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Abrogation of *Fam20c* altered cell behaviors and BMP signaling of immortalized dental mesenchymal cells

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Abstract

FAM20C mutations compromise the mineralization of skeleton and tooth in both human and mouse. Putatively, the mineralization disorder is attributed to the elevated fibroblast growth factor 23 (FGF23), which reduced the serum phosphorus by suppressing the reabsorption of phosphorus in kidney. Besides the regulation on systemic phosphorus homeostasis, *FAM20C* was also implicated to regulate cell behaviors and gene expression through a cell-autonomous manner. To identify the primary effects of *Fam20c* on dental mesenchymal cells, mouse *Fam20c*-deficient dental mesenchymal cells were generated by removing the floxed alleles from the immortalized mouse *Fam20c*$^{f/f}$ dental mesenchymal cells with *Cre*-expressing lentivirus. The removal of *Fam20c* exerted no impact on cell morphology, but suppressed the proliferation and mobility of the dental mesenchymal cells. *Fam20c* deficiency also significantly reduced the expression of *Osterix*, *Runx2*, *type I Collagen a 1 (Col1a1), Alkaline phosphatase (Alpl)* and the members of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family, but increased *Fgf23* expression. Consistently, the *in vitro* mineralization of *Fam20c*-deficient dental mesenchymal cells was severely disabled. However, supplements of the non-collagenous proteins from wild type rat dentin failed to rescue the compromised mineralization, suggesting that the roles of FAM20C in tooth mineralization are more than phosphorylating local matrices and regulating systemic phosphorus metabolism. Moreover, the down-regulated BMP signaling pathways in the *Fam20c* deficient dental mesenchymal cells revealed that the kinase activity of FAM20C might be required to maintain BMP signaling. In summary, our study discloses that *Fam20c* indeed regulates cell behaviors and cell signaling pathway in a cell-autonomous manner.

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Conflicts of interest

The authors declare no interest.

Keywords

FAM20C; mineralization; secretory proteins; phosphorylation; extracellular matrix

1. Introduction

FAM20C is a member of the Family with Sequence Similarity 20 (FAM20) and highly expressed in the mineralized tissues [1,2]. As the key kinase phosphorylating the matrix proteins in mineralized tissues [3], *FAM20C* deficiency causes lethal and non-lethal cases of Raine Syndrome in human and hypophosphataemia rickets in mouse [4,5,6], both of which impair the mineralization of skeleton and tooth. *Sox2-cre; Fam20c*$^{f/f}$ mice exhibit shorter incisors and smaller molars characterized by the thinner enamel and dentin layers, enlarged pulp chambers, undeveloped roots and hypomineralizaed alveolar bone along with the reduced expression of *dental matrix protein 1* (*Dmp1*) and *dentin sialophosphoprotein* (*Dspp*) [6,7]. In the global *Fam20c* deficient mice, *Fam20c* deficiency decreases the phosphorylation, but increases the O-glycosylation of FGF23, which prevents FGF23 from the degradation by Furin and in turn, results in hypophosphataemia by the accumulated FGF23 in circulation [8]. Therefore, the compromised dentin formation and mineralization are regarded as a result secondarily from hypophosphataemia. However, in the *K14-cre; Fam20c*$^{f/f}$ mice, which do not suffer from the reduced serum phosphorus or the impaired dentin and bone mineralization, the organization and thickness of enamel as well as the expression of *ameloblastin* (*AMBN*) and *amelotin* (*AMTN*) are remarkably impaired, while the expression of *Biglycan* and *bone sialoprotein* (*Bsp*) are up-regulated in the ameloblasts [9]. These phenomena strongly recommend that *Fam20c* can regulate the mineralization through the local effects which are independent of the systemic phosphorus homeostasis.

Recent studies revealed that the predicted substrates of FAM20C covers the majority of secreted phosphoproteins, including growth factors, components of lipid, calcium and phosphorus metabolism, neuropeptides, metalloprotease and their inhibitors [10]. This findings not only supports the notion that the biological roles of FAM20C are more than biomineralization, but also implicates that FAM20C could directly regulate the mineralization of bone and tooth via a cell autonomous manner besides systemic phosphorus metabolism. However, to date, there are few studies concerning the local effects of *Fam20c* during organogenesis because of the lack of a model to exclude the systemic influences. The specific cell line devoid of *Fam20c* is necessary to explore the cell autonomous regulation on mineralization or other biological processes.

We have generated the immortalized mouse *Fam20c*$^{f/f}$ dental mesenchymal cells [11]. To assess the cell behaviors and gene expression influenced by *Fam20c* deficiency during tooth development, we generated mouse dental mesenchymal cells deprived of *Fam20c* allele by lentivirus carrying *Cre* recombinase. By observing the gene expression profile, proliferation, migration, mineralization and BMP signaling pathway of the *Fam20c*-deficient dental mesenchymal cells, we can identify the cell autonomous regulation of *Fam20c* on the tooth formation and dentin mineralizaion, which will shed new light on the roles of phosphorylation of secreted proteins in tooth development.

2. Materials and Methods

2.1. Generation of mouse Fam20c deficient dental mesenchymal cell line

The immortalized *Fam20c*$^{f/f}$ dental mesenchymal cell line (designated as "DM *Fam20c*$^{f/f}$") has been established by immortalizing the primary molar mesenchymal cells from a postnatal 4-day *Fam20c*$^{f/f}$ mouse with SV40-T antigen [11]. To generate mouse *Fam20c*-deficient dental mesenchymal cells (referred to "DM *Fam20c*^{KO}"), *CMV-Cre-IRES-EGFP* lentivirus was employed to infect the DM *Fam20c*$^{f/f}$ as the manufacturer instructed (GenTarget Inc. San Diego, CA). After 24 hours of infection, the infected dental mesenchymal cells were replated at a low density for the growing up of single cell-derived clones. Only the clones in which all the offspring were EGFP positive after 3 passages were picked up for further investigation. The DM *Fam20c*^{KO} was cultured in αMEM supplemented with 10% fetal bovine serum, 100 unit/ml penicillin and 100ug/ml streptomycin.

2.2. Polymerase Chain Reaction (PCR) for genotyping, Reverse transcription-PCR (RT-PCR) and quantitative-PCR (Q-PCR)

To identify the removal of the floxed *Fam20c* alleles by *Cre* recombinase, the wild type (WT) *Fam20c* allele, the floxed *Fam20c* allele, the recombined *Fam20c* allele and *Cre* transgene were genotyped by PCR with the primers described previously [6].

RT-PCR was performed with the RT-PCR Kit (Life Technologies, Inc., Grand Island, NY) to ensure the deletion of full length *Fma20c* transcripts in DM *Fam20c*$^{f/f}$. Two set of primers were designed to distinguish the transcript of floxed *Fam20c* allele from that of recombined *Fam20c* allele. The first set of primers (Forward: 5'-TGCGGAGATCGCTGCCTTCC-3'; Reverse: 5'-GCCACTGTCGTAGGGTGGCA-3') that amplify the region from exon 5 to 8 produced a band of 388 bp for the floxed *Fam20c* transcript, but no band for the recombined transcript, whereas the second set of primers (Forward: 5'-GAGAGCAGGAGACGCCGCCT-3'; Reverse: 5'-CCACCACACTGCTCAGCCCG-3') targeting the fragment from exon 5 to 11 gave rise to a 820-bp product for the floxed *Fam20c* transcript, and a 431-bp band for the recombined transcript.

The relative gene expression was evaluated by Q-PCR following the protocol as described [11]. The primers for the transcripts of *activating transcriptional factor 4* (*Atf4*), *distal-less homeobox transcription factor 3* (*Dlx3*), *runt-related transcription factor 2* (*Runx2*), *osterix* (*Osx or Sp7*), *dentin matrix protein 1* (*Dmp1*), *dentin sialophosphateprotein* (*Dspp*), *bone sialoprotein* (*Bsp*), *matrix extracellular phosphoglycoprotein* (*Mepe*), *osteopotin* (*Opn or Spp1*), *alkaline phosphatase* (*Alpl*), *Type I Collagen, alpha-1*(*Col1a1*), *fibroblast growth factor 23* (*Fgf23*), *osteocalcin* (*Ocn or Bglap*), *osteonectin* (*Osn or Sparc*) and *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) were previously reported [6,11].

2.3. Cell morphology and proliferation

The morphologies of immortalized DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO} were compared under phase contrast microscope. The proliferation ratios were evaluated by 5-bromo-2'-deoxturidine (BrdU) labeling test by supplementing 30μM of BrdU into the culture medium

(Life Technologies, Inc., Grand Island, NY). After 4 hours of incorporation, the BrdU positive nuclei were detected by the BrdU assay Kit (Life Technologies, Inc., Grand Island, NY).

2.4. Cell migration

When DM *Fam20c*$^{f/f}$ or DM *Fam20c*^{KO} formed a monolayer, a scratch of 500 μm wide was made on the monolayer with a pipette tip. The width of scratches was compared after 24 hours of scratching. For cell expansion test, 1.0×10^7 DM *Fam20c*$^{f/f}$ or DM *Fam20c*^{KO} were concentrated into a drop of 100 μl. The diameter of the expanding colony was measured after 24 hours to compare the migration capability.

2.5. Immunocytochemistry

The antibody against BSP was gifted by Dr. Larry Fisher at National Institute of Dental and Craniofacial Research. The mouse antibodies against DMP1 and DSP were applied as previously described [11,12]. The polyclonal rabbit IgG against MEPE was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the polyclonal rabbit IgG against OPN/SPP1 from Abcam, Inc. (Cambridge, MA). The secondary antibodies were mouse and rabbit IgG conjugated with biotin (Vector Laboratories, Inc., Burlingame, CA). The antibody against biotin was conjugated with peroxidase horseradish for DAB color development (Vector Laboratories, Inc., Burlingame, CA).

2.6. Western blotting

To compare the FGF23 products secreted by DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}, the α-MEM supplemented with 10% fetal bovine serum (FBS) was replaced by α-MEM with 0.2% FBS when the cells reached confluent. After 24 hours of serum starvation, the media were supplemented with the protease inhibitor cocktail and centrifuged. The supernatant was further concentrated with Amicon Ultra Centrifugal Filters (EMD Millipore Company, Merck KGaA, Darmstadt, Germany). The final protein concentration in the supernatants was measured by the Bicinchoninic Acid assay (Pierce Biotechnology). Cell lyses containing equal amount of β-actin were loaded onto 5–15% SDS-PAGE to follow the protocol as previously described [13]. The the primary antibody against FGF23 was monoclonal mouse IgG [14]. The antibodies against the phosphorylated and total phosphorylated Smad1/5/8, p-38 and Erk were all polyclonal rabbit IgG purchased from Santa Cruz Biotechnology. The mouse β-actin antibody was obtained from Sigma-Aldrich, St. Louis, MO. Goat anti-mouse and goat anti-rabbit IgG conjugated with Alkaline Phosphatase were used as secondary antibodies (Vector Laboratories, Inc., Burlingame, CA). The CD star detection system for chemilumimescent detection was purchased from Sigma-Aldrich, St. Louis, MO.

2.7. Alkaline phosphatase (ALPL) activity and mineralization

To prepare the mineralization-inducing medium, 50mg/ml ascorbic acid and 10mM sodium β-glycerophosphate were added into the a-MEM supplemented with 10% fetal bovine serum,100 unit/ml pencillin,100mg/ml streptomycin. The protocols for ALPL and mineralization assay were previously described [11]. To rescue the disabled mineralization of DM Fam20cKO, the lyophilized non-collagenous proteins from WT rat incisors were

extracted and supplemented at the final concentration of 80 μg/ml into the mineralization-inducing medium [15]. After 1 week of induction, the culture was stained with Alizarin Red S (Sigma – Aldrich, St. Louis, MO) for mineralization assay.

2.8. Statistical Analysis

All results of the unpaired *student's* t-test were shown as the means with standard deviation (SD). The *p*-value less than 0.05 was regarded to be statistically significant.

3. Results

3.1. Deletion of Fam20c alleles from immortal DM Fam20c$^{f/f}$

CMV-Cre-IRES-EGFP lentivirus was incubated with DM *Fam20c*$^{f/f}$ to transfect *Cre* and *EGFP* transgenes into cells. After infection, the cells were re-plated for the formation of single cell-derived colonies. The colonies only producing EGFP positive offspring were picked up and regarded as DM *Fam20c*^{KO} for the following investigation (Fig. 1A). Genotyping PCR confirmed that *Cre* transgene was only detected in the genomic DNA of DM *Fam20c*^{KO}, instead of DM *Fam20c*$^{f/f}$ (Fig. 1B). Consistently, it was the recombined *Fam20c* null allele, as opposed of the floxed *Fam20c* allele that was detected in DM *Fam20c*^{KO}, whereas the DM *Fam20c*$^{f/f}$ only gave rise to the bands of floxed *Fam20c* allele (Fig. 1B). Since the exon 6 to 9 were flanked by *loxp* sites in the conditional *Fam20c* allele, RT-PCR with the 1st set primers (amplifying exon 5 to 8) only gave the product for DM *Fam20c*$^{f/f}$, but nothing for DM *Fam20c*^{KO} (Fig. 1C). In contrast, the 2nd set primers (amplifying exon 5 to 11) amplified an 820-bp band from the transcriptome of DM *Fam20c*$^{f/f}$, and a truncated transcript from DM *Fam20c*^{KO} (Fig. 1C). These results indicated an accomplished transformation of the floxed *Fam20c* allele into the recombined null allele in DM *Fam20c*^{KO}, in which a truncated *Fam20c* transcript lacking kinse domain was produced.

3.2. Fam20c deficiency decreased proliferation and mobility of dental mesenchymal cells

Compared with DM *Fam20c*$^{f/f}$, DM *Fam20c*^{KO} exhibited no difference in morphology under the bright field microscope (Fig. 2A). However, the ratio of BrdU positive cells to total cells declined from 52.84% in DM *Fam20c*$^{f/f}$ to the almost 40% in DM *Fam20c*^{KO} ($p<0.05$; Fig. 2B and C). Compared with DM *Fam20c*$^{f/f}$ which covered almost the entire scratches in 24 hours, only a few cells of DM *Fam20c*^{KO} migrated into the scratches in the same period (Fig. 3A). Similarly, the colony diameters from DM *Fam20c*$^{f/f}$ suspension were noticeably larger than those from the suspension containing equal amounts of DM *Fam20c*^{KO} cells (Fig.3B and C).

3.3. Abrogation of Fam20c changed gene expression of dental mesenchymal cells

The gene expression profile was quantitatively compared between *DM Fam20c*^{KO} and *DM Fam20c*$^{f/f}$. The transcriptions of *Atf4* and *Dlx3* in DM *Fam20c*^{KO} were comparable to DM *Fam20c*$^{f/f}$, while the expression of *Runx2* and *Osx/Sp7* were down-regulated (Fig. 4A). The transcriptions of *Dmp1*, *Dspp*, *Bsp*, *Mepe* and *Opn/Spp1* were all dramatically reduced in the DM *Fam20c*^{KO} (Fig. 4B). The mRNA amounts of *Alpl* and *Col1a1* were also decreased by *Fam20c* abrogation, whereas the decreases of *Ocn/Bglap* and *Osn/Sparc* were

insignificant (Fig. 4C). In the analyzed genes, *Fgf23* was the only one giving an elevated transcription in *DM Fam20c*^{KO} (Fig. 4C). Coinciding with the altered transcription in DM *Fam20c*^{KO}, the immunocytochemistry staining of SIBLINGs and the western blot of FGF23 in DM *Fam20c*$^{f/f}$ became weaker and stronger, respectively (Fig. 5A and B). These results suggested that *Fam20c* deficiency could affect gene expression in a cell autonomous manner.

3.4. Loss of Fam20c disabled the mineralization of dental mesenchymal cells

The ALPL activity of DM *Fam20c*^{KO} was still remarkably weaker than that of DM *Fam20c*$^{f/f}$ (Fig. 6A), which was consistent with the reduced transcription of *Alpl*. The formation of mineralized nodules was applied to evaluate the capability of mineralization. As the reduced ALPL activity predicted, after two weeks of the culture in osteogenic medium, DM *Fam20c*^{KO} produced fewer mineralized nodules than DM *Fam20c*$^{f/f}$ did (Fig. 6B). By supplement of non-collagenous proteins extracted from the rat dentin, the mineralization of DM *Fam20c*$^{f/f}$ was enhanced (Fig. 6C). Surprisingly, the supplement failed to rescue the declined mineralization in DM *Fam20c*^{KO} (Fig. 6D). Therefore, *Fam20c* was suggested to affect mineralization by the means more than phosphorus homeostasis.

3.5. Attenuated BMP signaling in Fam20c deficient dental mesenchymal cells

To further explore the role of *Fam20c* in cell behaviors, BMP signaling pathway was analyzed through western blots. Both the total (including phosphorylated and non-phosphorylated) Smad1/5/8 (pan-Smad1/5/8) and phosphorylated Smad1/5/8 (p-Smad1/5/8) in DM *Fam20c*^{KO} exhibited the weaker bands compared with DM *Fam20c*$^{f/f}$, though the ratios of phosphorylated to total Smad1/5/8 (p/pan-Smad1/5/8) gave no significant change (Fig.7A). In DM *Fam20c*^{KO}, the phosphorylated p-38 was comparable to DM *Fam20c*$^{f/f}$, but the ratio of p/pan-38 reduced dramatically because of the greatly increased pan-p-38 (Fig. 7B). Compared with DM *Fam20c*$^{f/f}$, the increased pan-Erk and decreased p-Erk made the ratio of p/pan-Erk reduced significantly (Fig.7C). Taken together, the BMP/Smad4 and BMP/Erk signaling in dental mesenchymal cells were attenuated by the deficiency of *Fam20c*. However, the Q-PCR revealed that the relative transcriptions of *Bmp2* and *Bmp7* in DM *Fam20c*^{KO} were higher than those in DM *Fam20c*$^{f/f}$ (Fig.7E). In contrast, the relative expression of *Bmp4* is comparable between DM *Fam20c*^{KO} and DM *Fam20c*$^{f/f}$ (Fig.7E). These results suggested that the attenuated BMP signaling in *Fam20c*-deficient dental mesenchymal cells may not result from the reduced expression of *Bmp* genes, but from the reduced phosphorylation of BMP ligands.

4. Discussion

Mutations of *FAM20C* compromise the mineralization of hard tissues in human and mouse [4,5,6,7,9,12]. Putatively, the compromised mineralization is attributed to the reduced serum phosphorus concentration that results from the decreased phosphorus reabsorption in kidney caused by elevated FGF23 [6]. However, specific deletion of *Fam20c* from epithelial tissues impairs the mineralization of enamel without elevating *Fgf23* expression, which suggests that *Fam20c* can regulate mineralization in the manner more than phosphorus homeostasis [9]. The latest discovery supports this hypothesis because FAM20C is predicted to phosphorylate the majority of secreted phosphoproteins, which covers a variety of biological

processes [10]. The DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO} were exploited in this study to examine if the cell behaviors and signaling pathways in the case of *Fam20c* deficiency would be affected the mechanism by other than phosphorus metabolism. Taking advantage of the *in vitro* model, the proliferation, migration and mineralization were significantly impaired when *Fam20c* was abrogated from the immortalized dental mesenchymal cells. Moreover, the BMP signaling pathways were also attenuated along with the deletion of *Fam20c*, implying that BMP ligands are the substrates of FAM20C and the phosphorylation of BMPs is critical for their activities.

Recent study reported that in the case of breast cancer cells, removal of *Fam20c* by CRISPR/Cas9 system reduced the metastasis by suppressing the phosphorylation of insulin-like growth factor binding protein-7 [10]. Our study indicates that *Fam20c* also exerts a regulation on migration of the non-tumor derived cell line. Coinciding with the *in vivo* phenotype, the mineralization of DM *Fam20c*^{KO} is severely disabled. The poor mineralization of DM *Fam20c*^{KO} can be attributed to the remarkably reduced expression of SIBLINGs, *Col1a1* and *Alpl*. The failure of mineralization rescue by the supplement of non-collagenous dentin extracts may result from the dramatic reduction of ALPL activity, which leads to the accumulation of pyro-phosphate on hydroxyapatite (HA) surface and inhibits the growth of HA crystals [16]. Since the *in vivo* ALPL activity in *Fam20c* knock-out mouse is increased, further investigation is required to clarify that *Fam20c* deficiency primarily results in hypo- or hyper-phosphatase. In contrast, the decreased *Runx2*, *Osx/Sp7*, *Dspp* and *Dmp1* expression as well as the increased *Fgf23* expression in the DM *Fam20c*^{KO} are consistent with the changes in *Fam20c* deficient mice. This agreement suggests that the regulation of *Fam20c* on the transcription of these genes is accomplished through a cell autonomous manner and independent of the systemic phosphorus homeostasis.

Since FAM20C phosphorylates the Ser residues in the Ser-X-Glu motif, which can be detected in BMP2, 4 and 7, the attenuated BMP signaling pathways in DM *Fam20c*^{KO} implies that the phosphorylation by FAM20C is critical for the activity of BMP ligands. Because BMP signaling pathways also play critical roles in dentinogenesis and dentin mineralization [17,18,19,20], the altered gene expression and disabled mineralization of DM *Fam20c*^{KO} may result from the attenuated BMP signaling caused by the insufficient phosphorylation of BMP ligands. Besides, the declined BMP signaling pathway may also result from the altered gene expression in DM *Fam20c*^{KO}. To date, there is no report on the phosphorylation of BMP ligands. Therefore, our following study will strike to provide the direct evidence for the phosphorylation of BMPs by FAM20C and if the phosphorylation is indeed essential for BMP activity.

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Highlights

- Deficiency of *Fam20c* decreases the proliferation, mobility and mineralization of mouse immortalized dental mesenchymal cells, indicating the primary effects of loss of *Fam20c* in mouse tooth.
- Abrogation of *Fam20c* not only decreased the expression of extracellular matrix genes, but also the genes related to the differentiation of dental mesenchymal cells, suggesting that Fam20c plays multiple roles in the development of dental mesenchymal cells.
- As the kinase phorsphorylating secreted growth factors, *Fam20c*-deficient dental mesenchyaml cells exhibited the declined canonical and non-canonical BMP signaling pathways, implying that the phosphorylation of BMP ligands is essential for normal BMP activity.

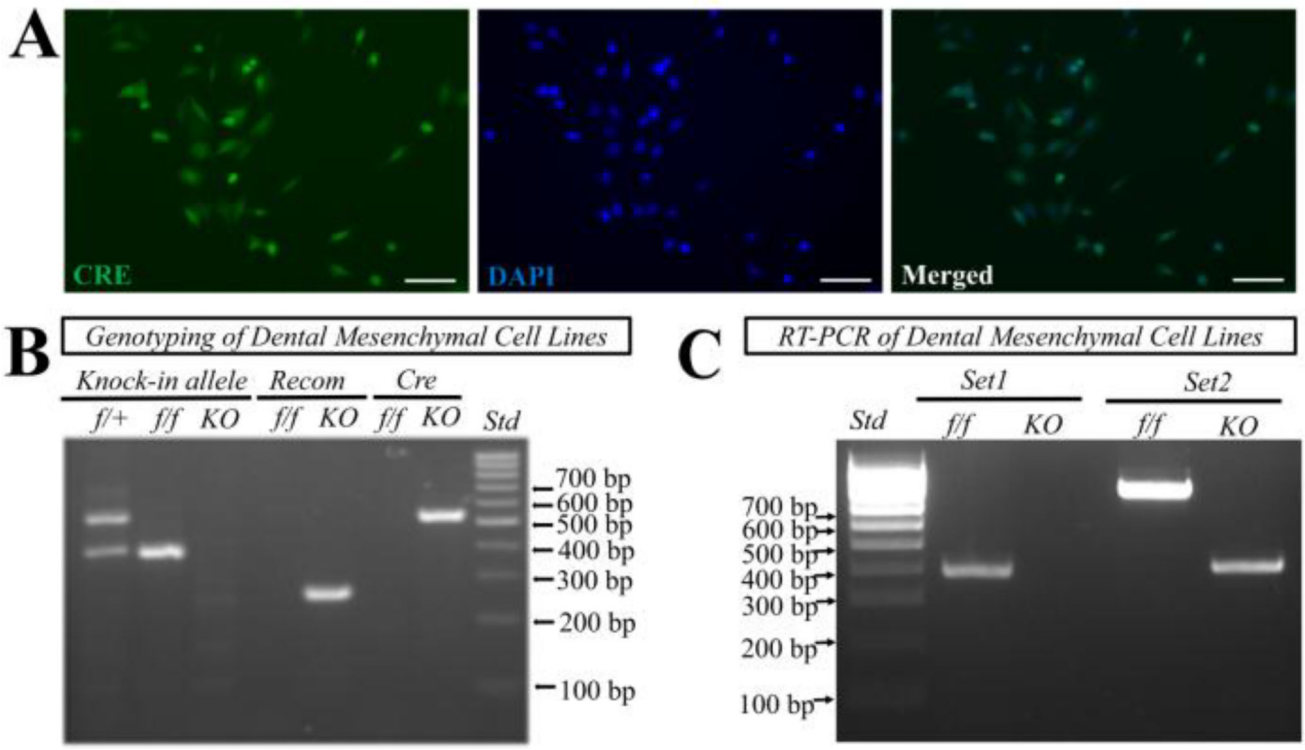

Fig. 1. Generation and identification of DM *Fam20c*^{KO}

(A) After infected by *CMV-Cre-IRES-EGFP* lentivirus, the immortalized DM *Fam20c*$^{f/f}$ formed single cell-derived clone and was passaged for 10 generations; All cells from the selected colony were positive for DAPI (blue) and EGFP (green), indicating that all the cells expressed the *Cre* transgene. **(B) Genotyping PCR for *Cre* transgen, the floxed and recombined *Fam20c* allele.** The genotyping for DM *Fam20c*$^{f/f}$ (*f/f*) and DM *Fam20c*^{KO} (*KO*) with specific primers for the floxed *Fam20c* allele (Knock-in allele), recombined *Fam20c* allele (Recom) and *Cre* transgene. The tail lystae from an identified *Fam20c*$^{f/+}$ mouse was employed as a positive control (*f/+*) with the primers for the floxed *Fam20c* allele (Knock-in allele). **(C) RT-PCR for the floxed and recombined *Fam20c* allele.** The Set 1 primers amplifying exon 5–8 produced a 388-bp band in DM *Fam20c*$^{f/f}$ (*f/f*), and no band in DM *Fam20c*^{KO} (*KO*), while the Set 2 primers for exon 5–11 gave an 820-bp band in DM *Fam20c*$^{f/f}$ (*f/f*), and a 421-bp band in DM *Fam20c*^{KO} (*KO*). (Std: DNA ladder; Scale bar:100 um)

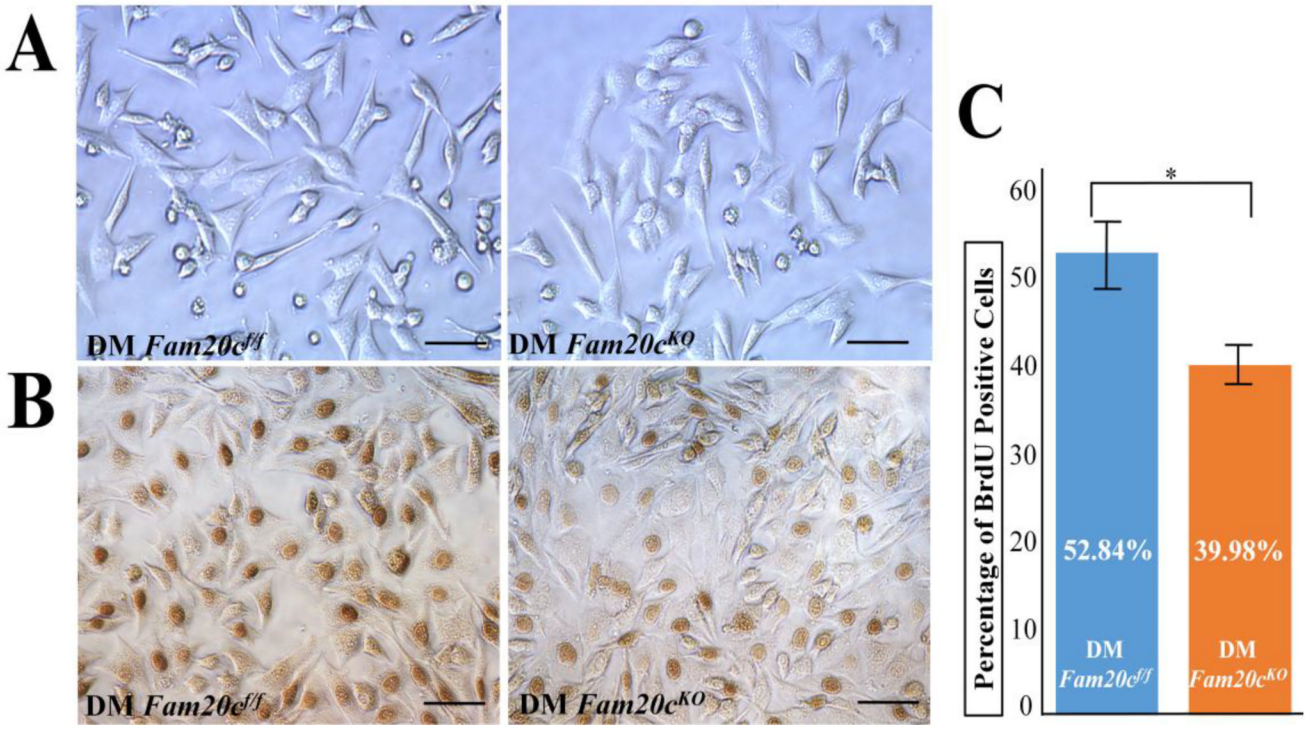

Fig. 2. Cell morphology and proliferation of the DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}

(A) The micrographs of DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO} under the Olympus reverse phase-contrast microscope. **(B)** BrdU-labeling images of DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO} were taken under the Olympus reverse phase-contrast microscope. **(C)** The statistical assay showed that the difference in the proliferation rate of DM *Fam20c*$^{f/f}$ (Mean=52.84%, SD=4.347%) was significantly higher than DM *Fam20c*^{KO} (Mean=39.98%, SD=5.423%) (p=0.0107).

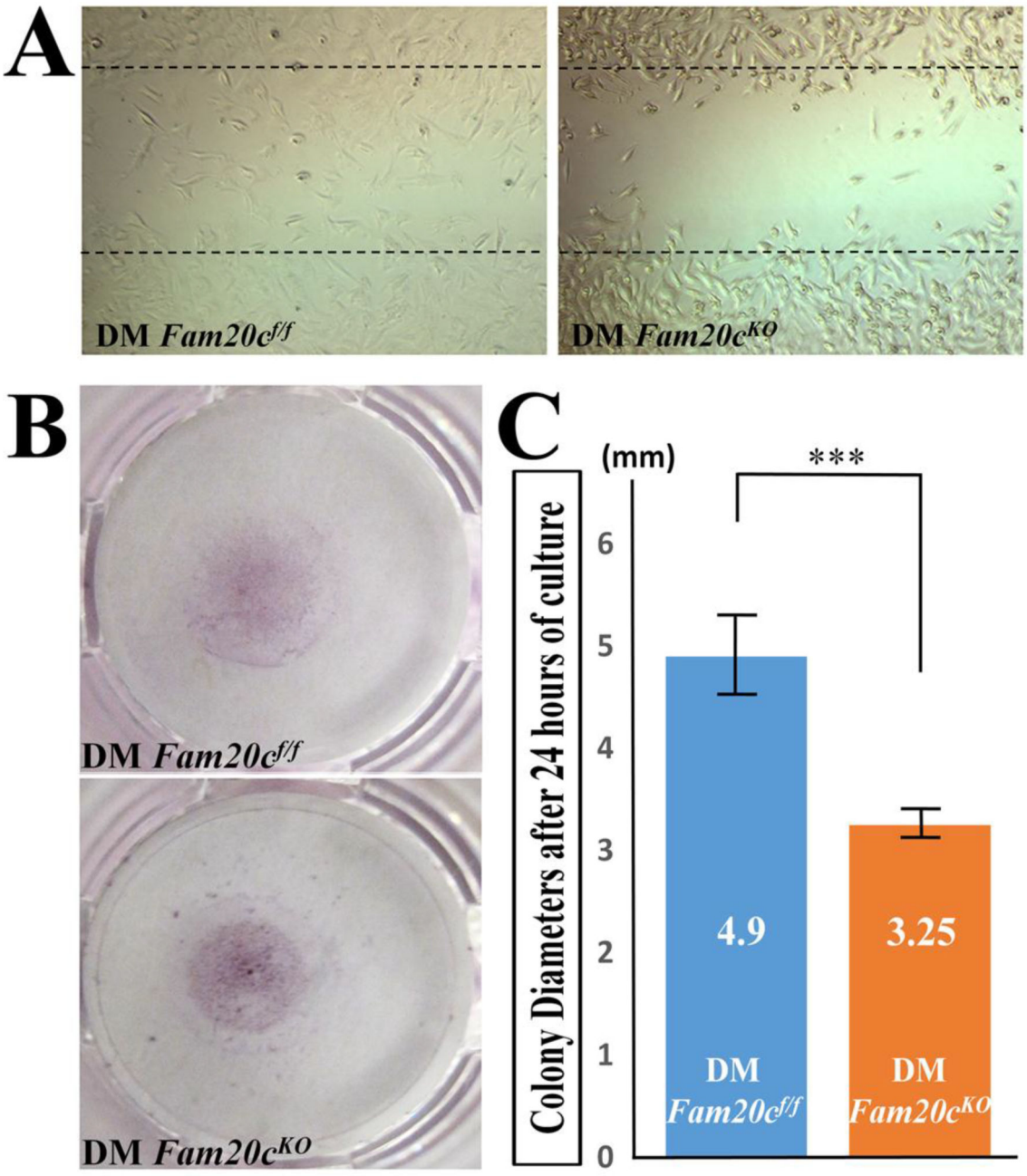

Fig. 3. Cell migration of the DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}

(A) Many DM *Fam20c*$^{f/f}$ migrated into the scratches after 24 hours of culture; In contrast, there were only a few DM *Fam20c*^{KO} in the scratches. **(B–C)** After 24 hours of culture, the average diameter of DM *Fam20c*$^{f/f}$ colonies from the suspension containing 1×10^7 of cells was 5.025 mm (Mean=5.025mm, SD=0.359mm), which was significantly larger than those of DM *Fam20c*^{KO} (Mean=3.98mm, SD=0.37mm) (***: $p<0.001$). Scale bar in (A) and (B) equals to100 μm).

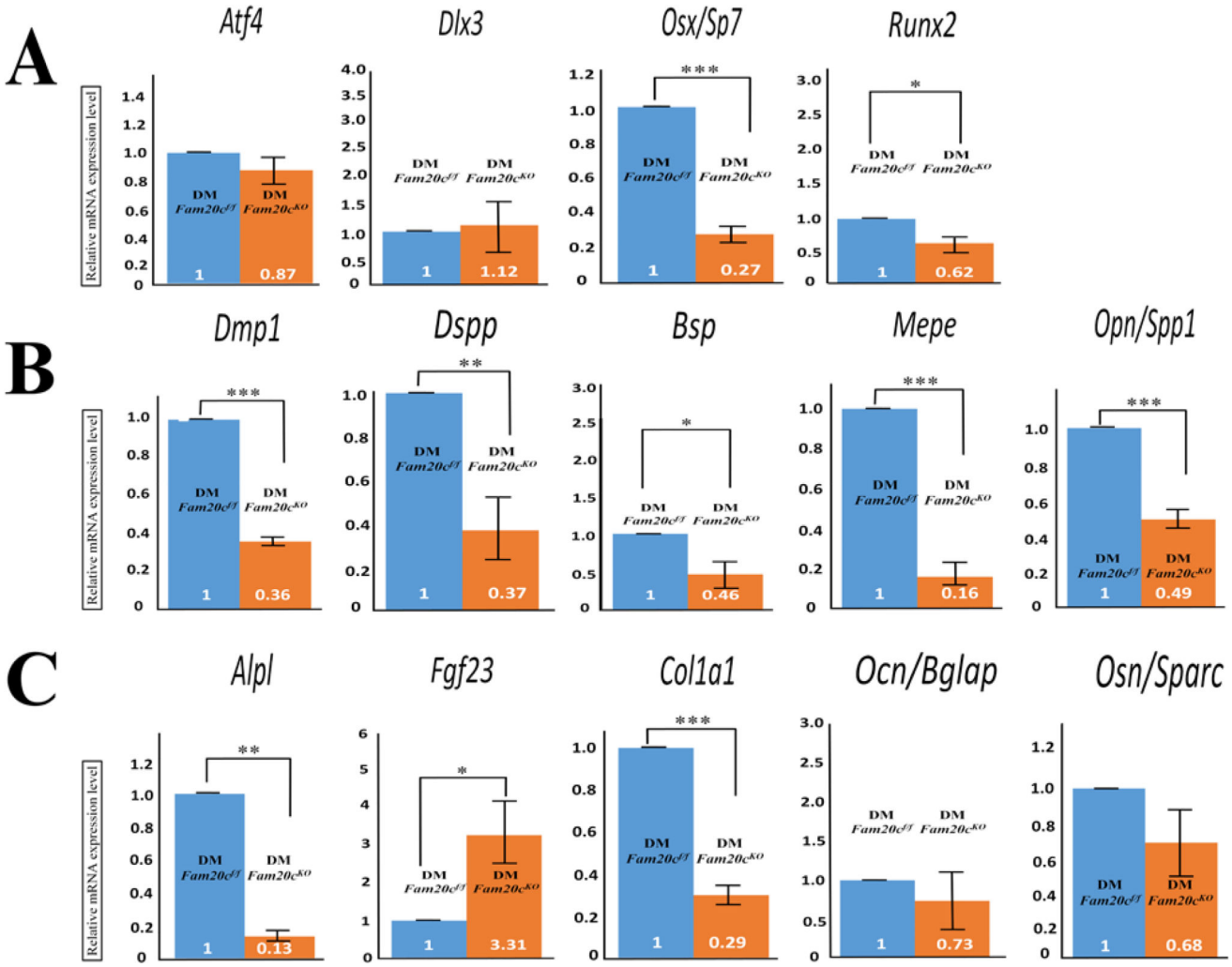

Fig. 4. Q-PCR for the transcription of bone-related genes in DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}

(A) The comparison of the relative transcription of *Atf4*, *Dlx3*, *Osx/Sp7* and *Runx2* between DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}. **(B)** The comparison of the relative transcription of *Dmp1*, *Dspp*, *Bsp*, *Mepe* and *Opn/Spp1* between DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}. **(C)** The comparison of the relative transcription of *Alpl*, *Fgf23*, *Col1a1*, *Ocn/Bglap* and *Osn/Sparc* between DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}. (*: p<0.05; **:p<0.01; ***: p<0.001.)

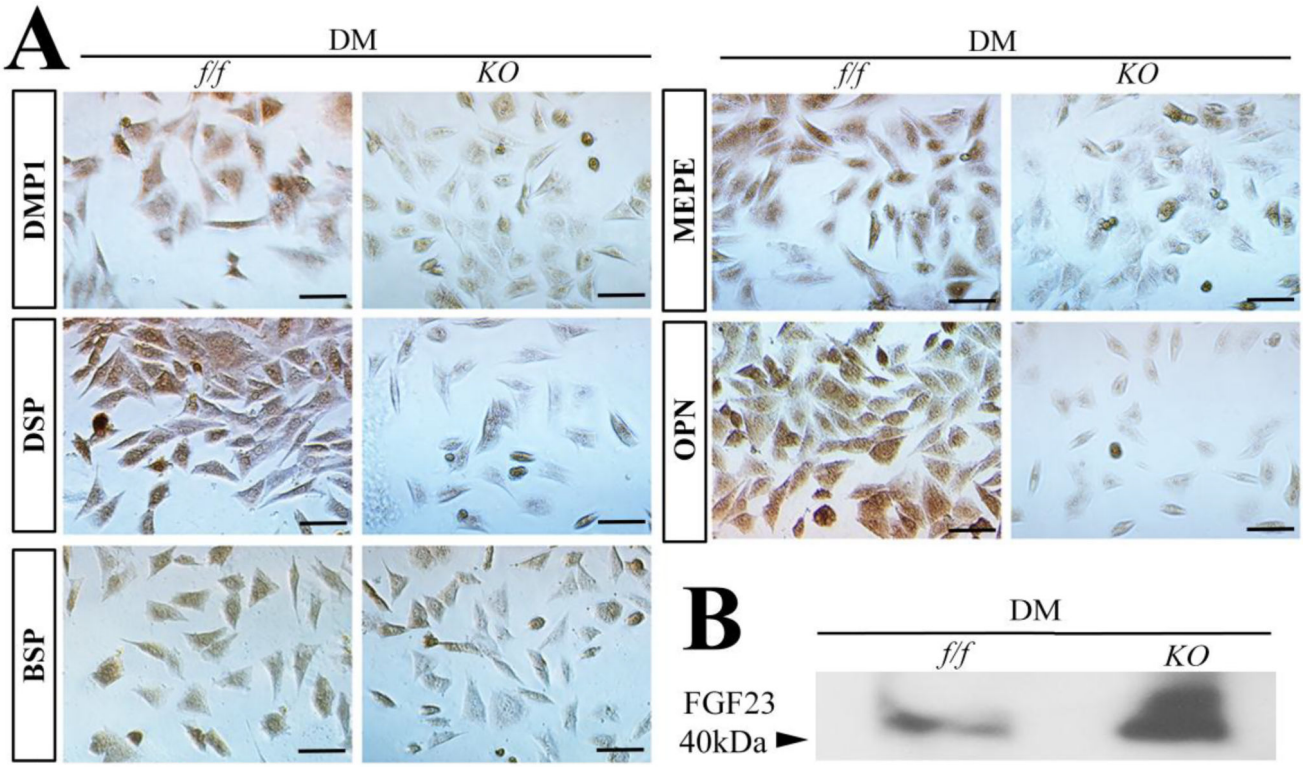

Fig. 5. Immunocytochemistry and Western blot for the bone-related genes in DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}

(A) Immunocytochemistry with the antibodies against DMP1, DSP, BSP, MEPE and OPN/SPP1 for their expression in DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}. **(B)** Western blotting with anti-FGF23 antibody for FGF23 concentration in the medium of DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}. (Scale bar: 100 um)

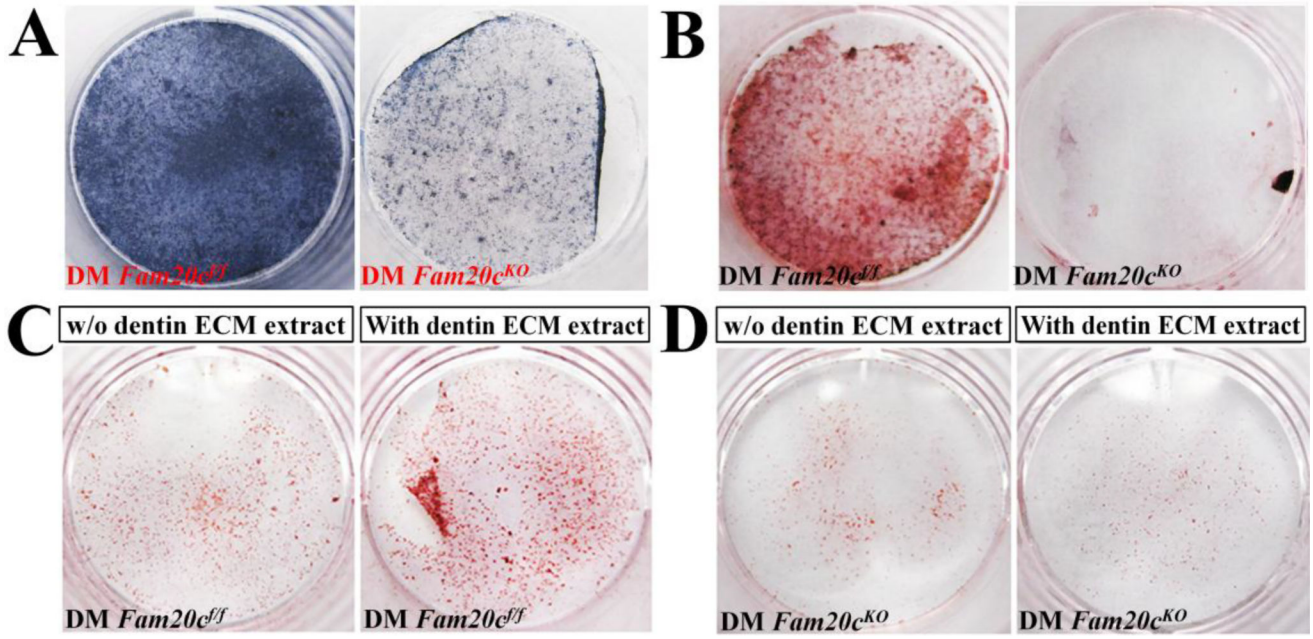

Fig. 6. Induced mineralized nodules in DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}

(A) The ALPL activity was assessed by *in situ* histochemistry assay. **(B)** After 3 weeks of osteogenic induction, the formation of mineralized nodules in DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO} was evaluated by Alizarin red staining. **(C)** After 1 week of osteogenic induction, the non-collagenous extract from rat dentin enhanced the formation of mineralized nodules in DM *Fam20c*$^{f/f}$. **(D)** The addition of non-collagenous extract from rat dentin failed to improve the formation of mineralized nodules in DM *Fam20c*^{KO}.

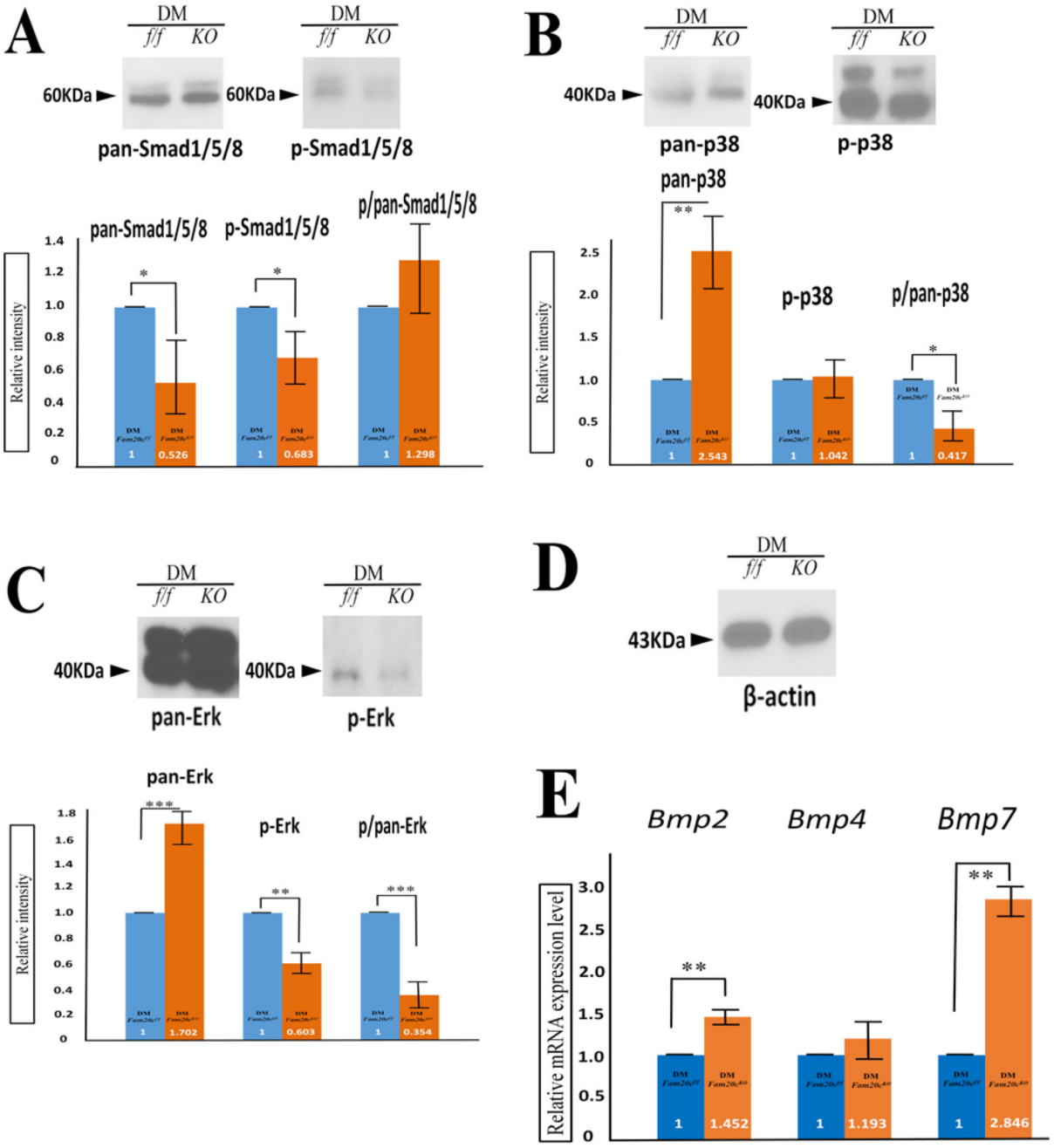

Fig. 7. Western blots for BMP signaling pathways in DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}

(A) Western blotting with antibodies against pan-Smad1/5/8 and p-Smad1/5/8 for canonical BMP signaling in DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}. **(B)** Western blotting with antibodies against pan-p38 and p-p38 for non-canonical BMP/p38 signaling in DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}. **(C)** Western blotting with antibodies against pan-Erk and p-Erk for non-canonical BMP/Erk signaling in DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}. **(D)** Western blot with antibody against β-actin was used as the internal control to normalize the amount of DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}. **(E)** Q-PCR revealed the relative expression of Bmp2, Bmp4

and Bmp7 between DM *Fam20c*$^{f/f}$ and DM *Fam20c*^{KO}. (*: $p<0.05$; **:$p<0.01$; ***: $p<0.001$.)