The importance of the SIBLING family of proteins on skeletal mineralisation and bone remodelling

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Abstract

The small integrin-binding ligand N-linked glycoprotein (SIBLING) family consists of osteopontin, bone sialoprotein, dentin matrix protein 1, dentin sialophosphoprotein and matrix extracellular phosphoglycoprotein. These proteins share many structural characteristics and are primarily located in bone and dentin. Accumulating evidence has implicated the SIBLING proteins in matrix mineralisation. Therefore, in this review, we discuss the individual role that each of the SIBLING proteins has in this highly orchestrated process. In particular, we emphasise how the nature and extent of their proteolytic processing and post-translational modification affect their functional role. Finally, we describe the likely roles of the SIBLING proteins in clinical disorders of hypophosphataemia and their potential therapeutic use.

Introduction

The skeleton is a highly intricate and complex organ that has a range of functions spanning from locomotion to ion homoeostasis. It is structurally adapted to suit its function: strong and stiff to withstand loading and yet light for movement and flexible to prevent fracture. The organic component of bone, termed the osteoid, comprises an extracellular matrix (ECM) primarily composed of collagen type I together with several non-collagenous proteins (NCPs).

One such family of NCPs is the small integrin-binding ligand N-linked glycoprotein (SIBLING) family. This consists of osteopontin (OPN), bone sialoprotein (BSP (IBSP)), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE). It is likely that this protein family arose from the secretory calcium-binding phosphoprotein family by gene duplication due to their apparent common evolutionary heritage, as is elegantly reviewed by Kawasaki & Weiss (2006), Kawasaki et al. (2007), Kawasaki (2011) and Rowe (2012).

All SIBLING proteins undergo similar post-translational modifications such as phosphorylation and glycosylation, the extent of which is crucial in determining their function (Boskey et al. 2009). It has long been known that the SIBLING proteins have an RGD sequence that facilitates cell attachment and cell signalling by binding to cell surface integrins (Fisher et al. 2001). More recently, work by Rowe et al. (2000, 2004), primarily focused on MEPE, has identified a new functional domain termed the acidic serine- and asparate-rich motif (ASARM) peptide, which is highly conserved across species. This peptide is proving critical in the functional activity of the SIBLING proteins, as is evidenced by the ASARM hypothesis proposed by Peter Rowe (Rowe 2004, David et al. 2010). This hypothesis describes the role of the SIBLING ASARM peptides, the cell membrane-associated glycoprotein phosphate-regulating endopeptidase homologue, X-linked (PHEX) and fibroblast growth factor 23 (FGF23) in bone renal phosphate (P_i) homoeostasis and mineralisation. This hypothesis can be used to explain numerous disorders of mineralisation including tumour-induced osteomalacia, autosomal-dominant...
hypophosphataemic rickets (ADHR) and X-linked hypophosphataemic rickets (XLH) and will be discussed in more detail in this review.

The SIBLING proteins have been extensively reviewed individually; however, in the present review, we focus on the role that each of the SIBLING proteins has on skeletal matrix mineralisation and bone remodelling, as well as their clinical relevance in disorders of bone matrix mineralisation and bone remodelling (Denhardt & Guo 1993, Ganss et al. 1999, Sodek et al. 2000, Fisher et al. 2001, Prasad et al. 2010).

Matrix mineralisation and bone remodelling

Endochondral ossification is a carefully orchestrated process responsible for the formation and postnatal linear growth of the long bones. It involves the replacement of a cartilage scaffold by mineralised bone. Integral to this process is the epiphysial growth plate, a highly specialised cartilaginous structure derived from a mesenchyme precursor that is located between the head and the shaft of the bone. The growth plate consists of chondrocytes arranged in columns that parallel the axis of the bone surrounded by their ECM that is rich in collagens, proteoglycans and numerous other NCPs (Ballock & O’Keefe 2003, Mackie et al. 2008, 2011, Gentili & Cancedda 2009, Heinegard 2009). Chondrocytes of the growth plate sit in distinct cellular zones of maturation and proceed through various stages of differentiation while maintaining their spatially fixed locations (Hunziker et al. 1987). It is the terminally differentiated hypertrophic chondrocyte that mineralises its surrounding ECM, localised to the longitudinal septa of the growth plate (Castagnola et al. 1988).

Chondrocyte, as well as osteoblast, mineralisation of the ECM is widely accepted to involve membrane-limited matrix vesicles (MV) within which calcium (Ca\(^{2+}\)) and inorganic P\(_i\) accumulate to initiate the biphasic process of mineralisation (Anderson 2003). When sufficient concentrations of both exist, Ca\(^{2+}\) and P\(_i\) begins to precipitate to form hydroxyapatite (HA) crystals. This initial stage of mineralisation is followed by the penetration of HA crystals through the MV trilaminar membrane and the modulation of ECM composition, promoting the propagation of HA outside of the MVs (Anderson 1995, 2003, Wu et al. 2002, Golub 2011). Mineralisation of the ECM is a tightly regulated process such that concentrations of Ca\(^{2+}\) and P\(_i\) are permissive for effective mineralisation and that the levels of mineralisation inhibitors such as inorganic pyrophosphate (PP\(_i\)) and matrix gela protein are balanced. Extracellular PP\(_i\) is a well-recognised and potent inhibitor of mineralisation that is regulated by ALP (Meyer 1984). In bone, ALP is an ectoenzyme located on the cell membrane’s outer surface of osteoblasts and chondrocytes as well as on the membrane of their MVs (Anderson 1995). Classically, ALP was thought to generate the P\(_i\) required for HA formation; however, it has since been shown to also hydrolyse PP\(_i\), thus achieving a ratio of P\(_i\)/PP\(_i\) permissive for HA crystal formation and growth (Moss et al. 1967, Majeska & Wuthier 1975, Hessle et al. 2002, Anderson 2003). PP\(_i\) inhibits the enzymatic activity of ALP, offering a feedback loop by which mineralisation is regulated (Addison et al. 2007).

Other regulators of ECM biomineralisation include nucleotide pyrophosphatase phosphodiesterase 1 (NPP1) and the ankylosis protein (ANK) that work in synergy to increase extracellular P\(_i\) levels. While NPP1 ectopically generates P\(_i\) from nucleoside triphosphates, ANK mediates its intracellular to extracellular channelling (Hakim et al. 1984, Terkeltaub et al. 1994, Ho et al. 2000). Analysis of mutant mice deficient in ALP function (Alp\(_{2}\)-/\(-/\) (Alp\(_{1}\)-/\(-/\))), which were surprisingly found to exhibit normal levels of bone mineralisation at birth, led us to search for other phosphatases that might also contribute to bone mineralisation, and this led to our description of PHOSPHO1 (Houston et al. 2002). As its discovery and characterisation, PHOSPHO1 has been proposed to play a crucial role in the accumulation of P\(_i\) within the MV and bone mineralisation (Houston et al. 2002, Stewart et al. 2006, Roberts et al. 2007, 2008, MacRae et al. 2010, Huesa et al. 2011). PHOSPHO1 has a non-redundant functional role during bone mineralisation, and the ablation of both PHOSPHO1 and ALP results in the complete lack of bone mineralisation throughout the whole skeleton (Yadav et al. 2011).

Mineralisation of the ECM not only facilitates the deposition of HA but also enables vascular invasion, a significant phase in endochondral ossification and the development of the skeleton. Hypertrophic chondrocytes express factors such as vascular endothelial growth factor (VEGF) that induce vascular invasion, allowing the infiltration of osteoclasts and differentiating osteoblasts that resorb the cartilaginous mineralised matrix and replace it with trabecular bone respectively (Zelzer et al. 2002). This process of bone remodelling continues throughout life and is responsible for the annual replacement of \(\sim 10\%\) of the adult skeleton (Frost 1990). Tight regulation of this process maintains an equilibrium such that disorders of bone mass, such as osteoporosis or osteopetrosis, do not occur (Manolagas 2000). During bone resorption, osteoclasts adhere to the bone surface forming a tight connection and allowing efficient resorption through extracellular acidification (Palokangas et al. 1997, Mellis et al. 2011). Like bone formation, this is under tight control by a variety of autocrine, paracrine, and endocrine factors and is thought to be primarily regulated by the terminally differentiated osteoblast, the osteocyte (Hill 1998, Manolagas 2000, Henriksen et al. 2009).

The SIBLING family of proteins

The SIBLING family of proteins consists of OPN, BSP, DMP1, DSPP and MEPE, all of which share common characteristics. Despite this, they display differential tissue distributions and functions that are highly dependent on their
post-translational modifications. The key role that each of the SIBLING proteins plays in biomineralisation is described in detail below (Fig. 1).

**Matrix extracellular phosphoglycoprotein**

MEPE, originally identified as a substrate for PHEX, is primarily expressed by osteocytes as well as by osteoblasts (Nampei et al. 2004). In the mouse skeleton, Mepe is detected as early as 2 days post partum, and several regulators of this expression have been documented in the literature (Lu et al. 2004). The addition of FGF2 to osteoblasts downregulates Mepe levels in a dose-dependent manner. The mechanism of action is part through the MAPK pathway (Zhang et al. 2004). Furthermore, osteoblasts stimulated by bone morphogenetic protein 2 (BMP2) also display a decreased Mepe expression level (Siggelkow et al. 2004). Recently, it has been shown that Wnt3a, a canonical Wnt signalling stimulator, induces this BMP2 signal and also as has its own direct stimulatory effects on Mepe expression through β-catenin and LEF1 (Cho et al. 2011).

The first evidence for a direct role of MEPE in bone mineralisation came from the increased mRNA expression levels of Mepe seen during osteoblast matrix mineralisation (Petersen et al. 2000, Argiro et al. 2001). The development of a Mepe null mouse further fuelled the proposed role of MEPE in mineralisation. This mouse model had increased bone mass with associated increased numbers and thickness of trabeculae. The mineral apposition rate (MAR) was dramatically increased as was the activity of Mepe null osteoblasts in culture (Gowen et al. 2003). Conversely, the overexpression of MEPE in mice, under the control of the col1a1 promoter, leads to a growth and mineralisation defect due to a decrease in bone remodelling. Mepe transgenic mice displayed wider epiphyseal growth plates and expanded primary spongiosa and a significant decrease in the MAR (David et al. 2009).

Like the other SIBLING proteins, the activity of MEPE is dependent on its state of cleavage and its phosphorylation. Recent work has identified the 2-2 kDa ASARM peptide of MEPE as the functional component of MEPE. This ASARM peptide is highly conserved across the SIBLING proteins, and in MEPE it is located immediately downstream of a small acidic serine- and asparagine-rich motif (ASARM) that undergoes post-translational phosphorylation. MEPE also inhibits the numbers and activities of osteoclasts. OPN has similar functional effects to MEPE in bone mineralisation; however, along with BSP, it is also expressed by osteoclasts. BSP is well established as a HA nucleator and is proving pivotal in diseases of increased bone formation as it increases osteoclastogenesis. DMP1 and DSPP are both expressed by bone and both are processed into numerous fragments. While DSPP promotes biomineralisation in both bones and teeth, DMP1 inhibits it. The full details of the cleavage products of the SIBLING proteins and their roles in biomineralisation are detailed in Table 1.
Table 1 The functional role of each of the SIBLING proteins: dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), bone sialoprotein (BSP), matrix extracellular phosphoglycoprotein (MEPE) and osteopontin (OPN), in biomineralisation and phosphate homeostasis, as is dependent on their cleavage and post-translational modification. Detailed are i) the cellular expression pattern, ii) the phenotype of the knockout mouse, iii) the phenotype of transgenic mice, iv) clinical conditions associated with mutation in this gene, v) cleavage product and post-translational modification, vi) known role of each cleavage product in ECM mineralisation, vii) role of cleavage product mineral metabolism, and viii) list of relevant references.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cellular expression pattern</th>
<th>Mouse bone phenotype</th>
<th>Cleavage product and post-translational modification</th>
<th>Role of cleavage products in mineralisation</th>
<th>Role of cleavage products in mineral metabolism</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>MEPE</td>
<td>Osteoblasts and osteocytes</td>
<td>Increased bone mass, MAR, trabecular number and thickness</td>
<td>Decreased MAR, bone remodelling, bone mass and increased growth plate widths</td>
<td>Unknown</td>
<td>ASARM peptide-3 serine phosphorylation</td>
<td>Inhibition</td>
</tr>
<tr>
<td>DMP1</td>
<td>Osteoblasts, osteoclasts, osteocytes, hypertrophic chondrocytes and dentin</td>
<td>Lower mineral content, defective cartilage formation resembling dwarfism with chondrodysplasia. Hypophosphataemia and increased FGF23</td>
<td>Narrow growth plate with accelerated mineralisation and increased bone turnover</td>
<td>Autosomal recessive hypophosphataemic rickets</td>
<td>Full-length, unphosphorylated DMP1</td>
<td>Promotion</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteoblasts, osteoclasts, osteocytes and hypertrophic chondrocytes</td>
<td>Increased mineral content and size</td>
<td>Bone phenotype not defined</td>
<td>Unknown</td>
<td>ASARM peptide-3 serine phosphorylation</td>
<td>Inhibition</td>
</tr>
<tr>
<td>BSP</td>
<td>Osteoblasts, osteoclasts, osteocytes, chondrocytes and dentin</td>
<td>Short hypomineralised bones with high trabecular bone mass and low bone turnover</td>
<td>Multi-dwarfism decreased BMD and decreased trabecular bone volume</td>
<td>Unknown</td>
<td>Bsp transgenic mice have increased Ca(^{2+}) levels but no difference in Pi levels compared with WT mice</td>
<td>Promoter</td>
</tr>
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</table>
The administration of the MEPE–ASARM peptide in vitro and in vivo can inhibit the uptake of P3. This is likely through a decreased expression of the type II sodium-dependent Pi cotransporter NPT2a, or through the promotion of FGF23 expression, a potent inhibitor of Pi (Liu et al. 2007, Dobbie et al. 2008, Marks et al. 2008, Martin et al. 2008, David et al. 2010, Shirley et al. 2010). MEPE may have a direct effect on matrix mineralisation outwith the supply and demand of Pi. The ASARM peptide of MEPE inhibits mineralisation by osteoblasts by directly binding to HA crystals (Addison et al. 2008, Martin et al. 2008). Integral to this inhibitory effect is the post-translational phosphorylation of the ASARM peptide at three serine residues. In osteoblasts, it appears that without this phosphorylation, the ASARM peptide has no effect on mineralisation (Addison et al. 2008, Martin et al. 2008). This is not the only evidence for a role for MEPE in the promotion of mineralisation. Recently, it has been shown that a truncated form of MEPE, which has the ASARM peptide removed, can promote bone mineralisation in culture and in mice (Sprowson et al. 2008). Furthermore, a mid-terminal fragment of MEPE (termed ‘AC100’) has been shown to enhance cell binding, through the stimulation of focal adhesion kinase and ERK (Hayashibara et al. 2004). Taken together, these results highlight the importance of post-translational processing in determining the functional role of MEPE.

The interaction between MEPE and PHEX is well documented in the literature. PHEX plays a central role in the protection of MEPE from proteolytic cleavage by cathepsin B; it can bind to MEPE and prevent the release of the ASARM peptide (Guo et al. 2002). The Hyp mouse, a spontaneous Phex knockout model, has an increased expression of cathepsin D, an upstream activator of cathepsin B (Rowe et al. 2006). This therefore suggests that PHEX can alter the activation of cathepsin B and therefore the cleavage of MEPE to the ASARM peptide. Furthermore, PHEX can bind to free ASARM peptides, therefore neutralising their activity by sequestration and hydrolysis (Liu et al. 2007, Addison et al. 2008, Martin et al. 2008). Recently, it has been shown that sclerostin (SCL), a potent inhibitor of the canonical Wnt signalling pathway, may act through the MEPE–PHEX axis, highlighting its significance in biomineralisation (Atkins et al. 2011).

Mepe transgenic mice display a decrease in ALP enzyme activity in both the growth plate and the primary spongiosa (David et al. 2009). In vivo, the addition of the phosphorylated ASARM peptide also reduced the number of ALP-positive cells in an osteoblast cell culture model (Martin et al. 2008). However, this remains controversial as normal ALP activity has been reported in osteoblasts treated with phosphorylated ASARM peptide (Addison et al. 2008). In the MEPE-overexpressing mouse, vascularisation is increased, as is VEGF expression, highlighting a role for MEPE in angiogenesis, an important stage in endochondral ossification (David et al. 2009). Consistent with angiogenesis is the cathepsin B cleavage site (Rowe et al. 2000). The administration of the MEPE–ASARM peptide in vitro and in vivo can inhibit the uptake of P3. This is likely through a decreased expression of the type II sodium-dependent Pi cotransporter NPT2a, or through the promotion of FGF23 expression, a potent inhibitor of Pi (Liu et al. 2007, Dobbie et al. 2008, Marks et al. 2008, Martin et al. 2008, David et al. 2010, Shirley et al. 2010).
infiltration of osteoclasts for bone resorption. Interestingly, mice administered with recombinant MEPE or transgenic for MEPE had a significant decrease in the numbers and activity of osteoclasts (Hayashibara et al. 2007, David et al. 2009). This therefore suggests that MEPE is highly relevant to both bone mineralisation and P₁, homeostasis. Future studies should focus on the interactions between MEPE and the Wnt signalling pathway due to its known implications in bone and cartilage mechanobiology.

**Osteopontin**

OPN, also known as secreted phosphoprotein 1 (SPP1), is a 34 kDa protein, originally identified as the bridge between the cells and HA in the ECM of bone (Sodek et al. 2000). The protein and gene structures, as well as the localisation, of OPN are well described in several excellent reviews (Denhardt & Guo 1993, Sodek et al. 2000, Fisher et al. 2001). In bone, OPN is produced by osteoblasts and osteocytes, as well as osteoclasts (Dodd et al. 1995, Sodek et al. 1995, Zohar et al. 1997). It has also been localised to hypertrophic cartilage of the growth plate (Landis et al. 2003). Several studies have documented the inhibitory role of OPN in HA formation and growth (Boskey et al. 1993, 2012, Hunter et al. 1994). It has also been shown to inhibit mineralisation in vascular smooth muscle cells (Wada et al. 1999, Jono et al. 2000). This inhibitory role of OPN is confirmed further by analysis of the Opn knockout mouse that has increased mineral content and size, as shown by Fourier transform infrared spectroscopy analysis in two different lines of Opn−/− mice at two different ages (Boskey et al. 2002). More specifically, it has recently been shown that the ASARM peptide of OPN inhibits ECM matrix mineralisation by binding to HA crystals (Addison et al. 2010, Boskey et al. 2012). Furthermore, a recent study by Boskey et al. showed the C- and N-terminal fragments of OPN, in this study, derived from milk OPN to promote de novo HA formation. Conversely, a central fragment inhibited it as is similar to bone OPN (Boskey et al. 2012). This highlights the importance of the post-translational fragmentation of OPN in determining its function. The study by Addison et al. (2010) also showed that, like MEPE, the ability of the OPN-ASARM to inhibit mineralisation is dependent on its phosphorylation at specific serine residues.

The importance of post-translational phosphorylation is further confirmed when examining the interaction between OPN, ALP and PP₁. Several studies have shown that ALP dephosphorylates OPN, thus preventing much of its inhibitory activity on HA formation and growth (Boskey et al. 1993, Hunter et al. 1994, Jono et al. 2000). Furthermore, PP₁, directly upregulates Opn expression in osteoblasts, and therefore the hydrolysis of PP₁ by ALP will have a significant effect on the expression levels of OPN (Addison et al. 2007). This is in concordance with the Enpp1-deficient mouse in which PP₁ deficiency brings about a deficiency in OPN (Johnson et al. 2003). The Akp2-deficient mouse displays a similar decreased PP₁ and OPN with an associated hypomineralisation. This hypomineralisation can be partially rescued by the double knockout: the Akp2−/−/Opn−/− mouse (Harney et al. 2006). Although previous studies have implicated a P₁-dependent mechanism (Beck et al. 2000, Beck & Knecht 2003), work by Addison et al. has implicated the MAPK signalling pathways responsible for the regulation of OPN by PP₁.

Analysis of the Opn−/− mouse has also indicated a role for OPN in the function and activity of osteoclasts. In these mice, there is an increase in osteoclast production, which could be a compensatory mechanism for the observed disabled motility and resorption activity of the osteoclast cells (Rittling et al. 1998, Chellaiah et al. 2003). Further studies have attempted to elucidate the precise role of OPN in bone resorption and have implicated CD44, a major cell surface receptor for hyaluronate (Aruffo et al. 1990) and a receptor for OPN (Suzuki et al. 2002, Chellaiah et al. 2003).

The loading of the skeleton in daily function results in the continuous modelling and remodelling of the skeleton (Frost 1990). This loading upregulates OPN expression in bone in vivo, and more recently it has been shown that the cyclical loading of rabbit joints has shown increased cellular OPN expression in the cartilage as well (Terai et al. 1999, Morinobu et al. 2003, Gross et al. 2005, King et al. 2005, Fujihara et al. 2006). This upregulation in response to loading has also been shown in in vitro cell cultures, and it is thought that MAPKs are involved in the transduction of the stimulus for OPN expression (Klein-Nulend et al. 1997, Owan et al. 1997, You et al. 2001). These intriguing results provide some clues into the molecular mechanisms underpinning adaptive bone remodelling.

**Bone sialoprotein**

BSP is a 70–80 kDa protein for which its gene and protein structures have been extensively reviewed (Ganss et al. 1999). The localisation of BSP is unique to the SIBLING family of proteins as it is exclusively located to the mineralised tissues such as bone, dentin and mineralising cartilage (Bianco et al. 1991, Chen et al. 1991). In bone, it is expressed in abundance by osteoblasts, as well as by osteoclasts, osteocytes and chondrocytes (Fisher & Fedarko 2003, Gordon et al. 2007).

During embryogenesis, BSP is first expressed at the onset of bone formation, thus suggesting it to be a strong candidate for a role in HA nucleation (Chen et al. 1992). This certainly seems convincing as numerous studies have documented BSP, which is localised to MVs, to be involved in the initial formation of HA (Harris et al. 2000, Fisher et al. 2001, Tye et al. 2003, Wang et al. 2006, Nahar et al. 2008). Indeed, the Bsp null mouse displays shorter, hypomineralised bones with associated higher trabecular bone mass with low bone turnover (Malaval et al. 2008). Moreover, it has been shown that as little as 9 nM BSP is required to nucleate HA.
Gordon et al. 2007). Similarly, osteoblast cultures grown in the presence of an anti-BSP antibody exhibit reduced mineralisation (Cooper et al. 1998, Mizuno et al. 2000). This nucleation potency is increased on BSP binding to collagen, suggesting a cooperative relationship (Baht et al. 2008).

The role of BSP as a HA nucleator is thought to involve the membrane-bound enzyme, ALP. Indeed, in the absence of BSP, high levels of ALP activity can promote the initiation of mineral deposition (Wang et al. 2006). This is further confirmed in BSP-overexpressing cell cultures that have a higher ALP activity (Valverde et al. 2008). It is likely that, like the other SIBLING proteins, the function of BSP is highly dependent on its post-translational modification (Stubbs et al. 1997).

BSP increases osteoclastogenesis and therefore bone resorption, making it crucial in the homoeostasis of bone remodelling (Ross et al. 1993, Raynal et al. 1996, Malaval et al. 2008, Valverde et al. 2008). This has been further examined in BSP transgenic mice in which an uncoupling of bone formation and resorption resulted in an osteopenia-like phenotype (Valverde et al. 2008). Furthermore, serum BSP expression in bone diseases characterised by excessive bone resorption, e.g. Paget’s disease, is abnormally high (Valverde et al. 2008). This highlights the need to investigate whether antibodies to BSP could decrease the pathological bone loss observed in the Bsp transgenic mouse and as such be an important therapeutic target for patients with bone diseases characterised by high BSP.

**Dentin sialophosphoprotein**

The role of DSPP in biominalisation has recently been reviewed (Prasad et al. 2010). Although originally thought to be exclusively expressed by dentin, DSPP is also expressed in bone, cementum and in non-mineralising tissues including the lung and kidney (Qin et al. 2002, Baba et al. 2004, Alvares et al. 2006, Ogbureke & Fisher 2007, Verdelis et al. 2008).

Analysis of the Dspp knockout mouse reveals defects in dentin mineralisation (Sreenath et al. 2003), as well as bone hypomineralisation (Verdelis et al. 2008). In humans, a mutation in the DSPP gene results in dentinogenesis imperfecta, characterised by dentin hypomineralisation and significant tooth decay (Kim et al. 2005). Of particular interest are the variations in the mineralisation properties observed at different ages in the Dspp−/− mouse. At 5 weeks of age, these mice displayed accelerated mineralisation, while at 9 months of age significant changes in bone structural properties were observed. This therefore suggests that DSPP has roles not only in the initial mineralisation of bone but also in the remodelling of the skeleton and therefore on bone turnover (Verdelis et al. 2008).

DSPP is proteolytically processed to two fragments: dentin phosphoprotein and dentin sialoprotein (DSP), both of which have important functions in mineralisation. Interestingly, a third fragment called dentin glycoprotein (DGP) has been identified as being cleaved from the C-terminal end of DSP by matrix metalloproteinase 2 (MMP2) and MMP20 (Yamakoshi et al. 2005). It has been suggested that the proteolytic processing of DSPP to DSP, DSP and DGP is the activating stage in the mechanism of DSPP function (Zheng et al. 2001, Qin et al. 2004, Prasad et al. 2010). The cleavage of DPP from DSPP is catalysed by a group of zinc metallopeptidases that includes BMP1, and it is this fragment of DSPP that contains the ASARM peptide (Tsuihysi et al. 2011). Various studies have shown DPP to be important in the formation and growth of HA as it has a strong affinity to Ca2+ when bound to collagen fibrils (Boskey et al. 1990, Saito et al. 1997, He et al. 2005). The phosphorylation of DPP is believed to be crucial to its function as removal of the phosphate groups results in a loss of its role in HA promotion (Saito et al. 1997). On the other hand, although DSP has been shown to be involved in the initialisation of mineralisation, it appears not to have a functional role in the maturation of the tissue (Suzuki et al. 2009). The mechanism by which DSPP regulates HA formation is thought to involve the canonical BMP2 signalling pathway as BMP2 has been shown to increase Dspp expression via BMPR Smads, Runx2 and Dlx5 (Iohara et al. 2004, Chen et al. 2008, Cho et al. 2010).

The vast information obtained about the DPP and DSP fragments over the past few decades serves to strengthen knowledge on the role of DSPP in biominalisation. Future studies should focus on the recently identified DGP fragment and its specific functional role, as well as further detailing the mechanisms of DSP and DPP functions.

**Dentin matrix protein 1**

DMP1 was first cloned from dentin and has since been identified in dentin, bone and cementum as well as in other non-mineralised tissues (George et al. 1993, MacDougall et al. 1998, Sun et al. 2011). In bone, DMP1 is primarily expressed not only by osteocytes but also by osteoblasts and hypertrophic chondrocytes (Toyosawa et al. 2001, Fen et al. 2002, Feng et al. 2003).

The first evidence of a role for DMP1 in biominalisation was its promotion of ECM mineralisation in MC3T3 cells overexpressing DMP1 (Narayanam et al. 2001). The generation of a Dmp1−/− mouse has further fuelled the potential role of DMP1 in bone mineralisation. The knockout mice have significantly lower mineral content when compared with their control counterparts (Ling et al. 2005). Interestingly, the re-expression of DMP1 in these Dmp1−/− mice rescues the skeletal defects seen (Lu et al. 2011).

Additionally, the Dmp1−/− deficient mice displayed a severe defect in cartilage formation as is similar to the human hereditary hypophosphatemic disease autosomal recessive hypophosphatemic rickets (ARHR) that is caused by mutations in Dmp1 (Feng et al. 2006, Farrow et al. 2009). These mice display a highly widened growth plate, suggesting an impairment of mineralisation at the chondro-osseous junction. Indeed, this cartilage defect results in a phenotype resembling dwarfism with chondrodysplasia (Ye et al. 2005).
It has since been shown that the distorted growth plates seen in the Dmp1 null mouse are in fact due to disorganisation as opposed to growth plate enlargement (Sun et al. 2010). Interestingly, the Dmp1 null mouse displays increased serum FGF23 levels and associated hypophosphataemia (Feng et al. 2006). Correction of this hypophosphataemia, by a high P_i diet, restored the Dmp1 null mouse growth plate defect (Feng et al. 2006). Furthermore, the DMP1+/− and FGF23+/− double knockout mice display growth plate widths similar to that seen in the single Fgf23 null mouse (Liu et al. 2008). This therefore suggests that the defective cartilage mineralisation observed in the Dmp1 null mouse is not simply a direct consequence of the lack of DMP1. More recently, a transgenic mouse has been developed the expresses a mutant form of Dmp1. The substitution of Asp213 with Ala213 blocks the processing of mouse Dmp1. Crossing this transgenic mouse with the Dmp1 null mouse recovered the growth plate disorganisation seen in the null mouse alone (Sun et al. 2011).

Like other SIBLING proteins, the proteolytic processing of DMP1 appears essential to its function and localisation. In bone and dentin, DMP1 is processed to two fragments: one 37 kDa fragment originating from the NH2-terminal and one 57 kDa fragment originating from the COOH-terminal (Qin et al. 2003). In DMP1, it is the COOH-terminal fragment that contains the ASARM peptide (Martin et al. 2008). The full-length DMP1 is expressed at much lower levels than its fragments, which themselves have different localisation patterns in bone (Huang et al. 2008, Maciejewska et al. 2008). In the growth plate, while the NH2-terminal fragment is localised to the resting, proliferation and pre-hypertrophic zones, the COOH-terminal fragment is found in the calcification front and ossification zone (Maciejewska et al. 2008).

The localisation of the COOH-terminal fragment is consistent with areas that are targets for the vascular invasion of the cartilage, a significant phase in matrix mineralisation. DMP1 has been postulated to play a role in angiogenesis as treatment with DMP1-induced vascular endothelial cadherin (VE-cadherin) and inhibited the VEGF-R2 activity, therefore suggesting DMP1 to be an inhibitor of VEGF-induced angiogenesis (Pirotte et al. 2011). The direct role of DMP1 on HA formation is highly dependent on its processing and its post-translational modification. When phosphorylated, full-length DMP1 has been shown to inhibit the formation and growth of HA; however, its dephosphorylated form and its two fragments are well-established nucleators of HA formation (He et al. 2003, Tartaix et al. 2004, Gericke et al. 2010). Thus, native DMP1 inhibits mineralisation unless it becomes cleaved or dephosphorylated, in which case it initiates mineralisation (Tartaix et al. 2004).

In addition to the ASARM peptide, signalling pathways are involved in DMP1 function and have recently been investigated in osteoblasts. Wu et al. (2011) showed that DMP1, through the activation of the αvβ3 integrin, activated the downstream effectors of the MAPK pathway, ERK and JNK (Wu et al. 2011). Concomitant to this is the stimulation of phosphorylated JNK translocation coupled with an upregulation of phosphorylated c-jun activation (Wu et al. 2011). Furthermore, it has been shown that the internalisation of DMP1 not only results in a release of stored Ca^{2+} but also activates p38 MAP kinase (Eapen et al. 2011). Dmp1 null mice have distinct abnormalities in the morphology and maturation of their osteocytes (Feng et al. 2006). The two DMP1 fragments also display differing localisation patterns in osteocytes (Maciejewska et al. 2009), suggesting that osteocytes may play a critical role in ECM mineralisation that involves DMP1. This is further supported by the stimulation of DMP1 expression in response to mechanical loading (Gluhak-Heinrich et al. 2007). Furthermore, the deletion of DMP1 leads to a dramatic increase in Fgf23 expression in the osteocytes, likely due to the defects seen in osteoblast-osteocyte transition (Feng et al. 2006, Qin et al. 2007). FGF23, a hormone produced by osteoblasts and osteocytes, has allowed the definition of bone as an endocrine organ as it targets the kidney to regulate P_i homeostasis. This therefore suggests that DMP1 can control P_i levels, as is consistent with the hypophosphataemia observed in the Dmp1 null mouse (Ye et al. 2005, Feng et al. 2006). This important discovery has allowed the further development of the ASARM hypothesis and has implicated DMP1 as central to biomineralisation and P_i homeostasis.

The ASARM hypothesis and bone diseases

Accumulating evidence has implicated the members of the SIBLING family of proteins in bone and mineralisation diseases. Their varying involvements in the process of matrix mineralisation make them potentially attractive candidates for therapeutic targets and therapies.

XLH is the most common form of inherited rickets, characterised by defective bone and tooth mineralisation, growth retardation and defective renal reabsorption of P_i (Carpenter et al. 2011). Mutations in PHEX have been associated with XLH in humans and have led to the development of the Hyp mouse (Holm et al. 1997). Hypophosphataemia alone is insufficient to explain the bone defect seen in the Hyp mouse as correction of the hypophosphataemia failed to correct the mineralisation defect observed (Ecarot et al. 1992, Rowe et al. 2006). Furthermore, when osteoblast cells from the Hyp mouse are grown in culture, they have defective ECM production and thus reduced mineralisation (Xiao et al. 1998). This therefore suggests that PHEX has multiple substrates that are involved in regulating mineralisation directly and this has allowed the creation of the ASARM hypothesis, as previously mentioned and as has recently been elegantly reviewed (Rowe 2004, 2012, David et al. 2010). The ASARM hypothesis is based on the concept of a minhibin, an unknown secreted factor that is a substrate for PHEX and therefore would accumulate in the Hyp mouse and in patients with XLH.

MEPE was first identified as a potential substrate for PHEX; however, in vitro studies have failed to demonstrate

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PHEX-dependent hydrolysis of MEPE (Guo et al. 2002). It has also been suggested that PHEX is likely responsible for the cleavage of DMP1 and DSPP, as it has a strong preference for cleaving bonds at the N-terminal of these two SIBLING proteins (Qin et al. 2004). However, analysis of the Hyp mouse indicated no differences in Dmp1 and Dspp expression in comparison with their WT controls, suggesting that DMP1 and DSPP are in fact properly processed in the Phex-deficient mouse (Zhang et al. 2010). In addition to this, there is an accumulation of SIBLING ASARM peptides in the Hyp mouse and patients with XLH (Liu et al. 2006). Accordingly, Fgf23 knockout reversed the hypophosphatemia observed in Hyp mice (Sitara et al. 2004). Although initial studies appeared to confirm Fgf23 as a substrate for PHEX, this has not been shown since (Bowe et al. 2001). Interestingly, a similar increase in FGF23 expression is observed in models of loss of DMP1, along with associated ARHR (Feng et al. 2006, Lorenz-Depiereux et al. 2006). This has led to the suggestion that a PHEX–DMP1 interaction is responsible for orchestrating mineralisation through decreasing FGF23 expression. Furthermore, current paradigm suggests that ASARM peptides can competitively displace this PHEX complex and this would therefore increase FGF23 activity, as is seen in the Hyp mouse and in patients with XLH (David et al. 2010, Martin et al. 2011, Boukessi et al. 2010).

It also appears that PHEX regulates Fgf23 expression as increased Fgf23 expression is observed in the Hyp mouse and patients with XLH (Liu et al. 2006). Accordingly, Fgf23 knockout reversed the hypophosphatemia observed in Hyp mice (Sitara et al. 2004). Although initial studies appeared to confirm Fgf23 as a substrate for PHEX, this has not been shown since (Bowe et al. 2001). Interestingly, a similar increase in FGF23 expression is observed in models of loss of DMP1, along with associated ARHR (Feng et al. 2006, Lorenz-Depiereux et al. 2006). This has led to the suggestion that a PHEX–DMP1 interaction is responsible for orchestrating mineralisation through decreasing FGF23 expression. Furthermore, current paradigm suggests that ASARM peptides can competitively displace this PHEX complex and this would therefore increase FGF23 activity, as is seen in the Hyp mouse and in patients with XLH (David et al. 2010, Martin et al. 2011, Boukessi et al. 2010).

Additionally, the accumulation of ASARM peptides can directly inhibit Na⁺-dependent P₃ uptake in the kidney, as has been shown both in vivo and in vitro, thus exacerbating the upregulation of FGF23 expression, the downregulation of 1,25(OH)₂D₃ and the inhibition of hypophosphatemia observed in XLH, ARHR and ADHR (Rowe et al. 2004, Dobbie et al. 2008, Marks et al. 2008, David et al. 2010, Shirley et al. 2010). The decrease in 1,25(OH)₂D₃ provides a feedback loop for increased PHEX expression through the increased expression of a 100 kDa transcription factor, a requirement for this PHEX expression (Ecarot & Desbarats 1999).

This regulatory loop of ASARM, PHEX and FGF23 expression and function highlights the multiple and complex functions of the SIBLING ASARM peptides in both P₃ homoeostasis and matrix mineralisation in disease and health. It is therefore vital that we endeavour to fully establish the interactions within this hypothesis to allow future therapeutic developments.

Certainly, much remains to be learnt regarding the in vivo role of the SIBLING proteins and the ASARM peptide in bone diseases. This is not just in disorders related to P₃ homoeostasis but also to other bone diseases such as osteoporosis and osteoarthritis (OA). Indeed, there are close links between the SIBLING proteins and OA, with serum BSP and OPN levels significantly correlating with OA disease severity (Petersson et al. 1998, Hasegawa et al. 2011). Furthermore, microarray data and gene analysis studies have highlighted MEPE and DMP1 as being differentially expressed in OA tissues (Hopwood et al. 2007, Sanchez et al. 2008). The interaction between MEPE and SCL, as described previously, is an exciting development due to the known anabolic effects of the SCL-neutralising antibodies on osteoporosis (Li et al. 2009, 2010, Atkins et al. 2011). This could therefore warrant investigation into the potential therapeutic use of MEPE in osteoporosis and potentially in OA due to the ever emerging role of SCL in this debilitating disease (Power et al. 2010, Chan et al. 2011, Delgado-Calle et al. 2011).

Conclusions

The aim of this review is to present an overview of the role of each member of the SIBLING family of proteins in matrix mineralisation. The SIBLING proteins are principally found in bone and dentin and are secreted into the ECM during its formation and subsequent mineralisation. It is apparent that the functional role of the SIBLING proteins is highly dependent on their state of cleavage and their post-translational modification (Table 1). Furthermore, the identification of the ASARM peptide, which is present across the SIBLING proteins, is proving critical in the functional activity of the SIBLING proteins. Future investigations should focus on determining the underpinning interactions between the SIBLING proteins and their place within the current ASARM hypothesis. This will allow the investigation into their potential therapeutic application to disorders of mineralisation including disorders of hypophosphataemia, osteoporosis and OA.

Declaration of interest

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

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Author contribution statement

K.S drafted the manuscript. K.S, V.M and C.F revised the manuscript content. K.S, V.M and C.F approved the final manuscript.
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