

Effect of diabetic osteoblasts on osteogenic differentiation of human umbilical cord mesenchymal stem cells

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Background. This study was aimed to investigate whether osteoblasts from diabetic patients have a promoting effect on osteogenesis of human umbilical cord mesenchymal stem cells (HUMSCs).

Methods. HUMSCs were co-cultured with osteoblasts of diabetic and non-diabetic patients. Morphological appearance and cytochemical characteristics of the non-diabetic osteoblasts and diabetic osteoblasts were observed by hematoxylin-eosin staining, type I collagen protein expression, alkaline phosphatase (ALP) staining and Alizarin Red S staining. Cell viability, type I collagen protein expression, ALP activity and osteocalcin mRNA expression in HUMSCs were investigated.

Results. Compared with negative control group, the cell proliferation, type I collagen protein expression, ALP activity and osteocalcin mRNA were increased in HUMSCs co-cultured with diabetic and non-diabetic osteoblasts ($P < 0.05$). There was no statistically significant difference in the HUMSCs cell proliferation, type I collagen protein expression, ALP activity and osteocalcin mRNA between the non-diabetic and diabetic group ($P > 0.05$).

Conclusions. Similar to osteoblasts from non-diabetic patients, osteoblasts from diabetic patients also have the ability to promote HUMSCs proliferation, and leading to HUMSCs exhibit some characteristic of osteoblasts.

Key words: diabetic, osteoblasts, mesenchymal stem cells, human umbilical cord, bone healing

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INTRODUCTION

Diabetes mellitus compromises the quality of bones and leads to a greater risk of bone fracture¹. Once a fracture has occurred, healing is delayed in patients with diabetes². The slower bone repair in diabetics is a consequence of a deficient function of osteoblasts³. Impaired bone and wound healing represents a major clinical problem in patients with diabetes⁴. Thus, it is important to explore novel modalities to facilitate fracture healing in diabetics.

Recently, cell therapy with bone marrow mesenchymal stem cells (MSCs) has been trialed to treat chronic wounds in diabetics⁵⁻⁷. The main functional characteristics of MSCs are their immunomodulatory ability, capacity for self-renewal, and differentiation into mesodermal tissues, such as bone cells⁸. However, the acquisition of bone MSCs from bone marrow is a relatively invasive procedure that may be associated with a small number of complications such as bleeding or infection. The number of bone MSCs and the proliferative or differentiation capacity is significantly decreased along with age⁹. There is growing evidence that Human Umbilical Cord Mesenchymal Stem Cells (HUMSCs) also exhibit pluripotent lineage differentiation potential¹⁰. This may be an alternative source

of MSCs which can be obtained by a simple, safe and painless procedure when the baby is delivered. HUMSCs showed a higher proliferative potential than bone MSCs and were capable of osteogenic and chondrogenic differentiation¹¹.

It has been reported that the tissue specific cells may cause or promote the differentiation of MSCs toward their cell type via a cell-to-cell interaction¹². In this study, osteoblasts derived from volunteer (non-diabetic or diabetic) cancellous bone were co-cultured with HUMSCs, and their ability to support HUMSCs proliferation, osteogenic differentiation were evaluated.

MATERIALS AND METHODS

Participants

The participants were recruited from patients undergoing bone fracture surgeries. Some had a history of type 2 diabetes. The age of the patients ranged between 50 and 70 years. The diagnosis of diabetes was based on the 2011 American Diabetes Association diagnostic criteria¹³. Participants were excluded from this study if they had a history of coronary heart disease, inherited disease, or any other chronic diseases. The experimental protocol

was approved by the Ethics Committee of our hospital and all participants gave written informed consent prior to sample collection.

Preparation of osteoblasts

Osteoblasts were isolated from cancellous bone of the femoral neck, femoral shaft or femoral condyle in patients undergoing hip joint or knee joint replacement. Cancellous were cut into small pieces, digested by 0.25% trypsin for 30 min, and by 1% collagenase for 3 h in Dulbecco's Modified Eagle Media/Nutrient Mixture F12 (DMEM/F12) at 37 °C. The released cells were separated from the bone chips by filtration through a 200 mesh grids. Cells were centrifugated and washed several times to remove excess collagenase, and were resuspended in DMEM/F12 (Hyclone) with 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂ at 37 °C. Co-cultures were initiated with second-passage osteoblasts.

The identification of osteoblasts

For the identification of osteoblasts isolated from cancellous of non-diabetic and diabetic patients, cell morphology was observed by hematoxylin-eosin staining. Alkaline phosphatase (ALP) staining was performed using the Gomori Calcium-Cobalt (Ca-Co) method. In brief, the osteoblasts were fixed with cold propyl alcohol for 10 min, and incubated in an incubator at 37 °C for 4-6 h. Then, the cells were stained with 2% cobalt nitrate for 3 min and 1% ammonium sulfide for 2 min. After being air-dried, the slides were finally mounted and used for microscopy.

The expression of type I collagen was detected by immunocytochemistry to identify osteoblasts. Briefly, cells were planted on coverslips. The coverslips were fixed in 4% formaldehyde for 10 min, 3% H₂O₂ for 15 min. The cells were then first permeabilized with 0.3% Triton X-100 in PBS for 20 min. After washing with PBS, cells were blocked with 5% BSA for 20 min, and incubated with primary antibody rabbit anti-type I collagen (Biosynthesis biotechnology Co., LTD. Beijing, China) at a ratio 1:100 at 4 °C overnight. After washing in PBS, cells were incubated with secondary antibody at 1:100 for 2 h at 4 °C. 3,3-diaminobenzidine (DAB) was prepared and applied to the coverslips for no more than 30 seconds. Images were taken using a digital camera under the microscope (Olympus).

Calcification nod staining was investigated by the Alizarin Red S staining method with slight modifications as described by Yun¹⁴. Alizarin red calcium deposition was measured at day 21. The cells were washed quickly with PBS and deionized water before 0.5% alizarin red solution was added. The solution was removed after 30 min and the cells were rinsed with deionized water until the water was clear. Mineralized matrix, indicated by bright red staining was identified by light microscopy.

Co-culture

HUMSCs were a kind gift from Professor Zhongchao Han (National Research Center for Stem Cell Engineering

and Technology, Tianjin, China). HUMSCs were cultured in DMEM/F12 in the lower compartment of a Millipore transwell-plate (Costar 3412, Corning Incorporated, NY, USA). After the HUMSCs were grown to confluence, different osteoblasts were placed onto 0.4 µm pore size polycarbonate membranes of the upper compartment of the transwell plate. Cells were evenly divided into four groups according to the types of osteoblasts in upper compartment: negative control group (only HUMSCs), positive control group (only non-diabetic osteoblasts), non-diabetic group (add non-diabetic osteoblasts in upper compartment), and diabetic group (add diabetic osteoblasts in upper compartment).

Cell proliferation assay

Cell proliferation was determined with a cell counting kit-8 (Beyotime Biotechnology, Jiangsu, China) according to the manufacturer's instructions. The same number of HUMSCs and osteoblasts (1×10⁵ cells per well, 500 µL medium/well) were cultured in a 24-well plate by placing transwell chambers as described above. After incubated 1, 2, 3, 4, 5, 6, and 7 days, cell counting reagent (50 µL) was added to lower compartment and incubated for 3 h.

2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium (WST-8) was reduced by dehydrogenases in cells yielding an orange-coloured product (formazan). The amount of formazan dye generated by the dehydrogenases in the cells was directly proportional to the number of living cells. The absorbance was measured at 450nm using STAKMAXTM microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Indirect Immunofluorescence

The HUMSCs were cultured on coverslips in the lower compartment of a transwell-plate. After being cultured for 10 days, the coverslips were fixed in 4% formaldehyde for 10 min. The cells were then first permeabilized with 0.3% Triton X-100 in PBS for 20 min. After wash with PBS, cells were blocked with 5% BSA for 30 min, and were incubated with primary antibodies of rabbit anti-type I collagen at dilution of 1:100 for 60 min. After another wash, cells were stained with goat anti-rabbit Alexa Fluor 568 secondary antibody at 1:100, which produces red fluorescence. Then the cells were counterstained with Hoechst333442 and observed under a fluorescent microscope. Five randomly selected immunoreactive fields were quantified by densitometric analysis with Image-Pro Plus 6 software (Media Cybernetics Inc., Silver Spring, MD, USA).

Alkaline phosphatase (ALP) activity in HUMSCs

ALP activity in HUMSCs was determined at 7, 10, and 14 days of incubation using alkaline phosphatase activity colorimetric assay kit (GENMED Scientifics inc. USA) according to the manufacturer's manual. The amount of *p*-nitrophenol liberated was measured at 405 nm by STAKMAXTM microplate reader. ALP activity was calculated according to the formula: [(Sample absorbance - background absorbance) × sample dilution factor

$\times 0.25] \div [0.005 \times 18.5 \times 0.6 \text{ (cm)} \times 5 \text{ (minutes)}] = \text{units/mL} \div (\text{sample protein concentration}) \text{ mg/mL} = \text{units/mg}$. Protein concentrations were measured using the Bio Rad protein assay kit with bovine serum albumin as a standard. The experiments were repeated three times.

Real time RT-PCR

To assess the osteogenesis of HUMSCs, osteocalcin (OCN) mRNA expression was detected by real time RT-PCR 21 days after cell co-culture. RNA was isolated from HUMSCs cell culture using TriZol Reagent (Tiangen Biotech Co., LTD, Beijing, China). Total RNA (1 μg) was converted to cDNA and amplification was performed using One-Step qPCR Kit (Tiangen Biotech Co., Ltd. Beijing, China). For the determination of OCN mRNA expression, SYBR Green detection was used and the val-

ues were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time quantitative polymerase chain reaction (PCR) was performed in a 7300 Sequence Detection System (Applied Biosystems). 5'-TAG TGA AGA GAC CCA GGC GC -3' and 5'-CAC AGT CCG GAT TGA GCT CA -3' for OCN ; 5'-GGT ATC GTC GAA GGA CTC ATG AC-3' and 5'-ATG CCA GTG AGC TTC CCG TTC AGC-3' for GAPDH. The thermal cycling conditions for the real-time PCR was 95 °C for 2 min followed by 45 cycles of 94 °C for 20 s, 60 °C for 20 s, and 68 °C for 20 s. Data were collected during the 68 °C. Relative expression levels for each primer set were normalized to the expression of GAPDH by the $2^{-\Delta\text{CT}}$ method.

Statistical analysis

Values were expressed as means \pm SD. The data were analyzed by one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Cell morphology and cytochemical characteristics

Osteoblasts were seen to adhere to the bottom of the culture dish after 4 to 24 h. On day 3, cells possessed typical shapes, such as fusiform, triangular, and polygonal, with one or two elliptical nuclei in the center of the cells. Cells were arranged linearly in a stepping-stone arrangement (Fig. 1A). Cell numbers increased gradually and reached confluence on day 6. Hematoxylin-eosin staining images showed that the nuclei were stained an amethyst color and the cytoplasm was stained pink (Fig. 1B). A brown-stained type I collagen positive reaction was observed by immunocytochemistry staining (Fig. 1C). Ca-Co and immunocytochemistry staining were applied to identify the osteoblastic cells. Cellular cytoplasm was stained grayish-black due to ALP staining. ALP-positive areas appeared on day 10 (Fig. 1D). The bright red staining was found in cells from non-diabetic osteoblasts and diabetic osteoblasts (Fig. 1E).

The influence of osteoblasts on the proliferation of HUMSCs

The proliferation of HUMSCs was increased gradually. Compared with negative control group, cell numbers increased faster in non-diabetic group and diabetic group. The exponential growth phase of non-diabetic and diabetic group began from day 2 to 4, reaching a maximum on day 5. In the negative control group, the exponential growth began from day 3 to 5, reaching a maximum on day 6 (Fig. 2).

The expression of type I collagen protein in HUMSCs co-cultured with osteoblasts

In the HUMSC co-cultured with different osteoblasts for 10 days, the human-specific type I collagen antibody clearly revealed type I collagen protein expression. The expression of type I collagen protein was indicated by

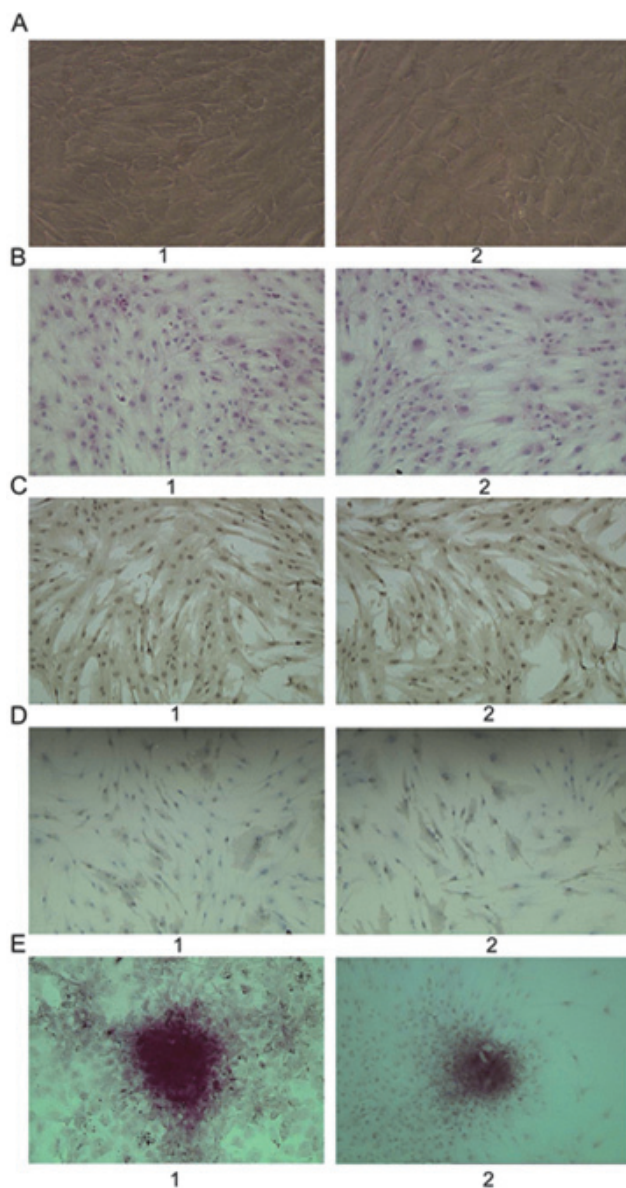


Fig. 1. Identification of osteoblasts (20 \times). A: morphology of osteoblasts; B: hematoxylin-eosin staining; C: expression of type I collagen; D: ALP staining; E: Alizarin Red S staining; 1: Non-diabetic group; 2: Diabetic group;

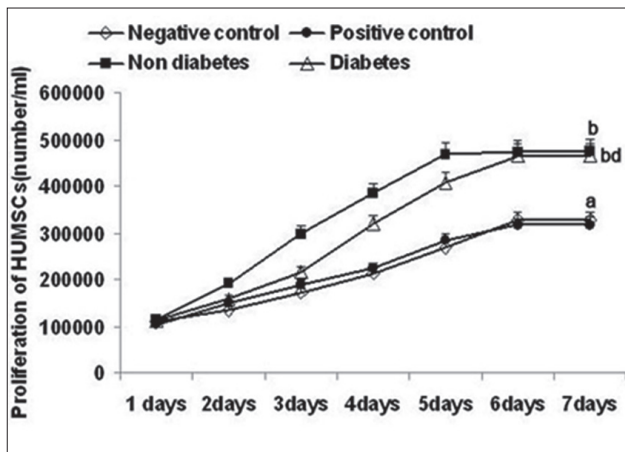


Fig. 2. Proliferation of HUMSCs co-cultured with osteoblasts. ^a $P > 0.05$, ^b $P < 0.05$ vs. negative control group ^d $P > 0.05$ vs. non-diabetic group.

bright red fluorescence, cell nuclear staining with Hoechst 33342 was indicated by blue fluorescence. Fig. 3 showed that there was no red fluorescence in negative control group, but a strong and homogenous signal for type I collagen antibody stained the cytoplasm was observed in non-diabetic group, diabetic group and positive control group. There was no significant difference in the expression of type I collagen protein between non-diabetic group and diabetic group.

Alkaline phosphatase (ALP) activity in HUMSCs co-cultured with osteoblasts

HUMSCs were examined for their capacity to differentiate when co-cultured with osteoblasts. Assay of ALP activity was used as an indicator of osteogenic differentiation. There was less ALP activity in negative control group which was not treated with osteoblasts, but ALP activity was increased when co-cultured with osteoblasts, compared with negative control group (Fig. 4). Co-culture with osteoblasts strongly enhanced the levels of ALP activity in the HUMSCs ($P < 0.01$). There was no statistically significant difference in ALP activity between non-diabetic group and diabetic group ($P > 0.05$).

The mRNA expression of OCN in HUMSCs co-cultured with osteoblasts

The mRNA expression of OCN, which is a terminal marker of osteoblasts differentiation, was assessed as definitive molecular markers for osteogenic differentiation (Fig. 5). HUMSCs co-culture with the osteoblasts induced the expression of OCN, which were nearly undetectable in the negative control group. The diabetic group showed less OCN expression than the non-diabetic group, but this difference did not reach statistical significant difference ($P > 0.05$).

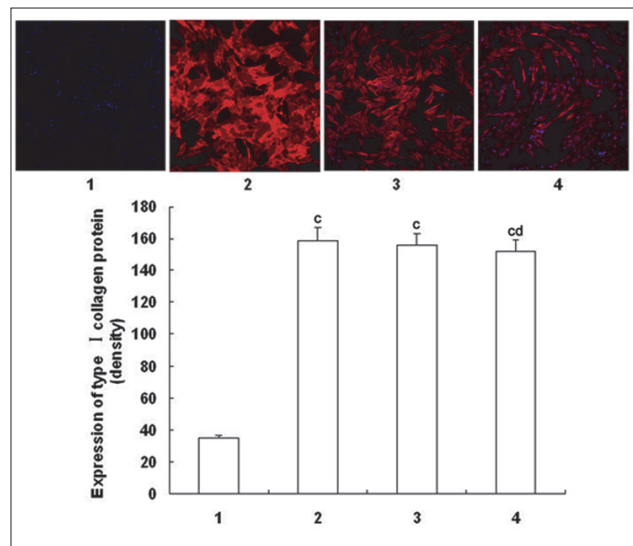


Fig. 3. Immunofluorescence staining for type I collagen in cultured HUMSCs. Anti-type I collagen polyclonal antibody stained cytoplasm showed red fluorescence in non-diabetic group and diabetic group, and no cytoplasmic staining was seen in negative control group. Counterstaining of the cell nuclei was performed with Hoechst 33342 (magnification 20 ×). 1: Negative control group; 2: Positive control group; 3: Non-diabetic group; 4: Diabetic group

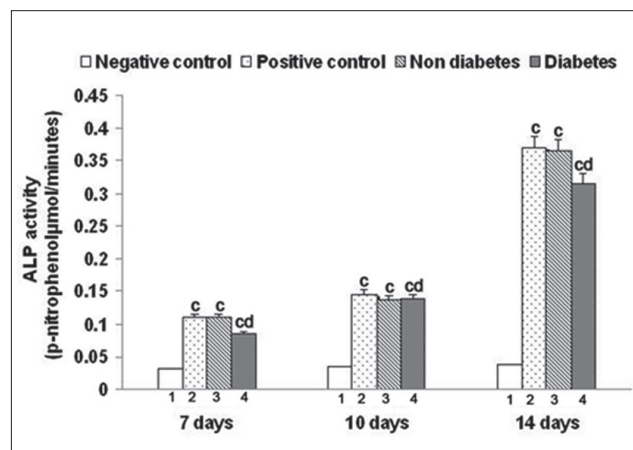


Fig. 4. Alkaline phosphatase activity in HUMSC co-cultured with osteoblasts. ALP activity was determined on day 7, 10 and 14. 1: Negative control group; 2: Positive control group; 3: Non-diabetic group; 4: Diabetic group; ^c $P < 0.01$ vs. negative control group ^d $P > 0.05$ vs. non-diabetic group.

DISCUSSION

HUMSCs have been shown to have a high proliferative potential, differentiate into an osteogenic phenotype¹⁵, and hold tremendous promise for tissue engineering and regenerative medicine¹⁶. As for delayed fracture healing in patients with diabetes, and chronic hyperglycemia that can modulate osteoblast gene expression¹⁷, whether diabetic osteoblasts have a promoting function in HUMSCs osteogenesis has not been studied. For this reason, we used non-diabetic and diabetic osteoblasts to induce the

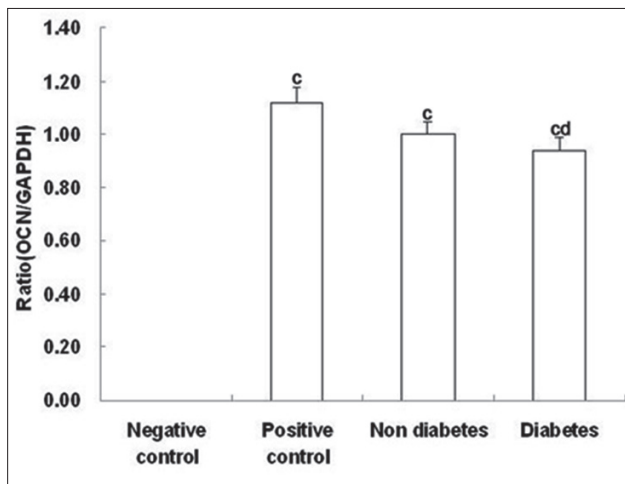


Fig. 5. OCN mRNA expression in HUMSC co-cultured with non-diabetic osteoblasts or diabetic osteoblasts for 21 days. There was no OCN mRNA expression in negative control group and taken as 0%. ^c $P < 0.01$ vs. control group ^d $P > 0.05$ vs. non-diabetic group. The experiment was performed three times.

HUMSCs to undergo osteogenic differentiation, and to assess the possible promoting function of diabetic osteoblasts on HUMSCs osteogenesis. Our results showed that diabetic osteoblasts promote HUMSCs proliferation and osteogenic differentiation.

In the present study, the isolated cells from non-diabetics and diabetics had the characteristics of osteoblasts, and were identical to those seen in primary osteoblasts cultures¹⁸. After 1-3 weeks, the isolated cells became positive for type I collagen expression, alkaline phosphatase and deposited mineralized matrix as shown with the Alizarin staining. The morphological appearance and cytochemical characteristics of the osteoblasts were identical to other studies characterizing osteoblasts histology¹⁹⁻²¹. The results testified that the isolated cells were osteoblasts.

Non-diabetic osteoblasts as well as diabetic osteoblasts can accelerate HUMSCs proliferation after co-culturing with isolated osteoblasts. These results are similar to the view that osteoblasts can promote MSC or hematopoietic progenitor cell proliferation²²⁻²³. Osteoblasts can also regulate osteogenic differentiation of MSCs in stimulating osteogenesis²⁴. Similar to this recent study, we found that osteoblasts co-cultured with HUMSC have a promoting function on HUMSC osteogenic differentiation. This was manifested by increased type I collagen protein, ALP activity and OCN mRNA expression.

The early time course of osteoblastic differentiation was characterized by early osteoblastic markers type I collagen and ALP. Type I collagen was strongly expressed on day 10. These results support the view that type I collagen is the main protein produced by osteoblasts at an early stage of maturation, and under appropriate conditions stem cells can be directed toward the osteoblasts lineage *in vitro*²⁵. ALP activity is known to have a close association with bone mineralization²⁶, its level commonly increases in the early period of osteoblastic differentiation and de-

creases in the mineralized period²⁷. In this study, ALP level gradually and significantly increased over the culture period. In the non-diabetic group and diabetic group, it was well expressed on day 7, reached a peak on day 14. Moreover, ALP activity was less detectable in negative control group which untreated with osteoblasts, further supporting that osteoblasts promote osteoblastic differentiation of the HUMSCs.

OCN is a specific bone marker at the late stage of bone formation²⁸. A recent study reported that co-cultures with more osteoblasts exhibited higher levels of osteogenic gene expression, such as OCN (ref.²⁹). Consistent with this result, our studies showed that OCN increased on day 21 in non-diabetic group and diabetic group with no significant difference between them, but there was no OCN expression in negative control group which was not treated with osteoblasts. The present study reveals that osteoblasts lead to an increase in osteogenic differentiation of HUMSCs toward its cell type, and this osteogenic differentiation maybe through cell-to-cell interaction and secrete type I collagen or OCN gene derived from osteoblasts.

CONCLUSION

Our data demonstrated that similar to the osteoblasts of non-diabetic patients, osteoblasts from diabetic patients also have a potential effect on promoting HUMSCs differentiation into osteoblasts.

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Author contributions: HCZ, CHY, CSF, WDW: study design; HCZ, WJ, LW, ZYX, WWH: data collection and analysis; HCZ, CHY, WLX, WDW, CSF: manuscript writing.

Conflict of interest statement: None declared.

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