Research Article

Synergistic Effects of BMP9 and miR-548d-5p on Promoting Osteogenic Differentiation of Mesenchymal Stem Cells

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Various stimulators have been reported to promote MSC osteogenic differentiation via different pathways such as bone morphogenetic protein 9 (BMP9) through influencing COX-2 and miR-548d-5p through targeting peroxisome proliferator-activated receptor-γ (PPARγ). Whether synergistic effects between BMP9 and miR-548d-5p existed in promoting osteogenesis from MSCs was unclear. In the study, the potential synergistic effects of BMP9 and miR-548d-5p on human MSC differentiation were investigated. Osteogenic differentiation of MSCs treated with BMP9 or miR-548d-5p was detected with multimodality of methods. The results demonstrated that BMP9 and miR-548d-5p significantly influenced COX-2 and PPARγ, respectively. BMP9 also influenced the expression of PPARγ, but no significant effect of miR-548d-5p on COX-2 was observed. When BMP9 and miR-548d-5p were combined, more potent effects on both COX-2 and PPARγ were observed than BMP9 or miR-548d-5p alone. Consistently, osteogenic analysis at different timepoints demonstrated that osteogenic genes, ALP activity, calcium deposition, OPN protein, and matrix mineralization were remarkably upregulated by BMP9/miR-548d-5p compared with BMP9 or miR-548d-5p alone, indicating the synergetic effects of BMP9 and miR-548d-5p on osteogenic differentiation of MSCs. Our study demonstrated that regulating different osteogenic regulators may be an effective strategy to promote bone tissue regeneration for bone defects.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that are able to differentiate into a series type of cells, such as osteoblasts, adipocytes, chondrocytes, or myocytes [1–4]. MSCs can be obtained from multiple sources, including adipose, periosteum, and bone marrow [5–8]. The fine-tuned balance between osteogenesis and adipogenesis of MSCs is important to a variety of human diseases, for instance, osteonecrosis, osteoporosis, and age-related bone loss [9, 10].

Bone morphogenetic proteins (BMPs), belonging to TGF-β superfamily, play a critical role in bone development and osteogenic differentiation [11, 12]. Among members of BMPs, BMP9 was reported to be one of the most potent BMPs to stimulate osteogenic differentiation of MSCs both *in vivo* and *in vitro* [13–16]. For BMP9 induced osteogenic

differentiation, a few important downstream targets were identified, including COX-2 [17, 18], Hey1 [19], and Creld2 [20]. COX-2 belongs to cyclooxygenase (COX) family, which produces prostaglandins (PGs) with arachidonic acid [18]. Among identified 3 types of COX, namely, COX-1, COX-2, and COX-3, COX-2 was demonstrated to be the only one that plays an important role in bone metabolism [21, 22]. Previous studies indicated that COX-2 can also promote BMP9 induced osteogenic differentiation through BMP9/Smads signal pathways [17, 18].

Peroxisome proliferator-activated receptor gamma (PPARγ), as an important cell-fate-defining factor, has been demonstrated to be able to promote adipogenesis [23, 24]. A novel strategy has been addressed that suppression of PPARγ could improve the bone regeneration [25] and MSCs osteogenic differentiation [26]. One effective method of

downregulating PPARγ was to introduce miRNAs. Previous study found that miR-548d-5p was able to downregulate PPARγ by targeting its mRNA 3′-UTR [27] and thus enhanced MSC osteogenic potential and blocked its adipogenesis.

The different mechanisms by BMP9 and miR-548d-5p in promoting MSC osteogenic differentiation made us hypothesize that simultaneously regulating different osteogenic regulators may produce more potent osteogenesis from MSCs, which, however, was not demonstrated. Therefore, we designed a series of experiments in the study to assess the effects of BMP9 and miR-548d-5p on osteogenic differentiation of human adipose-derived MSCs simultaneously.

2. Materials and Methods

2.1. Ethics Statement. To obtain adipose-derived MSCs, raw human adipose tissue collection and cell harvests were approved by the Chinese People's Liberation Army General Hospital's Protection of Human Subjects Committee. Subjects have received an explanation about the scope of the study and signed an informed consent statement before donation in the study.

2.2. Isolation of Adipose-Derived Mesenchymal Stems Cells (MSCs). The human adipose-derived MSCs were isolated from raw human lipoaspirates and cultured as the previous report [28, 29]. Briefly, clear lipoaspirates were firstly obtained through washing with phosphate buffer saline (PBS). After removing contaminating debris and red blood cells, 45 min digestion (0.1% collagenase I from Sigma in serum-free αMEM) was conducted. Then, equal volume of αMEM (10% fetal bovine serum from FBS, Gibco) was supplemented to inhibit the trypsin. The mixture was sieved through 80 μm mesh. The cell part was allowed to plate on tissue culture dishes and incubated at 37°C and 5% CO_2. The adherent cells were employed as ADSCs and passages of MSCs were limited to 5.

2.3. miR-548d-5p Transfection. The miR-548d-5p agomir (GMR-miR microRNA-548d-5p agomir, Shanghai GenePharma Co. Ltd.) was designed and synthesized. When cells confluence achieved ~50%, transfection of miR-548d-5p was performed using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer's instructions. The final concentration of miR-548d-5p agomir was set at 50 nM.

2.4. Recombinant Adenovirus Construction Expressing Bone Morphogenetic Protein 9 (BMP9). Recombinant BMP9 adenoviruses were obtained through AdEasy technology as described previously [30, 31]. The coding regions of BMP9 were amplified by PCR. The PCR products were cloned into an adenoviral shuttle vector. The vector was then applied to generate recombinant adenoviruses in HEK-293 cells. The prepared adenoviruses were designated as AdBMP9.

2.5. Quantitative RT-PCR. For cultured MSCs cells, total RNA was isolated from at least 1×10^5 cells by Total RNA Kit (OMEGA, Norcross, GA, USA), according to the manufacturer's protocol. After quantification, RNA samples with A260/A280 nm ratio more than 1.8 were retained and applied in the following experiments. 2 μg of total RNA was reversely transcribed into 1st-strand cDNA with PrimeScript RT reagent kit (Takara, Shiga, Japan). The obtained cDNA was then subjected to qRT-PCR analysis and the sequence of primers for PCR was as follows: GAPDH, 5′-ACC ACA GTC CAT GCC ATC AC-3′ (sense) and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (antisense); COX-2, 5′-GTC ACA AGA TGG CAA AAT GCT G-3′ (sense) and 5′-TAA GAT AAC ACT GCA GTG GCT C-3′ (antisense); PPARγ, 5′-ACT CTG GGA GAT TCT CCT ATT-3′ (sense) and 5′-CTC CAT AGT GAA ATC CAG AAG-3′ (antisense); Runx2, 5′-GCA CCG ACA GCC CCA ACT T-3′ (sense) and 5′-CCA CGG GCA GGG TCT TGT T-3′ (antisense); OCN, 5′-TGA GGA CCC TCT CTC TGC TC-3′ (sense) and 5′- GGG CTC CAA GTC CAT TGT T-3′ (antisense); OPN, 5′-ATC TGA GTC CTT CAC TG-3′ (sense) and 5′-GGG ATA CTG TTC ATC AGA AA-3′ (antisense); Col I, 5′-TGT TCG TGG TTC TCA GGG TAG-3′ (sense) and 5′-TTG TCG TAG CAG GGT TCT TTC-3′ (antisense); BSP, 5′-ATA GGC AAC GAG TAC AAC AC-3′ (sense) and 5′-GTA TCC AGA TGC AAA GAC AG-3′ (antisense). In addition, PCR products were analyzed with agarose gel electrophoresis.

2.6. Western Blotting Analysis. MSCs were lysed in Laemmli Sample Buffer (Bio-Rad). Proteins were collected by centrifugation and concentrations were determined by BCA Protein Assay Kit (Thermo Scientific). Proteins were loaded on sodium dodecyl sulfate polyacrylamide gel for electrophoresis (SDS-PAGE). After proteins were transferred to nitrocellulose membranes, primary antibodies against PPARγ (Abcam) and COX-2 (Abcam) were incubated overnight at 4°C. Then, corresponding secondary antibodies were incubated for 1 h at room temperature. GAPDH was used as internal standard.

2.7. Flow Cytometry Analysis. The MSCs of 10 days culture were rinsed with PBS and fixed with 4% paraformaldehyde. Subsequently, after treatment with 0.2% Triton X-100, 5% bovine serum albumin (BSA) was utilized for terminating the reaction. The cells were incubated with primary antibody specified for osteopontin (OPN) overnight at 4°C and then corresponding secondary antibodies conjugated FITC (Abcam) for 1 h at room temperature. Fluorescence-activated cell sorting caliber flow cytometry system (FACS Caliber BD Flow Cytometer) was used for data analysis.

2.8. Alkaline Phosphatase (ALP) Activity Assay. After osteogenic induction culturing for 3, 7, and 10 days, cells were rinsed twice and treated with 15 s sonication in 2 mL buffer (50 mM pH 7.2 Tris-HCl, 0.1% Triton X-100, and 2 mM $MgCl_2$). The measurement of ALP activity was performed with a previous method with minor modification using a commercial ALP Detection Kit (Nanjing Jiancheng

Bioengineering Ltd., Nanjing, China) [32]. The ALP data were described as nmol/15 min/mg protein.

2.9. Osteocalcin Content Analysis.
The culture mediums at osteogenic induction culturing for 3, 7, and 10 days were gathered. The detection of the concentration of osteocalcin was conducted through enzyme immunoassay (ELISA) using an osteocalcin kit as instructed (Immunodiagnostic Systems Ltd., Boldon, UK) [32].

2.10. Matrix Mineralization Assay.
Matrix mineralization was performed by alizarin red S staining, as described previously [33]. At 2 and 3 w after treatment, cells were fixed with 10% formaldehyde for 10 min, following incubation of 40 mM alizarin red S (Sigma) at 37°C for 1 h. After careful washing, the staining cultures were recorded by a light field microscope.

2.11. Measurement of Calcium Deposition Level.
At the 2 w for osteogenic induction culture, the cells were washed twice with PBS buffer and then supplemented with 1 mL of 1 M HCl, following cell incubation with gentle shaking overnight. The free $[Ca^{2+}]$ in the well was analyzed by the O-cresolphthalein complex method with Calcium Colorimetric Assay Kit (BioVision) [34].

2.12. Statistical Analysis.
Values were expressed as mean ± standard deviation (SD). P values < 0.05 were considered as statistically significant. Analysis between the groups was performed by using 1-way ANOVAs followed by Tukey's *post hoc* test for multiple pairwise examinations.

3. Results

3.1. The Effects of BMP9 and miR-548d-5p on COX-2 and PPARγ.
Adipose-derived MSCs were transfected with BMP9 and/or miR-548d-5p. Both gene expression and protein expression were analyzed by RT-PCR and western blotting, respectively. We first tested the effects of BMP9 and miR-548d-5p on COX-2 expression. Compared with the control, BMP9 infection could dominantly enhance the expression of COX-2 in accordance with previous study [18], while miR-548d-5p alone had negligible effects on COX-2. When BMP9 and miR-548d-5p were employed simultaneously, COX-2 was further enhanced compared with BMP9 alone (Figure 1(a)), $P < 0.01$). Then, the expression level of PPARγ was examined. As shown in Figure 1(b), both BMP9 and miR-548d-5p inhibited the expression of PPARγ, and more potent inhibition effect was observed by miR-548d-5p. When BMP9 and miR-548d-5p were combined, the expression of PPARγ was significantly lower than BMP9 and miR-548d-5p alone. These results indicated that BMP9 and miR-548d-5p may play synergetic roles in regulating osteogenic regulators, COX-2 and PPARγ. The effects of BMP9 and miR-548d-5p on COX-2 and PPARγ expression were further verified by western blotting. As shown in Figures 1(c) and 1(d), western blotting from protein levels achieved consistent results with RT-PCR from gene levels.

3.2. Expression of Osteogenic Genes.
In the different time points of osteogenic differentiation, osteogenic gene markers were analyzed by RT-PCR. The mRNA expression levels of Runx2, OCN, OPN, Col I, and BSP were analyzed at days 3, 7, and 10, respectively. As showed in Figure 2, miR-548d-5p infection alone could trigger a time-dependent minor increase in transcript expression of all these osteogenic markers. The transfection by BMP9 enhanced those mRNA expressions predominantly with differentiation time. Compared with BMP9 and miR-548d-5p alone, combination of BMP9 and miR-548d-5p treatment significantly enhanced the expression of all the five markers. These results suggested that BMP9 stimulation was a potent osteogenic factor, which directly promoted MSC osteogenic differentiation. Interestingly, we could presume that downregulation of PPARγ may indirectly reinforce the osteogenic potential of MSCs rather than directly promote their osteogenic differentiation. Thus, when cultured under osteogenic conditions, the potent effects of PPARγ downregulation on osteogenic differentiation could be manifested by synergism with osteogenic stimulators.

3.3. ALP Activity and Osteocalcin Content.
The activity of intracellular ALP was also investigated at days 3, 7, and 10. The activity of ALP in the treatment groups significantly increases with time. Both the solo treatment by BMP9 and combination of BMP9 and miR-548d-5p could enhance the activity largely, while miR-548d-5p infection alone could stimulate a relatively slight increase. Of the three ways of treating MSCs, the cotreatment showed a clear superiority compared to the other two, as shown in Figure 3(a). Then, the osteocalcin secretion was also examined at the same timepoint with ALP analysis (days 3, 7, and 10). As for osteocalcin contents, similar results were also obtained (Figure 3(b)). These findings further supported the assumption that BMP9 acted as a potent direct promoter of MSCs osteogenic differentiation which could be further strengthened by indirect enhancement of miR-548d-5p through inhibiting PPARγ.

3.4. Flow Cytometry of Osteopontin (OPN).
The combined impact of BMP9 and miR-548d-5p on protein expression of OPN, the late osteogenic marker, has then been investigated. According to FACS results of Figure 4, the expression of OPN was augmented about 2.5-fold by a separate administration of miR-548d-5p. However, a 9-fold increase of OPN expression was observed, after introduction of BMP9 alone. Moreover, the combination of BMP9 and miR-548d-5p could further enhance the expression of OPN and cause an over 14-fold augmentation of expression. The higher OPN overexpression induced by the combination of BMP9 and miR-548d-5p compared to simple summation of BMP9 and miR-548d-5p similarly suggested potential synergism of BMP9 and PPARγ inhibitor.

3.5. Matrix Mineralization and Calcium Deposition of MSCs.
Finally, we determined the synergistic effects of BMP9 and miR-548d-5p on matrix mineralization and calcium deposition. We found that matrix mineralization assessed by alizarin
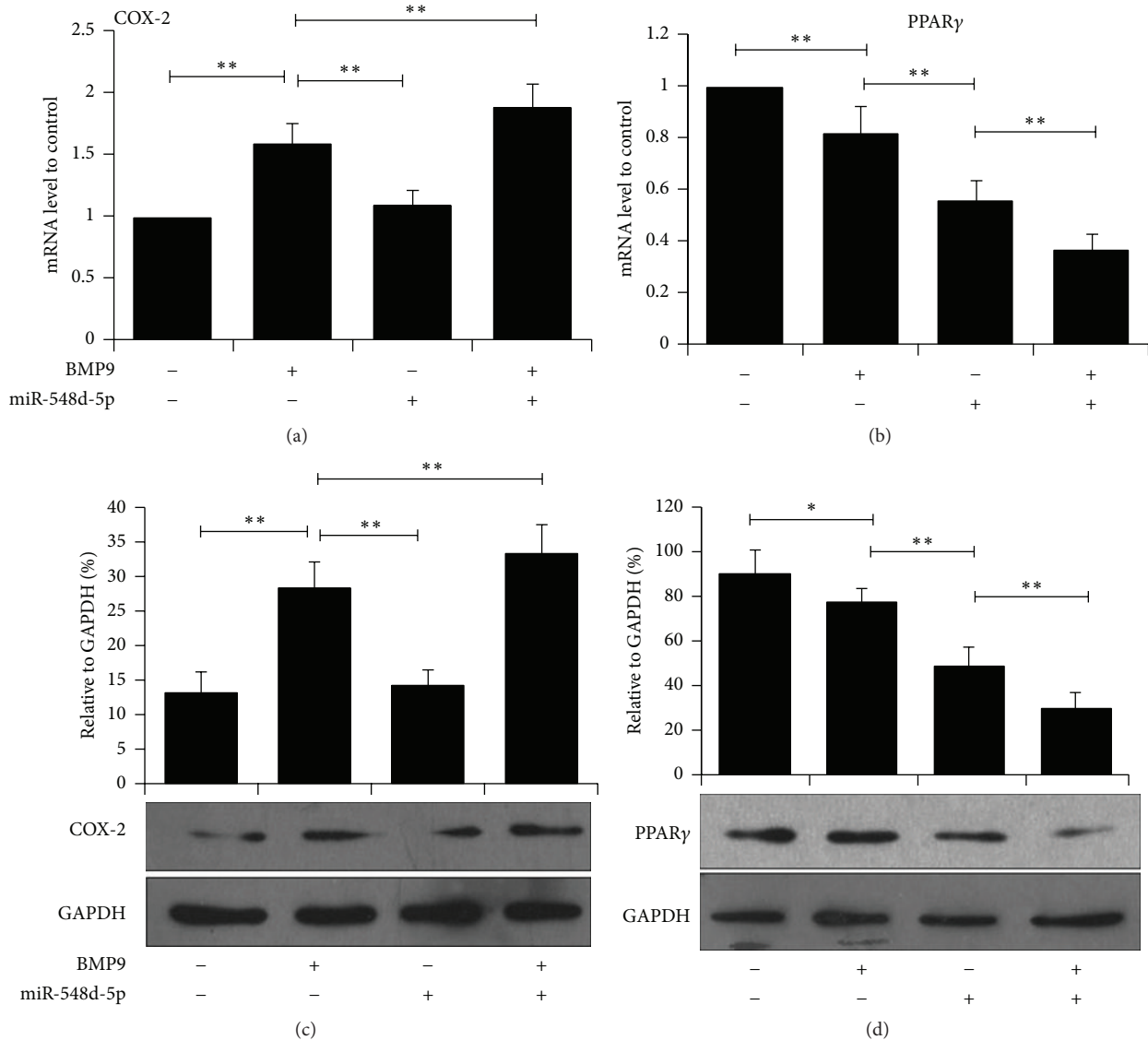
FIGURE 1: Combination of BMP9 and miR-548d-5p upregulates COX-2 expression and downregulates PPARγ. (a, b) RT-PCR shows the synergetic effects of BMP9 and miR-548d-5p on COX-2 and PPARγ in MSCs ($^{**}P < 0.01$). (c, d) Western blot assay shows the effects of BMP9 and miR-548d-5p on COX-2 and PPARγ in MSCs ($^{**}P < 0.01$).

red staining was remarkably promoted by the combination of BMP and miR-548d-5p, compared with their separate treatment (Figure 5(a)). Additionally, quantitative analysis of calcium deposition similarly showed that BMP9 induced apparently osteogenic calcium secretion compared with treatment by PPARγ inhibiting, and a more enhancement of calcium secretion was achieved when BMP9 accompanied with PPARγ inhibitor was introduced (Figure 5(b)). Taken together, the results of late stage in MSCs further suggested that inhibiting PPARγ could enforce BMP9 induced osteogenic differentiation.

4. Discussion

Osteogenic differentiation of stem cells is a highly orchestrated process in which a series of factors are involved.

BMPs [35–37], IGF [38], PI3K/Akt [17], miRNA [27, 39], drugs [33], and even microenvironment [40, 41] are able to regulate osteogenic differentiation. Albeit various, they could be mainly concluded into two pathways. The first one is to tempt stem cells to the way to osteogenic differentiation, such as BMP9. BMP9 belongs to the family of BMPs, which play an important role in both bone metabolism and tumor formation [11, 12]. Of all BMPs members, BMP9 was considered to be the most potential to enhance osteogenic differentiation [13–16]. As recent reports, the regulative function of BMP9 was fulfilled by possibly forming a loop structure with COX-2 and then orchestrating the BMP9/Smad signal pathway [18]. The other is to block stem cells to other ways of differentiation, for example, miR-548d-5p. miRNAs have been demonstrated to be potent to regulate stem cells differentiation [27, 39]. Of them, miR-548d-5p was found
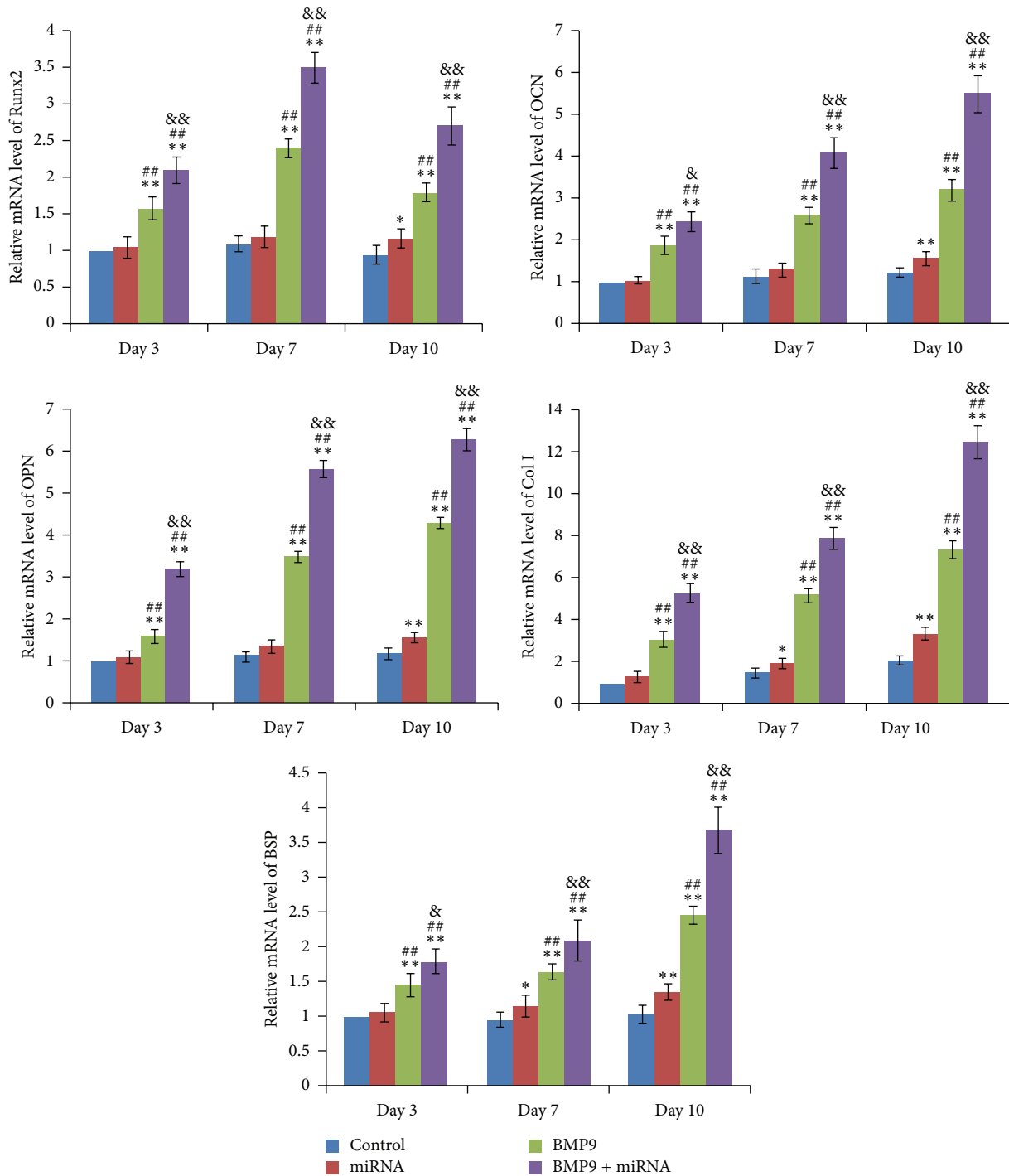
Figure 2: Combination of BMP9 and miR-548d-5p upregulates osteogenic differentiation-related factors in MSCs. RT-PCR demonstrated that Runx2, OPN, OCN, Col I, and BSP were significantly unregulated in BMP9 and miR-548d-5p treated MSCs at day 3, day 7, and day 10, respectively ($^{**}P < 0.01$ versus control; $^{##}P < 0.01$ versus miR-548d-5p; $^{\&\&}P < 0.01$ versus BMP9).

to be able to inhibit adipogenesis of MSCs by binding to the mRNA of PPARγ [27]. Thus, more cells were defined to osteogenesis. However, no such study as efforts from both two directions is reported. Therefore, we firstly choose BMP9 representing the tempting way and miR-548-5p representing

the blocking way to explore possible synergetic effects on MSCs osteogenic differentiation.

COX-2 plays a critical role in bone metabolism and has also been proved to be essential for BMP9 induced osteogenic differentiation [17, 18]. In current study, we found that
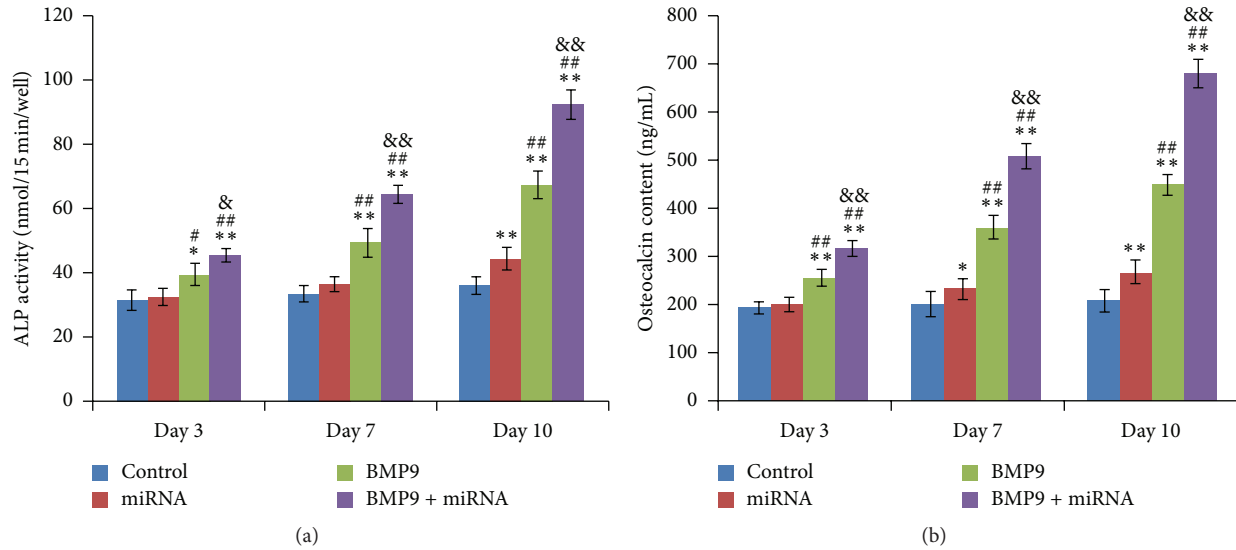
FIGURE 3: Combination of BMP9 and miR-548d-5p promotes ALP activities and augments OCN content in MSCs. (a) The synergetic effects of BMP9 and miR-548d-5p on ALP activities in MSCs at day 3, day 7, and day 10, respectively ($^{**}P < 0.01$ versus control; $^{##}P < 0.01$ versus miR-548d-5p; $^{\&\&}P < 0.01$ versus BMP9). (b) The synergic effects of BMP9 and miR-548d-5p on OCN content in MSCs at day 3, day 7, and day 10, respectively ($^{**}P < 0.01$ versus control; $^{##}P < 0.01$ versus miR-548d-5p; $^{\&\&}P < 0.01$ versus BMP9).

the combination of miR-548d-5p and BMP9 could further promote the gene and protein level expressions of COX-2 compared to BMP9 alone, while the separate miR-548d-5p treatment caused negligible impacts on it. This indicated that miR-548d-5p could possibly enhance the BMP9 induced upregulation of COX-2. In addition, the expression of PPARγ, one of most important factors to promote adipogenic differentiation in MSCs, was examined. PPARγ can be exclusively mutated by miR-548d-5p through targeting 3′-UTR of PPARγ. We found that BMP9 could also significantly, albeit not remarkably, reduce the expression of PPARγ. The downregulation of PPARγ by BMP9 possibly contributed to feedback effects associated with BMP9 induced osteogenic differentiation. And the combination of BMP9 and miR-548d-5p showed the strongest inhibiting effects on PPARγ.

We next found that the combination of BMP9 and miR-548d-5p could also improve early and late osteogenic differentiation markers of MSCs, as well as matrix mineralization, predominantly. In conformity with the results of COX-2, the expressions of BMP9 induced osteogenic differentiation downstream markers [42], including Runx2, OCN, OPN, Col I, and BSP, were remarkably upregulated by cotreatment of BMP9 and miR-548d-5p, while they were slightly enhanced in presence of miR-548d-5p alone. These findings further supported the hypothesis that miR-548d-5p promoted BMP9 induced osteogenic differentiation through silencing the expression of PPARγ. Moreover, similar results of ALP activity, calcium deposition, and matrix mineralization also corresponded to the hypothesis. It is needed to state that the terminal differentiation of the differentiated MSCs may last for longer time than we observed in the study. However, it was observed during our experiment that when differentiation was proceeded for 3 w or longer time, the

state of the differentiated MSCs would become much worse than that at 2 w due to the long-time culture, and some cells and calcified tubercles would easily fall off culture plate (this could be seen in Figure 5(a), alizarin red S staining at 3 w). This would result in large deviation for calcium deposition quantification. That was why we chose 2 w as the timepoint to quantify calcium deposition in the study (Figure 5(b)).

As mentioned above, the results of BMP9 induced osteogenic differentiation were in conformity with previous studies [11, 17, 18, 33]. BMP9 could apparently upregulate a series of osteogenesis factors in both early and late stage of MSCs, due to its direct involvement of several osteogenic differentiation pathways. However, taking the results of slight osteogenic enhancement of MSCs and large osteogenic improvement of BMP9 induced MSCs by PPARγ inhibiting together, we proposed the hypothesis that PPARγ inhibiting can promote osteogenic differentiation of those osteogenic induced MSCs but cannot stimulate those uncertain MSCs to osteogenic differentiation. PPARγ is one of the most important factors in balancing adipogenesis and osteogenesis in MSCs. Overexpression of PPARγ positively regulates adipogenesis but negatively regulates osteogenesis. However, downregulation of PPARγ alone is probably insufficient to effectively trigger osteogenesis-related pathways. Moreover, only under proper beneficial osteogenic conditions and osteogenesis-related pathways activated, PPARγ downregulation would ensure osteogenic differentiation thoroughly and successfully to some extent and thus largely promote MSCs osteogenic differentiation. Additionally, there were conflicts in whether PPARγ silencing was able to promote MSCs osteogenesis [26, 43]. Our assumption may serve to enlighten possible understanding of the contention. Although the *in vitro* data well confirmed the synergic effects of BMP9 and
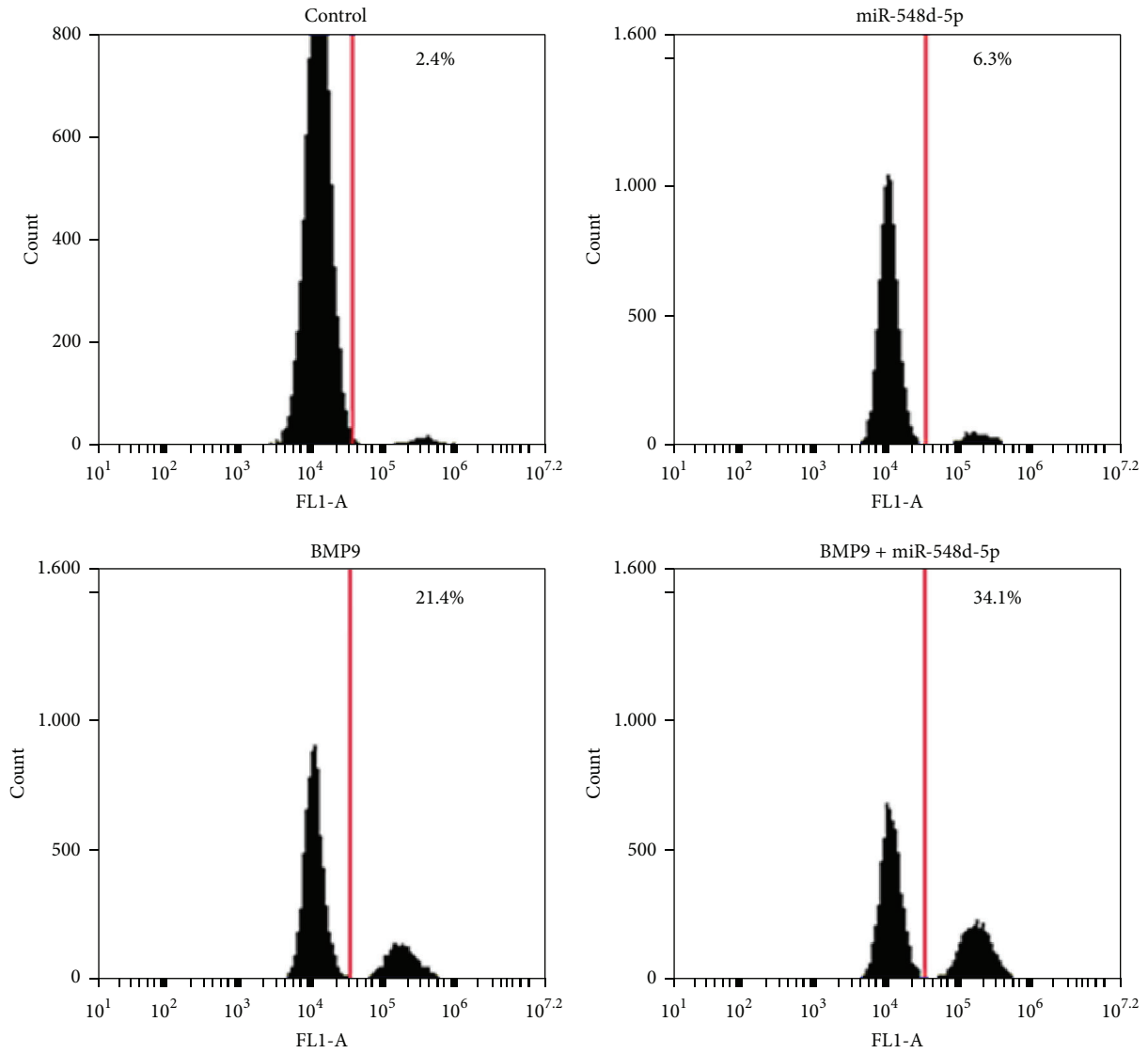
(a)
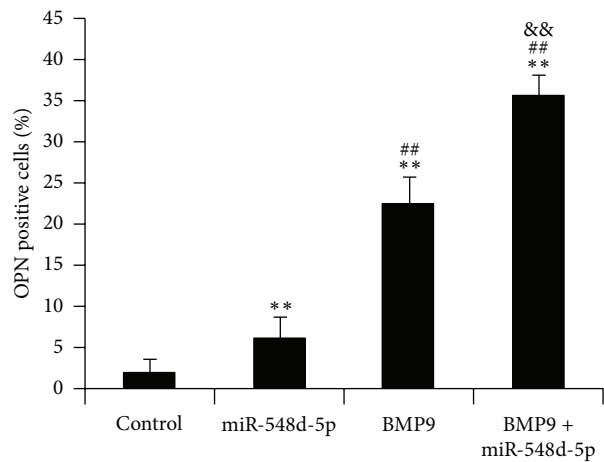
(b)

Figure 4: Fluorescence-activated cell sorting (FACS) analysis performed on the MSCs cultured for 10 days. OPN immunostained MSCs were measured by a flow cytometry analysis system (y-axis: the cell number; x-axis: fluorescence intensity). The data shows the synergetic effects of BMP9 and miR-548d-5p on OPN expression of MSCs at day 10 ($^{**}P < 0.01$ versus control; $^{##}P < 0.01$ versus miR-548d-5p; $^{\&\&}P < 0.01$ versus BMP9).
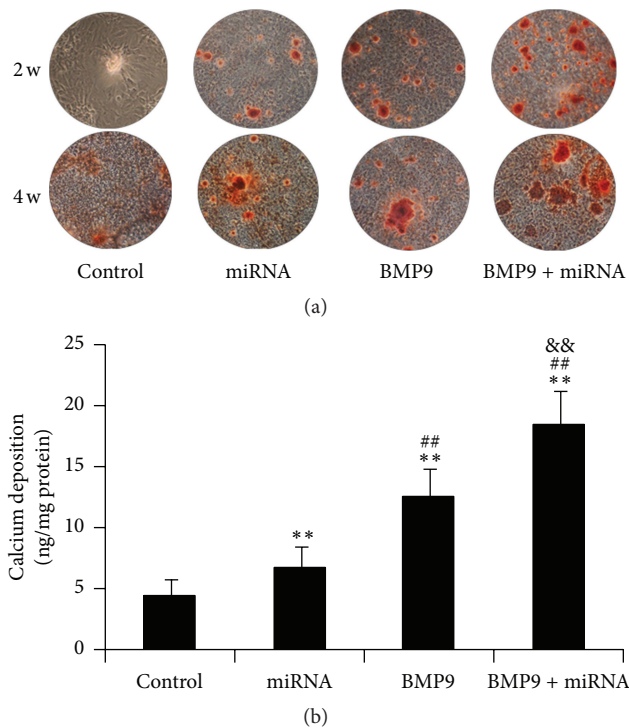
Figure 5: Combination of BMP9 and miR-548d-5p enhances matrix mineralization and calcium deposition in MSCs. (a) Alizarin red S staining at 2 and 3 w shows the synergetic effects of BMP9 and miR-548d-5p on matrix mineralization; (b) the synergetic effects of BMP9 and miR-548d-5p on calcium deposition in MSCs cultured for 14 days ($^{**}P < 0.01$ versus control; $^{##}P < 0.01$ versus miR-548d-5p; $^{\&\&}P < 0.01$ versus BMP9).

miR-548d-5p on promoting osteogenic differentiation of MSCs, we still cannot be sure that it would work well *in vivo* and this should be a future endeavor.

In conclusion, our study demonstrates that the combination of BMP9 and miR-548d-5p can promote osteogenic differentiation of MSCs. This synergic effect is probably due to the improvement of BMP9 induced osteogenic differentiation by mutated PPARγ with miR-548d-5p. And the subsequent upregulation of Runx2, OPN, OCN, Col I, and BSP was also enhanced. Our findings may not only shed light on the possible mechanism of synergic effect on osteogenic differentiation from two different directions but also provide an effective strategy to promote skeletal tissue regeneration of osteonecrosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Wei Zhang and LiCheng Zhang contributed equally to this work.

References

[1] D. J. Prockop, "Marrow stromal cells as stem cells for non-hematopoietic tissues," *Science*, vol. 276, no. 5309, pp. 71–74, 1997.

[2] M. F. Pittenger, A. M. Mackay, S. C. Beck et al., "Multilineage potential of adult human mesenchymal stem cells," *Science*, vol. 284, no. 5411, pp. 143–147, 1999.

[3] J. E. Aubin, "Advances in the osteoblast lineage," *Biochemistry and Cell Biology*, vol. 76, no. 6, pp. 899–910, 1998.

[4] Z.-L. Deng, K. A. Sharff, N. Tang et al., "Regulation of osteogenic differentiation during skeletal development," *Frontiers in Bioscience*, vol. 13, no. 6, pp. 2001–2021, 2008.

[5] J. Ringe, I. Leinhase, S. Stich et al., "Human mastoid periosteum-derived stem cells: promising candidates for skeletal tissue engineering," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 2, no. 2-3, pp. 136–146, 2008.

[6] P. A. Zuk, M. Zhu, H. Mizuno et al., "Multilineage cells from human adipose tissue: implications for cell-based therapies," *Tissue Engineering*, vol. 7, no. 2, pp. 211–228, 2001.

[7] L. da Silva Meirelles, P. C. Chagastelles, and N. B. Nardi, "Mesenchymal stem cells reside in virtually all post-natal organs and tissues," *Journal of Cell Science*, vol. 119, no. 11, pp. 2204–2213, 2006.

[8] Y. Amoh, L. Li, R. Campillo et al., "Implanted hair follicle stem cells form Schwann cells that support repair of severed peripheral nerves," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 49, pp. 17734–17738, 2005.

[9] J. Justesen, K. Stenderup, E. N. Ebbesen, L. Mosekilde, T. Steiniche, and M. Kassem, "Adipocyte tissue volume in bone marrow is increased with aging and in patients with osteoporosis," *Biogerontology*, vol. 2, no. 3, pp. 165–171, 2001.

[10] M. E. Nuttall and J. M. Gimble, "Is there a therapeutic opportunity to either prevent or treat osteopenic disorders by inhibiting marrow adipogenesis?" *Bone*, vol. 27, no. 2, pp. 177–184, 2000.

[11] J. D. Lamplot, J. Qin, G. Nan et al., "BMP9 signaling in stem cell differentiation and osteogenesis," *American Journal of Stem Cells*, vol. 2, no. 1, pp. 1–21, 2013.

[12] A. H. Reddi and A. Reddi, "Bone morphogenetic proteins (BMPs): from morphogens to metabologens," *Cytokine and Growth Factor Reviews*, vol. 20, no. 5-6, pp. 341–342, 2009.

[13] Q. Kang, W.-X. Song, Q. Luo et al., "A Comprehensive analysis of the dual roles of BMPs in regulating adipogenic and osteogenic differentiation of mesenchymal progenitor cells," *Stem Cells and Development*, vol. 18, no. 4, pp. 545–558, 2009.

[14] Q. Kang, M. H. Sun, H. Cheng et al., "Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery," *Gene Therapy*, vol. 11, no. 17, pp. 1312–1320, 2004.

[15] H. H. Luu, W.-X. Song, X. Luo et al., "Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells," *Journal of Orthopaedic Research*, vol. 25, no. 5, pp. 665–677, 2007.

[16] G. Luther, E. R. Wagner, G. Zhu et al., "BMP-9 induced osteogenic differentiation of mesenchymal stem cells: molecular mechanism and therapeutic potential," *Current Gene Therapy*, vol. 11, no. 3, pp. 229–240, 2011.

[17] J. Huang, S.-X. Yuan, D.-X. Wang et al., "The role of COX-2 in mediating the effect of PTEN on BMP9 induced osteogenic differentiation in mouse embryonic fibroblasts," *Biomaterials*, vol. 35, no. 36, pp. 9649–9659, 2014.

[18] J.-H. Wang, Y.-Z. Liu, L.-J. Yin et al., "BMP9 and COX-2 form an important regulatory loop in BMP9-induced osteogenic differentiation of mesenchymal stem cells," *Bone*, vol. 57, no. 1, pp. 311–321, 2013.

[19] K. A. Sharff, W.-X. Song, X. Luo et al., "Hey1 basic helix-loop-helix protein plays an important role in mediating BMP9-induced osteogenic differentiation of mesenchymal progenitor cells," *The Journal of Biological Chemistry*, vol. 284, no. 1, pp. 649–659, 2009.

[20] J. Zhang, Y. Weng, X. Liu et al., "Endoplasmic reticulum (ER) stress inducible factor cysteine-rich with EGF-like domains 2 (Creld2) is an important mediator of BMP9-regulated osteogenic differentiation of mesenchymal stem cells," *PLoS ONE*, vol. 8, no. 9, Article ID e73086, 2013.

[21] X. Zhang, E. M. Schwarz, D. A. Young, J. Edward Puzas, R. N. Rosier, and R. J. O'Keefe, "Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair," *The Journal of Clinical Investigation*, vol. 109, no. 11, pp. 1405–1415, 2002.

[22] A. M. Simon, M. B. Manigrasso, and J. P. O'Connor, "Cyclo-oxygenase 2 function is essential for bone fracture healing," *Journal of Bone and Mineral Research*, vol. 17, no. 6, pp. 963–976, 2002.

[23] S. Muruganandan, A. A. Roman, and C. J. Sinal, "Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program," *Cellular and Molecular Life Sciences*, vol. 66, no. 2, pp. 236–253, 2009.

[24] I. Takada, A. P. Kouzmenko, and S. Kato, "Wnt and PPARgamma signaling in osteoblastogenesis and adipogenesis.," *Nature reviews. Rheumatology*, vol. 5, no. 8, pp. 442–447, 2009.

[25] K. Arvidson, B. M. Abdallah, L. A. Applegate et al., "Bone regeneration and stem cells," *Journal of Cellular and Molecular Medicine*, vol. 15, no. 4, pp. 718–746, 2011.

[26] M.-J. Lee, H.-T. Chen, M.-L. Ho et al., "PPARγ silencing enhances osteogenic differentiation of human adipose-derived mesenchymal stem cells," *Journal of Cellular and Molecular Medicine*, vol. 17, no. 9, pp. 1188–1193, 2013.

[27] J. Sun, Y. Wang, Y. Li, and G. Zhao, "Downregulation of PPARgamma by miR-548d-5p suppresses the adipogenic differentiation of human bone marrow mesenchymal stem cells and enhances their osteogenic potential," *Journal of Translational Medicine*, vol. 12, article 168, 2014.

[28] P. A. Zuk, M. Zhu, P. Ashjian et al., "Human adipose tissue is a source of multipotent stem cells," *Molecular Biology of the Cell*, vol. 13, no. 12, pp. 4279–4295, 2002.

[29] Z. Liu, H. Wang, Y. Wang et al., "The influence of chitosan hydrogel on stem cell engraftment, survival and homing in the ischemic myocardial microenvironment," *Biomaterials*, vol. 33, no. 11, pp. 3093–3106, 2012.

[30] T.-C. He, S. Zhou, L. T. da Costa, J. Yu, K. W. Kinzler, and B. Vogelstein, "A simplified system for generating recombinant adenoviruses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 5, pp. 2509–2514, 1998.

[31] J. Luo, Z.-L. Deng, X. Luo et al., "A protocol for rapid generation of recombinant adenoviruses using the AdEasy system," *Nature Protocols*, vol. 2, no. 5, pp. 1236–1247, 2007.

[32] H.-P. Ma, L.-G. Ming, B.-F. Ge et al., "Icariin is more potent than genistein in promoting osteoblast differentiation and mineralization in vitro," *Journal of Cellular Biochemistry*, vol. 112, no. 3, pp. 916–923, 2011.

[33] Y.-K. Zhai, X.-Y. Guo, B.-F. Ge et al., "Icariin stimulates the osteogenic differentiation of rat bone marrow stromal cells via activating the PI3K-AKT-eNOS-NO-cGMP-PKG," *Bone*, vol. 66, pp. 189–198, 2014.

[34] K. M. Chen, B. F. Ge, H. P. Ma, and R. L. Zheng, "The serum of rats administered flavonoid extract from *Epimedium sagittatum* but not the extract itself enhances the development of rat calvarial osteoblast-like cells *in vitro*," *Pharmazie*, vol. 59, no. 1, pp. 61–64, 2004.

[35] J. A. Jane Jr., B. A. Dunford, A. Kron et al., "Ectopic osteogenesis using adenoviral bone morphogenetic protein (BMP)-4 and BMP-6 gene transfer," *Molecular Therapy*, vol. 6, no. 4, pp. 464–470, 2002.

[36] G. Li, H. Peng, K. Corsi, A. Usas, A. Olshanski, and J. Huard, "Differential effect of BMP4 on NIH/3T3 and C2C12 cells: implications for endochondral bone formation," *Journal of Bone and Mineral Research*, vol. 20, no. 9, pp. 1611–1623, 2005.

[37] R. T. Franceschi, D. Wang, P. H. Krebsbach, and R. B. Rutherford, "Gene therapy for bone formation: in vitro and in vivo osteogenic activity of an adenovirus expressing BMP7," *Journal of Cellular Biochemistry*, vol. 78, no. 3, pp. 476–486, 2000.

[38] L. Chen, W. Jiang, J. Huang et al., "Insulin-like growth factor 2 (IGF-2) potentiates BMP-9-induced osteogenic differentiation and bone formation," *Journal of Bone and Mineral Research*, vol. 25, no. 11, pp. 2447–2459, 2010.

[39] T. Itoh, Y. Nozawa, and Y. Akao, "MicroRNA-141 and -200a are involved in bone morphogenetic protein-2-induced mouse pre-osteoblast differentiation by targeting distal-less homeobox 5," *The Journal of Biological Chemistry*, vol. 284, no. 29, pp. 19272–19279, 2009.

[40] O. F. Zouani, L. Rami, Y. Lei, and M. Durrieu, "Insights into the osteoblast precursor differentiation towards mature osteoblasts induced by continuous BMP-2 signaling," *Biology Open*, vol. 2, no. 9, pp. 872–881, 2013.

[41] O. F. Zouani, J. Kalisky, E. Ibarboure, and M.-C. Durrieu, "Effect of BMP-2 from matrices of different stiffnesses for the modulation of stem cell fate," *Biomaterials*, vol. 34, no. 9, pp. 2157–2166, 2013.

[42] S.-A. Oh, H.-Y. Lee, J. H. Lee et al., "Collagen three-dimensional hydrogel matrix carrying basic fibroblast growth factor for the cultivation of mesenchymal stem cells and osteogenic differentiation," *Tissue Engineering Part A*, vol. 18, no. 9-10, pp. 1087–1100, 2012.

[43] W.-H. Yu, F.-G. Li, X.-Y. Chen et al., "PPARγ suppression inhibits adipogenesis but does not promote osteogenesis of human mesenchymal stem cells," *International Journal of Biochemistry and Cell Biology*, vol. 44, no. 2, pp. 377–384, 2012.