Combination of Runx2 and BMP2 increases conversion of human ligamentum flavum cells into osteoblastic cells

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The conversion of fibroblasts into osteoblasts requires the activation of key signaling pathways, including the BMP pathway. Although Runx2 is known to be a component of the BMP pathway, the combination of Runx2 and BMP2 has not yet been examined with respect to the conversion of fibroblasts into osteoblasts. Here, human ligamentum flavum (LF) fibroblast-like cells from six patients were tested for their conversion into osteoblasts using adenoviruses expressing Runx2 or BMP2. The forced expression of Runx2 or BMP2 in primary cultured LF cells resulted in a variety of proliferation and differentiation behaviors. Combined treatment of BMP2 plus Runx2 resulted in better osteoblastic differentiation than treatment with either component alone. These results indicate that the Runx2 and BMP2 pathways possess both common and independent target genes. Collectively, Runx2 plus BMP2 mediated efficient conversion of fibroblast-like LF cells into osteoblast-like cells, suggesting the possible use of these components for clinical applications such as spinal fusion. [BMB reports 2011; 44(7): 446-451]

INTRODUCTION

Osteogenesis is a complex process involving osteoprogenitor cell recruitment, proliferation, extracellular matrix (ECM) production and maturation, and ultimately, matrix mineralization by differentiated osteoblasts (1). Although the spatiotemporal processes responsible for osteoblastic differentiation from mesenchymal stem cells have not yet been completely elucidated, they are regulated by soluble factors, hormones, tissue-specific transcription factors, as well as cell-ECM and cell-cell interactions.

Osteogenic induction by bone morphogenetic proteins (BMPs) has been extensively studied. BMP2 binds to a heterotetrameric complex of types I and II transmembrane serine/threonine kinase receptor proteins. Upon activation by BMP2, these receptors phosphorylate the intracellular signaling molecule Smad1/5/8. Activated Smad1/5/8 then associates with Smad4 and translocates to the nucleus, whereupon it interacts with the transcription factor Runx2 and upregulates osteoblastic gene expression (2).

Runx2 is known to be a master gene for bone formation and also a downstream target gene of the BMP2 pathway (3-6). Runx2 is principally linked to osteoblast differentiation and is obligatory for the regulation of differentiating genes involved in both endochondral and intramembranous bone formation. Targeted disruption or C-terminus truncation of Runx2 in mice has revealed that Runx2 is required for bone development in vivo (3-5).

Injection of BMPs into soft tissues induces ectopic bone formation in subcutaneous tissues (7). Bone forming areas coincide with the presence of BMPs (8). Indeed, the activation of BMP signaling can convert fibroblasts into osteoblasts (9, 10). Hence, the BMP pathway is a critical signaling pathway for the initiation of bone formation in skeletal tissues. Full transaction of the BMP pathway involves several key transcription factors, including Dlx5, Dlx3, Runx2, Msx2, and Osterix, etc (11).

Many studies have tried to convert fibroblasts into osteoblasts by transfection of a single transcription factor, Runx2, or Osterix (12-14). In contrast to BMP2 activation, Runx2 or Osterix alone is not sufficient to convert fibroblasts into osteoblasts (14). Considering that Runx2 is a target gene of BMP2, we speculated that a combination of Runx2 and BMP2 may not be synergistic or additive with regard to conversion of fibroblasts into osteoblasts. To test this hypothesis, human ligamentum flavum (LF) fibroblast-like cells were examined for conversion into osteoblasts.

We found that combined treatment with Runx2 plus BMP2 resulted in the efficient conversion of fibroblast-like LF cells into osteoblast-like cells, suggesting the presence of an independent signaling pathway between Runx2 and BMP2 in...
addition to a common pathway.

RESULTS

Transduction of adenovirus expressing BMP2 or Runx2 in LF cells
Adenovirus expressing Runx2 or BMP2 was used to identify the synergistic, additive, or independent pathways between Runx2 and BMP2 signaling. To verify adenoviral transduction of LF cells, LF cells were transduced with adenovirus expressing LacZ reporter gene (Ad/LacZ). Microscopic examination showed an increase in the number of LacZ-positive cells with increasing multiplicity of infection (MOI) of Ad/LacZ (Fig. 1A). Ectopic expression of Runx2 or BMP2 in LF cells was detected by Western blot analysis (Fig. 1B). Adenoviral transduction did not produce morphological changes in the LF cells (Fig. 1C). Dose-dependent BMP2 expression increased alkaline phosphatase (ALP) activity in LF cells, suggesting that LF cells were converted into osteoblast-like cells (Fig. 1D).

ALP activity and Alizarin Red S (AR) staining in LF cells by expression of Runx2 and BMP2
To determine whether or not Runx2, BMP2, or Runx2 plus BMP2 could stimulate the differentiation of LF cells into osteoblasts, LF cells were cultured in osteogenic medium and evaluated for the activity of ALP, an early marker for osteoblasts. LF cells themselves were only weakly converted into osteoblast-like cells in osteogenic media. When the LF cells were infected by adenovirus expressing Runx2 or BMP2 for 2 weeks, the number of ALP-positive cells was much higher than that of the control cells (data not shown). There were three different groups comprising six total samples. Each group contained two samples and therefore, one representative sample was depicted. Analyses of the cells found the following results: First, LF cells from patient 1 (Pt 1) showed increased ALP activity after treatment with BMP2 but not after treatment with Runx2. Second, LF cells from patient 2 (Pt 2) showed increased ALP activity after treatment with either Runx2 or BMP2. Third, LF cells from patient 3 (Pt 3) showed increased ALP activity after treatment with Runx2, but less of an increase after treatment with BMP2 (Fig. 2A). Therefore, the forced expression of BMP2 increased ALP activity in cells from Pt 1 and Pt 2, but had a marginal effect on cells from Pt 3 (Fig. 2A). Although individual variations were observed in ALP activity, both Runx2 and BMP2 generally had positive effects on osteogenic differentiation, and the combined treatment with Runx2 and BMP2 led to higher ALP activity than either treatment alone.

Fig. 1. Adenovirus transduction into LF cells. (A) LF cells were transduced with LacZ-expressing adenovirus (Ad/LacZ) at different MOIs (0, 25, 50, 100, 150, 200, 500, and 1000) and then stained for LacZ activity at 2 days after transduction. The increased percentage of LacZ-positive cells was adenovirus dose-dependent. A concentration of 200 MOI resulted in a cell transduction rate of 65% (original magnification ×100). (B) Identification of proteins in adenovirus expressing Runx2 or BMP2. After transduction, proteins were identified by Western blot analysis. (C) Cell morphological observation after transduction of adenovirus expressing Runx2, BMP2, or a combination of Runx2 and BMP2 (R2-B2). (D) Effect of BMP2 on conversion of LF cells into osteoblast-like cells.

Fig. 2. Osteogenic differentiation of LF cells after expressing Runx2, BMP2, or a combination of Runx2 and BMP2. (A) ALP staining was performed on LF cells from three different representative groups at day 10. Runx2 transduction inhibited ALP staining in Pt 1 compared to Pt 2 and Pt 3. (B) AR staining was observed in LF cells at day 10. Runx2 transduction inhibited AR staining in Pt 1 compared to Pt 2. Runx2 showed no difference in Pt 3 compared to control. Combination of Runx2 and BMP2 increased the intensity of AR staining in all LF cells.
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Fig. 3. Effects of Runx2, BMP2, or a combination of Runx2 and BMP2 on LF cell proliferation. LF cells were transduced with adenovirus expressing Runx2, BMP2, or a combination of Runx2 and BMP2. Cell proliferation was determined by MTT assay. Relative proliferation was evaluated after normalization of each Ad/LacZ transduction sample against Ad/Runx2, Ad/BMP2, and Ad/Runx2/BMP2. The values represent the means ± SD. *P < 0.05, **P < 0.01, ***P < 0.005.

plus BMP2 enhanced ALP activity. According to the results, to a certain extent, Runx2 and BMP2 exerted their own signal transduction pathways.

We also examined calcium deposition of ECM during the late stage of osteoblast differentiation by AR staining. As shown in Fig. 2B, nodule-like AR-positive staining was observed in all LF cells at day 10, but staining was not observed at day 5 (data not shown). Although the intensity of AR staining was not increased in Runx2-expressing LF cells from Pt 1 at day 10, Runx2 increased the intensity of AR staining in cells from Pt 2 and Pt 3 (Fig. 2B). Treatment with BMP2 alone or with the combination of BMP2 plus Runx2 consistently increased AR staining, which is similar to the results for ALP activity among the three groups. These results confirm that, to a certain extent, Runx2 and BMP2 exerted their own signal transduction pathways. Taken together, the combined treatment using Runx2 plus BMP2 increased the conversion of LF cells into osteoblast-like cells.

Effects of BMP2 or Runx2 on LF cell proliferation
We tested the effects of Runx2 and BMP2 on LF cell proliferation using three representative samples as above. The transduction of adenovirus expressing Runx2 into LF cells produced different patterns of cell proliferation; cell proliferation was increased in some cells but not in others (Fig. 3). The differential effects of Runx2 on proliferation might have been due in part to the heterogeneity of LF cells. Although it has been reported that under serum deprivation conditions, Runx2 expression is increased in osteoblasts but not in chondrocytes (15), the precise mechanism of Runx2 activity on LF cell proliferation needs to be further studied. Transduction of adenovirus expressing BMP2 increased cell proliferation (Fig. 3). This result is very consistent with results from previous studies using primary cultured osteoblasts and chondrocytes (15). The combined treatment with Runx2 plus BMP2 promoted the proliferation of LF cells (Fig. 3). These results indicate that BMP2 increased cell proliferation in LF cells.

Expression of osteogenic markers by Runx2 and BMP2 in LF cells
Based on the positive responses to treatment with Runx2 or BMP2 (Fig. 2), the gene expression levels of various osteogenic markers were measured in LF cells from Pt 2 (Fig. 4). After transduction of adenoviruses, the expression level of each gene was determined by quantitative real-time PCR (Q-PCR) at day 10. The expression levels of osteogenic markers, ALP, type I collagen (Col1), B2 (BMP2), and R2 (Runx2), was evaluated by Q-PCR. Bar represents mean ± SD. **P < 0.01, ***P < 0.005.
pression of Runx2 in mature osteoblasts (18). Even though the exact mechanisms of BMP2 in LF cells are not clear, one possible explanation could involve the high expression of BMP antagonists. This hypothesis will be explored in future experiments with other osteogenic markers using this conversion model.

DISCUSSION

This study showed that LF cells could be converted into osteoblast-like cells by the combined expression of BMP2 and Runx2. The combined treatment increased osteoblastogenic differentiation of LF cells, which was reflected by upregulation of ALP activity and increased intensity of Alizarin Red S staining. Although BMP2 and Runx2 genes are known to share a common pathway, different phenotypes in the LF cells resulting from overexpression of each gene revealed the presence of independent signaling pathways in addition to a common pathway.

We used primary cultured LF cells from patients, and the cells showed heterogeneous characteristics in response to treatment with BMP2 or Runx2 alone and the combination of BMP2 plus Runx2. With respect to cell differentiation, forced expression of BMP2 or Runx2 resulted in heterogeneous effects in LF cells. Some LF cells were easily converted to osteoblast-like cells, whereas others became more resistant to conversion. However, the combination treatment of BMP2 and Runx2 increased the conversion of LF cells into osteoblast-like cells, resulting in upregulation of osteogenic bone markers.

We also tested the effects of BMP2 and Runx2 on proliferation of LF cells. Whereas BMP2 generally increased cell proliferation, Runx2 had no consistent effect. Specifically, Runx2 resulted in various levels of proliferation in LF cells from different patients. Runx2 is known as an important regulator of cell proliferation, and its ablation increases the growth of cultured cells (15, 19-21). On the other hand, forced expression of Runx2 suppresses proliferation of osteoblasts (15, 22) and non-osteogenic cells (23). Hence, Runx2 has been proposed as a gene related to cancer (24). Mechanistically, the levels of Runx2 oscillate during the cell cycle, and Runx2 inhibits p21, p27, and cyclin E expression in osteoblasts (15, 19). Although we do not know which factor(s) is/are responsible for cell proliferation induced by Runx2, such Runx2-sensitive genes might have been involved in the differential cell proliferation observed in this study.

With regard to signal transduction pathways, the BMP2 and Runx2 signaling pathways in LF cells do not completely overlap. Although Runx2 is a downstream target gene of BMP2, it has its own signaling pathway. Combination treatment converted LF cells into osteoblast-like cells, indicating that some factor(s) acting downstream of BMP2 may work synergistically with Runx2.

The requirement for Runx2 in osteoblastic differentiation and in vivo bone formation and maintenance has been studied (18). However, the combined treatment of Runx2 and BMP2 on human LF cells has not been determined with regard to the induction of osteoblast-specific gene expression and matrix mineralization. Here, the upregulated expression of osteoblastic markers in LF cells in response to exogenous Runx2 and BMP2 most likely resulted from enhanced differentiation of LF cells.

Collectively, the combined treatment using Runx2 plus BMP2 resulted in efficient conversion of fibroblast-like LF cells into osteoblast-like cells, suggesting the possible use of these components for clinical applications such as spinal fusion.

MATERIALS AND METHODS

Tissue acquisition

Specimens from the interlaminar portion of LF were obtained from patients during surgical spinal procedures. All protocols were approved by the Human Subjects Institutional Review Board of the institution. Six patients (age range, 51-71 years) had severe spinal stenosis of the lumbar spine. Thickened LF was noted in MRIs before surgery and by direct visualization intraoperatively. During the operations, surgeons tried to obtain tissue en bloc from the central portion of the LF while minimizing tissue damage, harvesting only the ligament proper, and including neither the ligament insertion site nor the origin site.

LF cell isolation and culture

LF cells were isolated from the ligament as previously described (25). Dissected specimens were minced with a scalpel into pieces of ~2 mm³. The LF tissues were then digested for 60 minutes at 37°C in 5% CO₂ with gentle agitation in Dulbecco’s Modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 250 U/ml of Type 1A collagenase (Sigma-Aldrich, Steinheim, Germany). Collagenase-treated ligament chips were removed, washed with serum-containing medium, and finally placed in a 25-mm culture flask with DMEM containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen) in a 5% CO₂ humidified incubator. The characterization of cell cultures was performed at two or third passages.

Adenoviral transduction

Cloning of the Runx2 adenoviral expression vector containing the murine type II (MASNSLF) Runx2 isoform, packaging of Runx2, and transduction of cells were performed as described in a previous study (19). At confluence, the cultured LF cells were rinsed with PBS three times and exposed to 50 μl of HBSS containing various doses of adenovirus expressing lacZ (Ad/LacZ), Runx2 (Ad/Runx2), and BMP2 (Ad/BMP2) for 1 hour. Then, culture medium (950 μl) was added to each well, and the cells were further incubated in a 5% CO₂ humid environment at 37°C.
Measurement of \( \beta \)-Galactosidase gene expression

\( \beta \)-Galactosidase gene expression was assessed at 2 days after transduction using 5-bromo-4-chloro-3-indolyl-\( \beta \)-Galactosidase (X-Gal, Sigma, St. Louis, MO, USA) staining. Briefly, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (Sigma-Aldrich) for 5 minutes, and rinsed in PBS containing 0.01% Na-deoxycholate, 0.02% Nonidet P-40, 5 mM EGTA, and 2 mM MgCl\(_2\). The cells were finally incubated with X-Gal substrate (0.5 mg/ml of X-Gal) in PBS containing 10 mM K\(_4\)Fe(CN)\(_6\), 10 mM K\(_3\)Fe(CN)\(_6\), 0.01% Na-deoxycholate, 0.02% Nonidet P-40, 5 mM EGTA, and 2 mM MgCl\(_2\) for 4 hours at 37°C.

Western blot analysis

Whole cells were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4, 0.15M NaCl, 0.5% SDS, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 μg/ml of pepstatin, and 1 μg/ml of leupeptin). Secretory protein was extracted by precipitation with trichloroacetic acid (TCA). Western blot analysis was performed with anti-BMP2 (Abcam, Cambridge, UK), anti-Runx2, or anti-\( \beta \)-Actin (Sigma-Aldrich) antibody.

ALP and alizarin red S staining

ALP staining was carried out according to standard techniques using a Sigma-Aldrich Alkaline phosphatase assay kit (Sigma-Aldrich). For AR staining, the cells were fixed with 70% ethanol for 1 hour at 4°C and washed three times with distilled water (D.W.). AR solution (40 mM) was added to cells for 10 min, rinsed with D.W., and finally washed with PBS.

Proliferation assay

LF cells were plated in 24-well plates at a cell density of 1 × 10\(^4\) per well. After adenovirus transduction, cells were incubated for 1, 2, 3, or 4 days. Cell proliferation was estimated by MTT assay. Briefly, cells were added to 500 μl of MTT solution and incubated for 4 hours. After removal of MTT solution, cells were washed with PBS and added to 500 μl of dimethyl sulphoxide. The optical density was measured at 570 nm by a Sunrise absorbance reader (Tecan Group Ltd. Männedorf, Switzerland).

Osteoblast-specific gene expression

Total RNA was isolated at 10 days after transduction using an easy-Blue total RNA Extraction kit (Intron Biotechnology, Kyunggi-do, Republic of Korea). cDNA synthesis was performed on DNase I-treated total RNA (4 μg) by Superscript II RT (Invitrogen) using oligo (dT) primer. Real-time PCR were as follows: Col1a2 (5'-TTG TTA CAG GAA GTC CCT TGC C-3' and 5'-ATG CTA CCA AAC TCC ACA GCC C-3'), osteocalcin (5'-AGC AAA GGT GCA GCC TTT GT-3' and 5'-TGA GCT GAG AGG GCA TAT GGC C-3'), Runx2 (5'-TGA GCT GAG AGG GCA TAT GGC C-3' and 5'-TAG ACA CCA AAC TCC ACA GCC C-3'), and \( \beta \)-Actin (5'-TTG TTA CAG GAA GTC CCT TGC C-3' and 5'-ATG CTA TCA CCT CCC CTG TGT G-3').

Statistical analysis

We performed all quantitative experiments at least in triplicate. Data were expressed as the mean ± standard deviation (SD) and were analyzed using Student's t test. A value of \( P \leq 0.05 \) was considered statistically significant.

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REFERENCES


