Noggin Resistance Contributes to the Potent Osteogenic Capability of BMP9 in Mesenchymal Stem Cells

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ABSTRACT: Mesenchymal stem cells (MSCs) are multipotent progenitors and can differentiate into osteogenic, chondrogenic, and adipogenic lineages. Bone morphogenetic proteins (BMPs) play important roles in stem cell proliferation and differentiation. We recently demonstrated that BMP9 is a potent but less understood osteogenic factor. We previously found that BMP9-induced ectopic bone formation is not inhibited by BMP3. Here, we investigate the effect of BMP antagonist noggin on BMP9-induced osteogenic differentiation. BMP antagonists noggin, chording, gremlin, follistatin, and BMP3 are highly expressed in MSCs, while noggin and follistatin are lowly expressed in more differentiated pre-osteoblast C2C12 cells. BMP9-induced osteogenic markers and matrix mineralization are not inhibited by noggin, while noggin blunts BMP2, BMP4, BMP6, and BMP7-induced osteogenic markers and mineralization. Likewise, ectopic bone formation by MSCs transduced with BMP9, but not the other four BMPs, is resistant to noggin inhibition. BMP9-induced nuclear translocation of Smad1/5/8 is not affected by noggin, while noggin blocks BMP2-induced activation of Smad1/5/8 in MSCs. Noggin fails to inhibit BMP9-induced expression of downstream targets in MSCs. Thus, our results strongly suggest that BMP9 may effectively overcome noggin inhibition, which should at least in part contribute to BMP9's potent osteogenic capability in MSCs. © 2013 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: BMP9; BMP signaling; mesenchymal stem cells; osteogenic differentiation; TGFβ/BMP antagonist

Bone morphogenetic proteins (BMPs) are members of the TGFβ superfamily, which play an important role in regulating stem cell proliferation and differentiation during development.[1–3] Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent cells and have the capacity to differentiate into multiple tissues.[4–6] MSCs can differentiate into osteoblastic, chondrogenic, and adipogenic lineages,[4–6] although it has recently been reported that MSCs are also able to differentiate into other lineages, including neuronal[7–9] and cardiomyogenic[10] lineages. It has been well established that BMPs regulate osteoblast differentiation and subsequent bone formation,[2,3,11] and genetic disruptions of these factors have resulted in skeletal and extraskeletal abnormalities during development.[12] We have conducted a comprehensive analysis of the osteogenic capacity of 14 human BMPs and demonstrated that BMP9 is one of the most potent, yet least characterized BMPs promoting osteogenic differentiation of MSCs both in vitro and in vivo.[3,13–15] We also demonstrated that BMP9 regulates a distinct set of downstream targets in MSCs.[3,15–20] BMPs initiate their signaling by binding to the plasma membrane TGFβ type I and type II receptors, leading to the nucleus gene regulation through both BMPR-Smad1/5/8-dependent and Smad-independent pathways.[2,15] We have demonstrated that ALK1 and ALK2 are required type I receptors for BMP9-induced osteogenic signaling.[21]

BMP9 (a.k.a., GDF2) was originally identified from fetal mouse liver cDNA libraries, and is a relatively uncharacterized member of the BMP family. BMP9 is highly expressed in the developing mouse liver, and recombinant human BMP9 stimulates hepatocyte proliferation.[22] BMP9 is a potent synergistic factor for hematopoietic progenitor-cell generation and colony formation and may play a role in the induction and maintenance of the neuronal cholinergic phenotype in the central nervous system.[23] The recombinant human BMP9 protein was shown to exert only mild osteoinductive activity in vivo,[22] while we and others have demonstrated that exogenously expressed BMP9 is highly capable of inducing osteogenic differentiation.[3,13–15] Nonetheless, many aspects of the molecular mechanism behind BMP9-regulated MSC differentiation remain to be fully elucidated. BMP signaling is negatively regulated at multiple cellular levels, including extracellular regulation (TGFβ/BMP antagonists, such as noggin), intracellular regulation (Smurf and inhibitory Smads), and transcriptional regulation (transcriptional repressors and epigenetic modifications).[24,25] We previously found that, unlike other osteogenic BMPs, BMP9-induced ectopic bone formation is not inhibited by BMP3,[14] a negative regulator

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of bone mineral density,[26] suggesting that BMP9 may exhibit unique capability to overcome BMP negative regulators in MSCs.

Here, we investigate effect of noggin on BMP9-induced osteogenic differentiation in MSCs. We find that several BMP antagonists are highly expressed in MSC lines, such as C3H10T1/2 and MEFs, while noggin and follistatin are lowly expressed in more differentiated pre-osteoblast C2C12 cells. BMP9-induced osteogenic markers and matrix mineralization are not blocked by noggin, whereas BMP2, 4, 6, and 7-induced osteogenic markers and mineralization are blunted by exogenous noggin. Furthermore, unlike the other four osteogenic BMPs, BMP9-transduced MSCs induce robust ectopic bone formation, which is not affected by exogenous noggin. Mechanistically, BMP9-induced nuclear translocation of Smad1/5/8 is not inhibited by noggin, while noggin blocks BMP2-induced nuclear translocation of Smad1/5/8 in MSCs. Noggin fails to inhibit BMP9-induced expression of downstream targets in MSCs. Taken together, our results strongly suggest that BMP9 may exhibit superior binding ability for its cognate receptors to its binding to noggin, leading to noggin resistance and contributing to BMP9's potent osteogenic capability in MSCs.

METHODS

Cell Culture and Chemicals

HEK-293, C2C12, C3H10T1/2, and HCT116 lines were obtained from the ATCC (Manassas, VA). HEK-293 and C2C12 cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM) as described.[13,16,27–31] C3H10T1/2 cells were maintained in complete Basal Medium Eagle. HCT116 cells were cultured in complete McCoy's 5A medium. Unless otherwise indicated, all chemicals were purchased from Sigma or Fisher Scientific.

Isolation of Mouse Embryonic Fibroblasts (MEFs)

MEFs were isolated from post coitus day 12.5–13.5 CD1 mice as described.[19,29,32] Each embryo was dissected into 10 ml sterile PBS, voided of its internal organs, and sheared through an 18-gauge syringe in the presence of 1 ml 0.25% trypsin and 1mM EDTA. After 15 min incubation with gentle shaking at 37°C, DMEM with 10% fetal bovine serum (FBS) was added to inactivate trypsin. Cells were plated on 100 mm dishes and incubated for 24 h at 37°C. Adherent cells were used as MEF cells. Aliquots were kept in a liquid nitrogen tank. All MEFs used in this study were within five passages.

Construction of Recombinant Adenoviruses Expressing BMP2, BMP4, BMP6, BMP7, BMP9 and Noggin

Recombinant adenoviruses were generated using the AdEasy technology as described.[13,33–36] The generation of adenoviruses expressing BMP2, BMP4, BMP6, BMP7, and BMP9 (e.g., AdBMPs) were previously described [13,14,37]. The coding region of mouse noggin was PCR amplified and cloned into an adenoviral shuttle vector and subsequently used to generate recombinant adenovirus in HEK293 cells. The resulting adenovirus was designated as Ad-Noggin, which also expresses red fluorescent protein (RFP). Analogous adenovirus expressing only green fluorescent protein (GFP) (AdGFP) or monomeric RFP (AdRFP) was used as controls.[13,29,30,33–36,38] All PCR-amplified fragments and cloning junctions were verified by DNA sequencing. Details about the vector construction are available upon request.

Total RNA Isolation and Semi-quantitative RT-PCR (sqPCR) Analysis

Total RNA was isolated using TRIZOL Reagents (Invitrogen). cDNA was generated by RT reaction with hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA), and used as PCR templates. Semi-quantitative RT-PCR was carried out as described.[19,20,27,29–31,37–43] PCR primers of 150–180 bp were designed by using *Primer3 Plus* program (http://www.bioinformatics.nl/cgi-bin/primer3-plus/primer3plus.cgi) to amplify the genes of interest (Table S1). A touchdown cycling program was as follows: 94°C for 2 min for 1 cycle; 92°C for 20 s, 68°C for 30 s, and 72°C for 12 cycles with a decrease in 1°C per cycle; and then at 92°C for 20 s, 57°C for 30 s, and 72°C for 20 s for 20–25 cycles, depending on the abundance of a given gene. PCR products were confirmed by resolving PCR products on 1.5% agarose gels. All samples were normalized with GAPDH expression.

Immunohistochemical Staining

Cultured cells were fixed with 10% formalin, permeabilized with 1% NP-40 and blocked with 10% goat serum, followed by incubation with an anti-osteocalcin, or osteopontin (OPN) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. After being washed, cells were incubated with biotin-labeled secondary antibody for 30 min, followed by incubating cells with streptavidin-HRP conjugate for 20 min. The presence of the expected proteins was visualized by DAB staining and examined under a microscope. Stains without the primary antibody, or with control IgG, were used as negative controls.

Immunofluorescence Staining

Immunofluorescence staining was performed as described.[27,29,37,39–41,43] Briefly, cells were fixed with methanol, permeabilized with 1% NP-40, and blocked with 10% BSA, followed by incubating with Smad1/5/8 antibody (Santa Cruz Biotechnology) for 1 h. After being washed, cells were incubated with FITC-labeled secondary antibody (Santa Cruz Biotechnology) for 30 min. Stains without primary antibodies, or with control IgG, were used as negative controls.

Alkaline Phosphatase (ALP) Assay

ALP activity was assessed quantitatively with a modified assay using the Great Escape SEAP Chemiluminescence assay kit (BD Clontech, Mountain View, CA) and qualitatively with histochemical staining assay as described.[13,14,17–20,27,29,32,37,38,42,43] Each assay condition was performed in triplicate.

Matrix Mineralization Assay (Alizarin Red S Staining)

MEFs were seeded in 24-well cell culture plates, infected with the indicated adenoviruses, and cultured in complete medium containing ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM). At day 14, mineralized nodules were detected with Alizarin Red S staining as described.[13,14,17–19,27,29,32,37,38]

Preparation of BMP Conditioned Medium

BMP2, BMP9, and GFP control conditioned media were prepared as described.[44] Briefly, subconfluent HCT116 cells were seeded in 75 cm^2 flaks and infected with the optimal titer of AdBMP2, AdBMP9, or AdGFP. At 15 h post-infection, the culture medium was changed to serum-free OPTI-MEM I (Invitrogen) medium. Conditioned media were collected at 48 h after infection (designated as cmBMP2, cmBMP9, or cmGFP) and used immediately.

Subcutaneous Stem Cell Implantation and Ectopic Bone Formation

The animal use and care were carried out according to the approved Institutional Animal Care and Use Committee. Stem cell-mediated ectopic bone formation was done as described.[13,14,19,20,29,32,37,42,43] Briefly, MEFs were infected with AdBMPs or AdRFP for 16 h, collected and resuspended in PBS for subcutaneous injection (5×10^6 cells/injection) into the flanks of athymic nude (nu/nu) mice (5 per group, 4–6 weeks old, female, Harlan Laboratories, Indianapolis, IN). At 4 weeks after implantation, animals were sacrificed. The implantation sites were retrieved for histologic evaluation, and bone/skeletal special staining.

Histologic Analysis and Bone-Specific Staining

Retrieved tissues were fixed, decalcified, and paraffin embedded. Serial sections were stained with hematoxylin and eosin (H&E). Masson's trichrome and alcian blue staining were carried out as described.[13,14,20,37,42,43]

Statistical Analysis

All quantitative experiments were performed in triplicate and/or repeated three times. Data were expressed as mean ± SD. Statistical significances between groups were determined by one-way analysis of variance and the Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Pre-Osteoblast Progenitors Express High Levels of BMP Antagonists

We first analyzed the endogenous expression status of several naturally occurring BMP antagonists, including noggin, chordin, gremlin, follistatin, and BMP3, in three pre-osteoblast progenitor cells lines (C3H10T1/2, MEFs, and C2C12). Under the same RT-PCR condition, all five inhibitors were shown highly expressed in

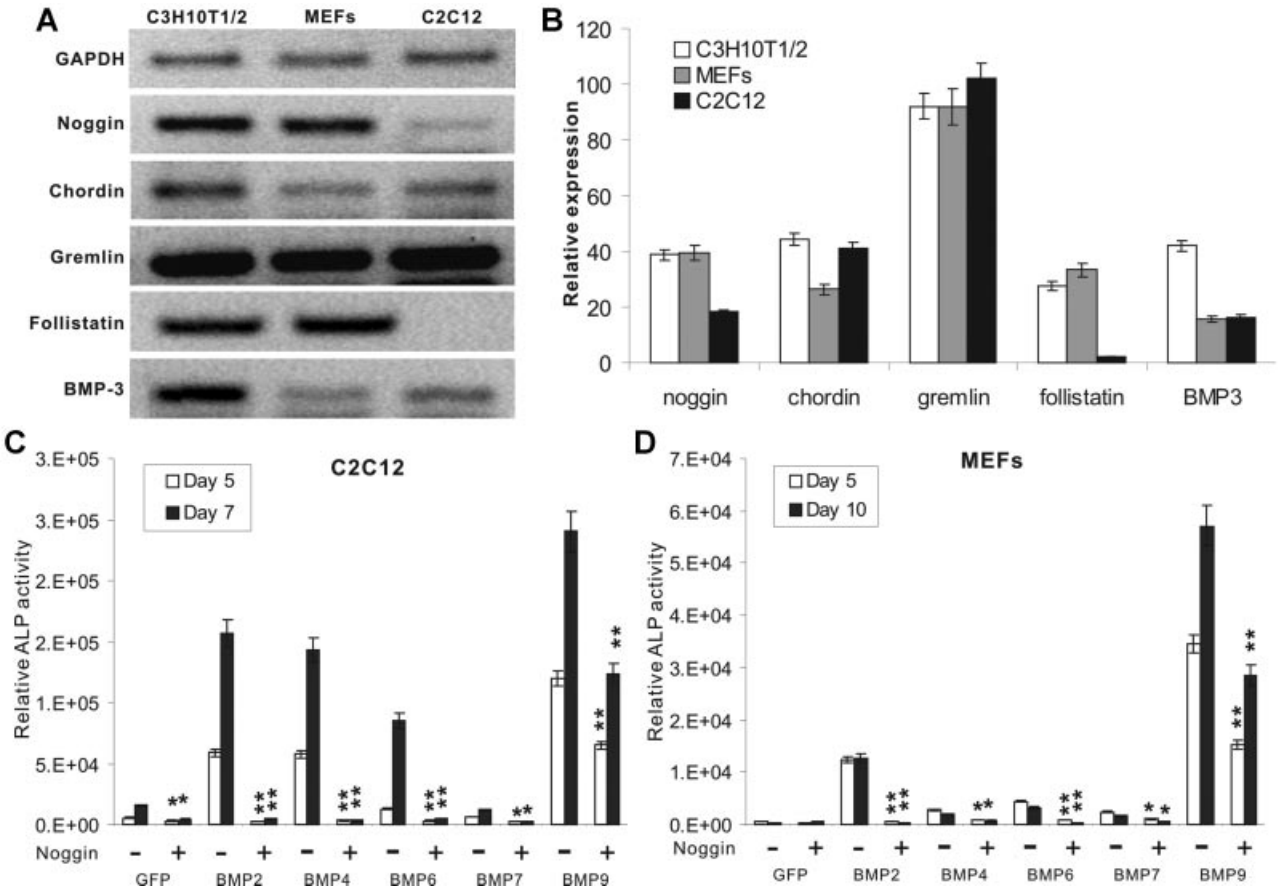

Figure 1. Noggin severely inhibits all osteogenic BMPs but BMP9-induced early marker of osteogenic differentiation of MSCs. (A and B) Endogenous expression of BMP inhibitors/antagonists in MSCs. (A) Total RNA was isolated from exponentially growing C3H10T1/2, MEFs, and C2C12 cells, and subjected to RT-PCR reverse transcription. The RT cDNA products were used as PCR templates for sqPCR analysis using specific primer pairs for mouse noggin, chording, gremlin, follistatin, and BMP3 (A). GAPDH was used as an internal normalization control. Representative results are shown. The sqPCR results were quantitatively analyzed with ImageJ software (B). (C and D) Noggin inhibition on osteogenic BMP-induced ALP activity in MSCs. C2C12 (C) and MEF (D) cells were co-infected with AdBMPs, AdGFP, and Ad-Noggin or AdRFP. ALP activity was measured at the indicated time points. Each assay condition was done in triplicate. The *p*-values were calculated by comparing the ALP activities in the absence (GFP group) versus in the presence of noggin. "*," $p < 0.05$; "**," $p < 0.01$.

C3H10T1/2 cells, while three of them were abundantly expressed in MEFs (Fig. 1A and B). Gremlin (chordin and BMP3 to a lesser extent) was highly expressed in the three lines, whereas the expression of noggin and follistatin was barely detectable in C2C12 cells (Fig. 1A and B). Interestingly, the expression pattern of these antagonists, especially noggin and follistatin, is seemingly consistent with the relative differentiation potential of the three lines, with C3H10T1/2 being the most undifferentiated, C2C12 the most differentiated, and MEFs in between. Thus, these results suggest that MSCs may express relatively high levels of BMP antagonists while these antagonists' role in regulating BMP-induced osteoblastic lineage-specific differentiation remains to be defined.

All Osteogenic BMP except BMP9 Induced-ALP Activity Is Severely Inhibited by Noggin in MSCs

We previously found that unlike other osteogenic BMPs (e.g., BMP2, BMP4, BMP6, and BMP7) BMP9-induced bone formation is not inhibited by BMP3.[14] We sought to investigate the effect of noggin on osteogenic BMPs. When pre-osteoblastic C2C12 cells were stimulated with osteogenic BMP2, BMP4, BMP6, BMP7, and BMP9 in the presence of noggin, the activity of early osteogenic marker ALP was remarkably inhibited in BMP2, BMP4, BMP6, and BMP7 transduced cells (Fig. 1C). However, in the BMP9-stimulated C2C12 cells the ALP activity remained at a high level although there was about 50% decrease in ALP activity (Fig. 1C). The effect of noggin on osteogenic BMP-induced ALP activity in C2C12 cells was consistent with ALP histochemical staining (Fig. S1A). Similar results were obtained in primary MEFs (Figs. 1D and S1B). Results from both cell lines indicate that noggin can effectively inhibit BMP2, BMP4, BMP6, and BMP7-induced osteogenic activity, while BMP9 is relatively resistant to noggin-mediated inhibition. This phenomenon was further confirmed in the commonly-used MSC line C3H10T1/2 cells (Fig. 2). Specifically, BMP2, 4, 6, and 7-induced ALP activity was significantly inhibited even in the lower titer group treated with Ad-Noggin (Fig. 2). Conversely, BMP9-induced ALP activity remained relatively high even in the high titer Ad-Noggin group (Fig. 2). Taken together, our results indicate that while noggin exhibits a profound inhibitory effect on four osteogenic BMP-induced osteoblastic lineage-specific commitment, BMP9 is resistant to noggin-induced inhibition of osteogenic differentiation in MSCs.

BMP9 Is Resistant to Noggin-Induced Inhibitory Effect on Late Osteogenic Markers and Mineralization of MSCs

We further analyzed the effect of noggin on BMP-induced late osteogenic markers and matrix mineralization. We infected subconfluent MEFs with AdBMPs and Ad-Noggin or AdRFP and performed immunohistochemical staining with an antibody against OPN (Fig. 3A) or osteocalcin (OCN) (Fig. 3B). The results

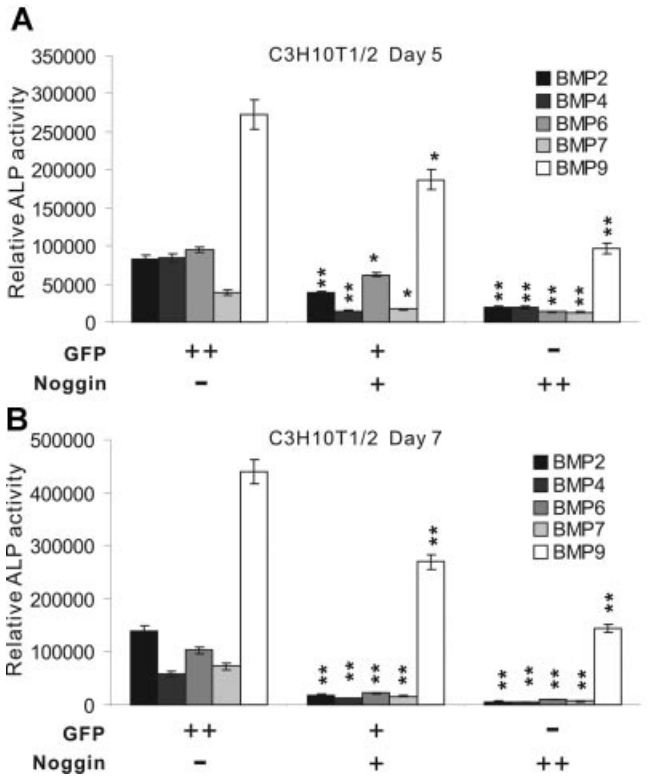

Figure 2. Dose-dependent inhibition of noggin on osteogenic BMPs induced osteogenic differentiation of MSCs. Subconfluent C3H10T1/2 cells were co-infected with a fixed titer of AdBMPs (MOI = 5) and various titers of Ad-Noggin or AdGFP. ALP activity was measured at day 5 (A) and day 7 (B) after infection. Each assay condition was done in triplicate. The *p*-values were calculated by comparing the ALP activities in the absence (GFP group) versus in the presence of noggin. "*," $p < 0.05$; "**," $p < 0.01$; "–," multiplicity of infection (MOI) = 0; "+," MOI = 5; "++," MOI = 10.

indicate that, while the expression of both late markers OPN and OCN was significantly decreased in the presence of noggin in BMP2, BMP4, BMP6, and BMP7-stimulated MSCs, BMP9-induced OPN and OCN expression was readily detectable (Fig. 3A and B). Accordingly, in vitro matrix mineralization induced by BMP2, BMP4, BMP6, and BMP7 was effectively inhibited by noggin in C2C12 (Fig. 3C) and C3H10T1/2 cells (Fig. 3D). However, BMP9-induced mineralization was not affected by noggin (Fig. 3C and D). Taken together, our in vitro results strongly suggest that BMP9 may be resistant to noggin-mediated inhibition of osteogenic differentiation of MSCs.

BMP9 Is Resistant to Noggin-Mediated Inhibition of Ectopic Bone Formation

We further examined the effect of noggin on osteogenic BMP-induced ectopic bone formation. Consistent with our previous studies,[3,14] BMP9-transduced C2C12 or MEFs were shown to form large ectopic bony masses, while these cells transduced with BMP2, BMP4, BMP6, and BMP7 formed much smaller masses (Fig. 4A). Noggin failed to inhibit BMP9-induced ectopic bone masses in both cell lines, while other four

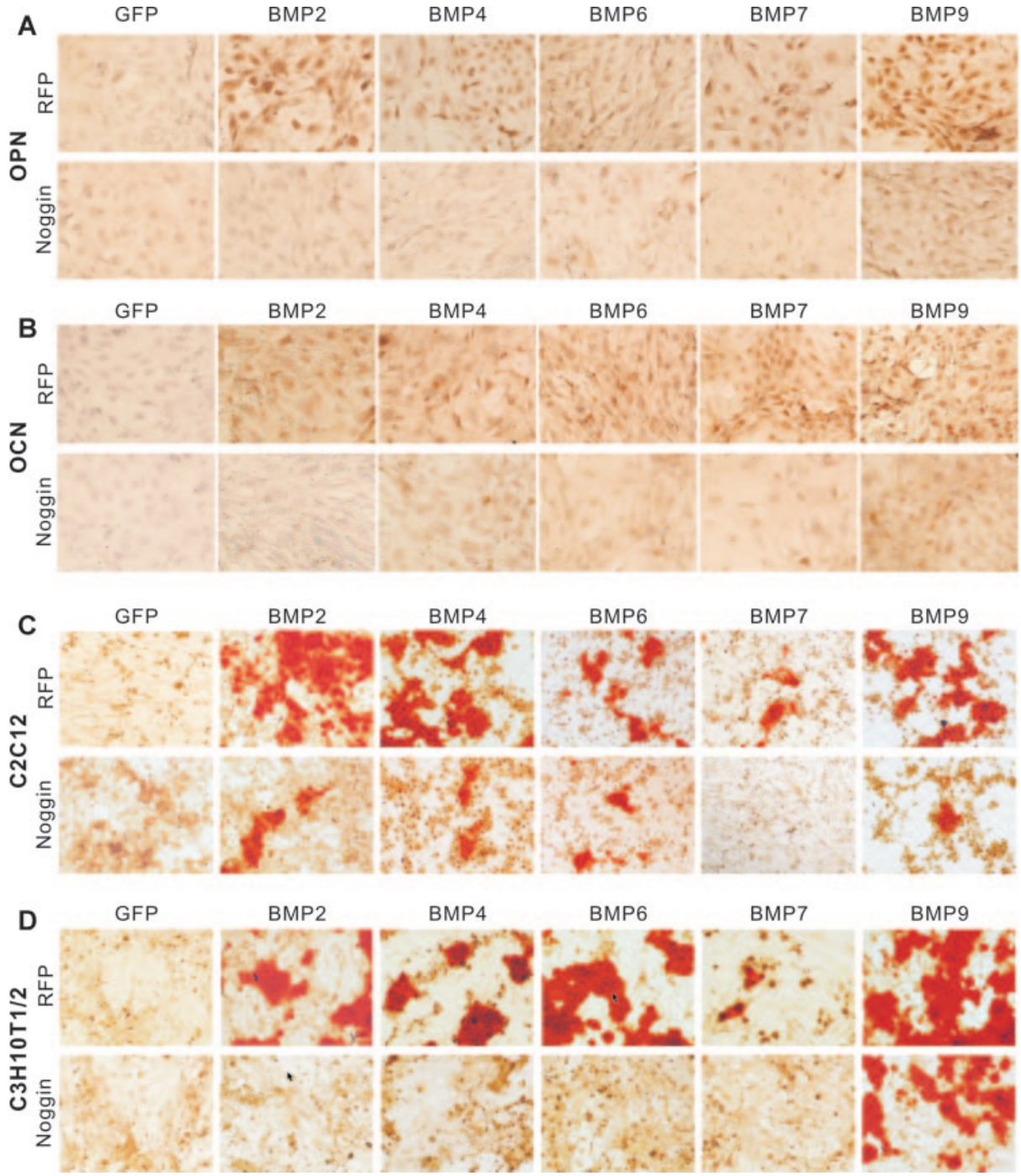

Figure 3. Noggin inhibits late osteogenic markers and mineralization induced by all osteogenic BMPs but BMP9. (A and B) Effect of noggin on BMP-induced osteopontin (OPN) and osteocalcin (OCN) expression in MEFs. Subconfluent MEFs were co-infected with AdBMPs and Ad-Noggin or AdRFP. At day 10 post infection, cells were subjected to immunohistochemical staining with an antibody against OPN (A) or OCN (B). Isotype IgG or without primary antibody staining was used as a negative control (data not shown). Representative images are shown. (C and D) Effect of noggin on BMP-induced matrix mineralization in C2C12 and C3H10T1/2 cells. Subconfluent C2C12 (C) and C3H10T1/2 (D) cells were co-infected with AdBMPs and Ad-Noggin or AdRFP. At day 14 post infection, cells were fixed and subjected to Alizarin Red S staining. Representative images are shown.

BMP-induced bone formation was effectively blunted (Fig. 4A). No detectable masses were recovered from the athymic mice injected with MEFs transduced with BMP2, 4, 6, and 7 in the presence of noggin, or transduced with RFP or noggin alone. Histologic evaluation revealed that BMP2, BMP6 and BMP7, but not BMP4, induced robust bone formation in C2C12 cells (Fig. S2A, a–d), consistent with our previous findings.[3,14] In MEFs, BMP2 and BMP6, to a lesser extent BMP7, were able to induce bone formation (Fig. S2B, a–d). However, BMP2, BMP6 and BMP7-induced bone formation was significantly inhibited by

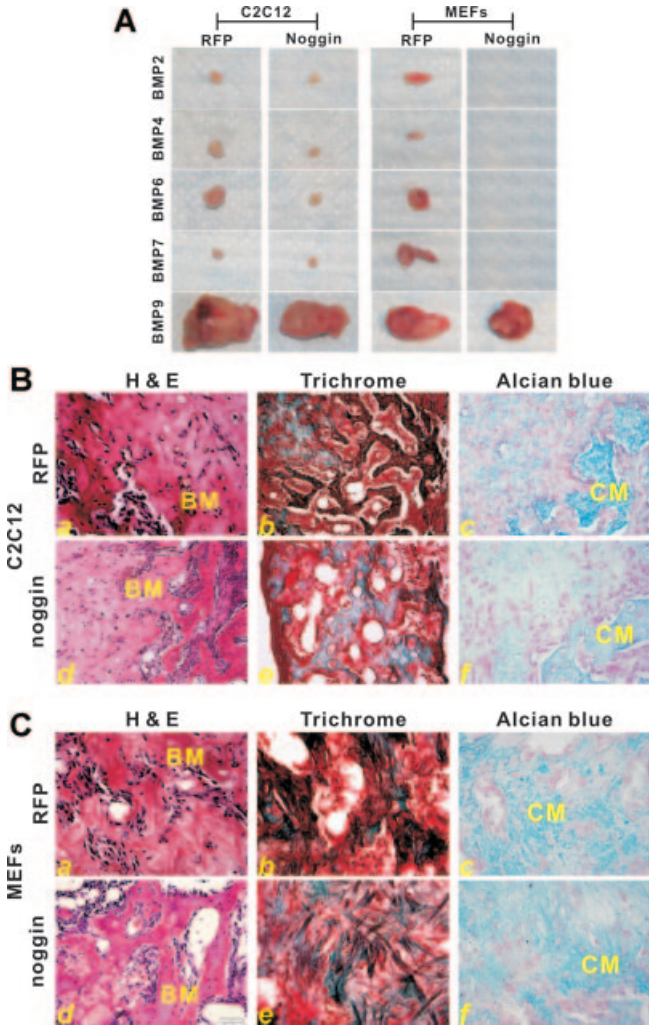

Figure 4. BMP9-induced ectopic bone formation is not inhibited by noggin. (A) Macrographic representation of the BMP-induced ectopic bone formation in the presence or absence of noggin. The subcutaneous injection sites with BMP-transduced C2C12 or MEF cells with or without noggin were retrieved at 4 weeks post injection. Note that no detectable masses were recovered from the athymic mice injected with MEFs transduced with BMP2, 4, 6, and 7 in the presence of noggin, or transduced with RFP or noggin alone. Representative images are shown. (B and C) Histologic analysis and bone-skeletal specific staining assays of the retrieved samples. Retrieved samples from the transduced C2C12 cells (B) and MEFs (C) were paraffin-embedded, sectioned, and subjected to H&E staining (a and d), trichrome staining (b and e), and alcian blue staining (c and f). Representative results are shown. Magnification, 200×; BM, bone matrix; CM, chondroid/cartilage matrix.

noggin in C2C12 cells (Fig. S2A, e–h) and almost completely inhibited in MEFs. BMP9 was shown to induce robust and mature bone formation in both C2C12 cells (Fig. 4B, a–c) and MEFs (Fig. 4C, a–c) as demonstrated by the H&E staining, trichrome staining and alcian blue staining. Unlike the above four BMPs, BMP9-induced ectopic bone formation was not inhibited by noggin in C2C12 cells (Fig. 4B, a vs. d) and MEFs (Fig. 4C,a vs. d), although there was a slight but noticeable decrease in bone matrix maturation as assessed by trichrome staining (Fig. 4B,C, b vs. e). Nonetheless, our in vivo results demonstrated that BMP9 is resistant to noggin-mediated inhibition of ectopic bone formation.

Noggin Is Unable to Inhibit BMP9-Activated Smad Signaling in MSCs

We sought to investigate the underlying molecular mechanism by examining the status of BMP-Smad signaling axis upon BMP and noggin stimulations in MSCs. BMP2 was shown to induce a significant nuclear accumulation of Smad1/5/8, which was completely blocked by noggin (Fig. 5A, a vs. b). Conversely, BMP9 was able to induce nuclear translocation of Smad1/5/8 (Fig. 5A, panel c), which was not affected by noggin (Fig. 5A, panel d). As expected, cmGFP did not induce any detectable nuclear translocation of BMP-R Smads (Fig. 5A, e vs. f). These results suggest that, while noggin can block BMP2 signaling at the initiation stage, BMP9 may overcome the inhibitory effect of noggin for BMP receptor binding and thus retain the capability to activate Smad signaling pathway. When C2C12 cells were stimulated with BMP2 or BMP9, both Smad6 and Smad7 were markedly up-regulated (Fig. 5B). However, BMP9-upregulated expression of Smad6 and Smad7 was not inhibited by noggin, whereas BMP2-induced expression of Smad6 and Smad7 was effectively inhibited by noggin (Fig. 5B). These results are consistent with the fact that noggin iwas unable to inhibit BMP9-induced nuclear translocation of Smad1/5/8 shown in Fig. 5A.

DISCUSSION

We previously conducted a comprehensive analysis of the osteogenic potential of 14 types of human BMPs and demonstrated that BMP9 is the most osteogenic BMP in MSCs, although BMP2 and BMP6 (BMP4 and BMP7, to a much lesser extent) also exhibit osteogenic capability.[3,13–15,37] As BMP9 is one of the least understood BMPs, we performed expression profiling analysis and have identified several important downstream targets of BMP9 signaling.[15–20] Furthermore, we have demonstrated that BMP9-induced osteogenic signaling can effectively synergize with other signaling pathways, including Wnt/β-catenin, retinoic acids, IGFs, and growth hormone.[20,29,32,42] BMP action is tightly regulated by BMP antagonists,[24,25] including the CAN (Cerberus and DAN) family of proteins that is made up of Gremlin and Cerberus, Twisted gastrulation, Chordin and Crossveinless 2, and Noggin.[24,25] It is conceivable that BMP9's potent osteogenic activity may be attributable to its ability to overcome the TGFβ/BMP negative regulators. Here, we demonstrate that BMP9 effectively antagonizes noggin-induced inhibition of osteogenic signaling in MSCs.

Noggin was originally identified as a protein that rescued dorsal development in embryos ventralized by UV irradiation in *Xenopus laevis*.[45] Noggin inhibits TGFβ/BMP signaling transduction by binding to the ligand domains that are required for BMPs to interact with BMP type I and type II receptors.[46] By diffusing

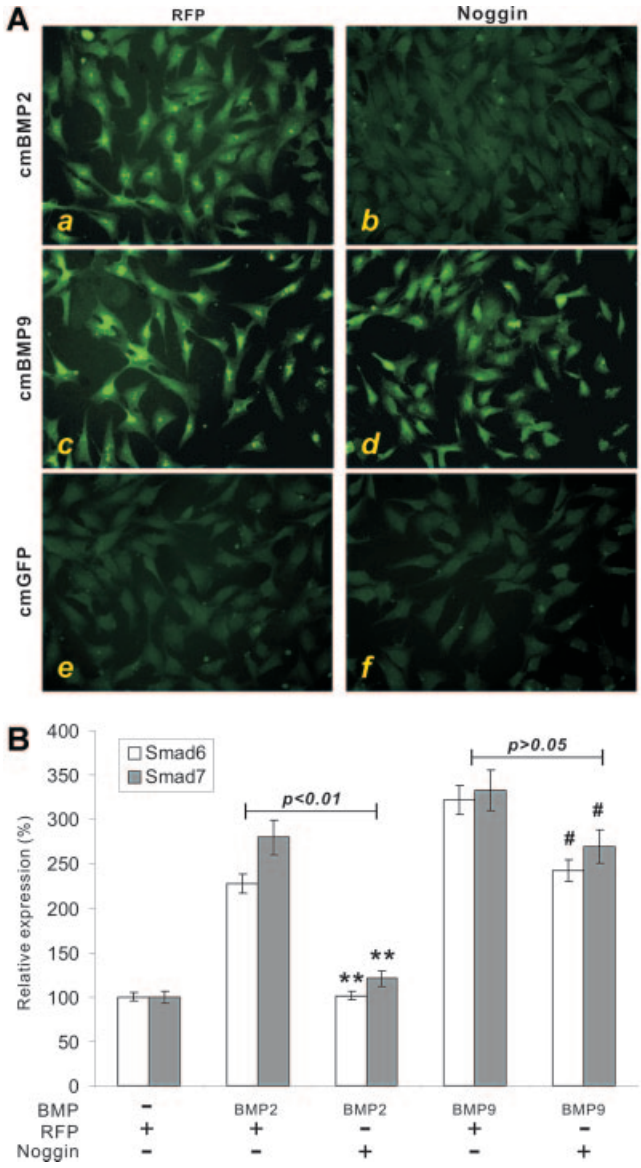

Figure 5. Noggin fails to inhibit BMP9-activated Smad signaling pathway in MSCs. (A) BMP2, but not BMP9, induced BMP R-Smad activation is inhibited by noggin. Subconfluent C2C12 cells were infected with Ad-Noggin or AdRFP and cultured in 1% FBS medium overnight. The transduced cells were then treated with conditioned medium cmBMP2 (a and b), cmBMP9 (c and d), or cmGFP control (e and f) for 2 h. The cells were fixed and subjected to immunofluorescence staining using an anti- Smad1/5/8 antibody (Santa Cruz Biotechnology). Control IgG or without primary antibody staining was used as a negative control (data not shown). Representative results are shown. (B) Noggin fails to inhibit BMP9-induced Smad6/7 expression. Subconfluent C2C12 cells were co-infected with AdBMPs and Ad-Noggin or AdRFP for 30 h. Total RNA was isolated from the infected cells and subjected to semi-quantitative RT-PCR analysis using PCR primers specific for mouse Smad6 and Smad7. GAPDH was used as a normalization control. Each assay condition was done in triplicate. The p-values were calculated by comparing the expression levels of Smad6 and Smad7 between the RFP group and the noggin group for each BMP treatment. "**," $p < 0.01$; "#," $p > 0.05$.

through extracellular matrices more efficiently than members of the TGFβ/BMP superfamily, noggin may have a principal role in creating morphogenic gradients. Noggin appears to have pleiotropic effect, both early in development as well as in later stages. *Nog* knockout mice display abnormalities at approximately E8.5, with a shortened body axis, smaller somites, malformed limbs, and neural tubes that fail to close.[47,48] However, the embryonic lethality of *Nog* null mice precludes analysis of its role in adult bone tissues.[24,25] Nonetheless, transgenic mice with overexpressed *Nog* in the bone microenvironment lead to phenotypes, including osteopenia, fractures, decreased trabecular bone volume and impaired osteoblastic function,[49,50] which is consistent with noggin's important role in bone and skeletal development.

In summary, our studies provide convincing in vitro and in vivo evidence showing that BMP9 is resistant to noggin inhibition of osteogenic differentiation of MSCs. Mechanistically, noggin fails to inhibit BMP9-induced Smad signaling pathway. These findings expand our knowledge about BMP9-mediated osteogenic signaling. Furthermore, noggin antagonism may at least in part account for the potent osteogenic capability of BMP9.

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REFERENCES

1. Attisano L, Wrana JL. 2002. Signal transduction by the TGF-beta superfamily. Science 296:1646–1647.
2. Deng ZL, Sharff KA, Tang N, et al. 2008. Regulation of osteogenic differentiation during skeletal development. Front Biosci 13:2001–2021.
3. Luu HH, Song WX, Luo X, et al. 2007. Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. J Orthop Res 25:665–677.
4. Prockop DJ. 1997. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74.
5. Rastegar F, Shenaq D, Huang J, et al. 2010. Mesenchymal stem cells: molecular characteristics and clinical applications. World J Stem Cells 2:67–80.
6. Shenaq DS, Rastegar F, Petkovic D, et al. 2010. Mesenchymal progenitor cells and their orthopedic applications: forging a path towards clinical trials. Stem Cells Int 2010: 519028.
7. Hermann A, Gastl R, Liebau S, et al. 2004. Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. J Cell Sci 117:4411–4422.
8. Keilhoff G, Goihl A, Langnase K, et al. 2006. Transdifferentiation of mesenchymal stem cells into Schwann cell-like myelinating cells. Eur J Cell Biol 85:11–24.
9. Wislet-Gendebien S, Wautier F, Leprince P, et al. 2005. Astrocytic and neuronal fate of mesenchymal stem cells expressing nestin. Brain Res Bull 68:95–102.
10. Makino S, Fukuda K, Miyoshi S, et al. 1999. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103:697–705.
11. Hogan BL. 1996. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev 10:1580–1594.

12. Zhao GQ. 2003. Consequences of knocking out BMP signaling in the mouse. Genesis 35:43–56.
13. Cheng H, Jiang W, Phillips FM, et al. 2003. Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). J Bone Joint Surg Am 85-A:1544–1552.
14. Kang Q, Sun MH, Cheng H, et al. 2004. Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. Gene Ther 11:1312–1320.
15. Luther G, Wagner ER, Zhu G, et al. 2011. BMP-9 Induced osteogenic differentiation of mesenchymal stem cells: molecular mechanism and therapeutic potentia. Curr Gene Ther 11:229–240.
16. Peng Y, Kang Q, Cheng H, et al. 2003. Transcriptional characterization of bone morphogenetic proteins (BMPs)-mediated osteogenic signaling. J Cell Biochem 90:1149–1165.
17. Peng Y, Kang Q, Luo Q, et al. 2004. Inhibitor of DNA binding/differentiation helix-loop-helix proteins mediate bone morphogenetic protein-induced osteoblast differentiation of mesenchymal stem cells. J Biol Chem 279:32941–32949.
18. Luo Q, Kang Q, Si W, et al. 2004. Connective tissue growth factor (CTGF) is regulated by Wnt and bone morphogenetic proteins signaling in osteoblast differentiation of mesenchymal stem cells. J Biol Chem 279:55958–55968.
19. Sharff KA, Song WX, Luo X, et al. 2009. Hey1 basic helix-loop-helix protein plays an important role in mediating BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. J Biol Chem 284:649–659.
20. Huang E, Zhu G, Jiang W, et al. 2012. Growth hormone synergizes with BMP9 in osteogenic differentiation by activating the JAK/STAT/IGF1 pathway in murine multilineage cells. J Bone Miner Res 27:1566–1575.
21. Luo J, Tang M, Huang J, et al. 2010. TGFbeta/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in mesenchymal stem cells. J Biol Chem 285:29588–29598.
22. Song JJ, Celeste AJ, Kong FM, et al. 1995. Bone morphogenetic protein-9 binds to liver cells and stimulates proliferation. Endocrinology 136:4293–4297.
23. Lopez-Coviella I, Berse B, Krauss R, et al. 2000. Induction and maintenance of the neuronal cholinergic phenotype in the central nervous system by BMP-9. Science 289:313–316.
24. Walsh DW, Godson C, Brazil DP, et al. 2010. Extracellular BMP-antagonist regulation in development and disease: tied up in knots. Trends Cell Biol 20:244–256.
25. Zakin L, De Robertis EM. 2010. Extracellular regulation of BMP signaling. Curr Biol 20:R89–R92.
26. Daluiski A, Engstrand T, Bahamonde ME, et al. 2001. Bone morphogenetic protein-3 is a negative regulator of bone density. Nat Genet 27:84–88.
27. Luo X, Chen J, Song WX, et al. 2008. Osteogenic BMPs promote tumor growth of human osteosarcomas that harbor differentiation defects. Lab Invest 88:1264–1277.
28. Haydon RC, Zhou L, Feng T, et al. 2002. Nuclear receptor agonists as potential differentiation therapy agents for human osteosarcoma. Clin Cancer Res 8:1288–1294.
29. Tang N, Song WX, Luo J, et al. 2009. BMP9-induced osteogenic differentiation of mesenchymal progenitors requires functional canonical Wnt/beta-catenin signaling. J Cell Mol Med 13:2448–2464.
30. He BC, Chen L, Zuo GW, et al. 2010. Synergistic antitumor effect of the activated PPAR gamma and retinoid receptors on human osteosarcoma. Clin Cancer Res 16:2235–2245.
31. He BC, Gao JL, Zhang BQ, et al. 2011. Tetrandrine inhibits Wnt/beta-catenin signaling and suppresses tumor growth of human colorectal cancer. Mol Pharmacol 79:211–219.
32. Zhang W, Deng ZL, Chen L, et al. 2010. Retinoic acids potentiate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. PloS ONE 5:e11917.
33. He TC, Chan TA, Vogelstein B, et al. 1999. PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell 99:335–345.
34. He TC, Sparks AB, Rago C, et al. 1998. Identification of c-MYC as a target of the APC pathway. Science 281:1509–1512.
35. He TC, Zhou S, da Costa LT, et al. 1998. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA 95:2509–2514.
36. Luo J, Deng ZL, Luo X, et al. 2007. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. Nat Protoc 2:1236–1247.
37. Kang Q, Song WX, Luo Q, et al. 2009. A comprehensive analysis of the dual roles of BMPs in regulating adipogenic and osteogenic differentiation of mesenchymal progenitor cells. Stem Cells Dev 18:545–559.
38. Si W, Kang Q, Luu HH, et al. 2006. CCN1/Cyr61 is regulated by the canonical Wnt signal and plays an important role in Wnt3A-induced osteoblast differentiation of mesenchymal stem cells. Mol Cell Biol 26:2955–2964.
39. Bi Y, Huang J, He Y, et al. 2009. Wnt antagonist SFRP3 inhibits the differentiation of mouse hepatic progenitor cells. J Cell Biochem 108:295–303.
40. Zhu GH, Huang J, Bi Y, et al. 2009. Activation of RXR and RAR signaling promotes myogenic differentiation of myoblastic C2C12 cells. Differentiation 78:195–204.
41. Huang J, Bi Y, Zhu GH, et al. 2009. Retinoic acid signalling induces the differentiation of mouse fetal liver-derived hepatic progenitor cells. Liver Int 29:1569–1581.
42. Chen L, Jiang W, Huang J, et al. 2010. Insulin-like growth factor 2 (IGF-2) potentiates BMP-9-induced osteogenic differentiation and bone formation. J Bone Miner Res 25:2447–2459.
43. Huang E, Bi Y, Jiang W, et al. 2012. Conditionally immortalized mouse embryonic fibroblasts retain proliferative activity without compromising multipotent differentiation potential. PloS ONE 7:e32428.
44. Zhou L, An N, Jiang W, et al. 2002. Fluorescence-based functional assay for Wnt/beta-catenin signaling activity. Biotechniques 33:1126–1128, 1130, 1132 passim.
45. Smith WC, Harland RM. 1992. Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. Cell 70:829–840.
46. Groppe J, Greenwald J, Wiater E, et al. 2002. Structural basis of BMP signalling inhibition by the cystine knot protein Noggin. Nature 420:636–642.
47. McMahon JA, Takada S, Zimmerman LB, et al. 1998. Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev 12:1438–1452.
48. Brunet LJ, McMahon JA, McMahon AP, et al. 1998. Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. Science 280:1455–1457.
49. Devlin RD, Du Z, Pereira RC, et al. 2003. Skeletal overexpression of noggin results in osteopenia and reduced bone formation. Endocrinology 144:1972–1978.
50. Wu XB, Li Y, Schneider A, et al. 2003. Impaired osteoblastic differentiation, reduced bone formation, and severe osteoporosis in noggin-overexpressing mice. J Clin Invest 112:924–934.