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Review Article

Characterisation of matrix vesicles in skeletal and soft tissue mineralisation

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ABSTRACT

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 MVs possess 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.

Contents

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, Gla-rich protein; HA, Hydroxyapatite; JNK, c-Jun N-terminal kinase; LPS, Lysophosphatidylserine; MEPE, Matrix extracellular phosphoglycoprotein; MGP, Matrix gla protein; miRNA, MicroRNA; MV, Matrix vesicle; NPP1, Ectonucleotide pyrophosphatase; nSMase2, Neutral sphingomyelinase 2; OPG, Osteoprotegerin; OPN, Osteopontin; PC, Phosphotidylcholine; PE, Phosphatidylethanolamine; P_iT, Pituitary-specific transcription factor; PP_i, Pyrophosphate; PS, Phosphatidylserine; PSS, Phosphatidylserine 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.

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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_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_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_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 haematopoetically 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 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 kappa-B 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,

Table 1
Some of the common proteins identified in MVs derived from VSMCs, mineralising osteoblasts, and femurs of chicken embryo [18,71,172].

Protein type/family	Protein name/family member
Calcium-binding proteins	Anx A1
	Anx A2
	Anx A5
	Anx A6
	Voltage-dependent anion channel-1 (VDAC1)
Phosphate transporter	5′-Nucleotidase
Oxidative stress	Peroxiredoxin 1
	Peroxiredoxin 2
Extracellular matrix	Collagen type VI, a1
Cytoskeletal and surface proteins	Actin-B
	Moesin
	Integrin, a3 isoform A
	Integrin, a5
	Integrin, b1 isoform 1A
	Sodium-potassium adenosine triphosphatase, a1
Chaperones	Calreticulin

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 "calcifying 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 P_i 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, as 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 enzymes and the Ca^{2+}-chelators carried by the MVs [180].

4. MV biogenesis and function

There have been several proposed mechanisms for the formation of MVs since their discovery. Even today, the exact process of MV assembly is still under debate. The following summarises the current theories.

4.1. Budding from the plasma membrane

One of the most discussed mechanisms of MV formation is through the "budding" process, where it is believed that MVs bud off the parental plasma membrane in a highly polarised manner [7,26]. Additional investigations have reported that microvilli on the cell surface of hypertrophic chondrocytes could be the precursors of MVs, and that the actin network is extensively involved during their formation [55,156,187]. More precisely, it has been shown that at the proliferative zone of the epiphyseal plate, chondrocytes are embedded in a highly impervious 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_i), 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_i 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_i and phosphatidylserine (PS) to form PS-Ca^{2+}-P_i complexes, and with PS and annexin (Anx) to form PS-Ca^{2+}-Anx complexes [170]. Anxs have also been shown to bind and regulate intravesicular 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 phosphatases, including: alkaline phosphatase, adenosine triphosphatase, pyrophosphatase, and PHOSPHO1 which generate and transport P_i; 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_i are attracted into the MVs by these compounds, until the threshold for Ca^{2+}-P_i 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 (Ca^{2+}/P_i) concentration, and the presence of mineralisation-regulating molecules present in the extracellular fluid [37]. Under calcifying conditions, the extracellular fluid contains sufficient Ca^{2+} and P_i to support further crystal propagation, with preformed HA serving as templates for new minerals to grow on [6].

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 Ca^{2+}-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-Ca^{2+}-P_i complexes [36,165]. *In vitro* formation of PS-Ca^{2+}-P_i 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-Ca^{2+}-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, vertebrae 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 Ca^{2+}, likely through the actions of Ca^{2+}-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 (PP_i) to P_i is of critical importance in the promotion or indeed restriction of mineral in physiological tissues. Although, the exact mechanism of P_i generation in MVs remains to be elucidated, several theories have been proposed (Fig. 1).

It has long been known that the glycosylphosphatidylinositol (GPI) anchored ectozyme, tissue-nonspecific alkaline phosphatase (TNAP), and ectonucleotide pyrophosphatase (NPP1)/phosphodiesterase 1 are the major regulators of the extracellular PP_i/P_i ratio. TNAP, encoded by *Alpl* (*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 PP_i 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 PP_i 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 *Alpl* [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 PP_i levels. Concomitant ablation of NPP1 on an *Akp2*$^{-/-}$ background partially restores the serum PP_i 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 PP_i/P_i 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 PP_i/P_i 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 PP_i 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

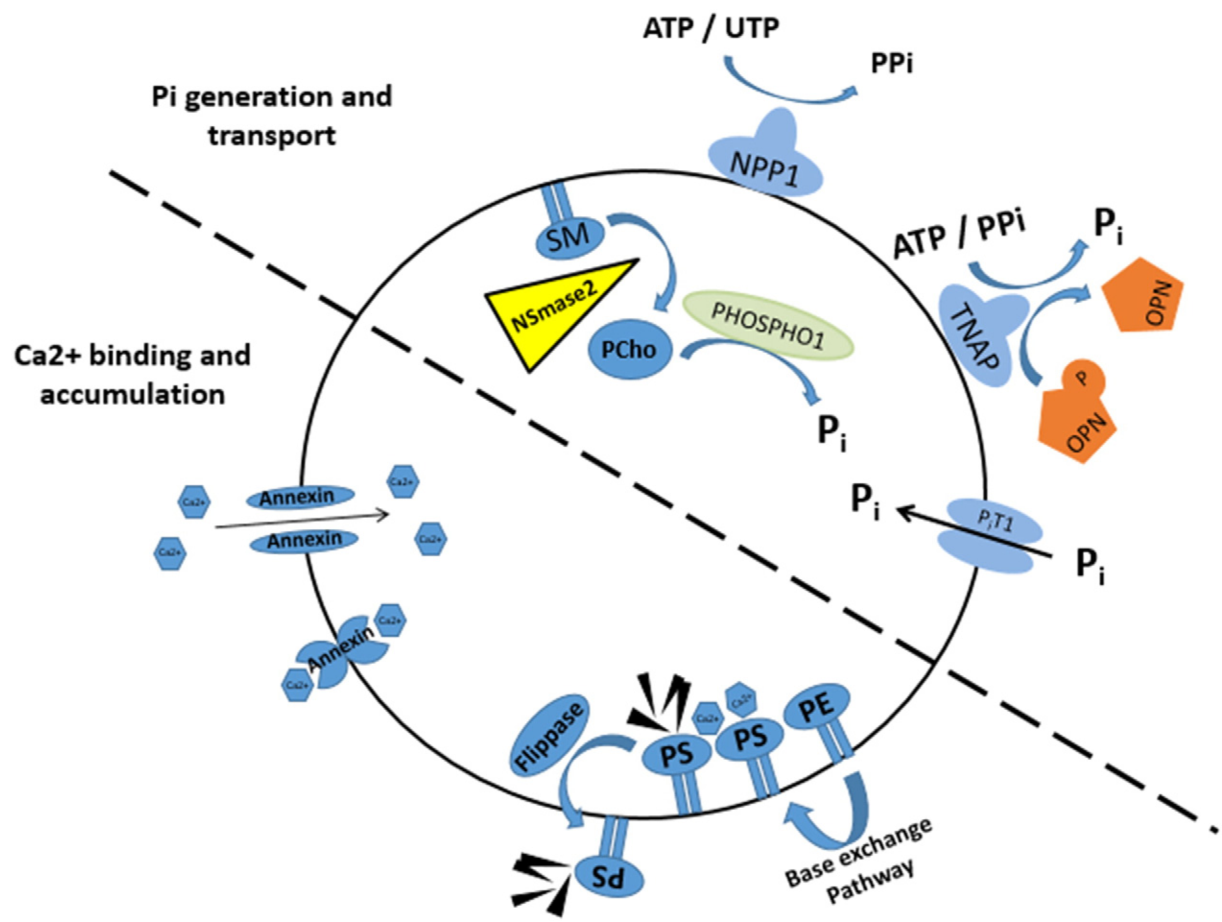

Fig. 1. Hypothetical generation of inorganic phosphate and the accumulation of calcium within MVs. The first stage in P_i generation is the production of PHOSPHO1 substrates, phosphocholine, and phosphoethanolamine, through the actions of nSMase2 and/or phospholipases. Once released from membrane lipid precursors, phosphocholine, and phosphoethanolamine undergo hydrolysis by PHOSPHO1 to generate intravesicular P_i. Further P_i accumulation within the MV may be facilitated by the phosphate transporter, P_iT1. Propagation of HA out with the confines of the MV is controlled by the local PP_i/P_i ratio. The main regulators of the extracellular PP_i/P_i ratio are NPP1 and TNAP.

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. P_iT1

Two related type III Na/Pi co-transporters, pituitary-specific transcription factor (P_iT-1)/Glvr1 and P_iT-2/Ram, encoded by *SLC20A1* and *SLC20A2* respectively, are both expressed by chondrocytes and osteoblasts, however P_iT-1 is the major mediator of P_i influx in these cell types [115,123,179]. Although ubiquitously expressed, P_iT1 shows enrichment in late hypertrophic chondrocytes, and as such has been associated with P_i enrichment of MVs to promote matrix mineralisation [121]. P_iT1 expression is stimulated by many classical osteotropic factors such as parathyroid hormone, Ca^{2+} and bone morphogenetic protein (BMP)-2. Indeed, it has been recently shown that the up-regulation of P_iT1 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 P_i 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 P_i 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 fractures and scoliosis [66,107,132,177,184]. Interestingly, recent data have confirmed that tibiae of PHOSPHO1 deficient mice are less stiff during growth but are eventually corrected in later life by alternative mechanisms [69]. Serum levels of PP_i and OPN are increased in *Phospho1*$^{-/-}$ mice but reducing the PP_i levels in *Phospho1*$^{-/-}$ mice by cross-breeding them to ApoE-TNAP transgenic mice, does not significantly improve the skeletal phenotype of *Phospho1*$^{-/-}$ mice [177]. In contrast, the ablation of the OPN gene, *Spp1*, completely prevents the scoliosis and improves the long bone defects characteristic of *Phospho1*$^{-/-}$ mice [176]. Inhibition of both PHOSPHO1 and TNAP activity by small molecule inhibitors significantly reduced ECM mineralization in osteoblast-like cell lines and metatarsals and this supports previous *in vivo* observations where the double ablation of *Phospho1* and *Akp2* led to a complete lack of skeletal mineralisation in embryonic mice [65,177]. Recent analysis of *Phospho1*$^{-/-}$/*P*$_it1^{col2/col2}$ double knockout mice has further revealed that MVs initiate mineralisation by a dual mechanism: PHOSPHO1-mediated intravesicular generation of P_i and phosphate transporter-mediated influx of P_i [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 P_i production has yet to be determined. The initial characterisation of this novel phosphatase revealed that PHOSPHO1 displayed high specific activity *in vitro* towards two products of phospholipid metabolism: phosphoethanolamine and phosphocholine [131]. As such, PHOSPHO1 may rely on MV membrane phospholipid metabolism in the provision of substrates for P_i generation.

Neutral sphingomyelinase 2 (nSMase2), encoded by the *Smpd3* gene, may provide a link between MV phospholipids and the PHOSPHO1 substrate phosphocholine.nSMase2, is recognised to break down the membrane lipid sphingomyelin to produce ceramide and phosphocholine; and has been identified in SaOS-2 derived MVs [154, 156]. The ablation of nSMase2 enzymatic activity by the chemically induced deletion of a major portion of *Smpd3*, as in the *fro/fro* mouse [17, 109] brings about a severe hypomineralisation of the skeletal and dental tissues ([76–78]. Rescue of the skeletal phenotype through overexpression of nSMase2 suggests that the hypomineralisation observed could be a consequence of deficient osteoblast mineralisation. This hypomineralisation may arise from a reduced production of PHOSPHO1 substrate (phosphocholine) in the absence of nSMase2 activity. Interestingly however, transmission electron microscopy analysis of mantle dentin from the *fro/fro* mouse revealed abundant MVs containing HA crystals as in heterozygous animals [76].

More recently, the role of choline kinase (CK) in bone formation has been highlighted. CK, encoded by *Chka* and *Chkb*, produces phosphocholine from choline, and the pharmacological inhibition of CK activity has resulted in decreased mineralisation capacity of human osteosarcoma MG-63 cells [96]. Similarly, another study showed *Chkb* mutant mice, *flp/flp*, exhibited reduced bone formation by osteoblasts. Osteoclasts from *flp/flp* mice also displayed lower sensitivity towards extracellular Ca^{2+} excessiveness which led to increased bone absorption and overall low bone mass [85]. Furthermore, inactivation of *Chkb* in mice led to a decrease in chondrocyte expression and phosphocholine concentration. Interestingly, PHOSPHO1 expression in these *Chkb*$^{-/-}$ mice is notably increased, possibly as to a compensatory mechanism to produce more P_i from less amount of substrate [95].

6. Pathological mineralisation

Pathological mineralisation is defined as the ectopic accumulation of minerals in non-skeletal tissues. There has been an emerging interest in the study of the mechanisms driving vascular calcification, which involves mineralisation of the blood vessels, valves or cardiac tissues. This is a condition commonly found in the elderly, diabetics, and patients who suffer from chronic kidney disease (CKD) [181]. The predominant mineral formed during vascular mineralisation is HA, although other types of biological minerals such as whitlockite have been found in diabetic arterial medial mineralisation caused by vitamin D toxicity [161].

The pathological process of vascular mineralisation was originally believed to be caused by passive deposition of calcium minerals as a consequence of ageing and vascular deterioration in the elderly. However, it has now been widely established that the driving force of vascular mineralisation is a complex network of tightly regulated active processes that resemble the physiological process of skeletal development (Fig. 2) [1,182]. Indeed, vascular cells are derived from the same pluripotent mesenchymal cells that give rise to osteoblasts, and they can spontaneously undergo bone-like transformation. These vascular cells may then release calcifying-competent ECM. In fact, the elastic lamellae which spans across arterial ECM can mineralise, leading to subsequent vessel rigidity and altered biomechanics, inducing cell transformation and differentiation [110]. Evidences of elastin involvement in mineralisation include its degradation during the pathogenesis of arterial calcification, with minerals forming along the elastin fibrils [20,68]. Moreover, *in vivo* studies conducted with Mgp-deficient mice (*mgp*^{m1}/*mgp*^{m1}) revealed extensive mineralisation of the elastic lamellae in the media of the aortic wall and aortic valves [99]. Cells which may readily undergo phenotypic to osteogenic transitions include: vascular smooth muscle cells (VSMCs) in the media, myofibroblasts in the adventitia, pericytes in microvessels, multipotent vascular mesenchymal progenitors, and valve interstitial cells (VICs) [29,53,120,124,188]. Alternatively, vascular mineralisation could occur independent of cell transdifferentiation. Past studies have shown that mutations in the genes encoding for mineralisation inhibitors, such as MGP, could also lead to vascular mineralisation without vascular cells trans-differentiating into cells of the osteoblastic phenotype [98,134].

7. MVs in ectopic vascular mineralisation

Amongst the many features that skeletogenesis and ectopic vascular mineralisation share, an emerging point of interest lies in the role of MVs during pathological mineralisation. In the recent decades, numerous studies have reported mineralising, MV-like structures released by mineralising VSMCs, in both *in vitro* and *in vivo* experiments [63,71, 128,136,140,144]. Interestingly, MV-like particles have also been found to be released by the vascular tissues under physiological conditions [71,99,128,129]. These bodies have been recently identified as exosomes with an endosomal origin that arise from multivesicular bodies ([70,142]. Below, we summarise some of the main components of vascular tissue-derived MVs and their potential role in promoting and inhibiting ectopic mineralisation.

7.1. VSMCs release MV-like structures under physiological conditions

As discussed previously, the function of MVs highly relies on their composition. Electron microscopy studies have revealed that MVs derived from VSMCs under physiological conditions do not contain crystals of HA [71]. Instead, MVs released by healthy VSMCs possess mineralisation inhibitors, such as the vitamin-K dependent protein, MGP, and fetuin-A, which prevent abnormal mineral deposition [72, 99,128]. Patients suffering from vascular mineralisation and ESRD show reduced expression of these inhibitors [29,75,159]. Indeed, MVs derived from VSMCs cultured under mineralising conditions contained less MGP [71]. Moreover, MVs harvested from mineralising VSMCs in both *in vitro* and *in vivo* conditions have a higher ability to nucleate Ca^{2+} and P_i when compared to MVs released under physiological conditions [63,128]. Conversely, the mineralisation of both VSMCs and

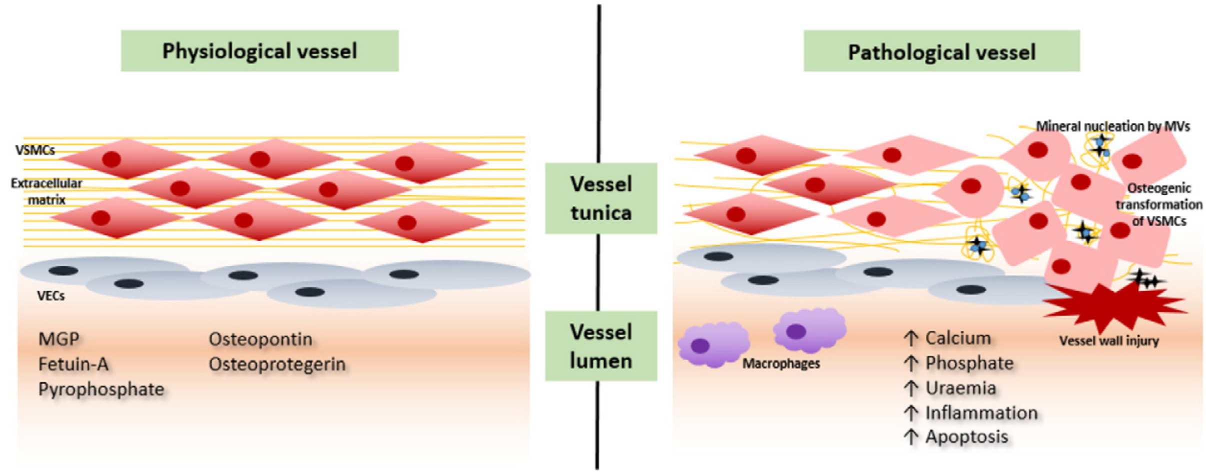

Fig. 2. Proposed mechanisms for vascular mineralisation. Under physiological conditions, VSMC phenotype is maintained by circulatory mineralisation inhibitors. Metabolic alterations such as inflammation, hypercalcemia or hyperphosphataemia induce the up-regulation of numerous osteogenic markers and loss of these mineralisation inhibitors. These events ultimately lead to osteogenic phenotypic transition and the secretion of calcifying MVs.

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 *Smpd3* 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 inhibitory 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 Ca^{2+} 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 regressed 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 Ca^{2+} 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 P_i-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)

MVs derived 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 Ca^{2+} 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 Ca^{2+} 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 for 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 PP_i, 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. P_iT1 is emerging as a key component in the pathogenesis of vascular mineralisation. Higher levels of P_i in the serum, due to the inability of the kidney to filter excess P_i, induces VSMCs to upregulate the expression of P_iT1, a predominant sodium-dependent phosphate co-transporter that leads to an accumulation of intracellular P_i. P_iT1 has been shown to induce VSMC osteogenic transition, marked by increased Runx2 expression [189]. Upstream regulation of P_iT1 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 P_i 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 mRNAs 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.

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