

# IGF1 potentiates BMP9-induced osteogenic differentiation in mesenchymal stem cells through the enhancement of BMP/Smad signaling

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**Engineered bone tissue is thought to be the ideal alternative for bone grafts in the treatment of related bone diseases. BMP9 has been demonstrated as one of the most osteogenic factors, and enhancement of BMP9-induced osteogenesis will greatly accelerate the development of bone tissue engineering. Here, we investigated the effect of insulin-like growth factor 1 (IGF1) on BMP9-induced osteogenic differentiation, and unveiled a possible molecular mechanism underlying this process. We found that IGF1 and BMP9 are both detectable in mesenchymal stem cells (MSCs). Exogenous expression of IGF1 potentiates BMP9-induced alkaline phosphatase (ALP), matrix mineralization, and ectopic bone formation. Similarly, IGF1 enhances BMP9-induced endochondral ossification. Mechanistically, we found that IGF1 increases BMP9-induced activation of BMP/Smad signaling in MSCs. Our findings demonstrate that IGF1 can enhance BMP9-induced osteogenic differentiation in MSCs, and that this effect may be mediated by the enhancement of the BMP/Smad signaling transduction triggered by BMP9. [BMB Reports 2016; 49(2): 122-127]**

## INTRODUCTION

Bone defects and non-union are very common diseases. Bone grafting is currently the main treatment for these diseases, although bone tissue engineering is the best alternative (1). One critical component of bone tissue engineering is growth factors, which should effectively promote differentiation of the seed cells towards an osteoblast lineage (2). The differentiation of precursor cells to osteoblasts is a well-regulated process, in which groups of factors interact with each other at critical

points to direct physiological bone formation. Among these factors, bone morphogenetic proteins (BMPs) are excellent candidates for study (3, 4). BMPs belong to the TGF- $\beta$  superfamily, and their osteogenic activities have been demonstrated in numerous preclinical and clinical studies (4). In clinical trials, BMP2 and BMP7 have been evaluated for the treatment of tibia fractures and spine fusion (5, 6). BMP9 is the most potent osteogenic BMP, however, the mechanism underlying this process remains unclear. The exploration of novel factors to potentiate BMP9-induced osteogenic differentiation will remarkably accelerate the development of bone tissue engineering.

It is conceivable that certain other factors are able to promote BMP9-induced osteogenesis. Indeed, a small number of factors and signaling pathways have been reported to be associated with the enhancement of BMP9-induced osteogenic differentiation in MSCs, such as Wnt/ $\beta$ -catenin (7), retinoic acids signaling (8), and COX-2 (9). Insulin-like growth factors (IGFs) are composed of two subtypes, IGF1 and IGF2. IGFs are well-known to regulate the proliferation and differentiation of muscle, cartilage, chondrocytes, and neurons (10-12). Moreover, IGFs have been shown to function cooperatively with BMP2 and/or BMP7 to regulate the osteogenic differentiation process (13, 14). This evidence suggests that IGFs may also have the potential to promote BMP9-induced osteogenesis in MSCs. IGF1 is secreted primarily in the liver in response to human growth hormone (GH) and acts systemically (15). However, IGF1 can also be found in osteocytes, where it regulates bone turnover during development (16). Although IGF1 can act synergistically with several BMPs in chondrocytes and preosteoblasts (13, 17), it remains unclear whether this effect could extend to BMP9-induced osteoblastic differentiation in MSCs.

In this investigation, we introduced a series of *in vitro* and *in vivo* experiments to study the effect of IGF1 on BMP9-induced osteogenic differentiation in MSCs, and unveiled a possible molecular mechanism underlying this effect. Our findings may provide another potential and efficacious strategy to enhance BMP9-induced osteogenesis in MSCs, which will greatly accelerate the development of bone tissue engineering.

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<http://dx.doi.org/10.5483/BMBRep.2016.49.2.228>

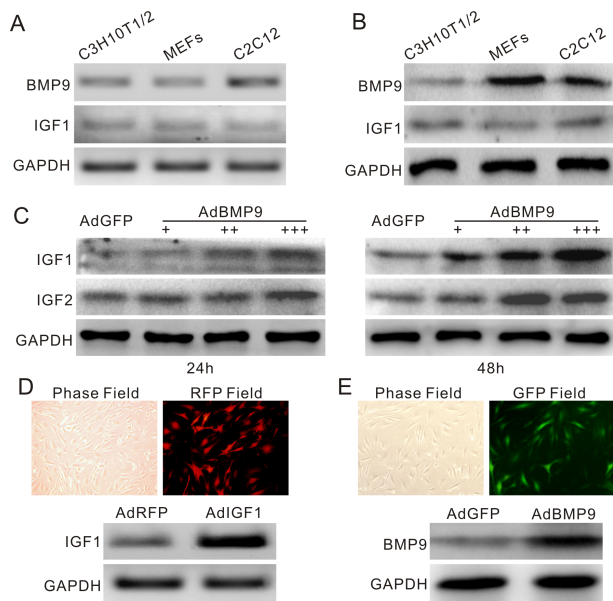
Received 4 November 2015, Revised 19 November 2015, Accepted 8 December 2015

**Keywords:** BMP9, BMPs/Smads, IGF1, MSCs, Osteogenic differentiation

## RESULTS

### IGF1 and BMP9 are detectable in MSCs, and BMP9 upregulates the expression of IGF1

Firstly, we examined the endogenous expression of IGF1 in MSCs using RT-PCR and Western blotting. We found that IGF1 was detectable in C3H10T1/2, MEF, and C2C12 cells (Fig. 1A and B), as is BMP9. To study the effect of IGF1 on BMP9-induced osteogenesis, we constructed recombinant adenoviruses for IGF1 and BMP9. Western blotting analysis showed that the respective recombinant adenoviruses greatly enhanced the expression of IGF1 (Fig. 1D) and BMP9 (Fig. 1E). Since C3H10T1/2 cells are one of the most commonly used progenitor cells for osteogenic differentiation research, this is the cell line we employed in the current investigation. Western blotting analysis showed that BMP9 markedly upregulated IGF1 in C3H10T1/2 cells (Fig. 1G), as it can IGF2. These data suggest that BMP9 and IGF1 are both present in MSCs, and that IGF1 may be associated with BMP9-induced osteogenesis in MSCs.



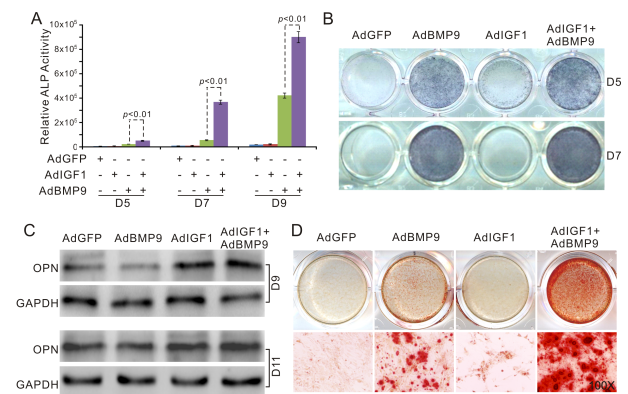
**Fig. 1.** Endogenous expression of BMP9 and IGF1 in progenitor cells and BMP9-induced expression of IGF1 in C3H10T1/2 cells. (A) PCR results show the endogenous expression of BMP9 and IGF1 in the three progenitor cells. GAPDH was used as a loading control. (B) Western blots show the endogenous expression of BMP9 and IGF1 in the three progenitor cells. GAPDH was used as a loading control. (C) Western blots show the BMP9-induced expression of IGF1 and IGF2 in C3H10T1/2 cells. GAPDH was used as a loading control. (D) Representative image and Western blot show the expression of IGF1 mediated by the adenovirus. GAPDH was used as loading control. (E) Representative image and Western blot show the expression of BMP9 mediated by the adenovirus. GAPDH was used as a loading control.

### Exogenous expression of IGF1 potentiates BMP9-induced ALP activity, osteopontin, and matrix mineralization in C3H10T1/2 cells

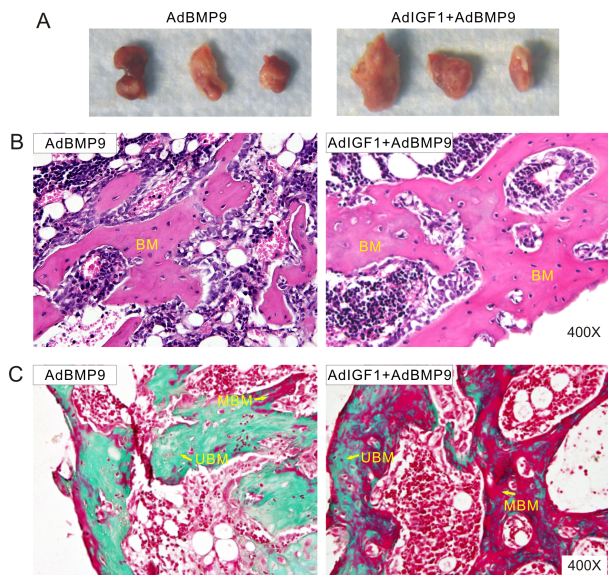
Next, we tested whether IGF1 could affect BMP9-induced osteogenic differentiation in MSCs. Subconfluent C3H10T1/2 cells were infected with AdGFP, AdBMP9, and/or AdIGF1. We found that exogenous expression of IGF1 exhibited no significant effect on ALP activity; BMP9 increased ALP activity in C3H10T1/2 cells, which could be strikingly enhanced when combined with IGF1 (Fig. 2A and B). Similar results were found with the expression of osteopontin (OPN) (Fig. 2C) on day 9. Alizarin Red S staining showed that the mineralized matrix nodules were readily detected in the BMP9-treated group, and the mineralized nodules in the BMP9-combined with IGF1-treated group were more pronounced than those in the group treated with BMP9 or IGF1 only (Fig. 2D). These data suggest that IGF1 may potentiate BMP9-induced osteogenic differentiation in MSCs, although IGF1 alone exerts no substantial osteogenic effects.

### IGF1 potentiates BMP9-induced ectopic bone formation

Next, we tested the effect of IGF1 on BMP9-induced osteogenesis *in vivo*. C3H10T1/2 cells were infected with AdRFP, AdBMP9, AdIGF1, or AdBMP9 and AdIGF1, collected, and subcutaneously injected into the flank of athymic nude mice. 5 weeks post-injection, the mice were sacrificed and the bone masses were retrieved. No bone masses were found in the AdRFP- or AdIGF1-treated groups. As a general observation, the volume of bone masses from the AdBMP9 combined with



**Fig. 2.** Effect of IGF1 on different BMP9-induced osteogenic markers in C3H10T1/2 cells. (A) ALP activity shows the effect of IGF1 on BMP9-induced osteogenic differentiation in C3H10T1/2 cells. (B) ALP staining shows the effect of IGF1 on BMP9-induced osteogenic differentiation in C3H10T1/2 cells. (C) Western blots show the effect of IGF1 on the expression of BMP9-induced OPN in C3H10T1/2 cells. GAPDH was used as a loading control. (D) Alizarin Red S staining shows the effect of IGF1 on BMP9-induced mineralization in C3H10T1/2 cells on day 20. The upper panel shows the scanning results, and the lower panel shows the images taken with the microscope.

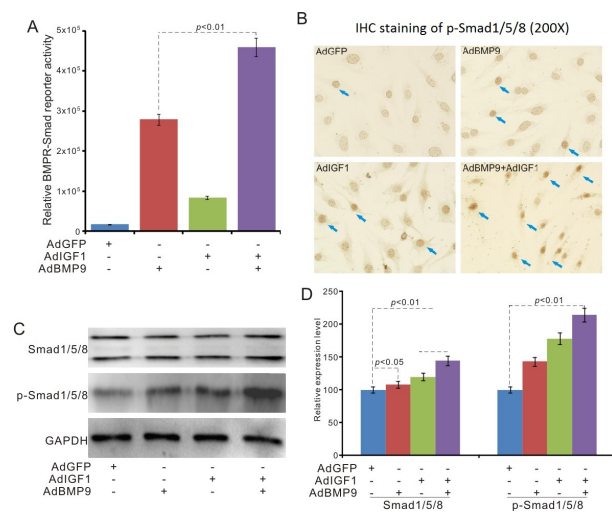


**Fig. 3.** Effect of IGF1 on BMP9-induced ectopic bone formation. (A) Representative retrieved bone masses show the effect of IGF1 on BMP9-induced ectopic bone formation. (B) H&E staining shows the effect of IGF1 on BMP9-induced ectopic bone formation. Representative images are shown (BM, bone matrix). (C) Masson's Trichrome staining shows the effect of IGF1 on BMP9-induced ectopic bone formation. Representative images are shown (MBM, matured bone matrix; UBM, ununmatured bone matrix).

AdIGF1 group were larger than those from the group treated with AdBMP9 only (Fig. 3A). H&E staining showed that a greater amount and thicker trabecular bone was present in the AdBMP9-combined with AdIGF1-treated group compared with the group treated with AdBMP9 only (Fig. 3B). Masson's trichrome staining showed that IGF1 promoted the maturation of BMP9-induced bone matrix (Fig. 3C). These data strongly suggest that IGF1 may be a strong enhancer for BMP9 in the induction of osteogenesis in MSCs.

#### IGF1 enhances BMP9-activated BMP/Smad signaling transduction in C3H10T1/2 cells

BMPs usually exert their physiological functions through BMP/Smad signaling, thus, we next investigated whether IGF1 could affect the BMP9-activated BMP/Smad signaling transduction in MSCs. The results showed that BMP9 alone could effectively promote the transcriptional activity of this BMP/Smad reporter, and that IGF1 alone could also moderately increase this activity. However, the transcriptional activity of this reporter was prominently enhanced when BMP9 was combined with IGF1 (Fig. 4A). Immunocytochemical staining and Western blotting showed that IGF1 could promote the BMP9-induced phosphorylation of Smad1/5/8 in C3H10T1/2 cells (Fig. 4B, C and D). These findings indicate that IGF1 may potentiate BMP9-induced activation of BMP/Smad signaling.



**Fig. 4.** Effect of IGF1 on BMP9-induced activation of BMP/Smad signaling in C3H10T1/2 cells. (A) Luciferase reporter activity shows IGF1 potentiates the activation of BMP9-induced BMP/Smad signaling. (B) Immunocytochemical staining shows the effect of IGF1 on BMP9-induced Smad1/5/8 phosphorylation (blue arrow shows the positive staining of Phosphorylated Smad1/5/8 in C3H10T1/2 cells). (C) Western blots show the effect of IGF1 on the level of BMP9-induced Smad1/5/8 and phosphorylation of Smad1/5/8. GAPDH was used as a loading control. (D) Quantification of Western blots shows the effect of IGF1 on the level of BMP9-induced Smad1/5/8 and phosphorylation of Smad1/5/8.

## DISCUSSION

BMP9, as one of the most potent osteogenic factors, may be widely used in bone tissue engineering for the treatment of bone defects, non-union, and fractures (4). In this investigation, we studied the effect of IGF1 on BMP9-induced osteogenic differentiation in MSCs, and found that IGF1 could substantially enhance BMP9-induced osteogenic differentiation. Mechanistically, we demonstrated that the effect of IGF1 on the promotion of BMP9-induced osteogenic differentiation may be mediated by the enhancement of BMP9-activated BMP/Smad signaling transduction in MSCs.

BMPs belong to the TGF- $\beta$  superfamily, of which there are approximately 20 members. Several BMPs such as BMP2 and BMP7 can induce MSCs to undergo osteogenic differentiation (18), with BMP9 being reported as the most potent osteogenic BMP to date (4). BMPs usually exert their physiological functions through the BMP/Smad pathway (19). Aside from this pathway, certain other essential factors or signaling pathways such as COX-2 (9), Wnt/ $\beta$ -catenin (7, 20), retinoic acids signaling (8, 20), and IGF2 (21) regulate or potentiate the osteogenic activity of BMP9 in MSCs.

The IGF system comprises two ligands (IGF1 and IGF2), two receptors (IGF1R and IGF2R), seven binding proteins (IGF binding proteins, IGFBP1 - IGFBP7) with high affinity for IGFs,

and IGFBPs degrading enzymes. IGF signaling is critical for the regulation of development and homeostasis, including differentiation, proliferation, and apoptosis (10-12, 22). In the skeletal system, IGFs are involved in the regulation of bone development, repair, and regeneration. Our previous study demonstrated that IGF2 could promote BMP9-induced osteogenesis (21), thus, theoretically, IGF2 may be used as a potent factor combined with BMP9 to induce osteogenesis in bone tissue engineering. However, IGF2 is expressed mainly during embryonic development, and is thought of as a major fetal growth factor (23). After birth, IGF2 remains at very low levels, and IGF2 overexpression may cause tissue hyperplasia (23, 24). For this reason, the combination of IGF2 and BMP9 may be high risk in clinical applications. In contrast, IGF1 has shown a different phenotype, being constantly secreted in response to growth hormones in the liver, and released systemically after birth (25, 26). IGF1 functions as a major growth factor in adults and is well-known to have anabolic effects on a wide variety of tissues including muscle, cartilage, and bone (22, 27-29). IGF1 null mice are born at approximately 60% birth weight compared to their littermates, but have longer life spans (29, 30). In the skeletal system, deletion or functional loss of IGF1 may contribute to age-related osteopenia (15). The level of IGF1 increases notably during bone regeneration or repair (31). When combined with VEGF, IGF1 can decoy progenitor cells towards osteogenic differentiation (32). This evidence implies that IGF1 may be more important and safe in the regulation of bone metabolism than IGF2. Although BMP9 was first found in hepatocytes, where IGF1 is also secreted, the effect of IGF1 on BMP9-induced osteogenic differentiation remains unknown.

Our findings suggest that IGF1 may potentiate BMP9-induced osteogenic differentiation in MSCs. Although IGF1 and BMP9 are both present in progenitor cells, osteogenic differentiation does not occur at this stage, which may be due to the fact that the endogenous levels of these two factors are far below the minimal levels needed to initialize osteogenic commitment. Using recombinant adenoviruses, we found that IGF1 enhanced BMP9-induced ALP activity and matrix mineralization in C3H10T1/2 cells (Fig. 2A-D). The results of the *in vivo* experiments showed that the bone volume and maturity of the BMP9-combined with IGF1-treated group was greater than that of the groups treated with IGF1 or BMP9 alone (Fig. 3B and C). BMP/Smad is the classical signaling pathway that mediates the osteogenic activity of BMP9 (33, 34). Our data indicates that IGF1 can enhance the activation of BMP9-induced BMP/Smad signaling in MSCs (Fig. 4A-D). All these data support the idea that IGF1 can potentiate BMP9-induced osteogenic differentiation in MSCs.

Taken together, the findings of this investigation strongly suggest that IGF1 is an excellent candidate enhancer for BMP9-induced osteogenic differentiation in MSCs, which may be mediated by the promotion of BMP9-initialized activation of BMP/Smad signaling transduction.

## MATERIALS AND METHODS

### Cell culture and chemicals

HEK293, C3H10T1/2, and C2C12 cells were purchased from ATCC (VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO<sub>2</sub>. All antibodies were purchased from Santa Cruz Biotechnology (CA, USA), and the PI3K inhibitor, LY294002, was bought from Selleck Chemicals (TX, USA).

### Construction of recombinant adenoviruses expressing RFP, GFP, BMP9, and IGF1

Recombinant adenoviruses were generated using AdEasy technology as previously described (35). The coding sequences of human BMP9 and IGF1 were amplified using PCR, cloned into an adenoviral shuttle vector, and subsequently used to generate recombinant adenoviruses in HEK293 cells. The recombinant adenoviruses were designated as AdBMP9 and AdIGF1. AdBMP9 was tagged with green fluorescent protein (GFP) and AdIGF1 was tagged with red fluorescent protein (RFP), as markers to track the viruses. Analogous adenovirus expressing only monomeric RFP (AdRFP) or GFP (AdGFP) were used as controls.

### Isolation of mouse embryonic fibroblasts (MEFs)

MEFs were isolated from mice on post-coitus day 13.5 as previously described (3, 8, 9). 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 1 mM EDTA. Following a 15-minute incubation with gentle shaking at 37°C, DMEM with 10% FBS was added to inactivate the trypsin. The cells were plated on 100-mm dishes and incubated for 24 hours at 37°C, following which adherent cells were used as MEF cells. Aliquots were kept in liquid nitrogen. MEFs used in this study were within 5 passages.

### RNA extraction and semi-quantitative RT-PCR analysis

Total RNA was isolated using TRIZOL reagents (Invitrogen), and used to generate cDNA templates by a RT reaction with hexamer and Superscript II RT (Invitrogen). The first strand cDNA products were further diluted 5-10 fold and used as PCR templates. Semi-quantitative RT-PCR was carried out as described. PCR primers were designed using the Primer3 program in order to detect the expression of genes of interest. All samples were normalized to the expression of GAPDH.

### Alkaline phosphatase (ALP) assay

ALP activity was assessed using a modified Great Escape SEAP chemiluminescence assay (BD Clontech, Mountain View, CA) and/or histochemical staining as described previously (4, 7, 9, 21). Cells were treated with the corresponding reagents as per the protocols. Each assay was performed in triplicate, and re-



peated in at least three independent experiments. ALP activity was normalized to the level of total cellular protein.

#### Transfection and luciferase reporter assay

Exponentially growing cells were seeded in T25 flasks and transfected with 2 µg/flask BMPR-Smad responsive element luciferase reporter (p12xSBE-Luc) (36), using Lipofectamine (Invitrogen). 16 hours post-transfection, cells were replated on 24-well plates and infected with AdBMP9, AdIGF1, and/or AdRFP at 4 hours after replating. 24 hours post-infection, cells were lysed and the lysates were collected for luciferase assays using a kit (Promega). Each assay condition was performed in triplicate.

#### Matrix mineralization assay (Alizarin Red S staining)

Cells were seeded on 24-well cell culture plates and infected with AdGFP, AdBMP9, and/or AdIGF1. Cells were cultured in the presence of ascorbic acid (50 µg/ml) and β-glycerophosphate (10 mM). 20 days post-infection, mineralized matrix nodules were stained with Alizarin Red S as described previously (4, 7, 20, 21). Cells were fixed with 0.05% (v/v) glutaraldehyde at room temperature for 10 minutes. After washing with distilled water, fixed cells were incubated with 0.4% Alizarin Red S (Sigma-Aldrich) for 5 minutes, followed by extensive washing with distilled water. The staining of mineralized matrix nodules was scanned and/or imaged under a microscope.

#### Immunocytochemical staining

Cultured cells were infected with the corresponding adenoviruses. Cells were fixed with 10% formalin and washed with PBS at the indicated time points. The fixed cells were permeabilized with 1% NP-40 and blocked with 10% goat serum, followed by incubation with a p-Smad1/5/8 primary antibody for 1 hour. Cells were then incubated with a biotin-labeled secondary antibody for 25 minutes, followed by incubation with streptavidin-HRP for 20 minutes at room temperature. The target protein was visualized by DAB staining and imaged under a microscope. Staining with the corresponding IgG was used as a negative control.

#### Ectopic bone formation

Cells were infected with adenoviruses as previously described (4, 8, 9, 20, 21). The animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Chongqing Medical University. 16 hours post-infection, cells were harvested and resuspended in PBS for subcutaneous injection ( $5 \times 10^6$ /injection) to the flanks of athymic nude mice (5 mice per group, 4-6 weeks old, female). 5 weeks post-injection, mice were sacrificed and the bone masses were retrieved for histological evaluation.

#### Histological evaluation and trichrome staining

Retrieved bone masses were fixed in 10% formalin (decalcified

if necessary) and embedded in paraffin. Paraffin-embedded sections were deparaffinized and then rehydrated in a graduated fashion. Deparaffinized sections were subjected to antigen retrieval and fixation, and then to hematoxylin and eosin (H&E) or Masson's Trichrome staining.

#### Statistical analysis

The results of all experiments are expressed as the mean ± standard deviation (SD) of at least three independent experiments. A Student's *t*-test was used for single-variable comparisons, and a *P* value <0.05 was considered statistically significant. Each assay condition was performed in triplicate, and the results were repeated in at least three independent experiments.

#### ACKNOWLEDGEMENTS

The authors wish to thank Dr. Di Chen of Rush University Medical Center for providing the p12xSBE-Luc plasmid. This work was supported by research grants from the Natural Science Foundation of China and Chongqing Science and Technology Commission (NSFC, 81272005; cstc2013jjB10021 to Zhong-liang Deng; cstc2015jcyjA10087 to Liang Chen).

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