Establishment of Immortalized BMP2/4 Double Knock-Out Osteoblastic Cells Is Essential for Study of Osteoblast Growth, Differentiation, and Osteogenesis

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Bone morphogenetic proteins 2 and 4 (BMP2/4) are essential for osteoblast differentiation and osteogenesis. Generation of a BMP2/4 dual knock-out (BMP2/4ko/ko) osteoblastic cell line is a valuable asset for studying effects of BMP2/4 on skeletal development. In this study, our goal was to create immortalized mouse deleted BMP2/4 osteoblasts by infecting adenoviruses with Cre recombinase and green fluorescent protein genes into immortalized murine floxed BMP2/4 osteoblasts. Transduced BMP2/4ko/ko cells were verified by green immunofluorescence and PCR. BMP2/4ko/ko osteoblasts exhibited small size, slow cell proliferation rate and cell growth was arrested in G1 and G2 phases. Expression of bone-related genes was reduced in the BMP2/4ko/ko cells, resulting in delay of cell differentiation and mineralization. Importantly, extracellular matrix remodeling was impaired in the BMP2/4ko/ko osteoblasts as reflected by decreased Mmp-2 and Mmp-9 expressions. Cell differentiation and mineralization were rescued by exogenous BMP2 and/or BMP4. Therefore, we for the first time described establishment of an immortalized deleted BMP2/4 osteoblast line useful for studying mechanisms in regulating osteoblast lineages.

J. Cell. Physiol. 231: 1189–1198, 2016. © 2015 The Authors. Journal of Cellular Physiology Published by Wiley Periodicals, Inc.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily. BMPs are initially identified by their capability to induce bone formation when implanted subcutaneously or intramuscularly in rodents (Urist, 1965; Wozney et al., 1988). To date, about 20 unique BMP ligands have been identified and compose at least four subgroups based on their amino acid sequence similarity (Sakou, 1998; Shi and Massague, 2003; Kishigami and Mishina, 2005). BMP2 and BMP4 are most similar to decapentaplegic (Dpp) in Drosophila melanogaster and belong to the BMP2/4 subclass as both of the two ligands exhibit a high affinity for the extracellular ligand binding domains of the type I BMP receptor (Hayward et al., 2002; Shi and Massague, 2003). The capacity of BMP2 to induce osteoblast differentiation has been rigorously demonstrated (Takuwa et al., 1991; Yamaguchi et al., 1991; Kubler et al., 1998; Welch et al., 1998; Bax et al., 1999; Chung et al., 1999; Wu et al., 2011). Moreover, BMP4 also plays an important role in osteogenesis (Martinoic et al., 2006; Wang et al., 2006; Luppen et al., 2008; Miyazaki et al., 2008). However, it is difficult to decipher unique roles of BMP2 and/or BMP4 during osteogenesis because of their functional redundancy each other (Selever et al., 2004). BMP2/4 are involved in organ development (Reversade et al., 2005; Ceyalo et al., 2007; Goldman et al., 2009; Uchimura et al., 2009). Mice with BMP2/4 conditional knock-out (cKO) exhibited severe impairments of osteogenesis and displayed different genotypic and phenotypic characteristics compared to that of BMP2 or BMP4 null mice (Bandyopadhyay et al., 2006). Furthermore, clinical investigations showed that variants in BMP2/4 genes are susceptible to otosclerosis and other diseases (Schrauwen et al., 2008; Tomlinson et al., 2011; Mu et al., 2012). Otosclerosis is a common form of adult-onset conductive hearing loss resulting from abnormal bone remodeling of the bony labyrinth that surrounds the inner ears. Genotyping pups bred between BMP2 and BMP4 heterozygous mice revealed that the ratio of adult compound heterozygous mice for BMP2/4 is much lower (Uchimura et al., 2009). Therefore, generation of a dual BMP2/4ko/ko osteoblastic cell line would be a valuable asset for studying the modulatory effects of BMP2/4 on osteoblast differentiation.
and relevant molecular events involved in bone-relate gene expression and extracellular matrix remodeling.

In the present study, we established an immortalized mouse deleted BMP2/4 osteoblast cell line using Cre-recombinase to simultaneously knock-out BMP2 and BMP4 genes in immortalized mouse floxed BMP2/4 osteoblastic cells and observed these cell behaviors. We further examined cell growth as well as their genotypic and phenotypic characteristics. Finally, we tested whether biological functions of these BMP2/4KO/KO cells were rescued by exogenous BMP2 and/or BMP4.

Materials and Methods

Generation of immortalized deleted BMP2/4 osteoblastic cells

The immortalized mouse floxed BMP2/4 osteoblasts (iBMP2/4fl/fl) ob were maintained in alpha minimum essential medium (a-MEM, Invitrogen, San Diego, CA) containing 10% fetal calf serum (FCS) plus penicillin (100 U/ml) and streptomycin (100 mg/ml) and cultured in 5% CO2 atmosphere at 37°C. Detail generation of iBMP2/4fl/fl ob cells were described by our previous study (Wu et al., 2009). For BMP2/4 double knock-out, adenoviruses with Cre recombinase and green fluorescent protein (Ad-Cre-GFP, Vector Biolabs, Malvern, PA) were added to the cells at 37°C. The cells were transduced overnight and then recovered in the cultured medium. GFP positive cells were observed using a Nikon inverted fluorescent microscope. The several GFP positive cells were selectively picked up and re-plated at low densities to obtain further cell growth. Genomic DNAs were isolated from the iBMP2/4fl/fl ob and immortalized mouse BMP2/4 knock-out osteoblasts (iBmp2/4ko/ko ob) using DNA purification kit, Wizard genomic (Promega, Madison, WI). PCR genotyping was performed by amplification of the BMP2/4fl/fl and BMP2/4ko/ko alleles using specific primers for BMP2 and BMP4 (Table I). PCR conditions: 4 min at 94°, 35 cycles of 1 min at 94°C, 1 min at 58–64°C and 2 min at 72°C, followed by 10 min at 72°C. The amplified products were run on 1% agarose gels.

Cell proliferation and morphology assays

Cell proliferation assay was identified by 5-bromo-2′-deoxyuridine (BrdU) incorporation and MTT method. Briefly, cells were plated into 6-well glass slides and incubated with 30 μM BrdU (Sigma–Aldrich, St. Louis, MO) in culture medium for 4 h. The cells were then treated with a mouse monoclonal anti-BrdU antibody (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA), followed by a 1:1,000 dilution of the secondary antibody with Alexa Fluor 488 green (Molecular Probes, Eugene, OR). For nucleus staining, cells were plated on coverslips and treated either with or without recombinant BMP2 (rBMP2, 100 ng/ml) and/or rBMP4 (20 ng/ml) (R&D Systems, Minneapolis, MN). After rBMPs induction, the cell differentiation and mineralization were detected using ALP and alizarin red S analyses.

Alkaline phosphatase (ALP) and mineralization assays

For detection of ALP activity, cultures of the iBMP2/4fl/fl and iBMP2/4ko/ko ob cells 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 MgCl2). In situ ALP staining was performed according to the supplier’s instructions (Bio-Rad Laboratories, Hercules, CA). For mineralization assay, these cells were seeded into 6-well plates and cultured in calcifying medium (α-MEM supplemented with 5% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, 10 mM dexamethasone and 10 mM sodium β-glycerophosphate) at 37°C on given time points. The cells were fixed in 10% formaldehyde neutral buffer and then stained with alizarin red S dye (Sigma–Aldrich). The amount of calcium deposition was quantified by de-staining with 10% cetylpyridinium chloride (Sigma–Aldrich) in 10 mM sodium phosphate at room temperature for 20 min. The absorbance was measured at 550 nm wavelength.

Induction of osteoblast differentiation and mineralization by recombinant BMP2 and/or BMP4

The iBMP2/4ko/ko ob cells were maintained in α-MEM medium with 10% FCS plus 100 unit/ml penicillin and 100 μg/ml streptomycin. The cells were then grown in α-MEM medium with 1% FCS plus 100 unit/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, 10 mM dexamethasone and 10 mM sodium β-glycerophosphate and treated either with or without recombinant BMP2 (rBMP2, 100 ng/ml) and/or rBMP4 (20 ng/ml) (R&D Systems, Minneapolis, MN). After rBMPs induction, the cell differentiation and mineralization were detected using ALP and alizarin red S analyses.

Quantitative real time polymerase chain reaction

Total RNA was extracted from the iBMP2/4fl/fl and iBMP2/4ko/ko ob cells using 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 OD260. The RNA was transcribed into cDNA by SuperScript II reverse transcriptase (Life technologies, Grand Island, NY). Specific primers for the quantitative real time polymerase chain reaction (qRT-PCR) were shown in Table I. qRT-PCR amplification reaction was analyzed in real time on an ABI 7500 (Applied Biosystems, Foster City, CA) using SYBR Green chemistry, and threshold values were calculated using SDSS2 software (Applied Biosystems). The ∆ΔCt method was used to calculate gene expression levels normalized to cyclophilin A value. The results were performed in triplicate of three separate experiments and expressed as a relative fold change in gene expression compared to the control.

Western blot analysis

Cells were maintained in α-MEM medium with 5% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, 10 mM dexamethasone and 10 mM sodium β-glycerophosphate and were then washed with PBS and lysed with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml and 1% Triton X-100). The cellular proteins were resolved using SDS–PAGE and transferred to nitrocellulose membranes for Western blot analyses. The membranes were probed with primary antibodies directed against p21, p27, and osterix. The intensity of the band was determined using the Odyssey Imaging System (LI-COR).
phenylmethylsulfonyl fluoride (PMSF), 50 KIU/ml aprotinin, 100 mM sodium orthovanadate; Santa Cruz Biotechnology, Inc.). Whole cell lysates were resolved by 7% SDS-PAGE gels and transferred to Trans-Blot membranes (Bio-Rad, Laboratories). Antibodies directed against mouse Bsp and Dmp1 (gifts from Dr. Larry Fisher, NIDCR), CREB-2 (ATF-4), Collα1, Mmp-2, Mmp-9, Oc, Opn, Osn (SPARC), Osx, PCNA, Rsk-2, Runx2 (Santa Cruz Biotechnology Inc.) and Dlx3 (Abcam, Cambridge, MA) were used as primary antibodies. The membranes were blocked with 5% non-fat milk in TBST buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20) for 60 min at room temperature. After washing, the membranes were incubated with primary antibodies against those proteins with appropriate dilution (1:500-1,000) overnight at 4°C. The GFP positive cells were observed under a Nikon inverted fluorescent microscope. (C and D) Genotyping and PCR strategy. Genomic DNAs from the iBMP2/4fx/fx and iBMP2/4ko/ko ob cells were isolated and amplified by the BMP2/4 specific primers shown in Table I. (C) primers, a and b, c and d, amplify fragments of 400, 403 bp from iBMP2/4fx/fx osteoblasts; primers, a and e, amplify fragment of 564 bp from iBMP2/4ko/ko osteoblasts. (D) primers, f and g, h and i, amplify fragments of 184, 216 bp from iBMP2/4fx/fx osteoblasts; primers, f and j, amplify fragment of 418 bp from iBMP2/4ko/ko osteoblasts. The amplified PCR products were run on 1% agarose gels and stained with ethidium bromide. Lane 1, lower molecular DNA marker; lane 2, negative control. Lanes 3 and 4. Genomic DNAs isolated from the iBMP2/4fx/fx and iBMP2/4ko/ko ob cells were amplified using the floxed BMP2 and BMP4 primers, respectively. Lanes 5 and 6. Genomic DNAs isolated from the iBMP2/4fx/fx and iBMP2/4ko/ko ob cells were amplified using the recombinant BMP2 and BMP4 primers, respectively. Lanes 7 and 8. Genomic DNAs isolated from the iBMP2/4fx/fx and iBMP2/4ko/ko ob cells were amplified using the BMP2 exon 3 and BMP4 exon 4 primers, respectively. (E) The iBMP2/4fx/fx and iBMP2/4ko/ko ob cells were photographed under a Nikon inverted microscope. (F) The iBmp2/4ko/ko ob cells were treated with or without BMP2 (100 ng/ml) plus BMP4 (20 ng/ml) for 48 h and cell morphology was observed under the microscope. (G) Cell length of iBMP2/4fx/fx, iBMP2/4ko/ko ob cells, and iBMP2/4ko/ko ob cells treated with BMP2/4 was quantitated. (H) Morphology of the iBMP2/4fx/fx and iBMP2/4ko/ko ob cells was observed using scanning electron microscope. Fx, floxed; Con, control; Rec, recombinant; Exon, BMP2 exon 3; BMP4 exon 4; ko, knock-out; n, number. *P < 0.01.
The DQ-FITC-collagen types I, IV, and DQ-FITC-gelatin were formaldehyde. After washing with PBS, the slides coated with washing with PBS, the slides were air dried and concentration of 40 ng/ml. Glass slides were pre-coated with DQ-FITC-collagen types I, IV, and DQ-FITC-gelatin (Life technologies) at a concentration of 40 ng/ml. In situ DQ-FITC-collagen types I, IV, and -gelatin degradation assays

Gelatin degradation was detected by Coomassie brilliant blue staining. Gelatinolytic activities of Mmps were analyzed using 10% SDS-PAGE gels and stained with Coomassie brilliant blue dye. Gelatinolytic activities of Mmps were analyzed using 10% SDS-PAGE gels and equilibrated with α-MEM without serum. The iBMP2/4ko/ko and iBMP2/4flox/flox cells were added to the plates containing the DQ-FITC-collagen type I or IV or DQ-FITC-gelatin coated slides and cultured for 12 h, respectively. The cells were fixed with 4% formaldehyde for 15 min and washed with PBS. Then, the cells were mounted using Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA). Images were taken using an inverted fluorescent microscope coupled to a CCD camera and NIH-GIEMENTS software. Spots of processing collagen type I, IV, and gelatin in the two cells were quantitated.

**Statistical analysis**

Quantitative data were presented as means ± S.D. with triplicate from three independent experiments and compared with the results of one-way ANOVA using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). The differences between groups were statistically significant at *P* < 0.05 and **P** < 0.01. We selected the transduced cells showed a high efficiency of infection observed under a Nikon fluorescent microscope.

**Results**

**Generation of immortalized mouse BMP2/4 knock-out osteoblastic cells**

To establish BMP2/4 knock-out osteoblasts (iBMP2/4ko/ko), the immortalized mouse floxed BMP2/4 osteoblastic cells (iBMP2/4flox/flox) were transduced with Ad-Cre-GFP and then selected. The transduced cells showed a high efficiency of infection observed under a Nikon fluorescent microscope.
Several GFP positive clones were selected and re-grown. Deletion of BMP2/4 genes by Cre recombinase in the iBMP2/4fx/fx ob cells was confirmed by using PCR (Fig. 1C and D; Table I). This result showed that Cre recombinase knocks out both BMP2/4 genes in the iBMP2/4fx/fx ob cells. Cell morphology between the iBMP2/4fx/fx and iBMP2/4ko/ko ob cells was observed using light inverted and scanning electron microscopes (Fig. 1E, G, and H). The iBMP2/4fx/fx ob cells show a spindle shape and long branches whereas the iBMP2/4ko/ko ob cells exhibit short branches. Also, size of the iBMP2/4ko/ko osteoblasts is smaller than their floxed counterparts. Morphology of the iBMP2/4ko/ko ob cells was changed by the recombinant BMP2/4 protein induction, displaying long branches similar to the iBMP2/4fx/fx ob cells (Fig. 1F and G).

Deletion of BMP2/4 leads to change of cell proliferation rate

To study the effect of BMP2/4 on mouse osteoblast proliferation, the iBMP2/4fx/fx and iBMP2/4ko/ko ob cell proliferation was analyzed using BrdU and MTT assays. This result showed that the iBMP2/4ko/ko ob cells display a slow growth rate compared to the iBMP2/4fx/fx ob cells (Fig. 2A–C). Exogenous BMP2/4 proteins were able to promote the knock-out osteoblast cell proliferation (Fig. 2D–F). To assess which mechanisms of BMP2/4 control cell proliferation, we analyzed the cell cycle distribution of the iBMP2/4fx/fx and iBMP2/4ko/ko ob cells. The data indicated that the G2 phase is delayed in the iBMP2/4ko/ko ob cells when these cells were cultured with 10% serum (Fig. 2Ga and b).
However, when both of the cells were grown without serum condition, cell cycles in the G1 and G2 phases were interfered in the iBMP2/4<sup>ko/ko</sup> ob cells (Fig. 2Gc and d). These results suggested that the slow growth of the iBMP2/4<sup>ko/ko</sup> ob cells is involved in the G1 and G2 phase arrest. Furthermore, we tested several genes related to cell proliferation and found that expression levels of the proliferating cell nuclear antigen (PCNA) and ribosomal S6 kinase-2 (Rsk-2) were decreased in the iBMP2/4<sup>ko/ko</sup> ob cells, whereas overexpression of BMP2/4 in the iBMP2/4 knock-out osteoblasts increased PCNA and Rsk-2 expression (Fig. 2H).

**Deletion of BMP2/4 causes delay of osteoblast differentiation and mineralization**

To assess the effect of BMP2/4 on cell differentiation and mineralization activities, we measured ALP activity by in situ ALP histochemistry as ALP is a marker of osteoblast differentiation. Both cells were cultured in calcifying medium in given time periods. This result showed that delayed cell differentiation is seen in the iBMP2/4<sup>ko/ko</sup> ob cells (Fig. 3A). Also, deletion of BMP2/4 genes led to low activity of cell mineralization by using alizarin red S staining (Fig. 3B and C). Furthermore, when exogenous BMP2 and/or BMP4 proteins were added to the iBMP2/4<sup>ko/ko</sup> ob cells, these cell differentiation and mineralization were rescued by either recombinant BMP2 or BMP4. Exogenous BMP2/4 had synergic effects on these osteoblast differentiation and mineralization (Fig. 3D and E).

**Knock-out of BMP2/4 down-regulates their downstream gene expression**

To further determine which gene expression is regulated by BMP2/4 in cell differentiation and mineralization, we measured bone-relate gene expression including transcription factors, extracellular matrix proteins and proteases. Using qRT-PCR, we observed decreases of bone-relate gene expressions in the BMP2/4<sup>ko/ko</sup> ob cells (Fig. 4A). It includes Bsp, Colla1, CREB-2, Dlx3, Dmp1, Oc, Osn, Oxs, and Runx2. Also, expression of matrix metalloproteinases, Mmp-2 and Mmp-9, was reduced. These results were also verified by Western blot assay (Fig. 4B and C). Furthermore, these downstream gene expressions were induced in the iBMP2/4<sup>ko/ko</sup> ob cells when BMP2/4 proteins were added into the deleted iBMP2/4 osteoblastic cells (Fig. 4D).
BMP2/4 activate extracellular matrix remodeling through Mmp-2 and Mmp-9

Expression of Mmp-2 and Mmp-9 was decreased in the iBMP2/4\(^{\text{ko/ko}}\) ob cells. As Mmp-2 and Mmp-9 are involved in physiological and pathological roles including extracellular matrix remodeling (Lund et al., 2011), we then investigated these proteinase secretion. Using zymography assay, we found that secretion of Mmp-2 and Mmp-9 in the iBMP2/4\(^{\text{ko/ko}}\) ob cells was lower than that of the iBmp2/4\(^{\text{flox/flox}}\) ob cells (Fig. 5A). To further investigate roles of Mmp-2 and Mmp-9 in extracellular matrix remodeling, in situ degradation of collagen types I, IV, and gelatin was measured as collagen types I, IV, and gelatin are substrates of Mmp-2 and Mmp-9 (Chaussain-Miller et al., 2006). The few and faint fluorescent spots of collagen types I, IV, and gelatin degradations were observed in the iBMP2/4\(^{\text{ko/ko}}\) ob cells compared to that of the iBMP2/4\(^{\text{flox/flox}}\) ob cells (Fig. 5Cc and d; Dc and d; Ec and d; F–H). This result indicates that BMP2/4 regulate extracellular matrix remodeling by regulating Mmp-2 and Mmp-9 activities.

Discussion

The results of this study have demonstrated, after infection of the immortalized murine iBMP-2/4\(^{\text{flox/flox}}\) osteoblasts with Ad-Cre-GFP, the creation of an immortalized iBMP2/4\(^{\text{ko/ko}}\) osteoblastic cell line. Successful infection was confirmed through GFP immunofluorescence of the cells, and BMP2/4 gene knock-out in the iBMP2/4\(^{\text{flox/flox}}\) ob cells was further verified using PCR assay. As the iBMP2/4\(^{\text{flox/flox}}\) osteoblasts show similar genotypic and phenotypic characteristics to the primary mouse normal osteoblasts (Wu et al., 2009), the advantage of
generation of these BMP2/4 cells is to obtain lot of BMP2/4fx/fx and BMP2/4ko/ko ob cells and used to study molecular mechanisms of BMP2/4 during osteoblast lineages and osteogenesis in vitro. In this study, we noted that the morphology of the iBMP2/4ko/ko osteoblasts in size is smaller than the iBMP2/4fx/fx ob cells and the iBMP2/4ko/ko osteoblast growth is slow compared to the floxed iBMP2/4 osteoblasts. Further study demonstrated that the cell cycle of the iBMP2/4ko/ko ob cells is arrested in the G1 and G2 phases, resulting from decreases of PCNA and Rsk-2 gene expression (Prosperi et al., 1994; Kawabe et al., 2002; Cude et al., 2007; Wu et al., 2014). Other studies have also shown that BMP2 and BMP4 are able to induce growth and differentiation of osteogenic cells in rats (Sakou, 1998). This finding demonstrates that BMPs are not only necessary for osteogenesis and ECM regulation, but also play a vital role in the life of the cell cycle and their ability to survive.

The iBMP2/4ko/ko osteoblasts demonstrated a diminished osteogenic phenotype as observed by lighter ALP activity and lowered capacity to mineralize as detected by alizarin red S staining. These results coincide with previous studies in which BMP2/4 cKO genes in mouse limbs caused severe skeletal defects (Bandyopadhyay et al., 2006). Rescue of the iBMP2/4 knock-out osteoblast differentiation and capacity for mineralization was noted with the addition of exogenous BMP2 and/or BMP4. The study further demonstrated that BMP2/4 knock-out osteoblast differentiation and capacity for mineralization was noted with the addition of exogenous BMP2 and/or BMP4. The study further demonstrated that BMP2/4 had synergic effects on the iBMP2/4ko/ko osteoblast differentiation and mineralization.

The iBMP2/4ko/ko osteoblasts, in conjunction with a reduced capacity for osteoblast differentiation and mineralization, also have a significant reduction in the expression of osteogenic genes. These include bone-relate extracellular matrix proteins, Bsp, Colla1, Dmp1, Oc, and Osn; transcription factors, CREB-2, Dlx3, Osx, and Runx2; and proteinases, Mmp-2 and

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**Fig. 5.** Deletion of BMP2/4 genes leads to impairment of extracellular matrix remodeling. (A) The equal amount of supernatant collected from the iBMP2/4fx/fx and iBMP2/4ko/ko cells was analyzed using gelatinolytic activities. Expression of Mmp-2 and Mmp-9 in the iBMP2/4ko/ko cells was decreased. (B) The equal amount of supernatant harvested from the iBMP2/4fx/fx and iBMP2/4ko/ko cells was loaded onto a 10% SDS-PAGE gel and stained by Coomassie brilliant blue dye. (C–E) The iBMP2/4fx/fx and iBMP2/4ko/ko ob cells were grown on the DQ-FITC-collagen types I, IV, and DQ-FITC-gelatin-coated slides for 12 h. The cells were fixed and degraded spots of the DQ-FITC-collagen types I, IV, and DQ-FITC-gelatin were observed using Nikon inverted fluorescent microscope (c and d). (a and b) The cells were photographed under a light inverted microscope. (e and f) The cells were treated with Hoechst dye for nuclei staining, (g and h) The images were merged. (F–H) Spots of the cleaved collagen type I, IV, and gelatin were quantitated from the iBMP2/4fx/fx and iBMP2/4ko/ko ob cells. There are significant differences between the iBMP2/4fx/fx and iBMP2/4ko/ko ob cells. *P < 0.05; **P < 0.01. Scale bars, 20 μm. fx, floxed; ko, BMP2/4 knock-out.
Chaussain-Miller C, Fioretti F, Goldberg M, Menashi S. 2006. The role of matrix
degradation and cell differentiation and mineralization. Knock-out of BMP2/4
mutations are associated with autosomal dominant genetic disorder called tricho-dento-osseous syndrome (Price et al., 1999). This reduction in gene expression explains the loss of osteogenic activity in the iBMP2/4+/− osteoblasts as compared to the iBMP2/4+/+ osteoblasts.

Along with a reduction in expression of genes involved in osteogenesis, there was also a decrease in genes associated with ECM formation and remodeling. CollaI, Mmp-2, and Mmp-9. Expression of Mmp-2 and Mmp-9 was dramatically reduced in the iBMP2/4+/− osteoblasts as detected by qRT-PCR. Western blot and zymography assays. In situ
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Conclusively, we have created an immortalized mouse deleted BMP2/4 osteoblast line for utilization in in vitro studies of BMP2/4 activity in osteogenesis. When characterized, the iBMP2/4+/− osteoblasts demonstrated delay of cell growth capability and cell cycle is arrested in the G1 and G2 phases as well as decreased expression of cell proliferation genes, PCNA and Rsk-2. Deletion of BMP2/4 caused decreased expression of osteogenic genes, resulting in retardation of cell differentiation and mineralization. Knockout of BMP-2 genes also reduced the osteoblast ability to degrade ECM via Mmp signaling, confirming BMP2/4 roles in regulating ECM formation. Therefore, the generated cell line would provide a useful tool for studies of the molecular mechanisms involved in regulating BMP2/4 osteoblast cell proliferation, differentiation and extracellular matrix remodeling during osteogenesis and bone regeneration.

Acknowledgments
We are grateful to core facility center at The University of Texas Health Center at San Antonio, Texas performed cell cycle experiments. This research was supported by the National Institutes of Health (NIH), National Institute of Dental and Craniofacial Research (NIDCR, DE19892) and partially by the Natural Science Foundation of China (81170929).

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zymography demonstrated in the iBMP2/4+/− osteoblasts weak
– strong activity of matrix metalloproteinase (MMP) 2 and 9. Changes in MMP expression reflect the role of MMPs in ECM remodeling during osteogenesis and bone regeneration.

Conclusively, we have created an immortalized mouse deleted BMP2/4 osteoblast line for utilization in in vitro studies of BMP2/4 activity in osteogenesis. When characterized, the iBMP2/4+/− osteoblasts demonstrated delay of cell growth capability and cell cycle is arrested in the G1 and G2 phases as well as decreased expression of cell proliferation genes, PCNA and Rsk-2. Deletion of BMP2/4 caused decreased expression of osteogenic genes, resulting in retardation of cell differentiation and mineralization. Knockout of BMP-2 genes also reduced the osteoblast ability to degrade ECM via Mmp signaling, confirming BMP2/4 roles in regulating ECM formation. Therefore, the generated cell line would provide a useful tool for studies of the molecular mechanisms involved in regulating BMP2/4 osteoblast cell proliferation, differentiation and extracellular matrix remodeling during osteogenesis and bone regeneration.

Acknowledgments
We are grateful to core facility center at The University of Texas Health Center at San Antonio, Texas performed cell cycle experiments. This research was supported by the National Institutes of Health (NIH), National Institute of Dental and Craniofacial Research (NIDCR, DE19892) and partially by the Natural Science Foundation of China (81170929).

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