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FAM20C regulates osteoblast behaviors and intracellular signaling pathways in a cell-autonomous manner

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Abstract

Recent studies indicate that Family with sequence similarity 20 member C (FAM20C) catalyzes the phosphorylation of secreted proteins, and participates in a variety of biological processes, including cell proliferation, migration, mineralization and phosphate homeostasis. To explore the local influences of FAM20C on osteoblast, *Fam20c*-deficient osteoblasts were generated by treating the immortalized *Fam20c^{fl/fl}* osteoblasts with *CMV-Cre-IRES-EGFP* lentivirus. Compared with the normal *Fam20c^{fl/fl}* osteoblasts, the expression of *Bone sialoprotein (Bsp)*, *Osteocalcin (Ocn)*, *Fibroblast growth factor 23 (Fgf23)* and transcription factors that promote osteoblast maturation were up-regulated in the *Fam20c*-deficient osteoblasts. In contrast, the expression of *Dental matrix protein 1 (Dmp1)*, *Dentin sialophosphoprotein (Dspp)*, *Osteopontin (Opn)*, *type I Collagen a 1 (Col1a1)* and *Alkine phosphatase (Alp)* were down-regulated in the *Fam20c*-deficient cells. These alterations disclosed the primary regulation of *Fam20c* on gene expression. The *Fam20c*-deficient osteoblasts showed a remarkable reduction in the ability of forming mineralized nodules. However, supplements of extracellular matrix proteins extracted from the normal bone failed to rescue the reduced mineralization, suggesting that FAM20C may affect the biomineralization by the means more than local phosphorylation of extracellular matrix proteins and systemic phosphorus homeostasis. Moreover, although *Fam20c* deficiency had little impact on cell proliferation, it significantly reduced cell migration and lowered the levels of p-Smad1/5/8, p-Erk and p-p38, suggesting that the kinase activity of FAM20C might be essential to cell mobility and the activity of BMP ligands. In summary, these findings provide evidences that FAM20C may regulate osteoblast maturation, migration, mineralization and BMP signaling pathways in a cell-autonomous manner.

Keywords

FAM20C; mineralization; secretory proteins; phosphorylation; osteoblast

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Conflict of interest

All the authors have no conflict of interest.

Introduction

FAM20C, previously known as Dentin matrix protein 4 (DMP4), was once regarded as an ECM component through immunohistochemical assay performed on mineralized tissues, but further investigations proved that FAM20C is an intracellular component enriched in Golgi apparatus (Hao et al., 2007; Ishikawa et al., 2012; Tagliabracci et al., 2012). At 2012, FAM20C was identified as the kinase phosphorylating the members of the small integrin-binding ligand, N-linked glycoproteins family (SIBLINGs, including DMP1, DSPP, BSP, OPN and MEPE [Matrix extracellular phosphoglycoprotein]), the major extracellular components regulating mineralization (Tagliabracci et al., 2012). The following studies indicated that FAM20C could specifically phosphorylate the serine residue at the Ser-X-Glu motif of secreted proteins (George and Veis, 2008; Tagliabracci et al., 2012; Tagliabracci et al., 2013; Tagliabracci et al., 2015). Latest study also predicted that in addition to the SIBLING proteins, the substrates of FAM20C may include growth factors, components of lipid, calcium and phosphorus metabolism, neuropeptides, protease inhibitors and metalloprotease and so on, which make up the majority of secreted phosphoproteins (Tagliabracci et al., 2015). Therefore, the kinase activity of FAM20C was suggested to play crucial roles in various biological and pathological processes.

Inactivating mutations in *FAM20C* in humans lead to Raine Syndrome characterized by lethal or non-lethal skeletal disorders (Simpson et al., 2007; Fradin et al., 2011). *Fam20c* knock-out mice phenocopy the non-lethal cases of human Raine Syndrome, which manifests hypophosphataemic rickets (Wang et al., 2012a; Rafaelsen et al., 2013). Similar to the human Raine Syndrome with hypophosphataemic rickets, *Fam20c*-deficient mice exhibit a retarded growth, widened growth plates, thin and porous cortex of long bones, which are believed to be partially attributed to the elevated FGF23 and reduced serum phosphorus (Wang et al., 2012a). Recent study revealed the regulatory role of FAM20C in the degradation of circulating FGF23 (Tagliabracci et al., 2014). During the degradation process of FGF23, the proteolytic cleavage occurs at the peptide bond between Arg¹⁷⁹ and Ser¹⁸⁰ of FGF23 (Bergwitz and Jüppner, 2010). The phosphorylation of Ser¹⁸⁰ (located in the Ser¹⁸⁰-Ala¹⁸¹-Glu¹⁸² motif) by FAM20C prevents the GalNac-T3-catalyzed O-glycosylation of Thr¹⁷⁸. The O-glycosylation at Thr¹⁷⁸ protects the proteolytic site of FGF23 from being exposed to the protease Furin and thus, prevents the degradation of FGF23 (Kato et al., 2006; Bergwitz et al., 2009). Therefore, the lack of phosphorylation at Ser¹⁸⁰ of FGF23 in *Fam20c*-deficient models promotes the O-glycosylation at Thr¹⁷⁸, which prevents FGF23 from being degraded and in turn, elevates plasma FGF23 concentration (Tagliabracci et al., 2014). Circulating FGF23 promotes the excretion of phosphorus by binding FGFR1 in the aid of Klotho to reduce sodium-phosphate co-transporters NaPi-2a/2c on renal proximal tubular cells (Kurosu et al., 2006; Farrow and White, 2010; Bhattacharyya et al., 2012). Although this study proposed a mechanism for the *in vivo* elevation of circulating FGF23, it could not explain the mechanism of *Fgf23* mRNA elevation in the *Fam20c*-deficient osteoblasts and osteocytes (Wang et al., 2012a). It is also unclear whether the altered expression of bone related genes, such as Osterix (OSX), DMP1 and OCN, directly results from *Fam20c* inactivation or is secondary to the systemic feedbacks, such as the reduction of serum phosphorus and elevation of serum parathyroid hormone (Wang et al., 2012a).

Moreover, since BMP ligands were speculated to be the substrates of FAM20C (Tagliabracci et al., 2015), it implies that FAM20C may directly regulate cell behaviors in a cell-autonomous manner by modifying paracrine or autocrine factors.

Previously, we reported the generation of immortalized *Fam20c^{fl/fl}* osteoblast cell line (Liu et al., 2015). Taking the advantages of osteoblasts carrying *Fam20c*-floxed allele and Cre recombinase, we generated osteoblast cells devoid of *Fam20c*, which provides a tool excluding the systemic influences on the individual cell. By exploring the cell behaviors and gene expression of the *Fam20c*-deficient osteoblasts, we can distinguish the cell-autonomous regulation of *Fam20c* on osteoblast from the systemic influences in the *in vivo* models.

Materials and methods

Lentivirus infection and establishment of OB *Fam20c^{KO}* osteoblast line

The immortalized *Fam20c^{fl/fl}* osteoblast cell line (designated as “OB *Fam20c^{fl/fl}*”) was generated in our previous study (Liu et al., 2015). When grown to 80% confluence, the OB *Fam20c^{fl/fl}* osteoblasts were infected by the *CMV-Cre-IRES-EGFP* lentivirus following the manufacture’s protocol (GenTarget Inc. San Diego, CA), to create *Fam20c*-deficient osteoblasts (referred to “OB *Fam20c^{KO}*”). After 24 hours of infection, the *Fam20c^{fl/fl}* osteoblasts were re-plated in a low density to form separated single cell-derived clones. The selected clones were examined under the Olympus microscope for *EGFP* expression and DAPI counter staining. Only the clones in which all cells were EGFP positive after 10 passages were characterized in this study.

Polymerase Chain Reaction (PCR) and primers

Genotyping PCR was performed to confirm the removal of the floxed *Fam20c* alleles by *Cre* recombinase. The protocols and primer sequences distinguishing the floxed *Fam20c* allele from the wild type (WT) allele were described previously (Wang et al., 2012a). The null allele recombined from the floxed *Fam20c* as well as the *Cre* transgene were also identified as the previously described (Wang et al., 2012a).

Reverse transcription-PCR (RT-PCR) was performed to detect the transcripts from the floxed and recombined *Fam20c* alleles. Total mRNAs were extracted with the RNA extraction kit and reversely transcribed into cDNA with the RT-PCR Kit (Life Technologies, Inc., Grand Island, NY). Two set of primers were used to distinguish the floxed *Fam20c* alleles from the recombined *Fam20c* alleles. The first set of primers (Forward: 5'-TGCGGAGATCGCTGCCTTCC-3'; Backward: 5'-GCCACT GTCGTAGGGTGGCA-3') that were designed to amplify a region from exon5 to exon 8 produced a band of 388 bp for the mRNA transcript from floxed *Fam20c* allele, and gave rise to no PCR band for the transcript of the recombined null allele. The second set of primers (Forward: 5'-GAGAGCAGGAGACGCCGCCT-3'; Backward: 5'-CCACCACACTGCTCAGCCCG-3') amplifying a fragment from exon 5 to exon 11 gave rise to a 820-bp product for the mRNA of floxed *Fam20c* allele, and a 431-bp band for that of the recombined null allele.

Quantitative-PCR (Q-PCR) was employed to evaluate the effects of *Fam20c* loss on gene expression. The protocols and primers for the amplification of activating transcriptional

factor 4 (*Atf4*), distal-less homeobox transcription factor 3 (*Dlx3*), runt-related transcription factor 2 (*Runx2*), osterix (*Osx*), dentin matrix protein 1 (*Dmp1*), dentin sialophosphoprotein (*Dspp*), bone sialoprotein (*Bsp*), matrix extracellular phosphoglycoprotein (*Mepe*), osteopontin (*Opn*), alkaline phosphatase (*Alp*), Type I Collagen, alpha-1 (*Col1a1*), fibroblast growth factor 23 (*Fgf23*), osteocalcin (*Ocn*), osteonectin (*Osn*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were described in the previous reports (Wu et al., 2010; Wang et al., 2012a; Liu et al., 2015). Q-PCR was also applied to compare the relative expression of *Bmp2* with primers (Forward: 5'-TGACTGGATCGTGGCACCTC-3'; Backward: 5'-CAGAGTCTGCACTATGGCATGGTTA-3'), of *Bmp4* with primers (Forward: 5'-ACAATGTGACACGGTGGGAAAC-3'; Backward: 5'-TGTGGGTGATGCTTG GGA CTAC-3') and of *Bmp7* with primers (Forward: 5'-ACATCCGGGAGCGATTG GAC-3'; Backward: 5'-TCCTCAGAAGCCCAGATGGTC-3').

Assay of cell morphology and cell proliferation

The morphologies of immortalized *OB Fam20c^{fl/fl}* and *OB Fam20c^{KO}* osteoblasts were examined under the Olympus phase contrast microscope. The 5-bromo -2'-deoxyuridine (BrdU) was supplemented into the culture medium in the concentration of 30μM to label the nuclei of proliferating cells (Life Technologies, Inc., Grand Island, NY). After 4 hours of incorporation of BrdU, the BrdU positive nuclei of the *Fam20c^{fl/fl}* and *Fam20c^{KO}* osteoblasts were detected by the BrdU assay Kit (Life Technologies, Inc., Grand Island, NY).

Immunocytochemistry

The antibody against BSP was a gift from Dr. Larry Fisher at National Institute of Dental and Craniofacial Research (Liu et al., 2014; Liu et al., 2015; Xie et al., 2016). The anti-DMP1 and anti-DSP antibodies were applied as previously described (Baba et al., 2004; Huang et al., 2008; Liu et al., 2014, Liu et al., 2015). The polyclonal rabbit IgG antibodies against MEPE and OPN were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Abcam, Inc. (Cambridge, MA), respectively. The anti-mouse and anti-rabbit IgG conjugated with biotin were applied as the secondary antibodies (Vector Laboratories, Inc., Burlingame, CA).

Western Blotting Assay

To compare the secreted FGF23 concentration between the culture media of *Fam20c^{fl/fl}* and *Fam20c^{KO}* osteoblasts, α-MEM with 0.2% fetal bovine serum (FBS) was applied to replace the α-MEM supplemented with 10% FBS, when the osteoblasts became confluent. After 24 hours of culture, the α-MEM with 0.2% FBS was mixed with the protease inhibitor cocktail and centrifuged. The supernatant was collected and then further concentrated with Amicon Ultra Centrifugal Filters (EMD Millipore Company, Merck KGaA, Darmstadt, Germany). The protein concentration in the supernatants was determined by the Bicinchoninic Acid assay (Pierce Biotechnology). Protein samples containing equal amount of β-actin were loaded onto 5–15% SDS-PAGE for Western blotting assay following the protocol as previously described (Xie et al., 2016). The monoclonal mouse anti-human FGF23 antibody (anti-FGF23-79) was used as the primary antibody (Jani et al., 2016). To evaluate the influence of FAM20C on BMP signaling pathways, the antibodies against the

phosphorylated Smad1/5/8 (p-Smad1/5/8), phosphorylated Erk (p-Erk), phosphorylated p-38 (p-p38), phosphorylated JNK (p-JNK), phosphorylated and non-phosphorylated Smad1/5/8 (pan-Smad1/5/8), phosphorylated and non-phosphorylated Erk (pan-Erk), phosphorylated and non-phosphorylated p-38 (pan-p38), and phosphorylated and non-phosphorylated JNK (pan-JNK) were all polyclonal rabbit IgG purchased from Santa Cruz Biotechnology. The mouse IgG against mouse β -actin was obtained from Sigma-Aldrich, St. Louis, MO. The secondary antibodies were goat anti-mouse and goat anti-rabbit IgG conjugated with Alkaline Phosphatase (Vector Laboratories, Inc., Burlingame, CA). The CD star detection system was applied for the detection of chemiluminescent bands (Sigma-Aldrich, St. Louis, MO).

Assay of alkaline phosphatase (ALP) activity and mineralization

The ALP activity assay and the mineralization assay followed the protocol previously described (Liu et al., 2015). The non-collagenous proteins in the bone matrix were extracted from the WT rat long bones as described in the previous reports (Qin et al., 2006). To rescue the incapacitated mineralization of *Fam20c*^{KO} osteoblasts, the lyophilized non-collagenous extracts from the bone matrix were supplemented at the final concentration of 80 μ g/ml into the mineralization-inducing medium (α -MEM supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100ug/ml streptomycin, 50 μ g/ml ascorbic acid and 10 μ M sodium β -glycerophosphate). After 1 week of induction, the cells and their secreted matrix were fixed with 4% PFA for 20 min, rinsed with phosphate buffered Saline (PBS) twice and then, stained with Alizarin Red S (Sigma–Aldrich, St. Louis, MO).

Cell migration assay

When the OB *Fam20c*^{f/f} and OB *Fam20c*^{KO} osteoblasts were grown to form a monolayer, a scratch of 500 μ m wide was made on the monolayer with a pipette tip. The images were taken at 0 and 24 hours after scratch was made. To further confirm the influence of FAM20C on cell migration, 1.0×10^7 osteoblasts were concentrated into a suspension of 100 μ l and dropped onto each well of a 24-well plate. At 2 hours after dropping the cell suspension, α -MEM with 10% fetal bovine serum was added to cover the surface of each well. The diameter of each cell mass was measured after 24 hours to evaluate the migration capability.

Statistical Analysis

All values were presented as the mean with standard deviation (SD). The p-value of <0.05 from the unpaired *student's* t-test was considered to be statistically significant.

Results

Generation of *Fam20c*-deficient osteoblast cell line

The OB *Fam20c*^{f/f} osteoblasts infected by *CMV-Cre-IRES-EGFP* lentivirus were re-plated in a very low density to produce the single cell-derived colonies. Only the colonies in which all the offspring cells were EGFP positive (i.e., with *Cre* integrated into the genomes of the infected osteoblasts) were selected and expanded for further investigation (Fig. 1A). Genotyping PCR confirmed that the genomic DNA extracted from OB *Fam20c*^{f/f} only gave rise to a 400-bp band for the floxed *Fam20c* allele (Fig. 1B). In contrast, the genomic DNA

of the infected *EGFP*-expressing osteoblasts were devoid of the bands for both the WT and floxed *Fam20c* alleles, but only gave rise to the bands for the recombined *Fam20c* null allele as well as *Cre* recombinase gene (Fig. 1B). RT-PCR with Set 1 primers (covering exon 5 to exon 8) detected a 381-bp band only in the transcriptome of the OB *Fam20c^{fl/fl}* (Fig. 1C). Meanwhile, Set 2 primers (from exon 5 to exon 11) were able to amplify an 820-bp band from the transcriptome of OB *Fam20c^{fl/fl}*, and a 431-bp band matching the truncated transcript from the mRNAs of the *EGFP*-expressing osteoblasts (Fig. 1C). These results indicated the removal of the fragment from exon 6 to exon 9 of *Fam20c* allele in the *EGFP*-expressing osteoblasts, and confirmed that the floxed *Fam20c* allele was successfully transformed into null allele by the transfected *Cre* in the *EGFP*-expressing osteoblasts.

***Fam20c*-deficient osteoblasts (OB *Fam20c^{KO}*) had no alteration in the morphology and proliferation**

We did not find significant morphological differences between the OB *Fam20c^{fl/fl}* and OB *Fam20c^{KO}* osteoblasts under the bright field microscope (Fig. 2A). Although in the OB *Fam20c^{KO}* group, the median value for the ratio of BrdU positive cell to total cells was slightly lower than that in the OB *Fam20c^{fl/fl}*, statistical analysis indicated that the difference between the proliferation ratio of these two lines was insignificant ($p > 0.05$; Fig. 2B&C), which was in agreement with the findings obtained from the *Fam20c*-deficient mice (Wang et al., 2012a).

Altered gene expression in *Fam20c*-deficient osteoblasts

Q-PCR was performed to compare the gene expression profile between OB *Fam20c^{KO}* and OB *Fam20c^{fl/fl}* cells. Compared with OB *Fam20c^{fl/fl}*, the transcription of *Runx2* and *Dlx3* were up-regulated, while the *Atf4* and *Osx* were down-regulated in the OB *Fam20c^{KO}* (Fig. 3A). The reduced transcription of *Dmp1*, *Dspp* and *Opn* were detected in the OB *Fam20c^{KO}*, but the elevated *Bsp* and unaltered *Mepe* transcription were observed in these *Fam20c*-deficient osteoblasts (Fig. 3B). The mRNA levels of *Alp* and *Col1a1* in the OB *Fam20c^{KO}* were lower than in OB *Fam20c^{fl/fl}* (Fig. 3C). In contrast, the mRNA levels of *Fgf23* and *Ocn* in the OB *Fam20c^{KO}* were higher than OB *Fam20c^{fl/fl}* (Fig. 3C). Immunocytochemistry analyses further revealed the effects of FAM20C loss on the expression of SIBLINGs. Consistent with the Q-PCR, the immunostaining for DMP1, DSP and OPN in OB *Fam20c^{KO}* were weaker than in OB *Fam20c^{fl/fl}* (Fig. 4A). The staining of MEPE in the *Fam20c^{KO}* was weaker than that in the OB *Fam20c^{fl/fl}*, and the BSP staining of *Fam20c^{KO}* was comparable to that in the OB *Fam20c^{fl/fl}* (Fig. 4A). Western blotting assay indicated that the level of FGF23 secreted by OB *Fam20c^{KO}* into culture medium was much higher than that of OB *Fam20c^{fl/fl}*, which was consistent with the *in vitro* Q-PCR and *in vivo* serum biochemistry data (Fig. 4B).

Impaired mineralization of *Fam20c*-deficient osteoblasts

In the mouse models, impaired mineralization in bone and tooth is the most evident defect caused by *Fam20c* deficiency (Wang et al., 2012a; Wang et al., 2012b). The capability of forming mineralized nodules of OB *Fam20c^{KO}* was evaluated to examine the influence of FAM20C loss on mineralization. The ALP activity in OB *Fam20c^{KO}* was remarkably lower than OB *Fam20c^{fl/fl}* (Fig. 5A), which was consistent with the Q-PCR analyses showing that

OB *Fam20c*^{KO} had a lower level of *Alp* mRNA. As expected, the number of mineralized nodules in OB *Fam20c*^{KO} was fewer than that of OB *Fam20c*^{f/f} (Fig. 5B), indicating that *Fam20c* deficiency impaired the mineralization in a cell autonomous manner. To address if the phosphorylation of the SIBLINGS and/or other non-collagenous proteins in ECM was essential for osteoblast mineralization, the non-collagenous proteins extracted from wild type rat long bone were supplemented into the culture medium. The addition of non-collagenous proteins extracted from the rat bone did not improve the formation of mineralized nodules in OB *Fam20c*^{KO} (Fig. 5D), whereas in OB *Fam20c*^{f/f}, supplementing the proteins extracted from the bone matrix enhanced the mineralized nodule formation (Fig. 5C). Thus, it implicates that *Fam20c* regulates mineralization by means more than phosphorylating SIBLINGS and/or ECM components.

Altered canonical and non-canonical BMP signaling pathways in *Fam20c*-deficient osteoblasts

As a class of secreted proteins with typical Ser-X-Glu motif, bone morphogenetic proteins (BMPs), especially BMP4, were also predicted to be substrates of FAM20C (Tagliabracci et al., 2015). However, up to date, there was no study clarifying that the secreted BMPs were phosphorylated and if phosphorylation played a role in BMP activity. In our study, Western blot analyses showed that both the pan-Smad1/5/8 and the p-Smad1/5/8 were significantly attenuated in the OB *Fam20c*^{KO}, though the ratios of p-Smad1/5/8 to pan-Smad1/5/8 changed insignificantly between OB *Fam20c*^{KO} and OB *Fam20c*^{f/f} (Fig. 6A). The pan-Erk showed no difference between OB *Fam20c*^{KO} and OB *Fam20c*^{f/f}, however, the ratio of p/pan-Erk in OB *Fam20c*^{KO} was dramatically decreased, because of the decreased p-Erk (Fig. 6B). In spite of the down-regulation in both the pan- and p-p38 levels, the ratio of p/pan-p38 increased significantly in OB *Fam20c*^{KO} (Fig. 6C). These results suggested that *Fam20c* deficiency attenuated both the canonical and non-canonical BMP signaling pathway. Intriguingly, the elevated transcription of *Bmp2* and *Bmp7*, along with the unaltered *Bmp4* transcription were detected in OB *Fam20c*^{KO} (Fig. 6E), which implied that the phosphorylation was essential for the activity of BMP ligands.

The attenuated migration of *Fam20c*-deficient osteoblasts

In the scratch tests, OB *Fam20c*^{f/f} almost covered the scratch area in 24 hours, whereas only a few OB *Fam20c*^{KO} osteoblasts migrated into the scratch (Fig. 7A). The OB *Fam20c*^{f/f} suspension developed colonies with larger diameters than OB *Fam20c*^{KO} suspension containing equal number of cells in 24 hours (Fig. 7B). The statistical analysis on the colony diameters confirmed that the diameter difference was statistically significant (Fig. 7C; $p < 0.001$).

Discussion

The *Fam20c*-deficient mice manifest rickets/osteomalacia along with the elevation of serum FGF23, reduction of serum phosphorus and increased level of serum parathyroid hormone (Wang et al., 2012a; Liu et al., 2014). However, it is still unclear whether these skeletal abnormalities in *Fam20c*-deficient mice are primarily attributed to hypophosphatemia or mainly due to the intrinsic defects of osteoblasts. The availability of OB *Fam20c*^{f/f} allows us

to assess the local effects of *Fam20c* deficiency on the osteoblasts, by excluding the influence of systemic factors, such as hypophosphatemia and altered serum level of parathyroid hormone.

The effects of *Fam20c* deficiency on the gene expression of osteoblasts

The reduced transcriptions of *Dmp1*, *Dspp* and *Opn*, and their relatively weaker immunocytochemical staining in *OB Fam20c^{KO}* indicate that the suppressed expression of these ECM molecules in the *Fam20c* knock-out mice results primarily from the loss of *Fam20c* (Wang et al., 2012a). Although the elevated *Bsp* transcription in *OB Fam20c^{KO}* suggests a negative regulation of FAM20C on *Bsp* transcription, the unchanged BSP staining in *OB Fam20c^{KO}* implicates that the phosphorylation on BSP may regulate its concentration post-transcriptionally, most likely by protein degradation. Moreover, the unchanged BSP staining in *OB Fam20c^{KO}* is controversial to the elevated BSP staining in *Fam20c* knock-out mouse tooth (Wang et al., 2012b), revealing a possible systemic feedback to *Fam20c* deficiency on BSP concentration. Similarly, the comparable *Mepe* transcription and the weaker MEPE staining in *OB Fam20c^{KO}* also suggested that the phosphorylation of MEPE could regulate its concentration post-translationally.

Since both the *in vitro* and *in vivo* data showed that the transcriptions of *Col1a1* are reduced in the context of *Fam20c* deficiency, it is believed that the primary regulation of FAM20C on *Col1a1* expression is negative. The reduced *Alp* transcription and ALP activity in *OB Fam20c^{KO}* consistently indicate a direct dependence of *Alp* expression on FAM20C activity. Both the mRNA and protein of *Fgf23* in the *OB Fam20c^{KO}* are remarkably increased, which is in agreement with the observation in *Fam20c*-deficient mice. In contrast, the transcription of *Ocn* was up-regulated in *OB Fam20c^{KO}*, which disagreed with the *in vivo* findings. The *in vitro* up-regulation of *Ocn* may result from the elevated *Runx2* and *Atf4*, which promotes *Ocn* expression *in vivo* (Ferron et al., 2010; Fulzele et al., 2010; Oury et al., 2011; Oury et al., 2013). Additionally, it also suggests that the decreased transcription of *Ocn* in *Fam20c* knock-out mice may be secondary to *Fam20c* deficiency and caused by systemic feedbacks.

The transcriptions of *Runx2* and *Dlx3* promote matrix production during the whole process of osteoblast maturation, while *Atf4* fulfills the same role only in late stage of osteoblast maturation (Hartmann, 2009). In *OB Fam20c^{KO}*, the transcriptions of *Runx2*, *Dlx3* and *Atf4* were all up-regulated. The transcription of *Osx* only ensures the differentiation from osteochondrogenic progenitors into immature osteoblasts (Hartmann, 2009), and was down-regulated in *OB Fam20c^{KO}*, which is consistent with the *in vivo* result (Wang et al., 2012a). Therefore, we speculate that the elevated transcriptions of *Runx2*, *Dlx3* and *Atf4* may be a compensatory response to the impaired osteoblast maturation caused by *Fam20c* deficiency. This speculation was also supported by the unaltered transcriptions of *Mepe* which is an inhibitor to osteogenic differentiation and mineralization (Gowen et al., 2003), and the unchanged *Osn* which works as an extracellular matrix down-regulated in mature bone in the *OB Fam20c^{KO}* (Rosset and Bradshaw, 2016).

The effects of *Fam20c* deficiency on *in vitro* mineralization

In this study, we observed that the capability of OB *Fam20c*^{KO} to form mineralized nodules was severely impaired. Since the influence of systemic factors, such as serum phosphorus, was excluded in these *in vitro* experiments, the impaired mineralization must be solely attributed to the intrinsic defects of the *Fam20c*-deficient osteoblasts. We once assume that the disabled phosphorylation of SIBLINGs and/or other non-collagenous components in *Fam20c*-deficient osteoblasts results in the impaired mineralization, and the phosphorylated non-collagenous components were sufficient to rescue the *Fam20c*-deficient osteoblast mineralization. To our surprise, the non-collagenous extracts from the normal bone matrix failed to rescue the mineralization defects of the *Fam20c*-deficient osteoblasts, suggesting that the loss or reduction of phosphorylation of the non-collagenous proteins was not the only cause responsible for the incapacitated mineralization of *Fam20c*-deficient osteoblasts. Since reduced ALP activity results in the accumulation of pyro-phosphate on the hydroxyapatite (HA) surface, which suppresses the further growth of HA crystals (Meyer, 1984), the significantly reduced *Alp* transcription and activity are likely to be one of the factors responsible for the impaired mineralization of the *Fam20c*-deficient osteoblasts. Obviously, further studies are needed to detect the concentration of pyro-phosphate in the *Fam20c*-deficient osteoblast cultured medium. Secondly, the non-collagenous proteins extracted in this manner do not include growth factors, such as BMP2 and BMP4. Thus, if the failure in phosphorylation for these growth factors contributes to the defective differentiation and maturation of *Fam20c*-deficient osteoblasts, and then the lack of such growth factors in our protein extract might be a factor for the rescue failure. Moreover, the reduced collagen expression was also supposed to impair the mineralization of OB *Fam20c*^{KO}.

Attenuated BMP signaling pathways in *Fam20c*-deficient osteoblasts

FAM20C catalyzes the attachment of phosphate to Ser residues in the Ser-X-Glu motifs of secretory proteins in the Golgi apparatus. The presence of Ser-X-Glu motifs in BMP2 (Ser⁴⁶-Asp⁴⁷-Glu⁴⁸, Ser¹¹⁷-Leu¹¹⁸-Glu¹¹⁹ and Ser¹⁴⁷-Ala¹⁴⁸-Glu¹⁴⁹), BMP4 (Ser⁵⁰-His⁵¹-Glu⁵², Ser⁹¹-Gly⁹²-Glu⁹³ and Ser¹⁵⁵-Ala¹⁵⁶-Glu¹⁵⁷) and BMP7 (Ser⁴⁸-Gln⁴⁹-Glu⁵⁰, Ser²¹⁹-Glu²²⁰-Glu²²¹ and Ser²⁴⁸-Val²⁴⁹-Glu²⁵⁰) implies that these proteins might be the potential substrates of FAM20C. In this study, we observed that both the canonical BMP signaling, and the non-canonical BMP/p38 and BMP/Erk signaling were attenuated in the OB *Fam20c*^{KO} osteoblasts, suggesting that FAM20C may enhance the BMP signaling pathways by phosphorylating the ligands, through which this kinase regulates osteoblast differentiation and maturation. Our further studies are warranted to provide more direct evidence as whether or not FAM20C phosphorylates BMPs and if the phosphorylation of BMPs by this kinase is indeed critical for their activities.

Since BMP ligands possess various biological functions, it implies that FAM20C can exert regulatory roles not only in mineralization, but also in many other biological processes. Although many studies revealed that BMP ligands promoted osteoblast differentiation and maturation by activating osteogenic transcription factors (Chen et al., 2012; Wu et al., 2016; Salazar et al., 2016;), there are few reports concerning the role of BMP signaling in the expression of ECM. In the OB *Fam20c*^{KO} osteoblasts, if the altered expression of SIBLINGs

and other ECM genes resulted from the attenuated BMP signaling pathways requires further investigation.

The effects of *Fam20c* deficiency on osteoblast migration

Latest study demonstrated that ablation of *Fam20c* by CRISPR/Cas9 technique in breast cancer cell migration revealed that cell migration relies on the FAM20C-dependent phosphorylation of insulin-like growth factor binding protein-7 (IGFBP-7) in an insulin-like growth factor-1 (IGF-1)-independent manner (Tagliabracci et al., 2015). In our study, the *Fam20c*-deficient osteoblasts are originated from the *Fam20c* knock-out mice generated by homologous recombination in embryonic stem cells (Wang et al., 2012a), which provides a non-tumor model and circumvents the off-target effects in CRISPR/Cas9 system. The *OB Fam20c^{KO}* osteoblasts exhibit a noticeably retarded capability of migration in both scratch wound healing and suspension drop expanding experiments. These data demonstrated that FAM20C may play a critical role in the migration capability and the phosphorylation of secreted proteins may be essential for cell migration.

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Fig 1. Removal of the floxed *Fam20c* allele by *CMV-Cre-IRES-EGFP* lentivirus

(A) The immortalized *Fam20c^{f/f}* osteoblasts were infected with *CMV-Cre-IRES-EGFP* lentivirus and cultured after 10 passages; note that all of the cells were positive for DAPI (blue) and EGFP (green), indicating an uniform infection of *CMV-Cre-IRES-EGFP* lentivirus in these osteoblasts. (B) The genomic DNA from *Fam20c^{f/+}* mouse tail (*f/+*), OB *Fam20c^{f/f}* (*f/f*; without infection by lentivirus) and OB *Fam20c^{KO}* (*KO*; after treatment with lentivirus) were genotyped with specific primers for the floxed *Fam20c* allele, recombined *Fam20c* allele and *Cre*, respectively. (C) RT-PCR was performed to identify the successful removal of the floxed *Fam20c* allele. The Set 1 primers for exon 5–8 gave a 388-bp band for OB *Fam20c^{f/f}* cells (*f/f*), but produced no band for OB *Fam20c^{KO}* cells (*KO*); the Set 2 primers for exon 5–11 of *Fam20c* transcript produced an 820-bp band for OB *Fam20c^{f/f}* (*f/f*), and a truncated 421-bp band for OB *Fam20c^{KO}* osteoblasts (*KO*). (Std: DNA ladder; Scale bar: 100 μm)

Fig 2. Cell morphology and proliferation of the OB *Fam20c^{KO}* cell line
(A) The micrographs OB *Fam20c^{fl/fl}* and OB *Fam20c^{KO}* osteoblasts were taken under the Olympus reverse phase-contrast microscope. **(B)** BrdU-labeling images of OB *Fam20c^{fl/fl}* and OB *Fam20c^{KO}* were photographed under the Olympus reverse phase-contrast microscope. **(C)** The statistical assay showed that there were no significant difference ($p>0.05$) in the proliferation rate between OB *Fam20c^{fl/fl}* (Mean=45.24%, SD=8.701%) and OB *Fam20c^{KO}* (Mean=37.83%, SD=5.814%). Scale bar in (A) and (B) equals to 100 μ m).

Fig 3. The mRNA levels of bone-related genes in OB *Fam20c^{fl/fl}* and OB *Fam20c^{KO}*
 Total mRNAs from OB *Fam20c^{fl/fl}* and OB *Fam20c^{KO}* were extracted and reversed transcribed into cDNAs. Then, the cDNAs were amplified by Q-PCR to calculate the relative expression levels of bone-related transcription factors: *Atf4*, *Dlx3*, *Osx* and *Runx2* (A), the SIBLING proteins: *Dmp1*, *Dspp*, *Bsp*, *Mepe* and *Opn* (B), and other bone-related genes: *Alp*, *Fgf23*, *Col1a1*, *Ocn* and *Osn* (C). (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.)

Fig 4. The levels of bone-related gene expression detected by immunocytochemistry and Western blot

(A) Immunocytochemistry with the antibodies against DMP1, DSP, BSP, MEPE and OPN were performed to assess the cultured OB *Fam20c^{ff}* and *Fam20c^{KO}* cells. (B) Western blotting with FGF23 antibody showed that the FGF23 concentration in the serum free medium of the OB *Fam20c^{KO}* was higher than in OB *Fam20c^{ff}*.

Fig 5. Impaired mineralization of OB *Fam20c^{KO}*

(A) *In situ* histochemistry assay showed a decrease in ALP activity in OB *Fam20c^{KO}* compared with OB *Fam20c^{fl/fl}*. (B) After 3 weeks of induction, Alizarin red staining showed that the formation of mineralized nodules was severely impaired in OB *Fam20c^{KO}*, compared to OB *Fam20c^{fl/fl}*. (C) After one week of culture in the osteogenic medium, the addition of non-collagenous proteins extracted from normal bone matrix of rat legs enhanced the formation of mineralization nodules in OB *Fam20c^{fl/fl}*. (D) There was no improvement on the formation of mineralization nodules by the addition of non-collagenous proteins extracted from normal bone matrix into OB *Fam20c^{KO}* osteoblasts.

Fig 6. The altered BMP signaling pathways in OB *Fam20c^{KO}*

Canonical and non-canonical BMP signaling pathways were analyzed by Western blotting with antibodies against pan-Smad1/5/8 and p-Smad1/5/8 for canonical BMP signaling (A), with antibodies against pan-Erk and p-Erk for BMP/p38 signaling (B), and with antibodies against pan-p38 and p-p38 (C) for BMP/p38 signaling. β-actin was used as the internal control to normalize the loading volume of OB *Fam20c^{ff}* and OB *Fam20c^{KO}* cell lysates (D). Q-PCR was applied to compare the relative transcription of *Bmp2*, *Bmp4* and *Bmp7* between OB *Fam20c^{ff}* and *Fam20c^{KO}* cells (E). (*: p<0.05; **: p<0.01.)

Fig 7. Reduced migration rate of OB *Fam20c^{KO}*

(A) After 24 hours of culture, the scratch in OB *Fam20c^{fl/fl}* was nearly fully covered by the cells. In contrast, there were only a few OB *Fam20c^{KO}* cells that had migrated into the scratch. (B) After 24 hours of dropping a suspension containing the 1×10^7 of cells into a culture dish, the diameter of OB *Fam20c^{KO}* colony was notably smaller than that of *Fam20c^{fl/fl}* osteoblasts. (C) The statistical assay showed a significant difference between the diameters of OB *Fam20c^{fl/fl}* colonies (Mean=5.025mm, SD=0.359mm) and OB *Fam20c^{KO}* colonies (Mean=3.98mm, SD=0.37mm). (***: $p < 0.001$).