Microcalcifications in breast cancer: Lessons from physiological mineralization.

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Abstract

Mammographic mammary microcalcifications are routinely used for the early detection of breast cancer, however the mechanisms by which they form remains unclear. Two species of mammary microcalcifications have been identified; calcium oxalate and hydroxyapatite. Calcium oxalate is mostly associated with benign lesions of the breast, whereas hydroxyapatite is associated with both benign and malignant tumors. The way in which hydroxyapatite forms within mammary tissue remains largely unexplored, however lessons can be learned from the process of physiological mineralization. Normal physiological mineralization by osteoblasts results in hydroxyapatite deposition in bone. This review brings together existing knowledge from the field of physiological mineralization and juxtaposes it with our current understanding of the genesis of mammary microcalcifications. As an increasing number of breast cancers are being detected in their non-palpable stage through mammographic microcalcifications, it is important that future studies investigate the underlying mechanisms of their formation in order to fully understand the significance of this unique early marker of breast cancer.

Keywords: microcalcifications; breast cancer; mineralization; calcifications; hydroxyapatite, mammography.

Abbreviations

ALP: alkaline phosphatase; ASARM: acidic serine- and aspartate-rich MEPE-associated motif; BI-RADS: breast imaging reporting and data system; βG: β-glycerophosphate; BMP2: bone morphogenic protein 2; BSP: bone sialoprotein; Ca: calcium; DMP1: dentin matrix protein 1; DSPP: dentin sialophosphoprotein; DPP: dentin phosphoprotein, DSP: dentin sialoprotein; ECM: extracellular matrix; G: glycerol; HA: hydroxyapatite; MEPE: matrix extracellular phosphoglycoprotein;MMP: matrix metalloproteinase; MMs: mammary microcalcifications; NPP1: nucleotide pyrophosphate phosphodiesterase 1; OPN: osteopontin; OSC: osteocalcin; OSN: osteonectin; PFA: phosphonoformic acid; Pi: inorganic phosphate; PPi: inorganic pyrophosphate; SIBLING: small integrin-binding ligand N-linked glycoprotein; .

INTRODUCTION

Breast cancer is a worldwide public health problem and is the most common cause of cancer deaths, accounting for approximately 16% of cancer deaths in adult women [1]. Mammography is used for the early detection of breast cancer and 30-50% of non-palpable breast cancers are detected solely through the appearance of microcalcifications during a mammogram scan [2]. Mammary microcalcifications are calcium deposits within the breast tissue and their mammographic appearance was first described in 1951 [3]. Mammary microcalcifications can be classified according to their appearance on a mammogram based on the Breast Imaging Reporting and Data system (BI-RADS) developed by the American College of Radiology. Some of the typical classifications include powdery, crushed stone-like and casting type calcifications, as shown in figure 1 [4].

There is mounting evidence to suggest that the morphological appearance of mammographic microcalcifications is associated with patient prognosis. Several studies have shown that breast cancer patients presenting with small tumors and mammographically detected casting type calcifications have a poor survival rate for this tumor size category [4-6]. There is also more recent evidence that invasive ductal carcinoma presenting with calcifications have a larger tumor size, increased lymph node involvement and decreased 8-year patient survival [7]. In addition, this study demonstrated that tumors with casting type

calcifications were associated with worse survival rates than those with non-casting type calcifications [7]. Studies have also suggested that clustering of microcalcifications could be used as a diagnostic tool to distinguish between benign and malignant lesions of the breast [8-10]. However, not all studies are in agreement that clustering of microcalcifications or casting type calcifications are related to patient outcome [11-13]. It is possible that the molecular structures of microcalcifications are a more important factor related to patient prognosis.

Two types of mammary microcalcifications have been identified and characterized on a molecular level; type I composed of calcium oxalate and type II composed of hydroxyapatite [14]. Calcium oxalate is associated with benign breast conditions or at most lobular carcinoma *in situ*, whereas hydroxyapatite is associated with both benign and malignant breast tissue [14-17]. Raman spectroscopy has been a useful tool to distinguish between hydroxyapatite found in benign breast tissue and hydroxyapatite associated with malignant breast cancer [17]. The carbonate content of hydroxyapatite is reduced significantly when progressing from benign to malignant breast disease [18]. Raman spectroscopy of mammary microcalcifications represents a novel non-invasive procedure that could be used in conjunction with mammography to aid in the detection of breast cancer [19].

Despite the importance of mammographic mammary microcalcifications for the initial detection of breast cancer and their potential prognostic value, limited research has been carried out to determine how and why these mammary microcalcifications are formed within the tumor microenvironment and it has been traditionally thought that they are formed by cellular degeneration. However, the process of physiological mineralization resulting in the hydroxyapatite deposition in bone is well documented and accepted as an active cell-mediated process [20]. This review brings together existing knowledge from the field of physiological mineralization and juxtaposes it with our current understanding of the genesis of mammary microcalcifications in order to better understand the significance of this unique early marker of breast cancer.

MODELS OF MINERALIZATION

Physiological mineralization is widely considered to be a regulated process and is restricted to specific sites in skeletal tissues, whereas pathological mineralization occurs in soft tissue [21]. It has been suggested that the mechanisms regulating pathological mineralization might be similar to those regulating physiological mineralization [21-23]. As limited research has been carried out on the molecular mechanisms involved in pathological mammary mineralization, lessons from other mineralization studies may be useful to establish whether a similar mechanism is taking place for mammary cells. In order to study the molecular process of hydroxyapatite deposition, *in vitro* models of mineralization have been developed. Originally used to investigate the physiological process of bone formation, these models have since been expanded to study pathological mineralization that occurs in soft tissue.

In vitro models of mineralization

The exogenous addition of organic phosphate to induce *in vitro* mineralization was first explored in the early 1920s using ossifying cartilage [24, 25] and β-glycerophosphate has become routinely used in mineralization studies in the years since these original experiments were carried out [26-28]. This is based on the concept that organic phosphate is broken down into inorganic phosphate and the phosphate ions produced can be used by the cells to create hydroxyapatite. The direct addition of inorganic phosphate has been used successfully in place of β-glycerophosphate as a source of phosphate for physiological and pathological models of mineralization *in vitro* [29, 30]. While β-glycerophosphate alone is sufficient to induce *in vitro* mineralization of osteoblast and chondrocyte cells [26, 28, 29], the addition of ascorbic acid is also routinely used to further enhance mineralization. This combination of β-glycerophosphate and ascorbic acid is often referred to in the literature as an osteogenic cocktail. As every cell culture system requires a different growth medium, the exact mineralizing conditions naturally varies between cell types and cell lines. However, it is the addition of β-glycerophosphate and ascorbic acid to a cell lines particular growth medium that has lead to the use of the generic term ‘osteogenic cocktail’ in the literature. Addition of ascorbic acid to the osteogenic cocktail is thought to enhance β-glycerophosphate induced mineralization through the upregulation of alkaline phosphatase activity and collagen

production [31, 32]. Dexamethasone, a synthetic glucocorticoid, is also sometimes added to enhance mineralization of certain cell lines [33-35]. It has been suggested that the effect of dexamethasone is species-specific, as in the mouse pre-osteoblast MC3T3-E1 cell line addition of dexamethasone decreases mineralization compared to the osteogenic cocktail alone [36].

Based on these studies, β-glycerophosphate and the osteogenic cocktail has been successfully used to study pathological mineralization of C4-2B prostate cancer cells, Madin-Darby canine kidney cells and bovine vascular smooth muscle cells *in vitro* [37-39]. More recently, treatment with an osteogenic cocktail to induce *in vitro* mineralization has also been applied to a panel of mammary cell lines [40]. Only the more aggressive mammary cell lines investigated had a tendency to mineralize *in vitro* when treated with the osteogenic cocktail. The addition of dexamethasone to the osteogenic cocktail enhanced mineralization of the Hs578Ts(i)$_8$ human mammary cell line, but decreased mineralization of the mouse mammary 4T1 adenocarcinoma cell line [40], further supporting the idea that dexamethasone has a species-specific effect. The type of mineral deposited by each mineralizing mammary cell line was identified by Raman spectroscopy as hydroxyapatite [40], which is a clinically relevant species of calcium found in breast tissue.

While β-glycerophosphate was used as a source of phosphate for these *in vitro* studies, the source of phosphate used by mammary cells to produce microcalcifications *in vivo* remains unexplored. However phosphate is abundant in the human body in the form of adenosine phosphates (AMP, ADP and ATP) in DNA and RNA and can be released by hydrolysis of ATP or ADP. It is possible that within the breast tumor microenvironment highly proliferating cells may cause a localized increase in phosphate, which could then be used by the cells to form hydroxyapatite. The idea that ATP acts as a source of phosphate for mineralization is supported by studies demonstrating that the addition of exogenous ATP to osteoblast cultures results in hydroxyapatite formation [41, 42], Inorganic pyrophosphate is another potential source of phosphate for hydoxyapatite production. Alkaline phosphatase is known to hydrolyze inorganic pyrophosphate [43], which produces inorganic phosphate that would be readily available for hydroxyapatite crystal formation. Polyphosphates have also been implicated as a source of phosphate during biomineralization. Polyphosphates are

located in areas of resorbing bone and calcifying cartilage [44] and there is evidence to suggest that polyphosphates induce osteoblast differentiation and bone mineralization [45]. It has been hypothesized that polyphosphates in the extracellular matrix may form a complex with calcium [44]. It is thought that alkaline phosphatase then cleaves the complex releasing calcium and phosphate, which are then available for hydroxyapatite formation [44]. While there is evidence to suggest that ATP, pyrophosphate and polyphosphates may act as sources of phosphate for physiological mineralization, the source of phosphate remains unexplored for mammary mineralization.

Bone morphogenetic protein 2

Bone morphogenetic protein 2 (BMP2) is a member of the transforming growth factor superfamily and has been added to cultures of osteoblasts to induce mineralization *in vitro* [46-50]. Enhanced mineralization induced by BMP2 has been shown to be associated with increased alkaline phosphatase activity [51, 52], which is essential for physiological mineralization [53]. The primary substrate of tissue-nonspecific alkaline phosphatase is inorganic pyrophosphate, which is a known inhibitor of hydroxyapatite formation [54]. Alkaline phosphatase has been shown to hydrolyze inorganic pyrophosphate, thereby removing its inhibitory effect [43]. The interactions between alkaline phosphatase and inorganic phosphate are discussed further in a subsequent section entitled ‘inorganic phosphate’.

The potential role of BMP2 in breast cancer and mammary microcalcifications is of particular interest in the literature, as BMP2 is also known to be expressed in human breast cancers [55]. There is evidence to suggest that BMP2 enhances angiogenesis and the survival of tumors *in vivo* [56, 57]. The overexpression of BMP2 in breast cancer MCF-7 cells increases the cells resistance to hypoxia-induced apoptosis [58]. Treatment with BMP2 has also been shown to increase the migration and invasive potential of numerous cell lines, including mammary cells [59-61]. BMP2 may also be an important mediator of mammary mineralization as BMP2 has recently been used in combination with the osteogenic cocktail to enhance mineralization of 4T1 mammary cells *in vitro* [62]. Studies by Liu *et al.* have also investigated the role of BMP2 in mammary microcalcifications by adapting rat breast tumors to cell culture, transducing them with adenoviral BMP2 and inoculation them into the

mammary fat pads of syngeneic Fisher 344 female rats. It was found that within 3 weeks of the study 100% of mammary tumors developed hydroxyapatite microcalcifications [63]. Additional work published by the same group has shown that a single intraperitoneal injection of recombinant BMP2 into rats bearing a syngeneic breast cancer produced dose-dependent and time-dependent microcalcifications in 100% of tumors [64].

LOCATION OF INITIAL MINERAL FORMATION

When investigating the molecular mechanisms of mineralization, one issue that arises is the location of initial crystal formation. The main candidates for nucleating hydroxyapatite are apoptotic bodies, extracellular matrix vesicles, intracellular vesicles and the extracellular matrix. Each of these are discussed below, however they are most likely not mutually exclusive. It is possible that each of these may contribute to both physiological and pathological mineralization.

Apoptotic bodies

Apoptotic bodies are membrane-bounded structures that result from programmed cell death [65]. Phosphate is mainly an intracellular ion, whereas calcium is mainly an extracellular ion. However, during cell injury or death the ion barrier formed by the cell membrane can be disrupted. It has been suggested that calcium and phosphate ions may then accumulate on the surface of apoptotic bodies through their external phosphatidylserine residues [22]. Apoptosis is known to be a regulated process involved in bone turnover [66], but there are conflicting reports as to the role of apoptosis in physiological mineralization itself [67, 68].

The potential role of apoptosis in pathological mineralization has been investigated in the literature. Mineralization in rat aortic tissue and vascular smooth muscle cells coincides with an increase of apoptosis [30, 69]. Inhibition of apoptosis using a caspase inhibitor resulted in a decrease in mineralization of vascular smooth muscle cells by ~40%. In addition, stimulation of apoptosis using anti-Fas IgM resulted in a 10-fold increase in calcification [70]. However, the presence of apoptotic bodies may merely contribute to mineralization by acting as a physical substrate for calcium and phosphate ions, which would be contributing more-so by default than an active regulated process. The role of apoptotic bodies during mammary

mineralization has not been specifically investigated. However, it has recently been show in a 3D model of mammary mineralization that the metabolic activity of the mammary cells increases over time coinciding with increased mineralization [62]. This suggests that an active regulated process may be underlying the formation of mammary microcalcifications, rather than a mechanism of cell death as has been previously assumed.

Matrix vesicles

It is generally accepted that matrix vesicles are more likely to be involved in the process of physiological mineralization than apoptosis. Matrix vesicles are membrane-bounded structures that are approximately 100nM in diameter [71]. They are released by budding from specific regions on the surface of cell membranes of chondrocytes, osteoblasts and odontoblasts [71]. Matrix vesicles have long been implicated in the role of biomineralization [72-74]. There is evidence to suggest that mineralization first occurs in matrix vesicles and subsequently spreads from the vesicles to the extravesicular interstices and then into adjacent collagen fibrils [75]. This spatial and temporal relationship of mineral deposition between matrix vesicles and collagen fibrils has been observed using electron microscopy techniques on turkey leg tendons [75]. It is thought that matrix vesicles accumulate calcium through calcium-channels, involving annexins, which are concentrated in the surface of matrix vesicles [71, 76]. The potential role of annexins in physiological mineralization is discussed in more detail below.

Matrix vesicles have also been implicated in the process of pathological mineralization. Calcifying human and bovine vascular smooth muscle cells have been shown to produce matrix vesicles *in vitro* [38, 77]. In addition, matrix vesicles have been visualized by transmission electron microscopy (TEM) in the case of tympanosclerosis, which is a pathological condition whereby mineral deposition occurs within the middle ear [78]. The human osteosarcoma cell lines Saos-2 and U-2 OS have both been shown to produce matrix vesicles *in vitro* [79, 80] and hydroxyapatite was confirmed within the matrix vesicles produced by Saos-2 cells [80]. While matrix vesicles are specifically functionalized to initiate mineralization many cell types shed membranous vesicles *in vitro*, although their exact relationship to matrix vesicles is unknown. Prostate cancer cells are capable of shedding

membrane vesicles from their cell surface in response to osteoblast conditioned media [81]. Shedding of membrane vesicles has also been observed *in vitro* for several mammary cell lines [82-84]. The number of membrane vesicles shed from human mammary cells correlates with their *in vitro* invasiveness [84]. However, the potential involvement of matrix vesicles in mammary mineralization has yet to be explored.

Intracellular mineralization

In addition to extracellular matrix vesicles, intracellular vesicles have also been implicated in the process of mineralization. In a study using mouse mandibular cartilage, intracellular vacuoles containing electron-dense granular material could be seen to occasionally open into the extracellular matrix [85]. Additional studies have reported similar findings for chondrocytes [86] and osteoblasts [87]. A mechanism for intracellular mineralization was proposed by Rohde *et al.* based on their TEM results [87]. It is proposed that intracellular calcium ions precipitate in the presence of phosphate, which results in the formation of needle-like crystals within the cytoplasm of the osteoblast. The crystals aggregates are surrounded by a membrane within the cell, migrate to the surface of the cell membrane and fuse to it, thereby releasing their contents into the extracellular space. Over time, mineral aggregates build up outside the cell and grow within the extracellular collagen matrix [87].

Intracellular mineralization has also been implicated in the process of pathological mineralization. Calcification of Purkinje cells of the cerebellar cortex in rats have been shown to possess electron-dense bodies containing calcium and phosphorus [88]. Hydroxyapatite nucleation within intracellular membrane-bound compartments has also been observed in Madin-Darby canine kidney epithelial cells [37]. Numerous small calcified vesicles were seen to be distributed throughout the cytoplasm and hydroxyapatite subsequently spread beyond individual organelles [37]. Intracellular hydroxyapatite crystals have also been detected in the sarcoplasm of mineralizing myocytes [89]. There is some evidence to suggest that intracellular vesicles are associated with mammary microcalcifications. Cytoplasmic calcification was observed in membrane-bound vesicles within breast tumor cells and the crystalline material was identified as hydroxyapatite [90], leading the authors to suggest that

the process of mammary mineralization is due to an active secretory process rather than necrosis or cellular degeneration [90].

Extracellular mineralization

It is thought that mineralization occurs in two phases; the initial formation of hydroxyapatite within vesicles followed by propagation in the extracellular matrix [75, 91]. However, an alternative view is that apatite is nucleated directly by matrix macromolecules, particularly collagen. Evidence obtained from electron microscopy imaging of calcifying leg tendons suggests that mineralization of collagen may occur on fibril surfaces [92]. More specifically, collagen hole zones have been implicated in accommodating apatite crystal nucleation [93, 94]. However, it has been suggested that non-collagenous macromolecules bound to fibrous collagen initiate mineral induction more intensely than collagen [95]. Non-collagenous matrix proteins implicated in direct nucleation includes phosphoproteins, phospholipids and proteolipids [91, 96-98]. Binding of phosphoproteins to collagen at hole zones is thought to be necessary for the initiation of mineralization [99]. It has been shown *in vitro* that dentin phosphoproteins must be irreversibly bound to collagen fibrils for mineralization of the collagen network to occur [100].

While the exact location of mammary mineralization has not been investigated, collagen is a major component of the extracellular matrix of mammary cells. The tumor microenvironment plays a critical role in the development and progression of cancer, by constantly modulating cell-matrix interactions [101]. It has been shown that tumor-initiating cells cultured on collagen induces a more differentiated phenotype [101]. The potential role of collagen in breast cancer and mammary microcalcification formation is discussed in more detail in a subsequent section of this review.

PHOSPHATE TRANSPORT

While physiological mineralization may be initiated in matrix vesicles and/or intracellularly, the question arises as to how phosphate is transported across the membrane into these structures in order to create hydroxyapatite? Sodium-phosphate (Na-Pi) cotransporter pumps are highly implicated in this process. There are three families of Na-Pi cotransporters; type I

(also known as SLC17), type II (also known as SLC34) and type III (also known as SLC20) [102]. The role of type I Na-Pi cotransporters has yet to be investigated in the process of physiological mineralization. However, there is evidence that both type II and type III cotransporters are involved. The type III Na-Pi cotransporter family consists of two forms of cotransporters; Pit1 and Pit2. It has been shown for the pre-osteoblast MC3T3-E1 cell line that the expression of Pit1 increases over time along with *in vitro* mineralization [49, 103]. The type II family of cotransporters have also been implicated in the process of cellular mineralization, which has been shown through numerous studies using phosphonoformic acid (PFA), which is known to inhibit all three members of the type II Na-Pi cotransporter family [104]. The addition of PFA to osteoblast cells *in vitro* inhibits inorganic phosphate induced, β-glycerophosphate induced and BMP2 induced mineralization [49, 105].

It has been suggested that phosphate transport also plays a physiological role in the mammary gland, as the NaPi-IIb cotransporter is highly expressed in lactating mammary gland tissue of mice and goats [106, 107]. This transporter is also associated with membrane vesicles isolated from goats milk [107]. Therefore dysregulation of this system may result in pathological conditions. One such study found elevated expression of the type II NaPi-IIb (SLC34A2) cotransporter in the mRNA of breast cancer tumors compared to their normal adjacent tissue [108]. In addition, it has recently been shown that β-glycerophosphate induced and inorganic phosphate induced mineralization of the 4T1 mouse mammary cell line *in vitro* is inhibited by treatment with PFA [40]. This indicates that phosphate transport may play an important role in mammary cell mineralization.

CALCIUM TRANSPORT

The source of calcium and its transport to potential sites of physiological mineralization is not fully understood, but there are two possible explanations that have been explored in the literature: (i) calcium may be released from intracellular stores such as mitochondria and (ii) calcium channel forming annexins may transport calcium into matrix vesicles. It is well known that mitochondria store calcium intracellularly [109]. The uptake of calcium into mitochondria occurs through the 40kD uniporter MCU [110] and calcium release occurs through the Na^{+}/Ca^{2+} exchanger NCLX [111]. There is evidence to suggest that inorganic phosphate

accompanies calcium on its way into the mitochondrial matrix, where it forms a precipitate [112] mostly consisting of hydroxyapatite [113]. More recently, during the *in vitro* mineralization of osteoblasts, calcium phosphate was identified within osteoblast mitochondrial granules and intracellular vesicles that transport material to the extracellular matrix [114]. In addition, the authors observed calcium-containing vesicles conjoining mitochondria and suggested a mechanism may exist in osteogenic cells whereby ionic calcium and perhaps phosphate are transferred from mitochondria to intracellular vesicles [114].

Calcium channel forming annexins may be another key factor involved in calcium transport during physiological mineralization. The annexin calcium channel blocker K-201 inhibits calcium uptake into matrix vesicles isolated from mineralizing chrondrocytes [115]. These vesicles contain annexins II, V and VI [116] and calcium uptake is inhibited by antibodies specific for annexin V [116]. Annexins V and VI also enhance mineral formation when incorporated into synthetic nucleation complexes, whereas annexin II has no significant effect [117]. However, despite these compelling findings *in vitro*, there is conflicting evidence for an essential role of annexins in biomineralization *in vivo*. Annexin V deficient mice do not display any impairment of skeletal development [118] and mice lacking annexin V, annexin VI and collagen X display no change in biomineralization in femora at 1 month and 1 year old [119]. Similarly, no obvious mineralization related phenotype was observed in 13 day old mice deficient for both annexins V and VI [120]. However, age may be an important consideration. Annexin VI knockout mice have delayed mineralization in growth plates, as shown in embryonic and newborn mice [121, 122]. However, mineralization is less affected in the growth plate during later postnatal bone development and in adult bone [121, 122].

The role of annexins has also been explored in breast cancer and annexin VI in particular is thought to act as both a tumor suppressor and a motility promoting factor [123, 124]. Annexins I and II may also be involved in breast cancer cell mobility and invasion [125-128]. While annexin I expression is strongest in non-tumor and benign breast lesions, expression is higher in lymph node metastases compared to corresponding primary breast cancer [129]. In addition, overexpression of annexin I in invasive cancer is related to an unfavourable prognosis [130]. This suggests that annexins may play a multifaceted role in

cancer progression. While the role of annexins has not been specifically explored in relation to mammary mineralization, it has been shown that MCF-10a mammary cells do not express annexin VI [123]. A separate study has shown that MCF-10a cells are not capable of mineralizing *in vitro* [40]. It is possible that the lack of annexin VI in this cell line impairs calcium transport and thereby inhibits mineralization. Therefore further studies should investigate the possibility that expression of annexins may correlated with mineralization potential of mammary cell lines.

INDUCERS AND INHIBITORS OF BIOMINERALIZATION

Once hydroxyapatite crystals have been nucleated, further growth and proliferation of the crystals takes place within the extracellular matrix. This is a tightly regulated process that can be enhanced or inhibited by a number of bone matrix proteins. Three phases are involved in *in vitro* osteoblast mineralization; proliferation, matrix development and maturation and mineralization [20]. During these two later stages a number of non-collagenous proteins are upregulated, including but not limited to osteopontin, bone sialoprotein and alkaline phosphatase [20]. The roles of several of these bone matrix proteins in physiological mineralization and potential involvement in breast cancer and mammary mineralization are discussed below.

Collagen type 1

Collagen is the most abundant protein in the extracellular bone matrix and is essential for bone formation. Collagen type 1 is the most well documented form associated with physiological mineralization and is secreted in the form of a precursor from osteoblasts. Extracellular processing results in mature three-chained collagen type 1 molecules, which assemble into collagen fibrils [131]. The fibrils then combine to form larger fibers. This network of collagen fibers acts as a natural scaffold for hydroxyapatite crystals to grow between [22]. Collagen is also a major component of the extracellular matrix (ECM) of breast tissue. Breast cancer cells are known to penetrate through tissue barriers into the ECM and degrade the collagen in the ECM [132]. Alteration of the shape of collagen fibers is associated with breast disease. Malignant breast tissue contains long, straight collagen fibers exhibiting

minimal curvature, whereas normal breast tissue contains collagen fibers with a greater degree of curvature [133]. Collagen may be a useful biomarker for the diagnosis of bone metastasis in breast cancer, as peptides of collagen type 1 are elevated in the serum from breast cancer patients with bone metastasis [134, 135]. Malignant breast tumors themselves have also been shown to have increased expression of type I and type III collagen mRNA compared to benign tissue [136]. There is some evidence to suggest that collagen may be involved in the formation of mammary microcalcifications. It has been reported that new fine microcalcifications form within breast tissue at biopsy sites following surgical implantation of a bioresorbable collagen plug [137]. This indicates that the presence of collagen may contribute to the formation of mammary microcalcifications. In addition, it has recently been show that there is an upregulation of collagen type 1 mRNA during the process of *in vitro* mammary mineralization [62]. Collagen fibers in the ECM could act as a substrate for hydroxyapatite crystal growth as observed in the extracellular bone matrix. This concept has been explored using 3D collagen scaffolds, which were found to support the growth and mineralization of breast cancer cells *in vitro* [62].

SIBLING proteins

The extracellular matrix of bone also contains non-collagenous proteins that are involved in mineralization. One such category of non-collagenous proteins is the SIBLING (Small Integrin-Binding LIgand, N-linked Glycoprotein) family, which consists of osteopontin, bone sialoprotein, dentin matrix protein 1, dentin sialophosphoprotein and matrix extracellular phosphoglycoprotein [138]. The role of each of these in physiological mineralization and their potential role in breast cancer is discussed below.

Osteopontin

Osteopontin (OPN) is a non-collagenous phosphoprotein that is abundant in bone [139]. OPN is generally thought to be a negative regulator in the process of physiological mineralization, as mineralization is completely inhibited in osteoblast cultures that overexpress OPN [140]. Phosphorylation of OPN is important in this process. The exogenous addition of OPN inhibits mineralization of osteoblasts *in vitro*, but this process is reversed by the addition of alkaline

phosphatase (ALP), which dephosphorylates OPN [54]. Similarly, phosphorylated OPN peptides have been shown to inhibit *in vitro* mineralization, whereas non-phosphorylated peptides have no effect. This inhibition may be due to the high affinity of OPN to hydroxyapatite [141]. OPN can bind to hydroxyapatite but it is not capable of precipitating hydroxyapatite from an agarose-gel system containing free calcium and phosphate [142]. Conversely, OPN has been shown to bind to hydroxyapatite and prevent further crystal growth *in vitro* [143]. However, it has also been shown that under certain conditions OPN can act as a hydroxyapatite nucleator. OPN is capable of undergoing conformational changes and it is thought that this may expose sites on the OPN structure that promotes hydroxyapatite formation [144]. Therefore under certain circumstances, such as dephosphorylation and conformational changes, OPN may act as an enhancer of mineralization. However OPN is largely regarded primarily as an inhibitor of physiological mineralization. There is also evidence to suggest that OPN is involved in osteoclast activity, which would result in increased bone resorption [145, 146].

OPN is known to be expressed in human breast cancers and is associated with a poor prognosis for breast cancer patients. In human breast tumor samples, increased expression of OPN has been documented for invasive breast cancer samples compared to both benign and normal tissue and OPN staining increases in intensity with increasing tumor grades [147, 148]. Injection of siRNA against OPN into the breast tumors of nude mice results in decreased tumor weight and reduced angiogenesis [147]. A more recent study has shown that an RNA aptamer against OPN is capable of reversing breast tumor growth in mice [149]. There is also evidence to suggest that there is an association between OPN and bone metastasis [150]. Antisenses designed against OPN resulted in a decrease of tumor bone metastasis in mice [151]. OPN is also a secreted protein and elevated levels of OPN have been detected in the serum of diagnosed breast cancer patients compared to normal volunteers [152]. In addition, elevated plasma levels of OPN has been associated with a shorter survival for patients with metastatic breast cancer [153]. The negative associations of OPN with breast cancer progression may be due to its ability to enhance migration and survival of cells [154]. OPN siRNA decreases mammary cell motility *in vitro* [147]. There is also evidence that OPN is capable of enhancing proliferation of various cell lines [155, 156].

Breast cancer MDA-MB-231 cells transfected with OPN shRNA have significantly reduced proliferation and increased apoptosis [157]. These studies suggest that overexpression of OPN within the tumor microenvironment may exacerbate tumor growth and survival.

OPN has also been shown to be associated with calcifications of human breast cancer samples. This was shown in a study by Bellahcene *et al.,* in which high expression of OPN was associated with frequent microcalcification deposition in breast cancer lesions [148]. This work is supported by other studies, which have also suggested an association between OPN and mammary microcalcifications [158, 159]. The role of OPN was recently investigated using an *in vitro* model of mammary mineralization [40]. An increase in OPN mRNA was detected during the process of *in vitro* mineralization, however the addition of exogenous OPN to the osteogenic cocktail did not inhibit mineralization [40] as has been reported for osteoblasts [54]. The reason exogenous OPN has no effect in this model of mammary mineralization may be due to high endogenous levels of ALP in the 4T1 cell line used [40]. ALP is known to dephosphorylate OPN, thereby removing its inhibitory effects [143, 144].

Bone sialoprotein

Bone sialoprotein (BSP) is a highly abundant non-collagenous bone matrix protein comprising ~15% of the total non-collagenous proteins in bone [160]. It has been shown in fetal porcine calvarial bone that BSP mRNA expression is restricted to differentiated osteoblasts, with particularly strong signals at the site of *de novo* bone formation [161]. However, in older osteoblasts and in some of the newly-entrapped osteocytes BSP mRNA expression was more modest [161]. This suggests a role for BSP in bone formation [161]. BSP contains an RGD (Arg-Gly-Asp) cell-attachment sequence and there is evidence to suggest that BSP could mediate the attachment of osteoblasts to the bone matrix during the process of bone remodeling [162]. BSP has also been implicated in the nucleation of hydroxyapatite. This has been shown using a steady-state agarose-gel system, in which BSP containing gels were capable of precipitating hydroxyapatite from a solution of phosphate and calcium [142].

BSP expression is also documented in human breast cancers. It has been reported that there is significant overexpression of BSP in malignant breast lesions compared to

normal and benign breast tissue [163]. Breast carcinomas with microcalcifications had the highest immunoreactivity to BSP antibodies [163]. In addition, it was recently shown that BSP mRNA is upregulated during the process of *in vitro* mammary mineralization [62]. BSP overexpression is also associated with lymph node involvement and a poorer long-term survival rate for breast cancer patients [164]. Several studies of breast cancer patients have shown that overexpression of BSP could be a potential marker to indicate whether bone metastasis will manifest at a later time point [164, 165]. The reason for this may be due to the osteomimetic properties of mammary cells, which could result in the preferential metastasis to the bone, as the cells would be more innately adapted to flourish within this microenvironment.

Dentin Matrix Protein 1

Dentin Matrix Protein 1 (DMP1) is a non-collagenous protein thought to be involved in physiological mineralization. DMP1-deficient mice develop severe defects in cartilage formation during postnatal chondrogenesis [166]. In addition, it has been shown *in vitro* that transgenic MC3T3-E1 cells overexpressing DMP1 have an earlier onset of mineralization and produce larger mineralization nodules compared non-transgenic cells [167]. The role of DMP1 in promoting mineralization is thought to be through the nucleation of hydroxyapatite. DMP1 is known to bind with a high affinity to collagen fibrils [168] and it has been shown that DMP1 is capable of nucleating hydroxyapatite in the presence of type 1 collagen [169]. *In vitro* studies have implicated a role for DMP1 in mineral formation in both intra [170] and extra-fibrillar spaces [171].

To the best of our knowledge, no studies have investigated if DMP1 is associated with mammary microcalcifications. However, DMP1 has been investigated in relation to breast cancer progression. High expression of DMP1 in primary breast lesions is associated with a lower risk of developing skeletal metastasis [172]. In addition, breast tumors with high levels of DMP1 had a significantly higher disease-free survival rate [172]. This indicates a role for DMP1 as a tumor suppressor, which is supported by findings that mammary tumorgenesis is accelerated in $DMP1^{-/-}$ mice [173]. DMP1 may act by suppressing the motility of tumor cells, as it has been shown that treatment of MCF-7 cells with siRNA against DMP1 promotes the

cells migratory ability [172]. The inverse relationship between DMP1 expression and breast cancer progression is in contrast with what is known about OPN and BSP. As previously discussed, OPN and BSP overexpression in breast cancer is associated with a poor prognosis [147, 148, 164]. The differing role of DMP1 as a tumor suppressor may be due to the matrix metalloproteinase (MMP) upon which it acts. DMP1, OPN and BSP are known to bind and activate pro-MMP-9, pro-MMP-3 and pro-MMP-2 respectively [174]. Although MMP-2 and MMP-9 are known to be involved in invasion, metastasis and angiogenesis [175], their overexpression in primary breast tumors is not associated with a poorer outcome. In contrast, MMP-9 expression has been shown to be an independent predictor of relapse-free survival [176].

Dentin sialophosphoprotein

Dentin sialophosphoprotein (DSPP) is another member of the SIBLING family of proteins that is involved in biomineralization. DSPP is thought to be critical for bone and dentin formation, as DSPP-deficient mice have defects in the mineralization of bone [177] and dentin [178]. The role of DSPP in physiological mineralization resides in its cleaved products; dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) [179]. DPP is known to have a high affinity for type 1 collagen [180], where it binds and promotes the formation of initial apatite crystals. As mineralization proceeds, DPP and other proteins bind to growing hydroxyapatite faces and inhibit or slow crystal growth, which influences the size and shape of the crystals [179]. The function of DSP is less well understood compared to DPP. DSP has little or no effect on *in vitro* mineralization and does not promote cell attachment [181]. However, *in vivo* studies indicate that DSP may be involved in the initiation of dentin mineralization, but not in the maturation of this tissue [182].

To the best of our knowledge, the potential role of DSPP and its cleavage products has not been investigated for the process of mammary mineralization. In addition, very little research has focused on DSPP in breast cancer. However, one study has shown that DSPP is upregulated in breast cancer [183]. In addition, DSPP has also shown to be upregulated in prostate [184], lung [183] and oral cancers [185]. This is an area that warrants further investigation.

Matrix extracellular phosphoglycoprotein

Matrix extracellular phosphoglycoprotein (MEPE) is a SIBLING protein expressed in odontoblasts, osteoblasts and osteocytes [186, 187]. In bone, MEPE expression reaches its maximum during bone matrix maturation [188] and is thought to be a negative regulator of physiological mineralization. This is supported by *in vivo* studies demonstrating that MEPE null mice have increased bone density [189]. In addition, MEPE overexpression in mice results in decreased mineralization and low bone mass [190]. Negative regulation of mineralization has also been demonstrated *in vitro*, as ATDC5 chondrogenic cells overexpressing MEPE results in decreased mineralization [191]. *In vitro* studies have also investigated whether it is the intact protein or its cleaved product, acidic, serine- and aspartate-rich MEPE-associated motif (ASARM), that are responsible for inhibition of mineralization. Phosphorylated intact MEPE was found to promote mineralization in a gelatin diffusion system, whereas the ASARM peptide inhibited mineralization [192]. When both MEPE and the ASARM peptide were dephosphorylated, neither had an effect on mineralization within this *in vitro* system [192]. This indicates the importance of posttranslational modification for the activity of MEPE in mineralization [192].

As far as we are aware, there is currently no evidence to implicate a role for MEPE in mammary mineralization. Very little research has investigated the involvement of MEPE in cancer, as MEPE expression is much more restricted than other SIBLING proteins [193, 194]. Only tumors that result in oncogenic hypophosphataemic osteomalacia appear to express MEPE [195]. Elevation of MEPE expression at both the transcription and translational levels has been detected in oncogenic osteomalacia tumors [196]. In a collection of human normal and cancer tissues, including breast cancer samples, minimal expression of MEPE mRNA was detectable [183]. This indicates that MEPE is not involved in cancer progression.

Alkaline phosphatase

Alkaline phosphatase (ALP) is commonly used as a biochemical marker of osteoblast mineralization. ALP is an ectoprotein that is membrane-bound to osteoblasts by a glycosylphosphatidylinositol anchor [197] and is also associated with matrix vesicles [198]. It

is well documented that ALP is upregulated during the process of osteoblast mineralization [20, 199]. ALP enhances mineralization mainly through its ability to hydrolyze sources of phosphate, liberating inorganic phosphate for subsequent incorporation into hydroxyapatite. ALP has been shown to hydrolyze β-glycerophosphate *in vitro* [24, 197]. There is also some evidence to suggest that ALP inhibits osteopontin (OPN), a known inhibitor of mineralization, by dephosphorylating OPN and thereby impairing its functionality *in vitro* [54, 143, 144]. ALP is also capable of hydrolyzing inorganic pyrophosphate (PPi), an endogenous inhibitor of hydroxyapatite formation [197].

Due to the well established role of ALP in physiological mineralization, the potential role of ALP in mammary mineralization was recently investigated using an *in vitro* model involving mouse mammary 4T1 cells [40]. During this process, there was increased expression of ALP mRNA and the exogenous addition of ALP to the osteogenic cocktail significantly enhanced mammary mineralization [40]. Levamisole, a known inhibitor of ALP, inhibited β-glycerophosphate induced mineralization, indicating that ALP is essential for mammary mineralization within this *in vitro* model [40]. In addition, while the tumorgenic 4T1 cell line were found to express ALP, little or no expression was detectable in the non-tumorgenic and non-mineralizing MCF10a mammary cell line [40]. This suggests that ALP expression and mineralization potential may be associated with more aggressive mammary cell phenotypes.

Inorganic pyrophosphate

Inorganic pyrophosphate (PPi) is a molecule composed of two phosphate ions that are coupled by an ester bond. PPi is a by-product of many metabolic reactions and is present in the extracellular matrix of most tissues and bodily fluids. PPi is a well documented inhibitor of physiological mineralization and is thought to antagonise the ability of inorganic phosphate to bind calcium to form hydroxyapatite [200]. Elevated levels of PPi have been detected in the plasma of patients with hypophosphatasia, a condition characterised by skeletal hypomineralization [201]. The exogenous addition of PPi to osteoblast cultures also inhibits mineralization *in vitro* [54]. A balance between PPi and inorganic phosphate is an important factor in maintaining normal physiological mineralization. If this balance is disturbed, it can

lead to pathological mineralization [202]. PPi is generated by nucleotide pyrophosphate phosphodiesterase 1 (NPP1) and transported by ANK, which are located in the membrane of osteoblasts. NPP1 is also located on matrix vesicles released from osteoblasts. Mice that are deficient in either *NPP1* or *ANK* exhibit decreased extracellular PPi and also have hypermineralized bones [43].

The role of PPi in limiting bone formation is interlinked with both ALP and OPN. *NPP1* and *ANK* deficient mice have decreased OPN expression [43]. The exogenous addition of PPi to osteoblast cultures results in the upregulation of OPN as well as a decrease in *Enpp1* and *ANK* gene expression [43, 54]. PPi has been shown to inhibit the ability of ALP to mediate the release of inorganic phosphate from β-glycerophosphate. A balance between PPi and ALP is important, as the inhibitory effect of PPi is reversed by the addition of ALP [54]. A mechanism for the role of PPi during mineralization was proposed by Harmey *et al* [43]. A negative feedback loop exists, in which increased levels of PPi inhibits the gene expression of *ANK* and *Enpp1*. PPi also enhances the transcription of *OPN* and through these mechanisms inhibits the formation of hydroxyapatite. In order for physiological mineralization to take place, ALP must be present to hydrolyze PPi and remove its inhibitory effects [43].

The potential role of PPi in mammary mineralization has recently been investigated using the *in vitro* model of 4T1 mammary cell mineralization [40]. However, it was found that the addition of exogenous PPi to the osteogenic cocktail did not inhibit mammary mineralization as expected. This may be due to high endogenous levels of ALP in the 4T1 cell line. ALP is capable of hydrolyzing PPi [197], which would remove its inhibitory effect. This provides further evidence that there may be similarities between the process of mammary cell mineralization and that of physiological mineralization[40].

Fetuin-A

Fetuin-A is a glycoprotein belonging to the cystatin group of protease inhibitors [203]. Fetuin-A is thought to be a circulating inhibitor of mineralization and this concept is supported by *in vivo* studies in which fetuin-A deficient mice present with growth plate defects and increased bone formation with age [204]. Due to the size of Fetuin-A (>40 kDa), it is likely to be excluded from the interior of tightly packed collagen fibrils [205]. However, Fetuin-A is thought

to exert its effect in the extracellular fluid in the form of colloidal calciprotein particles. Fetuin-A forms these particles by forming a complex with matrix gla protein, BMP2 and small calcium phosphate particles [206, 207]. It is thought that the formation of these colloidal calciprotein particles reduces the concentration of free calcium in the extracellular fluid, thereby inhibiting further growth of calcium phosphate crystals [207].

Fetuin-A has also been implicated in pathological mineralization. By combining fetuin-A deficient mice with the calcification-prone DBA/2 genetic background, virtually all of these mice displayed massive soft tissue mineralization [208]. Areas of the bodies affected included myocardium, kidney, lung, skin, brown fat, pancreas and reproductive organs [208]. Despite these findings, fetuin-A has not been investigated in relation to mammary microcalcifications. However, limited studies have explored its potential involvement in breast cancer progression. Fet-null mice have been crossed with a polyoma middle T antigen (PyMT) transgenic mouse model [209]. The PyMT model is known to recapitulate many processes found in human breast cancer progression [210]. The control group (PyMT/Fet$^{+/+}$) formed mammary tumors 90 days after birth, whereas tumor latency was prolonged in PyMT/Fet$^{-/-}$ and PyMT/Fet$^{+/-}$ mice [209]. The majority of PyMT/Fet$^{-/-}$ mice were tumor free by the end of the study at approximately 40 weeks, indicating that fetuin-A may be involved in breast cancer progression [209]. It has been suggested that fetuin-A and anti-fetuin-A autoantibody may be useful serum biomarkers for breast cancer screening and diagnosis [211]. Fetuin-A has been identified as a tumor antigen in the urine of breast cancer patients [211]. In addition, the authors detected fetuin-A autoantibody in the peripheral blood of 79.1% of breast cancer patients compared to only 9.6% of controls [211]. However, a more recent study has shown that fetuin-A is decreased in the serum from breast cancer patients compared to controls [212]. Therefore more research will be required to elucidate the potential role of fetuin-A in breast cancer.

Matrix Gla Protein

Matrix gla protein (MGP) is an extracellular matrix glycoprotein containing γ-carboxyl groups, allowing it to chelate calcium and function as a mineralization inhibitor [213]. Mice lacking MGP present with inappropriate calcification of various cartilages, which causes short stature,

osteopenia and fractures [214]. In addition, these mice die within two months due to arterial calcification, which leads to blood vessel rupture [214]. In this MGP knockout model it is the extracellular matrix of the blood vessel (i.e. the elastin and collagen) which mineralizes. As previously discussed MGP binds with fetuin-A [206, 207] in addition to calcium, but there is also evidence to suggest that it binds with BMP2 [215]. It has been hypothesized that MGP not only inhibits the formation of hydroxyapatite crystals, but also plays a role in local regulation of BMP signalling [207]. This may occur by trapping BMPs on the surface of growing hydroxyapatite crystals by binding to fetuin-A [207].

A very limited number of studies have investigated the potential role of MGP in mammary mineralization or breast cancer progression. Only one study has looked at the possible role of MGP in relation to mammary microcalcifications [216]. Hirota *et al* have shown that MGP mRNA expressing cells did not correlate with the deposition of calcium phosphate in calcifying foci in human breast cancers [216]. However upregulation of the *MGP* gene is associated with breast cancer cases where prognosis is poor, but there was no correlation between MGP protein expression and overall survival [217]. This study indicates that in human breast cancer, the mRNA level of MGP may be a marker indicating poor prognosis, whereas MGP protein expression is not [217]. It should be noted that this study only contained a small sample set of 9 patient samples. Therefore further studies are required to clarify the potential role of MGP in breast cancer and mammary microcalcification formation.

Osteocalcin

Osteocalcin (OSC also known as bone Gla protein) is a major non-collagenous protein that is synthesised by osteoblasts and upregulated during bone formation [20]. OSC contains γ-carboxyglutamic acid (Gla) residues, which promotes the adsorption of the protein to hydroxyapatite [218]. Post translational modification of OSC by γ-glutamyl carboxylase is an essential process in order for OSC to attract calcium ions, which can then be incorporated into hydroxyapatite crystals [219, 220]. Due to the role of OSC during bone formation, the potential of this protein for bone healing has also been investigated. It has been documented that cylindrical nanocrystalline hydroxyapatite implants containing OSC accelerate bone

regeneration in Wistar rats [221]. Therefore OSC represents a potential therapeutic agent for bone healing and regeneration.

The level of OSC in the serum of breast cancer patients has been a subject of interest in the literature. It has been shown that the serum levels of OSC are elevated in patients with primary breast cancer [222]. In addition, elevated OSC serum levels were successfully used to identify breast cancer patients with bone metastasis [222]. Circulating OSC is also elevated in breast cancer patients presenting with bone metastasis following routine 4`-epidoxorubin treatment. This was accompanied by bone pain remission and regression of bone lesions in these patients. Therefore OSC appears to act as a biological marker of recovered osteoblast activity [223]. Despite this research, OSC has never been examined in the breast tumor microenvironment or in relation to mammary microcalcifications.

Osteonectin

Osteonectin (OSN) is a glycoprotein that is also highly abundant in bone. Newly differentiated osteoblasts synthesize and secrete OSN, which is then incorporated into the mineralized bone matrix [224]. OSN has been shown to enhance the ability of hydroxyapatite to bind to collagen type 1 [225]. OSN is also expressed in human breast cancers and high expression is associated with frequent microcalcification deposition [148]. OSN has also been implicated as a biomarker for tumor development, however this is a controversial topic with conflicting findings in the literature [226-228].

A PROPOSED MECHANISM OF MAMMARY CELL MINERLIZATION

Numerous proteins are known to be involved in physiological mineralization and some of the key regulators have been discussed in detail here. Several of these have also been implicated in the progression of breast cancer. The role of these regulators in physiological mineralization and breast cancer are summarized on table 1. A mechanism of mammary cell mineralization has been proposed (Figure 2), which is based on an imbalance between the enhancers and inhibitors of physiological mineralization [40]. It has been suggested that ALP on the surface of mammary cells hydrolyzes organic phosphate to inorganic phosphate. The type II family of Na-Pi cotransporters then transports the inorganic phosphate inside the

mammary cell. Once inside the cell, inorganic phosphate could combine with calcium to form hydroxyapatite, which then leaves the cell by an unknown mechanism. Once hydroxyapatite is deposited in the extracellular matrix, it is possible that collagen acts as a substrate for further crystal growth, as has been demonstrated for physiological mineralization [22]. OPN production is upregulated in an attempt to limit crystal growth; however it has no effect, possibly due to dephosphorylation by ALP. PPi may also be present in the extracellular matrix, however the upregulation of ALP may hydrolyze PPi to inorganic phosphate thereby removing its inhibitory effect. Therefore the production of pathological mammary microcalcifications may be due to an imbalance of these regulators of physiological mineralization within the tumor microenvironment [40]. To the best of our knowledge this is the only detailed mechanism that has been proposed for the process of mammary mineralization. As this mechanism is largely based on findings from cell culture systems, a substantial amount of research is still required to verify this mechanism. It is likely that as research progresses in this field that the proposed mechanism will develop and evolve over time. Due to the large volume of evidence supporting the role of matrix vesicles in physiological mineralization [71], future investigations should also focus on their potential role in nucleation of hydroxyapatite during mammary mineralization. Also as a collagenous extracellular matrix is abundant in breast tissue, more specific studies should look at the potential role of these collagen fibrils in mineral deposition and propagation.

CONCLUSION

Mammographic microcalcifications are important for the early detection of breast cancer, however the mechanisms by which they form remains poorly understood. Based on the literature discussed here, it is hypothesized that hydroxyapatite deposition in the soft tissue of the breast may occur in a similar manner to the formation of hydroxyapatite in bone. There is mounting evidence to demonstrate that the bone matrix proteins involved in osteoblast mineralization are also expressed in mammary cells and their expression is associated with various aspects of breast cancer including mammary mineralization. As more breast cancers are being detected at their early stages, largely through mammographic microcalcifications, it

is important that the underlying mechanisms of their biology are investigated to understand their significance and impact within the tumor microenvironment.

Figure legends

Figure 1. Mammographic features from samples that were histologically proven,1-14mm invasive breast carcinoma cases. Primary calcifications are visible as powdery, crushed stone-like and casting type calcifications taken from Tabar et al., 2004 [4].

Figure 2. A proposed mechanism of *in vitro* mammary cell mineralization [40]. β-glycerophosphate (βG) is hydrolyzed by alkaline phosphatase (ALP) to glycerol (G) and inorganic phosphate (Pi). Pi is internalized by the type II family of Na-Pi cotransporters and combines with calcium (Ca) inside the cell to form hydroxyapatite (HA). Upregulation of ALP mRNA occurs. Hydroxyapatite enters the extracellular matrix. Inorganic pyrophosphate (PPi) is a natural inhibitor of HA formation. However, the overexpression of ALP cause PPi to be hydrolyzed to Pi, which can subsequently be incorporated into growing HA crystals. Osteopontin (OPN) is a natural inhibitor of HA crystal growth, however overexpression of ALP may dephosphorylate OPN, thereby removing its inhibitory effect. Hydroxyapatite crystals in the extracellular matrix of the breast tissue may enhance the proliferation and migration of surrounding cells and further aggravate tumor growth and metastasis [40].

Table 1. Summary of mediators of physiological mineralization and their association with breast cancer

Bone matrix protein	***Role in physiological mineralization***	***Implications in breast cancer***
Collagen type 1	- Collagen fibers form a network in the extracellular matrix for hydroxyapatite crystals to grow between [22]	- Degradation of collagen in the extracellular matrix is evident during breast cancer progression [132] and long, straight collagen fibers are associated with malignant breast cancer [133] - Elevation of collagen peptides in the serum of breast cancer patients with bone metastasis [134, 135] - Microcalcifications form around surgically implanted collagen plugs [137] - Increased mRNA expression is associated with *in vitro* 4T1 mammary cell mineralization [62]
Osteopontin (OPN)	- A negative regulator of mineralization in its phosphorylated form [54] - Binds hydroxyapatite and prevents further crystal growth, when in its phosphorylated form [143]	- Increased protein expression in human breast cancer tumors and in plasma is associated with a poorer prognosis [147, 148] - Expression associated with mammary microcalcifications [148] - Increased expression associated with *in vitro* 4T1 mammary cell mineralization, however exogenous OPN has no effect. This may be due to overexpression of ALP [40]
Bone sialoprotein (BSP)	- Elevation of BSP mRNA at sites of bone formation [199] - Nucleates hydroxyapatite [142] - May mediate attachment of osteoblasts to bone matrix [162]	- Overexpression in malignant breast lesions [163] and node positive tumors [164] - Overexpression associated with a poorer long-term survival [164] - High expression associated with mammary microcalcifications [163] - Increased mRNA expression is associated with *in vitro* 4T1 mammary cell mineralization [62]
Dentin matrix protein 1 (DMP1)	- Promotes mineral formation [170, 171] - Binds to collagen fibrils [168] - Nucleates hydroxyapatite in the presence of collagen type 1 [169]	- Breast tumors with high levels of DMP1 are associated with a lower risk of developing skeletal metastasis and a higher disease-free survival [172] - Suppresses the motility of tumor cells [172]
Dentin sialophosphoprotein (DSPP)	- Produces dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) as cleaved products [179] - DPP binds collagen type 1 [180] - DPP promotes the initial formation of apatite and later inhibits or slows crystal growth, which influences the size and shape of crystals [179] - DSP may be involved in the initiation of dentin mineralization [182]	- Upregulated in breast cancer [183]
Matrix extracellular phosphoglycoprotein (MEPE)	- Thought to be a negative regulator of physiological mineralization [188-191]	- MEPE is minimally expressed in breast cancer [183]
Alkaline	- Upregulated during osteoblast mineralization [20, 199]	- Increased serum levels associated with bone metastasis [229]

phosphatase (ALP)	- Enhances mineralization by hydrolyzing organic phosphate to inorganic phosphate [24, 197] - Inhibits OPN by dephosphorylation [143, 144] - Hydrolyzes PPi to inorganic phosphate [197]	- Essential for *in vitro* 4T1 mammary cell mineralization [40]
Inorganic pyrophosphate (PPi)	- An inhibitor of osteoblast mineralization [54] - Inhibits the ability of ALP to hydrolyze organic phosphate [54] - Enhances transcription of OPN [43]	- Exogenous PPi has no effect on *in vitro* 4T1 mammary cell mineralization, however this may be due to overexpression of ALP [40] -
Fetuin-A	- A circulating inhibitor of mineralization [204] - Forms colloidal calciprotein particles, resulting in reduced calcium concentration in the extracellular fluid [207]	- Fet-null mice crossed with PyMT mice results in prolonged tumor latency [209] - Fetuin-A may be a useful serum biomarker for breast cancer screening and prognosis [211], but not all studies are in agreement [212]
Matrix gla protein (MGP)	- An inhibitor of mineralization [213] - MGP forms a complex with fetuin-A, calcium and BMP [206, 207], reducing the concentration of free calcium in the extracellular fluid [207]	- MGP mRNA expressing cells do not correlate with the deposition of calcium phosphate in calcifying foci in human breast cancers [216] - Upregulation of MGP mRNA is associated with a poor prognosis for breast cancer [217]
Osteocalcin (OSC)	- Synthesized by osteoblasts and upregulated during bone formation [20] - OSC attracts calcium ions, which can then be incorporated into hydroxyapatite crystals [219, 220]	- Serum levels are elevated in the serum of patients with primary breast cancer [222] - Elevated serum levels associated with bone metastasis in breast cancer patients [222]
Osteonectin (OSN)	- Synthesized and secreted by osteoblasts and incorporated into the mineralized bone matrix [224] - Enhances the ability of hydroxyapatite to bind to collagen type 1 [225]	- May be a biomarker of tumor progression, but this is a subject of debate in the literature [226-228] - High expression in tumors associated with mammary microcalcifications [148]

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