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Immortalized Mouse Floxed *Fam20c* Dental Papillar Mesenchymal and Osteoblast Cell Lines Retain Their Primary Characteristics

Chao Liu[1,*], **Xiaofang Wang**[1], **Hua Zhang**[1], **Xiaohua Xie**[1,2], **Peihong Liu**[1,3], **Ying Liu**[1], **Priyam H. Jani**[1], **Yongbo Lu**[1], **Shuo Chen**[4], and **Chunlin Qin**[1,*]

[1]Department of Biomedical Sciences and Center for Craniofacial Research and Diagnosis, Texas A&M University Baylor College of Dentistry, Dallas, Texas

[2]Department of Endodontics, The Institute of Hard Tissue Development and Regeneration, the 2nd Affiliated Hospital of Harbin Medical University, Harbin, China

[3]Department of Periodontics, Harbin Medical University School of Stomatology, Harbin, China

[4]Department of Pediatric Dentistry, Dental School, The University of Texas Health Science Center at San Antonio, San Antonio, Texas

Abstract

Fam20c is essential for the normal mineralization of dentin and bone. The generation of odontoblast and osteoblast cell lines carrying floxed *Fam20c* allele can offer valuable tools for the study of the roles of *Fam20c* in the mineralization of dentin and bone. The limited capability of the primary odontoblasts and osteoblasts to proliferate necessitates the development of odontoblast and osteoblast cell lines serving as substitutes for the study of differentiation and mineralization of the odontoblasts and osteoblasts. In this study, we established and characterized immortalized mouse floxed *Fam20c* dental papilla mesenchymal and osteoblast cell lines. The isolated primary mouse floxed *Fam20c* dental papilla mesenchymal cells and osteoblasts were immortalized by the infection of lentivirus containing Simian Virus 40 T-antigen (SV40 T-Ag). The immortalization of floxed *Fam20c* dental papilla mesenchymal cells and osteoblasts was verified by the long-term passages and genomic integration of SV40 T-Ag. The immortalized floxed *Fam20c* dental papilla mesenchymal and osteoblast cell lines not only proliferated at a high rate and retained the morphology of their primary counterparts, but also preserved the dentin and bone specific gene expression as the primary dental papilla mesenchymal cells and osteoblasts did. Consistently, the capability of the primary floxed *Fam20c* dental papilla mesenchymal cells and osteoblasts to mineralize was also inherited by the immortalized dental papilla mesenchymal and osteoblast cell lines. Thus, we have successfully generated the immortalized mouse floxed *Fam20c* dental papilla mesenchymal and osteoblast cell lines.

Dentin and bone are two mineralized tissues that resemble each other in composition and mechanisms of formation. Odontoblasts and osteoblasts are the major cells necessary for the

[*]Correspondence to: Chunlin Qin, Department of Biomedical Sciences and Center for Craniofacial Research and Diagnosis, Texas A&M University Baylor College of Dentistry, 3302 Gaston Avenue Dallas, TX 75246. cqin@bcd.tamhsc.edu.

morphogenesis, maturation, and mineralization of dentin and bone, respectively. Odontoblasts and osteoblasts synthesize a series of extracellular matrix (ECM) proteins, that include type I collagen and SIBLINGs [Small Integrin-Binding Ligand N-linked Glycoproteins consisting of dentin sialophosphoprotein (DSPP), dental matrix protein 1 (DMP1), osteopontin (OPN), bone sialoprotein (BSP), and matrix extracellular phosphoglycoprotein (MEPE)] (Qin et al., 2004; Chen et al., 2008). These ECM molecules undergo post-translational modifications essential for the formation of dentin and bone. For example, disorders in the post-translational hydroxylation of type I pro-collagen resulting from the mutations of prolyl-3-hydroxylase-1 (P3H1, encoded by LEPRE1 gene) and/or cartilage-associated protein (CRTAP) cause autosomal recessive osteogenesis imperfecta (Vranka et al., 2004; Barnes et al., 2006; Morello et al., 2006). As one of the most important post-translational modifications, phosphorylation of SIBLINGs is essential for the normal mineralization of dentin and bone (Razzouk et al., 2002; Qin et al., 2004; Tartaix et al., 2004; Gericke et al., 2005).

FAM20C is a kinase that phosphorylates a series of secretory proteins including the SIBLINGs (Tagliabracci et al., 2012). FAM20C belongs to the “family with sequence similarity 20 (FAM20)” and is highly expressed in the differentiating and matured odontoblasts and osteoblasts (Wang et al., 2010). Deficiencies in human FAM20C cause Raine Syndrome manifesting as lethal osteosclerotic bone dysplasia or hypophosphatemic Rickets (Simpson et al., 2007; Simpson et al., 2009). Inactivation of *Fam20c* in mice results in mineralization disorders in dentin and bone. The defective mineralization in *Fam20c*-deficient mice is accompanied by altered phosphorus metabolism, reduced DMP1 expression and elevated serum FGF23 level (Wang et al., 2012a). A more recent study suggests that FAM20C phosphorylates Ser^{180} of FGF23, which prevents the O-glycosylation on Thr^{178} and allows the proteolysis of FGF23 (Tagliabracci et al., 2014). However, the understanding of the biochemical mechanisms by which FAM20C phosphorylates its substrates is still limited, partially due to the lack of valuable tools such as *Fam20c*-deficient cell lines. Mouse cell lines without the *Fam20c* allele derived from cells that form the presumptive mineralized tissues are the ideal tools to study the kinase roles of normal and mutant FAM20C.

In the present study, we developed and characterized the dental papilla mesenchymal (which can differentiate into odontoblasts) and osteoblast cell lines carrying floxed *Fam20c* allele. The primary dental papilla mesenchymal cells and osteoblasts isolated from $Fam20c^{f/f}$ mice were transformed into immortal cell lines by SV40T-Ag transfection (Wu et al., 2010;Wu et al., 2011). These cell lines displayed a stable capability for expansion as well as an identical gene expression profile to their primary cells. Using the benefit of the floxed *Fam20c* allele (Wang et al., 2012a), exon 6–9 can be excised by *Cre* recombinase to generate cell lines with the null *Fam20c* allele, which can be used for further experiments in the studies on FAM20C.

Materials and Methods

Genotyping of mice carrying floxed *Fam20c* allele (*Fam20c*$^{f/f}$ mouse)

The floxed *Fam20c* allele was generated by inserting Cre recombinase recognition sites (*loxp*) upstream to exon 6 and downstream to exon 9 into the mouse *Fam20c* gene. The *Fam20c*$^{f/f}$ mice were genotyped by polymerase chain reaction (PCR) assay as described previously. The floxed *Fam20c* allele produced a band of 400 bp, which was 100 bp shorter than that of the WT *Fam20c* allele (Wang et al., 2012a). The protocols for mouse utilization were approved by the Institutional Animal Care and Use Committee of Baylor College of Dentistry of Texas A&M University Health Sciences Center, TX, USA.

Primary dental papilla mesenchymal cells and calvarial osteoblasts from *Fam20c*$^{f/f}$ mouse

The first molars of postnatal 4-day *Fam20c*$^{f/f}$ mice were collected for the isolation of dental papilla mesenchyme, which was digested in 3 mg/ml collagenase type I and 4 mg/ml of dispase for 30 min at 37°C. The digested dental mesenchyme was dispersed in a single-cell suspension by mechanical aspiration up and down for further ex vivo expansion. The calvarial bones of postnatal 1 day *Fam20c*$^{f/f}$ mice were isolated and cut into small 0.1 cm^3 pieces. These pieces were attached to petri dishes for the emigration of osteoblasts from the calvarial bones. Both the primary dental papilla mesenchymal cells and calvarial osteoblasts were cultured with αMEM supplemented with 10% fetal bovine serum, 100 unit/ml pencillin and 100 μg/ml streptomycin at 37°C in humidified air containing 5% CO_2. The medium was refreshed every 3 days until the cells reached confluence.

Lentivirus infection and selection of immortalized cells

The primary cells of passage 3 were infected by lentivirus containing SV40 T-Ag following the manufacturer's protocol (Applied Biological Materials, In., Richmond, BC). After infection, the primary cells were re-plated at a low density to get separated clones from a single cell. Twelve clones from infected dental papilla mesenchymal cells and calvarial osteoblasts were selected for further examination and passages, respectively. After 30 passages over 6 months, all the selected clones were expanded into cell lines. One of the 12 clones was selected randomly from the immortalized dental papilla mesenchymal and osteoblast cell lines for the further analysis.

Cell morphology and proliferation

The primary cells of passage 3 and the immortal cell lines after passage 40 were selected for cell morphology and proliferation evaluation. The morphologies of the primary cells and immortalized cell lines were observed by a light inverted microscope. 5-bromo-2′-deoxyuridine (BrdU) was added to the medium in the 30 μM concentration to label the nuclei of the proliferating cells (Life technologies, Inc., Grand Island, NY). Four hours after the incorporation of BrdU, testing for BrdU positive nuclei in the primary cells and immortalized cell lines was performed with the BrdU assay Kit (Life technologies, Inc.).

RNA extraction and reverse transcription-PCR (RT-PCR)

Total RNA was extracted from passage 3 primary cells and passage 40 cell lines with RNA extraction Kit and total mRNA was reverse transcribed into cDNA with the RT-PCR Kit (Life Technologies, Inc.). The protocol for the amplification of Alp (Alkaline phosphatase), Atf4 (Activating transcription factor 4), Bsp (Bone sialoprotein), Col1a1 (Alpha 1 collagen type 1), Dlx3 (Distal-less homeobox 3), Dmp1(Dental matrix protein 1), Dspp (Dentin sialophosphoprotein), Gapdh (Glyceraldehyde-3-phosphate dehydrogenase), Mepe (Matrix extracellular phosphoglycoprotein), Ocn (Osteocalcin), Opn (Osteopontin), Osn (Osteonectin), Osx (Osterix), Runx2 (Runt-related transcription factor 2), and SV40 were reported previously (Wu et al., 2010; Wu et al., 2011). The cDNA of FAM20C transcripts was amplified with the primers (Forward: 5′-TGCGGAGATCGCTGCCTTCC-3′; Reverse: 5′-GCCACTGTCGTAGG GTGG CA-3′) at the annealing temperature of 55°C; the size of the PCR product was expected to be 450 bp.

Immunohistochemistry

For the detection of SV40 T-Ag and the dentin or bone-specific proteins, polyclonal (rabbit) antibodies against ATF4, DLX3, MEPE, OSN, OCN (Santa Cruz Biotechnology, Inc., Snata Cruz, CA), OPN and OSX (Abcam, Cambridge, MA), and the monoclonal (mouse) antibodies against SV40 T-Ag, RUNX2 and COL1α1 were applied (Abcam). The antibodies against BSP (a gift from Dr. Larry Fisher, National Institue of Dental and Craniofacial Research), DMP1 and DSP were applied as previously described (Baba et al., 2004; Huang et al., 2008; Liu et al., 2014). The secondary antibodies were goat anti-rabbit or goat anti-mouse antibodies conjugated with Biotin (Vector Laboratories, Inc., Burlingame, CA). In the negative control groups, the primary antibodies were not used, while the secondary antibodies were the same as in the experimental groups.

Alkaline phosphatase (ALP) assay and the mineralization assay

For the ALP activity assay, the primary cells and immortalized cell lines were cultured in the mineralization-inducing medium for a week (α-MEM supplemented with 10% fetal bovine serum, 100 unit/ml pencillin,100 μg/ml streptomycin, 50 μg/ml ascorbic acid and 10 mM sodium β-glycerophosphate). The in situ ALP staining was performed following the protocol described in the assay kit (Bio-Rad, Hercules, CA). For the mineralization assay, the primary cells and immortalized cell lines were cultured for 2–3 weeks in the mineralization-inducing medium. The mineralized cell culture was fixed with 4% PFA for 10 min, rinsed with phosphate buffered saline (PBS) and stained with Alizarin Red S (Sigma–Aldrich, St. Louis, MO).

Results

Immortalization of floxed *Fam20c* dental papilla mesenchymal cells and osteoblasts

The homozygous *Fam20c*$^{f/f}$ mice used for cell isolation were confirmed by PCR as previously described (Wang et al., 2012a). The primary mesenchymal cells from the dental papilla and osteoblasts from the calvarium were isolated and re-confirmed by genotyping for their homozygous floxed *Fam20c* allele (Fig. 1A). To immortalize the dental papilla

mesenchymal cells and osteoblasts, the lentivirus containing SV40 T-Ag gene was applied (Wu et al., 2010; Wu et al., 2011). After 10–14 days of the infection, colonies formed from the infected dental papilla mesenchymal cell and osteoblast culture. The colonies selected for the further 40 passages bypassed senescence without evidence of growth retardation in the following 5 months. One of the dental papilla mesenchymal cell lines and one of the osteoblast cell lines were selected for detailed characterization. Both the genotyping PCR with specific primers and immunohistochemistry with antibody against SV40 T-Ag demonstrated that SV40 T-Ag was detected only in the passaged dental papilla mesenchymal and osteoblast cell lines, but not in the primary cells (Fig. 1B and C).

Morphology and proliferation of the primary and immortalized dental papilla mesenchymal and osteoblast cell lines

Under the light-inverted microscope, the immortalized dental papilla mesenchymal cell lines after passage 40 exhibited a fibroblast-like morphology similar to that of the primary *Fam20c*$^{f/f}$ dental papilla mesenchymal cells (Fig. 2A and B). Similarly, both the primary *Fam20c*$^{f/f}$ calvarial osteoblasts and the immortalized osteoblasts showed the typical fibroblast shape (Fig. 2C and D). However, both the immortalized dental papilla mesenchymal cells and osteoblasts proliferated at a significantly higher rate compared with their primary counterparts in the BrdU-labelling assays (Fig. 2E–I).

Tissue-specific gene expression in the primary and immortalized cells

To examine if the immortalized cell lines have maintained their dentin-specific and bone-specific gene expression profile, RT-PCR assay was performed to detect the gene expression associated with the differentiation and mineralization of odontoblast and osteoblasts. The transcripts of *Alp, Fam20c, Bsp, Dmp1, Mepe, Dspp, Ocn, Opn, Osn, Col1*α*1, Runx2, Dlx3, Osx*, and *Atf4* were detected in the primary dental papilla mesenchymal cells as well as in the immortalized dental papilla mesenchymal cell line (Fig. 3A). The proteins encoded by these genes were also detected by immunohistochemistry in the primary and immortal dental papilla mesenchymal cells (Fig. 3B and C). Both RT-PCR and immunohistochemistry results consistently confirmed the expression of *Alp, Fam20c, Bsp, Dmp1, Mepe, Dspp, Ocn, Opn, Osn, Col1*α*1, Runx2, Dlx3, Osx*, and *Atf4* in the primary calvarial osteoblasts and immortalized osteoblasts (Fig. 3A–C). These data confirmed that the immortalized *Fam20c*$^{f/f}$ dental papilla mesenchymal and osteoblast cell lines have sustained their dentinogenic and osteogenic characteristics, respectively, even after a long term passage and culture.

Induced differentiation and mineralization of the immortalized *Fam20c*$^{f/f}$ dental papilla mesenchymal and osteoblast cell lines

To further confirm the dentinogenic and osteogenic characteristics in the transformed *Fam20c*$^{f/f}$ dental papilla mesenchymal cells and osteoblasts, the activity of ALP, the marker for both the odontoblast and osteoblast differentiation, was examined in the immortalized cell lines. After one week's culture in the mineralization-inducing medium, positive staining of ALP activity was found in the primary dental papilla mesenchymal cells, primary calvarial osteoblasts, the induced immortalized dental papilla mesenchymal and osteoblast cell lines (Fig. 4A). When the mineralization-inducing culture extended to 2 weeks, the

primary dental papilla mesenchymal cells, primary calvarial osteoblasts, the induced immortalized dental papilla mesenchymal and osteoblast cell lines formed mineralized nodules (Fig. 4B).

Discussion

Odontoblasts and osteoblasts play important roles in the formation, mineralization and repair of dentin and bone, respectively. However, due to the Hayflick limit (Hayflick and Moorhead, 1961), the cultured primary cells undergo senescence rapidly and are often unable to provide an adequate number of cells for analyses. Quite a few odontoblast and osteoblast cell lines have been developed from human and mouse, which are used to elucidate the mechanism of the formation and mineralization of dentin and bone under physiological and pathological condition (McKay et al., 1996; Hulley et al., 1998; MacDougall et al., 1998; Costa et al., 1999; Shea et al., 2000; He et al., 2004). Using the benefits of the SV40 T-antigen and the lentivirus vectors, the primary cells were reported to be transformed into immortal cell lines that still retained the original genotypic and phenotypic characteristics (Wu et al., 2010; Wu et al., 2011). The immortalized dental papilla mesenchymal and osteoblast cell lines transformed by SV40 T-antigen have been applied in the physiological and pathological research in the cell differentiation, mineralization, regeneration, gene expression regulation, and crosstalk of signaling pathways in dentin and bone (Galler et al., 2006; Iwata et al., 2007; Hoffman et al., 2010).

The *Fam20c*-deficient mice suffered from hypophosphataemic rickets which affected dentin and bone (Wang et al., 2012a, b). Several studies indicated that FAM20C regulates mineralization by phosphorylating the SIBLING proteins and FGF23 (Ishikawa et al., 2012; Tagliabracci et al., 2012; Tagliabracci et al., 2014). However, the exact mechanisms of how FAM20C acts in regulating dentinogenesis and osteogenesis are unclear, and there is a discrepancy between the osteosclerotic phenotype associated with some human *FAM20C* mutations and the rickets phenotype of *Fam20c*-deficient mice. By introducing the *Cre* recombinase, the $Fam20c^{f/f}$ dental papilla mesenchymal and osteoblast cell lines can be transformed into *Fam20c*-deficient dental papilla mesenchymal and osteoblast cell lines which can thus serve as a valuable tool to study the mechanisms of how FAM20C works in the phosphorylation of secretory proteins, cell differentiation and the potential crosstalk among different signaling pathways as well as in the elucidation of pathogenesis underlying the development of Raine syndrome. For example, by transfecting the constructs expressing *FAM20C* with different mutations identified in Raine syndrome patients into the *Fam20c-null* dental papilla mesenchymal and osteoblast cell lines, investigators can determine the effects of various mutations on the kinase activity of the different mutant FAM20C enzymes. To ensure that the information obtained from the immortalized cell lines is applicable to the primary cells, the genotypes and phenotypes of the primary cells must be maintained in the transformed (immortalized) cell lines. In our study, the primary and the immortalized dental papilla mesenchymal and osteoblasts exhibited similar morphologies. The strong ALP activity and capability of forming mineralized nodules were also retained in the SV40 T-antigen-positive dental papilla mesenchymal and osteoblast cell lines. The gene expression assay showed that both the immortalized dental papilla mesenchymal cells and the osteoblasts expressed osteonectin, osteocalcin, collagen Type I, and SIBLINGs, which are

the main components of the ECM molecules involved in the differentiation and mineralization of odontoblasts and osteoblasts. The accelerated proliferation rate in the transformed cell lines, which was also observed in other SV40 T-antigen transformed cell lines, is attributed to the promotion of SV40 T-antigen on the cell cycle as described previously (Porcu et al., 1992). Moreover, the transcription factors key to dentinogenesis and osteogenesis, including *Runx2, Osx, Atf4*, and *Dlx3,* were expressed in the immortalized dental papilla mesenchymal and osteoblasts as well as in their primary counterparts. Taken together, we concluded that the immortalized *Fam20c*$^{f/f}$ dental papilla mesenchymal and osteoblast cell lines have maintained the characteristics of the primary dental papilla mesenchymal cells and osteoblasts. We envision that these *Fam20c*$^{f/f}$ cell lines will serve as an excellent tool for the study of FAM20C function in dentinogenesis and osteogenesis.

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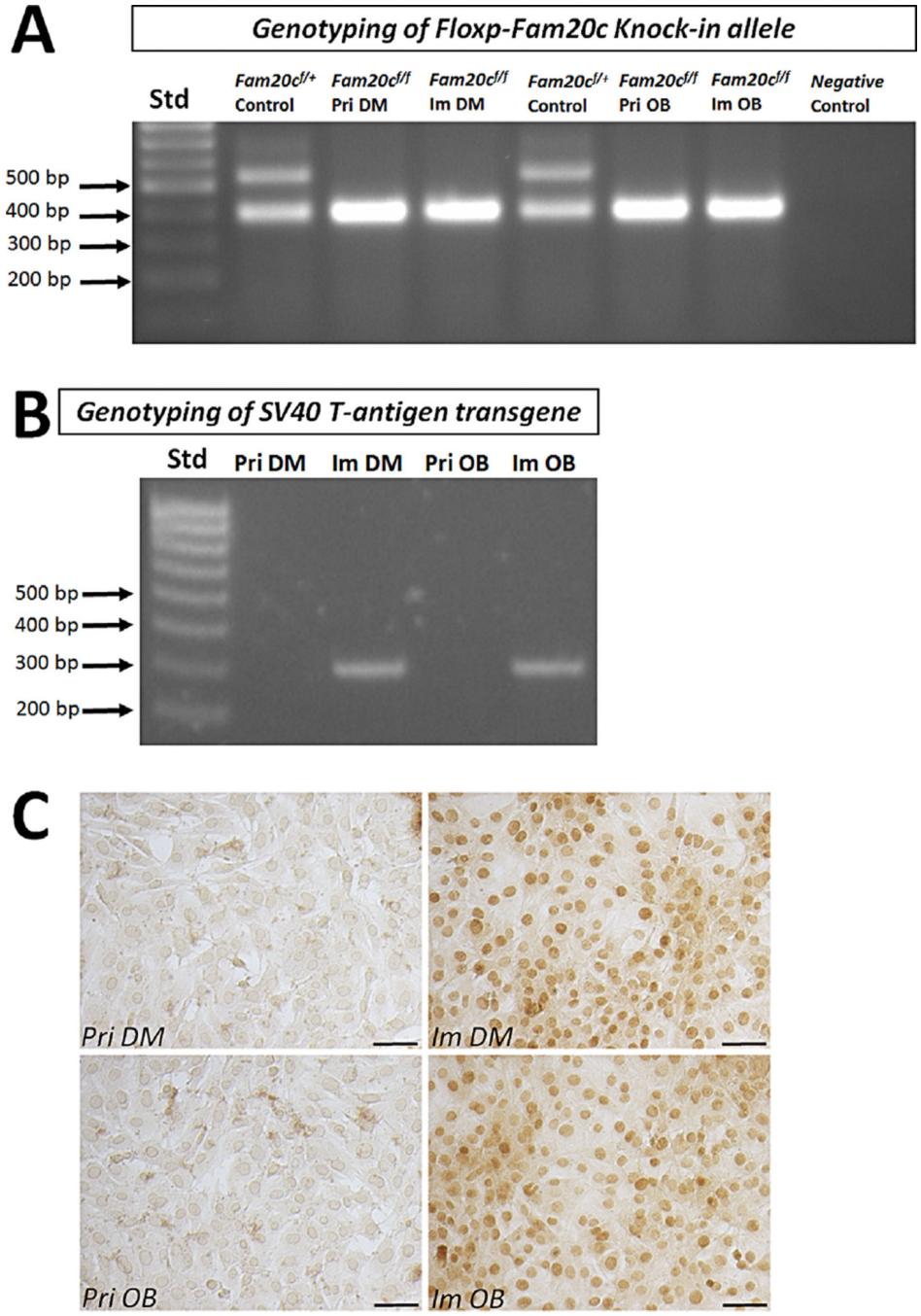

Fig. 1.

Identification of floxed *Fam20c* alleles and SV40 transformation. A: Genomic DNA isolated from *Fam20c*$^{f/+}$ control mouse (Lane 2 and 5), primary dental papilla (Lane 3, Pri DM) and calvarium (Lane 6, Pri OB) from *Fam20c*$^{f/f}$ mouse, and immortalized dental papilla mesenchymal cells (Lane 4, Im DM) and immortalized osteoblasts (Lane 7, Im OB) from the *Fam20c*$^{f/f}$ dental papilla mesenchyme and calvarium were amplified by PCR using primers specific for the floxed *Fam20c* alleles. (Lane 1 is DNA ladder labeled as Std; Lane 8 is negative control that contained only primers specific for the floxed *Fam20c* and no

genomic DNA). B: Genomic DNA extracted from primary dental papilla (Lane 2) and calvarium (Lane 4) of *Fam20c*$^{f/f}$ mouse, and immortalized dental papilla mesenchymal cells (Lane 3) and osteoblasts (Lane 5) were amplified by primers specific for *SV40 T-Ag*. (Lane 1 is DNA ladder labeled as Std). C: Immunohistochemical staining with the antibody against SV40 T-Ag. The immunostaining of SV40 T-Ag was only present in the nuclei of immortalized dental papilla mesenchymal and osteoblast cell lines, but absent in the nuclei of primary dental papilla mesenchymal cells and calvarial osteoblasts. (Pri DM, primary dental papilla mesenchymal cells; Pri OB, primary calvarial osteoblasts; Im DM, immortalized dental papilla mesenchymal cell line; Im OB, immortalized osteoblast cell line; Scale bar, 100 μm).

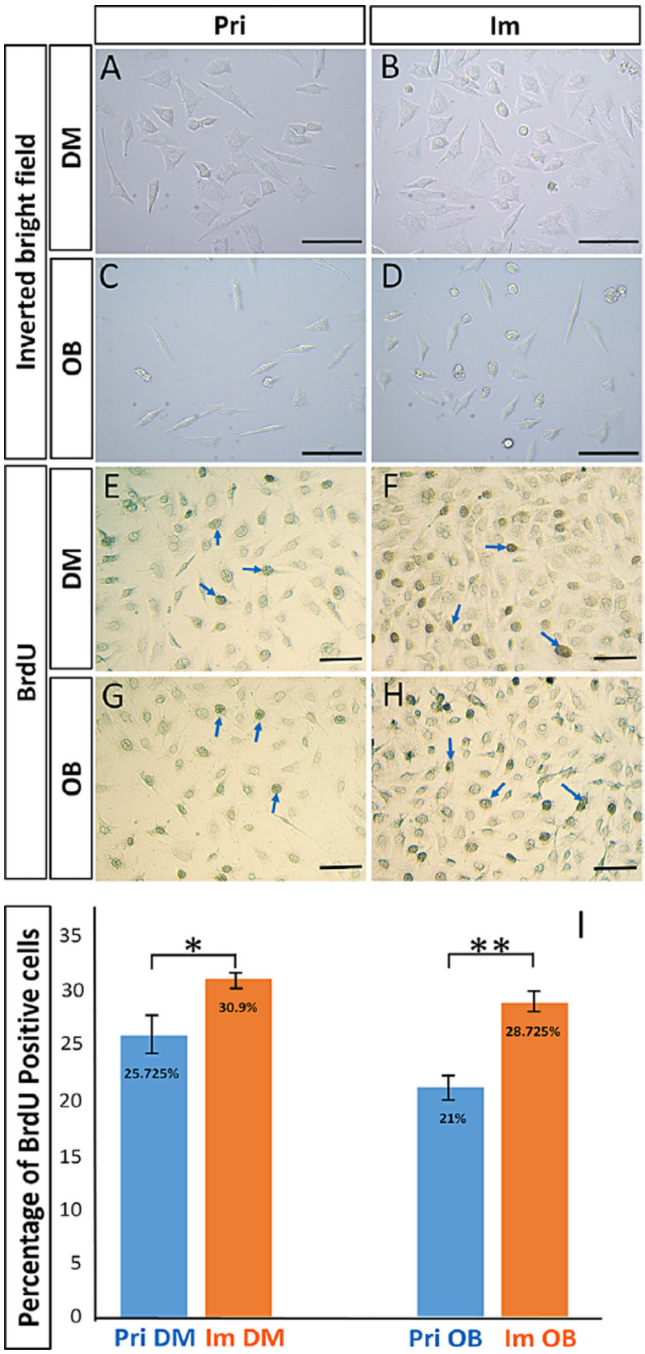

Fig. 2.

Comparison of cell morphologies and proliferation rates between *Fam20c*$^{f/f}$ primary and immortalized cell lines. A and B: The immortalized dental papilla mesenchymal cell line (B) showed a similar fibroblast-like morphology as to their primary dental papilla mesenchymal cells (A). C and D: The primary calvarial osteoblasts (C) and the immortalized osteoblast cell line (D) had the same fibroblast shape. E–H: Immunohistochemistry with the antibody against BrdU showed the BrdU positive nuclei (blue arrows) in the primary dental papilla mesenchymal cells (E), immortalized dental papilla mesenchymal cell line (F), primary

calvarial osteoblasts (G) and immortalized osteoblast cell line (H). I: Statistical comparison of proliferation rates. The percentage of BrdU positive nuclei in the immortalized dental papilla mesenchymal cell line was 30.9% (SD = 2.228%), which was significantly higher than that of primary dental papilla mesenchymal cells (25.725%, SD = 3.3% $P < 0.05$); the difference in BrdU positive percentage between the immortalized osteoblast cell line (28.725%, SD = 0.95%) and primary calvarial osteoblasts (21.0%, SD = 3.915%) was also significant ($P < 0.01$). (Scale bar, 100 μm).

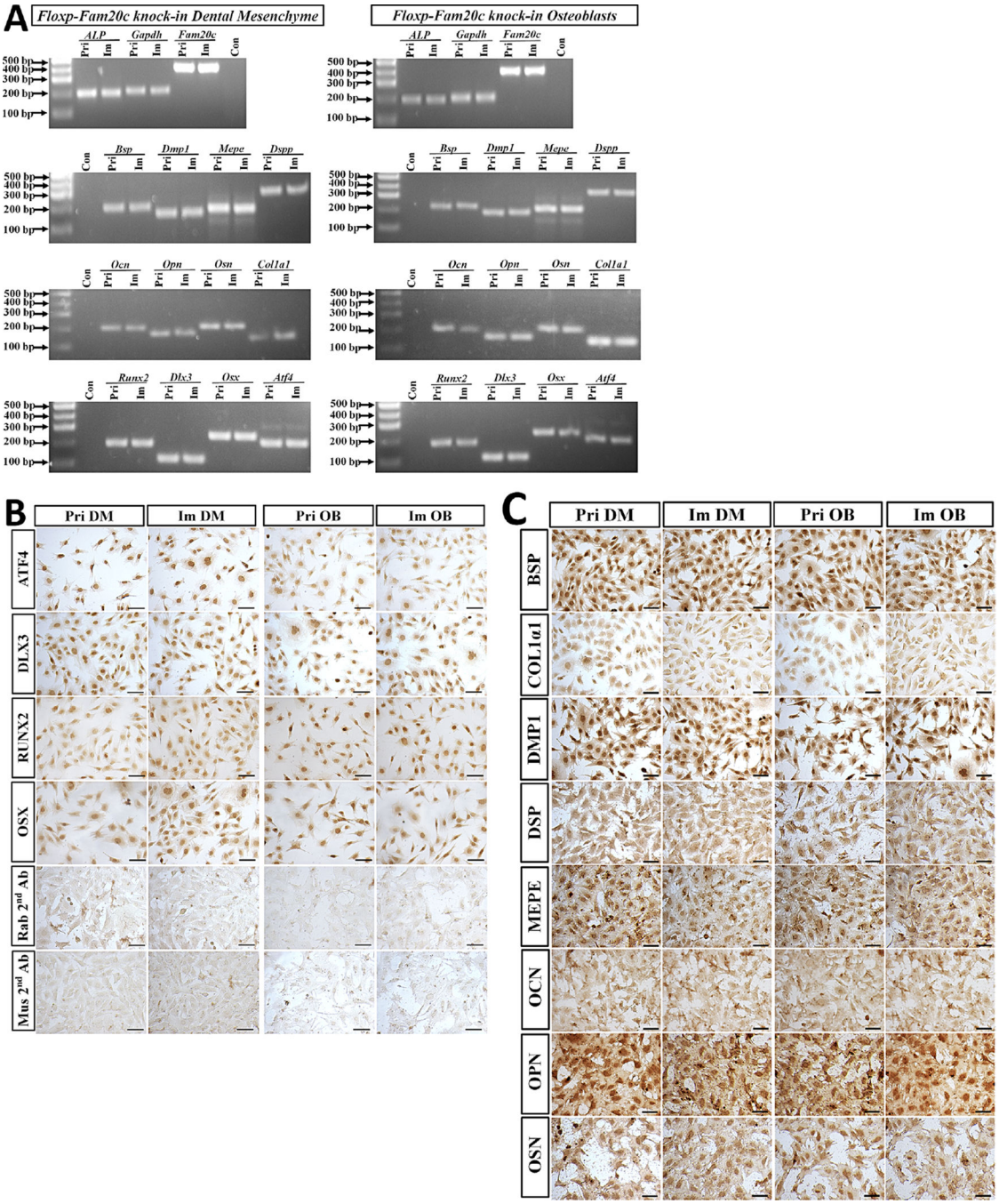

Fig. 3.

Dentin-specific and bone-specific gene expression in the primary and immortalized cells. A: Total RNA extracted from primary dental papilla mesenchymal cells, primary calvarial osteoblasts, immortalized dental papilla mesenchymal and osteoblast cell lines were reversely transcribed into cDNA, and then, amplified by PCR using the primers specific for *Alp, Fam20c, Bsp, Dmp1, Mepe, Dspp, Ocn, Opn, Osn, Col1, Runx2, Dlx3, Osx*, and *Atf4*. B and C: Immunohistochemistry with antibodies against ATF4, DLX3, RUNX2, OSX, BSP, COL1A1, DMP1, DSP, MEPE, OCN, OPN, and OSN in the primary dental papilla

mesenchymal cells, primary calvarial osteoblasts, immortalized dental papilla mesenchymal, and osteoblast cell lines. In the negative controls, only the secondary antibodies against Rabbit (Rab 2nd Ab) and mouse (Mus 2nd Ab) IgG, while no primary antibodies were applied. (Pri DM, primary dental papilla mesenchymal cells; Pri OB, primary calvarial osteoblasts; Im DM, immortalized dental papilla mesenchymal cell line; Im OB, immortalized osteoblast cell line; Scale bar = 100 μm).

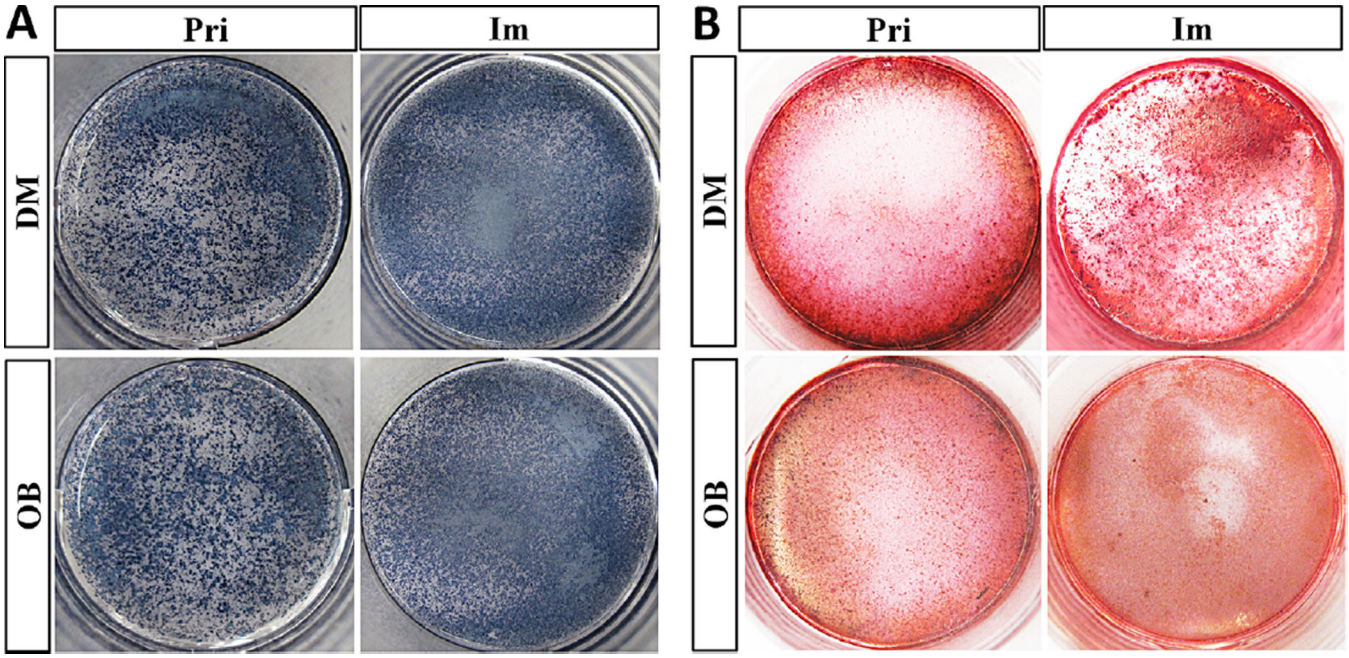

Fig. 4.

Alkaline phosphatase (ALP) activity and the formation of mineralized nodules in the *Fam20c*$^{f/f}$ primary and immortalized cell lines. A: After 1 week's culture in the mineralization-inducing medium, in situ cytochemsitry of ALP activity showed positive staining in the primary dental papilla mesenchymal cells, primary calvarial osteoblasts, and the induced immortalized dental papilla mesenchymal and osteoblast cell lines. B: After 2 weeks of mineralization-inducing culture, condensed mineralized nodules were also detected by Alizarin Red S staining in the primary dental papilla mesenchymal cells, primary calvarial osteoblasts, the induced immortalized dental papilla mesenchymal and osteoblast cell lines.