Activation of JNKs is essential for BMP9-induced osteogenic differentiation of mesenchymal stem cells

Yan-fang Zhao, Jing Xu, Wen-juan Wang, Jin Wang, Juan-wen He, Li Li, Qian Dong, Yan Xiao, Xing-lian Duan, Xue Yang, Yi-wen Liang, Tao Song, Min Tang, Dan Zhao & Jin-yong Luo*

Key Laboratory of Diagnostic Medicine Designated by the Chinese Ministry of Education, Chongqing Medical University, Chongqing, PR China

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent cells present not only in the bone marrow, but in a number of other tissues (1). MSCs can self-renew and differentiate into osteoblasts, chondrocytes, myoblasts or adipocytes (1). Bone morphogenetic proteins (BMPs) belong to the transforming growth factors (TGF-β), and have been proved to play pivotal roles in the processes such as embryogenesis, hematopoiesis, neurogenesis and osteogenesis (2, 3). Several forms of recombinant BMPs, in particular BMP2 and BMP7, have been extensively used to augment bony healing in the clinical setting (4, 5). Though much attention has been directed to BMP2 and BMP7 in bone regeneration, it is unclear whether these are actually the most osteogenic BMPs.

Although BMP9 is highly capable of promoting osteogenic differentiation of mesenchymal stem cell (MSCs), the molecular mechanism involved remains to be fully elucidated. Here, we explore the possible involvement and detail role of JNKs (c-Jun N-terminal kinases) in BMP9-induced osteogenic differentiation of MSCs. BMP9-stimulated the activation of JNKs in MSCs. BMP9-induced osteogenic differentiation of MSCs was dramatically inhibited by JNKs inhibitor SP600125. Moreover, BMP9-activated Smads signaling was decreased by SP600125 treatment in MSCs. The effects of inhibitor are reproducible with adeno viruses expressing siRNA targeted JNKs. Taken together, our results revealed that JNKs was activated in BMP9-induced osteogenic differentiation of MSCs. What is most noteworthy, however, is that inhibition of JNKs activity resulted in reduction of BMP9-induced osteogenic differentiation of MSCs, implying that activation of JNKs is essential for BMP9 osteoinductive activity. [BMB Reports 2013; 46(8): 422-427]

RESULTS

BMP9 induced phosphorylation/activation of JNKs in MSCs

To determine if JNKs can be activated by BMP9 in MSCs, C3H10T1/2 cells were infected with Ad-BMP9 or Ad-GFP with infection efficiency at 40% (Fig. 1A). As illustrated in Fig. 1B, BMP9 significantly increased the levels of phosphorylated JNKs, without altering the total amounts of JNKs proteins. However, SP600125, which is a selective inhibitor for JNKs activation, was able to suppress BMP9-induced phosphorylation of JNKs. Similar results were also observed in C2C12 (Fig. 1C)
JNKs in BMP9-induced osteogenic differentiation
Yan-fang Zhao, et al.

Fig. 1. BMP9 stimulated phosphorylation of JNKs in MSCs. (A) Infection efficiency of Ad-BMP9 in C3H10T1/2 cells. Magnification, 100. GFF: Green Fluorescence Field; BLF: Bright Light Field. (B) C3H10T1/2 cells were infected with Ad-BMP9 or Ad-GFP, at 24 hours post infection, total amount and phosphorylated forms of JNKs were analyzed by western blotting. SP: abbreviation of SP600125. (C) C2C12 cells were infected with Ad-BMP9 or Ad-GFP, at 24 hours post infection, total amount and phosphorylated forms of JNKs was analyzed by western blotting. (D) BMSCs were infected with Ad-BMP9 or Ad-GFP, at 24 hours post infection, total amount and phosphorylation forms of JNKs was analyzed by western blotting. (E) C3H10T1/2 cells were treated with BMP9-CM, total amount and phosphorylated forms of JNKs were analyzed by western blotting at indicated time points. (F) C3H10T1/2 cells were infected with Ad-BMP9, JNKs-dependent reporter activity was quantitatively assessed at 24 and 36 hours post BMP9-stimulation. Data were means ± SD of three experiments. **P < 0.01 VS GFP; ##P < 0.01 VS BMP9.

and BMSCs (Fig. 1D). Moreover, we tested the effect of BMP9-conditioned medium (BMP9-CM) on activation of JNKs in C3H10T1/2 cells. As illustrated in Fig. 1E, an increased level of JNKs phosphorylation was first increased at 5min, and peaked at 30 min post BMP9-CM treatment. These findings indicated that BMP9 was capable of effectively promoting phosphorylation of JNKs in MSCs.

JNKs can phosphorylate and then activate transcription factor AP-1 (16). Therefore, using a JNKs-dependent AP-1 promoter reporter pB2-AP1-Luc, which contains JNKs-responsive elements and reflects the level of JNKs activation, we found that BMP9 was capable of increasing JNKs-regulated AP-1 transcription activity (Fig. 1F). However, SP600125 was able to effectively inhibit BMP9-induced reporter activity. These data implied that BMP9 can activate JNKs, and subsequently induce JNKs-dependent transcription activity. Together, these above results strongly suggested that BMP9 can effectively induce activation of JNKs in MSCs.

BMP9-induced osteogenic differentiation of MSCs was dramatically inhibited by SP600125, a selective inhibitor of JNKs

Next, to further document the detail role of JNKs in BMP9-induced osteogenic differentiation of MSCs, C3H10T1/2 cells were exposed to BMP9 in the presence of varying concentrations of SP600125 (0, 10, 20 and 30 μM). SP600125 was able to inhibit BMP9-induced ALP activity of C3H10T1/2 cells mostly in a dose-dependent manner (Fig. 2A and B). Similar phenomena were also observed in C2C12 and BMSCs (Fig. 2C).

Moreover, we found that SP600125 treatment resulted in a significant decrease in BMP9-induced matrix mineralization (Fig. 2D), as well as OCN protein expression of MSCs (Fig. 2E). Collectively, these results strongly implied us that inhibition of JNKs can suppress BMP9-induced osteogenic differentiation of MSCs.

It has been evidenced in our previous studies that Id1, Id2, Id3 and Runx2 are targets of BMP9, and are critical to BMP9-induced osteogenic differentiation of MSCs (7). Using semi-quantitative RT-PCR analysis, we found that BMP9-induced gene expression of Id1, Id2, Id3 and Runx2 was strongly decreased by SP600125 (Fig. 2F). We further examined the Runx2 protein expression by western blotting. As shown in Fig. 2G, BMP9-induced Runx2 protein expression was decreased by SP600125 treatment. Next, using a commonly used Runx2-regulated reporter (p6OSE-Luc), we found that SP600125 was able to inhibit BMP9-induced p6xOSE-Luc activity, which contains Runx2-responsive elements and reflects Runx2 transcription activity (Fig. 2H). These findings revealed that inhibition of JNKs suppressed BMP9-induced activity of Runx2, a key transcription factor associated with osteoblast differentiation.

Blocking of JNKs activity decreased BMP9-induced activation of Smads signaling

We next sought to probe the possible mechanism behind the effects of JNKs on BMP9-induced osteogenic differentiation of MSCs. Our previous study has demonstrated that p38 and ERK1/2 are likely to regulate BMP9-induced osteogenic differ-
Fig. 2. Inhibition of JNKs activity suppressed BMP9-induced osteogenic differentiation of MSCs. (A) and (B) C3H10T1/2 cells were infected with Ad-BMP9 in the presence of SP600125 (0, 10, 20 and 30 μM). ALP activity was assessed by quantitative assay and staining assay at 7 days post BMP9-stimulation. Data were means ± SD of three experiments. **P < 0.01 VS GFP; ##P < 0.01 VS BMP9. (C) C2C12 and BMSCs cells were infected with Ad-BMP9 in the presence of SP600125 (30 μM). ALP activity was quantitatively assessed at 7 days post BMP9-stimulation. Data were means ± SD of three experiments. **P < 0.01 VS GFP; ##P < 0.01 VS BMP9. (D) C3H10T1/2 and C2C12 cells were infected with Ad-BMP9 in the presence of SP600125 (30 μM), matrix mineralization was assessed by Alizarin Red S staining at 21 days post BMP9-stimulation. (E) C3H10T1/2 cells were infected with Ad-BMP9 in the presence of SP600125 (30 μM). Osteocalcin (OCN) expression was assessed at 12 days by immunocytochemical staining post infection. Magnification, 100. (F) C3H10T1/2 cells were infected with Ad-BMP9 in the presence of SP600125 (30 μM), the gene expression level of Id1, Id2, Id3 and Runx2 was assessed by RT-PCR at 24 hours post infection by RT-PCR. (G) C3H10T1/2 cells were infected with Ad-BMP9 in the presence of SP600125 (30 μM), the protein expression level of Runx2 was assessed by western blotting at 24 hours post infection. (H) C3H10T1/2 cells were infected with Ad-BMP9 in the presence of SP600125 (30 μM), Runx2-regulated reporter activity was quantitatively assessed at 24 and 36 hours post infection. Data were means ± SD of three experiments. **P < 0.01 VS GFP; ##P < 0.01 VS BMP9.

Fig. 3. Blocking of JNKs led to suppression on BMP9-activated Smads signaling in MSCs. (A) C3H10T1/2 cells were infected with Ad-BMP9 in the presence of SP600125 (30 mM), total amount and phosphorylated forms of Smad1/5/8 was analyzed by western blotting. (B) C3 H10T1/2 cells were treated with BMP9-CM in the presence of SP600125 (30 mM), nuclear translocation of Smad1/5/8 were detected by immunochemistry staining at 4 hours post BMP9-stimulation, Magnification, 100. (C) C3H10T1/2 cells were infected with Ad-BMP9 in the presence of SP600125 (30 μM), SBE reporter activity was quantitatively assessed at 24 and 36 hours post infection. Data were means ± SD of three experiments. **P < 0.01 VS GFP; ##P < 0.01 VS BMP9.
JNKs in BMP9-induced osteogenic differentiation
Yan-fang Zhao, et al.

Silence of JNKs led to a decrease in BMP9-induced osteogenic differentiation of MSCs

Using specific inhibitor for JNKs, we found that inhibition of JNKs activity decreased BMP9-induced osteogenic differentiation of MSCs. To confirm that the effects of the inhibitor was truly due to JNKs inhibition and not a nonspecific drug effect, we employed adenovirus expressing siRNA targeted JNKs to infect C3H10T1/2 cells. Western blotting was carried out to assess the inhibitory efficiency of the siRNA on expressions of JNKs (Fig. 4A). Then, the effect of JNKs knockdown on BMP9-induced osteogenic differentiation of MSCs was assessed. As shown in Fig. 4B-D, gene silence of JNKs diminished BMP9-induced ALP activity and matrix mineralization of C3H10T1/2 cells. Furthermore, knockdown of JNKs effectively decreased BMP9-activated Smad signaling, leading to reduction of BMP9-induced Smad1/5/8 transcriptional activity (Fig. 4E) and less phosphorylation of Smad1/5/8 (Fig. 4F). Collectively, these above results implied us that JNKs may play a private role in regulating BMP9-induced osteogenic differentiation and Smads signal activation.

DISCUSSION

BMP9 was originally isolated from fetal mouse liver and is a potent stimulant of hepatocyte proliferation (17). Other roles of BMP9 include inducing the cholinergic phenotype of embryonic basal forebrain cholinergic neurons (18), regulating glucose and lipid metabolism in liver (19), and maintaining homeostasis of iron metabolism (20). Also, BMP9 is a potent inducer for osteogenic differentiation of rat dental follicle stem cells (21). In previous studies, BMP9 has been proved to play a critical role in the processes by which MSCs undergo commitment to the osteoblast lineage (6-9). However, little is known about the downstream signaling pathway(s) involved.

In addition to the Smad1/5/8 transcription factors, MAPKs are also involved in BMPs osteogenic signaling transduction (11-14). Recently, we have reported that p38 and ERK1/2 MAPKs act in opposition to regulate BMP9-induced osteogenic differentiation of MSCs (15). In this current study, we examined the ability of JNKs, another important member of MAPKs, in regulating BMP9-induced osteogenic differentiation of MSCs. The results obtained here implied that BMP9 was able to activate JNKs. Importantly, blocking of JNKs activity led to reduction on BMP9-induced osteogenic differentiation of MSCs.
JNKs in osteogenic differentiation and bone formation (11,13), and others proposing that JNKs is inhibitory (14). Although these studies about the precise role of JNKs in skeletal development didn’t lead to complete unanimity, it is well accepted that JNKs plays a functional role in osteogenesis and bone metabolism.

In addition to Smads, MAPKs can also be activated by BMPs stimulation (2,11-14), which represents an important mechanism for non-Smads pathway(s) of BMPs signaling. It has been described by various studies that JNKs can be activated by BMPs and lead to various effects depending on the cell context (11,14, 22, 23) However, the exact role of JNKs on BMPs-induced osteogenic differentiation is diverse and disputable. By now, the studies about JNKs on BMPs-induced osteogenesis were mainly focus on BMP2, and these studies did not reach on consensus. Several studies agree on the notion that JNKs play a positive role in BMP2-induced osteogenic differentiation (11, 13). However, other study obtained opposite results, showing that JNKs have a negative role in BMP2-induced osteogenic differentiation (14). All these experiments did not ascertain the exact role of JNKs in BMPs-induced osteogenic differentiation and bone formation because of the controversial results. However, it is convincingly supported that JNKs play essential roles in regulating osteogenic activity of BMPs, with positive or negative effects. In the current study, we found that BMP9 was capable of activating JNKs to induce osteogenic differentiation of MSCs, and supposed that JNKs are positive regulatory components in BMP9 osteoinductive activity.

In conclusion, we have found that BMP9 can activate JNKs in the induction of the osteogenic differentiation of MSCs. Notably, using specific inhibitor and siRNA for JNKs, we found that activation of JNKs are essential for BMP9-induced osteogenic differentiation of MSCs. Furthermore, we found that JNKs are likely to regulate BMP9-induced osteogenic differentiations of MSCs via influence on Smads signaling. This knowledge will provide insights into the molecular mechanisms by which BMP9 induces osteogenic differentiation of MSCs.

MATERIALS AND METHODS

Cell culture and chemicals
C3H10T1/2, C2C12 and HCT116 cells were obtained from ATCC and maintained in complete DMEM supplemented with 10% fetal bovine serum and 100 units/ml streptomycin/penicillin at 37°C in a humidified atmosphere of 5% CO2.

Isolation of primary bone marrow stromal cells (BMSCs)
A single-step primary BMSCs purification method using adhesion to cell culture plastic was employed as described in the reference (8).

Construction of recombinant adenoviruses
Recombinant adenovirus expressing BMP9 (Ad-BMP9) were generated previously (6, 15). Recombinant adenoviruses expressing siRNA targeted JNKs (AdR-si-JNKs) was kindly provided by Dr. Tong-chuan He of University of Chicago Medical Center.

Preparation of BMP9-conditioned medium
BMP9-conditioned medium (BMP9-CM) were prepared as described (15). Briefly, HCT116 cells were infected with an optimal titer of Ad-BMP9. At 24 hour after infection, the culture medium was changed to serum-free DMEM. Conditioned medium was collected at 48 hrs after infection and used immediately.

Determination of ALP activity
ALP activity was assessed by a modified Great Escape SEAP chemiluminescence quantitative assay and a histochemical staining assay as described (15).

Alizarin Red S staining
Matrix mineralization was detected by Alizarin Red S stain, as described previously (15). The staining of matrix mineralization was recorded under bright field microscopy.

Western blotting
Western blotting was performed as previously described (15). Primary antibodies were obtained from Santa Cruz, as follows: anti-phosphor-JNKs, anti-JNKs, anti-phosphor-Smad1/5/8, anti-Smad1/5/8, and anti-β-actin.

Immunocytochemical staining
Immunocytochemical staining was performed as previously described (15). The presence of the expected protein was visualized by DAB staining and examined under a microscope.

RNA isolation and semiquantitative RT-PCR analysis
Total RNA was isolated using TRIZOL Reagents. Total RNA was used to generate cDNA templates by RT reaction with hexamer and SuperScript II RT. Semiquantitative RT-PCR was carried out as described previously (15).

Immunofluorescence staining
Immunofluorescence staining was performed as previously described (15). Fluorescence signal was recorded under a fluorescence microscope.

Luciferase reporter assay
Luciferase reporter assay was performed as previously described (15). Cells were seeded in 25 cm² cell culture flasks and transfected with 2 mg per flask of luciferase reporter plasmid using Lipofectamine. At 16 hr after transfection, cells were replated to 24 well plates. Cells were infected with Ad-BMP9 in the presence of SP600125 or co-infected with Ad-BMP9 and AdR-si-JNKs. Cells were lysed and subjected to luciferase assays at indicated time points.

Statistical analysis
Data are expressed as means ± SD. Statistical analysis was performed using SAS (version 8.1; SAS Institute, Cary, NC), P <
0.05 was taken as the level of significance.

Acknowledgements
This work was supported by grants from the National Natural Science Foundation of China (No.31071304, 81272006).

REFERENCES
1. Deng, Z. L., Sharff, K. A., Tang, N., Song, W. X., Luo, J., Luo, X., Chen, Bennett, E., Reid, R., Manning, D., Xue, A., Montag, A. G., Luo, H. H., Haydon, R. C. and He, T. C. (2008) Regulation of osteogenic differentiation during skeletal development. Front. Biosci. 13, 2001-2021.
2. Hogan, B. L. (1996) Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. 10, 1580-1594.
3. Chen, D., Zhao, M. and Mundy, G. R. (2004) Bone morphogenetic proteins. Growth Factors 22, 233-241.
4. Borai, S., Paul, O., Hawkes, D., Wickham, M. and Lorich, D. G. (2009) Complications of recombinant human BMP-2 for treating complex tibial plateau fractures: a preliminary report. Clin. Orthop. Relat. Res. 467, 3257-3262.
5. Rutherford, R. B., Nussenbaum, B. and Krebsbach, P. H. (2003) Bone morphogenetic protein 7 ex vivo gene therapy. Drug. News Perspect. 16, 5-10.
6. Kang, Q., Sun, M. H., Cheng, H., Peng, Y., Montag, A. G., Deyrup, A. T., Jiang, W., Luo, H. H., Luo, J., Szatkowski, J. P., Vanichakarn, P., Park, J. Y., Li, Y., Haydon, R. C. and He, T. C. (2004) Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. Gene Ther. 11, 1312-1320.
7. Luu, H. H., Song, W. X., Luo, X., Manning, D., Luo, J., Deng, Z. L., Sharff, K. A., Montag, A. G., Haydon, R. C. and He, T. C. (2007) Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. J. Orthop. Res. 25, 665-677.
8. Luo, J., Tang, M., Huang, J., He, B. C., Gao, J. L., Chen, L., Zuo, G. W., Zhang, W., Luo, Q., Shi, Z., Zhang, B. Q., Bi, Y., Luo, X., Jiang, W., Su, Y., Shen, J., Kim, S. H., Huang, E., Gao, Y., Zhou, J. Z., Yang, K., Luo, H. H., Pan, X., Haydon, R. C., Deng, Z. L. and He, T. C. (2010) TGFbeta/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in mesenchymal stem cells. J. Biol. Chem. 285, 29508-29518.
9. Wu, N., Zhao, Y., Yin, Y., Zhang, Y. and Luo, J. (2010) Identification and analysis of type II TGF-[beta] receptors in BMP-9-induced osteogenic differentiation of C3H10T1/2 mesenchymal stem cells. Acta. Biochim. Biophys. Sin. (Shanghai). 42, 699-708.
10. Helder, C. H., Miyazono, K. and ten Dijke, P. (1997) TGF-beta signaling from cell membrane to nucleus through SMAD proteins. Nature 390, 465-471.
11. Guicheux, J., Lemmonnier, J., Ghayor, C., Suzuki, A., Palmer, G. and Cavazzoni, J. (2003) Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. J. Bone Miner. Res. 18, 2060-2068.
12. Gallea, S., Lallemand, F., Atti, A., Rawadi, G., Ramez, V., Spinella-Jaegle, S., Kawai, S., Faucheu, C., Huet, L., Baron, R. and Roman-Roman, S. (2001) Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells. Bone 28, 491-498.
13. Liu, H., Liu, Y., Viggesswarup, M., Zheng, Z., Titus, L. and Borden, S. D. (2011) Activation of c-Jun NH2-terminal kinase 1 increases cellular responsiveness to BMP-2 and decreases binding of inhibitory Smad6 to the type 1 BMP receptor. J. Bone Miner. Res. 26, 1122-1132.
14. Huang, Y. F., Lin, J. J., Lin, C. H., Su, Y. and Hung, S. C. (2012) c-Jun N-terminal kinase 1 negatively regulates osteoblastic differentiation induced by BMP2 via phosphorylation of Runx2 at Ser104. J. Bone Miner. Res. 27, 1093-1105.
15. Zhao, Y., Song, T., Wang, W., Wang, J., He, J., Wu, N., Tang, M., He, B. and Luo, J. (2012) P38 and ERK1/2 MAPKs act in opposition to regulate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. PLoS One 7, e43383.
16. Wiedmann, C., Gibson, S., Jarpe, M. B. and Johnson, G. L. (1999) Mitogen-activated protein kinase: conservation of a three-module from yeast to human. Physiol. Rev. 79, 143-180.
17. Song, J. I., Celeste, A. J., Kong, F. M., Jirtle, R. L., Rosen, V. and Thies, R. S. (1995) Bone morphogenetic protein-9 binds to liver cells and stimulates proliferation. Endocrinology 136, 4293-4297.
18. López-Coviella, I., Berse, B., Krauss, R., Thies, R. S. and Blusztajn, J. K. (2000) Induction and maintenance of the neuronal cholinergic phenotype in the central nervous system by BMP9. Science 289, 313-316.
19. Chen, C., Grzegorzezski, K. J., Baras, S., Zhao, Q., Schneider, H., Wang, Q., Singh, M., Pukac, L., Bell, A. C., Duan, R., Coleman, T., Duttaroy, A., Cheng, S., Hirsch, J., Zhang, L., Lazard, Y., Fischer, C., Barber, M. C., Ma, Z. D., Zhang, Y. Q., Reavey, P., Zhong, L., Teng, B., Sanyal, I., Ruben, S. M., Blondel, O. and Birsie, C. E. (2003) An integrated functional genomics screening program reveals a role for BMP-9 in glucose homeostasis. Nat. Biotechnol. 21, 294-301.
20. Truksa, J., Peng, H., Lee, P. and Beutler, E. (2006) Bone morphogenetic proteins 2, 4, and 9 stimulate murine hepatic cell 1 expression independently of Hif, transferring receptor 2 (Trf2), and IL-6. Proc. Natl. Acad. Sci. U.S.A. 103, 10289-10291.
21. Li, C., Yang, X., He, Y., Ye, G., Li, X., Zhang, X., Zhou, L. and Deng, F. (2012) Bone morphogenetic protein-9 induces osteogenic differentiation of rat dental follicle stem cells in P38 and ERK1/2 MAPK dependent manner. Int. J. Med. Sci. 9, 862-871.
22. Tian, X. Y., Yung, L. H., Wong, W. T., Liu, J., Leung, F. P., Liu, L., Chen, Y., Kong, S. K., Kwan, K. M., Ng, S. M., Lai, P. B., Yung, L. M., Yao, X. and Huang, Y. (2012) Bone morphogenic protein-4 induces endothelial cell apoptosis through oxidative stress-dependent p38MAPK and JNK pathway. J. Mol. Cell Cardiol. 52, 237-244.
23. Sovershav, M. A., Egorina, E. M., Sovershav, T. A., Svensson, B. and Hansen, J. B. (2011) Increased expression of TF in BMP7-treated human mononuclear cells depends on activation of select MAPK signaling pathways. Thromb. Res. 128, e154-159.