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Metformin Enhances Osteogenesis and Suppresses Adipogenesis of Human Chorionic Villous Mesenchymal Stem Cells

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Metformin is the first-line anti-hyperglycemic drugs commonly used to treat type 2 diabetes. Recent studies have shown that metformin can enhance bone formation through induction of endothelial nitric oxide synthase (eNOS). Human chorionic villous mesenchymal stem cells (CV-MSCs) are promising candidates for regenerative medicine. The present study aimed to investigate the effects of metformin on the osteogenic and adipocytic differentiation of human CV-MSCs, and to elucidate the underlying mechanism. CV-MSCs, prepared from human term placentae, were cultured with different concentrations of metformin. Treatment for 72 hours with 0.05 mM metformin had no noticeable effect on the proliferation of CV-MSCs. Consequently, CV-MSCs were cultured for seven or 14 days in the osteogenic medium supplemented with 0.05 mM metformin. Treatment for seven days with metformin increased the expression levels of osteogenic protein mRNAs, including alkaline phosphatase, runt-related transcription factor 2, and osteopontin. Metformin also enhanced the mineralization of CV-MSCs. Furthermore, metformin induced the expression of eNOS in CV-MSCs during osteogenic differentiation. By contrast, when CV-MSCs were cultured for 14 days in the adipogenic medium, 0.05 mM metformin inhibited the expression of adipogenic protein mRNAs, including proliferators-activated receptor-γ and CCAAT/enhancer binding protein-α. The lipid droplet accumulation was also reduced on 28 days after metformin treatment. These findings indicate that metformin can enhance osteogenic differentiation of CV-MSCs and reduce adipocyte formation. The effect of metformin on osteogenic differentiation of CV-MSCs may be associated with eNOS expression. Our findings will highlight the therapeutic potential of metformin in osteoporosis and bone fracture.

Keywords: adipocyte differentiation; chorionic villous mesenchymal stem cells; eNOS; metformin; osteogenic differentiation
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Introduction

Metformin is the first-line anti-hyperglycemic drugs commonly used to treat type 2 diabetes (Kirpichnikov et al. 2002). It is considered to be an insulin-sensitizing drug that can inhibit the production of hepatic glucose and increase peripheral glucose uptake. Apart from diabetes, metformin also possesses positive effects on cardiovascular disease and nerve regeneration (Hettich et al. 2014; Fung et al. 2015). In recent years, the effects of metformin on bone metabolism have received considerable attention; there is a close relationship between glucose and bone metabolism (Meier et al. 2016; Starup-Linde and Vestergaard 2016). Bone metabolism includes two steps: osteoclastic bone resorption and osteoblastic bone formation. This process is important for bone repair and mineral homeostasis (Raggatt and Partridge 2010). Diabetes can induce osteoporosis and increase the risk of bone fractures (Mai et al. 2011; Tsentidis et al. 2016; Wang et al. 2016). Mesenchymal stem cells (MSCs) possess multiple differentiation abilities and have great therapeutic potentials for bone repair (Doulatov and Daley 2013). Osteogenic genes including runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and osteopontin (OPN) are critical for osteogenic differentiation of MSCs (Ryoo et al. 2006; Vimalraj et al. 2015). Recent studies have shown that metformin can decrease fracture risk in patients with diabetes (Vestergaard et al. 2005). In addition, metformin can induce the osteogenic differentiation of MC3T3E1 cells (clonal osteoblast-like cells derived from mouse calvaria) and bone marrow (BM) progenitor cells (Gao et al. 2010; Jang et al. 2011). However, the effect of metformin on the differentiation of human chorionic villous mesenchymal stem cells (CV-MSCs) is still unknown.

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The beneficial effects of metformin are associated with phosphorylation of AMP-activated protein kinase (AMPK) (Musi et al. 2002; Duca et al. 2015). AMPK is important in regulating the glucose and fatty acid metabolism. Metformin can augment the differentiation of endothelial progenitor cells through AMPK/endothelial nitric oxide synthase (eNOS)/nitric oxide pathway (Li et al. 2015). Takahashi et al. (2015) reported that metformin can treat ischemic peripheral artery diseases by increasing the activation of AMPK and eNOS.

In the present study, we investigate the effects of metformin on the differentiation of human CV-MSCs and explored the underlying mechanisms. The results showed that metformin can promote osteogenic differentiation of human CV-MSCs and inhibit adipocyte formation. The effect of metformin on osteogenic differentiation of CV-MSCs may be associated with metformin-induced eNOS expression.

Materials and Methods

Human CV-MSC isolation

MSCs were isolated from the chorionic villi of human term placenta and characterized as we and others previously described (Park et al. 2011; Pilz et al. 2011; Gu et al. 2016). Chorionic villi were minced and digested. Cells were filtered by a nylon tissue mesh to obtain a single cell suspension. Cells were centrifuged with a Percoll (Sigma Aldrich, St. Louis, USA) discontinuous gradient and then seeded in a 10-cm Petri dish using Dulbecco's modified Eagle's medium (DMEM) (HyClone, South Logan, USA) containing 10% fetal calf serum and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY). After 4 days, media were changed, and nonadherent cells were washed. This study was approved by the ethics committee of the First Affiliated Hospital of Soochow University.

Cell proliferation assay

Cell proliferation was detected using a CCK8 kit (Dojindo, Tokyo, Japan) according to the manufacture's instruction. Briefly, cells were cultured in 96-well plates. Metformin (0, 0.05, 0.1, 0.5, and 1.0 mM) (Sigma Aldrich) was added to the cells 24 h after plating and incubated for 72 h. 10 μl CCK-8 solution was added to the wells and the cells were incubated for 2.5 h in the incubator. The Multi-Volume Spectrophotometer System (BioTek Epoch, Vermont, USA) was used to measure the absorbance at 450 nm.

Osteogenic differentiation of human CV-MSCs

Human CV-MSCs were cultured in DMEM medium supplemented with 10% fetal calf serum, 10 mM β-glycerol phosphate (Sigma Aldrich), 0.1 mM L-ascorbic acid (Sigma Aldrich), and 10 nM dexamethasone (Sigma Aldrich), 2 mM glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). In some experiments, metformin (0.05 mM) was added once every 3 days during osteogenic differentiation. Cells were collected at days 7 and 14 during osteogenic differentiation.

Alizarin red staining

Calcium deposits were detected by alizarin red S staining after 28 days of osteogenic induction. Cells were incubated with 2% alizarin red (pH 4.2) (Sigma Aldrich) for 10 min and washed with distilled water. Cells were detected by phase-contrast microscopy at day 28 to verify the presence of mineralized nodules.

Adipocyte differentiation of human CV-MSCs

Human CV-MSCs were cultured in adipogenic medium (Cyagen Biosciences Inc., USA) with or without metformin. Cells were detected by phase-contrast microscopy at day 28 to verify the presence of lipid droplets.

Oil red staining

At day 28 after adipocyte differentiation, cells were fixed in 1% formaldehyde, incubated with 0.5% Oil red solution (Sigma Aldrich) for 20 min, rinsed with 8% propylene glycol (Sigma Aldrich) for 3 min, washed in distilled water and mounted with aqueous mounting medium.

Real-time quantitative PCR

Total RNA was extracted from cells using TRIzol (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer's instructions and reverse transcribed. Real-time PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) and conducted with the Biorad CFX96 (Bio-Rad). Primer sequences for real-time PCR were listed in Table 1. Glyceraldehyde-3-phosphate dehy-

Table 1. Oligonucleotides used in real-time PCR.

Gene	Primer	
OPN	Forward	GAGGGCCAAGACGAAGACATC
	Reverse	CAGATCACGTCATCGCACAAC
OCN	Forward	CACTCCTCGCCCTATTGGC
	Reverse	CCCTCCTGCTTGGACACAAAG
RUNX2	Forward	TGGTTACTGTCATGGCGGGTA
	Reverse	TCTCAGATCGTTGAACCTTGCTA
ALP	Forward	GTGAACCGCAACTGGTACTC
	Reverse	GAGCTGCGTAGCGATGTCC
eNOS	Forward	TGATGGCGAAGCGAGTGAAG
	Reverse	ACTCATCCATACACAGGACCC
FABP4	Forward	ACTGGGCCAGGAATTTGACG
	Reverse	CTCGTGGAAGTGACGCCTT
PPARγ	Forward	GGGATCAGCTCCGTGGATCT
	Reverse	TGCACTTTGGTACTCTTGAAGTT
C/EBPα	Forward	GTGGAGACGCAGCAGAAG
	Reverse	TTCCAAGGCACAAGGTTATC
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG

drogenase (GAPDH) mRNA was used as an endogenous control.

Western-blot analysis

Protein extracts were resolved on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% freshly prepared milk-TBST (Tris-buffered saline with Tween 20) for two hours at room temperature, and then incubated overnight at 4°C with antibodies specific for eNOS and β-actin (Abcam, Cambridge, MA). Signals were detected with horseradish peroxidase-labeled secondary antibodies using chemiluminescence labeling.

Statistical analysis

SPSS statistical software (version 16.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data were presented as means ± SEM. The significance between two groups was determined using Student's *t* test for normally distributed variables. One-way ANOVAs were used for multiple comparisons. *P* values less than 0.05 were considered as statistically significant.

Results

Effects of metformin on the proliferation of human CV-MSCs

Human CV-MSCs were treated with different concentrations of metformin for 48 h, and proliferation was examined using a CCK-8 kit. As shown in Fig. 1, at the concentration of 0.1-1 mM, metformin significantly inhibited the proliferation of CV-MSCs compared to untreated group ($p < 0.01$). No significant difference was found among the three groups ($p > 0.05$). However, 0.05 mM metformin almost had no effect on the proliferation of CV-MSCs ($p > 0.05$). Based on these results, we used 0.05 mM metformin in the subsequent experiments.

Metformin increases the osteogenic differentiation of human CV-MSCs

To investigate the effects of metformin on the osteogenic differentiation of CV-MSCs, metformin (0.05 mM) was added into the osteogenic medium. Real-time PCR was performed at days 7 and 14. Compared to the untreated control, metformin significantly increased the expression of ALP, RUNX2, and OPN at days 7 and 14 ($p < 0.05$, $p < 0.01$; Fig. 2A-C). However, metformin had no significant effect on the expression of osteocalcin (OCN) (Fig. 2D). To further confirm the effect of metformin on osteogenic differentiation of human CV-MSCs, Alizarin red staining was performed after 28 days of osteogenic induction. The results showed metformin increased the mineralization of human CV-MSCs at day 28 (Fig. 2E).

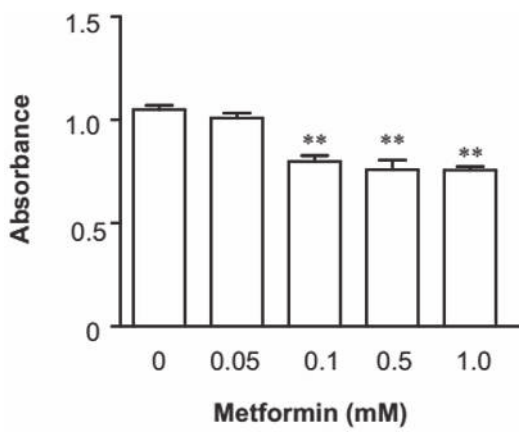

Fig. 1. The effects of metformin on the proliferation of human CV-MSCs
Human CV-MSCs were cultured with metformin (0, 0.05, 0.1, 0.5, and 1.0 mM) for 72 h. The proliferation of CV-MSCs was detected using a CCK8 kit. The data were shown as means ± SEM (n = 3). $^{**}p < 0.01$.

Metformin inhibits the adipocyte differentiation of human CV-MSCs

To investigate the effects of metformin on adipocyte differentiation of human CV-MSCs, metformin was added into the adipogenic medium. Real-time PCR showed that metformin did not affect the expression of adipogenic genes at day 7 compared to untreated control. However, the expression of proliferators-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα) was significantly reduced by metformin at day 14 (Fig. 3A, B). Moreover, metformin almost had no effect on the expression of fatty acid binding protein 4 (FABP4) at day 14 (Fig. 3C). Oil red O staining showed that metformin significantly inhibited the lipid droplet accumulation of CV-MSCs at day 28 (Fig. 3D).

Metformin induces eNOS expression

Nitric oxide is known to be involved in bone metabolism. To investigate the mechanism by which metformin affects osteogenic differentiation, we analyzed the expression of eNOS. Real-time PCR was performed at day 3. The results showed that metformin increased the mRNA expression of eNOS compared to untreated control (Fig. 4A). Moreover, western blot showed that metformin treatment increased the protein expression of eNOS at day 3 (Fig. 4B).

Discussion

Diabetes is considered to be a global health problem. Most diabetes patients have impaired bone formation and remodeling. The role of anti-diabetes drugs in the bone metabolism has received extensive attention in recent years (see Introduction). In the present study, we investigated the role of metformin in the differentiation of human CV-MSCs. Our results showed that metformin treatment could promote the osteogenesis but suppress adipogenesis of human CV-MSCs.

Metformin is an insulin sensitizing drug commonly used to treat type 2 diabetes. It has been reported that metformin can improve insulin resistance and lipid profiles (Kirpichnikov et al. 2002). Recently, *in vivo* and *in vitro* studies have suggested a role for metformin in regulating cell proliferation. High dose metformin can inhibit the

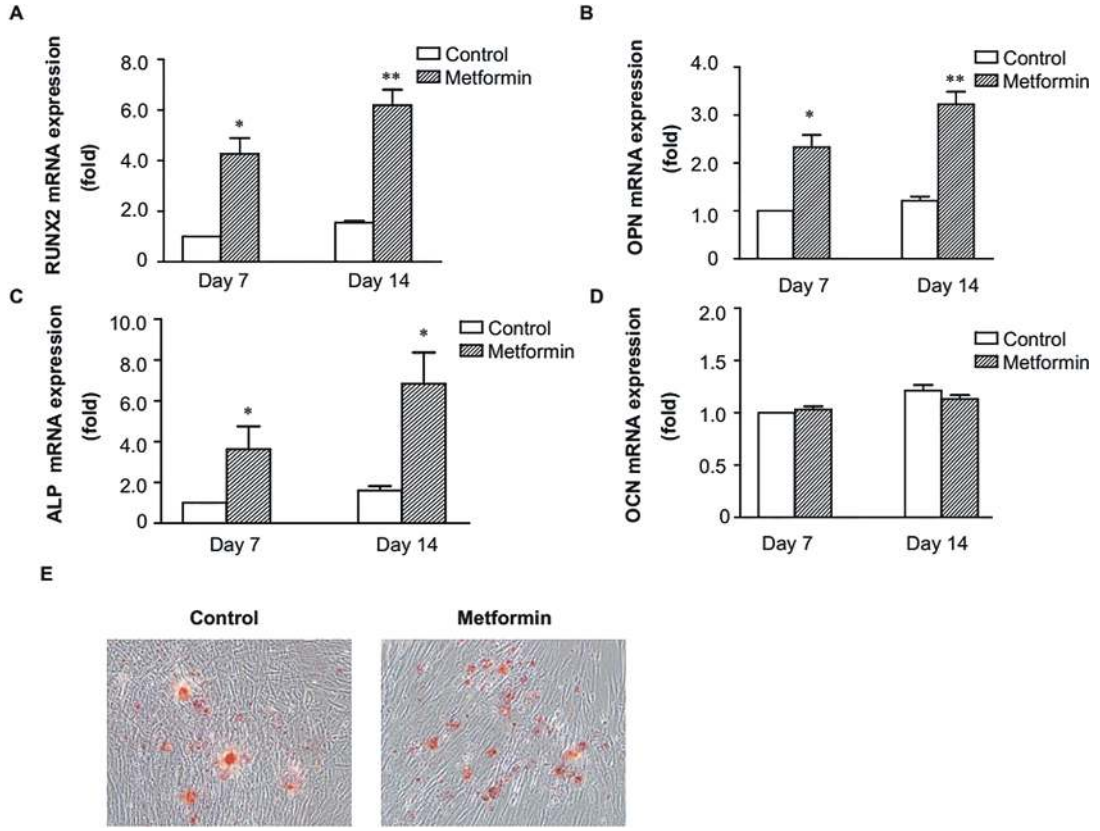

Fig. 2. The effects of metformin on the osteogenic differentiation of human CV-MSCs. Human CV-MSCs were cultured in osteogenic medium supplemented with 0.05 mM metformin. (A-D) The mRNA levels of RUNX2, OPN, OCN, and ALP were analyzed by real-time PCR at days 7 and 14. Data were shown as means ± SEM (n = 3). $*p < 0.05$, $**p < 0.01$, (E) Alizarin Red staining was performed at day 28.

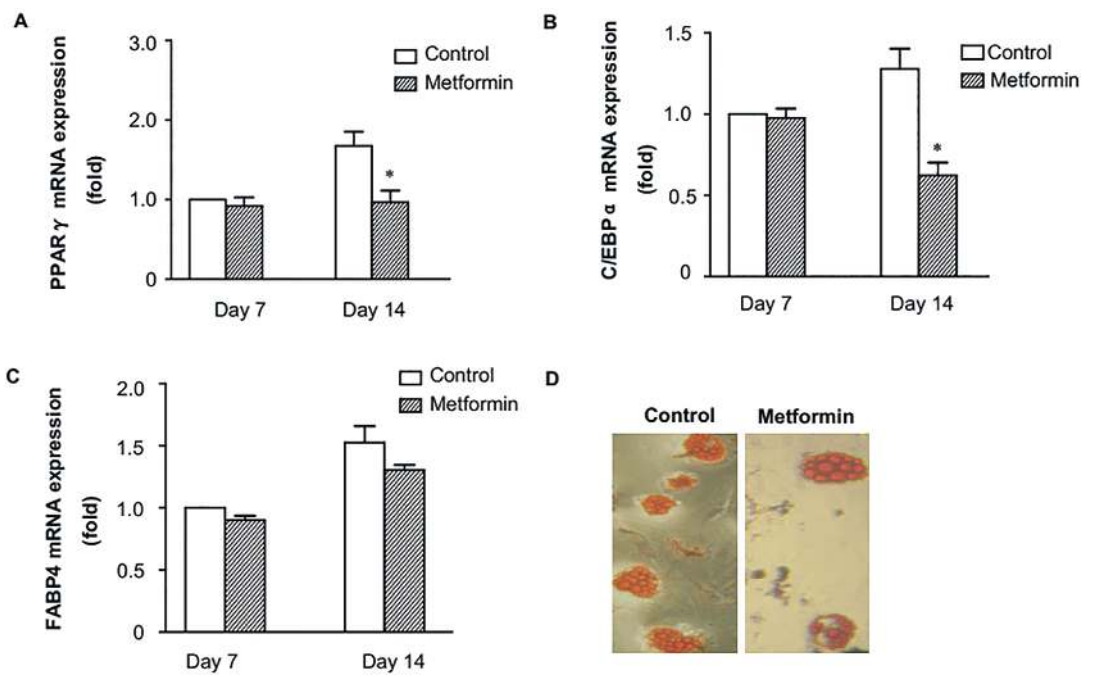

Fig. 3. The effects of metformin on the adipogenic differentiation of human CV-MSCs.
Human CV-MSCs were cultured in adipogenic medium supplemented with 0.05 mM metformin. (A-C) The mRNA levels of PPARγ, C/EBPα, and FABP4 were analyzed by real-time PCR at days 7 and 14. Data were shown as means ± SEM (n = 3). $*p < 0.05$, $**p < 0.01$, (D) Lipid droplets were stained with oil red O at day 28.

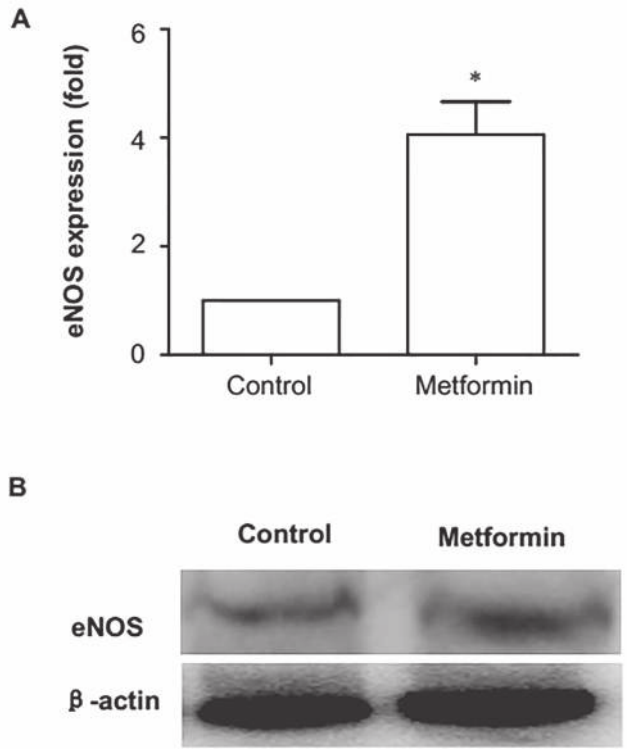

Fig. 4. The effects of metformin on the expression of eNOS. Human CV-MSCs were cultured in osteogenic medium supplemented with 0.05 mM metformin for 3 days. (A) The mRNA level of eNOS was analyzed by real-time PCR. Data were shown as means ± SEM (n = 3). *$p <$ 0.05. (B) Western blot analysis was performed to detect the expression of eNOS.

growth of tumor cells (He et al. 2015). In contrast, low dose metformin (0.64 μM) can increase the proliferation of rat primary osteoblasts (Zhen et al. 2010). In the present study, our data clearly showed that low dose (0.05 mM) metformin exerted no noticeable effects on the proliferation of human CV-MSCs. At the concentration range of 0.1-1 mM, metformin significantly reduced the proliferation of human CV-MSCs. Interestingly, Li et al. (2015) reported that 0.5-1 mM metformin did not affect the proliferation of endothelial progenitor cells. Wu et al. (2011) found that 1 mM metformin just slightly increased the proliferation of human amnion-derived MSCs and had no effect on the proliferation of rabbit BM-derived MSCs. These results suggest that the effect of metformin may dependent on different doses and cell sources.

We found that metformin promotes the osteogenic differentiation of human CV-MSCs. Metformin can increase the expression of osteogenic genes, RUNX2, ALP, and OPN, suggesting an increase of osteogenesis. However, there was no obvious change in the expression of OCN. In addition, we observed a significant increase in the amount of calcium deposition after metformin treatment, which further confirmed that metformin increased the osteogenic differentiation of human CV-MSCs. Molinuevo et al. (2010) found that 10 μM metformin can increase ALP activity and mineralization in rat BM progenitor cells. Cortizo et al. (2006) showed that metformin (25-100 μM) can increase type I collagen production and ALP activity in MC3T3E1 cells. Moreover, metformin can reverse rosiglitazone-induced bone loss in rats (Sedlinsky et al. 2011). Our results were consistent with these reports and confirmed that metformin possessed beneficial effects on bone formation. In contrast, others reported that metformin (1 mM) had no effect on the osteogenic differentiation of MSCs when cultured in β-glycerol phosphate and ascorbic2-phosphate medium (Wu et al. 2011). Cortizo et al. (2006) also found that metformin had no effect on ALP activity in UMR106 rat osteosarcoma cells. At the concentration of 2 mM, metformin reduced mineralization of osteoblasts (Kasai et al. 2009). These conflicting results may be attributed to the different culture conditions and cell sources.

We also found that metformin reduced the expression of C/EBPα and PPARγ in human CV-MSCs at day 14 after adipogenic induction. However, metformin did not affect the expression of C/EBPα and PPARγ at day 7 after adipogenic induction. C/EBPα and PPARγ are critical factors in adipogenic differentiation. Our results suggested that metformin did not affect adipogenesis at an early stage of differentiation. Metformin only inhibited the adipogenesis of human CV-MSCs at a later stage of differentiation. Previous studies have shown that metformin can suppress the adipogenic differentiation of murine MC3T3E1 cells in combination with atorvastatin (Kim et al. 2015). In addition, metformin can decrease adipogenic gene expression in subcutaneous adipose tissue explants (Moreno-Navarrete et al. 2011). Our findings were in agreement with their results. Interestingly, we found that the expression of FABP4 was just slightly reduced after metformin treatment. FABP4 is a target gene of PPARγ. PPARγ can induce the expression of FABP4 in adipocytes. However, Garin-Shkolnik et al. (2014) have shown that FABP4 can negatively regulate PPARγ expression and adipocyte differentiation. We believe that there may be a balance between FABP4 and PPARγ. Metformin can maintain the expression of FABP4. FABP4 then reduced the expression of PPARγ, which may help to suppress adipogenesis in human CV-MSCs.

We next investigated the possible factors which contribute to metformin-induced osteogenic differentiation of human CV-MSCs. It has been accepted that eNOS partipicates in the regulation of bone homeostasis (Wimalawansa 2010). eNOS knockout mouse showed impaired osteogenic differentiation (Afzal et al. 2004). Bezafibrate can promote the osteogenic differentiation of MC3T3E1 cells through regulating eNOS (Zhong et al. 2011). In the present study, we found that treatment of human CV-MSCs with metformin led to an increase in the expression of eNOS. These data suggest that eNOS may play an important role in metformin-induced osteogenic differentiation of human CV-MSCs. Bone morphogenetic protein 2 (BMP2) is another regulator that can stimulate osteoblast differentia-

tion (Salazar et al. 2016). BMP2 overexpression can augment osteogenic differentiation of human periodontal ligament stem cells *in vivo* (Yi et al. 2016). Metformin can stimulate the expression of BMP2 in MC3T3E1 cells (Kanazawa et al. 2008). However, Wang et al. (2012) reported that metformin can suppress the expression of BMP2 in insulin-resistant mice. In the present study, we did not observe an obvious change in the expression of BMP2 in metformin-treated CV-MSCs (data not shown). Previous studies have shown that AMPK can stimulate the osteogenic differentiation of MC3T3E1 cells (Kanazawa et al. 2007). AMPK is an energy regulator that regulates glucose metabolism. Molinuevo et al. (2010) have reported that metformin can activate AMPK signaling pathway in BM progenitor cells, followed by increased expression of eNOS and BMP2. It is possible that metformin can promote osteogenic differentiation through activation of AMPK /eNOS pathway in human CV-MSCs.

There are some limitations in this study. The effect of metformin on AMPK activation was not evaluated. Additional experiments need to be done to demonstrate the relationship between metformin-induced eNOS expression and osteogenic differentiation in human CV-MSCs.

In the present study, we investigated the effects of metformin on the differentiation of human placental CV-MSCs. Our results demonstrated that metformin can promote the osteogenic differentiation of human CV-MSCs and inhibit adipocyte formation. Moreover, metformin can induce eNOS expression in human CV-MSCs, suggesting a possible involvement of eNOS in metformin-induced osteogenesis of CV-MSCs *in vitro*.

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Conflict of Interest

The authors declare no conflict of interest.

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