O45-REGULATION OF OSTEOBLAST DIFFERENTIATION AND ECM REMODELING BY BMP2/4 IN VITRO

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Absract

Both of bone morphogenetic proteins 2 and 4 (Bmp2 and Bmp4) are two closely related members of the transforming growth factor beta superfamily and play diverse roles in normal and pathological processes. However, detail understandings of mechanisms through which Bmp2 and Bmp4 exert their effects remain elusive due to their functional compensations each other. To study roles of Bmp2/Bmp4 in osteoblast differentiation and extracellular matrix (ECM) remodeling, calvarial osteoblasts from Bmp2/4 conditional mice with Cre recombinase recognition site (loxP) were isolated and transfected with simian virus 40 large T antigen to generate immortalized BMP2$^{C/C}$4$^{C/C}$ (iBMP2 $^{C/C}$/4$^{C/C}$) osteoblast lines. The BMP2/4 genes in the iBMP2 $^{C/C}$/4$^{C/C}$ cells were double knocked out by Ad-Cre recombinase infection. Differentiation and mineralization of iBMP2$^{C/C}$/4$^{C/C}$ knock-out (iBmp2$^{C/C}$/4$^{C/C}$ KO) cells were detected by alkaline phosphatase (ALP) and alizarin (ALZ) red S staining analyses. ECM remodeling was also observed in fluorescent microscope. Cell differentiation was dramatically decreased in the iBMP2$^{C/C}$/4$^{C/C}$ KO cells compared to that of the iBMP2$^{C/C}$/4$^{C/C}$ osteoblasts. Mineralization was also reduced in these KO cells by ALZ staining. Furthermore, Bmp2/4 double knockout cells have major defects in remodeling the ECM as reflected by changes in collagen type I processing. Here we for the first time demonstrate the establishment of iBmp2$^{C/C}$/4$^{C/C}$ KO osteoblasts. Cell differentiation and mineralization in the iBmp2$^{C/C}$/4$^{C/C}$ KO cells were decreased. Furthermore, ECM processing in these KO cells was impaired. This indicates that BMP2/4 play important roles in osteoblast differentiation and ECM remodeling.

Key words: BMP2/4; Conditional Knockout; Osteoblast; Differentiation; Extracellular matrix; Remodeling;

Introduction

Bone morphogenetic proteins (BMPs) were discovered as potent inducers of ectopic bone formation when implanted subcutaneously (Urist, 1965) [1]. They are structurally related to transforming growth factor-β and contain over 20 different BMPs as well as can be subclassified into four groups depending on their structures [2]. BMP2 and BMP4 are highly similar to each other and most similar to Decapentaplegic in Drosophi, which belong to the BMP2/4 subclass [3]. The capacity of both BMP2/4 to induce osteoblastic differentiation has been rigorously demonstrated by recombinant BMP2/4 or BMP2/4 overexpression [4, 5]. However, it has been difficult to decipher the specific roles of BMP2 or BMP4 during osteoblast differentiation, and a thorough understanding of the mechanism through which BMP2/4 affect osteoblasts remains elusive due to their functional compensations [6, 7].

Although removal of any individual ligand has not displayed any defects in skeletal differentiation, mice simultaneously lacking BMP2 and BMP4 displayed severe impairment of osteogenesis[6,7]. This indicates that knock out of both BMP2 and BMP4 would be an efficient way for studying the modulatory effects of BMP2 and BMP4 on osteoblast differentiation, and generation of a BMP2/4 double knockout osteoblast cell line would be a valuable tool for role and mechanism studies in vitro.

Our objectives were to establish an immortalized Bmp2/4 double null (iBmp2$^{C/C}$/4$^{C/C}$ KO) osteoblast cell line and study the effects of Bmp2/4 double knockout on osteoblast differentiation and extracellular matrix (ECM) remodeling.

Material and Methods

Cell culture, adenovirus infection and colony selection. iBmp2$^{C/C}$/4$^{C/C}$ (immortalized floxed Bmp2/4) cells [8]were plated in 6-well plates at a density of 1 10^4 per well, grown with alpha minimum essential medium (a-MEM, Invitrogen, San Diego, CA) containing 10% fe-

tal 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. Four hours later, the cells were infected with Ad-Cre-GFP (Vector Biolabs, Philadelphia, PA) according to the manufacturer's instructions with 1000 MOI. Forty eight hours after infection, GFP signals were observed under a fluorescence microscope (Nikon TE62000), the well-isolated colonies with GFP positive were removed selectively and seeded at low densities to obtain the secondary selection. One of the selected secondary colonies, named as iBmp2$^{C/C}$/4$^{C/C}$ KO (immortalized BMP2$^{C/C}$/4$^{C/C}$ knock-out) cells, was expanded after reaching confluence and used for the following experiments.

Detection of BMP2/4 Knockout. Specific primers for BMP2 and BMP4 were synthesized with annealing temperatures listed in table 1. Genomic DNA was isolated from iBmp2$^{C/C}$/4$^{C/C}$ and iBmp2$^{C/C}$/4$^{C/C}$ KO cells. Two-hundred nanograms of DNA were diluted in a 25 μl polymerase chain reaction (PCR) mix (Sigma-Aldrich). Five μl of PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Correct DNA was verified by DNA sequencing.

Detection of SV40 expression. For detection of SV40 gene and protein expression in iBmp2$^{C/C}$/4$^{C/C}$ KO cells, PCR and fluorescent immunohistochemistry were performed as indicated previously [8].

Alkaline phosphatase(ALP) enzyme in situ histochemistry. For detection of ALP activity, cultures of both iBmp2$^{C/C}$/4$^{C/C}$ and iBmp2$^{C/C}$/4$^{C/C}$ KO cells osteoblast cells for 2 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).

Mineraliztaion assay. iBmp2$^{C/C}$/4$^{C/C}$ and iBmp2$^{C/C}$/4$^{C/C}$ KO 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 unit/ml) and streptomycin (100 μg/ml), 50 μg/ml ascorbic acid, 10 nM dexamethasone and 10 mM sodium β-glycerophosphate) at 37°C for 2 weeks respectively. The cells were fixed in 10% formaldehyde neutral buffer solution and then stained with Alizarin Red S (Sigma-Aldrich).

In Situ DQ-FITC-Collagen Degradation Imaging. Six chamber glass slides were pre-coated with DQ-fluorescein-collagen type I (Invitrogen) at a final concentration of 25 ng/μl for 2 h at 37 °C. After washing with phosphate-buffered saline, the slides were air dried and fixed with 2% formaldehyde. After extensive washing with phosphate-buffered saline, the coated slides were equilibrated with no serum medium. iBMP2 $^{C/C}$/4$^{C/C}$ and iBmp2$^{C/C}$/4$^{C/C}$ KO cells were then plated at the density of 1 10^4/ml and cultured in a-MEM medium for 48 h. The cells were fixed for 15 min in 3.7% formaldehyde, washed with phosphate-buffered saline, and mounted. Images were taken with an inverted fluorescent microscope (Nikon Cool pix 4500) coupled to a digital camera under the same parameters.

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

Results

Generation of iBmp2$^{C/C}$/4$^{C/C}$ KO cell line. To establish immortalized Bmp2 and Bmp4 double knockout osteoblast cells, iBmp2$^{C/C}$/4$^{C/C}$ cells were infected with Ad-Cre-GFP virus particles, and then selected under a fluorescence microscope with GFP positive. The double knockout of Bmp2/4 in the osteoblasts was further confirmed by PCR using specific Bmp2 and Bmp4 primers. PCR analysis shows that BMP2 and BMP4 genes were only detected in iBmp2$^{C/C}$/4$^{C/C}$ cells, but not seen in iBmp2$^{C/C}$/4$^{C/C}$ KO cells (Figure 1). Immunohistochemistry confirmed that simian virus 40 T-Ag was expressed in all of i*Bmp2/4* cells and iBmp2$^{C/C}$/4$^{C/C}$ KO cells.

iBmp2$^{C/C}$/4$^{C/C}$ KO cell differentiation and mineralization. To evaluate the effects of BMP2/4 knockout on differentiation and mineralization activities of these cells, we examined ALP activity, one of osteoblast differentiation markers, by *in situ* ALP histochemistry. It shows that the positive expression of ALP in both the iBmp2$^{C/C}$/4$^{C/C}$ and iBmp2$^{C/C}$/4$^{C/C}$ KO cells after a culture of 2 weeks in calcifying media. However, the ALP density was dramatically decreased in the iBMP2$^{C/C}$/4$^{C/C}$ KO cells compared to that of the iBMP2$^{C/C}$/4$^{C/C}$ osteoblasts. Also, mineralized nodules in both osteoblast cells were seen by Alizarin Red S staining, but mineralization was obviously reduced in these KO cells (Figure 2).

In Situ Zymography and ECM remodeling. To assess the changes in MMP activities and

ECM remodeling induced by BMP2/4 knockout, in situ collagen degradation was imaged using iBMP2$^{C/C}$/4$^{C/C}$ and iBMP2$^{C/C}$/4$^{C/C}$ KO grown on DQ-FITC-collagen type I-coated slides. iBMP2$^{C/C}$/4$^{C/C}$ osteoblasts exhibited a bright collagen degradation spots in close contact with the collagen matrix. In contrast, in iBMP2$^{C/C}$/4$^{C/C}$ KO cells, a faint and intracellular vesicular staining was observed, which was mainly due to the uptake of DQ-FITC-collagen and its intracellular degradation as reported in various cell types [9], indicative of decreased collagenase activity at focal sites and defects in remodeling the ECM(Figure 3).

Discussion

In this study, we established an immortalized cell line iBMP2$^{C/C}$/4$^{C/C}$ KO from mouse iBMP2$^{C/C}$/4$^{C/C}$ osteoblasts by infection with recombinant adenovirus Ad-Cre-GFP expressing both GFP as marker and Cre recombinase, a type I topoisomerase that catalyzes site-specific recombination of DNA between loxp sites. The iBMP2$^{C/C}$/4$^{C/C}$ KO cells do not express BMP2 and BMP4 and show decreased differentiation and mineralization ability compared with the iBMP2$^{C/C}$/4$^{C/C}$ cells. Also, iBMP2$^{C/C}$/4$^{C/C}$ KO cells have defects in remodeling the ECM as reflected by changes in collagen type I processing. These data suggest that the cell line iBMP2$^{C/C}$/4$^{C/C}$ KO will be a valuable asset for studying signaling pathways of bone development and metabolism mediated by *BMP2 and BMP4* and their down stream signaling pathways.

Osteoblasts are an important player in skeletal development, bone formation, remodeling and fracture repair. BMPs are key regulators of osetoblasts growth and differentiation. BMP signaling pathways have been identified and shown to mediate the osteoinductive signals of BMPs. These include the Smad-dependent, Smad-independent p38 mitogen-activated protein kinase pathway and the phosphatidylinositol 3-kinase/AKT pathway [10, 11]. BMP target genes include a growing number of tissue-determining transcription factors, such as Runx2 and Osx, which promote differentiation of mesenchymal precursors toward the osseous cell phenotypes [12].

Mutations of components in BMP signaling pathways result in abnormal bone formation. For instance, if mice lose both Alk3 (Bmprla) and Alk6 (Bmplb), two of the three type I receptors used in BMP signal transduction, it shows a dramatic decrease in the size of skeletal primordial due to a reduction of proliferation and increase in apoptosis[13]. For Osterix knockout mice [14], it exhibited severe defects in osteoblastic differentiation, and the phenotype observed in mice limbs of Bmp2$^{C/C}$; Bmp4$^{C/C}$; Prx1::cre mice is similar to the phenotype reported for Osterix knockout mice, since one important role of BMP2 and BMP4 during endochondral ossification may be to induce osterix gene expression in osteoprogenitors[7]. In our experiments, iBMP2$^{C/C}$/4$^{C/C}$ KO cells without both BMP2 and BMP4 showed dramatically decreased differentiation and mineralization ability, which coincides with the in vivo evidence. Further studies will be required to elucidate the participation of these agents such as Runx2 and Osx in this process.

It is well known that bone formation and remodeling are a highly coordinated process which involves a series of successive events including extracellular matrix destruction and turnover [15] (18, 62, 71). Here we showed that iBMP2$^{C/C}$/4$^{C/C}$ KO cells have defects in processing the ECM component collagen type I. These results deomonstate essential roles for BMP2/4 signaling in bone metabolism and remodeling.

To our knowledge, this is the first report of the development of the mouse iBMP2$^{C/C}$/4$^{C/C}$ KO osetoblast cell line. One of the advantages of establishing such a stable BMP2/4 knockout osteoblast cell line is to provide a large consistent source for biochemical and molecular analyses. Therefore, the cell line iBMP2$^{C/C}$/4$^{C/C}$ KO will be a valuable asset for studying signaling pathways of bone formation and development mediated by *BMP2 and BMP4* and their down stream signaling pathways.

Conclussions

We for the first time demonstrate the establishment of immortalized Bmp2/4 double null iBmp2$^{C/C}$/4$^{C/C}$ KO cells. Cell differentiation and mineralization in the iBmp2$^{C/C}$/4$^{C/C}$ KO cells were decreased. Moreover, ECM processing in these KO cells was impaired. These results indicate that BMP2/4 play important roles in osteoblast differentiation and ECM remodeling, and iBmp2C$^{/C}$/4$^{C/C}$ KO cells are useful models for studying the mechanism of Bmp2/4 in osteoblast proliferation, differentiation, and mineralization.

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