Published in final edited form as:

Exp Cell Res. 2014 July 15; 325(2): 148–154. doi:10.1016/j.yexcr.2014.01.018.

Multifunctional ECM proteins in bone and teeth

Sriram Ravindran and Anne George*

Brodie Tooth Development Genetics & Regenerative Medicine Research Laboratory

Department of Oral Biology, University of Illinois at Chicago, Chicago, IL 60612, USA

Abstract

The extracellular matrix (ECM) of all tissues and organs is a highly organized and complex structure unique to the specific organ type. The ECM contains structural and functional proteins that define cellular function, organization, behavior and ultimately organ characteristics and function. The ECM was initially thought to contain only a specific set of secretory proteins. However, our group and several other groups have shown that the ECM contains functional proteins that have been previously defined as solely intracellular. In the present review, we have focused on the ECM of mineralized tissues namely: bone and dentin. We provide here, a brief review of some non-classical ECM proteins that have been shown to possess both intra and extracellular roles in the formation of these mineralized matrices.

Introduction

Most of the tissues that constitute a living organism consist of live cells embedded within an extracellular matrix (ECM). ECM constitutes the non-cellular component of tissues and is composed of structural and functional proteins (1). Each tissue has a unique ECM signature that provides instructional cues for cellular differentiation, migration, wound healing and immune response. Briefly, the ECM determines tissue functionality. Recent tissue engineering strategies have focused on mimicking the composition of the ECM to achieve desired cellular behavior and lineage specific differentiation to facilitate regeneration of tissues such as bone, dental pulp and cartilage (2–5). The primary reason behind such approaches lies in the complex nature of the ECM. As the ECM is dynamic, it is difficult to determine the exact components and composition of the ECM matrix. Recently, several unknown and multifunctional proteins previously thought to be intracellular are being identified and classified as functional proteins in the ECM. The goal of this review is to focus on novel multi-functional extracellular matrix proteins that contribute to the formation of mineralized tissues namely; bone and dentin.

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*To whom correspondence should be addressed. Department of Oral Biology, University of Illinois at Chicago, Chicago, IL 60612, USA, Tel: 1-312-413-0738; Fax: 1-312-996-6044; anneg@uic.edu.

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Bone and dentin are complex mineralized tissues generated by osteoblasts and odontoblasts respectively. The main function of the osteoblast and odontoblast cells is to form mineralized matrices by secretion of collagenous and noncollagenous proteins as well as mediators of mineralization. Although similar in many ways, the ECM of bone and dentin has several significant differences with respect to their composition and function. Bone has recently been characterized as an endocrine organ (6). Osteoblasts and osteocytes produce two hormones, namely, fibroblast growth factor 23 (FGF23) and osteocalcin that function as endocrine hormones regulating kidney function and insulin secretion respectively. Although not characterized as an endocrine organ, odontoblast cells have also been shown to express FGF23 (7) and osteocalcin. Similarly, both tissues express growth factors belonging to the bone morphogenetic protein/transforming growth factor beta (BMP/TGF β) superfamily, fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs) (8,9), phosphatases and metalloproteases. However, the responses of osteoblasts and odontoblasts to these growth factors are different. For example, expression of the transcription factor Runx2 is triggered by treatment of osteoblasts with BMP/TGF β superfamily of proteins. However, the same response is elicited by odontoblasts when treated with FGFs.

In this review, we have attempted to stress the importance of multifunctional proteins in the formation and maintenance of mineralized tissues. Advances in science dictate that nature behaves in a conservative manner. This implies that large amount of work is accomplished with minimal effort. In the biological system, this is facilitated by the use of multifunctional proteins. Therefore, this review focuses on the multifunctional proteins highlighted below that influences stem cell differentiation and matrix mineralization.

Dentin matrix protein 1 (DMP1)

DMP1 is an acidic phosphorylated non-collagenous protein (NCP) that is present in the ECM of bone and dentin. Although initially isolated from the dentin matrix, DMP1 is present in all mineralized tissue matrices. Loss of DMP1 function causes rickets and osteomalacia (10) characterized by hypo-mineralized tissues and abnormal phosphate regulation influenced by altered FGF23 expression (10). In bone, DMP1 is a target molecule for the important osteoblast transcription factor runx 2 (11). In the ECM, DMP1 binds calcium and initiates nucleation of crystalline hydroxyapatite (12,13).

Apart from its role in matrix mineralization, DMP1 can act as both intra and extracellular signaling molecule impacting osteoblast and odontoblast differentiation at several stages of development. DMP1 possesses a functional nuclear localizing signal (NLS) that enables intracellular nuclear translocation of the protein (14). In the nucleus, DMP1 binds to DSPP promoter and influences odontogenic differentiation of precursor odontoblasts by controlling DSPP expression (15). As an extracellular signaling molecule, MMP2 cleaved fragments of DMP1 influence stem cell differentiation (16). Extracellular DMP1 is endocytosed via the cell surface receptor GRP78 (17). Endocytosis of DMP1 triggers calcium mediated signaling resulting in differentiation of precursor mesenchymal cells (18). DMP1 tethered to the ECM can initiate integrin mediated signaling via the $\alpha 5 \beta 3$ cell surface integrin and contribute to the ECM-osteocyte signaling mechanisms via the mitogen activated protein kinase (MAPK) pathway (19).

Collectively, the published data on this multi-functional protein indicates that DMP1 can play several roles at various stages of development of mineralized tissues starting from modulation of lineage specific differentiation of mesenchymal stem cells to nucleation of crystalline hydroxyapatite.

Dentin Phosphophoryn (DPP)

DPP is a member of the small integrin binding ligand, N-linked glycoproteins (SIBLING). DPP is predominantly expressed by odontoblasts and is the most abundantly expressed non-collagenous protein in the dentin matrix. DPP is also present in the bone matrix, but in extremely small amounts (20). DPP and dentin sialoprotein (DSP) are both encoded by the gene DSPP (21). The creation of DPP protein from DSPP mRNA is a topic of debate with several suggested mechanisms (22–24). We have recently identified an active internal ribosomal entry site (IRES) in the DSPP gene that might result in the production of DPP protein. As a result of this, the DPP protein would lack a signal peptide and therefore, will have to be secreted via non-classical secretory pathways.

The primary function of DPP in the matrix is to initiate nucleation of hydroxyapatite crystals (25–28). However, recently, DPP has been shown to possess signaling functions that can initiate lineage specific differentiation of mesenchymal stem cells (29). DPP in the ECM can mediate cell adhesion and initiate integrin mediated signaling (30,31). Additionally, DPP can also initiate calcium and calmodulin dependent signaling to promote differentiation of precursor mesenchymal cells (32). The acidic domain in DPP facilitates non-classical endocytosis of the protein (33). Although the acidic domain by itself does not possess any signaling bioactivity, we can speculate that some of the signaling roles of DPP can be attributed to the endocytosis of the protein.

Therefore, DPP is a multi faceted protein with proven roles in matrix mineralization and signaling. Further studies are required to completely understand its intracellular role in preodontoblasts.

Osteopontin (OPN)

Osteopontin is a highly phosphorylated sialoprotein found in the ECM of bone and teeth (34). Similar to DMP1 and DPP, It is a member of the SIBLING family of noncollagenous proteins (35). Although initially identified in the ECM of bone, OPN is one of the most extensively studied SIBLING proteins and performs several functions in several tissues ranging from development to signaling roles in cancer based on its phosphorylation state (36,37). With respect to matrix mineralization, OPN binds calcium avidly and is a key regulator of hydroxyapatite nucleation.

OPN is an interfacial ECM protein and can be localized to the interfaces of cell and ECM and also in the cement lines (matrix-matrix interface) (38). This specific localization pattern was postulated to be important for minimizing strain induced fatigue damage and micro crack propagation (38) and has recently been verified experimentally using a knock-out mouse model (39). The contribution of OPN to mineralized matrix formation lies in its ability to control bone quality and not just bone mass (39). OPN can influence osteoclast

attachment and facilitate matrix mineralization by influencing the shape and size of the hydroxyapatite crystals (40–42).

Phosphorylation and other post-translational modifications control the various functions of OPN. For example, with respect to matrix mineralization, OPN from bone containing 13 phosphorylated serines inhibit hydroxyapatite nucleation. On the other hand, milk derived OPN containing 28 phosphorylated serines promote nucleation (43). Additionally, OPN can also be proteolytically processed by preteases such as PHEX, thrombin, matrix metalloprotease 2 (MMP2), MMP3 and MMP7 giving rise to several bioactive peptides with distinct functions (44,45).

Apart from its direct role in matrix mineralization, OPN also possesses signaling functions. The RGD domain in OPN binds to $\alpha 4 \beta 1$ integrin and triggers a cell type specific integrin mediated signaling cascade. Interestingly, OPN possesses a transglutaminase (TG) binding domain and a recent study shows that oligomerization of OPN by TG2 enhances its ability to promote integrin mediated osteoblast attachment (46). Apart from outside-in signaling functions, intracellular isoforms of OPN generated by alternate translation can initiate intracellular signaling functions in several cell types such as macrophages, dendritic cells, lymphocytes, T-cells and osteoclasts and via its interactions with CD44 intra and extracellularly (47–50). Finally, OPN has been localized to the nucleus of 293T cells linked to polio-like kinase-1 playing a role in duplication of the 293T cells (51).

OPN is one of the most extensively studied ECM proteins that has been shown to be truly multifunctional with several functional roles in various cell types. With respect to mineralized tissues, OPN serves to both promote and inhibit nucleation of hydroxyapatite at various stages and synergistically performs its signaling functions using its RGD motif and proteolytic fragments to promote osteoclast or osteoblast activity depending on the biological context.

Periostin

Periostin is a 90KDa matricellular protein expressed by preodontoblasts, osteoblasts and osteocytes. Originally, periostin was identified in osteoblasts as osteoblast specific factor -2 as a protein that plays a role in preosteoblast cell adhesion (52). Although initially localized in the periosteum of bone, cell types subjected to mechanical strains such as the periodontal ligament and tendons express this protein (53).

The localization of periostin preferentially in collagen rich tissues such as bone and dentin implicates a role for this protein in bone biology and regeneration (53). In bone periostin is expressed during embryonic development and bone growth in the periosteum and plays an important role in controlling the cortical thickness of the bone. In adult animals, periostin expression is observed in bone fractures or mechanical injury (53).

In the tooth, periostin is expressed by ameloblasts, preodontoblasts, odontoblasts and periodontal ligament cells (54). Periostin knockout mice display ectopic mineralization and hypermineralized dentin (54) suggesting that periostin is a negative regulator of matrix mineralization. Additionally, periostin is negatively regulated upon *in vitro* mechanical

loading and TGF β stimulation further confirming its role as a negative regulator of matrix mineralization.

In all mineralizing tissues, periostin plays a significant role in the crosslinking of collagen fibrils and ECM organization by binding directly to type I collagen and also to fibronectin and tenascin-C in the matrix (53). Periostin possesses a heparin-binding domain. This fact coupled with its association with fibronectin presents an interesting possibility of periostin serving as a reservoir for heparin binding growth factors, glycoproteins and proteoglycans (55).

From a signaling perspective, periostin regulates MMP-2 expression via the $\alpha 5 \beta 3$ integrin mediated signaling pathway in periodontal ligament cells. Additionally, an isoform of periostin (isoform 3) has been localized in the nucleus of developing tissues (56). A role for periostin in the nucleus is yet to be determined.

All of these evidences suggest that periostin functions as a negative regulator of mineralization, however, its signaling function is responsible for the organization of the ECM matrix.

Glucose regulated protein 78 (GRP78)

GRP78, also known as heat shock protein $\alpha 5$ (hspa5) is a member of the 70KDa heat shock protein family. GRP78 is an endoplasmic reticulum (ER) stress response protein that facilitates folding and assembly of secretory and membrane proteins. Published reports indicate increased production and membrane localization of GRP78 by cells that have been challenged with toxins, cancer, calcium stress or apoptotic agents (57–60). On the plasma membrane, GRP78 resides within lipid rich surfaces and localizes to detergent resistant membrane fractions during purification (61,62). Although GRP78 has been extensively studied for its role in cancer cell survival and as a target for cancer therapy, its role in mesenchymal cells and mineralized tissue formation has only recently been studied.

Cells involved in the formation of a mineralized matrix are under constant calcium induced stress leading to increased production of the stress response genes including GRP78. We have shown that GRP78 can be localized to the plasma membrane of both pre-osteoblast and pre-odontoblast cells. As a membrane resident protein, GRP78 functions as a receptor for DMPI facilitating its endocytosis and the subsequent calcium related signaling events associated with it (17,18). This is a recently identified role for this already multi- functional protein in mesenchymal cells.

Further, we have shown that GRP78 secretion also increased upon induction of osteogenic or odontogenic differentiation of respective precursor mesenchymal cells (63). *In vitro* binding experiments indicated that GRP78 binds to type I collagen and DMPI. When both ligands are present together, its binding affinity to type I collagen is greater and saturates first before binding to DMPI saturates. Subsequent calcium phosphate nucleation experiments indicated that GRP78 induces nucleation of amorphous calcium phosphate by itself, when bound to type I collagen and when bound to demineralized dentin wafers (63).

These results corroborated well with the developmental expression pattern of GRP78 confirming its role in the formation of mineralized matrices (64).

Additionally, we have observed the presence of GRP78 in the nucleus of terminally differentiated odontoblasts. Further investigations are necessary to ascertain its role in the nucleus of differentiated cells. Overall, this stress chaperone that was once thought to be an intracellular heat shock protein has emerged as one with very diverse roles in several cell types ranging from survival to matrix mineralization. In mineralizing tissues, we envision that GRP78 plays different roles during various developmental stages. GRP78 by itself does not possess any bioactivity as a signaling molecule, but can guide differentiation of precursor mesenchymal cells by serving as a receptor for DMP1. During later stages of matrix mineralization GRP78 can bind to type I collagen matrix directly or to DMP1 bound to the collagen matrix to initiate or support matrix mineralization.

TGF β receptor II (TGF β R2) interacting protein 1 (TRIP1)

TRIP1 is a member of the WD40 repeat containing family of proteins. This group of proteins play important roles in signal transduction, vesicular trafficking and cell cycle regulation (65,66). TRIP1 binds to the cytoplasmic domain of TGF β R2 and influences TGF β signaling by acting as a negative regulator and inhibiting positive regulation of TGF β target genes (67). Published data shows that overexpression of TRIP1 in mesenchymal cells triggered better differentiation of these cells under mineralization conditions in the presence of ascorbic acid, organic β -glycerophosphate and dexamethasone (68). Additionally, TRIP1 can be detected in the secretome of mesenchymal cells and can initiate nucleation of calcium phosphate polymorphs *in vitro* (68–69).

TRIP1 plays an active role in TGF β mediated signaling. Therefore, it is conceivable that TRIP1 might affect this signaling pathway. Further studies are required to explore the possibilities of its effect on TGF β signaling on mesenchymal stem cell differentiation and matrix mineralization.

Identifying the localization and function of TRIP1 in mineralized matrix formation has paved the way for inclusion of TRIP1 into the group of multifunctional proteins that possess extra and intracellular roles in controlling the complex process of stem cell differentiation and matrix mineralization.

Concluding Remarks

With respect to mineralized tissue biology, the ECM of bone and dentin are comprised of a multitude of proteins. Using the present technology it will be a daunting task to identify the exact composition of these matrices. Recent proteomic studies have shown that the ECM of mineralized tissues is comprised of thousands of proteins ranging from structural proteins to transcription factors (70). Recently proteins that have been previously thought to be intracellular only have been identified in the ECM with unique functions that complement their intracellular roles.

With the focus of this review being multifunctional ECM proteins in mineralized tissues, we have described the multifunctional roles of DMP1 and DPP that serve as positive regulators of mineralization and also are involved in several signaling mechanisms across different stages of development. On the other hand, we have also provided an overview of the multifunctional OPN and periostin that are considered to be negative regulators of hydroxyapatite nucleation and also possess a multitude of signaling functions. OPN although, is an extremely complex protein with several isoforms, proteolytic fragments and post-translational modifications that can function in contrasting roles in different conditions.

Finally, we have described two novel matricellular proteins, namely GRP78 and TRIP. The intracellular functions of these proteins have been studied extensively. However, we have recently unraveled some interesting extracellular functions for these proteins in mineralized tissue formation.

In conclusion, the various proteins and their functions reviewed here serve as an example of the complex nature of the ECM of mineralized tissues. Identifying and understanding the role of such multifaceted proteins would help in the treatment of mineralized tissue disorders such as osteoporosis, osteogenesis and dentinogenesis imperfecta.

Acknowledgments

We acknowledge the support of NIH grants DE 19633, DE11657 and the Brodie Endowment Fund.

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Figure 1.

Immunohistochemical localization of DMP1, GRP-78 and Tripl: The figure represents the localization of DMP1, GRP-78 and Tripl in the calvaria and femur of 30 day wild type mice. The images are 3D reconstruction of z-stack confocal images of the respective sections immunostained with antibodies to the respective proteins. The area within the white lines in the femur section images represents the bone marrow. The white arrows in all images show the extracellular localization of the proteins.

Figure 2.

Hypothetical figure showing the role of multifunctional proteins in the formation of mineralized tissues:

Mesenchymal cells and their secreted ECM (A) are required for the formation of mineralized tissues. During development, the mesenchymal cells interact with the ECM (B) leading to ECM modification and differentiation of the cells into osteoblasts and osteocytes. This process results in the secretion of classical and non-classical ECM proteins that favor nucleation of hydroxyapatite such as DMP1 (C) and also ones that control nucleation such as OPN (C) on the collagen matrix. These proteins orchestrate the process of hydroxyapatite nucleation on the collagen matrix (D) leading to the formation of mineralized tissues such as bone (E).