Review Article

Characterisation of matrix vesicles in skeletal and soft tissue mineralisation

L. Cui *, D.A. Houston, C. Farquharson, V.E. MacRae

The Roslin Institute and Royal (Dick) School of Veterinary Studies, The University of Edinburgh Easter Bush Campus, Edinburgh, Midlothian, EH25 9RG, UK

A R T I C L E   I N F O

Article history:
Received 9th October 2015
Revised 25th March 2016
Accepted 6th April 2016
Available online 9th April 2016

Keywords:
Matrix vesicles
Vascular calcification
Mineralisation
Phosphate
Mechanisms

A B S T R A C T

The importance of matrix vesicles (MVs) has been repeatedly highlighted in the formation of cartilage, bone, and dentin since their discovery in 1967. These nano-vesicular structures, which are found in the extracellular matrix, are believed to be one of the sites of mineral nucleation that occurs in the organic matrix of the skeletal tissues. In the more recent years, there have been numerous reports on the observation of MV-like particles in calcified vascular tissues that could be playing a similar role. Therefore, here, we review the characteristics of MVs that enable them to participate in mineral deposition. Additionally, we outline the content of skeletal tissue- and soft tissue-derived MVs, and discuss their key mineralisation mediators that could be targeted for future therapeutic use.

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Abbreviations: AB, Apoptotic body; ADP, Adenosine diphosphate; Anx, Annexin; ASARM, Acidic serine- and aspartate-rich motif; ATP, Adenosine triphosphate; BMP, Bone morphogenetic protein; CK, Choline kinase; CKD, Chronic kidney disease; ECM, Extracellular matrix; GPI, Glycosylphosphatidylinositol; GRP, Glu-rich protein; HA, Hydroxyapatite; JNK, c-Jun N-terminal kinase; LPS, Lipopolysaccharide; MEPE, Matrix extracellular phosphoglycoprotein; MGP, Matrix gla protein; mRNA, MicroRNA; MV, Matrix vesicle; NPP1, Ectonucleotide pyrophosphatase; nMase2, Neutral sphingomyelinase 2; OPG, Osteoprotegerin; OPN, Osteopontin; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PT, Pituitary-specific transcription factor; PPi, Pyrophosphate; PS, Phosphatidyserine; PSS, Phosphatidyserine synthase; RANKL, Receptor activator of nuclear factor kappa-B ligand; RGD, Arginine-glycine-aspartic acid; Runx2, Runt-related transcription factor 2; SIBLING, Small integrin-binding ligand N-linked glycoprotein; siRNA, Small interfering RNA; TGM2, Transglutaminase 2; TNAP, Tissue nonspecific alkaline phosphatase; VDAC1, Voltage-dependent anion channel 1; VIC, Valve interstitial cell; VSMC, Vascular smooth muscle cell.

* Corresponding author.

E-mail address: Cui.Lin@roslin.ed.ac.uk (L. Cui).

http://dx.doi.org/10.1016/j.bone.2016.04.007
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1. Introduction

The skeleton encompasses bone and cartilage. It is a multifunctional and highly specialised system which comprises both mechanical and biochemical properties that provide the basis for its roles in locomotion, growth, and protection [44]. The skeleton also stores 98% and 85% of the total body calcium (Ca^{2+}) and phosphate (P\textsubscript{i}), respectively [52,66]. Furthermore, in recent years, research has uncovered the emerging role of bone as an endocrine organ that regulates development and energy homeostasis [74,118]. The development and lifelong maintenance of the skeletal tissues is tightly regulated through the actions of distinct cell types. Hypertrophic chondrocytes in the epiphyseal plate mineralise the extracellular matrix (ECM) through specialised structures named matrix vesicles (MVs). The first hydroxyapatite (HA) depositions are located within the confinement of these nano-spherical bodies. MVs are membrane-bound particles of cellular origin, that range from 100 to 200 nm in diameter [8,45]. The ability for MVs to calcify is dependent on their content. Mineralising MVs typically contain abundant proteins and lipids that are known to chelate P\textsubscript{i} and Ca^{2+}. MVs have also been reported in osteoid, mantle dentin, and calcifying tendons [7,10,24,86,89,158,178]. However, the density of these particles appear to decrease with the increasing compactness of collagen fibrils in the mature bone [25]. Therefore, MVs may be attributed a role in the mineralisation of the embryonic bone, rather than the mature lamellar bone [88]. Indeed, mineral nucleation is a complex process, and whilst MVs are important for this process they are unlikely to be the sole mechanism responsible for the first steps of skeletal mineralisation. Throughout the years, there have been many studies conducted with knockout models on various proteins implicated in the initiation of mineralisation, that consistently show different levels of mineralisation [13,66,113,147]. These studies have provided in vivo proof that mineralisation can be achieved through various means. Hence multiple rational theories which describe mineral crystallisation exist. One of the most discussed theories is the nucleation of apatite through collagen polypeptide stereochemistry with Ca^{2+} and P\textsubscript{i}, where apatite crystals precipitate and propagate from an amorphous phase, in the gap zone of collagen fibrils [49,87,91,117]. In contrast, studies conducted using electron microscopy and X-ray diffraction analysis on human cortical femur bone, revealed that the majority of the mineral is present outside of collagen fibrils and in the interfibrillar compartment in the form of elongated mineral plate structures [102,103,139]. However, the present review focuses on our current knowledge and understanding of the role of MVs in the mineralisation process. During recent decades, the role of MVs in the pathogenesis of vascular mineralisation has become increasingly apparent, with a number of studies reporting the presence of vesicles in vascular tissues that are comparable in both structure and content to skeletal MVs (Table 1). However, the exact mechanisms through which MVs orchestrate the mineralisation process remain unclear. This review presents a summary of our current knowledge to date on the secretion, function, and content of MVs during both physiological and pathological mineralisation.

2. Bone formation

Bones develop through two different mechanisms. Mesenchymal stem cells can directly differentiate into osteoblasts through intramembranous ossification. This process is responsible for the formation of flat bones such as the cranium, sternum, and rib cage. Alternatively, the mesenchymal stem cells may differentiate into chondrocytes, which serve as templates for bone formation by endochondral ossification that leads to the development of long bones [119]. Endochondral ossification begins with a primary centre in the diaphysis consisting of a cartilage model, hypertrophic chondrocytes and vascular invasion. This is followed by the extension into secondary centres in the epiphyseal plate, which are responsible for longitudinal growth. Concomitant invasion of the cartilaginous scaffold occurs accompanied by haematopoietically derived bone resorbing cells, known as osteoclasts. The latter resorb the mineralised chondrocyte remnants and much of the cartilaginous matrix [41]. Furthermore, mesenchymal cells in the cartilage perichondrium begin to differentiate into osteoblasts, directed by the expression of the transcription factors, Runt-related transcription factor 2 (Runx2) and osterix [83]. These bone forming cells deposit a bone-specific matrix, rich in type I collagen, on remnants of chondrocyte ECM and in the perichondrium, which are subsequently mineralised [125]. Throughout lifetime, synchronised actions of osteoblasts and osteoclasts continue to remodel the bone, allowing growth and adaptation in response to mechanical loading. The most abundant cellular component of mature bone are the terminally differentiated osteoblasts, known as osteocytes [82], which reside deep within the bone matrix. The osteocytes orchestrate the actions of the osteoblasts and osteoclasts through relaying of external mechanical signals, to trigger deposition or resorption of bone possibly via the expression osteoprotegerin (OPG) and receptor activator of nuclear factor kappab ligand (RANKL) [23,111,152,173].

The intricate process of skeletogenesis can be clearly observed in the formation of the appendicular skeleton, which proceeds via a cartilage primordium [84]. Under the influence of the transcription factor Sox9, mesenchymal stem cells differentiate into chondrocytes that proliferate and generate type II collagen and a proteoglycan-rich ECM [125]. The chondrocytes within the prospective bone progress through morphologically distinct zones, co-ordinated by sequential expression of transcription and growth factors [101]. Chondrocytes in the most advanced region of the epiphyseal plate exit the cell cycle and become hypertrophic. The hypertrophic chondrocytes,
osteoblasts and odontoblasts, release MVs into the ECM [6]. The first mineral deposits are formed within the protective confines of these nano-particles.

3. Origin and discovery of MVs

Initial identification of MVs took place in 1967, in studies performed independently by H. Clarke Anderson [9], and Ermanno Bonucci [185]. The MVs were considered of cellular origin and were found in conjunction with mineral deposition, during rat epiphyseal growth plate development. The discovery of MVs, then termed “vesicles” or “calciﬁying globules”, was initially received with scepticism. The relationship between MVs and the nucleation of HA was, and remains, controversial. Some believe that the nucleation of HA takes place through the deposition of Ca\(^{2+}\) and Pi in the “hole zone” regions of collagen fibrils within the organic matrix [50,58,87,90]. Others have disregarded MVs as cellular debris or artefacts due to sample preparation which would otherwise seldom be seen [88]. Indeed, it is important to stress that, with many techniques, there are certain limitations to the identiﬁcation of MVs by electron microscopy. Apart from artefacts that could accumulate during specimen preparation, the localisation of MVs could be complicated by the presence of other cellular membrane-bound, micro-structures due to their similarity in size and shape. However, the identiﬁcation of MVs can be facilitated by their co-localisation with mineral crystals present within the confines of the limiting membranes, and also those extruding out and rupturing the membrane. Through the evidence obtained from a series of in vivo and in vitro studies, which also revealed that these structures are typically surrounded by a trilaminar membrane, their existence became more widely accepted [105,170,186].

Nevertheless, current methodology makes it impossible to isolate populations of pure MVs. The obtained vesicle population is naturally heterogeneous, and may include bodies that arise from physiological and/or non-physiological backgrounds. Moreover, unique markers for MVs have yet to be identiﬁed, therefore it is not presently possible to explicitly distinguish MVs from other vesicle populations. Indeed, many of those who initially questioned the existence of MVs later published data on the function of these particles [86,142].

There have been recurrent observations that the earliest recognisable crystal structures in the growth cartilage, bone and dentin to be found in the MV interior [12], thus suggesting MVs are responsible for the initiation of mineralisation. The competence of MVs to direct this process depends on many factors, amongst which include the type of matrix, which is composed mainly of collagen fibrils and proteoglycans. The latter effectively slows down the diffusion of oxygen and depletes the proliferating cells from other nutrients, thus making them hypoxic [80]. As a result, the chondrocytes adapt to secrete high levels of glycolytic enzymes [80] and adopt anaerobic glycolysis for respiration [127]. Upon the penetration of blood vessels in the proliferative zone, the hypoxic cells receive a sudden delivery of nutrients, electrolytes (e.g. Ca\(^{2+}\) and P\(_4\)), and oxygen leading to a state of oxidative stress. Their mitochondria become fully loaded with Ca\(^{2+}\), and can no longer produce adenosine triphosphate (ATP). This causes the cells to swell. Reactive oxygen species are consequentially generated, and along with the elevated level of P\(_4\), as a result of ATP hydrolysis, the opening of mitochondrial permeability transition pores is induced [141]. At this stage, vesicles that are loaded with Ca\(^{2+}\) are released from the mitochondria into the cytosol, whereby the released Ca\(^{2+}\) interact with P\(_4\) and phosphatidylserine (PS) to form PS-Ca\(^{2+}\)-P\(_4\) complexes, and with PS and annexin (Anx) to form PS-Ca\(^{2+}\)-Anx complexes [170]. Annexes have also been shown to bind and regulate intra-vascular Ca\(^{2+}\), inhibition of Anx activity decreasing chondrocyte mineralisation [164]. These PS-Anx complexes attach to the cytoplasmic leaflet of the plasma membrane of the chondrocytes. Due to most of the Ca\(^{2+}\) having been incorporated into these complexes, depletion of Ca\(^{2+}\) in the cytosol occurs. As a result, cytoskeletal proteins such as actin depolymerises, and blebbings are formed at the plasma membrane which eventually detach to allow for the MVs to travel to the ECM [167,170].

Similarly, other studies have supported this mechanism of MV formation by comparing the lipid and protein composition of MVs and the plasma membrane of chondrocyte and osteoblasts. These membranes were indeed similar in composition, albeit they possessed different levels of structural lipids and proteins [105]. In contrast, there has also been a study reporting distinct membrane compositions in MVs and cellular plasma membranes, but this observation has remained highly controversial to this date [92].

Further studies employing the SaOS-2 osteoblastic-like cells have shown that MVs are originated from speciﬁc regions of the plasma membrane, and that they bud off in the same orientation as the parental membrane [45]. More recently, MVs released by SaOSLM2 cells (a cell line derived from SaOs-2 with a p53 deletion) have been found to be derived from microvilli structures at the apical plasma membrane [157], supporting previous ultrastructural observations noted in studies of long bone mineralisation [21,55].

4.2. Assembly of vesicles through apoptotic cell membrane rearrangement

An alternative hypothesis for MV formation suggests that the vesicles are assembled due to the rearrangement of the apoptotic cell membrane [73]. However, it has been subsequently shown that MVs and apoptotic cell membranes are morphologically and functionally different as osteoblasts and growth plate chondrocytes have been observed to be intact post-MV release, suggesting apoptosis-independent mechanisms are responsible [81,172]. Nevertheless, MV formation and apoptosis are likely to occur simultaneously during cell differentiation as apoptotic vesicles are still capable of accumulating mineral deposition, and this may be a contributing factor in ectopic mineralisation.

While these theories are still topical, it is very likely that the release of MVs involve both cell membrane rearrangement, budding, and further additional mechanisms that have yet to be elucidated.

4.3. Mineral formation in MVs

As mentioned previously, MVs have been recognised to nucleate hydroxyapatite through a biphasic phenomenon and is divided between mineral crystallisation within the MVs and subsequent mineral propagation [7]. During Phase I, there is an increase in activity of the MV phosphates, including: alkaline phosphatase, adenosine triphosphatase, pyrophosphatase, and PHOSPHO1 which generate and transport P\(_4\), as well as Ca\(^{2+}\)-binding compounds such as the Anx family and PS [6]. The location of these molecules are generally found near the MV membrane [6]. PHOSPHO1 is found inside the MVs [107]. Ca\(^{2+}\) and P\(_4\) are attracted into the MVs by these compounds, until the threshold for Ca\(^{2+}\)-P\(_4\) precipitation is reached [7]. The enzyme carbonic anhydrase,
which is also found inside the MVs, stabilises the initial crystals. The precipitation is at first converted into an intermediate octacalcium-phosphate before being transformed into the more insoluble HA [135]. In Phase II, the crystals of HA have accumulated sufficiently to penetrate through the MV membrane to reach the extracellular fluid, ultimately destroying the MVs. The rate of mineral deposition is controlled by the pH of the extracellular fluid, its ion (Ca²⁺/Pi) concentration, and the presence of mineralisation-regulating molecules present in the extracellular fluid [37]. Under calcifying conditions, the extracellular fluid contains sufficient Ca²⁺ and Pi to support further crystal propagation, with preformed HA serving as templates for new minerals to grow on.

5. The importance of MV constituents for skeletal tissues

The composition of MVs directs the mineralisation nature of MVs. The variable regulation of proteins and lipids of MVs that promote mineralisation depends on the mineralising nature of their parental cells, as well as the local environment.

5.1. Phosphatidylserine (PS)

The presence of extracellular lipid material at the mineralisation front of calcifying tissues was identified over 50 years ago [169]. The source of this material can now be attributed to the Ca²⁺-binding, acidic phospholipids of the MV membrane [170]. Since these early discoveries, a wealth of information surrounding the lipid components of MV has been revealed, from which the critical role of lipids in MV-mediated mineralisation can be appreciated.

The anionic phospholipid, PS, shows selective enrichment in the inner leaflet of MV membranes, where it is typically found as PS-Ca²⁺–Pi complexes [36,165]. In vitro formation of PS-Ca²⁺–Pi complexes show a potent ability to induce HA precipitation when incubated in synthetic cartilage lymph [166]. Indeed, early transmission electron microscopy studies revealed an association between the inner leaflet membrane and primitive mineral formation [11]. PS-Ca²⁺–Pi complexes have now been identified at the initial stages of growth plate cartilage [168], bone [28], dentin [143] and tumour mineralisation [15]. Furthermore, studies utilising high performance thin layer chromatography identified an increase in the levels of PS and lysophosphatidylserine (LPS) during the in vitro mineralisation of chick growth plate MVs, attributable to the ATP-independent base exchange of ethanolamine for serine in phosphatidylethanolamine (PE) [165,171]. The maintenance of high levels of PS in MV membranes, and the nucleation capacity that it brings, may be a necessary component of mineralisation.

Gain of function mutations in the PS synthase (PSS) 1 gene has been shown to cause the rare Lenz–Majewski syndrome, which is associated with hyperostosis of the cranium, vertebral and diaphysis of tubular bones [146]. Excessive PS accumulation (via PSS1 mediated exchange of serine with the choline moiety of phosphatidylcholine (PC)), and thus enhanced nucleation of HA by MVs may contribute to the phenotype observed in this condition. However, mice deficient in PSS1 and PSS2, which mediate the exchange of ethanolamine for serine in PE, show no perturbations of mineralisation [16,22]. Not only is PS involved in the formation of mineral, but it may additionally play a role in the externalisation of immature mineral from MVs. Indeed, the externalisation of PS is induced in response to increases in intracellular Ca²⁺, likely through the actions of Ca²⁺-dependent phospholipid scramblases, a process which has been observed in the plasma membranes of hypertrophic chondrocytes [40] and osteoblasts [42].

5.2. TNAP and NPP1

The ratio of inorganic pyrophosphate (PPi) to Pi is of critical importance in the promotion or indeed restriction of mineral in physiological tissues. Although, the exact mechanism of Pi generation in MVs remains to be elucidated, several theories have been proposed (Fig. 1).

It has long been known that the glycosylphosphatidylinositol (GPI) anchored ectoenzyme, tissue-nonspecific alkaline phosphatase (TNAP), and ectonucleotide pyrophosphatase (NPP1)/phosphodiesterase 1 are the major regulators of the extracellular PPi/Pi ratio. TNAP, encoded by Aplp (Akp2 in mice), is abundant on the surface of MVs derived from osteoblasts, hypertrophic chondrocytes, and odontoblasts [108]. Moreover, a study has identified the phosphosubstrate utilisation of TNAP and NPP1 (encoded by Enpp1) at the level of the MV [35]. Analysis of the catalysis of ATP, adenosine diphosphate (ADP) and PPi, by osteoblast-derived MVs from wild-type (WT), Akp2–/– and Enpp1–/– mice highlighted that TNAP is the major phosphatase of these vesicles with its absence producing the largest deficit in substrate hydrolysis. Interestingly, the absence of NPP1 from MVs did not affect the hydrolysis of the tested substrates indicating that when associated with MVs, NPP1 does not have major PPi generating role, but rather can act as a “back-up” phosphatase in the absence of TNAP. This role as a “plan B” phosphatase is proposed as the reason why in the Phospho1−/−: Akp2−/− double knockout mouse, mineralisation of the axial skeleton can be occasionally observed [177].

Hypophosphatasia, a condition of defective TNAP activity, commonly resulting from missense mutations in Aplp [57], demonstrating the importance of TNAP in skeletal mineralisation. Akp2−/− mice phenocopy hypophosphatasia with hypomineralisation of the skeleton and teeth ensuing after birth [175] and evidence of craniosynostosis [97]. Both patients with hypophosphatasia and Akp2−/− mice exhibit raised serum PPi levels. Concomitant ablation of NPP1 on an Akp2−/−/− background partially restores the serum PPi levels and skeletal mineralisation [56]. Despite this, pioneering studies utilising electron microscopy revealed that MVs from patients with hypophosphatasia and Akp2−/−/− mice possess crystals of HA within their interiors [13,14]. These findings highlight alternative mechanisms of generating a PPi/Pi ratio conducive to mineral formation within the interior of MVs.

TNAP produces an environment surrounding MVs conducive to mineralisation not only through regulation of the PPi/Pi ratio, but also through modulating the phosphorylation status of osteopontin (OPN). OPN is a major non-collagenous bone protein which inhibits the nucleation and growth of HA, through binding to nascent crystals by means of the phosphorylated residues of the protein. Indeed, dephosphorylation of OPN results in the loss of its inhibitory properties [2,27]. More recently, significant increases in OPN transcript and protein in the plasma and skeleton of the Akp2−/−/− mouse have been noted [113]. Furthermore, the skeletal over-expression of TNAP in Akp2−/−/− mice decreased the phosphorylation status of OPN within long bones. These novel data suggest that the pro-mineralisation role of TNAP may be related not only to its accepted PPi activity but also to its ability to modify the phosphorylation status of OPN.

5.3. SIBLING proteins

OPN, bone sialoprotein, matrix extracellular phosphoglycoprotein (MEPE), dentin matrix protein and dentin sialoprotein make up a group of non-collagenous extracellular mineralisation-regulating proteins termed SIBLING (small integrin-binding ligand N-linked glycoprotein) proteins [150]. These proteins share a conserved arginine-glycine-aspartic acid (RGD) motif which mediates their cell attachment and signalling functions [46]. The conserved acidic serine- and aspartate-rich motif (ASARM) peptide region within this family of proteins appears however, to be a key determinant of their role in mineralisation [133]. In particular, it is the post-translational modifications of this motif, through enzymatic cleavage and phosphorylation, which dictates its function. Indeed, the ASARM peptide of OPN has been shown to inhibit the ECM mineralisation of osteoblast-like cells through the binding of HA [4]. This inhibition of mineralisation was dependent on the number of phosphorylated serine residues, with non-phosphorylated ASARM
A peptide showing no inhibition of mineralisation [4]. OPN has also emerged as a potent inhibitor of ectopic, pathological mineralisation [48]. This effect was most clearly demonstrated by a study which showed the exacerbation of vascular calcification in the matrix gla protein (MGP) null mouse through the simultaneous ablation of OPN [147]. The phosphorylated ASARM peptide of MEPE has also been shown to inhibit the mineralisation of osteoblast-like cell and bone marrow stromal cell cultures [3,106].

More recently, inhibition of mineralisation by the phosphorylated, but not non-phosphorylated, MEPE-ASARM peptide in cultured murine embryonic metatarsals, a model of chondrocyte mineralisation, has been observed [149]. To our knowledge, the ability of TNAP to dephosphorylate the OPN ASARM peptide as previously mentioned [2,4,113], has yet to be shown in other SIBLING protein derived ASARM peptides.

5.4. PiT1

Two related type III Na/Pi co-transporters, pituitary-specific transcription factor (Pit1)/Glvr1 and Pit2/Ram, encoded by SLC20A1 and SLC20A2 respectively, are both expressed by chondrocytes and osteoblasts, however Pit-1 is the major mediator of Pi influx in these cell types [115,123,179]. Although ubiquitously expressed, Pit1 shows enrichment in late hypertrophic chondrocytes, and as such has been associated with Pit1 enrichment of MVs to promote matrix mineralisation [121]. Pit1 expression is stimulated by many classical osteotropic factors such as parathyroid hormone, Ca²⁺ and bone morphogenetic protein (BMP)-2. Indeed, it has been recently shown that the up-regulation of Pit1 in response to BMP-2 in MC3T3 osteoblast-like cells is mediated through c-Jun N-terminal kinase (JNK) pathway stimulation [155].

5.5. PHOSPHO1

First identified in chick growth plate chondrocytes, PHOSPHO1, is a phosphatase and a member of the haloacid dehalogenase superfamily [60]. PHOSPHO1 is essential for the initiation of skeletal mineralisation. The expression of PHOSPHO1 has been shown to be around 120-fold higher in growth plate chondrocytes compared to non-skeletal tissues and the soluble protein has been identified in MV extracts from chick growth plate cartilage [153] and murine hypertrophic chondrocytes and osteoblasts [130]. Furthermore, through analysis of the ability of intact and disrupted Akp2−/− MVs to generate Pi from phospholipid precursors, PHOSPHO1 has been localised to the sheltered interior of MVs [130], a location critical to its key function in the initiation of mineral formation. To date PHOSPHO1 has been identified in the MVs derived from osteoblasts, chondrocytes, and odontoblasts [61,107,130] and a number of studies unequivocally provide evidence for its key role in intravesicular Pi generation. A reduction in ECM mineralisation was observed in MVs from murine and chick epiphyseal cartilage after treated with the PHOSPHO1 small molecule inhibitor, lansoprazole [130]. Treatment of 5-day old chick embryos with lansoprazole similarly abolished the mineralisation of both wing and leg bones of young chicks [104].

The pivotal role of PHOSPHO1 was recently highlighted by the generation and characterisation of the Phospho1−/− mouse. Phospho1−/− mice display severe bone and tooth abnormalities including hypomineralisation, bowed long bones, spontaneous

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**Fig. 1.** Hypothetical generation of inorganic phosphate and the accumulation of calcium within MVs. The first stage in Pi generation is the production of PHOSPHO1 substrates, phosphocholine, and phosphoethanolamine, through the actions of nSMase2 and/or phospholipases. Once released from membrane lipid precursors, phosphocholine, and phosphoethanolamine undergo hydrolisis by PHOSPHO1 to generate intravesicular Pi. Further Pi accumulation within the MV may be facilitated by the phosphate transporter, PiT1. Propagation of HA out with the confines of the MV is controlled by the local Pi/Pi ratio. The main regulators of the extracellular Pi/Pi ratio are NPP1 and TNAP.
tissues (76). Reduction of a major portion of 156. The ablation of nSMase2 enzymatic activity by the chemically in-

34x259]nestingly however, transmission electron microscopy analysis of mantle substrate (phosphocholine) in the absence of nSMase2 activity. Inter-

34x385]PHOSPHO1 substrate phosphocholine. nSMase2, is recognised to break

PHOSPHO1−/− mice [176]. Inhibition of both PHOSPHO1 and TNAP activity by small molecule inhibitors signi-

34x573]ux of Pi [174] (Fig. 1). Interestingly, in the PHOSPHO1−/− murine model, hydrolysis of ATP is reduced not directly due to the absence of PHOSPHO1 expression, but an apparent downregulation of TNAP expression [35].

Despite a comprehensive appreciation of the resultant phenotype of PHOSPHO1 deficiency, the precise molecular and biochemical mechanisms underpinning PHOSPHO1-mediated intravesicular Pi production has yet to be determined. The initial characterisation of this novel phosphatase revealed that PHOSPHO1 displayed high spe-

34x489]fi

437x291]in vivo

437x291]and

437x291]mgpm1

534x259]ed as
isolated vesicles was significantly decreased when cultured with serum [128]. A possible explanation has implicated the role of fetuin-A, a natural mineralisation inhibitor secreted by the liver that circulates in the serum [151]. A recent study suggests that nSMase2 controls the loading of fetuin-A and exosome secretion in VSMCs [70]. Depletion of Smad3 by small interfering RNA (siRNA) resulted in a decrease in exosome secretion and calcification, suggesting a direct role in regulating vascular mineralisation. Further studies are clearly needed to examine the clinical potential of nSMase2-specific inhibitors for blocking exosome release and phosphocholine generation.

A novel role for gla-rich protein (GRP) in vascular mineralisation inhibition has also been recently highlighted [162]. It has been proposed that GRP, another vitamin-K dependent protein, may prevent calcium-induced signalling pathways and direct mineral binding to inhibit crystal formation. Moreover, GRP up-regulation in mineralisation competent MVs derived from VSMCs has been demonstrated, and may be associated with the fetuin-A-MGP mineralisation inhibitor system [162]. Intriguingly, GRP activity has been shown to be dependent on its γ-carboxylation status. Likewise, the reduced loading of MGP into MVs may be due to an accumulation of uncarboxylated MGP as a result of elevated Ca²⁺ levels which could readily impair the functionality of the endoplasmic reticulum [144]. These observations suggest that both the local environment and the MV content are crucial in determining the fate of MVs in soft tissues.

The vitamin K-dependent proteins, MGP and GRP, represent exciting potential therapeutic targets for the inhibition of vascular mineralisation. In rats, treatment with the vitamin K antagonist, warfarin leads to rapid mineralisation of the arteries. This can be reversed by a vitamin K-rich diet [138]. Specifically, Vitamin K2 supplementation prevents arterial mineralisation, yet vitamin K1 does not [62,148]. Furthermore, in the population based Rotterdam study, increased intake of vitamin K2, but not K1, was shown to be inversely related to all-cause mortality and severe aortic mineralisation [47].

7.2. Cell death

It has been proposed that MV secretion is a result of an adaptive response to normalise the presence of mineral imbalance [145] as ectopic mineralisation is thought to be initiated by apoptotic bodies (ABs) released during VSMC necrosis [124]. Furthermore, in vitro studies using human VSMCs have revealed that ABs are able to accumulate Ca²⁺ in a similar manner to MVs [124]. ABs released by tissue necrosis, along with MVs derived from viable mineralising vascular cells, may induce an imminent pathological mineralisation site, as they accumulate mineral deposition.

Autophagy is a dynamic and highly regulated process of self-digestion responsible for cell survival and reaction to oxidative stress. Recent research has highlighted autophagy as a novel adaptive mechanism that protects against P₄-induced VSMC mineralisation, by acting to regulate apoptosis and the release of mineralising MVs from VSMCs [39]. Further studies are required to fully understand the mechanisms driving the autophagic response in VSMCs.

7.3. Annexins (Anxs)

MV secretion from calcifying VSMCs share similarities with chondrocyte-derived MVs, with enrichment of Anx A2, A5, and A6 [34,71,164]. Anx A6 has been shown to be abundant at sites of vascular mineralisation in vivo, and siRNA depletion of Anx A6 reduces VSMC mineralisation. Furthermore, biotin cross-linking and flow cytometry studies have demonstrated that Anx A6 shuttles to the plasma membrane in response to elevated calcium levels in vitro and forms Anx A6-PS nucleation complexes within MVs [71]. Fetuin-A has also been found to bind Anx A2 in a Ca²⁺ dependent manner, with membrane fraction immunoprecipitation revealing the binding to take place at the surface of the cell [34]. This suggests a possible mechanism for fetuin-A mediated inhibition of vascular mineralisation. However, the function of fetuin-A could be ultimately overwhelmed by Ca²⁺ overload and other mineralisation-regulating protein activity.

7.4. Macrophage-derived MVs

During the initial phase of atherosclerosis development, inflammation and local stress call for an accumulation of macrophages to the pathogenic areas, implicating a direct role that these white cells could play during the antecedent of mineralisation. Indeed, early mineralisation of atherosclerotic plaques has been shown to directly associate with macrophage accumulation [5]. Recent research has highlighted the first time that macrophages have the ability to release mineralising MVs enriched in the calcium binding proteins, S100A9 and Anx A5, which contribute to accelerated microcalcification in VSMCs [114]. These data further emphasise the importance of calcium-chelating proteins on MV mineralisation.
7.5. Osteogenic markers in vascular cell-derived MVs

Proteomic analysis, such as mass spectrometry has been used to identify the protein composition of MVs released by vascular tissue [71]. The results have been compared to previous mass spectrometry data of MVs derived from osteoblasts and chondrocytes [18,172]. Interestingly, MVs derived from these three cell-types share similar surface receptors, Ca\(^{2+}\)-binding proteins, ECM components, and cytoskeletal proteins (Table 1).

Interestingly, the concentration of TNAP has been shown to be either lowered or unchanged upon the addition of extracellular Ca\(^{2+}\) or Ca\(^{2+}\)-chelator, suggesting that TNAP may not be a key mediator of calcium-induced VSMC mineralisation [71]. However, chemical inhibition of TNAP activity has been shown to suppress VSMC mineralisation in vitro [112]. Moreover, it has been revealed that transglutaminase 2 (TGM2), a calcium-dependent enzyme that can cross-link ECM proteins, was found in MVs during aortic mineralisation [33]. The latter study showed decreased TNAP activity, and reduced ability for MVs to calcify type I collagen in CKD rats following TGM2 inhibition. A link has also been established between TNAP and the hydrolysis of circulatory PPi, an endogenous vascular mineralisation inhibitor [163]. These findings suggest that TNAP could have multiple roles in ectopic mineralisation depending on the inhibitors and inducers of mineralisation that are present in the microenvironment.

OPG, which is known to be a soluble decoy receptor for RANKL, the principal regulator of osteoclast function [59]. Deficiency of OPG in mice results in mineralisation of the aorta and renal arteries [30], and RANKL administration increases VSMC mineralisation in vitro [122]. OPG has been detected in VSMC derived MVs, and has been shown to co-localise immunohistochemically with Anx A6 [137]. It has been proposed that at physiological concentrations, OPG directly inhibits VSMC mineralisation, potentially by a mechanism whereby OPG is secreted via vesicle release from viable or apoptotic VSMCs, limiting the MV-driven mineral nucleation and deposition of HA in the vascular wall.

There are a number of key osteogenic markers that have been detected in calcified vascular cells, whose roles in MVs have yet to be examined. PiT1 is emerging as a key component in the pathogenesis of vascular mineralisation. Higher levels of Pi, in the serum, due to the inability of the kidney to filter excess Pi, induces VSMCs to upregulate the expression of PiT1, a predominant sodium-dependent phosphate co-transporter that leads to an accumulation of intracellular Pi. PiT1 has been shown to induce VSMC osteogenic transition, marked by increased Runx2 expression [189]. Upstream regulation of PiT1 in VSMCs has also been demonstrated in response to treatment with BMP-9, a potent inducer of VSMC mineralisation [183]. Recent studies have shown that the bone specific phosphatase PHOSPHO1 also plays a critical role in VSMC mineralisation, and that “phosphatase inhibition” may be a useful therapeutic strategy to reduce vascular mineralisation [79,112]. However, it has yet to be determined whether PHOSPHO1 is present in VSMC-derived MVs. As previously stressed, nSMase2 hydrolyses sphingomyelin to phosphocholine [154], which may be subsequently hydrolysed into choline and Pi by PHOSPHO1. These data, together with the recently elucidated role of nSMase2 in MV-mediated VSMC mineralisation [70] highlight the need for further investigations into the actions of PHOSPHO1 in vascular cell-derived MVs.

7.6. microRNAs (miRNAs)

MicroRNAs (miRNAs) are an important class of endogenous, single stranded, non-coding RNAs, which are involved in the regulation of gene expression and translation. miRNAs suppress gene expression through imperfect base pairing to the \(^{3}′\) untranslated region of target miRNAs leading to repression of protein production or mRNA degradation. Importantly, a single miRNA may affect the transcription of multiple genes involved in common pathways. miRNAs upregulated during vascular mineralisation include miRNA-221, −222 [100], −762, −714, −712 [54], −210 [43,93,126]. Conversely, several miRNAs that are involved in mineralisation inhibition are downregulated during vascular mineralisation, including miRNA-125b [31,51], −30 [19,94], −204 [38,64], −26 [67,116].

Intriguingly, vesicle-like structures derived from non-mineralising cells have the ability to transfer RNA or miRNA to new cells facilitating cell-to-cell or cell-to-ECM communication [160]. Recently, miRNA microarray analysis has identified for the first time a number of dysregulated miRNAs in MVs derived from CKD rats showing aortic mineralisation, including miRNA-667, −702, −3562, −3568 and −3584 [32]. A fuller understanding of the functional role of miRNAs in MVs may provide insight into the cellular regulation of MV packaging of miRNA and help to determine the post-transcriptional networks involved in vascular mineralisation.

8. Concluding remarks and future directions

The pathogenesis and physiology of mineralisation is a result of a network of active cell signalling and differentiation, orchestrated by the microenvironment. Our current knowledge of MVs is undoubtedly building towards a foundation in understanding the complex mechanisms underpinning the development of matrix mineralisation. Further insights into MV function may also enable the identification of effective targets for the development of novel therapeutics for the treatment of skeletal disorders and vascular mineralisation.

Acknowledgements

This study was supported by funding from the Biotechnology and Biological Sciences Research Council (BBSRC) in the form of an Institute Strategic Programme Grant (BB/J004316/1) (VEM and CF), an Institute Career Path Fellowship (BB/F023928/1, VEM), and studentship funding via a BBSRC CASE Studentship (BB/K011618/1, LC) and the East of Scotland BioScience Doctoral Training Partnership (EASTBIO DTP; BB/J01446X/1, DAH).

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