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Development and characterization of a mouse floxed *Bmp2* osteoblast cell line that retains osteoblast genotype and phenotype

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Abstract

Bone morphogenetic protein 2 (*Bmp2*) is essential for osteoblast differentiation and osteogenesis. Generation of floxed *Bmp2* osteoblast cell lines is a valuable tool for studying the effects of *Bmp2*

on osteoblast differentiation and its signaling pathways during skeletal metabolism. Due to relatively limited sources of primary osteoblasts, we have developed cell lines that serve as good surrogate models for the study of osteoblast cell differentiation and bone mineralization. In this study, we established and characterized immortalized mouse floxed *Bmp2* osteoblast cell lines. Primary mouse floxed *Bmp2* osteoblasts were transfected with pSV3-neo and clonally selected. These transfected cells were verified by PCR and immunohistochemistry. To determine the genotype and phenotype of the immortalized cells, cell morphology, proliferation, differentiation and mineralization were analyzed. Also, expression of osteoblast-related gene markers including *Runx2*, *Osx*, *ATF4*, *Dlx3*, bone sialoprotein, dentin matrix protein 1, osteonectin, osteocalcin and osteopontin were examined by quantitative RT-PCR and immunohistochemistry. These results showed that immortalized floxed *Bmp2* osteoblasts had a higher proliferation rate but preserved their genotypic and phenotypic characteristics similar to the primary cells. Thus, we, for the first time, describe the development of immortalized mouse floxed *Bmp2* osteoblast cell lines and present a useful model to study osteoblast biology mediated by *BMP2* and its downstream signaling transduction pathways.

Keywords

Floxed *Bmp2*; Immortalization; Osteoblast; Gene expression; SV40-T-Ag

Introduction

Skeletal development is a multi-step process that involves the recruitment and proliferation of osteoprogenitor cells and their differentiation into osteoblasts (Aubin and Liu 1996; Marie 1998; Stein et al. 1996). Osteoblasts are derived from undifferentiated mesenchymal cells and their differentiation and bone mineralization are under the control of various osteoblast-specific transcriptional and growth factors (Franceschi et al. 2007; Karsenty 2008). Bone morphogenetic proteins (*BMPs*) are members of the transforming growth factor beta (*TGF-β*) family that play pivotal roles in the commitment and differentiation of cells of osteoblastic lineage (Chen et al. 2004; Rosen 2009). Among *BMP* members, *BMP2* is a potent stimulator of osteogenesis both in vitro and in vivo (Bax et al. 1999; Chung et al. 1999; Kubler et al. 1998; Takuwa et al. 1991; Welch et al. 1998; Yamaguchi et al. 1991) and can induce new bone formation (Urist 1965; Wozney et al. 1988). Moreover, *BMP2* promotes osteoblast maturation by increasing the expression of the transcription factors and osteoblast marker genes (Ducy and Karsenty 2000; Hogan 1996; Reddi 1997; Wu et al. 2003). However, detailed understandings of the molecular mechanisms of *Bmp2* in exerting its effects on bone development and formation are not fully known, in particular during postnatal bone development as homozygous null embryos for *Bmp2* showed developmental abnormalities and died at embryonic day 9.5 (Zhang and Bradley 1996). Recently, conditional *Bmp2* knockout (*cBmp2*-KO) mice have been generated and have revealed important roles of *Bmp2* in later stages of osteogenesis (Bandyopadhyay et al. 2006) and bone fracture healing (Tsuji et al. 2006) as well as other organ development (Lee et al. 2007; Ma et al. 2005; Rivera-Feliciano and Tabin 2006; Singh et al. 2008). Therefore, generation of a floxed *Bmp2* osteoblast cell line would be a valuable tool for studying the effects of *Bmp2* on osteoblast cell lineage as well as relevant molecular events involved in extracellular matrix mineralization and bone regeneration. Such information will help to realize the potential of *BMP2* as a therapeutic agent and for the rational targeting of *BMP2* to the appropriate clinical indication.

In the present study, we report the development and characterization of an immortalized osteoblast cell line from floxed *Bmp2* mice. We chose these cells because a conditional allele of the mouse *Bmp2* gene was created by Cre recombinase recognition sites (*loxP*)

between intron 2 and intron 3 of the mouse *Bmp2* gene and the *Bmp2* gene can be excised by introducing *Cre* recombinase. The primary cells were transfected with simian virus 40 T-antigen (SV40). These transformed cells in extended culture showed stable growth and continued to express osteoblast-related genes similar to the primary cells.

Materials and methods

Generation of *Bmp2* conditional mice

A conditional allele of the mouse *Bmp2* gene was created by introducing *Cre* recombinase recognition sites (loxP), which were placed upstream and downstream of exon 3 to excise the protein-coding region in exon 3 of the *Bmp2* gene (Ma and Martin 2005). Genotyping of wild-type (WT) C57BL/6 and floxed *Bmp2* mice was performed by PCR analyses using WT *Bmp2* and floxed *Bmp2* specific primers (Table 1). Genomic DNA was isolated from the mouse tails by using DNA purification kit, Wizard® Genomic (Promega, Madison, WI, USA). For *Bmp2*, the floxed *Bmp2* and WT *Bmp2* alleles were amplified as 400- and 200-bp products, respectively. Protocols utilized for mouse experiments were approved by the Animal Care and Use for Research of the University of Texas Health Science Center at San Antonio (UTHSCSA), TX, USA.

Primary cell culture of mouse floxed *Bmp2* osteoblasts

The calvarial bones of 1-day-old floxed *Bmp2* mice were isolated and washed with phosphate-buffered saline (PBS), cut into small pieces (about 0.1 cm³), attached to a flask with Minimum Essential Medium Alpha Medium (α-MEM; Invitrogen, San Diego, CA, USA) containing 10% fetal calf serum plus penicillin (100 unit/ml) and streptomycin (100 µg/ml) and cultured at 37° C in a humidified atmosphere of air containing 5% CO₂. The medium was refreshed every 2 days and cells were spread after reaching confluence.

Gene transfer and selection of immortalized cells

Primary cells in passage 3 were transfected with pSV3neo (ATCC, Manassas, VA, USA), a plasmid containing coding sequences of SV40 T-Ag and a neomycin (G418)-resistance gene as described previously (Wu et al. 2009). Two days after transfection, cells were re-plated at a low density and then selected with 600 µg/ml of G418 (Sigma-Aldrich, St. Louis, MO, USA) in order to select transfected cells. Several colonies were formed and well-isolated colonies were removed selectively and re-plated at low densities to obtain the secondary selection. Several single cells that grew were expanded into cell lines and passaged at least 30–50 times over a 7- to 12-month period before further analysis. A cell line *iBmp2-ob* (immortalized floxed *Bmp2* calvarial osteoblast) of passage 50 from the several secondary cell lines established and primary floxed *Bmp2* osteoblast cells of passage 3 were used for the following characterization.

Detection of transformation

Simian virus 40 sequences were accessed in Genbank (Accession No. J02400) and specific primers were synthesized (Table 1). Genomic DNA was isolated from *iBmp2-ob* cells of passage 50 and from primary cells of passage 3 as described above. Two hundred nanograms of DNA was diluted in a 25-µl polymerase chain reaction (PCR) mix of 1× PCR buffer containing 10 pmol of forward and reverse primers, 1 U Red Taq DNA Polymerase and 2.5 mM dNTPs (Sigma-Aldrich). The reactions were carried out at 95° C for 5 min for one cycle and then at 95° C for 30 s, 55° C for 60 s and 72° C for 60 s for 30 cycles, with a final 10 min extension at 72° C. After extension, 5 µl of PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. For detection of SV40 protein expression, *iBmp2-ob* and primary cells were seeded on coverslips in a 6-well

plate and cultured for 48 h in α -MEM medium supplemented with 10% FBS, penicillin (100 unit/ml) and streptomycin (100 μ g/ml). The coverslips were rinsed with 1 $\times$ PBS and fixed with cold acetone and methanol (1:1). The cells were blocked with 10% goat serum (Sigma-Aldrich) and incubated with a primary anti-SV40 large T-antigen monoclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at 37°C. Then the cells were washed and incubated with the secondary antibody with Alexa Fluo® 568 red (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. For negative control, the primary SV40 antibody was replaced by mouse IgG I (Dakocytomation, Carpinteria, CA, USA). For cell nucleus staining, the cells were treated with Hoechst (Sigma-Aldrich). Images of Alexa Fluo® 568 staining of the SV40 protein were obtained at the Core Optical Imaging Facility at UTHSCSA under the same parameters in a Nikon inverted microscope and quantitated by means of NIS-GIEMENTS software.

Primary and transfected cell morphology and proliferation

Morphology of *iBmp2-ob* cells of passage 50 and primary floxed *Bmp2* osteoblasts of passage 3 was observed by a light-inverted microscope. For cell proliferation, *iBmp2-ob* and primary cells were treated with 30 μ M of 5-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich) in culture medium for 4 h. After BrdU incorporation, cells were rinsed with 1 $\times$ cold PBS and fixed for 10 min with methanol/acetone (1:1). After washing with 1 $\times$ PBS, cells were treated with 10% normal goat serum (Sigma-Aldrich) for 60 min at room temperature and followed by washing with PBS. The cells were then incubated with a mouse monoclonal anti-BrdU antibody (1:100; Santa Cruz Biotechnology), followed by a 1:1,000 dilution of the secondary antibodies (goat-anti-mouse) with Alexa Fluo® 488 green (Molecular Probes). For nucleus staining, the cells were incubated with a 1:5,000 dilution of Hoechst (Sigma-Aldrich). Images were obtained using a Nikon inverted microscope and proliferative cells were expressed as a percentage of the number of BrdU positive cells relative to the total number of Hoechst positive nuclei.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from *iBmp2-ob* and primary cells by using RNA STAT-60 kit (Tel-Test, Friendswood, TX, USA), treated with DNase I (Promega) and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA concentration was determined at an optical density of OD₂₆₀. The RNA was transcribed into cDNA by SuperScript II reverse transcriptase (Invitrogen). Specific primers used for the PCR reaction are shown in Table 1. These included ALP, ATF4, *Bmp2*, *Bsp*, *Dlx3*, *Dmp1*, *Oc*, *Opn*, *Osn*, *Osx*, *Runx2*, *Gapdh* and collagen type I. The PCR reaction was first denatured at 95° C for 5 min and then carried out at 95° C for 60 s, at 52–60°C for 60 s and at 72° C for 60 s for 35 cycles and with a final 10 min extension at 72° C. Five μ l of PCR products were analyzed by agarose gels with ethidium bromide staining. DNA was verified by DNA sequencing. For quantitative real-time PCR (qRT-PCR), amplification reactions were analyzed in real time on an ABI 7500 (Applied Biosystems, Foster City, CA, USA) using SYBR Green chemistry and the threshold values were calculated using SDS2 software (Applied Biosystems) as described earlier (Chen et al. 2005). The primer sequences used for qRT-PCR are shown in Table 1.

Immunohistochemistry

For detection of bone-related proteins, *iBmp2-ob* and primary cells were fluorescently immunostained by antibodies against mouse *Bsp* and *Dmp1* (gifts from Dr. Larry Fisher, NIDCR), *Runx2*, *Osx*, (*Sp7*), *OpnOc* and *Col1a1* (Santa Cruz Biotechnology) and *Dlx3* (Abcam, Cambridge, MA, USA). Negative control of mouse IgG was purchased from Dakocytomation. Immunohistochemical assays were performed with corresponding secondary antibodies with Alexa Fluo® 488 green fluorescent labeling (Molecular Probes). For nucleus staining, cells were treated with 1:1,000 dilution of propidium iodide (Sigma-

Aldrich) for 10 min followed by washing with PBS. Microphotographs were obtained under a Nikon microscope using a Nikon Coolpix 4500 digital camera.

Alkaline phosphatase (ALP) assay and mineralization assay

For detection of ALP activity, primary and immortalized osteoblast cells were cultured in calcifying medium [α -MEM supplemented with 10% FBS, penicillin (100 unit/ml) and streptomycin (100 μ g/ml), 50 μ g/ml ascorbic acid and 10 mM sodium β -glycerophosphate] for 1 and 2 weeks, respectively. Cells were then fixed with 70% ethanol for 5 min and washed in the buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$). In situ ALP staining was performed according to the supplier's instructions (Bio-Rad, Hercules, CA, USA). For mineralization assays, *iBmp2-ob* and primary floxed *Bmp2* cells were plated in a 12-well plate at a density of 4×10^5 per well and cultured in calcifying medium at 37° C for 2 and 3 weeks. The cells were fixed in 10% formaldehyde neutral buffer solution and then stained with Alizarin Red S (Sigma-Aldrich). Calcium content was measured according to the manufacturer's instructions using Osteogenesis Assay kits (Millipore, Temecula, CA, USA). Briefly, 400 μ l of 10% acetic acid was added to each well and incubated for 30 min. Cells were scraped and transferred to microcentrifuge tubes. The tubes were heated to 85°C for 10 min and then transferred to ice for 5 min, followed by centrifugation. The supernatants were transferred to new microcentrifuge tubes and neutralized with 10% ammonium hydroxide. Calcium content in the supernatants was determined by the Osteogenesis Assay. Protein concentration was measured using the micro BCA Assay Reagent Kit (Pierce, Rockford, IL, USA).

Results

Immortalization of mouse floxed *Bmp2* osteoblasts

A conditional allele of the mouse *Bmp2* gene was generated as described previously (Ma and Martin 2005) by introducing *Cre* recombinase sites (*loxP*) into regions between intron 2 and intron 3 of the *Bmp2* gene. The homozygous floxed *Bmp2* mice were confirmed by PCR using specific floxed *Bmp2* primers (Fig. 1a, line 4; Table 1) and Southern blot analysis (data not shown), whereas PCR product generated by the floxed *Bmp2* primers was not detected in the WT mice (Fig. 1a, line 3). However, both WT and homozygous floxed *Bmp2* mice contained an intact *Bmp2* gene with primers specific for *Bmp2* exon 3 region (Fig. 1a, lines 7 and 8). To generate immortalization of mouse floxed *Bmp2* cell lines, primary calvarial osteoblast cells were isolated and transfected with SV3 neo plasmid containing SV40 T-Ag gene and then selected with G418. The colonies were formed after 2- to 3-week selections and transfected cells from these colonies grew over 50 population doublings (PDLs) without significant growth retardation. One of the transfected cell lines, termed *iBmp2-ob* (immortalized floxed *Bmp2* osteoblasts), was passaged at 50 generations and used for detailed characterization. SV40 T-Ag gene expression was seen in *iBmp2-ob* cells but not observed in the primary cells by PCR analyses (Fig. 1b). Immunohistochemistry further confirmed that simian virus 40 T-Ag was expressed in all *iBmp2-ob* cells, whereas immunostaining was not present in the primary cells (Fig. 2).

Morphology and proliferation of primary and immortalized osteoblast cells

Cell morphology was studied using a light-inverted microscope (Fig. 3a, b). *iBmp2-ob* cell morphology resembled the primary cells, displaying a spindle shape. There were no microscopical findings showing senescence or neoplastic nature in the *iBmp2-ob* cells. Proliferation of the primary and *iBmp2-ob* cells appeared stable. Compared to the primary osteoblasts, the *iBmp2-ob* cells exhibited a more rapid growth rate as revealed by BrdU assay (Figs. 4f and 5).

Expression of osteoblast-related genes in primary and *iBmp2-ob* cells

To determine whether *iBmp2-ob* cells express bone-related genes similar to the primary osteoblast cells, we tested an expression of various bone-related genes using RT-PCR assay. Figure 6a showed that the transformed cell line synthesized collagenous and non-collagenous protein genes such as collagen type I, *ALP*, *Bsp*, *Dmp1*, *Oc*, *Opn*, *Osn*. Also, these transformed cells expressed *Bmp2*, *ATF4*, *Dlx3*, *Osx* and *Runx2* that are involved in osteoblast differentiation and bone mineralization (Fig. 6a). To further assess whether there are different expressional levels of bone-related genes between the primary and immortalized cells, qRT-PCR assay was performed. These results showed that there were no significant differences between the primary and immortalized cells except with *Bsp* (Fig. 6b). Furthermore, immunohistochemistry studies confirmed that these cells express the proteins as well (Figs. 7, 8, 9 and 10). These results indicate that *iBmp2-ob* cells, with a long-term culture, retain their genotypic and phenotypic characteristics similar to the primary osteoblast cells.

Osteoblast cell differentiation and mineralization

To determine the differentiation and mineralization activities of these primary and *iBmp2-ob* cells, we examined *ALP* activity by in situ *ALP* histochemistry as *ALP* is a marker of osteoblast cell differentiation (Katagiri et al. 1990; Yamaguchi et al. 1991). The positive expression of *ALP* in both the primary and *iBmp2-ob* cells was seen at 1 and 2 weeks of culture in calcifying medium (Fig. 11a–d). However, higher *ALP* activity was observed in both the primary and immortalized cells at the 2-week culture (Fig. 11c, d). Also, mineralized nodule formation in the primary and immortalized cells was observed at 2 and 3 weeks of culture by using Alizarin Red S staining (Fig. 11e–h). At the 3-week culture, higher density was seen in the primary and transformed cells (Fig. 11g, h). Quantitative measurement of the mineralization deposit showed no significant difference between the primary and immortalized cells at 3-week culture (Fig. 11i).

Discussion

In this study, we established and characterized an immortalized cell line *iBmp2-ob* from primary mouse floxed *Bmp2* calvarial osteoblasts at postnatal day 1 by transfection with SV40 T-Ag, a viral oncogene. The immortalized cells expressed SV40 and preserved high *ALP* activity and strong mineralization ability. Also, *iBmp2-ob* cells expressed osteoblast-related gene markers. These data suggest that the cell line *iBmp2-ob* is functionally active and displays all the genotypic and phenotypic characteristics similar to the primary cells.

Osteoblasts are important players in skeletal development, bone formation, bone remodeling and fracture repair. Therefore, various osteoblast culture procedures have been used to elucidate the molecular and cellular mechanisms of these cells and their roles in osteoblast differentiation and bone metabolism during physiological and pathological processes. Osteoblast cell lines have been developed from various species including human (Bodine et al. 1996; Darimont et al. 2002; Winn et al. 1999) and mouse (Divieti et al. 1998; Takeshita et al. 2001). One of the criteria used for selection of these immortalized osteoblast cell lines is that those transformed cells are able to maintain the osteoblast genotypic and phenotypic characteristics in a cultured system so that the information derived from these cells is applicable to primary osteoblasts. The strategy for these studies was to introduce SV40 T-Ag into primary osteoblast cells, as SV40 T-Ag has been demonstrated to be an effective agent for immortalization manipulation in a variety of studies. Moreover, numerous reports have shown that the viral SV40 T-Ag is capable of retaining these immortalized cell genotype and phenotype similar to those of primary cells (Galler et al. 2006; Hoffman et al. 2010; Iwata et al. 2007). These cell lines have been used in many applications, including the

study of normal and pathological cell differentiation and mineralization, bone tissue repair and regeneration, gene regulation and signaling pathway during bone development.

To our knowledge, this is the first report of the development of a mouse floxed *Bmp2* osteoblast cell line. One of the advantages of establishing such a stable osteoblast cell line is to provide a large, consistent source of homogenous cell products for biochemical and molecular analyses. Another reason is that they provide an excellent resource suitable for *Bmp2* knockout experiments with the introduction of *Cre* recombinase (Gagneten et al. 1997; Matsuda and Cepko 2007), as *Bmp2* plays an essential role in osteoblast differentiation, bone formation and bone remodeling (Chen et al. 2004; Kubler et al. 1998; Rosen 2009; Takuwa et al. 1991).

Gene expression studies on the floxed *Bmp2* immortalized cells showed that these cells synthesize osteoblast-related gene markers including collagenous and non-collagenous proteins. Both these groups of proteins are essential components of bone extracellular matrix and are involved in osteoblast differentiation and bone mineralization processes (Aubin and Liu 1996; Marie 1998; Wu et al. 2003). Also, we observed that these immortalized osteoblast cells were able to differentiate and form mineralized nodules when cultured in calcifying medium. With a longer culture in calcifying medium, *ALP* activity was increased and larger sizes and higher density of the mineralized nodules were visualized in both the primary and immortalized cells. In addition, cell morphology of these primary and immortalized cells are very similar, although the growth pattern of the transfected cells display a more rapid proliferation rate. Protein products coded for the SV40 genes are highly multifunctional for cell proliferation. The large antigen (Tag) is able to interact with a large number of cellular proteins including DNA polymerase α (Gannon and Lane, 1987), TATA binding protein (TBP) and associate factor (TAFs) (Damania and Alwine 1996) and the transcriptional co-activator, histone acetyltransferase p300/CBP (Avantaggiati et al. 1996), thus promoting the transactivation of a number of cellular proliferation genes such as insulin-like growth factor (IGF-1) and its receptor (Porcu et al. 1992; Carbone et al. 2008) and the cyclin-dependent kinase *cdc2* (Chen et al. 1996). These signaling pathways are critical for SV40-mediated transformation because SV4- induced cell proliferation requires the interaction of these proteins, forcing the cell to enter the cell cycle (Porcu et al. 1992). In addition, Tag interaction with the two major cellular tumor suppressor proteins, p53 and pBR, causes the activation of E2F/DP transcription factors that in turn promote transcription of genes causing cell cycle progression (Michalovitz et al. 1986; Ludlow et al. 1990; Royzman et al. 1997; Herzig et al. 1999).

Moreover, we observed expression of the growth factor *Bmp2* and several transcription factors known to be necessary for bone development including *ATF4*, *Dlx3*, *Osx* and *Runx2*. Both *Runx2* and *Osx* are important factors for osteogenesis and chondrogenesis (Ducy et al. 1997; Komori et al. 1997; Mundlos et al. 1997; Nakashima et al. 2002; Otto et al. 1997). Mutations of either *Runx2* or *Osx* gene resulted in abnormal bone formation and development (Lee et al. 1997; Nakashima et al. 2002; Otto et al. 1997; Quack et al. 1999). *Runx2*-deficient mice displayed a complete lack of both intramembranous and endochondral ossification and showed an absence of osteoblast differentiation, resembling phenotypic aspects of human *Runx2* mutations that cause cleidocranial dysplasia (CCD) (Lee et al. 1997; Mundlos et al. 1997; Otto et al. 1997; Quack et al. 1999). *Dlx3* is a homeodomain transcriptional factor essential for bone, hair and tooth development. Mutations of the *Dlx3* gene are associated with the autosomal dominant genetic disorder called tricho-dento-osseous syndrome (TDO) characterized by abnormalities in hair, tooth and bone development (Dong et al. 2005; Duverger et al. 2008; Price et al. 1998; Wright et al. 2008). *ATF4* is necessary for osteoblast differentiation and skeletal development and *ATF4* mutations are associated with Coffin–Lowry syndrome (Ameri and Harris 2008; Xiao et al.

2005; Yang et al. 2004). Furthermore, it has been shown that these transcriptional factors are regulated by *Bmp2* and other growth factors (Fei et al. 2010; Hassan et al. 2006; Javed et al. 2008; Matsubara et al. 2008; Park and Morasso 2002; Ulsamer et al. 2008). Therefore, the cell line *iBmp2-ob* or knock out of *Bmp2* gene by *Cre* recombinase in the *iBmp2-ob* cells will be a valuable asset for studying signaling pathways of bone formation and development mediated by *Bmp2* and its down stream signaling pathways.

Conclusion

We have established and characterized an osteoblast cell line from floxed *Bmp2* mice. This cell line preserves the osteoblast genotype and phenotype through long-term culture procedures even though these immortalized cells proliferate rapidly compared to the primary cells. Thus, the *iBmp2-ob* cells and these cells with *Bmp2* knock-out by *Cre* recombinase in vitro can be useful cell models for studying the mechanism of *Bmp2* effects on osteoblast cell proliferation, differentiation and mineralization as well as the potential application of these cells for bone regeneration.

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Abbreviations

Bmp2	Bone morphogenetic protein 2
BrdU	5-bromo-2'-deoxyuridine
ALP	Alkaline phosphatase
ATF4	Activating transcription factor 4
Bsp	Bone sialoprotein
Col1α1	Alpha 1 collagen type
Dlx3	Distal-less 3
Dmp1	Dentin matrix protein 1
Gadph	Glyceraldehyde-3-phosphate dehydrogenase
Oc	Osteocalcin
Opn	Osteopontin
Osn	Osteonectin
Osx	Osterix
SV40	SV40 large T-antigen.

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Fig. 1.

Identification of floxed alleles in *Bmp2* gene and SV40 transformation. **a** Genomic DNAs from wild-type (WT) and floxed *Bmp2* mice were isolated and amplified by the WT *Bmp2*, floxed *Bmp2* and *Bmp2* exon 3 specific primers shown in Table 1. PCR products were run on agarose gels and stained with ethidium bromide. Lane 1 Low DNA mass ladder (Invitrogen); lane 2 Con, negative control; lane 3 genomic DNA of WT mice amplified by floxed *Bmp2* primers; lane 4 genomic DNA of floxed *Bmp2* mice amplified by floxed *Bmp2* primers; lane 5 genomic DNA of WT mice amplified by WT *Bmp2* primers; lane 6 genomic DNA of floxed *Bmp2* mice amplified by WT *Bmp2* primers; lane 7 genomic DNA of WT mice amplified by *Bmp2* exon 3 primers; lane 8 genomic DNA of floxed *Bmp2* mice amplified by *Bmp2* exon 3 primers. **b** Genomic DNAs from the primary and *iBmp2-ob* osteoblast cells were isolated and amplified by the floxed *Bmp2* and SV40 specific primers, respectively. PCR products were run on agarose gels and stained with ethidium bromide. Lane 1 pGEM DNA markers (Promega); lane 2 Con, negative control; lane 3 primary *Bmp2 loxp*; lane 4 immortalized *Bmp2 loxp*; lane 5 primary DNA for SV40; lane 6 immortalized DNA for SV40. Pri Primary, Imm immortalized

Fig. 2.

Expression of simian virus 40 large T-antigen in immortalized cells by immunohistochemistry. Immunohistochemical staining with antibody against simian virus 40 large T-antigen (SV40 T-Ag) in the primary and *iBmp2-ob* cells. Immunolabeling of SV40 T-Ag was mostly present in the nucleus whereas immunostaining was not seen in the primary cells. Scale bar 10 μ M

Fig. 3. Morphology of mouse primary and immortalized osteoblast cells. Primary and immortalized cells were photographed under a light microscope using a Nikon Coolpix digital camera. The immortalized cell morphology resembles that of the primary osteoblasts, displaying a spindle shape. *Scale bar* 10 μ M

Fig. 4.

Proliferation of mouse primary and immortalized osteoblast cells. Proliferation of primary and immortalized cells was immunostained using BrdU antibody after a 4-h BrdU incorporation (30 μ M). The immortalized osteoblast cells showed a higher proliferation rate (**f**) than that of the primary cells (**b**). *BrdU*⁺ (**b, f**) and *BrdU*⁻ (**j, n**) show cells stained with and without BrdU antibody. *Scale bar* 10 μ M

Fig. 5.

Mouse *primary* and *immortalized* osteoblast cell proliferation. Proliferation of primary and immortalized cells was immunostained using a mouse monoclonal anti-BrdU antibody (1:100 dilution) after a 4-h BrdU incorporation (30 μ M), followed by a 1:1,000 dilution of the secondary antibody (goat-anti-mouse) with Alexa Fluo® 488 green. For nucleus staining, the cells were incubated with a 1:5,000 dilution of Hoechst. Images were obtained by a Nikon inverted microscope and proliferative cells were expressed as a percentage of the number of BrdU positive cells relative to the total number of Hoechst positive nuclei. BrdU positive staining of the primary cells acts as 100% and *asterisk* (*) shows significant difference between the primary and immortalized cells ($p < 0.05$)

Fig. 6.

Expression of bone-related genes in the primary and immortalized osteoblast cells by polymerase chain reaction. Total RNAs from the primary and immortalized cells were extracted and reversely transcribed. The cDNAs were amplified by RT-PCR (**a**) and qRT-PCR (**b**) using specific primers shown in Table 1. PCR analysis of mRNA expression of *ALP*, *ATF4*, *Bmp2*, *Bsp*, *Colla1*, *cyclophilin A*, *Dmp1*, *Oc*, *Opn*, *Osn*, *Osx* and *Runx2* genes. For qRT-PCR analysis, expression of those mRNAs in the primary cells acts as 1.0-fold increase. Asterisks (*) show significant differences between the two cells ($p < 0.05$). *Pri* Primary, *Imm* immortalized

Fig. 7.

Expression of *Bsp* and *Dlx3* genes in the primary and immortalized osteoblast cells by immunohistochemistry. *Bright* photos of the primary and immortalized cells were photographed under a light microscope using a Nikon camera. The expression of *Bsp* and *Dlx3* in those primary and immortalized cells were stained with antibodies against *Bsp* and *Dlx3*, respectively. Cells were stained with propidium iodide for the nucleus. *Scale bar* 10 μ M

Fig. 8.

Expression of collagen type 1 (*Colla1*) and *Dmp1* genes in the primary and immortalized osteoblast cells by immunohistochemistry. *Bright* photos of the primary and immortalized cells were photographed under a light microscope using a Nikon camera. The expression of *Colla1* and *Dmp1* in those primary and immortalized cells were stained with antibodies against *Colla1* and *Dmp1*, respectively. Cells were stained with propidium iodide for the nucleus. Scale bar 10 μ M

Fig. 9.

Expression of *Oc* and *Opn* genes in the primary and immortalized osteoblast cells by immunohistochemistry. *Bright* photos of the primary and immortalized cells were photographed under a light microscope using a Nikon camera. The expression of *Oc* and *Opn* in those primary and immortalized cells were stained with antibodies against *Oc* and *Opn*, respectively. Cells were stained with propidium iodide for the nucleus. *Scale bar* 10 μ M

Fig. 10.

Expression of *Osx* and *Runx2* genes in the primary and immortalized osteoblast cells by immunohistochemistry. *Bright* photos of the primary and immortalized cells were photographed under a light microscope using a Nikon camera. The expression of *Osx* and *Runx2* in those primary and immortalized cells were stained with antibodies against *Osx* and *Runx2*, respectively. Cells were stained with propidium iodide for the nucleus. *Scale bar* 10 μM

Fig. 11.

Alkaline phosphatase (*ALP*) activity and mineralized nodule formation in the primary and transformed osteoblast cells. **a–d** In situ histochemistry of *ALP* activity in the primary (**a, c**) and immortalized (**b, d**) osteoblast cells were visualized after a culture of 1 (**a, b**) and 2 (**c, d**) weeks in calcifying medium. **e–h** the primary and immortalized cells were treated with calcifying medium for 2 (**e, f**) and 3 (**g, h**) weeks. With a longer culture, *ALP* activity and density of mineralized nodules were increased in both the primary and immortalized cells. **i** Cells were cultured in calcifying medium for 3 weeks and calcium deposits were determined by Osteogenesis Assay kit described in “Materials and Methods”. Data represent mean $\pm$ SD of three independent experiments. *Immor* Immortalized

Table 1

Primer sequences used for polymerase chain reactions

Gene	Primer sequences	Product size (bp)	T _m (°C)
<i>ALP</i>	Forward: 5'-CGGGACTGGTACTCGGATAA-3' Reversed: 5'-TGAGATCCAGGCCATCTAGC-3'	209	55
<i>ATF4</i>	Forward: 5'-GAAACCTCATGGGTTCTCCA-3' Reversed: 5'-AGAGCTCATCTGGCATGGTT-3'	203	58
<i>Bsp</i>	Forward: 5'-AAAGTGAAGGAAAGCGACGA-3' Reversed: 5'-GTTCTTCTGCACCTGCTTC-3'	215	52
<i>Collα1</i>	Forward: 5'-CCTGACGCATGGCCAAAGAA-3' Reversed: 5'-GCATTGCACGTCATCGCAC-3'	145	60
<i>Cyclo</i>	Forward: 5'-GAGCTCTGAGCACTGGAGAGA-3' Reversed: 5'-GATGCCAGGACCTGTATGCT-3'	181	58
<i>Dlx3</i>	Forward: 5'-GCGACACTCAGGAATCATTTG-3' Reversed: 5'-CGGTCCATGCAATTGTTATC-3'	108	50
<i>Dmp1</i>	Forward: 5'-CAGTGAGGATGAGGCAGACA-3' Reversed: 5'-TCGATCGCTCCTGTTACTCT-3'	175	54
Wild-type <i>Bmp2</i>	Forward: 5'-CGGGAAACAGATACAGGAAGC-3' Reversed: 5'-GCTGTTTGTGTTGGCTTGA-3'	200	59
<i>Bmp2</i> Exon III	Forward: 5'-AGGGTTTCAGGTCAGTTTCCG-3' Reversed: 5'-TCCGAAGGTAAAGTGTGCTTGG-3'	403	60
Floxed <i>Bmp2</i>	Forward: 5'-GATGATGAGGTTCTTGGCGG-3' Reversed: 5'-AGGGTTTCAGGTCAGTTTCCG-3'	400	60
<i>Gapdh</i>	Forward: 5'-CAAAGTTGTCATGGATGACC-3' Reversed: 5'-CCATGGAGAAAGCTGGGG-3'	195	56
<i>Oc</i>	Forward: 5'-CTTGGTGCACACCTAGCAGA-3' Reversed: 5'-TTCGTGTTCTCCCTGCTGT-3'	208	54
<i>Opm</i>	Forward: 5'-TCTGATGAGACCGTCACATGC-3' Reversed: 5'-AGGTCCTCATCTGTGGCATC-3'	170	53
<i>Onn</i>	Forward: 5'-AAACATGGCAAGGTGTGTGA-3' Reversed: 5'-TGCAATGGTCCGATGTAGTC-3'	218	54
<i>Oxx</i>	Forward: 5'-ACTCATCCCTATGGCTCTGTG-3' Reversed: 5'-GGTAGGGAGCTGGGTTAAGG-3'	238	55
<i>Runx2</i>	Forward: 5'-TACAAACCATACCCAGTCCCTGTTT-3' Reversed: 5'-AGTGCTCTAACCACAGTCCATGCA-3'	197	55
SV40	Forward: 5'-AGCAGACACTCTATGCCCTGTGTGGAGTAAG-3' Reversed: 5'-GACTTTGGAGGCTTCTGGATGCAACTGAG-3'	751	55

ALP Alkaline phosphatase, *ATF4* activating transcription factor 4, *Bmp2* bone morphogenetic protein 2, *Bsp* bone sialoprotein, *Collα1* alpha 1 collagen type, *Cyclo* Cyclophilin A, *Dlx3* Distal-less 3, *Dmp1* dentin matrix protein 1, *Gapdh* glyceraldehyde-3-phosphate dehydrogenase, *Oc* osteocalcin, *Opm* osteopontin, *Oxx* Osterix, SV40 SV40 large T-antigen