Published in final edited form as:

Adv Exp Med Biol. 2015 ; 881: 129–142. doi:10.1007/978-3-319-22345-2_8.

Dentin Matrix Proteins in Bone Tissue Engineering

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Abstract

Dentin and bone are mineralized tissue matrices comprised of collagen fibrils and reinforced with oriented crystalline hydroxyapatite. Although both tissues perform different functionalities, they are assembled and orchestrated by mesenchymal cells that synthesize both collagenous and noncollagenous proteins albeit in different proportions. The dentin matrix proteins (DMPs) have been studied in great detail in recent years due to its inherent calcium binding properties in the extracellular matrix resulting in tissue calcification. Recent studies have shown that these proteins can serve both as intracellular signaling proteins leading to induction of stem cell differentiation and also function as nucleating proteins in the extracellular matrix. These properties make the DMPs attractive candidates for bone and dentin tissue regeneration. This chapter will provide an overview of the DMPs, their functionality and their proven and possible applications with respect to bone tissue engineering.

Keywords

Dentin matrix proteins; DMP1; DPP; DSP; FAM20C; DMP4; Tissue engineering; Bone regeneration

8.1 Introduction

Bone and dentin are mineralized hard tissues. The primary inorganic component of these two tissues is crystalline hydroxyapatite and the primary organic component is type I collagen. Type I collagen forms a dynamic and instructive template for the deposition of calcium phosphate polymorphs and their subsequent transformation into crystalline hydroxyapatite crystals. However, the process of hydroxyapatite nucleation and collagen mineralization is highly complex and is controlled by proteins that are categorized as noncollagenous proteins (NCPs) (Young et al. 1992). Although they only constitute a fraction of the bone and dentin volume, without the NCPs, it would be impossible to achieve bone formation and remodeling. Dentin matrix proteins (DMPs), osteocalcin, osteopontin and bone sialoprotein (BSP) are some examples of NCPs found in bone and dentin.

Bone tissue engineering, which consists of repair and regeneration, is an area that has generated a lot of interest. The fast pace of development of materials for bone tissue

engineering suggests that the potential to be clinically viable would be sooner than other fields of tissue engineering. Products such as demineralized bone matrices (DBMs) and other allograft bone materials are already in clinical use (Campana et al. 2014; Wang et al. 2007). On the other hand, the incidence of bone disorders and the repair of deformed bones is an ever-increasing problem owing to many factors such as, lifestyles, global wars and increase in devastating accidents worldwide. Current clinical practices cannot address many of the regenerative aspects of bone injuries satisfactorily and tissue engineering has been seen as the logical alternative (Amini et al. 2012).

As proposed more than two decades ago by Langer and Vacanti, tissue engineering of any tissue requires three components, namely: Cells, scaffolds and growth factors (Langer and Vacanti 1993). These components are often referred to as the tissue engineering triad. Several types of scaffolds have been used for bone tissue engineering including artificial polymers, natural polymers and synthetic self-assembling peptides (George and Ravindran 2010). The focus of this chapter will be on the use of DMPs as signaling molecules and hydroxyapatite nucleating agents for bone tissue engineering.

Dentin matrix proteins (DMPs) are a group of NCPs found in the extracellular matrix of dentin and bone albeit in different quantities. Currently, this group consists of four different proteins, namely: Dentin matrix protein 1 (DMP1), dentin phosphophoryn (DPP) or dentin matrix protein 2 (DMP2), dentin sialoprotein (DSP) and DMP 4 (also known as Fam20C) (Hao et al. 2007, 2009). Over the years, these DMPs have been shown to play multiple roles that control important functions ranging from attachment, proliferation and differentiation of stem cells and preosteoblasts to matrix mineralization. The multifunctional properties of these DMPs make them as attractive agents to be incorporated within an appropriate scaffold to enable stem cell based bone tissue engineering. Although primarily isolated as calcium binding and hydroxyapatite nucleating proteins, the DMPs can be used successfully as signaling molecules to direct stem cell recruitment, attachment and differentiation.

The overall goal of this chapter is to highlight the important functions of these DMPs and provide an overview of their utilization in bone tissue engineering.

8.2 Expression and Localization of DMPs in Bone

During bone repair several of the development processes are recapitulated. Therefore, it is important to identify the localization pattern of the dentin matrix proteins during bone development, as it is indicative of its function. In general, the dentin matrix proteins are expressed both intracellularly and extracellularly during development. Based on their localization patterns each of these molecules perform different functions. Such knowledge would be useful when designing strategies for bone tissue engineering.

8.2.1 DMP1

DMP1 was the first of the DMPs to be discovered and was initially thought to be specific to the dentin matrix (George et al. 1993, 1994). However, several studies confirmed that DMP1 is expressed in many tissues at a basal level and in high quantities by tissues undergoing biomineralization such as bone and dentin (Hao et al. 2004; Huang et al. 2008). Figure 8.1

shows the expression of DMP1 in 3-month-old mouse femur section. The figure is a 3D reconstruction of z stack confocal images. The red staining represents DMP1. Note the high expression levels in the bone matrix and also in the osteoblasts. The DMP1 protein in the extracellular matrix (ECM) is seen as two processed fragments: the 37 KDa N-terminal fragment and the 57 Kda C-terminal fragment (Qin et al. 2003). The N-terminal fragment of DMP1 is glycosylated at the Ser74 residue and is important for DMP1 to carry out its functions *in vivo* (Qin et al. 2006). However, this glycosylated form of DMP1 fragment is a negative regulator of hydroxyapatite nucleation (Gericke et al. 2010). Processing of DMP1 into the two fragments is critical for its function *in vivo*.

Overexpression of DMP1 that cannot be processed in DMP1 null mice failed to rescue the defective mineralization of dentin, bone and cementum observed in the knockout mice, showing the need for proteolytic processing of DMP1 for it to be functional *in vivo* (Sun et al. 2011).

Of the two fragments of DMP1, the 57 KDa C-terminal fragment is the most biologically active. Studies with knockout mouse models revealed that this fragment alone could restore the functionality of full-length DMP1 (Lu et al. 2011). The two fragments of DMP1 also show varied distribution in intracellular compartments as well as in the extracellular space (Maciejewska et al. 2009). The C-terminal fragment of DMP1 is primarily localized to the mineralizing areas of bone and dentin in the ECM. Inside the cell, the C-terminal fragment accumulates in the nucleus of the mesenchymal cells. On the other hand, the N-terminal fragment of DMP1 is localized to the pre-dentin, a non-mineralizing tissue and in the articular cartilage in the ECM. Intracellularly, it was predominantly localized to the cytosol and plasma membrane. This varied distribution suggests that both fragments of DMP1 may play different biological roles in the intra and extracellular environments.

8.2.2 DPP or DMP2

DPP is expressed by odontoblasts and is considered as a marker for terminally differentiated odontoblasts. Recent report indicates that DPP is expressed using an internal ribosome entry site (IRES) present in the DSPP gene (Zhang et al. 2014). However, several other studies have shown that DSPP is a substrate for proteases such as bone morphogenetic protein 1 a tolloid-related protein to be proteolytically processed into dentin sialoprotein (DSP), DPP and dentin glycoprotein DGP (Ritchie et al. 2012). In reality, it could be a combination of both processes. Interestingly, the proteolytic cleavage process does not account for the increased presence of DPP and the absence of DSPP precursor protein in the matrix. On the other hand, the IRES theory cannot account for the presence of the small DGP protein. Therefore, it is reasonable to propose that a combination of processing methodologies might account for the abundant presence of DPP in the matrix.

During development and maturation of the odontoblasts, the expression level of DPP increased and was maintained at high levels in the dentin matrix (Hao et al. 2004, 2009). Although initially thought to be uniquely specific to the dentin matrix, DPP has also been identified in the bone matrix (Qin et al. 2002). The red immunofluorescence staining in Fig. 8.2 shows the expression of DPP in the mineralizing dentin (marked as D in the figure).

Furthermore, DPP has also been identified in other non-mineralizing tissues albeit in very small amounts (Prasad et al. 2011).

8.2.3 DSP

DSP is the second abundant noncollagenous protein next to DPP and along with DPP is considered as a phenotypic marker of dentin and secretory odontoblasts. DSP and DPP are proteins encoded by the gene DSPP. Although encoded by the same gene, the amounts of protein present at any given time vary significantly. DPP accounts for about 50 % of the total NCPs present in dentin whereas DSP constitutes only 5 % (Qin et al. 2001; Yamakoshi et al. 2006). As they are expressed via the same gene, the expression patterns of DSP and DPP coincide although the protein half-life of DSP may be shorter due to its susceptibility to proteolytic cleavage. Thus the difference in the amount of DPP and DSP protein levels could be a direct demonstration of the functional difference between these two proteins. Our experiments also indicate a differential expression of DSP compared to DPP. In Fig. 8.2, the blue immunohistochemical staining shows predominant DSP expression in the odontoblast cells and to some extent in the dental pulp. However, it is not observed in the dentin matrix.

8.2.4 DMP4/FAM20C

DMP4 is the newest member of the DMP family of proteins. It was discovered as a novel protein via a subtractive hybridization technique (Hao et al. 2007). It is a 579 amino acid long secretory protein that contains a known calcium-binding domain. In situ hybridization techniques showed that the mRNA for DMP4 is highly expressed in odontoblasts and osteoblasts.

Localization studies by in situ hybridization and immunohistochemistry identified the spatial and temporal expression patterns of this protein during development of osteogenesis and odontogenesis (Wang et al. 2010). These studies revealed that during osteogenesis, DMP4/Fam20C is expressed in the chondrocytes, osteoblasts and osteocytes. The intracellular localization is predominantly cytoplasmic. In the ECM, the protein was localized in the long bone as well as in the alveolar bone matrices. During odontogenesis, the mRNA expression was noted in odontoblasts, ameloblasts, cementoblasts and periodontal ligament fibroblasts. The protein expression was however, noted only in odontoblasts, ameloblasts and cementoblasts (cytoplasmic localization). In the ECM, the protein has been identified in dentin, enamel, cementum and bone. Figure 8.3 shows immunohistochemical localization of Fam20C in the femur of 30-day-old mice. The white arrows in the image point to ECM localization. Figure 8.3b is a zoomed in image of the boxed area in Fig. 8.3a. Overall, the expression pattern of this protein suggests a strong role for this protein in biomineralization.

Recently, it was shown that DMP4 is a kinase that phosphorylates the family of secreted calcium binding proteins that consists of the matrix proteins osteopontin, DMP1 and DSPP (Tagliabracci et al. 2012, 2013). This further suggests that phosphorylation events, catalyzed by secreted kinases may be a critical component of regulatory mechanisms in the ECM.

From the localization patterns of the dentin matrix proteins, it is clear that the organic matrix synthesized by either osteoblasts or odontoblasts is not homogenous in composition and that in a mineralizing tissue there are well-defined zones with different properties. The dentin

matrix proteins are a heterogeneous mixture and they operate within a well-defined and controlled program to facilitate cell adhesion and differentiation and in the extracellular matrix to nucleate, direct crystal placement, stabilize the mineral phase and regulate the crystal size.

8.3 Functions of DMPs

The localization pattern of the DMPs suggests that these proteins are multifunctional. In this section, we will elucidate the multifunctional

8.3.1 DMP1

The first multifunctional protein identified from the dentin matrix is DMP1. DMP1 belongs to a family of non-collagenous proteins categorized as small integrin-binding ligand, N-linked glycoproteins (SIBLING) (Ravindran and George 2014). Loss of DMP1 results in defective biomineralization of bone and dentin, particularly the phenotype exhibits hypophosphatemic rickets and osteomalacia (Feng et al. 2006). Recent studies have shown that DMP1 regulates the hormone FGF23 (Feng et al. 2006) and plays critical role in osteoblast differentiation and biomineralization. Both of these functions will be highlighted below.

8.3.1.1 Biomineralization Function of DMP1—DMP1 is a highly phosphorylated protein with a strong affinity for calcium. As a result, its primary function in the ECM is to facilitate nucleation of hydroxyapatite crystals. In vitro experiments indicate that DMP1 can self assemble into a β -sheet template and facilitate the nucleation of hydroxyapatite (He et al. 2003; Bhatia et al. 2012). Additionally, DMP1 can bind specifically to the N-telopeptide region of type I collagen. Nucleation of hydroxyapatite was exclusively found in regions where DMP1 bound to type I collagen (He and George 2004). Thus, DMP1 functions as a nucleating protein in the extracellular matrix of bone and dentin. Further, overexpression of DMP1 accelerated biomineralization and significantly altered the biomechanical properties of cortical bone (Bhatia et al. 2012). The active HAP nucleating domain was found to reside at the C-terminal end of the molecule and overexpression of this fragment alone, could rescue the defective mineralization phenotype observed in DMP1 null mice (Lu et al. 2011).

8.3.1.2 DMP1 and Stem Cell Differentiation—DMP1 contains an RGD tripeptide. RGD domain serves as ligands for integrin receptors on the cell membrane and can regulate cell adhesion and cytoskeletal dynamics. Published studies show that the RGD domain in DMP1 promotes selective cell attachment (Kulkarni et al. 2000). Furthermore, the RGD domain in DMP1 binds to $\alpha 5 \beta 1$ integrin on the cell surface and activates the extracellular signal-regulated kinase (ERK) 1/2 pathway to stimulate osteoblastic differentiation of mesenchymal stem cells including periodontal ligament stem cells (PDLSCs) (Chandrasekaran et al. 2013; Eapen et al. 2011). This DMP1 mediated signaling cascade can occur via DMP1 tethered to the ECM via its collagen binding domains facilitating cell attachment and differentiation.

DMP1 in the ECM can undergo receptor-mediated endocytosis via its receptor GRP-78 (Ravindran et al. 2008). Endocytosis of DMP1 triggers calcium mediated regulation leading

to the activation of the mitogen activated protein kinase (MAPK) signaling cascade resulting in osteogenic differentiation of mesenchymal stem cells (Eapen et al. 2010). GRP-78 is also a multi- functional protein. GRP-78 binds type I collagen and DMP1 in the ECM and in conjunction with DMP1 can initiate the nucleation of calcium phosphate to facilitate matrix mineralization (Ravindran et al. 2011).

DMP1 is a substrate for the matrix metalloprotease 2 (MMP2) enzyme. The proteolytic fragments of DMP1 generated via MMP2 cleavage also have the potential to trigger mesenchymal stem cell differentiation (Chaussain et al. 2009). This scenario can be envisaged as one wherein bone remodeling is occurring resulting in release of several NCPs that are acted upon by proteases. These proteolytic fragments can then prove to be useful in recruiting and directing differentiation of stem cells enabling new bone formation.

Apart from its obvious roles in stem cell differentiation and in biomineralization, DMP1 has an intriguing presence in the nucleus of mesenchymal cells (Siyam et al. 2012; Narayanan et al. 2003). Studies show that nuclear DMP1 can bind to DSPP promoter and initiate transcription of DSPP gene (Narayanan et al. 2006), which in turn can stimulate DPP secretion resulting in accelerated mineralization of the organic extra-cellular matrix.

Collectively, DMP1 is a multi-faceted protein that plays a critical role in native bone and dentin physiology. Using DMP1 for bone tissue engineering may promote accelerated bone formation and differentiation of mesenchymal stem cells to osteoblast precursors and fully functional osteo-blasts facilitating biomineralization.

8.3.2 DPP

Like DMP1, DPP also belongs to the SIBLING family of proteins and contains an RGD sequence for integrin binding and this domain is conserved among most species. DPP is the most acidic protein ever discovered with an isoelectric point of 1. DPP is highly phosphorylated and contains multiple aspartic acid-serine-serine repeats (DSS repeats) contributing to its acidic nature (George et al. 1996). DPP is also a multi-functional protein with both intra and extra cellular functions.

8.3.2.1 DPP Mediated Hydroxyapatite Nucleation—DPP is the most abundant noncollagenous protein in the dentin matrix and it is also present in bone albeit in relatively smaller quantity. Studies have shown that the abundant levels of phosphoserine and aspartic acid in DPP might be responsible for calcium binding in an ordered manner. The calcium phosphate clusters initially formed by immobilized phosphate esters have short range order based on the ionic and coordination binding of the successive layers. These clusters grow until they coalesce with adjacent clusters into apatite nuclei (George and Hao 2005; Veis 1993; Prasad et al. 2010). Presence of DPP in the alveolar bone at later stages of development suggests that this ECM protein might be involved in regulating crystal size at later stages of osteogenesis (Hao et al. 2004). However, phosphorylation of DPP is essential for its function as a mediator of biomineralization (He et al. 2005).

8.3.2.2 Signaling Roles of DPP—Recently several studies have identified many signaling functions for DPP resulting in mesenchymal stem cell differentiation. In the ECM,

DPP can mediate cell adhesion and integrin signaling via the RGD peptide (Eapen et al. 2012a, b). Integrin signaling in turn plays a significant role in stem cell differentiation and cytoskeletal rearrangement. Additionally, DPP can also trigger calcium and calmodulin mediated signaling cascades in undifferentiated mesenchymal cells guiding their differentiation process (Eapen et al. 2013). All of these signaling roles of DPP will prove to be crucial if it has to be utilized effectively as a component of the tissue engineering triad.

Recently, the acidic domain of DPP consisting of multiple DSS repeats was identified as an endocytic peptide capable of cellular endocytosis via a pathway that is not receptor mediated (Ravindran et al. 2013). The acidic domain by itself does not possess any signaling function and hence it can be used as a carrier molecule for targeted protein delivery.

Collectively, all these studies point to yet another multi-functional DMP that is an enticing target both as a signaling molecule as well as a facilitator of targeted protein delivery.

8.3.3 DSP

As discussed previously, the 5' region of DSPP encodes DSP. Although DSP is expressed at the same time as DPP, its quantity in mineralized tissues constitutes only a fraction of the amount of DPP. On the other hand, functional studies have identified that DSP serves as a negative regulator of biomineralization and thereby bringing balance and control to the action of DPP (Gibson et al. 2013). Published reports also confirm that DSP is involved in the formation and maintenance of tooth structures such as the peridontium (Baba et al. 2004).

Although DSP is not as functionally diverse as DMP1 and DPP, it is still an important contributor to biomineralization by regulating mineralization. It is therefore, important to consider the use of this protein in tissue engineering applications from this perspective to achieve a biomimetic setup.

8.3.4 DMP4/Fam20C

8.3.4.1 Calcium-Binding Property of DMP4/Fam20c—A characteristic functional domain identified in the DMP4 sequence is the Greek-key calcium-binding motif located at the C-terminal end of the molecule. Conformational analysis indicates that the entire molecule adopts a flexible random structure, but in the presence of calcium, the Greek key domain could bind calcium ions resulting in ordered structures.

A role for DMP4 in the extracellular matrix was confirmed by mineral nodule formation assay after its overexpression. Silencing DMP4 inhibited mineralization of the ECM organic matrix. Phenotype of the DMP4 null mice exhibited hypophosphatemic rickets confirming a significant role that this molecule plays in the formation of physiological bone (Wang et al. 2012). Published studies also indicate that Fam20C is a new regulator of FGF23 along with DMP1 and plays a significant role in biomineralization. However, when DMP1 is overexpressed in the Fam20C knockout mice, the phenotype is not rescued indicating that DMP1 and Fam20C do not perform redundant functions *in vivo* (Wang et al. 2014). Mutations in Fam20C are associated with Raine syndrome, an autosomal recessive skeletal dysplasia characterized by osteosclerosis and ectopic calcifications often leading to neonatal

death (Simpson et al. 2007). This further attests to the importance and uniqueness of Fam20C function during biomineralization.

8.3.4.2 Fam20C/DMP4 and Osteoblast Differentiation—Gain of function studies were performed to elucidate the function of DMP4 in pre-osteoblasts and undifferentiated mesenchymal stem cells. Results from these studies found an increase in osteogenic gene expression levels. Similarly, silencing DMP4 showed down-regulation in the mineralization cascade (Hao et al. 2007). A recent report suggests that Fam20C is also a secreted kinase that phosphorylates extracellular proteins that regulate biomineralization (Tagliabracci et al. 2012, 2013).

Apart from its role in dentin mineralization, Fam20C is also involved in the process of enamel formation and amelogenesis (Wang et al. 2013). This fact shows that its role in biomineralization is ubiquitous and is not limited to type I collagen-based biomineralization. It is to be noted that enamel formation and biomineralization is a completely different phenomenon when compared to bone and dentin. The primary difference being the complete absence of type I collagen in enamel. However, in dentin and bone, type I collagen is the primary organic component. Further studies are required to understand the complexities of this novel biomineralization regulator.

Overall, the dentin matrix proteins are unique and play a regulatory role in calcified tissue formation. The calcium binding domains in each of these proteins are different and play a combinatorial and important role in the assembly of hard tissues. Besides the extracellular function they initiate intracellular events that aid in cell differentiation and synthesis of a functional bone matrix.

8.4 Tissue Engineering Strategies Using DMPs

Tissue engineering bone requires recreating the complexities that nature has devised for physiological bone formation. Several studies have shown that the bone repair processes resemble normal development of the skeleton during embryogenesis. Therefore, bone repair and regeneration should be greatly influenced by the intimate interactions between the cells and the matrix. There are several signaling molecules used for inductive bone regenerative engineering. Among them recombinant bone morphogenetic proteins have been used clinically. However, several detrimental clinical outcomes raise concerns. In the previous sections, we introduced and briefly summarized the various functionalities of the members of the DMP family. In this section, we will look at how these proteins can be exploited in bone repair and regeneration.

8.4.1 DMP1

Due to its multi-functional nature, DMP1 is a very attractive protein to be used as native protein in its entirety or its processed forms in tissue engineering applications. To this end, DMP1 has been used as a signaling molecule in biomimetic collagen constructs to regenerate soft tissues such as the dental pulp tissue (Alsanea et al. 2011; Prescott et al. 2008). With respect to bone regeneration, biomineralization motifs from DMP1 have been used to generate engineered peptides with the ability to directly transform amorphous

calcium phosphate (ACP) to crystalline hydroxyapatite (HAP) (Tsuji et al. 2008). Nucleation of crystalline HAP with a calcium to phosphorus ratio of 1.6 similar to that of native bone is absolutely critical for bone tissue engineering applications to generate high quality engineered tissues capable of withstanding the mechanical loading that bones are normally subjected to. In this regard, the ability of DMP1 derived peptides to induce transformation of ACP to crystalline HAP is a handy tool in our quest for a signaling molecule to be incorporated within the biomimetic scaffold.

Along similar lines, genetically engineered non toxic phage bio-nanofibers containing the nucleating motifs from DMP1 have been generated to serve as building blocks of self assembling fibers that can be used to recreate the complex structure of bone (Xu et al. 2011). In addition, the presence of DMP1 motifs in these constructs facilitated the nucleation of crystalline HAP.

More recently, synthetic peptides that contain the type I collagen binding domain and the nucleating domains of DMP1 were generated for repair of carious dentin (unpublished study). The function of the collagen-binding domain was to tether the peptides to the N-telopeptide region of type I collagen and the nucleating domain would facilitate calcium binding and subsequent transformation to crystalline hydroxyapatite. These peptides are non-toxic, easy to synthesize and can be incorporated into any collagen based scaffold designed for bone tissue engineering.

In a recent study, using biomimetic strategy, we have developed a self-assembling leucine zipper chimeric protein that has the ability to self-assemble into a biomimetic scaffold for tissue engineering applications (Huang et al. 2014). The primary feature of this molecule is its ability to be customized for several tissue-engineering applications. One of the variants that we have developed for bone tissue regeneration is the leucine zipper chimeric protein that contains the DMP1 nucleating motifs. In vivo and in vitro studies show that these scaffolds can support cell differentiation and facilitate hydroxyapatite nucleation (Padovano et al. 2015). Figure 8.4 shows the ability of the self-assembling, DMP1 calcium-binding motif containing leucine zipper hydrogel to nucleate crystalline hydroxyapatite. When subjected to in vitro nucleation, these the nucleated particles contained hydroxyapatite morphology (Fig. 8.4a). Element analysis (arrows in Fig. 8.4b) revealed the presence of calcium and phosphorus and finally, small angle diffraction experiments using TEM confirmed the crystalline nature of the nucleated calcium phosphate (Fig. 8.4c).

One underrated function of DMP1 that has not been significantly explored in bone tissue engineering is its signaling function in osteogenesis. Osteogenesis is a dynamic process initiated by mesenchymal stem cells in coordination with signaling molecules. As DMP1 can stimulate MSCs to differentiate into functional osteoblasts, therefore incorporating the osteogenic signaling portion of DMP1 might be of importance to induce osteogenic differentiation of adult stem cells. These signaling properties of DMP1 are recent discoveries that will in time find its way into tissue engineering applications much like its nucleating properties. Depending on the end use, various combination of the functional domains of DMP1 can be developed into powerful tools that can be incorporated in scaffolds to elicit responses for mineralized tissue regeneration.

8.4.2 DPP

Similar to DMP1, the primary function of DPP is biomineralization. Recent studies have shown that type I collagen scaffolds containing DPP was able to generate hydroxyapatite containing mineralized nodules similar to those found in bone when incubated with simulated body fluid solution at 37 °C (Zurick et al. 2013). The other NCPs used in this study were osteopontin (OPN) and bone sialoprotein (BSP). It is to be noted that although the other NCPs generated calcium phosphate deposits, none of them resembled mineral nodules present in bone. From this study it is clear that DPP would be a prime candidate to be incorporated in bone tissue engineering scaffolds to facilitate nucleation and growth of HAP.

Our recent report shows the ability of the DSS polypeptide domain from DPP to penetrate the plasma membrane of several cell types including mesenchymal, epithelial and endothelial cell types (Ravindran et al. 2013). This DSS domain is endocytosed in a non-classical manner that is not receptor mediated. Additionally, the mechanism of endocytosis is also not via clathrin or caveolar endocytic pathways. These properties provide the DSS polypeptide with the ideal characteristics of a carrier protein. Results show the ability of this polypeptide to transport the osteoblast transcription factor Runx2 to the nucleus of mesenchymal cells (arrows in Fig. 8.5a). Further, the transcriptional activity of Runx2 remains intact upon cell entry and nuclear translocation (Fig. 8.5b). These results show the utility of this acidic polypeptide to function as a carrier molecule for targeted protein delivery to induce osteogenic differentiation of mesenchymal stem cells.

The minimum DSS repeats required to achieve endocytosis was found to be 6. Therefore, the carrier peptide is easy to synthesize, and when conjugated with a target protein forms a potent tool for direct intracellular transport of transcription factors to elicit required cell response. Another key feature of this study is that the DSS polypeptide by itself does not trigger cellular differentiation. Therefore, it can serve as an effective transport molecule not only for Runx2, but several other factors as well. In this regard, the tissue engineering implications for this polypeptide are far reaching. It is possible to envisage the use of multiple fusion proteins consisting of early and late transcription factors tagged to the DSS polypeptide. These fusion proteins can be delivered in a controlled manner at different stages to effectively control stem cell fate and tissue regeneration. Such approaches will be at the forefront of bone tissue engineering applications and further studies are required to identify and standardize the different parameters that might influence the success of this approach.

8.4.3 DSP

DSP is one of the least studied DMPs and its primary function seems to be inhibition of mineralization and influencing the role of DPP. DSP has so far not been used in any tissue engineering applications. However, one possible application of DSP could be its use along with DPP to control the rate and quality of biomineralization. Several NCPs existing in the bone and dentin matrix function as negative regulators of biomineralization. Their primary function is to work in tandem with the positive regulators to ensure appropriate and high quality mineral deposition. The function of these inhibitors is just as important as other

positive regulators and cannot be ignored when designing biomimetic scaffolds for bone tissue engineering.

We therefore envisage DSP working in tandem with DPP in the same ratio as they exist in vivo. The appropriate physiological concentrations of these molecules might be necessary to control the rate and quality of biomineralization bringing balance and protecting against hyper mineralization events. However, additional research needs to be performed to validate this hypothesis before DSP can be used as a regulatory molecule in bone tissue engineering.

8.4.4 DMP4/Fam20C

Like the other DMPs such as DMP1 and DPP, Fam20C is a positive regulator of biomineralization. The recent finding that Fam20c is a Golgi kinase opens up avenues for the use of this protein in tissue engineering applications. Protein phosphorylation is one of the most important cellular control mechanisms. Several of the proteins that function to regulate biomineralization are phosphoproteins. Phosphorylation of these proteins makes the proteins highly polyanionic and are thought to play a role in regulating biomineral morphology. The current theory is that the negatively charged proteins could selectively interact with the crystallographic faces that would alter the growth kinetics at the growing step edges in specific directions thereby altering the crystal shape. Therefore, kinases that could phosphorylate matrix proteins could have potential applications in tissue engineering bone. It is therefore possible to envision the use of this protein for such a purpose.

8.5 Conclusion

Formation of the vertebrate biomineral requires the structural arrangement of the HAP crystals. At the nanostructural level the HAP crystals are crystallographically oriented nearly parallel to the long axis of the collagen fibrils. This structural arrangement leads to the formation of intertwined organic-inorganic composite that imparts bone with high strength and rigidity. The noncollagenous proteins of which the dentin matrix proteins form a subset of proteins play an important role in the biomineralization process. These proteins can help in the orientation and reinforcement of the nanocrystals by the formation of a transient amorphous precursor phase leading to their transformation into a crystalline phase.

The use of the dentin matrix proteins in bone tissue engineering applications can help recreate the architecture of the mineralized collagen fibril by regulating the kinetics of the amorphous to crystalline transition. This is an important step toward reproducing the hierarchical organization of natural bone. Nature uses multiple proteins to achieve controlled mineralization. Understanding the physiological function of the dentin matrix proteins would help in tissue engineering bone by promoting crystallographic alignment of apatite crystals as well as facilitating spatial control of mineralization in specific locations on the organic collagenous matrix. Our understanding of the biomineralization process can help in providing cues for bone tissue repair and regeneration.

Acknowledgments

We are pleased to acknowledge the long-term support from the National Institutes of Health, DE 19633 and DE 11657 for financial support of our own contributions in this area.

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Fig. 8.1.

Expression of DMP1 in the femur of 3-month- old mouse: 3D rendering of z-stack confocal micrographs showing immunofluorescence staining of DMP1 (*red stained areas*). *M* denotes the bone marrow and *B* represents bone matrix

Fig. 8.2.

Expression of DPP and DSP in the unerupted molar of 7 day old mice: Images are 3D rendering of z-stack confocal images of sections stained with the DPP (*red*) and DSP (*blue*) antibodies. Note the difference in the expression pattern of the two proteins. *DP* represents the dental pulp and *D* represents dentin. Note the predominant expression of DPP in the mineralizing dentin. (a) Expression of DPP; (b) Expression of DSP; (c) Merged image of (a) and (b)

Fig. 8.3.

Expression of DMP4/Fam20C in the femur of 1-month-old mouse. *Arrows in (a) point to positive staining in the ECM. (b) Is an enlarged image of the boxed area in ((a)) properties of the DMPs that justify them as potential candidates for use in bone tissue engineering applications.*

Fig. 8.4. Nucleation of crystalline hydroxyapatite by 3D self-assembling leucine zipper scaffolds containing the nucleating domains of DMP1. (a) is an SEM image of the nucleated crystals. (b) shows energy dispersive X-ray element analysis of the crystals showing the presence of calcium and phosphorus ions (*white arrows*). (c) is a small angle diffraction image from a TEM showing the presence of crystalline calcium phosphate (*arrows pointing to dots* represent crystals)

Fig. 8.5.

Protein delivery using the DSS polypeptide: (a) represents 3D reconstruction of z-stack confocal images showing endocytosis of quantum dot tagged DSS poly-peptide expressed as a fusion protein with the osteoblast transcription factor Runx2 (red). Note the nuclear localization of the transcription factor (arrows). The green staining represents EEA1 (early endosome antigen 1) staining, a marker for endosomes. Note that the endocytosed protein is not routed to the endosomes for degradation. (b) represents the qRT PCR data showing expression levels of alkaline phosphatase (ALP), SMAD 4 and DMP1 that are down stream targets for Runx2. Increase in gene expression at 48 h post endocytosis indicates that the endocytosed Runx2 retains its transcriptional activity