2016

Identification of FAM20C binding proteins

https://hdl.handle.net/2144/16124

Boston University
IDENTIFICATION of FAM20C BINDING PROTEINS

I PING LIN

Doctoral of Dental Surgery (D.D.S), National Taiwan University, 2009

Submitted in partial fulfillment of the requirements for the degree of 
Masters of Science in Dentistry 
In the Department of Periodontology 
2016
First Reader _____________________________ Date __________

Dr. Yoshiyuki Mochida, DDS, Ph.D.
Associate Professor
Department of Molecular and Cell Biology

Second Reader _____________________________ Date __________

Dr. Philip C. Trackman, Ph.D.
Professor, Director of Oral Biology Research
Department of Molecular and Cell Biology

Department Chairman _____________________________ Date __________

Dr. Serge Dibart, DMD
Professor
Department of Periodontology
IDENTIFICATION of FAM20C BINDING PROTEINS

I PING LIN

Henry M. Goldman School of Dental Medicine, Boston University, 2016

Major Professor: Dr. Yoshiyuki Mochida, DDS, Ph.D.,
Associate Professor of Molecular and Cell Biology

ABSTRACT

FAM20 (family with sequence similarity 20) members in humans consist of
FAM20A, FAM20B and FAM20C. The mutations of FAM20A in humans lead to
Amelogenesis Imperfecta (AI), gingival hyperplasia and enamel renal syndrome
(ERS) in humans. Mutations of FAM20B in Danio rerio result in decreased cartilage
matrix production and skeletal defects. Mutations in FAM20C leads to neonatal
lethal osteosclerotic bone dysplasia in humans, known as Raine syndrome. One of
the mutants is FAM20C-D478A. FAM20C intracellularly functions as a Golgi casein
kinase. It phosphorylates secretory pathway proteins within S-x-E motif (where S is
Ser, X is any amino acid, and E is Glu). Extracellular role of FAM20C has also been
suggested as a growth and differentiation factor, and the exogenous FAM20C
treatment accelerates MC3T3-E1 osteoblast differentiation and mineralization in
vitro. The first purpose of this study was to purify FAM20C protein. HEK 293 cells
were transfected with FAM20C expression vectors, cell clones that overexpress
FAM20C were isolated and FAM20C protein was collected and purified. The
Western blot results of purified FAM20C showed higher bands, around 100 kDa and
170 kDa, than expected molecular weight, 66 kDa. Post-translational modification was thought to be the possible reason. Therefore, the second purpose was to find binding proteins of FAM20C by mass spectrometry protein identification analysis to check if FAM20C has other post-modifications, such as glycosylation. FAM20C-WT and FAM20C-D478A proteins were chosen to perform the study since FAM20C-D478A was investigated in previous studies of Golgi casein kinase, FAM20C, and was found to have no kinase activity.

In this study, periostin was identified to bind to FAM20C and the binding of these 2 proteins was confirmed by immunoprecipitation and Western blot analysis. Since FAM20C functions as a secretory kinase, it is suggested that periostin may be a substrate for FAM20C kinase. Further investigation is needed to determine the presence of phosphorylation in periostin and its role in periosteum and periodontal ligament.
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1. Introduction

Periodontium

Periodontium consists of gingiva, alveolar mucosa, alveolar bone, cementum and periodontal ligament. It is a supporting apparatus for the teeth in function and occlusion [1. Antonio Nanci, Dieter D. Bosshardt, 2006, 2. Anthony Palumbo].

The gingiva is firmly bound to the underlying bone and is continuous with the alveolar mucosa which is located apically and movable. Type I collagen is the predominant component in the connective tissue of gingiva. There are also cells, nerves, blood vessels and ground substance in the connective tissue. The cells are mainly fibroblasts, mast cells and immunologic cells. The ground substance is composed of glycosaminoglycans and proteoglycans. It helps water retention which can support the functional force and maintain the shape and structure of the gingival tissue [2. Anthony Palumbo].

Alveolar bone is the osseous tissue in the maxilla and mandible which houses and supports the sockets of the teeth. Bones are constantly remodeled since they have to respond to the forces of mastication. It is created by osteoblasts and continues remodeling throughout life. Osteoblasts produce type I collagen, the major bone matrix protein which is then mineralized with calcium and phosphate, forming hydroxyapatite [2. Anthony Palumbo]. Type I collagen is the predominant organic component, up to 90%. Many noncollagenous proteins are found in bone, such as bone sialoprotein, dentin matrix protein1, osteopontin, osteocalcin and proteoglycans, etc [1. Antonio Nanci, Dieter D. Bosshardt, 2006].

Cementum is the hard, avascular connective tissue that coats the root of teeth. It is classified into acellular and cellular types and serves primarily to attach the principal periodontal ligament fibers. It is made of collagen fibers within a mineralized matrix [1. Antonio Nanci, Dieter D. Bosshardt, 2006]. The composition of cementum is similar to bone. Cementum is composed of 50% mineral and 50% organic matrix [2. Anthony Palumbo].

Periodontal ligament is the soft, unmineralized connective tissue between the cementum and the alveolar bone. It is a connective tissue well adapted to its main function, supporting teeth in their sockets and as a shock absorber against mastication impact. It is also a sensory receptor and a cell reservoir for tissue homeostasis, repair and regeneration [1. Antonio Nanci, Dieter D. Bosshardt, 2006]. Periodontal ligament cells are a heterogeneous population of cells that can differentiate into fibroblasts, cementoblasts, and osteoblasts [3. Nohutcu RM, McCauley LK, 1997, 4. McCulloch CA, Bordin S, 1991]. Several noncollagenous proteins are found in periodontal ligament, including alkaline phosphatase, proteoglycans, and glycoproteins. Interestingly, despite the mechanical stress of mastication or orthodontic forces under physiological conditions, the periodontal ligament always maintains its width unmineralized. The issue of how periodontal ligament maintains uncalcified between two calcified tissues remains unsolved and

**Components involving in mineralization**

Considering the mechanism of bone mineralization, three main components are involved: inorganic substance, collagen fibrils, and non-collagenous proteins, constituting bone organic matrix. The forth component is cells that can produce organic matrix, control the flux of ions into the extracellular matrix and release signals to begin or end the mineralization process [6. Ermanno Bonucci, 2013. ].

**Mineral (HA, Hydroxyapatite)**

The term apatite indicates a mineral structure. Biologically mineralized crystals are typically formed in an organic matrix with precise regulation of synthetic mechanisms through proteins. Biogenic apatite varies in several ways from the geologically produced mineral. First, biogenic apatite has a smaller crystal size, which has a higher surface area, thus permitting additional adsorption of ions and molecules on the apatite surface. Second, the biogenic apatite contains significant carbonate substitutions. F ions, for example, are readily incorporated into the HA lattice, forming a less soluble phase of calcium phosphate. Finally, biological mineral tends to attain high crystallinity and a more organized structure on the time scale of days or months rather than years [7. Liam C. Palmer, Christina J. Newcomb, 2008].

HA is the calcium phosphate mineral found in vertebrate bones and teeth. In the early 1900s, X-ray diffraction patterns identified ground bone to be similar to geological HA, Ca$_5$(PO$_4$)$_3$(OH). Later studies have shown that the ratios of Ca/P in bone can vary significantly because the body utilizes bone as a reservoir to maintain homeostasis with respect to calcium, magnesium, and phosphate ions. Carbonated HA, Ca$_{10}$(PO$_4$,CO$_3$)$_6$(OH)$_2$, is the most abundantly produced phosphate mineral in bones and teeth. The ratios of Ca/P in enamel and dentin more closely resemble the stoichiometric ratio, since teeth are not typically involved in maintaining ion homeostasis [7. Liam C. Palmer, Christina J. Newcomb, 2008].

The inorganic substance of bone is a calcium phosphate, regarded as hydroxyapatite with the formula Ca$_{10}$(PO$_4$)$_6$(OH)$_2$. The size and composition of bone apatite changes with age, so does Ca/P ratio [6. Ermanno Bonucci, 2013, 8. Adele L. Boskey, 2007].

**Collagen fibrils**

Collagens comprise a large family of triple helical proteins and are the most abundant proteins in vertebrates. There are now at least 29 genetically distinct types of collagen. Type I collagen is the most abundant structural protein in vertebrates. It is composed of two $\alpha$ 1 chains and one $\alpha$ 2 chain, forming a long uninterrupted triple helical structure with short non-triple helical telopeptides at both the N- and C- termini. One of the critical factors for the structural and biomechanical
functions of type I collagen fibrils are the PTMs (post-translational modifications) of peptidyl lysine residues [9. Mitsuo Yamauchi, Marnisa Sricholpech, 2012].

In the cell, specific peptidyl lysine residues both in the helical and nonhelical domains can be hydroxylated forming 5-hydroxylysine, which then can be glycosylated. The type and extent of collagen glycosylation varies among different collagen types and varies depending on the functional regions within the tissue, maturation and pathological conditions. In bone, for instance, alterations in the levels of collagen glycosylation have been reported in several bone/skeletal disorders, such as osteogenesis imperfecta [81. Tenni R, Valli M, 1993. 82. Brenner RE, Vetter U, 1990. 83. Cetta G, De Luca G, 1983.] and postmenopausal osteoporosis [84. Michalsky M, Norris-Suarez K, 1993. 85. Moro L, Bettica P, 1997. 86. Moro L, Suarez KN, 1997], suggesting a role of collagen glycosylation in bone mineralization [9. Mitsuo Yamauchi, Marnisa Sricholpech, 2012].

Outside the cell, an enzymatic oxidative deamination occurs on some telopeptidyl lysine and hydroxylysine residues producing the reactive aldehydic residues. The aldehydes can initiate a series of condensation reactions to form extensive covalent intra- and intermolecular cross-links. The initial intra- and intermolecular crosslinks can then further mature into multivalent crosslinks, which demonstrate the highly specific molecular packing arrangement in the fibril and show tissue specificity. The cross-links are the final step of collagen biosynthesis and are critical in providing the fibril with stability and the biomechanical functions [9. Mitsuo Yamauchi, Marnisa Sricholpech, 2012].

Collagen is composed largely of the amino acids glycine, proline, and hydroxyproline, often as Gly-X-Y repeats (where X and Y are either proline or hydroxyproline). Tropocollagen, the subunit of collagen fibrils formed of three polypeptide strands, assemble in a parallel, quarter-staggered arrangement. There is a 40 nm gap, also called the “hole zone” with 27 nm of overlap between adjacent units. This spacing give rise to the basic 67 nm repeat unit and banding observed by electron microscopy, also known as the D-period. The hole zones are critical in mineralization, as they appear to be the site of mineral nucleation. The crystals appear to grow and proliferate from this area. The size of this gap also appears to constrain the mineral growth [7. Liam C. Palmer, Christina J. Newcomb, 2008].

Type I collagen is the most abundant component of the bone matrix and may be able to induce the process of calcification, especially in combination with phosphoproteins. That is, the collagen-phosphoproteins complex facilitate calcification better than collagen alone [10. Glimcher MJ, 1989]. Collagen provides the template for mineral deposition in bone and dentin.

Non-collagenous components

Non-collagenous components include proteoglycans, phosphoproteins, and phospholipids. In addition to type I collagen, the ECM of bone and dentin contains several noncollagenous proteins (NCPs). These NCPs are believed to actively promote and control mineralization of collagen fibers and crystal growth within osteoid and predentin. Most of the phosphoproteins are SIBLINGs (Small Integrin-Binding Ligand, N-linked Glycoproteins), genes of which are on the same chromosome. The polyanionic SIBLINGs all interact with collagen fibrils and can be
cleaved into small fragments by enzymes. The posttranslational modifications, such as phosphorylation, binding and fragmentation may affect mineralization [11. C. Qin, O Baba, 2004].

The SIBLING proteins include osteopontin (OPN), bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE).

Osteopontin (OPN) is a secreted 60-kDa phosphoprotein. The name was introduced to reflect the potential function in bone to serve as a bridge between cells and hydroxyapatite through RGD and polyaspartic acid motifs. OPN is present in bone in large quantities, but it is also expressed in a variety of tissues and cells [11. C. Qin, O Baba, 2004]. Several animal studies show that OPN may be a major inhibitory factor on mineralization [66. Boskey AL, Spevak L, 2002] and its deficiency affects the function of osteoclasts [67. Razzouk S, Brunn JC, 2002].

BSP (Bone sialoprotein), unlike OPN, is found almost exclusively in mineralized tissues including bone, dentin, mineralizing cartilage, and cementum. Analysis of some data suggests that BSP acts as a nucleator of the initial apatite crystals, and then acts as inhibitor in directing the growth of the crystals [11. C. Qin, O Baba, 2004]. Adult mice with a knockout of the Ibsp gene (Bsp-/-) are shorter than their wild type counterparts and display a low level of bone remodeling [87. Malaval L, Wade-Gueye NM, 2008]. Another study further showed that Bsp-/- mice are born with their shorter stature and that the lack of Bsp alters long bone growth, membranous/cortical primary bone formation and mineralization, as well as cartilage and osteoblast gene expression, with low bone Igf-1 and high levels of Opn [88. Wafa Bouleftour, Maya Boudiffa, 2014].

DMP1(dentin matrix protein 1) is found in dentin, bone and soft tissue. It plays an important role in mineralization of bone and dentin supported by many studies [89. Shigeki Suzuki, Naoto Haruyama, 2012]. MC3T3-E1 cells overexpressing DMP1 showed earlier onset of mineralization and produced mineralized nodules [90. Narayanan K, Srinivasas R, 2001]. Dmp1 knockout mice showed delayed conversion of osteoid to bone and predentin to dentin [91. Feng JQ, Ye L, 2002].11. C. Qin, O Baba, 2004]. The full length form of DMP1 inhibits calcification, but becomes a promoter of calcification when cleaved or dephosphorylated [31.Tartaix et al(2004)].

Dentin sialophosphoprotein (DSPP), the precursor protein, gives rise to two proteins, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP). DPP is the most abundant NCP in the ECM of dentin. It contains large amounts of aspartic acid and phosphoserine. DPP is an important initiator and modulator of dentin apatite crystal formation. Dspp knockout mice show defective mineralization of dentin. DSPP is present not only in teeth but also in bone and osteoblasts but a much lower levels than in teeth [11. C. Qin, O Baba, 2004].

MEPE (Matrix extracellular phosphoglycoprotein) is an inhibitor of mineralization is situ. MEPE exists as smaller peptides, including the C-terminal ASARM (acidic, serine-, and aspartic acid-rich MEPE motif) peptide, which is
analogous to the C terminus of the salivary protein statherin. In bone, MEPE is mainly expressed by osteocytes [12. Adele L. Boskey, 2010].

Differences in collagen between skeletal and nonskeletal tissues

Skeletal tissues

The intra- and intermolecular crosslinks show a highly specific molecular packing arrangement in the fibril and tissue specificity. One of the most well-investigated pathways is the one leading to a Pyr(pyridinoline) crosslinks. It starts with the Hylald residue in the telopeptides, which then reacts with the neighboring helical hydroxylysine residue producing an intermolecular aldimine cross-link. The aldimine can rearrange to form a ketoamine form. Both forms are reducible with reducing agents and can condense with each other forming a trifunctional cross-link, Pyr. A pyrrolic compound is a trifunctional maturation product of the ketoamines. Pyrrole cross-links appear to be restricted to bone and high-load tendons [13. David R. Eyre, Mary Ann Weis, 2013, 9. Mitsuo Yamauchi, Marnisa Sricholpech, 2012]. The pyrrole cross-link is of greater biomechanical importance than the pyridinoline cross-links in mature bone, more amount of pyrrole correlated with higher bone strength [14. L.Knott, A.J.Bailey, 1998]. Pyridinoline and pyrrole are predominant in skeletal tissues. Although Pyr is present in most skeletal and connective tissues, such as cartilage, bone, dentin, tendon, aorta and ligament, it is almost absent from skin and cornea.

(a.) (b.) (c.)

Illustration 1. Structures of pyridinoline and pyrrole cross-links. (a.) The structure of hydroxylysyl pyridinoline (HL-Pyr) cross-link. (b.) The structure of lysyl pyridinoline (L-Pyr) cross-link (C.) The structure of pyrrole cross-link. (Adopted from L.Knott et. al. Bone.1998)

Non-skeletal tissues
HHL (histidino-hydroxylsine-nonoleucine) is predominant in skin and cornea and are suitable for these two tissues because it is a more UV-resistant stable tri-functional cross-link. The major cross-link in soft-tissue is derived from an ACP (aldol condensation product) which can further condense with hydroxylysine forming an aldimine bond. This cross-link, which involves four amino acids, has the most complex chemical structure in all of the known collagen cross-links. [9. Mitsuo Yamauchi, Marnisa Sricholpech, 2012].

In mineralizing tissues, bone specific lysyl pyridinoline and pyrolic cross-links are in a large amount. The collagen post-translational modifications appear to play an integral role in matrix mineralization. Disturbances in the post-translational modification of collagen can affect the mineralization density and crystal structure of the tissue [14. L.Knott, A.J.Bailey, 1998].

**Mechanism of mineralization of hard tissues**

The mechanism of mineralization in bone and other hard tissues is still uncertain. While investigators in the one-mineralization field are divided between accepting collagen-mediated and matrix vesicle mediated mechanisms of mineralization, there is no obligatory incompatibility between these two mechanisms. Matrix vesicle-mediated and collagen-mediated models of mineralization are two separate, but linked steps during osteogenesis and dentinogenesis [15. Marc D.McKee, Betty Hoac, 2013].

Matrix vesicles are formed and released from the outer membranes of osteoblasts and related cells. It is believed that HA is first nucleated within the vesicle. As the crystallite grows bigger, it breaks through the vesicle and is exposed to the extracellular fluid. Extravesicular mineralization is then driven by the extracellular phosphate: pyrophosphate ratio and by the presence of a collagenous fibrillar scaffold, with further regulation of crystal growth by noncollagenous proteins of the small integrin-binding ligand N-linked glycoprotein family [15. Marc D.McKee, Betty Hoac, 2013, 7. Liam C. Palmer, Christina J. Newcomb, 2008].

According to the theory, the initial mineral formation (phase 1) is under cellular control, whereas mineral propagation (phase 2) is mediated by collagen in the extracellular matrix. The hole zones of collagens are critical in mineralization, as they appear to be the site of mineral nucleation. The crystals appear to grow and proliferate from this area. The size of this gap also appears to constrain the mineral growth. In fact, matrix vesicles are not the only site of mineral nucleation. Numerous studies have shown that a wide variety of matrix proteins can also nucleate and control the growth or agglomeration of these crystals [15. Marc D.McKee, Betty Hoac, 2013, 7. Liam C. Palmer, Christina J. Newcomb, 2008].

The biologic control regulating crystal growth in mineralized tissues appears to control the level of mineral-nucleating and mineral-inhibiting noncollagenous proteins to transform unmineralized matrix into mineralized matrix. Many of the noncollagenous proteins are highly acidic phosphoproteins that bind strongly to mineral to regulate crystal growth. Small integrin-binding ligand N-linked glycoproteins are associated with specific sites on collagen molecules, possibly to

**Mechanism of maintaining unmineralized in saliva and periodontal ligament**

Calcium and inorganic phosphate ions are essential to hard tissue formation since they are the components of crystals. Phosphoproteins/phosphopeptides with clusters of acidic residues aid in the prevention of unwanted precipitation of solid calcium phosphates. The acidic residues, particularly phosphoserine, interact with calcium and stabilize clusters of calcium and phosphate. Statherin and acidic proline-rich proteins in saliva have a distinct similarity of structure, with all containing clusters of negatively charged residues such as phosphoserine, glutamate and aspartate. These residues interact with calcium and phosphate ion clusters to stabilize them in aqueous environments, that is, inhibiting precipitation and crystal growth of hydroxyapatite from supersaturated solutions of calcium phosphate [17. Cochrane NJ, Cai F, 2010, 18. Cochrane NJ, Reynolds, 2012, 19. Raj PA, Johnsson M, 1992].

Evidence shows that cells in the periodontal ligament secrete molecules to regulate the extent of mineralization and prevent the fusion of tooth root with surrounding bone, e.g. ankylosis [1. Antonio Nanci, Dieter D. Bosshardt, 2006, 20. Michele M. Fong, M. Ali Darendelier, 2007]. Balancing between the activities of bone sialoprotein and osteopontin may play a role in establishing and maintaining an unmineralized periodontal ligament region. Matrix "Gla" protein is an inhibitor of mineralization (See below). Matrix Gla protein also may function to preserve the periodontal ligament width. It has been reported that Msx2 prevents the osteogenic differentiation of periodontal ligament fibroblasts by repressing Runx2 transcriptional activity and may play a vital role in preventing ligaments and tendons, in general, from mineralization [21. Yoshizawa T, Takizawa F, 2004]. It also been claimed that glycosaminoglycans [22. Kirkham J, Brookes SJ, 1995] or RGD-cementum attachment protein [23. Ohno S, Doi T, Fujimoto K, 2002] may also play a role in maintaining the unmineralized state of the periodontal ligament.

A novel protein, follicular dendritic cell secreted protein (FDC-SP) has been identified in human periodontal ligament. FDC-SP is not PDL specific. It is a small secreted protein of unknown function expressed in human tonsillar germinal centers. FDC-SP may function in part by regulating B cell chemotaxis and be a regulator of B cell responses [24. Monther Al-Alwan, Qiujiang Du, 2007]. The molecular properties, organization and function of FDC-SP are very similar to statherin, which plays an important role in the inhibition of spontaneous precipitation of calcium salts in saliva [25. Sayaka Nakamura, Tatsuo Terashima, 2005]. Recent study shows that FDC-SP is a negative regulator of PDL cell differentiation into osteoblastic cells and maintains the PDL fibroblastic phenotype, which may be through an EGFR pathway [5. Na Wei, Haiyang Yu, 2011]. It has also been proven that FDC-SP overexpression inhibits osteogenic differentiation of human periodontal ligament cells [26. Xiang L, Ma L, He Y, 2013].
Inhibitors of mineralization

Soft tissues do not normally mineralize under a physiological condition, partly because they lack an abundant scaffolding ECM and consist mostly of cells or because potent mineralization-inhibiting molecules may be present when ECM is present [27. Schinke T, McKee MD, 1999 ].

Vitamin K-dependent carboxylation/γ-carboxyglutamic (GLA) domain is a protein domain that contains post-translational modifications of many glutamate residues by vitamin K-dependent carboxylation to form γ-carboxyglutamate (Gla). Proteins with this domain are known as Gla-proteins. Gla-proteins are mainly found in blood plasma and calcified tissues, like bone and dentin. Blood coagulation proteins are Gla-proteins in blood plasma. Matrix Gla protein and osteocalcin are two Gla-proteins found in bone with sufficient amount. The Gla residues have high-affinity of binding to calcium ions. [57. Vermeer, 1990]

Matrix Gla protein (MGP)

MGP is a 14-kDa ECM protein which contains 4 γ-carboxylated glutamic acid (Gla) residues in mice. It is originally isolated from bone and may also result from cartilage and blood vessels. Mgp-deficient mice show two major phenotypic abnormalities: extensive mineralization of the ECM in arteries, and premature mineralization of cartilage, supporting that MGP functions as a potent mineralization inhibitor in blood vessels and cartilage [28. Luo G, Ducy P, 1997, 29. N.R.Kaipatur, M. Murshed, 2008]. Another study of transgenic mice expressing MGP ectopically in bones and teeth show extensive hypomineralization, which is four to eight-fold higher than wild-type tissues. This result also strengthen the view that physiologic mineralization of bone and tooth ECMs is critically regulated by potent mineralization inhibitors [29. N.R.Kaipatur, M. Murshed, 2008].

Osteocalcin (OC)

OC is the most abundant noncollagenous protein in bone. OC needs calcium to associate with the ECM and the calcium can trigger its conformational change. The known functions of OC are to inhibit hydroxyapatite nucleation and activate mature osteoclasts. OC is a potent chemoattractant to osteoclast precursors [30. Hauschka, P.V., Lian, B.J., 1989, 31. Daniel L. Coutu, Jian Hui Wu, 2008].

Blood coagulation proteins

Blood coagulation proteins, such as prothrombin (factor II), factor VII, IX, and X, the anticoagulant proteins C and S, contain Gla domain and play a role in coagulation [57. Vermeer, 1990]. MGP and osteocalcin are both calcium-binding proteins that may participate in the organization of bone tissue. Both have glutamate residues that are post-translationally carboxylated by the enzyme gamma-glutamyl
carboxylase in a reaction that requires Vitamin K hydroquinone. This process also occurs with a number of proteins involved in coagulation: prothrombin, factor VII, factor IX and factor X, protein C, protein S and protein Z [58. U Grober, J Reichrath, 2014]. Increased carboxylation of vitamin K–dependent proteins within the arterial vessel wall suggests the presence of the more active coagulation factors. These proteins have been shown to be present in all stages of atherosclerosis [59. Henri, 2006]

**Genetic disease**

One or more abnormalities in the genome may cause genetic diseases. Some genetic diseases are inherited and the mutated genes are passed down from their parents or ancestors. Some genetic diseases caused by new mutations or changes to the DNA, such as lifestyle and environmental factors, are not hereditary. There are many types of single gene disorders: autosomal dominant, autosomal recessive, X-linked dominant, X-linked recessive, Y-linked, and mitochondrial disorder.

**FAM20 family**

FAM20 (family with sequence similarity 20) members in humans consist of FAM20A, FAM20B and FAM20C. By database analysis with the mouse Fam20a sequence, Nalbant et al. (2005) identified a family of genes with related sequence that included 2 additional members in mammals, FAM20B and FAM20C. These proteins are highly conserved in human, mouse, and rat. All contain a conserved putative signal sequence and a conserved C-terminal domain (CCD). Expression analysis revealed that Fam20a, Fam20b and Fam20c were expressed during hematopoiesis. The FAM20 family represents a new family of secreted proteins with potential functions in regulating differentiation and function of hematopoietic tissues [63. Naibant D, Youn H, 2005].


FAM20C has been shown to be a secreted, calcium-binding protein [65. Hao J, Narayanan K, 2007]. Mutations of FAM20C result in Raine syndrome, autosomal-recessive disorder. One of the mutants is D478A which indicates that aspartic acid at position 478 was replaced by alanine. Patients with Raine syndrome may display lethal osteosclerotic bone dysplasia characterized by generalized osteosclerosis, ectopic calcifications and characteristic facial features often die shortly after birth. The nonlethal patients, manifesting bone sclerosis, hypophosphatemic rickets/osteomalacia may live into an adulthood [43. Takeyari S, Yamamoto T, 2014, 44. Peihong Liu, Hua Zhang, 2014]. Few nonlethal cases have dental abnormalities, such as high palate, small teeth with enamel dysplasia, and clinical features of hypophosphatemia [33. Simpson MA, et al. (2009). 45. Rafaelsen SH, et al. (2013). 46. Junyu Xiao, Vincent S (2013). 34. Fradin M, Stoetzel C (2010)]. In Fam20C-deficient mice, a remarkable loss of bone, cementum, along with inflammation of the periodontal ligament and formation of periodontal pockets were developed [44. Peihong Liu, Hua Zhang]. The phenotypes of these patients with mutated FAM20C indicate the important role of FAM20C in mineralization.

The first reported case of Raine syndrome was presented in 1989; a female neonate, who died at the age of 86min [32. Raine J, Winter RM, 1989]. Kan and Kozlowski suggested the name of Raine syndrome to describe this new lethal osteosclerotic bone dysplasia. Since the first case reported in 1989 by Raine et al., 23 cases have been published. All the cases described were lethal during the neonatal period except for the last two reported patients, aged 8 and 11 years [33. Simpson MA, et al., 2009] and two sisters, aged 1 and 4 years [34. Fradin M, Stoetzel C, Muller J, 2010]. Individuals with lethal osteosclerotic bone dysplasia characterized by generalized osteosclerosis with periosteal bone formation. Affected individuals survive only days or weeks, with the cause of death most commonly reported as respiratory failure, which may be due to thoracic malformation. Radiography shows that generalized increase in the density of all bones and obvious increase in the ossification of the skull and facial bones. The ossification leads to characteristic facial features, including narrow prominent forehead, proptosis of the eyes, depressed nasal bridge, and mid-facial hypoplasia. The periosteal bone formation, which differentiates the Raine syndrome from osteopetrosis and other known osteosclerotic dysplasias, is also characteristic of this disorder [35. M. A. Simpson, R. Hsu, 2007]. The two nonlethal patients reported in 2009 presented severe mental retardation, whereas the other two nonlethal sisters reported in 2010 presented unexpectedly normal psychomotor development at ages 4 and 1. Identification of mutations in nonlethal patients confirms a broader phenotypic spectrum. The survival of these four patients may be due to milder underlying genotype or the more aggressive treatments they have received. These case reports motivated us to revisit FAM20 gene classification [33. Simpson MA, et al., 2009, 34. Fradin M, Stoetzel C, Muller J, 2010]

Mutations of the FAM20C gene were identified in six subjects with lethal osteosclerotic bone dysplasia. Homozygous changes were identified in four subjects, three of whom were offspring of consanguineous unions and comprised
three nonsynonymous base changes (1093G→A [Gly365Arg], 112T→G [Leu374-Arg], and 1603C→T[Arg535Trp]) and an intron 4/exon 5 acceptor splice-site change (c915-3C→G). Heterozygous changes were identified in two subjects. One heterozygous change was nonsynonymous base change (1094G→A [Gly365-Glu]) and an intron 7/exon8 acceptor splice-site change (c1322-2A→G). The other heterozygous change was the exon4/intron4 donor splice site (c914+5G→C) and the intron8/exon9 acceptor splice site (c1404-1G→A) [35. M. A. Simpson, R. Hsu, 2007]. Mutations of FAM20C gene were also identified in four subjects with nonlethal osteosclerotic bone dysplasia. The two nonlethal patients with severe mental retardation, one was a homozygous nonsynonymous mutation in exon 7(1309G>A D437N) and the other was heterozygous nonsynonymous mutation in exon 2(731T>A I244N) and in exon 3(796G>A G266R) [ 33. Simpson MA, et al., 2009.] The newly discovered two nonlethal patients with normal psychomotor development was homozygous mutation in c.940C>T(p.P314S) [ 34. Fradin M, Stoetzel C, Muller J, 2010]

Identification of FAM20C as the Golgi-casein kinase

The discovery of protein phosphorylation was first reported over a hundred years ago by the characteristics of the milk protein Casein [60.Hammarsten O,1883 ]. Two families of expressed protein kinases have been termed Casein kinase1(CK1) and Casein kinase 2(CK2). However, Casein is a secreted protein whereas CK1 and CK2 are cytoplasmic and nuclear. CK1 and CK2 don’t contribute the phosphorylation of Casein. Golgi casein kinase (G-CK), first describing in lactating mammary glands, had been termed due to the contribution of endogenous Casein phosphorylation [61. Bingham EW, Farrel HM Jr, 1974].

Drosophila Four-jointed (Fj) was identified as the first molecularly characterized Golgi-localized protein kinase [62. Ishikawa HO, Takeuchi H, 2008]. The protein sequences related to Fj and its mammalian homologue, Fjx1. The closest homologues are encoded by Family with sequence similarity 20 (FAM20), which in humans comprises FAM20A, FAM20B, and FAM20C [63. Nalbant D, Youn H, 2005].

Golgi Casein kinase (G-CK) was first described in lactating mammary glands and has been enzymatically characterized. FAM20C was identified as the G-CK for the following evidence. First, FAM20C was present both in the medium and cell lysate confirmed with the Western blotting and exhibited extensive overlap with a Golgi marker by immunolocalization. It shows that FAM20C is a Golgi-localized protein. Second, the Km for ATP of FAM20C (78µM) is similar to G-CK (80µM)[61. Bingham EW, Farrel HM Jr, 1974]. Third, both of them have the ability to phosphorylate Casein. Furthermore, G-CK exhibits a preference for Mn++ over Mg++ as a cofactor, same as FAM20C [64. Bingham EW, Groves ML,1979 ]. FAM20C exhibited a strong activity on the G-CK peptide and only a very weak activity on the other CK1 and CK2 peptides, supporting its responsibility for G-CK activity. For the

**FAM20C substrates**

FAM20C is the Golgi casein kinase that phosphorylates secreted phosphoproteins with Ser-x-Glu/pSer (S-X-E/pS) motifs. FAM20C substrates include both Casein and members of the SIBLINGs (small integrin-binding ligand, N-linked glycoproteins consisting of five identically oriented tandem genes clustered within an ~375 kb span of nucleotides on human chromosome 4) [47. Hiroyuki O. Ishikawa, Aiguo Xu, 2012. 70. Vincent S Tagliabracci, 2013]. As mentioned above, the SIBLINGs include osteopontin (OPN), bone sialoprotein (BSP), dentin matrix protein 1(DMP1), dentin sialophophoprotein (DSPP), and matrix extracellularphosphoglycoprotein (MEPE). Evidence has supported that the SIBLINGs have important roles in regulating HA formation in bone and dentin, implicating the role of FAM20C in biomineralization [15. Marc D. McKee, Betty Hoac, 2013, 7. Liam C. Palmer, Christina J. Newcomb, 2008, 70. Vincent S Tagliabracci, 2013].


Phosphorylation of OPN occurs mainly on serine residues and is principally catalyzed by casein kinases. OPN has a high level of serine conservation and half of the conserved serine residues are potential phosphorylation sites for casein kinases I and II. Phosphorylation of OPN is essential for its inhibitory role on
mineralization [66. Boskey et al, 2002]. Studies showed that phosphates on OPN may be involved in regulating the resorptive activity of osteoclasts [67. Razzouk et al, 2002. 11. C. Qin, O Baba, 2004].

Phosphorylation in BSP, like OPN, occurs mostly at serine residues. Human BSP contains 5 phosphates, 4 of which are located in the central region of its primary sequence. Removal of the phosphates has no apparent influence on the ability of BSP to promote crystal formation, but they may affect crystal growth [68. Hunter and Goldberg, 1994. 11. C. Qin, O Baba, 2004].

DMP1 has 65 Ser/thr that are potential casein kinases I and II phosphorylation sites. Although the functions of phosphates on DMP1 are unknown, they may serve as sequestering groups for recruiting calcium ions, similar to the roles of phosphates on DPP [11. C. Qin, O Baba, 2004].

DPP, derived from DSPP, is the most abundant NCP in dentin and is highly phosphorylated. About 45% of the total sequences is serine residues and most of them are phosphorylated. The large number of phosphates is vital to the functions of DPP. Removal of phosphate groups results in the loss of its role in the nucleation of apatite onto collagen [69. Saito et al., 1997. 11. C. Qin, O Baba, 2004].

MEPE (Matrix extracellular phosphoglycoprotein) is regulated by posttranslational modifications. The phosphorylated protein is an effective promoter of mineralization, while the associated ASARM peptide (acidic serine-aspartate-rich MEPE-associated motif) is an effective inhibitor [12. Adele L. Boskey, 2010].

FAM20C substrates are highly expressed in bone and teeth and their ability to modulate mineralization possibly depending on their phosphorylation status [2. Anthony Palumbo. 47. Hiroyuki O. Ishikawa, Aiguo Xu(2012)] .

2. Materials and methods

Reagents

- Human embryonic kidney 293 cells were purchased from Clontech (Mountain View, CA, USA).
- Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (1X), liquid (4.5 g/L D-Glucose, L-Glutamine, 110mg/L Sodium Pyruvate) purchased from Life Technologies (Carlsbad, CA, USA).
- Penicillin and streptomycin mixture was added to DMEM medium using Penicillin-Streptomycin Solution, 100X, 10,000 I.U. Penicillin 10,000 µg/mL and Streptomycin purchased from Mediatech, Inc. (Manassas, VA., USA).
- Fetal Bovine Serum which was purchased from Invitrogen (Carlsbad, CA, USA) and also added to DMEM.
- Cells were detached from culture plates using Trypsin, 0.25% 1X, with 2.5g porcine trypsin (1:250/L gamma irradiated) in HBSS with 1g/L EDTA, without calcium, magnesium (liquid) purchased from Thermo Scientific (Waltham, MA, USA).
- PMSF (phenylmethylsulfonyl fluoride) was purchased from MP Biomedicals. The stock concentration is 0.1M and the working concentration is 1mM.
Anti-V5 antibody was purchased from Life-Technologies and protein A-Sepharose 4B conjugated was from Life Technologies.
Anti-V5 AP antibody was purchased from Invitrogen.
Anti-FAM20C antibody was purchased from Sigma-Aldrich.
Rabbit IgG was purchased from Thermo Scientific.
SDS NuPAGE® MOPS SDS Running Buffer (for Bis-Tris Gels only) (20X) was purchased from Invitrogen (Carlsbad, CA, USA).
NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm, 12 well purchased from Invitrogen (Carlsbad, CA, USA).
Immobilon-P Membrane purchased from Millipore (Billerica, MA, USA) was used to transfer western blot gel.
Nonfat dry milk was purchased from Lab Scientific (Livingston, NJ, USA).
Alkaline phosphatase conjugate substrate kit from Bio-Rad laboratories (Hercules, CA, USA).
Chemiluminescent HRP Antibody Detection Reagent was purchased from Denville Scientific Incorporation.
HyBlot CL autoradiography film was purchased from Denville Scientific Incorporation.
For purification of polyhistidine-containing recombinant proteins, Ni-NTA agarose was purchased from Qiagen and columns was purchased from Bio-Rad.
For dialysis, Float-A-Lyzer was purchased from Spectrum Laboratories, Inc.
Coomassie brilliant blue (CBB R-250) was purchased from Bio-Rad.
Western Blot Stripping Buffer was purchased from Thermo Scientific.

Buffers

- Lysis buffer containing 150mM NaCl, 20mM Tris-HCl (pH7.5), 10mM EDTA, 1% Triton-X 100 and 1% deoxycholate was prepared.
- Transfer buffer containing 0.025M Tris-HCl (pH 8.3), 0.192M glycine, and 20% methanol was prepared.
- For purification of polyhistidine-containing recombinant proteins, stock solution A (10X) containing 200mM sodium phosphate, monobasic (NaH2PO4) and 5M NaCl was prepared. Stock solution B (10X) containing 200mM sodium phosphate, dibasic (Na2HPO4) and 5M NaCl was prepared. 200 ml of 5x Native purification buffer was prepared with 7 g monobasic sodium phosphate, 29.2g NaCl and deionized water. One hundred ml of 3M Imidazole was prepared with 20.6g Imidazole, 8.77 ml stock solution A (10X) and 1.23 ml stock solution B (10X) and deionized water, adjusted the pH to 6.0.
- 50 ml Native Wash Buffer containing 50 ml of 1X Native purification buffer and 335 µl of 3M Imidazole, pH6.0 was prepared.
- 15 ml Native Elution Buffer containing 13.75 ml of 1X Native purification buffer and 1.25 ml of 3M Imidazole, pH6.0 was prepared.
- Tris-buffered saline (TBS) (1X) containing 20 mM Tris (pH 7.5) and 137 mM NaCl was prepared.
- SDS-Sample buffer containing 100 mM Tris-HCl (pH8.8), 0.01% bromophenol blue, 36% glycerol and 4% SDS was prepared.
- CBB staining buffer containing 45% methanol, 10% glacial acetic acid, 45% water and 3 g/L of Coomassie Brilliant Blue R250

**Plasmid construction**

Human FAM20C expression vector constructs including wild-type (WT) and a mutant form lacking its kinase activity (D478A, (Tagliabracci, 2012 #213)) expression vector were generated by PCR methods. The plasmids containing full length of coding sequence for human FAM20C was purchased (Open Biosystems) and used as PCR template. The sequences of the primers were as follows; (for FAM20C-WT) forward primer: 5’-GCGGTACCGCCATGAAGATGATGCTGG-3’ and reverse primer: 5’-GCCTCGAGCGCTCGAGGCCGCGTCTTG-3’, (for FAM20C-D478A) mutagenesis forward primer: 5’-CAGTATTGGCCCTCGAG-3’ and mutagenesis reverse primer: 5’-CCCTCTTCCATTGCTAAGTG-3’. The PCR products of each FAM20C form were ligated into pcDNA3.1-V5/His mammalian expression vector, sequenced and the plasmids harboring FAM20C-WT and FAM20C-D478A cDNAs followed by V5/His- (pcDNA3.1-FAM20C-WT-V5/His and pcDNA3.1-FAM20C-D478A-V5/His) were successfully generated.

**Cell Culture**

Human embryonic kidney (HEK) 293 cells were maintained in DMEM containing a high concentration of glucose (4.5 mg/ml), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin in a 5% CO₂ atmosphere at 37 °C.

**Transfection to generate FAM20C-stably transfected clones**

HEK 293 cells were plated onto 6-well culture plates at a concentration of 3 \( \times 10^5 \) cells / well. On the following day, 293 cells were transiently transfected in duplicate using X-treme GENE 9 DNA transfection reagent (Roche applied sciences) with pcDNA3.1-empty-V5/His, pcDNA3.1- human FAM20C-WT-V5/His and pcDNA3.1- human FAM20C-D478A-V5/His according to the manufacturer’s protocol.

**Immunoprecipitation and Western Blot (WB) Analysis**

After 24 hours of transfection, the transfected HEK 293 cells were lysed using lysis buffer and the cell lysates were collected. Cellular debris was removed by centrifugation at 12,000 rpm for 5 min at 4 °C and the supernatant was collected. Anti-V5 antibody was added to each lysate sample for immunoprecipitation (1:500) overnight at 4 °C. On the following day, 25ul of protein A-Sepharose 4 B conjugate was added to each immunoprecipitated sample and samples were further incubated.
for 30 minutes at 4 °C with gentle rocking. Samples were then centrifuged, the supernatant was discarded and beads were washed with lysis buffer twice.

Proteins bound to the beads were solubilized in SDS sample buffer, separated by SDS-polyacrylamide gel electrophoresis using NuPAGE® Novex® 4-12% Bis-Tris Gels. Proteins were then transferred to Immobilon-P membrane in transfer buffer using Mini-PROTEAN® Tetra Cell system at 50 volts for 2 hours. The membrane was blocked with 10% skim milk in TBS overnight at 4 °C. The membrane was then washed 3 times with TBS and incubated with anti-V5 AP antibody (1:2000) overnight at 4 °C. The membrane was washed 3 times with TBS and the proteins of interest, FAM20C-WT and FAM20C-D478A, were visualized using alkaline phosphatase conjugate substrate kit.

**Generation of stable FAM20C-WT and FAM20C-D478A-overexpressing clones**

The transfected HEK 293 cells were trypsinized from one confluent well of a 6-well plate and replated onto 15 cm culture dishes. Four hundred µg/mL of G418 neomycin analogue was added on the following day and the cells were further cultured. Ten single colony-derived clones transfected with either FAM20C-WT or FAM20C-D478A were isolated, transferred to 24-well culture plates and further cultured. Cells were expanded and replated to 6-well plate. Once cells became confluent, two ml of CM was collected and immunoprecipitated with anti-V5 antibody. WB with anti-V5 AP antibody for each clone was performed. The clones were chosen with stronger bands of interest by WB. The following clones, empty vector(EV)-clone #5, FAM20C-WT-clone #4, and FAM20C-D478A –clone #2 were further cultured in a larger scale for protein production.

**Purification of FAM20C proteins from conditioned media using Ni-NTA purification system**

The clones were grown on ten of 15 cm culture plates and three of 10cm culture plates, and the conditioned media of FAM20C-WT and FAM20C-D478A were collected. The conditioned media were centrifuged at 1,500 rpm for 5 min to remove any floating/dead cells and the aliquot was made into several 50 ml tubes. The total volume of each media collected was around 450 ml, thus 9 tubes. Then, 500 µl of Ni-NTA agarose was added to each 50 ml tube and incubated overnight at 4 °C. After incubation, the conditioned media were poured into the columns to trap the agarose beads and washed with 500 ml of native wash buffer. Proteins were then eluted into 22 fractions by 1.7 ml of native elution buffer for each fraction. Forty µl of the eluted sample from each fraction was taken, mixed with SDS sample buffer, applied to SDS-PAGE, and Western blotting was performed with anti-V5 AP antibody to identify the positive fractions.

After the fractions with FAM20C expression performed by Western Blotting were identified, these fractions were combined and dialyzed against distilled water.
using Float-A-Lyzer for 3 days at 4 °C. Distilled water was changed twice a day. After dialysis, samples were lyophilized and resuspended in distilled water. The protein concentration was measured and the purified protein was kept at -20 °C until use. Purified proteins were taken and Western blotting was performed with anti-V5 AP antibody and anti-FAM20C antibody.

Purification of FAM20C proteins from cell lysates using Ni-NTA purification system

The clones were grown on ten of 15 cm culture plates. The cell pellets of FAM20C-WT and FAM20C-D478A were collected after trypsin treatment and stored at -20 °C until use. Forty ml of Native Binding Buffer (1x Native purification buffer) with protease inhibitors was used to resuspend cells from one 15cm dish. Cells were frozen at -20 °C and thawed at room temperature for two cycles. Cells were then sheared by passing the preparation through 16-gauge needle four times. The cell lysates were centrifuged at 3,000 rpm for 15 min and the supernatant was transferred to new tubes. Then, 400 µl of Ni-NTA agarose was added to each 40 ml of the clear supernatant and incubated overnight at 4 °C. After incubation, the lysates were poured into the columns and washed with 500 ml of Native Wash Buffer. Proteins were then eluted into 15 fractions by 1ml of Native Elution Buffer for each fraction. Forty µl of samples from each fraction were taken, mixed with SDS sample buffer, applied to SDS-PAGE, and Western blotting was performed with anti-V5 AP antibody to identify the positive fractions.

Expression of purified FAM20C proteins from conditioned media and cell lysates

After the protein concentration was measured with each type of FAM20C proteins derived from conditioned media or cell lysates (i.e. FAM20C-WT from conditioned media or lysates, FAM20C-D478A from conditioned media or lysates), various amounts of FAM20C-WT or FAM20C-D478A from conditioned media and cell lysates were prepared to quantify their expression levels. Purified proteins were prepared with SDS sample buffer, applied to SDS-PAGE. The expression levels were verified by Western blotting using anti-V5 antibody.

Protein identification by Mass Spectrometry (MS) analysis

Various amounts of purified FAM20C-WT (2.5, 5 and 7.5 µg) and FAM20C-D478A (10 and 15 µg) proteins were prepared and applied to the SDS-PAGE. After the gel electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB). CBB staining positive bands were cut into segments. The segmented gels were sent to Taplin Mass Spectrometry Facility at Harvard Medical School for protein identification analysis.

Binding of FAM20C-V5 to periostin
The mammalian expression vector constructs of mouse periostin with C-terminal HA tag (pCAGI Puro delta periostin (b-)-WT-HA (periostin-WT), pCAGI Puro delta periostin-delta-CTR-HA (periostin-dCTR-HA), pCAGI Puro delta periostin-delta-EMI-HA (Periostin-dEMI-HA), pCAGI Puro delta periostin-delta-EMI-CTR-HA (periostin-dEMI-CTR-HA) were kindly provided by Dr. Akira Kudo (Tokyo Institute of Technology, Japan).

Illustration 2. **Domain structures of intact and domain deletion forms of periostin.** There were three deletion forms of periostin. CTR was cleaved as delta CTR. EMI was cleaved as delta EMI. EMI and CTR were cleaved as delta EMI CTR. (Adopted from Kii et. al. J. Biol.Chem. 2010)

HEK 293 cells were plated onto 6-well culture plates at a concentration of 3 x10^5 cells / well. On the following day, cells were transiently transfected in duplicate using X-tremeGENE 9 DNA transfection reagent with an empty vector (EV) of pcDNA3-3'HA, periostin-WT-HA, periostin-dCTR-HA, periostin-dEMI-HA, periostin-dEMI-CTR-HA and FAM20C-V5. After 24 hours of transfection, cell lysates and conditioned media were collected. Twenty µl of lysates were taken, and conditioned media or the rest of lysates was immunoprecipitated (IP) with anti-V5 antibody. Western blotting (WB) using anti-HA antibody was performed to all samples from lysates with IP, lysates without IP, and conditioned media with IP. The membrane of lysates with IP was then stripped by stripping buffer and WB with anti-V5 antibody was performed to verify the expression of FAM20C.

3. Results

The goal of the study was to purify the FAM20C protein and then to find its binding proteins. The hypothesis was that FAM20C protein has post-modification, such as glycosylation.

Protein sequence of FAM20C-WT and FAM20C-D478A with V5 tag was shown in Fig. 1.
Transfection to generate FAM20C-stably transfected clones

To identify the binding proteins of FAM20C, FAM20C stably transfected clones were first generated. HEK 293 cells were selected for their higher transfection efficiency and transfected using X-tremeGENE 9 DNA transfection reagent with EV, FAM20C-WT and FAM20C-D478A. It was transfected in duplicate. One was lysed for the protein verification and the other was lysed for further culture to 15 cm-plates. The lysates was collected and immunoprecipitated (IP) with anti-V5 antibody. To determine whether the transfection with FAM20C-V5 was successful, Western blot analysis with anti-V5 antibody was performed. The expression of FAM20C-WT (Fig. 2, lane 2) and FAM20C-D478A (Fig. 2, lane 3) was confirmed by Western blot analysis.

Cell cloning

To clone a single cell-derived population of either FAM20C-WT or FAM20C-D478A transfected cells, the transfected HEK 293 cells were trypsinized from 1 confluent well of 24-well plate and replated onto 15 cm culture dishes. Ten single colony-derived clones transfected with either FAM20C-WT or FAM20C-D478A were isolated and transferred to 24-well plates. WB with anti-V5 AP antibody for CM of each clone was performed. The clone that exhibited the strongest FAM20C expression among all ten clones was selected (Fig. 3A and 3B) and reconfirmed by another Western Blotting (Fig 3C.). The expected molecular weight of FAM20C is 66 kDa. In the study, the main band of FAM20C-WT clone was located slightly lower than 100 kDa. The expression of Clone #4 was stronger than clone #1 and #5. The main band of FAM20C-D478A was also located slightly lower 100 kDa. The expression of Clone #2 was stronger than clone #7 and #8. The following clones, empty vector (EV)-clone #5, FAM20C-WT-clone #4, and FAM20C-D478A –clone #2, were chosen (Fig 3C.). Extra bands around 170 kDa was both noted at FAM20C-Wt and FAM20C-D478A.

Purification of FAM20C proteins from conditioned media using Ni-NTA purification system

To obtain FAM20C proteins from conditioned media, Ni-NTA purification system was used. The conditioned media of FAM20C-WT and FAM20C-D478A were collected. Ni-NTA agarose (1/100 volume) was added to conditioned media and incubated overnight at 4 °C. The conditioned media was washed with Native Wash Buffer after poring into the columns. Proteins were eluted into fractions by Native Elution Buffer. Sample from each fraction was taken and applied to SDS-PAGE, and Western Blotting was done with anti-V5 AP antibody. Immunoreactive bands to anti-V5 antibody were detected at around 100 kDa, which is the expected size of FAM20C, and 170 kDa in all fractions tested. Fraction 3 had the strongest expression compared with other fractions. In Fraction 3, one obvious
immunoreactive band is at around 100 kDa, and two additional bands are at 170 kDa. No other obvious bands in each lane indicated successful purification by means of Ni-NTA purification system. Fractions containing FAM20C-WT and FAM20C-D478A were identified by WB analysis (Fig. 4).

In order to obtain FAM20C proteins, fractions positive for V5 antibody were combined and dialyzed against distilled water. After dialysis, samples were lyophilized and resuspended in distilled water.

To further confirm the expression of the dialyzed and concentrated FAM20C protein samples, the protein concentration was measured, various amounts were taken, and analyzed by Western blotting (WB) using anti-V5 AP antibody and anti-FAM20C antibody. The results that showed immunoreactive bands to anti-V5 antibody were around 100 kDa and 170 kDa (Fig. 5, lanes 1 and 2). These bands were also immunoreactive to anti-FAM20C antibody (Fig. 5, lanes 3 and 4). FAM20C-WT had stronger expression than FAM20C-D478A. The expression of FAM20C-WT and –D478A was confirmed by Western blotting with both anti-V5 and anti-FAM20C antibodies after purification (Fig. 5).

**Purification of FAM20C proteins from cell lysates using Ni-NTA purification system**

To obtain FAM20C proteins from cell lysates and compare them to the proteins from CM, Ni-NTA purification system was used. Cells were grown on ten of 15 cm culture plates. The cell pellets of FAM20C-WT and FAM20C-D478A were collected with trypsin and resuspended in Native Binding Buffer with protease inhibitors. Cells were sheared by being frozen and thawed and by passing through 16-gauge needle. The supernatant was transferred to new tubes after centrifugation. Ni-NTA agarose was then added to lysates and incubated. The lysates was then poured into the columns and washed with native wash buffer. Proteins were then recollected into 15 fractions by 1mL native elution buffer for each fraction. Forty µl of sample from each fraction was taken, applied to SDS-PAGE, mixed with SDS sample buffer and Western Blotting was performed with anti-V5 AP antibody.

Fractions containing FAM20C-WT and FAM20C-D478A were identified by WB analysis (Fig.6). The expression of FAM20C-WT of the later fraction was stronger than early ones. Fraction 13 had the strongest intensity of expression compared with earlier fractions. Fraction 3 of FAM20C-D478A had the strongest intensity of expression among all the fractions. The expression of FAM20C-D478A of the next fraction was weaker than the earlier ones. Compared the FAM20C proteins from cell lysates to the ones from conditioned media, three obvious immunoreactive bands to anti-V5 antibody, one around 100 kDa and the other two near 170 kDa, were still noted in cell lysates (Fig.3 and 5, arrows). Many immunoreactive bands to anti-V5 antibody ranging from 55 kDa to 130 kDa, were noted in lysate samples, much different from conditioned media samples (Fig.4 and 6).
Expression of purified FAM20C proteins from conditioned media and cell lysates

To obtain FAM20C proteins from cell lysates, all fractions with positive expression were combined and dialyzed against distilled water at 4 °C. After dialysis, samples were lyophilized and resuspended in distilled water. To further confirm and compare the expression of the purified FAM20C protein samples from conditioned media and cell lysates, the protein concentration was measured. Various amounts of the purified FAM20C proteins from conditioned media and cell lysates were taken and the expression was confirmed by Western blotting using anti-V5 antibody(Fig.7). Obvious immunoreactive bands to anti-V5 antibody were clearly shown, indicating purification of FAM20C was achieved. Ten ng of purified FAM20C-WT protein from conditioned media was needed to be detected in the Western blot, while 0.4µg of purified FAM20C-WT protein from cell lysates was needed. Similarly, 75 ng of purified FAM20C-D478A proteins from conditioned media was needed to be detected in the Western blot, while 2.5 µg of FAM20C-D478A proteins from cell lysates was needed. FAM20C proteins were able to be collected from both purification methods. It also showed that FAM20C proteins were purified more easily from conditioned media than from lysates. Therefore, we decided to use FAM20C proteins from conditioned media (Fig.7).

Protein identification by Mass Spectrometry (MS) analysis

In order to examine the binding proteins of FAM20C, purified FAM20C-WT (2.5, 5 and 7.5 µg) and FAM20C-D478A (10 and 15 µg) proteins were prepared, electrophoresed, and the gel was stained with Coomassie Brilliant Blue (CBB). The CBB-positive bands were identified (see below), cut into segments, and these samples were sent to Taplin Mass Spectrometry Facility at Harvard Medical School for protein identification analysis.

The gel stained by CBB showed three clear bands, one close to 85 kDa and the other two close to 170 kDa, in each lane (Fig.8, arrows). The data was analyzed after being filtered by the setting that Xcorr2 is 2.2 and Xcorr3 is 3.5 [48. Ming Zhou et al.(2004)]. Analysis was performed by excluding the proteins located in cytoplasm first (Figs. 9-14).

<table>
<thead>
<tr>
<th>Band 1 (170 kDa upper band)</th>
<th>FAM20C-WT</th>
<th>FAM20C-D478A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FAM20C,</td>
<td>HSPG2,</td>
</tr>
<tr>
<td></td>
<td>HSPG2,</td>
<td>A2MG,</td>
</tr>
<tr>
<td></td>
<td>TNC,</td>
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<td></td>
<td>FN1,</td>
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<td>IGHG1,</td>
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<td>LAMB2,</td>
</tr>
</tbody>
</table>

| Band 4 (170 kDa upper band) | HSPG2, | FAM20C, |
|                            | A2MG,  | TNC,    |
|                            | FN1,   | PRP1,   |
|                            | IGHG1, | LAMB2,  |
| Band 2  
(170 kDa lower band) | FAM20C, HSPG2, TNC, IGHG1, FN1, NID2 | Band 5  
(170 kDa lower band) | FAM20C, HSPG2, A2MG, FN1, NID2, TNC |
|------------------------|-----------------------------------|------------------------|---------------------------------|
| Band 3  
(90~75 kDa)       | FAM20C, POSTN                      | Band 6  
(85~75 kDa)       | FAM20C, HSPG2, PRP1, POSTN, ITIH2 GRN |

Table 1. **Protein identification by Mass Spectrometry (MS) analysis.** The extracellular proteins that appeared most times in each segment were summarized.

HSPG2, HEPARAN SULFATE PROTEOGLYCAN 2  
NID2, NIDOGEN 2  
LAMB2, LAMININ, BELTA-2  
A2MG, ALPHA-2-MACROGLOBULIN  
PRP1, PROLINE-RICH PEPTIDE-1  
POSTN, PERIOSTIN  
GRN, GRANULIN  
TNC, TENASCIN C  
FN1, FIBRONECTIN 1  
IGHG1, IMMUNOGLOBULIN HEAVY CONSTANT GAMMA1  
ITIH2, INTER-ALPHA-TRYPSIN INHIBITOR HEAVY CHAIN 2

We first rule out the proteins that are localized in cytoplasm. Considering the number of unique peptides found by MS analysis, proteins with more than three of unique peptides are considered as the binding proteins. Proteins listed above are candidate FAM20C-binding proteins. According to the MS analysis, POSTN (periostin) is the only protein that found in band 3 and band 6. HSPG2 is present in all bands except band 3. It is known that HSPG2, NID2 and LAMB2 are basement membrane proteins. Besides periostin, HSPG2 and NID2, LAMB2, A2MG, PRP1, GRN, TNC, FN1, IGHG1 and ITIH2 were noted in band 4, 5 and 6. Since periostin is known to be a secretory protein and predominantly expressed in collagen-rich fibrous connective tissue and have several S-X-E motifs in their amino acid sequences, periostin may be one of the substrates of FAM20C [49. Russell A.Norris, Brook Damon, 2007, 44. Peihong Liu, Hua Zhang,2014 ]. Therefore, we decided to further investigate the association between FAM20C and POSTN.

**Binding of FAM20C to periostin (POSTN)**
To verify the interaction between FAM20C and periostin, HEK 293 cells were transiently transfected with various forms of periostin-HA expression vectors and FAM20C-V5. Immunoprecipitation (IP)-Western blotting (WB) analyses were performed to identify the interaction between periostin and FAM20C from conditioned media. The expression of FAM20C-WT was confirmed by Western blotting with anti-V5 antibody (Fig. 15B). The expression of periostin -HA was confirmed by Western blotting with anti-HA antibody (Fig. 15D).

In the presence of FAM20C-V5 (Fig. 15A, lane 3, 7, 9, and 11), immunoreactive bands to anti-HA antibody were shown after IP with V5. periostin -HA was expressed in the presence of FAM20C-V5, indicating that FAM20C associates with periostin. All forms of periostin, i.e. periostin -HA, periostin dCTR-HA, periostin dEMI-HA and periostin dEMI -CTR-HA had strong expression in the presence of FAM20C-V5 after IP with V5. The results demonstrated that all forms of periostin bound to FAM20C in HEK293 cells.

4. Discussion

Protein identification by Mass Spectrometry (MS) analysis

Proteins in the CBB gel were sent to Taplin Mass Spectrometry Facility at Harvard Medical School for protein identification analysis. The facility utilizes Orbitrap mass spectrometers from Thermo Scientific for high throughput analyses of gel bands.

Mass spectrometers are used either to measure simply the molecular mass of a polypeptide or to determine additional structural features including the amino acid sequence or the site of attachment and type of posttranslational modifications. For simply measuring the molecular mass of a polypeptide, single-stage mass spectrometers are used, acting essentially as balances to weigh molecules. Tandem mass spectrometry (MS/MS) is commonly used in the product ion mode to determine the amino acid sequence of a specific peptide. For determining additional structural features, specific ions are selected and subjected to fragmentation through collision after the initial mass determination. Every mass spectrometry consists of three distinct stages. First, protein samples are isolated and optionally fractionated. The final protein sample is then digested and the resulting peptide sample is further fractionated. Second, the peptides are subjected to qualitative and quantitative mass-spectrometric analysis. Finally, the large data sets generated are analyzed by suitable software tools to deduce the amino acid sequence. The peptide identity is assigned to the MS/MS spectra through database searching [50. Bruno Domon, Ruedi Aebersold, 2006].

According to the MS analysis, FAM20C-WT is found in all samples, band 1, 2 and 3. FAM20C-D478A is also found in all samples, band 4, 5 and 6. Ruling out the
proteins in cytoplasm and considering the number of unique peptides found by MS analysis, proteins with more than three of unique peptides are considered as the binding proteins. The possible FAM20C-binding proteins are POSTN (periostin), HSPG2, LAMB2, A2MG, PRP1, GRN, TNC, FN1, IGHG1 and ITIH2.

HSPG2 protein is a large multidomain proteoglycan and a major component of basement membranes. HSPG2 plays an important role in maintaining endothelial barrier function and vascular homeostasis. It is also involved in the stabilization of other molecules and cell adhesion [http://www.ncbi.nlm.nih.gov/gene/3339].

Nidogen protein is a basement membrane protein that binds collagens I, IV and laminin. Nidogen may be involved in maintaining the structure of the basement membrane [http://www-ncbi.nlm-nih.gov.ezproxy.bu.edu/gene/22795].

Laminins are the major noncollagenous constituent of basement membranes. Laminins, composed of 3 non identical chains: laminin alpha, beta and gamma (formerly A, B1, and B2, respectively). Laminin, beta 2 has a more restricted tissue distribution. It is enriched in the basement membrane of muscles at the neuromuscular junctions, kidney glomerulus and vascular smooth muscle. Transgenic mice in which the beta 2 chain gene was inactivated, showed defects in the maturation of neuromuscular junctions and impairment of glomerular filtration [http://www-ncbi.nlm-nih.gov.ezproxy.bu.edu/gene/3913].

Alpha-2-macroglobulin is a protease inhibitor. It inhibits many proteases, including trypsin, thrombin and collagenase. A2M can degrade A-beta, the major component of beta-amyloid deposits, and may play a role in Alzheimer disease [http://www.genecards.org/cgi-bin/carddisp.pl?gene=A2M].

Proline-rich proteins are major components of parotid and submandibular saliva in humans and other animals. They can be divided into acidic, basic and glycosylated proteins. The acidic proline-rich proteins bind calcium strongly and may be important in maintaining the concentration of ionic calcium in saliva. Also, they can inhibit formation of hydroxyapatite, whereby growth of hydroxyapatite crystals on the tooth surface in vivo may be avoided [92. Bennick A, 1982].

Granulins are a family of secreted, glycosylated peptides that are cleaved from a single precursor protein, progranulin. Cleavage of the signal peptide produces mature granulin which can be further cleaved into a variety of active peptides. The peptides and intact granulin proteins both regulate cell growth by inhibiting or stimulating. Granulin family members are important in normal development, wound healing, and tumorigenesis [http://www-ncbi.nlm-nih.gov.ezproxy.bu.edu/gene/2896]

Tenascin C protein contains multiple EGF-like and fibronectin type-III domains. It is implicated in guidance of migrating neurons as well as axons during


The inter-alpha-trypsin inhibitors (ITI) are a family of structurally related plasma serine protease inhibitors involved in extracellular matrix stabilization and in prevention of tumor metastasis. The ITI family contains multiple proteins made up of a light chain and a variable number of heavy chains. ITIH2 stands for inter-alpha-trypsin inhibitor heavy chain 2. [http://www.ncbi.nlm.nih.gov/gene/3698]

Periostin (POSTN) is a 90 kDa secreted protein. It is the first protein to be described with tissue specificity to the periosteum and the periodontal ligament. High levels of expression of periostin are observed in periosteum and periodontal ligament. The periostin may be secreted into the surrounding extracellular matrix possibly by osteoblasts, osteoblast precursors or periodontal fibroblasts. Periostin is mainly expressed in collagen-rich fibrous connective tissues, such as periodontium, periodontal ligament, aorta and heart valve. It appears to play an essential role in response to mechanical strains from physical exercise, mastication, blood flow and pressure. [53. Isao Kii, Takashi Nishiyama .(2010).] The localization of periostin is unique and no other glycoprotein has a similar distribution pattern. The expression of periostin is also regulated by TGF-β, functioning significantly on periosteal expansion and the recruitment of osteoblast precursors. Periostin has the potential to be a tissue-specific mediator of the effects of TGF-β on new bone formation and play a role in the recruitment and attachment of osteoblast precursors in the periosteum [54. Keisuke Horiuchi, Norio Amizuka.(1999)]. Periostin is found binding to type I collagen directly. Periostin null mice demonstrated a reduction in collagen fibril diameter in its skin resulting in a decrease in overall stiffness and a significant reduction in collagen cross-linking, associated with improper collagen fibril formation [49. Russell A.Norris, Brook Damon,2007]. Periostin is also shown to support BMP-1 mediated proteolytic activation of lysyl oxidase on the extracellular matrix, which promotes collagen cross-linking and determines the properties of connective tissues that are constantly subjected to mechanical stress [55.Takumi Maruhashi, Isao Kii.(2010)]. Periostin (POSTN) is discovered as a member of vitamin K-dependent γ -carboxylated protein family characterized by the presence of fasciclin domains. The most abundant Gla-containing protein secreted by bone marrow derived mesenchymal stromal cells. Periostin may be the bone-associated Gla-containing protein with similar function to Matrix Gla Protein (MGP) and Osteocalcin (OC), both of them are considered to be inhibitors of mineralization [28. Luo G, Ducy P,1997. 29. N.R.Kaipatur, M. Murshed, 2008. 30. Hauschka, P.V., Lian, B.J. 1989. 31. Daniel L. Coutu, Jian Hui Wu, 2008]. The γ -carboxylation on the fasciclin domain of periostin may modulate cell-ECM interaction and provide hydroxyapatite binding properties [31. Daniel L. Coutu, Jian Hui Wu,2008]. One experiment demonstrated
that loss of Fam20C function in mice led to a remarkable loss of alveolar bone and cementum, inflammation of the periodontal ligament, severe periodontal disease, along with a significant reduction in the SIBLING proteins and periostin [44. Peihong Liu, Hua Zhang, 2014].

In this study, FAM20C is the most abundant peptides found in the MS. FAM20C is a secreted Golgi casein kinase. It phosphorylates secretory pathway proteins within S-x-E motifs. The secreted proteins include a family of secretory calcium-binding phosphoproteins (SCPP) that have a high affinity for calcium, and regulate biomineralization. Phosphorylated S-x-E motifs bind calcium and regulate calcium phosphate precipitation as hydroxyapatite (HA). Raine syndrome, as the increased bone mass, could be explained by decreased phosphorylation of proteins and peptides that inhibit biomineralization. Mutations in FAM20C, incapable of limiting bone formation, leads to osteosclerotic bone dysplasia in humans with neonatal lethal outcome, known as Raine syndrome. It can be concluded that phosphorylation plays an important role in regulating biomineralization and inhibiting abnormal precipitation in humans [51. Vincent S. Tagliabracci, James L. Engel, 2012. 34. Fradin M, Stoetzel C, Muller J.(2010). 52. Sharon Grubner, Jung Hsin Lin.(2013.)] One of the mutants is D478A. It was used in this study because previous studies of Golgi casein kinase showed FAM20C-D478A doesn’t have kinase activity. Also, using the FAM20C-WT and FAM20C-D478A proteins to find binding proteins of FAM20C, kinase-dependent and kinase-independent binding proteins might be able to differentiate. In the study, more amounts of FAM20C-D478A were required to achieve the similar expression to FAM20C-WT. It’s probably because FAM20C-D478A lacks kinase activity and have more binding proteins. This may support that FAM20C protein have post-modifications, such as phosphorylation and glycosylation.

Periostin (POSTN) is the only protein that is found in band 3 and band 6. HSPG2 is present in all bands except band 3. Besides periostin, HSPG2 and NID2, LAMB2, A2MG, PRP1, and GRN were noted in band 4, 5 and 6. There are more proteins binding to FAM20C-D478A than FAM20C-WT. It is assumed that FAM20C-D478A lacks the kinase activity so that its substrates could not be phosphorylated and retained with FAM20C-D478A. For example, PRP1, a secreted phosphoprotein found in saliva, still binds to FAM20C-D478A. Ruling out the proteins in cytoplasm and proteins with less frequency in the analysis, periostin and HSPG2 are highly suspected as the binding proteins of FAM20C. Therefore, the following experiment was decided to check whether or not FAM20C binds to periostin and determine the possible interaction between these two proteins. Periostin can regulate collagen fibrillogenesis and determine the biomechanical properties of the connective tissues by regulating the crosslinking of collagen fibrils, which also play a vital role in the mechanism of mineralization. It is reasonable to speculate that FAM20C-periostin interaction is important for PDL integrity. FAM20C might phosphorylate periostin and upregulate periostin to control the unmineralized status in the connective tissues, such as periodontal ligament and periosteum. Future studies should be
warranted to determine the possible phosphorylation site(s) of periostin by FAM20C and the functional role of phosphorylation in periostin in PDL tissues.

**Binding of FAM20C to periostin (POSTN)**

Based on the binding experiment of FAM20C-V5 and periostin -HA in this study, the result shows that all forms of periostin, i.e. periostin -HA, periostin dCTR-HA, periostin dEMI-HA, and periostin dEMI-CTR-HA were bound to FAM20C. Periostin may be phosphorylated by FAM20C and the phosphorylated sites may locate in the middle four fasciclin domains. It coincides with the amino acid sequences of mPeriostin isoform1 that has four sites that are S-x-E motifs and might be phosphorylated by FAM20C (Fig.16).

Analyzing the amino acid sequence from previous study [Coutu et al, JBC, 2008], periostin in human beings has 3 probable FAM20C phosphorylation sites (Fig.16A), while periostin isoform in mice has 4 probable phosphorylation sites (Fig.16B). To compare the amino acid sequence from MS analysis to the sequence in human periostin, the sequence derived from MS analysis was underlined in the human periostin. The underlined peptides, the segments of periostin found in band 3 and band 6, didn’t contain any of these phosphorylation sites (Fig.17).

It is known that some noncollagenous proteins play important roles in maintaining the unmineralized status in saliva and periodontal ligament by means of posttranslational modification, such as phosphorylation. For example, statherin and acidic proline-rich proteins in saliva interact with calcium and phosphate ion clusters with their negatively charged residues, like phosphoserine, to inhibit precipitation and crystal growth of hydroxyapatite [19. Raj PA, Johnsson M, 1992]. Several noncollagenous proteins [11. C. Qin, O Baba, 2004], such as bone sialoprotein, osteopontin and matrix”Gla” protein [28. Luo G, Ducy P,1997. 29. N.R.Kaipatur, M. Murshed, 2008], have been indicated to attract calcium and control mineralization process. A novel protein, follicular dendritic cell secreted protein (FDC-SP) has also been identified in human periodontal ligament [24. Monther Al-Alwan, Qiujiang Du, 2007. 25. Sayaka Nakamura, Tatsuo Terashima, 2005. 26. Xiang L, Ma L, 2013]. The molecular properties, organization and function of FDC-SP are very similar to statherin. It has been proven that FDC-SP overexpression inhibits osteogenic differentiation of human periodontal ligament cells. Phosphorylation plays an important role in regulating biomineralization and inhibiting abnormal precipitation in humans based on the characteristics of Raine syndrome, which FAM20C is mutated and not phosphorylated.

The suspected of molecular weight of FAM20C is 66 Dka. After purification of FAM20C from conditioned media in the study, strong expression was noted at around 100 kDa and less strong expression at around 170 kDa. Bands at 170 kDa were observed in all the results. Covalent binding of FAM20C proteins or post-modification may be the possible explanations. Post-modification, such as
glycosylation, of FAM20C was hypothesized and finding the binding proteins of FAM20C was the second goal of the experiment.

In this study, it is concluded that periostin is one of the FAM20C binding proteins. Further studies are required to verify the binding is direct or indirect. Moreover, periostin may be phosphorylated by FAM20C and the phosphorylation may regulate the biomineralization of extracellular matrix where periostin is located. It will be an interesting future aim to test whether periostin is phosphorylated by FAM20C or not and to further investigate the role of phosphorylation for periostin.
>human FAM20C-WT
MKMLVRRFR VLILMVFLVA CALHIALDLL PRLERRGARP SGEPCSCAQ
PAAEVAAPGW AQVRGRPGEP PAASSAAGDA GWPNKHTLRI QDFSSDPSS 100
NLSSHLEKL PAAEPAERA LRGRDGALR PHDPAPRLL RDPGPRRSES
PPPGGDDASL LARLFEHPLY RVAVPPLTEE DVLFNVNSDT RLSPKAANP 200
DWPAGAGEA EFLSPGGEAV DSYPNLKFW IGINRYELYS RHNPAAEALL
HDLSSQRITS VAMKSGGTQL KLMTFQNYG QALFKPMKQT REQUETPDFF 300
YFSYERHNA EIAAFFLDRU LDFRVRVPVA GRNVMTKEI RVTRDGDLLW
RTFISPANN ICFYGECSSY CSTEHALCGK PDQIEGLLAA FLPDSLAKR 400
KTWRNPWRSS YHKKKAEEWE VDPDYCEEVK QTPYDSSHR ILDVMDDTIF
DLMGNMDRH YYETFEKFGN ETFIHLDNG RFGKYSHEDE LSLVPLQQC 500
CRIRKSTYLR LQLLAKEEKY LSLLMAESLR GDQVAPVLYQ PHEALDRRL
RVVLKAVRDC VERNGLHSVV DDDLDTEHRA ASARLESRGP FEKPIPNPL
LGLDSTRTG HHHHH

>human FAM20C-D478A
MKMLVRRFR VLILMVFLVA CALHIALDLL PRLERRGARP SGEPCSCAQ
PAAEVAAPGW AQVRGRPGEP PAASSAAGDA GWPNKHTLRI QDFSSDPSS 100
NLSSHLEKL PAAEPAERA LRGRDGALR PHDPAPRLL RDPGPRRSES
PPPGGDDASL LARLFEHPLY RVAVPPLTEE DVLFNVNSDT RLSPKAANP 200
DWPAGAGEA EFLSPGGEAV DSYPNLKFW IGINRYELYS RHNPAAEALL
HDLSSQRITS VAMKSGGTQL KLMTFQNYG QALFKPMKQT REQUETPDFF 300
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DLMGNMDRH YYETFEKFGN ETFIHLDNG RFGKYSHEDE LSLVPLQQC 500
CRIRKSTYLR LQLLAKEEKY LSLLMAESLR GDQVAPVLYQ PHEALDRRL
RVVLKAVRDC VERNGLHSVV DDDLDTEHRA ASARLESRGP FEKPIPNPL
LGLDSTRTG HHHHH

FIGURE 1. Protein sequence of FAM20C-WT and FAM20C-D478A with V5/His tag. Note that Aspartic acid at position 478 of FAM20C-D478A was replaced by alanine, which is highlighted in red, and V5 tag with 6 X Histidine regions were shown in bold letters.
FIGURE 2. Expression of FAM20C-WT or FAM20C-D478A protein. The 293 cells were transfected with pcDNA3.1-empty-V5/His, pcDNA3.1- human FAM20C WT-V5/His and pcDNA3.1- human FAM20C D478A-V5/His. The cell lysate was collected and was immunoprecipitated with anti-V5 antibody. The expression of FAM20C-WT and –D478A was confirmed by Western blotting using anti-V5 AP antibody.
FIGURE 3. Western blot analysis of stably transfected clones expressing FAM20C-WT and –D478A. A & B, 10 single colony-derived clones transfected with either FAM20C-WT or FAM20C-D478A were isolated and transferred to 24-well plates. Western blotting with anti-V5 AP antibody for these clones was performed. C, Three clones with higher FAM20C expression were selected and the expression was confirmed.
FIGURE 4. **Purification of FAM20C proteins from conditioned media using Ni-NTA system.** Conditioned media from one of the selected FAM20C-WT and FAM20C-D478A clones were collected and Ni-NTA agarose was added to purify the FAM20C-WT-V5/His and –D478A-V5/His proteins. Proteins were then eluted into fractions with Native Elution Buffer. Forty µl of sample from each fraction was taken, applied to SDS-PAGE, and Western Blotting was performed with anti-V5 AP antibody. The immunoreactive bands are observed at around 100kDa and some additional bands at ~170 kDa.
FIGURE 5. **Western blot analysis with anti-FAM20C antibody.** The positive fractions (Fig. 4) were combined, dialyzed against distilled water, samples were lyophilized and resuspended in distilled water. The protein concentration was measured. The expression of FAM20C-WT and –D478A was confirmed by Western blotting (WB) using anti-V5 AP antibody and anti-FAM20C antibody after purification.
FIGURE 6. **Purification of FAM20C proteins from cell lysates using Ni-NTA system.** The cell pellets of FAM20C-WT and FAM20C-D478A were collected after trypsin treatment and were resuspended in Native Binding Buffer with protease inhibitors. Cells were sheared by two cycles of frozen-thawed treatment and by passing through 16-gauge needle four times. After centrifuge, the supernatant was taken, Ni-NTA agarose was added to purify the FAM20C-WT-V5/His and –D478A-V5/His proteins and was then under incubation. After incubation, the cell lysate was poured into the columns and washed with Native Wash Buffer. Then, proteins were eluted into fractions with Native Elution Buffer. Forty µl of sample from each fraction was taken, applied to SDS-PAGE, and Western Blotting was done with anti-V5 AP antibody. The immunoreactive bands are observed at around 100 kDa and some additional bands at ~170 kDa.
FIGURE 7. **Expression of purified FAM20C proteins from conditioned media and cell lysates.** Purified FAM20C proteins from conditioned media and from lysates were taken and confirmed by Western blotting (WB) using anti-V5 antibody. Ten ng of purified FAM20C –WT proteins from conditioned media was needed to be detected in the Western blot but 0.4 µg of purified FAM20C-WT proteins from the lysate was needed. Similarly, 75 ng of purified FAM20C-D478A proteins from conditioned media was needed to be detected obviously in the Western blot but 2.5 µg of FAM20C-D478A proteins from the lysate was needed. It proved that FAM20C proteins can be purified from both purification methods. It also showed that FAM20C proteins were more easily purified from conditioned media than from cell lysates.
FIGURE 8. **Protein identification by Mass Spectrometry (MS) analysis.** 2.5µg, 5µg and 7.5µg of FAM20C-WT and 10µg and 15µg of FAM20C-D478A purified proteins from conditioned media were taken and loaded to the SDS-PAGE. The gel was stained by CBB and the bands stained were cut into segments. The segmented gels were subjected to Mass spectrometric analysis. Arrow 1-3 indicates the bands from FAM20C-WT proteins. Arrow 1 indicates a band detected slightly above 170 kDa, arrow 2 slightly below 170 kDa and arrow 3 near 85 kDa. Arrow 4-6 indicates the bands from FAM20C-D478A proteins. Arrow4 indicates a band detected slightly above 170 kDa, arrow 5 slightly below 170 kDa, and arrow 6 close to 85 kDa. Bovine serum albumin (BSA) proteins were used as a control group and no obvious band was noted. It was not shown in the picture, which is a missing part in this thesis.
FIGURE 9. **Protein identification derived from band 1.** Analysis was performed by excluding the proteins located in cytoplasm first. HSPG2, TNC, FN1, IGHG1 and ITIH2 are all extracellular proteins and possible FAM20 binding proteins. Compared the band 1 to the other bands, HSPG2 is the most frequently possible binding protein and may be a stronger binding substrate.
FIGURE 10. **Protein identification derived from band 2.** HSPG2, TNC, IGHG1, FN1, NID2 are all extracellular proteins. The most frequently protein is HSPG2 and the second most is TNC.

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FIGURE 11. Protein identification derived from band 3. POSTN (periostin) is the extracellular protein that may be the binding protein of FAM20C.
FIGURE 12. **Protein identification derived from band 4.** Compared with other bands, HSPG2, A2MG, TNC, FN1, PRP1, IGHG1, LAMB2, and NID2 are extracellular proteins and are more likely to be the binding proteins of FAM20C.
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**FIGURE 13.** Protein identification derived from band 5. HSPG2, A2MG, FN1, NID2, and TNC are extracellular proteins that may be the binding proteins of FAM20C.
FIGURE 4. Protein identification derived from band 6. HSPG2, PRP1, POSTN (periostin), ITIH2, and GRN are extracellular proteins that may be the binding proteins of FAM20C.
FIGURE 15. Binding of FAM20C to peristin (POSTN). HEK 293 cells were transiently transfected with peristin-WT-HA, peristin-dCTR-HA, peristin-DMI-HA, peristin-DMI-CTR-HA and FAM20C-V5. The binding between peristin and FAM20C was investigated by immunoprecipitation (IP) with anti-V5 antibody and Western blotting (WB) using anti-HA antibody. The result showed that peristin-HA, peristin-dCTR-HA, peristin-DMI-HA, peristin-DMI-CTR-HA all bound to FAM20C.
A.

MIPFLPMFSL LLLIVNFPIN ANNHYDKILA HSRIRGRDQG PNVCALQQIL
GTKKYYFSTC KNWYKKSICG QKTTVLYECC PYGMRMEGMA GCPAVLPIDH 100
VYCTLGIVGA TTTOYRESSAS KLREEIEKGQ SFTYFAPE NE AWDNLDSDIR
RGLESNVNVE LLNLHALSHMII NKRMOLTDLK NGMIPPSYMN NLGLYPHYP 200
NGVVTNVCAR IIHGNUQATN GVCHRVIDRVL TQIGTSDQDF IEAEDLLSSF
RAAAITSDSL EALGGRDGHFT LFAPTEAEFE KLFRGVLERI MGDKVASEAL 300
MKYHILTVLQ CSESIMGGAV FETLEGNTIE IGCDGDSTTV NGIKMVNKKD
IVTNNGVIIH IDQVLIPQSDA KQVIELAQGQ QTTFDCTDVAQ LGKALSALPD 400
GETLLAVPN NAFFDIDLSTM DQRLLKLLIQ NHILKVKVGL NELYNGQILE
TIGKQQLKRF VRYRTAVCIE SCMEIKGSKQQ RNGAIHIFRE IIKPAEKSLL 500
EKLQYKQKRF TFLSLEEDAD LKELLTPQPD WTLFVPTNDA FKGMTSEEKE
ILIRDNALKQ NIILYHTLPG VFIGKGFEPG VTNILKTTQG SKIFLKEVND 600
TLLVNELKIE EDSINTMNGV IHVDKLLYP ADTFVGNQDL LEILNKLKLYI
IQIKFVRGST FKEIPVTVYT ITKivistKVP KIKVIEGSIQ PIKTEGPTL 700
TKVIEKGEPE FRLIKEGETI TEVIKGEPII KKYTKIIIDGV PVEITEKETR
EERIITGPEI KYTRISTGGG ETETTLKKLL QEVEITKTKF IEGGGHLFE 800
DEEIKRLQQG DTPVRLQAN KKVQGSRRRL REGRSQ

B.

MVPLLPLYAL LLLLVNOLDNP ANANSYDKV LAHRSRIRGRD QGSNVCAQLQ
ILGTKKYYFCS SCKNYYQGAI CGKTTVLVE CCYPYMRMEG MKGCPAVMP 100
DHVYTGLKIV GATTQHYSDY VSLRLEEGY KGSYTYFAPE NEAWELEDS
IRRGLENDV VENLALHSH MNVKRMTDK LKHGVMVPSM YNNLQLFHN 200
YPGVYVTVRCV ARVINGQQA TNGVHVIDR VLTQIGTSIQ DLFEAEDLDS
SFPAATTSD LLESLRGDRH PTLFAPTEAE FEKLPRYVNLE RIMGKVASE 300
AKMYHILINT LQCSEAITGG AVFTETMEGTS IEIICEGDSI SINGIKMVNK
KDIVKNGVT HLIHEVLIPD SAKQVIELAG KQQTTETDLV AQLGLASSKL 400
LDHKLIRQQK RFSFILPLEA ADLKLIDTLP GDWTLFAPE DAFKMGIEE
RELLIKONNA LQNIILYHTL PGYVYIKGPE PGVTNILKTQ QGSKYLKGRV 600
NETLLVNELK SKESSIMTN GVHHVVDKLL YPADFPGVDQ LEILNKLKLYI
KYIQIKFVRST FSTEKKMTVT YRPAMTQIQI EGDNPFRPLL EGETVTEYVH 700
GEPVKKVTYK IDGVPVETT EKQTREERI TGPEIKYTRI STGGGETGET
LQKFLQKEVV KVTKFIQEG GDHLFEDDEEK RLLQGDTPAK KIPANKVQG 800
PREREGRSQ

1-23: Signal peptide
42-89: EMI
111-232: Fasciclin 1
247-369: Fasciclin 2
384-496: Fasciclin 3
509-633: Fasciclin 4
634-811: CTR

A.

MIPFLPMFSL LLLLIVNPIP ANNHYDKILA HSRIRGRDQG PNVCALQQIL
GTKKKYFSTC KNWYKSCICG QKTTVLYECC PGYMRMEGMK GCPAVLPIHD 100
VYGTLGIVGA TTTQFRYSDAS KLREIEGKQ SFTYFAPSE AWDLNLSDIR
RGLESNVNVE LLNALHSAMI NRKLMTKDLK NGMIIIPSMYN NLGLFINHYIP 200
NGVVTVCNAR IHHGNIQATN GVIVHIDRVL TQIGTISQDF IEAEddlSSF
RAAATSDIL EALGRDGHT LFAPNFAF KLFPRGVLREI MGDKVASEAL 300
MKYHILNTLQ CSES1MGGAV PETLEGNTIE IGCDSITV NGIKMVNKD
IVTNNGVHIK IDQVLIPSQA KQVIELAGKQ QTFTDILVAQ LGLASALRPD 400
GEYTLAPVNL NAFSDTLLSM DQRLKLLILQ NHPILKKVGL NELYNGQILE
TIGGKQRLVF VYRTAVCIE SCMEKSGKQG RNGAIHIFRE IIPKAESLHI 500
EKLQKDFFS TFLSLEAAD LKELTQPFGD WTLFVPTNDA FKGMTEEKEE
ILIIRDNLALQ NIILYHLTPG VFIGKGFPEG VTNILKTTGQ SKIFLKEVND 600
TLLVNLKESQ ESDMTNGV IIVVDKLLLYP ADTVGNQDL LEILNKLKY
IQIIFVRGST FKEIPVTVVT TKIITKVQVE KKVIEESLQ PIITGEPTFL 700
TKVKEGFEPE FRLLKEGETI TEEHEGPII KKVTKIDGV PVEITEKETR
EERIITGPEI KYTRISTGGG ETETTLLKLL QEEVTKVTWF IEGGDGHLFE 800
DEEIKRLQG DTPVRKQLAN KKVQQSGRRRL REGRSQ

B.

MIPFLPMFSL LLLLIVNPIP ANNHYDKILA HSRIRGRDQG PNVCALQQIL
GTKKKYFSTC KNWYKSCICG QKTTVLYECC PGYMRMEGMK GCPAVLPIHD 100
VYGTLGIVGA TTTQFRYSDAS KLREIEGKQ SFTYFAPSE AWDLNLSDIR
RGLESNVNVE LLNALHSAMI NRKLMTKDLK NGMIIIPSMYN NLGLFINHYIP 200
NGVVTVCNAR IHHGNIQATN GVIVHIDRVL TQIGTISQDF IEAEddlSSF
RAAATSDIL EALGRDGHT LFAPNFAF KLFPRGVLREI MGDKVASEAL 300
MKYHILNTLQ CSES1MGGAV PETLEGNTIE IGCDSITV NGIKMVNKD
IVTNNGVHIK IDQVLIPSQA KQVIELAGKQ QTFTDILVAQ LGLASALRPD 400
GEYTLAPVNL NAFSDTLLSM DQRLKLLILQ NHPILKKVGL NELYNGQILE
TIGGKQRLVF VYRTAVCIE SCMEKSGKQG RNGAIHIFRE IIPKAESLHI 500
EKLQKDFFS TFLSLEAAD LKELTQPFGD WTLFVPTNDA FKGMTEEKEE
ILIIRDNLALQ NIILYHLTPG VFIGKGFPEG VTNILKTTGQ SKIFLKEVND 600
TLLVNLKESQ ESDMTNGV IIVVDKLLLYP ADTVGNQDL LEILNKLKY
IQIIFVRGST FKEIPVTVVT TKIITKVQVE KKVIEESLQ PIITGEPTFL 700
TKVKEGFEPE FRLLKEGETI TEEHEGPII KKVTKIDGV PVEITEKETR
EERIITGPEI KYTRISTGGG ETETTLLKLL QEEVTKVTWF IEGGDGHLFE 800
DEEIKRLQG DTPVRKQLAN KKVQQSGRRRL REGRSQ

FIGURE 17. Peptides of human periostin found in band 3 and band 6. A., The underlined peptides are the segments of periostin found in band 3. B., The underlined peptides are segments of periostin found in band 6. No segments included the possible phosphorylation sites, highlighted letter S. Both segments showed that there is no possible phosphorylation site in the fragmented periostin binding to FAM20C-WT or FAM20C-D478A in the purified samples from conditioned media.
5. References


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6. Vita

**Personal Information**

I Ping, Lin

Date of Birth: November 23, 1984

700 Albany Street, W231
Boston, MA. 02118

Email: ehping13@bu.edu

**Education**

Henry M. Goldman School of Dental Medicine, Boston University

Masters of Science in Dentistry (MSD) and Certificate of Advanced Graduate Study (CAGS) in Periodontology

September 2013-Present (Expected graduation is June, 2016)

National Taiwan University

Doctoral of Dental Surgery (D.D.S)

August 2003-May 2009