during the neural differentiation of 46C ESCs (Pickford et al., 2011). This study went onto show that specific heparin/HS polysaccharides can support the formation of neural progenitor cells from HS-competent 46C ESCs, and this effect was dependent on the sulphation pattern, concentration and the length of the saccharide. FGF-2 activity is also crucial during osteogenesis (Marie et al., 2012; Ornitz and Marie, 2015), and FGF-2-induced osteopontin expression was reduced in rat calvarial osteoblast cells treated with sodium chlorate to inhibit GAG sulphation (Ling et al., 2006). However, these results should be treated with caution because sodium chlorate inhibits all sulphation in a cell; thus, we cannot interpret if the results of the latter study reflect changes in the sulphation patterning of HS and/or CS/DS, or other components entirely.

CHO cells (pgsD-677) deficient in HS still exhibit FGF-2 signalling, although at a lower level than controls (Ashikari-Hada et al., 2009), indicating that the remaining CS/DS can support FGF-2 signalling in these cells. Consistent with this suggestion, CS/DS binds FGF-2, and FGF-2 binding and FGF-2-induced Erk1/2 activation is regulated by CS/DS chain sulphation (Ramachandra et al., 2014; Nikolovska et al., 2015). In this regard, knocking-down UST expression (adds a sulphate group to the 2-OH position of GlcA or IdoA in CS/DS) reduced FGF-2 binding and FGF-2-induced Erk1/2 activation in CHO cells (Nikolovska et al., 2015). Brittle star (class Ophiuroidea) 2-O sulphated CS/DS has a high amount of IdoA and is able to activate Erk1/2 in the presence of FGF-2 in CHO cells that lack any GAGs (Ramachandra et al., 2014). DS preparations also bind FGF-2, and DS enhances FGF-2 induced cell proliferation in F32 lymphoid cells (Penc et al., 1998; Taylor et al., 2005). Together these studies suggest that the domain organisation and/or sulphation patterning of CS/DS modulates FGF-2 signalling, as has previously been shown for HS (Jastrebova et al., 2010).

Regulation of FGF signalling by syndecans, and by HS and CS/DS specifically, in vascular calcification is currently unknown; this aim will therefore be addressed in this thesis.

1.5.3 Exogenous GAGs can be used to modulate growth factor signalling and osteoblast mineralisation

It is clear that the BMPs and FGFs interact in a specific fashion with GAGs and studies in ESCs are providing evidence to suggest that specific GAG oligosaccharides of defined pattern and known ligand activity can be used to selectively activate these signalling pathways. For example, relatively short GAG oligosaccharides containing N-, 2-O and to a lesser extent 6-O-sulphation are crucial for the support of FGF-mediated formation of neuroectodermal precursors from mouse ESCs, whilst longer sequences (~16-24 saccharides) containing N- and 6-O-sulfate groups are needed to support mesodermal differentiation. It is likely that these results reflect the
different signalling pathways involved in each event with BMP-4 signalling more important in the formation of haemangioblast than neural precursors (Holley et al., 2011b; Pickford et al., 2011). Given that both FGF and BMP signalling regulate both osteogenesis and vascular calcification, these findings suggest that specific GAG oligosaccharides (either purified from biological sources or generated by chemo-enzymatic methods (Xu et al., 2011)) could provide a novel mechanism to inhibit cell differentiation, and possibly mineralisation; this aim will therefore be addressed in this thesis. Indeed, heparin has been previously reported to inhibit bovine VSMC mineralisation in vitro (Yang et al., 2005).

The ability for GAGs to either support or inhibit signalling by distinct ligands is also concentration-dependent, as low concentrations of exogenous GAGs potentiate a GAG-dependent signalling pathway, whilst high concentrations prevent access of the ligand to the cell surface receptor, switching to an inhibitory effect (Holley et al., 2011b; Pickford et al., 2011). In this regard, heparin introduced at 10 ng/ml was sufficient to rescue haemangioblast formation from ESCs, whereas at higher concentrations, heparin dose-dependently inhibited this process (Holley et al., 2011b). Taken together, these findings suggest that much care will need to be taken when using GAGs to inhibit cell differentiation, and possibly mineralisation. Indeed, both exogenous HS (Dombrowski et al., 2009) and enzymatic removal of HS chains (Manton et al., 2007) increased mineral deposition by MSCs, suggesting GAG levels may need to be finely balanced in this setting.

Several studies have also implicated exogenous CS/DS in the regulation of osteoblast differentiation and/or mineralisation. For example, exogenous CS or DS enhances the osteogenic differentiation and/or mineralisation of human MSCs (Wollenweber et al., 2006; Mathews et al., 2014), and mouse MC3T3-E1 osteoblasts (Miyazaki et al., 2008). These studies suggest that CS/DS plays a pro-calcific role in osteoblasts. In further support of this suggestion, chondroitinase ABC treatment (which digests both CS and DS) inhibited matrix mineralisation by MC3T3-E1 osteoblast cells (Miyazaki et al., 2008).

In summary, GAG synthesis is non-template driven however control is exerted due to the activity of the biosynthetic enzymes themselves. Many of these enzymes create motifs that act as substrates for the next enzyme in the biosynthetic pathway, and in HS, this leads to areas of highly modified sequence which alternate with areas of intermediate or no modification. This domain structure is less clear in CS/DS, but the incomplete yet interdependent activity of the biosynthetic enzymes still leads to highly heterogeneous chains containing 3D motifs which have been shown to be tissue and developmental/disease-state specific (Zhang, 2010). These motifs are functional as they can regulate BMP and FGF signalling, both of which are important regulators of bone formation and vascular calcification. Hereditary multiple osteochondroma (HMO), which has been linked to aberrant HS synthesis, is associated with a loss of control of osteogenesis and the formation of cartilage-capped bony outgrowths at the ends of long bones. The decrease in HS chain length and probable increase in proximal HS sulphation in this disease has been linked to disturbances in hedgehog gradients across the growth plate (Roehl
and Pacifici, 2010). Whilst aberrant GAG synthesis has only been associated with deregulated bone formation in HMO, it does pose the possibility that GAG synthesis is altered in other disease contexts where abnormal bone remodelling occurs, such as vascular calcification. This hypothesis will be tested in this thesis.

1.6 Summary

It is well recognised that PG core proteins and their GAGs play a critical role in the regulation of osteogenic signalling pathways (e.g. BMPs, FGFs) and thus, bone formation (Rodgers et al., 2008). Given the similarities between bone formation and vascular calcification, it is intuitive to consider that GAGs and their PG cores may also play a role in regulating vascular calcification. Consistent with this suggestion, decorin (Fischer et al., 2004; Yan et al., 2011) and biglycan (Song et al., 2012) have been reported to play a stimulatory role in vascular calcification (see section 1.2.5); however, much discord currently exists over the expression levels of these PGs in vascular and/or cardiac valve calcification (summarised in Table 1.2). Disparities in the expression levels of other PGs in vascular calcification have also been observed, including versican, lumican and osteoglycin (summarised in Table 1.2). Therefore, in this thesis, the expression profile of several PG core proteins during the osteogenic differentiation and mineralisation of VSMCs will be examined simultaneously in order to highlight other PGs (e.g. syndecans) with a novel role in this process.

The ability of PGs to regulate growth factor signalling is primarily (although not entirely) a function of their GAG chains, which can act as co-receptors, stabilise gradients and/or protect ligands from degradation (Roehl and Pacifici, 2010). GAGs are abundantly expressed in bone (Prince and Navia, 1983; Waddington et al., 1989; Waddington and Embery, 1991; Duer et al., 2008), and they have also been identified as a major component of human calcified atherosclerotic plaques (Duer et al., 2008). However, the composition of GAGs in calcified vessels has not been determined. The size and sulphation patterning of GAG chains regulates growth factor signalling (see section 1.5), and several growth factors play a pro- or anti-calcific role in the VSMC mineralisation process, including BMPs and FGFs (Table 1.1). Indeed, the GAG chains displayed on decorin are vital to its role in accelerating human VSMC mineralisation in vitro, likely through the up-regulation of TGF-β1 signalling in these cells (Yan et al., 2011). The sulphation patterning of HS and CS/DS during VSMC mineralisation will therefore be investigated in this thesis.

The role of GAGs in vascular calcification is largely unexplored, with the exception of two studies. Firstly, heparin has been reported to inhibit the mineralisation of bovine VSMCs in vitro (Yang et al., 2005). A report published during the course of this thesis also revealed that exostosin-like glycosyltransferase 2 (EXTL2)-null mice produce high levels of HS and CS/DS, and CKD- and high phosphate-induced aortic calcification was increased in these mice (Purnomo et al., 2013). However, these results must be treated with caution, as there were also significant differences in the aortic CS/DS disaccharide composition of EXTL2-null mice when compared to wild-type controls. Therefore, it is difficult to interpret if the results of this study...
reflect changes in the sulphation patterning of CS/DS, or increases in total levels of HS and CS/DS. The role of GAGs in VSMC mineralisation will therefore be investigated in this thesis.

1.7 Hypothesis and Aims
The evidence discussed in this Introduction led to the hypothesis that PGs (and their GAG chains) play a role in regulating vascular calcification by fine-tuning the signalling pathways that promote VSMC mineralisation. To test this hypothesis, an unbiased approach was used in order to identify a PG with a novel role in the regulation of VSMC mineralisation. The following aims were therefore addressed in this study:

1. Determine the expression profile of PGs at confluence, and during VSMC osteogenic differentiation and mineralisation.

2. Profile the GAG composition of VSMCs at confluence, and during VSMC osteogenic differentiation and mineralisation.

3. Identify a PG that plays a novel role in the regulation of VSMC mineralisation, and determine the function of this PG using knock-down and over-expression approaches.
## 2 Materials and Methods

### 2.1 Materials

All reagents were analytical grade and obtained from Sigma Aldrich (UK) unless otherwise stated.

### 2.2 Cell culture

All cell culture experiments and maintenance were carried out in a class II biological safety cabinet under aseptic conditions. Cells were grown and maintained in a Heracell incubator (Thermo Scientific) at 37°C in a humidified atmosphere containing 5% (v/v) CO₂.

#### 2.2.1 Bovine VSMCs

Bovine VSMCs were isolated from bovine aortas using the explant culture method (Leik et al., 2004; Churchman and Siow, 2009). In brief, bovine aortas were obtained from a local abattoir and the perivascular fat and connective tissue surrounding the aorta was removed by blunt dissection. Aortas were cut open longitudinally and the luminal side of the vessel wall washed with sterile phosphate buffered saline (PBS) containing 200 U/ml penicillin and 2.8 µM streptomycin. The endothelial layer was removed from the lumen using a scalpel blade and 2 mm² sections from the aortic medial layer were placed medial side down in a sterile petri dish. Aortic explants were cultured for 2 weeks in high glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 200 U/ml penicillin, 2.8 µM streptomycin, 1 mM sodium pyruvate, 1x non-essential amino acids and 10% (v/v) FCS. Fungazone (1%(v/v)) was also included in the culture medium for the first week to prevent fungal infections. Cells were first seen to migrate from the explants after 5-7 days, at which point explants were removed. These cells had a spindle-like morphology and were characterised as VSMCs on the basis of positive immunostaining for the VSMC markers αSMA (Sigma #A257; 1:200 dilution) and SM22α (Abcam #Ab89989; 1:100 dilution) (staining protocol described in (Taylor, 2014)); 100% of stained VSMCs were positive for αSMA and SM22α. Other populations of VSMCs used in this study were isolated by Thomas Morris, Dr Rebecca Taylor or Dr. Smeeta Sinha (University of Manchester, UK), and were characterised as described above.

Bovine VSMCs were routinely cultured in high glucose DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 1.4 µM streptomycin, 1 mM sodium pyruvate, 1x non-essential amino acids and 10% (v/v) fetal calf serum (FCS) (hereafter referred to as 10% FCS-DMEM). Medium was changed every 2-3 days, and cells were split in a 1:3 ratio when ~90% confluent. Four different preparations of cells were used in this study, VSMC-A, VSMC-B, VSMC-C and VSMC-D, which were isolated from four different bovine aortas. The VSMC preparation used in individual experiments are indicated in the figure legends. Cells were used between passage 10 and 13.

#### 2.2.2 Human coronary artery smooth muscle cells (SMCs)

Human coronary artery SMCs were purchased from Gibco®, Life Technologies, UK (Lot no:
886619, 36 year-old woman) and routinely cultured in medium 231 with smooth muscle growth supplement (SMGS) (both from Gibco®, Life Technologies, UK). Cells were cultured on tissue culture plastic treated with 0.1% (v/v) gelatin in PBS for 20 minutes and washed once with PBS before use. Media was changed every 2-3 days, and split in a 1:3 ratio when ~90% confluent. Cells were used at passage 7 or 8.

2.2.3 Human MSCs
Human MSCs were purchased from Lonza (Lot no: 7F3915, 21 year-old male) and routinely cultured in MesenPro RS™ medium (Gibco®, Life Technologies, UK). Cells were cultured on tissue culture plastic treated with 0.1% (v/v) gelatin in PBS for 20 minutes and washed once with PBS before use. Media was changed every 3-4 days, and split in a 1:5 ratio when ~80-90% confluent. Cells were used at passage 5.

2.2.4 Human embryonic kidney (HEK) 293Ts
HEK293Ts were routinely cultured in high glucose DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 1.4 µM streptomycin. Medium was changed every 2-3 days, and cells were split in a 1:10 ratio when ~90% confluent. Cells were used between passage 5 and 10.

2.3 VSMC mineralisation assays
Bovine VSMCs were seeded in 6-well plates or 25 cm² tissue culture flasks at $2 \times 10^4$ cells/cm² and cultured in 10% FCS-DMEM until confluent (day 0). Cells were then cultured in 10% FCS-DMEM and 3 or 5 mM β-GP to induce calcification. Controls were cultured in the absence of β-GP. Medium was changed every 2-3 days over the course of the experiment.

To analyse the gene expression profile (including PG core proteins, GAG biosynthetic enzymes, osteogenic and SMC markers) of control and mineralising VSMC-A by quantitative polymerase chain reaction (qPCR), RNA was isolated from cells as detailed in section 2.10. For stages 1 and 2 of VSMC-A mineralisation, the experiment was performed twice, with triplicate samples for each treatment group (i.e. three 25 cm² tissue culture flasks of control or β-GP-treated VSMCs at each timepoint). For reference, gene expression analyses carried out on control or β-GP-treated VSMCs at stages 1 and 2 of VSMC-A mineralisation were combined as no differences in gene expression were observed between the treatment groups ($n=3$ samples per timepoint, per experiment, to give a total of $n=12$ per treatment group). For stages 3-5 of VSMC-A mineralisation, 3 independent experiments were performed, with triplicate samples for each treatment group, at each timepoint. For reference, all gene expression analyses of control and mineralising VSMCs-A were performed on the same pool of samples.

To analyse the protein expression profile (FGF-2, syndecan 1, syndecan 4) of control and mineralising VSMC-A by western blotting, protein lysates were isolated from cells as detailed in section 2.9. For stages 3-5 of VSMC-A mineralisation, 3 independent experiments were performed. For reference, protein lysates were collected from mineralisation experiments performed separately to the gene expression analysis experiments described above.
2.4 Cell treatments

2.4.1 TNF, TGF-β1, IL-1β
Lyophilised recombinant TNF (#210-TA), TGF-β1 (#240-B) and IL-1β (#201-LB) (all from R&D Systems, Abingdon, UK) were reconstituted in sterile PBS containing 0.1% (w/v) bovine serum albumin (BSA) to give a stock concentration of 1 µg/ml (TGF-β1, IL-1β) or 10 µg/ml (TNF). Confluent VSMCs were incubated with 10 ng/ml TNF, TGF-β1 or IL-1β in 10% FCS-DMEM for the times indicated in the figure legends, and RNA collected from the cells as detailed in section 2.10. An equivalent volume of PBS containing 0.1% (w/v) BSA used as a vehicle control. RNA was also collected from cells before the addition of TNF, TGF-β1, IL-1β or vehicle (‘0 hours’).

2.4.2 Exogenous FGF-2
Lyophilised recombinant FGF-2 (Peprotech, UK) was reconstituted in sterile 5 mM Tris (pH 7.6) with 0.1% (w/v) BSA to give a stock concentration of 100 µg/ml. VSMCs were grown to confluence (day 0) and incubated with 25 or 50 ng/ml FGF-2 in 10% FCS-DMEM ± 3 mM β-GP for up to 11 days. An equivalent volume of 5 mM Tris (pH 7.6) with 0.1% (w/v) BSA was used as a vehicle control. Medium was changed every 2-3 days.

2.4.3 Heparin
(i) Culturing VSMCs with heparin from confluence
Lyophilised heparin isolated from intestinal pig mucosa (#HEP001, Iduron, UK) was reconstituted in sterile PBS to give a stock concentration of 3 mg/ml. To analyse the effects of heparin on VSMC mineralisation, VSMCs were grown to confluence (day 0) and incubated with 3 mM β-GP and heparin (2, 10 or 50 µg/ml) for up to 10 days. An equivalent volume of sterile PBS was used as a vehicle control. Medium was changed every 2-3 days.

(ii) Culturing VSMCs with heparin 24 hours post-plating
Twenty-four hours after seeding VSMCs into 6-well plates at 2 X 10⁵ cells/cm², cells were cultured with heparin (10 µg/ml or 50 µg/ml) in 10% FCS-DMEM ± 3 mM β-GP for up to 16 days. The first day of culture in the calcification medium was defined as day 0. Sterile PBS was used as a vehicle control.

2.4.4 BGJ398 (FGFR inhibitor)
BGJ398 (sc-364430, Santa Cruz, UK) was reconstituted in dimethyl sulfoxide (DMSO) warmed to 37°C to give a stock concentration of 1 mM. To analyse FGF-2 signalling following BGJ398 treatment, VSMC-A were grown to confluence and incubated with BGJ398 (10 nM, 100 nM, 250 nM, 500 nM or 1 µM) in 10% FCS-DMEM for 48 hours. An equivalent volume of DMSO was used as a vehicle control. After this time, cells were serum starved for 2 hours with DMSO or BGJ398 (10 nM, 100 nM, 250 nM, 500 nM or 1 µM) then incubated with FGF-2 (25 or 50 ng/ml) for 5 minutes. Protein cell lysates were then collected and analysed as detailed in section 2.9.

To analyse the effects of BGJ398 on VSMC mineralisation, VSMCs were grown to confluence (day 0) and incubated with BGJ398 (10 nM, 100 nM or 1 µM) in 10% FCS-DMEM ± 3 or 5 mM
β-GP for up to 11 days. An equivalent volume of DMSO was used as a vehicle control. Medium was changed every 2 days.

2.4.5 Gó6976 (PKC inhibitor)
Gó6976 (#12060S, Cell Signalling, UK) was reconstituted in DMSO to give a stock concentration of 1 mM. To analyse the effects of Gó6976 on VSMC mineralisation, VSMCs were grown to confluence (day 0) and incubated with 1 µM Gó6976 in 10% FCS-DMEM ± 3 mM β-GP for up to 11 days. An equivalent volume of DMSO was used as a vehicle control. Medium was changed every 2 days.

2.5 Gene silencing using siRNA
2.5.1 siRNAs
Syndecan 4 siRNA-1 (bovine-specific) and syndecan 4 siRNA-2 (human and bovine-specific) were pre-designed and purchased from Ambion, Life Technologies (UK) and Fischer Scientific (UK), respectively. Syndecan 1 siRNA was designed using Block-iT RNAi designer (Life Technologies) and synthesised by Eurofins (Luxembourg). The sequences for EXT1 siRNA-1 and EXT1 siRNA-2 were obtained from (Busse et al., 2007), and synthesised by Eurofins (Luxembourg). PKCα siRNA-1 and PKCα siRNA-2 were pre-designed and purchased from Qiagen (UK) and Ambion, Life Technologies (UK), respectively. See Table 2.1 for siRNA sequences. A negative control siRNA was purchased from Qiagen, UK (#1027281). Lyophilised siRNAs were reconstituted in nuclease-free H₂O at 50 µM and stored at -20°C until use. The final siRNA concentrations used in experiments are indicated in the figure legends.

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sense strand (5’-3’)</th>
<th>Anti-sense strand (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syndecan 4 siRNA-1</td>
<td>AUACUUUUCGGGAGACUUAtt</td>
<td>UAAGUCUCCGGAAAGUAAUtg</td>
</tr>
<tr>
<td>Syndecan 4 siRNA-2</td>
<td>GCAAGAAACCCAUCUACAAAtt</td>
<td>UUGUAGAUGGGAUUCUGCtt</td>
</tr>
<tr>
<td>Syndecan 1 siRNA</td>
<td>UCAUGCUGUACCGGAGAAttt</td>
<td>UUCAUCCGGUAGCAUAGAtt</td>
</tr>
<tr>
<td>EXT1 siRNA-1</td>
<td>GGAUCAUCCCCAGAGCAGAttt</td>
<td>UCCUGUCCUGGGAUAGUCCtt</td>
</tr>
<tr>
<td>EXT1 siRNA-2</td>
<td>GGCUUAUUUUCUCAGUUttt</td>
<td>AUCUGAAGAAAAAUAGCCtt</td>
</tr>
<tr>
<td>PKCα siRNA-1</td>
<td>GTCTTTTCAGTTCATAGAtt</td>
<td>TTTAATTGGAGCTGAAGGac</td>
</tr>
<tr>
<td>PKCα siRNA-2</td>
<td>GGCUGUACUCGUAGCAGAtt</td>
<td>UCCUGAGCAAGUAGCACGCGa</td>
</tr>
</tbody>
</table>

2.5.2 VSMC mineralisation assays
VSMCs were seeded in 6-well plates at 2 X 10⁴ cells/cm² and cultured in 10% FCS-DMEM until ~80% confluent. Cells were then transfected with the siRNAs as follows. The siRNA and 2.5 µl Lipofectamine RNAiMAX (Life Technologies, UK) was added to 500 µl Optimem I reduced serum medium (Gibco®, Life Technologies, UK). The solution was gently mixed and incubated
for 20 minutes at room temperature (RT). To the transfection mixture, 2 ml 10% FCS-DMEM was added, and the resulting solution was added to one well of a 6-well plate. Forty-eight hours after the first siRNA transfection when the cells had reached confluence (day 0), the cells were transfected in 10% FCS-DMEM with β-GP to induce calcification as follows. The siRNA transfection mixture was prepared as described above and incubated for 20 minutes at RT. To the transfection mixture, 2 ml 10% FCS-DMEM with 3.75 or 5.75 mM β-GP was added, and the resulting mixture was added to one well of a 6-well plate. The transfection agents were removed after 4 hours and the media replaced with 2 ml 10% FCS-DMEM containing 3 or 5 mM β-GP. The siRNA transfection agents were removed after 4 hours as previous studies in this lab have shown that Lipofectamine RNAiMAX and/or Optimem I reduced serum medium inhibit VSMC mineralisation when included in the cell culture medium between transfections (Taylor, 2014). Controls were transfected in the absence of β-GP. Cells were transfected every 48-72 hours during the course of the experiment.

2.5.3 Effects of FGF-2, TNF and TGF-β1 on VSMC mineralisation with siRNA-treated cells

VSMCs were seeded in 6-well plates at 2 X 10^4 cells/cm^2 and cultured in 10% FCS-DMEM until ~80% confluent. Cells were transfected with the siRNAs in 10% FCS-DMEM as described in section 2.5.2. Forty-eight hours after the second siRNA transfection (day 0), the cells were transfected in 10% FCS-DMEM with β-GP ± FGF-2, TNF or TGF-β1 as follows. The siRNA transfection mixture was prepared as described in section 2.5.2 and 2 ml 10% FCS-DMEM containing 3.75 mM β-GP ± FGF-2 (31.25 or 62.5 ng/ml), TNF (1.25 or 12.5 ng/ml) or TGF-β1 (12.5 ng/ml) was added. The resulting mixture was then added to one well of a 6-well plate. The final concentration of β-GP in the media was 3 mM and the final concentration of ligands was as follows: FGF-2 (25 or 50 ng/ml), TNF (1 or 10 ng/ml), and TGF-β1 (10 ng/ml). After 4 hours, the transfection agents were removed and the media replaced with 2 ml 10% FCS-DMEM containing 3 mM β-GP ± FGF-2 (25 or 50 ng/ml), TNF (1 or 10 ng/ml) or TGF-β1 (10 ng/ml). Cells were transfected every 48-72 hours during the course of the experiment. Fresh FGF-2, TNF and TGF-β1 was added each time the culture medium was changed.

2.5.4 Signalling assays with siRNA-treated VSMCs

VSMCs were seeded in 6-well plates at 2 X 10^4 cells/cm^2 and cultured in 10% FCS-DMEM until ~80% confluent. Cells were transfected with the siRNAs in 10% FCS-DMEM as described in section 2.5.2, with the exception that the siRNA transfection agents were not removed between transfections. Cells were transfected every 48-72 hours. Forty-eight hours after the third siRNA transfection, cells were washed twice with PBS and incubated with serum free-DMEM for 2 hours, then treated with TGF-β1 (10 ng/ml) or FGF-2 (25 or 50 ng/ml) in serum free-DMEM for the times indicated in the figure legends. Protein lysates were collected as detailed in section 2.9.1. An equivalent volume of vehicle (PBS with 0.1% (w/v) BSA for TGF-β1 experiments; 5
mM Tris (pH 7.6) with 0.1% (w/v) BSA for FGF-2 experiments) was used as a control. Protein lysates were also collected from cells before the addition of TGF-β, FGF-2 or vehicle (‘0 hours’).

2.5.5 siRNA transfections in human coronary artery SMCs and MSCs
Human coronary artery SMCs or human MSCs were seeded into 6-well plates treated with 0.1% (v/v) gelatin at 2 X 10^4 cells/cm^2 and 6 X 10^3 cells/cm^2, respectively. Cells were transfected with negative control siRNA or syndecan 4 siRNA-2 as described in section 2.5.2, with the exception that the siRNA transfection agents were not removed between transfections. Cells were transfected every 48-72 hours during the course of the experiment.

2.6 Generation of syndecan 4 over-expression pCDH-EF1-T2A-puro vector
2.6.1 Amplification of syndecan 4 insert using PCR primers containing the Nhel and BamHI restriction sites
The retroviral vector pBabe Puro, containing the full-length human syndecan 4 gene with an extracellular influenza hemagglutinin (HA)-tag (herein referred to as pBabe_SDC4) was a kind gift from Dr Mark Morgan (University of Liverpool, UK). PCR was used to clone the Nhel and BamHI restriction sites onto the 5’ and 3’ ends, respectively, of the full-length human syndecan 4 gene with extracellular HA-tag (without the stop codon) in the following reaction: 0.1 ng, 1 ng or 10 ng pBabe Puro_SDC4, 0.4 µM sense primer, 0.4 µM anti-sense primer, 1x Phusion high-fidelity buffer (New England Biolabs, UK), 1.5 U Phusion high-fidelity DNA polymerase (New England Biolabs, UK) and 200 µM deoxyribonucleotide triphosphates (dNTPs). The total reaction volume was adjusted to 50 µl with nuclease-free water. Primer sequences are shown in Table 2.2. The PCR reaction was carried out using a Mastercycler gradient PCR machine (Eppendorf, UK) and the following heat cycles: 95°C for 2 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, and one cycle of 72°C for 30 seconds.

Following PCR, 8 µl DNA loading buffer (0.25% (w/v) bromophenol blue, 30% (v/v) glycerol) was added to the PCR reaction mixture and 26 µl of the PCR/DNA loading buffer mixture was separated by gel electrophoresis on a 1% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 10 mM EDTA and 0.35% (v/v) acetic acid, pH 8.5) with 1x GelRed Nucleic Acid Gel Stain (Biotium, USA). The PCR product was visualised using an UVitec transilluminator and images captured with a UVitec camera (both UVitec, Cambridge, UK); a single band of the predicted size (~639 bp) was observed. The remaining PCR/DNA loading buffer mixture was separated by gel electrophoresis on a 1% (w/v) agarose gel in TAE buffer with 1x GelRed Nucleic Acid Gel Stain and the PCR product was excised from the gel using a clean blade and a Dark Reader transilluminator (Clare Chemical Research, Dolores, USA) to visualise the PCR product. The DNA was subsequently purified using the QIAEX II gel extraction kit (Qiagen, UK) according to the manufacturer’s instructions.
Table 2.2. Primers used to amplify the full-length human syndecan 4 gene with extracellular HA-tag out of the pBabe_SDC4 vector

<table>
<thead>
<tr>
<th>Sense primer (5’-3’)</th>
<th>Anti-sense primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TATGCTAGC GCCACCATGGCCCCCGCCGTCT</td>
<td>ATAGGATCCCGCTAGAACTCATTGGTG</td>
</tr>
</tbody>
</table>

The NheI (sense primer) and BanHI (anti-sense primer) restriction sites are highlighted in green. The Kozac sequence on the sense primer is highlighted in red.

2.6.2 Restriction enzyme digests

To insert the syndecan 4 PCR product into the pCDH-EF1-MCS-T2A-puro vector (System Biosciences, USA), both the PCR product and pCDH vector were digested with the NheI and BamHI restriction enzymes (both from New England Biolabs, UK) in the following reaction: 10 µl purified PCR product (~0.5 µg DNA) or 1 µg pCDH vector, 1x CutSmart® buffer (New England Biolabs, UK), 10 U NheI and 10 U BanHI. The total reaction volume was adjusted to 20 µl with nuclease-free water. The mixture was incubated for 2.5 hours at 37°C. After this time, 3 µl DNA loading buffer (0.25% (w/v) bromophenol blue, 30% (v/v) glycerol) was added to the digested PCR product and pCDH-EF1-T2A-puro vector and the samples separated by gel electrophoresis on a 1% (w/v) agarose gel in TAE buffer with 1x GelRed Nucleic Acid Gel Stain. The DNA was extracted from the agarose gel using the QIAEX II gel extraction kit (Qiagen, UK) according to the manufacturer’s instructions.

2.6.3 Ligation

The digested syndecan 4 PCR product was ligated into the corresponding sites of the digested pCDH-EF1-T2A-puro vector in the following reaction: 1x T4 buffer, 400 U T4 DNA Ligase (both from New England Biolabs, UK), 1µl digested pCDH-EF1-MCS-T2A-puro vector (~6 ng) and 1 µl (~6 ng) or 3 µl (~18 ng) digested syndecan 4 PCR product. The total reaction volume was adjusted to 10 µl with nuclease-free water. A negative control reaction was set up with no syndecan 4 insert to determine if any uncut or self-ligating recipient pCDH vector was present. Samples were incubated overnight at 4°C.

2.6.4 Transformation of E-coli with pCDH_SDC4

To transform E-coli (XL-1 blue competent cells; Agilent Technologies, UK) with the ligation reaction mixture, cells were thawed on ice and 50 µl of cells were added to the 10 µl ligation reaction mixtures under aseptic conditions. The transformation mixture was then incubated on ice for 30 minutes, heat shocked for 45 seconds at 42°C and incubated on ice for a further 2 minutes. After this time, 200 µl super optimal broth with catabolite repression (SOC) medium (Invitrogen, Life Technologies, USA) was added to the transformation mixture, and incubated for 1 hour at 37 °C under agitation. Following this incubation period, all of the transformation mixture was transferred to a lysogeny broth (LB) agar plate containing 100 µg/ml carbenicillin.
(Melford Laboratories Ltd., UK) and the mixture was spread evenly across the agar plate. The plates were incubated overnight at 37°C.

2.6.5 Amplification of plasmid DNA
The following day, individual colonies were isolated from the agar plates using sterile loops and transferred to 5 ml sterile 20% (w/v) LB broth containing 100 µg/ml carbenicillin. The cultures were incubated overnight at 37°C under agitation. The next day, the bacterial cultures were collected by centrifugation at 10,000 xg for 5 minutes at RT, and DNA was purified using the QIAprep Spin Miniprep kit (Qiagen, UK) according to the manufacturer’s instructions. DNA concentrations were quantified using a Nanodrop 2000 spectrophotometer (Pierce Thermo Scientific, UK) and quality confirmed by a 260/280 ratio of approximately 1.8.

To ensure that the syndecan 4 insert had been incorporated into the pCDH vector, the DNA purified from each bacterial culture was digested with the NheI and BamHI restriction enzymes in the following reaction: ~500 ng DNA, 1x CutSmart® buffer, 20 U NheI, 20 U BamHI, and made up to a total reaction volume of 20 µl with nuclease-free water. The mixture was incubated for 2 hours at 37°C and the digested products were separated on a 1% (w/v) agarose gel in TAE buffer with 1x GelRed Nucleic Acid Gel Stain. The PCR product was visualised using an Uvitec transilluminator and images captured with a UVItech camera (both UVItec, Cambridge, UK).

2.6.6 Plasmid DNA sequencing
The purified DNA was sequenced by the DNA Sequencing Facility (University of Manchester, UK) in the following reaction: ~2 µg DNA, 0.33 µM sense or anti-sense primer, and made up to a total reaction volume of 10 µl with nuclease-free water. Two sequencing primers were used: the sense primer was designed against a region within the EF1α promoter, and the anti-sense primer against a region within the puromycin-resistance gene in the pCDH-EF1-T2A-puro vector. Primer sequences are shown in Table 2.3.

<table>
<thead>
<tr>
<th>Sense primer (5’-3’)</th>
<th>Anti-sense primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCCACGCTTTGCTGACCTGCTT</td>
<td>TTCTTGAGCTCGGTGAC</td>
</tr>
</tbody>
</table>

The sense primer binds within the elongation factor 1α (EF1) promoter, and the anti-sense primer binds within the puromycin-resistance sequence.

2.6.7 Expansion of pCDH_SDC4
Purified syndecan 4 over-expression vector (pCDH_SDC4) obtained in section 2.6.5 was transformed into E. coli to obtain higher DNA concentrations. XL-1 blue competent cells were thawed on ice and 50 µl of cells were added to 2 µl purified plasmid DNA. The transformation
mixture was then incubated on ice for 30 minutes, heat shocked for 45 seconds at 42°C, then incubated on ice. After 2 minutes, 200 µl SOC medium was added to the transformation mixture and incubated for 1 hour at 37 °C under agitation. Following this incubation period, 40 µl of the transformation mixture was transferred to a LB agar plate containing 100 μg/ml carbenicillin and the mixture was spread evenly across the agar plate. The plates were incubated overnight at 37°C.

The following day, a single bacterial colony was isolated from the agar plate using a sterile loop and transferred to 50 ml 20% (w/v) LB broth containing 100 μg/ml carbenicillin. The cultures were incubated overnight at 37°C under agitation. The next day, the bacterial cultures were collected by centrifugation at 4,000 xg for 10 minutes at RT, and DNA was purified using the HiSpeed Plasmid Midi Kit (Qiagen, UK). The kit was used according to the manufacturer’s instructions with the following modifications. Following elution of the DNA from the HiSpeed Tip, the DNA was precipitated by adding 3.5 ml isopropanol. The resulting mixture was then divided between six 1.5 ml eppendorfs and centrifuged at 19,000 xg for 30 minutes at 4°C. The supernatant was discarded and the DNA pellet was washed with 333 µl 70% (v/v) ethanol. Following centrifugation at 19,000 xg for 10 minutes at 4°C, the DNA pellet was air-dried at RT and re-suspended in 20 µl nuclease-free water. The pellets were then incubated overnight at 4°C. The next day, the eluted DNA was combined and DNA concentrations and quality were quantified as described in section 2.6.5.

2.6.8 Expansion of psPAX2 and pMD2.G from bacterial glycerol stocks

The packaging vectors, psPAX2 and pMD2.G were both kind gifts from Dr Andrew Gilmore (University of Manchester, UK). A sterile 200 µl tip was used to collect frozen E-coli glycerol stocks (XL-1 blue competent cells; Agilent Technologies, UK) containing psPAX2 or pMD2.G. E-coli containing either psPAX2 or pMD2.G was transferred to a LB agar plate containing 100 μg/ml carbenicillin and the plate was streaked using standard procedures and incubated overnight at 37°C. The following day, a single bacterial colony was isolated from the agar plate using a sterile loop and transferred to 50 ml 20% (w/v) LB broth containing 100 μg/ml carbenicillin. The cultures were incubated overnight at 37°C under agitation and DNA was purified as described in section 2.6.7.

2.6.9 Generation of lentivirus using HEK293Ts

Empty pCDH vector (pCDH_empty) or pCDH_SDC4 were combined with the two packaging vectors, psPAX2 and pMD2.G, and transfected into HEK293Ts to generate lentiviral particles using the following protocol. High-glucose DMEM (250 µl) was combined with 6 µg pCDH_empty or pCDH_SDC4, 4.5 µg psPAX2 and 3 µg pMD2.G in a single eppendorf. In a separate eppendorf, 1x polyethylenimine (PEI) in 150 mM NaCl was added to 223 µl high-glucose DMEM. Both mixtures were incubated for 2 minutes at RT, after which the PEI-DMEM mixture was added to the eppendorf containing the DNA. The resulting mixture was vortexed for 2 minutes and then incubated for 20 minutes at RT. The mixture was then added to HEK293Ts grown to ~70% confluency in a 75 cm² tissue culture flask containing 5 ml of fresh medium (high
glucose DMEM, 10% FCS, 100 U/ml penicillin, 1.4 µM streptomycin). Cells were incubated overnight at 37°C, 5% (v/v) CO₂.

The following day, the media was replaced with 10 ml of fresh medium containing 10 mM sodium butyrate (Merck Millipore, UK) and the cells were incubated at 37°C, 5% (v/v) CO₂ for 6 hours. After this time, 5-7 ml of fresh medium was added and the cells were incubated at 37°C, 5% (v/v) CO₂ for a further 48 hours. The cell medium was then collected and centrifuged at 450 xg (C28 BOECO, Hamburg, Germany) for 5 minutes and the supernatant filtered through a 0.45 µm low protein binding filter (Millex, Millipore, UK). The conditioned medium containing lentivirus was then stored at 4°C or used immediately.

### 2.6.10 Over-expression of human syndecan 4 using lentivirus

(i) **HEK293Ts**

HEK293Ts were grown to ~30% confluency in 25 cm² tissue culture flasks and 7 ml of lentivirus-containing medium (from one 75 cm² flask of HEK293Ts producing either pCDH_empty or pCDH_SDC4 lentivirus) was added to the cells with 8 µg/ml hexadimethrine bromide. Cells incubated with 8 µg/ml hexadimethrine bromide only served as controls. All cells were incubated at 37°C, 5% (v/v) CO₂ for 17 hours. After this time, the medium was removed, the cells were washed once with PBS and 5 ml of fresh medium was added. The cells were then cultured for 3 days before being treated with 3 µg/ml puromycin dihydrochloride for 3 days to select for transduced cells. Cells treated with hexadimethrine bromide only were also cultured with puromycin dihydrochloride in order to determine when all the non-transduced cells had been killed. After the puromycin selection, cells were cultured and passaged according to standard procedures (see section 2.2.4).

(ii) **VSMCs**

VSMCs (5 X 10⁵ cells) were pelleted by centrifugation at 800 xg for 5 minutes and re-suspended in lentivirus-containing medium with 8 µg/ml hexadimethrine bromide. The volumes of lentivirus used are detailed in Table 2.4. Cells incubated with 8 µg/ml hexadimethrine bromide only served as controls. Re-suspended cells were transferred to one well of a 6-well plate and incubated at 37°C, 5% (v/v) CO₂ for 17 hours. After this time, the media was removed, the cells were washed once with PBS and 2 ml of fresh media was added. After 24 hours, the cells were incubated with lentivirus-containing medium and 8 µg/ml hexadimethrine bromide for a second time in order to increase transduction efficiency. The volumes of lentivirus used are detailed in Table 2.4. Cells were incubated overnight at 37°C, 5% (v/v) CO₂ for 17 hours, after which, the media was removed, cells were washed once with PBS and 2 ml of fresh media was added. Once the cells had reached 70-90% confluence, cells were treated with trypsin for 5 minutes and seeded at 2.2 X 10⁴ cells/cm² in 25 cm² tissue culture flasks. The cells were then cultured for 24 hours before being treated with 3 µg/ml puromycin dihydrochloride for 5 days to select for transduced cells. Cells treated with hexadimethrine bromide only were also cultured with puromycin dihydrochloride in order to determine when all the non-transduced cells had been
killed. After the puromycin selection, cells were cultured and passaged according to standard procedures (see section 2.2.1).

Table 2.4. Volume of lentivirus used to transduce VSMC-A

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<thead>
<tr>
<th>VSMC</th>
<th>Transfection 1 (volume of lentivirus)</th>
<th>Transfection 2 (volume of lentivirus)</th>
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</thead>
<tbody>
<tr>
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<td>2 ml</td>
<td>0</td>
</tr>
<tr>
<td>Ctrl-2</td>
<td>1 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Ctrl-3</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Ctrl-4</td>
<td>5 ml</td>
<td>2 ml</td>
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<tr>
<td>SDC4 o/e-1</td>
<td>5 ml</td>
<td>0</td>
</tr>
<tr>
<td>SDC4 o/e-2</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>SDC4 o/e-3</td>
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<td>5 ml</td>
</tr>
<tr>
<td>SDC4 o/e-4</td>
<td>5 ml</td>
<td>2 ml</td>
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</tbody>
</table>

2.6.11 Amplification of full-length human syndecan 4 with extracellular HA-tag from lentivirus-transduced cells

(i) PCR

RNA was extracted from lentivirus-treated HEK293Ts or VSMCs as described in section 2.10. PCR was used then to amplify the full-length human syndecan 4 gene with extracellular HA-tag from lentivirus-transduced cells in the following reaction: 1 µg cDNA, 0.4 µM sense primer, 0.4 µM anti-sense primer, 1x Phusion high-fidelity buffer (New England Biolabs, UK), 1.5 U Phusion high-fidelity DNA polymerase (New England Biolabs, UK) and 200 µM deoxyribonucleotide triphosphates (dNTPs). The total reaction volume was adjusted to 50 µl with nuclease-free water. Primer sequences are shown in Table 2.2. The PCR reaction was carried out using a Mastercycler gradient PCR machine (Eppendorf, UK) and the following heat cycles: 95°C for 2 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, and one cycle of 72°C for 30 seconds.

Following PCR, 8 µl DNA loading buffer (0.25% (w/v) bromophenol blue, 30% (v/v) glycerol) was added to the PCR reaction mixture and 26 µl of the PCR/DNA loading buffer mixture were separated by gel electrophoresis on a 1% (w/v) agarose gel in TAE buffer (40 mM Tris, 10 mM EDTA and 0.35% (v/v) acetic acid, pH 8.5) with 1x GelRed Nucleic Acid Gel Stain (Biotium, USA). The PCR product was visualised using an UVitec transilluminator and images captured with a UVitec camera (both UVitec, Cambridge, UK). The PCR product was then excised from the gel using a clean blade and a Dark Reader transilluminator (Clare Chemical Research, Dolores, USA) to visualise the PCR product. The DNA was subsequently purified using the QIAEX II gel extraction kit (Qiagen, UK) according to the manufacturer’s instructions.
(ii) **DNA sequencing**

Purified DNA was sequenced by the DNA Sequencing Facility (University of Manchester, UK) in the following reaction: ~40 ng DNA, 0.33 µM sense or anti-sense primer, and made up to a total reaction volume of 10 µl with nuclease-free water. Primer sequences are shown in Table 2.2.

### 2.7 Alizarin red staining of cultured VSMCs

Mineral deposition by cultured VSMCs was confirmed by staining with alizarin red. Cells were washed twice with PBS and fixed for 20 minutes in 2% (v/v) formaldehyde with 1% (w/v) sucrose, in PBS. Following a single wash in ddH$_2$O, cells were stained under agitation with 40 mM alizarin red (pH 4.1) for 20 minutes. Cells were then washed a further 4-6 times in ddH$_2$O and allowed to dry overnight. Images were captured using a digital camera (Olympus DP70, USA) attached to an IX51 inverted microscope (Olympus, USA).

### 2.8 Alizarin red dye elution

To elute the alizarin red stain and quantify mineral deposition, cells were placed on a shaking platform for 30 minutes in 10% (v/v) acetic acid. Cells were collected, and with the acetic acid, heated at 85°C for 10 minutes. Samples were then cooled on ice and centrifuged at 16,000 xg for 15 minutes at 4°C. The supernatant was collected and 4% (v/v) sodium hydroxide was added to each sample to neutralise the acid. Absorbance was read at 405 nM using an MRX II absorbance reader (Dynex Technologies, UK).

### 2.9 Western blotting

#### 2.9.1 Sample preparation

Protein was collected using lysis buffer (20 mM Tris-HCl pH 7.6, 150 mM sodium chloride, 1 mM EDTA, 1% (v/v) Igepal, 50 mM sodium flouride, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, and 1x protease inhibitor cocktail set 1 (Calbiochem, Nottingham, UK)) and samples were centrifuged at 16,000 xg for 20 minutes at 4°C to pellet any cell debris. The supernatant was transferred into a clean eppendorf tube and protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce Thermo Scientific, UK). Samples were normalised for protein content (15-50 µg, protein loading is indicated in the figure legends) and then heated to 95°C for 10 minutes with 1x Laemml buffer (60 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.01% (v/v) bromophenol blue).

#### 2.9.2 SDS-PAGE and blotting

Protein samples were separated using denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% Bis-Tris gel, or 4-20% Bis-Tris gradient gel (Bio-Rad, UK). Precision Plus Protein Standards All Blue (Bio-Rad, 10 µl) were also run on each gel. Proteins were then transferred to Hybond-P polyvinylidene membranes (GE Healthcare, UK) using the Trans-blot semi-dry transfer cell (Bio-Rad, UK) and semi-wet transfer buffer (38 mM glycine, 48 mM Tris, 0.028% (w/v) SDS in 20% (v/v) methanol, pH 8.3) for 70 minutes at 0.15 A. Alternatively, when blotting for syndecan 4, proteins were transferred to 0.45 µm nitrocellulose...
membranes (Bio-Rad, UK) using the Dual Run & Blot System (Expedeon, UK) and wet transfer buffer (38 mM glycine, 48 mM Tris, 0.038% (v/v) SDS in 20% (v/v) methanol) for 2 hours at 300 V and 230 A.

Following transfer, membranes were blocked (see Table 2.5 for blocking solutions) for 1 hour at RT and incubated with primary antibody (diluted in the same solution used to block) overnight at 4°C (see Table 2.5). The next day, membranes were washed three times in Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST) for 20 minutes each and incubated with secondary antibody (diluted in the same solution used to block) for 1 hour at RT. Membranes were washed a further three times, for 20 minutes each, in TBST and then incubated with Enhanced Chemiluminescence Western Blotting Substrate or Supersignal West PICO Chemiluminescence substrate (both Pierce Thermo Scientific, UK) for 5 minutes. Membranes were exposed to Amersham Hyperfilm ECL (GE Healthcare, UK) in a dark room or imaged on the ChemiDoc XRS System (Bio-Rad, UK).

Membranes were stripped in 25 mM glycine in 1% (w/v) SDS (pH 2) for 30 minutes at RT and re-probed for a second primary antibody if required. All membranes were stripped and re-probed for β-actin to confirm equal protein loading (see Table 2.5 for β-actin antibody details).

2.10 RNA isolation and generation of complementary DNA (cDNA)

Total RNA was extracted from VSMCs using the RNeasy Mini Kit (Qiagen, UK) and any remaining genomic DNA was removed by DNase treatment (Ambion, Life Technologies, UK); both kits were used according to the manufacturer’s instructions. RNA concentrations were quantified using a Nanodrop 2000 spectrophotometer (Pierce Thermo Scientific, UK) and quality confirmed by a 260/280 ratio of approximately 2.0.

RNA was reverse transcribed to generate complementary DNA (cDNA) using a Taqman® kit in the following reaction: 1 µg RNA, 1x reverse transcriptase buffer, 5 mM MgCl₂, 2 mM dNTP mix, 1.67 µM random hexamers, 0.5 U/ml RNase inhibitor, 1.2 U/µl reverse transcriptase. RNA was reversed transcribed using the Mastercycler gradient PCR machine (Eppendorf, Cambridge, UK) and the following heat cycles: 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5 minutes. Samples processed without reverse transcriptase were used as a negative control. All cDNA was stored at -20°C before analysis.
Table 2.5. Western blotting antibodies

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (kDa)</th>
<th>Blocking agent</th>
<th>Primary antibody</th>
<th>Dilution and duration</th>
<th>Secondary antibody</th>
<th>Dilution and duration</th>
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</thead>
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<tr>
<td>Akt</td>
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<td>5% (w/v) milk in TBST</td>
<td>Cell Signalling (#9272)</td>
<td>1:1000; o/n 4°C</td>
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<td>1:1000; 1 hour RT</td>
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<td>pErk1/2</td>
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<td>5% (w/v) BSA in TBST</td>
<td>Cell Signalling (#4377)</td>
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<td>Cell Signalling (#4695)</td>
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2.11 cDNA analysis

2.11.1 Primer design

With the exception of peptidylprolyl isomerise A (PPIA), Runx2, Msx2, BMP-2, αSMA and SM22α, all primers were designed using Primer3 (Just Bio) and purchased from Eurogentec (Southampton, UK). Primers were designed across two coding exons for a given gene sequence, with the exception of syndecan 1, NDST3, CHSY-2 and CHSY-3 which were designed within a single coding exon. Primer pairs were validated to ensure linear amplification over several orders of magnitude of template dilutions (see section 2.11.2) and single product formation (see section 2.11.3). Primer pairs for Runx2, Msx2, BMP-2, αSMA and SM22α were designed and optimised by Dr Gareth Hyde (Imperial College London, UK). The primer set for PPIA was designed by and purchased from Primer Design (Southampton, UK). Primer sequences are shown in Table 2.6.

2.11.2 Confirmation of primer efficiency

To confirm that the primers used for qPCR were efficient, cDNA was serially diluted in DEPC-treated ddH2O as follows: neat cDNA, 1:4 cDNA, 1:16 cDNA, 1:64 cDNA. Using these cDNA dilutions, qPCR was performed using 1x SYBR Green PCR master mix, 0.8 µM of sense primer, 0.8 µM anti-sense primer and 1.5 µl of cDNA per reaction. Samples were processed using a CFX96 or CFX384 Real-Time PCR system (Bio-Rad, UK) and the following heat cycles: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 1 minute and 72°C for 15 seconds, followed by 1 cycle of 95°C for 15 seconds, 60°C for 20 seconds and 95°C for 15 seconds. All samples were amplified in duplicates, and the averaged C\textsubscript{t} value was plotted against the [–Log] cDNA input. A linear line of best fit was generated using Microsoft Excel 2007 software and the gradient of the line of best fit (‘slope’) was converted into an efficiency value using Equation 2.1:

\textbf{Equation 2.1:} 
\textit{Primer efficiency} = 10^{\frac{\text{Slope}}{1}} - 1

Primer efficiencies are shown in Table 2.6. Primers were deemed suitable for qPCR if the efficiency was between 90-110% (Taylor et al., 2010). The syndecan 3 and PKCα primers were the only primers to slightly fall outside of the recommended efficiency range, with efficiency values of 86% and 118%, respectively. The coefficient of determination (r\textsuperscript{2}) was also deduced to determine how well the data fitted to the line of best fit. All primers analysed had r\textsuperscript{2} values between 0.98-1. Primer efficiency could not be calculated for PKCβ, NDST3, NDST4, C6ST2, glypicans 2, 3, 5 and 6 as these genes could not be detected or were detected at the lower limits of detection by qPCR.

2.11.3 Confirmation of primer specificity

To confirm that the primers used for qPCR were specific to their intended target, 0.5 µl of 0.25% (w/v) bromophenol blue, 30% (v/v) glycerol was added to primer products obtained from the qPCR reaction and separated on a 2% (w/v) agarose, in Tris-acetate-EDTA (TAE) buffer (40 mM Tris base, 10 mM EDTA and 0.35% (v/v) acetic acid, pH 8.5) gel with 1x GelRed Nucleic
Table 2.6. Primer sequences and efficiency for qPCR

<table>
<thead>
<tr>
<th>Gene family</th>
<th>Gene</th>
<th>Gene accession number</th>
<th>Sense primer (5’-3’)</th>
<th>Anti-sense primer (5’-3’)</th>
<th>Product size (bp)</th>
<th>Efficiency (%)</th>
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<td>HS enzymes</td>
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<td>Primer Sequence (Reverse)</td>
<td>Efficiency</td>
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Unless otherwise stated, all primers have been designed against the bovine genome. Primer efficiency could not be tested for primers against PKCβ, NDST3, NDST4, C6ST2, glypicans 2, 3, 5 and 6 as they could not be detected or were detected at the lower limits of detection by qPCR.
Acid Gel Stain (Biotium, California, USA) using gel electrophoresis. Following separation by gel electrophoresis, primer products were visualised using a UVitec transilluminator (UVitec, Cambridge, UK). Primers were deemed specific for their intended targets if a single band of the expected amplicon size was observed (data not shown).

2.11.4 qPCR

qPCR was performed using 1x SYBR Green PCR master mix, 0.8 µM of sense primer, 0.8 µM anti-sense primer and 1.5 µl of cDNA per reaction. Samples were processed using a CFX96 or CFX384 Real-Time PCR system (Bio-Rad, UK) and the following heat cycles: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 1 minute and 72°C for 15 seconds, followed by 1 cycle of 95°C for 15 seconds, 60°C for 20 seconds and 95°C for 15 seconds. All samples were amplified in duplicates and averaged. The expression of the tested gene was then normalised to the reference genes (ribosomal protein L12 (RPL12) and PPIA in bovine samples; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin and/or TATA-binding protein (TBP) in human samples) using the comparative Ct method (Applied Biosystems, UK). SYBR green is a double-stranded DNA intercalating dye, that fluoresces once bound to the DNA. The PCR cycle number when the signal is detected above the threshold level (i.e. background) represents the Ct value for a given mRNA transcript. This Ct value is normalised by subtracting the average Ct value of the appropriate reference genes. The normalised target amount in the sample is then equal to $2^{\Delta Ct}$. Pilot experiments confirmed that the expression levels of the reference genes, RLP12 and PPIA, are consistent during the osteogenic differentiation and mineralisation of bovine VSMCs (Figure 2.1).

Figure 2.1. The expression levels of the reference genes, RPL12 and PPIA, are consistent during the osteogenic differentiation and mineralisation of bovine VSMCs. Confluent VSMC-A (day 0) were cultured with ± 3 mM β-GP for up to 14 days. RNA was collected at day 0, then at specific stages of the mineralisation process (stages 1-5). The expression levels of the reference genes, RPL12 and PPIA, were measured by qPCR. Results displayed as mean ± SEM. Day 0 ($n=13$ samples from 4 independent experiments); Stage 1-2 ($n=6$ samples from 2 independent experiments); Stage 3-5 ($n=9$ samples from 3 independent experiments).
2.12 Isolation of metabolically radiolabelled GAGs

2.12.1 Confluent VSMCs

To generate radiolabelled GAGs, two separate preparations of VSMCs (VSMC-A and VSMC-B) were grown to ~80-90% confluence in 175 cm$^2$ tissue culture flasks and incubated with 23.3-40 µCi/ml D-[6-$^3$H]glucosamine (PerkinElmer Life Sciences, UK) for 48 hours. The culture medium (20 ml), plus a 15 ml PBS wash, was then removed and incubated with 100 µg/ml pronase (Roche, UK) for 4 hours at 37°C. The cell/matrix layer was extracted with 0.1% (v/v) Triton X-100 in PBS for 2 hours at RT, removed and treated with pronase as described above.

2.12.2 Mineralising VSMCs

Bovine VSMCs (VSMC-A) were seeded in 175 cm$^2$ tissue culture flasks at 2 X 10$^4$ cells/cm$^2$ and cultured in 10% FCS-DMEM until confluent (day 0). VSMCs were then cultured in the presence of 3 mM β-GP for 10 days until the early stages of VSMC mineralisation. At this time, cells were incubated with 28 µCi/ml D-[6-$^3$H]glucosamine for 48 hours. The culture medium (20 ml), plus a 15 ml PBS wash, was removed and treated with 10 µg/ml DNase (Roche, UK) for 30 minutes at 37°C, followed by 100 µg/ml pronase for 4 hours at 37°C. The cell/matrix layer was extracted with 1% (v/v) Triton X-100, 6M urea in dH$_2$O for 48 hours at 4°C. The urea was de-ionised using mixed resin beads (Sigma Aldrich, UK) prior to use. The extracted cells and matrix were collected and passed through a needle several times to break up the matrix, buffer-exchanged into 1% (v/v) Triton X-100 in PBS using anion-exchange chromatography and treated with DNase and pronase as described above.

2.12.3 Purification of GAGs using ion-exchange chromatography and gel filtration

To purify $^3$H-labelled GAG chains from the pronase-treated fractions, anion-exchange chromatography was performed. A 5 ml diethylaminoethanol (DEAE)-Sephacel (Sigma Aldrich, UK) column was prepared and equilibrated with 10 column volumes (cv) PBS, 5 cv 1.5 M sodium chloride (NaCl), 20 mM sodium phosphate (NaP) in dH$_2$O (pH 7) and 10 cv PBS before use. Pronase-treated fractions were loaded onto the DEAE-Sephacel column and washed with 100 ml PBS to remove material non-specifically bound to the column. Bound material was then eluted with a gradient of 0.15-0.825 M NaCl, 20 mM NaP in dH$_2$O (pH 7). Fractions of 2 ml were collected, and aliquots (20 µl for confluent and mineralising VSMC-A; 50 µl for confluent VSMC-B) were taken for scintillation counting. Fractions were pooled as appropriate, and diluted three times in dH$_2$O.

To concentrate samples into a smaller working volume, the pooled fractions were loaded onto a 1 ml DEAE-Sephacel column equilibrated as described above. Bound material was eluted with 15 washes in 1 ml 1.5 M NaCl, 20 mM NaP in dH$_2$O (pH 7). Fractions of 1 ml were collected and 1 µl aliquots were taken for scintillation counting. The fractions that contained the majority of the radiolabelled material were pooled and de-salted using a PD10 column (GE Healthcare Life Sciences, UK) in 1 ml fractions. The resulting material was then lyophilised and re-suspended in 1 ml ddH$_2$O.
2.12.4 Purification of HS and CS/DS chains using enzymatic digestion and size-exclusion chromatography

(i) Purification of HS

To resolve intact HS chains from CS/DS, samples were lyophilised, re-suspended in 50 mM Tris, 50 mM NaCl (pH 7.9) and digested with chondroitinase ABC (two aliquots of 2 mlU overnight at 37°C). The following day, digested samples were loaded onto a Sepharose CL-6B (Fischer Scientific, UK) size-exclusion column and eluted with 200 mM ammonium bicarbonate at a flow rate of 12 ml/hour. Fractions of 1 ml were collected and 20 µl aliquots were taken for scintillation counting. The fractions that contained intact HS chains were pooled, lyophilised twice and re-suspended in 1 ml ddH₂O. To ensure no salt was left in the samples, intact HS chains were de-salted using a PD10 column in 0.5 ml fractions and the resulting material was lyophilised and re-suspended in 1 ml ddH₂O.

(ii) Purification of CS/DS

To resolve intact CS/DS chains from HS, samples were lyophilised, re-suspended in 100 µL 100 mM sodium acetate, 0.1 mM calcium acetate (pH 7) and digested with heparinases I, II, and III (used at 2 mlU each, all from Iduron, UK) overnight at RT. The different substrate specificities of the heparinase enzymes for HS are shown in Table 2.7. Two aliquots of each enzyme were added over the digest period. The following day, digested samples were loaded onto a Sepharose CL-6B size-exclusion column and eluted as described in section 2.12.4i. The fractions that contained intact CS chains were pooled, lyophilised and de-salted as described in section 2.12.4i.

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<th>Enzyme</th>
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<td>GlcNSO₃ (±3 6S)-IdoA (2S)</td>
<td>Cleaves within S domains</td>
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<tr>
<td>Heparinase II</td>
<td>GlcNSO₃/Ac (±6S)-IdoA(±2S)</td>
<td>Extensive breakdown of the chain.</td>
</tr>
<tr>
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<td>GlcNSO₃/Ac (±6S)-GlcA</td>
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2.12.5 Characterisation of radiolabelled HS

(i) HS chain length

An aliquot (50,000 cpm of ³H) of HS was lyophilised and re-suspended in equal volumes (200 µl) of 2 M sodium borohydride and 100 mM NaOH for 48 hours at 45°C to liberate HS chains from the attached peptide core. Phenol red (20 µl) was then added to the sample to indicate pH and the reaction was neutralised by dropwise addition of glacial acetic acid. Samples were loaded onto a Sepharose CL-6B column with 50 µ1 sodium dichromate to indicate the total
volume ($V_t$) of the column and eluted with 200 mM ammonium bicarbonate at a flow rate of 12 ml/hour. Fractions of 1 ml were collected and analysed by scintillation counting.

To confirm the HS chains had been liberated from the attached peptide cores during alkaline borohydride treatment, and the estimated MW of the GAG chain was accurate, un-treated HS was loaded onto the Sepharose CL-6B column with 50 µl sodium dichromate and eluted as described above. Collected fractions were analysed by scintillation counting, and plotted against $K_{av}$ (see below). The $^3$H cpm peak was shifted to the right (indicating smaller material) in alkaline borohydride-treated HS samples when compared against the un-treated control.

For the determination of average HS chain length, $K_{av}$ values (calculated using Equation 2.2) were compared with a calibration curve (Wasteson, 1971a). The elution of material from a size-exclusion column can be influenced by the total volume of the packed bed (in this instance, the total volume of Sepharose CL-6B resin in the column) and the way in which the column has been packed. Therefore, the $K_{av}$ value is used to standardise a size-exclusion column as it eliminates the effects of these two parameters on the elution profile of HS samples (and also other material, e.g. CS/DS, proteins). Using the $K_{av}$ of a column also means that the Wasteson (1971a) calibration curve can be used to determine the MW of unknown sized HS samples as Wasteson plotted the elution of sized CS oligosaccharides against the $K_{av}$ on a Sepharose CL-6B column.

To calculate the $K_{av}$ (Equation 2.2) of the Sepharose CL-6B column used in this study, the $V_o$ was taken as the fraction number at which the largest material eluted from the Sepharose CL-6B size-exclusion column. Previous work in our laboratory has identified that untreated HS is high MW, and will start to elute at the void volume of the Sepharose CL-6B column. Therefore, in this study, the $V_o$ was taken as the first fraction number at which untreated HS was eluted from the column. When running untreated HS on the Sepharose CL-6B column, the $V_o$ of the column remained relatively consistent between runs. The $V_t$ is the point at which the smallest material will elute, and was taken as the fraction number half way between the first and last fraction containing sodium dichromate. Sodium dichromate is a small, yellow-coloured salt, and will elute in the total volume of the column ($V_t$). Sodium dichromate was run alongside both untreated and sodium borohydride-treated HS on the CL-6B to monitor the $V_t$ of the Sepharose CL-6B column between runs, and hence the $K_{av}$ of the column.

Equation 2.2: $K_{av} = (\text{fraction number}-V_o)/(V_t-V_o)$

(ii) Low pH nitrous acid depolymerisation
An aliquot (10,000 cpm of $^3$H) of HS was lyophilised and re-suspended in 100 µl nitrous acid (from 0.114g barium nitrite dissolved in 500 µl ddH$_2$O + 500 µl 1N sulphuric acid) for 30 minutes at RT. Phenol red (20 µl) was then added to the sample to indicate pH and the reaction was neutralised by dropwise addition of saturated sodium carbonate. Oligosaccharide products were fractionated on a Superdex 30 (Fischer Scientific, UK) size-exclusion column and eluted with
100 mM ammonium bicarbonate at a flow rate of 4 ml/hour, with collection of 0.5 ml fractions. Oligosaccharide products were analysed by scintillation counting.

The Superdex 30 size-exclusion columns used in this study were calibrated with sized-material as follows. Radiolabelled HS disaccharides that had been previously generated by low pH-nitrous acid depolymerisation (and separated on a Bio-Gel P-10 column and pooled; this material was kindly provided by Dr Rebecca Holley, University of Manchester) were separated on the Superdex 30 column to indicate the elution point(s) of disaccharides from this column. As HS disaccharides will elute last from the column due to their small size (~450 daltons), the size of the larger oligosaccharides generated by low pH nitrous acid depolymerisation could be inferred by working back from the elution point of the disaccharides i.e. dp2<dp4<dp6<dp8. The $V_t$ of the Superdex 30 column (indicated by sodium dichromate) was also used to monitor the point at which the smallest HS material (in this instance, HS disaccharides) eluted.

The number of linkages susceptible to low pH nitrous acid depolymerisation was calculated as follows. The total $^3$H cpm eluting in a specific peak was adjusted for the number of $^3$H-labelled GlcN sugars in the oligosaccharide corresponding to that peak. The percentage susceptibility is calculated as the percentage of total susceptible linkages (i.e. adjusted counts) in the total $^3$H cpm (i.e. unadjusted counts). The percentage of contiguous linkages (i.e. the disaccharide peak) was calculated as the percentage of $^3$H (adjusted) counts under the disaccharide peak in the total number of susceptible linkages. Tetrasaccharides have an internal GlcNAc and therefore correspond to alternate susceptible linkages, and the percentage is calculated in an equivalent process to the contiguous linkages.

(iii) Heparinase I and III digests

An aliquot (10,000 cpm of $^3$H) of HS was lyophilised, re-suspended in 100 µl 100 mM sodium acetate, 0.1 mM calcium acetate (pH 7) and digested with heparinase III (two aliquots of 2 mIU over digest period) overnight at 37°C. Oligosaccharide products were fractionated on a Superdex 30 size-exclusion column as described above and analysed by scintillation counting. Alternatively, an aliquot (10,000 cpm of $^3$H) of HS was digested with heparinase I (two aliquots of 5 mIU over digest period) and oligosaccharide products were fractionated on a Superdex 30 size-exclusion column as described above and analysed by scintillation counting.

The number of linkages susceptible to heparinase III treatment was calculated as follows. The total $^3$H counts eluting in a specific peak were adjusted for the number of $^3$H-labelled GlcN sugars in the oligosaccharide corresponding to that peak. The percentage susceptibility is calculated as the percentage of total susceptible linkages (i.e. adjusted counts) in the total $^3$H counts (i.e. unadjusted counts). The percentage of contiguous linkages (i.e. disaccharides) was calculated as the percentage of $^3$H (adjusted) counts under the disaccharide peak in the total number of susceptible linkages. Tetrasaccharides have an internal IdoA(±2S) and therefore correspond to alternate susceptible linkages, and the percentage is calculated in an equivalent process to the contiguous linkages.
(iv) Propac PA-1 SAX HPLC
Radiolabelled HS (50,000 cpm of $^3$H) was lyophilised, re-suspended in 100 µl 100 mM sodium acetate, 0.1 mM calcium acetate (pH 7) and digested with heparinas I, II, and III (used at 2 mIU each) overnight at RT. Two aliquots of each enzyme were added over the digest period. Disaccharides were recovered by size-exclusion chromatography (Superdex 30 resin), lyophilised twice and re-suspended in 1 ml ddH$_2$O.

Disaccharides (5000 cpm of $^3$H) were loaded onto a ProPac PA1 SAX-column (Dionex, Camberley, UK) and analysed by HPLC. Disaccharides were eluted with a 45 minute linear gradient of 0-1 M NaCl in HPLC-grade water (pH 3.5) at a flow rate of 1 ml/minute. Fractions of 0.5 ml were collected and analysed by scintillation counting. Disaccharides were identified by comparison with known standards (Iduron, UK). The relative amount of each disaccharide was expressed as a percentage of the total counts.

2.12.6 Characterisation of radiolabelled CS/DS

(i) CS/DS chain length
CS/DS chain length was determined as described for HS in section 2.12.5i, with the following modification. The number of disaccharides per CS/DS chain was calculated based on the assumption that the average MW of a CS/DS disaccharide is 503 daltons.

(ii) Chondroitinase B
An aliquot (10,000 cpm of $^3$H) of CS/DS was lyophilised, re-suspended in 100 µl 50 mM Tris, 50 mM NaCl (pH 7.9) and digested with chondroitinase B (two aliquots of 2 mIU over digest period, Iduron, UK) overnight at 37°C. DS oligosaccharides were fractionated on a Superdex 30 size-exclusion column and eluted with 100 mM ammonium bicarbonate at a flow rate of 4 ml/hour, with collection of 0.5 ml fractions. The relative amount of each species was measured by scintillation counting and expressed as a percentage of the total counts.

The number of linkages susceptible to chondroitinase B (digests disaccharides containing IdoA-GalNAc) was calculated as follows. The total $^3$H cpm eluting in a specific peak were adjusted for the number of $^3$H-labelled GalNAc sugars in the oligosaccharide corresponding to that peak. The percentage susceptibility is calculated as the percentage of total susceptible linkages (i.e. adjusted counts) in the total $^3$H cpm (i.e. unadjusted counts). The percentage of contiguous linkages susceptible to chondroitinase B was calculated as the percentage of $^3$H (adjusted) counts under the disaccharide peak in the total number of susceptible linkages. Tetrasaccharides have an internal GlcA and therefore correspond to alternate susceptible linkages, and the percentage is calculated in an equivalent process to the contiguous linkages.

(iii) Hypersil SAX-HPLC
Radiolabelled CS/DS (50,000 cpm of $^3$H) was lyophilised, re-suspended in 500 µl 50 mM Tris, 50 mM NaCl (pH 7.9) and digested with chondroitinase ABC (two aliquots of 2 mIU over digest
period) overnight at 37°C. Disaccharides were recovered by size-exclusion chromatography (Superdex 30 resin), then lyophilised twice and re-suspended in 1 ml ddH$_2$O.

Disaccharides (5000 cpm of $^3$H) were loaded onto a Hypersil 5 µm SAX-column (Thermo Scientific, UK) and analysed by HPLC. Disaccharides were eluted with a 45 minute linear gradient of 0-0.7 M NaCl in HPLC-grade water (pH 3.5) at a flow rate of 1 ml/minute. Fractions of 0.5 ml were collected and analysed by scintillation counting. Disaccharides were identified by comparison with known standards (Iduron, UK). The relative amount of each disaccharide was expressed as a percentage of the total counts.

(iv) Confirming the identification of UA-GalNAc(6S), UA-GalNAc(4S) and UA(2S)-GalNAc

To confirm the identification of the UA-GalNAc(6S), UA-GalNAc(4S) and UA(2S)-GalNAc disaccharides, 15,000 cpm of $^3$H-labelled CS/DS disaccharides were loaded onto the Hypersil SAX-HPLC column along with 5 µg UA-GalNAc(6S), 5 µg UA-GalNAc(4S) and 5 µg UA(2S)-GalNAc disaccharide standards (all from Iduron, UK). Disaccharides were eluted a 45 minute linear gradient of 0-0.7 M NaCl in HPLC-grade water (pH 3.5) at a flow rate of 1 ml/minute, and fractions were collected manually by monitoring the fluorescence of the UA-GalNAc(6S), UA-GalNAc(4S) and UA(2S)-GalNAc disaccharide standards at 232nm. The fractions of interest were re-loaded onto the Hypersil SAX-HPLC column and eluted as described above. Fractions of 0.5 ml were collected and analysed by scintillation counting.

2.13 Statistics

Data are presented as the mean ± standard deviation (SD) or standard error of the mean (SEM). Statistical comparisons of unpaired data were made with Student’s t-tests or one-way ANOVA and multiple comparison post-hoc tests. Data with two or more variables were analysed with a 2-way ANOVA and multiple comparison post-hoc tests, as indicated in the figure legends. All statistical comparisons were made using GraphPad Prism 6 software and probabilities of $P<0.05$ were considered statistically significant. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. 
3 Determining the expression profile of PGs during VSMC osteogenic differentiation and mineralisation

3.1 Introduction

Vascular calcification involves the osteogenic differentiation of VSMCs, VSMC apoptosis, calcifying matrix vesicle release and ECM mineralisation (reviewed in section 1.1). As discussed in Chapter 1, this disease process has the potential to be regulated by PGs and by their GAG chains (see section 1.6). Whilst some PGs have previously been implicated in the development of vascular calcification, such as decorin (Fischer et al., 2004; Yan et al., 2011) and biglycan (Song et al., 2012), conflicting results have often been obtained for their expression levels in both in vitro and in vivo models of the disease (summarised in Table 1.2). In addition, previous studies have often limited their analyses of VSMC mineralisation to a single PG and have not considered the coordinated up- or down-regulation of all PGs. Therefore, there is a great need to characterise the expression profile of different PG core proteins during the osteogenic differentiation and mineralisation of VSMCs.

The studies described in this chapter will begin to investigate a role for PGs in vascular calcification by addressing the following aims:

1) Determine the osteogenic differentiation and mineralisation potential of different VSMC preparations.
2) Characterise the PG profile of VSMCs at confluence.
3) Characterise the expression profile of different PGs during VSMC osteogenic differentiation and mineralisation.
3.2 Results

3.2.1 Characterisation of different VSMC preparations

Three preparations of VSMCs were isolated from three different bovine aortas by the explant culture method (section 2.2.1) and are referred to here as VSMC-A, VSMC-B and VSMC-C. To determine the osteogenic differentiation and mineralisation potential of these three VSMC preparations, a well-established *in vitro* model of vascular calcification was used (Collett et al., 2007; Alam et al., 2009). VSMCs were grown to confluence (day 0) and cultured with 3 or 5 mM β-GP for 12-14 days to induce mineralisation. Controls were cultured without β-GP. The extent of mineralisation was determined by staining cells with alizarin red at specific time-points between days 5-14. RNA was also isolated from the cells at day 0, and at specific time-points between days 5-14. The mRNA levels of specific osteogenic (Runx2, Msx2 and/or BMP-2) and SMC (αSMA and/or SM22α) markers were then determined using qPCR.

(i) VSMC-A

VSMC-A deposited a mineralised matrix when cultured in the presence of 3 mM β-GP, and the extent of mineralisation was increased with time (Figure 3.1A). No mineralisation was detected in cells cultured without β-GP (Figure 3.1A). The extent of mineralisation was quantified by dye elution that demonstrated that the level of mineralisation present at each stage of VSMC-A mineralisation was similar in four independent experiments (Figure 3.1B).

RNA was isolated from these cells at specific time-points over the course of the experiment: stage 1 (no mineral deposition, day 5), stage 2 (initial formation of mineral deposits, day 7), stage 3 (early mineralisation, days 9-10), stage 4 (mid mineralisation, days 10-12) and stage 5 (late mineralisation, days 12-14). RNA was also isolated from controls at these same time-points. At stages 1 and 2 of VSMC-A mineralisation, there was no change in the mRNA levels of Runx2, Msx2, BMP-2, αSMA and SM22α in β-GP-treated VSMC-A when compared to controls. For this reason, the expression levels of these mRNA transcripts at stage 1 and 2 of VSMC-A mineralisation were combined in all future analyses (Figure 3.1C). At the early stages (stage 3) of mineralisation, Runx2 (~2-fold increase; P<0.01) and Msx2 (~2-fold increase; P<0.01) mRNA expression was significantly up-regulated in β-GP-treated VSMC-A when compared to controls at the same time-point (Figure 3.1C). Runx2 and Msx2 remained significantly up-regulated in β-GP–treated VSMC-A at stages 4 and 5 of mineralisation when compared to controls (Figure 3.1C). The mRNA expression of BMP-2, a growth factor involved in the osteogenic differentiation and mineralisation of VSMCs (Li et al., 2008; Liberman et al., 2011; Balderman et al., 2012), was also up-regulated (~1.8-fold increase, P<0.01) in β-GP-treated VSMC-A at stages 3, 4 and 5 of mineralisation when compared to controls at the same time-point (Figure 3.1C). In contrast, αSMA (~3-fold decrease, P<0.001) and SM22α (~2.5-fold decrease, P<0.01) mRNA expression was significantly down-regulated in β-GP-treated VSMC-A at stages 4 and 5 of mineralisation, suggesting the differentiation of these cells from a smooth muscle to an osteoblast-like phenotype.
Figure 3.1. VSMC-A undergo osteogenic differentiation and mineralisation in the presence of β-GP. Confluent VSMC-A (day 0) were cultured ± 3 mM β-GP for up to 14 days. (A) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. Red staining indicates mineralisation. Arrow indicates early mineral deposits. (B) Mineral deposition was quantified by dye elution. Results displayed as mean ± SEM (n=4-6 samples from 4 independent experiments). (C) RNA was collected at day 0, then at specific stages of the mineralisation process (stages 1-5). The expression levels of osteogenic (Runx2, Msx2, BMP-2) and SMC (αSMA, SM22α) markers were measured using qPCR. Expression is shown relative to day 0, which is equal to 1. mRNA expression levels at stages 1 and 2 were combined. Results displayed as mean ± SEM. Stages 1&2 (n=12 samples from 2 independent experiments); Stages 3-5 (n=9 samples from 3 independent experiments). Data presented in (A) and (B) were analysed using a 2-way ANOVA with Sidak post-hoc tests. * P<0.05; ** P<0.01; *** P<0.001 against same time-point control.
(ii) **VSMC-B**
In contrast to VSMC-A, VSMC-B formed large multicellular nodules in the presence of 3 mM β-GP thereby resembling a subpopulation of VSMCs known as CVCs (Watson et al., 1994; Tintut et al., 2003). Cells cultured without β-GP also formed multicellular nodules in culture, suggesting that high phosphate levels were not responsible for this process. No mineral deposition was detected in these cells upon staining with alizarin red (Figure 3.2A). As phosphate levels in the culture medium determine VSMC mineralisation potential (Wada et al., 1999; Jono et al., 2000), VSMC-B were also cultured in 5 mM β-GP for up to 14 days. In the presence of this higher concentration of β-GP, VSMC-B formed large multicellular nodules with time in culture but still did not deposit a mineralised matrix (Figure 3.2B). To determine if VSMC-B were able to undergo osteogenic differentiation in the presence of β-GP, RNA was also isolated from these cells at days 0, 9, 11 and 14. These time-points were chosen as they corresponded to the time taken for VSMC-A to reach stages 3, 4 and 5 of mineralisation. The mRNA expression levels of specific osteogenic (Msx2) and SMC (αSMA) markers were then measured by qPCR. There was no change in the mRNA expression levels of Msx2 and αSMA in β-GP-treated VSMC-B when compared to controls (Figure 3.2C), suggesting that VSMC-B did not undergo osteogenic differentiation or mineralise in the presence of β-GP.

(iii) **VSMC-C**
VSMC-C formed multilayer ridges with time in culture that is characteristic of the ‘hill and valley’ morphology of previously characterised VSMCs (Collett et al., 2007; Alam et al., 2009). These ridges were formed in the presence or absence of β-GP suggesting that high phosphate levels were not responsible for this process (Figure 3.3A). VSMC-C did not deposit a mineralised matrix in the presence of 3 mM (data not shown) or 5 mM β-GP (Figure 3.3A). Mineralisation was also not detected in control cells cultured without β-GP (Figure 3.3A). To determine if VSMC-C were able to undergo osteogenic differentiation in the presence of β-GP, the mRNA expression levels of specific osteogenic (Msx2) and SMC (αSMA) markers were measured by qPCR at days 0, 9, 11 and 14. These time-points were chosen as they corresponded to the time taken for VSMC-A to reach stages 3, 4 and 5 of mineralisation. There was no change in the mRNA expression levels of Msx2 and αSMA in β-GP-treated VSMC-B when compared to controls (Figure 3.3B), suggesting that VSMC-C were unable to undergo osteogenic differentiation or mineralise in the presence of β-GP.
Figure 3.2. VSMC-B do not undergo osteogenic differentiation and mineralisation in the presence of β-GP. (A) Confluent VSMC-B (day 0) were cultured ± 3 mM β-GP for 14 days. Phase-contrast images of VSMCs stained with alizarin red (bar = 500 µm). (B) Confluent VSMC-B (day 0) were cultured ± 5 mM β-GP for up to 14 days. Phase-contrast images of VSMCs stained with alizarin red. Scale bar = 500 µm. Images are representative of 2 independent experiments. (C) RNA was collected from VSMCs at days 0, 9, 11 and 14. The expression levels of specific osteogenic (Msx2) and smooth muscle cell (αSMA) markers were measured using qPCR. Expression is shown relative to day 0, which is equal to 1. Results displayed as mean ± SEM and were derived from three wells of cells from one experiment. The data were analysed using a 2-way ANOVA with Sidak post-hoc tests.
Figure 3.3. VSMC-C do not undergo osteogenic differentiation and mineralisation in the presence of β-GP. Confluent VSMC-C (day 0) were cultured ± 5 mM β-GP for 14 days. (A) Phase-contrast images of VSMCs stained with alizarin red. Scale bar = 500 µm. Images are representative of 2 independent experiments. (B) RNA was collected from VSMCs at days 0, 9, 11 and 14. The expression levels of specific osteogenic (Msx2) and smooth muscle cell (αSMA) markers were measured using qPCR. Expression is shown relative to day 0, which is equal to 1. Results displayed as mean ± SEM and were derived from three wells of cells from one experiment. The data were analysed using a 2-way ANOVA with Sidak post-hoc tests.
3.2.2 PG core protein profile of VSMCs at confluence

To examine the PG profile of VSMCs as they undergo osteogenic differentiation and mineralisation, the PGs expressed by confluent VSMCs were first characterised. RNA was isolated from confluent VSMC-A, VSMC-B and VSMC-C and the mRNA expression levels of a panel of PGs were determined using qPCR. All three preparations of VSMCs expressed the four syndecan genes (Figure 3.4A). Whilst syndecan 1 and syndecan 4 were the most highly expressed syndecan isoforms in all three VSMC preparations, the relative expression of these syndecan isoforms differed slightly between VSMC-A, VSMC-B and VSMC-C. These preparations of VSMCs also expressed a variety of other HS and/or CS/DS core proteins, including perlecan, agrin, betaglycan, glypican 1 and glypican 4 (Figure 3.4B). Glypican 5 was also detected, although it was at the lower limits of detection when measured by qPCR (data not shown). In contrast, glypicans 2, 3 and 6 mRNA transcripts could not be detected in all three VSMC preparations. To determine if this observation was due to poor primer efficiency, another primer set was designed for each gene. Neither primer set detected glypicans 2, 3 or 6 mRNA in VSMCs (data not shown), indicating that these genes may not be expressed and/or are undetectable at the mRNA level in these cells.

The CS/DS core proteins, biglycan, versican and decorin, were also expressed by confluent VSMC-A, VSMC-B and VSMC-C (Figure 3.4B). The relative expression levels of these CS/DS core proteins were markedly higher than that of the HS core proteins analysed. The keratan sulphate core proteins, lumican and osteoglycin, were also expressed by all three preparations of VSMCs (Figure 3.4B).

3.2.3 PG core protein profile of VSMCs during osteogenic differentiation and mineralisation

The results presented in section 3.2.2 demonstrated that VSMCs express a panel of HS, CS/DS and KS core proteins, including syndecan 4. To determine the expression profile of these PGs during VSMC osteogenic differentiation and mineralisation, VSMC-A were cultured with 3 mM β-GP to induce mineralisation. Controls were cultured without β-GP. RNA and protein were isolated from these cells at specific time-points over the course of the experiment, as described in section 3.2.1i (see Figure 3.5A). The mRNA expression levels of several PGs (syndecan 1, syndecan 2, syndecan 3, syndecan 4, glypican 1, glypican 4, perlecan, agrin, betaglycan, biglycan, decorin, versican, osteoglycin, lumican) were then determined by qPCR. The protein expression levels of specific PGs (syndecan 1 and 4) during VSMC osteogenic differentiation and mineralisation were also analysed by western blotting. For reference, phase-contrast images of alizarin red-stained VSMC-A during the different stages of osteogenic differentiation and mineralisation are shown in Figure 3.1A.
Figure 3.4. PG core protein profile of confluent VSMCs. RNA was isolated from confluent VSMC-A, VSMC-B and VSMC-C and the mRNA expression levels of (A) the four syndecan genes and (B) a panel of PG core proteins, including the syndecans for reference, were measured using qPCR. Expression is shown relative to the reference genes, RPL12 and PPIA. The experiment was performed 2-4 times in triplicate for VSMC-A (n=6-12 samples), and once in triplicate for VSMC-B (n=3 samples) and VSMC-C (n=3 samples). Results are displayed as mean ± SEM. Glypicans 2, 3 and 6 could not be detected by qPCR. Glypican 5 was detected, but at the lower limits of detection of qPCR (data not shown).
(i) **Syndecans 1-4**

The four members of the syndecan family (syndecans 1-4) were differentially expressed during VSMC-A osteogenic differentiation and mineralisation. There was no change in syndecan 2 and syndecan 3 mRNA expression during VSMC-A osteogenic differentiation and mineralisation (Figure 3.5). Syndecan 1 mRNA expression was also unchanged at stages 1-3 of VSMC-A mineralisation, however, there was a trend for syndecan 1 mRNA to be down-regulated (~1.8-fold decrease, p=0.06, Figure 3.5) in β-GP-treated VSMC-A at stage 4 of mineralisation when compared to controls. The down-regulation of syndecan 1 mRNA in β-GP-treated VSMC-A reached significance at stage 5 of mineralisation (~2-fold decrease, P<0.01, Figure 3.5). To determine if the changes in syndecan 1 mRNA expression corresponded to changes in protein, syndecan 1 protein levels were analysed during the VSMC-A mineralisation time-course by western blotting. These studies demonstrated that syndecan 1 protein expression was also down-regulated in β-GP-treated VSMC-A at stage 4 and 5 of mineralisation when compared to controls at the same time-point (Figure 3.6A).

There was no change in syndecan 4 mRNA expression at stages 1-4 of VSMC-A mineralisation when compared to controls at the same time-points. However, syndecan 4 mRNA was significantly up-regulated (~5-fold increase; P<0.001, Figure 3.5) in β-GP-treated VSMC-A at the late stages of VSMC mineralisation (stage 5) when compared to controls. To determine if the changes in syndecan 4 mRNA expression corresponded to changes in protein, syndecan 4 protein levels were also analysed during the VSMC-A mineralisation time-course by western blotting. However, changes in the protein expression levels of syndecan 4 during VSMC-A mineralisation were not consistent between experiments. In one experiment, syndecan 4 protein levels were increased in β-GP-treated VSMCs when compared to controls at stage 4 of VSMC mineralisation, but there was no difference in syndecan 4 protein levels in a second experiment (Figure 3.6B).

(ii) **Other PGs**

The mRNA levels of the HSPG, glypican 4, were not changed at stages 1-4 of VSMC-A mineralisation when compared to controls (Figure 3.7A). However, glypican 4 mRNA expression was significantly up-regulated (~2-fold increase; P<0.01, Figure 3.7A) in β-GP-treated VSMC-A at stage 5 of mineralisation when compared to controls at this time-point. In contrast, glypican 5 mRNA was expressed in VSMC-A at the lower limits of detection when measured by qPCR and could not be consistently measured during the VSMC-A mineralisation time-course. Therefore, this PG was not analysed any further.
Figure 3.5. Syndecan 4 mRNA is up-regulated during VSMC-A osteogenic differentiation and mineralisation. Confluent VSMC-A (day 0) were cultured ± 3 mM β-GP for up to 14 days. RNA was collected from cells at day 0, then at specific stages of the mineralisation process (stages 1-5). The expression levels of syndecans 1-4 were measured using qPCR. Expression is shown relative to day 0, which is equal to 1. mRNA expression levels at stages 1 and 2 were combined. Results displayed as mean ± SEM. Stages 1&2 (n=12 samples from 2 independent experiments); Stages 3-5 (n=9 samples from 3 independent experiments). The data were analysed using a 2-way ANOVA with Sidak post-hoc tests. ** P<0.01; *** P<0.001 against same time-point control.
Figure 3.6. Syndecan 4 protein expression is not consistently up-regulated during VSMC-A osteogenic differentiation and mineralisation. Confluent VSMC-A (day 0) were cultured ± 3 mM β-GP for up to 14 days. Protein lysates (25 µg) were collected from cells at day 0, then at specific stages of the mineralisation process (stages 3, 4 and 5). The protein expression levels of (A) syndecan 1 (blot representative of 2 independent experiments) and (B) syndecan 4 (blots from 2 independent experiments are shown) was measured using western blotting. β-actin was used a loading control.
There was no change in perlecan mRNA expression at stages 1 and 2 of VSMC osteogenic differentiation and mineralisation. In individual experiments, the mRNA expression levels of perlecan were significantly down-regulated in β-GP-treated VSMC-A at stage 3 of mineralisation, but when these data were combined, this difference in perlecan mRNA expression did not reach statistical significance (p=0.07, Figure 3.7A). At stages 4 and 5 of VSMC-A mineralisation, there was no difference in perlecan expression between control and β-GP-treated VSMC-A (Figure 3.7A). The mRNA levels of betaglycan were not changed during VSMC-A osteogenic differentiation and mineralisation (Figure 3.7A).

VSMC-A osteogenic differentiation and mineralisation was also associated with changes in the expression of the CS/DS core proteins, biglycan, decorin and versican. There was no change in the mRNA levels of biglycan and versican at stage 1 and 2 of VSMC mineralisation (Figure 3.7B). However, the expression levels of biglycan (~2-fold decrease, P<0.001, Figure 3.7B) and versican (~2-fold decrease, P<0.01, Figure 3.7B) were significantly down-regulated in β-GP-treated VSMC-A at stage 3, 4 and 5 of mineralisation when compared to controls. There was no change in the mRNA levels of decorin at stage 1, 2 and 3 of VSMC-A mineralisation (Figure 3.7B). However, decorin expression was significantly, and transiently, down-regulated (~2-fold decrease, P<0.01, Figure 3.7B) in β-GP-treated VSMC-A at stage 4 of mineralisation when compared to controls. At stage 5 of VSMC-A mineralisation, decorin expression levels were decreased in both control and β-GP-treated VSMC-A, but were not significantly different at this time-point (p=0.27).

The expression levels of the keratan sulphate core proteins, lumican and osteoglycin, were also analysed during VSMC-A osteogenic differentiation and mineralisation. There was no change in the expression levels of lumican and osteoglycin in β-GP-treated VSMC-A at stage 1, 2 and 3 of mineralisation when compared to controls (Figure 3.7C). However, the later stages of VSMC-A mineralisation (stage 4 and 5) were associated with the down-regulation of both lumican (~3-fold decrease, P<0.001) and osteoglycin (~6-fold decrease, P<0.01) mRNA in β-GP-treated VSMC-A when compared to controls at these time-points (Figure 3.7C).

Together, these studies demonstrate that syndecan 4 mRNA is increased during the late stages of VSMC-A osteogenic differentiation and mineralisation, and that this change is accompanied by the differential regulation of other PGs.
Figure 3.7. Differential regulation of PG core proteins during VSMC-A osteogenic differentiation and mineralisation. Confluent VSMC-A (day 0) were cultured ± 3 mM β-GP for up to 14 days. RNA was collected from cells at day 0, then at specific stages of the mineralisation process (stages 1-5). The expression levels of (A) HS and/or CS/DS, (B) CS/DS and, (C) keratan sulphate PG core proteins were measured using qPCR. Expression is shown relative to day 0, which is equal to 1. mRNA expression levels at stages 1 and 2 were combined. Results displayed as mean ± SEM. Stages 1&2 (n=12 samples from 2 independent experiments); Stages 3-5 (n=9 samples from 3 independent experiments). The data were analysed using a 2-way ANOVA with Sidak post-hoc tests. * P<0.05; ** P<0.01; *** P<0.001 against same time-point control.
3.2.4 PG core protein profile of VSMCs that do not mineralise in the presence of β-GP

To determine whether the changes in mRNA expression of the genes encoding PGs were due to extended culture times in the presence of β-GP, similar studies were performed using the two VSMC preparations that do not undergo osteogenic differentiation and mineralisation with β-GP (VSMC-B (Figure 3.2) and VSMC-C (Figure 3.3)). These cells were cultured with 5 mM β-GP for up to 14 days; controls were cultured without β-GP. RNA was isolated from these cells at days 9, 11 and 14, as these time-points corresponded to the time taken for VSMC-A to reach stages 3, 4 and 5 of mineralisation. The expression levels of syndecan 1, syndecan 4, glypican 4, perlecan, biglycan, decorin, versican, osteoglycin and lumican were then measured by qPCR. For reference, the analyses completed on VSMC-B and VSMC-C were combined to increase ‘n’ numbers for statistical analyses as no changes in gene expression were detected between control and β-GP-treated cells over the course of the experiment for both VSMC populations.

There were no significant changes in the mRNA levels of syndecan 1, syndecan 4, glypican 4, biglycan, decorin, versican, osteoglycin and lumican in β-GP-treated VSMC-B and VSMC-C following extended culture when compared to controls at the same time-point (Figure 3.8A). In contrast, although the mRNA expression levels of perlecan were not consistent over the course of the experiment, there was a trend for perlecan mRNA expression to be down-regulated in β-GP-treated VSMC-B and VSMC-C when compared to controls (Figure 3.8B).
Figure 3.8. PG core protein expression is not changed in non-mineralising VSMCs in the presence of β-GP. Confluent VSMC-B and VSMC-C (day 0) were cultured ± 5 mM β-GP for up to 14 days. RNA was collected from cells at day 0, then at days 9, 11 and 14. The expression levels of (A) syndecan 1, syndecan 4, glypican 4, biglycan, decorin, versican, osteoglycin, lumican and (B) perlecan were measured using qPCR. Expression is shown relative to day 0, which is equal to 1. This experiment was performed once in triplicate for both VSMC-B and VSMC-C, and the data were combined. Results displayed as mean ± SEM (n=6 for each treatment group at all time-points analysed). The data were analysed using a 2-way ANOVA with Sidak post-hoc tests.
3.3 Discussion

The results presented in this chapter demonstrate that different preparations of VSMCs have different abilities to undergo osteogenic differentiation and deposit a mineralised matrix in the presence of β-GP. Syndecan 4 mRNA expression increases at the later stages of VSMC mineralisation, and the mRNA expression levels of several other PGs are also differentially regulated during this process. Notably, glypican 4 mRNA expression is up-regulated, and syndecan 1, biglycan, decorin, versican, osteoglycin and lumican mRNAs are all down-regulated. These changes in gene expression do not occur in preparations of VSMCs that do not deposit a mineralised matrix in the presence of β-GP were analysed.

3.3.1 VSMCs display different abilities to undergo osteogenic differentiation and mineralisation

Three preparations of VSMCs were isolated from the tunica media of bovine aortas by the explant culture method (Leik et al., 2004; Churchman and Siow, 2009). Cells were first seen to migrate out of the tissue explants after 5-7 days in culture and were characterised by the expression of the SMC markers, αSMA and SM22α. These data are consistent with other studies which have shown that it takes approximately 5-7 days for VSMCs to adhere, proliferate and migrate using this method (Bostrom et al., 1993), and VSMCs have been characterised by the expression of the SMC markers described above (Shioi et al., 1995; Yang et al., 2005).

It is well established that some, but not all, populations of VSMCs can be induced to differentiate into osteoblast-like cells both in vitro and in vivo (reviewed in Chapter 1). Furthermore, the rate of mineralisation is variable between different preparations of cells (Hyde and Canfield, unpublished data; Watson et al., 1994). In addition, VSMC mineralisation is not always accompanied by the differentiation of VSMCs from a smooth muscle to osteoblast-like phenotype (Canfield, unpublished data) (Alves et al., 2014; Khavandgar et al., 2014; O’Neill and Adams, 2014). Therefore, in this study, the osteogenic differentiation and mineralisation potential of three different preparations of VSMCs that were isolated from three different bovine aortas was assessed. VSMC-A underwent osteogenic differentiation (as demonstrated by the up-regulation of Runx2, Msx2, BMP-2, and down-regulation of αSMA, SM22α) and deposited a mineralised matrix in the presence of β-GP. In contrast, VSMC-B formed large multicellular nodules but did not undergo osteogenic differentiation or mineralise in the presence of β-GP. The multicellular nodular structures formed by VSMC-B resembled those formed by a subpopulation of VSMCs known as CVCs (Bostrom et al., 1993; Watson et al., 1994; Tintut et al., 2003). Interestingly, only 20-40% of cloned CVC populations form nodules that become mineralised (Watson et al., 1994). Consistent with this result, VSMC-B did not spontaneously mineralise with time in culture, even when maintained for up to 30 days (data not shown). In contrast to VSMC-A and VSMC-B, VSMC-C formed multilayer ridges, characteristic of the ‘hill and valley’ morphology of previously characterised VSMCs (Collett et al., 2007; Alam et al., 2009). However, these cells did not deposit a mineralised matrix or undergo osteogenic differentiation in the presence of β-GP. It is not known why some VSMC preparations deposit a
mineralised matrix in the presence of β-GP, while others do not, although it has been reported that only 204 out of 398 bovine VSMC preparations stained positive for ALP (Jono et al., 1997). β-GP is an organic phosphate donor that leads to elevated phosphate levels through the action of ALP, and in turn, raised phosphate levels stimulate the osteogenic differentiation and mineralisation of VSMCs (reviewed in Shanahan et al., 2011). It is therefore possible that ALP activity is reduced in VSMC-B and VSMC-C when compared to the mineralising VSMC preparation, VSMC-A.

Overall, these studies demonstrate that VSMCs are a heterogeneous population of cells that display different abilities to undergo osteogenic differentiation and mineralisation in vitro.

### 3.3.2 Differential modulation of syndecan core proteins during the osteogenic differentiation and mineralisation of VSMCs: up-regulation of syndecan 4

The syndecans are a family of transmembrane PGs, and in mammals, four syndecan genes exist. Consistent with previous reports (Rauch et al., 2005; Shibata et al., 2010), syndecan 1, 2 and 4 were expressed by all three preparations of VSMCs examined in this thesis. Syndecan 3 was also expressed at the mRNA level in these preparations of VSMCs. Whilst syndecan 3 has been detected in the mouse aorta (Adhikari et al., 2011), this syndecan isoform has not been reported to be expressed by VSMCs specifically.

Syndecan 4 mRNA expression was increased at the late stages (stage 5) of VSMC osteogenic differentiation and mineralisation (Figure 3.5), and this result was verified in a second preparation of mineralising VSMCs (data not shown). Furthermore, the mRNA expression levels of syndecan 4 were not changed in two separate preparations of VSMCs that do not undergo osteogenic differentiation and mineralisation in the presence of β-GP (Figure 3.8A), suggesting that the changes observed in syndecan 4 during VSMC mineralisation are either necessary for, or are a consequence of, these processes rather than due to extended culture times in the presence of β-GP. Consistent with these findings, syndecan 4 mRNA expression is increased in calcified vessels isolated from a rat model of hyperphosphatemia and hyperparathyroidism (Shibata et al., 2010). Syndecan 4 is also expressed by osteoblasts (Molteni et al., 1999; Jackson et al., 2007; Song et al., 2007; Wang et al., 2011b) and syndecan 4 mRNA expression is increased during the osteogenic differentiation of the mouse osteoblastic cell line, MC3T3-E1 (Jackson et al., 2007). However, the role of syndecan 4 in osteogenesis in vivo is somewhat unclear. Bone formation is not altered in syndecan 4 null-nice, although this result may be confounded by a compensatory increase in syndecan 2 expression in these mice (Bertrand et al., 2013). Indeed, syndecan 2 has been postulated to play an important role in osteoblast differentiation independent of the other syndecans (discussed further below).

Syndecan 4 regulates several signalling pathways that are known to play important roles in vascular calcification, which suggests that this PG may be important in the regulation of this disease. For example, previous studies have shown that syndecan 4 is required for FGF-2-induced Akt activation in vitro (Partovian et al., 2008; Ju and Simons, 2013) and in vivo (Zhang
et al., 2003; Partovian et al., 2008; Ju and Simons, 2013), and activated Akt can phosphorylate a number of substrates that play a role in the regulation of apoptosis. VSMC apoptosis promotes vascular calcification (Proudfoot et al., 2000; Collett et al., 2007; Clarke et al., 2008), and reduced activation of Akt has been observed during VSMC osteogenic differentiation and mineralisation (Ponnusamy, 2013) (Hyde and Canfield, unpublished data). In light of this evidence, it is possible that syndecan 4 expression is up-regulated during the later stages of VSMC osteogenic differentiation and mineralisation to maintain activation of Akt and inhibit VSMC apoptosis. Consistent with this hypothesis, cardiac myocyte apoptosis is reduced when syndecan 4 is over-expressed in the hearts of rat 1 day following the induction of myocardial infarction in these animals (Xie et al., 2012). Alternatively, syndecan 4 may just be a marker of VSMC mineralisation. The role of syndecan 4 in VSMC mineralisation will be investigated further in Chapters 5 and 6.

In contrast to the results obtained for mRNA expression using qPCR, the protein profile of syndecan 4 during VSMC mineralisation was not consistent between different experiments. In some experiments, syndecan 4 protein appeared to be increased in mineralised cultures; in others experiments, syndecan 4 protein was not changed. The antibody used in these studies was raised against the extracellular domain of syndecan 4 (5G9, Santa-Cruz), and this domain is susceptible to shedding from the cell surface by proteases like the MMPs (Fitzgerald et al., 2000; Manon-Jensen et al., 2010; Manon-Jensen et al., 2013; Ramnath et al., 2014). MMP expression is increased during VSMC osteogenic differentiation and mineralisation (Kapustin et al., 2011; Chen et al., 2011; Pai et al., 2011; Taylor, 2014), suggesting that there could be increased shedding of syndecan 4 during this process. However, syndecan 4 which had been shed from the cell surface could not be detected in the cell culture medium of mineralising VSMCs by western blotting (data not shown). In these studies, the cell culture medium was not concentrated before the detection of shed syndecan 4 by western blotting, but concentrating the medium may be one method that would enable the detection of shed syndecan 4. Previous work in this lab has shown that the protein in the cell culture medium can be concentrated via pull-down with Strataclean resin prior to western blotting to enable the detection of the secreted inflammatory protein, TSG-6 (Taylor, 2014). However, the presence of FCS in the cell culture medium prevented sufficient collection of TSG-6 onto the Strataclean resin, likely due to saturation of the beads with serum proteins (Taylor, 2014). As VSMCs will not calcify in the absence of FCS (data not shown), this method was not suitable for the detection of shed syndecan 4 in the cell culture medium of mineralising VSMCs. Detection of syndecan 4 in the cell culture medium of VSMCs using an ELSIA could be an appropriate alternative in future studies.

A second complication in these studies was the sensitivity of different batches of the syndecan 4 antibody. The western blots presented in Figure 3.6 used the same lot number of syndecan 4 antibody (5G9, Santa-Cruz), however a different lot number of this antibody had to be used in subsequent experiments which had much poorer sensitivity for the detection of syndecan 4 in VSMC-A. Different blocking agents (e.g. milk, BSA) and antibody dilutions (1:50-1:500) were
used to optimise this new lot of syndecan 4 antibody in VSMC-A, but this could not be achieved within the timeframe of this project. A second syndecan 4 antibody raised against the cytoplasmic domain of syndecan 4 (C-20, Santa-Cruz) was also used in these studies, but the high background associated with this antibody prevented the reliable detection of syndecan 4 in these cells (data not shown). The use of different syndecan 4 antibodies or different protein detection methods (e.g. mass spectrometry) would be advantageous in future studies.

In contrast to syndecan 4, syndecan 1 expression was down-regulated at the mRNA and protein level at stages 4 and 5 of VSMC osteogenic differentiation and mineralisation, corresponding with the differentiation of these cells into an osteoblast-like phenotype. The mRNA expression levels of syndecan 1 were also not changed in two separate preparations of VSMCs that do not undergo osteogenic differentiation and mineralisation in the presence of β-GP (Figure 3.8A), suggesting that the changes observed in syndecan 1 expression during VSMC mineralisation are not due to extended culture in the presence of β-GP. Syndecan 1 has been implicated as a regulator of mature VSMC phenotype, and the expression of VSMC markers (αSMA and calponin) are reduced in syndecan 1-null mouse VSMCs (Chaterji et al., 2014). Pilot studies were therefore performed to determine if knocking-down syndecan 1 expression increases VSMC osteogenic differentiation and mineralisation. Knocking-down syndecan 1 expression using siRNA had no effect on VSMC-A mineralisation (Appendix, Figure 10.1), suggesting that the down-regulation of syndecan 1 expression during this process may reflect the differentiation of these cells from a smooth muscle to an osteoblast-like phenotype. Consistent with this hypothesis, syndecan 1 expression is decreased during osteoblast differentiation (Birch and Skerry, 1999). Alternatively, it is possible that syndecan 1 expression may decrease during VSMC mineralisation to compensate for increases in the mRNA expression levels of syndecan 4. Indeed, some redundancy is believed to exist between the four syndecan genes, but the overlap between syndecans 1 and 4 is not fully understood.

As discussed above, some redundancy is believed to exist between the four syndecan genes and changes in the expression of one syndecan family member may be compensated for by another (Bertrand et al., 2013). Whilst the expression levels of syndecan 1 and 4 were changed during VSMC osteogenic differentiation and mineralisation, no changes were detected in syndecan 2 or 3 mRNA expression. Syndecan 2 mRNA is expressed during osteoblast differentiation (Molteni et al., 1999; Birch and Skerry, 1999), but its role in osteoblast mineralisation is currently unknown. As there was no change in syndecan 2 expression during VSMC osteogenic differentiation and mineralisation, these findings could reflect tissue-specific regulation of syndecan 2 expression in VSMCs and osteoblasts during osteogenic differentiation. Consistent with this hypothesis, distinct gene expression profiles have been observed during the mineralisation of human MSCs and VSMCs (Alves et al., 2014).

The expression levels of syndecan 3 have not been documented during osteoblast differentiation, although low mRNA levels have been detected in cavarial osteoblasts (Song et al., 2007; Teplyuk et al., 2009). Therefore, the finding that syndecan 3 mRNA expression is not
changed during the osteogenic differentiation and mineralisation of VSMCs may be consistent with the suggestion that it has no role in this process.

Together, these studies demonstrate that the four syndecan genes are differentially regulated during VSMC osteogenic differentiation and mineralisation, and of particular interest to this study, the mRNA expression levels of syndecan 4 are increased during the late stages of VSMC mineralisation.

3.3.3 VSMC osteogenic differentiation and mineralisation is also associated with the up-regulation of glypican 4 and down-regulation of several PG core proteins

Conflicting results have often been obtained for the expression levels of several PGs, including decorin, biglycan, lumican, versican and osteoglycin, in both in vitro and in vivo models of vascular calcification (Table 1.2). In addition, previous studies have often limited their analyses of VSMC mineralisation to a single PG and have not considered the coordinated up- or down-regulation of other PGs. Therefore, this study sought to characterise the expression profile of several PGs, in addition to syndecans 1-4, during the osteogenic differentiation and mineralisation of VSMCs.

Glypican 4 is a HSPG, and along with syndecan 4, it was the only PG to be up-regulated during VSMC osteogenic differentiation and mineralisation (Figure 3.7A). Glypican 4 mRNA is also increased in the MG-63 osteosarcoma cell line during osteogenic differentiation and mineralisation (Kumarasuriyar et al., 2009), and gene knock-out studies in zebrafish have demonstrated that glypican 4 deficiency causes craniofacial defects, including a smaller head and shorter jawbones (LeClair et al., 2009). The authors of this study speculated that glypican 4 may cause these effects via Wnt signalling, as loss of the Wnt ligand, Wnt5b, causes similar cartilage defects. Wnt signalling is also a key regulator of vascular calcification (Shao et al., 2005; Al-Aly et al., 2007; Shao et al., 2007; Kirton et al., 2007), therefore, glypican 4 expression may be up-regulated in mineralising VSMCs to enhance the activation of Wnt signalling, and in turn, the osteogenic differentiation of VSMCs. Consistent with this hypothesis, the mRNA expression levels of glypican 4 were not changed in two separate preparations of VSMCs that do not undergo osteogenic differentiation and mineralisation in the presence of β-GP (Figure 3.8A), suggesting that the changes observed in glypican 4 during VSMC osteogenic differentiation and mineralisation are associated with this process, and are not due to extended culture in the presence of β-GP.

During the osteogenic differentiation and mineralisation of VSMCs, the mRNA expression levels of several PG core proteins (including biglycan, decorin, versican, osteoglycin and lumican) were reduced in mineralising VSMCs when compared to controls (Figure 3.7B-C). Importantly, the expression levels of these PGs were not changed in two separate preparations of VSMCs that do not undergo osteogenic differentiation and mineralisation in the presence of β-GP (Figure 3.8A), suggesting that the changes observed in the expression levels of these PGs are either necessary for, or are a consequence of, these processes rather than due to extended
culture times in the presence of β-GP. Furthermore, the mRNA expression levels of several PGs were not changed during VSMC osteogenic differentiation and mineralisation, including syndecan 2, syndecan 3 (Figure 3.5) and betaglycan (Figure 3.7A), providing further evidence that only specific PGs are associated with the osteogenic differentiation and mineralisation of VSMCs.

Biglycan is important for postnatal bone development (Xu et al., 1998), and it may also play a stimulatory role in vascular calcification (Song et al., 2012). In light of this evidence, it is perhaps surprising that the mRNA expression levels of this PG core protein were reduced in mineralising VSMCs, although this result is consistent with a previous study which also used mineralising bovine VSMCs (Fischer et al., 2004). It is possible that biglycan modulates osteogenic differentiation and/or mineralisation in a species and/or cell type-dependent manner, and may not be required for the differentiation of VSMCs into an osteoblast-like phenotype. Consistent with this hypothesis, it is well known that some proteins can have opposite functions in different cell types. For example, BMP-7 promotes the osteogenic differentiation of osteoblastic precursors (Cheng et al., 2003), but inhibits VSMC osteogenic differentiation and mineralisation (Davies et al., 2005; Kang et al., 2010). It is also important to note that it could not be determined if these changes in biglycan mRNA expression corresponded to changes in protein levels as, unfortunately, attempts to optimise a biglycan antibody for western blotting were unsuccessful. This was also the case for antibodies against decorin, versican, osteoglycin and lumican (data not shown).

Decorin is a regulator of VSMC mineralisation (Fischer et al., 2004; Yan et al., 2011), and its expression was down-regulated in β-GP-treated VSMCs at stage 4 of mineralisation when compared to controls (Figure 3.7B). Whilst decorin expression has also been shown to be decreased in human calcified aortic valves (Matsumoto et al., 2012b), other studies have reported that decorin mRNA and/or protein expression are increased in in vitro (Fischer et al., 2004; Alves et al., 2014) and ex vivo (Shibata et al., 2010) models of vascular calcification. It has been proposed decorin may promote vascular calcification as adenovirus-mediated over-expression of decorin has been shown to accelerate the deposition of a mineralised matrix by VSMCs in vitro (Fischer et al., 2004; Yan et al., 2011). Decreases in decorin expression during VSMC osteogenic differentiation and mineralisation could therefore reflect a feedback mechanism to prevent further mineralisation in this preparation of VSMCs.

Consistent with previous reports in human calcified aortic valves (Matsumoto et al., 2012b), osteoglycin and lumican mRNA expression was down-regulated in β-GP-treated VSMCs from stage 3 of matrix mineralisation, corresponding with the up-regulation of the osteogenic markers Runx2 and Msx2 and down-regulation of smooth muscle cell markers (Figure 3.1C and 3.7C). Previous studies have implicated osteoglycin as a marker of differentiated VSMCs (Shanahan et al., 1997), but it has no role in the development of aortic calcification in ApoE-null mice (Moncayo-Arlandi et al., 2014), suggesting the down-regulation of osteoglycin expression in mineralising VSMCs may simply be a marker of VSMC osteogenic differentiation. In agreement
with this hypothesis, osteoglycin was not associated with sites of calcification within atherosclerotic plaques (Shanahan et al., 1997). In contrast, the role of lumican in vascular calcification is currently unknown.

Versican expression is down-regulated upon the deposition of a mineralised matrix by MSCs (Hoshiba et al., 2009), and it is also down-regulated in human calcified thoracic aorta aneurysms (Matsumoto et al., 2012a) and calcified aortic valves (Matsumoto et al., 2012b). Consistent with these reports, the mRNA expression level of versican was reduced in mineralising VSMCs (Figure 3.7B). Interestingly, elastin degradation promotes vascular calcification (Hosaka et al., 2009; Aikawa et al., 2009) and over-expressing versican inhibits elastin assembly in VSMCs (Huang et al., 2006), therefore versican expression may be down-regulated in mineralising VSMCs to promote elastin assembly and slow down the mineralisation process. Like decorin, decreases in versican expression during VSMC osteogenic differentiation and mineralisation may reflect a feedback mechanism to prevent further mineralisation in this preparation of VSMCs.

Perlecan is major HSPG of the arterial basement membrane and the mRNA expression levels of this PG were decreased in calcified vessels taken from a rat model of hyperphosphatemia and hyperparathyroidism (Shibata et al., 2010). In agreement with this result, there was a trend for perlecan mRNA expression to be transiently down-regulated in β-GP-treated VSMC-A at stage 3 of VSMC mineralisation when compared to controls (p=0.07, Figure 3.7A). Surprisingly, there was also a trend for perlecan mRNA expression to be down-regulated in two separate preparations of VSMCs that do not undergo osteogenic differentiation and mineralisation in the presence of β-GP (Figure 3.8B), suggesting that perlecan does not have a specific role in the osteogenic differentiation and mineralisation of VSMCs. Indeed, perlecan plays a vital role in cartilage development (Arikawa-Hirasawa et al., 1999), but its role in osteogenesis is less clear. Further work is required to determine if perlecan plays a functional role in the osteogenic differentiation and mineralisation of VSMCs.

Together these studies demonstrate that in addition to the up-regulation of syndecan 4 mRNA during the osteogenic differentiation and mineralisation of VSMCs, other specific PG core proteins are differentially expressed. Whilst there are some discrepancies between the literature and the present study on the expression levels of specific PC core proteins during VSMC mineralisation, it is possible that these conflicting results could reflect the requirement of different PGs at specific stages of the osteogenic differentiation and mineralisation process, or reflect tissue- and/or species-specific roles of these different PGs during mineralisation.

### 3.4 Conclusion

The following conclusions can be drawn from the results presented in this chapter:

- Syndecan 4 mRNA expression is increased at the later stages of VSMC osteogenic differentiation and mineralisation.
The mRNA expression levels of several other PGs are also differentially regulated during VSMC osteogenic differentiation and mineralisation. Notably, glypican 4 mRNA expression is up-regulated, and syndecan 1, biglycan, decorin, versican, osteoglycin and lumican mRNAs are all down-regulated.
4 Profiling the changes in GAG composition during VSMC osteogenic differentiation and mineralisation

4.1 Introduction
The results presented in Chapter 3 demonstrated that VSMC osteogenic differentiation and mineralisation was associated with several changes in the different PG core proteins expressed by VSMCs. Of particular interest, the late stages of VSMC osteogenic differentiation and mineralisation were associated with the up-regulation of syndecan 4 mRNA (Figure 3.5), a PG that can display HS or CS/DS chains (Shworak et al., 1994; Ueno et al., 2001; Deepa et al., 2004; Gopal et al., 2010). In addition, the mRNA expression levels of several other PGs were also differentially regulated during VSMC osteogenic differentiation and mineralisation. Notably, glypican 4 mRNA expression is up-regulated, and syndecan 1, biglycan, decorin, versican, osteoglycin and lumican mRNAs are all down-regulated (Figure 3.7).

The ability of PGs to regulate growth factor signalling is primarily (although not entirely) a function of their GAG chains, which can act as co-receptors, stabilise gradients and/or protect ligands from degradation (Roehl and Pacifici, 2010). GAGs are abundantly expressed in bone (Prince and Navia, 1983; Waddington et al., 1989; Waddington and Embery, 1991; Duer et al., 2008) and they have been implicated in the regulation of osteoblast differentiation and/or mineralisation (Manton et al., 2007; Miyazaki et al., 2008; Mathews et al., 2014). The size and sulphation patterning of GAG chains regulates growth factor signalling (Holley et al., 2011b; Pickford et al., 2011), and several growth factors play a pro- or anti-calcific role in the VSMC mineralisation process (Table 1.1). Indeed, the GAG chains displayed on decorin are vital to this PG’s role in accelerating human VSMC mineralisation in vitro, likely through the regulation of TGF-β1 signalling in these cells (Yan et al., 2011). GAGs are also abundantly expressed in calcified atherosclerotic plaques (Duer et al., 2008), but the composition of GAGs in calcified vessels/mineralising VSMCs, and their role in this process has not been examined. Therefore, as well as examining changes in PG core protein expression (see Chapter 3), there is also a need to define changes in GAG patterning during VSMC mineralisation. The studies described in this chapter will address the following aims:

1. Characterise the HS and CS/DS chain composition of VSMCs at confluence, and during VSMC osteogenic differentiation and mineralisation.
2. Characterise the HS and CS/DS biosynthetic enzyme profile of VSMCs at confluence, and during osteogenic differentiation and mineralisation.
4.2 Results

4.2.1 Structural analysis of GAGs synthesised by VSMCs at confluence and during VSMC osteogenic differentiation and mineralisation

Metabolically $^3$H-labelled GAGs were isolated from VSMC-B (Figure 4.1Ai&ii) and VSMC-A (Figure 4.1Bi&ii) at confluence, and during VSMC-A osteogenic differentiation and mineralisation. For the mineralising VSMC-A preparation, cells were cultured in the presence of 3 mM $\beta$-GP for 10 days until stage 3 of mineralisation. At this time, cells were incubated with D-[6-$^3$H]glucosamine for 48 hours to generate radiolabelled GAGs (Figure 4.1Ci). By the end of the radiolabelling period, cells were at stage 4/5 of mineralisation (Figure 4.1Cii). The stage of VSMC mineralisation observed at the start and end of radiolabelling period was determined by eluting the alizarin red stain from parallel cultures which were stained with this dye (Figure 4.1Ciii), and matching these results against the VSMC-A mineralisation time-course presented in Figure 3.1B.

Two fractions of GAGs were extracted from all three VSMC preparations: secreted GAGs were isolated from the culture medium, while cell-surface and matrix-associated GAGs (hereafter referred to as the cellular/matrix extract) were isolated as described in section 2.12. Samples were digested with pronase to release peptides carrying one or two GAG chains and charged species were separated by gradient salt elution from a DEAE anion-exchange column. $^3$H-labelled material in the medium (Figure 4.1Ai,Bi&Di) and cellular/matrix (Figure 4.1Aii,Bii&Dii) extracts were separated into 4 pools according to the DEAE salt gradient profile, and pools 2, 3 and 4 were concentrated and desalted as described in section 2.12.3. $^3$H-labelled material in pool 1 from both the medium and cellular/matrix extracts of all three VSMC preparations could not be concentrated to allow for further analysis. It is possible that this radiolabelled material comprised carbohydrates and/or N-linked glycans that had bound non-specifically to the DEAE column.

A series of enzyme digests and fractionation of oligosaccharide products by gel filtration were used to confirm the presence of HS or CS/DS chains in each pool of $^3$H-labelled material. Chondroitinase ABC and a combination of heparinase enzymes (I, II and III) digest CS/DS and HS chains to disaccharides, respectively. $^3$H-labelled material in pool 2 from both the medium and cellular/matrix extracts of all three VSMC preparations was resistant to digestion with chondroitinase ABC and hepaninase I, II and III, indicating that these pools of $^3$H-labelled material did not contain CS/DS or HS (data not shown). It is possible that these pools of radiolabelled material contained some hyaluronan, keratan sulphate and/or potentially highly sialylated N-linked glycans that had bound to the DEAE. These fractions were not investigated further in this study.
(A) VSMC-B

(Ci) Stage 3: start of radiolabel

(ii) Stage 4/5: end of radiolabel

(iii) Quantification of mineralisation

(D) Mineralising VSMC-A
Figure 4.1. Isolation of $^3$H-labelled GAGs from confluent and mineralising VSMCs. (A-B) VSMC-B and VSMC-A were grown to 80-90% confluence and $^3$H-glucosamine was introduced into the medium for 48 hours to generate radiolabelled GAGs. The culture medium was collected and the cellular/matrix extract was released using PBS, 1% (v/v) Triton X-100. Radiolabelled material was pronase-treated, loaded onto a 5 ml DEAE column and washed with 100 ml PBS to remove material which had bound non-specifically to the DEAE. Bound material was eluted using an increasing salt gradient of 0.15-0.82 M, collecting 2 ml fractions. Aliquots were taken from the (A-Bi) medium and (A-Bii) cellular/matrix fractions of (A) confluent VSMC-B and (B) confluent VSMC-A for scintillation counting. Due to technical difficulties, (Bi) confluent VSMC-A medium DEAE-gradient had to be stopped at fraction 9 and left overnight. A large amount of radiolabelled material was eluted off the DEAE when the DEAE-gradient was restarted the following day (fraction 14). (C) VSMC-A were cultured with 3 mM β-GP for 10 days to induce calcification. At stage 3 of VSMC mineralisation, $^3$H-glucosamine was introduced into the medium for 48 hours. Phase-contrast images of VSMC-A stained with alizarin red at the (Ci) start and (Cii) end of the radiolabelling period. Scale bar = 500 µm. (Ciii) Mineral deposition was quantified by dye elution ($n$=1). The culture medium was collected from mineralising VSMC-A and the cellular/matrix extract was released using 6M Urea, 1% (v/v) Triton X-100. Radiolabelled material was treated with pronase and DNase, loaded onto a 5 ml DEAE column and eluted using an increasing salt gradient as described above. Aliquots were taken from the (Di) medium and (Dii) cellular/matrix fractions of mineralising VSMC-A for scintillation counting. For all three VSMC preparations, the radiolabel was carried out once.

Approximately 50% of radiolabelled material eluted in pool 3 of the cellular/matrix extract, but this pool represented only a minor component (6-11%) of the medium-derived GAGs. $^3$H-labelled material in pool 3 from both the medium and cellular/matrix extracts of confluent VSMC-A and VSMC-B was 80-90% resistant to digestion with chondroitinase ABC (data not shown). $^3$H-labelled material in pool 3 from the medium extract of mineralising VSMC-A was also 85% resistant to digestion with chondroitinase ABC, however pool 3 of the cellular/matrix extract was only 56% resistant to digestion with this enzyme (data not shown). The chondroitinase ABC-resistant chains were confirmed to be intact HS chains as they were digested to disaccharides when treated with heparinase I, II and III (data not shown). The HS-containing pools from the medium (Figure 4.2Bi,Ci&Di) and cellular/matrix (Figure 4.2Bii,Cii&Dii) GAG extracts of all three VSMC preparations were therefore digested with chondroitinase ABC and CS/DS disaccharides were fractionated from intact HS chains using size-exclusion chromatography. The purified intact HS chains were then characterised as described in section 4.2.2.

The majority (75-87%) of radiolabelled material eluted in pool 4 from the medium extract of all three VSMC preparations, but this pool represented only ~50% of the total GAGs in the cellular/matrix extract of these cells (Figure 4.1Bii,Cii&Dii). $^3$H-labelled material in pool 4 from both the medium and cellular/matrix extracts of all three VSMC preparations was resistant to digestion with heparinase I, II and III (data not shown). The heparinase-resistant chains were confirmed to be intact CS/DS chains as they were digested to disaccharides when treated with chondroitinase ABC (data not shown). As these pools of $^3$H-labelled material were a pure population of CS/DS chains, no additional purification was carried out on this material before further analysis (see section 4.2.3).
Figure 4.2. Purification of HS. Intact HS chains were isolated from Pool 3 of the (A-Ci) medium and (A-Cii) cellular/matrix extracts of (A) confluent VSMC-B, (B) confluent VSMC-A and (C) mineralising VSMC-A. 3H-labelled material was treated with chondroitinase ABC and fractionated by gel filtration on a Sepharose CL-6B column. Material was eluted at a flow rate of 12 ml/hour, and 1 ml fractions were collected. Aliquots were taken for scintillation counting and compared against the gel filtration profile of untreated GAG. Untreated GAG is plotted on the left y-axis, and chondroitinase ABC-treated GAG is plotted on the right y-axis. Void volume ($V_0$) = fraction 40; Total volume ($V_t$) = fraction 130-160.
4.2.2 HS characterisation

Intact HS chains purified from both the medium and cellular/matrix extracts of all three VSMC preparations were characterised using specific scission agents as described below.

(i) Chain length

HS chain length was determined following alkaline release from the peptide core and estimated according to the Wasteson calibration (Wasteson, 1971b). These studies demonstrated that HS chain length was very similar between confluent VSMC-B and VSMC-A, although some small differences were noted. For example, both medium- and cellular/matrix-derived HS chains were slightly smaller in confluent VSMC-A compared to confluent VSMC-B, (Table 4.1). In both cases, the cellular/matrix-derived HS was longer than medium-derived HS (Table 4.1).

Cellular/matrix-derived HS chains from mineralising VSMC-A also appeared shorter when compared to confluent VSMC-A (Table 4.1). Medium-derived HS chains from mineralising VSMC-A also appeared slightly shorter when compared to confluent VSMC-A (Table 4.1).

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<th>Table 4.1. HS chain length</th>
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<td>Medium-derived HS</td>
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<tr>
<td>VSMC-B</td>
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<tr>
<td>Kav</td>
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<tr>
<td>Chain length (kDa)</td>
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<tr>
<td>Approx no. of dp2s per chain</td>
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<tr>
<td>Cellular/matrix-derived HS</td>
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<td>VSMC-B</td>
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<td>Kav</td>
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<tr>
<td>Chain length (kDa)</td>
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<td>Approx no. of dp2s per chain</td>
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Chain length was determined following alkaline release from the peptide core and estimated according to the Wasteson calibration (Wasteson, 1971b). The number of disaccharides (dp2) per chain was calculated based on the assumption that the average molecular weight of a HS disaccharide is 450 daltons.

(ii) N-sulphation domain distribution

When used at low pH (pH 1.5), nitrous acid depolymerises regions of contiguous N-sulphated residues within HS chains (S domains) to disaccharides, and regions of alternating GlcNS and GlcNAc residues (NA/NS domains) within HS chains to tetrasaccharides (Shively and Conrad, 1976). Therefore, this method is extremely useful for generating information about the content and positioning of N-sulphated sequences within HS chains. Nitrous acid treatment of HS from the medium (Figure 4.3Ai,Bi&Ci) and cellular/matrix GAG (Figure 4.3Ai,Bii&Cii) extracts from all three preparations of VSMCs produced a characteristic oligosaccharide profile when analysed by gel filtration. However, it is worth noting that ~20% of HS from the cellular/matrix extract of confluent VSMC-A was not depolymerised with low pH nitrous acid (indicated by arrow in Figure 4.3Bii). This material was also resistant to digestion with a combination of heparinases (I, II and III) and chondroitinase ABC (data not shown). These data suggest that this material may
comprise of hyaluronan, keratan sulphate and/or potentially highly sialylated N-linked glycans which had co-eluted with the HS.

The low pH nitrous acid profile was used to calculate the percentage of N-sulphated residues present within HS chains, as summarised in Table 4.2. These studies demonstrated that the proportion of N-sulphate groups present in contiguous sequences was relatively consistent between confluent VSMC-B and VSMC-A medium- (VSMC-B, 44%; VSMC-A, 42%) and cellular/matrix-derived (VSMC-B, 42%; VSMC-A, 40%) HS chains. Whilst the proportion of GlcNS in alternating sequences with GlcNAc was similar in cellular/matrix-derived HS chains derived from confluent VSMC-B and VSMC-A, the proportion of GlcNS in alternating sequences with GlcNAc was slightly higher in medium-derived HS chains from confluent VSMC-B compared to confluent VSMC-A (VSMC-B, 26%; VSMC-A, 20%). The remainder of GlcNS-containing disaccharides were separated by longer sequences of N-acetylated disaccharide units in the intact polymer, with products ranging from hexasaccharides to oligosaccharides about 6-8 disaccharide units in length (Figure 4.3Ai&ii, Bi&ii).

The proportion of both total and contiguous GlcNS residues within HS chains from mineralising VSMC-A did not dramatically change when compared to confluent VSMC-A (Table 4.2). However, the proportion of GlcNS in alternating sequence with GlcNAc in cellular/matrix-derived HS was slightly reduced in mineralising VSMC-A when compared to confluent VSMC-A (23% and 29%, respectively). The proportion of GlcNS in alternating sequence with GlcNAc was similar between medium-derived HS chains from confluent and mineralising VSMC-A (Table 4.2).

Table 4.2. N-sulphation domain distribution

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<th>Medium-derived HS</th>
<th>Cellular/matrix-derived HS</th>
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<tr>
<td></td>
<td>VSMC-B</td>
<td>VSMC-A</td>
</tr>
<tr>
<td>% N-sulphation</td>
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<td>39</td>
</tr>
<tr>
<td>% contiguous N-sulphation</td>
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<td>42</td>
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<tr>
<td>% alternate N-sulphation</td>
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<td>20</td>
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</table>

The number of linkages in HS that were susceptible to low pH nitrous acid depolymerisation was calculated as follows. The total $^3$H counts eluting in a specific peak were adjusted for the number of $^3$H-labelled GlcN sugars in the oligosaccharide corresponding to that peak. The percentage N-sulphation is calculated as the percentage of total susceptible linkages (i.e. adjusted counts) in the total $^3$H counts (i.e. unadjusted counts). The percentage of contiguous linkages of N-sulphation (i.e. disaccharides) was calculated as the percentage of $^3$H (adjusted) counts under the disaccharide peak in the total number of susceptible linkages. Tetrasaccharides have an internal GlcNAc and therefore correspond to alternate N-sulphation linkages, and the percentage is calculated in an equivalent process to the contiguous linkages.
Figure 4.3. N-sulphation domain analysis of confluent and mineralising VSMC HS chains using low pH nitrous acid depolymerisation. HS from the (A-Ci) medium and (A-Cii) cellular/matrix extracts of (A) confluent VSMC-B, (B) confluent VSMC-A and (C) mineralising VSMC-A were treated with nitrous acid (pH 1.5) and oligosaccharide products were fractionated on a Superdex 30 size-exclusion column at a flow rate of 4 ml/hour. Fractions (0.5 ml) were collected and analysed by scintillation counting, and compared against the gel filtration profile of untreated HS. Untreated HS is plotted on the left y-axis, and low pH nitrous acid-treated HS is plotted on the right y-axis. (Bii) Blue arrow indicates radiolabelled material (20%) which was resistant to low pH nitrous acid depolymerisation. dp8, octosaccharides; dp6, hexasaccharides; dp4, tetrasaccharides; dp2, disaccharides. Void volume ($V_0$) = fraction 60; Total volume ($V_t$) = fraction 180.
(iii) **Heparinase I and III**

To further investigate the sulphated domain structure of HS, the medium- and cellular/matrix-derived HS chains were treated with heparinase III (which cleaves GlcA-GlcNAc/NS(±6S)) and fractionated by gel filtration (Figure 4.4). Un-treated HS was also fractionated by gel filtration as a control (Figure 4.4). Heparinase III treatment of medium (Figure 4.4Ai&Bi) and cellular/matrix HS (Figure 4.4Aii&Bii) from confluent VSMC-A and VSMC-B generated disaccharides (dp2) predominantly. Longer oligosaccharides (tetrasaccharides, dp4; hexasaccharides, dp6; octosaccharides, dp8) were also produced following heparinase III treatment, indicating the presence of Ido(±2S)-containing disaccharides which are resistant to heparinase III (Table 4.3). The heparinase III oligosaccharide profile of medium- and cellular/matrix-derived HS from confluent VSMC-A (Figure 4.4Bi&Bii) was similar to the profile from mineralising VSMC-A (Figure 4.4Ci&ii). The heparinase III profile was used to calculate the percentage of susceptible linkages, as summarised in Table 4.3.

Heparinase I has a more restricted site of action, cleaving HS at IdoA(2S)-GlcNS/NAc(±6S), and can therefore reveal the proportion of IdoA(2S) residues present within HS chains. Therefore, HS derived from the medium- and cellular/matrix-extracts of all three VSMC preparations were treated with heparinase I and fractionated by gel filtration (Figure 4.5). These studies demonstrated that less than 10% of both medium- (Figure 4.5Ai&Bi) and cellular/matrix-derived (Figure 4.45Aii&Bii) HS chains from confluent VSMC-B and VSMC-A was susceptible to heparinase I treatment. The heparinase I oligosaccharide profile was similar in HS isolated from mineralising VSMC-A (Figure 4.5C).

### Table 4.3. Heparinase III oligosaccharide distribution

<table>
<thead>
<tr>
<th></th>
<th>Medium-derived HS</th>
<th>Cellular/matrix-derived HS</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>VSMC-B</td>
<td>VSMC-A</td>
</tr>
<tr>
<td>% susceptible</td>
<td>82</td>
<td>86</td>
</tr>
<tr>
<td>% dp2</td>
<td>88</td>
<td>90</td>
</tr>
<tr>
<td>% dp4</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>% dp6</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>% dp8</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

*The number of linkages that were susceptible to heparinise III was calculated as follows. The total \(^3\)H counts eluting in a specific peak were adjusted for the number of \(^3\)H-labelled GlcN sugars in the oligosaccharide corresponding to that peak. The percentage susceptibility is calculated as the percentage of total susceptible linkages (i.e. adjusted counts) in the total \(^3\)H counts (i.e. unadjusted counts). The percentage of contiguous linkages (i.e. disaccharides, dp2) was calculated as the percentage of \(^3\)H (adjusted) counts under the disaccharide peak in the total number of susceptible linkages. The percentage of tetrasaccharides (dp4), hexasaccharides (dp6) and octosaccharides (dp8) was calculated in an equivalent process to the contiguous linkages.*
Figure 4.4. Sulphation domain analysis of confluent and mineralising VSMC HS chains using heparinase III. HS from the (A-Ci) medium and (A-Cii) cellular/matrix extracts of (A) confluent VSMC-B, (B) confluent VSMC-A and (C) mineralising VSMC-A were treated with heparinase III and oligosaccharide products were fractionated on a Superdex 30 size-exclusion column at a flow rate of 4 ml/hour. Fractions (0.5 ml) were collected and analysed by scintillation counting, and compared against the gel filtration profile of untreated HS. Untreated HS is plotted on the left y-axis, and heparinase III-treated HS is plotted on the right y-axis. dp8, octosaccharides; dp6, hexa saccharides; dp4, tetrascarharides; dp2, disaccharides. Void volume ($V_0$) = fraction 60; Total volume ($V_t$) = fraction 150-160.
Figure 4.5. Sulphation domain analysis of confluent and mineralising VSMC HS chains using heparinase I. HS from the (A-Ci) medium and (A-Cii) cellular/matrix extracts of (A) confluent VSMC-B, (B) confluent VSMC-A and (C) mineralising VSMC-A were treated with heparinase I and oligosaccharide products were fractionated on a Superdex 30 size-exclusion column at a flow rate of 4 ml/hour. Fractions (0.5 ml) were collected and analysed by scintillation counting. Void volume ($V_0$) = fraction 60; Total volume ($V_t$) = fraction 150-160.
(iv) **Disaccharide analysis**

To analyse the disaccharide composition of HS chains isolated from confluent VSMC-B, confluent VSMC-A and mineralising VSMC-A, HS was digested with heparinase I, II and III, and disaccharides were analysed using ProPac PA1 SAX-HPLC; disaccharide species were identifiable by comparison with known standards (Figure 4.6). Medium- (Figure 4.7Ai,Bi&Ci) and cellular/matrix-derived (Figure 4.7Aii,Bii&Cii) HS from all three preparations of VSMCs gave typical disaccharide profiles. The percentage contribution of each disaccharide species to the HS composition was calculated as described in section 2.12.5iv. The HS disaccharide composition of medium- and cellular/matrix-derived HS was very similar between confluent VSMC-B and confluent VSMC-A (Figure 4.8A). The total levels of \( N \), 2-O- and 6-O-sulphation were also calculated from the individual disaccharide species for medium- and cellular/matrix-derived HS (Figure 4.8B). This calculation revealed that approximately 56% of GalNAc residues were converted to GlcNS, while 15% of HS disaccharides were 2-O-sulphated and less than 7% were 6-O-sulphated.

The percentage contribution of each disaccharide species to the composition of medium- and cellular/matrix-derived HS was very similar between confluent and mineralising VSMC-A (Figure 4.8A). The total level of \( N \), 2-O- and 6-O-sulphation in medium- and cellular/matrix-derived HS chains was therefore also very similar between confluent and mineralising VSMC-A (Figure 4.8B).

![Figure 4.6. HS disaccharide standards profile.](image-url) Representative elution profile of HS disaccharide standards. Disaccharide standards were applied to a ProPac PA1 SAX-HPLC column and eluted with a linear gradient of 0-1 M NaCl at a flow rate of 1 ml/minute. The elution profile was monitored by fluorescence at 232 nm.
Figure 4.7. SAX-HPLC separation of HS disaccharides generated by multi-heparinase enzyme digest. HS from the (A-Ci) medium and (A-Cii) cellular/matrix extracts of (A) confluent VSMC-B, (B) confluent VSMC-A and (C) mineralising VSMC-A were digested with heparinase I, II and III. Disaccharides were applied to a ProPac PA1 SAX-HPLC column and eluted with a linear gradient of 0-1 M NaCl at a flow rate of 1 ml/minute, with collection of 0.5 ml fractions. Fractions were analysed by scintillation counting. Peak 1, $\Delta$UA-GlcNAc; Peak 2, $\Delta$UA-GlcNS; Peak 3, $\Delta$UA-GlcNAc(6S); Peak 4, $\Delta$UA(2S)-GlcNAc; Peak 5, $\Delta$UA-GlcNS(6S); Peak 6, $\Delta$UA(2S)-GlcNS; Peak 7, $\Delta$UA(2S)-GlcNS(6S). Profiles are representative of HS disaccharides generated from 2 independent multiple heparinase digests of the same HS samples, and 3 independent SAX-HPLC runs of each disaccharide sample.
Figure 4.8. Analysis of HS disaccharide composition in confluent and mineralising VSMCs. HS from the medium and cellular/matrix fractions of confluent VSMCs (VSMC-B and VSMC-A) and mineralising VSMC-A were digested with heparinase I, II and III, and disaccharides were analysed ProPac PA1 SAX-HPLC. (A) The total $^3$H counts under each disaccharide peak for medium- and cellular/matrix-derived HS was expressed as a percentage of the total counts. (B) The overall sulphation composition of medium- and cellular/matrix-derived HS. 0S ($\Delta$UA-GlcNAc), NS [$\Delta$UA-GlcNS, $\Delta$UA-GlcNS(6S), $\Delta$UA(2S)-GlcNS, $\Delta$UA(2S)-GlcNS(6S)], 2S [$\Delta$UA(2S)-GlcNS, $\Delta$UA(2S)-GlcNS(6S), $\Delta$UA(2S)-GlcNAc] and 6S [$\Delta$UA-GlcNAc(6S), $\Delta$UA-GlcNS(6S), $\Delta$UA(2S)-GlcNS(6S)]. Results displayed as mean ± SD ($n=6$ HPLC runs from 2 independent heparinase digests of each preparation of VSMCs).
4.2.3 CS/DS characterisation

Intact CS/DS chains purified from both the medium and cellular/matrix extracts of all three VSMC preparations were characterised using specific scission agents as described below.

(i) Chain length

CS/DS chain length was determined following alkaline release from the peptide core and estimated according to the Wasteson calibration (Wasteson, 1971b). In confluent VSMCs, both cellular/matrix- and medium-derived CS/DS chains were shorter in confluent VSMC-B compared to confluent VSMC-A (Table 4.4). In both confluent VSMC preparations, medium-derived CS/DS chains were longer than cellular/matrix-derived CS/DS (Table 4.4).

Cellular/matrix-derived CS/DS chains were slightly shorter in mineralising VSMC-A when compared to confluent VSMC-A (Table 4.4). In contrast, medium-derived CS/DS chains from confluent and mineralising VSMC-A were the same size (Table 4.4).

Table 4.4. CS/DS chain length

<table>
<thead>
<tr>
<th>Medium-derived CS/DS</th>
<th>Cellular/matrix-derived CS/DS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VSMC-B</td>
</tr>
<tr>
<td>Kav</td>
<td>0.45</td>
</tr>
<tr>
<td>Chain length (kDa)</td>
<td>30</td>
</tr>
<tr>
<td>No. dp2 per chain</td>
<td>60</td>
</tr>
</tbody>
</table>

Chain length was determined following alkaline release from the peptide core and estimated according to the Wasteson calibration (Wasteson, 1971b). The number of disaccharides (dp2) per chain was calculated based on the assumption that the average molecular weight of a CS/DS disaccharide is 503 daltons.

(ii) Ratio of DS to CS

Chondroitinase B cleaves CS/DS chains at IdoA-GalNAc and fractionation of these oligosaccharides can reveal the proportion of IdoA-containing disaccharides (i.e. DS) present within contiguous (which will generate disaccharides) and alternating sequences (which will generate tetrasaccharides). This method is therefore extremely useful for generating information about the content and positioning of IdoA within CS/DS chains (Maccarana et al., 2009). The proportion of IdoA-containing disaccharides in both contiguous and alternating sequences was determined from the oligosaccharides profiles presented in Figure 4.9. Medium-derived CS/DS chains from VSMC-B and VSMC-A at confluence contained 12 and 14% IdoA, respectively (Table 4.5). The proportion of IdoA-containing disaccharides in both contiguous and alternating sequences was relatively consistent in medium-derived CS/DS chains from confluent VSMC-B and VSMC-A (Table 4.5). In contrast, the proportion of IdoA in cellular/matrix-derived CS/DS
Figure 4.9. Analysis of the DS to CS ratio in confluent and mineralising VSMCs. CS/DS from the (A-Ci) medium and (A-Cii) cellular/matrix extracts of (A) confluent VSMC-B, (B) confluent VSMC-A and (C) mineralising VSMC-A were digested with chondroitinase B and DS oligosaccharides were separated from undigested material by gel filtration on a Superdex 30 size-exclusion column at a flow rate of 4 ml/hour. Fractions (0.5 ml) were collected and analysed by scintillation counting. Profiles are representative of 2 independent chondroitinase B digests of the same material. CS, chondroitin sulphate; DS dp6, DS hexasaccharides; DS dp4, DS tetrasaccharides; DS dp2, DS disaccharides. Void volume \( (V_0) \) = fraction 60; Total volume \( (V_t) \) = fraction 150.
chains from confluent VSMC-A was higher when compared to confluent VSMC-B (37% compared to 20%, respectively). Despite a difference in total IdoA content, the proportion of IdoA in both contiguous and alternating sequences was relatively similar in cellular/matrix-derived CS/DS chains from these cells (Table 4.5). The remainder of IdoA-containing disaccharides were present in hexasaccharides.

In both medium- and cellular/matrix-derived CS/DS chains from mineralising VSMC-A, there was an increase in IdoA content when compared to confluent VSMC-A (7% increase in medium-derived CS/DS, 10% increase in cellular/matrix-derived CS/DS). However, no difference was detected in the proportion of IdoA-containing disaccharides in both contiguous and alternating sequences in medium- and cellular/matrix-derived CS/DS chains from confluent and mineralising VSMC-A (Table 4.5).

Table 4.5. Chondroitinase B oligosaccharide distribution

<table>
<thead>
<tr>
<th></th>
<th>Medium-derived CS/DS</th>
<th>Cellular/matrix-derived CS/DS</th>
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<tbody>
<tr>
<td></td>
<td>VSMC-B</td>
<td>VSMC-A</td>
</tr>
<tr>
<td>% susceptible</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>% contiguous</td>
<td>49</td>
<td>47</td>
</tr>
<tr>
<td>% alternate</td>
<td>41</td>
<td>41</td>
</tr>
</tbody>
</table>

The number of linkages in CS/DS that were susceptible to chondroitinase B was calculated as follows. The total $^3$H counts eluting in a specific peak were adjusted for the number of $^3$H-labelled GalNAc sugars in the oligosaccharide corresponding to that peak. The percentage susceptibility is calculated as the percentage of total susceptible linkages (i.e. adjusted counts) in the total $^3$H counts (i.e. unadjusted counts). The percentage of contiguous linkages (i.e. disaccharides) was calculated as the percentage of $^3$H (adjusted) counts under the disaccharide peak in the total number of susceptible linkages. Tetrasaccharides have an internal GlcA(±2S) and therefore correspond to alternate susceptible linkages, and the percentage is calculated in an equivalent process to the contiguous linkages.

(iii) Analysis of CS/DS disaccharides from confluent and mineralising VSMCs

To further analyse the composition of $^3$H-labelled CS/DS disaccharides, samples were analysed using a Hypersil SAX-HPLC. All of the $^3$H-labelled CS/DS disaccharides analysed in this study contained 2 major disaccharide species which eluted where the $\Delta$UA-GalNAc(6S), $\Delta$UA-GalNAc(4S) and $\Delta$UA(2S)-GalNAc standards should elute (Figure 4.10). However, as the $\Delta$UA-GalNAc(6S), $\Delta$UA-GalNAc(4S) and $\Delta$UA(2S)-GalNAc disaccharide standards eluted very close together (see Figure 4.11A), it was difficult to confirm which disaccharide species were present in the radiolabelled samples. Therefore, to determine which CS/DS disaccharide species are expressed by VSMCs, the following approach was used as summarised in Figure 4.11B. Both the $^3$H-labelled CS/DS disaccharides and CS/DS disaccharide standards were generated by chondroitinase ABC digestion. This enzyme generates a double bond between the C4 and C5
of the non-reducing terminal uronate residue, which in turn generates a useful and sensitive UV chromophore (maximum absorption at 232nm) which can be detected by a fluorescent reader. However, only the CS/DS disaccharide standards can be detected by fluorescence in this way as abundant material is required. Therefore, 15,000 cpm of $^3$H-labelled CS/DS disaccharides from mineralising VSMC-A were loaded onto the Hypersil SAX-HPLC column along with the following CS/DS disaccharide standards: $\Delta$UA-GalNAc(6S), $\Delta$UA-GalNAc(4S) and $\Delta$UA(2S)-GalNAc. The $^3$H-labelled CS/DS disaccharides and CS/DS disaccharide standards were eluted together with a 45 minute linear gradient of 0-0.7 M NaCl. By monitoring the fluorescence of the $\Delta$UA-GalNAc(6S), $\Delta$UA-GalNAc(4S) and $\Delta$UA(2S)-GalNAc disaccharide standards at 232nm, $^3$H-labelled material was collected manually from the HPLC into three individual collection tubes when each of these CS/DS disaccharide standards were detected by fluorescence.

The three fractions of $^3$H-labelled material corresponding to the positions in which $\Delta$UA-GalNAc(6S), $\Delta$UA-GalNAc(4S) and $\Delta$UA(2S)-GalNAc disaccharide standards were eluted, were then re-loaded separately onto the Hypersil SAX-HPLC and eluted with a 45 minute linear gradient of 0-0.7 M NaCl at a flow rate of 1 ml/minute, with collection of 0.5 ml fractions. The fractions were then analysed by scintillation counting. This protocol identified the two major CS/DS disaccharide species expressed by VSMCs as $\Delta$UA-GalNAc(6S) and UA-GalNAc(4S) (Figure 4.12). The $^3$H-labelled material which had eluted with the $\Delta$UA(2S)-GalNAc standard appeared to be carried-over from $\Delta$UA-GalNAc(4S), as $^3$H-labelled material corresponding to the $\Delta$UA(2S)-GalNAc standard eluted in the same position as $\Delta$UA-GalNAc(4S) when analysed by scintillation counting (Figure 4.12). To conclude, these studies demonstrate that the CS/DS synthesised by VSMCs contains $\Delta$UA-GalNAc(6S) and UA-GalNAc(4S); $\Delta$UA(2S)-GalNAc was not detected.

In all three preparations of VSMCs, less than 10% of CS/DS disaccharides were not modified by sulphation ($\Delta$UA-GalNAc), whilst the sulphated CS/DS disaccharide species, $\Delta$UA(2S)-GalNAc(6S) and $\Delta$UA(2S)-GalNAc(4S), were detected at very low levels in these cells (Figure 4.10). As $\Delta$UA(2S)-GalNAc, $\Delta$UA-GalNAc(4S,6S) and $\Delta$UA(2S)-GalNAc(4S,6S) were not consistently identified in different HPLC runs, these disaccharide species were therefore not included the sulphation analysis of CS/DS chains from the three VSMC preparations.
Figure 4.10. SAX-HPLC separation of CS/DS disaccharides generated by chondroitinase ABC digest. CS/DS from the (A-Ci) medium and (A-Cii) cellular/matrix extracts of (A) confluent VSMC-B, (B) confluent VSMC-A and (C) mineralising VSMC-A were digested with chondroitinase ABC. Disaccharides were applied to a Hypersil SAX-HPLC column and eluted with a linear gradient of 0-0.7 M NaCl at a flow rate of 1 ml/minute, with collection of 0.5 ml fractions. Fractions were analysed by scintillation counting. Peak 1, ΔUA-GalNAc; Peak 2, ΔUA-GalNAc(6S); Peak 3, ΔUA-GalNAc(4S); Peak 4, ΔUA(2S)-GalNAc(6S); Peak 5, ΔUA(2S)-GalNAc(4S). The following disaccharides species were not consistently identified between HPLC runs and samples: ΔUA(2S)-GalNAc, ΔUA-GalNAc(4S,6S), ΔUA(2S)-GalNAc(4S,6S). Profiles are representative of 3 independent SAX-HPLC runs of each CS/DS sample.
Elution of CS/DS dp2 standards was monitored by fluorescence [see (A)].

H-labelled CS/DS dp2s associated with each CS/DS dp2 standard was re-loaded onto the Hypersil SAX-HPLC and eluted with a 45 minute linear gradient of 0.0-0.7 M NaCl at a flow rate of 1 ml/min.

Fractions analysed by scintillation counting.

15K cpm 3H-labelled CS/DS dp2s plus CS/DS dp2 standards (\(\Delta\text{UA-GalNAc}(6S)\), \(\Delta\text{UA-GalNAc}(4S)\) and \(\Delta\text{UA}(2S)\)-GalNAc) were loaded onto the Hypersil SAX-HPLC and eluted with a 45 minute linear gradient of 0-0.7 M NaCl at a flow rate of 1 ml/min.

3H-labelled CS/DS dp2s were manually collected from the HPLC as each CS/DS dp2 standard was detected.

Figure 4.1. Confirming the identification of \(\Delta\text{UA-GalNAc}(6S)\) and \(\Delta\text{UA-GalNAc}(4S)\) in 3H-labelled CS/DS disaccharide samples. (A) Representative elution profile of CS/DS disaccharide standards. Disaccharide standards were applied to a Hypersil SAX-HPLC column and eluted with a linear gradient of 0-1 M NaCl at a flow rate of 1 ml/minute. The elution profile was monitored by fluorescence at 232 nm. (B) Schematic overview of the approach used to confirm the identification of \(\Delta\text{UA-GalNAc}(6S)\) and \(\Delta\text{UA-GalNAc}(4S)\) in 3H-labelled CS/DS disaccharide samples.
The disaccharide composition (Figure 4.13A) and total levels of 2-, 4- and 6-O-sulphation (Figure 4.13B) in medium-derived CS/DS chains were very similar in confluent VSMC-B and confluent VSMC-A. In contrast, the proportion of UA-GalNAc(4S) (Figure 4.13A) and total level of 4-O-sulphation (Figure 4.13B) in cellular/matrix-derived CS/DS chains was increased in confluent VSMC-A when compared to confluent VSMC-B. This change was associated with lower levels of total 6-O-sulphation in cellular/matrix-derived CS/DS chains from confluent VSMC-A (VSMC-A, 23% 6S; VSMC-B, 38% 6S). The CS/DS 4S:6S ratio was also calculated from the total level of 4- and 6-O-sulphation in CS/DS chains (Figure 4.14). Whilst the medium-derived CS/DS 4S:6S ratio was very similar in the two preparations of confluent VSMCs, there was a 2-fold increase in the cellular/matrix-derived CS/DS 4S:6S ratio in confluent VSMC-A when compared to confluent VSMC-B (Figure 4.14).

The disaccharide composition of medium- and cellular/matrix-derived CS/DS (Figure 4.13A) and total levels of 4- and 6-O-sulphation (Figure 4.13B) were markedly different in confluent and mineralising VSMC-A. Specifically, there was a ~10% increase in ΔUA-GalNAc(4S) and a ~10% decrease in ΔUA-GalNAc(6S) in CS/DS chains from mineralising compared to confluent VSMC-A (Figure 4.13A). This change corresponded to a ~2-fold increase in the medium- and cellular/matrix-derived CS/DS 4S:6S ratio in mineralising compared to confluent VSMC-A (Figure 4.14).
Figure 4.13. Analysis of CS/DS chain composition in confluent and mineralising VSMCs. CS/DS from the medium and cellular/matrix fractions of confluent VSMCs (VSMC-B and VSMC-A) and mineralising VSMC-A were digested with chondroitinase ABC and disaccharides were analysed by Hypersil SAX-HPLC. (A) The total $^3$H counts under each disaccharide peak for medium- and cellular/matrix-derived CS/DS was expressed as a percentage of the total counts. The following disaccharides species were not detected (see Figure 4.10): ∆UA(2S)-GalNAc, ∆UA-GalNAc(4S,6S), ∆UA(2S)-GalNAc(4S,6S). (B) The overall sulphation composition of medium- and cellular/matrix-derived CS/DS. 0S (∆UA-GalNAc), 2S [∆UA(2S)-GalNAc, ∆UA(2S)-GalNAc(6S), ∆UA(2S)-GalNAc(4S, 6S)], 4S [∆UA-GalNAc(4S), ∆UA-GalNAc(4S, 6S), ∆UA(2S)-GalNAc(4S), ∆UA(2S)-GalNAc(4S, 6S)] and 6S [∆UA-GalNAc(6S), ∆UA(2S)-GalNAc(6S), ∆UA-GalNAc(4S, 6S), ∆UA(2S)-GalNAc(4S, 6S)]. Results displayed as mean ± SD (n=3 HPLC runs from a single digest of each preparation of VSMCs).
Figure 4.14. CS/DS 4S:6S ratio is increased in mineralising VSMCs. CS/DS from the medium and cellular/matrix fractions of confluent VSMCs (VSMC-B and VSMC-A) and mineralising VSMC-A were digested with chondroitinase ABC. Disaccharides were applied to a Hypersil SAX-HPLC column and eluted with a linear gradient of 0-0.7 M NaCl at a flow rate of 1 ml/minute, with collection of 0.5 ml fractions. Fractions were analysed by scintillation counting. \(^3\)H counts under each peak was counted and expressed as a percentage of the total counts. The 4S:6S ratio was calculated from these counts. Results displayed as mean ± SD (n=3 HPLC runs for each preparation of VSMCs).

4.2.4 HS and CS/DS biosynthetic enzyme profile of VSMCs at confluence

The results presented in this Chapter have demonstrated that whilst the HS chain composition of VSMC-A and VSMC-B at confluence is consistent (Figure 4.9), there were differences in CS/DS chain composition between these cells (Figures 4.13 and 4.14). Therefore, to determine if these differences were associated with changes in the glycomic transcription profile of these cells, RNA was isolated from confluent VSMC-A and VSMC-B and the expression levels of both HS and CS/DS biosynthetic enzymes were measured using qPCR.

Both preparations of VSMCs at confluence were shown to express a wide range of HS and CS/DS biosynthetic enzymes (Figure 4.15A&B). The majority of HS biosynthetic enzymes were expressed at a similar level in confluent VSMC-A and VSMC-B, with the exception of heparinase, which was more abundantly expressed in VSMC-A compared to VSMC-B (P<0.01, Figure 4.15A). The majority of CS/DS biosynthetic enzymes were also expressed at a similar level in confluent VSMC-A and VSMC-B (Figure 4.15B).

HS and CS/DS biosynthetic enzymes that belonged to multi-isoform families had the same pattern of expression in confluent VSMC-A and VSMC-B (Figure 4.15A&B). For example, NDST1 and NDST2 were expressed by confluent VSMC-A and VSMC-B, whereas NDST3 and NDST4 could not be detected at the mRNA level in either VSMC preparation. In addition, of the two CS 6-O-sulfotransferases analysed in this study, only C6ST1 could be detected at the mRNA level in confluent VSMC-A and VSMC-B by qPCR. To determine if these observations were due to poor primer efficiency or design, another primer set for each gene was designed. However, neither primer set could detect NDST3, NDST4 or C6ST2 mRNA in confluent VSMC-A and VSMC-B (data not shown), indicating that these genes may not be expressed and/or are undetectable at the mRNA level in these cells.
Figure 4.15. GAG biosynthetic enzyme profile of confluent VSMCs. RNA was isolated from confluent VSMC-A and VSMC-B and the mRNA expression levels of (A) HS biosynthetic enzymes and (B) CS/DS biosynthetic enzymes were measured using qPCR. Expression is shown relative to the reference genes, RPL12 and PPIA. Results are displayed as mean ± SEM. The experiment was performed 2-4 times in triplicate for VSMC-A (n=6-12 samples), and once in triplicate for VSMC-B (n=3 samples). The data were analysed using an unpaired t-test for each gene. ** P<0.01. The following GAG biosynthetic enzymes could not be detected in VSMC-A and VSMC-B by qPCR: NDST3, NDST4, C6ST2.
4.2.5 HS and CS/DS biosynthetic enzyme profile during VSMC osteogenic differentiation and mineralisation

VSMC-A osteogenic differentiation and mineralisation is associated with a shortening of HS chains (Table 4.1), but no change in disaccharide composition (Figure 4.8). In addition, both the DS:CS (Table 4.5) and CS/DS 4S:6S ratios (Figure 4.14) were increased in mineralising VSMCs. Therefore, to determine the expression profile of HS and CS/DS biosynthetic enzymes during the osteogenic differentiation and mineralisation of VSMCs, VSMC-A were cultured with 3 mM β-GP to induce calcification. Controls were cultured without β-GP. RNA was then isolated from these cells at specific time-points over the course of the experiment: stage 1 (no mineral deposition), stage 2 (initial formation of mineral deposits), stage 3 (early mineralisation), stage 4 (mid mineralisation) and stage 5 (late mineralisation). For reference, phase-contrast images of alizarin red-stained VSMC-A during the different stages of osteogenic differentiation and mineralisation are shown in Figure 3.1A.

(i) HS biosynthetic enzymes

HS chains undergo a series of modifications involving NDSTs, epimerases, sulphotransferases and Sulfs (Figure 1.9). There was no change in the mRNA levels of NDST1 and NDST2 at stage 1 and 2 of VSMC-A mineralisation (Figure 4.16). However, NDST1 mRNA was significantly up-regulated (~3 fold increase, P<0.001, Figure 4.16) in β-GP-treated VSMCs at stage 3, 4 and 5 of VSMC-A mineralisation. In contrast, only the later stages of VSMC-A mineralisation (stage 4 and 5) were associated with a significant increase (~1.5 fold increase, P<0.001, Figure 4.16) in NDST2 mRNA expression in β-GP-treated VSMC-A when compared to controls at the same time-point. The mRNA expression levels of C5 epimerase were not changed during VSMC-A osteogenic differentiation and mineralisation (data not shown). As HS disaccharide composition was not significantly altered during VSMC-A mineralisation (section 4.2.2iv), the mRNA levels of the HS sulphotransferases were not examined in this study.

Heparanase is an endogenously expressed endo-β-D-glucuronidase which degrades HS chains into active fragments that are 10–20 sugar units long (Vlodavsky et al., 1999). As HS chain length was reduced in mineralising VSMC-A, the expression levels of heparanase were also quantified during the osteogenic differentiation and mineralisation of VSMC-A. These studies demonstrated that there was no change in the mRNA expression levels of heparanase in β-GP-treated VSMC-A when compared to controls (Figure 4.16).
Figure 4.16. HS biosynthetic enzyme profile of VSMCs during osteogenic differentiation and mineralisation. Confluent VSMC-A (day 0) were cultured ± 3 mM β-GP for up to 14 days. RNA was collected from cells at day 0, then at specific stages of the mineralisation process (stages 1-5). The expression levels of NDST1, NDST2 and heparanase were quantified using qPCR. Expression is shown relative to day 0, which is equal to 1. mRNA expression levels at stages 1 and 2 were combined. Results displayed as mean ± SEM. Stages 1&2 (n=12 samples from 2 independent experiments); Stages 3-5 (n=9 samples from 3 independent experiments). The data were analysed using a 2-way ANOVA with Sidak post-hoc tests. *** P<0.001 against same time-point control.

(ii) CS/DS biosynthetic enzymes

CS/DS biosynthesis involves a series of polymerases, epimerases and sulphotransferases (Figure 1.11). The osteogenic differentiation and mineralisation of VSMC-A was associated with changes in the mRNA expression levels of the CS/DS chain polymerising enzymes, CHGN2 and CHSY-1. CHGN2 mRNA was significantly up-regulated (~2-fold increase, P<0.001, Figure 4.17) in β-GP-treated VSMC-A at stage 4 and 5 of mineralisation when compared to controls. In contrast, CHSY-1 mRNA was up-regulated (~2.5-fold increase, P<0.001, Figure 4.17) in β-GP-treated VSMC-A at stage 5 of VSMC mineralisation. The mRNA expression levels of CHSY-2, CHSY-3 and ChPF2 were not changed during VSMC-A osteogenic differentiation and mineralisation (data not shown).

The two DS epimerases, DS epimerase-1 and -2, were differentially expressed during VSMC-A osteogenic differentiation and mineralisation. DS epimerase-1 mRNA was significantly up-regulated (~2-fold increase, P<0.01, Figure 4.17) in β-GP-treated VSMC-A at stage 3 of mineralisation, corresponding with the stage of mineralisation at which Runx2, BMP-2 and Msx2 mRNA were also up-regulated (Figure 3.1). Importantly, stage 3 of VSMC mineralisation was also the time-point at which ³H-glucosamine was introduced into the media of mineralising cells to generate radiolabelled GAGs. DS epimerase-1 mRNA expression then returned to control levels at stage 4 and 5 of VSMC-A mineralisation (Figure 4.17). In contrast, DS epimerase-2 mRNA was up-regulated (~3-fold increase, P<0.001, Figure 4.17) in β-GP-treated VSMC-A at stage 5 of mineralisation when compared to controls at the same time-point.
The osteogenic differentiation and mineralisation of VSMC-A was also associated with changes in the mRNA levels of the CS/DS sulphotransferases (Figure 4.17). There was no change in the mRNA levels of C4ST1 and C4ST2 at stage 1 and 2 of VSMC-A mineralisation (Figure 4.17). However, C4ST1 mRNA was significantly up-regulated (~3-fold increase, \( P<0.001 \), Figure 4.17) in β-GP-treated VSMC-A at stage 3, 4 and 5 of mineralisation. In contrast, only the late stages of VSMC-A mineralisation (stage 5) were associated with a significant increase (~2-fold increase, \( P<0.01 \), Figure 4.17) in C4ST2 mRNA in β-GP-treated VSMC-A when compared to controls. The mRNA levels of C6ST1 and D4ST1 were not changed during the osteogenic differentiation and mineralisation of VSMC-A when compared to controls at the same time-point (Figure 4.17).

4.2.6 GAG biosynthetic enzyme profile of VSMCs that do not mineralise in the presence of β-GP

In the presence of β-GP, the expression levels of specific HS (NDST1 and NDST2) and CS/DS (CHSY1, CHGN2, C4ST1, C4ST2, DS epimerase-1 and -2) biosynthetic enzymes were up-regulated in VSMC-A (Figures 4.16 and 4.17). Therefore, to determine if these changes were due to extended culture times in the presence of β-GP, two separate VSMC preparations that do not undergo osteogenic differentiation and mineralisation (VSMC-B (Figure 3.2) and VSMC-C (Figure 3.3)) were cultured with 5 mM β-GP for up to 14 days. Controls were cultured without β-GP. RNA was isolated from these cells at days 9, 11 and 14, as these time-points corresponded to the time taken for VSMC-A to reach stages 3, 4 and 5 of mineralisation. The expression levels of NDST1, NDST2, CHSY1, CHGN2, C4ST1, C4ST2, DS epimerase-1 and -2 were then measured by qPCR.

There were no significant changes in the mRNA expression levels of NDST1, NDST2, CHSY1, CHGN2, C4ST1, C4ST2, DS epimerase-1 and -2 in β-GP-treated VSMC-B and VSMC-C when compared to controls at the same time-point (Figure 4.18).
Figure 4.17. CS/DS biosynthetic enzyme profile of VSMCs during osteogenic differentiation and mineralisation. Confluent VSMC-A (day 0) were cultured ± 3 mM β-GP for up to 14 days. RNA was collected from cells at day 0, then at specific stages of the mineralisation process (stages 1-5). The expression levels of specific CS/DS chain polymerising enzymes (CHSY1, CHGN2), DS epimerases (DS epimerase-1, -2), CS sulphotransferases (C4ST1, C4ST2, C6ST1) and DS sulphotransferase (D4ST1) were quantified using qPCR. Expression is shown relative to day 0, which is equal to 1. mRNA expression levels at stages 1 and 2 were combined. Results displayed as mean ± SEM. Stages 1&2 (n=12 samples from 2 independent experiments); Stages 3-5 (n=9 samples from 3 independent experiments). The data were analysed using a 2-way ANOVA with Sidak post-hoc tests. ** P<0.01; *** P<0.001 against same time-point control.
Figure 4.18. GAG biosynthetic enzyme expression is not changed in non-mineralising VSMCs in the presence of β-GP. Confluent VSMC-B and VSMC-C (day 0) were cultured ± 5 mM β-GP for up to 14 days. RNA was collected from cells at day 0, then at days 9, 11 and 14. The expression levels of various HS and CS/DS biosynthetic enzymes were quantified using qPCR. Expression is shown relative to day 0, which is equal to 1. This experiment was performed once in triplicate for both VSMC-B and VSMC-C, and the data were combined. Results displayed as mean ± SEM (n=6 for each treatment group at all time-points analysed). The data were analysed using a 2-way ANOVA with Sidak post-hoc tests.
4.3 Discussion

The results presented in this chapter demonstrate that the composition of HS chains synthesised by two separate preparations of VSMCs (VSMC-A and VSMC-B) is very similar. In contrast, the CS/DS synthesised by these cells was distinct: both in terms of IdoA content and total 4-O- and 6-O-sulphation. During VSMC-A osteogenic differentiation and mineralisation, NDST1 and NDST2 mRNA expression increases in mineralising VSMC-A but there is no marked change in HS disaccharide composition. HS chains isolated from the cellular/matrix extract of mineralising VSMC-A were smaller in size when compared to confluent VSMC-A, although this did not appear to be related to changes in heparanase mRNA. In contrast, the mRNA levels of specific CS/DS 4-O-sulphotransferases (C4ST1, C4ST2) were up-regulated in mineralising VSMC-A, correlating with increased CS/DS 4-O-sulphation. DS epimerase-1 and -2 mRNA were also significantly increased in mineralising VSMC-A, correlating with an increase in the DS to CS ratio in these cells. Finally, the expression levels of C4ST1, C4ST2, DS epimerase-1 and -2 were not changed in two separate preparations of VSMCs that do not undergo osteogenic differentiation and mineralisation in the presence of β-GP.

4.3.1 HS chain structure is consistent between different VSMC preparations at confluence

The level and distribution of sulphation within HS chains is tightly regulated leading to tissue-specific patterning (Maccarana et al., 1996; Ledin et al., 2004). At confluence, VSMC-A and VSMC-B produced HS and CS/DS, both secreted into the medium and displayed on PG core proteins at the cell surface and/or in the cell matrix. HS was predominantly associated with the cellular/matrix extract of both preparations of confluent VSMCs, whilst CS/DS represented >75% of the total GAGs in the culture medium. The HS chains present in the culture medium of both confluent VSMC-A and VSMC-B were slightly smaller than the HS isolated from the cellular/matrix-extract. It is possible that the HS detected in the culture medium could arise from shedding of PGs at the cell surface and/or ECM, or by heparanase-mediated cleavage of HS chains (Vlodavsky et al., 1999).

Differences in HS chain length can have functional consequences as they can differentially regulate growth factor binding, and in turn, downstream signalling events (Holley et al., 2011b; Pickford et al., 2011). Both medium- and cellular/matrix-derived HS chains were slightly smaller in confluent VSMC-A compared to confluent VSMC-B (Table 4.1), although it could not be determined if this difference was significant as these experiments were only carried out once. Interestingly, the mRNA levels of the endogenously expressed HS-degrading enzyme, heparanase, were significantly higher in confluent VSMC-A compared to confluent VSMC-B (Figure ) which may in part explain the small differences observed in HS chain length between the two preparations of confluent VSMCs.

The composition of HS chains from both preparations of confluent VSMCs was further analysed by chemical depolymerisation with low pH nitrous acid, release of NA domains by digestion with heparinase III, release of NS domains by digestion with heparinase I and disaccharide...
compositional analysis by SAX-HPLC. Low pH nitrous acid depolymerisation of the HS synthesised by confluent VSMC-A and VSMC-B demonstrated that 39-43% of GlcNAc residues were converted to GlcNS. In good agreement with this result, compositional analysis of HS disaccharides by SAX-HPLC demonstrated that 38-39% of disaccharides were N-sulphated. As HS is generally characterised to have approximately 50% of GlcNAc residues converted to GlcNS (Maccarana et al., 1996; Ledin et al., 2004), these results suggest that VSMC HS is characteristic of a slightly under-sulphated form of HS.

Approximately 83% of linkages in confluent VSMC-A and VSMC-B HS chains were susceptible to cleavage by heparinase III (Table 4.3), which cleaves HS at GlcNac/NS(±6S)-GlcA. In contrast, heparinase I cleaves disaccharides containing IdoA(2S) and only <10% of HS chains derived from the medium and cellular/matrix extract of confluent VSMC-A and VSMC-B were susceptible to heparinase I scission (Figure 4.5). The majority of these HS chains were separated by large resistant sequences. It has been widely reported that HS from other cell types such as bovine aortic endothelial cells (Pye and Kumar, 1995) and human skin fibroblasts (Turnbull and Gallagher, 1990) has limited to susceptibility to heparinase I treatment, demonstrating the compositional difference between HS and heparin which consists of multiple sequences of IdoA(2S) disaccharides. Approximately 70% of IdoA(2S)-containing disaccharides were present in contiguous sequences in confluent VSMC-A and VSMC-B HS. Together with the heparinase III profile, these data demonstrate that IdoA(2S)-containing disaccharides are present in contiguous sequences in a few sites in well spaced domains in the intact HS chain from VSMCs. This structural organisation of sulphated domains within HS chains is common and has been also observed in a number of cell types including bovine endothelial cells (Pye and Kumar, 1995), mouse 3T3 fibroblasts (Merry et al., 1999; Murphy et al., 2004) and human skin fibroblasts (Turnbull and Gallagher, 1990).

HS disaccharide composition is tissue specific (Maccarana et al., 1996; Ledin et al., 2004). Consistent with this finding, the disaccharide composition of medium-derived and cellular/matrix HS from confluent VSMC-A and VSMC-B was very similar (Figure 4.8). The disaccharide profile of VSMC HS is, however, distinct from other cell types present in the blood vessel wall, such as endothelial cells (Pye and Kumar, 1998). It is also important to note that in contrast to the heparinase I profile, SAX-HPLC analysis of HS disaccharide composition demonstrated that both preparations of confluent VSMCs contained 14-16% 2-O-sulphation. There could be several reasons for this discrepancy. For example, it is possible that the size-exclusion resin used in the current study was unable to completely resolve heparinase I-treated HS. Alternatively, it may indicate the presence of the rare GlcA(2S) disaccharide (Bienkowski and Conrad, 1985). These analyses demonstrate HS scission agents should not be used in isolation, and instead they should be used together to fully characterise both the domain structure and disaccharide composition of HS.
4.3.2 VSMC mineralisation is associated with subtle changes in HS

The osteogenic differentiation and mineralisation of VSMC-A was not associated with any significant changes in the proportions of HS and CS/DS in the medium extract, but there was a decrease in the cellular/matrix HS component when compared to confluent VSMC-A. This observation could reflect a decrease in HSPGs during VSMC mineralisation, and/or a switch from HS to CS/DS chains on PG core proteins capable of carrying both GAG chains such as the syndecans (Shworak et al., 1994; Ueno et al., 2001; Deepa et al., 2004).

Subtle changes in HS chain structure were also observed during VSMC osteogenic differentiation and mineralisation. The disaccharide composition and chain length of medium-derived HS chains was very similar between confluent and mineralising VSMC-A (Figure 4.8). However, the osteogenic differentiation and mineralisation of VSMC-A was accompanied by a ~56% decrease in cellular/matrix HS chain length when compared to confluent VSMC-A (Table 4.1). This is not the first study to report differences in the composition of medium-derived and cellular/matrix HS (Hampson et al., 1984; Johnson et al., 2007). Indeed, these compositional differences may be functionally important as HS from MC3T3-E1 pre-osteoblasts can induce proliferation or differentiation when added back to the same cells, depending on whether the HS was sourced from the conditioned media or the cell layer (Jackson et al., 2007).

It is possible that the decrease in HS chain length during VSMC osteogenic differentiation and mineralisation was due to the action of heparanase, an endogenously expressed endo-β-D-glucuronidase which degrades HS chains into active fragments that are 10–20 sugar units long (Vlodavsky et al., 1999). However, no change in heparanase mRNA expression was detected during VSMC osteogenic differentiation and mineralisation. Whilst there is good correlation between heparanase mRNA levels and activity in human breast cancer cells (Vlodavsky et al., 1999) and osteoblasts (Smith et al., 2010), the heparanase gene encodes for a latent 65 kDa proheparanase which must undergo proteolytic processing to generate 8 kDa and 50 kDa subunits that heterodimerise to form the active heparanase enzyme (Fairbanks et al., 1999). Generation of the active heparanase enzyme from proheparanase is tightly regulated by various factors, including the proteinase cathepsin L (Abboud-Jarrous et al., 2008). It is possible that proteolytic processing of proheparanase is altered in mineralising VSMCs, leading to increased active heparanase levels and shorter HS chains. To determine if this is the case, it would be important for future studies to determine if heparanase activity is changed during the osteogenic differentiation and mineralisation of VSMC-A.

Differences in HS chain length between the medium- and cellular/matrix-extract of mineralising VSMC-A could also reflect small compositional differences between the HS attached to HSPGs in this cell system, such as the syndecans and glypicans. The glypicans are membrane-associated via a lipid moiety, so the HS chains present on the glypicans are likely to be found in the cellular/matrix GAG extract. In contrast, the transmembrane-spanning syndecans are known to display HS and/or CS/DS chains (Shworak et al., 1994; Ueno et al., 2001; Deepa et al., 2004; Gopal et al., 2010), and the extracellular domain of the syndecans are also susceptible to
sheding from the cell surface (Fitzgerald et al., 2000; Manon-Jensen et al., 2010; Manon-Jensen et al., 2013; Ramnath et al., 2014). Analysis of the PG core proteins in the medium-derived and cellular/matrix HS fractions would be required to determine in which fractions the different PG core proteins were recovered.

The mRNA levels of several HS biosynthetic enzymes were analysed during VSMC osteogenic differentiation and mineralisation, and of all the genes analysed, only NDST1 and NDST2 mRNA expression were significantly increased in mineralising cells when compared to controls (Figure 4.16). Interestingly, the mRNA expression levels of NDST1 and NDST2 were not changed in two separate preparations of VSMCs that do not undergo osteogenic differentiation and mineralisation in the presence of β-GP, suggesting that the changes observed in the expression levels of NDST1 and NDST2 are either necessary for, or are a consequence of, these processes rather than due to extended culture times in the presence of β-GP.

Whilst the mRNA levels of NDST1 and NDST2 were significantly up-regulated in mineralising VSMCs, there was no change in HS chain N-sulphation (Table 4.2 and Figure 4.8). It is possible that this finding reflects the heterogeneous nature of this population of cells, which are composed of VSMCs that have differentiated to an osteoblast-like phenotype, are in the process of undergoing osteogenic differentiation, or have not differentiated. Alternatively, it could suggest that changes in the mRNA levels of NDST1 and NDST2 do not necessarily correlate with changes in HS chain N-sulphation. Indeed, NDST activity and HS chain N-sulphation are increased in the brains of α-l-iduronidase-null mice (a model of mucopolysaccharide I Hurler), but this increase did not correlate with increased mRNA expression levels of the four NDST genes in the brains of these mice (Holley et al., 2011a). HS chain modifications are highly dependent on the interactions between specific HS biosynthetic enzymes, and this forms the basis of the GAGosome model (Esko and Selleck, 2002). Compatible with the GAGosome model, NDST1 activity is regulated by the expression levels of the HS chain polymerising enzyme EXT2 (Presto et al., 2008) and the sulphate donor PAPS (Carlsson et al., 2008). Whilst the levels of 3'-phosphoadenosine 5'- phosphosulfate (PAPS) were not quantified in this study, there was no change in EXT2 mRNA expression during the osteogenic differentiation and mineralisation of VSMC-A (data not shown). In light of this evidence, it is possible that increases in NDST1 and NDST2 mRNA during the osteogenic differentiation and mineralisation of VSMCs could not produce increases in HS chain N-sulphation without a concomitant increase in the expression levels of EXT2. These studies importantly highlight the need to correlate changes in the mRNA expressions levels of the GAG biosynthetic enzymes to changes in GAG chain composition.

Collectively, these analyses demonstrate that HS chain composition is very similar in two separate preparations of VSMCs at confluence, and also during the osteogenic differentiation and mineralisation of VSMCs. However, HS chains isolated from the cellular/matrix extract are smaller in size during VSMC the osteogenic differentiation and mineralisation. A limitation of these findings is that the structural characterisation of HS chains was from one experiment and
these experiments would therefore need to be repeated to determine if this finding is reproducible. A second limitation of these studies is that it is possible not all radiolabelled GAGs were recovered from both confluent and mineralising VSMCs. In this study, radiolabelled GAGs were extracted from the cell and matrix layer of confluent VSMCs using 0.1% (v/v) Triton X-100 in PBS. Attempts were made to recover any remaining ECM deposited on the tissue culture plastic by the cells using 6 M urea and 0.1% (v/v) Triton X-100; however no additional radiolabelled material was recovered in this extract (data not shown). These results suggest that 0.1% (v/v) Triton X-100 in PBS was sufficient to extract both cell- and matrix-associated GAGs from confluent VSMCs. When the GAGs synthesised by mineralising VSMCs were analysed, 6 M urea and 0.1% (v/v) Triton X-100 was used to extract radiolabelled GAGs from the cells and matrix initially. A harsher extraction agent was used for mineralising VSMC-A as these cells were 12 days post-confluent and a dense matrix had been deposited. The extracted cells and matrix were also passed through a needle several times to break up the matrix. Even with this approach, it is of course possible that not all radiolabelled GAGs were recovered. Future studies would perhaps benefit from using a harsher extraction buffer, such as urea and guanidine HCl, to ensure all GAGs are recovered from the cells and associated ECM.

4.3.3 CS/DS chain structure is not consistent between different VSMC preparations at confluence
Although less well characterised than HS, there is increasing evidence to implicate CS/DS as an important regulator of osteoblast differentiation and/or mineralisation (Wollenweber et al., 2006; Miyazaki et al., 2008; Mathews et al., 2014). Consistent with previous reports (Karousou et al., 2004), CS/DS represented >75% of the total GAGs in the medium of both preparations of confluent VSMCs, but only ~50% of the total GAGs in the cellular/matrix extract of these cells. The CS/DS chains present in the culture medium were larger than the CS/DS isolated from the cellular/matrix-extract, suggesting that there are differences in the CS/DS chains attached to different PG core proteins in these cells (discussed further below). In addition, whilst the IdoA content and disaccharide composition of medium-derived CS/DS chains from both preparations of confluent VSMCs was very similar, there were significant differences in the composition of cellular/matrix CS/DS chains. Specifically, the IdoA content and CS/DS 4S:6S ratio was decreased in confluent VSMC-B compared to VSMC-A (Table 4.5 and Figure 4.14).

It is well established that the level and distribution of sulphation within HS chains is tightly regulated leading to tissue-specific patterning (Maccarana et al., 1996; Ledin et al., 2004), but it is not known if this level of regulation also applies to CS/DS. The differences between CS/DS chains derived from the medium-derived and cellular/matrix fractions of both preparations of VSMCs at confluence may suggest that there are compositional differences between the CS/DS chains attached to different PG core proteins in these cells, as well as between different VSMC preparations. For example, the transmembrane-spanning syndecans are known to display HS and/or CS/DS chains (Shworak et al., 1994; Ueno et al., 2001; Deepa et al., 2004). In contrast, the CS/DS PGs decorin, biglycan and versican, are secreted into the cell matrix and may
therefore be present in both the cell culture medium and/or the cellular/matrix extract. Consistent with the hypothesis that a cell can produce different CS/DS chains depending on the core protein, human skin fibroblasts produce decorin/biglycan with 60% IdoA content, whilst the versican produced by these cells only contains 7% IdoA content (Pacheco et al., 2009b). Analysis of the PG core proteins in the medium-derived and cellular/matrix CS/DS fractions would be required to determine which fractions the different PG core proteins were recovered.

The major CS/DS disaccharide species identified in both preparations of confluent VSMCs were ΔUA-GalNAc(4S) and ΔUA-GalNAc(6S); in addition, less than 10% of the CS/DS disaccharides were not modified by sulphation (ΔUA-GalNAc) (Figure 4.13). In contrast to this finding, ΔUA-GalNAc was identified as the major (~80%) disaccharide species expressed by human VSMCs isolated from tibial arteries (Yan et al., 2011). Whilst these differences in CS/DS disaccharide composition reported in this thesis and Yan et al (2011) may be due to the different origins of the VSMCs (aorta vs. tibial arteries), there does appear to be several question marks concerning the analyses carried out by Yan et al (2011). For example, VSMCs were treated ± Ox-LDL, but the media used (i.e. control or calcification media) and the time-point at which CS/DS was isolated from these cells was not specified. In addition, CS/DS disaccharide composition was analysed using reverse-phase HPLC and AMAC-tagged disaccharide identification, but there were discrepancies between the CS/DS disaccharide standards and analysed samples elution profiles i.e. there were several unidentified peaks in the CS/DS samples. A further point is that previous work in our lab has shown that it is difficult to identify the ΔUA-GalNAc disaccharide using reverse-phase HPLC and AMAC-tagged disaccharide identification as the ΔUA-GalNAc disaccharide often elutes with unbound AMAC (Dr Rebecca Holley, unpublished data). However, Yan et al (2011) made no reference to the unbound AMAC peak in their analyses.

### 4.3.4 VSMC mineralisation is associated with an increase in the DS:CS ratio and CS/DS 4-O-sulphation

During the osteogenic differentiation and mineralisation of VSMCs, there was a marked increase in the ratio of DS to CS that also correlated with a transient increase in DS epimerase-1 and -2 mRNA (Table 4.5 and Figure 4.17). Notably, DS epimerase-1 was up-regulated in β-GP-treated VSMC-A at stage 3 of VSMC mineralisation, corresponding to the time-point at which 3H-glucosamine was introduced into the media of these cells to generate radiolabelled GAGs. Furthermore, there was no change in DS epimerase-1 and -2 mRNA expression in two separate preparations of VSMCs that do not undergo osteogenic differentiation or mineralise in the presence of β-GP, suggesting that the changes observed in DS epimerase-1 and -2 during VSMC mineralisation are associated with the osteogenic differentiation and mineralisation of these cells, and are not due to extended culture in the presence of β-GP.

The DS-specific 4-O-sulphotransferase, D4ST1, is also required to generate IdoA-containing disaccharides (Pacheco et al., 2009a), but there was no change in D4ST1 mRNA in β-GP-treated VSMC-A when compared to controls (Figure 4.17). It is possible that whilst D4ST1 is
required to generate DS disaccharides, D4ST1 is already expressed highly enough in VSMCs to facilitate the epimerisation of GlcA to IdoA in the presence of increased DS epimerase-1 and -2 expression.

VSMC osteogenic differentiation and mineralisation also appears to be associated with a switch from CS to DS on CS/DS PG core proteins, such as decorin and biglycan. This finding is perhaps surprising as CS is the predominant GAG species expressed in bone, whilst DS represents only a minor component (Prince and Navia, 1983; Waddington et al., 1989; Waddington and Embery, 1991). It is possible that differences in IdoA content between mineralising VSMCs and bone could reflect differences in the mineralised matrix formed in vitro and in vivo. In support of this hypothesis, DS containing PGs have also been isolated from the mouse osteoblastic cell line, MC3T3-E1, both before and during mineralisation in vitro (Ecarot-Charrier and Broekhuysen, 1987; Takeuchi et al., 1990). Whilst DS may not represent a major GAG component of bone in vivo, there are several lines of evidence to suggest that DS may play a role in the mineralisation process. For example, exogenous DS increased the mineralisation of human MSCs (Mathews et al., 2014) and the mouse MC3T3-E1 osteoblastic cell line (Miyazaki et al., 2008). Conversely, chondroitinase ABC treatment (which digests both CS and DS) inhibited the mineralisation of MC3T3-E1, whereas chondroitinase AC (which does not digest DS) had a weaker inhibitory effect on the mineralisation of these cells (Miyazaki et al., 2008). Collectively these findings suggest that an increase in DS chain synthesis during VSMC osteogenic differentiation and mineralisation may play a role in regulating this process.

During the osteogenic differentiation and mineralisation of VSMC-A, there was also a marked increase in the CS/DS monosulphated disaccharide species, ∆UA-GalNAc(4S) and a corresponding decrease in ∆UA-GalNAc(6S) (Figure 4.13). This compositional modification correlated with increased C4ST1 and C4ST2 mRNA in mineralising VSMCs when compared to controls, but there was no change in C6ST1 mRNA (Figure 4.17). Interestingly, small increases in C4ST1 and C4ST2 mRNA have also been reported in the MG-63 osteosarcoma cell line during differentiation and mineralisation (Kumarasuriyar et al., 2009), and ∆UA-GalNAc(4S) is the major sulphated CS/DS disaccharide species expressed by the mouse osteoblastic cell line, MC3T3-E1 (Miyazaki et al., 2008). 4-O-sulphated CS also represents >90% of GAGs in bone (Prince and Navia, 1983; Waddington et al., 1989). Taken together, these findings therefore suggest that increased CS/DS 4-O-sulphation during VSMC mineralisation may correlate with the differentiation of VSMCs to an osteoblast-like phenotype. In further support of this hypothesis, there was no change in C4ST1 or C4ST2 mRNA expression in two separate preparations of VSMCs that do not undergo osteogenic differentiation and mineralisation in the presence of β-GP.

The results presented in this thesis demonstrate that the DS:CS ratio and CS/DS 4-O-sulphation are increased during VSMC osteogenic differentiation and mineralisation. A limitation of these findings is that the structural characterisation of CS/DS chains was from one experiment. However, the mRNA levels of the DS epimerases (DS epimerase-1 and -2) and 4-
4.4 Conclusion

The following conclusions can be drawn from the results presented in this chapter:

- HS chains isolated from the cellular/matrix extract of mineralising VSMC-A are smaller in size when compared to confluent VSMC-A, but there is no change in HS disaccharide composition during VSMC-A mineralisation.

- The mRNA levels of specific CS/DS 4-O-sulphotransferases (C4ST1, C4ST2) are up-regulated in mineralising VSMC-A when compared to controls, correlating with an increase in CS/DS 4-O-sulphation.

- DS epimerase-1 and -2 mRNA are also significantly increased in mineralising VSMC-A when compared to controls, correlating with an increase in the DS to CS ratio in these cells.
5 Determining the effects of modulating syndecan 4 expression on VSMC mineralisation

5.1 Introduction

The results presented in Chapters 3 and 4 have demonstrated that syndecan 4 mRNA expression is increased during the late stages of VSMC osteogenic differentiation and mineralisation (Figure 3.5), and this process is also associated with compositional changes in the CS/DS chains synthesised by mineralising VSMCs (Figures 4.9, 4.13 and 4.14). In contrast, there is no change in HS chain composition in mineralising VSMCs (Figures 4.8).

Syndecan 4 can interact with a variety of ligands, both through its GAG chains and core protein, thereby regulating many aspects of cell behaviour, including adhesion and apoptosis (Tkachenko et al., 2005; Xie et al., 2012; Echtermeyer et al., 2011; Elfenbein and Simons, 2013; Couchman et al., 2015). Both growth factor signalling (Table 1.1) and apoptosis (Proudfoot et al., 2000; Collett et al., 2007; Clarke et al., 2008) regulate vascular calcification, thereby suggesting that syndecan 4 may also play a role in this disease. However, it is currently unknown if syndecan 4 stimulates mineralisation, or whether it is induced to prevent mineralisation. Alternatively, syndecan 4 may just be a marker of VSMC mineralisation. The role of syndecan 4 in VSMC mineralisation will therefore be investigated in this chapter.

Inflammation is associated with vascular calcification (Aikawa et al., 2007; Abdelbaky et al., 2013), and a variety of inflammatory mediators such as TNF (Tintut et al., 2000; Son et al., 2008; Aoshima et al., 2012; Masuda et al., 2013) and IL-1β (Parhami et al., 2002) accelerate VSMC mineralisation. Syndecan 4 mRNA expression is regulated by TNF and/or IL-1β in a number of cell types, such as chondrocytes (Bertrand et al., 2013), bronchial smooth muscle cells (Tan et al., 2012), nucleus pulposus cells (Wang et al., 2011a), endothelial cells (Zhang et al., 1999; Ramnath et al., 2014; Vuong et al., 2015), cardiac myocytes and cardiac fibroblasts (Strand et al., 2013). However, the effects of TNF and IL-1β on syndecan 4 expression in VSMCs are not known; it is also not known whether syndecan 4 regulates the inflammatory responses associated with vascular calcification.

The studies described in this chapter will determine whether syndecan 4 regulates VSMC mineralisation by addressing the following aims:

1) Determine the effects of knocking-down syndecan 4 expression on VSMC mineralisation.
2) Generate a VSMC cell line stably over-expressing syndecan 4.
3) Determine the effects of over-expressing syndecan 4 on VSMC mineralisation.
4) Elucidate the effect of a range of inflammatory mediators on syndecan 4 expression in VSMCs.
5) Examine effects of knocking-down syndecan 4 expression on the induction of calcification by TNF and TGF-β1.
5.2 Results

5.2.1 Investigating the effects of knocking-down syndecan 4 expression on VSMC mineralisation

To determine the role of syndecan 4 in VSMC mineralisation, a bovine-specific syndecan 4 siRNA (siRNA-1) was used to knock-down syndecan 4 expression in VSMC-A, which was verified at the mRNA (~91% knock-down, P<0.001; Figure 5.1A) and protein level (Figure 5.1B). A random negative control siRNA was used as a control. VSMC-A were transfected with negative control siRNA or syndecan 4 siRNA-1 and cultured with 3 mM β-GP for up to 12 days to induce calcification (Figure 5.2A). Negative control siRNA-treated VSMC-A cultured without 3 mM β-GP were used as an additional control (Figure 5.2A). Knocking-down syndecan 4 expression with siRNA-1 significantly increased (~2-fold, P<0.01; Figure 5.2B) the later stages of (stage 4 and 5) of VSMC mineralisation when compared to negative control siRNA-treated cells cultured with β-GP.

Figure 5.1. Confirming knock-down of syndecan 4 with syndecan 4 siRNA-1. VSMC-A were transfected with negative control or syndecan 4 siRNA-1 at a final concentration of 20 nM. Knock-down of syndecan 4 expression using siRNA-1 was confirmed using (A) qPCR and (B) western blotting. In (A), syndecan 4 mRNA expression is shown relative to the reference genes, RPL12 and PPIA. Results displayed as mean ± SEM and were derived from 3 wells of cells from 3 independent experiments. The data were analysed using an unpaired t-test. *** P<0.001.
Figure 5.2. Syndecan 4 siRNA-1 increases VSMC mineralisation. VSMC-A transfected with negative control siRNA or syndecan 4 siRNA-1 were cultured with 3 mM β-GP for up to 12 days. Negative control siRNA-treated cells cultured without β-GP were used as an additional control. Both siRNAs were used at a final concentration of 20 nM. siRNA transfections and medium changes were carried out every 48-72 hours. (A) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (B) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 8 wells of cells from 4 independent experiments. The data were analysed using a 2-way ANOVA with Sidak post-hoc tests. ** P<0.01.
Some redundancy exists between the four syndecan genes, and changes in the expression levels of one syndecan gene may be compensated for by another syndecan (Bertrand et al., 2013). Therefore, to determine whether knocking-down syndecan 4 expression with siRNA-1 had any effect on the mRNA expression levels of syndecans 1-3, VSMC-A were transfected with negative control siRNA or syndecan 4 siRNA-1 and the expression levels of syndecans 1-3 were determined by qPCR at 6, 24, 48 and 96 hours after the first siRNA transfection. Knocking-down syndecan 4 expression with siRNA-1 had no significant effect on the mRNA expression levels of syndecans 1, 2 and 3 when compared to negative control siRNA-treated cells (Figure 5.3).

![Graph showing mRNA expression levels of syndecans 1, 2, and 3](image)

**Figure 5.3.** Syndecan 4 siRNA-1 has no effect on the mRNA expression levels of syndecan 1, 2 or 3 in VSMCs. VSMC-A were transfected twice with negative control or syndecan 4 siRNA-1, with 48 hours between transfections. Both siRNAs were used at a final concentration of 20 nM. RNA was isolated from cells at 6, 24, 48 and 96 hours after the first siRNA transfection and the mRNA levels of syndecans 1-3 were measured by qPCR. Expression is shown relative to the reference genes, RPL12 and PPIA. Results displayed as mean ± SEM and were derived from 3 wells of cells from 3 independent experiments. The data were analysed using a 2-way ANOVA with Sidak post-hoc tests.

It has been reported that siRNAs can have off-target effects (Birmingham et al., 2006), therefore, a second siRNA (syndecan 4 siRNA-2) that targets a different region of the syndecan 4 open reading frame was also used in these studies (Figure 5.4). Using syndecan 4 siRNA-2 at the same concentration as siRNA-1 (20 nM), syndecan 4 mRNA was knocked down by 70% when compared to negative control siRNA-treated cells (data not shown). Given the comparatively poor knock-down efficiency of syndecan 4 siRNA-2 at this concentration, this experiment was repeated using 40 nM siRNA-2. Syndecan 4 mRNA expression was knocked down by 85% (P<0.01) with this concentration of syndecan 4 siRNA-2 when compared to
negative control siRNA-treated cells (Figure 5.5A). Both syndecan 4 siRNA-2 and the negative control siRNA were therefore used at 40 nM in all subsequent experiments.

VSMC-A were transfected with negative control siRNA or syndecan 4 siRNA-2 and cultured with 3 mM β-GP for up to 12 days to induce calcification (Figure 5.5B). Negative control siRNA-treated VSMC-A cultured without 3 mM β-GP were used as an additional control (Figure 5.5B). Knocking-down syndecan 4 expression with siRNA-2 significantly increased (~2-fold, P<0.01; Figure 5.5C) stage 4/5 of VSMC mineralisation when compared to negative control siRNA-treated cells cultured with β-GP.

Figure 5.4. Syndecan 4 siRNA-1 and siRNA-2 target different regions of the bovine syndecan 4 ORF. Bovine syndecan 4 mRNA sequence is shown in grey. Syndecan 4 siRNA-1 (red) and syndecan 4 siRNA-2 (blue) target different regions of the syndecan 4 sequence.
Figure 5.5. Syndecan 4 siRNA-2 increases VSMC mineralisation. VSMC-A were transfected with negative control or syndecan 4 siRNA-2 at a final concentration of 40 nM. (A) Knock-down of syndecan 4 expression using siRNA-2 was confirmed using qPCR. mRNA expression is shown relative to the reference genes, RPL12 and PPIA. Results displayed as mean ± SEM and were derived from 3 wells of cells from 3 independent experiments. The data were analysed using an unpaired t-test. ** P<0.01. (B) VSMC-A transfected with negative control siRNA or syndecan 4 siRNA-2 were cultured with 3 mM β-GP for up to 12 days. Negative control siRNA-treated cells cultured without β-GP were used as an additional control. siRNA transfections and medium changes were carried out every 48-72 hours. Cells were stained with alizarin red. Scale bar = 500 µm. (C) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM (n=8 wells of cells from 4 independent experiments). The data were analysed using a 2-way ANOVA with Sidak post-hoc tests. ** P<0.01.
5.2.2 Investigating the effects of knocking-down syndecan 4 expression in non-mineralising VSMCs

Knocking-down syndecan 4 expression with two separate siRNAs increases mineralisation in a preparation of VSMCs that mineralise in the presence of β-GP (Figures 5.2 and 5.5). To further investigate the role of syndecan 4 in VSMC mineralisation, syndecan 4 expression was knocked-down in the non-mineralising, nodule forming VSMC-B using syndecan 4 siRNA-1. Knock-down of syndecan 4 expression in VSMC-B using siRNA-1 was confirmed at the mRNA level using qPCR (~87% knock-down, Figure 5.6A). In the presence of β-GP, syndecan 4 siRNA-1 did not induce mineralisation in VSMC-B (Figure 5.6B).

![Figure 5.6. Syndecan 4 siRNA-1 does not induce mineralisation in VSMC-B. (A) VSMC-B were transfected twice with negative control or syndecan 4 siRNA-1, with 48 hours between transfections. Both siRNAs were used at a final concentration of 20 nM. Forty-eight hours after the 2nd transfection, RNA was isolated from the cells and syndecan 4 mRNA expression was measured by qPCR. Expression is shown relative to the reference genes, RPL12 and PPIA (n=1). (B) VSMC-B were transfected with negative control siRNA or syndecan 4 siRNA-1 were cultured with 5 mM β-GP for 12 days. Both siRNA's were used at 20 nM. siRNA transfections and medium changes were carried out every 48-72 hours. Phase-contrast images of cells stained with alizarin red after 12 days. Scale bar = 500 µm. This experiment was performed once.](image-url)
5.2.3 Generation of a syndecan 4 over-expression vector

Knocking-down syndecan 4 expression using two separate siRNAs increases VSMC mineralisation (Figures 5.2 and 5.5), but it is not able to induce mineralisation in a preparation of VSMCs that do not mineralise in the presence of β-GP (Figure 5.6). To determine the effects of over-expressing syndecan 4 on VSMC mineralisation, an over-expression lentiviral construct was generated. The procedure used to generate the construct is detailed in section 2.6. In brief, NheI and BamHI restriction sites were cloned onto the 5’ and 3’ ends, respectively, of the full-length human syndecan 4 gene containing an extracellular HA-tag (a kind gift from Dr Mark Morgan, University of Liverpool) using PCR. The syndecan 4 sequence was amplified without the stop codon to allow the elongation factor 1α (EF1) promoter to drive the expression of the syndecan 4 gene and run directly into a self-cleaving T2A peptide and puromycin-resistance gene in the new recipient vector (pCDH-EF1-MCS-T2A-puro). The PCR product was separated by gel electrophoresis, and a single band of the predicted size (~639 bp) was observed (Figure 5.7). The PCR product was then excised and the DNA purified as described in section 2.6.1.

The syndecan 4 PCR product and the new recipient vector (pCDH-EF1-MCS-T2A-puro) were digested with NheI and BamHI to generate ‘sticky’ ends, followed by ligation of the PCR product into the corresponding sites of the digested pCDH vector. The ligation mixture was then transformed into E-coli (XL-1 blue competent cells); 5 individual colonies were collected the next day and expanded in LB broth cultures overnight. Plasmid DNA was purified as described in section 2.6.5. A negative control ligation reaction was set up with no syndecan 4 insert. No colonies were observed on the negative control plate, suggesting that no uncut or self-ligated pCDH-EF1-MCS-T2A-puro vector was present in the ligation reactions.
To confirm the syndecan 4 insert had been incorporated into the pCDH vector, the purified plasmid DNA was digested with NheI and BamHI and the resulting products separated by gel electrophoresis. Two bands were observed in all 5 colonies analysed (Figure 5.8). The band at ~700 bp corresponds to the syndecan 4 insert (expected fragment size = 637 bp), while the larger band at 8000 bp corresponds to the digested pCDH vector (expected fragment size = 7079 bp). To further verify that the syndecan 4 insert had correctly incorporated into the pCDH vector, the purified plasmid DNA was sequenced as described in section 2.6.6. No mutations were observed in any of the 5 colonies analysed (data not shown). Three purified syndecan 4 over-expression (pCDH_SDC4) plasmid DNA preps (2, 3 and 4) were selected to be expanded and generate lentivirus using HEK293Ts.

![Figure 5.8. Restriction enzyme digest of plasmid DNA.](image)

Figure 5.8. Restriction enzyme digest of plasmid DNA. Plasmid DNA samples were digested with NheI and BamHI restriction enzymes for 2 hours at 37°C and then run on a 1% (w/v) agarose gel. Hyperladder 1 was also run on the gel as a molecular weight marker (not shown). 1:1 and 1:3 represent the vector:insert ratio used during the vector/insert ligation. 3 colonies were analysed from the 1:1 vector:insert ratio, and 2 colonies were analysed from 1:3 vector:insert ratio. The expected sizes of the digested pCDH vector and syndecan 4 insert was 7079 bp and 637 bp, respectively.

Initial experiments sought to confirm that the syndecan 4 over-expression vector generated in this study could be used to produce lentivirus and to generate a HEK293T cell line stably over-expressing syndecan 4. HEK293Ts were selected for use in these pilot studies as they are a commonly used cell line for lentivirus transfections. In brief, three preparations of syndecan 4 over-expression lentivirus were generated from pCDH_SDC4 vector 2, 3 or 4 as described in section 2.6.9. Control lentivirus was generated from the pCDH_empty vector. HEK293Ts were grown to ~30% confluence and transduced with control or syndecan 4 over-expression lentivirus overnight. After this time, the culture medium was replaced and the cells were cultured until confluent. The pCDH vector contains a puromycin resistance gene which allows transduced cells to be selected. All HEK293Ts not treated with lentivirus were killed after 3 days in culture with 3 µg/ml puromycin dihydrochloride (Figure 5.9A). No cell death was observed in HEK293Ts
treated with control or syndecan 4 over-expression lentivirus (Figure 5.9A). Using antibodies against syndecan 4 (5G9, Santa-Cruz) and the HA-tag, western blotting confirmed that cells transduced with syndecan 4 over-expression lentivirus were over-expressing syndecan 4 when compared to control lentivirus-treated cells (Figure 5.9B). As the highest syndecan 4 over-expression was achieved in cells treated with syndecan 4 over-expression (vector 3) lentivirus (Figure 5.9B), the syndecan 4 over-expression lentivirus was generated from pCDH_SDC4 vector (3) for use in subsequent studies with VSMCs.

5.2.4 Generation of a VSMC cell line stably over-expressing syndecan 4 using lentivirus

To generate a VSMC cell line stably over-expressing syndecan 4, VSMC-A (mineralising VSMC prep, see Figure 3.1) were seeded into 6-well plates and incubated with control or syndecan 4 over-expression lentivirus overnight. To increase transduction efficiency, the cells were transduced again 24 hours later with either control or syndecan 4 over-expression lentivirus overnight. The volume of lentivirus used to transduce VSMC-A is shown in Table 2.4. Forty-eight hours after the 2nd lentivirus infection, cells not treated with lentivirus, as well as control lentivirus-treated cells, had reached confluence (Figure 5.10A, top panel). Whilst some cell death was observed in VSMCs exposed to one round of infection with the syndecan 4 over-expression lentivirus (SDC4 o/e-1 VSMC-A), the extent of cell death was very high in cells exposed to two separate rounds of syndecan 4 over-expression lentivirus infection (SDC4 o/e-2 VSMC-A and SDC4 o/e-3 VSMC-A; Figure 5.10A, lower panel). Consequently, SDC4 o/e-2 VSMC-A and SDC4 o/e-3 VSMC-A could not be further cultured in these experiments.

To select for transduced cells, control lentivirus (Ctrl-1, Ctrl-2, Ctrl-3) and syndecan 4 over-expression lentivirus (SDC4 o/e-1) VSMCs were cultured with 3 μg/ml puromycin dihydrochloride for 5 days. All cells not treated with lentivirus were killed after 5 days in culture with 3 μg/ml puromycin dihydrochloride (Figure 5.10B). In contrast, no cell death was observed in Ctrl-1, Ctrl-2, Ctrl-3 or SDC4 o/e-1 VSMC-A (Figure 5.10B). To determine if syndecan 4 was over-expressed by SDC4 o/e-1 VSMC-A, semi-quantitative RT-PCR and western blotting were used. The full-length human syndecan 4 gene containing an extracellular HA-tag was detected in SDC4 o/e-1 VSMC-A at the mRNA level (Figure 5.11A). The PCR product was sequenced (as detailed in section 2.6.11), which confirmed the human syndecan 4 gene with extracellular HA-tag was expressed with no mutations (Figure 5.11B). As expected, no mRNA expression of the human syndecan 4 gene with extracellular HA-tag was detected in any of the control lentivirus-treated VSMCs using semi-quantitative RT-PCR (Figure 5.11B).

Using antibodies against syndecan 4 (5G9, Santa-Cruz) and the HA-tag, western blotting confirmed that SDC4 o/e-1 VSMC-A were over-expressing the human syndecan 4 protein when compared to control lentivirus-treated cells (Ctrl-1, Ctrl-2 and Ctrl-3 VSMC-A) (Figure 5.11C). The HA-tag antibody did not react with protein isolated from any of the three lines of control lentivirus-treated VSMCs (Figure 5.11C).
Figure 5.9. Syndecan 4 can be over-expressed in HEK293Ts using lentivirus. Three preparations of syndecan 4 over-expression lentivirus were generated from pCDH_SDC4 vector 2, 3 or 4 as described in the Materials and Methods (section 2.6.9). Control lentivirus was generated from the pCDH empty vector. HEK293Ts were grown to ~30% confluence and transduced with control lentivirus (Ctrl) or syndecan 4 over-expression (SDC4 o/e) lentivirus (2, 3 or 4) overnight. Cells not transduced with lentivirus (untreated) were used as a control. After this time, the medium was replaced and the cells were cultured until ~80-90% confluent. (A) Transduced cells were selected with 3 µg/ml puromycin for 3 days. Cells not transduced with lentivirus (untreated) were used as a positive control to confirm when all cells not transduced with lentivirus were killed. Scale bar = 500 µm. (B) Protein lysates (25 µg) were collected from puromycin-resistant cells and syndecan 4 over-expression was confirmed using antibodies raised against syndecan 4 (Santa Cruz, 5G9) and the extracellular HA-tag. Blots were stripped and re-probed for β-actin to confirm equal protein loading.
Figure 5.10. Infecting VSMCs with syndecan 4 over-expression lentivirus results in substantial cell death. VSMC-A were seeded into 6-well plates at 2 X 10^4 cells/cm^2 with either control (Ctrl-1, -2 or -3) or syndecan 4 over-expression (SDC4 o/e-1) lentivirus (generated from the pCDH_empty and pCDH_SDC4 vector 3, respectively) and incubated overnight. Cells not treated with lentivirus were used as a control (untreated). After 24 hours, fresh 10% FCS-DMEM was added. After a further 24 hours, VSMC-A were transduced for a second time with either control or syndecan 4 over-expression lentivirus for 24 hours. The volume of lentivirus added to cells is detailed in Table 2.4. (A) Phase-contrast images of cells 48 hours after the 2nd lentivirus infection. Scale bar = 500 µm. Cells were passaged and seeded at 2.2 X 10^4 cells/cm^2. (B) Transduced cells were selected with 3 µg/ml puromycin for 5 days. Cells not transduced with lentivirus (untreated) were used as a positive control to confirm when all cells not transduced with lentivirus were killed. Scale bar = 500 µm.
Figure 5.11. Syndecan 4 can be over-expressed in VSMC-A using lentivirus. (A) RNA was isolated from puromycin-resistant Ctrl-1, -2, -3 or SDC4 o/e-1 lentivirus VSMCs-A. PCR was used to confirm the full-length human syndecan 4 gene with HA-tag was expressed by puromycin-resistant SDC4 o/e-1 VSMC-A. RNA isolated from HEK293Ts over-expressing syndecan 4 was used as a positive control. The resulting PCR products were separated on a 1% (w/v) agarose gel using electrophoresis. Hyperladder 1 was run alongside the PCR products as a molecular weight marker (not shown). The expected size of the PCR product was 639 bp. (B) The PCR product from SDC4 o/e-1 VSMC-A was extracted from the agarose gel and the PCR product was sequenced. The generated sequence was compared against the human syndecan 4 gene with extracellular HA-tag using Multalin software (http://multalin.toulouse.inra.fr/multalin/). (C) Protein lysates (50 µg) were collected from puromycin-resistant Ctrl-1, -2, -3 or SDC4 o/e-1 lentivirus VSMCs-A, and syndecan 4 over-expression was confirmed using antibodies raised against syndecan 4 (Santa Cruz, 5G9) and the extracellular HA-tag. Blots were stripped and re-probed for β-actin to confirm equal protein loading.
5.2.5 Investigating the effects of over-expressing syndecan 4 on VSMC mineralisation

To determine whether over-expressing syndecan 4 in VSMCs modulates matrix mineralisation, passage-matched Ctrl-1 VSMC-A and cells over-expressing syndecan 4 (SDC4 o/e-1 VSMC-A) were cultured in the presence of 5 mM β-GP\(^1\) for up to 12 days to induce mineralisation. Ctrl-1 VSMC-A and SDC4 o/e-1 VSMC-A were chosen as they both had a single round of lentivirus infection. During the expansion of lentivirus-treated VSMCs, cells over-expressing syndecan 4 grew at a slightly slower rate than control lentivirus-treated cells (Figure 5.12A). Therefore, SDC4 o/e-1 VSMC-A were seeded at a higher plating density than Ctrl-1 VSMC-A (as detailed in the legend to Figure 5.12B) to ensure cells had reached the same confluency before the addition of 5 mM β-GP to the culture medium.

VSMC-A do not normally form ridges with time in culture, but in cells over-expressing syndecan 4, β-GP-induced mineralisation was localised to ridges (Figure 5.12B). In contrast, mineralisation occurred in a more diffuse and widespread pattern in Ctrl-1 VSMC-A (Figure 5.12B). Over-expressing syndecan 4 in VSMC-A significantly increased (~2-fold increase, \(P<0.001\); Figure 5.12C) β-GP-induced matrix mineralisation when compared to control lentivirus-treated cells.

Another preparation of control (Ctrl-4) and syndecan 4 over-expressing VSMCs-A (SDC4 o/e-4) were generated using lentivirus on a separate occasion. Syndecan 4 over-expression in SDC4 o/e-4 was confirmed at the mRNA (Figure 5.13A) and protein level (Figure 5.13B). β-GP-induced matrix mineralisation was increased in VSMCs over-expressing syndecan 4 when compared to control lentivirus-treated cells (Figure 5.13C&D).

Together these studies demonstrate that over-expressing syndecan 4 in VSMCs may slow cell proliferation, but increases matrix mineralisation when compared to control lentivirus-treated VSMCs.

\(^1\) A higher concentration of β-GP was used in these studies as preliminary studies indicated that mineralisation was delayed in control lentivirus-treated VSMCs when compared to passage-matched untreated cells (data not shown).
Figure 5.12. Over-expressing syndecan 4 in VSMC-A reduces cell proliferation but increases matrix mineralisation. (A) Cell proliferation in passage-matched control lentivirus (Ctrl-1, Ctrl-2 and Ctrl-3 VSMC-A) and syndecan 4 over-expression lentivirus-treated VSMC-A (SDC4 o/e-1) was analysed by seeding cells at $0.9 \times 10^4$ cells/cm$^2$ into 25 cm$^2$ tissue culture flasks. Cells were trypsinised and counted 5 days after plating using the Countess™ Automated Cell Counter. Results displayed as mean ± SD and were derived from duplicate counts (technical repeats) of each cell type. Data are representative of 2 independent experiments. (B) Passage-matched confluent Ctrl-1 VSMC-A and SDC4 o/e-1 VSMC-A were seeded at $2 \times 10^4$ cells/cm$^2$ and $3 \times 10^4$ cells/cm$^2$, respectively. After 5 days when the cells had reached confluence, cells were cultured with 5 mM β-GP for up to 12 days. Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (C) The alizarin red stain was eluted and quantified by absorbance at 405 nm. Results displayed as mean ± SEM and were derived from 6 (Ctrl-1) or 5 (SDC4 o/e-1) wells of cells from 3 independent experiments. The data were analysed using an unpaired t-test. *** P<0.001. A higher concentration of β-GP was used in these studies as it was noted that mineralisation was delayed in control lentivirus-treated VSMCs when compared to untreated cells (data not shown).
Figure 5.13. VSMC mineralisation is increased in a second preparation of VSMCs over-expressing syndecan 4. VSMC-A were seeded into 6-well plates at 2 \times 10^4 cells/cm^2 with either control or syndecan 4 over-expression lentivirus (generated from the pCDH_empty and pCDH_SDC4 vector, respectively) and incubated overnight. After 24 hours, fresh 10% FCS-DMEM was added. After a further 24 hours, VSMC-A were transduced for a second time with either control or syndecan 4 over-expression lentivirus for 24 hours. (A) RNA was isolated from control lentivirus (Ctrl-4) and syndecan 4 over-expression lentivirus (SDC4 o/e-4) VSMC-A. PCR was used to confirm the full-length human syndecan 4 gene with HA-tag was expressed by puromycin-resistant syndecan 4 over-expression VSMC-A. The resulting PCR product was separated on a 1% (w/v) agarose gel using electrophoresis. Hyperladder 1 was run alongside the PCR products as a molecular weight marker (not shown). (B) Protein lysates (20 µg) were collected from puromycin-resistant Ctrl-4 or SDC4 o/e-4 lentivirus VSMCs-A, and syndecan 4 over-expression was confirmed using an antibody against the HA-tag. Blots were stripped and re-probed for β-actin to confirm equal protein loading. (C) Passage-matched confluent Ctrl-4 VSMC-A and SDC4 o/e-4 VSMC-A were cultured with 5 mM β-GP for 12 days. Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (D) The extent of mineralisation was quantified by elution of the alizarin red stain. This experiment was performed once.
5.2.6 Determining the effects of TNF, IL-1β and TGF-β1 on syndecans 1-4 mRNA expression in VSMCs

Syndecan 4 mRNA expression is regulated by TNF and IL-1β in a number of cell types (Zhang et al., 1999; Wang et al., 2011a; Tan et al., 2012; Strand et al., 2013; Bertrand et al., 2013; Ramnath et al., 2014; Vuong et al., 2015), but the effects of these inflammatory mediators on syndecan 4 expression in VSMCs have not been determined. Therefore, confluent VSMC-D (a different preparation of VSMCs which also undergo osteogenic differentiation and mineralisation in the presence of β-GP (Taylor, 2014)) were incubated with 10 ng/ml TNF, 10 ng/ml IL-1β or vehicle for 24 or 48 hours. These doses of TNF and IL-1β were selected as they have been previously shown to induce syndecan 4 mRNA expression in other cell types (Tan et al., 2012; Strand et al., 2013; Bertrand et al., 2013; Ramnath et al., 2014). Previous studies have also shown that the pro-calcific growth factor, TGF-β1, has no effect on syndecan 4 mRNA expression in a human umbilical cord endothelial cells (Zhang et al., 1999) and mouse cardiac myocytes and fibroblasts (Strand et al., 2013). Therefore, VSMC-D were also incubated with 10 ng/ml TGF-β1 for 24 or 48 hours. RNA was collected from the cells and the mRNA expression levels of syndecans 1-4 were determined by qPCR and compared with expression levels in vehicle-treated controls at the same time-points.

IL-1β had no effect on syndecan 1, 2, 3 and 4 expression when compared to vehicle-treated controls (Figure 5.14). In contrast, TNF significantly increased syndecan 1 (3-fold increase; P<0.001), syndecan 2 (2-fold increase; P<0.001) and syndecan 4 (~9-fold increase; P<0.001) mRNA expression after 48 hours, but had no effect on syndecan 3 mRNA expression (Figure 5.14).

TGF-β1 significantly increased syndecan 1 (7-fold increase; P<0.01) and syndecan 3 (3-fold increase; P<0.05) mRNA expression in VSMCs after 24 hours when compared to vehicle-treated controls (Figure 5.14). Syndecan 1 and syndecan 3 mRNA expression remained significantly up-regulated in TGF-β1-treated VSMCs after 48 hours (Figure 5.14). TGF-β1 also elicited a significant increase in syndecan 4 mRNA expression (~4-fold; P<0.05) after 24 hours when compared to vehicle-treated controls (Figure 5.14). Syndecan 4 mRNA expression was then down-regulated to the levels observed in vehicle-treated VSMCs at 48 hours. In contrast, TGF-β1 had no effect on syndecan 2 mRNA expression in VSMCs at either time-point (Figure 5.14).
Figure 5.14. Syndecan 4 mRNA expression is regulated by TNF and TGF-β1, but not IL-1β, in VSMCs. VSMC-D were grown to ~90% confluence and treated with TNF, TGF-β1, IL-1β (all used at 10 ng/ml) or vehicle (PBS + 0.1% (w/v) BSA) for up to 48 hours in the presence of 10% (v/v) FCS. Syndecans 1-4 mRNA expression was measured using qPCR. Expression is shown relative to the reference genes, RPL12 and PPIA. Results are displayed as mean ± SEM. This experiment was performed twice in triplicate for 10 ng/ml TNF, 10 ng/ml TGF-β1 and vehicle (both n=6), and once in triplicate for 10 ng/ml IL-1β (n=3). The data were analysed using a 2-way ANOVA with Sidak post-hoc tests. * P<0.05; ** P<0.01; *** P<0.001 compared to the vehicle control at the same time-point.
Together, these data demonstrate that syndecan 4 mRNA expression is regulated by TNF and TGF-β1, but not IL-1β, in VSMCs. These changes in syndecan 4 mRNA expression could not be correlated to changes in protein in these studies. Whist a syndecan 4 antibody (5G9, Santa Cruz) was used to detect endogenous syndecan 4 in VSMC-A (see Figure 3.6), a different lot number of this antibody was used in these experiments which had much poorer sensitivity for the detection of syndecan 4 in these cells. Different blocking agents (e.g. milk, BSA) and antibody dilutions (1:50-1:500) were used to optimise this new lot of syndecan 4 antibody in VSMC-A, but this could not be achieved within the timeframe of this project. The use of different syndecan 4 antibodies or different protein detection methods (e.g. mass spectrometry) would be advantageous in future studies to determine if TNF- and TGF-β1-induced changes in syndecan 4 mRNA expression correlate to changes in protein. Indeed, with a few exceptions (Wang et al., 2011a; Tan et al., 2012; Strand et al., 2013), the majority of studies have examined the effects of TNF, TGF-β1 and IL-1β on syndecan 4 mRNA expression only.

5.2.7 Investigating the effects of knocking-down syndecan 4 on VSMC mineralisation in the presence of TNF and TGF-β1

TNF (Tintut et al., 2000; Masuda et al., 2013) and TGF-β1 (Kanno et al., 2008; Wang et al., 2010) accelerate VSMC mineralisation, and they can both stimulate syndecan 4 mRNA expression in VSMCs (Figure 5.14). To determine if syndecan 4 mediates VSMC mineralisation induced by TNF or TGF-β1, VSMC-A were transfected with syndecan 4 siRNA-1 or negative control siRNA and cultured with 3 mM β-GP and TNF (1 or 10 ng/ml) or TGF-β1 (10 ng/ml). Negative control siRNA-treated VSMC-A cultured with 3 mM β-GP and vehicle were used as an additional control.

In the presence of β-GP, 10 ng/ml TNF accelerated VSMC mineralisation in negative control siRNA-treated VSMC-A when compared to vehicle-treated negative control siRNA-treated cells cultured with β-GP (Figure 5.15Ai). TNF did not stimulate VSMC mineralisation in the absence of β-GP (data not shown). The extent of mineralisation was similar in negative control siRNA- and syndecan 4 siRNA-1-treated VSMC-A cultured with β-GP and 10 ng/ml TNF (Figure 5.15Aii). As TNF is a potent accelerator of VSMC mineralisation it is possible that the effects of knocking-down syndecan 4 expression during this process could not be observed at this high dose of TNF. Therefore, in the next series of experiments, negative control siRNA-treated VSMC-A were cultured with β-GP and 1 ng/ml TNF. In the presence of β-GP, 1 ng/ml TNF also accelerated VSMC mineralisation in negative control siRNA-treated VSMC-A when compared to the vehicle control (Figure 5.15Bi). Knocking-down syndecan 4 expression with siRNA-1 slightly increased VSMC mineralisation in the presence of β-GP and 1 ng/ml TNF when compared to negative control siRNA-treated cells cultured with β-GP and 1 ng/ml TNF (Figure 5.15Bii). However, when this data was combined from two independent experiments, this difference did not reach statistical significance (Figure 5.15Bii).
Figure 5.15. The effects of TNF on VSMC mineralisation in the absence of syndecan 4. (A) VSMC-A were transfected twice with negative control or syndecan 4 siRNA-1, with 48 hours between transfections. Both siRNAs were used at a final concentration of 20 nM. Forty-eight hours after the 2nd transfection, negative control-treated VSMC-A were cultured with 3 mM β-GP and vehicle (PBS with 0.1% (w/v) BSA) or 10 ng/ml TNF. Syndecan 4 siRNA-1-treated VSMC-A were cultured with 3 mM β-GP and 10 ng/ml TNF. siRNA transfections and medium changes were carried out every 48-72 hours. (Ai) After 4 days, cells were stained with alizarin red. Scale bar = 500 µm. (Aii) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 3 wells of cells from 2 independent experiments. The data were analysed using a 1-way ANOVA with Tukey post-hoc tests. (B) VSMC-A were transfected as described in (A). Forty eight hours after the 2nd transfection, negative control-treated VSMC-A were cultured with 3 mM β-GP and vehicle (PBS with 0.1% (w/v) BSA) or 1 ng/ml TNF. Syndecan 4 siRNA-1-treated VSMC-A were cultured with 3 mM β-GP and 1 ng/ml TNF. (Bi) After 4 days, cells were stained with alizarin red. Scale bar = 500 µm. (Bii) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SD and were derived from 2 independent experiments.
In the presence of β-GP, 10 ng/ml TGF-β1 slightly accelerated VSMC mineralisation in negative control siRNA-treated VSMC-A when compared to vehicle-treated negative control siRNA-treated cells (Figure 5.16A-B). TGF-β1 did not stimulate VSMC mineralisation in the absence of β-GP (data not shown). In the presence of β-GP and TGF-β1, knocking-down syndecan 4 expression with siRNA-1 significantly increased VSMC mineralisation when compared to negative control siRNA-treated cells also cultured with β-GP and TGF-β1 (2-fold increase, P<0.05; Figure 5.16A&B).

Together, these data demonstrate that syndecan 4 regulates VSMC mineralisation both in the presence of β-GP and the pro-calcific factor, TGF-β1. However, knocking-down syndecan 4 expression does not appear to affect the ability of TNF to accelerate VSMC mineralisation.

![Figure 5.16. The effects of TGF-β1 on VSMC mineralisation in the absence of syndecan 4. VSMC-A were transfected twice with negative control or syndecan 4 siRNA-1, with 48 hours between transfections. Both siRNAs were used at a final concentration of 20 nM. Forty-eight hours after the 2nd transfection, negative control-treated VSMC-A were cultured with 3 mM β-GP and vehicle (PBS with 0.1% (w/v) BSA) or 10 ng/ml TGF-β1. Syndecan 4 siRNA-1-treated VSMC-A were cultured with 3 mM β-GP and 10 ng/ml TGF-β1. siRNA transfections and medium changes were carried out every 48-72 hours. (A) After 5 days, cells were stained with alizarin red. Scale bar = 500 µm. (B) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 5 wells of cells from 2 independent experiments. The data were analysed using a 1-way ANOVA with Tukey post-hoc tests. * P<0.05.](image)
5.2.8 Investigating the role of syndecan 4 in TGF-β1 signalling

Syndecan 4 can interact with a variety of ligands through its GAG chains, and syndecan 4 therefore has the potential to regulate the signalling of a variety of factors that accelerate VSMC mineralisation, such as TGF-β1 (Kanno et al., 2008; Wang et al., 2010). As knocking down syndecan 4 expression increased VSMC mineralisation induced by β-GP (Figures 5.2 and 5.5), and by β-GP and TGF-β1 (Figure 5.16), one could hypothesise that knocking-down syndecan 4 expression may modulate TGF-β1 signalling in VSMCs leading to increased matrix mineralisation. Therefore, to determine if knocking-down syndecan 4 expression in VSMCs modulates TGF-β1 signalling, VSMC-A were transfected with negative control siRNA or syndecan 4 siRNA-1 for up to 5 days. Cells were serum-starved for 2 hours and then incubated with vehicle or 10 ng/ml TGF-β1 for 15 minutes. Protein lysates were also collected from cells before the addition of vehicle or TGF-β1 ('0 hours').

Smad2 is phosphorylated down-stream of TGF-β1 receptor activation (Ross and Hill, 2008). In negative control siRNA-treated VSMC-A, TGF-β1-induced phosphorylation of Smad2 (Figure 5.17). Smad2 was not phosphorylated in negative control siRNA-treated cells in the presence of vehicle alone (Figure 5.17). No difference was detected in the level of TGF-β1-induced Smad2 phosphorylation in negative control siRNA- and syndecan 4 siRNA-1-treated VSMC-A (Figure 5.17).

Figure 5.17. Investigating the effects of knocking-down syndecan 4 expression on TGF-β1 signalling in VSMCs. VSMC-A were transfected twice with negative control or syndecan 4 siRNA-1, with 48 hours between transfections. Both siRNAs were used at a final concentration of 20 nM. Forty-eight hours after the 2nd transfection, cells were serum-starved for 2 hours and stimulated with vehicle (PBS with 0.1% (w/v) BSA) or TGF-β1 (10 ng/ml) for 15 minutes. Protein lysates were also collected from cells before the addition of vehicle or TGF-β1 ('0 hours'). (A) Phosphorylation of Smad2 and expression of total Smad2 was assessed by western blotting (25 µg protein loaded per lane). β-actin was used as a loading control. Blots are representative of 2 independent experiments.
5.3 Discussion
The results presented in this chapter demonstrate for the first time that knocking-down syndecan 4 expression using siRNA increases VSMC mineralisation. In contrast, knocking-down syndecan 4 expression in a preparation of VSMCs which do not mineralise in the presence of β-GP does not stimulate the deposition of a mineralised matrix by these cells, suggesting that syndecan 4 may act as a ‘brake’, rather than a driver of VSMC mineralisation. Over-expressing syndecan 4 in VSMCs also increases β-GP-induced matrix mineralisation, suggesting that the expression of syndecan 4 must be finely balanced to regulate VSMC mineralisation. Finally, syndecan 4 mRNA expression in VSMCs is regulated by TNF and TGF-β1, but not IL-1β. Syndecan 4 also regulates VSMC mineralisation induced by β-GP and TGF-β1, but this is not due to increased TGF-β1 signalling in syndecan 4 knock-down VSMCs.

5.3.1 Modulating syndecan 4 expression in VSMCs regulates matrix mineralisation
Syndecan 4 mRNA expression is increase during the late stages of VSMC mineralisation (Figure 3.5), and knocking-down its expression using 2 different siRNAs significantly increased β-GP-induced VSMC mineralisation when compared to negative-control siRNA-treated cells (Figures 5.2 and 5.5). Whilst knocking-down syndecan 4 expression increased matrix mineralisation in a preparation of mineralising VSMCs (VSMC-A), knocking-down syndecan 4 expression in a preparation of cells which do not mineralise in the presence of β-GP (VSMC-B), had no effect on the ability of these cells to mineralise (Figure 5.6). Importantly, syndecan 4 expression was also not changed when VSMC-B were cultured in the presence of β-GP (Figure 3.8A). Therefore, these data suggest that syndecan 4 may act as a brake on the VSMC mineralisation process, but this may be limited to cells that are already ‘primed’ to undergo osteogenic differentiation and mineralisation.

The role of syndecan 4 in osteogenesis in vivo is somewhat unclear. Bone formation is not altered in syndecan 4 null-mice, although this was confounded by a compensatory increase in syndecan 2 expression during development in these mice (Bertrand et al., 2013). Indeed, syndecan 2 has been postulated to play an important role in osteoblast differentiation independent of the other syndecan genes (discussed in section 3.3.3). Importantly, no compensatory increases were detected in the other syndecan genes when syndecan 4 expression was knocked-down in VSMCs, further supporting the conclusion that the effects of knocking-down syndecan 4 expression in VSMC mineralisation are due to this specific syndecan gene.

Interestingly, knocking-down syndecan 4 expression increases VSMC mineralisation in the presence of β-GP and the same effect was observed when syndecan 4 was over-expressed in VSMCs (Figures 5.12 and 5.13). This result is consistent with several other studies which have reported that similar effects are observed when syndecan 4 expression is knocked-down and over-expressed (Matsui et al., 2011; Astudillo et al., 2014; Baeyens et al., 2014). For example, Wnt3a signalling is enhanced in syndecan 4 siRNA-treated HeLa cells, and this effect was also
observed when syndecan 4 was over-expressed in these cells (Astudillo et al., 2014). The authors of this study speculated that syndecan 4 may exert a dominant-negative effect on endogenous syndecan 4 signalling when it is over-expressed, a hypothesis also proposed by Matsui et al (2011). In the latter study, cardiac rupture and mortality was increased in syndecan 4-null compared to wild-type mice following myocardial infarction, but over-expressing the shed form of syndecan 4 in the hearts of wild-type mice using adenovirus also increased mortality in these animals (Matsui et al., 2011). In another study laminar flow-induced cell alignment was inhibited in syndecan 4-null HUVECs, and this effect could be rescued by the re-expression of syndecan 4 in these cells. However, re-expression of syndecan 4 above endogenous levels (5- to 10-fold increase) in syndecan 4-null HUVECs inhibited laminar flow-induced cell alignment, as observed when syndecan 4 was not present (Baeyens et al., 2014). Importantly, these findings do not appear to be restricted to the syndecan 4 gene, as wound healing is delayed when syndecan 1 expression is both knocked-down or over-expressed in mice (Stepp et al., 2002; Elenius et al., 2004).

In contrast to the studies described above, it has also been reported that knocking-down and over-expressing syndecan 4 in other model systems can elicit opposite effects. For example, the transient receptor potential canonical 6 (TRPC6) channel mediates the entry of calcium into cells, and in the kidney, TRPC6 regulates the glomerular filtration barrier and serve as an important determinant of glomerular permeability (Möller et al., 2007; Krall et al., 2010). siRNA-induced knockdown of syndecan 4 expression in a mouse podocyte cell line reduced the expression levels of TRPC6, and in turn, TRPC6-mediated cation influx and currents in these cells. In contrast, over-expressing full-length syndecan 4 increased TRPC6 expression and TRPC6-mediated cation influx and currents in these cells, although the level of syndecan 4 over-expression was not described (Liu et al., 2012). Syndecan 4 has also been shown to play a role in the liver and syndecan 4 null-mice are more susceptible to concanavalin-A-induced hepatic injury through increased activity of the pro-inflammatory cytokine, osteopontin (Kon et al., 2008). In wild-type mice however, intraperitoneal administration of the extracellular domain of syndecan-4 markedly reduced the development of concanavalin-A-induced hepatic injury, although the serum level and half-life of the administered syndecan 4 IgG in these animal were not described (Kon et al., 2008). Together these studies suggest that the effects of knocking-down and over-expressing/exogenous administration of syndecan 4 are highly dependent on the in vitro and in vivo model used, and in some cases, the expression levels of the syndecan genes.

There are limitations in the over-expression experiments presented in this chapter that must be considered. Firstly, marked cell death was observed following two rounds of infection with the syndecan 4 over-expression lentivirus in VSMC-A (SDC4 o/e-2 and -3). Some cell death was also observed in SDC4 o/e-1 VSMC-A, cells which were exposed to a single round of infection with the syndecan 4 over-expression lentivirus. As no cell death was observed in control lentivirus-treated VSMCs, it did not appear that these cells were sensitive to lentivirus infection. Furthermore, no cell death was observed in HEK293Ts treated with either control or syndecan 4
over-expression lentivirus. These findings could therefore suggest that over-expressing syndecan 4 in VSMCs is detrimental to VSMC viability. Interestingly, marked cell death has also been observed when syndecan 4 was over-expressed in HEK293 EBNA cells using lentivirus (S. A. Cain and C. Kielty, University of Manchester; unpublished findings). Together these results suggest that some cell types are sensitive to syndecan 4 over-expression.

A second limitation of the over-expression experiments detailed in this chapter is that it was not known whether syndecan 4 was appropriately glycosylated in cells over-expressing this PG. Protein lysates from Ctrl-1, Ctrl-2, Ctrl-3 and SDC4 o/e-1 were digested with HS and CS/DS degrading enzymes (chondroitinase ABC, heparinase I, II and III) for 2 hours at 37°C prior to separation by gel electrophoresis. There was no shift in the molecular weight of the protein band detected by the HA-tag antibody following HS and CS/DS degrading enzyme treatment (data not shown), suggesting that syndecan 4 may not be glycosylated. Glycosylation of the syndecan 4 core protein is functionally important as the GAG chains can regulate growth factor signalling, and consistent with this, the GAG chains displayed on decorin are vital to this PGs role in accelerating human VSMC mineralisation in vitro (Yan et al., 2011). Because of these limitations, the mechanism by which syndecan 4 regulates VSMC mineralisation will be investigated in subsequent chapters using siRNA-treated cells only.

Overall these studies suggest that the expression levels of syndecan 4 must be finely balanced to regulate VSMC mineralisation. Furthermore, increases in the level of syndecan 4 above this ‘critical window’ of expression may exert the same effect as when syndecan 4 is not present.

5.3.2 TNF and TGF-β1 regulate syndecan 4 expression in VSMCs, and syndecan 4 regulates TGF-β1-induced VSMC mineralisation

IL-1β has also been reported to induce syndecan 4 mRNA expression in a variety of cell types including bronchial smooth muscle cells (Tan et al., 2012), nucleus pulposus cells (Wang et al., 2011a), and cardiac myocytes and fibroblasts (Strand et al., 2013). However, IL-1β had no effect on syndecan 4 mRNA expression in VSMCs (Figure 5.14). Previous reports have shown that the dose of IL-1β used in this study (10 ng/ml) can induce syndecan 4 mRNA expression in other cell types (Strand et al., 2013), suggesting that this result was not simply a dose-related effect. Furthermore, the IL-1β used in this study was biologically active at this dose as the expression of the secreted inflammatory protein, TNF-stimulated gene 6 (TSG-6), was shown to be up-regulated in IL-1β-treated VSMCs in these experiments (Taylor, 2014). It is therefore possible that the effects of IL-1β on syndecan 4 expression are cell type and/or species-specific.

TNF induces syndecan 4 mRNA expression in VSMCs (Figure 5.14), and consistent with this finding, TNF has been shown to regulate syndecan 4 expression in a number of other cell types (Zhang et al., 1999; Wang et al., 2011a; Tan et al., 2012; Strand et al., 2013; Bertrand et al., 2013; Ramnath et al., 2014). Interestingly syndecan 4 is capable of exerting anti-inflammatory effects in models of pulmonary inflammation (Tanino et al., 2012), myocardial infarction (Xie et al., 2012) and pressure-overload (Strand et al., 2013), therefore, the induction of syndecan 4
expression in response to TNF may represent a negative feedback response to help to dampen the inflammation present.

TGF-β1 accelerates the osteogenic differentiation and mineralisation of rat VSMCs (Wang et al., 2010), and blocking endogenous TGF-β1 signalling attenuates the expression of osteogenic differentiation markers in VSMCs cultured in osteogenic-inducing medium (Kanno et al., 2008; Wang et al., 2010). TGF-β1 is also thought to be, at least in part, responsible for the accelerated mineralisation of human VSMCs in the presence of ox-LDLs (Yan et al., 2011). TGF-β1 elicited a small, but significant, increase in syndecan 4 mRNA expression in VSMCs after 24 hours (Figure 5.14). In contrast to these results, TGF-β1 had no effect on syndecan 4 mRNA expression in HUVECs (Zhang et al., 1999) and mouse cardiac myocytes and fibroblasts (Strand et al., 2013). It is important to note that the results presented in this chapter and Strand et al (2013) analysed syndecan 4 mRNA expression after 24 hours TGF-β1 treatment, and also used the same concentration of TGF-β1 (10 ng/ml). Therefore, like IL-1β, the effects of TGF-β1 on syndecan 4 mRNA expression may be cell type-specific.

In the presence of TGF-β1 and β-GP, knocking-down syndecan 4 expression with siRNA-1 further accelerated VSMC mineralisation when compared to negative control siRNA-treated cells. In contrast, knocking-down syndecan 4 expression using siRNA-1 had no effect on the ability of TNF to accelerate VSMC mineralisation in β-GP-treated cells. TNF is a more potent accelerator of VSMC mineralisation compared to TGF-β1 in this cell system (Figures 5.15 and 5.16), so it is possible that the effects of knocking-down syndecan 4 expression in the presence of TNF could not be observed at the doses used in this study. Alternatively, these findings could suggest that the regulatory role of syndecan 4 in VSMC mineralisation is specific to the pro-calcific stimuli present.

Syndecan 4 has been shown to bind TGF-β1 through its GAG chains (Ishiguro et al., 2001a), thereby protecting TGF-β1 from degradation (Lyon et al., 1997). Consistent with this, active TGF-β1 levels were reduced in the kidneys of syndecan 4-null mice with CKD (Scarpellini et al., 2014). Knocking-down syndecan 4 expression in VSMCs had no effect on TGF-β1-induced Smad2 phosphorylation when compared to negative control siRNA-treated cells (Figure 5.17). These findings could therefore suggest that although syndecan 4 is required for the sequestration of active TGF-β1, it does not appear to be required for TGF-β1-induced Smad2 phosphorylation. Consistent with this, TGF-β1-induced Smad2 phosphorylation was similar between wild-type and syndecan 4-null cardiac fibroblasts (Matsui et al., 2011). As TGF-β1 signalling is not enhanced in syndecan 4 siRNA-1-treated VSMCs, these findings could therefore suggest that syndecan 4 may be regulating VSMC mineralisation by an alternative signalling mechanism. The mechanism by which syndecan 4 regulates VSMC mineralisation will be explored in Chapters 6 and 7.
5.4 Conclusion

The following conclusions can be drawn from the results presented in this chapter:

- Knocking-down syndecan 4 expression increases VSMC mineralisation.
- Over-expressing syndecan 4 using lentivirus also increases VSMC mineralisation, suggesting that syndecan 4 expression must be finely balanced to regulate VSMC mineralisation.
- The pro-calcific factors, TNF and TGF-β1, induce syndecan 4 expression in VSMCs.
- Knocking-down syndecan 4 expression increases VSMC mineralisation induced by TGF-β1, but this cannot be accounted for by changes in TGF-β1 signalling in VSMCs where syndecan 4 expression had been knocked-down.
6 Investigating the signalling pathways regulated by syndecan 4 during VSMC mineralisation

6.1 Introduction
The results presented in Chapters 3 and 5 have demonstrated that syndecan 4 mRNA expression is increased during the late stages of VSMC osteogenic differentiation and mineralisation (Figure 3.5), and knocking-down syndecan 4 expression using siRNA increases VSMC mineralisation (Figures 5.2 and 5.5). Together, these studies suggest that syndecan 4 expression is up-regulated during VSMC osteogenic differentiation and mineralisation to act as a ‘brake’/slow down the mineralisation process.

Previous studies have shown that syndecan 4 regulates FGF-2-induced activation of Akt (Zhang et al., 2003; Partovian et al., 2008; Elfenbein et al., 2012; Ju and Simons, 2013). FGF-2 plays an important role in bone formation, although its role in this process is complex, as it appears to be highly dependent on the timing and duration of FGF-2 signalling. For example, bone formation and mineralisation is reduced in FGF-2-null mice, in addition to cells isolated from these mice (Montero et al., 2000; Naganawa et al., 2006). In vitro, whilst short-term FGF-2 treatment for the first 3-8 days of a 9-14 days osteogenic-inducing protocol stimulates matrix mineralisation in calvarial osteoblasts (Fakhry et al., 2005; Ling et al., 2006) and MSCs (Dombrowski et al., 2009), continuous FGF-2 treatment inhibits mineralisation in these cells (Debiais et al., 1998; Fakhry et al., 2005; Ling et al., 2006; Dombrowski et al., 2009; Biver et al., 2012). Together these studies could suggest that FGF-2 is required initially to promote mineralisation, but then must be down-regulated so mineralisation can proceed.

Whilst short-term FGF-2 treatment (up to 12 hours) stimulates the expression of the osteogenic markers, osteopontin and osteocalcin, in rat VSMCs (Nakahara et al., 2010), the role of FGF-2 in VSMC mineralisation is currently unknown. Therefore, the studies described in this chapter will investigate the role of FGF-2 and syndecan 4 in VSMC mineralisation by addressing the following aims:

1) Characterise the expression of FGF-2 during VSMC osteogenic differentiation and mineralisation.
2) Investigate the role of FGF-2 in VSMC mineralisation.
3) Determine the role of syndecan 4 in regulating FGF-2 signalling in VSMCs.
4) Determine if FGF-2 regulates syndecan 4 expression in VSMCs.
6.2 Results

6.2.1 FGF-2 expression profile during VSMC osteogenic differentiation and mineralisation

The literature lacks a thorough investigation of the expression and role of FGF-2 in VSMC mineralisation. To determine the expression profile of FGF-2 during VSMC osteogenic differentiation and mineralisation, three different preparations of VSMCs were used: VSMC-A undergo osteogenic differentiation and mineralisation in the presence of β-GP (Figure 3.1), whilst VSMC-B (Figure 3.2) and VSMC-C (Figure 3.3) do not undergo osteogenic differentiation or mineralise when cultured with β-GP.

(i) VSMC-A

Confluent VSMC-A were cultured with 3 mM β-GP to induce mineralisation. Controls were cultured without β-GP. RNA was isolated from these cells at specific time-points over the course of the experiment: stage 1 (no mineral deposition), stage 2 (initial formation of mineral deposits), stage 3 (early mineralisation), stage 4 (mid mineralisation) and stage 5 (late mineralisation) (Figure 6.1A). The mRNA expression levels of FGF-2 were then determined by qPCR. FGF-2 expression was significantly increased (~40-fold, P<0.001) in β-GP-treated VSMC-A during the late stages (stage 5) of mineralisation when compared to controls at the same time-point (Figure 6.1A). This increase in FGF-2 mRNA expression during VSMC osteogenic differentiation and mineralisation also coincided with increased syndecan 4 mRNA expression these cells (Figure 6.1A).

To determine if increases in FGF-2 mRNA during the late stages of VSMC osteogenic differentiation and mineralisation corresponded to changes in FGF-2 protein, the protein expression levels of FGF-2 in cell/matrix lysates from control and β-GP-treated VSMC-A were analysed by western blotting (Figure 6.1B&C). FGF-2 exists in several isoforms: low molecular weight (LMW) FGF-2 (18 kDa) and higher molecular weight (HMW) FGF-2 variants (22, 22.5, 24 and 34 kDa). LMW FGF-2 was the predominant FGF-2 variant expressed by VSMCs (Figure 6.1B). Two HMW FGF-2 variants were also detected (although faintly) in VSMCs, which appeared to correspond to the 22/22.5 kDa and 24 kDa FGF-2 variants (Figure 6.1B). LMW FGF-2 was increased in β-GP-treated VSMC-A during the late stages (stage 5) of VSMC osteogenic differentiation and mineralisation when compared to the same time-point control (Figure 6.1B). This was confirmed using densitometry to quantify the LMW FGF-2/β-actin ratio in control and β-GP-treated VSMCs (~2.5-fold increase; Figure 6.1C).
Figure 6.1. FGF-2 expression is increased in mineralising VSMCs. Confluent VSMC-A (day 0) were cultured ± 3 mM β-GP for up to 14 days. (A) RNA was collected from cells at day 0, then at specific stages of the mineralisation process (stages 1-5). The mRNA expression levels of syndecan 4 (reproduced from Figure 3.5) and FGF-2 were measured using qPCR. Expression is shown relative to day 0, which is equal to 1. mRNA expression levels at stages 1 and 2 were combined. Results displayed as mean ± SEM. Stages 1&2 (n=12 samples from 2 independent experiments); Stages 3-5 (n=9 samples from 3 independent experiments). The data were analysed using a 2-way ANOVA with Sidak post-hoc tests. *** P<0.001 against same time-point control. (B) Protein lysates (25 µg) were collected from cells at day 0, then at specific stages of the mineralisation process (stages 3, 4 and 5). The expression of FGF-2 was measured using western blotting. β-actin was used a loading control. Blots are representative of 3 independent experiments. (C) Low-MW FGF-2 (18 kDa) protein expression was quantified using densitometry with ImageJ software. Results displayed as mean ± SEM and were derived from 3 independent experiments.
(ii) VSMC-B and VSMC-C

To determine if these changes in FGF-2 expression during VSMC osteogenic differentiation and mineralisation were due to extended culture times in the presence of β-GP, VSMC-B and VSMC-C were also cultured with 5 mM β-GP for up to 14 days. Controls were cultured without β-GP. RNA was isolated from these cells at days 9, 11 and 14, as these time-points corresponded to the time taken to reach stages 3, 4 and 5 of mineralisation in β-GP-treated VSMC-A. The expression levels of FGF-2 were then measured by qPCR. These studies demonstrated that there was no change in FGF-2 expression in β-GP-treated VSMC-B and VSMC-C when compared to controls at the same time-points (Figure 6.2).

6.2.2 Effects of exogenous FGF-2 on VSMC mineralisation

To determine whether exogenous FGF-2 regulates VSMC mineralisation, VSMC-A were cultured with 3 mM β-GP and vehicle, 25 or 50 ng/ml FGF-2 for up to 12 days. Cells cultured with vehicle alone were used as a control. FGF-2 significantly reduced VSMC mineralisation in a dose-dependent manner when compared to β-GP-treated cells (Figure 6.3A); this decrease was quantified by dye elution (P<0.01, Figure 6.3B).
6.2.3 Investigating the role of endogenous FGF-2 signalling in VSMC mineralisation

(i) Effects of BGJ398 on FGF-2 signalling in VSMCs

Exogenous FGF-2 inhibits VSMC mineralisation (Figure 6.3); whereas endogenous FGF-2 levels increase in mineralising VSMCs (Figure 6.1). Therefore, to determine the role of endogenous FGF-2 signalling in VSMC mineralisation, the pan-FGFR inhibitor BGJ398 was used (Guagnano et al., 2011).

To first confirm BGJ398 inhibits FGF-2 signalling in VSMCs, confluent VSMC-A were cultured with vehicle or BGJ398 (10 nM, 100 nM, 250 nM, 500 nM or 1 µM) for 48 hours. Cells were then serum-starved for 2 hours with vehicle or BGJ-398 (10 nM, 100 nM, 250 nM, 500 nM or 1 µM), and stimulated with FGF-2 (25 or 50 ng/ml) for 5 minutes. Vehicle-treated cells not stimulated with FGF-2 were used as controls. Akt and Erk1/2 are phosphorylated down-stream of FGF-2 receptor activation (see Figure 1.5), therefore, the phosphorylation of these proteins was assessed by western blotting. These studies showed that 25 ng/ml FGF-2 (Figure 6.4A) and 50 ng/ml FGF-2 (Figure 6.4B) increased Akt and Erk1/2 phosphorylation when compared to vehicle-treated cells not stimulated with FGF-2. BGJ398 blocked FGF-2-induced Akt and Erk1/2 phosphorylation in the presence of both 25 ng/ml FGF-2 (Figure 6.4A) and 50 ng/ml FGF-2 (Figure 6.4B). These studies confirm that BGJ398 blocks FGF-2 signalling in VSMCs.

(ii) Effects of BGJ398 on VSMC mineralisation

To determine the effects of BGJ398 in VSMC mineralisation, VSMC-A were cultured with vehicle or BGJ398 (10 nM, 100 nM or 1 µM) ± 3 mM β-GP for up to 12 days. At the highest concentration used in these studies, 1 µM BGJ398 significantly increased VSMC mineralisation by ~3-fold when compared to the vehicle control (P<0.05, Figure 6.5A&B). In the absence of β-GP, BGJ398 (100 nM and 1 µM) did not stimulate mineralisation in VSMC-A (Figure 6.5C).

To confirm that BGJ398 also blocks the inhibitory effects of exogenous FGF-2 on VSMC mineralisation, confluent VSMC-A were cultured with 5 mM β-GP and FGF-2 (25 or 50 ng/ml) ± 1 µM BGJ398 for up to 8 days. Cells cultured with 5 mM β-GP and vehicle were used as a control. A higher concentration of β-GP was used in these studies as pilot studies had indicated that the β-GP powder had lost potency during storage and a similar time-course for VSMC mineralisation was required. BGJ398 (1 µM) significantly increased VSMC mineralisation when compared to the vehicle control (P<0.05, Figure 6.6A&B). FGF-2 reduced VSMC mineralisation, and this effect was lost when cells were co-incubated with BGJ398 (Figure 6.6A&B).
Figure 6.3. Exogenous FGF-2 inhibits VSMC mineralisation. Confluent VSMC-A were cultured ± 3 mM β-GP with vehicle (5 mM TRIS + 0.1% (w/v) BSA), or with 3 mM β-GP and FGF-2 (25 or 50 ng/ml) for up to 12 days. (A) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (B) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 10 wells of cells at stages 3 or 4 of VSMC mineralisation from 3 independent experiments. The data were analysed using a 1-way ANOVA with Dunnet post-hoc tests. ** P<0.01.
Figure 6.4. The FGFR inhibitor, BGJ398, inhibits FGF-2 signalling in VSMCs. Confluent VSMC-A were cultured with vehicle (DMSO) or BGJ398 (10 nM, 100 nM, 250 nM, 500 nM or 1 µM) for 48 hours. Cells were then serum-starved for 2 hours with vehicle (Veh) or BGJ398 (10 nM, 100 nM, 250 nM, 500 nM or 1 µM), and stimulated with (A) 25 ng/ml FGF-2 or, (B) 50 ng/ml for 5 minutes. FGF-2. Vehicle-treated VSMCs not stimulated with FGF-2 were used as a control. Phosphorylation of Akt (pAkt) and Erk1/2 (pErk1/2), and expression of total Akt and total Erk1/2 were assessed by western blotting (25 µg protein loaded per lane). β-actin was used as a loading control. Blots are representative of 2 independent experiments for both concentrations of FGF-2.
Figure 6.5. The FGFR inhibitor, BGJ398, increases VSMC mineralisation. (A-B) Confluent VSMC-A were cultured ± 3 mM β-GP with vehicle (DMSO), or with 3 mM β-GP and BGJ398 (10 nM, 100 nM or 1 µM) for up to 12 days. (A) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (B) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 3 wells of cells from 3 independent experiments. The data were analysed using a 1-way ANOVA with Dunnet post-hoc tests. * P<0.05. (C) Confluent VSMC-A were cultured with BGJ398 (10 nM, 100 nM or 1 µM), or with 3 mM β-GP and vehicle (DMSO) for up to 12 days. Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. Images are representative of 2 independent experiments.
Figure 6.6. BGJ398 blocks the inhibitory effect of exogenous FGF-2 on VSMC mineralisation. Confluent VSMC-A were cultured with 5 mM β-GP and FGF-2 (25 or 50 ng/ml) ± 1 µM BGJ398 for up to 8 days. Cells cultured with 5 mM β-GP and vehicle (DMSO) were used as a control. (A) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (B) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 3 wells of cells from 2 independent experiments. The data were analysed using a 1-way ANOVA with Dunnet post-hoc tests. ns, not significant; * P<0.05. † A higher concentration of β-GP was used in these studies as pilot studies had indicated that the β-GP powder had lost potency during storage and a similar time-course for VSMC mineralisation was required.
In addition to its inhibitory effects on the FGFRs, BGJ398 has been reported to inhibit vascular endothelial growth factor receptor 2 (VEGFR2) signalling (Guagnano et al., 2011). Therefore, the presence of the main VEGFR (VEGFR2) in VSMCs was investigated by western blotting using a VEGFR2 antibody (Cell Signalling, #9698); protein lysates from bovine aortic endothelial cells were used as a positive control. These studies demonstrated that VEGFR2 could not be detected at the protein level in VSMC-A (Figure 6.7). A second antibody (Santa-Cruz, sc-6251) could also not detect VEGFR2 in bovine VSMCs (data not shown). These data suggest that the effects of BGJ398 on VSMC mineralisation are due to its effects on FGF-2 signalling in these cells, and are not due to off-target effects on the VEGFR.

![Figure 6.7. VEGFR2 cannot be detected in VSMCs.](image)

Protein lysates (20 µg) from confluent bovine VSMC-A were probed for VEGR2 by western blotting. Protein lysates (20 µg) from bovine aortic endothelial cells ('Endo') were used as a positive control (kind gift from Thomas Morris, University of Manchester, UK). β-actin was used as a loading control.

6.2.4 Role of syndecan 4 in FGF-2 signalling in VSMCs

Both syndecan 4 and FGF-2 expression are up-regulated during the late stages of VSMC osteogenic differentiation and mineralisation (Figure 6.1). Either knocking-down syndecan 4 expression with siRNA (Figures 5.2 and 5.5), or inhibiting FGF-2 signalling with BGJ398 (Figure 6.5), increases VSMC mineralisation suggesting that syndecan 4 and FGF-2 expression are increased in mineralising VSMCs to put a ‘brake’/slow down the mineralisation process. Previous studies have shown that FGF-induced Akt phosphorylation is reduced in syndecan 4-null endothelial cells (Partovian et al., 2008; Ju and Simons, 2013). Therefore, to determine if syndecan 4 also regulates FGF-2 signalling in VSMCs, VSMC-A were transfected with syndecan 4 siRNA-1 or syndecan 4 siRNA-2 for 6 days and serum-starved for 2 hours. The cells were then incubated with vehicle or FGF-2, and the levels of Akt and Erk1/2 phosphorylation were assessed by western blotting. VSMC-A treated with negative control siRNA were used as controls.
(i) Akt

Pilot studies were first performed to choose the optimal time-point at which Akt is phosphorylated in response to FGF-2. VSMC-A were transfected with negative control siRNA and serum-starved as described above, then stimulated with vehicle or FGF-2 (50 ng/ml) for 5, 15 or 30 minutes. FGF-2-induced Akt phosphorylation was maximal after 5 minutes when compared to vehicle-treated cells (Figure 6.8). This time-point was therefore selected to analyse the effects of knocking-down VSMC syndecan 4 expression on FGF-2-induced Akt phosphorylation.

VSMC-A were transfected with negative control siRNA, syndecan 4 siRNA-1 or syndecan 4 siRNA-2, and serum-starved as described above. Cells were then stimulated with vehicle or increasing concentrations of FGF-2 (25 or 50 ng/ml) for 5 minutes. FGF-2-induced Akt phosphorylation was reduced in syndecan 4 siRNA-1-treated VSMCs when compared to negative control siRNA-treated cells (Figure 6.9A). FGF-2-induced Akt phosphorylation was also reduced in VSMCs when syndecan 4 was knocked-down using syndecan 4 siRNA-2 (Figure 6.9B).

For both syndecan 4 siRNA-1 and siRNA-2, blots shown in Figure 6.9 are representative of 2 independent experiments. However, the variability in Akt phosphorylation between individual experiments, meant that the phosphorylated Akt:total Akt ratios were not significantly different when the syndecan 4 siRNA-treated samples were compared to controls (data not shown).

![Figure 6.8. FGF-2-induced pAKT is maximal after 5 minutes. VSMC-A were transfected with negative control siRNA every 48 hours. Forty-eight hours after the 2nd siRNA transfection, cells were serum-starved for 2 hours and stimulated with vehicle (5 mM TRIS + 0.1% (w/v) BSA) or 50 ng/ml FGF-2 for 5, 15 or 30 minutes. Protein lysates were also collected from cells before the addition of vehicle or FGF-2 (‘0 hours’). Phosphorylation of Akt (pAkt) and expression of total Akt were assessed by western blotting (25 µg protein loaded per lane). β-actin was used as a loading control.](image-url)
**Figure 6.9.** FGF-2-induced pAKT is regulated by syndecan 4 in VSMCs. VSMC-A were transfected with negative control siRNA, (A) syndecan 4 siRNA-1 or, (B) syndecan 4 siRNA-2 every 48 hours. Forty-eight hours after the 2nd siRNA transfection, cells were serum-starved for 2 hours and stimulated with vehicle (5 mM TRIS + 0.1% (w/v) BSA) or FGF-2 (25 or 50 ng/ml) for 5 minutes. Protein lysates were also collected from cells before the addition of vehicle or FGF-2 (‘0 hours’). Phosphorylation of Akt (pAkt) and expression of total Akt were assessed by western blotting (25 µg protein loaded per lane). β-actin was used as a loading control. Blots are representative of 2 independent experiments for both syndecan 4 siRNA-1 and siRNA-2.
(ii) Erk1/2

Erk1/2 is also phosphorylated down-stream of FGF-2 receptor activation (see Figure 1.5). Pilot studies were therefore performed to choose optimal time-point at which Erk1/2 is phosphorylated in response to FGF-2. Negative control siRNA-treated VSMC-A were transfected and serum-starved as described above, then stimulated with vehicle or FGF-2 (50 ng/ml) for 5, 15 or 30 minutes. Erk1/2 phosphorylation was maximal after 15 minutes stimulation with FGF-2 (Figure 6.10). FGF-2-induced Erk1/2 phosphorylation was slightly increased in syndecan 4 siRNA-1-treated VSMCs when compared to FGF-2-stimulated negative control siRNA-treated cells (Figure 6.11A). In contrast, FGF-2-induced Erk1/2 phosphorylation was slightly decreased/unchanged in syndecan 4 siRNA-2 VSMCs when compared to negative control siRNA-treated cells stimulated with FGF-2 (Figure 6.11B).

Taken together, these studies suggest that knocking-down syndecan 4 expression reduces FGF-2-induced Akt activation in VSMCs, but it has variable effects on FGF-2-induced Erk1/2 activation in the same cells.

![Figure 6.10. FGF-2-induced pERK is maximal after 15 minutes. VSMC-A were transfected with negative control siRNA every 48 hours. Forty-eight hours after the 2nd siRNA transfection, cells were serum-starved for 2 hours and stimulated with vehicle (5 mM TRIS + 0.1% (w/v) BSA) or 50 ng/ml FGF-2 for 5, 15 or 30 minutes. Protein lysates were also collected from cells before the addition of vehicle or FGF-2 (‘0 hours’). Phosphorylation of Erk1/2 (pErk1/2) and expression of total Erk1/2 were assessed by western blotting (25 µg protein loaded per lane). β-actin was used as a loading control.](image-url)
Figure 6.11. FGF-2-induced pERK does not appear to be regulated by syndecan 4 in VSMCs. VSMC-A were transfected with negative control siRNA, (A) syndecan 4 siRNA-1 or, (B) syndecan 4 siRNA-2 every 48 hours. Forty-eight hours after the 2nd siRNA transfection, cells were serum-starved for 2 hours and stimulated with vehicle (5 mM TRIS + 0.1% (w/v) BSA) or FGF-2 (25 or 50 ng/ml) for 15 minutes. Protein lysates were also collected from cells before the addition of vehicle or FGF-2 ('0 hours'). Phosphorylation of Erk1/2 (pErk1/2) and expression of total Erk1/2 were assessed by western blotting (25 µg protein loaded per lane). β-actin was used as a loading control. Blots are representative of 2 independent experiments for both syndecan 4 siRNA-1 and siRNA-2.
6.2.5 Investigating the effects of exogenous FGF-2 on VSMC mineralisation in the absence of syndecan 4

Syndecan 4 regulates FGF-2-induced Akt activation in VSMCs (Figures 6.9). To determine whether syndecan 4 is also required for the inhibitory effect of FGF-2 on VSMC mineralisation, VSMC-A were transected with syndecan 4 siRNA-1 or negative control siRNA and cultured with 3 mM β-GP and 25 ng/ml FGF-2. Cells transfected with negative control siRNA and cultured with 3 mM β-GP and vehicle were used as a control (Figure 6.12A). These studies confirmed that 25 ng/ml FGF-2 significantly reduced mineralisation in VSMCs-A transfected with negative control siRNA, when compared to the vehicle control (P<0.05, Figure 6.12A&B). Furthermore, the inhibitory effect of FGF-2 on mineralisation was prevented when syndecan 4 expression was knocked-down using syndecan 4 siRNA-1 (Figure 6.12A &B).

To determine if syndecan 4 knock-down can also prevent the induction of mineralisation by higher concentrations of FGF-2, experiments were repeated using 50 ng/ml FGF-2. Using this higher concentration of FGF-2, VSMC mineralisation was significantly reduced in negative control siRNA-treated VSMCs-A when compared to the vehicle control (P<0.001; Figure 6.13A&B). However, the extent of mineralisation was also significantly reduced in syndecan 4 siRNA-treated VSMCs cultured with β-GP and 50 ng/ml FGF-2 when compared to the vehicle control (P<0.001; Figure 6.13A&B).

Together, these data suggest that syndecan 4 is required for the inhibition of VSMC mineralisation by exogenous FGF-2. However, at high concentrations of FGF-2, this growth factor can still inhibit VSMC mineralisation in the absence of syndecan 4.

6.2.6 Investigating the effects of exogenous FGF-2 on syndecan 4 expression in VSMCs

During the late stages of VSMC mineralisation, the mRNA expression levels of syndecan 4 and FGF-2 are both increased (Figure 6.1). Previous studies have shown that FGF-2 induces syndecan 4 mRNA expression in rat aortic VSMCs (Cizmeci-Smith et al., 1997), as well as rat (Song et al., 2007) and mouse calvarial osteoblasts (Teplyuk et al., 2009). Therefore, to determine if exogenous FGF-2 can also induce syndecan 4 expression in bovine VSMCs, VSMC-A were serum-starved for 2 hours and then treated with vehicle or 10 ng/ml FGF-2 for up to 24 hours. This dose of FGF-2 was selected as it had been shown to induce syndecan 4 mRNA expression in rat calvarial osteoblasts (Song et al., 2007). RNA was collected from these cells at 0, 1, 3, 6 and 24 hours after the addition of vehicle or FGF-2, and the mRNA expression levels of syndecan 4 were determined by qPCR. These studies demonstrated that FGF-2 (10 ng/ml) had no significant effect on syndecan 4 mRNA expression in VSMC-A when compared to vehicle-treated cells at the same time-point (Figure 6.14A).

To determine if the concentration of FGF-2 influences the ability of FGF-2 to induce syndecan 4 expression in bovine VSMCs, the experiments were repeated using 50 ng/ml FGF-2. Even at higher concentrations (50 ng/ml), FGF-2 had no effect on syndecan 4 mRNA expression in VSMC-A when compared to vehicle-treated cells at the same time-point (Figure 6.14B).
Figure 6.12. Knocking-down syndecan 4 rescues the inhibitory effect of FGF-2 on VSMC mineralisation. VSMC-A were transfected twice with negative control or syndecan 4 siRNA-1, with 48 hours between transfections. Both siRNAs were used at a final concentration of 20 nM. Forty-eight hours after the 2nd transfection, negative control-treated VSMC-A were cultured with 3 mM β-GP and vehicle (5 mM TRIS + 0.1% (w/v) BSA) or FGF-2 (25 ng/ml). Syndecan 4 siRNA-1-treated VSMC-A were cultured with 3 mM β-GP and FGF-2 (25 ng/ml). siRNA transfections and medium changes were carried out every 48-72 hours. (A) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (B) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 6 wells of cells from 3 independent experiments. The data were analysed using a 1-way ANOVA with Tukey post-hoc tests. ns, not significant; * P<0.05.
Figure 6.13. Knocking-down syndecan 4 cannot completely rescue the inhibitory effect of higher concentrations of FGF-2 on VSMC mineralisation. VSMC-A were transfected twice with negative control or syndecan 4 siRNA-1, with 48 hours between transfections. Both siRNAs were used at a final concentration of 20 nM. Forty-eight hours after the 2nd transfection, negative control-treated VSMC-A were cultured with 3 mM β-GP and vehicle (5 mM TRIS + 0.1% (w/v) BSA) or FGF-2 (50 ng/ml). Syndecan 4 siRNA-1-treated VSMC-A were cultured with 3 mM β-GP and FGF-2 (50 ng/ml). siRNA transfections and medium changes were carried out every 48-72 hours. (A) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (B) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 5 wells of cells from 3 independent experiments. The data were analysed using a 1-way ANOVA with Tukey post-hoc tests. *** P<0.001.
Figure 6.14. FGF-2 does not regulate syndecan 4 mRNA expression in VSMCs. Confluent VSMC-A were serum-starved for 2 hours and then treated with (A) 10 ng/ml FGF-2 or (B) 50 ng/ml FGF-2 for up to 24 hours. Cells treated with an equivalent volume of vehicle (5 mM TRIS + 0.1% (w/v) BSA) were used as a control. RNA was isolated before the addition of vehicle or FGF-2 (0 hours), then at 1, 3, 6 and 24 hours after the addition of vehicle or FGF-2. Syndecan 4 mRNA expression was measured using qPCR and is shown relative to the housekeeping genes, RPL12 and PPIA. For (A), results are displayed as mean ± SEM and were derived from 4 wells of cells from 2 independent experiments. For (B), results are displayed as mean ± SEM and were derived from 3 wells of cells from 3 independent experiments. Data presented in (A) and (B) were analysed using a 2-way ANOVA with Sidak post-hoc tests.
6.3 Discussion

The results presented in this chapter demonstrate for the first time that FGF-2 mRNA and protein expression increases during the late stages of VSMC osteogenic differentiation and mineralisation. Exogenous FGF-2 inhibits VSMC mineralisation, and blocking endogenous FGF-2 signalling with a pan-FGFR inhibitor (BGJ398) increases VSMC mineralisation, suggesting that FGF-2 plays an inhibitory role in the mineralisation process. Syndecan 4 regulates FGF-2-induced Akt activation in VSMCs, and syndecan 4 is required for the inhibition of VSMC mineralisation by FGF-2. Together, these studies suggest that syndecan 4 expression increases in mineralising VSMC to maintain FGF-2 signalling, and in turn, slow down the mineralisation process.

6.3.1 FGF-2 expression is increased during VSMC mineralisation, and it may play an inhibitory role in the mineralisation process

FGF-2 mRNA and protein expression is increased in β-GP-treated VSMCs during the late stages of VSMC osteogenic differentiation and mineralisation, coinciding with increased syndecan 4 mRNA expression in these cells (Figure 6.1). FGF-2 mRNA expression is also increased during the osteogenic differentiation and mineralisation of osteoprogenitors (Huang et al., 2007) and it is expressed at sites of bone formation \textit{in vivo} (Gonzalez et al., 1990), suggesting that changes in FGF-2 expression during osteogenic differentiation and mineralisation may play an important role in both VSMCs and osteoprogenitors. Indeed, the mRNA expression levels of FGF-2 were not changed in two separate preparations of VSMCs that do not undergo osteogenic differentiation and mineralisation in the presence of β-GP (Figure 6.2), suggesting that changes in FGF-2 expression are associated with the osteogenic differentiation and mineralisation of these cells, and are not due to extended culture times in the presence of β-GP.

Exogenous FGF-2 inhibited VSMC mineralisation, whilst blocking endogenous FGF-2 signalling with a pan-FGFR inhibitor (BGJ398) increased VSMC mineralisation, suggesting that FGF-2 may play an inhibitory role in this process (Figures 6.3 and 6.5). Consistent with this, the FGFR1 inhibitor SU5402 increased rat MSC mineralisation in the presence of raised phosphate levels (Dombrowski et al., 2009). Importantly, BGJ398 did not stimulate VSMC mineralisation in the absence of β-GP, suggesting that raised phosphate levels are also required for BGJ398 to exert pro-calcific effects. Furthermore, BGJ398 did not stimulate mineralisation in a non-mineralising VSMC preparation (VSMC-B) (data not shown). Together these studies demonstrate that FGF-2 plays an inhibitory role in VSMC mineralisation, but this is limited to cells that are already ‘primed’ to undergo osteogenic differentiation and mineralisation. Therefore, like syndecan 4, FGF-2 expression may be increased in mineralising VSMCs to act as a ‘brake’/slow down the mineralisation process. In support of this hypothesis, FGFR1 expression (an FGFR through which FGF-2 signals through) is co-localised to Runx2-positive VSMCs in human calcified blood vessels (Nakahara et al., 2010).
Only one previous study has examined the effect of exogenous FGF-2 on osteogenic marker expression in VSMCs (Nakahara et al., 2010). In this study, short-term (up to 12 hours) FGF-2 treatment stimulated the expression of osteopontin and osteocalcin mRNA in rat VSMCs in vitro (Nakahara et al., 2010). It was also stated that FGF-2 did not induce mineralisation in these cells, although how these studies were performed was not clear (Nakahara et al., 2010).

Although VSMC osteogenic differentiation and mineralisation often occurs simultaneously (as in the case of VSMC-A, see Figure 3.1), there is substantial evidence to suggest that VSMC osteogenic differentiation may in fact occur as a consequence of mineralisation (Sage et al., 2011; Khavandgar et al., 2014), and in some arterial beds, VSMC osteogenic differentiation may not even be required for the deposition of a mineralised matrix (O’Neill and Adams, 2014). The effects of FGF-2 on VSMC osteogenic differentiation were not studied in this thesis, but require investigation to determine the effects of FGF-2 on VSMC osteogenic differentiation in long-term mineralisation experiments.

FGF-2 signalling has also been shown to regulate the osteogenic differentiation and/or mineralisation of other cell types, including MSCs (Dombrowski et al., 2009; Biver et al., 2012) and calvarial osteoblasts (Debiais et al., 1998; Fakhry et al., 2005; Ling et al., 2006; Song et al., 2007). The role of FGF-2 in this process is however complex, as it appears to be highly dependent on the timing and duration of FGF-2 signalling (discussed in section 6.1). Consistent with this study (Figure 6.3), continuous FGF-2 treatment inhibits the deposition of a mineralised matrix by MSCs (Dombrowski et al., 2009; Biver et al., 2012) and calvarial osteoblasts (Debiais et al., 1998; Fakhry et al., 2005; Ling et al., 2006). It has been suggested that FGF-2 stimulates the osteogenic potential of osteoprogenitor cells following short-term treatment (3-8 days of a 9-14 day protocol), but it must then be down-regulated to allow mineralisation to proceed (Ling et al., 2006; Fakhry et al., 2005; Dombrowski et al., 2009). It is possible that increases in FGF-2 expression during the later of stages of VSMC osteogenic differentiation and mineralisation may play an inhibitory role in the deposition of a mineralised matrix by VSMCs. Consistent with this, exogenous FGF-2 inhibited VSMC mineralisation when included in the cell culture medium from 2 and 4 days after the commencement of the β-GP-treatment protocol, whilst FGF-2 increased mineralisation when included in the cell culture medium for the first 4 days of β-GP-treatment only (Appendix, Figure 10.2).

The mechanism by which FGF-2 inhibits VSMC mineralisation is currently unknown. It has been suggested that FGF signalling acts to repress BMP activity through transcriptional inhibition of BMP-4, and transcriptional activation of the BMP antagonist, noggin (Pouget et al., 2014). In contrast, FGF-2 increases BMP-2 signalling during skull development (Warren et al., 2003) and enhances BMP-2-induced ectopic bone formation in mice (Nakamura et al., 2005b). Both BMP-2 (Li et al., 2008; Liberman et al., 2011) and BMP-4 (Yao et al., 2008; Yao et al., 2009; Panizo et al., 2009) are pro-calcific factors in vascular calcification, and their expression has been localised to sites of calcification in human vessels (Bostrom et al., 1993; Dhore et al., 2001). Whilst FGF-2 could inhibit BMP-4 signalling and VSMC mineralisation, its reported effects on BMP-2 signalling could suggest that BMP-2 signalling is also enhanced in the presence of FGF-
Further work is therefore required to determine if there is cross-talk between BMP-2, BMP-4 and FGF-2 signalling during the deposition of a mineralised matrix by VSMCs.

A limitation of these findings is that a pan-FGFR inhibitor (BGJ398) was used to determine the role of endogenous FGF-2 in VSMC mineralisation. FGF-2 signals through all four FGFRs, and whilst BGJ398 inhibits FGF-2 signalling in VSMCs (Figures 6.4 and 6.6), signalling by the other FGFs could also be inhibited. An alternative approach would be to knock-down FGF-2 expression in VSMCs using siRNA, and induce these cells to mineralise in the presence of β-GP.

6.3.2 Syndecan 4 regulates FGF-2-induced Akt activation in VSMCs, and is required for the inhibitory effect of FGF-2 on VSMC mineralisation

Akt and Erk1/2 are phosphorylated down-stream of FGF-2 receptor activation (Figure 1.5). Knocking-down VSMC syndecan 4 expression did not have a consistent effect on FGF-2-induced Erk1/2 phosphorylation (Figure 6.11). Previous studies have shown that in the absence of syndecan 4, FGF-2-induced Erk1/2 phosphorylation is enhanced in mouse lung endothelial cells (Elfenbein et al., 2012), but reduced in syndecan 4-deficient HUVECs (Matsui et al., 2011) and mouse VSMCs (Ikese et al., 2011). These differences may represent disparities in the signalling between cells derived from different tissues and species.

Syndecan 4 is required for FGF-2-induced Akt phosphorylation in vitro (Partovian et al., 2008; Ju and Simons, 2013) and in vivo (Zhang et al., 2003; Partovian et al., 2008; Ju and Simons, 2013). Consistent with these studies, knocking-down VSMC syndecan 4 expression reduced FGF-2-induced Akt phosphorylation (Figure 6.9). This effect was observed with two separate syndecan 4 siRNA’s that target different regions of the syndecan 4 ORF. Syndecan 4-dependent activation of PKCα has a crucial role in the assembly of mTORC2 (also called PDK2) and activation of PDK1 (Partovian et al., 2008; Ju and Simons, 2013). Both mTORC2 and PDK1, in turn, control the activation of Akt by two phosphorylation events: Thr308 phosphorylation is accomplished PDK1, and Ser473 phosphorylation by mTORC2. In the absence of syndecan 4, PKCα activation is reduced, leading to impaired assembly of mTORC2 (Partovian et al., 2008) and diminished activation of PDK1, which it controls through Pak1/2 (Ju and Simons, 2013) (see Figure 1.8). PKCα is crucial to the effects of syndecan 4 in FGF-2-induced Akt activation (Partovian et al., 2008; Ju and Simons, 2013); the role of PKCα in VSMC mineralisation will therefore be explored in Chapter 7.

Syndecan 4 is required for FGF-2-induced Akt phosphorylation in VSMCs, and it is also required for the inhibition of VSMC mineralisation by low concentrations of exogenous FGF-2 (Figure 6.12). Together, these studies suggest that an important feedback mechanism may occur during VSMC mineralisation to prevent further mineral deposition. In this regard, syndecan 4 expression may increase in mineralising cells to maintain FGF-2 signalling, and in turn, slow down the mineralisation process (see Figure 6.15). FGF-2 also plays an important role in neointimal hyperplasia, but wire-induced vascular injury was reduced in syndecan 4-null mice.
(Ikesue et al., 2011). These data could suggest that syndecan 4 may play an injury-specific role in vascular disease. In this regard, a loss of FGF-2-induced VSMC proliferation and migration is beneficial in neointimal formation (Ikesue et al., 2011), but FGF-2 signalling inhibits VSMC mineralisation. Similar observations have reported for the Gas6 receptor, Axl, which inhibits VSMC mineralisation in vitro (Collett et al., 2007) but plays a stimulatory role in flow-induced intima-media thickening in mice (Korshunov et al., 2006).

Figure 6.15. Schematic overview of the proposed role of FGF-2 and syndecan 4 in VSMC mineralisation. Syndecan 4 expression is increased in mineralising VSMCs, and knocking-down syndecan 4 expression using siRNA increases VSMC mineralisation, suggesting that syndecan 4 acts as a brake on the mineralisation process. FGF-2 expression is also increased in mineralising VSMCs, and FGF-2 inhibits VSMC mineralisation. Syndecan 4 is required for the inhibitory effect of FGF-2 on VSMC mineralisation, suggesting that syndecan 4 and FGF-2 act in a synergistic manner to slow down the mineralisation process.

Whilst syndecan 4 was required for the inhibition of VSMC mineralisation by exogenous FGF-2 at low concentrations (25 ng/ml), at higher concentrations of FGF-2 (50 ng/ml), this growth factor could inhibit VSMC mineralisation in the absence of syndecan 4; although to a lesser extent (Figure 6.13). Whilst the FGFs are able to bind to FGFRs with high affinity, this interaction and the subsequent signaling events are amplified in the presence of HS which is thought to act as a co-receptor for FGF/FGFR binding (Yayon et al., 1991; Rapraeger et al., 1991; Kan et al., 1999). HS serves to concentrate FGFs around the cell surface and increases the probability and stability of FGF/FGFR interactions (Kan et al., 1999). Therefore, when used at high concentrations, FGF-2 may be available in excess and does not require syndecan 4 to act as a co-receptor. As syndecan 4 has the potential to regulate FGF-2 signalling through the HS chains displayed on its extracellular domain, the role of HS in VSMC mineralisation will be explored in Chapter 7.
The requirement for syndecan 4 in FGF-2-induced inhibition of VSMC mineralisation may also have important implications for the syndecan 4 over-expression studies presented in Chapter 5. As discussed above, the ability of FGFs to bind to their FGFR are amplified in the presence of HS, but the ability of GAGs to potentiate growth factor signalling is highly concentration-dependent. In this regard, low concentrations of heparin (a highly sulphated form of HS) potentiated the hematopoietic differentiation of ESCs, whilst high concentrations were inhibitory, likely by preventing access of the necessary growth factors to its cell surface receptor (Holley et al., 2011b). In light of this evidence, it is possible that over-expressing syndecan 4 in VSMCs may compete with endogenous syndecan 4 to sequester FGF-2, preventing access of this growth factor to its FGFR and leading to increased VSMC mineralisation in these cells (Figures 5.12 and 5.13). It would therefore be of interest for future studies to determine if FGF-2 can inhibit VSMC mineralisation in cells over-expressing syndecan 4.

VSMC apoptosis promotes VSMC mineralisation (Proudfoot et al., 2000; Collett et al., 2007; Clarke et al., 2008), therefore syndecan 4 expression may be up-regulated during VSMC osteogenic differentiation and mineralisation to maintain FGF-2-induced activation of Akt and inhibit VSMC apoptosis. Indeed, cardiac myocyte apoptosis is increased in syndecan 4-null mice following myocardial infarction (Echtermeyer et al., 2011), whilst it is reduced when syndecan 4 is over-expressed in the hearts of rats 1 day following the induction of myocardial infarction in these animals (Xie et al., 2012). Therefore, to begin to examine the role of syndecan 4 in regulating VSMC apoptosis, preliminary experiments were performed to knock-down syndecan 4 expression in human coronary artery SMCs as these cells are more susceptible to phosphate-induced cell death when compared to bovine VSMCs (Hyde and Canfield, unpublished findings). Human coronary artery SMCs were plated onto 0.1% (v/v) gelatin-coated tissue culture plastic in order for them to adhere, and knocking-down syndecan 4 expression in these cells using siRNA caused a massive loss of cells from the tissue culture plastic during media changes, which prevented the analysis of phosphate-induced cell death in these cells (Appendix, Figure 10.3). Similar results were also obtained when syndecan 4 expression was knocked-down in human MSCs using siRNA; these cells are also maintained on gelatin-coated tissue culture plastic (Appendix, Figure 10.4) (S. A. Cain and C. Kielty, University of Manchester; unpublished findings). In contrast, bovine VSMCs can adhere to untreated tissue culture plastic and knocking-down syndecan 4 expression in these cells had no discernible effect on adhesion. Syndecan 4 is known to play a role in cell adhesion, although this appears to be dependent on the cell-type used (Whiteford et al., 2007). Focal adhesion formation was not examined in syndecan 4 siRNA-treated cells, but requires further investigation to determine why the adhesion phenotype differs between human coronary artery and bovine VSMCs. The possible link between FGF2, Akt phosphorylation, apoptosis of VSMC and mineralisation also still remains to be defined.
6.3.3 FGF-2 does not induce syndecan 4 expression in VSMCs

FGF-2 and syndecan 4 expression is increased in mineralising VSMCs, but the mechanism by which the expression levels of both genes are increased in mineralising VSMCs was unknown. Previous studies have shown that FGF-2 (10-20 ng/ml) induces syndecan 4 mRNA expression in different cell types (Cizmeci-Smith et al., 1997; Song et al., 2007; Teplyuk et al., 2009), but FGF-2 (10 and 50 ng/ml) had no effect on syndecan 4 mRNA expression in bovine VSMCs (Figure 6.14). There could be several reasons for this discrepancy in results. For example, FGF-2 synergises with Runx2 to enhance syndecan 4 mRNA expression in mouse calvarial osteoblasts (Teplyuk et al., 2009). Basal Runx2 mRNA levels are very low in VSMCs (~0.0002), so it is possible that raised levels of this osteogenic transcription factor are required for FGF-2 to induce syndecan 4 expression in bovine VSMCs e.g. as observed during VSMC osteogenic differentiation and mineralisation (Figure 3.1). Alternatively, FGF-2 may regulate syndecan 4 expression in a species-specific manner. The mechanism by which both syndecan 4 and FGF-2 expression is increased in mineralising VSMCs therefore, still remains to be defined.

6.4 Conclusion

The following conclusions can be drawn from the results presented in this chapter:

- FGF-2 mRNA and protein expression is increased in mineralising VSMCs, coinciding with increased syndecan 4 mRNA expression in these cells.
- FGF-2 inhibits VSMC mineralisation, and syndecan 4 is required for the inhibitory effect of FGF-2 on VSMC mineralisation.
- Syndecan 4 expression may be increased in mineralising VSMC to maintain FGF-2 signalling, and in turn, slow down the mineralisation process.
7 Investigating the role of extracellular and cytoplasmic domain of syndecan 4 in VSMC mineralisation

7.1 Introduction
The results presented in this thesis have demonstrated for the first time that syndecan 4 mRNA expression is increased during the late stages of VSMC osteogenic differentiation and mineralisation (Figure 3.5), and knocking-down syndecan 4 expression using siRNA increased VSMC mineralisation (Figures 5.2 and 5.5). FGF-2 mRNA and protein is also increased during the late stages of VSMC osteogenic differentiation and mineralisation (Figure 6.1), and FGF-2 plays an inhibitory role in the matrix mineralisation process (Figures 6.3 and 6.5). Finally, syndecan 4 regulates FGF-2-induced Akt activation in VSMCs (Figures 6.9), and syndecan 4 is required for the inhibition of VSMC mineralisation by low concentrations of FGF-2 (Figure 6.12). Together, these data suggest that syndecan 4 expression is up-regulated in mineralising VSMCs to maintain the inhibitory effect of FGF-2 on VSMC mineralisation and, in turn, puts a ‘brake’ on the mineralisation process. Syndecan 4 has the potential to regulate FGF-2 signalling through the GAG chains displayed on its extracellular domain, and also through its cytoplasmic domain which interacts with PKCα. The contributions of these two domains to the role of the syndecan 4 in VSMC mineralisation are, however, unknown and will be investigated in this chapter.

7.1.1 The GAG chains displayed on syndecan 4 can regulate growth factor signalling
The GAG chains displayed on the extracellular domain of PGs can regulate growth factor signalling by acting as co-receptors, stabilising signalling gradients and/or by protecting ligands from degradation (Roehl and Pacifici, 2010). Whilst the FGFs are able to bind to FGFRs with high affinity, this interaction and the subsequent signalling events are amplified in the presence of HS which is thought to act as a co-receptor for FGF/FGFR binding (Yayon et al., 1991; Rapraeger et al., 1991; Kan et al., 1999). The functional significance of this interaction has been demonstrated using EXT1-null ESCs. EXT1 is an early enzyme in the HS biosynthetic pathways, and EXT1 depletion has been previously shown to reduce HS biosynthesis, often with only short sulphated HS chains generated (Busse and Kusche-Gullberg, 2003; Osterholm et al., 2009; Pickford et al., 2011). FGF signaling is disrupted in EXT1-null ESCs, and Nanog mRNA expression is also increased (Kraushaar et al., 2010). Enhanced Nanog expression retains self-renewal and inhibits cell fate commitment of ESCs (Mitsui et al., 2003), suggesting that HS enhances FGF signaling to facilitate the transition of ESCs from self-renewal to cell differentiation commitment. In light of this evidence, one could hypothesise that syndecan 4 may regulate the effects of FGF-2 on VSMC mineralisation through its HS chains; this hypothesis will be tested in this chapter.
7.1.2 Interaction between syndecan 4 and PKCα also regulates regulate growth factor signalling

In addition to its GAG chains, syndecan 4 can regulate FGF-2-induced Akt activation through its interaction with PKCα (Figure 1.8). PKCα belongs to the PKC family of serine/threonine kinases, that comprise 10 isoenzymes which can be sub-grouped into three classes: (i) conventional PKCs (α, β1, β2, γ) which are activated by calcium, DAG or phosphatidylserine; (ii) novel PKCs (δ, ε, η, θ) which lack the calcium binding property, but can be activated by DAG or phosphatidylserine; (iii) atypical PKCs (ζ, ι) which are only activated by phosphatidylserine.

All PKCs have a conserved N-terminal regulatory domain, containing several shared sub-regions. The C1 domain present in all of the PKC isoforms is involved in recognition of DAG. DAG is a lipid second messenger that is generated in the plasma membrane as a consequence of the activation of membrane-associated PLCγ (discussed further below). The C1 domain is functional in both conventional and novel PKC isoforms, but is limited in atypical PKCs. The C2 domain acts as a calcium sensor and is present in both conventional and novel PKC isoenzymes. PKCs also contain a pseudosubstrate motif that blocks the catalytic site of the C-terminal catalytic domain of the non-activated kinase (Newton, 2001). The V5 domain, located in the C-terminal of PKCs, share little to no sequence homology between PKC isoenzymes (Steinberg, 2008). This domain contains a conserved threonine phosphorylation site in the activation loop, in addition to threonine or serine phosphorylation sites at the turn- and hydrophobic motifs. The structure of PKCα is shown in Figure 7.1. As syndecan 4 only interacts with PKCα, this PKC isoform will be discussed in more detail below; the other PKC isoforms are reviewed in detail elsewhere (Steinberg, 2008).

Figure 7.1. Structure of PKCα. PKCs have a conserved kinase domain (shown in blue), but the regulatory domain varies between PKC isoenzymes. All PKC regulatory domains have a pseudosubstrate motif (shown in green) next to the C1 domain (shown in red). The C1 domain binds diacylglycerol (DAG) and phosphatidylserine (PtdSer) in PKCα. The C2 domain (shown in yellow) function as a calcium (Ca^{2+})-dependent phosphatidylinositol 4,5-bisphosphate (PIP2) binding module. The C-terminal of PKCs contain the activation loop (AL), turn motif (TM) and hydrophobic motif (HM). The figure is not drawn to scale. Figure adapted from (Steinberg, 2008).
Calcium and DAG are second messengers that are generated following the binding of certain growth factors (e.g. FGF-2, Figure 1.5), hormones or neurotransmitters to their corresponding receptors. The consequent activation of membrane-associated PLCγ results in hydrolysis of the membrane phospholipid, PIP$_2$ to DAG and inositol trisphosphate (IP$_3$). IP$_3$ diffuse through the cell to bind to IP$_3$-sensitive calcium channels on the endoplasmic reticulum, releasing calcium ions into the cytosol. PKCα binds to these calcium ions, triggering the translocation of PKCα to the cell membrane where it interacts with DAG and the anchoring protein, receptor for activated C kinase (RACK). As a result, PKCα expels the pseudosubstrate motif from the substrate-binding pocket, and becomes activated; PKCα can then regulate diverse biological functions such as osteoblast differentiation and mineralisation (Nakura et al., 2011; Jeong et al., 2012; Galea et al., 2014) (discussed further below).

The activation of PKCα is not limited to the pathway described above, as it can also be regulated by syndecan 4. In this regard, syndecan 4 can recruit and mediate the subsequent activation of PKCα at the plasma membrane in the absence of increased cytosolic calcium levels (Oh et al., 1997b; Oh et al., 1997a; Oh et al., 1998; Horowitz and Simons, 1998b; Horowitz and Simons, 1998a; Keum et al., 2004; Partovian et al., 2008). The ability of syndecan 4 to activate PKCα is dependent on the binding of syndecan 4 to cytoplasmic PIP$_2$. Under normal circumstances, PKCα is phosphorylated at serine 183 by PKCδ, which inhibits the binding of PIP$_2$ and prevents PKCα activation (Couchman et al., 2002; Murakami et al., 2002). In response to growth factor stimulation (e.g. via FGF-2), the serine 183 site is dephosphorylated by a type I/Iia serine phosphatase, thereby promoting syndecan 4 oligomerisation and PIP$_2$ binding (Oh et al., 1997a; Horowitz and Simons, 1998b). As a result, the catalytic domain of PKCα binds to the cytoplasmic domain of syndecan 4 and PKCα-mediated signalling is activated in the cell, including activation of Akt (Figure 1.8). The importance of syndecan 4 in the activation of PKCα has been confirmed using gene knock-out and over-expression strategies. For example, PKCα activation is reduced in the absence of syndecan 4 (Oh et al., 1997b; Oh et al., 1997a; Oh et al., 1998; Horowitz and Simons, 1998b; Horowitz and Simons, 1998a; Keum et al., 2004; Partovian et al., 2008), whilst over-expressing syndecan 4 in fibroblasts, but not a mutant lacking its cytoplasmic domain, enhances the translocation of PKCα in the plasma cell membrane, and increases the activity of membrane PKCα (Keum et al., 2004).

PKCα has been implicated in the regulation of a number of biological functions, including osteoblast differentiation and mineralisation. Specifically, knocking-down PKCα expression or inhibiting PKCα activity with the pharmacological inhibitor G6976, promotes osteogenic differentiation and/or mineralisation in vitro (Nakura et al., 2011; Jeong et al., 2012; Galea et al., 2014) and in vivo (Galea et al., 2014), suggesting PKCα normally acts to suppress bone formation. PKCα may also serve a role in vascular calcification, as a report published during the course of this thesis demonstrated that inhibiting PKCα activity with G6976, or knocking-down PKCα expression using siRNA, increased VSMC mineralisation (Lee et al., 2014b). In light of this evidence, one could hypothesise that knocking-down syndecan 4 expression in VSMCs
may reduce FGF-2-induced Akt activation and increase matrix mineralisation through a loss of PKCα activity. This hypothesis will also be tested in this chapter.

This chapter will investigate the role of HS and PKCα in VSMC mineralisation by addressing the following aims:

1. Determine the effects of modulating HS expression on matrix mineralisation and FGF-2 signalling in VSMCs.

2. Determine the effects of inhibiting or knocking-down PKCα expression in VSMC mineralisation.
7.2 Results

7.2.1 Investigating the effects of knocking-down EXT1 expression on VSMC mineralisation

To determine the role of HS in VSMC mineralisation, EXT1 expression was knocked-down in VSMCs using siRNA; two separate siRNAs (EXT1 siRNA-1 and siRNA-2) were used in combination to increase knock-down efficiency. EXT1 mRNA expression was knocked-down by ~66% in cells transfected with EXT1 siRNA-1 and siRNA-2 when compared to negative control siRNA-treated VSMC-A (Figure 7.2A). The transfection efficiency could not be improved by using higher concentrations of siRNA (data not shown).

To investigate the effects of reduced HS expression on VSMC mineralisation, VSMC-A were transfected with negative control siRNA or EXT1 siRNA-1 and siRNA-2, and cultured with 3 mM β-GP to induce calcification (Figure 7.2B). Knocking-down EXT1 expression significantly increased VSMC mineralisation (~2.5-fold increase, P<0.001; Figure 7.2C) when compared to negative control siRNA-treated cells cultured with β-GP at the same time-point.

7.2.2 Investigating the effects of knocking-down EXT1 expression on FGF-2-induced Akt activation in VSMCs

Syndecan 4 regulates FGF-2-induced Akt activation in VSMCs (Figure 6.9). Previous studies have shown that HS also regulates FGF-2 signalling (Kraushaar et al., 2010; Kraushaar et al., 2012). Therefore, to determine if HS regulates FGF-2 signalling in VSMCs, VSMC-A were transfected with EXT-1 siRNA-1 and siRNA-2 for 5 days. Forty-eight hours after the 2nd siRNA transfection, cells were serum-starved for 2 hours and then incubated with vehicle or FGF-2 (25 or 50 ng/ml) for 5 minutes. VSMC-A transfected with negative control siRNA were used as controls.

In negative control siRNA-treated VSMCs, FGF-2 stimulated Akt phosphorylation (Ser473) when compared to the vehicle control (Figure 7.3). FGF-2-induced Akt phosphorylation was reduced in EXT1 siRNA-treated VSMCs when compared to negative control siRNA-treated cells (Figure 7.3). Blots shown in Figure 7.3 are representative of 2 independent experiments. However, the variability in Akt phosphorylation between individual experiments, meant that the phosphorylated Akt:total Akt ratios were not significantly different when the EXT1 siRNA-treated samples were compared to controls (data not shown).
**Figure 7.2. Knocking-down EXT1 increases VSMC mineralisation.** (A) VSMC-A were transfected with negative control siRNA, or with EXT1 siRNA-1 and siRNA-2, at a final concentration of 20 nM. Knock-down of EXT1 mRNA expression was confirmed using qPCR. EXT1 mRNA expression is shown relative to the reference genes, RPL12 and PPIA. Results displayed as mean ± SEM and were derived from 3 wells of cells from 3 independent experiments. The data were analysed using an unpaired t-test. ** P<0.01. (B-C) VSMC-A transfected with negative control siRNA, or with EXT1 siRNA-1 and siRNA-2, were cultured with 3 mM β-GP for up to 12 days. All siRNAs were used at a final concentration of 20 nM. siRNA transfections and medium changes were carried out every 48-72 hours. (B) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (C) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 8 wells of cells from 2 independent experiments. The data were analysed using an unpaired t-test. *** P<0.001.
Figure 7.3. Knocking down EXT1 in VSMCs reduces FGF-2-induced Akt activation in VSMCs. VSMC-A were transfected twice with negative control siRNA or EXT1 siRNA-1 and siRNA-2, with 48 hours between transfections. Forty-eight hours after the 2nd transfection, cells were serum-starved for 2 hours and stimulated with vehicle (5 mM TRIS + 0.1% (w/v) BSA) or FGF-2 (25 or 50 ng/ml) for 5 or minutes. Protein lysates were also collected from cells before the addition of vehicle or FGF-2 (0 hours'). Phosphorylation of Akt (Ser473) and expression of total Akt were assessed by western blotting (25 µg protein loaded per lane). β-actin was used as a loading control. Blots are representative of 2 independent experiments.

7.2.3 Determining the effects of exogenous heparin on VSMC mineralisation

(i) Addition of heparin from confluence

Knocking-down EXT1 expression increases VSMC mineralisation (Figure 7.2) and reduces FGF-2-induced Akt activation (Figure 7.3), suggesting that like syndecan 4, HS is required to put a ‘brake'/slow down the mineralisation process. Complementing these findings, previous studies have shown that heparin (a highly sulphated form of HS) inhibits bovine aortic VSMC mineralisation in vitro (Yang et al., 2005). Therefore, to confirm the reported effects of heparin on VSMC mineralisation, VSMC-A were cultured with 3 mM β-GP and 2, 10 or 50 µg/ml heparin for up to 10 days. Cells cultured with 3 mM β-GP ± vehicle were used as controls. These doses of heparin were selected as 10 and 50 µg/ml heparin has been reported to inhibit VSMC mineralisation (Yang et al., 2005); this effect has not been observed at lower concentrations of heparin (Yang et al., 2005).

Heparin (2 and 10 µg/ml) had no effect on stages 3 and 4 of VSMC mineralisation when compared to vehicle-treated VSMC-A at the same time-point (Figure 7.4A&B). At higher concentrations (50 µg/ml), heparin had no effect on the early stages (stage 3) of VSMC mineralisation (Figure 7.4A&B). However, 50 µg/ml heparin significantly increased (~1.5-fold, \( P<0.05 \)) the later stages (stage 4) of VSMC mineralisation when compared to β-GP and vehicle-treated VSMC-A at the same time-point (Figure 7.4A&B).
Together these studies suggest that low concentrations (2 and 10 µg/ml) of heparin have no effect on the early stages of VSMC mineralisation. However, when used at higher concentrations (50 µg/ml), heparin increased the later stages of VSMC mineralisation when compared to controls.

(2) Addition of heparin one day after plating

In the previous report published by Yang et al (2005), it was unclear whether heparin and β-GP were added to the culture medium when cells reached confluence, or before. Therefore, to determine whether time of addition of these reagents is important, 24 hours after seeding VSMCs into 6-well plates, cells were cultured with 3 mM β-GP and 10 or 50 µg/ml heparin for up to 16 days. Cells cultured with vehicle ± 3 mM β-GP were used as controls.

Preliminary experiments revealed that four-days after the addition of vehicle ± β-GP to the culture medium, VSMC-A had reached confluence (Figure 7.5A). However, in the presence of both concentrations of heparin (10 and 50 µg/ml) and β-GP, the rate of cell proliferation appeared reduced when compared to vehicle-treated VSMC-A at the same time-point (Figure 7.5A). Cells treated with the highest concentration of heparin (50 µg/ml) and β-GP had still not reached confluence 8 days after the addition of vehicle ± β-GP to the culture medium of VSMC-A (Figure 7.5A).

No mineralisation was detected with alizarin red staining in cells cultured with vehicle alone (Figure 7.5B). At the early stages (stage 3) of VSMC mineralisation, 10 µg/ml heparin had no effect on the extent of mineralisation when compared to β-GP-treated controls at the same time-point (Figure 7.5B&C). However, in cells treated with the highest concentration of heparin (50 µg/ml) and β-GP, the early stages (stage 3) of VSMC mineralisation were markedly reduced when compared to vehicle-treated cells cultured with β-GP (Figure 7.5B&C). At the later stages of VSMC mineralisation, both concentrations of heparin (10 and 50 µg/ml) appeared to markedly reduce matrix mineralisation when compared to β-GP and vehicle-treated cells at the same time-point (Figure 7.5B&C). The mineralisation that occurred in heparin-treated cells was also restricted to ridges that formed with time in culture, in contrast to β-GP- and vehicle-treated VSMC-A where mineralisation occurred in a diffuse pattern (Figure 7.5B).
Figure 7.4. Investigating the effects of heparin on VSMC mineralisation. Confluent VSMC-
A were cultured with vehicle (PBS) ± 3 mM β-GP, or with 3 mM β-GP and increasing
concentrations of heparin (2, 10 or 50 µg/ml) for up to 10 days. (A) Phase-contrast images of
cells stained with alizarin red. Scale bar = 500 µm. (B) The extent of mineralisation was
quantified by dye elution. The experiment was performed twice in duplicate or triplicate, with the
exception of 3 mM β-GP + 2 µg/ml heparin which was performed once in duplicate. Results are
displayed as mean ± SEM. The data were analysed using a 2-way ANOVA with Tukey post-hoc
tests. ** P<0.01.
Figure 7.5. Heparin inhibits VSMC proliferation and mineralisation when it is added to the culture medium 24 hours after seeding cells. Twenty-four hours after seeding VSMC-A at $2 \times 10^5$ cells/cm$^2$, cells were cultured with 3 mM β-GP and 10 or 50 µg/ml heparin for up to 16 days. Cells cultured with vehicle (PBS) ± 3 mM β-GP were used as controls. (A) Phase-contrast images of cells were taken 4 and 8 days after the first addition of 3 mM β-GP and vehicle or heparin to the culture medium. Scale bar = 500 µm. (B) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (C) The extent of mineralisation was quantified by dye elution. Results are displayed as mean ± SD. The experiment was performed once in duplicate.
7.2.4 Determining the effects of Gö6976 on VSMC mineralisation

Syndecan 4 can recruit and mediate the subsequent activation of PKCα at the plasma membrane in the absence of increased cytosolic calcium levels (Oh et al., 1997b; Oh et al., 1997a; Oh et al., 1998; Horowitz and Simons, 1998b; Horowitz and Simons, 1998a; Keum et al., 2004; Partovian et al., 2008). To determine the role of PKCα in VSMC mineralisation, the PKCα inhibitor Gö6976 was used. VSMC-A were cultured with vehicle or 1 µM Gö6976 ± 3 mM β-GP for up to 12 days. This dose of Gö6976 was selected as it promoted matrix mineralisation in mouse VSMCs (Lee et al., 2014b) and the pre-osteoblastic mouse MC3T3-E1 cell line (Nakura et al., 2011). In the presence of Gö6976, VSMC mineralisation was significantly increased (~5-fold, P<0.05) when compared to vehicle-treated VSMC-A at the same time-point (Figure 7.6A&B). In the absence of β-GP, Gö6976 did not stimulate mineralisation in VSMC-A (Figure 7.6C).

Gö6976 is not a specific PKCα inhibitor as it also inhibits PKCβ. As PKCβ has been detected at the mRNA level in mouse VSMCs (Lee et al., 2014b), the presence of this isoform in bovine VSMCs was investigated using both qPCR and western blotting. PKCβ could not be detected at the mRNA level in VSMC-A using qPCR. Importantly, two separate primer sets were designed for the bovine PKCβ gene and neither primer set detected PKCβ mRNA in VSMC-A (data not shown). PKCβ could also not be detected at the protein level using western blotting (data not shown). Therefore, these results suggest that PKCβ may not be expressed and/or is undetectable at the mRNA and protein level in bovine VSMCs.

7.2.5 Determining the effects of knocking-down PKCα expression on VSMC mineralisation

Gö6976 is not a specific inhibitor of PKCα. Therefore, to confirm that PKCα regulates VSMC mineralisation, PKCα expression was knocked-down in VSMC-A using two separate siRNAs (PKCα siRNA-1 and PKCα siRNA-2) that targeted different regions of the PKCα open reading frame (Figure 7.7). A random negative control siRNA was used as a control. Compared to negative control siRNA-treated VSMC-A, PKCα mRNA expression was knocked-down by 95% and 88% with PKCα siRNA-1 and PKCα siRNA-2, respectively (Figure 7.8A). PKCα siRNA-2 reduced PKCα protein by ~70% in VSMCs (Figure 7.8B). In contrast, PKCα siRNA-1 almost completely removed detectable PKCα protein (~95% decrease compared to negative control siRNA) (Figure 7.8B). Therefore, the effects of knocking-down PKCα expression on VSMC mineralisation were investigated using PKCα siRNA-1 only.

VSMC-A were transfected with negative control siRNA or PKCα siRNA-1, and cultured with 5 mM β-GP for up to 12 days to induce calcification. A higher concentration of β-GP was used in these studies as pilot studies had indicated that the β-GP powder had lost potency during storage and a similar time-course for VSMC mineralisation was required. Knocking-down PKCα expression with PKCα siRNA-1 significantly increased (~2.5-fold increase, P<0.05) VSMC mineralisation when compared to negative control siRNA-treated cells cultured with β-GP (Figure 7.9A&B).
Figure 7.6. The PKCα and PKCβ inhibitor, Gö6976, increases VSMC mineralisation. Confluent VSMC-A were cultured ± 3 mM β-GP with vehicle (DMSO), or with 3 mM β-GP and 1 µM Gö6976 for up to 12 days. (A) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (B) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 3 wells of cells from 3 independent experiments. The data were analysed using an unpaired t-test. ** P<0.01. (C) Confluent VSMC-A were cultured with 1 µM Gö6976, or with 3 mM β-GP and vehicle (DMSO) for 12 days. Phase-contrast images of VSMCs stained with alizarin red. Scale bar = 500 µm. This experiment was performed once.
Figure 7.7. PKCa siRNA-1 and siRNA-2 target different regions of the bovine syndecan 4 ORF. Bovine PKCa mRNA sequence (NM_174435.1) is shown in grey. PKCa siRNA-1 (red) and syndecan 4 siRNA-2 (blue) target different regions of the PKCa sequence.
Figure 7.8. Knocking-down PKCa expression in VSMCs using siRNA. (A) VSMC-A were transfected with negative control siRNA, PKCa siRNA-1 or PKCa siRNA-2 at a final concentration of 20 nM. Knock-down of PKCa expression was analysed using qPCR. PKCa mRNA expression is shown relative to the reference genes, RPL12 and PPIA. Results displayed as mean ± SEM and were derived from 3 wells of cell s from 3 independent experiments. The data were analysed using a 1-way ANOVA with Tukey post-hoc tests. ** P<0.01. (B) Protein lysates were also collected from negative control siRNA-, PKCa siRNA-1- or PKCa siRNA-2-treated VSMC-A, and PKCa protein expression was analysed by western blotting (25 µg protein loaded per lane). β-actin was used as a loading control. Blots are representative of 2 independent experiments. (C) Knock-down of PKCa protein with PKCa siRNA-1 and PKCa siRNA-2 was quantified using densitometry with ImageJ software. Results displayed as mean ± SD and were derived from 2 independent experiments.
Figure 7.9. Knocking-down PKCα expression using siRNA increases VSMC mineralisation. VSMC-Å were transfected with negative control siRNA or PKCα siRNA-1, and cultured with 5 mM β-GP¹ for up to 12 days. All siRNAs were used at a final concentration of 20 nM. siRNA transfections and medium changes were carried out every 48-72 hours. (A) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (B) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 3 wells of cells from 3 independent experiments. The data were analysed using an unpaired t-test. * P<0.05. ¹ A higher concentration of β-GP was used in these studies as pilot studies had indicated that the β-GP powder had lost potency during storage and a similar time-course for VSMC mineralisation was required.
7.3 Discussion

The results presented in this chapter demonstrate that knocking-down EXT1 expression increases VSMC mineralisation, and at high concentrations, the addition of heparin (a highly sulphated form of HS) also increases VSMC mineralisation. Previous studies have established that syndecan 4 regulates PKCα activation (Oh et al., 1997b; Oh et al., 1997a; Oh et al., 1998; Horowitz and Simons, 1998b; Horowitz and Simons, 1998a; Keum et al., 2004; Partovian et al., 2008), and inhibiting PKCα activity with Gö6976 or knocking-down PKCα expression using siRNA increases VSMC mineralisation. Taken together, these data demonstrate that the effects of knocking-down syndecan 4 expression on VSMC mineralisation can be reproduced by knocking-down EXT1 or PKCα expression, suggesting that the GAG chains displayed by syndecan 4 and this protein’s interaction with PKCα may both contribute to the effects of syndecan 4 on VSMC mineralisation. These data also suggest that the levels of HS must be finely balanced to regulate VSMC mineralisation.

7.3.1 HS regulates VSMC mineralisation

Syndecan 4 has the potential to regulate FGF-2 signalling through its GAG chains. Knocking-down EXT1 expression in VSMCs increased VSMC mineralisation, reproducing the observations made in syndecan 4 siRNA-treated VSMCs (Figures 5.2 and 5.5). HS acts as a co-receptor for FGF/FGFR binding (Yayon et al., 1991; Rapraeger et al., 1991; Kan et al., 1999), and the IdoA(2S) modification in HS chains has been shown to be essential for high-affinity binding of FGF-2 to HS (Turnbull et al., 1992). The addition of an adjacent 6-O-sulfate group is also necessary for FGF-dependent receptor dimerisation and tyrosine kinase activation to allow signal transduction (Pye et al., 1998; Schlessinger et al., 2000). The HS chains synthesised by VSMCs contain ∆UA(2S)-GlcNS(6S) (Figure 4.8), suggesting that VSMC HS could be essential for FGF signalling in these cells. Indeed, this suggestion is supported by the demonstration that FGF-2 induced Akt activation is reduced in EXT1 siRNA-treated VSMCs. However, a limitation of these findings is that the level of HS knock-down in EXT1 siRNA-transfected VSMCs was not confirmed in this thesis using 10E4 staining, an antibody which detects GlcNS epitopes which are present in all HS chains.

Heparin is a highly-sulphated structural relative of HS, readily available as a low-cost animal derivative drug with synthetic mimetics also widely available. Heparin is additionally being investigated as a culture additive to regulate stem cell fate decisions in vitro (Holley et al., 2011b; Pickford et al., 2011). At low concentrations (2 and 10 µg/ml), exogenous heparin had no effect on VSMC mineralisation when included in the culture medium of VSMCs from confluence. However, at higher concentrations (50 µg/ml), heparin increased the later stages of VSMC mineralisation when compared to vehicle-treated controls at the same time-point (Figure 7.4). Consistent with these results, both exogenous HS (Dombrowski et al., 2009) and enzymatic removal of HS chains (Manton et al., 2007) increased mineral deposition by MSCs. Moreover, exogenous heparin (4 and 20 µg/ml) increased the deposition of mineralised matrix by the osteoblastic MC3T3-E1 cell line (Miyazaki et al., 2008). Together, these data suggest
that the presence of HS, rather than an increase in its levels, is required to put a ‘brake’ on the mineralisation process. Indeed, the ability of heparin to support or inhibit signalling by distinct ligands is concentration-dependent, as low concentrations of heparin can potentiate a GAG-dependent signalling pathway, whilst high concentrations prevent access of the ligand to the cell surface receptor, switching to an inhibitory effect (Holley et al., 2011b). This may also be the case for syndecan 4, as both knocking-down (Figures 5.2 and 5.5) and over-expressing syndecan 4 in VSMCs increased matrix mineralisation (Figures 5.12 and 5.13) (discussed in section 5.3.2).

In contrast to the results presented in this chapter, another study reported that heparin (12.5-50 µg/ml) inhibits bovine VSMC mineralisation in a dose-dependent manner (Yang et al., 2005). There could be several reasons for this discrepancy in results. For example, it was unclear in the Yang et al. (2005) study if heparin was added to the culture medium when the cells reached confluence, or before. It is well established that heparin inhibits VSMC proliferation (Guyton et al., 1980; Pukac et al., 1992), and this finding was confirmed in preliminary studies which showed that heparin inhibited both VSMC proliferation and VSMC mineralisation when VSMCs were cultured with β-GP and heparin 24 hours after seeding cells into 6-well plates (Figure 7.5). Whilst the effects of heparin on VSMC proliferation in the Yang et al. (2005) study were not described, it is possible that their results were a consequence of a decrease in VSMC proliferation, rather than a direct effect on the VSMC mineralisation process.

7.3.2 PKCα activation is reduced in syndecan 4 siRNA-treated VSMCs and knocking-down PKCα expression increases VSMC mineralisation

Previous studies have shown that PKCα activation is reduced in syndecan 4-null cells (Oh et al., 1997b; Oh et al., 1997a; Oh et al., 1998; Horowitz and Simons, 1998b; Horowitz and Simons, 1998a; Keum et al., 2004; Partovian et al., 2008), and PKCα is required for FGF-2 induced Akt activation (Partovian et al., 2008; Ju and Simons, 2013), suggesting that the interaction between syndecan 4 and PKCα could be important to the ability of this PG to act as a brake on VSMC mineralisation. Inhibiting PKCα activity with Gö6976 increased VSMC mineralisation (Figure 7.6). The increase in VSMC mineralisation observed with Gö6976 was subsequently verified using PKCα siRNA-1 to knock-down PKCα expression in VSMCs (Figure 7.9). Knocking-down PKCα expression using siRNA, or inhibiting PKCα activity with Gö6976, has also been shown to increase matrix mineralisation in mouse VSMCs (Lee et al., 2014b). However, whilst Gö6976 increased VSMC mineralisation in the presence of β-GP, Gö6976 alone did not induce VSMC mineralisation. Similarly, Gö6976 did not induce the deposition of mineralised matrix by mouse VSMCs in the presence of normal phosphate levels (~0.9 mM) (Lee et al., 2014b). Gö6976 also did not stimulate mineralisation in a non-mineralising VSMC preparation (VSMC-B) (data not shown). Taken together, these results suggest that PKCα acts as ‘brake’ on VSMC mineralisation, but it may not be a driver of the mineralisation process as it cannot stimulate mineralisation in VSMCs that do not mineralise in the presence of raised of phosphate levels.
Syndecan 4 can regulate FGF-2-induced activation of Akt through its interaction with PKCα, and in the absence of syndecan 4, PKCα activation is reduced (Zhang et al., 2003; Partovian et al., 2008; Ju and Simons, 2013). The effects of knocking-down syndecan 4 expression on PKCα activation in VSMCs were not analysed in this study due to time constraints. Therefore, future studies could determine if syndecan 4 regulates the localisation and subsequent activation PKCα at the cell membrane in VSMCs. To do this, negative control- and syndecan 4 siRNA-treated VSMCs could be stimulated with FGF-2, and the phosphorylation of PKCα and expression of total PKCα at the cell membrane could be assessed by western blotting.

The results presented in Chapter 6 suggested that syndecan 4 is required for the inhibition of VSMC mineralisation by FGF-2 (Figure 6.12). In light of this evidence, one could hypothesise that PKCα also required for the inhibitory effect of FGF-2 on VSMC mineralisation. To determine the role of PKCα in FGF-2 signalling in VSMCs, FGF-2-induced Akt activation could be assessed in PKCα siRNA-treated VSMCs. Furthermore, the effects of FGF-2 on VSMC mineralisation could be assessed in PKCα siRNA-treated VSMCs to determine the role of PKCα in FGF-2-induced inhibition of VSMC mineralisation.

In addition to the effects of PKCα on FGF-2 signalling, it has been reported that PKCα may regulate VSMC mineralisation through the stabilisation of the microtubule network (Lee et al., 2014b). Microtubule stabilisation is reduced in high phosphate-treated mouse VSMCs (Lee et al., 2014a; Lee et al., 2014b), and stabilisation of the microtubule network with the anti-cancer drug, paclitaxel, reduced VSMC mineralisation (Lee et al., 2014a). Whilst the polymerised tubulin/free tubulin ratio (used in the Lee et al (2014b) study as an indicator of microtubule stabilisation) was reduced in Gö6976- and high phosphate-treated VSMCs when compared to cells cultured with high phosphate alone, it was not determined if this was a consequence of enhanced mineral deposition in Gö6976-treated cells, or if this was due to direct inhibition of PKCα activity. Changes in microtubule stabilisation during VSMC mineralisation were not investigated in this thesis, but as it has been proposed that stabilisation of the microtubule network reduces calcifying MV release (Lee et al., 2014a), it would be of interest to determine the role of this pathway in the regulation of VSMC mineralisation by syndecan 4/PKCα.

It has also been suggested that PKCα regulates osteoblast differentiation and mineralisation though the suppression of Wnt target gene expression (Galea et al., 2014). Syndecan 4 can also regulate Wnt signaling as syndecan 4 over-expression and fibronectin inhibited the Wnt/β-catenin pathway (Astudillo et al., 2014). Wnt signalling has a role in vascular calcification, as Wnt3a-induced β-catenin signalling enhances the osteogenic differentiation of VSMCs (Faverman et al., 2008), and both aortic mineral deposition and β-catenin signalling are inhibited by SMC-specific activation of parathyroid hormone signalling in LDLR-null mice fed a high-fat diet (Cheng et al., 2010). Taken together, these data suggest that knocking-down syndecan 4 or PKCα expression in VSMCs could lead to increased VSMC mineralisation through the regulation of Wnt and/or FGF-2 signalling. This interesting hypothesis requires further investigation.
Whilst over-expressing PKCα in the osteoblastic MC3T3-E1 cell line reduces ALP activity and the expression of several osteogenic marker genes in these cells (Nakura et al., 2011), the effects of over-expressing PKCα on osteoblast or VSMC mineralisation are unknown. Previous studies have shown that activation of PKC with the phorbol ester, phorbol-12-myristate-13-acetate (PMA), has no effect on matrix mineralisation in MC3T3-E1 cells (Nakura et al., 2011); however there are several limitations regarding the experimental design of this study to consider. Firstly, PMA is a pan-PKC activator and is not specific to the PKCα isoform. Secondly, it has been well established that prolonged or repetitive treatment with phorbol esters leads to the de-phosphorylation and subsequent degradation of PKC, thereby depleting PKC from cells (Blumberg, 1980; Nelson and Alkon, 2009). In the Nakura et al (2011) study, MC3T3s were treated with differentiation media and PMA for 28 days; therefore it is possible that PKC was depleted in these cells. Together, these results bring into question whether matrix mineralisation is unaffected in PMA-treated MC3T3s due to the activation or loss of PKC. Moreover, it is unclear which PKC isoform was modulated in these experiments. Over-expression studies are therefore required to determine if PKCα can inhibit VSMC mineralisation.

7.4 Conclusions

The following conclusions can be drawn from the results presented in this chapter:

- Knocking-down EXT1 expression increased VSMC mineralisation, but at high concentrations, the addition of heparin also increased VSMC mineralisation. These results may therefore suggest that the presence of cell surface/matrix HS at a regulated level is required to put a ‘brake’ on the mineralisation process.
- Inhibiting PKCα activity with Gö6976, or knocking-down PKCα expression using siRNA, increased VSMC mineralisation suggesting that like syndecan 4, PKCα acts as a ‘brake’ on the mineralisation process.
8  General discussion

Vascular calcification is the formation of mineralised tissue, bone and/or cartilage within the vessel wall. Most patients with cardiovascular disease have some degree of calcification, although it is most prevalent in patients with CKD, type 2 diabetes mellitus and atherosclerosis (Lehto et al., 1996; Nakayama et al., 2011). Calcification is not only highly prevalent in these diseases, but there is now substantial evidence that it contributes to the morbidity and mortality associated with these common conditions (Rennenberg et al., 2009; London et al., 2011). Existing approaches for the prevention of vascular calcification are limited (Wu et al., 2013), therefore, there is a great need to find new therapeutic targets for the treatment of this devastating pathology.

The first aim of this study was to characterise the expression profile of different PGs during the osteogenic differentiation and mineralisation of VSMCs. Interestingly, syndecan 4 mRNA expression was increased during the later stages of VSMC osteogenic differentiation and mineralisation. The mRNA expression levels of several other PGs were also differentially regulated during this process. Notably, glypican 4 mRNA expression was up-regulated, and syndecan 1, biglycan, decorin, versican, osteoglycin and lumican mRNAs were all down-regulated. These changes in gene expression did not occur when preparations of VSMCs that do not deposit a mineralised matrix in the presence of $\beta$-GP were analysed. Together, these data suggest that the changes observed in the expression levels of syndecan 4, and other PGs, are associated with the osteogenic differentiation and mineralisation of VSMCs, and are not due to extended culture in the presence of $\beta$-GP.

Syndecan 4 is a type I transmembrane glycoprotein that is capable of displaying HS and/or CS/DS chains on its extracellular domain (Shworak et al., 1994; Ueno et al., 2001; Deepa et al., 2004; Gopal et al., 2010). This PG can interact with a variety of growth factors, thereby regulating many aspects of cell behaviour, including apoptosis (Tkachenko et al., 2005; Echtermeyer et al., 2011; Xie et al., 2012; Elfenbein and Simons, 2013; Couchman et al., 2015). Both growth factor signalling (Table 1.1) and apoptosis (Proudfoot et al., 2000; Collett et al., 2007; Clarke et al., 2008) regulate vascular calcification, suggesting that syndecan 4 may also play a role in this condition. This study demonstrated for the first time that knocking-down syndecan 4 expression using siRNA increased VSMC mineralisation, both in the presence of $\beta$-GP and in the presence of $\beta$-GP and TGF-$\beta 1$. In contrast, knocking-down syndecan 4 expression in a preparation of VSMCs that do not mineralise in the presence of $\beta$-GP did not stimulate the deposition of a mineralised matrix by these cells. Together these data suggest that syndecan 4 may act as a brake on the VSMC mineralisation process, but importantly, it is not a driver of the mineralisation process on its own.

Whilst knocking-down syndecan 4 expression increased VSMC mineralisation, the signalling pathways regulated by syndecan 4 during this process were unknown. Syndecan 4 has been previously established as a regulator of FGF-2 signalling (Zhang et al., 2003; Partovian et al., ....
therefore, the role of FGF-2 in VSMC mineralisation was determined. This study demonstrated for the first time that FGF-2 mRNA and protein expression were increased during the late stages of VSMC osteogenic differentiation and mineralisation, coinciding with increased syndecan 4 mRNA expression in these cells. Interestingly, exogenous FGF-2 inhibited VSMC mineralisation and blocking endogenous FGF-2 signalling with an FGFR inhibitor (BGJ398) increased VSMC mineralisation, suggesting that FGF-2 plays an inhibitory role in the mineralisation process. Furthermore, syndecan 4 regulated FGF-2-induced Akt activation in VSMCs, and syndecan 4 was required for the inhibition of VSMC mineralisation by low concentrations (25 ng/ml) of FGF-2. Therefore, syndecan 4 expression may be increased in mineralising VSMC to maintain FGF-2 signalling, and in turn, slow down the mineralisation process.

It is important to note that blocking endogenous FGF signalling using BGJ398 in VSMCs was not enough to induce mineralisation in the absence of raised phosphate levels, or in a preparation of VSMCs that do not mineralise even in the presence of β-GP. Similar results have been observed with the soluble elastin-derived peptide α-elastin, which accelerated human VSMC mineralisation in the presence of high (2.5 mM) phosphate levels, but did not stimulate mineralisation under normal phosphate load (1.4 mM) (Hosaka et al., 2009). This was a recurring theme in this thesis; neither syndecan 4, FGF-2 nor PKCα (see below) appear to act as drivers of the VSMC mineralisation process, and instead they may act as ‘brakes’. These findings are perhaps not surprising as syndecan 4- (Echtermeyer et al., 2001), FGF-2- (Zhou et al., 1998) and PKCα-null mice (Leitges et al., 2002) are viable and fertile, and do not have an overt phenotype unless challenged in a disease model. It is possible that the other members of the syndecan, FGF and PKC family are able to compensate for a loss of syndecan 4, FGF-2 or PKCα during development in vivo; it is also possible that this may occur to some extent in the in vitro model of vascular calcification used in this thesis.

The HS chains displayed on PG core proteins can regulate growth factor signalling (including FGF-2) by acting as co-receptors, stabilising signalling gradients and/or by protecting ligands from degradation (Roehl and Pacifici, 2010). Therefore, in addition to the PG analysis carried out in this study, the GAGs synthesised by VSMCs at confluence and during VSMC mineralisation were also analysed. These studies demonstrated that the composition of HS chains synthesised by two separate preparations of VSMCs were very similar, and there were no marked changes in HS disaccharide composition during VSMC osteogenic differentiation and mineralisation. However, HS chains isolated from the cellular/matrix extract of mineralising VSMCs were smaller in size when compared to confluent cells, although this did not appear to be related to changes in heparanase mRNA expression.

To determine if the HS chains displayed on syndecan 4 (and other PGs) regulate VSMC mineralisation, siRNAs against EXT1 were used. The EXT1 gene encodes a HS biosynthetic enzyme that is responsible for polymerising the HS chain backbone, and knocking-down EXT1 expression using siRNA has been shown to reduce HS biosynthesis in vitro (Busse et al., 2007;
Knocking-down EXT1 expression in VSMCs increased matrix mineralisation and reduced FGF-2-induced Akt activation when compared to negative control siRNA-treated cells. These findings reproduced the results obtained with syndecan 4 siRNA-treated VSMCs, suggesting that the HS chains displayed on the extracellular domain of syndecan 4 (and likely other PGs) are important to its role in the VSMC mineralisation process. Indeed, the FGFs are able to bind to FGFRs with high affinity, but this interaction and subsequent signaling events are amplified in the presence of HS, which is thought to act as a co-receptor for FGF/FGFR binding (Yayon et al., 1991; Rapraeger et al., 1991; Kan et al., 1999).

Although syndecan 4 is predominantly thought to display to HS chains on its extracellular domain, there is also evidence to suggest that CS/DS chains can be synthesised onto the syndecan 4 core protein (Shworak et al., 1994; Ueno et al., 2001; Deepa et al., 2004; Gopal et al., 2010). The studies presented in this thesis demonstrated that the mRNA levels of specific CS/DS 4-O-sulphotransferases (C4ST1, C4ST2) were up-regulated in mineralising VSMCs, correlating with an increase in CS/DS 4-O-sulphation. DS epimerase-1 and -2 mRNA were also significantly increased in mineralising VSMCs, correlating with an increase in the DS to CS ratio in these cells. Interestingly, CHO cells (pgSD-677) deficient in HS still exhibit FGF-2 signalling (Ashikari-Hada et al., 2009), indicating that the remaining CS/DS can support FGF-2 signalling in these cells. Indeed, CS/DS binds FGF-2, and like HS, FGF-2 binding and FGF-2-induced Erk1/2 activation is regulated by CS/DS chain sulphation (Ramachandra et al., 2014; Nikolovska et al., 2015). DS preparations also bind FGF-2, and DS enhances FGF-2 induced cell proliferation in F32 lymphoid cells (Penc et al., 1998; Taylor et al., 2005). Therefore, increases in the DS to CS ratio and CS/DS 4-O-sulphation in mineralising VSMCs could act to modulate FGF-2 activity in these cells. In light of these findings, future studies could determine if CS/DS chains are displayed on the syndecan 4 core protein isolated from VSMCs, and if these GAGs are biologically active with respect to FGF-2 signalling in these cells. Indeed, FGF-2 binding to the syndecan 4 core protein is only maximal when the HS and CS/DS chains are both present on the core protein (Deepa et al., 2004), suggesting that CS/DS and HS act co-operatively on syndecan 4 to bind FGF-2 and enhance FGF-2 signalling.

In light of the changes in CS/DS chain composition during VSMC mineralisation, it would also be of interest to determine the role of CS/DS in VSMC mineralisation. For example, siRNA could be used to knock-down DS epimerase-1 and -2 expression, thereby preventing the epimerisation of CS to DS. The effects of knocking-down DS biosynthesis on VSMC mineralisation and FGF-2 signalling could then be determined to deduce if DS plays an inhibitory or stimulatory role in this process.

Whilst knocking-down syndecan 4 expression increased VSMC mineralisation, over-expressing syndecan 4 in VSMCs also increased β-GP-induced matrix mineralisation. Similarly, knocking-down HS synthesis using siRNA against EXT1 or the addition of heparin both increased VSMC mineralisation. The concentration-dependent effects of both HS and syndecan 4 on FGF-2
signalling may explain these apparently contradictory results. In this regard, the ability of heparin to support or inhibit signalling by distinct ligands is concentration-dependent, as low concentrations of heparin can potentiate a GAG-dependent signalling pathway, whilst high concentrations prevent access of the ligand to the cell surface receptor, switching to an inhibitory effect (demonstrated in differentiating mouse ESCs by (Holley et al., 2011b)). Therefore, the presence of HS and syndecan 4, rather than an increase above their endogenous levels, may be required to put a ‘brake’ on VSMC mineralisation. Indeed, a U-curve relationship has also been proposed between vitamin D and vascular calcification, with high calcification scores associated with both very high and very low levels of vitamin D (Shroff et al., 2008a; Drüeke and Massy, 2012). HS and syndecan 4 may therefore not be a suitable therapeutic target in the treatment of vascular calcification as their expression levels have to be finely regulated during this process.

The *in vitro* model of vascular calcification used in this study is predominantly driven by high phosphate, mimicking one of factors that drives medial calcification in CKD patients (Shigematsu et al., 2003); however, additional factors may drive mineralisation in these patients such as inflammation (Shao et al., 2010). Syndecan 4 exerts anti-inflammatory effects in models of pulmonary inflammation (Tanino et al., 2012), hepatic injury (Kon et al., 2008), myocardial infarction (Xie et al., 2012) and pressure-overload (Strand et al., 2013). In addition, inflammatory mediators such as TNF regulate syndecan 4 expression in VSMCs (this study), as well as in other cell types (Zhang et al., 1999; Wang et al., 2011a; Tan et al., 2012; Strand et al., 2013; Bertrand et al., 2013; Ramnath et al., 2014). In light of the evidence, the induction of syndecan 4 expression during VSMC mineralisation may represent a negative feedback mechanism to help to dampen the inflammatory response in mineralising VSMCs. Therefore, the presence of syndecan 4 may also be influential during the progression of vascular calcification in a model where inflammatory factors play more of a prominent role (discussed further in section 8.1.3).

One of the final aims of this study was to determine the role of PKCα in VSMC mineralisation. Previous studies have shown that PKCα activation is reduced in syndecan 4-null endothelial cells (Partovian et al., 2008; Ju and Simons, 2013), and PKCα is required for FGF-2 induced Akt activation (Partovian et al., 2008; Ju and Simons, 2013), suggesting that the interaction between syndecan 4 and PKCα could be important to the ability of this PG to regulate VSMC mineralisation. Inhibiting PKCα activity with Gö6976, or knocking-down PKCα expression using siRNA, increased VSMC mineralisation. As the effects of knocking-down syndecan 4 expression on VSMC mineralisation were reproduced by knocking-down PKCα expression in these cells, these data could suggest that syndecan 4’s interaction with PKCα is required for the inhibitory effect of this PG on VSMC mineralisation.

Whilst there are no specific activators of PKCα currently available, there is increasing interest in generating PKCα-specific activators for the treatment of cancer (Antal et al., 2015). To determine if PKCα could also be a suitable therapeutic target in the treatment of vascular calcification, a VSMC cell line stably over-expressing PKCα could be used to determine if
increasing PKCα expression inhibits the deposition of a mineralised matrix by VSMCs. In vivo studies are also required to determine the effects of knocking-down PKCα expression in vascular calcification.

8.1 Future work

8.1.1 Verifying the role of endogenous FGF-2 signalling in VSMC mineralisation
A limitation of this study is that a pan-FGFR inhibitor (BGJ398) was used to determine the role of endogenous FGF-2 signalling in VSMC mineralisation. Whilst FGF-2 signals through all four FGFRs, signalling by the other FGFs could also be inhibited when using BGJ398. An alternative approach would be to knock-down FGF-2 expression in VSMCs using siRNA, and induce these cells to mineralise in the presence of β-GP.

8.1.2 Investigating the other signaling pathways regulated by syndecan 4 during VSMC mineralisation
The effects of knocking-down syndecan 4 expression on VSMC mineralisation were linked to FGF-2 signalling in this study; however, syndecan 4 may also regulate other signaling pathways important in this process. For example, syndecan 4 has been proposed to inhibit Wnt/β-catenin signalling (Astudillo et al., 2014), and Wnts play a pro-calcific role in vascular calcification (Shao et al., 2005; Faverman et al., 2008; Cheng et al., 2010). Therefore, one could hypothesise that VSMC mineralisation is increased in the absence of syndecan 4 due to increased Wnt/β-catenin signalling in these cells. To begin to investigate this hypothesis, Wnt-induced β-catenin activation could be assessed in negative control siRNA and syndecan 4 siRNA-treated VSMCs. If syndecan 4 regulates Wnt-induced β-catenin activation in VSMCs, the effects of inhibiting Wnt signalling during VSMC mineralisation (e.g. using pyrvinium (Thorne et al., 2010)) in the presence and absence of syndecan 4 could be assessed. In this regard, inhibition of Wnt signalling in syndecan 4 siRNA-treated VSMCs could prevent the increase in matrix mineralisation observed in these cells.

8.1.3 Investigating the role of syndecan 4 in vascular calcification in vivo
Knocking-down syndecan 4 expression in an in vitro model of vascular calcification increased mineral deposition when compared to controls. Of course, in vitro models cannot always recapitulate the disease in vivo, therefore investigating the role of syndecan 4 in an in vivo model of vascular calcification is required. For example, vascular calcification could be induced in syndecan 4-null mice using the 5/6 nephrectomy and high phosphate diet model (Lau et al., 2012; Hyde et al., 2014), which recapitulates vascular calcification in CKD. Alternatively, syndecan 4-null mice could be crossed onto the ApoE-null background, which develop atherosclerosis with calcification when fed a high-fat diet (Naik et al., 2012). If syndecan 4 can act as a brake on VSMC mineralisation and exert anti-inflammatory effects, the double knock-out mice should have faster lesion development and/or more extensive disease. Indeed, atherosclerotic lesion development is enhanced in double knock-out syndecan 4/ApoE-null mice.
(Baeyens et al., 2014), but the effects on vascular calcification were not investigated in this study.

To further verify a role for syndecan 4 in vascular calcification, a detailed analysis of syndecan 4 localisation and expression in human calcified arteries is also required to determine if syndecan 4 co-localises to areas of calcification in the vessel wall.

8.1.4 Role of PKCα in VSMC mineralisation in vitro

Based on the evidence presented in thesis and published findings (Oh et al., 1997b; Oh et al., 1997a; Oh et al., 1998; Horowitz and Simons, 1998b; Horowitz and Simons, 1998a; Keum et al., 2004; Partovian et al., 2008), it was eluded that syndecan 4 could regulate VSMC mineralisation through its interaction with PKCα. To confirm syndecan 4 regulates the localisation of and subsequent activation PKCα at the cell membrane in VSMCs, negative control- and syndecan 4 siRNA-treated VSMCs could be stimulated with FGF-2; the phosphorylation of PKCα and expression of total PKCα at the cell membrane could then be assessed by western blotting.

Knocking-down PKCα expression in VSMCs increased β-GP-induced matrix mineralisation, but the effects of over-expressing PKCα are currently unknown. Therefore, PKCα could be over-expressed in VSMCs using lentivirus, and passage-matched control lentivirus and PKCα over-expressing VSMCs could be induced to mineralise in the presence of β-GP.

PKCα has the potential to regulate VSMC mineralisation via several signalling pathways, including FGF-2 (Partovian et al., 2008; Ju and Simons, 2013), Wnts (Galea et al., 2014) and microtubule stabilisation (Lee et al., 2014b). To determine if these signalling pathways are regulated by PKCα in VSMC mineralisation, Wnt-induced β-catenin activation and FGF-2-induced Akt activation could be assessed in PKCα siRNA-treated VSMCs and/or VSMCs isolated from PKC-null mice.

8.1.5 Investigating the role of PKCα in vascular calcification in vivo

As discussed in section 8.1.1, the in vitro model of vascular calcification used in this study is predominantly driven by high phosphate and cannot therefore reflect the influence of other factors in the mineralisation process, such as inflammation and oxidative stress. Therefore, to determine the role of PKCα in vascular calcification in vivo, a model of CKD and vascular calcification could be used in wild-type and PKCα-null mice, such as the 5/6 nephrectomy and high phosphate diet mouse model (Lau et al., 2012; Hyde et al., 2014). Alternatively, a model of vascular calcification in atherosclerosis could be used, such as the ApoE-null mouse with high-fat diet (Naik et al., 2012).

If over-expressing PKCα inhibits VSMC mineralisation in vitro (see section 8.1.4), a PKCα-specific activator or adenoviral delivery of PKCα in mice could also be used to determine if increased PKCα activity is able to inhibit VSMC mineralisation in vivo.
8.2 Overall conclusion

Overall this study has shown that several PG core proteins are differentially regulated during VSMC mineralisation, and this is associated with compositional changes in the CS/DS GAG chains synthesised by mineralising VSMCs. This study has also shown that the expression of one specific PG, syndecan 4, may be increased in mineralising VSMCs to maintain FGF-2 signalling and slow down calcification. The HS chains displayed on syndecan 4 (and likely other PGs), and its interaction with PKCα, are likely required for the inhibitory effect of syndecan 4 on VSMC mineralisation. Therefore, the FGF-2/syndecan 4/PKCα signalling axis may form the basis for novel therapies in the treatment of vascular calcification.


smooth muscle cells during neointima formation and in atherosclerotic plaques. *Arterioscler Thromb Vasc Biol*, 17(11), 2437-47.


Figure 10.1. Knocking-down syndecan 1 expression has no effect on VSMC mineralisation. VSMC-A transfected with negative control siRNA or syndecan 1 siRNA were cultured with 3 mM β-GP for up to 12 days. Negative control siRNA-treated cells cultured without β-GP were used as an additional control. Both siRNAs were used at a final concentration of 40 nM. siRNA transfections and medium changes were carried out every 48-72 hours. (A) Phase-contrast images of cells stained with alizarin red. Scale bar = 500 µm. (B) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 3 wells of cells from 3 independent experiments.
Figure 10.2. FGF-2 modulates VSMC mineralisation in a time-dependent manner. Confluent VSMC-A were cultured with 5 mM β-GP ± 25 ng/ml FGF-2. FGF-2 was included in the culture medium of β-GP-treated cells continuously (Days 0-12) or for specific time-points during the β-GP-treatment protocol (refer to text in Figure). (A) Phase-contrast images of cells stained with alizarin red on day 12. Scale bar = 500 µm. (B) The extent of mineralisation was quantified by dye elution. Results displayed as mean ± SEM and were derived from 3 wells of cells from 2 independent experiments. The data were analysed using a 1-way ANOVA with Dunnet post-hoc tests. * P<0.05; ** P<0.01; *** P<0.001 against β-GP control.
Figure 10.3. Knocking-down syndecan 4 expression in human coronary artery SMCs. (A) Human coronary artery SMCs were seeded into 6-well plates treated with 0.1% (v/v) gelatin at 2 X 10^4 cells/cm^2. Cells were transfected with negative control siRNA or syndecan 4 siRNA-2 at a final concentration of 20 nM. Forty-eight hours after the 2nd siRNA transfection, the cells were harvested for RNA. Knock-down of syndecan 4 expression was then confirmed using qPCR. Syndecan 4 mRNA expression is shown relative to the reference genes, GAPDH and β-actin. (B) Phase-contrast images of cells were taken (Bi) before the commencement of siRNA-treatment (day 0), and (Bii) 48 hours after the 2nd siRNA transfection. Scale bar = 500 µm.
Figure 10.4. Knocking-down syndecan 4 expression in human MSCs. (A) Human MSCs were seeded into 6-well plates treated with 0.1% (v/v) gelatin at $6 \times 10^3$ cells/cm$^2$. Cells were transfected with negative control siRNA or syndecan 4 siRNA-2 at a final concentration of 20 nM. Knock-down of syndecan 4 expression was confirmed using qPCR. Syndecan 4 mRNA expression is shown relative to the reference genes, TBP and GAPDH. (B) Phase-contrast images of cells before the commencement of siRNA treatment (day 0). Phase-contrast images of cells were then taken 48 hours after the 1$^{st}$ siRNA transfection (day 2), and 48 hours after the 2$^{nd}$ siRNA transfection (day 4). Scale bar = 500 µm.