REVIEW

Regulation of mineralisation in bone and vascular tissue: a comparative review

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Abstract

Biom mineralisation, the deposition of mineral onto a matrix, can be both a physiological and pathological process. Bone formation involves the secretion of an extracellular matrix (ECM) by osteoblasts and subsequent mineralisation of that matrix. It is regulated by a number of local and systemic factors and is necessary for maintenance of normal bone health. Conversely, mineralisation (or calcification) of soft tissues, including the vasculature, is detrimental to that tissue, leading to diseases such as arterial medial calcification (AMC). The mechanisms underlying AMC development are not fully defined, though it is thought that vascular smooth muscle cells (VSMCs) drive this complex, cell-mediated process. Similarly, AMC is regulated by a variety of enzymes and molecules, many of which have already been implicated in the regulation of bone mineralisation. This review will provide an overview of the similar, and sometimes opposing effects of these signalling molecules on the regulation of bone mineralisation and AMC.

Key Words

- ▶ bone formation
- ▶ vascular calcification
- ▶ osteoblast
- ▶ vascular smooth muscle cells

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Introduction

Biom mineralisation is considered to be the deposition of mineral in an organised manner onto a matrix (Boskey 1998). In living tissues this can be either a physiological or a pathological process. The skeleton is characterised by its mineralised composition, a property that is vital for its structure and function. Conversely, the deposition of mineral in soft tissues is often pathological and usually detrimental to the function of that tissue. The mineralisation (or calcification) of the vasculature is particularly damaging as it is associated with an increased risk of adverse cardiovascular events (Hutcheson *et al.* 2017). At present there are no effective therapeutic options that can prevent or regress vascular calcification.

In journal articles, the terms ‘calcification’ and ‘mineralisation’ are used interchangeably. Nonetheless, mineralisation is most often used in the context of bone and refers to the process of laying down minerals within an extracellular matrix (ECM). Calcification, by definition,

refers to the accumulation of calcium salts in a tissue and this is a commonly used term to describe the pathological processes occurring in soft tissues (Orriss 2020). This review compares the processes of physiological bone formation and pathological vascular calcification, and provides an overview of how these can be regulated by local and systemic factors.

Bone formation

Bone is a composite tissue comprised of both organic and mineral components, specialised cells and water. As a result, bone formation is a two-step process and begins with synthesis and deposition of the ECM (also known as osteoid) followed by mineralisation. The ECM is formed primarily of type I collagen (~90%) along with non-collagenous proteins, growth factors

and glycoproteins. The mineral component of bone is hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH}_2)$) which is formed from inorganic phosphate (PO_4^{3-} or P_i) and calcium (Ca^{2+}) ions. Osteoblasts, which are derived from mesenchymal stromal cells (MSCs), are responsible for bone formation. Due to the large number of ECM proteins they synthesise and export, osteoblasts contain a large golgi apparatus, secretory vesicles and abundant rough endoplasmic reticulum and mitochondria (Dudley & Spiro 1961).

Bone mineralisation: how does it occur?

Bone mineralisation is essential for maintaining the stiffness and rigidity of bone but despite decades of research the exact mechanisms by which mineralisation occurs remains an area of debate. Currently, there are two main theories, matrix vesicle-mediated and collagen template, though other processes may also contribute to mineralisation. Matrix vesicles (MVs) are a small (100–200 nm), specialised form of extracellular vesicle that are secreted into the osteoid by osteoblasts to facilitate mineralisation (Anderson 2003). The concept of MVs was first proposed in the late 1960s following independent work by Anderson and Bonucci which discovered that nanoparticles were involved in mineralisation (Bonucci 1967). The contents of MVs include Ca^{2+} and P_i ions, enzymes, lipids and miRNAs (Anderson 1969, Cui *et al.* 2016). The accumulation of Ca^{2+} and P_i within the MV, via the actions of phosphatases and transporters, is thought to increase the concentration of these ions enough to begin the initial phase of mineralisation. To begin with, these ions combine to produce an octacalcium-phosphate intermediate prior to the formation of hydroxyapatite (Anderson 1969). The continuing growth of the hydroxyapatite crystals within the MV will eventually lead to membrane rupture and release of the crystals into the extracellular environment. Subsequent growth of the hydroxyapatite crystals is then dependent on the extracellular concentrations of Ca^{2+} and P_i and on the levels of local mineralisation inhibitors (Anderson 1969, Cui *et al.* 2016).

The theory of collagen template-mediated mineralisation was first suggested in the early 1990s (Landis *et al.* 1993). This idea proposes that the 40 nm hole regions within the collagen fibrils allow Ca^{2+} and P_i to accumulate, precipitate and form hydroxyapatite crystals (Landis *et al.* 1993). The structure and assembly of the collagen fibres then dictates the subsequent crystal growth. Whilst there is evidence to support both

theories, it remains unclear which of these mechanisms is primarily responsible for the initiation and propagation of hydroxyapatite formation. However, the end result is the same, a mineralised bone matrix. Furthermore, it is likely that these two processes of mineralisation are not mutually exclusive. Whilst it is possible that they could both occur at the same time, no conclusive evidence appears to suggest that this is the case.

One complication is that it appears highly unlikely that hydroxyapatite released from MVs is able to navigate its way into the specific hole regions of the collagen fibril unaided, after bursting open the MV. As with many techniques that study processes at the nanoscale, artefactual evidence of hydroxyapatite mineral in MVs may be as result of specimen preparation for electron microscopy. Amorphous calcium phosphate (ACP) is thermodynamically unstable, its stabilisation is controlled by pH and is solution-mediated, which is likely to result in conversion to the more stable crystalline hydroxyapatite after chemical fixation (Boskey & Posner 1973). More recently it was proposed that MVs released from osteoblasts during mineralisation contain an ACP precursor that is transported to the hole region of the collagen fibril (Boonrungsiman *et al.* 2012). Nucleation sites within the gap region initiate crystallisation and allow crystal growth to spread along the fibril surface. The finding that ACPs are a precursor to the formation of mature hydroxyapatite has been supported by several studies (Mahamid *et al.* 2008, Dey *et al.* 2010, Nudelman *et al.* 2010, Mahamid *et al.* 2010). Lotsari *et al.* suggested that deposited ACP granules undergo a step growth which enables the ACP precursors to crystallise and form apatite platelets (Lotsari *et al.* 2018). However, it remains unclear how MVs spatially recognise where to transport their contents to ensure that hydroxyapatite formation occurs.

Other studies have eluded to the presence of mineral in the interfibrillar compartment and therefore outside of the collagen fibrils, suggesting alternative theories of bone structure and subsequently, mineralisation (McNally *et al.* 2012, 2013, Schwarcz *et al.* 2020). For example, processes including (1) mineral growth beyond the gap regions along the fibril, (2) growth between the fibrils and, (3) growth external to the fibrils have all been proposed. All of which would ultimately require different mechanisms (Landis *et al.* 1993, Lees *et al.* 1994, Probst & Lees 1996, Su *et al.* 2003, Nudelman *et al.* 2010, Silver & Landis 2011, McNally *et al.* 2012). Together, evidence from the literature presents a complex and currently incomplete understanding of the process of bone mineralisation.

Regulation of bone mineralisation

The process of bone mineralisation is tightly regulated by a variety of local and systemic factors. The coordinated action of these prevents hypo- or hypermineralisation, both of which are detrimental to skeletal function. Some of the key regulators of bone mineralisation are described subsequently (Fig. 1).

Pyrophosphate (PP_i)

The key role of PP_i as a local inhibitor of mineralisation was first discovered by the seminal work of Fleisch and colleagues in the 1960s (Fleisch & Bisaz 1962a, Fleisch & Bisaz 1962b). Extracellular PP_i potently inhibits the ability of Ca²⁺ to crystallise with P_i to form hydroxyapatite; it also binds strongly to the surface of hydroxyapatite and prevents further crystal growth (Fleisch & Bisaz 1962a, Fleisch & Bisaz 1962b, Fleisch *et al.* 1966). This initial work has helped to establish the idea that PP_i acts as the body's 'water softener' and acts locally to control mineralisation processes (Orriss 2020).

Ecto-nucleotides pyrophosphatases/ phosphodiesterases (NPPs)

The NPP family of enzymes primarily hydrolyse the phosphodiester bond in nucleotide triphosphates (e.g. ATP, UTP) to generate PP_i and the corresponding monophosphate. Of the seven members of the NPP family, it is NPP1 which has been shown to be particularly important in bone (Hessle *et al.* 2002, Mackenzie *et al.* 2012). NPP1 knockout mice (*Enpp1*^{-/-}) have reduced extracellular PP_i levels and display aberrant calcification of the spine, joints, tendons and other collagen-rich soft tissues (Hessle *et al.* 2002, Johnson *et al.* 2003, Mackenzie *et al.* 2012, Hajjawi *et al.* 2014). *Enpp1*^{-/-} animals also display reduced trabecular and cortical bone; the cause of this surprising observation is unclear but it could be a consequence of reduced mobility and/or defects in other tissues (Mackenzie *et al.* 2012, Hajjawi *et al.* 2014).

Tissue non-specific alkaline phosphatase (TNSALP)

TNSALP is the form of alkaline phosphatase that is found in the bone, liver and kidney. It is a broad spectrum ecto-nucleotidase that can hydrolyse numerous phosphate containing molecules including PP_i (Zimmermann *et al.* 2012). The ability of TNSALP to hydrolyse PP_i to generate P_i means that it plays a central role in driving bone mineralisation (Millan 2013). In keeping with this

function, TNSALP knockout mice have skeletal deformities, defective bone mineralisation and die before weaning of epileptic seizures (Waymire *et al.* 1995, Narisawa *et al.* 1997, Fedde *et al.* 1999). The antagonistic actions of TNSALP and NPP1 are now known to be critical in determining the local PP_i and P_i levels and, as a result, the levels of bone mineralisation (Johnson *et al.* 2000, Harmey *et al.* 2004).

PHOSPHO1

Another phosphatase critical for bone mineralisation is PHOSPHO1. This enzyme hydrolyses phosphoethanolamine and phosphocholine to release P_i and is thought to contribute to the initiation of mineralisation (Houston *et al.* 2004, Roberts *et al.* 2007). Consistent with this role, PHOSPHO1 knockout mice display reduced skeletal mineralisation, growth plate abnormalities and spinal deformities (Yadav *et al.* 2011, Dillon *et al.* 2019). Interestingly, deletion for both PHOSPHO1 and TNSALP results in mice with an entirely unmineralised skeleton, further highlighting the pivotal roles of both enzymes in regulating mineralisation (Yadav *et al.* 2011).

Non-collagenous proteins (NCPs)

NCPs account for approximately 10% of the ECM and the majority of them are synthesised by osteoblasts. Many of these proteins can regulate skeletal mineralisation to some extent but of particular importance are matrix gla protein (MGP), osteocalcin (OCN) and osteopontin (OPN). MGP and OCN belong to the gla-containing protein family, members of which require post-translational vitamin K-dependent γ -carboxylation for activation. MGP acts via gla residues, which have a high affinity for Ca²⁺, to bind hydroxyapatite and prevent crystal growth (Luo *et al.* 1997, O'Young *et al.* 2011). Accordingly, mice lacking MGP display widespread ectopic calcification, particularly in the aorta (Luo *et al.* 1997).

OCN is highly expressed by mature osteoblasts and is often used as a marker of differentiation. However, its exact role in bone turnover remains unclear. Studies of the first OCN knockout mice reported increased bone mass but no gross skeletal abnormalities (Ducy *et al.* 1996). More recently it has been suggested that the carboxylated form of OCN is required for binding to hydroxyapatite, whereas uncarboxylated OCN has an endocrine function, particularly for energy metabolism in bone (Oldknow *et al.* 2015). Two recent studies have questioned this role, demonstrating that the debate about the exact function of OCN remains ongoing (Diegel *et al.* 2020, Moriishi & Komori 2020).

Figure 1

Comparison of the processes regulating bone formation and AMC. In bone, NPP1 and TNSALP regulate extracellular P_i /PP $_i$ levels to allow for regulated mineralisation. Osteoblasts also secrete matrix vesicles containing enzymes, Ca^{2+} and P_i to facilitate the mineralisation of the deposited osteoid. The processes mediating the development of AMC are multifaceted and involve the loss of calcification inhibitors, gain of calcification inducers, increased apoptosis and VSMC transdifferentiation. Release of mineralisation competent extracellular vesicles may also promote calcification.

OPN, which belongs to the small integrin binding ligand, N-glycosylated (SIBLING) protein family, is also often used as a marker of osteoblast differentiation. OPN contains an acidic serine-rich motif (ASARM) that binds hydroxyapatite and prevents crystal growth (Addison *et al.* 2010). In keeping with this inhibitory role, OPN knockout mice display increased levels of bone mineral (Boskey *et al.* 2002).

Extracellular nucleotides

It is well established that extracellular nucleotides (e.g. ATP, UTP, ADP), signalling via P2 receptors, can modulate osteoblast differentiation, proliferation and function (Orriss 2015). In particular, ATP and UTP act locally to inhibit bone mineralisation and reduce TNSALP expression and activity, effects that are mediated by a number of P2 receptor subtypes (Orriss *et al.* 2007, 2012, 2013).

Vitamin D

In its active form vitamin D₃ (1,23-dihydroxyvitamin D₃), along with parathyroid hormone (PTH) and FGF23, plays a central role in the regulation of Ca^{2+} and P_i homeostasis. Acting via its receptor, vitamin D₃ directly promotes

osteoblast differentiation and regulates the expression of proteins including Runx2, OCN and OPN (Price & Baukol 1980, Prince & Butler 1987, Gardiner *et al.* 2000, Drissi *et al.* 2002). Vitamin D₃ deficiency is most commonly associated with impaired bone mineralisation leading to rickets in children and osteomalacia in adults.

Ion transporters

During the mineralisation process, the formation of hydroxyapatite from Ca^{2+} and P_i results in the production of H^+ ions in the ECM that require removal since small reductions in pH can inhibit the mineralisation process (Brandao-Burch *et al.* 2005). Chloride-proton antiporters such as CIC3 and CIC5 facilitate H^+ entry into osteoblasts to remove them from the extracellular environment. Deletion of both CIC3 and CLC5 results in a lack of mineralisation illustrating the importance of chloride-proton exchange in this process (Larrouture *et al.* 2015).

Vascular calcification

Vascular calcification is the pathological deposition of calcium phosphate, often as hydroxyapatite, in the

intimal and/or medial layer of the arteries and the heart valves. It is a common consequence of atherosclerosis, diabetes, chronic kidney disease (CKD) and ageing and is associated with an increased risk of having a future adverse cardiovascular event (e.g. heart attack, stroke) (Zhu *et al.* 2012). Arterial medial calcification (AMC) refers to the calcification that develops within the tunica media of blood vessels and is characterised by increased vessel stiffness and reduced blood flow. Traditionally, AMC was thought to be a passive process caused solely by high serum levels of P_i and Ca^{2+} . However, it is now accepted that the development of AMC is a complex, cell-mediated process that is predominantly driven by vascular smooth muscle cells (VSMCs) (Shanahan *et al.* 2011).

The development of AMC

The underlying processes which mediate the development of AMC are multifaceted and not fully characterised. Nevertheless, there have been significant advances in our understanding of these in the last few years. Under normal conditions, the actions of local and circulating inhibitors prevent pathological calcification from developing (Zhu *et al.* 2012, Orriss 2020). However, when the balance between these inhibitors and calcification inducers becomes dysregulated, as occurs in some diseases, mineral deposition can occur. This imbalance can also trigger VSMC transdifferentiation and increased apoptosis. Although a pathological event, the development of AMC is thought to share some similarities with physiological bone formation. As such, many of the enzymes and molecules that regulate bone mineralisation have also been associated with AMC (Fig. 1). These are discussed in more detail below:

Calcification inducers

Molecules that have been associated with inducing calcification include Ca^{2+} , P_i , reactive oxygen species (ROS), uremic toxins and glucose (Chen *et al.* 2006, Byon *et al.* 2008, Covic *et al.* 2010, Shanahan *et al.* 2011). Increased levels of one or more of these inducers can trigger phenotypic changes in VSMCs as well as promote apoptosis. Due to their central role in AMC, *in vitro* studies typically use increased P_i and/or Ca^{2+} levels to model the changes which occur in VSMCs. Mineralisation competent extracellular vesicles, DNA

damage and premature VSMC senescence have also been shown to promote calcification (Reynolds *et al.* 2004, Liu *et al.* 2013).

The central role of TNSALP in producing P_i and driving bone mineralisation means this enzyme has been an area of interest in the context of AMC. Studies have shown that TNSALP expression increases in a calcifying environment and that enzyme inhibition reduces VSMC calcification (Shioi *et al.* 1995, Narisawa *et al.* 2007). Furthermore, overexpression of TNSALP *in vivo* leads to extensive AMC (Sheen *et al.* 2015). In contrast, other work has reported that enzyme inhibition has no effect on VSMC calcification (Shroff *et al.* 2010, Morony *et al.* 2012, Patel *et al.* 2019). Additionally, comparative studies have shown that, *in vitro*, TNSALP activity is up to 100 times lower in calcifying VSMCs than mineralising osteoblasts (Patel *et al.* 2018, 2019). Despite these conflicting data, *in vivo* studies have reported beneficial effects of TNSALP inhibitors on AMC; however, these also exerted deleterious actions on bone (Pinkerton *et al.* 2018, Opdebeeck *et al.* 2020). Thus, it is clear that targeting TNSALP to treat AMC is not without its complications. Inhibition of PHOSPHO1 also reduces VSMC calcification (Kiffer-Moreira *et al.* 2013). Nevertheless, given the fundamental role of PHOSPHO1 in bone development inhibiting this enzyme therapeutically may also cause adverse skeletal effects.

Calcification inhibitors

Numerous proteins and small molecules, many of which also inhibit bone mineralisation, have also been shown to block AMC; these include PP_i , ATP, MGP, sclerostin, fetuin A and osteoprotegerin (OPG) (Luo *et al.* 1997, Min *et al.* 2000, Ketteler *et al.* 2002, Patel *et al.* 2018, De Mare *et al.* 2019, Orriss 2020). Global knockout mouse models for MGP and OPG develop extensive AMC, highlighting the importance of these proteins in preventing pathological calcification (Luo *et al.* 1997, Bucay *et al.* 1998). Generalised calcification of infancy (GACI) is a disease characterised by widespread medial calcification. GACI is primarily caused by mutations in the *Enpp1* gene which results in a loss of NPP1 and, consequently, less extracellular PP_i (Rutsch *et al.* 2001). Consistent with the human condition, *Enpp1*^{-/-} mice display aortic calcification and VSMCs derived from these animals show a greater ability to calcify (Johnson *et al.* 2005).

VSMC transdifferentiation: how similar are they to osteoblasts?

In the early 1990s, several groups demonstrated the expression of osteoblast genes (e.g. OPN, BMP2) in calcified deposits and atherosclerotic plaques (Bostrom *et al.* 1993, Giachelli *et al.* 1993). Later, bone-associated proteins were also detected in the calcified arteries of patients with end stage renal disease (Moe *et al.* 2002, Moe *et al.* 2003). There are now numerous studies reporting that, in a calcifying environment, VSMCs undergo a transdifferentiation to become more osteoblast-like. Specifically, this is associated with a loss of typical VSMC markers (e.g. SM22 α , α SMA, CALD1) and a gain of osteoblast markers (e.g. Runx2, OPN, TNSALP) (Shioi *et al.* 1995, Shanahan *et al.* 1999, Jono *et al.* 2000, Steitz *et al.* 2001, Zhu *et al.* 2011). However, despite these changes the cells may not become as osteoblast-like as is widely thought. A recent *in vitro* study, which directly compared calcifying VSMCs and osteoblasts, showed that whilst calcifying VSMCs express typical osteoblast genes, the levels were significantly lower than those seen in the mature osteoblast (Patel *et al.* 2019). Other work has also reported that calcifying VSMCs have a transcriptional profile that is only partially similar to osteoblasts and that calcification can occur without changes to VSMC marker gene expression (Alves *et al.* 2014). Interestingly, VSMCs cultured on hydroxyapatite without calcifying conditions also display increased expression of Runx2 and TNSALP (Nahar-Gohad *et al.* 2015), demonstrating that the matrix on which a cell is grown can also influence phenotype.

In vitro VSMC calcification also differs significantly in morphological appearance to *in vitro* bone formation (Fig. 2). Osteoblasts are able to secrete large amounts of collagenous matrix which is then mineralised to form large 'trabecular-shaped' bone nodules. In contrast, calcifying VSMCs tend to form small discrete regions of calcification that do not appear to be associated with deposited ECM and instead are often associated with cellular debris (Patel *et al.* 2019). However, it is also worth noting that in both osteoblast and calcifying VSMC cultures, the use of excessive phosphate levels (≥ 5 mM) results in widespread, non-specific mineral deposition that is not a cell-mediated process (Hortells *et al.* 2015, Patel *et al.* 2019).

The role of cell death and senescence in bone formation and AMC

Physiological bone formation is not associated with significant cell death; instead it is thought that apoptosis only occurs to terminate the bone-forming activity of osteoblasts. Approximately 60–90% of osteoblasts are estimated to die by apoptosis at the end of the bone remodelling phase (i.e. once bone formation is complete) with many of the remaining cells undergoing a terminal differentiation into an osteocyte (Jilka *et al.* 2007). Ageing is associated with decreased osteoblast proliferation and bone formation and increased senescence, all of which are thought to contribute to age-associated bone loss (Roholl *et al.* 1994, Jilka *et al.* 1996, Yudoh *et al.* 2001).

Figure 2

In vitro bone formation and VSMC calcification have different morphological appearances. Phase contrast light microscopy images showing (A) matrix deposition in differentiating osteoblasts and (B) discrete mineralised nodule formation in mature, bone forming osteoblasts. (C) In calcifying VSMCs, regions of calcification are smaller and not obviously associated with deposited matrix. Transmission electron microscopy images show (D) collagen deposition and (E) mineralised matrix in osteoblast cultures. (F) No matrix deposition is apparent in calcifying VSMCs. Scale bars: A–C = 200 μ m, D–E = 1 μ m, F = 2 μ m.

A number of studies have linked the initiation of AMC with increased VSMC apoptosis (Proudfoot *et al.* 2000, Clarke *et al.* 2008, Ciceri *et al.* 2016). Whilst the mechanisms are not fully characterised, it is thought that a calcifying environment can induce apoptosis and that the VSMC-derived apoptotic bodies act as a nucleation site for calcification (Proudfoot *et al.* 2000). In agreement, pharmacological inhibition of apoptosis has been shown to decrease VSMC calcification (Ciceri *et al.* 2016, Patel *et al.* 2018). AMC is also associated with accumulation of prelamin A, premature senescence and the senescence associated secretory phenotype (SASP) (Liu *et al.* 2013, Sanchis *et al.* 2019). *In vitro*, senescent VSMCs show an increased capacity to calcify, apparently as a result of increased expression of osteoblast marker genes (e.g. Runx2, TNSALP) (Nakano-Kurimoto *et al.* 2009). However, this seems counter-intuitive given that senescence is associated with decreased TNSALP activity and collagen production in osteoblasts (Kassem *et al.* 1997). Taken together these findings show that whilst senescence is detrimental to both osteoblasts and VSMCs, the overall end effect of this process differs. Ultimately, it leads to reduced bone formation in the skeleton but increased calcification in the arteries.

The effect of ageing on bone formation and AMC

An intriguing observation is that AMC and dysregulated bone homeostasis, leading to conditions such as osteoporosis, are diseases of old age. The association between opposing mineralisation processes in ageing was first identified in patients with reduced spinal bone mineral density (BMD) who also presented with AMC (Banks *et al.* 1994). This phenomenon is also prominent in patients with accelerated ageing diseases such as Werner's syndrome and Hutchinson–Gilford progeria syndrome (HGPS); sufferers of these conditions display increased AMC but reduced BMD (Salamat *et al.* 2010, Gordon *et al.* 2011, Pescatore *et al.* 2019).

Age-related changes are observed in both osteoblasts and VSMCs but to differing effects. In older cells, the capacity to differentiate from MSCs into osteoblasts is reduced and instead the adipogenic lineage is favoured. Ageing is also associated with reduced cell adhesion, bone mineralisation and Wnt expression (Rauner *et al.* 2008, Coipeau *et al.* 2009, Lai *et al.* 2016, Becerikli *et al.* 2017). In contrast, VSMC ageing is associated with the reverse. Together with switching from a contractile to a synthetic

phenotype, increased migration and proliferation, aged VSMCs also demonstrate increased calcification and ECM changes (Bochaton-Piallat *et al.* 1993, Jiang *et al.* 2012, Monk & George 2015). Increased markers of osteogenic differentiation and calcification have also been associated with premature ageing of VSMCs, particularly in children on dialysis or in cells which accumulate prelamin A (Liu *et al.* 2013, Sanchis *et al.* 2019). *In vivo*, rat models of AMC also display more severe calcification and greater changes in VSMC phenotype in aged animals compared to young (Chen *et al.* 2020).

Bone formation and AMC can be differentially regulated by local and systemic signalling molecules

The development and maintenance of the skeleton and cardiovascular system are regulated by a vast array of hormones, peptides and local signalling molecules. These can act in an endocrine, paracrine or autocrine manner to directly regulate cell differentiation, function and/or survival. Many of these have been studied for their effects on bone formation and/or AMC *in vitro* and *in vivo*. Importantly, there are now numerous publications describing opposing actions of local and systemic signalling factors on osteoblasts and calcifying VSMCs. Responses of these two cell types to cell and environmental stresses also appear to be different. Some of the signalling systems where differential effects have been reported are discussed below (Fig. 3).

ROS and antioxidants

The effects of ROS and antioxidants on the bone and the vasculature are well studied. ROS include reactive molecules (e.g. hydrogen peroxide (H₂O₂)) and free radicals (e.g. superoxide (O₂[•]), hydroxyl (OH[•])) that oxidise other molecules in order to stabilise themselves. ROS are primarily involved in DNA, mitochondrial and cell damage although these oxidising agents also have beneficial actions such as intracellular signalling and neurotransmitter degradation (Zuo *et al.* 2015). Antioxidants inhibit the oxidation of these molecules and generally exert beneficial effects on cells. Oxidative stress arises when the levels of ROS exceed those of the endogenous antioxidants, overwhelming the cells' resistance to oxidative damage.

In calcifying VSMCs, different ROS species have been shown to induce VSMC calcification and the expression of

↑ bone formation and ↓ AMC	↓ bone formation and ↑ AMC	Same effect
<ul style="list-style-type: none"> Antioxidants IGF-1 Ghrelin PGE₂ FGF2 Nitric oxide Hydrogen sulphide 	<ul style="list-style-type: none"> ROS High glucose 	<i>Inhibitory:</i> <ul style="list-style-type: none"> MGP PP_i ATP / UTP Sclerostin <i>Stimulatory:</i> <ul style="list-style-type: none"> BMP-2 Fibronectin
↑ bone formation, effects on AMC unclear	↓ bone formation, effects on AMC unclear	
<ul style="list-style-type: none"> PTH (intermittent) Estrogen / Testosterone 	<ul style="list-style-type: none"> FGF-23 	

Figure 3

Local and systemic molecules regulating bone formation and AMC. A number of molecules can exert differential effects on bone formation and AMC *in vitro* and/or *in vivo*. There are also a number of compounds which exert the same functional effect, be it inhibitory or stimulatory.

osteoblast-associated genes and decrease levels of VSMC-related markers (Mody *et al.* 2001, Byon *et al.* 2008, Liu *et al.* 2014, Sanchis *et al.* 2019). In contrast, there is good evidence that ROS inhibit osteoblast differentiation and bone formation (Mody *et al.* 2001, Fatokun *et al.* 2008, Kim *et al.* 2010). One of these studies directly compared VSMCs with MC3T3-E1 osteoblast-like cells and found clear differential effects of ROS on VSMC calcification and bone formation (Mody *et al.* 2001). Thus, much like senescence, the detrimental effects on cell function induced by increased ROS are different in VSMCs and osteoblasts.

In keeping with the beneficial cellular effects of antioxidants, the actions of these compounds on calcifying VSMCs and osteoblasts are also opposing. Numerous studies have reported these differential effects of antioxidants *in vitro* and *in vivo*. For example, resveratrol, quercetin, curcumin and allopurinol all reduce VSMC calcification, apoptosis and osteoblast marker gene expression (Hou *et al.* 2016, Zhang *et al.* 2016, Cui *et al.* 2017, Yan *et al.* 2019). In contrast, these same compounds can increase osteoblast differentiation, TNSALP activity and promote bone formation (Mizutani *et al.* 1998, Dai *et al.* 2007, Chen *et al.* 2016, Orriss *et al.* 2016, Pang *et al.* 2018, Son *et al.* 2018).

Interestingly, a recent paper reported that poly(ADP-ribose) (PAR) plays a key role in driving ECM mineralisation in bone and vascular tissue (Muller *et al.* 2019). Müller *et al.* report that PAR production usually occurs in response to DNA damage and oxidative stress and that this signalling pathway can promote osteogenic differentiation (Muller *et al.* 2019). Numerous studies show that DNA damage promotes the development of AMC (Liu *et al.* 2013, Sanchis *et al.* 2019, Bartoli-Leonard *et al.* 2020) but inhibits bone formation (Kim *et al.* 2017, da Luz Dias *et al.* 2018, Qiao *et al.* 2020, Zhou *et al.* 2020).

Thus the notion that the beneficial effects of PAR on bone mineralisation are as a result of DNA damage in osteoblasts appears contradictory. However, it is clear that PAR plays an important physiochemical role in driving mineralisation processes and further work is now required to fully reconcile these opposing findings.

PTH, FGF23 and the regulation of Ca²⁺ and P_i homeostasis

The skeleton acts as a major reservoir for the storage of both Ca²⁺ and P_i. Regulation of blood Ca²⁺ and P_i levels is a complex process and involves a number of tissues (bone, kidney, intestine) and hormones (PTH, vitamin D₃, FGF23). Dysregulation in this system can have a significant effect on both the bone and the vasculature. CKD, in particular, is associated with hyperphosphatemia and hypocalcaemia leading to the development of AMC and impaired bone turnover (Yamada & Giachelli 2017).

PTH is secreted by the parathyroid glands in response to low serum Ca²⁺ levels. It regulates bone remodelling directly via actions on osteoblasts and indirectly by actions in other tissues (such as increasing the formation of active vitamin D₃ in the kidney). The effects of PTH on osteoblasts are complex and depend on duration of exposure and the differentiation state of the cells (Isogai *et al.* 1996). Whilst continuous PTH treatment is catabolic, intermittent PTH treatment stimulates osteoblast differentiation and bone formation, and is used as a treatment for osteoporosis (Qin *et al.* 2004). PTH can also act on osteoblasts to directly regulate the expression of genes important in bone remodelling (e.g. RANKL, OPG) (Lee & Lorenzo 1999). The actions of PTH on VSMCs and its role in AMC are less well defined. Although hyperparathyroidism is associated with AMC and mice with PTH replacement develop aortic calcifications, addition of PTH to VSMCs

did not alter Ca^{2+} deposition (Lomashvili *et al.* 2006, Neves *et al.* 2007). Thus, it remains unclear whether the effects of PTH on AMC are a consequence of increased P_i levels or the result of direct actions on VSMCs.

FGF23 is released by osteocytes and osteoblasts in response to high P_i levels. It acts in association with its co-factor α -klotho to inhibit P_i reabsorption in the kidneys. FGF23 knockout mice display hyperphosphatemia, skeletal abnormalities and AMC (Sitara *et al.* 2004). Equally, mutations in α -klotho result in osteoporosis and widespread medial calcification (Kuro-o *et al.* 1997). Treatment of the osteoblast cell line, MC3T3-E1, with FGF23 and α -klotho has been shown to inhibit bone mineralisation (Shalhoub *et al.* 2011). In agreement, overexpression of FGF23 in osteoblasts can inhibit differentiation and matrix mineralisation (Wang *et al.* 2008). In contrast, the effects of FGF23 on VSMC calcification are undefined with studies reporting no effects or an increase in calcification (Lindberg *et al.* 2013, Jimbo *et al.* 2014). In haemodialysis patients, high FGF-23 levels have been associated with AMC (Turan *et al.* 2016). Taken together the studies to date suggest that, whilst not fully understood, the direct actions of PTH and FGF23 on osteoblasts and calcifying VSMCs may differ.

Other hormones and local signalling molecules

Several other signalling molecules and hormones have been shown, albeit in independent studies, to promote osteoblast differentiation and bone formation but inhibit AMC *in vitro* and/or *in vivo*. These include IGF-1 (Zhang *et al.* 2002, Radcliff *et al.* 2005), ghrelin (Li *et al.* 2005, Liang *et al.* 2012), PGE_2 (Machwate *et al.* 2001, Gao *et al.* 2016), FGF-2 (Canalis *et al.* 1988, Montero *et al.* 2000, Borland *et al.* 2017), nitric oxide (NO) (van't Hof & Ralston 2001, Kanno *et al.* 2008) and hydrogen sulphide (H_2S) (Zavaczki *et al.* 2011, Grassi *et al.* 2016). In contrast, high glucose levels, as seen in diabetes, inhibit bone formation and increase fracture risk but promote AMC (Chen *et al.* 2006, Jiao *et al.* 2015).

The beneficial effects of oestrogen and testosterone on the skeleton are well documented and these actions include stimulating osteoblast differentiation and bone formation (Clarke & Khosla 2009). In contrast, the influence of these hormones on AMC is less clear since studies have shown both inhibitory (Osako *et al.* 2010, Son *et al.* 2010) and stimulatory actions (Balica *et al.* 1997, Zhu *et al.* 2016). Thus, further work is required to determine whether oestrogen and testosterone have opposing actions on bone formation and AMC.

The same functional effect, different cellular mechanism

It is important to acknowledge that not all proteins and signalling molecules exert opposing effects on bone formation and AMC. Molecules including MGP, PP_i , extracellular nucleotides and sclerostin, are inhibitory to both processes (Moe *et al.* 2005, Patel *et al.* 2018, De Mare *et al.* 2019, Orriss 2020) whilst proteins such as fibronectin, BMP-2 and PAR are stimulatory (Ding *et al.* 2006, Li *et al.* 2008, Muller *et al.* 2019). In some cases, the mechanisms mediating these actions are similar; for example, PP_i acts to directly prevent hydroxyapatite crystal formation and propagation in the same way in any tissue (Orriss 2020). However, the cellular mechanisms mediating these functional effects can also differ. For example, ATP and UTP inhibit both bone mineralisation and VSMC calcification (Orriss *et al.* 2007, Patel *et al.* 2018). In osteoblasts, these effects are mediated by multiple P2 receptor subtypes (P2Y_2 , P2X_1 , P2X_7) and receptor activation leads to a reduction in TNSALP expression and activity and alterations in endogenous ATP release (Orriss *et al.* 2007, 2017). In VSMCs, ATP and UTP inhibit VSMC calcification via a reduction in apoptosis (Patel *et al.* 2018). In contrast, ATP and UTP have no effect on osteoblast survival and so the inhibitory effects of these nucleotides on bone mineralisation do not involve alterations in cell death (Patel *et al.* 2018).

Conclusions: the calcification paradox

As discussed, it is established that ageing and diseases can lead to diminished cell function. In osteoblasts, this can result in reduced differentiation and impaired bone formation whereas dysfunctional VSMCs can increase their propensity to calcify leading to AMC development. These cellular changes are reflected systematically by the observation that ageing is associated with an increased risk of developing both AMC and osteoporosis. Furthermore, diseases which cause AMC, such as CKD and diabetes, also induce bone loss and/or abnormalities in bone turnover (Jiao *et al.* 2015, Neven *et al.* 2015, Yamada & Giachelli 2017). Thus, it is important that a treatment for AMC does not induce any further deleterious effects on the bone.

Many of the factors identified here as having opposing effects are anabolic for bone but able to inhibit AMC. Whilst it is acknowledged that very few published

studies have directly compared the two processes, the evidence overall indicates that it should be feasible to develop a treatment that can prevent or regress AMC without exerting negative actions on the skeleton. Further comparative work is now essential to fully understand these differential actions on osteoblasts and calcifying VSMCs. This will pave the way for developing a bone-preserving therapeutic treatment for AMC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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