Biochemical and Biophysical Research Communications 386 (2009) 89–95

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Immortalization and characterization of mouse floxed *Bmp2/4* osteoblasts

Li-An Wu [a,c], Guohua Yuan [a,d], Guobin Yang [a,d], Iris Ortiz-Gonzalez [a], Wuchen Yang [b], Yong Cui [b], Mary MacDougall [e], Kevin J. Donly [a], Stephen Harris [b], Shuo Chen [a,*]

[a] Department of Pediatric Dentistry, The University of Texas Health Science Center at San Antonio, TX, USA
[b] Department of Periodontics, Dental School, The University of Texas Health Science Center at San Antonio, TX, USA
[c] Department of Pediatric Dentistry, School of Stomatology, The Fourth Military Medical University, Xi-an, China
[d] Key Laboratory of Oral Biomedical Engineering Ministry of Education, Wuhan, China
[e] Department of Oral/Maxillofacial Surgery, University of Alabama, Birmingham, AL, USA

ARTICLE INFO

Article history:
Received 28 May 2009
Available online 6 June 2009

Keywords:
Immortalization
Floxed *Bmp2/4*
Gene expression
Osteoblasts
SV40 T-Ag

ABSTRACT

Generation of a floxed *Bmp2/4* osteoblast cell line is a valuable tool for studying the modulatory effects of *Bmp2* and *Bmp4* on osteoblast differentiation as well as relevant molecular events. In this study, primary floxed *Bmp2/4* mouse osteoblasts were cultured and transfected with simian virus 40 large T-antigen. Transfection was verified by polymerase chain reaction (PCR) and immunohistochemistry. To examine the characteristics of the transfected cells, morphology, proliferation and mineralization were analyzed, expression of cell-specific genes including *Runx2*, *ATF4*, *Dlx3*, *Osx*, dentin matrix protein 1, bone sialoprotein, osteopontin, osteocalcin, osteonectin and collagen type I was detected. These results show that transfected floxed *Bmp2/4* osteoblasts bypassed senescence with a higher proliferation rate, but retain the genotypic and phenotypic characteristics similar to the primary cells. Thus, we for the first time demonstrate the establishment of an immortalized mouse floxed *Bmp2/4* osteoblast cell line.

Introduction

Bone morphogenetic proteins (*Bmps*) were initially identified by their ability to induce bone and cartilage formation when implanted subcutaneously or intramuscularly in animals [1]. The *Bmps* are structurally related to transforming growth factor-β (TGF-β). They contain over 20 different *Bmps* and are subclassified into at least four groups depending on their structures [2]. *Bmp2* and *Bmp4* are most similar to Decapentaplegic (*Dpp*) in *Drosophila* and belong to the *Bmp2/4* subclass [3]. Among *Bmp* family proteins, *Bmp2* is one of the best characterized molecules. Recombinant *Bmp2* has been shown to be a potent stimulator of osteogenesis both *in vitro* and *in vivo* [4–9]. Overexpression *Bmp2* induces expression of osteocalcin and other genes that are important for osteoblast differentiation [10]. Moreover, there is growing evidence indicating that *Bmp4* also plays an essential role in osteoblast differentiation, bone formation and fracture healing [11–14]. However, detail understandings of the mechanisms through which *Bmp2* and *Bmp4* exert their effects on osteoblast functions remain elusive as it has been difficult to decipher the specific roles of *Bmp2* or *Bmp4* during osteogenesis because of functional redundancy between them [15]. Recently, mice simultaneously lacking *Bmp2* and *Bmp4* have been produced and displayed severe impairment of osteogenesis as well as exhibited different genotypic and phenotypic characteristics compared to either that of *Bmp2* or *Bmp4* null mice [16]. Thus, generation of a floxed *Bmp2/4* osteoblast cell line would be a valuable tool for studying the modulatory effects of *Bmp2* and *Bmp4* on osteoblast differentiation as well as relevant molecular events involved in matrix mineralization *in vitro*. Such information will help to realize the potential of *Bmps* as therapeutic agents and for the rational targeting of specific *Bmps* to the appropriate clinical indication.

In the present study, we established an immortalized mouse floxed *Bmp2/4* osteoblast cell line using transfection of simian virus 40 T-antigen (SV40). The cells show stable growth and retain the phenotypic and genotypic characteristics similar to primary floxed *Bmp2/4* osteoblasts *in vitro*.

Materials and methods

Generation of Bmp2 and Bmp4 double conditional mice. A conditional allele of the mouse *Bmp2* gene was created by introducing Cre recombinase recognition sites (*loxP*), which were integrated to excise the protein-coding region in exon 3 of the *Bmp2* gene. Mice carrying floxed *Bmp4* allele were kindly provided by Hogan [17]. The *loxP* sites were placed upstream and downstream of exons 3 and 4, within intron 2 and 3′ of the poly-adenylation site,

* Corresponding author. Address: Department of Pediatric Dentistry, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA. Fax: +1 210 567 6603.
E-mail addresses: chens0@uthscsa.edu, ailingcun@yahoo.com.cn (S. Chen).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.05.144

and the entire *Bmp4* protein coding sequence was excised by the Cre recombinase [17]. For generation of *Bmp2* and *Bmp4* double conditional mice, $Bmp2^{C/C}$ animals were crossed with $Bmp4^{C/C}$ animals to generate $Bmp2^{C/+}/Bmp4^{C/+}$ animals. These animals were crossed with $Bmp2^{C/C}$ or $Bmp4^{C/C}$ animals to generate $Bmp2^{C/C}/Bmp4^{C/+}$ or $Bmp2^{C/+}/Bmp4^{C/C}$ animals, respectively. $Bmp2^{C/C}/Bmp4^{C/+}$ or $Bmp2^{C/+}/Bmp4^{C/C}$ animals were crossed with each other to generate $Bmp2^{C/C}/Bmp4^{C/C}$ animals. Genotyping of $Bmp2^{C/C}/Bmp4^{C/C}$ animals were done by PCR analyses using floxed *Bmp2* and *Bmp4* specific primers (Table 1). Genomic DNA was isolated from the mouse tails by DNA purification kit, Wizard® Genomic (Promega, Madison, WI). For *Bmp2*, the floxed allele was amplified as a 400 bp product, while for *Bmp4*, the floxed allele amplified as a 184 bp band. 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, TX, USA.

Primary cell culture. The calvarial bones of 1-day floxed *Bmp2/4* mice were isolated and washed with phosphate buffered saline (PBS), cut into pieces (about 0.1 cm^3), attached to a flask with Minimum Essential Medium Alpha Medium (a-MEM, Invitrogen, San Diego, CA) containing 10% fetal calf serum plus penicillin (100 U/ml) and streptomycin (100 μg/ml) and cultured at 37 °C in a humidified atmosphere of air containing 5% CO_2.

Gene transfer and selection of immortalized cells. Primary floxed *Bmp2/4* osteoblast cells in passage 3 were transfected with pSV3neo (ATCC, No. 37150, Manassas, VA), a plasmid containing coding sequences of SV40 T-Ag and a neomycin (G418)-resistance gene by electroporation [18]. One day after transfection, cells were cultured in standard medium supplemented with 600 μg/ml of G418 (Sigma–Aldrich, St. Louis, MO). Several colonies were formed, and well-isolated colonies were removed selectively and replated at low densities to obtain the secondary selection. One of the selected secondary colonies was named i*Bmp2/4* (immortalized floxed *Bmp2/4*). i*Bmp2/4* cells of passage 50 and primary floxed *Bmp2/4* osteoblast cells of passage 3 were used for the following characterization.

Cell morphology and proliferation assays. Morphology of i*Bmp2/4* and primary cells was observed by a light inverted microscope. Cell proliferation assay was performed by direct cell counting and MTT method. Briefly, cells were seeded into 6-well plates at 5×10^4 cells per well. The cells were trypsinized and counted daily using a hemocytometer for up to 4 days. For MTT assay, cells were seeded into 96-well plates with 1.5×10^3 cells per well and detected at day 1, 2, 3, 4, respectively, by MTT cell proliferation assay kit (ATCC, No. 30-1010K, Manassas, VA).

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 i*Bmp2/4* and primary cells. pSV3 neo plasmid was used as positive control. Two-hundred nanograms of DNA (for pSV3 neo plasmid DNA 10 ng) was diluted in a 25 μl polymerase chain reaction (PCR) mix (Sigma–Aldrich). Five microliters of PCR products was analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. For detection of SV40 protein expression, i*Bmp2/4* and primary cells were seeded on coverslips in 6-well plates and cultured for 48 h in standard a-MEM medium. The coverslips were rinsed with PBS and fixed with cold acetone and methanol (1:1). The cells were blocked with 10% goat serum and incubated with

Table 1
Primer sequences used for polymerase chain reaction.

Gene	Primers sequence	Product size	T_m (°C)
Runx2	Forward: 5′-TACAAACCATACCCAGTCCCTGTTT-3′ Reversed: 5′-AGTGCTCTAACCACAGTCCATGCA-3′	197	55
Osx	Forward: 5′-ACTCATCCCTATGGCTCGTG-3′ Reversed: 5′-GGTAGGGAGCTGGGTTAAGG-3′	238	55
Dlx3	Forward: 5′-GCGACACTCAGGAATCATTG-3′ Reversed: 5′-CGGTCCATGCATTTGTTATC-3′	108	50
Col1α1	Forward: 5′-CCTGACGCATGGCCAAGAAGA-3′ Reversed: 5′-GCATTGCACGTCATCGCACA-3′	145	60
Oc	Forward: 5′-CTTGGTGCACACCTAGCAGA-3′ Reversed: 5′-TTCTGTTTCCTCCCTGCTGT-3′	208	54
ALP	Forward: 5′-CGGGACTGGTACTCGGATAA-3′ Reversed: 5′-TGAGATCCAGGCCATCTAGC-3′	208	55
Bsp	Forward: 5′-AAAGTGAAGGAAAGCGACGA-3′ Reversed: 5′-GTTCCTTCTGCACCTGCTTC-3′	215	52
Dmp1	Forward: 5′-FCAGTGAGGATGAGGCAGACA-3′ Reversed: 5′-TCGATCGCTCCTGGTACTCT-3′	175	54
Opn	Forward: 5′-TCTGATGAGACCGTCACTGC-3′ Reversed: 5′-AGGTCCTCATCTGTGGCATC-3′	170	53
Gapdh	Forward: 5′-CAAAGTTGTCATGGATGACC-3′ Reversed: 5′-CCATGGAGAAGGCTGGGG-3′	195	56
ATF4	Forward: 5′-GAAACCTCATGGGTTCTCCA-3′ Reversed: 5′-AGAGCTCATCTGGCATGGTT-3′	203	58
Osn	Forward: 5′-AAACATGGCAAGGTGTGTGA-3′ Reversed: 5′-TTGCATGGTCCGATGTAGTC-3′	218	54
Floxed *Bmp2*	Forward: 5′-GATGATGAGGTTCTTGGCGG-3′ Reversed: 5′-AGGGTTTCAGGTCAGTTTCCG-3′	400	60
Floxed *Bmp4*	Forward: 5′-AGACTCTTTAGTGAGCATTTTCAAC-3′ Reversed: 5′-AGCCCAATTTCCACAACTTC-3′	184	55
SV40	Forward: 5′-AGCAGACACTCTATGCCTGTGTGGAGTAAG-3′ Reversed: 5′-GACTTTGGAGGCTTCTGGATGCAACTGAG-3′	751	55

Osx, Osterix; ALP, alkaline phosphatase; Gadph, glyceraldhyde-3-phosphate dehydrogenase; *Osn*, osteonectin; *Oc*, osteocalcin; *Bsp*, bone sialoprotein; *Dmp1*, dentin matrix protein 1; SV40, SV40 large T-antigen; *Opn*, osteopontin; Col1A1, alpha 1 collagen type I.

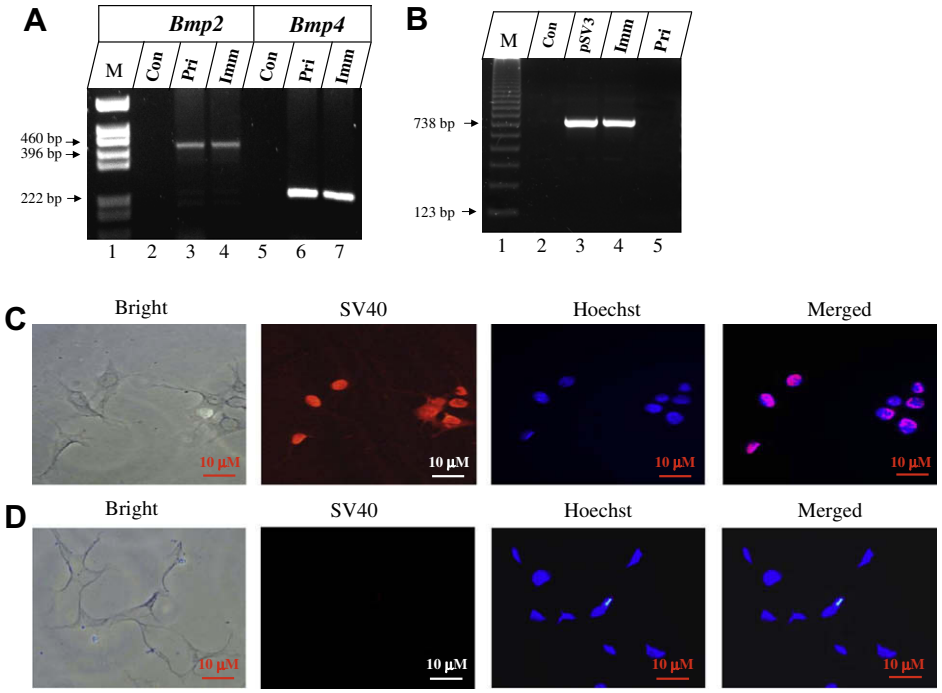

Fig. 1. Identification of floxed alleles in *Bmp2* and *Bmp4* genes and SV40 transformation. (A) Genomic DNA in the primary and i*Bmp2/4* osteoblast cells was isolated and amplified by the floxed *Bmp2* and *Bmp4* specific primers, respectively. PCR products were run on agarose gels and stained with ethidium bromide. Lane 1, pGEM DNA markers (Promega); lane 2, control; lane 3, primary *Bmp2* loxp; lane 4, immortalized *Bmp2* loxp; lane 5, control; lane 6, primary *Bmp4* loxp; lane 7, immortalized *Bmp4* loxp. Pri, primary; Imm, immortalized. (B) Gel electrophoretical analysis of PCR amplification products with specific primers for SV40 T-Ag. Lane 1, 123 bp DNA ladder (Invitrogen); lane 2, control; lane 3, pSV3 neo; lane 4, immortalized; lane 5, primary. (C, D) Immunohistochemical staining with antibody against simian virus 40 large T-antigen (SV40 T-Ag) in i*Bmp2/4* cells and immunolabeling was mostly present in the nucleus (C) whereas immunostaining was not seen in the primary osteoblast cells (D).

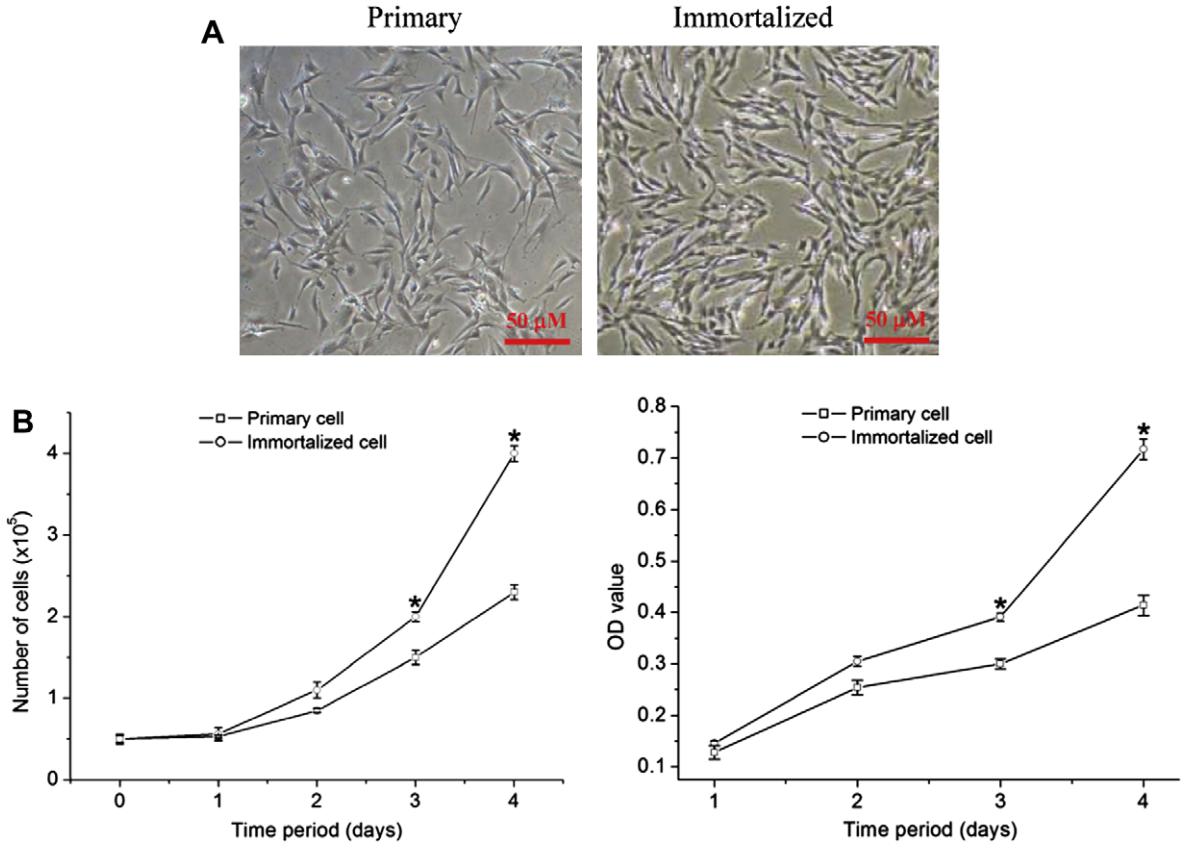

Fig. 2. Cell morphology and proliferation of mouse primary and immortalized osteoblast cells. (A) Primary and immortalized osteoblast cells were photographed under a light microscope using a Nikon Coolpix 4500 digital camera. (B) Proliferation data of primary and i*Bmp2/4* cells by cell counting and MTT assay. i*Bmp2/4* cells showed higher proliferation rate than primary cells after 3-day culture. Asterisk shows significant differences between the primary and immortalized cells ($p < 0.05$).

a primary anti-SV40 large T-antigen monoclonal antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 37 °C. Then the cells were washed with PBS containing 0.1% goat serum and incubated with the secondary antibody conjugated with Alexa Fluo® 568 red (Molecular Probes, Eugene, OR) for 1 h at room temperature. For negative control, the primary SV40 antibody was replaced by mouse IgG I (Dakocytomation, Carpinteria, CA). For cell nucleus staining, the cells were treated with Hoechst (Sigma–Aldrich). Images of Alexa Fluo® 568 red staining of the SV40 protein were obtained at the Core Optical Imaging Facility at UTHSCSA under the same parameters in a Nikon inverted microscope.

Alkaline phosphatase (ALP) assay. For detection of ALP activity, cultures of both primary and immortalized osteoblast cells for 3 weeks were 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).

Mineralization assay. i*Bmp2/4* and primary cells were plated in 6-well plates at a density of 4×10^5 per well and cultured in calcifying medium (a-MEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml), 50 μg/ml ascorbic acid, 10 nM dexamethasone and 10 mM sodium β-glycerophosphate) at 37 °C for 3 weeks, respectively. The cells were fixed in 10% formaldehyde neutral buffer solution and then stained with Alizarin Red S (Sigma–Aldrich).

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from i*Bmp2/4* and primary cells by RNA STAT-60 kit (Tel-Test, Inc., Friendswood, TX), treated with DNase I (Promega), and purified with the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). RNA concentration was determined at an optical density of OD_{260}. The RNA was transcribed into cDNA by SuperScript II reverse transcriptase (Invitrogen). Specific primers for the RT-PCR were shown in Table 1, and these included Gapdh, *Runx2*, *Dlx3*, *Osx, ATF4, Dmp1, Bsp*, *Opn*, *Oc*, *Osn*, ALP and collagen type I. The PCR was first denatured at 95 °C for 10 min, and then carried out at 95 °C for 60 s, at 50–60 °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 microliters of PCR products was analyzed by agarose gel with ethidium bromide staining. Corrective DNA was verified by DNA sequencing.

Immunohistochemistry. i*Bmp2/4* and primary cells were prepared as described above. For detection of osteoblast-related proteins, fluorescent immunohistochemistry was performed using antibodies directed against mouse *Bsp* and *Dmp1* (gifts from Dr. Larry Fisher, NIDCR), *Runx2*, *Osx*, *Opn*, *Oc*, and Col1A1 (Santa Cruz Biotechnology Inc.) and *Dlx3* (Abcam, Cambridge, MA). Negative control of mouse IgG I was purchased from Dakocytomation (Carpinteria, CA). Immunohistochemical assay was performed as described above with corresponding secondary antibodies conjugated with Alexa Fluor 488 green fluorescent labeling (Molecular Probes). Microphotographs were obtained under a Nikon microscope using a Nikon Cool pix 4500 digital camera.

Statistical analysis. All values were represented as the mean ± standard deviation (SD). Statistical significance was determined using the unpaired Student's *t*-test with a *p*-value of <0.05 being statistical significant.

Results

Generation of Bmp2 and Bmp4 double conditional mice

To establish *Bmp2* and *Bmp4* conditional mice, *Bmp2* conditional mice were crossed with *Bmp4* conditional animals. The floxed *Bmp2/4* mice were confirmed by PCR using specific *Bmp2* and *Bmp4* primers (Fig. 1A and Table 1) and Southern blot analysis (data not shown).

Immortalization of mouse floxed Bmp2/4 osteoblasts

To create immortalization of mouse floxed *Bmp2/4* osteoblast cell lines, primary cells were transfected with pSV3 neo plasmid containing SV40 T-Ag gene and then selected with G418. The G418 resistant cells were formed after 2–3 week selections. These

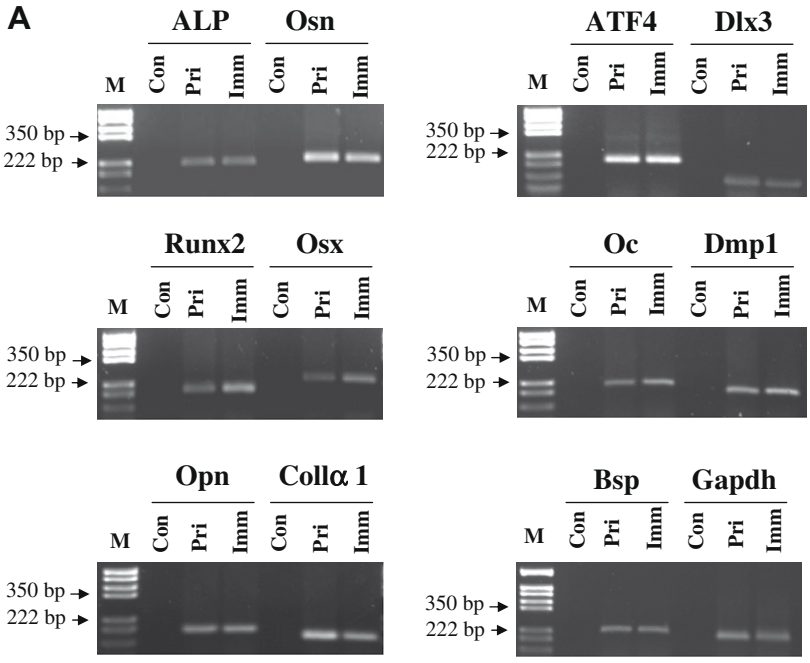

Fig. 3. Expression of bone-related genes in the primary and immortalized osteoblast cells. (A) Total RNAs from the primary and immortalized cells were extracted and reversely transcribed. The cDNAs were amplified by PCR using specific primers shown in Table 1. The PCR products were run on 1.2% agarose gels and stained with ethidium bromide. (B) Immunohistochemical staining with antibody to *Dlx3*, *Osx*, *Runx2*, *Bsp*, *Opn*, *Dmp1*, *Oc*, and collagen type I in primary mouse osteoblast cells at passage 3 and immortalized mouse i*Bmp2/4* cells at passage 50.

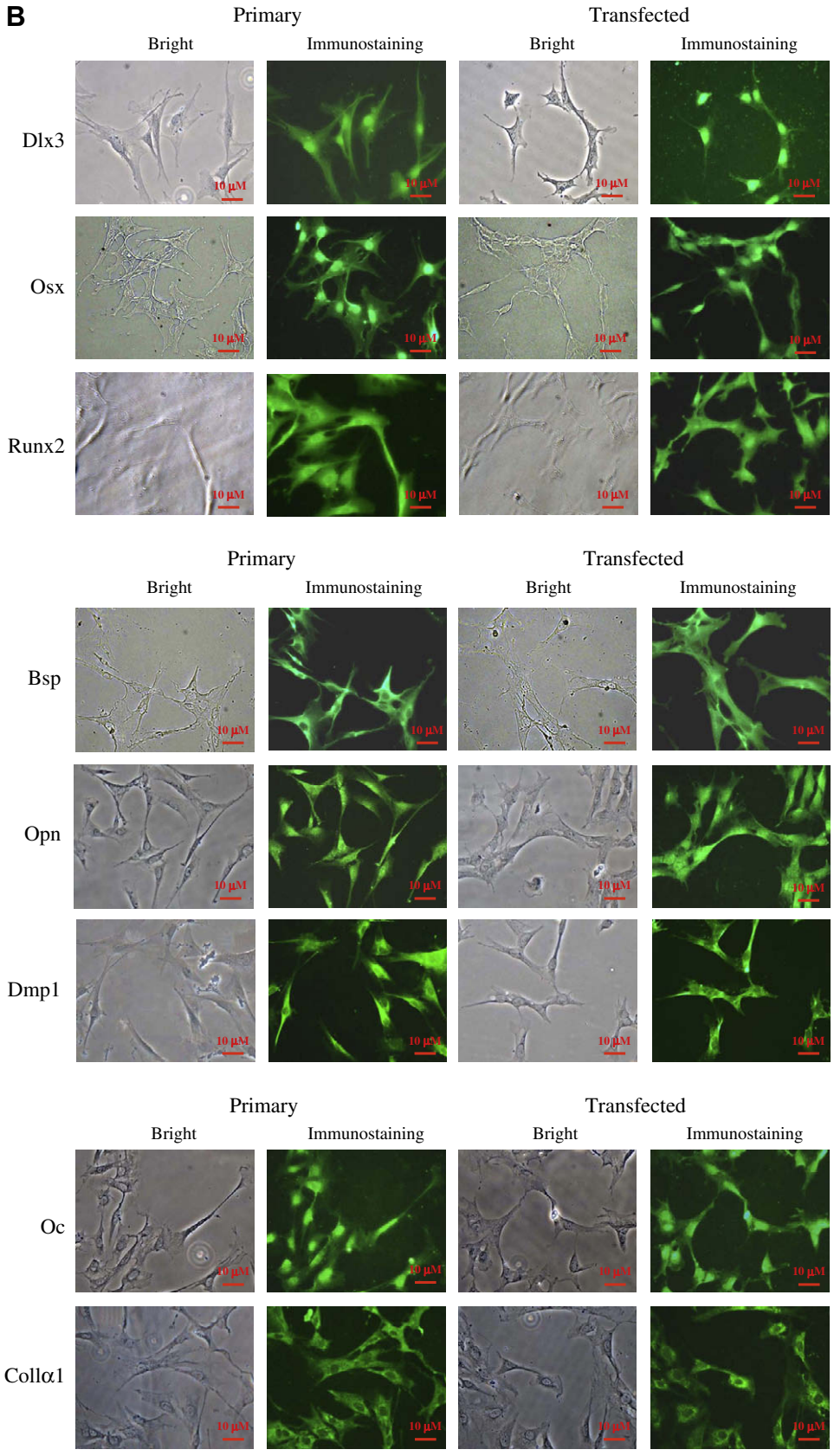

Fig. 3 (*continued*)

cells bypassed senescence and grew over 50 population doublings (PDLs) without significant growth retardation and termed as i*Bmp2/4*. On the other hand, non-transfected primary cells entered crisis at about 5 PDLs. The i*Bmp2/4* cells were passaged at 50 generation and used for detail characterization. PCR analysis shows that SV40 T-Ag gene was detected in i*Bmp2/4* cells and pSV3 neo

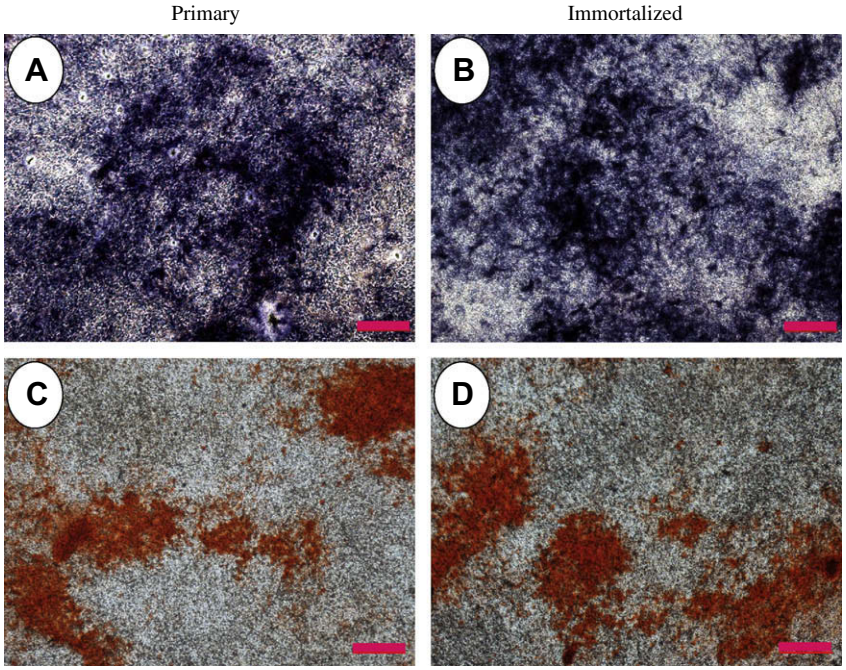

Fig. 4. Alkaline phosphatase (ALP) activity and mineralized nodule formation in the primary and immortalized osteoblast cells. (A, B) *In situ* histochemistry of ALP activity in the primary (A) and immortalized (B) cells was visualized after a culture of 3 weeks in calcifying media. (C, D) Both the primary and immortalized cells were treated with calcifying medium for 3 weeks. Nodule formation in the primary (C) and immortalized (D) cells was seen with Alizarin red staining. Scale bars show 100 μM.

plasmid, but not seen in the primary cells (Fig. 1B). Immunohistochemistry further confirmed that simian virus 40 T-Ag was expressed in all of i*Bmp2/4* cells whereas immunostaining was not present in the primary cells (Fig. 1C and D).

Morphology and proliferation of primary and immortalized cells

Cell morphology was studied using a light inverted microscopy (Fig. 2A). i*Bmp2/4* cells display spindle shape similar to that of the primary cells. There was no microscopical finding indicating senescence or neoplastic nature in i*Bmp2/4* cells. Proliferation of both the primary and i*Bmp2/4* cells appeared stable. However, the i*Bmp2/4* cells displayed a more rapid growth rate than the primary cells as revealed by daily cell counting and MTT assay (Fig. 2B).

Expression of tissue-specific genes in iBmp2/4 cells

To assess bone-related gene expressions in i*Bmp2/4* and primary cells, we studied expression of collagenous and non-collagenous as well as transcription factor genes using RT-PCR assay. Fig. 3A shows that both of the transformed and primary cells synthesized collagenous and non-collagenous protein genes such as collagen type I, ALP, *Bsp*, *Dmp1*, *Oc*, *Osn*, *Opn*. These cells also expressed osteoblast-specific transcription factors, *Runx2*, *Osx* and *ATF4* (Fig. 3A). Immunohistochemistry studies further confirmed that these cells expressed those proteins (Fig. 3B). These results indicate that i*Bmp2/4* cells retain their genotypic characteristics similar to the primary osteoblastic cells.

ALP expression and mineralization nodule formation

To evaluate the differentiation and mineralization activities of these cells, we examined ALP activity, one of osteoblast differentiation markers, by *in situ* ALP histochemistry. Fig. 4A shows that the positive expression of ALP in both the primary and i*Bmp2/4* cells after a culture of 3 weeks in calcifying media. Also, mineralized nodules in both the primary and immortalized osteoblast cells were seen by Alizarin Red S staining (Fig. 4B).

Discussion

In this study, we established an immortalized mouse floxed *Bmp2/4* osteoblast cell line, named i*Bmp2/4*, from primary mouse floxed *Bmp2/4* osteoblasts by transfection of SV40 T-Ag, a viral oncogene. i*Bmp2/4* cells present SV40 positive and retain high ALP activity and strong mineralization ability. Moreover, they express all of the identification markers of osteoblasts. These data suggest that the cell line i*Bmp2/4* is functionally active and displays genotypic and phenotypic characteristics similar to that of the primary osteoblast cells.

The strategy for this study was to introduce a pSV3 neo, a plasmid containing coding sequences of SV40 T-Ag and a neomycin-resistance gene into primary floxed *Bmp2/4* osteoblasts, as pSV3 neo has been demonstrated to be an effective agent for immortalization manipulation in a variety of studies [18–20]. Moreover, numerous studies have shown that the viral oncogene SV40 large T-antigen is capable of immortalizing various cells including bone-derived cell line RCT3 [21–24]. In the present experiment, we transfected SV40 into the mouse primary floxed *Bmp2/4* osteoblast cells, and the transfected i*Bmp2/4* cells express SV40 detected by PCR and immunostaining. The i*Bmp2/4* cells have been continuously cultured for over one year, indicating a stable genomic integration of SV40 T-antigen coding sequences.

Although it was reported that SV40 might alter the nature of cells such as growth with extension and cell morphology [25], cell morphology of immortalized cells transfected by SV40 similar to the primary ones was also observed [19,26,27]. In the present experiment, the growth pattern of the transfected cells is altered with a more rapid proliferation rate. However, the i*Bmp2/4* cells retain phenotypic and genomic characteristics with no obvious cell morphology changes. Osteoblast cells immortalized with SV40 large T-antigen are capable of maintaining the differentiated fea-

tures were also reported [28,29]. These characteristics include ALP activity, type I collagen synthesis, presence of osteopontin transcripts which could be stimulated with $1,25(OH)_2D_3$, and ability of producing mineralizing extracellular matrix. In this study, we examined the ALP activity, mineralized nodule formation and the expression of bone-related genes that act as osteoblast terminal phenotype identification markers [30,31]. Our results show that both the primary and transfected i*Bmp2/4* cells displayed strong mineralization ability and characteristics of bone-related gene expression.

In summary, we were able to establish a cell line of immortalized mouse floxed *Bmp2/4* osteoblast cells with differentiation and mineralization capability. The cell line can provide an excellent resource of large amounts suitable for *Bmp2/4* knock out experiments with the addition of Cre recombinase *in vitro*. Thus, we for the first time established the i*Bmp2/4* cells that can be a useful cell model for investigating the mechanism of *Bmp2/4* effects on osteoblast proliferation, differentiation and mineralization.

Acknowledgments

This work was supported in part by National Institutes of Health Grants DE019802 (S.C.), AR054616 (S.H.) and the grant from the Natural Science Foundation of China 30801293 (LA Wu) and 2009-10 3M ESPE Preventative Pediatric Dentistry Postdoctoral Research Fellowship of AAPD (LA Wu).

References

[1] A.H. Reddi, Bone morphogenetic proteins: an unconventional approach to isolation of first mammalian morphogens, Cytokine Growth Factor Rev. 8 (1997) 11–20.
[2] T. Sakou, Bone morphogenetic proteins: from basic studies to clinical approaches, Bone 22 (1998) 591–603.
[3] R.J. Wordinger, A.F. Clark, Bone morphogenetic proteins and their receptors in the eye, Exp. Biol. Med. 232 (2007) 979–992.
[4] N.R. Kubler, J.F. Reuther, G. Faller, T. Kirchner, R. Ruppert, W. Sebald, Inductive properties of recombinant human BMP-2 produced in a bacterial expression system, Int. J. Oral Maxillofac. Surg. 27 (1998) 305–309.
[5] Y. Takuwa, C. Ohse, E.A. Wang, J.M. Wozney, K. Yamashita, Bone morphogenetic protein-2 stimulates alkaline phosphatase activity and collagen synthesis in cultured osteoblastic cells, MC3T3-E1, Biochem. Biophys. Res. Commun. 174 (1991) 96–101.
[6] A. Yamaguchi, T. Katagiri, T. Ikeda, J.M. Wozney, V. Rosen, E.A. Wang, A.J. Kahn, T. Suda, S. Yoshiki, Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro, J. Cell Biol. 113 (1991) 681–687.
[7] R.D. Welch, A.L. Jones, R.W. Bucholz, C.M. Reinert, J.S. Tjia, W.A. Pierce, J.M. Wozney, X.J. Li, Effect of recombinant human bone morphogenetic protein-2 on fracture healing in a goat tibial fracture model, J. Bone Miner. Res. 13 (1998) 1483–1490.
[8] B.E. Bax, J.M. Wozney, D.E. Ashhurst, Bone morphogenetic protein-2 increases the rate of callus formation after fracture of the rabbit tibia, Calcif. Tissue Int. 65 (1999) 83–89.
[9] C.Y. Chung, A. Iida-Klein, L.E. Wyatt, G.H. Rudkin, K. Ishida, D.T. Yamaguchi, T.A. Miller, Serial passage of MC3T3-E1 cells alters osteoblastic function and responsiveness to transforming growth factor-beta1 and bone morphogenetic protein-2, Biochem. Biophys. Res. Commun. 265 (1999) 246–251.
[10] W. Huang, G.H. Rudkin, B. Carlsen, K. Ishida, P. Ghasri, B. Anvar, D.T. Yamaguchi, T.A. Miller, Overexpression of BMP-2 modulates morphology, growth, and gene expression in osteoblastic cells, Exp. Cell Res. 274 (2002) 226–234.
[11] C.A. Luppen, R.L. Chandler, T. Noh, D.P. Mortlock, B. Frenkel, BMP-2 vs. BMP-4 expression and activity in glucocorticoid-arrested MC3T3-E1 osteoblasts: Smad signaling, not alkaline phosphatase activity, predicts rescue of mineralization, Growth Factors 26 (2008) 226–237.
[12] T. Miyazaki, S. Miyauchi, A. Tawada, T. Anada, S. Matsuzaka, O. Suzuki, Oversulfated chondroitin sulfate-E binds to BMP-4 and enhances osteoblast differentiation, J. Cell. Physiol. 217 (2008) 769–777.
[13] S. Martinovic, F. Borovecki, V. Miljavac, V. Kisic, D. Maticic, I. Francetic, S. Vukicevic, Requirement of a bone morphogenetic protein for the maintenance and stimulation of osteoblast differentiation, Arch. Histol. Cytol. 69 (2006) 23–36.
[14] Z. Wang, C.C. Clark, C.T. Brighton, Up-regulation of bone morphogenetic proteins in cultured murine bone cells with use of specific electric fields, J. Bone Joint Surg. Am. 88 (2006) 1053–1065.
[15] J. Selever, W. Liu, M.F. Lu, R.R. Behringer, J.F. Martin, Bmp4 in limb bud mesoderm regulates digit pattern by controlling AER development, Dev. Biol. 276 (2004) 268–279.
[16] A. Bandyopadhyay, K. Tsuji, K. Cox, B.D. Harfe, V. Rosen, C.J. Tabin, Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis, PLoS Genet. 2 (2006) 2117–2130.
[17] H. Kulessa, B.L. Hogan, Generation of a loxP flanked bmp4loxP–lacZ allele marked by conditional lacZ expression, Genesis 32 (2002) 66–68.
[18] B. Thonemann, G. Schmalz, Immortalization of bovine dental papilla cells with simian virus 40 large t antigen, Arch. Oral Biol. 45 (2000) 857–869.
[19] K.M. Galler, H. Schweikl, B. Thonemann, R.N. D'Souza, G. Schmalz, Human pulp-derived cells immortalized with Simian Virus 40 T-antigen, Eur. J. Oral Sci. 114 (2006) 138–146.
[20] Y. Inoshima, N. Ishiguro, Establishment of vascular endothelial cell lines from the aortas of wild Japanese serows (*Capricornis crispus*), Cell Biol. Int. 33 (2009) 617–620.
[21] P.S. Jat, P.A. Sharp, Large T antigens of simian virus 40 and polyomavirus efficiently establish primary fibroblasts, J. Virol. 59 (1986) 746–750.
[22] C.D. Woodworth, H.C. Isolm, Regulation of albumin gene expression in a series of rat hepatocyte cell lines immortalized by simian virus 40 and maintained in chemically defined medium, Mol. Cell. Biol. 7 (1987) 3740–3748.
[23] G.I. Tennekoon, J. Yoshino, K.W. Peden, J. Bigbee, J.L. Rutkowski, Y. Kishimito, G.H. DeVries, G.M. McKhann, Transfection of neonatal rat Schwann cells with SV-40 large T antigen gene under control of the metallothionein promoter, J. Cell Biol. 105 (1987) 2315–2325.
[24] D.S. Neufield, S. Ripley, A. Hendersen, H.L. Ozer, Immortalization of human fibroblasts transformed by origin-defective simian virus 40, Mol. Cell. Biol. 7 (1987) 2794–2802.
[25] G.H. Yoo, J. Washington, J. Oliver, M. Piechocki, H. Kim, J. Foster-Nora, T.Y. Shibuya, D.R. Wilson, J.F. Ensley, The effects of exogenous p53 overexpression on HPV-immortalized and carcinogen transformed oral keratinocytes, Cancer 94 (2002) 159–166.
[26] M. MacDougall, F. Thiemann, H. Ta, P. Hsu, L.S. Chen, M.L. Snead, Temperature sensitive simian virus 40 large T antigen immortalization of murine odontoblast cell cultures: establishment of clonal odontoblast cell line, Connect. Tissue Res. 33 (1995) 97–103.
[27] T. Iwata, Y. Yamakoshi, J.P. Simmer, I. Ishikawa, J.C. Hu, Establishment of porcine pulp-derived cell lines and expression of recombinant dentin sialoprotein and recombinant dentin matrix protein-1, Eur. J. Oral Sci. 115 (2007) 48–56.
[28] J.K. Heath, S.B. Rodan, K. Yoon, G. Rodan, Rat calvarial cell lines immortalized with SV-40 large T antigen: constitutive and retinoic acid-inducible expression of osteoblastic features, Endocrinology 124 (1989) 3060–3068.
[29] J.K. Heath, L.J. Suva, K. Yoon, M. Kiledjian, T.J. Martin, G.A. Rodan, Retinoic acid stimulates transcriptional activity from the alkaline phosphatase promoter in the immortalized rat calvarial cell line, RCT-1, Mol. Endocrinol. 6 (1992) 636–646.
[30] F. Lecanda, D.A. Towler, K. Ziambaras, S.L. Cheng, M. Koval, T.H. Steinberg, R. Civitelli, Gap junctional communication modulates gene expression in osteoblastic cells, Mol. Biol. Cell 9 (1998) 2249–2258.
[31] E. Bergeron, H. Senta, A. Mailloux, H. Park, E. Lord, N. Faucheux, Murine preosteoblast differentiation induced by a peptide derived from BMP-9, Tissue Eng. Part A (2009) [Epub ahead of print].