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Metformin promotes differentiation of human bone marrow derived mesenchymal stem cells into osteoblast via GSK3β inhibition

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Abstract. – OBJECTIVE: Metformin, a common and first-line drug for diabetes mellitus, is widely used in the world. Recently, many studies have documented that osteogenesis could be mediated by metformin. However, the specific mechanism by which metformin affects osteogenesis has not been clearly identified. Therefore, the aim of this study is to evaluate the role of GSK3β in metformin-induced osteogenic differentiation of mesenchymal stem cells (MSCs).

MATERIALS AND METHODS: Osteoblast-marker genes, including Col-1, OCN, and RUNX2, were measured by RT-PCR in differentiated MSCs treated with Metformin. Osteogenic differentiation viability was measured by Alkaline phosphatase (ALP) assays and Alizarin Red Staining. The expression of GSK3β, β-catenin and AMPK were measured by Western blotting in MSCs treated with metformin.

RESULTS: We found that metformin at 100 μM significantly promoted osteogenic differentiation of human mesenchymal stem cells (hBMSCs). Next, we showed that GSK3β and Wnt signaling pathway are involved in metformin-induced osteogenic differentiation of hBMSCs. Furthermore, osteogenic differentiation of hBMSCs induced by metformin could be eliminated by inhibiting phosphorylation of GSK3β.

CONCLUSIONS: The data suggested that metformin promoted the osteoblast differentiation of MSCs by, at least partly, inhibiting GSK3β activity. Additionally, we also found that AMPK plays an essential role in the inhibition of GSK3β by metformin.

Key Words:
Metformin, Mesenchymal stem cells, GSK3Ð, AMPK.

Introduction

The incidence of diabetes has increased rapidly all over the world, especially in China following improvements in living standards and extension of life spans. Many researches showed that patients with a metabolic disease, diabetes mellitus, have been confirmed to associate with various skeletal disorders, including a reduction in bone strength, an increase in bone fracture rate and impairment in bone healing[1]. Diabetes mellitus is considered as a risk factor for osteoporosis and bone fracture. The effect on diabetes patients of oral antidiabetic drugs (OADs) like metformin on bone mineral density (BMD) and fractures has been focused on in recent decades[2]. Metformin, a common and first-line drug, is widely used in a world for treating diabetes mellitus by suppressing glucose production[3]. Additionally, metformin may be a potential application for therapy of various cancers by inhibiting growth of tumor cells[4]. Recently, several studies suggested that osteogenesis can be mediated by metformin. Some clinical studies[5-7] have shown that patients treated with metformin had a reduction in fracture risk. Bone is a complex tissue containing several cell types that is continuously undergoing a process of self-renewal and repair, termed bone-remodeling [8]. Further investigations[9,10] showed that metformin improved bone structure and induced osteoblastic cell differentiation in *vivo* and *vitro*, respectively, by multiple mechanisms. Gao et al[9] have shown that metformin improved bone mass and bone quality via regulation of osteoblast markers core binding factor a1 and by expression of low density lipoprotein (LDL) receptor-related protein 5. Molinuevo et al[10] have found that the action of metformin in a shift toward the osteoblastic differentiation of bone marrow progenitor cells (BMPCs) may be caused by mediating the AMPK and Runx2/Cbfa1 pathway. Bone marrow derived mesenchy-

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mal stem cells (MSCs) are multi potent stromal cells that play a fundamental role in bone biology and osteogenesis. Gao et al[11] reported that the anti-diabetic drug metformin has a direct inhibition effect on bone loss and this action might be partly mediated through induction of Cbfa1 and Lrp5 of bone marrow cells. However, the exact mechanisms by which metformin regulate osteogenic differentiation need to be further explored. Multi complex signaling pathways have been confirmed to involve in the regulation of osteogenic differentiation of human mesenchymal stem cells (hBMSCs) by metformin, including Wnt/β-catenin pathway. The activation of Wnt/β-catenin pathway by wnts (eg, Wnt3a) or other chemicals causes dephosphorylation and release of cytoplasmic β-catenin from a protein complex consisting of Axin1/2, APC, CK1 and GSK3β[12]. Subsequently, it translocates into the nucleus to activate Wnt/β-catenin-responsive genes, such as c-Myc, CyclinD1, TCF-1 and LEF-1, to regulate various developmental processes, including osteogenic differentiation. The proteasomal degradation of β-catenin mediated by GSK-3β in destruction complex is the central step in the canonical Wnt signaling pathway[13]. However, whether GSK3β is involved in the promotion of osteogenic differentiation of hBMSCs by metformin is unclear. In the present study, we demonstrate for the first time that metformin promotes osteogenic differentiation of hBMSCs by inhibiting GSK3β.

Materials and Methods

Cell Culture

All experiments were approved by the Medical Ethics Committee of the Second Military Medical University. hBMSCs were purchased from Cyagen Biosciences Inc (Suzhou, China) and cultured in ORICell™ human mesenchymal stem cell growth medium (Cyagen, Suzhou, China). hBMSCs from passage 3 to passage 5 were utilized for osteogenic differentiation as protocol. Briefly, hBMSCs were cultured in human mesenchymal stem cell growth medium at 37°C in a 5% CO_2 humidified incubator. When cells were approximately 80-90% confluent, they could be dissociated and replaced in growth medium at 1×10^6 cells in 6-well tissue culture plates pre-coated with Gelatin Solution. After 24 h, the growth medium from each well was carefully aspirated off and 2 ml human mesenchymal stem cell osteogenic differentiation medium (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 microg/ mL streptomycin, 1% Glutamine, 10 nM dexamethasone, 0.2 mM L-ascorbic acid, and 10 mM β-glycerophosphate) (Cyagen, Suzhou, China) were added to induce osteogenic differentiation. The cells were cultured in differentiation medium for 15 days with a medium change every 3 days.

Transfection

A constitutive active form of GSK3b (S9A, CA-GSK3b) was obtained by Addgene (Cambridge, MA, USA)[14]. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instruction from the manufacturer. In brief, cells were seeded on 6-well tissue culture plates to reach 90% confluence; 24 h before transfection, cells were changed to no-antibiotic medium, and the DNA:Lipofectamine ratio at 1:3 was used. The co-transfection of a GFP plasmid was used to monitor the transfection efficiency. Cells were incubated at 37°C in 5% CO_2. The empty plasmid, pCDNA3.1, was used for the mock transfection.

Alkaline Phosphatase Assays

ALP enzyme activity was measured by alkaline phosphatase detection kit (Genmed Scientifics, Shanghai, China) according to the manufacturer's protocol. In brief, cultured cells were washed with PBS and cracked with a solution containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.02% NaN_3 and 1 μg/ml aprotinin. Lysates were homogenized and assayed for ALP activity by incubating with a pipette for 15 min at 37°C using a spectrophotometer. Results were normalized to total cellular protein content.

Alizarin Red Staining

The matrix mineralization was measured by Alizarin Red staining (BioVision, San Francisco, CA, USA). Briefly, cells were fixed with 70% ethanol for 1 h and rinsed with PBS, and then treated with 40 mM ARS solution at pH 4.2 for 10 min. After washing with phosphate-buffered saline (PBS) for 15 min, the stained cells were photographed. For quantification of staining, the Alizarin Red S stain was released from the cell matrix by incubation in cetylpyridinium chloride for 15 min and the amount of released dye was measured by spectrophotometry at 540 nm. The results were normalized to total cellular protein content.

Western Blot Analysis

The whole-cell lysates were prepared with cell lysis buffer (Beyotime, Shanghai, China). The protein content was quantified by BCA methods. Equal protein from samples was electrophoresed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the nitrocellulose membrane. The membrane was incubated with primary antibodies against GSK3β and p-GSK3β-Ser9 (Cell Signaling Technology, Danvers, MA, USA), AMPK and p172-AMPK (Cell Signaling Technology, Danvers, MA, USA), anti-β-actin (Abcam, Cambridge, MA, USA), anti-β-catenin (Abcam, Cambridge, MA, USA), active anti-β-catenin (Millipore, Massachusetts, USA) and secondary horseradish peroxidase (HRP)-conjugated antibody. β-actin was used as an internal control.

RNA Preparation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from cells using TRIzol reagent (Life Technologies, New York, NY, USA) and miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Next, cDNA was synthesized with the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany), and qPCR was carried out using miScript SYBR Green PCR Kit (Life Technologies, New York, NY, USA) and miScript/QuantiTect Peimer assay (Qiagen, Hilden, Germany). For each sample, real time PCR (RT-PCR) was performed in triplicate. The expression levels of miRNAs and genes are presented as values normalized against U6 and β-actin transcripts, respectively. Primer sequences (Table I) used for qRT-PCR were obtained from Sangon Company (Shanghai, China).

Statistical Analysis

Data were collected from three or more independent experiments and are shown as means ± standard deviation (SD). Statistical analyses were performed by one-way ANOVA followed by Tukey's test using GraphPad Prism software (Version 5.01, La Jolla, CA, USA). Statistical significance was set at $p<0.05$.

Results

Effects of Metformin on the Osteoblastic Differentiation and Mineralization

To investigate the effect of metformin on osteogenic differentiation, hBMSCs were cultured in osteogenic differentiation medium with metformin. As shown in Figure 1A, the expression of osteoblastic marker genes, including Col-1, OCN and RUNX2, were significantly increased in a dose-dependent manner. Correspondingly, metformin (100 μM) also significantly increased ALP activities at day 14 (Figure 1B). Alizarin Red staining showed that osteoblastic differentiation and mineralization were significantly enhanced in hBMSCs treated with metformin (100 μM) under osteogenic differentiation medium for 21 days (Figure 1C). Taken together, these results suggested that metformin could regulate osteoblastic differentiation and mineralization.

Effect of Metformin on the GSK3β and Wnt Signaling Pathway in hBMSCs

It has been shown that GSK3β activity was a vital component of the Wnt signaling pathway, playing a critical role of osteogenic differentiation of various stem cells[12,13]. To investigate the effect of metformin on GSK3β activity, Western blot analysis was used to examine the expression of GSK3β. The results showed that metformin significantly increased the phosphorylation of GSK3β at serine 9 residue (Figure 2A). Next, we found that metformin increased the expression of activated β-catenin (β-catenin*; Figure 2B) and TOPFlash activity (Figure 2C). These results suggested that GSK3β and Wnt signaling pathway may be involved in metformin-induced osteogenic differentiation of hBMSCs.

Testing the Role of GSK3β in Metformin-Induced Osteogenic Differentiation by Using a Construct Encoding CA-GSK3β

To estimate the role of metformin-induced GSK3β phosphorylation in osteogenic differentiation of hBMSCs, constitutive active form of GSK3β (S9A, CA-GSK3β) was constructed.

Table I. Primer sequences and used for qRT-PCR

Gene	Primer sequence (5'-3')
Col-I	F: CCAGAAGAACTGGTACATCAGCAA
	R: CGCCATACTCGAACTGGAATC
OCN	F: AGCAAAGGTGCAGCCTTTGT
	R: GCGCCTGGGTCTCTTCACT
RUNX2	F: CCCGTGGCCTTCAAGGT
	R: CGTTACCCGCCATGACAGTA
β-Actin	F: CAGGCTGTGCTATCCCTGTA
	R: CATACCCCTCGTAGATGGGC

Note: F, Forward; R, Reverse

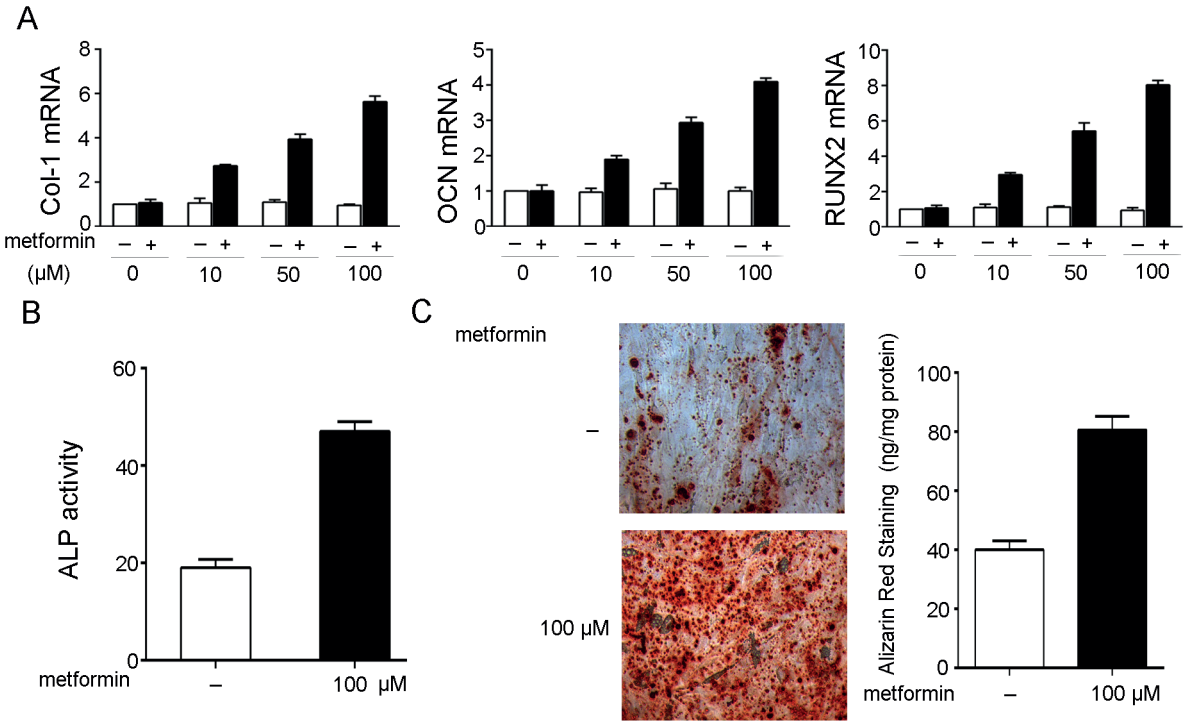

Figure 1. Effect of metformin on the differentiation and mineralization of hBMSCs. ***(A)*** Metformin (0, 10, 50, 100 μM) was added after the cells reached confluency. After 14 days, the osteoblastic marker genes (Col-1, OCN and RUNX2) were measured to evaluate osteoblastic differentiation by qRT-PCR with β-actin as control. Results were expressed as fold increase over the control values. ***(B)*** After 14 days, ALP activity of hBMSCs by metformin (100 μM) treatment was measured. ***(C)*** After 21 days, Alizarin Rea staining was measured and quantification was shown on the right. All data are expressed as mean ± SD. * $p<0.05$, ** $p<0.01$.

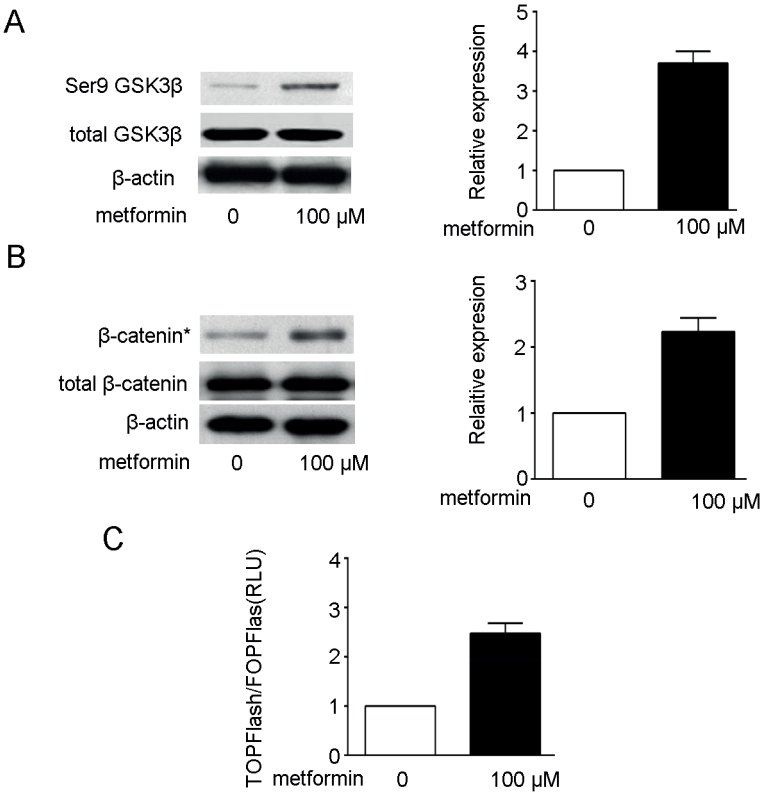

Figure 2. Effect of metformin on the GSK3β signaling pathway in hBMSCs. Metformin (100 μM) was added after the cells reached confluency. After 14 days, serine 9-phosphorylated GSK3β ***(A)*** and active β-catenin (β-catenin*) ***(B)*** were performed by immunoblotting, and Luciferase activity of TOPFlash/FOPFlash ***(C)*** was measured. All data are expressed as mean ± SD. * $p<0.05$, ** $p<0.01$.

Figure 3A shows that inhibition of GSK3β phosphorylation significantly decreased the expression of Col-1, OCN and RUNX2. Furthermore, expression of activated β-catenin (β-catenin*) and the TOPFlash activity were decreased in CA-GSK3β (Figures 3B and C). Therefore, we considered that the induction of osteogenic differentiation by metformin could be alleviated by inhibiting phosphorylation of GSK3β.

Testing the Role of AMPK in Inhibition of GSK3β by Metformin

Previous investigations[15] showed that AMPK plays a key role in metformin-induced cell growth, apoptosis, or differentiation. So, we tested the role of AMPK in inhibition of GSK3β by metformin. Metformin at 100 μM significantly increased the phosphorylation of AMPK at 6 h, which could be inhibited by compound C at a dose-dependent manner (Figure 4). Further studies showed that metformin significantly increased the phosphorylation of GSK3β at serine 9 residue, which could be inhibited by compound C through AMPK (Figure 4). These data suggested that AMPK play an essential role in inhibition of GSK3β by metformin.

Discussion

In the present study, we investigated that effect of metformin on differentiation of human bone marrow derived MSC into osteoblasts. We found that metformin significantly enhanced the MSC osteogenic differentiation at a dose-dependent manner. We further demonstrated that phosphorylation of GSK3β, which has a vital role in cell growth, differentiation, and apoptosis, was activated by metformin. Inhibition of GSK3β by metformin resulted in accumulation of cytosolic β-catenin and activation of Wnt signaling pathway. Next, a plasmid containing a constitutive active form of GSK3β (S9A, CA-GSK3β) was transfected to constitutively activate GSK3β. Series analysis showed that GSK3β activation, whereas the effect on osteogenic differentiation of MSCs

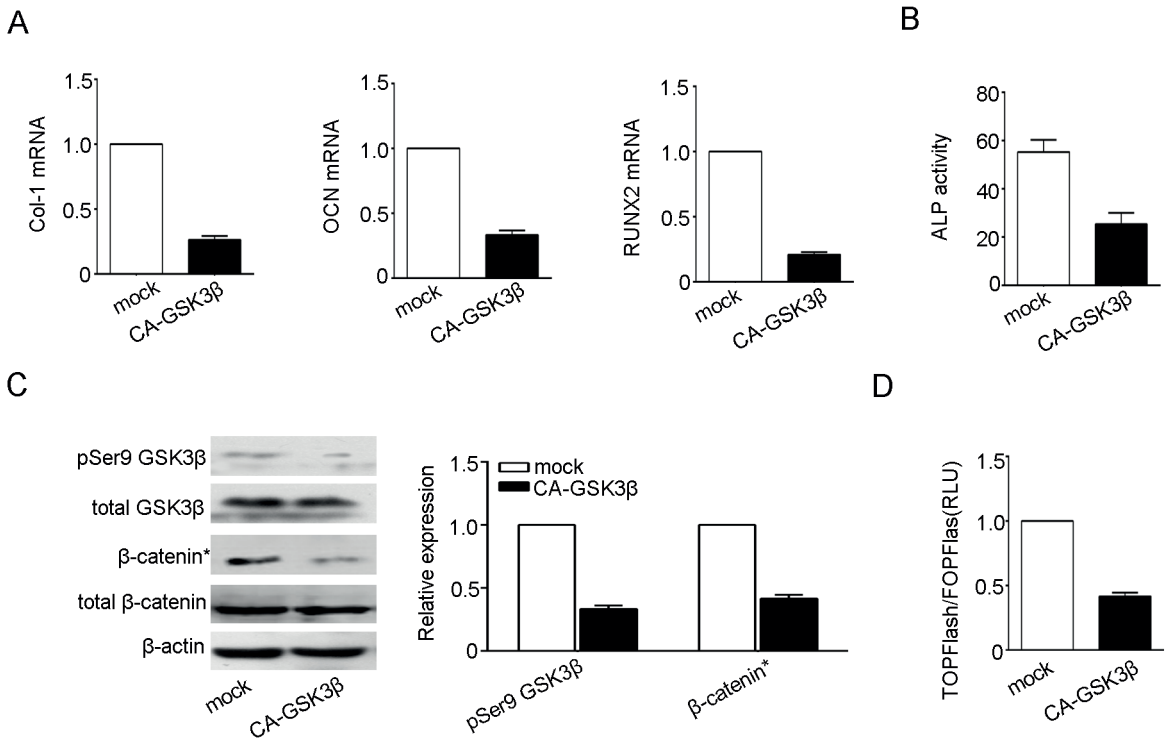

Figure 3. Effect of GSK3β activation on the differentiation of hBMSCs induced by metformin. Metformin (100 μM) was added after the cells reached confluency. After 14 days, ***(A)*** the osteoblastic marker genes (Col-1, OCN and RUNX2) were measured to evaluate osteoblastic differentiation by qRT-PCR with β-actin as control. Results were expressed as fold increase over the control values. ***(B)*** ALP activity of hBMSCs by metformin (100 μM) treatment was measured. ***(C)*** Serine 9-phosphorylated GSK3β and active β-catenin (β-catenin*) were performed by immunoblotting ***(D)*** Luciferase activity of TOPFlash/FOPFlash. All data are expressed as mean ± SD. *$p<0.05$, ** $p<0.01$.

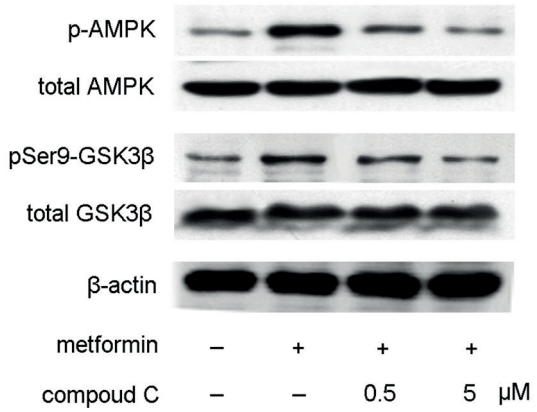

Figure 4. To test the role of AMPK in metformin-induced GSK3β phosphorylation. Inhibition of metformin (100 μM) induced GSK3β phosphorylation by compound C (0.5, 5 μM) treatment. Cells were incubated with metformin for 6 h following treatment with compound C for 30 min. Serine 9-phosphorylated GSK3β and p-AMPK were performed by immunoblotting. All data are expressed as mean ± SD. * $p<0.05$, ** $p<0.01$.

promoted by metformin was abolished, suggesting that metformin promoted the osteogenic differentiation of MSCs by suppressing GSK3β. Bone remodeling is a dynamic and continuous process involving a balance between formation of new bone, mediated by osteoblasts, and removal of old bone, regulated by osteoclasts[16]. This process requires the interaction between various cell types such as osteoblasts, osteoclasts, and various stem cells capable of differentiation, including MSCs[17]. The differentiation of human MSCs into osteoblasts plays an important in formation of new bone, which is regulated by a variety of drugs, including metformin. The positive effect of metformin on osteogenic differentiation has been reported by previous studies. However, little is known about the mechanisms of metformin-induced osteogenic differentiation of MSCs. Metformin, as a classic anti-hyperglycemic agent, is commonly used in treatment of type 2 diabetes by improving insulin resistance[18]. Recently, its positive effect of mediating osteogenic differentiation has been focused and the underlying mechanism also been preliminarily explored. Consistent with our results, Gao et al[11] reported that metformin promoted osteoblast differentiation and inhibited adipocyte differentiation of MSCs. Furthermore, the effect of metformin in a shift toward the osteoblastic differentiation of BMPCs is possibly mediated by AMPK activation[10]. On this basis, various studies have shown that activation of the AMPK signaling pathway is a relevant mechanism of action for metformin on osteogenic differentiation both *in vivo* and *ex vivo*[1]. Particularly, metformin may stimulate osteoblast differentiation through the transactivation of Runx2 via AMPK/USF-1/SHP regulatory cascade in mouse calvaria-derived cells[19]. In addition, metformin promoted osteoblastic differentiation by inducing activation and redistribution of phosphorylated extracellular signal-regulated kinase (P-ERK)[20]. In our study, we found that GSK3β may be a critical factor for metformin-induced osteoblast differentiation of MSCs. GSK3β is a regulatory switch that plays a crucial role in various signaling pathways that are involved in many important biological processes, including cell survival and osteogenic differentiation[21,22]. Recent works[23,24] reported that multiple chemicals could regulate the osteogenic differentiation of stem cells through activation of GSK3β. TNF-α inhibited the osteogenesis of MSCs by GSK3β inhibition[23]. GSK3β-inhibitor (2'Z,3'E)-6-Bromoindirubin-3'-oxime (BIO) enhances osteoblast differentiation on titanium surfaces[24]. In this study, we found that metformin promotes osteoblast differentiation of MSCs by, at least partly, inhibiting GSK3β activity. Our data further demonstrate that metformin could inhibit GSK3β activity, resulting in activation of Wnt/β-catenin signaling in hBMSCs. Reduction of GSK3β activity lead to dephosphorylation and release of cytoplasmic β-catenin from a protein complex consisting of Axin1/2, APC, CK1 and GSK3β increased accumulation of β-catenin. Nuclear accumulation of β-catenin follows the stimulatory activation of Wnt/β-catenin-responsive genes to regulate various developmental processes, including osteogenic differentiation. Activated β-catenin expression was up-regulated in metformin-mediated osteogenic differentiation. When GSK3β was constitutively active, activated β-catenin expression was changed. These results suggested that Wnt/β-catenin signaling participated in metformin-mediated osteogenic differentiation of hBMSCs.

Conclusions

We identified metformin as a positive regulator in human osteogenesis, acting by inhibiting GSK3β activity. Wnt/β-catenin signaling may participate in metformin-mediated osteogenic differentiation of hBMSCs. Our findings suggest that metformin may be a useful target in the treatment of pathological conditions of bone loss.

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Conflict of Interest
The Authors declare that they have no conflict of interest.

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