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THE ROLE OF OSTEOCYTE MARKERS IN MEDIAL VASCULAR CALCIFICATION

Dongxing Zhu
This thesis is presented for the degree of
Doctor of Philosophy at University of
Edinburgh
2013
Declaration

I declare that this thesis has been composed entirely by the candidate, Dongxing Zhu. This thesis has not been previously submitted for a Doctor of Philosophy, a degree or any professional qualification. I have done all the work, unless acknowledged otherwise. All source of information has been acknowledged.

Dongxing Zhu
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Finally, a big thank you must be paid to my friends and family, especially my parents for their support and encouragement.
Abstract

Vascular calcification is prevalent in ageing, in atherosclerosis, and especially in patients with Chronic Kidney Disease (CKD), with associated increased morbidity and mortality. The phenotypic transition of Vascular Smooth Muscle Cells (VSMCs) into osteoblastic/chondrogenic-like cells is critical for the development of calcification in CKD patients. Osteocytes, terminally differentiated osteoblasts, have recently emerged as major regulators of calcification in bone. Recently, osteocyte-like cells have been observed in human peripheral arteries with medial vascular calcification. However, it remains undetermined as to whether VSMCs can undergo osteocytic differentiation within a calcifying environment and the functional role of osteocyte formation in the development of medial vascular calcification.

Initial studies have characterised the ectonucleotide pyrophosphatase/phosphodiesterase 1 knockout (Enpp1−/−) mouse as a valid model of medial vascular calcification, which is employed throughout this thesis. This thesis has compared VSMCs to osteoblasts undergoing osteocytic differentiation in vitro. VSMC in vitro calcification was accompanied by up-regulated expression of osteocyte markers, including Sost, E11, Dmp1, Phex, Mepe and Fgf23. Immunohistochemistry confirmed the appearance of sclerostin and E11 in calcified aortae from the Enpp1−/− mouse. Further studies have identified a direct inhibitory role for the osteocyte specific gene FGF23 in modulating vascular calcification. The inhibitory effect of FGF23 on VSMC calcification was mediated through the MAPK/ERK signalling pathway. This thesis has also determined the role of BMP9, a highly osteogenic bone morphogenic protein, in vascular calcification, which induces VSMC calcification through a Smad signalling mechanism. Furthermore, VSMC expression of the osteocytic marker Sost was markedly increased following BMP9 treatment. Intriguingly, BMP9 was markedly elevated in serum from dialysis patients and a significant correlation was observed between dialysis time and BMP9 concentration in patients receiving haemodialysis.

The work described herein has demonstrated that vascular calcification is associated
with an osteocyte phenotype, and reports a direct inhibitory effect of the osteocyte specific gene FGF23 on vascular calcification. Furthermore, this thesis has shown that BMP9 induces the expression of the osteocytic marker *Sost* in VSMCs, and appears to play a critical role in vascular calcification.
Publications

Original peer reviewed papers arising from this thesis


Additional publications


Mackenzie NCW, Staines KA, Zhu D, Genever P, MacRae VE (2013). miRNA-221 and miRNA-222 synergistically function to promote vascular calcification. (Submitted to *Cell Biochemistry & Function*)

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**Published Meeting Abstracts**

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*Bone Research Society Annual Meeting* - Cambridge June 2011  
New Investigator Award for the abstract entitled “The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells”

*European Calcified Tissue Society* - Lisbon May 2013  
Young Investigator Award for the abstract entitled “A protective role for FGF-23 in local defence against disrupted arterial wall integrity”

*Bone Research Society Annual Meeting* - Oxford September 2013  
New Investigator Award for the abstract entitled “BMP9 induces the calcification of vascular smooth muscle cells”
### Abbreviations

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<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>4-PL</td>
<td>Four parameter logistic</td>
</tr>
<tr>
<td>ADHR</td>
<td>Autosomal dominant hypophosphotemic rickets</td>
</tr>
<tr>
<td>ALK1</td>
<td>Activin receptor-like kinase 1</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APRT</td>
<td>Adenine phosphoribosyltransferase</td>
</tr>
<tr>
<td>ASARM</td>
<td>Acidic serine- and aspartate-rich motif</td>
</tr>
<tr>
<td>AV</td>
<td>Atrioventricular</td>
</tr>
<tr>
<td>BMPs</td>
<td>Bone morphogenic proteins</td>
</tr>
<tr>
<td>Bsp</td>
<td>Bone sialoprotein</td>
</tr>
<tr>
<td>CAVS</td>
<td>Calcific aortic valve stenosis</td>
</tr>
<tr>
<td>Cbf2β</td>
<td>Transcriptional co-activator core binding protein 2β</td>
</tr>
<tr>
<td>CMD</td>
<td>Cranio-metaphyseal dysplasia</td>
</tr>
<tr>
<td>CVCs</td>
<td>Calcifying vascular cells</td>
</tr>
<tr>
<td>DMP1</td>
<td>Dentin matrix protein 1</td>
</tr>
<tr>
<td>DNB</td>
<td>Dimethoxy-N-(quinolin-3-yl) benzenesulfonamide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ENPP1</td>
<td>Ecto-nucleotide pyrophosphatase/phosphodiesterase 1</td>
</tr>
<tr>
<td>ESRD</td>
<td>End stage renal disease</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FGF23</td>
<td>Fibroblast growth factor 23</td>
</tr>
<tr>
<td>GACI</td>
<td>Generalized arterial calcification of infancy</td>
</tr>
<tr>
<td>Gas6</td>
<td>Growth arrest specific gene 6</td>
</tr>
<tr>
<td>GLA</td>
<td>Gamma-carboxyglutamic acid</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
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<tr>
<td>HBSS</td>
<td>Hanks buffered saline solution</td>
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<td>HHT</td>
<td>Hereditary hemorrhagic telangiectasia</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoproteins</td>
</tr>
<tr>
<td>Igf1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low-density lipoprotein receptor</td>
</tr>
<tr>
<td>LDS</td>
<td>Lithium dodecyl sulphate</td>
</tr>
<tr>
<td>LRP5</td>
<td>Lipoprotein receptor-related protein 5</td>
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<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MGP</td>
<td>Matrix Gla Protein</td>
</tr>
<tr>
<td>MEPE</td>
<td>Matrix extracellular phosphoglycoprotein</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino) ethanesulfonic acid</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanesulfonic acid</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>Membrane-type matrix metalloproteinase 1</td>
</tr>
<tr>
<td>MVs</td>
<td>Matrix vesicles</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-Steroidal Anti-Inflammatory Drugs</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleoside triphosphate</td>
</tr>
<tr>
<td>Ocn</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>Opn</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>Osx</td>
<td>Osterix</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PHEX</td>
<td>Phosphate-regulating gene with homologies to endopeptidases on the X chromosome</td>
</tr>
<tr>
<td>Pi</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PPi</td>
<td>Pyrophosphate</td>
</tr>
<tr>
<td>Prrx1</td>
<td>Paired related homeobox 1</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear-κB</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear-κB ligand</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>ROCK</td>
<td>RhoA/Rho-kinase</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SL</td>
<td>Semilunar</td>
</tr>
<tr>
<td>SMA</td>
<td>α-smooth muscle-actin</td>
</tr>
<tr>
<td>SMM-HC</td>
<td>SM-myosin heavy chain</td>
</tr>
<tr>
<td>SOC</td>
<td>Super Optimal Broth with Catabolite repression</td>
</tr>
<tr>
<td>SOST</td>
<td>Sclerostin</td>
</tr>
<tr>
<td>SPC</td>
<td>Subtilisin-like proprotein convertases</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline containing 0.1% Tween 20</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TIO</td>
<td>Tumor-induced osteomalacia</td>
</tr>
<tr>
<td>TNAP</td>
<td>Tissue non-specific alkaline phosphatase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VICs</td>
<td>Valve interstitial cells</td>
</tr>
<tr>
<td>VKDPs</td>
<td>The vitamin K-dependent proteins</td>
</tr>
<tr>
<td>VKOR</td>
<td>Vitamin K epoxide reductase</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>VSMCs</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>XLH</td>
<td>X-linked hypophosphatemia</td>
</tr>
<tr>
<td>α-MEM</td>
<td>Minimum essential medium alpha</td>
</tr>
<tr>
<td>µCT</td>
<td>Micro-computed tomography</td>
</tr>
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1 Introduction

1.1 Overview

Vascular calcification was first documented by Mönckeberg in the 19th century, and for many years, it was regarded as a passive and degenerative disease without conservative treatment options (Virchow, 1899). Over the past two decades, more and more scientists have focused their research interest on this subject, as vascular calcification has severe clinical outcomes. Extensive research has shown that vascular calcification is a tightly regulated, cell-mediated process that shares many similarities with bone mineralisation (Abedin et al., 2004; Shroff and Shanahan, 2007). Although the precise mechanisms through which vascular calcification occurs remain unclear, its incidence highly correlated with mortality and morbidity of cardiovascular disease, especially in patients with atherosclerosis, end stage renal disease (ESRD) and diabetes (Giachelli, 2004; Shroff and Shanahan, 2007). By understanding better the molecular pathways and genetic circuitry responsible for the pathological mineralisation process, novel drug targets may be identified and exploited to combat and reduce the detrimental effects of vascular calcification on human health. This thesis will identify novel pathways associated with vascular calcification, using an in vitro Vascular Smooth Muscle Cell (VSMC) calcification model in conjunction with an animal model of medial vascular calcification, ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1-/-) mice. It should be noted that many sections of this introduction are based on my published review on the mechanisms and clinical consequences of vascular calcification (Zhu et al., 2012).

1.2 Blood vessel structure and function

The blood vessels are the part of circulatory system that transports blood throughout the body. There are three major types of blood vessels: arteries (carrying the blood away from the heart), capillaries (exchanging water and chemicals between the blood and the tissues) and veins (carrying blood from capillaries back to the heart). Large vessels (arteries and veins) consist of three main layers, which are the tunica intima, the tunica media and the tunica adventitia. The tunica intima is the innermost layer of endothelial cells in direct contact with blood. The tunica media consists of VSMCs
and elastic tissue. VSMCs change volume of blood vessels and local blood pressure via contraction and relaxation. The outermost layer of large vessels is the tunica adventitia, which is made up of collagen fibres. It protects blood vessels and anchors it to surrounding structures (Figure 1.1). Different to large vessels, capillaries only have a single layer of endothelial cells which permits exchanges of material between the contents of capillaries and the surrounding tissue. Calcification can occur in all large of arteries.

1.3 Vascular calcification

1.3.1 Types of vascular calcification

Vascular calcification can be categorised into four main types according to its location: atherosclerotic intimal calcification, medial calcification (Mönckeberg’s sclerosis), cardiac valve calcification and calcific uremic arteriolopathy (Vattikuti and Towler, 2004) (Figure 1.2). Histologically, calcified deposits may be amorphic, chondromorphic or osteomorphic in structure, and may be characterised as metastatic or dystrophic.

1.3.1.1 Atherosclerotic intimal calcification

Atherosclerosis is the development of plaques within the intimal layer of large vessels, and underlies coronary artery disease and cerebrovascular disease, the most common forms of life threatening cardiovascular disorders (Doherty et al., 2004). Atherosclerosis can be induced by chronic inflammation and lipid deposition, with dyslipidemia frequently linked to the severity of calcium deposition (Pohle et al., 2001; Schmermund et al., 2001). Atherosclerotic calcification is the most common form of calcific vasculopathy, and occurs as early as the second decade of life just after fatty streak formation (Stary et al., 1995). Small aggregates of crystalline calcium can be detected in developing lesions, and in adults past the fourth decade of life, greater lesion areas may be calcified (Stary, 2000). The degree of calcification correlates with the extent of atherosclerosis, with age and hypertension as dominant risk factors for systemic calcified atherosclerosis (Allison et al., 2004).
Figure 1.1 Diagrammatical representation of the structure of arteries. This diagram summarises the structure of a normal large artery which consists of three distinct layers: intima, media and adventitia (Lusis et al., 2000).

Figure 1.2 Types of vascular calcification. (A), Atherosclerotic intimal calcification. Diagram showing a ruptured atherosclerotic plaque, areas of calcification are indicated. The arrow indicates cholesterol crystals. (B), Medial calcification. Abundant calcification in the medial area of this artery is indicated by H & E staining. (C), Cardiac valve calcification. (D), Calcific uremic arteriolopathy (Johnson et al., 2006).
The predominant mineral form in calcified lesions is hydroxyapatite (HA) (Yu, 1974). In addition to mineral, these lesions also contain matrix vesicles (MVs) as well as outright bone and cartilage (Tanimura et al., 1983; Mohler et al., 2001; Hunt et al., 2002).

### 1.3.1.2 Medial calcification

Medial calcification, also termed Mönckeberg’s sclerosis, occurs in the tunica media of blood vessels. It is a characteristic feature of Generalized Arterial Calcification of Infancy (GACI), diabetes and Chronic Kidney Disease (CKD) (Chen and Moe, 2003; Giachelli, 2004; London et al., 2005; Shroff and Shanahan, 2007), and is associated with increased cardiovascular mortality and amputation risk (Chantelau et al., 1995; Lehto et al., 1996; London et al., 2003). Medial calcification occurs independently of atherosclerotic calcification and is a process similar to intramembranous bone formation, with no cartilaginous precursor required (Vattikuti and Towler, 2004). Calcium deposition can be observed throughout most of the medial width in the early stage of disease. At later stages of disease, the media is filled with circumferential rings of mineral. In some cases, osteocytes and bone trabeculae can also be observed (Shanahan et al., 1999).

### 1.3.1.3 Cardiac valve calcification

Heart valves allow unidirectional blood flow through the heart. The four main valves of the mammalian heart are: the two atrioventricular (AV) valves and the two semilunar (SL) valves. The AV valves including the mitral valve and the tricuspid valve are located between the atria and the ventricles. The SL valves are the aortic valve and the pulmonary valve and are located in the arteries leaving the heart. Calcific aortic valve disease is identified by thickening and calcification of the aortic valve leaflets (Figure 1.3) in the absence of rheumatic heart disease. It is divided into aortic sclerosis, in which the leaflets do not obstruct left ventricular outflow, and aortic stenosis, in which obstruction to the left ventricular outflow is present. A number of recent studies have shown that calcific aortic valve lesions have many features characteristic of an active cell-regulated process, including lipoprotein deposition (O’Brien et al., 1996; Olsson et al., 1999); chronic inflammation (Olsson
et al., 1994; Otto et al., 1994) and active calcification (Hunt et al., 2002; Rajamannan et al., 2003) and shares similar underlying mechanisms with atherosclerotic intimal calcification (Salhiyyah et al., 2011).

1.3.1.4 Calcific uremic arteriolopathy
Calcific uremic arteriolopathy is a severe type of widespread medial vascular calcification which occurs in blood vessels of patients with ESRD (often referred to as stage 5 CKD) (Qunibi et al., 2002). It affects cutaneous and subcutaneous arteries and arterioles, leading to intimal proliferation, fibrosis and thrombosis (Qunibi et al., 2002; Mwipatayi et al., 2007).

1.3.2 The cellular source of vascular calcification
Cells that are involved in vascular calcification include VSMCs, pericytes, Calcifying Vascular Cells (CVCs) and Valve Interstitial Cells (VICs) (Figure 1.4). These cells can produce calcified matrix and undergo osteo/chondrogenic differentiation in vitro under calcifying conditions. These cell types are closely related and may be variant phenotypes of one another (Minasi et al., 2002; Tintut et al., 2003).

1.3.2.1 Vascular Smooth Muscle Cells (VSMCs)
VSMCs normally reside in the media of blood vessels and are responsible for regulating vascular tone. VSMCs exhibit a contractile phenotype and highly express genes which are involved in maintaining myofilament structure and function. These genes include α-smooth muscle-actin (SMA), SM22α and SM-myosin heavy chain (SMM-HC) (Shanahan et al., 1993). VSMCs can be activated from a quiescent, differentiated state into an active proliferating and synthesising phenotype (Hedin et al., 1999). This phenotypic change is associated with loss of smooth muscle cell markers and can be induced by various stimuli in vitro, including various growth factors, injury or mechanical stress (Thyberg, 1996; Worth et al., 2001). VSMCs are thought to be the predominant cells associated with medial calcification (Essalihi et al., 2004; Narisawa et al., 2007), in contrast to intimal calcification which also involves lipids and inflammatory cells (Pohle et al., 2001; Schmermund et al., 2001).
Figure 1.3 Extensive calcific aortic valve stenosis in the valve leaflets of a patient undergoing valve replacement surgery. Abundant calcification is indicated by arrow.

Figure 1.4 Origin of osteoblastic-cells in the vascular wall. Several cell types may differentiate into osteoblast-type cells within the vessel wall under appropriate conditions, including VSMCs, pericytes and CVCs (Johnson et al., 2006). ROS indicates reactive oxygen species, PPI indicates pyrophosphate, and Runx2 indicates runt-related transcription factor 2.
Vascular calcification is prevalent in patients with CKD, especially those with ESRD. ESRD patients normally have hyperphosphatemia (2mM phosphate) compared to healthy control (1-1.5mM phosphate) (Giachelli, 2009). In mice, serum phosphate (Pi) is higher at baseline (2.6mM). However, serum Pi of those mice with surgically induced CKD can reach as high as 3.5mM (Mathew et al., 2008). A number of studies have shown that VSMCs cultured with high Pi can undergo calcification in vitro, and that this process involves the osteo/chondrogenic differentiation of VSMCs (Steitz et al., 2001; Speer et al., 2009) (Figure 1.5). This in vitro model has been widely used for investigating the cellular and molecular mechanisms of vascular calcification.

Figure 1.5 Morphology of calcification of human vascular smooth muscle cell cultures (HVSMCs). HVSMCs were cultured under high Pi or normal Pi for 10 days. Calcium deposition was examined by Von kossa staining. (A) No deposits were observed in the normal Pi culture (1.4mM). (B) Black calcium deposits were found in the extracellular regions of the high Pi culture (2.4mM) (Jono et al, 2000).

1.3.2.2 Pericytes

Perivascular cells were firstly described by Eberth (Eberth, 1871; cited in Collett et al., 2005) and Rouget (Rouget, 1879; cited in Collett et al., 2005) almost 150 years ago. Since then, these cells have been given many different names including Rouget cells, mural cells, deep cells and perivascular cells. In 1923, Zimmerman termed these cells “pericytes” (Zimmermann, 1923; cited in Collett et al., 2005). Pericytes are elongated, contractile cells found wrapped about precapillary arterioles outside the basement membrane and are present in veins, arteries and capillaries (Andreeva et al., 1998). Several pericycle markers have been identified, including SMA, non-
muscle actin, non-muscle and muscle myosin, amino peptidase-N, amino peptidase-A and a cell surface ganglioside (3G5) (Andreeva et al., 1998). Previous studies have shown that pericytes can differentiate into osteoblasts (bone-forming cells) and chondrocytes (cartilage cells) (Doherty et al., 1998; Farrington-Rock et al., 2004), suggesting that pericytes may be central to the aetiology of vascular calcification. Further studies have shown that signalling through the wingless-type MMTV integration site family (Wnt)/β-catenin pathway stimulates chondrogenic and inhibits adipogenic differentiation of pericytes (Kirton et al., 2007), which may directly contribute to the development and progression of calcium deposition. In addition, pericytes can form multicellular nodules that contain a mineralised matrix, similar to those found in calcified aortae (Doherty and Canfield, 1999; Cola et al., 2004). Molecules associated with bone development and formation have been observed in these mineralised nodules, emphasising the regulatory similarities between vascular and bone calcification (Doherty et al., 1998; Canfield et al., 2000).

1.3.2.3 Calcifying Vascular Cells (CVCs)
CVCs are a subpopulation of smooth muscle cells and exhibit osteoblastic characteristics and undergo spontaneous calcification in vitro (Watson et al., 1994; Balica et al., 1997; Tintut et al., 1998; Radcliff et al., 2005). CVCs have features in common with pericytes including similar shape and osteoblastic characteristics as well as positive staining with 3G5 antibody (Watson et al., 1994). During osteogenic differentiation, CVCs accumulate not only minerals but also lipids such as triglycerides. Indeed, the induction of de novo lipogenesis promotes the calcification of CVCs under pro-osteogenic conditions such as high Pi levels (Ting et al., 2011). Studies characterising the calcific nodules produced by CVCs in Apolipoprotein E (ApoE)-null mice have revealed that the nodules resemble calcific atherosclerotic plaque and can be destabilised in the presence of active lipids and monocytes (Li et al., 2012), providing a novel animal model of vulnerable plaque dynamics.

1.3.2.4 Valve Interstitial Cells (VICs)
Calcification of the aortic valve occurs following trans-differentiation of VICs through a myofibroblast stage into osteoblast-like cells (Liu et al., 2007a). VICs are
present in all three layers of the aortic valve and can be induced to differentiate into myofibroblasts by inflammatory response (often caused by endothelial damage) (Liu et al., 2007) and the release of angiotensin, transforming growth factor-β (TGF-β) and matrix metalloproteinases (MMPs) (Zhou et al., 1996; Kaden et al., 2003; Kaden et al., 2005). After further accumulation of lipids, changes in structure and fibrosis, differentiation to an osteoblast phenotype is thought to occur via the Wnt3/low-density lipoprotein receptor-related protein 5 (Lrp5)/β-catenin signalling pathway (Cosmi et al., 2002; Osman et al., 2006; Rajamannan, 2009). Osteoblastic cells then mediate deposition of mineral by processes associated with bone formation (Rajamannan et al., 2003). In addition, the receptor activator of nuclear-κB ligand/receptor activator of nuclear-κB/osteoprotegerin (RANKL/RANK/OPG)-axis, which regulates osteoclast differentiation, activation and survival, has also been shown to play an important role in aortic valve calcification (Kaden et al., 2004), which will be described in section 1.3.5.2.9.

1.3.3 Clinical consequences of vascular calcification

Calcification of blood vessels is a common consequence of ageing, ESRD, diabetes and atherosclerosis (Allison et al., 2004; Shroff and Shanahan, 2007; Kestenbaum et al., 2009) and is associated with significant mortality and morbidity of cardiovascular disease (Arad et al., 2000; Rosenhek et al., 2000; Keelan et al., 2001; Wayhs et al., 2002). Indeed clinically, vascular calcification is now accepted as a valuable predictor of coronary heart disease (Greenland et al., 2007). The clinical ramifications of vascular calcification in CKD, atherosclerosis and cardiac valve calcification are described here in more detail.

1.3.3.1 Chronic Kidney Disease (CKD)

It has been reported that approximately 40% of patients with CKD have vascular calcification compared with 13% of age-matched controls with normal renal function (Russo et al., 2004). Kramer and colleagues demonstrated a positive association between the presence of vascular calcification and renal failure, and that this association increased markedly in CKD diabetic patients (Kramer et al., 2005). Converging evidence from clinical, epidemiological and translational research
studies has suggested that vascular calcification progresses inexorably during dialysis and may only partially reverse after successful transplantation (Ossareh, 2011; Shroff, 2011). Medial calcification leads to vascular stiffness and decreases the compliance of blood vessels. These changes result in both increased pulse pressure (Dao et al., 2005) and left ventricular hypertrophy (Speer and Giachelli, 2004). In dialysis patients, medial calcification contributes to calcific uremic arteriolopathy, a necrotising skin condition with high mortality rates (Coates et al., 1998).

1.3.3.2 Generalized Arterial Calcification of Infancy (GACI)
GACI is a rare autosomal recessive disease which is characterised by the calcification of arteries, in conjunction with arterial stenosis caused by intimal proliferation. The majority of affected children die within the first six months of life as the result of end-organ damage. In a subset of patients, peri-articular calcification of joints also occurs (Rutsch et al., 2003; Rutsch et al., 2008).

1.3.3.3 Atherosclerosis
Previous studies have shown that intimal calcification is positively correlated with atherosclerotic plaque burden (Rumberger et al., 1995; Sangiorgi et al., 1998), increased risk of myocardial infarction (Beadenkopf et al., 1964; Loecker et al., 1992) and plaque instability (Fitzgerald et al., 1992; Burke et al., 2000). Furthermore, calcium deposits may directly alter atherosclerotic plaque stability (Wong et al., 2012).

1.3.3.4 Cardiac valve calcification
In the aortic valve, calcification gives rise to life-threatening stenosis. Calcific aortic valve stenosis (CAVS) is the leading reason for valve replacement in Europe and North America, and is considered to be a major mode of failure of native as well as bioprosthetic valves (O'Keefe et al., 1991). CAVS is also correlated with a high risk of cardiovascular dysfunction, and is the third leading cause of cardiovascular disease (Ribeiro et al., 1998).
1.3.4 Risk factors for vascular calcification

Elevated serum Pi levels are recognised as a major risk factor for cardiovascular events in the general population (Dhingra et al., 2007; Kestenbaum et al., 2009) and in CKD (Young et al., 2005; Adeney et al., 2009). Serum Pi levels greater than 5.5 mg/dL are strongly correlated with mortality in ESRD patients (Block et al., 2004; Tentori et al., 2008). Furthermore, relatively small increases in serum Pi (3.5–4.5 mg/dL) have also been correlated with increased risk of cardiovascular and all-cause mortality in CKD patients (Kestenbaum et al., 2005) and the general population with normal renal function (Tonelli et al., 2005). Increased susceptibility of CKD patients to vascular calcification likely underlies this high risk of cardiovascular disease-related deaths in CKD patients.

A number of clinical studies have also shown an association between elevated serum calcium and increased risk of myocardial infarction and vascular calcification in both CKD patients and in the general population (Yamada et al., 2007a; Kovesdy et al., 2010; Larsson et al., 2010; West et al., 2010). Furthermore, a recent meta-analysis has reported that dietary calcium supplementation is associated with a significantly increased risk of myocardial infarction (Bolland et al., 2010). Clinical studies investigating the patterns of systemic atherosclerotic calcification have further revealed age and hypertension as the dominant risk factors for calcification (Allison et al., 2004).

In recent years, several studies have demonstrated the positive relationship between vascular calcification and bone health (Frye et al., 1992; Kiel et al., 2001). Vascular calcification is often accompanied by either decreased bone mineral density or disturbed bone turnover. This association has been observed in general populations (Hyder et al., 2007) and also in patients with osteoporosis, Paget’s disease and CKD (Raggi et al., 2007; Toussaint et al., 2008). It appears that in patients with CKD that both extremes of bone remodelling, low turnover (adynamic bone) and hyperparathyroid bone, may accelerate vascular calcification by not allowing calcium or phosphorus into bone, or resorbing it out of bone, respectively (Moe and Chen, 2004). In genetically altered animals with deletions of OPG and Klotho, a
combined osteoporosis-arterial calcification phenotype has been observed (Bucay et al., 1998; Nabeshima, 2002). Furthermore, bone loss and vascular calcification share various common mechanisms, including estrogen deficiency, vitamin D and K abnormalities, chronic inflammation and oxidative stress (Hofbauer et al., 2007).

CAVS is associated with classic atherosclerotic risk factors, including hypercholesterolaemia, hypertension, smoking and male gender (Mohler et al., 1991; Stewart et al., 1997). A faster disease progression has also been reported in patients with a metabolic syndrome (Briand et al., 2006). Lifestyle modifications are therefore likely to be advantageous, however a beneficial effect of controlling cardiovascular risk factors has yet to be demonstrated in CAVS.

1.3.5 Mechanisms of vascular calcification

In the past decades, a series of clinical and basic science studies performed have underscored the biological complexity of the process driving vascular calcification. Accumulating evidence has shown that vascular calcification is a tightly regulated active process (Speer and Giachelli, 2004), rather than a consequence of ageing and vascular deterioration in the elderly. As shown in Figure 1.6, four main mechanisms regarding the cause of vascular calcification have been proposed: (1) induction of bone formation; (2) loss of inhibition; (3) circulating nucleational complexes and (4) cell death. Each mechanism is discussed below.

1.3.5.1 Induction of bone formation

The pathological cell-mediated process of vascular calcification shares many similarities with that of the physiological matrix mineralisation during skeletal development. Therefore, a brief introduction of bone growth and formation is described below.
1.3.5.1.1 The skeleton: bone and cartilage

The skeleton is the internal structure of vertebrate animals, which supports the body, protects the vital organs and functions as a framework for the attachment of muscles. The skeleton consists of two tissues: bone and cartilage. It has six main functions: to provide the framework for the body; to permit movement; to protect many vital organs; to produce blood cells; to store calcium and phosphorus; and to regulate endocrine function.

Bone is a specialised connective tissue which has two forms: cortical (compact) bone and trabecular (cancellous) bone. Cortical bone accounts for 80% of the total bone mass and provide bone with a smooth, white and solid appearance. It consists of
haversian sites and osteons. Trabecular bone only makes up 20% of the total skeleton mass, but 80% of the bone surface. It is less dense and more elastic and has a higher turnover rate than compact bone and always occupies the interior region of bone. Trabecular bone forms the interior scaffolding to maintain bone’s shape. Its centre contains red and yellow marrow, bone cells and other tissues (Figure 1.7).

![Structure and components of bone](http://www.wisegeek.com/what-is-bone-marrow-edema.htm).

Cartilage is a type of flexible connective tissue, which functions in providing structural support for the early embryo, forming a template for bone development and repairing fractured bones (Shum and Nuckolls, 2002). Cartilage is composed of chondrocytes. Chondrocytes produce a large number of collagen fibres, proteoglycan and elastin fibres. There are three main types of cartilage: hyaline or articular cartilage, elastic cartilage, and fibrocartilage. Hyaline cartilage consists predominantly of collagen type II, functioning as a centre of ossification and bone growth. Elastic cartilage contains elastic fibre networks and collagen fibres, which give support to external structures. Fibrocartilage consists of a mixture of fibrous tissue and cartilaginous tissue. In various proportions, in addition to collagen type II, it also contains collagen type I.
1.3.5.1.2 Bone formation

Bones develop through two different mechanisms: mesenchymal stem cells can directly differentiate into bone-forming osteoblasts by a process named intramembranous ossification, which is responsible for the formation of flat bones or these cells can differentiate into chondrocytes, the primary cell type of cartilage, to provide a template for bone formation named endochondral ossification, which is responsible for the formation of long bones (Ortega et al., 2004). Endochondral ossification occurs in two distinct sites: the diaphysis (primary sites of ossification) and the epiphysis (secondary sites of ossification).

The growth plate is formed between these two sites by the segregation of chondrocytes at different stages of differentiation (Ortega et al., 2004). In the growth plate, chondrocytes mineralise their extracellular matrix by promoting the formation of crystalline HA through a series of physico-chemical and biochemical processes. Three conditions are required for HA formation, including the presence of MVs, low proteoglycan concentrations and a source of calcium and Pi ions (Ali, 1985). The initial stage of mineral formation taking place in MVs, which are extracellular membrane enclosed particles budding on the membrane surfaces of osteoblasts, chondrocytes and ondotoblasts (Anderson, 1995). This biomineralisation is a biophasic phenomenon, including mineral formation within MVs and mineral propagation (Anderson, 1995) (Figure 1.8).
Phase one is concerned with the formation of the first crystals of mineral within MVs. Calcium and Pi are attracted into MVs until sufficient amounts present for precipitation (Wuthier, 1975; Anderson, 1995). In addition, Phosphatase, Orphan 1 (PHOSPHO1), a novel phosphatase, increases the intravesicular concentration of Pi inside MVs and thus control the first step of HA crystal deposition inside MVs (Stewart et al., 2006). This precipitation is then converted to an intermediate octacalcium-phosphate whose crystals are transformed finally into the more insoluble HA (Sauer and Wuthier, 1988). Calcium accumulation is regulated by calcium-binding phospholipids, calcium binding-proteins in MVs, and the action of Ca$^{2+}$ ion channels, such as annexin I, phosphatidylserine (Rojas et al., 1992; Wu et al., 1995). Pi accumulation is associated with the action of phosphatases, especially tissue non-specific alkaline phosphatase (TNAP) and PHOSPHO1 (Anderson, 1995; Stewart et al., 2006). In addition, sodium dependent phosphate transporters also play an important role in uptake of Pi (Montessuit et al., 1991).

Phase two is mineral propagation. When the mineral crystals grow to an extent, they penetrate the MV membrane and are exposed to the extracellular fluid. The rate of mineral crystal proliferation is controlled by extravesicular fluid conditions (Ca$^{2+}$ and Pi), the pH of the extracellular fluid, and the presence of molecules in the extracellular fluid (Howell, 1971). Mineralisation is dependent on the concentrations
of Ca\(^{2+}\) and Pi within MVs, and on the levels of mineralisation inhibitors such as inorganic pyrophosphate (PPi). Therefore, the ratio of Pi and PPi controls the formation and deposition of HA within extracellular matrix. Three molecules have been identified to regulate the ratio of Pi and PPi including TNAP, NPP1 (nucleotide pyrophosphatase/phosphodiesterase isozyme) and Ankyrin (ANK) (which forms a PPi channel on the surface of mineralising cells). Recently, PHOSPHO1, a novel phosphatase, has also been shown to regulate matrix mineralisation (Houston et al., 2004). These key regulators of calcification are further described in detail in section 1.3.5.2.

1.3.5.1.3 Bone cells
There are three main types of cells that make up bone: osteoblasts, osteocytes and osteoclasts. Osteoblasts originate from mesenchymal stem cells. They are immature bone cells, and can make a protein mixture called osteoid, which is mainly composed of collagen type I. They also produce hormones, alkaline phosphatase, and many matrix proteins (Prockop, 1997; Ducy et al., 2000). Osteocytes are derived from osteoblasts, and are mature bone cells. Their main functions include matrix maintenance and calcium homeostasis (Knothe Tate et al., 2004). Osteoclasts are the cells responsible for bone resorption by removing the mineralised matrix (Teitelbaum, 2000). These three cell types participate in a variety of important biological processes during embryonic development and in postnatal life.

1.3.5.1.4 Osteoblasts
Osteoblasts are derived from pluripotent precursor cells (stromal stem cells) which also have the ability to become adipocytes, chondrocytes, and myoblasts (Prockop, 1997; Ducy et al., 2000). Osteoblasts are responsible for bone formation, which is a physiological process involving skeleton growth, bone remodelling, and fracture repair (Erlebacher et al., 1995). A number of hormones and local factors regulate osteoblast differentiation such as Runx-related transcription factor 2 (Runx2) and Osterix (Osx).
Runx2, also named Chfa1, has been shown to play an important role in osteoblast differentiation. Runx2 is a transcription factor that belongs to the Runx family, which also include Runx1 and Runx3 (Yoshida et al., 2004). Runx2 acquires DNA binding activity via forming heterodimers with transcriptional co-activator core binding protein 2β (Cbf2β) (Kundu et al., 2002). Targeted deletion of Runx2 causes a complete lack of bone formation due to the absence of osteoblast differentiation, indicating that Runx2 is required for osteoblast differentiation (Komori et al., 1997; Otto et al., 1997). Maeno et al have produced Runx2 transgenic mice generated under the control of paired related homeobox 1 (Prrx1) promoter, which allows Runx2 expressed in mesenchymal cells before the onset of bone development. The authors have demonstrated that Runx2 is sufficient to direct mesenchymal cells to osteoblasts and lead to intramembranous bone formation during embryogenesis (Maeno et al., 2011). In vitro studies have also suggested the crucial role of Runx2 in osteoblast differentiation. Overexpression of Runx2 in non-osteogenic cells induces osteoblast-related gene expression (Ducy et al., 1997; Harada et al., 1999). Knockdown of Runx2 in primary rat osteoblast cells inhibits osteoblast differentiation and reduces mineralised nodule formation (Banerjee et al., 1997). Runx2 can regulate the expression of osteoblast-predominant genes, including osteocalcin (Ocn), osteopontin (Opn) and bone sialaprotein (Bsp) via directly binding to the promoter of these genes (Ducy et al., 1999). However, overexpression of Runx2 in transgenic mice leads to the inhibition of the late-stage osteoblast differentiation and osteopenia (Liu et al., 2001; Geoffroy et al., 2002). These data suggest that Runx2 is essential for early osteoblast differentiation but subsequent down-regulation of Runx2 is required for terminal differentiation of osteoblasts into osteocytes.

Osx is another transcription factor involved in osteoblast differentiation. Osx knockout mice have no mineralised skeleton and no mature osteoblasts. The mesenchymal cells from these mice express Runx2, but those from Runx2 knockout mice do not express Osx, indicating that Osx acts downstream of Runx2 (Nakashima et al., 2002). Osteoblast specific Osx null mice exhibit osteopenia caused by the inhibition of osteoblast maturation. The accumulation of immature osteoblasts in bone has been observed in these adult mice (Baek et al., 2009). Similar to Runx2,
transgenic mice overexpressing Osx also show osteopenia, and osteoblast differentiation at a late stage is inhibited in these mice (Yoshida et al., 2012). In addition, Wnt (Day and Yang, 2008), Hedgehog (Ehlen et al., 2006) and TGF-β/Bone morphogenic protein (BMP) signalling pathways (Franco et al., 2004) are also involved in osteoblast differentiation (Figure 1.9).

**Figure 1.9 Regulation of osteoblast differentiation.** Runx2 and Osx promote mesenchymal stem cells differentiation into preosteoblasts but inhibit preosteoblasts differentiation into mature osteoblasts. Wnt, Hedgehog and TGF-β/BMP signalling pathways are also involved in the regulation of osteoblast differentiation.

### 1.3.5.1.5 Osteocytes

Osteocytes make up approximately 90% of all bone cells in adult animals, and are embedded in the mineralised bone matrix. However, little is known about their function. Osteocytes are derived from osteoblasts or bone forming cells, and are regularly dispersed throughout the mineralised bone matrix within caves called lacunae. They form a syncytial network and communicate with neighbouring osteocytes and other cells via cell processes within canaliculi. Osteocytes are
considered as a likely candidate for the role of a mechanosensor/transducer in bone because of their location within a fluid filled network in the matrix, their extensive communication with other bone cells and their responsiveness to mechanical stimuli (Lanyon, 1992; Klein-Nulend et al., 1995; Pitsillides et al., 1995). It has been proposed that osteocytes sense the need for bone turnover and control this by conveying local signals directly or otherwise to osteoblasts and osteoclasts (Klein-Nulend et al., 1995; Maejima-Ikeda et al., 1997). Recent studies have suggested that osteocytes play an important role in regulating Pi homeostasis and mineralisation (Feng et al., 2009). There are a number of markers for osteocytes. E11 is the earliest and most specific marker for embedding osteocytes. The major function of E11 may be in the maintenance the dendritic morphology of osteocytes (Tanaka-Kamioka et al., 1998). Other osteocyte markers include sclerostin (SOST), dentin matrix protein 1 (DMP1), phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX), matrix extracellular phosphoglycoprotein (MEPE) and fibroblast growth factor 23 (FGF23) (Bonewald, 2011) (Figure 1.10). These molecules are mainly expressed by osteocytes and play an important role in mineralisation and Pi homeostasis. The function of these molecules is described below.

The transition of osteoblasts to osteocytes (osteocytogenesis) is essential for normal human bone formation. The cellular and molecular mechanisms that regulate this process are not fully understood, but there have been some important observations, suggesting that osteocytogenesis is an active invasive process. Holmbeck and colleagues have demonstrated that osteocyte phenotype and the formation of osteocyte dendritic processes are dependent on continuous cleavage of type I collagen, which is the most abundant matrix protein in the osteocyte environment (Holmbeck et al., 2005). Membrane-type matrix metalloproteinase 1 (MT1-MMP) is a membrane-anchored proteinase that is capable of cleaving collagens type I, II, III, fibrin, fibronectin and other matrix molecules (Ohuchi et al., 1997; Hiraoka et al., 1998; Hotary et al., 2003). MT1-MMP knockout mice show loss of formation of osteocyte processes, due to disruption of collagen cleavage in osteocytes (Holmbeck et al., 2005). Osteocyte morphology is also controlled by E11, a marker of the
embedding osteoid osteocyte. Targeted deletion of E11 in bone cells results in decreased canaliculi and increased trabecular bone (Bonewald, 2011). In addition to MT1-MMP and E11, the organised expression of tubulin, vimentin, and actin in cell bodies and dendrites of osteocytes is crucial to osteocyte morphology (Tanaka-Kamioka et al., 1998). Previous studies have also demonstrated that DMP1 expression in the extracellular matrix is crucial for normal osteoblast to osteocyte differentiation via down-regulation of osteoblast markers (Feng et al., 2006). In addition, bone morphogenic protein-9 (BMP9) has also been suggested to play an important role in osteocytogenesis. Injection of BMP9 adenoviral vector into athymic nude rats results in the appearance of a large number of osteocytes at the injection site after 35 days (Li et al., 2003).

1.3.5.1.6 The osteocyte markers
DMP1 is an acidic protein that was first cloned from the mineralised dentin matrix. It belongs to the small integrin-binding ligand, N-linked glycoprotein family (SIBLINGS) (Fisher et al., 2001; Fisher and Fedarko, 2003), which can bind to HA and can be detected in blood (Fedarko et al., 2000; Fisher et al., 2001; Jain et al., 2002). DMP1 is expressed in all mineralised tissue, with the highest expression present in osteocytes (Toyosawa et al., 2004). Low levels of DMP1 expression have also been reported in non-mineralised tissues, such as brain, kidney and pancreas (Terasawa et al., 2004). DMP1 induces the differentiation of MC3T3-E1 cells to osteoblast-like cells and enhances mineralisation (Narayanan et al., 2001). Feng and colleagues have also demonstrated that the expression of DMP1 is highly associated with “bone nodule” formation and mineralisation in primary rat calvarial cells (Feng et al., 2002). It has been reported that DMP1 acts as a transcriptional component to target the nucleus and activate the osteoblast specific genes (Narayanan et al., 2003). DMP1 null mice are normal at birth, but post-natally develop abnormalities, including typical rickets (delayed secondary ossification, enlarged growth plates and short limbs) and osteomalacia (Ling et al., 2005; Feng et al., 2006). All of these abnormalities are associated with a defect in the maturation of osteoblasts into osteocytes, as well as increased FGF23 expression (Feng et al., 2006).
Figure 1.10 Morphology of the osteocyte and expression of markers during the osteoblast to osteocyte transition. (A) Scanning electron micrograph of the osteocyte. (B) The osteocyte in mouse tibia was immunolabeled with sclerostin, a specific mature osteocyte marker (Arrows). (C) Specific markers are expressed during the process of osteoblast to osteocyte differentiation. PHEX, MEPE and DMP1 are important regulators of biomineralisation and FGF23 regulates renal Pi excretion. Sclerostin is a mature osteocyte marker and functions as a negative regulator of bone formation (Bonewald., 2011).
Sclerostin, encoded by the SOST gene, is a secreted glycoprotein among the Differential screening-selected gene Aberrative in Neuroblastoma (DAN) family, which includes proteins that inhibit BMP and Wnt signalling (Brunkow et al., 2001). It is primarily expressed in bone, specifically by osteocytes (Winkler et al., 2003; van Bezooijen et al., 2004; Yuce et al., 2010). The expression of sclerostin has also been observed within the medial vessel wall of the great arteries during embryogenic and neonatal cardiovascular development (van Bezooijen et al., 2007). Sclerostin expression is suppressed by parathyroid hormone (PTH) (Leupin et al., 2007).

Mutations within the SOST gene cause sclerosteosis and van Buchem’s disease, which are both associated with a general skeletal overgrowth and sclerosis of the axial and appendicular skeleton (Balemans et al., 2001; Brunkow et al., 2001; Loots et al., 2005). Targeted deletion of SOST in mice enhances bone formation and bone strength (Li et al., 2008a). Interestingly, sclerostin antibody treatment increases bone formation and restores bone loss in osteoporotic rats (Li et al., 2009). In addition, administration of sclerostin antibody enhances metaphyseal bone healing in rats (Agholme et al., 2010). Sclerostin inhibits BMP signalling and inhibits the mineralisation of osteoblastic cells by competing with type I and II BMP receptors for binding to BMPs (Winkler et al., 2003). Recombinant sclerostin can bind to noggin, a BMP antagonist, to form the sclerostin-noggin complex. This complex is competitive with type I and II BMP receptors for binding to BMPs (Winkler et al., 2004). In addition, sclerostin inhibits proliferation and alkaline phosphatase activity, and induces apoptosis in human mesenchymal stem cells (Sutherland et al., 2004a).

However, in some bone-derived cells, sclerostin may not function as a classical BMP antagonist, suggesting an alternative pathway mediating sclerostin activity (van Bezooijen et al., 2004). Previous studies have shown that sclerostin can bind to the extracellular domain of LRP5 and LRP6 and inhibit Wnt signalling by disrupting frizzled-LRP complex formation (Semenov et al., 2005). LRP5 mutations linked to the high bone mineral density are associated with decreased binding of sclerostin with LRP5 and a reduction of sclerostin-induced inhibition of Wnt signalling (Li et al., 2005; Ellies et al., 2006; Semenov and He, 2006; Balemans et al., 2008). Recent studies by Craig et al have shown that sclerostin can bind and regulate the activity of cysteine-rich protein 61, a protein which regulate bone cell function and
angiogenesis (Craig et al., 2010).

E11 was first described in 1990 as an unknown phorbol ester-inducible gene in MCT3T3 osteoblast-like cells, named OTS-8 (Nose et al., 1990). Since then, this gene has been given several names depending on the tissue of expression. In rat type I epithelial alveolar lung cells, this gene is known as T1alpha (Rishi et al., 1995) and the protein is known as RTI40 (Gonzalez and Dobbs, 1998). T1alpha or RTI40 is expressed on the apical surface of lung epithelial cells, which form the air-blood barrier (Dobbs et al., 1988; Rishi et al., 1995). Targeted deletion of this gene results in mice that die at birth due to respiratory failure. This is caused by a failure of type II alveolar lung cells to differentiate into type I cells (Ramirez et al., 2003). T1alpha is also expressed in the lymphatic endothelium. T1alpha null mice have abnormal lymphatic vasculature formation and lymphedema (Schacht et al., 2003). Hypoxia induces expression of T1alpha through the transcription factor SP1, while normoxia inhibits its expression in type I alveolar lung cells (Cao et al., 2003). It is also regulated by interleukin-3 in endothelial cells (Hong and Detmar, 2003). In osteocytes, T1alpha is known as E11 (Wetterwald et al., 1996). E11 is detected in the plasma membranes of osteocytes and their processes, but not in osteoblasts (Schulze et al., 1999). However, overexpression of E11 in an osteoblast-like cell line (ROS 17/2.8 cells) results in the formation of extended cytoplasmic processes (Sprague., et al., 1996). A more recent study has shown that E11 is osteocyte selective compared to osteoblasts and it is induced by mechanical strain in vitro and in vivo. In addition, E11 is necessary for the elongation of dendritic processes (Zhang et al., 2006). In addition, it has been shown that extracellular matrix mineralisation promotes the expression of E11 and osteocytogenesis, suggesting the involvement of E11 during osteocyte differentiation (Prideaux et al., 2012).

PHEX is a member of the M13 family of type II cell-surface membrane zinc-dependent proteases (Beck et al., 1997). In humans, inactivating mutations in Phex leads to X-linked hypophosphatemia (XLH), a dominantly inherited X-linked disorder of Pi homeostasis, and the most frequently inherited form of vitamin D-resistant rickets in humans (The Hype Consortium, 1995). XLH is characterised by
growth retardation, rachitic and osteomalacic bone disease, hypophosphatemia, and aberrant vitamin D metabolism (Econs and Francis, 1997; Tenenhouse, 1999). The Hyp mouse carrying a 3’ deletion in the Phex gene provides an animal model for human XLH (Beck et al., 1997). Phex is predominantly expressed in bone and teeth cells (Du et al., 1996; Beck et al., 1997) and has peptidase activity in vitro (Boileau et al., 2001; Addison et al., 2008), suggesting that Phex may regulate mineralisation via controlling the functional bioactive peptide. Previous studies have shown that Phex regulates FGF23 expression, which is one of the major regulators of Pi homeostasis in mice and humans (Liu et al., 2003; Nakatani et al., 2009; Brownstein et al., 2010). Loss of function of Phex in the Hyp mouse leads to increased circulating FGF23 levels, which induces renal Pi wasting and osteomalacia (Onishi et al., 2008; Liu et al., 2009) via the inhibition of renal proximal tubular cells type II sodium-phosphate cotransporters (Farrow and White, 2010) and the modulation of fibroblast growth factor receptor (FGFR) signalling in osteocytes (Martin et al., 2011). In addition, recent studies have demonstrated that osteopontin (OPN), a mineralisation inhibitor, can be proteolytically cleaved by Phex (Martin et al., 2011). The accumulated OPN fragments may contribute to the mineralisation defects seen in the Hyp mouse (Martin et al., 2011). It has been shown that PTH inhibits osteoblast Phex expression (Alos and Ecarot, 2005).

MEPE belongs to the SIBLING family and is highly expressed in osteocytes (Rowe et al., 2000). Increased bone formation and bone mass has been observed in MEPE knockout mice, which show increased osteoblast number and osteoblast activity but unaltered osteoclast number and osteoclast surface (Gowen et al., 2003). However, MEPE knockout mice have normal vitamin D and serum Pi (Gowen et al., 2003), suggesting MEPE may not directly regulate Pi homeostasis and vitamin D metabolism. The overexpression of MEPE under the control of the col1a1 promoter in mice causes a growth and mineralisation defect, due to a decrease in bone remodelling (David et al., 2009). These MEPE transgenic mice show wider epiphyseal growth plates, expanded primary spongiosa and decreased bone formation and mineralisation (David et al., 2009). Interestingly, MEPE transgenic mice are resistant to diet-induced renal calcification, which is mediated by increased MEPE
protein-derived urinary acidic serine- and aspartate-rich motif (ASARM) peptides and reduced urinary Ca X PO$_4$ product (David et al., 2009). Further studies have demonstrated that the inhibitory effect of MEPE on mineralisation is dependent upon its state of cleavage and its phosphorylation (Rowe et al., 2004; Martin et al., 2008). MEPE can be cleaved by cathepsin B to release a 2.2 KD carboxy-terminal ASARM peptide (Rowe et al., 2004). This ASARM peptide inhibits mineralisation in osteoblasts by directly binding to HA (Rowe et al., 2004; Rowe et al., 2005; Addison et al., 2008). The post translational phosphorylation of the ASARM peptide of MEPE is essential for its inhibitory effect on mineralisation (Addison et al., 2008). Interestingly, PHEX can inhibit MEPE cleavage by blocking cathepsin-like enzyme activity (Guo et al., 2002). Further studies have shown that MEPE directly binds to PHEX through the ASARM motif, thus preventing MEPE cleavage and release of ASARM peptide (Rowe et al., 2005). In addition, recombinant MEPE and its ASARM peptide inhibit mineralisation and PHEX activity, leading to increased FGF23 expression in bone marrow stromal cell (BMSC) cultures (Liu et al., 2007b). Recent studies have demonstrated that MEPE also inhibits growth plate chondrocyte matrix mineralisation through its ASARM peptide (Staines et al., 2012).

FGF23 was first identified as a gene responsible for tumor-induced osteomalacia (TIO) and autosomal dominant hypophosphatemic rickets (ADHR) (Shimada et al., 2001). FGF23 is a hormone that is synthesised primarily by osteocytes (Ubaidus et al., 2009). It is a 30 KD protein, and can be processed between arginine 179 and serine 180 to generate smaller N-terminal (18KD) and C-terminal (13KD) fragments. Studies have shown that it is likely to be cleaved by subtilisin-like proprotein convertases (SPC) (Benet-Pages et al., 2004). FGF23 mutations R176Q, R179Q and R179W have been demonstrated to be responsible for human ADHR (White, 2000). Further studies have shown that these mutations protect FGF23 from proteolysis, thereby elevating FGF23 circulating levels, leading to renal Pi wasting in ADHR patients (White et al., 2001). It has been shown that FGF23 regulates serum Pi levels via inhibiting the expression of Na/Pi-2a and Na/Pi-2c cotransporters that mediate phosphate uptake in proximal tubular epithelial cells (Shimada et al., 2004a). In addition, FGF23 can reduce vitamin D activity, resulting in decreased serum Pi levels.
by inhibiting intestine Pi absorption (Shimada et al., 2004a). Interestingly, FGF23 inhibits PTH secretion, which is a primary regulator of serum Pi (Krajisnik et al., 2007). Independent of its effect on Pi homeostasis, it has been reported that FGF23 directly inhibits osteoblast differentiation and matrix calcification in vitro (Wang et al., 2008). FGF23 knockout mice display hyperphosphatemia in early life, excessive skeletal mineralisation and soft tissue calcification (Sitara et al., 2004; Razzaque et al., 2005). FGF23 transgenic mice develop severe hypophosphatemia rickets, due to increased renal Pi wasting and inappropriately low serum 1,25-dihydroxyvitamin D levels (Shimada et al., 2004b).

1.3.5.1.7 Osteoclasts

Osteoclasts are responsible for bone resorption, which is necessary for bone remodelling and the maintenance of appropriate blood calcium levels. Osteoclasts are multinuclear cells derived from hematopoietic stem cells and share a common differentiation pathway with macrophages and dendritic cells (Suda et al., 1992). Macrophage colony-stimulating factor (M-CSF) and the RANKL/RANK/OPG axis are two major factors that regulate osteoclast differentiation. M-CSF is mainly expressed by osteoblasts and stromal cells, which binds to its receptor CSF-1R, thereby providing signals for the maturation and commitment of osteoclast precursor cells, as well as their survival and proliferation (Yoshida et al., 1990; Fuller et al., 1993). RANKL, which belongs to the tumor necrosis factor-α (TNF-α) superfamily, is expressed by osteoblasts and stromal cells (Suda et al., 1999). RANKL binds to its receptor RANK on osteoclast precursor cells to activate the expression of a number of osteoclast genes, including tartrate-resistant acid phosphatase (TRAP), cathepsin K, calcitonin receptor, αvβ3-integrin and matrix metalloproteinase-9 (MMP-9) (Nakagawa et al., 1998). OPG is a soluble decoy receptor that prevents RANKL binding to its receptor RANK, thereby inhibiting osteoclastogenesis (Simonet et al., 1997). Therefore, the ratio of OPG and RANKL is crucial for osteoclastogenesis. Osteoclasts migrate to the resorption site, dissolve the mineral and degrade the organic bone matrix. Initially, cytokines such as M-CSF and vascular endothelial growth factor (VEGF) recruit the osteoclasts to the bone resorption site (Niida et al., 1999). Following recruitment, osteoclasts attach tightly to the resorption site through
a specific membrane domain called the sealing zone (Vaaninen and Horton, 1995; Vaaninen et al., 2000) and release proteolytic enzymes that degrade the organic bone matrix, including lysosomal cysteine proteinases and MMPs. The high levels of expression of MMP-9 and cathepsin K, and their secretion into bone resorption lacunae suggests that they play a central role in the process of bone resorption (Tezuka et al., 1994; Drake et al., 1996). After matrix degradation, the degradation products are removed from the resorption lacuna through a transcytotic vesicular pathway (Nesbitt and Horton, 1997). TRAP is localised in the transcytotic vesicles of resorbing osteoclasts, and it can generate highly destructive reactive oxygen species, which are able to destroy collagen (Halleen et al., 1999). TRAP knockout mice display osteopetrosis, suggesting that TRAP functions in the further destruction of matrix-degradation products in the transcytotic vesicles (Hayman et al., 1996).

1.3.5.1.8 Vascular calcification resembles bone formation

The initiation sites for calcification in bone and cartilage are MVs (Anderson, 1995). These are membrane-bound vesicles that are produced by budding from osteoblasts, chondrocytes and odontoblasts (Anderson et al., 1990; Anderson, 1995). MVs contain the necessary calcium-binding proteins and phosphatase for nucleation of HA crystals (Anderson et al., 1990; Nahar et al., 2008). As described in section 1.3.5.1.2, the mineralisation occurring within MVs is a biphasic phenomenon (Figure 1.8): Phase I is the formation of the first crystals of mineral within MVs. Calcium ions and inorganic Pi accumulate within MVs. When sufficient concentrations of both exist, calcium Pi begins to precipitate. This precipitation is firstly non-crystalline, but then converted into a series of intermediates and finally transformed into HA crystals. Phase II is the mineral propagation. The mineral crystals grow within the MVs until they penetrate the MV membrane and are exposed to the extracellular fluid, permitting further growth and development (Anderson, 1995).

MV-like structures have been found in calcified arteries and heart valves (Kim, 1976; Tanimura et al., 1983). These MV-like structures are derived from VSMCs and may be the remnants of apoptotic bodies (Kim, 1995) (Figure 1.11).
Similar to chondrocyte-derived vesicles, MVs isolated from calcifying VSMCs or atherosclerotic arteries have the ability to nucleate calcium and Pi (Hsu and Camacho, 1999; Reynolds et al., 2004). Using mass spectrometry, Kapustin et al have identified common components between VSMC-derived and chondrocyte-derived MVs. These include ECM components, plasma membrane receptors and enzymes, and proteins involved in the regulation of oxidative stress and protein folding, as well as intracellular vesicle trafficking and biogenesis (Balcerzak et al., 2008; Kapustin et al., 2011). However, under normal conditions, VSMC-derived MVs do not calcify, because they are loaded with negative regulators of HA crystal nucleation and growth, such as fetuin-A and matrix gla protein (MGP) (Reynolds et al., 2004; Kapustin et al., 2011). In cooperation with local mediators such as PPI, these molecules protect the arteries from deposition and growth of minerals (Luo et al., 1997; Harmey et al., 2004; Jahnen-Dechent et al., 2011). Under calcifying conditions, disturbed intracellular calcium leads to inhibitor depletion and formation of nucleation complexes within VSMC-derived vesicles (Kapustin et al., 2011). In

![Figure 1.11 VSMCs release MVs under calcifying conditions.](image)

Electron microscopy (EM) analysis of calcified VSMCs showing calcification within (arrows) and on the surface of vesicles (arrowheads in i). Some calcified intracellular vesicles were observed (arrow in ii) (Reynolds et al., 2004).
the absence of these inhibitors, or following the stimulation of cell death-related processes, together with the bone-like activity of vascular cells, calcification is readily induced (Canfield et al., 2002; Speer et al., 2002; Shroff et al., 2008).

Osteoblasts and chondrocytes are responsible for bone and cartilage formation and calcification within the skeleton. Normal VSMC populations contain cells that undergo phenotypic transition to osteocytic, osteoblastic and chondrocytic cells in a calcified environment (Shanahan et al., 1999; Steitz et al., 2001; Johnson et al., 2005; Speer et al., 2009). Chondro-osseous and calcification promoting genes reported in calcifying VSMCs include the transcription factor Msx2 which promotes osteoblastogenesis (Shao et al., 2005), the osteoblast master transcription factor Runx2 (Speer et al., 2009), the chondrocyte specific extracellular matrix constituent Aggrecan and Collagen Types I, II, IX and XI (Johnson et al., 2008). The phosphate transporter PiT-1 is the predominant sodium-dependent phosphate co-transporter expressed in human VSMCs. Pi increases PiT-1 expression, which leads to increased levels of intracellular Pi. This induces Runx2 expression and the osteogenic conversion of VSMCs (Li et al., 2006). Smooth muscle cell-specific Runx2 deficiency inhibits atherosclerostic calcification in vivo, suggesting the importance of osteogenic transition of VSMCs in the pathogenesis of vascular calcification (Sun et al., 2012). BMPs are highly potent regulators of osteoblast differentiation and bone formation (Yamaguchi et al., 2000). Several BMPs have been implicated in vascular calcification, including BMP2, BMP4 and BMP7. Expression of BMP2 is found in calcified atherosclerotic lesions (Bostrom et al., 1993; Cheng et al., 2003). BMP2 treatment enhances VSMC calcification in vitro through promoting Pi uptake via up-regulating PiT-1 expression and induction of osteogenic phenotype modulation (Li et al., 2008b). BMP2 has also been reported to up-regulate Runx2 expression through inhibition of microRNA miR-30b and miR-30c and promotes calcification (Balderman et al., 2012). BMP4 is up-regulated in calcified atherosclerotic lesions (Dhore et al., 2001), involved in the osteogenic transition of VSMCs (Hayashi et al., 2006) and increases VSMC calcification in vitro (Mikhaylova et al., 2007). Conversely, BMP7 is an inhibitor of vascular calcification (Davies et al., 2003; Kang et al., 2010). Inhibition of BMPs in vivo either by chemical inhibition or by
overexpression of MGP, a BMP antagonist, protects against vascular calcification and atherosclerosis (Yao et al., 2010; Derwall et al., 2012).

1.3.5.2 Loss of inhibition
1.3.5.2.1 Pyrophosphate (PPi)
Bone mineralisation is a tightly regulated process and it is initiated in osteoblast- or chondrocyte-derived MVs (Anderson, 1995). MVs contain inorganic Pi and calcium (Ca\(^{2+}\)) ions, which are crystallised into HA in the MVs and then released into the extracellular matrix (ECM) for further growth and proliferation (Anderson, 1969; Ali et al., 1970). PPi consists of two molecules of inorganic Pi which are joined by a hydrolysable high energy ester bond (Meyer, 1984). It antagonises the ability of Pi to crystallise with calcium to form HA and thereby prevents HA propagation (Harmey et al., 2004). Therefore, the ratio of Pi/PPi is important for normal mineral deposition. PPi levels are tightly regulated by TNA P, NPP1 and ANK (Harmey et al., 2004). These three molecules will be discussed in the following sections. The role of PPi in vascular calcification has clearly been demonstrated. Rat aortic rings cultured under high Pi conditions exhibit no calcification unless the PPi produced by vessels is removed, suggesting PPi is an inhibitor of calcification (Lomashvili et al., 2004). Patients deficient in Enpp1, an enzyme that generates PPi, develop severe medial calcification at an early age (Rutsch et al., 2001; Rutsch et al., 2003). Mice lacking Enpp1 also show ectopic artery calcification (Sali et al., 1999). Furthermore, reduced plasma PPi levels are observed in haemodialysis patients with vascular calcification (de Vries and Sperling, 1977). Interestingly, treatment with PPi inhibits uremic vascular calcification without adverse effects on bones (O'Neill et al., 2011).

1.3.5.2.2 Tissue Non-specific Alkaline Phosphatase (TNAP) and Phosphatase, Orphan 1 (PHOSPHO1)
TNAP (Akp2), one of a family of four homologous human alkaline phosphatase enzymes, is predominantly expressed in bone, kidney and liver, but it is also found in other tissues (Whyte, 1994). The role of TNAP in bone mineralisation has been well studied. Deletion of TNAP (Akp2\(^{-/-}\)) in mice leads to hypophosphatasia characterised by rickets, osteomalacia, bone fractures and increased PPi levels (Whyte, 1994;
Narisawa et al., 1997; Fedde et al., 1999). Osteoblasts from $Akp2^{-/-}$ mice cannot initiate mineralisation, although differentiate normally (Wennberg et al., 2000). In humans, inactivated mutations of $Akp2$ have been reported to cause hypophosphatasia (Whyte, 1994; Chang et al., 2012). The role of TNAP in bone mineralisation may provide Pi by hydrolysing phosphorylated substrates and removing PPI, which is the natural substrate of TNAP and a key inhibitor of mineralisation (Robison and Soames, 1924; Ecarot-Charrier et al., 1983; Tenenbaum, 1987). Therefore, TNAP decreases the PPI/Pi ratio to supply a suitable environment for mineralisation (Harmey et al., 2004). However, the skeletons of $Akp2^{-/-}$ mice are normally mineralised at birth, suggesting the existence of other phosphatases responsible for MV-mediated ECM mineralization (Narisawa et al., 1997; Fedde et al., 1999). PHOSPHO1 is a strong candidate for this missing phosphatase, which is described below. TNAP also plays an important role in vascular calcification. Narisawa et al indicated that chemical inhibitors of TNAP could suppress VSMC *in vitro* calcification (Narisawa et al., 2007). Lomashvili et al have also reported up-regulation of TNAP activity and PPI hydrolysis in uremic vascular calcification (Lomashvili et al., 2008).

PHOSPHO1 is a novel phosphatase with up-regulated expression in mineralising chondrocytes and osteoblasts (Houston et al., 1999). Immunolocalisation studies have shown that PHOSPHO1 is specifically present in active sites of skeletal mineralisation, suggesting that it plays a key role in the mineralisation process (Bas et al., 2006). PHOSPHO1 is a member of the haloacid dehalogense (HAD) superfamily of magnesium-dependent hydrolases, and shows high specific activity towards the membrane phospholipids, phosphoethanolamine (PEA) and phosphocholine (PCho). PHOSPHO1 can generate phosphorus in mineralising cells by degrading phospholipids (Hunt et al., 2002; Roberts et al., 2004). Stewart et al have demonstrated that PHOSPHO1 is present in MVs (Stewart et al., 2006). PHOSPHO1-specific inhibitors, lansoprazole and SCH202676, can inhibit the mineralisation of MVs from $Akp2$ null osteoblasts, indicating that PHOSPHO1 plays a role in the initiation of matrix mineralisation (Roberts et al., 2007). Recently, it has been reported that inhibition of PHOSPHO1 activity with lansoprazole results in
impaired skeletal mineralisation in developing chick long bones (Macrae et al., 2010). The role of PHOSPHO1 in mineralisation was further confirmed in a comparison of the bone phenotype of $\text{Phospho}1^{-/-}$, $\text{Akp2}^{-/-}$ and $\text{Phospho}1^{-/-}\text{Akp2}^{-/-}$ double knockout mice (Yadav et al., 2011). Both $\text{Phospho}1^{-/-}$ and $\text{Akp2}^{-/-}$ mice develop lower mineralised skeleton, whereas the double ablation of PHOSPHO1 and TNAP leads to the complete absence of skeletal mineralisation. These data suggest both phosphatases play independent, non-redundant roles in the mineralisation process (Yadav et al., 2011). Further studies have demonstrated that PHOSPHO1 is required for the proper formation of mechanically competent bones that are able to withstand habitual load (Huesa et al., 2011). Furthermore, pharmacological inhibition of PHOSPHO1 suppresses vascular calcification (Kiffer-Moreira et al., 2013).

1.3.5.2.3 Ectonucleotide pyrophosphatase/phosphodiesterase-1 (NPP1)

NPP1, also known as PC1, is a transmembrane glycoprotein that acts as a phosphodiesterase (pyrophosphatase) and is a member of the NPP (nucleotide pyrophosphatases/phosphodiesterases) family (Oda et al., 1991). The main role of this enzyme is to cleave nucleoside triphosphate (NTP) to generate PPi (Bollen et al., 2000). NPP1 is expressed in various tissues including bone and cartilage (Caswell and Russell, 1988; Harahap and Goding, 1988; Huang et al., 1994). It has been shown that overexpression of $\text{Enpp1}$ inhibits mineralisation of murine osteoblastic MC3T3 cells in vitro (Johnson et al., 1999). Further studies have demonstrated that NPP1 acts as a mineralisation inhibitor in vivo. $\text{Enpp1}^{-/-}$ osteoblasts show a markedly lowered extracellular PPi concentration and increased calcification (Johnson et al., 2003). Similarly, VSMCs from $\text{Enpp1}^{-/-}$ mice exhibit enhanced in vitro calcification compared to wild types (Narisawa et al., 2007). Both the $\text{Enpp1}$ knockout mouse and the tiptoe walking ($ttw/ttw$) mouse, (which has an autosomal recessive inheritance of a naturally occurring nonsense mutation in $\text{Enpp1}$) spontaneously develop ankylosing intervertebral and peripheral joint hyperostosis, articular cartilage calcification and vascular calcification (Sakamoto et al., 1994; Okawa et al., 1998; Johnson et al., 2003). Further studies by Johnson et al have demonstrated that NPP1 and PPi deficiencies promote vascular calcification by modulating the trans-differentiation of VSMCs and intra-arterial chondrogenic.
differentiation rather than simply inhibiting mineral formation (Johnson et al., 2005). In addition, inactivating mutations of Enpp1 are also associated with the human disease GACI (Rutsch et al., 2001; Rutsch et al., 2003; Lorenz-Depiereux et al., 2010). Enpp1 knockout mice have been used as an animal model of medial arterial vascular calcification in this thesis (Chapters 3-5).

1.3.5.2.4 Ankylosis protein (ANK)

ANK is a 492 amino acid protein with a molecular mass of 54.5 KD and contains three N-linked glycosylation sites and multiple phosphorylation sites (Ho et al., 2000). ANK is expressed in many tissues in adult mice including heart, brain, lung, liver, spleen, muscle, and kidney and in developing articular cartilage (Sohn et al., 2002). It transports PPI from the cytoplasm to the extracellular space (Ho et al., 2000; Nurnberg et al., 2001). Ank mutant mice (ank/ank) create a non-functional ANK protein, which causes a significant increase in intracellular PPI concentration and a decrease in extracellular PPI levels in homozygous mice. These mice show severe joint calcification and arthritic disease including ectopic vascular calcification, cartilage erosion and vertebral fusion (Ho et al., 2000; Johnson et al., 2005). Ank null mice display the same phenotype (Gurley et al., 2006). Interestingly, VSMCs isolated from ank/ank mutant mice exhibit enhanced in vitro calcification and TNAP activity compared to controls (Narisawa et al., 2007). In addition, recent studies have suggested that Ank plays an important role in bone formation and bone remodelling. Human mutations in Ank cause craniometaphyseal dysplasia (CMD), which is a rare genetic disorder with hyperostosis of craniofacial bones and widened metaphyses in long bones (Nurnberg et al., 2001; Reichenberger et al., 2001). These mutations occur in the form of point mutations and one-amino-acid insertions or deletions that cluster mostly in the cytoplasmic domains close to the C-terminus (Gurley et al., 2006). Chen et al created a knock-in mouse model for CMD that express a human mutation (Phe377 deletion) in Ank. These mice show increased bone mass in craniofacial bones, especially the mandibles and excessive trabecular bone in diaphyses of long bones, however, the cortex of long bones is hypomineralised (Chen et al., 2009). Further studies have demonstrated that the Phe377del mutation in Ank leads to impaired osteoblastogenesis and
osteoclastogenesis, resulting in hypomineralisation and a high bone mass phenotype (Chen et al., 2011a). Kim et al have demonstrated that ANK function deficiency leads to impaired bone formation and bone resorption via directly affecting osteoblast and osteoclast differentiation (Kim et al., 2010).

1.3.5.2.5 Regulation of Pyrophosphate (PPI) levels

As described above, PPI is a crucial inhibitor of mineralisation. Its levels are tightly controlled for normal bone formation during development. Previous studies have shown that Akp2, Enpp1, and Ank are involved in regulating PPI levels (Figure 1.12). Altered PPI levels in Akp2, Enpp1, or Ank deficient mice lead to abnormal mineralisation. Interestingly, crossbreeding the Akp2^{+/−} and Enpp1^{+/−} mice can rescue the PPI levels and mineralisation abnormalities in the single-deficient animals (Hessle et al., 2002). [Akp2^{−/−}, ank/ank] mice show a partial normalisation of the calcification phenotypes. The calcification inhibitor OPN is increased in Akp2 knockout mice and decreased in ank/ank mutant mice. Both PPI levels and Opn mRNA are normal in [Akp2^{+/−}, ank/ank] and [Akp2^{+/−}, Enpp1^{+/−}] mice (Harmey et al., 2004).
Figure 1.12 Role of NPP1, ANK and TNAP in the regulation of HA deposition. NPP1 hydrolyses ATP into AMP and PPI. PPI is converted into Pi by TNAP. The transport of PPI and Pi through the cell membrane is mediated by ANK and PiT-1, respectively. PPI acts to inhibit HA formation, while Pi promotes this process. Thus the ratio of PPI/Pi is highly important in regulating mineralisation (Harmey et al., 2004).

1.3.5.2.6 Matrix Gla Protein (MGP)

MGP is a vitamin-K dependent calcium-binding protein which binds calcium with its gamma-carboxyglutamic acid (GLA) residues (Schinke and Karsenty, 2000). Mgp-deficient mice die within the first two months of age as a result of extensive arterial calcification, inappropriate cartilage calcification and osteopenia, suggesting that MGP is an inhibitor of calcification (Luo et al., 1997). These abnormalities in Mgp-deficient mice are similar to the phenotype of a rare human disease Singleton-Merten Syndrom, which also displays artery calcification (Luo et al., 1997). Expression of MGP has been observed in human calcified atherosclerotic aortae (Canfield et al.,
2002; Mongin and Kimelberg, 2005), and its expression is confined to endothelial cells and smooth muscle cells (Engelse et al., 2001). A potential mechanism through which MGP inhibits calcification may involve calcium chelation (Schinke and Karsenty, 2000), as evidenced by the presence of circulating complexes of MGP and calcium Pi (Price et al., 2002b). However, previous studies have also demonstrated the important role of MGP in cell differentiation (Canfield et al., 2000). Bostrom et al have shown that MGP modulates BMP2 activity by directly binding to BMP2 and that its effect on calcification and osteogenic differentiation is determined by the availability of BMP2 (Fye et al., 1993; Bostrom et al., 2001). Recently, Yao et al have demonstrated that MGP overexpression in ApoE knockout mice maintained on a high fat diet (a model for atherosclerosis) reduces BMP activity and atherosclerotic lesion size, as well as intimal and medial calcification (Yao et al., 2010). Clinical studies have shown that serum MGP levels are associated with vascular calcification (Di Paola et al., 2011). The association of MGP gene polymorphisms and increased risk of myocardial infarction and cardiovascular mortality in CKD patients has also been noted (Brancaccio et al., 2005).

1.3.5.2.7 Osteopontin (OPN)

OPN is a secreted phosphorylated glycoprotein and its expression is normally limited to the bone, kidney and epithelial linings (Chen et al., 1993). It regulates mineralisation by inhibiting HA growth and promoting osteoclast function through $\alpha_v\beta_3$ integrin (Giachelli and Steitz, 2000). OPN is abundant at the sites of calcification in human atherosclerotic plaques and calcified aortic valves (Giachelli et al., 1993; Fitzpatrick et al., 1994), suggesting that OPN may be involved in vascular calcification. It has been reported that OPN inhibits calcium deposition in calcifying bovine smooth muscle cells in vitro and that phosphorylation of OPN is required for its inhibitory effects (Wada et al., 1999; Jono et al., 2000b). Up-regulation of OPN has also been observed in the calcified aortae of $Mgp^{-/-}$ mice (Steitz et al., 2001). $Opn$ knockout mice do not develop vascular calcification, however VSMCs isolated from $Opn$ null mice show increased calcium deposition compared to wild type controls in response to high Pi (Speer et al., 2005). Additionally, deletion of $Opn$ in $Mgp^{-/-}$ mice enhances vascular calcification (Speer et
Furthermore, Matsui et al. have reported that deletion of *Opn* in ApoE<sup>−/−</sup> mice fed on a high fat diet results in reduced atherosclerotic lesions and increased vascular mineral-deposited areas, suggesting that *Opn* is a negative regulator of vascular calcification (Zhuang et al., 1996). In addition to inhibiting calcification, OPN also mediates diverse biological functions, including inflammation and tissue remodelling (Lund et al., 2009).

1.3.5.2.8 Fetuin-A

Fetuin-A is an abundant serum protein in mammals and is expressed in the adult liver and many other organs during embryogenesis (Liu et al., 1995). Fetuin-A inhibits the formation of HA crystals *in vitro*, but has no effect after the crystals are formed (Heiss et al., 2003). In addition, fetuin-A circulates in a complex of MGP, calcium and Pi, suggesting that it is associated with mineral clearance (Price et al., 2002b). Mice lacking fetuin-A develop severe calcification of soft tissue on a calcium, Pi and vitamin D rich diet but notably not of the vasculature (Schafer *et al.*, 2003). However, fetuin-A deficiency in ApoE<sup>−/−</sup> mice fed on a high fat diet enhances vascular calcification (Pitsillides *et al.*, 1995), suggesting that it is an important inhibitor of vascular calcification. In addition, fetuin-A concentrations are significantly lower in patients on dialysis and correlate with the incidence of cardiovascular disease mortality (Ketteler *et al.*, 2003).

1.3.5.2.9 Osteoprotegerin (OPG)/ Receptor activator of NF-κB ligand (RANKL)/Receptor activator of NF-κB (RANK) axis

As described in section 1.3.5.1.7, the OPG/RANKL/RANK axis plays an important role in osteoclastogenesis. *Opg* knockout mice develop osteoporosis (Bucay *et al.*, 1998), while RANK knockout mice (Dougall *et al.*, 1999), RANKL knockout mice (Kong *et al.*, 1999) and OPG overexpressing mice (Simonet *et al.*, 1997) show osteopetrosis, due to altered osteoclast activity. Unexpectedly, *Opg* null mice also show vascular calcification in some arteries (Bucay *et al.*, 1998), suggesting that the OPG/RANKL/RANK axis may also function in vascular calcification. Indeed, expression of OPG has been detected in the media of large arteries (Simonet *et al.*, 1997), coronary smooth muscle cells (Hofbauer and Schoppet, 2001) and endothelial
cells (Malyankar et al., 2000). Interestingly, OPG treatment prevents warfarin-induced vascular calcification (Price et al., 2001b). The inhibitory effect of OPG on vascular calcification has also been reported by several other groups (Bennett et al., 2006; Morony et al., 2008; Di Bartolo et al., 2011), and elevated OPG serum levels have also been shown to be a predictor of future adverse cardiovascular events (Browner et al., 2001; Nishiura et al., 2009). RANK and RANKL expression are upregulated in calcified aortae (Min et al., 2000). It has been shown that RANKL enhances vascular calcification via binding to RANK and increasing the expression of BMP4 through the nuclear factor kappa beta (NFκB) pathway (Panizo et al., 2009). Recent studies have shown that RANKL cross-talks with the renin–angiotensin II system to promote vascular calcification (Osako et al., 2013). Interestingly, increased RANKL and decreased OPG expression have also been observed in calcific aortic stenosis (Kaden et al., 2004). The OPG-RANK-RANKL axis may therefore play an important role in vascular calcification (Figure 1.13).

**1.3.5.3 Circulating nucleational complexes**

A number of studies have linked bone remodelling, specifically osteoclastic resorption activity, with vascular calcification. Osteoporosis and vascular diseases are commonly found together and shares many of the same risk factors, such as ageing, inflammatory disease, systemic glucocorticoid use, chronic renal failure or estrogen deficiency (Sattler et al., 2004; Ryerson et al., 2010). Postmenopausal osteoporotic patients have a higher risk of vascular calcification and calcium mineral deposits of the aorta locate adjacent to osteopenic vertebrae (Tschumper et al., 1994; Kiel et al., 2001). In addition, postmenopausal women with vascular calcification lose more metacarpal bone than those without vascular calcification, leading to speculation that the formation of bone occurs in the vessels while it is lost in the skeleton (Hak et al., 2000). Moreover, Opg<sup>−/−</sup> mice show early onset osteoporosis, exhibit vascular calcification in the aorta and renal arteries and have increased osteoclast activity (Bucay et al., 1998). Increased bone resorption and increased vascular calcification have also been seen in the vitamin-D treated rat (Price et al., 2000). In this model, the vascular calcification observed is reduced by treatment of bone resorption inhibitors, such as bisphosphonates alendronate, ibandronate and
OPG (Price et al., 2001a; Heuser et al., 2010). It has been proposed that bone resorption at the sites of bone generates crystal nuclei that move from bone to the vascular wall through blood, which triggers vascular calcification (Price et al., 2002a). Under certain circumstances, a complex of calcium mineral and proteins including fetuin-A and MGP can be observed in blood. Interestingly, the release of this complex can be suppressed by osteoclastic inhibition (Banks et al., 1994).

**1.3.5.4 Cell death**

As described in section 1.3.5.1.2, MVs initiate calcification in forming bone and mineralising cartilage (Anderson, 1995), which are budding from osteoblasts or chondrocytes and contains calcium-binding proteins and phosphatases for nucleation of HA (Anderson, 1995). MV-like structures have been observed in calcified arteries and heart valves (Kim, 1976; Tanimura et al., 1983). Kockx et al have reported that these structures are derived from VSMCs and contain B-cell lymphoma 2-associated
(BAX) protein, which is a proapoptotic member of the B-cell lymphoma 2 (Bcl2) family. These data suggest that MVs may be the remnants of apoptotic cells (Kockx et al., 1998). In addition, previous studies have shown that cell death may promote MV generation and that chondrocyte apoptotic bodies share similarities with MVs (Kim, 1995; Cansu et al., 2010). Proudfoot et al investigated the association of cell death with calcification of VSMCs in vitro (Proudfoot et al., 2000). They reported that apoptosis occurred before the onset of calcification in VSMC nodules, and that stimulation or inhibition of apoptosis increased or decreased the calcification, respectively. Interestingly, like MVs, VSMC-derived apoptotic bodies concentrate calcium in crystallised forms (Proudfoot et al., 2000). Clarke et al also examined the role of apoptosis in vascular calcification. By using a mouse model of inducible VSMC-specific apoptosis, they demonstrated that chronic apoptosis of VSMCs accelerated atherosclerosis, calcification and medial degeneration (Rutsch et al., 2003). These observations provide evidence that VSMC-derived apoptotic bodies can initiate vascular calcification. In addition, Shroff et al have reported that apoptosis of VSMCs plays an important role in dialysis induced medial vascular calcification (Shroff et al., 2008).

Factors that promote cell survival have also been implicated in the control of vascular calcification (Figure 1.14). The growth arrest specific gene 6 (Gas6)/tyrosine kinase receptor Ax1 signalling pathway has been demonstrated to play an important role in preventing vascular calcification (Wax et al., 2001) (Figure 1.15). Gas6 binds to Ax1 (the predominant receptor of Gas6) on the cell surface and activates a number of downstream signalling pathways. The “canonical” pathway is considered to be the phosphatidylinositol 3-kinase (PI3K) pathway, resulting in cell survival (Wax et al., 2001; Melaragno et al., 2004). It has been reported that Gas6 inhibits the apoptosis of endothelial cells (Hasanbasic et al., 2004) and VSMCs (Melaragno et al., 2004). Collett et al have demonstrated that activation of Gas6/Ax1 signalling inhibits the osteogenic differentiation of vascular pericytes (Collett et al., 2003) and prevents VSMC in vitro calcification (Collett et al., 2007). This inhibitory effect requires the activation of PI3K signalling to prevent the apoptosis of VSMCs (Collett et al., 2007). Insulin-like growth factor 1 (Igf1), which promotes
proliferation, survival and migration of VSMCs, has been shown to inhibit the osteoblastic differentiation and mineralisation of vascular cells through both ERK and PI3K signalling pathways (Klein-Nulend et al., 1995). Further studies have shown that the post-translational glycosylation of the IGF receptor is crucial for the protective role of IGF1 on vascular calcification (Siddals et al., 2011).

**Figure 1.14 The role of cell death in vascular calcification.** Gas6/Ax1 activates PI3K signalling and promotes VSMC proliferation and survival, thereby inhibits vascular calcification. IGF-1 promotes proliferation and inhibits osteoblastic differentiation and calcification of VSMCs via ERK and PI3K signalling pathways.

### 1.3.5.5 Role of phosphate and calcium in vascular calcification

As described in section 1.3.3.1, vascular calcification is a common complication in CKD and increases cardiovascular morbidity and mortality in these patients. There is a strong link between the development of calcification in CKD patients and dysregulated mineral metabolism characterised by hyperphosphatemia and
hypercalcemia. Phosphorus, mainly in the form of inorganic Pi, is a key mineral present in bone, phospholipids and nucleic acids. It is essential for adenosine triphosphate (ATP) generation, intracellular signalling and pH buffering. In humans, 85% of Pi is present in bone, 14% is intracellular and only 1% is extracellular fluid (Goldman et al., 2007). The kidney is the major regulator of Pi homeostasis. Elevated serum Pi is a major risk factor for cardiovascular events in CKD patients (Block et al., 1998; Brancaccio et al., 2005; Adeney et al., 2009) as well as the general population (Dhingra et al., 2007; Kestenbaum et al., 2009; Tonelli et al., 2009). Serum Pi greater than 5.5mg/dL is strongly correlated with mortality in ESRD patient (Block et al., 2004; Noordzij et al., 2006; Tentori et al., 2008). In addition, a relatively small elevation in serum Pi (3.5-4.5 mg/dL) has also been correlated with increased risk of cardiovascular and all-cause mortality in CKD patients (Kestenbaum et al., 2005) and in the general population with normal renal function (Tonelli et al., 2005). The high risk of mortality is likely due to increased vascular calcification in CKD. In addition, elevated Pi has direct effect on VSMCs. Numerous studies have shown that increasing inorganic Pi in the culture media to levels comparable to those observed in hyperphosphatemia (≥2.4mM) results in matrix calcification in VSMCs cultured in vitro (Wada et al., 1999; Jono et al., 2000a; Steitz et al., 2001; Chen et al., 2002). Concomitant with calcification, the VSMCs undergo osteo/chondrogenic differentiation (Steitz et al., 2001). This phenotypic transition has also been observed in humans as well as in animal models of vascular calcification (Steitz et al., 2001; Moe et al., 2002; Moe et al., 2003). High Pi induced VSMC calcification is dependent on a sodium phosphate cotransporter \( \text{PiT-1} \) (Jono et al., 2000a). However, recent studies have shown that hyperphosphatemia-induced nanocrystals are responsible for the phenotypic transition of VSMCs, rather than soluble Pi (Sage et al., 2011). Although most studies focus on the role of elevated serum Pi in vascular calcification in CKD patients, evidence also indicates an important role of elevated calcium and an elevated calcium x Pi product in driving calcification. A number of studies have shown that elevated calcium is associated with calcification in CKD patients (Yamada et al., 2007a; Larsson et al., 2010; West et al., 2010). Likewise, elevated calcium levels in the culture media comparable to those seen in hypercalcemia (≥2.6mM) also induce the calcification and phenotypic
transition of VSMCs (Yang et al., 2004). Interestingly, elevated levels of extracellular calcium has been shown to induce the production of calcifying vesicles that contain preformed apatite- a hallmark of mineralisation-competent matrix MVs in VSMCs. However, high Pi could not induce the same effect on MV mineralisation (Reynolds et al., 2004; Reynolds et al., 2005). Further studies have indicated that elevated calcium regulates key components of VSMC-derived vesicles to enhance calcification (Kapustin et al., 2011).

1.4 Potential treatment of vascular calcification

Although currently there are no specific drugs that inhibit vascular calcification, several potential strategies have recently been investigated, including the administration of vitamin K, statins, bisphosphonates, TNAP inhibitors and Non-Steroidal Anti-Inflammatory Drugs (Figure 1.16).

1.4.1 Vitamin K

The vitamin K-dependent proteins (VKDPs) MGP and Gas6 are produced by VSMCs and pericytes. As described in section 1.3.5.2.6 and section 1.3.5.4, MGP and GAS6 are key inhibitors of vascular calcification. The process of converting VKDPs to their biologically active forms requires the carboxylation of glutamic acid residues by vitamin K (Furie et al., 1999). In rats, inactivation of MGP by treatment with the vitamin K antagonist warfarin leads to rapid calcification of the arteries. This can be regressed by a vitamin K-rich diet (Schurgers et al., 2007). Specifically, vitamin K2 supplementation prevents arterial calcification, yet vitamin K1 does not (Howe and Webster, 2000; Spronk et al., 2003). In the population based Rotterdam study, increased intake of vitamin K2, but not K1, was shown to be inversely related to all-cause mortality (relative risk = 0.91) and severe aortic calcification (relative risk = 0.74) (Geleijnse et al., 2004). A more recent investigation examined the association of vitamin K1 and vitamin K2 intake with coronary calcification in a cross-sectional study among 564 postmenopausal women (Beulens et al., 2009). 62% of the women had coronary calcification. Vitamin K2 intake was again associated with decreased coronary calcification (relative risk =0.80). Interestingly, one of the major dietary sources of vitamin K2 is cheese (Schurgers and Vermeer, 2000), which
although is not related to a healthy lifestyle or diet, has yet to be established as a dietary risk factor for cardiovascular disease. It is therefore possible that cheese could exert a beneficial effect in the cardiovascular system and that the high cheese consumption in France and the Mediterranean countries may possibly account for the lower prevalence of cardiovascular disease.

**Figure 1.15 Potential treatments for vascular calcification.** Medial vascular calcification is an active regulated process that involves the balance of calcification promoters and inhibitors. (1) Vitamin-K may inhibit vascular calcification via activation of MGP, a recognised calcification inhibitor. (2) Hydroxyapatite formation is normally inhibited by PPI, which is generated from ATP by ENPP1. Large doses of PPI have been shown to inhibit vascular calcification in Vitamin-D-toxic rats. Bisphosphonates are non-hydrolysable analogue of PPI that also inhibit vascular calcification. (3) TNAP hydrolyses PPI and this could be targeted by newly developed TNAP inhibitors.

### 1.4.2 Statins

The mechanism attributed to the pleiotrophic effects of statins involves the inhibition of RhoA/Rho-kinase (ROCK) activity (Rikitake and Liao, 2005). Inhibition of ROCK with the inhibitor Y-27632 or siRNA significantly increased TNAP activity and calcification of bovine VSMCs and rat aorta organ cultures (Chen et al., 2010b).
Furthermore, MVs isolated from bovine VSMCs incubated with Y-27632 showed increased TNAP activity and increased ability of MVs to subsequently calcify collagen by 66% (Chen et al., 2010). Together these data clearly demonstrate that the RhoA/ROCK signaling pathway is an important negative regulator of vascular calcification. Exposure to fluvastatin has been shown to directly inhibit calcification in VSMCs in vitro, with warfarin treatment abolishing this beneficial effect (Nakano-Kurimoto et al., 2009). Atorvastatin has also been shown to protect cultured VSMCs from Pi-induced calcification by inhibiting apoptosis via restoration of the Gas6-Axl pathway (Son et al., 2007). However, the clinical use of statins has yet to be shown to effectively inhibit vascular calcification, with neither fulvastatin (Forbat et al., 1998) nor atorvastatin (Schmermund et al., 2006) therapy able to attenuate coronary artery calcification progression. Furthermore, a recent clinical trial focusing on changes in coronary artery plaque composition and plaque volume during aggressive dual lipid-lowering therapy with atorvastatin and ezetimibe demonstrated no significant differences in plaque calcification (Kovarnik et al., 2012). These clinical data may be due to statins inhibiting the initiation rather than the progression of vascular calcification.

1.4.3 Bisphosphonates
Bisphosphonates are used as a standard therapy for osteoporosis. Studies in rats have shown that alendronate and ibandronate inhibit warfarin and uremia induced media calcification at doses that inhibit bone resorption (Price et al., 2001a; Price et al., 2006). However, it has recently been reported that whilst etidronate prevents the development of vascular calcification in rats with adenine-induced chronic renal failure, bone formation and mineralisation are adversely affected (Lomashvili et al., 2009). These findings support and extend previous results showing that the most effective etidronate dose for the prevention of arterial calcification also reduced bone mineral density in 5/6-nephrectomized rats (Tamura et al., 2007). In 2008, a multicentre genetic study and retrospective observational analysis of subjects affected by GACI revealed a positive association between survival and bisphosphonate treatment (Rutsch et al., 2008). More recently, the long-term survival of a severe case of GACI diagnosed prenatally and treated with etidronate over a 2
year period has been reported. Progressive resolution of arterial calcification was seen by 3 months of age, which was maintained until 2 years of age. Throughout the 2-year follow-up the patient developed mild hypophosphatemia, due to renal Pi wasting, without signs of rickets (Edouard et al., 2011). This study supports the development of a formalised approach for the treatment of GACI with bisphosphonates.

1.4.4 Phosphatase inhibitors
As described in section 1.3.5.2.2, TNAP and PHOSPHO1 play an important role in mineralisation. Novel chemical TNAP inhibitors have been generated by Narisawa et al (Narisawa et al., 2007). These chemical compounds have been shown to inhibit in vitro VSMC calcification (Narisawa et al., 2007). Recently, a PHOSPHO1 inhibitor has also been developed, which has been shown to suppress VSMC calcification (Kiffer-Moreira et al., 2013). Indeed, the combination of these two inhibitors further reduced calcification compared to either inhibitor alone (Kiffer-Moreira et al., 2013). Therefore, TNAP and PHOSPHO1 inhibitors may serve as scaffolds for future efforts to develop novel drugs to treat vascular calcification.

1.4.5 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)
Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are commonly used for anti-inflammation and analgesia post-operatively in orthopaedic patients. However, several studies have demonstrated that these drugs suppress bone growth, remodelling and repair (Nilsson et al., 1986; Keller et al., 1987; Ho et al., 1995) through mechanisms including cell cycle arrest and cell death induction (Chang et al., 2005). The administration of Tanshinone IIA, one of the major lipophilic components extracted from the root of Salvia miltiorrhiza Bunge (Shang et al., 2012), attenuates atherosclerotic calcification in a rat model, through inhibition of oxidative stress (Tang et al., 2007). Furthermore, the natural antioxidants curcumin and silybin inhibit VSMC calcification in vitro (Roman-Garcia et al., 2011). However, the cyclooxygenase-2 inhibitor Celecoxib induced no significant changes in atherosclerotic calcification in a mouse model of atherosclerosis (Bea et al., 2003).
Further studies are therefore required to more fully investigate the potential therapeutic applications of NSAIDs in suppressing vascular calcification.

1.5 Aims and strategy

Vascular calcification is now recognised as a common and clinically important risk marker of cardiovascular disease. Although vascular calcification shares many similarities with bone formation, further investigations are required to study the precise mechanisms of vascular calcification that will inform the development of novel therapeutics to treat vascular calcification. Shanahan and colleagues have shown that osteocytes are present in human vessels with medial calcification (Shanahan et al., 1999). However, this observation has yet to be investigated in detail. Therefore, the aim of this project was to examine whether VSMCs can undergo osteocytic differentiation within a calcifying environment and to assess the role of the osteocytic hormone FGF23 in modulation of VSMC calcification. Furthermore, I have also investigated the role of BMP9, a well-known osteogenic modulator, in vascular calcification. To achieve these aims, an in vitro murine VSMC calcification model combined with an animal model of medial vascular calcification (Enpp1−/− mice) has been used. During my PhD, I have completed the following aims:

1. Characterised the phenotype of the Enpp1−/− mouse as a medial vascular calcification model.
2. Determined the osteocyte phenotypic transition of VSMCs within a calcifying environment and in the calcified aortae of the Enpp1−/− mouse.
3. Examined the direct role of FGF23 in vascular calcification.
4. Defined the role of the osteogenic promoter BMP9 in vascular calcification.
2 Materials and Methods

2.1 Reagents
All chemicals were obtained from Sigma Aldrich (Poole, UK) unless otherwise stated. PCR primers were obtained from Qiagen Ltd (West Sussex, UK), Eurofins MWG Biotech (Ebersberg, Germany), or Primer Design (Southampton, UK). Antibodies were from R&D System (Minneapolis, USA), Cell Signalling (Herts, UK) or Abcam (Cambridge, UK) unless otherwise stated. Buffer recipes are listed in Appendix 1.

2.2 Cell culture

2.2.1 Preparation of cell culture medium
Minimum Essential Medium Alpha (α-MEM) was obtained from Life Technologies Ltd (Paisley, UK). α-MEM was supplemented with 1% gentamicin (Life Technologies Ltd), 10% foetal bovine serum (FBS) (heat-inactivated, Life Technologies) before use (referred to hereafter as growth medium). Media was then sterilised by passage through a 0.22µm filter and stored at 4°C. All tissue culture reagents were prepared in a category 2 culture hood with heat-sterilised equipment.

2.2.2 Isolation of primary calvarial osteoblasts
Primary calvarial osteoblasts were isolated as previously described (Hessle et al., 2002). Briefly, mouse pups at three days of age were sacrificed by cervical dislocation and the frontal calvariae were dissected. After suture removal, the calvariae were washed in hanks buffered saline solution (HBSS) (Life Technologies Ltd), followed by serial collagenase digestion using a four step process at 37°C in a shaking incubator:

1. 10min incubation in 1mg/ml collagenase type II (Worthington, New Jersey, USA). The supernatant was discarded.
2. The calvariae were incubated for a further 30min in 1mg/ml collagenase type II. The supernatant was retained and termed fraction 1.
3. The calvariae were incubated for 10min in 4mM ethylenediaminetetraacetic acid (EDTA). The supernatant was harvested and termed fraction 2.
4. The calvariae were finally digested in 1mg/ml collagenase type II for 30min and the supernatant was collected and termed fraction 3.

The supernatants from fractions 1, 2, 3 were combined and centrifuged at 2,000rpm for 5min and the cells were resuspended in 1ml growth medium. 1ml of cell suspension was added to T75 flasks (Greiner Bio-One, GmbH, Frickenhausen, Baden- Württemberg, Germany) containing 12ml growth medium. The flasks were incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂ for 3h. The medium was then changed to remove dead cells and cellular debris. The cells were then incubated for a further 3d until 75% confluency, before sub-culturing and use in other experiments (Figure 2.1A).

2.2.3 Isolation of primary murine Vascular Smooth Muscle Cells (VSMCs)
As previously described (Johnson et al., 2008), VSMCs were isolated from five week old male C57BL/6 mouse aortae, the adventitia was removed and the aorta was cut open to expose the endothelial layer. Aortae from eight mice were pooled for digestion with 1mg/ml trypsin at 37°C for 10min to remove any remaining adventitia and endothelium, and then cultured in growth medium overnight in a humidified atmosphere of 95% air/5% CO₂ at 37°C. Tissues were then digested in 425U/ml collagenase type II for 4h at 37°C. Tissues together with the supernatant were then transferred to a 25ml universal tube containing 20ml growth medium and centrifuged at 2,000rpm for 5min. The cells were resuspended in 1ml growth medium. The 500µl of cell suspension was added to a T25 flask containing 6ml of growth medium, which was coated with 0.25µg/cm² murine laminin to promote maintenance of the contractile differentiation state. VSMCs were expanded for two passages in T25 flasks and used for further experiments (Figure 2.2A). Isolated VSMCs exhibited positive staining for SMA, a specific marker of VSMCs (Figure 2.2B).
Figure 2.1 Isolated mouse calvarial osteoblasts. (A) Morphology of mouse calvarial osteoblasts at confluence. (B) Mineralised nodules were formed after 28d when cultured with 50µg/ml ascorbic acid and 2.5mM βGP, as revealed by Alizarin Red S staining. Scale bars=50µm.

Figure 2.2 Isolated mouse VSMCs. (A) Morphology of VSMCs at confluence. (B) Isolated VSMCs exhibited positive staining SMA (Green). Nucleus was stained with DAPI (Life Technologies) (Blue). Scale bars=50µm.
2.2.4 Induction of calcification
Primary murine calvarial osteoblasts and murine VSMCs were seeded at a density of $1.5 \times 10^4$/cm$^2$ and cultured in growth medium at 37°C in a humidified 5% CO$_2$ atmosphere. As previously described (Hessle et al., 2002), matrix calcification of osteoblasts was induced by adding 50µg/ml ascorbic acid (AA) and 2.5mM βGP to the growth medium for up to 28d (Figure 2.1B). Matrix calcification of VSMCs was induced by adding either 50µg/ml AA and 2.5mM βGP (Steitz et al., 2001) or 2mM Pi (a mixture of Na$_2$HPO$_4$ and NaH$_2$PO$_4$, pH=7.4) to reach a final concentration of 3mM Pi (Montes de Oca et al., 2010). Media was changed every third/fourth day.

2.2.5 Quantification of calcification
Calcium deposition by osteoblasts and VSMCs was evaluated by Alizarin Red S staining, as previously described (Zavaczki et al., 2011). Briefly, cells were washed twice with phosphate buffered saline (PBS) (Life Technologies Ltd) and fixed in 4% paraformaldehyde (PFA) for 5min at 4°C. 2% Alizarin Red S (pH=4.2) was used to stain cell layers for 10min at room temperature. Staining was quantified by the addition of 1ml 10% cetylpyridinium chloride for 10min to leach the stain. The optical density of the resultant solution was analysed using a Multiskan Ascent plate reader (Thermo Scientific, Northumberland, UK) at a wavelength of 570nm. 10% cetylpyridinium chloride was used as a blank.

As previously described, calcium deposition by VSMCs was also assessed using an acid leaching protocol (Zavaczki et al., 2011). Cells were washed twice with PBS and decalcified in 0.6N HCL for 24h at room temperature. Calcium content of the supernatants was colorimetrically determined by the Randox Calcium Assay Kit (Randox Ltd, Crumlin, UK). After decalcification, cells were washed three times with PBS and harvested in 0.1M NaOH/0.1% sodium dodecyl sulphate (SDS). Protein content of the samples was measured using the Bio-Rad protein assay reagent (Bio-Rad, Hertfordshire, UK) based on the Bradford dye binding procedure and gamma globulin was used as standard. The total calcium content was normalised with regard to the total protein as µg/mg protein.
2.2.6 Determination of alkaline phosphatase (TNAP) activity
Primary murine calvarial osteoblasts and VSMCs were harvested in 300µl 1% Triton-x-100 with 1mM MgCl₂. TNAP activity was analysed using Thermo-line TNAP reagent (Thermo-line, Melbourne, Australia). The enzyme activity was measured by using p-nitrophenyl phosphate (pNPP) as a substrate, which is converted to the yellow chromogen p-nitrophenyl in the presence of TNAP (Orriss et al., 2007). The optical density was read at 405nm by a Multiskan Ascent plate reader. Protein content was measured with the Bio-Rad protein assay. Total TNAP activity was expressed as nmoles pNPP hydrolysed/min/mg protein.

2.2.7 Determination of cell viability
Cell viability was measured using Alamar Blue (Life Technologies). At 0, 7, 14, 21 and 28d, Alamar Blue dye (10µl per 100µl medium) was incubated with cells at 37°C for 4h in 96-well plates. The plates were processed for optical density absorbance analysis using a Multiskan Ascent plate reader at a dual wavelength of 575nm and 600nm. Culture medium was used as a blank.

2.3 Transfecting Vascular Smooth Muscle Cells (VSMCs)
2.3.1 Plasmids
pBV-SBE4-Luc (Figure 2.3) containing four copies of the Smad-binding site (GTCTAGAC) was produced by Zawel et al (Zawel et al., 1998) and was purchased from Addgene (Cambridge, USA; Addgene database: 16495). A Renilla luciferase expression plasmid pEF1-RLuc was a kind gift from Dr. Tom Burdon (The Roslin Institute, The University of Edinburgh, UK).

2.3.2 Transformation of Escherichia coli (E.coli)
The two plasmids described in section 2.3.1 were transformed into E.coli to enable DNA amplification. XL-10 Gold Ultracompotent E. coli strain was used (Stratagene, Santa Clara, USA). The XL-10 gold cells were thawed on ice and a 40µl aliquot was then incubated with 1.6µl of β-mercaptoethanol for 10min in a 1.5ml micro-centrifuge tube. 1µl of plasmid, typically 5-500ng of DNA, was then added to the cells, mixed gently and incubated on ice for 30min. The cells were then heat-shocked
at 42°C for 30s and returned to ice for a further 2min. 450µl of autoclaved pre-warmed Super Optimal Broth with Catabolite repression (SOC) medium was then added to the transformation reaction and the cells were grown for 1h at 37°C in an orbital shaker at 200rpm. 100µl of the transformed culture was spread on Lysogeny broth (LB) agar plates supplemented with 100µg/ml of ampicillin. Plates were inverted and incubated at 37°C overnight.

2.3.3 Maxi-preparation of plasmid DNA

Individual colonies were picked from the agar plates of transformed bacteria and placed into a 5ml LB culture medium containing 100µg/ml ampicillin. This was incubated overnight at 37°C with constant agitation. The bacteria culture was then transferred to 250ml LB culture medium with 100µg/ml of ampicillin in glass erlenmeyer flasks and grown at 37°C overnight with constant agitation. The plasmid DNA was purified using the PureLink HiPure Plasmid Filter Maxiprep Kit (Life Technologies). Briefly, the bacterial cells were harvested by centrifugation at 4,000rpm for 60min at 4°C. The bacterial pellet was resuspended in 10ml resuspension buffer R3 containing RNase A, the cells were then lysed by addition of 10ml lysis buffer L7, mixed thoroughly by inverting and incubated at room temperature for 5min. 10ml precipitation buffer N3 was added to the lysate and mixed thoroughly, leading to precipitation of the genomic DNA, proteins, cell debris, and SDS. The lysate was then poured into a HiPure Filter Midi Column equilibrated with buffer EQ1 and allowed the column to drain by gravity flow. The column was then washed with 20ml wash buffer W8. The DNA was then eluted by 5ml elution buffer E4, precipitated through the addition of 0.7volumes of room temperature isopropanol and pelleted by 30min centrifugation at 12,000rpm at 4°C. The supernatant was discarded and the DNA pellet washed in 70% ethanol and centrifuged at 12,000rpm for a further 30min. The supernatant was discarded and the pellet left to air dry for 10min. The DNA pellet was then resuspended in 100µl TE buffer. DNA concentration and quality was determined on a Nanodrop spectrophotometer (ND-1000). Quality was assessed by the A260/A280 ratio, where 1.8 was considered optimum. The plasmid DNA was stored at -20°C until required. The plasmid pBV-SBE4-Luc was sequenced by Eurofins MWG Biotech (Figure 2.4).
Figure 2.3 Map of pBV-SBE4-Luc: four copies of the Smad4 binding element are cloned to pBV-Luc

Figure 2.4 The sequence of pBV-SBE4-Luc. The Smad4 binding sites are indicated in red font.

5’AGACTTTCTCTATCGATAGGTACTAA GTCTAGACGGCCA GTCTAGACGTACT
AAGTCTAGACGCGAGCTAGACGTACCAGCCTTACGCTGCTAGCCCGG
GCTCGAGATCTGCGATCTGCATCTCAATTAGTCAGCAACCATAGTCCCGCC
CCTAACCTCGCCATCCGCCCCCTAATTAGTCAGCGCCTCCTAGTTTTTT
GCCCCATCGCTGACTATTCCAGAAGTTTTTTATTTATTCAGAGGCCAGGCCAGCCGCTC
CCTCTGAGCTATCCAGAAAGTAG3’
2.3.4 Transient transfection of plasmid DNA
VSMCs were seeded into 12-well plate at a density of 1.5 x 10^4/cm^2 and cultured overnight to reach approximately 60% confluency. Cells were then transfected with 0.4µg/ml pBV-SBE4-Luc and 8ng/ml internal control plasmid pEF1-RLuc (for each well to be transfected) using FuGENE6 transfection reagent (Roche, West Sussex, UK) according to the manufacturer’s instructions. Briefly, 1.2µl FuGENE6 was diluted in 98.8µl serum-free α-MEM medium in a sterile tube (for each well to be transfected). 0.4µg pBV-SBE4-Luc and 8ng pEF1-RLuc were added to the α-MEM/FuGENE6 mixture, mixed gently and incubated for 15min at room temperature. The transfection reagent: DNA complex was then added to the VSMCs in a drop-wise manner. Control cells were incubated with α-MEM/FuGENE6 alone. The cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO2 for 24h.

2.3.5 Luciferase activity assay
Luciferase was performed as previously described (Amarzguioui et al., 2000) using the Dual-Luciferase® Reporter Assay System kit (Promega, Southampton, UK). The transfected cells were washed with ice cold PBS and incubated with 250µl passive lysis buffer for 30min on ice. Following brief centrifugation to pellet cell debris, luciferase assay was performed in white non-transparent 96-well plates (Thermo Scientific) using a GloMax 96 Microplate Luminometer (Promega) equipped with two injectors. Dual-luciferase assays were performed on 20µl lysate supernatant. Firefly and Renilla luciferase activities were read following the respective injections of 100µl LAR II and 100µl Stop & Glo (Figure 2.5). The instrumental background levels of luminescence were recorded using untransfected cells. The relative luciferase activity was calculated as a ratio of the Firefly activity to the Renilla activity.
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Figure 2.5 Format of the Dual Luciferase Assay using GloMax 96 Microplate Luminometer equipped with two injectors.

20µl PLB lysate

100µl LARII

First measurement Firefly luciferase

100µl Stop & Glo

Second measurement *Renilla* luciferase
2.3.6 Transfection of Small interfering RNA (siRNA)
FlexiTube GeneSolution for Smad4 containing four preselected siRNAs and AllStars Negative Control siRNA (referred to hereafter as scramble siRNA) were purchased from Qiagen Ltd. On the day of transfection, VSMCs were seeded into 12-well plate at a density of 80,000 cells/well. siRNAs were diluted to 20nM in 200ul α-MEM culture medium without serum and mixed by vortexing. 6µl of Hyperfect transfection reagent (Qiagen Ltd) was added to the diluted siRNA, mixed by vortexing and incubated for 10min at room temperature to allow formation of transfection complexes. The complexes were then dropped onto cells. The plate was gently swirled to ensure uniform distribution of the transfection complexes and incubated at 37°C with 5% CO₂ for 48h or 96h. qPCR and western blot was used to confirm the knockdown efficiency of siRNA.

2.4 RNA methods
2.4.1 RNA isolation
Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen Ltd), according to the manufacturer’s instructions. Briefly, cells were directly lysed in 350µl RLT buffer (Qiagen) containing 3.5µl β-mercaptoethanol and transferred to a nuclease free centrifuge tube. The cells were homogenised with an electronic homogeniser for 30s. 350µl of 70% ethanol was then added into the homogenised lysate and mixed well by pipetting. The samples were loaded on Qiagen Rneasy columns, followed by centrifugation. The flow-through was discarded and 700µl buffer RW1 was added to the columns. After centrifugation, the columns were washed twice with RPE buffer and placed in a new 1.5ml collection tube. Total RNA was finally eluted with 35µl nuclease-free water. For each sample, total RNA content was analysed by absorbance at 260nm and purity by 260/280 ratios using a Nanodrop spectrophotometer. Samples were then diluted to the same concentration (that of the lowest sample) in nuclease-free water.

2.4.2 Reverse transcription
20µl diluted RNA was incubated with 4µl random primers (random primers diluted 1:60; Life Technologies) at 70°C for 10min in a Hybaid PCR Express Thermal
cycler (Thermo Scientific). The sample was then rapidly cooled on ice. A master mix of 4µl 5 x First-Strand buffer (Life Technologies Ltd), 2µl dithiothreitol (DTT) (0.1M; Life Technologies Ltd), 2µl dNTP mix (10mM; Life Technologies Ltd) and 1µl SuperscriptTM II RT (Life Technologies Ltd) was prepared and 16µl of master mix was added to each sample. The samples were run on the following program in a Hybaid PCR machine (Thermo Scientific): 25°C for 10min; 42°C for 50min; 70°C for 15min and held at 4°C. The cDNA samples were stored at -20°C. For semi-quantitative RT-PCR or quantitative RT-PCR reactions, the cDNA was diluted to 5ng/µl in nuclease-free water.

2.4.3 Semi-quantitative polymerase chain reaction (PCR)

PCR was performed on cDNA to examine gene expression levels. PCR reactions were prepared in a total volume of 30µl, containing 2.5µl 10 x PCR buffer minus Mg, 0.5µl dNTP mix (10mM; Life Technologies Ltd), 0.6µl MgCl₂ (50mM; Life Technologies Ltd), 1.25µl primer mix, 5µl template cDNA (5ng/µl), 0.125 µl Taq polymerase (5U/µl; Life Technologies Ltd) and 15.025µl nuclease-free water. The mixture was cycled in a Hybaid PCR Express Thermal cycler (Thermo Scientific) under the following conditions: 1 cycle of 94°C for 3min, 30-35 cycles of 94°C for 30s, 52-60°C for 45s and 72°C for 1min, followed by a final cycle of 72°C for 7min. The number of cycles and annealing temperatures varied depending on the primers being used. PCR products were then run on a 1.5% agarose gel. 18s primers (Primer Design) were used as a loading control. Nuclease-free water was used instead of cDNA template as negative control.

2.4.4 Agarose gel electrophoresis

The PCR products obtained from section 2.4.3 were diluted 5:1 with 5 x blue loading buffer (Bioline Reagents Ltd, London, UK). 20µl was loaded per sample in separate wells in 1.5% Agarose/1 x Tris-acetate-EDTA (TAE) (Ambion, Cambridge, UK) gels containing 0.1µl/ml SYBR-Safe (Life Technologies Ltd). Gels were run in TAE buffer in an electrophoresis tank at 100V. HyperLadder™ 100bp (Bioline Reagents Ltd) were used as molecular weight markers. Gels were imaged under UV light using a Gel Logic 200 Imaging System and software (Kodak, Herts, UK).
2.4.5 Quantitative PCR (SYBR Green)
The qPCR reactions were conducted in a 96-well plate (Thermo Scientific). Each reaction consists of 5µl cDNA (5ng/µl) plus 15µl master mix. Master mixes contained 1µl primers (10µM), 4µl nuclease-free water and 10µl of SYBR Green (Roche). A reaction containing H₂O in place of cDNA was also amplified as a negative control. The qPCR reaction was cycled in a Stratagene Mx3000P PCR cycler (Agilent Technologies, Santa Clara, USA) using the following protocol: 1 cycle of 95°C for 10min followed by 50 cycles of 95°C for 15s and 60°C for 1min. Each sample was tested in triplicate. Primers were designed to span an exon to avoid amplifying genomic DNA. Primers were tested for efficiency using serial dilutions of cDNA (known to express the gene of interest) to create a standard curve. Primers were considered acceptable if the amplification efficiency was within the range of 90-110%, with an R² value between 0.99 and 1.00. Primer specificity was demonstrated by the generation of a single peak in the dissociation curve (Figure 2.6). The relative expression of the analysed genes compared to the housekeeping gene Gapdh (Primer Design) was calculated using the ∆∆Ct method (Livak and Schmittgen, 2001). The primers used in this study are listed in Appendix 2.

2.5 Protein Methods
2.5.1 Determination of protein concentration
2.5.1.1 Detergent compatible (DC) protein assay
Cells were washed with ice cold PBS and lysed in an appropriate volume of radioimmunoprecipitation assay (RIPA) buffer containing 0.15 x volume of complete mini protease inhibitor cocktail (Roche). The samples were centrifuged at 12,000 rpm for 5min to remove cell debris and the supernatant was transferred to a fresh centrifuge tube. DC protein assay (Bio-Rad) was used to measure the protein concentration according to manufacturer’s instructions. Briefly, bovine γ-globulin protein standards ranging from 0.125-2 mg/ml were prepared in RIPA buffer. 5µl of standard or sample was added into a well of a 96-well plate in duplicate followed by 25µl alkaline copper tartrate (Reagent A) and 200µl Folin’s reagent (Reagent B). The plate was shaken and incubated at room temperature for 15min. The absorbance at 690nm was read on a Multiskan Ascent plate reader and the protein concentration
Figure 2.6 Validation of qPCR primer efficiency and specificity. Representative image of (A) standard curve and (B) dissociation curve.
2.5.1.2 Bradford protein assay
Bradford protein assay was also used to measure protein concentration of certain samples. Briefly, nine $\gamma$-globulin protein standards ranging from 10µg/ml-90µg/ml were prepared. 160µl of standard or diluted sample was added into a well of a 96-well plate in duplicate followed by 40µl Bradford dye reagent concentrate (Bio-Rad). The plate was shaken and incubated at room temperature for 5min and the absorbance was read at 595nm on a Multiskan Ascent plate reader. The protein concentration was determined by reading from the standard curve.

2.5.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)
As described in section 2.5.1.1, cells were scraped in RIPA buffer and protein concentration was determined by DC protein assay. Samples were prepared as follows: The volume of sample required for 10µg of total protein was calculated. Appropriate volumes of 4 x lithium dodecyl sulphate (LDS) sample buffer (Life Technologies Ltd) and reducing agent dithiothreitol (DTT) (Life Technologies Ltd) were added to the protein samples and denatured at 70°C for 10min. Proteins were then separated according to weight on 10% Novex Bis-tris gel (Life Technologies Ltd). The comb was removed from pre-cast gel and the wells were rinsed with 1 x 3-(N-morpholino) propanesulfonic acid (MOPS) or 2-(N-morpholino) ethanesulfonic acid (MES) running buffer (Life Technologies). The gel was then placed into a tank filled with 1 x MOPS or MES running buffer (Life Technologies Ltd) and the centre of the tank was filled with an anti-oxidant (Life Technologies Ltd) to maintain proteins in their reduced state. The denatured protein samples and a pre-stained molecular weight marker All Blue (Bio-Rad) were then loaded onto the gel. The gel was ran at 200V for 50min to separate the proteins.

2.5.3 Western blotting
Following electrophoresis, the gel was removed from the cassette and immersed in transfer buffer. The protein was transferred onto a nitrocellulose membrane (Amersham Hybond ECL, GE Healthcare, Buckinghamshire, UK), which had been
pre-soaked with transfer buffer. Transfer was carried out in ice cold transfer buffer using the X-blot module system (Life Technologies Ltd) at a current of 30V for 2h. The nitrocellulose membrane was blocked for 1h at room temperature in 5% BSA in Tris-Buffered Saline containing 0.1% Tween 20 (TBST). The membranes were incubated with primary antibody (at an appropriate dilution in blocking buffer) overnight at 4°C. The membrane was washed in TBST to remove any unbound primary antibody and then incubated with corresponding (horseradish peroxidase) HRP-conjugated secondary antibody at an appropriate dilution in blocking buffer (DAKO, Glostrup, Denmark) for 1h. The membrane was washed with TBST three times and the immune complexes were visualised by enhanced chemiluminescence (ECL) (GE Healthcare). The membranes were then stripped with “stripping buffer” (Pierce, Rockford, USA) at room temperature for 1h and 20min and reprobed for β-actin expression (1:25000 dilution in 5%BSA), and the immune complexes were visualised by ECL. Where required, quantitative analysis of protein expression was performed by digitally scanning the developed ECL films and measuring protein density using Image J software (Wayne Rasband, National Institute of Health, USA). The antibodies used and their dilution are listed in Appendix 3.

2.6 In vivo methods

2.6.1 Generation of Enpp1\(^{-/-}\) mice

Enpp1\(^{-/-}\) (encoding NPP1 protein) mice were originally created by Sali et al (Sali et al., 1999).

2.6.2 Animal maintenance and breeding

The Enpp1\(^{-/-}\) mouse line was obtained through a Material Transfer Agreement (MTA) from Professor José Luis Millán (Sanford-Burnham Medical Research Institute, California, US). The Enpp1\(^{-/-}\) mouse is on a mixed background of 129 sv\(^{+/+}\) (ES cells), C57BL/6 (blastocyst donor and G1 production) and 129J. Enpp1\(^{+-}\) mice were mated together in the Small Animal Unit at The Roslin Institute to expand transgenic lines. Offspring carrying the mutant Enpp1 gene were identified by genotyping (section 2.6.3). All animals were maintained under conventional housing
conditions with a 12h light/dark cycle and were given free access to tap water and standard rodent feed.

2.6.3 Genotyping of Enpp1\(^{-/-}\) mice

Genotyping of Enpp1\(^{-/-}\) mice was performed by Genetyper (Genetyper, New York, US). Briefly, total RNA was extracted from mouse ear samples and reverse transcribed. RT-PCR was used to genotype Enpp1\(^{-/-}\) and wild-type samples. Primers and PCR conditions used were developed by Genetyper and details have not been disclosed. As demonstrated in Figure 2.7, RT-PCR analysis shows the 160bp product from a wild-type mouse and a product of 220bp from a homozygous mutant mouse. Both 160bp and 220bp products are seen in a heterozygous mutant mouse.

![Figure 2.7 Genotyping of Enpp1\(^{-/-}\) and wild-type mice using RT-PCR. RT-PCR analysis shows the 160bp product from a wild-type mouse, a product of 220bp from a homozygous mutant mouse, both 160bp and 220bp products from a heterozygous.](image)

![Figure 2.8 The \(\mu\)CT analysis for cortical and trabecular bone of Enpp1\(^{-/-}\) and wild-type mice. Pictures describing the scanned position for trabecular and cortical bone analysis.](image)
2.6.4 Micro-computed tomography (µCT)

All µCT analysis and data interpretation was done according to advice from Dr. Rob van’t Hof (Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK). Tibiae and femurs were dissected from female wild-type and Enpp1⁻/⁻ mice at the age of 22 weeks and stored in distilled water at -20°C. In order to analyse trabecular architecture and cortical bone geometry, the bones were scanned with a µCT system (Skyscan 1172 X-Ray microtomograph, Aartselaar, Belgium). High-resolution scans with an isotropic voxel size of 5µm were obtained (60kV, 0.5mm aluminium filter, 0.5° rotation angle). Two images at each rotation angle were averaged in order to reduce signal noise and improve the accuracy of the BMD measurements. Scan time was approximately 30min per bone. The scans were then reconstructed with NRecon software (Skyscan, Belgium). For each bone, a 1mm section of the metaphysis was used for analysis of trabecular bone. The base of growth plate was used as a standard reference point. A further 1500µm below the base of the metaphysis section a 500µm section of the mid-diaphysis was used to analysis of cortical structure (Figure 2.8).

A median filter (radius=1) was applied to reduce the noise in the reconstructed images. Bone tissue was identified by thresholding. Threshold is used to segment “bone” from “non-bone” to create a binary image based on a two-dimensional CT scan. The optimal threshold was determined from image histograms and was set to exclude soft tissue but to include poorly mineralised bone. All samples were analysed with the same threshold. The BMD of the bone structures were measured using the thresholded images as a mask. The unfiltered image data were used as input. For accurate calculation of BMD appropriate calibration of the Skyscan CT analyser was carried out with known density calcium HA phantoms. The following parameters were analysed using CTAn software (Skyscan, Belgium). For trabecular bone, percent bone volume (%BV/TV), trabecular number (Tb.N; /mm), bone mineral density (BMD; g/cm³), trabecular thickness (Tb.Th; mm), trabecular separation (Tb.Sp) and structure model index (SMI) were analysed. For cortical bone, %BV/TV, BMD (g/cm³), cortical thickness, percentage of closed pores were evaluated.
2.7 Histology

2.7.1 Tissue processing and sectioning

Mice were culled by cervical dislocation and tissue was immediately harvested. Bone and soft tissues were fixed in 4% PFA for 24h at 4°C. The bone tissue samples were then decalcified in 10% EDTA (pH 7.4) for 2-4 weeks at 4°C, EDTA was changed every 3 days. Decalcified bone and soft tissues were washed in distilled H$_2$O and processed into wax using the following protocol: two changes of each of the following: 70% ethanol for 1h; 80% ethanol for 1h; 95% ethanol for 1h; 100% ethanol for 1h; xylene (VWR, Leicester, UK) for 1h; and overnight in paraffin wax (VWR) at 60°C, followed by fresh paraffin wax for 1h. The processed tissue was then embedded in paraffin wax and allowed to cool. Wax blocks were cooled an ice for 30min and sectioned at a thickness of 5µm. The sections were floated onto a waterbath at 40°C and transferred to electrostatically-charged slides (VWR). The slides were dried at 37°C overnight to ensure attachment of the sections to the slide.

2.7.2 Alizarin Red S staining for calcified aortae

Paraffin sections were de-waxed in xylene and rehydrated through a series of graded alcohols to distilled H$_2$O. The sections were stained with Alizarin Red S for 10-30min at room temperature and the intensity of the orange red staining was checked by microscopy. After removing excess dye, the sections were rinsed in acetone for several seconds and then washes with 50:50 acetone:xylene. Finally, the sections were cleared with xylene for 5min and mounted in DePeX (VWR).

2.7.3 Immunohistochemical staining

Immunohistochemical staining was performed using the Vectastain Elite ABC kit (Vector Labs, Peterborough, UK) according to manufacturer’s instructions. Briefly, paraffin sections were de-waxed in xylene and rehydrated through a series of graded alcohols to distilled H$_2$O. Bone sections were pre-treated with 1mg/ml trypsin at 37°C for 30min and soft tissues were incubated in the citric acid buffer (0.01M, pH=6.0) for 20min at 100°C to unmask epitopes blocked during fixation. Exogenous peroxidise activity was quenched by incubating the sections in 0.3% H$_2$O$_2$ in PBS for 30min at room temperature. Sections were washed with PBS (3 x 5min) and blocked
in blocking buffer (1:100 dilution of blocking serum from donor of secondary antibody in PBS for 30min at room temperature. The sections were then incubated in primary antibody (Appendix 3), which was diluted to an appropriate concentration in blocking buffer at 4°C overnight. Unbound primary antibody was washed with PBS (3 x 5min) and the sections were then incubated in biotinylated labelled secondary antibody (1:200 dilution in blocking buffer) at room temperature for 30min. Following by 3 x 5min washes in PBS, the sections were incubated with ABC reagent (avidin and HRP) for 30min at room temperature. After 3 x 5min washes with PBS, staining was developed using diaminobenzidine (DAB, Sigma) solution (0.06% DAB in 0.1% H₂O₂ in PBS) as substrate until the desired reaction intensity was reached. The sections were rinsed in tap water and counterstained with haematoxylin (VWR). Finally, sections were dehydrated through graded alcohols, cleared with xylene and mounted in DePeX (VWR).

2.8 Enzyme-linked immunosorbent assay (ELISA)

2.8.1 Sample collection
Sera from children with CKD (10 dialysis and 10 predialysis) were kindly provided by Dr. Rukshana C. Shroff (Renal Unit, Great Ormond Street Hospital for Children, London, UK) and Professor Cathy Shanahan (Division of Cardiovascular Medicine, King’s College London, London, UK) and collected as previously described (Shroff et al., 2008).

2.8.2 BMP9 ELISA
100µl of human serum was used to measure BMP9 concentrations using the human BMP9 DuoSet Kit (R&D systems) according to manufacturer’s instructions. In brief, a 96-well microplate was coated with 100µl diluted capture antibody (1:180 dilution in 1% BSA in PBS) overnight at room temperature. Following by washing with PBS containing 0.05% Tween 20 (3 x 400µl), the plate was blocked with 300µl 1% BSA in PBS for 1h. BMP9 recombinant protein standards ranging from 0-1000pg/ml were prepared in 1% BSA in PBS. The plate was washed with PBS containing 0.05% Tween 20 (3 x 400µl) and incubated with 100µl standard or sample for 2h at room temperature. After washing, 100µl detection antibody (1:180 dilution in 1% BSA in
PBS) was added to the plate and incubated for 2h at room temperature. After further washing with PBS containing 0.05% Tween 20 (3 x 400µl), the plate was incubated with Streptavidin-HRP (1:200 dilution in 1% BSA in PBS) for 20min before further washes with PBS containing 0.05% Tween 20 (3 x 400µl). Finally 100µl substrate solution (1:1 mixture of Color Reagent A and Color Reagent B, R&D systems) was added for 20min in the dark room, and thereafter 50µl stopping solution was finally added to the plate. The plate was processed for optical density absorbance analysis using a BioTek plate reader (BioTek, Bedfordshire, UK) at a dual wavelength of 450nm and 540nm. The BMP9 concentration was determined by reading from the standard curve, which was created by Gen5 software (BioTek) using a four parameter logistic (4-PL) curve-fit.

2.9 Statistics
Statistical analysis was performed using Minitab16 (Coventry, UK). Kolmogorov-Smirnov normality test was performed to check whether experimental data were normally distributed. For normally distributed experimental data, the Student’s t-test was performed to analyse significance between two data groups. To compare more than two data groups, one-way analysis of variance (ANOVA) using a general linear model incorporating pair wise comparisons was performed. For experimental data that were not normally distributed, a suitable non-parametric test such as the Mann-Whitney test was performed. All data are expressed as the mean +/- standard error of the mean (SEM). P<0.05 was considered to be significant.
3 Assessment of the Enpp1\textsuperscript{−/−} mouse as a model of vascular calcification

3.1 Introduction

Animal models of human disease mimic pathological states known to occur in humans and are used in basic as well as pre-clinical research. Through using animal models, scientists can investigate the cellular and molecular mechanisms underlying a specific pathological process. In addition, animal models are also used to develop and evaluate new therapeutics. As described in Chapter 1, vascular calcification has significant clinical consequences. Over the past two decades, a variety of rat and mouse models have been developed in order to investigate the cellular and molecular mechanisms of vascular calcification.

Gene deletion (knockout) and gene over-expression in mice have been reported as commonly employed techniques to show the function of a particular protein in the pathological process of vascular calcification. Several murine knockout models of genes that regulate bone formation have led to new insights into the pathogenesis of arterial calcification. For instance, the osteocyte hormone FGF23 and its cofactor Klotho have been implicated in the pathological process of vascular calcification. FGF23 null mice (Razzaque et al., 2006; Stubbs et al., 2007) and Klotho deficient mice (Kuro-o et al., 1997) develop extensive vascular and soft tissue calcification. MGP (Luo et al., 1997), Fetuin A (Schafer et al., 2003), OPG (Bucay et al., 1998), NPP1 (Johnson et al., 2005) deficient mice, as well as ANK (Ho et al., 2000) mutant mice also display extensive vascular calcification, revealing important roles for these proteins in the calcification process, as described in Chapter 1.

The ApoE knockout mouse and the Low-Density Lipoprotein Receptor (LDLR) knockout mouse are two well-established animal models for studying atherosclerosis (Nakashima et al., 1994; Ma et al., 2012). ApoE, a glycoprotein and constituent of all lipoproteins except LDL, is mainly produced in the liver and brain. It is a ligand of receptors that are responsible for clearing chylomicrons and very low–density lipoprotein (VLDL) remnants (Meir and Leitersdorf, 2004). ApoE\textsuperscript{−/−} mice show
increased plasma VLDL levels and LDL concentrations as well as decreased HDL levels, leading to the development of atherosclerotic lesions in the aortic root and the ascending aorta (Jawien et al., 2004). Interestingly, ApoE−/− mice develop all phases of atherosclerotic lesions that are observed in humans, including early fatty streaks comprised of foam cells and migrating smooth muscle cells over more advanced lesions with a necrotic core and proliferating smooth muscle cells surrounded with a fibrous cap as well as calcified plaques (Nakashima et al., 1994). Administration of a western diet (high fat diet) strikingly increases VLDL and LDL levels and accelerates the formation and size of lesion in ApoE−/− mice (Plump et al., 1992; Nakashima et al., 1994). It has been reported that accelerated atherosclerosis significantly increases mortality and morbidity in patients with CKD (London and Drueke, 1997; Foley et al., 1998). The most marked difference of atherosclerotic plaques in patients with or without CKD is not in plaque size but their calcification extent (Schwarz et al., 2000; Moe et al., 2002). CKD induced by 5/6 nephrectomy in ApoE−/− mice significantly accelerates atherosclerosis and both intimal and medial vascular calcification (Bro et al., 2003; Massy et al., 2005).

LDLR is a cell-surface receptor that removes cholesterol-rich intermediate density lipoproteins (IDL) and LDL from plasma and thereby regulates the plasma cholesterol level (Brown and Goldstein, 1986). Inactivating mutations of LDLR in humans result in familial hypercholesterolemia characterised by significantly increased LDL plasma levels, which is a significant risk factor of atherosclerosis and heart disease (Laios and Drogari, 2006). Ldlr−/− mice show increased plasma cholesterol, mainly due to elevated LDL levels. These mice develop no or mild atherosclerosis, with only small fat deposits in the aorta (Ishibashi et al., 1994). However, when fed with high cholesterol diet, these mice display a dramatic increase in cholesterol levels and show rapidly developing, large atherosclerotic lesions (Ishibashi et al., 1994; Ma et al., 2012). In addition, high cholesterol and high fat fed Ldlr−/− mice exhibit a 2-fold increase in aortic calcification and a decreased bone formation rate (Davies et al., 2003; Davies et al., 2005). Vascular calcification in Ldlr−/− mice is primarily associated with atherosclerotic lesions, although punctate medial calcification also occurs (Mathew et al., 2008). Similar to ApoE−/− mice,
induction of CKD in the high fat fed Ldlr<sup>−/−</sup> mice significantly increases aortic calcification (Davies et al., 2003), as well as low bone turnover disease (Davies et al., 2005). Compared to ApoE<sup>−/−</sup> mice, Ldlr<sup>−/−</sup> mice display more moderate atherosclerosis and intimal calcification. However, the largest proportion of the plasma cholesterol levels in high cholesterol fed Ldlr<sup>−/−</sup> mice consists of LDL, which is similar to that of humans, whereas VLDL carries the most plasma cholesterol in ApoE<sup>−/−</sup> mice (Neven and D'Haese, 2011). ApoE<sup>−/−</sup> mice or Ldlr<sup>−/−</sup> mice combined with CKD are less suitable to study medial vascular calcification but provide useful tools to study the accelerated atherosclerosis and intimal vascular calcification in CKD.

Alternatively, pharmacological treatment including calcitriol- (Niederhoffer et al., 1997), warfarin- (Price et al., 1998) and adenine- (Katsumata et al., 2003) induced vascular calcification in rats have also been developed. Calcitriol (also termed 1,25-dihydroxyvitamin D<sub>3</sub>) is the active form of vitamin D. Calcitriol and other vitamin D analogs are currently used for treatment of osteoporosis (Ringe and Schacht, 2004; Wu-Wong et al., 2004), secondary hyperparathyroidism (Moe and Drueke, 2003) and CKD (Wu-Wong et al., 2004), in which vascular calcification is prevalent. Paradoxically, it has been reported that calcitriol induces vascular calcification itself (Price et al., 2004). The effects of calcitriol on vascular calcification are both indirect and direct. Calcitriol treatment often results in hypercalcemia and hyperphosphatemia in CKD patients, due to increased intestinal absorption of calcium and Pi (Sochorova et al., 2009). As described in Chapter 1, both hypercalcemia and hyperphosphatemia are significant risk factors for vascular calcification. Vitamin D receptors are expressed by VSMCs. Calcitriol treatment induces in vitro matrix calcification of VSMCs and regulates the expression of calcification regulators (Inoue and Kawashima, 1988; Jono et al., 1998). Bas and colleagues have demonstrated that calcitriol administration (1µg/kg) time dependently induces medial vascular calcification in rats with intact renal function, with aortic mineral content being significantly up-regulated as early as 6 days (Bas et al., 2006). Interestingly, after calcitriol withdrawal, vascular calcification regresses rapidly, which seems to be an active cellular process in which cells from the monocytic/macrophage lineage are involved (Bas et al., 2006).
As described in section 1.4.1, vascular calcification inhibitors MGP and GAS6 are VKDPs, which requires carboxylation to become biologically active in the presence of a reduced form of vitamin K. Vitamin K naturally occurs in the oxidised form and must be converted into a reduced form by vitamin K epoxide reductase (VKOR). Warfarin shares a common ring structure with vitamin K, interferes with VKOR, preventing the conversion of vitamin K into the reduced form, and ultimately inhibits carboxylation and leads to a deficiency of active MGP and GAS6 (Danziger, 2008).

Cross-sectional studies have demonstrated an association between anticoagulant therapy with coumadin (warfarin) and calcification in arterial media (Price et al., 1998; Palaniswamy et al., 2011). Warfarin treatment induces rapid vascular calcification in the rat model (Price et al., 1998; Dao et al., 2002).

I have used the \textit{Enpp1}^{−/−} mouse in this thesis, which shows extensive medial vascular calcification. The \textit{Enpp1}^{−/−} mouse has been described in detail in section 1.3.5.2.3.

As described in Chapter 1, GACI is a rare autosomal-recessive disorder, associated
with a high mortality rate due to the development of severe cardiovascular complication in early life. The typical phenotype of GACI includes calcification of large and medium-sized arteries and arterial stenosis caused by intimal proliferation (Moran, 1975). Peri-articular calcification of the greater joint is also observed in a subset of GACI patients. Most affected infants die within the first 6 months of life from sequelae of vascular occlusion, typically myocardial infarction or congestive heart failure due to hypertension (Moran, 1975; Rutsch et al., 2000). The finding of systematic lowering NPP1 activity leading to low extracellular P Pi (ePPi) levels prompted the search for Enpp1 mutations, the gene encoding NPP1 (Rutsch et al., 2000). In fact, most of GACI patients were found to carry inactivating mutations in Enpp1 (Rutsch et al., 2008). The understanding of GACI caused by deficiency of ePPi, an inhibitor of HA crystal formation, leads to the use of bisphosphonates (synthetic analogues of P Pi) to effectively treat GACI patients (Rutsch et al., 2008; Ramjan et al., 2009). GACI patients treated with bisphosphonates showed a regression of calcification and an increased survival rate (Rutsch et al., 2008).

Previous studies have demonstrated that Enpp1−/− mice develop extensive medial calcification (Johnson et al., 2005) at 22 weeks of age. In order to evaluate the validity of the Enpp1−/− mouse as a model for medial vascular calcification, I examined the extent of artery calcification in the Enpp1−/− mouse and confirmed Johnson’s findings. Furthermore, as described in Chapter 1, because of the important role of the osteogenic transition of VSMCs in vascular calcification, I also examined whether Enpp1−/− VSMCs showed altered expression of osteogenic genes. Interestingly, recent studies have shown that loss-of-function Enpp1 mutations result in both GACI and hypophosphatemic rickets due to elevated levels of FGF23, which is an important regulator of systematic Pi homeostasis (Lorenz-Depiereux et al., 2010). This suggests that NPP1 also plays an important role in bone development. To date, the studies of Enpp1 ablation on bone development have been restricted to the long bones of 10-day-old mice (Anderson et al., 2005). There are no studies about the phenotype of adult Enpp1−/− skeleton. Therefore, a detailed phenotypic assessment of the skeleton of Enpp1−/− mice at age of 22 weeks was also performed.
3.2 Hypothesis

Adult Enpp1\(^{-/-}\) mice show altered bone phenotype and soft tissue calcification compared to wild-type controls, thereby establishing the Enpp1\(^{-/-}\) mouse as a valid animal model for studying medial vascular calcification.

3.3 Aims

1. To assess the phenotype of long bones of adult Enpp1\(^{-/-}\) mice.
2. To examine the extent of calcification in soft tissues, including arteries (aorta) and kidney of adult Enpp1\(^{-/-}\) mice.
3. To compare the expression of osteogenic genes between Enpp1\(^{-/-}\) and wild-type VSMCs.

3.4 Materials and Methods

3.4.1 Enpp1\(^{-/-}\) mice

The generation and genotyping of Enpp1\(^{-/-}\) mice has been described in section 2.6.1 and section 2.6.3. Mice were culled at 22 weeks of age (adult). Gender and number of mice studied are specified in each individual experiment.

3.4.2 Gross analysis

At the age of 22 weeks, male and female Enpp1\(^{-/-}\) and wild-type mice were weighed (n=8 for each group). Following euthanasia, body length (crown-rump) measurements were recorded on each mouse. Femur and tibia length and width were measured using DigiMax digital vernier callipers (R.S Components Ltd, Corby, Northants, UK). Thereafter, radiographic assessment of left hind-limb was made from X-Ray images (Faxitron, Wheeling, IL, USA).

3.4.3 Tissue preparation for microscopic analysis

Tissue was prepared as described in section 2.7.1. Long bones and femorotibial and talocrural joints were fixed with 10% neutral buffered formalin (NBF) overnight and decalcified with 10% EDTA for 14 days at 4\(^{\circ}\)C and embedded in wax according to standard procedures as described in section 2.7.1. Kidney and aorta were fixed with 10% NBF overnight, embedded in paraffin wax and 4\(\mu\)M sections were stained with
Chapter 3  Assessment of the *Enpp1*\(^{-/}\) mouse as a model of vascular calcification

Hematoxylin and Eosin (H & E) to assess morphology and pathology, Alizarin Red S and von Kossa to assess calcification status of aorta and kidney (Pathological assessment was performed by Professor Elspeth Milne, Veterinary Pathology Unit, Royal (Dick) School of Veterinary Studies, The University of Edinburgh).

### 3.4.4 μCT analysis

Tibiae and femurs were dissected from female wild-type and *Enpp1*\(^{-/}\) mice at the age of 22 weeks and stored in distilled water at -20°C. To analyse trabecular architecture and cortical bone geometry, the bones were scanned with a μCT system as described in section 2.6.4.

### 3.4.5 Isolation of VSMCs from wild-type and *Enpp1*\(^{-/}\) mice

Primary VSMCs were isolated as described in section 2.2.3 from two wild-type and two *Enpp1*\(^{-/}\) mice at 22-weeks of age. Isolated VSMCs were expanded in T25 tissue culture flasks (Greiner Bio-One) as previously described (section 2.2.3).

### 3.4.6 Induction and characterisation of calcification

Wild-type and *Enpp1*\(^{-/}\) VSMCs were seeded at a density of 50,000 cells/well in a 12-well plate. At 80% confluence, calcification was induced by adding 2mM Pi (Na\(_2\)HPO\(_4\)/NaH\(_2\)PO\(_4\)) as described in section 2.2.4. Calcium deposition was confirmed by Alizarin Red S staining as previously described (section 2.2.5).

### 3.4.7 Analysis of gene expression in wild-type and *Enpp1*\(^{-/}\) VSMCs

Wild-type and *Enpp1*\(^{-/}\) VSMCs were seeded at a density of 50,000 cells/well in a 12-well plate. After confluence, RNA was extracted and cDNA was prepared as previously described (section 2.4.1 & section 2.4.2). qPCR was performed as described in section 2.4.5. Due to limited RNA, the expression of the following genes: *E11*, *Ank*, *PiT-1*, *Fgf23*, *Mgp* and *Akp2* were examined. The sequences of these primers are listed in Appendix 2.
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Assessment of the $Enpp1^\text{+/+}$ mouse as a model of vascular calcification

3.5 Results

3.5.1 Reduced growth of $Enpp1^{\text{+/+}}$ mice

Initial studies were performed to address whether adult $Enpp1^{\text{+/+}}$ mice displayed a reduced growth phenotype resembling that previously reported for 10-day-old $Enpp1^{\text{+/+}}$ mice (Anderson et al., 2005). Male and female 22-week-old $Enpp1^{\text{+/+}}$ mice were lighter (83.5%; P<0.001 & 65.7%; P<0.001 respectively) and shorter (91.7%; P<0.01 & 91.3%; P<0.001 respectively) (Table 3.1). The femurs of both male and female 22-week-old $Enpp1^{\text{+/+}}$ mice were significantly shorter compared to that of wild-type mice (94%; P<0.05 & 96.1%; P<0.01 respectively). Interestingly, the tibiae of male 22-week-old $Enpp1^{\text{+/+}}$ mice was significantly longer (103.1%, P<0.01), however no differences were observed in female 22-week-old $Enpp1^{\text{+/+}}$ mice (Table 3.1).

### Table 3.1 Body weight, body length and long bone length at 22 weeks of age from $Enpp1^{\text{+/+}}$ and wild-type (WT) mice. Data are presented as mean +/- SEM (n=10). Significance is denoted by *P<0.05, **P<0.01, ***P<0.001.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (week)</th>
<th>Genotype</th>
<th>Body weight (g)</th>
<th>Body length (cm)</th>
<th>Femur length (mm)</th>
<th>Tibia length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>22</td>
<td>WT</td>
<td>32.1 (0.7)</td>
<td>9.92 (0.13)</td>
<td>16.11 (0.42)</td>
<td>18.85 (0.25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$Enpp1^{\text{+/+}}$</td>
<td>21.1 (0.7)**</td>
<td>9.06 (0.14)**</td>
<td>15.47 (0.13)**</td>
<td>19.26 (0.13)**</td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>WT</td>
<td>31.0 (0.8)</td>
<td>9.72 (0.18)</td>
<td>16.06 (0.16)</td>
<td>18.63 (0.11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$Enpp1^{\text{+/+}}$</td>
<td>25.9 (1.0)**</td>
<td>8.91 (0.15)**</td>
<td>15.09 (0.59)*</td>
<td>19.21 (0.23)**</td>
</tr>
</tbody>
</table>

3.5.2 Altered bone development of $Enpp1^{\text{+/+}}$ mice

To further understand the physiological role of NPP1 on skeletal development and mineralisation, a detailed histological assessment of adult $Enpp1^{\text{+/+}}$ mice at the age of 22 weeks was performed. A striking hyperostosis (excessive bone growth) of the cervical (Figure 3.1A) and thoracic (Figure 3.1C) vertebrae, as well as the interphalangeal and femorotibial (Figure 3.1E) joint was observed in $Enpp1^{\text{+/+}}$ mice compared to wild-type controls (Figure 3.1B, D & F respectively). In addition, the encroachment of bone lesions onto the spinal cord was observed, which may be associated with the abnormal gait observed in some $Enpp1^{\text{+/+}}$ mice. The femorotibial
joint of the \textit{Enpp1\textsuperscript{-/-}} mice also showed some defects, including increased presence of blood cells, thinner articular cartilage, misshappen and disorganised heads of the tibiae and femora and ectopic cartilage deposition (Figure 3.1E). Furthermore, \textit{Enpp1\textsuperscript{-/-}} mice showed increased mineralisation of the knee and ankle joints but reduced mineralisation of femur and tibia (Figure 3.1G) compared to wild-type controls ((Figure 3.1H), as revealed by radiography of the hind-limb. These data suggest that \textit{Enpp1\textsuperscript{-/-}} mice show significantly increased mineralisation within the vertebrae and interphalangeal, femorotibial and talocrural joints and a reduced long bone mineralisation.

\subsection*{3.5.3 Reduced trabecular bone mass of \textit{Enpp1\textsuperscript{-/-}} mice}

It has been reported that \textit{Enpp1\textsuperscript{-/-}} mice have reduced mineral content in both growth plate and adjacent bone (Anderson et al., 2005). \textit{\textmu}CT analysis showed that a moderate decrease in bone mineral density, bone volume fraction and average trabecular thickness was observed in tibiae of \textit{Enpp1\textsuperscript{-/-}} mice at the age of 10 days (Anderson et al., 2005). In order to extend these observations, the present study used high resolution \textit{\textmu}CT analysis of the tibia and femur of female adult mice to investigate the role of \textit{Enpp1} in normal bone development.

22-week-old female \textit{Enpp1\textsuperscript{-/-}} mice had reduced bone volume in the trabecular compartment of the tibia and femur. \textit{Enpp1\textsuperscript{-/-}} mice had significantly reduced BV/TV and trabecular number in both tibia and femur compared to wild-type controls (P<0.01, Table 3.2 & Figure 3.2). Trabecular thickness was significantly decreased in the tibia of female \textit{Enpp1\textsuperscript{-/-}} mice at the age of 22 weeks (P<0.001, Table 3.2). A reduction in trabecular thickness in the femur of female \textit{Enpp1\textsuperscript{-/-}} mice was also recorded, although it was not significant (Table 3.2).
Figure 3.1 Histological and radiographic assessment of skeleton from wild-type and Enpp1<sup>−/−</sup> mice. H & E staining of transverse section of the neck of the Enpp1<sup>−/−</sup> mice (A) showed an enlarged cervical vertebra and increased deposition of cartilage (arrows) compared to wild-type control (B). H & E staining of longitudinal section of mid-thoracic vertebrae of the Enpp1<sup>−/−</sup> mice (C) showing a severely increased deposition of cartilage in the connective tissue and incursion into the spinal cord (arrows) compared to wild-type controls (D). H & E staining of sagittal section of the femorotibial joint of the Enpp1<sup>−/−</sup> mice (E) showed remodelling of the femur and over-growth of ectopic cartilage (arrows) compared to wild-type controls (F). Radiographs indicate calcification of the femorotibial and tarsocrural joint (asterisk), and reduced mineralisation in the tibia and femur (arrows) of Enpp1<sup>−/−</sup> mice (G) compared to wild-type controls (H).
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The Structural Model Index (SMI) quantifies the characteristic of 3D structure in terms of amounts of plates and rods (Hidebrand and Ruegsegger, 1997). The SMI was significantly higher in tibiae (P<0.05) and femurs (P<0.01) from 22-week-old female *Enpp1*−/− mice, revealing that the trabecular in *Enpp1*−/− mice are less ‘plate-like’ and less connected (Table 3.2). These data confirm that *Enpp1*−/− mice have decreased trabecular bone, which are consistent with previous reports that mice with *Enpp1* deficiency display an osteopenic phenotype (Anderson et al., 2005). My colleague Dr. Neil Mackenzie has recently reported that 22-week-old male *Enpp1*−/− mice also had significant reduction in trabecular bone mass, with decreased bone volume, BV/TV, trabecular number and trabecular thickness. The SMI in tibiae and femurs from male *Enpp1*−/− mice was also significantly increased compared to that of wild-type controls (Mackenzie et al., 2012b).

<table>
<thead>
<tr>
<th>Bone</th>
<th>Age (weeks)</th>
<th>Genotype</th>
<th>%BV/TV</th>
<th>BMD (g/cm³)</th>
<th>Trab. Thickness (μm)</th>
<th>Trab. Number (TbN/μm)</th>
<th>Structure Model Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibia</td>
<td>22</td>
<td>WT</td>
<td>14.31(1.81)</td>
<td>1.20(0.01)</td>
<td>13.95(0.35)</td>
<td>0.01037(0.001436)</td>
<td>2.11(0.19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Enpp1</em>−/−</td>
<td>5.07(0.38)**</td>
<td>1.20(0.02)</td>
<td>11.60(0.33)**</td>
<td>0.004364(0.000304)**</td>
<td>2.60(0.07)*</td>
</tr>
<tr>
<td>Femur</td>
<td>22</td>
<td>WT</td>
<td>15.03(1.57)</td>
<td>1.15(0.02)</td>
<td>12.50(0.24)</td>
<td>0.01191(0.00114)</td>
<td>1.91(0.15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Enpp1</em>−/−</td>
<td>5.49(0.55)**</td>
<td>1.14(0.02)</td>
<td>11.43(0.58)</td>
<td>0.0048(0.0004)**</td>
<td>2.57(0.04)**</td>
</tr>
</tbody>
</table>

Table 3.2 µCT analysis of trabecular bone in female wild-type and *Enpp1*−/− mice. 22 week femur (WT n=6 & KO n=9) and tibia (WT n=7 & KO n=9) were examined. SEM is shown in brackets, significance is denoted by *P<0.05, **P<0.01, ***P<0.001.

3.5.4 Reduced cortical thickness of *Enpp1*−/− mice

A significant reduction in cortical thickness was observed in the tibia of 22-week-old female *Enpp1*−/− mice (P<0.05, Table 3.3). 22-week-old *Enpp1*−/− femur also showed decreased cortical thickness, although this was not significant. The percentage closed porosity (a measure of the connectivity of the pores) in the cortical bone was significantly decreased in 22-week-old female *Enpp1*−/− tibia (P<0.01, Table 3.3). No significant change was observed in 22-week-old female *Enpp1*−/− femur.
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Unexpectedly, bone mineral density remained unchanged in both tibia and femur of female $Enpp1^{+/−}$ mice. However, due to decreased cortical thickness, it is still possible that 22-week-old female $Enpp1^{+/−}$ mice show an overall reduction in the mineral content. Comparable changes in male $Enpp1^{+/−}$ mice at 22 weeks of age was also observed by my colleague Dr. Neil Mackenzie (Mackenzie et al., 2012b).

![3D reconstruction of the trabecular bone scanned using µCT.](image)

Figure 3.2 3D reconstruction of the trabecular bone scanned using µCT. (A) Tibia and (B) femur from female wild-type and $Enpp1^{+/−}$ mice at the age of 22 weeks. These 3D data demonstrated the reduction of trabecular number in the $Enpp1^{+/−}$ mice.

<table>
<thead>
<tr>
<th>Bone</th>
<th>Age (weeks)</th>
<th>Genotype</th>
<th>%BV/TV (SEM)</th>
<th>BMD (g/cm²) (SEM)</th>
<th>Cortical Thickness (µm) (SEM)</th>
<th>Closed Porosity (%)</th>
<th>Total Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibia</td>
<td>22</td>
<td>WT</td>
<td>98.18 (0.27)</td>
<td>1.32 (0.01)</td>
<td>44.57 (2.14)</td>
<td>0.09 (0.01)</td>
<td>1.82 (0.27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$Enpp1^{−/−}$</td>
<td>98.28 (0.14)</td>
<td>1.32 (0.01)</td>
<td>37.71 (0.95)*</td>
<td>0.04 (0.01)**</td>
<td>1.72 (0.14)</td>
</tr>
<tr>
<td>Femur</td>
<td>22</td>
<td>WT</td>
<td>98.94 (0.33)</td>
<td>1.39 (0.029)</td>
<td>44.89 (3.44)</td>
<td>0.06 (0.023)</td>
<td>1.06 (0.33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$Enpp1^{−/−}$</td>
<td>98.95 (0.05)</td>
<td>1.40 (0.005)</td>
<td>43.32 (0.47)</td>
<td>0.05 (0.006)</td>
<td>1.05 (0.05)</td>
</tr>
</tbody>
</table>

Table 3.3 µCT analysis of cortical bone in female wild-type and $Enpp1^{+/−}$ mice. 22 week femur (WT n=6 & KO n=8) and tibia (WT n=7 & KO n=9) were tested. SEM is shown in brackets, significance is denoted by *P<0.05, **P<0.01, ***P<0.001.
3.5.5 Extensive soft tissue calcification of Enpp1<sup>−/−</sup> mice

To test whether the Enpp1<sup>−/−</sup> mouse is a valid animal model for medial vascular calcification, the extent of artery calcification was examined in 22-week old female wild-type control and Enpp1<sup>−/−</sup> mice. Consistent with previous studies, Alizarin Red S staining and von Kossa staining showed that ectopic calcification occurred in the coronary artery, the medial layer of the ascending, aortic arch and the brachiocephalic artery of Enpp1<sup>−/−</sup> mice (Figure 3.3A). Significant calcification was also observed in the cortex of kidney of Enpp1<sup>−/−</sup> mice (Figure 3.3C). No calcification was found in wild-type controls (Figure 3.1B & D).

![Figure 3.3](image)

**Figure 3.3 Extensive soft tissue calcification in Enpp1<sup>−/−</sup> mice.** Alizarin Red S staining (arrows) showed calcification of the tunica media of the aorta from Enpp1<sup>−/−</sup> mice (A) and wild-type controls (B). von Kossa staining (arrows) showed ectopic calcification of the kidney in Enpp1<sup>−/−</sup> mice (C) compared to wild-type controls (D).

3.5.6 Altered calcification and differentiation of Enpp1<sup>−/−</sup> VSMCs cultured in calcifying medium

Enpp1<sup>−/−</sup> VSMCs showed a significant increase in calcium deposition compared to wild-type controls, as revealed by Alizarin Red S staining (Figure 3.4A). In addition, Akp2 mRNA expression was also significantly up-regulated (1.6 fold, P<0.001) in
**Chapter 3 Assessment of the Enpp1\(^{-/-}\) mouse as a model of vascular calcification**

Enpp1\(^{-/-}\) VSMCs relative to wild-type controls (Figure 3.4B). These data are consistent with previous studies (Johnson et al., 2005; Narisawa et al., 2007). In addition, Enpp1\(^{-/-}\) VSMCs showed significantly decreased expression of the calcification inhibitor Mgp (39%, P<0.001) (Figure 3.4C) as well as the PPI transporter Ank (76%, P<0.001) (Figure 3.4D). No significant change was observed in PiT-1 expression (Figure 3.4E). Interestingly, the osteocyte specific genes Fgf23 (2.9 fold, P<0.001) (Figure 3.4F) and E11 (3.0 fold, P<0.001) (Figure 3.4G) were dramatically increased in Enpp1\(^{-/-}\) VSMCs compared to wild-type controls. These data suggest that NPP1 and PPI depletion directly modulate VSMCs towards the osteoblastic/osteocytic phenotype, contributing to increased calcification observed in Enpp1\(^{-/-}\) VSMCs.

### 3.6 Discussion

The deposition of HA, is essential for the mineralisation of bone and cartilage, and for normal skeletal development. Impaired HA deposition leads to multiple bone frailties, including osteomalacia, rickets and hypophosphatasia. PPI is a key inhibitor of HA crystal formation. Previous studies have identified three molecules that play an important role in regulating PPI levels, including TNAP (Hessle et al., 2002), which hydrolyses PPI, NPP1, which generates ePPI from NTPs (Johnson et al., 1999) and ANK (Ho et al., 2000), which mediates intracellular to extracellular transport of PPI. Inactivating mutations in Akp2 result in the inborn-error-metabolism known as hypophosphatasia, characterised by poorly mineralised cartilages and bones (Henthorn and Whyte, 1992). This loss of TNAP activity leads to an increase in its substrate, ePPI (Addison et al., 2007). Conversely, NPP1 and PPI depletion results in ectopic bone and soft tissue calcification. Mice lacking NPP1 have severe mineralisation defects, which are associated with abnormally low ePPI levels (Sali, 1999; Ho et al., 2000; Harmey et al., 2004; Anderson et al., 2005). Therefore Enpp1\(^{-/-}\) mice are a valuable tool to investigate the role of NPP1 in controlling physiological and pathological mineralisation. The present study is the first evaluation of hyperostosis of vertebrae and joints and soft tissue calcification of adult Enpp1\(^{-/-}\) mice. My data confirms and extends previous reports (Johnson et al., 2001; Harmey et al., 2004; Johnson et al., 2005) and support the important role of NPP1 in matrix
Chapter 3  Assessment of the Enpp1\textsuperscript{\textminus\textminus} mouse as a model of vascular calcification

mineralisation through the generation of ePPI (Anderson et al., 2005; Babij et al., 2009).

Figure 3.4 Altered calcification and differentiation of Enpp1\textsuperscript{\textminus\textminus} VSMCs. (A) Alizarin Red S staining showed up-regulated calcium deposition in Enpp1\textsuperscript{\textminus\textminus} VSMCs. mRNA expression of (B) Akp2, (C) Mgp, (D) Ank, (E) Pit-1, (F) Fgf23 and (G) E11 in Enpp1\textsuperscript{\textminus\textminus} VSMCs and wild-type controls. Results are presented as mean\textpm SEM. Significance is represented compared to 0d as:***P<0.001.
The data here demonstrate that 22-week-old female Enpp1^{−/−} mice have reduced trabecular bone mass and cortical thickness in both tibia and femur. This is likely to be a direct effect of loss of NPP1 activity, however reduced body weight in Enpp1^{−/−} mice will change the loading on bones and thus may also have an effect on their structures. Interestingly, 22-week-old Enpp1^{−/−} mice have shorter femurs but longer tibiae compared to wild-type controls, indicating that changes in bone structure due to different effects of loading may be occurring and requires further investigation.

Previous studies have analysed the mineralisation of bone from 10-day-old Enpp1^{−/−} mice and [Enpp1^{−/−}; Akp2^{−/−}] double knockout mice and indicated that the effects of Enpp1 ablation on an Akp2 null background is site-specific (Anderson et al., 2005b). Therefore, in contrast to normal mineralisation observed in calvaria, vertebrae and soft tissue in [Enpp1^{−/−}; Akp2^{−/−}] double knockout mice, long bones of these double knockout mice remained hypomineralised. This study by Anderson and colleagues suggests that hypomineralisation of long bones of Enpp1^{−/−} mice and [Enpp1^{−/−}; Akp2^{−/−}] double knockout mice may be related to relatively low levels of endogenous NPP1 expression throughout the long bone when compared to the calvaria (Anderson et al., 2005b). Thus, in long bones, the complete loss of NPP1 activity would further decrease ePPi to abnormally low levels, resulting in insufficient PPI substrate for TNAP to generate Pi for normal mineral formation. Interestingly, it has been demonstrated that NPP1 can directly regulate osteoblastic gene expression and cellular differentiation in calvarial osteoblasts (Nam et al., 2011). Furthermore, an accumulation of NTPs due to lack of hydrolysis by NPP1 (Prosdocimo et al., 2009) may have a downstream effect on bone remodelling via purinergic signalling (Orriss et al., 2010).

My data has shown that adult Enpp1^{−/−} mice have severe hyperostosis of the vertebrae and disorganisation and excessive bone production in the femorotibial joint. These data are consistent with previous reports that adult Enpp1^{−/−} mice show an osteopenic phenotype, where hypermineralisation of the soft tissues and certain skeletal sites was observed (Anderson et al., 2005b). In addition, loss of NPP1 activity affects skeletal sites in a site-specific manner, as calcified nodule formation and mineral
deposition are inhibited to a higher extent in osteoblasts isolated from \textit{Enpp1}^{-/-} bone marrow than calvarial osteoblasts isolated from the same animal (Anderson et al., 2005b). It has also been reported that NPP1 is an important regulator of insulin signalling in various tissue types including adipose and muscle (Di Paola et al., 2011). The effect of glucose regulation may also contribute to the hypomineralisation observed in \textit{Enpp1}^{-/-} mice. The present study has shown that \textit{Enpp1}^{-/-} mice display significantly reduced body weight in adult mice. This may indicate that reduced fat accumulation associated with insulin sensitivity in \textit{Enpp1}^{-/-} mice. Previous studies have demonstrated that over-expression of hepatic NPP1 in mice results in insulin resistance and glucose intolerance (Dong et al., 2005). Further studies are required to investigate the regulation of insulin signalling in bone by NPP1.

To evaluate the validity of the \textit{Enpp1}^{-/-} mouse as an animal model for medial vascular calcification, the present study detected the extent of vascular calcification in these mice. Severe arterial calcification was observed in adult \textit{Enpp1}^{-/-} mice. In addition, \textit{Enpp1}^{-/-} VSMCs showed dramatically increased calcification compared to wild-type controls. These data are consistent with previous reports (Johnson et al., 2005; Narisawa et al., 2007). \textit{Enpp1}^{-/-} VSMCs showed significantly up-regulated \textit{Akp2} expression, a bone-specific phosphatase, which plays an important role in vascular calcification. Up-regulated \textit{Akp2} expression has been observed in aortae from uremic rats, another animal model of vascular calcification (Lomashvili et al., 2008). In addition, \textit{Mgp} and \textit{Ank} were significantly decreased in \textit{Enpp1}^{-/-} VSMCs. MGP serves as a calcification inhibitor expressed by blood vessels to limit calcium phosphate deposition (Luo et al., 1997; Shanahan et al., 1999; Tyson et al., 2003). MGP-null mice develop extensive calcification (Luo et al., 1997). This inhibitory effect of MGP on vascular calcification has been attributed partly to its ability to antagonise the activity of BMP2, which is an important promoter of vascular calcification (Bostrom et al., 2001; Zebboudj et al., 2002). As described in Chapter 1, \textit{Ank} transports PPi into the extracellular space, where it inhibits the formation of HA crystals. \textit{Ank} mutant mice develop aortic medial calcification due to impaired PPi channelling (Ho et al., 2000; Johnson et al., 2005). Unexpectedly, significant expression of osteocyte genes \textit{Fgf23} and \textit{E11} was observed in \textit{Enpp1}^{-/-} VSMCs.
These data suggest that the osteocytic transition of VSMCs may be involved in vascular calcification in Enpp1−/− mice. Further studies will be described in Chapter 4 which specifically study whether vascular calcification is associated with an osteocyte phenotype. Interestingly, my colleague Dr. Neil Mackenzie demonstrated increased circulating FGF23 levels in Enpp1−/− mice compared to wild-type controls (Mackenzie et al., 2012b). Furthermore, FGF23 is primarily expressed by osteocytes and acts as a phosphaturic hormone that regulates Pi homeostasis, calcium homeostasis and bone mineralisation (Quarles, 2003; Razzaque, 2009). FGF23 binds to FGF receptors (mainly FGFR1) and the co-receptor Klotho in the kidney and promotes excretion of Pi, resulting in reduced serum Pi (Kurosu et al., 2006; Ben-Dov et al., 2007). In addition, recent human genetic studies have shown that Enpp1, if mutated, causes hypophosphatemic rickets as a result of increased FGF23 (Lorenz-Depiereux et al., 2010). However, further studies are required to investigate the mechanisms whereby Enpp1 inactivation stimulates Fgf23 gene expression and whether increased FGF23 levels have a direct effect on vascular calcification in Enpp1−/− mice. Further studies will be described in Chapter 5 which investigate whether FGF23 has a direct role in vascular calcification.

Loss-of-function of inactivating mutations in Enpp1 leads to GACI in human infants, which is characterised by the extensive artery calcification as well as arterial stenosis caused by intimal proliferation. Most of the affected patients die within the first six month (Rutsch et al., 2003; Rutsch et al 2008). Enpp1−/− mice therefore are a useful animal model which mimics GACI in humans. However, the present study provided several lines of evidence to support that Enpp1−/− mice shares many similarities with other animal models of medial vascular calcification, such as Mgp−/− mice and uremic rats:

1. The adult Enpp1−/− mice developed severe medial vascular calcification.
2. Increased circulating FGF23 levels were observed in Enpp1−/− mice (Mackenzie et al., 2012a).
3. The osteogenic/osteocytic phenotype transition of VSMCs was recorded in Enpp1−/− mice.

Therefore, the studies in this chapter have shown that the Enpp1−/− mouse is a valid
animal model to investigate the molecular and cellular mechanisms of medial vascular calcification.

I have also confirmed that Enpp1−/− mice display severe disruption to the structural properties of long bones. Furthermore, hypercalcification in joints and soft tissues confirms that NPP1 plays a key role in soft tissue calcification, as well as maintaining skeletal structure and function. As Enpp1−/− mice show comparable pathologies to GACI in humans, they are an essential model with which to study this devastating disease. In addition, Enpp1−/− mice display severe medial vascular calcification and shares many similarities to other rodent vascular calcification models. These mice are therefore an applicable model for the study of medial vascular calcification and are employed in Chapters 4, 5 and 6.
Chapter 4 The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells

4 The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells

4.1 Introduction
As described in Chapter 1, vascular calcification is an active cell-regulated process that resembles bone formation. In bone, osteoblasts and chondrocytes calcify their extracellular matrix by promoting the formation of crystalline HA, through a series of physico-chemical and biochemical processes.

Osteocytes are terminally differentiated osteoblasts and occupy approximately 90% of all bone cells in human and animal skeletons, and are embedded in the mineralised bone matrix. Although the precise function of osteocytes remains largely unknown, it is recognised that these cells are important players that direct bone remodelling in response to load-bearing (Burger and Klein-Nulen, 1999). Indeed, osteocytes have recently been shown to be the major bone cells responsible for the synthesis of both RANKL and OPG, which have traditionally been considered osteoblast specific proteins (Nakashima et al., 2011; Xiong et al., 2011). In addition, osteocytes also play an important role in calcium and Pi homeostasis. They are capable of modifying the matrix environment around them (Aarden et al., 1996) and producing calcification modifying hormones and growth factors (Bonewald and Johnson, 2008; Bonewald, 2011). There is also a requirement for local production of MMPs and modulators of calcification in the osteocyte’s canalicul-lacuna environment for healthy osteocyte function (Karsdal et al., 2004). Within the past two decades, a number of osteocyte markers have been identified, including DMP1, sclerostin, PHEX, MEPE, FGF23 and E11/podoplanin/gp130 (Bonewald, 2006). These molecules have been described in detail in section 1.3.5.1.6 and play an important role in regulating calcification and Pi homeostasis.

A number of studies have reported that VSMCs, the predominant cell type involved in vascular calcification, can undergo phenotypic transition to osteoblastic and
Chapter 4 The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells

chondrocytic cells in a calcified environment (Steitz et al., 2001; Abedin et al., 2004; Yang et al., 2004; Johnson et al., 2005; Speer et al., 2005). The presence of osteocytes has been observed in Mönckeberg’s sclerosis lesions, as well as in human calcified carotid atherosclerotic plaques (Shanahan et al., 1999; Hunt et al., 2002). However, it has yet to be established whether VSMC calcification involves transition to an osteocyte phenotype. Therefore, in the present study, I have undertaken in vitro VSMC calcification studies, in conjunction with in vivo analysis of the Enpp1−/− mouse model of medial calcification and demonstrated that vascular calcification is associated with the appearance of an osteocyte phenotype.

4.2 Hypothesis
VSMCs undergo osteocytic differentiation during the calcification process.

4.3 Aims
1. To compare VSMCs to osteoblasts undergoing osteocytic transition in vitro.
2. To investigate the expression profiles of osteocyte markers during the osteoblast and VSMC calcification process in vitro.
3. To compare the osteocyte marker expression in VSMCs cultured in a calcifying versus non-calcifying environment.
4. To examine the expression of osteocyte markers in vivo in the Enpp1−/− mouse model of vascular calcification.

4.4 Materials and Methods
4.4.1 Cell culture
Primary calvarial osteoblasts and aortic VSMCs were isolated as described in section 2.2.2 and section 2.2.3, respectively. Cells were grown to 75% confluence (termed 0d) and cultured in calcifying medium containing 2.5mM βGP and 50µg/ml AA for 28d. These culture conditions have been previously described to induce matrix calcification for both calvarial osteoblasts and VSMCs (Steitz et al., 2001; Collett et al., 2007).
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4.4.2 Characterisation of calcification
Primary calvarial osteoblasts and VSMCs were cultured in calcifying medium and fixed in 4% PFA for 10min at 0, 7, 14, 21 and 28d, as described in section 2.2.4. Calcium deposition was identified with Alizarin Red S staining as described in section 2.2.5. Quantification of calcium deposition was also detected by HCL leaching as described in section 2.2.5.

4.4.3 Tissue Non-specific Alkaline Phosphatase (TNAP) activity and cell viability
Primary calvarial osteoblasts and VSMCs were cultured with calcifying medium for up to 28d. TNAP activity were analysed as described in section 2.2.6. Cell viability was also detected as described in section 2.2.7.

4.4.4 qPCR analysis
Calvarial osteoblasts or VSMCs samples were collected at the defined time points and total RNA was extracted as described in section 2.4.1. cDNA was prepared from RNA by reverse transcription as described in section 2.4.2 and was used to perform qPCR analysis for the osteoblast marker genes Akp2, Runx2, Osteocalcin (Ocn), Collagen Type Iα (Col1a1), the osteocyte markers Sost, Dmp1, Phex, Mepe, E11 and Fgf23, as well as the calcification regulators PiT-1 and Ank. Each PCR was performed in triplicate. The results were normalised with Gapdh and relative expression levels were calculated using the ∆∆Ct method as described in section 2.4.4.

4.4.5 Analysis of protein expression
Osteoblasts and VSMCs were grown with calcifying medium for up to 28 days. Proteins were extracted from cultures at 0, 7, 14, 21 and 28d in an appropriate volume of RIPA buffer, subjected to SDS-PAGE and transferred onto a nitrocellulose membrane as outlined in section 2.5.2. The membrane was probed with FGF23, RUNX2, E11, sclerostin and β-actin antisera as described in section 2.5.3.
4.4.6 Immunohistochemistry

Tibiae and aortae were dissected from 22 week-old wild-type and $Enpp1^{-/-}$ mice. Tissues were prepared as described in section 2.7.1. Calcified aorta was confirmed by Alizarin Red S staining as described in section 2.7.2. Immunohistochemistry was performed to examine the expression of sclerostin and E11 as previously described (section 2.7.3).

4.5 Results

4.5.1 Differentiation of osteoblasts into osteocytes in vitro

4.5.1.1 Characterisation of osteoblast matrix calcification in vitro

Calvarial osteoblasts had negligible amounts of Alizarin Red S staining (calcium deposition) (Figure 4.1A) and TNAP activity (Figure 4.1B) at 0d. Further incubation with calcifying medium for an additional 7, 14, 21 and 28d resulted in significant increases in Alizarin Red S staining (Figure 4.1A) and TNAP activity (Figure 4.1B). No alterations in cell viability were observed in these cells (Figure 4.1C). $Akp2$ mRNA expression was also examined by qPCR and a significant increase was observed at 7, 14, 21 and 28d compared to 0d (Figure 4.2A). The mRNA expression of the type III sodium-dependent Pi transporter $PiT-1$ and $Ank$, which are recognised regulators of osteoblast mineralisation, was significantly up-regulated at 28d (4.4 fold, $P<0.001$ & 8.2 fold, $P<0.001$, respectively; Figure 4.2B & C). These data confirm the formation of calcified nodules in calvarial osteoblasts under calcifying conditions.

4.5.1.2 Up-regulation of osteoblast markers during the process of osteoblast matrix calcification in vitro

Next, the expression of specific osteoblast markers was evaluated by qPCR. Significant increases in mRNA expression of $Ocn$ (40.5 fold, $P<0.05$) (Figure 4.3A), $Runx2$ (3.1 fold, $P<0.001$) (Figure 4.3B) and $Col1a1$ (2.16 fold, $P<0.01$) (Figure 4.3C) was observed at 7d. At 28d, significant increases in mRNA expression of $Ocn$ (2966 fold, $P<0.001$) (Figure 4.3A), $Runx2$ (2.7 fold, $P<0.001$) (Figure 4.3B) and $Col1a1$ (4.6 fold, $P<0.001$) (Figure 4.3C) were recorded.
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Figure 4.1 In vitro matrix calcification of murine calvarial osteoblasts cultured for 28d under calcifying conditions. (A) Representative images and quantification of Alizarin Red S staining, (B) TNAP activity and (C) cell viability. Results are presented as mean±SEM. Significance is represented compared to 0d as:***P<0.001.
Figure 4.2 Up-regulation of calcification regulators during *in vitro* matrix calcification of calvarial osteoblasts cultured for 28d under calcifying conditions. Fold changes in mRNA expression of (A) *Akp2*, (B) *PiT-1* and (C) *Ank*. Results are presented as mean +/- SEM. Significance is represented compared to 0d as: ***P<0.001
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Figure 4.3 mRNA expression of osteoblast markers was significantly upregulated during calvarial osteoblast matrix calcification *in vitro*. Fold changes in mRNA expression of (A) Ocn, (B) Runx2 and (C) Col1a1. Results are presented as mean±SEM. Significance is represented compared to 0d as: *P<0.05, **P<0.01, ***P<0.001.
Chapter 4 The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells

4.5.1.3 Up-regulation of osteocyte markers during the process of osteoblast matrix calcification \textit{in vitro}

To determine whether osteoblasts terminally differentiate into osteocytes, the expression of osteocyte markers were examined during the culture period. At 7d, significant increases in mRNA expression of \textit{Sost} (3.4 fold, \(P<0.001\)) (Figure 4.4A), \textit{Dmp1} (29.5 fold, \(P<0.001\)) (Figure 4.4B), \textit{Phex} (236.5 fold, \(P<0.001\)) (Figure 4.4C), \textit{Mepe} (64.3 fold, \(P<0.001\)) (Figure 4.4D), \textit{E11} (2.2 fold, \(P<0.001\)) (Figure 4.4E) and \textit{Fgf23} (16.7 fold, \(P<0.001\)) (Figure 4.4F) were observed. Genes continued to increase significantly with culture time. By 28d, significant increases in mRNA expression of \textit{Sost} (401.6 fold, \(P<0.001\)) (Figure 4.4A), \textit{Dmp1} (922.6 fold, \(P<0.001\)) (Figure 4.4B), \textit{Phex} (548.1 fold, \(P<0.001\)) (Figure 4.4C), \textit{Mepe} (321.3 fold, \(P<0.001\)) (Figure 4.4D), \textit{E11} (4.4 fold, \(P<0.001\)) (Figure 4.4E) and \textit{Fgf23} (664.1 fold, \(P<0.001\)) (Figure 4.4F) were recorded.

Comparable changes in protein expression of sclerostin and FGF23 were observed. E11 protein expression appeared to reduce following 28d culture, which may be due to post-transcriptional or post-translational regulation (Figure 4.5). Previous studies have documented that E11 protein is degraded by the calpain family of proteinase (Martin-Villar et al., 2009). These observations indicate that calvarial osteoblasts can differentiate into osteocyte phenotype \textit{in vitro}. 

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Figure 4.4 Expression of osteocyte markers was significantly up-regulated during calvarial osteoblast matrix calcification in vitro. Fold changes in mRNA expression of (A) Sost, (B) Dmp1, (C) Phex, (D) Mepe, (E) E11 and (F) Fgf23. Results are presented as mean±SEM. Significance is represented compared to 0d as: *P<0.05, **P<0.01, ***P<0.001.
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![Time in culture (Days)]

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Figure 4.5 Protein expression of FGF23, sclerostin and E11 during the osteoblast in vitro differentiation process. FGF23 and sclerostin protein expression was increased over the 28d culture period. E11 protein expression was increased at 7d, but reduced at 14, 21 and 28d.

4.5.2 VSMC matrix calcification in vitro is associated with an osteocyte phenotype

4.5.2.1 Characterisation of VSMC matrix calcification

Alizarin Red S staining (Figure 4.6A) and TNAP (Figure 4.6B) activity in murine aortic VSMCs were negligible at 0d of culture. Significant increases in calcium deposition (Figure 4.6A), and TNAP activity (Figure 4.6B) were observed following 7, 14, 21 and 28d of culture in calcifying culture medium. No alterations in cell viability were observed in these cultures (Figure 4.6C). Akp2 mRNA expression was unchanged at 7d, 14d and 21d but significantly decreased at 28d (77%) (Figure 4.7A). Calcification regulators including PiT-1 (3.0 fold, P<0.001) (Figure 4.7B) and Ank (6.0 fold, P<0.001) (Figure 4.7C) mRNA expression were significantly increased at 7d. Up-regulated gene expression was maintained with time in culture.

4.5.2.2 Osteogenic differentiation of VSMCs under calcifying conditions

Previous studies have shown VSMCs undergo osteogenic differentiation when cultured in calcifying conditions (Bostrom et al., 1993; Tyson et al., 2003; Speer et al., 2009). To test whether my in vitro calcification of VSMC model is consistent with previous studies, several osteoblast markers Ocn, Runx2 and Colla1 expression were examined during murine aortic VSMC matrix calcification in vitro. mRNA
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Expression of *Ocn* (3.5 fold, P<0.001) (Figure 4.8A) and *Runx2* (4.8 fold, P<0.001) (Figure 4.8B) significantly increased at 7d. A significant increase in mRNA expression of *Ocn* (2.6 fold, P<0.001) (Figure 4.8A) and *Runx2* (4.9 fold, P<0.001) (Figure 4.8B) was observed at 28d. Significant increase in *Col1a1* (4.6 fold, P<0.001) mRNA expression was observed at 7d (Figure 4.8C). *Col1a1* mRNA expression remained unchanged at 14, 21 and 28d. RUNX2 protein expression was markedly increased at 7, 14, 21 and 28d compared to 0d (Figure 4.8D).

![Graph showing Alizarin Red S staining](image)

**Figure 4.6** *In vitro* matrix calcification of aortic VSMCs cultured for 28d under calcifying conditions. (A) Representative images and quantification of Alizarin Red S staining (calcium deposition), (B) TNAP activity and (C) cell viability. Results are presented as mean±SEM. Significance is represented compared to 0d as: ***P<0.001
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Figure 4.7 Up-regulation of calcification regulators during *in vitro* matrix calcification of aortic VSMCs cultured for 28d under calcifying conditions. Fold changes in mRNA expression of (A) Akp2, (B) Pit-1, and (C) Ank. Results are presented as mean±/SEM. Significance is represented compared to 0d as: *P<0.05, **P<0.01, ***P<0.001
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Figure 4.8 mRNA expression of osteoblast markers and chondrocyte markers during VSMC matrix calcification in vitro. Fold changes in mRNA expression of (A) Ocn, (B) Runx2 and (C) Col1a1. (D) RUNX2 protein expression. Results are presented as mean+/-SEM. Significance is represented compared to 0d as: *P<0.05, **P<0.01, ***P<0.001
4.5.2.3 VSMC matrix calcification in vitro is associated with an osteocyte phenotype

To investigate whether VSMC calcification is associated with an osteocyte phenotype, osteocyte marker (Sost, Dmp1, Phex, Mepe, E11 and Fgf23) mRNA expression was examined. By 7d, significant increases in mRNA expression of the osteocyte marker Sost (234.8 fold; P<0.001) (Figure 4.9A), Dmp1 (35.7 fold; P<0.001) (Figure 4.9B), Phex (5.3 fold; P<0.001) (Figure 4.9C), Mepe (2.8 fold; P<0.05) (Figure 4.9D) and E11 (2.2 fold; P<0.05) (Figure 4.9E) were observed, which was maintained throughout the 28d culture period. By 28d, significant increases in mRNA expression of Sost (401.5 fold; P<0.001) (Figure 4.9A), Dmp1 (46.7 fold; P<0.001) (Figure 4.9B), Phex (6.4 fold; P<0.001) (Figure 4.9C), Mepe (2.6 fold; P<0.05) (Figure 4.9D) and E11 (1.4 fold, P<0.01) (Figure 4.9E) were recorded. Fgf23 mRNA expression was significantly increased at 14d (3.9 fold, P<0.05) and 21d (10.8 fold; P<0.001) (Figure 4.9F). Significantly increased E11 protein expression was noted from 7d onwards. However, sclerostin protein expression was only observed at 28d (Figure 4.10). These data suggest that VSMCs obtain an osteocyte phenotype when cultured under calcifying conditions in vitro.

To further address whether matrix calcification promotes osteocytic differentiation of VSMCs, VSMCs were cultured with 50µg/ml AA in the presence or absence of 2.5mM βGP for 21d, as βGP provides the source of Pi for calcification. βGP treatment induced a significant increase in calcium deposition (P<0.05) (Figure 4.11A). At 21d, Runx2 gene expression was up-regulated in both treatments, with no significant difference between treatments, demonstrating that the osteoblastic transition of VSMCs occurs in the absence of βGP. However, significant increases were observed following 21d βGP treatment in the expression of the later osteoblast marker Colla1, calcification regulators PiT-1 and Ank, as well as osteocyte markers Dmp1, Sost and E11. No differences in gene expression were observed in Phex and Mepe (Figure 4.11B). No significant change in RUNX2 protein expression was observed. Osteocyte markers sclerostin and E11 protein expression markedly increased in the presence of βGP at 21d (Figure 4.11C). These studies further
demonstrate that Pi induced *in vitro* VSMC matrix calcification is associated with the expression of markers of the osteocyte phenotype.

**Figure 4.9** mRNA expression of osteocyte markers was significantly up-regulated during VSMC matrix calcification *in vitro*. Fold changes in mRNA expression of (A) *Sost*, (B) *Dmp1*, (C) *Phex*, (D) *Mepe*, (E) *E11* and (F) *Fgf23*. Results are presented as mean+/-SEM. Significance is represented compared to 0d as: *P<0.05, **P<0.01, ***P<0.001.
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Figure 4.10 Protein expression of sclerostin and E11 during the *in vitro* VSMC matrix calcification process. Sclerostin and E11 protein expression was increased over the 28d culture period.

Figure 4.11 VSMC matrix calcification was associated with an osteocyte phenotype. (A) Fold change in calcium deposition by HCL leaching. (B) Fold changes in the mRNA expression of osteoblast markers, calcification regulators and osteocyte markers. (C) Protein expression of RUNX2, E11 and sclerostin. Significance is represented compared to 21d (AA: Ascorbic acid) as: *P<0.05, **P<0.01, ***P<0.001.
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4.5.2.4 Osteocyte markers are expressed by the calcified aortae from $Enpp1^{-/-}$ mouse

Further investigations were performed to identify whether vascular calcification is associated with an osteocyte phenotype in vivo by detecting the expression of osteocyte markers in calcified aortae from $Enpp1^{-/-}$ mice. As shown in Chapter 3, this mouse shows significant alterations in bone mineralisation and severe soft tissue and medial arterial calcification. Alizarin Red S staining confirmed calcification in the medial layer of the $Enpp1^{-/-}$ aorta (Figure 4.12A). No staining was observed in wild-type control (Figure 4.12B). As expected, expression of sclerostin (Figure 4.13A) and E11 (Figure 4.13B) was detected in osteocytes and associated canaliculi within cortical bone, which was used as a positive control. Both E11 (Figure 4.12C & D) and sclerostin (Figure 4.12 G & H) were also detected in the $Enpp1^{-/-}$ calcified aortic media. No E11 and sclerostin positive staining was observed in wild-type mice (Figure 4.12E & F, Figure 4.12 I & J) or control sections incubated with IgG only (Figure 4.12 K & L). These data verified the in vitro data and confirmed the increased expression of osteocyte markers during the vascular calcification process in vivo.

4.6 Discussion

As described in section 1.3.5.1.8, previous studies have shown that VSMCs, the predominant cell type involving vascular calcification, can undergo osteo/chondrogenic differentiation in calcified conditions (Steitz et al., 2001; Abedin et al., 2004; Yang et al., 2004; Johnson et al., 2005; Speer et al., 2005), but it is still unknown whether vascular calcification involves the terminal differentiation of the nascent osteoblasts to an osteocyte phenotype.

In the present study, consistent with previous studies, Alizarin Red S staining showed calvarial osteoblasts formed calcified multicellular nodules under calcifying conditions in vitro (Hessle et al., 2002; Narisawa et al., 2007). In addition, these calcified osteoblasts showed increased TNAP enzyme activity and $Akp2$, $Ank$ and $PiT-1$ mRNA expression, which are well recognised calcification regulators (Hessle
et al., 2002; Yang et al., 2004; Narisawa et al., 2007). Furthermore, several osteoblast (Ocn, Runx2 and Coll1a1) and osteocyte (Sost, Dmp1, Phex, Mepe, E11 and Fgf23) markers were also increased significantly during osteoblast differentiation. These are consistent with previous studies (Johnson et al., 2005; Bonewald, 2006).

Figure 4.12 Expression of osteocytic markers in the calcified aorta from the Enpp1<sup>−/−</sup> mouse in vivo. Medial aortic calcification was identified by alizarin red staining (arrows) in (A) the Enpp1<sup>−/−</sup> aorta compared to wild-type control (B). Increased expression of (C, D) E11 and (G, H) sclerostin was detected in the calcified media of Enpp1<sup>−/−</sup> aorta (arrows). No expression of (E, F) E11 and (I, J) sclerostin was observed in the wild-type aorta. Representative images of negative control of Enpp1<sup>−/−</sup> aorta (K) and wild-type aorta (L). Scale bar = 50 µm.
Interestingly, the formation of matrix calcification was also observed in cultured murine aortic VSMCs under the same calcifying conditions that promoted osteoblast matrix calcification. Calcified VSMCs also showed a significant increase in TNAP enzyme activity which has a critical role in vascular calcification. Expression of TNAP is observed in calcified aortae in ESRD patients and it co-localises with other bone matrix proteins, such as osteopontin, bone sialoprotein and collagen Type I (Moe et al., 2002). TNAP promotes the calcification of rat aorta rings in vitro by hydrolysing the inhibitory PPI that is generated by the aortae (Lomashvili et al., 2004), and TNAP inhibitors suppress VSMC matrix calcification in vitro (Narisawa et al., 2007). TNAP expression is also up-regulated in aortae from uremic rats, resulting in increased PPI hydrolysis (Lomashvili et al., 2008). Overexpression of TNAP significantly increased the calcification of aortic ring cultures (Villa-Bellosta et al., 2011).

Ank is a multiple-pass transmembrane protein that transports PPI from the cytoplasm into extracellular space to inhibit HA formation (Hakim et al., 1984). Ectopic calcification of aortae was observed in ank/ank mutant mice, which has a C-terminal intracellular domain truncation (Ho et al., 2000). Unexpectedly, Ank expression was significantly up-regulated during the VSMC calcification process, which may be a cellular defence mechanism against rapidly progressing calcification. Increased
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mRNA expression of calcification regulators PiT-1 was also observed. Interestingly, there were notable differences in the temporal expression pattern of PiT-1 between osteoblasts and VSMCs in vitro. This may be because PiT-1 is a predominant sodium-dependent Pi co-transporter expressed in VSMCs (Li et al., 2006). Increased expression of PiT-1 leads to elevated intracellular Pi level and induces osteogenic transition of VSMCs (Li et al., 2006). Conversely, knockdown of PiT-1 expression by gene silencing methods inhibits Pi uptake by VSMCs and its osteogenic transition and matrix calcification (Muller et al., 2008). Consistent with previous studies detailing molecular changes that occur during osteoblast and VSMC matrix calcification, these data presented here further demonstrate that vascular calcification is a tightly regulated process that shares many similarities with bone formation (Jono et al., 2000a; Steitz et al., 2001; Tyson et al., 2003; Speer et al., 2009).

In order to test whether VSMC calcification is associated with an osteocyte phenotype transition, the expression patterns of Sost, Dmp1, Phex, Mepe, E11 and Fgf23 were investigated. Increased expression of these genes was observed during the osteoblast and VSMC matrix calcification process in vitro. Temporal differences in Sost and E11 between osteoblasts and VSMCs may reflect the likelihood of osteoblasts more readily undergoing osteocytic transition compared to VSMCs. E11 protein expression in osteoblasts decreased at 21d and this may be associated with post-transcriptional or post-translational regulation (Martin-Villar et al., 2009). In addition, Sost, E11 and Dmp1 expression was dramatically increased at 21d in VSMCs in the presence of βGP and AA compared to non-calcifying conditions (AA only), suggesting that these genes are associated with VSMC matrix calcification. These studies confirm that elevated Pi induces in vitro VSMC matrix calcification which is accompanied by the increased expression of a number of osteocyte markers. This is in agreement with studies in bone, which have shown that calcification drives osteocyte formation (Irie et al., 2008). In addition, Prideaux and colleagues have shown that extracellular matrix calcification promotes E11 expression and drives osteocyte formation (Prideaux et al., 2012). However, it has also been proposed cells that are already differentiating into osteocyte-like cells may direct calcification...
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(Lefebvre et al., 2001). Further investigations are needed to address whether calcification is controlled by or directly regulates the osteocytic phenotype transition of VSMCs. Whichever mechanism exists, it is clear that matrix mineralisation and osteocytogenesis are very closely entwined.

This is the first report indicating the up-regulation of expression of osteocyte markers Dmp1, E11 and Sost in VSMCs during in vitro matrix calcification. Studying an in vivo mouse model for vascular calcification – the Enpp1−/− mouse - further confirmed and extended these in vitro data. Enpp1−/− mice lacking ePPi, a major calcification inhibitor, develop articular, perispinal and medial aortic calcification (as described in Chapter 3). These Enpp1−/− mice share certain phenotypic features with human idiopathic infantile arterial calcification (Rutsch et al., 2001; Rutsch et al., 2003). My immunohistochemistry data demonstrated increased expression of both sclerostin and E11 in the calcified media of Enpp1−/− mice. This observation is consistent with increased expression of sclerostin in CMV-Msx2 transgenic mice, which show significant cardiovascular calcification (Shao et al., 2005). Furthermore, a recent proteomic study has demonstrated the expression of sclerostin in aortic extracts (David et al., 2007). Interestingly, it has been suggested that sclerostin negatively regulates bone formation via the inhibition of Wnt pathway (Li et al., 2008a), a pathway which has also been implicated in the pathobiology of vascular calcification (Shao et al., 2005). In addition, sclerostin has been shown to potentiate cysteine-rich protein 61 (Cyr61)-mediated cell growth and vascular migration and alters Cyr61-mediated cell adhesion by directly binding to Cyr61 (Craig et al., 2010). Therefore, sclerostin may be an important regulator of vascular calcification in various animal models. A proposed mechanism is shown in Figure 4.14.

In conclusion, this study has confirmed that VSMC matrix calcification resembles bone formation. Most importantly, this is the first study to demonstrate that the increased expression of osteocyte markers during VSMC calcification in vitro and in vivo. However, further in vivo studies are required to confirm the phenotype transition of VSMCs to osteocytes in the vascular calcification process using
additional pathological animal models, such as atherosclerosis and CKD. A fuller understanding of the osteocytic phenotype transition of VSMCs in the calcification process and in particular functional studies to determine the role of Sost, E11 and Dmp1 may develop novel potential therapeutic strategies for the inhibition of vascular calcification.

Figure 4.14 SOST and bone-vascular axis in CKD. Reduced bone mineral density and vascular calcification are common features of CKD patients. In CKD patients, osteocytes produce elevated sclerostin in response to an increased serum phosphate concentration, where sclerostin inhibits bone formation via the inhibition of Wnt pathway and results in reduced bone mineral density. On the other hand, sclerostin is also expressed by VSMCs in response to elevated serum phosphate levels. VSMC-derived sclerostin possibly together with bone-derived sclerostin may counteract the Wnt pathway in VSMCs and protect them against rapid progression of calcification.
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5 FGF23 protects against vascular calcification via MAPK/ERK signalling pathway

5.1 Introduction
In Chapter 3, I have shown that FGF23 is up-regulated in Enpp1⁻/⁻ VSMCs, which exhibit increased calcification compared to wild-type controls. In addition, my colleague Dr. Neil Mackenzie has recently reported that FGF23 circulating levels are significantly increased in the Enpp1⁻/⁻ mouse (Mackenzie et al., 2012a). Moreover, in Chapter 4, I have demonstrated that vascular calcification is associated with an osteocyte phenotype. Therefore, further studies were undertaken to investigate the role of the osteocyte-specific gene FGF23 in vascular calcification.

The Fibroblast Growth Factor family contains 23 members that regulate cell proliferation, migration, differentiation and survival (Eswarakumar et al., 2005). FGF23 is the most recently identified FGF and is mainly produced by osteocytes in bone. FGF23 has been shown to play an important role in regulating Pi homeostasis via its receptors (mainly FGFR1) in the presence of its cofactor, Klotho (Kurosu et al., 2006). The primary physiological actions of FGF23 are to exaggerate phosphaturia (Pi wasting) by downregulating the type IIa and IIc sodium-Pi transporters expression within the renal proximal tubular cells and to decrease the circulating concentrations of 1,25-dihydroxyvitamin D₃ via inhibition of 1α hydroxylase activity (Kurosu et al., 2006; Wolf, 2010). Interestingly, FGF23 is also a negative regulator of parathyroid hormone (PTH) secretion, which is a major controller of bone remodelling and calcium homeostasis (Ben-Dov et al., 2007) (Figure 5.1).

Whilst increasing interest is focusing on the role of FGF23/Klotho axis in regulating vascular calcification, little was known about these molecules in vascular calcification at the start of my project. Both FGF23 and Klotho deficient mice develop extensive vascular calcification (Sitara et al., 2006; Ohnishi et al., 2009). In support of this data, FGF23 antibody treatment increases vascular calcification in CKD rats (Shalhoub et al., 2012). It has also been reported that circulating FGF23
concentrations are highly correlated with the extent of calcium deposition in arteries in mice fed with high Pi diet (El-Abbadi et al., 2009). An association between FGF23 levels and calcium accumulation in the arteries of dialysis patients has also been reported (Srivaths et al., 2011). In addition, it has also been demonstrated that circulating FGF23 levels are increased in the Enpp1−/− mouse model of medial vascular calcification (Mackenzie et al., 2012a), as well as in patients with hypophosphatemic rickets resulting from a loss of function mutation in the ENPP1 gene (Lorenz-Depiereux et al., 2010). In patients with CKD, increased FGF23 plasma levels have been linked to a decrease in kidney function, the presence of vascular damage and an increased risk of cardiovascular mortality (Nasrallah et al., 2010; Yilmaz et al., 2010; Isakova et al., 2011; Srivaths et al., 2011). Interestingly, it has been shown that Klotho and two cognate receptors for FGF23 (FGFR1 and FGFR3) are expressed by aortic vascular tissues (Donate-Correa et al., 2011), suggesting that the aorta is a target organ of FGF23. In addition, recent studies have shown that the loss of vascular Klotho promotes vascular calcification (Hu et al., 2011; Lim et al., 2012). However, there is conflicting evidence as to whether FGF23 has a direct role in vascular calcification. It has been reported that FGF23 directly inhibits vascular calcification (Razzaque and Lanske, 2007; Lim et al., 2012; Shalhoub et al., 2012). However, it has also been suggested that increasing FGF23 concentrations may stimulate vascular calcification by acting directly on vascular wall to reduce local Klotho expression (Donate-Correa et al., 2011). Furthermore, a recent study has suggested that FGF23 is not associated with and does not induce vascular calcification (Scialla et al., 2013).

5.2 Hypothesis

FGF23 directly regulates vascular calcification

5.3 Aims

1. To examine the expression profiles of the components of the FGF23/Klotho signalling pathway during the VSMC in vitro matrix calcification process.
2. To investigate the functional role of FGF23 in VSMC matrix calcification.
3. To dissect the mechanisms whereby FGF23 mediates VSMC matrix calcification.
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Figure 5.1 The role of FGF23 in regulating Pi homeostasis. FGF23 is mainly produced by osteocytes in bone. FGF23 inhibits renal Pi transporter expression, which results in increased renal Pi excretion. In addition, FGF23 can suppress renal expression of 1-α hydroxylase, which leads to reduced calcitriol synthesis and decreased intestinal Pi absorption, and subsequent reduced serum Pi levels (Razzaque et al., 2009).

5.4 Materials and Methods
5.4.1 Enpp1<sup>-/-</sup> mice

Enpp1<sup>-/-</sup> mice were generated and characterised as previously described in section 2.6.1 and section 2.6.3.

5.4.2 Cell culture

Primary VSMCs were isolated as previously described (section 2.2.3). Cells were seeded in growth medium at a density of 1.5x10<sup>4</sup>/cm<sup>2</sup> in multi-well plates. Calcification was induced by incubation with growth medium containing 50μg/ml AA and 2.5mM βGP or 3mM inorganic Pi (Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>) as described in section 2.2.4. The medium was changed every third/fourth day. Recombinant mouse FGF23 (R&D Systems) at 10-50ng/ml was added to cultures at confluence. PD98059 (Sigma) at 10μM and PD173074 (Source Bioscience, Nottingham, UK) at 10 and 50nM were also added at confluence in 0.1% DMSO to inhibit Erk1/2 signalling and
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FGFR1, respectively. Control cultures received 0.1% DMSO only.

5.4.3 Detection of calcification
Calcium deposition was evaluated by Alizarin Red S staining (Sigma) or Randox calcium assay kit (Randox) as previous described (section 2.2.5).

5.4.4 TNAP activity
TNAP activity was determined as described in section 2.2.6. Total TNAP activity was expressed as nmoles pNPP hydrolysed/min/mg protein.

5.4.5 Analysis of gene expression by qPCR
Total RNA was extracted and reverse transcribed as previous described (section 2.4.1 and section 2.4.2). qPCR was performed as previously mentioned in section 2.4.4 for the genes Akp2, Ocn, Runx2, Pit-1, Fgf23, Klotho, FgfR1 and Gapdh. Semi-quantitative PCR was performed to examine FgfR3 expression, as described in section 2.4.3.

5.4.6 Western blotting for protein expression
Cells were lysed in an appropriate volume of RIPA buffer (Life Technologies Ltd) containing “complete” protease inhibitor cocktail according to manufacturer’s instructions (Roche). Immunoblotting was undertaken as previously described (section 2.5.3). Nitrocellulose membranes were probed overnight at 4°C with anti-FGF23 (R&D Systems), anti-Klotho (Abcam) or anti-FGFR1 (Cell Signaling Technology) primary antibody (1:1000 dilution), washed in TBST and incubated with goat anti-rat (FGF23) or goat anti-rabbit (Klotho and FGFR1) IgG peroxidase secondary antibody (DAKO) for 1h (1:3000 dilution in 5% BSA). The immune complexes were visualised using the enhanced ECL Western Blotting Detection System (GE Healthcare). Membranes were then washed in Restore acidic antibody removal buffer (Pierce) and re-probed for 1h for β-actin expression (1:5000 dilution in 5% milk; anti β-actin-peroxidase clone AC15; Sigma). Semi-quantitative assessment of band intensity was achieved using image-J software (NIH).
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5.4.7 Western blotting for cell signalling studies

Following confluency, VSMCs were incubated for 24h in serum free medium, and then either lysed immediately or stimulated with FGF23 (10ng/ml) for 10-60min before lysis. Cells were lysed in PhosphoSafe extraction buffer (Merck Biosciences Ltd, Nottingham, UK) containing “Complete” protease inhibitor cocktail (Roche) according to manufacturer's instructions. Immunoblotting was undertaken as previously described (section 2.5.3). The membranes were probed for 1h at room temperature with primary rabbit antibodies (all 1:1000 dilution in 5% BSA) against phospho-Akt (Ser 473), total Akt, phospho-Erk1/2 (Thr202/Tyr204) and total Erk1/2 (Cell Signaling Technology). The membranes were then incubated with anti-rabbit IgG-peroxidase (Cell Signalling Technology) for 1h (1:1000 dilution in 5% milk). The immune complexes were visualised and semi-quantitative assessment of band intensity achieved as described above.

5.4.8 Histology and immunohistochemistry

Aortae dissected from 22-week-old Enpp1⁻/⁻ and WT mice were processed as previously described (section 2.7.1). Briefly, tissues were fixed in 4% PFA for 24h before being dehydrated and embedded in paraffin wax and sectioning at 4µm using standard procedures. Sections were de-waxed in xylene and stained with Alizarin Red S to visualize calcium deposition as described in section 2.7.2. Immunohistochemistry was performed as described in section 2.7.3. In brief, de-waxed sections were de-masked for 10min in 0.1% trypsin. Endogenous peroxidises and non-specific antibody binding were blocked before overnight incubation at 4°C with 5µg IgG/ml anti-FGF23 antibody (R&D Systems) or 10µg IgG/ml anti-Klotho antibody (Abcam). The sections were then washed in PBS, incubated with goat anti-rat or goat anti-rabbit IgG peroxidise respectively (1:100 dilution, R&D Systems) for 1h, and incubated with DAB substrate reagent (0.06% DAB, 0.1% H₂O₂ in PBS) for 5min. The sections were finally dehydrated, counter stained with haematoxylin and eosin and mounted in DePeX. Control sections were incubated with non-immune rat IgG (5µg IgG/ml) or non-immune rabbit IgG (10µg IgG/ml) in place of the primary FGF23 or Klotho antibody, respectively.
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5.5 Results

5.5.1 VSMC in vitro matrix calcification is associated with increased FGF23 expression

5.5.1.1 Validation of VSMC in vitro matrix calcification

AA and βGP treatment significantly induced calcium deposition and TNAP activity in VSMCs, as described in section 4.5.2.1. In addition, mRNA expression of Akp2 (1.6 fold; P<0.001; Figure 5.2A), Runx2 (4.8 fold; P<0.001; Figure 5.2B), PiT-1 (3.0 fold; P<0.001; Figure 5.2C) and Ocn (2.5 fold, P<0.01; Figure 5.2D) was significantly increased at 7d and maintained throughout the culture period. Furthermore these genes were significantly elevated in VSMCs cultured in calcifying medium, compared to those cultured in control medium (Figure 5.2A, B, C & D, P<0.001). These results confirm the validity of this in vitro model to study in vitro matrix calcification of aortic VSMCs over an extended culture period.

5.5.1.2 Up-regulation of FGF23 during the VSMC in vitro matrix calcification process

Fgf23, Klotho and FgfR1 mRNA expression by VSMC was noted at all time-points during culture in calcifying medium containing βGP and AA (Figure 5.3). At 21d, a significant increase in Fgf23 expression (10.8 fold; P<0.05; Figure 5.3A) was observed compared to 0d. Klotho (2.1 fold; P<0.05; Figure 5.3B) and FgfR1 (5.4 fold; P<0.001; Figure 5.3C) mRNA expression was also significantly increased at 7d compared to 0d, and maintained throughout the 21d culture period. Furthermore, Fgf23, Klotho and FgfR1 mRNA expression was significantly increased in cells cultured under calcifying conditions compared to VSMCs cultured under control conditions (P<0.001). The specificity of commercial antibodies was confirmed by examining recombinant FGF23 and Klotho (Figure 5.4A). Bands corresponding to the size of both the FGF23 and Klotho recombinant proteins were observed (30 and 120KD respectively), alongside the full length proteins present in VSMCs (37KD, 118KD respectively). The temporal FGF23, FGFR1 and Klotho protein expression was generally comparable to corresponding gene expression (Figure 5.4B). However, whilst FgfR1 mRNA expression increased throughout the time course, no marked
changes in FGFR1 protein were observed. This may be due to post-transcriptional or post-translational regulation of FGFR1 expression.

As previously described in section 2.2.1, VSMC *in vitro* matrix calcification was induced by AA and βGP. However, this method has several disadvantages:

1. It takes up to 28 days to induce calcification.
2. The extent of calcification induced by this method is variable. Negligible levels of calcification are often seen.
3. βGP is an organic Pi donor, which is not a naturally occurring molecule.
4. TNAP activity is required for βGP-induced calcification.

**Figure 5.2 In vitro matrix calcification of murine aortic VSMCs cultured for 21d under calcifying conditions.** Fold change in (A) Akp2, (B) Runx2, (C) PiT-1 and (D) Ocn expression in VSMCs cultured with 2.5mM βGP and 50µg/ml AA or control medium. Results are presented as mean±/ SEM. **P<0.01; ***P<0.001 compared with 0d.
Figure 5.3 Up-regulation of \( Fgf23 \) expression during \textit{in vitro} calcification of murine aortic VSMCs cultured for 21d with \( \beta \)GP and AA. Fold change in the mRNA expression of (A) \( Fgf23 \), (B) \( Klotho \) and (C) \( Fgfr1 \). Results are presented as mean +/- SEM., \( n=4 \) *P<0.05; **P<0.01; ***P<0.001 compared with 0d.
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Therefore, I developed protocols that utilised inorganic Pi (3mM) to induce VSMC in vitro matrix calcification, as medial vascular calcification is prevalent in CKD and is often due to increased Pi levels (as described in section 1.3.4 and section 1.3.2.1).

VSMCs were cultured in growth medium containing high Pi (3mM Pi) for up to 14 days. High Pi resulted in a significant increase in VSMC calcium deposition as determined by Alizarin Red S staining (Figure 5.5A) and HCL leaching (Figure 5.5B) at 7d and 14d, compared to cells cultured in control medium (1mM Pi) (P<0.001; Figure 5.5A & B). A significant increase in mRNA expression of Runx2 (P<0.01; Figure 5.6A) and Akp2 (P<0.01; Figure 5.6B) was seen by 7d and maintained for the duration of culture. PiT-1 mRNA expression was significantly increased by 14d (P<0.001; Figure 5.6C). Fgf23 (P<0.05; Figure 5.7A) mRNA was significantly increased at 7d and maintained for the duration of culture. However, FGF23 protein levels remained unchanged, probably due to post-translational modification. It has been reported that FGF23 can be cleaved into N-terminal and C-terminal fragments (White et al., 2001). Protein expression of Klotho and FGFR1 was observed at 7d and 14d in both high Pi and control cultures (Figure 5.7B). FgfR3 mRNA expression was seen at 7d and 14d in both high Pi and control cultures, as revealed by semi-quantitative RT-PCR (Figure 5.7C). Taken together, these data

Figure 5.4 Up-regulated protein expression of FGF23, Klotho and FGFR1 during the in vitro VSMC calcification process. (A) Recombinant mouse FGF23 (rFGF23) and Klotho (rKlotho) were used to verify specificity of commercial antibodies in VSMCs. (B) FGF23, Klotho and FGFR1 protein expression in corresponding VSMCs cultured with 2.5mM βGP and 50µg/ml AA.
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suggest that high Pi induced VSMC calcification is associated with the up-regulation of FGF23 expression in VSMCs, in the presence of FGFR1, FGFR3 and the cofactor Klotho.

Figure 5.5 High Pi (3mM) induced VSMC in vitro matrix calcification. (A) Alizarin Red S staining, (B) Calcium content was determined by quantitative HCL leaching (µg/mg protein). Results are presented as mean+/− SEM. ***P<0.001 compared with control.
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Figure 5.6 High Pi induced osteogenic differentiation of VSMCs in vitro. Fold change in the mRNA expression of (A) Runx2, (B) PiT-1 and (C) Akp2. Results are presented as mean +/- SEM. *P<0.05; **P<0.01; ***P<0.001 compared with control.
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Figure 5.7 Up-regulation of FGF23 expression during in vitro matrix calcification of murine aortic VSMCs cultured for 14d with 3mM Pi. (A) Fold change in the mRNA expression of Fgf23. (B) Klotho and FGFR1 protein expression during the in vitro VSMC matrix calcification process. (C) Fgfr3 mRNA expression during the in vitro matrix calcification process. Results are presented as mean±SEM. *P<0.05; **P<0.01; ***P<0.001 compared with control.
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5.5.1.3 Up-regulated expression of FGF23 and Klotho expression in calcified aortae from Enpp1⁻/⁻ mice

The association of FGF23 with vascular calcification was further strengthened by examination of calcified aortae from Enpp1⁻/⁻ mice, which show significant alterations in bone mineralisation and medial vascular calcification (as described in Chapter 3). Calcium deposition was observed in Enpp1⁻/⁻ aortae (Figure 5.8A & Figure 5.9A), as revealed by Alizarin Red S staining, with no staining was observed in WT controls (Figure 5.8B & Figure 5.9B). Immunohistochemistry was performed to evaluate the expression of FGF23 and Klotho in the Enpp1⁻/⁻ calcified aorta. Increased expression of FGF23 and Klotho was observed in Enpp1⁻/⁻ calcified aortae (Figure 5.8C & Figure 5.9C) compared to WT controls (Figure 5.8D & Figure 5.9D). No positive staining was observed in negative controls (Figure 5.8E & F and Figure 5.9 E & F). Unfortunately, I was unable to optimise the protocol for immunostaining of FGFR1 and FGFR3 in Enpp1⁻/⁻ and WT aortae. Therefore, expression of Fgfr1 and FgfR3 was confirmed in Enpp1⁻/⁻ and WT aortae by qPCR. Interestingly, reduced Fgfr1 expression was noted in the Enpp1⁻/⁻ tissue (Figure 5.10A). No significant change was observed in FgfR3 expression in Enpp1⁻/⁻ and WT aortae (Figure 5.10B). These studies verify our in vitro data and confirm that the up-regulation of FGF23 and Klotho is associated with vascular calcification.
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Figure 5.8 Emergence of FGF23 in the calcified aorta from the Enpp1<sup>−/−</sup> mouse in vivo. Medial aortic calcification was detected by Alizarin Red S staining in (A) Enpp1<sup>−/−</sup> tissue compared to (B) WT control. (C) Increased protein expression of FGF23 was observed in the calcified media of Enpp1<sup>−/−</sup> aortic tissue. (D) No expression of FGF23 was detected in the non-calcified media of the WT control. Representative image of (E) Enpp1<sup>−/−</sup> compared to (F) WT negative control tissue. Scale bars = 50µm.
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Figure 5.9 Emergence of Klotho in the calcified aorta from the *Enpp1<sup>−/−</sup>* mouse in vivo. Medial aortic calcification was detected by Alizarin Red S staining in (A) *Enpp1<sup>−/−</sup>* tissue compared to (B) WT control. (C) Increased protein expression of Klotho was observed in the calcified media of *Enpp1<sup>−/−</sup>* aortic tissue. (D) Basal expression levels of Klotho were detected in the non-calcified media of the WT control. Representative image of (E) *Enpp1<sup>−/−</sup>* compared to (F) WT negative control tissue. Scale bars = 50µm.
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5.5.2 FGF23 directly protects VSMCs against calcification

It has been established that FGF23 is up-regulated during the vascular calcification process (as shown in section 5.5.1). To further investigate whether FGF23 promotes or inhibits vascular calcification, VSMCs were treated with FGF23 recombinant protein in the presence of control medium (1mM Pi) or high Pi medium (3mM Pi). High Pi dramatically induced calcium deposition in VSMCs (Figure 5.1A & B), which is consistent with previous results (Figure 5.5). No calcium deposition was observed in control medium (Figure 5.11B). Interestingly, a significant decrease in calcium deposition was observed in VSMCs cultured with high Pi in the presence of 10ng/ml (28.1% decrease; P<0.01) or 50ng/ml (28.8% decrease; P<0.01) FGF23 recombinant protein (Figure 5.11B). FGF23 treatment had no effect on cell viability (Figure 5.11C) or cleaved caspase-3 (a marker of apoptosis) expression (Figure 5.11D).

To further investigate the functional role of FGF23 signalling in vascular calcification, VSMCs were treated with the FGFR inhibitor PD173074 in the presence of high Pi. PD173074 treatment significantly enhanced VSMC matrix calcification at both 10nM (37.6% increase; P<0.01) and 50nM (87.8% increase; P<0.001) (Figure 5.12B). Increased calcification was also confirmed by Alizarin Red S staining (Figure 5.12A). No differences in cell viability were observed following...
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PD173074 treatment (Figure 5.12C). These data suggest FGF23 signalling protects VSMCs against calcification.

**Figure 5.11 Effect of FGF23 treatment on the in vitro matrix calcification of murine aortic VSMCs.** VSMCs were incubated with FGF23 (10-50ng/ml) in the presence of 3mM Pi for 9 days. (A) Alizarin Red S staining, (B) Calcium content was determined by quantitative HCL leaching (µg/mg protein), (C) Cell viability was assessed using the Alamar blue assay (OD570-OD620) and (D) Expression of cleaved caspase-3 protein as an indication of apoptosis. Results are presented as mean +/- SEM. *P<0.05; **P<0.01 compared with high Pi.
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5.5.3 FGF23 directly inhibits the osteogenic conversion of VSMCs

To further investigate whether FGF23 has a direct effect on the osteogenic differentiation of VSMCs, several osteogenic markers were evaluated by qPCR following FGF23 treatment. FGF23 treatment significantly inhibited the mRNA expression of Ocn (P<0.05), and PiT-1 (P<0.05) (Figure 5.13A & B). No significant difference in the mRNA expression of Runx2, Akp2 and ColIa1 was observed (Figure 5.13C, D & E).
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daypathway

A  
Ocn expression

B  
PiT-1 expression

C
Runx2

D  
Akp2 expression

E
Col1a1 expression

Figure 5.13 Effect of FGF23 on the expression of osteogenic markers in VSMCs. VSMCs were treated with 10ng/ml and 50ng/ml FGF23 for 48h, mRNA expression of (A) Ocn, (B) PiT-1, (C) Runx2, (D) Akp2 and (E) Col1a1. Results are presented as mean±SEM. *P<0.05 compared with control.
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5.5.4 FGF23 inhibits VSMC matrix calcification through the Erk1/2 signalling pathway

Previous studies have shown that FGF23 induces phosphorylation of Akt and Erk1/2 in both renal proximal tubule epithelial cells (Medici et al., 2008) and human aortic smooth muscle cells (Lim et al., 2012). Signal transduction studies were therefore completed to disclose the FGF23 initiated signaling mechanism by which this ligand prevents VSMC calcification. FGF23 significantly induced phosphorylation of Erk1/2, after 10 min (P<0.01) and 30 min (P<0.05) but not after 60 min exposure (Figure 5.14A & B). In contrast, Akt phosphorylation was not induced following FGF23 treatment at any of the time points studied (Figure 5.14A & C). These data potentially suggest that FGF23 may prevent VSMC calcification via Erk1/2 signaling.

To directly test whether the inhibitory effect of FGF23 on VSMC calcification was mediated via Erk1/2 signalling, VSMCs were treated with FGF23 alone or in combination with PD98059 (10μM), an Erk1/2 inhibitor, in the presence of high Pi. As previously shown (Figure 5.11A & B), FGF23 significantly prevented VSMC calcification in comparison to control cultures (Figure 5.14D) and this reduction was tempered when PD98059 was also present, resulting in a complete ablation of the protective effect afforded by FGF23 treatment (Figure 5.14D). PD98059 had no significant effect on VSMC calcification when added alone (Figure 5.14D). Taken together, these data strongly suggest that activation of the Erk1/2 pathway is a key event that mediates the protective effect of FGF23 against VSMC calcification.

5.6 Discussion

Currently, there is conflicting evidences as to whether FGF23 has a protective or harmful role on vasculature, with recent studies suggesting a direct inhibitory effect of FGF23 on vascular calcification (Razzaque and Lanske, 2007; Lim et al., 2012; Shalhoub et al., 2012). This present study in mice supports and extends these findings, and indicates that FGF23 is directly involved in modulating the pathogenesis of vascular calcification by exerting a protective effect on arterial wall integrity through activation of the Erk1/2 pathway.
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Figure 5.14 FGF23 stimulated Erk1/2 activation but not Akt activation in VSMCs. Effect of FGF23 (10ng/ml) on the phosphorylation of Erk1/2 compared with total Erk1/2, and phosphorylated Akt compared with total Akt shown by (A) western blot analysis and (B,C) densitometry quantification. (D) Effect of PD98059 (10µM) on calcium content (µg/mg protein) of VSMCs in the presence/absence of FGF23 (10ng/ml). Results are presented as mean+/- SEM. *P<0.05, **P<0.01 compared with a 0 exposure time (mins); b control and c FGF23.
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In the present study, AA and βGP treatment induced the formation of calcified matrix and TNAP activity in murine VSMC cultures, which has been described in section 4.5.2.1. In addition, VSMCs cultured under calcifying conditions showed increased expression of Akp2, Runx2, PiT-1 and Ocn, which are recognised regulators of vascular calcification.

The expression patterns of FGF23 and its cofactor Klotho during the VSMC matrix calcification in vitro process were detected in order to investigate the association between FGF23 and vascular calcification. Increased expression levels of Fgf23 and Klotho mRNA were noted after 7 days of culture in calcifying VSMCs. This increased expression was maintained throughout the culture period. Comparable changes in protein expression were also observed. FGFR1 expression was also confirmed in these cells, establishing the potential for FGF23 to directly function in VSMCs through binding to FGFR1 in the presence of Klotho.

This is the first report indicating that FGF23 and Klotho are up-regulated during murine VSMC matrix calcification in vitro. These data were further confirmed by studying an in vivo mouse model of vascular calcification. Mice lacking NPP1, a major generator of extracellular PPi, spontaneously develop articular cartilage, perispinal, and medial aortic calcification at a young age (Sali et al., 1999). These NPP1 knockout mice (Enpp1−/−) share phenotypic features with a human disease, GACI, and interestingly these mice show elevated levels of circulating FGF23 (Rutsch et al., 2001; Rutsch et al., 2003; Mackenzie et al., 2012a). A fully detailed characterisation of the phenotype of Enpp1−/− mice was described in Chapter 3. In the present study, an immunohistochemical approach demonstrated increased expression of FGF23 and Klotho in the calcified media of Enpp1−/− aortic tissue. This data is supported by the expression of FGF23 within calcified areas of atherosclerotic lesions (Voigt et al., 2010). However, the reduced levels of PPi in this mouse model may generate different effects on the vascular phenotype to that seen in alternative animal models of vascular calcification, such as kidney failure models. Additionally, the demonstration of the osteocytic hormone FGF23 in Enpp1−/− tissue in the present...
study confirms the up-regulation of molecules such as sclerostin associated with the osteocyte phenotype in the Enpp1−/− mouse model of aortic medial calcification in Chapter 4. The demonstration of Klotho expression in the present study in the rodent vasculature extends recent data describing Klotho expression in human arteries and aortic smooth muscle cells (Donate-Correa et al., 2011; Lim et al., 2012). It has recently been demonstrated that CKD is a state of vascular Klotho deficiency promoted by chronic circulating stress factors, including pro-inflammatory, uremic, and disordered metabolic conditions (Lim et al., 2012). Vascular produced Klotho has been shown to be an endogenous inhibitor of calcification (Lim et al., 2012), and may therefore be up-regulated in an attempt to impart, together with FGF23, a protective function in Enpp1−/− tissue. Indeed emerging evidence now suggests that Klotho exerts direct cardiovasculo-protective effects (Saito et al., 2000; Yamamoto et al., 2005; Rakugi et al., 2007; Hu et al., 2011; Liu et al., 2011), revealing a new mechanism by which Klotho may exert anti-aging effects in the arterial system.

In order to establish whether FGF23 is directly involved in modulating the pathogenesis of vascular calcification, functional studies on VSMCs were undertaken in vitro. Administration of the FGFR inhibitor PD173074 to cultured VSMCs promoted Pi-stimulated calcification. PD173074 has been previously shown to completely block the stimulation of the Fgf23 promoter in osteoblastic cells (Liu et al., 2009). These data therefore suggest that the augmented FGF23 produced during VSMC calcification exerts a protective effect through binding to FGF receptors. Further studies demonstrated a direct inhibitory action of FGF23 treatment on VSMC calcification in vitro. These findings are consistent with recent studies that have demonstrated that FGF23 is able to significantly inhibit extracellular calcium deposition in human aortic VSMCs after pre-treatment with calcitrol (Lim et al., 2012). Notably, Lim and colleagues also found that these beneficial effects were shown to be reversed after suppressing Klotho protein synthesis with Klotho siRNA. A vasculo-protective role for FGF23 is supported by the observation that Fgf23 null mice show extensive vascular and soft tissue calcification, together with severe hyperphosphatemia (Shimada et al., 2001; Sitara et al., 2004; Sitara et al., 2008).
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There is also evidence that FGF23 may directly inhibit skeletal calcification, independent of Pi homeostasis (Sitara et al., 2008). Furthermore, inactivating mutations of Fgf23 in diseases such as familial tumoral calcinosis manifest with severe ectopic calcification (Razzaque and Lanske, 2007). However, contrary to these reports there is the observation that increased FGF23 plasma levels in patients with CKD, have been linked to decreased kidney and vascular function, and increased risk of cardiovascular mortality (Nasrallah et al., 2010; Parker et al., 2010; Isakova et al., 2011; Srivaths et al., 2011). It has also been proposed that reduced Klotho tissue levels cause vascular resistance to FGF23 in CKD, thus masking FGF23’s protective effects on the vasculature (Lim et al., 2012).

In order to elucidate the potential mechanism through which FGF23 may be exerting it’s protective effect on VSMCs, modulation of the PI3-kinase/Akt and MAPK/Erk1/2 signalling pathways were examined. These pathways are involved in a wide range of cellular functions, including transcription, proliferation, migration, survival, differentiation and calcification (Roy et al., 2001; Kok et al., 2009). The present study revealed that FGF23 only induced the phosphorylation of Erk1/2 in murine VSMCs, corroborating the recent report describing FGF23 activation of this pathway in human aortic smooth muscle cells (Lim et al., 2012). Interestingly, in contrast to the studies undertaken by Lim and colleagues (Lim et al., 2012), no induction of Akt phosphorylation was noted. This may be due to the differences in cell culture conditions and/or divergent human and mouse VSMC responses to FGF23. The present study has demonstrated that the inhibition of the Erk1/2 pathway results in the complete loss of the protective effect against VSMC calcification afforded by FGF23 treatment. The Erk1/2 pathway has been shown to regulate calcification in various osteoblast and osteoblast precursor cell types (Salasznyk et al., 2004; Franceschi et al., 2007; Khatiwala et al., 2007). Consistent with these actions, several studies have shown that Erk1/2 activation regulates calcification and osteoblastic differentiation in vascular smooth muscle cell cultures (Roy et al., 2001; Simmons et al., 2004; Ding et al., 2006). These data therefore supports and extend these previous reports demonstrating the importance of the Erk1/2 pathway in
Chapter 5 FGF23 protects against vascular calcification via MAPK/ERK signalling pathway regulating vascular calcification.

The present study has built on the findings of Chapter 4, providing fundamental insights into the expression profiles of FGF23 during vascular calcification. These data suggest that the Erk1/2 signalling pathway is essential for FGF23 to prevent murine VSMC calcification in vitro. FGF23 therefore appears to play a critical role in vascular calcification, and may represent a novel potential therapeutic strategy for clinical intervention.
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6 BMP9 induces the osteogenic differentiation and calcification of VSMCs

6.1 Introduction

As described in Chapter 3, the key calcification inhibitor MGP was significantly decreased in Enpp1⁻/⁻ VSMCs (section 3.5.6). Previous studies have demonstrated that MGP inhibits vascular calcification partially through antagonising the activity of BMPs, as described in section 1.3.5.2.6. In Chapter 4, I have shown that the osteocyte marker sclerostin was significantly up-regulated in Enpp1⁻/⁻ calcified aortae, which is a secreted inhibitor of Wnt signalling and plays an important role in inhibiting bone accretion (Delgado-Calle et al., 2013). Interestingly, the expression of sclerostin is modulated by BMPs (Sutherland et al., 2004b; Delgado-Calle et al., 2013). These data suggest that up-regulated activity of BMPs may contribute to the medial vascular calcification observed in Enpp1⁻/⁻ mice. BMP9 (also known as growth differentiation factor 2, GDF2) is a recently identified BMP. In this chapter, I have investigated the potential role of BMP9 in medial vascular calcification.

BMPs belong to the TGF-β superfamily and play an important role in regulating cell proliferation and differentiation during development (Attisano and Wrana, 2002; Shi and Massague, 2003). The BMP family consists of at least 15 members in humans, including BMP2, 4, 6 and 7 (Kang et al., 2004). BMPs exert their downstream signalling by binding to the heterodimeric complex of two transmembrane serine/threonine kinase receptors, BMPR type I and BMPR type II. The activated receptor kinases phosphorylate specific intracellular proteins, called Smads (Miyazono et al., 2005). Smad proteins consist of three distinct subclasses based on their function: Receptor-regulated Smads (R-Smads), Common-mediator Smads (Co-Smads) and Inhibitory Smads (I-Smads). R-Smads in the BMP2, 4 and 7 signalling pathways are Smad1, Smad5 and Smad8, while R-Smads in the TGF-β signalling pathway are Smad2 and Smad3 (Yamashita et al., 1996). The phosphorylated R-Smads translocate into the nucleus and form a heterodimeric complex with Smad4 (Co-Smads) to activate target genes (Miyazono, 1999). The inhibitory Smads (Smad6 and Smad7) are potent intracellular antagonists of R-Smads. Smad7 is an inhibitor of
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the BMP, TGF-β and activin signalling pathways, while Smad6 specifically inhibits the BMP signalling pathway (Yamashita et al., 1996; Miyazono, 1999). BMPs are important regulators of osteoblast differentiation and bone formation (Hogan, 1996; Luu et al., 2007; Deng et al., 2008). Genetic disruption of BMPs results in severe skeletal and extraskeletal abnormalities (Zhao, 2003).

A number of *in vitro* and *in vivo* studies have suggested the important role of BMPs in vascular calcification. Several BMPs have been detected in calcified atherosclerotic plaques, including BMP2, 4 and 6 (Schluesener and Meyermann, 1995; Dhore et al., 2001). In addition, BMP2 and BMP4 treatment results in enhanced calcification *in vitro* (Mikhaylova et al., 2007; Li et al., 2008b). The type III sodium-dependent cotransporter PiT-1 is crucial in BMP2 regulated calcification (Li et al., 2008b). SMC-specific BMP2 overexpression accelerates vascular calcification in ApoE<sup>−/−</sup> mice (Nakagawa et al., 2010). Similarly, mice lacking the endogenous BMP inhibitor MGP develop profound vascular calcification (Luo et al., 1997; Zebboudj et al., 2002). Conversely, inhibition of BMPs either by the pharmacological inhibitor LDN-193189 or by overexpression of the local BMP inhibitor MGP protects against vascular calcification and atherosclerosis (Yao et al., 2010; Derwall et al., 2012).

BMP9 is produced by hepatocytes and is a new member of the BMP family (Bidart et al., 2012). Several studies have shown that BMP9 plays an important role in inducing and maintaining the cholinergic phenotype of embryonic basal forebrain cholinergic neurons (Lopez-Coviella et al., 2000); inhibiting hepatic glucose production and inducing the expression of key enzymes of lipid metabolism (Chen et al., 2003) and stimulating murine hepcidin 1 expression (Truksa et al., 2006). BMP9 is also present in adult blood of rodents and humans and acts as a specific activin receptor-like kinase 1 (ALK1) ligand (David et al., 2007). BMP9 is involved in angiogenesis, although there has been debate as to whether it inhibits or stimulates this process (David et al., 2007; Scharpfenecker et al., 2007; Suzuki et al., 2010). Hereditary Hemorrhagic Telangiectasia (HHT, Rendu-Osler disease) is an autosomal dominant genetically inheritable vascular dysplasia (Johnson et al., 1996), which is
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characterised by direct arteriovenous connections without an intervening capillary bed (Shovlin, 1997; 2010). Inactivating mutations in ENG, which encodes the TGF-β receptor endoglin, result in HHT type 1 (McAllister et al., 1994); whereas those seen in HHT type 2 are due to mutations in ACVRL1, which encodes ALK1 (Johnson et al., 1996). Interestingly, ACVRL1 mutations result in an altered BMP9 response in NIH-3T3 fibroblasts (Ricard et al., 2010). These data suggest the important role of BMP9 in angiogenesis and vascular remodelling. In addition, it has been reported that BMP9 is an important regulator of osteoblast differentiation and bone formation (Sharff et al., 2009; Luo et al., 2010; Bidart et al., 2012). However, the role of BMP9 in regulating the phenotypic trans-differentiation of VSMCs during calcification has yet to be investigated. Due to its presence in blood, BMP9 may play a key role in modulating blood vessel calcification in diseases such as CKD.

6.2 Hypothesis
BMP9 promotes the osteogenic differentiation and calcification of VSMCs and the serum concentration of BMP9 is altered in CKD patients.

6.3 Aims
1. To examine the expression of BMP9 and its receptors during the VSMC matrix calcification process.
2. To investigate whether BMP9 enhances VSMC matrix calcification and osteogenic differentiation in vitro.
3. To dissect the molecular mechanisms whereby BMP9 promotes vascular calcification.
4. To compare serum concentrations of BMP9 in predialysis and dialysis CKD patients.

6.4 Materials and Methods
6.4.1 Cell culture and induction of calcification
VSMCs were isolated as described in section 2.2.3 and cultured in growth medium. Matrix calcification was induced by 3mM inorganic Pi (a mixture of Na₂HPO₄ and NaH₂PO₄, pH=7.4) as previously described (section 2.2.4). Medium was changed
Chapter 6 BMP9 induces the osteogenic differentiation and calcification of VSMCs every third/fourth day. In all experiments, unless otherwise stated, recombinant mouse BMP9 (R&D Systems) was added to VSMC cultures at confluence for up to 14 days at a final concentration of 0.5, 5 and 50ng/ml. Activin receptor–like kinase-1 fusion protein (ALK1-Fc) (R&D Systems) was added to cells at 250ng/ml and 1µg/ml in the presence of BMP9 (50ng/ml). Additionally, the TNAP inhibitor 2,5-Dimethoxy-N-(quinolin-3-yl) benzenesulfonamide (DNB) (Merk, KGaA, Darmstadt, Germany) was added to cells at 3µM in the presence of BMP9 (50ng/ml).

6.4.2 Detection of calcification
Calcium deposition was stained by Alizarin Red S and quantified by HCL leaching as previously described (section 2.2.5).

6.4.3 TNAP activity
TNAP activity was assessed as previously described (section 2.2.6).

6.4.4 Semi-quantitative PCR
RNA was extracted and reverse transcribed into cDNA as described in section 2.4.1 and section 2.4.2. Semi-quantitative PCR was performed as described in section 2.4.3 to examine the expression of BMP receptors ALK1, ALK2, ActRII-A, ActRII-B and BMPRII in VSMCs. PCR products were analysed on a 1.5% agarose gel as described in section 2.4.4. Primers used are listed in Appendix 2.

6.4.5 Gene expression analysis by qPCR
qPCR was performed as described in section 2.4.5 to evaluate the expression of the following genes: Runx2, Osterix, Akp2, PiT-1, Mgp, Sost, Bmp2, Bmp9, Smad4, Msx2, Ocn, E11, Coll1a1, Enpp1, Ank, Sox5, Sox6 and Sox9. Primers used are listed in Appendix 2.

6.4.6 Western blotting for cell signalling
VSMCs were grown to confluence and serum starved for 24h. Cells were stimulated with 0-50ng/ml recombinant mouse BMP9 for 30min or 50ng/ml BMP9 for 0-60min (R&D Systems). Cells were lysed in PhosphoSafe extraction buffer (Merck)
containing “Complete” protease inhibitor cocktail (Roche) according to manufacturer's instructions. Western blotting was performed as previously described (section 2.5.3). Briefly, nitrocellulose membranes were probed overnight at 4°C with the rabbit primary antibodies against phospho-Smad1/5/8, phospho-Smad2,3, total Smad2, 3 & 4, phospho-Akt (Ser 473), total Akt, phospho-Erk1/2 (Thr202/Tyr204) and total Erk1/2 (Cell Signaling Technology) and total Smad1/5/8 (Santa Cruz). The membranes were then incubated with anti-rabbit IgG-peroxidase (Cell Signalling Technology) for 1h (1:1000 dilution in 5% milk). The immune complexes were visualised using the enhanced chemi-luminescence (ECL) Western Blotting Detection System (GE Healthcare).

6.4.7 Fluorescent immunocytochemical staining
VSMCs were seeded on glass coverslips in 12-well plates at a density of 50,000 cells/well. Following confluence, VSMCs were serum-restricted for 24h and stimulated with 0-50ng/ml recombinant mouse BMP9 (R&D Systems) for 30min. Cells were fixed with 4% PFA and washed with PBS. The fixed cells were permeabilised with 0.3% Triton-X 100 (Sigma) and incubated with anti-alpha-SMA (Sigma), anti-CD31 (Abcam), anti-phospho-Smad1/5/8 antibody (Cell Signalling Technology) overnight. After washing, cells were incubated with Alexa Fluor@488 goat-anti mouse antibody or Alexa Fluor@594 goat-anti rabbit antibody (Life Technologies Ltd) for 1h in the dark. Glass coverslips were then mounted onto slides with Prolong® Gold Anti-Fade Reagent contained DAPI (Life Technologies Ltd). Fluorescence signal was detected using a Leica fluorescence microscope.

6.4.8 Luciferase assay
VSMCs were seeded in 12-well plates at a density of 50,000 cells/well. 0.4µg pBV-SBE4-Luc and 8ng pEF1-RLuc were transfected with FuGENE6 (Roche), as described in section 2.3.4. Cells were then incubated with 0-50ng/ml BMP9 recombinant protein (R&D systems) for 24h and luciferase assay was performed as described in section 2.3.5.
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6.4.9 Smad4 siRNA transfection

Flexitube gene solution for Smad4 was purchased from Qiagen and transfected as described in section 2.3.6. Briefly, on the day of transfection, VSMCs were seeded in 12-well plate at a density of 80,000 cells/well and transfected with 20nM Allstars Negative Control siRNA (Scrambled siRNA, Qiagen) or 20nM Smad4 siRNAs (Qiagen) with 6ul Hyperfect transfection reagent (Qiagen) according to manufacturer’s instructions. Cells 48h or 96h after transfection are used for experiments. The knockdown efficiency of Smad4 was verified by qPCR and western blot. For long-term VSMC calcification assay, cells were re-transfected with Smad4 siRNA at 4d and cultured in the presence of calcifying medium for up to 9 days.

6.4.10 ELISA analysis of BMP9 expression

ELISA was performed as previously described (section 2.8.2) to evaluate circulating levels of BMP9 in sera from children with CKD (10 dialysis and 10 predialysis).

6.5 Results

6.5.1 VSMC in vitro matrix calcification is associated with increased Bmp9 expression

As described in section 2.2.4, VSMC in vitro matrix calcification was induced by 3mM inorganic Pi (a mixture of Na$_2$HPO$_4$ and NaH$_2$PO$_4$). Consistent with previous observations (section 4.5.2.1), high Pi (3mM Pi) significantly induced VSMC in vitro matrix calcification at 14d, as revealed by Alizarin Red S staining (Figure 6.1A). No staining was observed in VSMCs cultured with control medium (1mM Pi) (Figure 6.1A). Quantitative HCL leaching showed that calcium deposition in VSMCs was significantly up-regulated at 7d (19.6 fold, P<0.01) and 14d (246.7 fold, P<0.001) in the presence of high Pi compared to control medium (Figure 6.1B).
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In addition, a significant increase in mRNA expression of osteogenic markers Bmp2 (2.9 fold, P<0.001, Figure 6.2A) and PiT-1 (1.5 fold, P<0.01, Figure 6.2B) was observed by 14d in VSMCs cultured with high Pi medium compared to normal Pi medium, which was consistent with previous studies (Hasaneen et al., 2005). Runx2 mRNA expression was significantly up-regulated at 7d in the presence of high Pi compared to control medium (1.2 fold, P<0.05, Figure 6.2C). Bmp9 mRNA expression (3.3 fold, P<0.001) was also significantly increased at 14d in VSMCs cultured in calcifying medium (Figure 6.2D). Together these data show that matrix

Figure 6.1 High Pi (3mM) induced VSMC in vitro matrix calcification. (A) Alizarin Red S staining, (B) Calcium content was determined by quantitative HCL leaching (μg/mg protein). Results are presented as mean+/− SEM. **P<0.01; ***P<0.001 compared with control.
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calcification caused by elevated Pi levels is associated with the up-regulation of Bmp9 expression in VSMCs.

Figure 6.2 Up-regulation of Bmp9 during the VSMC in vitro matrix calcification process. Fold change in the mRNA expression of (A) Bmp2, (B) P iT-1, (C) Runx2 and (D) Bmp9. Results are presented as mean +/-SEM. *P<0.05, ***P<0.001 compared with control.
6.5.2 BMP9 directly modulates VSMC matrix calcification

Having established the increased expression of Bmp9 during the VSMC matrix calcification process, I sought to establish whether BMP9 promotes vascular calcification by direct treatment of VSMCs with recombinant BMP9. Studies were undertaken in VSMCs cultured for 9 days in growth medium containing 3mM Pi in the presence of BMP9 (0.5-50ng/ml). No effect of treatment was noted in low Pi medium (1mM Pi). A significant increase in calcium deposition was observed following BMP9 treatment at 50ng/ml, as determined by Alizarin Red S staining (Figure 6.3A) and HCL leaching (3.4 fold; P<0.01; Figure 6.3B). Furthermore, in the presence of calcifying medium, a minimum concentration of 5ng/ml BMP9 treatment induced a significant increase in the mRNA expression of the osteogenic markers Runx2 (1.4 fold; P<0.05; Figure 6.4A), Osterix (2.8 fold; P<0.05; Figure 6.4B) and Akp2 (2.6 fold; P<0.05; Figure 6.4C). Increased mRNA expression of Runx2, Osterix and Akp2 was also observed following BMP9 treatment in the presence of control medium (Figure 6.4 A, B & C).

Figure 6.3 BMP9 treatment promoted VSMC in vitro matrix calcification. (A) Alizarin Red S staining, (B) Calcium content was determined by quantitative HCL leaching (µg/mg protein). Results are presented as mean+/− SEM. *P<0.05, **P<0.01.
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Figure 6.4 BMP9 induced osteogenic differentiation of VSMCs. Fold change in mRNA expression of (A) Runx2, (B) Osterix and (C) Akp2. Results are presented as mean±SEM.  
*P<0.05, **P<0.01, ***P<0.001.
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Sox5, Sox6 and Sox9 are key transcription factors that determine chondrocyte differentiation (Lefebvre et al., 2001). No significant changes were observed in mRNA expression of Sox5 (Figure 6.5A), Sox6 (Figure 6.5B) and Sox9 (Figure 6.5C) following BMP9 treatment either in the presence of calcifying medium or control medium, suggesting that BMP9 induces the direct differentiation of osteoblasts from VSMCs and not via intermediary chondrocytic cells. This suggests an intramembranous rather than endochondral mechanism. Notably, up-regulation of Pit-1 was induced by 5ng/ml BMP9 in the presence of calcifying medium (Figure 6.6A). No significant changes were seen in the presence of control medium. Furthermore, a concomitant reduction in the mRNA expression of the mineralisation inhibitor Mgp was observed following treatment of VSMCs with 50ng/ml BMP9 in the presence of calcifying medium (29%; P<0.05; Figure 6.6B), as well as control medium (25%, P<0.01; Figure 6.6B). As described in Chapter 4, vascular calcification is associated with an osteocyte phenotype. E11 is an early osteocyte marker, and sclerostin is expressed by more mature osteocytes, as described in section 1.3.5.1.6. BMP9 treatment had no effect on E11 mRNA expression (Figure 6.6C). Notably, the up-regulation of the osteocyte gene Sost was induced by 5ng/ml BMP9 (1.8 fold; P<0.001; Figure 6.6D) in the presence of calcifying medium, as well as control medium (1.9 fold, P<0.05; Figure 6.6D). Furthermore, 50ng/ml BMP9 treatment induced a significant increase in Sost mRNA expression in the presence of calcifying medium (5.1 fold, P<0.001; Figure 6.6D) and control medium (6.2 fold, P<0.001; Figure 6.6D).

6.5.3 BMP9 induces VSMC matrix calcification through a TNAP-dependent mechanism

Previous studies have shown that TNAP is a key regulator of vascular calcification through its ability to hydrolyse PPi, a key inhibitor of calcification (Shaffer et al., 2007; Yamada et al., 2007b; Jensky et al., 2011). Studies were therefore undertaken to establish whether BMP9 modulates VSMC matrix calcification through a TNAP-dependent mechanism. A minimum concentration of 5ng/ml BMP9 treatment resulted in significantly increased TNAP activity in VSMCs (2.7 fold; P<0.001; Figure 6.7A) in the presence of high Pi, as well as control medium (3.0 fold,
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P<0.001; Figure 6.7A). 50ng/mg BMP9 treatment induced a considerably greater increase in TNAP activity in the presence of high Pi (10.1 fold, P<0.001; Figure 6.7A) and control medium (10.1 fold, P<0.001; Figure 6.7A). Furthermore, co-treatment with the TNAP inhibitor DNB (3µM) (Sioka et al., 2007) significantly reduced the pro-calcificatory effects of BMP9 (68%; P<0.001; Figure 6.7B).

Figure 6.5 BMP9 had no effect on chondrogenic differentiation of VSMCs. Fold change in mRNA expression of (A) Sox5, (B) Sox6 and (C) Sox9. Results are presented as mean±/ SEM.
Figure 6.6 Effect of BMP9 on the expression of calcification regulators and osteocyte markers. Fold change in mRNA expression of (A) *Pit-1*, (B) *Mgp*, (C) *E11* and (D) *Sost*. Results are presented as mean±SEM. *P<0.05, **P<0.01, ***P<0.001.
6.5.4 BMP9 signals though the ALK1 receptor to promote VSMC matrix calcification

In order to determine the profile of BMP receptors expressed in murine VSMCs, RT-PCR was performed. Strong bands with similar intensity were obtained using primers for \textit{ALK1}, \textit{ALK2}, \textit{BMPR-II}, \textit{ActR-IIA} and \textit{ActR-IIB} (Figure 6.8A). These results indicate that both type I and type II BMP receptors are expressed in cultured murine VSMCs. It has been shown that BMP9 preferentially binds to ALK1 (David et al., 2007; Hyder et al., 2007). Having established ALK1 expression in murine VSMCs, I next sought to examine the effect of inhibiting ALK1 signaling on BMP9-induced VSMC matrix calcification using a soluble chimeric protein (ALK1-Fc) (Ranalletta et al., 2006). ALK1-Fc (1µg/ml) significantly inhibited BMP9-induced TNAP activity (33%; P<0.01; Figure 6.8B) and markedly reduced the pro-calcificatory actions of BMP9 on VSMCs (89%; P<0.001; Figure 6.8C). These data are the first to...
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demonstrate that BMP9 signals via ALK1 to promote the osteogenic differentiation
and matrix calcification of VSMCs.

**Figure 6.8** BMP9 signals through the ALK1 receptor to promote VSMC matrix
calcification. (A) Expression of BMP receptors during VSMC in vitro matrix calcification. (B)
VSMCs were cultured with 50ng/ml BMP9 in combination with 250ng/ml or 1µg/ml ALK1-Fc
for 4d in the presence of control medium and high Pi. TNAP activity was determined. (C)
VSMCs were cultured with 50ng/ml BMP9 in combination with 250ng/ml or 1µg/ml ALK1-Fc
for 9d in the presence of high Pi. Calcium content was determined by Alizarin Red S staining
and quantitative HCL leaching (µg/mg protein). Results are presented as mean+/SEM. **P<0.01, ***P<0.001.
6.5.5 BMP9 induces VSMC matrix calcification through activation of the Smad signalling pathway

Recent studies have shown that BMP9 promotes the activation of Smad1/5/8 and Erk1/2 during osteogenic differentiation (Xu et al., 2012; Zhao et al., 2012). Signal transduction studies were therefore completed to disclose the BMP9 initiated signaling mechanism by which this ligand induces VSMC matrix calcification. BMP9 (0.5-50ng/ml) markedly induced phosphorylation of Smad1/5/8 following treatment for 10, 30 and 60min (Figure 6.9A & B). VSMCs were positively stained for anti-smooth muscle α-actin (SMA), a VSMC specific marker, but no staining was observed when CD31, an endothelial cell marker, was used (Figure 6.10A). Immunofluorescent staining confirmed the translocation of p-Smad1/5/8 to the nucleus following BMP9 exposure (Figure 6.10B). Concomitantly, the phosphorylation of Smad2, Smad3 and Erk1/2 was weakly induced by BMP9 (Figure 6.9A & B). BMP9 treatment had no effect on Akt phosphorylation (Figure 6.9A & B). The common partner Smad, Smad4 protein expression remained unchanged, as shown in Figure 6.9A. To further investigate whether BMP9 activates the Smad signalling pathway, VSMCs were transfected with a previously reported BMPR-Smad luciferase reporter, pBV-SBE4 (Zawel et al., 1998), and treated with 0-5ng/ml BMP9 recombinant protein. As expected, 5ng/ml BMP9 treatment significantly induced pBV-SBE4 reporter activity (1.7 fold, P<0.05; Figure 6.10C). These data suggest that signalling through Smad1/5/8 phosphorylation mediates the principal effects of BMP9 on VSMC matrix calcification, with activation of Smad2, Smad3 and Erk1/2 pathways potentially refining cellular effects.

6.5.6 BMP9-mediated matrix calcification of VSMCs is Smad4 dependent

Smad1/5/8, Smad2 and Smad3 form complexes with the common-partner Smad, Smad4. These complexes translocate and accumulate in the nucleus and regulate the transcription of target genes. Therefore, to directly test whether BMP9 promotes VSMC matrix calcification through a Smad signalling mechanism, I examined the effect of Smad4 siRNA knockdown on BMP9-induced matrix calcification of VSMCs. qPCR and western blotting were used to validate the knockdown efficiency of Smad4 siRNAs after a 48h or 96h transfection period. Smad4 siRNA1, 2, 3 and 4
Chapter 6 BMP9 induces the osteogenic differentiation and calcification of VSMCs resulted in a significant reduction of Smad4 mRNA expression at 48h after transfection, which was sustained for 96h (Figure 6.11A). A comparable decrease in Smad4 protein expression at 48h or 96h was also observed (Figure 6.11B). Allstars negative control (Scramble siRNA) had no effect on Smad4 mRNA and protein expression (Figure 6.11A & B). Smad4 siRNA2 was used for further studies as it induced the greatest decrease in Smad4 mRNA expression. Transfection of VSMCs with Smad4 siRNA2 significantly inhibited BMP9-induced TNAP activity in the presence of high Pi (72%; P<0.001; Figure 6.12A), as well as control medium (66%; P<0.001; Figure 6.12A), and markedly reduced the pro-calcificatory actions of BMP9 on VSMCs (61%; P<0.001; Figure 6.12B). These results are the first to show that BMP9 signals through Smad4 to promote the osteogenic differentiation and matrix calcification of VSMCs.

Figure 6.9 Effect of BMP9 on phosphorylation of Smad1/5/8, Smad2, Smad3, Erk and Akt. (A) VSMCs were starved with serum-free medium for 24h, and then treated with 0-50ng/ml BMP9 for 30min. (B) VSMCs were starved with serum-free medium for 24h, and then treated with 50ng/ml BMP9 for 0-60min.
Figure 6.10 BMP9 stimulated translocation of p-Smad1/5/8 into the nucleus and upregulated BMPR-Smad luciferase activity. (A) VSMC was positively stained with SMA antibody (Green). (B) VSMCs were starved with serum free medium for 24h and then treated with 0-50ng/ml BMP9 for 30min. Immunofluorescence staining was performed to detect the translocation of p-Smad1/5/8 (Red). (C) VSMCs were transfected with pBV-SBE4 and pEF1-Rluc. After 24h, cells were treated with 0-5ng/ml BMP9 for 24h, and a luciferase assay was performed. Results are presented as mean +/- SEM. *P<0.05 compared with control.
Figure 6.11 Validation of Smad4 siRNAs. VSMCs were transfected with Mock (Transfection reagent only), 20nM Allstars negative control (Scramble siRNA) or 20 nM Smad4 siRNA1, 2, 3 and 4 for 48h and 96h. (A) Fold change in Smad4 mRNA expression. (B) Smad4 protein expression. Results are presented as mean +/- SEM. ***P<0.001, compared with Allstars negative control.
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Figure 6.12 BMP9-induced TNAP activity and matrix calcification of VSMCs was Smad4 dependent. VSMCs were transfected with 20nM Smad4 siRNA. (A) TNAP activity in VSMCs transfected with scramble siRNA or Smad4 siRNA for 4 days in the presence or absence of 50ng/ml BMP9 under control medium or high Pi medium. (B) VSMCs were transfected with scramble siRNA or Smad4 siRNA and treated for 9 days with high Pi medium in the presence or absence of 50ng/ml BMP9. Alizarin Red S staining and quantitative HCL leaching of calcium deposition. Results are presented as mean+/-.SEM. *P<0.05, **P<0.01, ***P<0.001.
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6.5.7 BMP9 serum levels are up-regulated in CKD patients with dialysis

Investigations in children with CKD have provided evidence that vessel wall calcification begins predialysis, with factors specific to the dialysis milieu triggering accelerated calcification (Shroff et al., 2008). Following reports that BMP9 circulates in a biologically active form (David et al., 2008), I sought to compare BMP9 levels in predialysis and dialysis serum from children with CKD. Intriguingly, BMP9 was markedly elevated in serum from dialysis patients (2.3fold; P<0.001; Figure 6.13A). Whilst no correlation between serum BMP9 concentration and calcium/phosphate concentration was noted, a significant correlation (Pearson Correlation = 0.712, P<0.05; Figure 6.13B) was observed between dialysis time and BMP9 concentration in patients receiving haemodialysis, suggesting that this highly osteogenic BMP may contribute to the accelerated calcification associated with dialysis.

![BMP9 concentration in CKD and dialysis](image1.png)

**Figure 6.13 Assessment of BMP9 in serum from CKD dialysis patients.** (A) BMP9 concentrations (pg/ml) in predialysis and dialysis serum from children with CKD. (B) Correlation of BMP9 serum concentrations (pg/ml) with dialysis time (yrs). **P<0.01.
6.6. Discussion

Previous studies have shown that vascular calcification is an active cell-regulated process and several molecular mechanisms have been demonstrated, including abnormal calcium and Pi metabolism, osteo/chondrogenic transition of VSMCs, apoptosis, oxidative stress, inflammation, and loss of inhibitors of vascular calcification (Craig et al., 2010; Zhang et al., 2010). However, the precise mechanisms of vascular calcification are not yet fully understood. This Chapter expands the current knowledge of the importance of BMP activity in vascular calcification. Here I provide the first evidence to suggest that BMP9, one of the most osteogenic BMPs, may also contribute to this pathological process.

Hyperphosphatemia is a significant contributor to vascular calcification and cardiovascular mortality in CKD patients, with the control of serum Pi now recognised as a crucial strategy to prevent arterial calcification. In the present study, quantitative alizarin red staining of calcium deposition confirmed the formation of calcified matrix in murine VSMC cultures, induced by high Pi treatment. This is in agreement with previous reports (Luo et al., 2010; Ricard et al., 2010; Schnitzler et al., 2010). Furthermore, VSMCs cultured under calcifying conditions showed increased expression of Runx2 and PiT-1, which are recognised regulators of vascular calcification. In order to investigate the association between BMP9 and vascular calcification, the expression profile of BMP9 was determined during VSMC matrix calcification in vitro. Increased Bmp9 mRNA expression was noted after 14 days of culture in calcifying murine VSMCs. In agreement with previous studies in human vascular smooth muscle cells (Li et al., 2008b), expression of type I and type II BMP receptors was also noted, establishing the potential for BMP9 to directly function in VSMCs.

In order to establish whether BMP9 is directly involved in modulating the pathogenesis of vascular calcification, functional studies on VSMCs were undertaken. BMP9 administration increased calcium deposition, with a concomitant upregulation in osteogenic marker expression. Interestingly, the osteocyte marker sclerostin was also induced by BMP9 treatment. Sclerostin is a recognised inhibitor
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of bone formation (ten Dijke et al., 2008), and has been shown in Chapter 4 to be upregulated during VSMC matrix calcification. Furthermore, elevated serum sclerostin levels have recently been reported in patients with CKD ((Pelletier et al., 2013; Viaene et al., 2013), highlighting the requirement for further studies to establish the clinical relevance of sclerostin in vascular calcification. It should be noted that BMP9 treatment had no effect on VSMC matrix calcification when cultured with normal Pi medium. However, BMP9 treatment induced the osteogenic differentiation of VSMCs under normal Pi conditions. These data suggest that elevated Pi levels are crucial for BMP9-induced VSMC matrix calcification, with BMP9 treatment increasing the sensitivity of VSMCs to calcification by inducing the osteogenic transition of VSMCs.

It has been well established that BMP9 induces TNAP activity in mesenchymal stem cells (MSCs), muscle-derived stem cells and C3H10T1/2 cells (Chen et al., 2010a; Xiang et al., 2012; Xu et al., 2012). In the present study, BMP9 treatment induced TNAP activity in calcifying VSMCs, with co-treatment with the TNAP inhibitor DNB preventing the pro-calcificatory effects of BMP9. These data suggest that BMP9 stimulates VSMC matrix calcification through a TNAP-dependent mechanism clearly emphasising the importance of TNAP in mediating this pathological process, as highlighted in previous studies (Lopez-Coviella et al., 2006).

BMPs initiate their actions by binding to transmembrane serine/threonine receptors, belonging to the TGF-β/BMP receptor superfamily. ALK1 is a type I cell surface receptor expressed predominantly in vascular cells and is implicated in blood vessel formation and organisation (Mitchell et al., 2010). BMP9 has been reported to preferentially bind to the ALK1 receptor (David et al., 2006), with previous studies demonstrating an important role for ALK1 in mediating BMP9-induced osteogenic differentiation (Luo et al., 2010). Therefore, following confirmation of ALK1 mRNA expression in VSMCs in the present study, the consequences of ALK1 inhibition using an ALK1-Fc chimera were assessed. ALK1-Fc treatment abolished BMP9 induced VSMC matrix calcification, and significantly reduced BMP9 mediated TNAP activity. These findings support previous data showing that ALK1-Fc inhibits
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BMP9-mediated TNAP expression in C2C12 cells (Brown et al., 2005), and show for the first time the use of the ALK1-Fc chimera to mediate significant loss of VSMC matrix calcification.

It has been well established that BMPs can initiate intracellular signalling through the activation of Smads. Mechanistically, my data shows that in VSMCs, BMP9 stimulates the phosphorylation of Smad1/5/8, which form a heterodimeric complex with Smad4 in the nucleus and induces VSMC matrix calcification. This highlights for the first time the Smad signalling pathway as a hub in driving BMP9 induced gene expression changes to initiate aortic calcification. These data are in agreement with recent studies showing increased Smad1/5/8 phosphorylation in atherosclerotic lesions and calcified aortic valves (Yao et al., 2010; Ankeny et al., 2011; Derwall et al., 2012). My data further suggest that activation of Smad2, Smad3 and Erk1/2 pathways may also refine the effects of BMP9 on VSMC matrix calcification.

Investigations into the natural history of vascular calcification in children with CKD have provided evidence that dialysis promotes vascular calcification (Shroff et al., 2008). Following reports that BMP9 circulates in a biologically active form (David et al., 2007), I sought to compare BMP9 levels in predialysis and dialysis serum from children. One of the most exciting findings of this study was the observation that BMP9 was markedly elevated in serum from dialysis patients, suggesting that this highly osteogenic BMP may contribute to the accelerated calcification associated with dialysis. Further studies are required to fully elucidate the mechanisms underpinning this novel finding.

In conclusion, I have undertaken in vitro VSMC matrix calcification studies, in conjunction with clinical analysis, to provide fundamental insights into the role of BMP9 as a potent osteogenic inducer of vascular calcification. My findings suggest that BMP9 binds to the ALK-1 receptor to stimulate the phosphorylation of Smad1/5/8, which forms a heterodimeric complex with Smad4 in the nucleus, inducing increased TNAP activity and VSMC matrix calcification. Furthermore, I report novel data suggesting that BMP9 may contribute to the accelerated
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calcification associated with dialysis. BMP9 therefore appears to play a critical role in vascular calcification, and may represent a novel potential therapeutic strategy for clinical intervention.
7 Final discussion

7.1 General discussion

Matrix calcification of osteoblasts and chondrocytes is a key process allowing normal bone development. However, in some soft-tissue pathological conditions, calcification also occurs in arteries and cardiac valves. This pathological calcification results in a number of clinical disorders which have been described in section 1.3.3, and is the leading cause of death in CKD patients. Currently, there is no effective treatment of vascular calcification. Therefore, it is imperative to unravel the precise mechanisms controlling this complex process. Recent advances in the field have demonstrated similarities between vascular calcification and bone formation (Vattikuti and Towler, 2004; Johnson et al., 2006). Key cellular and molecular elements involved in bone formation also participate in the regulation of calcification in blood vessels. For example, the transcription factors \textit{Runx2} and \textit{Msx2} are essential for bone formation, and also contribute to vascular calcification (Shao et al., 2005; Sun et al., 2012). A better understanding of the relationship between bone formation and vascular calcification may therefore be helpful for the identification of potential therapeutic targets to prevent vascular calcification.

Osteocytes are the most abundant cells in bone, and are terminally differentiated osteoblasts. Emerging evidence has demonstrated the crucial role of osteocyte specific proteins in controlling cellular differentiation, Pi homeostasis and matrix calcification, and these include sclerostin, E11, MEPE, DMP1, PHEX and FGF23 (Bonewald, 2011; Kennedy and Schaffler, 2012). Interestingly, osteocyte-like cells have been observed in human calcified aortae (Shanahan et al., 1999; Hunt et al., 2002). These data suggest that vascular calcification may be associated with the emergence of an osteocyte phenotype. By comparing the cellular and molecular events during the matrix calcification process of osteoblasts and VSMCs \textit{in vitro}, the work reported in this thesis has demonstrated that VSMCs undergo osteocytic differentiation within a calcifying environment, which is characterised by the up-regulation of osteocyte markers sclerostin, E11 and FGF23. This thesis has further identified the inhibitory effect of the osteocyte specific marker FGF23 on vascular
calcification. In addition, the role of BMP9, a highly osteogenic bone morphogenic protein, in vascular calcification has also been reported.

It is now recognised that elevated serum Pi is a major risk factor for medial vascular calcification in CKD patients (Alfrey and Ibels, 1978; Block et al., 1998). High Pi at the concentrations similar to human hyperphosphatemic CKD patients triggers matrix calcification and osteo/chondrogenic differentiation of VSMCs in vitro (Steitz et al., 2001; Shanahan et al., 2011). I have used this in vitro VSMC matrix calcification model throughout this thesis. Under control conditions, VSMCs express a number of endogenous inhibitors of matrix calcification. Loss of these inhibitors results in vascular calcification. One of these inhibitors is NPP1, which is an enzyme that generates PPI through hydrolysing ATP (Lohman et al., 2012). The multiple-pass transmembrane protein ANK transports PPI to extracellular matrix, where it directly inhibits HA crystal formation (Costello et al., 2011). Loss of NPP1 leads to extensive medial vascular calcification in Enpp1−/− mice and children with GACI (Rutsch et al., 2001; Johnson et al., 2005). The studies presented in Chapter 3 confirm that the Enpp1−/− mouse is an appropriate animal model of medial vascular calcification and provide robust justification for its use in subsequent experiments. The studies in Chapter 3 show that the Enpp1−/− mouse develops ectopic medial vascular calcification. In addition, Enpp1−/− VSMCs exhibit significantly increased calcification and up-regulated expression of osteogenic marker Akp2. These data are in agreement with previous studies (Johnson et al., 2005; Narisawa et al., 2007), suggesting that VSMCs in calcifying arteries from Enpp1−/− mice give rise to osteogenic-like cells, which is comparable to the Mgp−/− mouse model (Speer et al., 2009) and uremic rat model (Lomashvili et al., 2008) of vascular calcification. Interestingly, the expression of calcification inhibitor Mgp was significantly down-regulated in Enpp1−/− VSMCs compared to wild-type controls. The inhibitory effect of MGP on vascular calcification not only involves calcium chelation (Schinke and Karsenty, 2000) but also antagonises BMP signalling (Wallin et al., 2000; Yao et al., 2006). Previous studies have shown that BMP signalling is preferably up-regulated in calcified aortae from Ldlr−/− (Derwall et al., 2012) and ApoE−/− mice (Yao et al., 2010), as well as calcified human aortic valves (Ankeny et al., 2011). It is reasonable to
speculate that decreased expression of MGP in \( \textit{Enpp1}^{-/-} \) aortae leads to up-regulation of BMP signalling, which may contribute to medial vascular calcification that is observed in the \( \textit{Enpp1}^{-/-} \) mouse. Moreover, the data presented in Chapter 3 show the up-regulated expression of the osteocyte specific markers \( Fgf23 \) and \( E11 \) in \( \textit{Enpp1}^{-/-} \) VSMCs. This is consistent with previous studies that have demonstrated the appearance of osteocyte-like cells in human arteries with medial vascular calcification (Shanahan et al., 1999). In addition, the data presented here show that \( \textit{Enpp1}^{-/-} \) mice exhibit severe disruption to the architecture and mineralisation of long bones.

A number of studies have shown that VMSCs, the predominant cells involved in medial vascular calcification, can differentiate into osteoblastic and chondrocytic cells in a calcified environment (Steitz et al., 2001; Yang et al., 2004; Speer et al., 2005). Whether high Pi induced matrix calcification also drives the osteocytic differentiation of VSMCs has been investigated in Chapter 4. The data presented in this chapter further strengthen the association between matrix calcification and the osteocyte phenotype transition in VSMCs. The osteocyte markers \( Sost, E11, Dmp1, Phex, Mepe \) and \( Fgf23 \) were significantly up-regulated during the process of VSMC matrix calcification. Increased protein expression of sclerostin and E11 was also observed in calcified aortae from the \( \textit{Enpp1}^{-/-} \) mouse. These data have demonstrated that vascular calcification is associated with an osteocyte phenotype. In agreement with my observations, recent studies have demonstrated that sclerostin is significantly up-regulated in the aortic wall of CKD rats compared to controls (Kramann et al., 2013). Interestingly, clinical studies have also shown that higher sclerostin levels are associated with less aortic calcification in CKD patients (Claes et al., 2013). My studies are the first to show that matrix calcification up-regulates the expression of the osteocyte markers, however the precise role of these osteocyte markers in vascular calcification remains unclear and requires further investigation. Sclerostin is an attractive target for further study due to its previously identified function as a key inhibitor of Wnt signalling, which plays an important role in osteoblast differentiation (Semenov et al., 2005), and its expression in CMV-Msx2 transgenic mice, which show extensive cardiovascular calcification (Shao et al.,
2005). Interestingly, clinical studies have shown that high circulating sclerostin levels are associated with improved survival in haemodialysis patients, in which vascular calcification is frequently seen and contributes to increased mortality and morbidity in these patients (Viaene et al., 2013). Additionally, sclerostin directly interacts with Cyr61, potentiating Cyr61-mediated cell growth and vascular migration and altering Cyr61-mediated cellular adhesion (Craig et al., 2010).

The Wnt family are a group of 19 secreted glycoproteins that act as important extracellular signalling ligands (Mikels and Nusse, 2006). The Wnt signalling pathway is highly conserved in eukaryotes and plays a crucial role in embryogenesis, postnatal development and adult tissue homeostasis (Nusse, 2005). The canonical Wnt signalling pathway involves the multifunctional protein β-catenin. Wnt ligands bind to the Frizzled–LRP5/6-receptor complex, preventing the degradation of β-catenin. The accumulated β-catenin subsequently translocates from the cytoplasm to the nucleus, cooperates with the T-cell factor (TCF) or lymphoid enhancer-binding factor (LEF) transcriptional factors and activates Wnt target genes (Baron and Rawadi, 2007; Gessert and Kuhl, 2010; Kim et al., 2013). A number of studies have demonstrated the important role of canonical Wnt/β-catenin signalling in vascular and cardiac valve calcification. Increased expression of the Wnt coreceptor LRP5, the ligand Wnt3, as well as β-catenin has been observed in calcified valves (Caira et al., 2006). Transgenic overexpression of Msx2 in aortic valves and vascular tissue increases arterial calcium deposition, and up-regulates the expression of Wnt ligands Wnt3a and Wnt7a (Shao et al., 2005). Concomitant up-regulation of Wnt/β-catenin signalling has also been observed in calcifying aortic valves as well as in the arterial tunica media of these mice (Shao et al., 2005). In agreement with these findings, β-catenin has also been shown to synergise with TGF-β to induce myofibroblastic conversion of VICs (Chen et al., 2011b).

Sclerostin directly binds to LRP5/6 to antagonise the canonical Wnt/β-catenin signalling pathway (Li et al., 2005; Holdsworth et al., 2012). Therefore, the inhibitory effect of sclerostin on vascular calcification may act through inhibition of Wnt/β-catenin signalling. The data presented in Chapter 4 show significantly up-
regulated expression of sclerostin in calcified VSMCs, as well as calcified aortae from the \textit{Enpp1}^{−/+} mouse. These data are consistent with previous studies (Shao et al., 2005; Roman-Garcia et al., 2010). Taken together, the up-regulation of sclerostin in calcifying vascular tissue most probably acts as a cell defensive response that blocks Wnt/\(\beta\)-catenin signalling in order to retard further calcification in vascular tissue. Furthermore, vasculature-derived sclerostin may be secreted into blood and affect bone. Indeed, clinical studies have shown that serum sclerostin levels are positively associated with abdominal aortic calcification in post-menopausal women (Hampson et al., 2013). Therefore, sclerostin may act as a key negative regulator of calcification both in bone and in vasculature, and may represent an important effector in the bone-vascular axis.

Having established the up-regulated expression of osteocyte markers during the process of VSMC matrix calcification, my studies in Chapter 5 further identified the direct inhibitory role of the osteocyte specific marker FGF23 in vascular calcification. Although a number of clinical and basic studies have demonstrated the association between FGF23 and vascular calcification (El-Abbadi et al., 2009; Srivaths et al., 2011; Lim et al., 2012; Mackenzie et al., 2012a), it remains unclear as to whether it directly modulates the calcification and osteogenic differentiation of VSMCs. The novel data presented in Chapter 5 reports increased expression of FGF23 and its cofactor Klotho in calcified VSMCs, as well as in calcified aortae from the \textit{Enpp1}^{−/+} mouse. Interestingly, my colleague Dr. Neil Mackenzie has reported that FGF23 serum levels are significantly up-regulated in the \textit{Enpp1}^{−/+} mouse compared to wild-type controls. The increased circulating levels may be due to increased \textit{Fgf23} gene transcription in calvarial osteoblasts in the \textit{Enpp1}^{−/+} mouse. My data suggests that vasculature-derived FGF23 may also contribute to increased FGF23 circulating levels. It has not been determined whereby \textit{Fgf23} gene transcription in bone and vasculature is stimulated by \textit{Enpp1} inactivation in my studies. However, recent data has shown that inactivating mutations in \textit{Phex} and \textit{Dmp1} result in impaired matrix calcification and stimulates \textit{Fgf23} expression via FGF receptor activation (Martin et al., 2011). Further studies will be required to determine if the increase in FGF23 observed in \textit{Enpp1}^{−/+} bone and vasculature is
intrinsic and due to pathways similar to *Phex* and *Dmp1* mutations or as a result of distinct signalling pathways.

Interestingly, a recent study has shown that FGF23 has no effect on matrix calcification of human VSMCs (Scialla et al., 2013), whereas my data presented here has demonstrated that FGF23 directly inhibits matrix calcification and osteogenic phenotypic transition of mouse VSMCs. These differences may be due to different culture conditions. However, my data here are supported by previous studies in which overexpression of FGF23 directly suppressed osteoblast differentiation and matrix calcification *in vitro* (Wang et al., 2008), as well as studies performed by Lim et al. which demonstrated an inhibitory effect of FGF23 on vascular calcification (Lim et al., 2012). The data presented are also consistent with a recent study which describes FGF23 antibody treatment increasing vascular calcification in the uremic rat model of CKD (Shalhoub et al., 2012). Taken together, these data suggest that increased expression of FGF23 in the vasculature may act as a cell adaptive mechanism that directly prevents vascular tissue from further calcification. In addition, FGF23 may also indirectly inhibit vascular calcification due to its role in systemic Pi homeostasis. Therefore, it is reasonable to speculate that high Pi loading in CKD patients induces bone-derived and vasculature-derived FGF23 to be secreted into the blood, resulting in increased circulating FGF23 levels, which indirectly and directly inhibit vascular calcification. However, increased circulating FGF23 levels may also lead to adverse outcomes, including left ventricular hypertrophy (Mirza et al., 2009; Faul et al., 2011), hypomineralisation of long bones (Mackenzie et al., 2012a), and atherosclerosis (Ashikaga et al., 2010). FGF23 therefore represents a key effector in the kidney-bone-vascular axis.

My studies have also identified the important role of BMP9 in vascular calcification. The data presented in Chapter 6 have shown that BMP9 significantly promotes matrix calcification and osteogenic/osteocytic differentiation of VSMCs. This observation is consistent with previous studies that report BMP9 inducing the osteogenic differentiation and matrix calcification of mesenchymal stem cells (Luo et al., 2010), and muscle resident stromal cells (Leblanc et al., 2011). Additionally,
these data further support my findings in Chapters 3, 4 and 5 that vascular calcification is associated with an osteocyte phenotype.

Furthermore, my data have demonstrated that BMP9-induced vascular calcification is TNAP dependent and mediated via the ALK1-Smad1/5/8-Smad4 signalling pathway. It has been reported that TNAP is a key regulator of vascular calcification, which hydrolyses PPI into inorganic Pi (Narisawa et al., 2007). TNAP specific inhibition has been shown to significantly prevent vascular calcification as described previously in Chapter 6 and by others (Narisawa et al., 2007). Clinical studies have also shown that serum TNAP levels are associated with vascular calcification in CKD patients (Shantouf et al., 2009). Therefore, TNAP emerges as a potential therapeutic target for vascular calcification. However, it should be taken into consideration that inhibition of TNAP activity may also lead to adverse outcomes for physiological bone mineralisation. Blood vessel specific targeted strategies may therefore be required.

Inactivating mutations in ALK1 leads to HHT in humans, an autosomal dominant genetically inheritable vascular dysplasia (Johnson et al., 1996; Dupuis-Girod et al., 2010). These mutations result in a functional haploinsufficiency model affecting BMP9 signalling (Ricard et al., 2010). Ablation of ALK1 in mouse embryos results in death at midgestation, with severe vascular abnormalities characterised by excessive fusion of capillary plexes into cavernous vessels and hyperdilation of large vessels (Oh et al., 2000). These data have demonstrated the crucial role of ALK1 in vascular function. My data here have demonstrated for the first time that BMP9 induces TNAP activity and matrix calcification in VSMCs via the ALK1 receptor. This induction can be prevented by a specific ALK1 receptor inhibitor, ALK1-Fc. This observation is supported by previous observations reporting that ALK1 is required for BMP9-induced osteogenic differentiation and matrix calcification in mesenchymal stem cells (Luo et al., 2010). Recently an interesting study has demonstrated that LDN-193189, a small molecule inhibitor of BMP type I receptor kinases, effectively attenuated vascular inflammation, atherosclerosis, and vascular...
calcification in \textit{Ldlr}^{-/-} mice (Derwall et al., 2012). Therefore, ALK1 may represent a novel therapeutic target for vascular calcification.

Previous studies have shown that smooth muscle-targeted overexpression of BMP2 accelerates vascular calcification in ApoE^{-/-} mice, but not atherosclerosis (Nakagawa et al., 2010). Conversely, inhibition of BMP signalling using MGP overexpression in ApoE^{-/-} mice inhibits vascular calcification, as well as antecedent vascular inflammation and atherosclerosis (Yao et al., 2010). My data here confirm the crucial role of BMP signalling in regulating vascular calcification and identify BMP9, a novel BMP, as a key promoter of vascular calcification. My data here show BMP9 significantly stimulates the phosphorylation of p-Smad1/5/8. \textit{Smad4} siRNA significantly prevented BMP9-induced TNAP activity and matrix calcification. These data suggest that BMP9 induces vascular calcification through canonical BMP signalling and further strengthen the concept that BMPs regulate vascular calcification. Interestingly, it has been reported that Smad1/5/8 is preferably activated in calcified human aortic valves (Ankeny et al., 2011), as well as in the calcified aortae of \textit{Ldlr}^{-/-} mice (Derwall et al., 2012). BMP9 is present in a biologically active form and stimulates phosphorylation of Smad1/5/8 via ALK1 (David et al., 2007; Bidart et al., 2012). Whether BMP9 contributes to the activation of Smad1/5/8 observed in calcified valves and aortae requires further investigation.

The most novel finding in Chapter 6 is that BMP9 serum concentration is significantly up-regulated in dialysis CKD patients compared to predialysis CKD patients. Therefore, BMP9 may contribute to dialysis-induced vascular calcification and osteogenic differentiation of blood vessels as described previously (Shroff et al., 2008).

In conclusion, the work described in this thesis has demonstrated that vascular calcification is associated with an osteocyte phenotype, and reports a direct inhibitory effect of the osteocyte specific gene FGF23 on vascular calcification. Furthermore, this thesis has shown that BMP9 induces the expression of the osteocytic marker \textit{Sost} in VSMCs, and appears to play a critical role in vascular
calcification. A proposed model for the role of osteocyte markers in vascular calcification has been shown in Figure 7.1.

Figure 7.1 A proposed model for the role of osteocyte markers in vascular calcification. Vascular calcification is commonly observed in Chronic Kidney Disease. Increased serum calcium and phosphate levels in CKD patients result in calcification and osteo/chondrogenic differentiation of vascular smooth muscle cells in the vascular wall. In the later phase of vascular calcification, the upregulation of osteocyte markers sclerostin and FGF23 is observed, as shown in chapter 4. The upregulation of sclerostin and FGF23 may act as a defensive response in order to reduce the mineralization in the vascular tissue. Sclerostin and FGF23 may spill over to the circulation and may reciprocally inhibit bone metabolism. Chapter 6 has shown that BMP9 directly promotes calcification and osteogenic conversion of VSMCs and may be involved in dialysis-induced vascular calcification.
7.2 Directions for future research

The results presented in this thesis have increased our knowledge of the association of osteocyte markers with vascular calcification. They have revealed that vascular calcification is accompanied by the up-regulation of osteocyte markers, such as E11, sclerostin and FGF23. This thesis has also demonstrated the direct inhibitory role of the osteocyte marker FGF23 in vascular calcification. In addition, they have shown that BMP9 promotes vascular calcification and the osteocytic differentiation of VSMCs. However, further studies are required to verify the phenotypic transition of VSMCs to osteocytes in the vascular calcification process using additional pathological animal models of vascular calcification, such as the ApoE/− mouse and CKD rat models. Further work is also required to fully elucidate the mechanism by which matrix calcification drives the osteocytic differentiation of VSMCs and the precise role of other osteocyte markers, such as sclerostin, in vascular calcification.

Sclerostin is the most attractive target for further studies. It would be interesting to investigate whether sclerostin directly inhibits the osteoblast/osteocytic transition, as well as matrix calcification of VSMCs. Studies employing the in vitro VSMC matrix calcification model would allow examination of this. Canonical Wnt signalling is a potent regulator of vascular calcification and it has been suggested that sclerostin is a key inhibitor of Wnt signalling. It would be certainly interesting to clarify whether sclerostin inhibits vascular calcification via antagonising the Wnt signalling pathway. In addition, the detailed functional studies of aortae and VSMCs derived from Sost knockout and overexpressing transgenic mouse models would be of great benefit in furthering our understanding of the role of sclerostin in vascular calcification in vivo. Both of these transgenic models have been generated but were unattainable in this PhD. The Sost/− mouse shows increased bone formation and bone strength (Li et al., 2008a), however it is not known whether this mouse model develops vascular calcification. In addition, whether Sost/− aortae or VSMCs show increased calcification has yet to be examined. The vascular phenotype of transgenic mice overexpressing Sost, which show decreased bone formation (Winkler et al., 2003), has also yet to be studied. Treatment of animal models of vascular calcification with sclerostin recombinant protein would also be useful to examine the role of sclerostin
in vascular calcification. However, due to the known role of sclerostin in regulating osteoblast mineralisation, overexpressing sclerostin under the control of an SMC-specific promoter in animal models of vascular calcification would be the logic way forward. Clinical trials have been initiated to use sclerostin antibody to treat patients with postmenopausal osteoporosis, and the potential adverse effects of increased vascular calcification should therefore be considered.

In this thesis, a direct inhibitory effect of FGF23 on vascular calcification was reported. Further studies are required to investigate whether FGF23 recombinant protein treatment reduces vascular calcification in vivo in animal models of vascular calcification, such as uremic rats and the Enpp1−/− mouse. However, FGF23 recombinant protein should be cleavage resistant. In addition, due to the known role of FGF23 in regulating Pi homeostasis, VSMC specific over-expression of FGF23 in animal models of vascular calcification (the Enpp1−/− mouse) would advance our understanding of the role of FGF23 in vascular calcification. Further studies are also required to investigate the mechanisms whereby Enpp1 inactivation results in increased expression of Fgf23 in bone and vasculature.

A number of non-invasive imaging techniques have been established during the past two decades to detect and quantify vascular calcification, such as micro-computed tomography (Neven et al., 2010; Awan et al., 2011) and macroscopic fluorescence reflectance imaging (Aikawa et al., 2009). It would be therefore useful to employ these novel techniques to monitor calcification progression and assess the effect of sclerostin and FGF23 treatment on vascular calcification in animal models in vivo.

This thesis has also demonstrated that BMP9 promotes vascular calcification and that BMP9 serum concentration is up-regulated in dialysis CKD patients compared to predialysis CKD patients. Larger cohorts of patients are required to confirm these observations. Further studies are also required to compare the phosphorylation status of Smad1/5/8 in vessels from these patients. It would also be interesting to examine whether inhibition of BMP9 signalling using a BMP9 neutralising antibody or
ALK1-Fc recombinant protein would reduce vascular calcification in animal models of vascular calcification *in vivo*.

Lastly, the combination of *in vitro*, *ex vivo* and *in vivo* experimental approaches has largely increased our understanding the mechanisms underlying vascular calcification. As described in Chapter 3, a number of rodent models of vascular calcification have been developed. However, one should be cautious when translating data directly from rodents to humans, due to the markedly different cardiovascular characteristics between these species. Therefore, large animal models of vascular calcification which more closely simulate human cardiovascular physiology, anatomy and function are essential to develop the discoveries from rodent models into clinical therapies and interventions for vascular calcification. For example, the pig is an excellent model of vascular biology due to the shared similarities with humans in heart physiology, coronary vasculature, and blood flow. Novel studies have recently described the generation of gene knockout pigs using transcription activator-like effector nucleases (TALENs) (Carlson et al., 2012). Therefore, the generation of porcine models of vascular calcification, such as the *Enpp1* or *Ldlr* pig would confirm my findings in rodents in this thesis and enable the testing of potential therapeutics such as FGF23 and sclerostin recombinant proteins.
Reference List


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is increased by a low protein diet and prevented by treatment with ibandronate. *Kidney Int* 70:1577-83.


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Appendix 1

**Cell culture buffers**

Hanks buffered saline solution (HBSS)
1.26mM CaCl$_2$, 0.493mM MgCl$_2$, 0.407mM MgSO$_4$, 5.33mM KCl, 0.441mM KH$_2$PO$_4$, 4.17mM NaHCO$_3$, 137.93mM NaCl, 0.338mM Na$_2$HPO$_4$, 5.56mM D-Glucose

Phosphate buffered saline (PBS)
140mM NaCl, 2.5mM KCl, 10mM Na$_2$HPO$_4$, 1.8mM KH$_2$PO$_4$

Radio-immunoprecipitation assay (RIPA) buffer
150mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0

**Bacterial culture**

Lysogeny broth (LB) media
1% bacto-tryptone, 0.5% bacto-yeast extract, 150mM NaCl, adjusted to pH 7.5

LB agar
LB supplemented with 1.5% bactoagar

Super optimal broth with catabolite repression (SOC) media
2% bacto-tryptone, 0.5% bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl$_2$, 10mM MgSO$_4$, 20mM glucose

**Gel electrophoresis**

Tris-acetetic acid-EDTA (TAE)
40mM Tris, 1mM EDTA, 0.1% acetic acid

**PureLink HiPure Plasmid Filter Maxiprep Kit buffer compositions**

Resuspension Buffer (R3)
50mM Tris-HCl, pH 8.0, 10mM EDTA

RNase A
20mg/mL in Resuspension Buffer (R3)

Lysis Buffer (L7)
Appendix 1

Buffer recipes

0.2M NaOH 1% (w/v) SDS

Precipitation Buffer (N3)

3.1M Potassium acetate, pH 5.5

Equilibration Buffer (EQ1)

0.1M Sodium acetate, pH 5.0, 0.6M NaCl, 0.15% (v/v) Triton® X-100

Wash Buffer (W8)

0.1M Sodium acetate, pH 5.0, 825mM NaCl

Elution Buffer (E4)

100mM Tris-HCl, pH 8.5, 1.25M NaCl

TE Buffer (TE)

10mM Tris-HCl, pH 8.0, 0.1mM EDTA

SDS-PAGE gel running buffer

MOPS running buffer

50 mM MOPS pH 7.7, 50 mM Tris, 0.1% SDS, 1mM EDTA

MES running buffer

50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3

Transfer buffer

25 mM Tris, 192 mM glycine, 10% ethanol 0.1% SDS

LDS sample buffer

10% glycerol, 141 mM Tris base, 106 mM Tris HCl, 2% LDS, 0.51 mM EDTA, 0.22 mM SERVA® Blue G250, 0.175 mM phenol red, pH 8.5

Sample reducing agent

500mM dithiothreitol

Western blotting

Tris-buffered saline with Tween 20 (TBST)

10mM Tris HCl pH8.0, 150mM NaCl, 0.1% Tween-20

Blocking solution

5% (w/v) bovine serum albumin (BSA) in TBST

Immunohistochemistry
Appendix 1

Solutions and Reagents

Wash buffer

1xPBS

Antibody Diluent

1xPBS/5% normal goat/rabbit serum

Antigen Unmasking

Citrate: 10mM Sodium Citrate Buffer: To prepare 1L add 2.94g sodium citrate trisodium salt dihydrate (C_6H_5Na_3O_7•2H_2O) to 1L dH_2O. Adjust pH to 6.0.

Trypsin: 1mg/ml in 1xPBS

0.3% Hydrogen Peroxide

To prepare, add 1 ml 30% H_2O_2 to 99 ml dH_2O

DAB Reagent

100mg DAB, 100ml 1XPBS, 100μl H_2O_2

BMP9 ELISA buffers

Capture Antibody

360μg/mL of mouse anti-human BMP9

Detection Antibody

72μg/mL of biotinylated goat anti-human BMP9

Wash Buffer

0.05% Tween 20 in PBS, pH 7.2 - 7.4

Reagent Diluent

1% BSA in PBS, pH 7.2 - 7.4, 0.2μm filtered

Substrate Solution

1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine)

Stop Solution

2 N H_2SO_4
## Appendix 2

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