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Original Article

Human umbilical cord mesenchymal stem cells pretreated with angiotensin-II facilitates angiogenesis by improving the function of endothelial cells

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Abstract: Mesenchymal stem cells (MSCs) pretreatment is an effective route for improving cell-based therapy of skin wounds. This study aimed to investigate angiotensin-II (Ang-II) pretreatment of human umbilical cord-MSCs (hUC-MSCs) to facilitate the therapeutic efficacy of hUC-MSCs in skin wounds. 5 cm^2 full-thickness excisional skin wounds were created on each side of the midline of the male pig. The wounds were treated with hUC-MSCs after 100 ng/ml Ang-II pretreatment (Ang-II-MSCs, 1 × 10^7 in 1 mL), hUC-MSCs (MSCs, 1 × 10^7 in 1 mL), or the control (essential medium 1 mL). Conditioned medium of hUC-MSCs (MSC-CM) or hUC-MSCs pretreated by 100 ng/ml Ang-II (Ang-II-MSC-CM) was also used to treat HUVECs or HUVECs transfectants (siRNA-control, siVEGFR2). The results showed that the rate of wound closure, vascular reconstruction, and the expression of CK19 and collagen arrangement were increased after Ang-II-MSCs transplantation. The apoptotic rate of HUVECs after Ang-II-MSC-CM administration was decreased, and their capacity of tube formation was enhanced. However, knockdown of VEGFR2 blocked the function of Ang-II-MSC-CM on preserving HUVECs from apoptosis, and the capacity of tube formation was also suppressed. The increase of Bcl-2 and alleviation of caspase 3 were observed in HUVECs or HUVECs transfectants exposure to Ang-II-MSC-CM. In summary, our results elucidated that the pretreatment of hUC-MSCs with Ang-II improved the outcome of MSC-based therapy for skin wounds via enhancing angiogenesis and ameliorating endothelial cell dysfunction in a VEGFR2 dependent manner.

Keywords: Angiotensin-II, hUC-MSCs, wound healing, angiogenesis, endothelial cells

Introduction

Wound healing is a very complicated pathophysiologic process, including cell migration, proliferation, angiogenesis and tissue remodeling [1]. Angiogenesis is an essential step in the wound healing, since the formation of new blood vessels ensures the delivery of nutrition and oxygen. The dysregulation of vascular growth is induced by inflammatory factors and results in damage of endothelial cells (ECs) [2]. The dysfunction of ECs decreased responsiveness to impaired neovascularization and endothelial regeneration in skin wounds [3]. Thus, a key strategy to restore blood flow serves essential role in the repair of dysfunctional ECs.

Abundant experimental evidences have recently demonstrated the therapeutic potentials of mesenchymal stem cells (MSCs) for the regeneration and repair of damaged tissue in almost all of the major organs, including the brain, eye, heart, lung, liver, kidney, and skin [4-10]. Therefore, BM-MSCs provide an attractive source of cells for tissue repair and regeneration. Previous studies have indicated the MSCs could repair endothelial dysfunction [11-13], played important roles during wound healing, thus via influencing neo-vascularization [14-17]. However, in some settings, the number of surviving MSCs is, in fact, too low to explain the significant functional improvements observed following cell engraftment [18]. And the viability of the transplanted MSCs is poor when exposed to harsh, proapoptotic microenvironments, such as the presence of excessive inflammatory stimuli, cytotoxic free radicals or oxidative stress. In order to increase the therapeutic

potentials of MSCs and enhance their ability to promote angiogenesis, the researchers made a lot of efforts. For instance, the transplantation of VEGF-1α and gene-transfected MSCs [19, 20]. To avoid the risk of genetic modification, another way to enhance stem cell survival, promoted ischemic tissue angiogenesis and repaired endothelial dysfunction of MSCs needs to further explore.

Researchers are attempting to use the "adaptive cytoprotection" to overcome this problem. The protection elicited by mild irritants is called "adaptive cytoprotection which has been put forward by the famous America digestion physiologist Robert in 1983." Many studies have found that the use of pretreated technology not only can increase the capacity of anti-apoptosis and proliferation, but also can strengthen the ability of hUC-MSC to accelerate the repair of damaged tissues [21-24]. Previous study from our group has showed repetitive exposure to LPS protects hUC-MSCs against the apoptotic consequences of subsequent endotoxin insults [25]. Other studies reveal that a suitable physiologic niche treatment of 5% O_2 for 48 h for BM-MSCs significantly increased the secretion of proangiogenesis factors and enhanced angiogenesis in ischemic tissue by ameliorating endothelial dysfunction for DLLI treatment [26].

A local RAS is expressed in many tissues [27-30]. The ischemia wound can activate the local RAS. In the ischemic tissues, the activation of RAS and elevated Ang-II induces cells injury. However, in vitro, it has been reported that Ang-II is a good trigger for pretreating BMSCs to enhance the paracrine release of growth factors (GFs). MSCs exposed to Ang-II can increase paracrine production of VEGF, prevent or attenuate ischemic tissue injury, lead to accelerating the wound healing [31, 32]. Therefore, it is significant to explore the effects of an appropriate physiological environment on the ability of MSCs to attenuate ischemic tissue injury and promote angiogenesis when transplanted into the skin wounds.

In our study, we identified that a suitable treatment of 100 ng/mL Ang-II for hUC-MSCs significantly enhanced angiogenesis by ameliorating endothelial dysfunction for the skin wounds treatment and Ang-II-induced cytoprotection is associated with VEGFR2.

Materials and methods

Cell culture

hUC-MSCs were isolated as previously described [33]. Umbilical cord tissues from three full-term healthy babies delivered by caesarean section at the First Affiliated Hospital of PLA General Hospital (Beijing, China), were cut into 1-mm^3 samples and thoroughly rinsed with phosphate-buffered saline (PBS), following removal of the umbilical vessels and external membrane. The tissues were placed in culture flasks (Corning, Tewksbury, MA, USA) at a distance of 0.5 cm with DMEM/F12, supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2. To ensure that fixation of the tissue, the medium was replaced every 3 days. The tissues were removed when the cells in the tissue samples reached 80-85% confluence. The cells were digested with trypsin-EDTA (Gibco Life Technologies) and transferred to T-75 culture flasks for propagation and culture. The 3^{th}-8^{th} cells were stored for use in subsequent studies. The protocol of the current study was approved by the ethics committee of the First Affiliated Hospital of PLA General Hospital (Beijing, China). HUVEC was purchased from China Center of Type Culture Collection (CCTCC) in Beijing and cultured in RPMI 1640 medium with 5% CO_2 at 37°C.

VEGFR2 knockdown

MISSION® VEGFR2 small interference RNA (Sigma-Aldrich, St. Louis, MO, USA) was purchased and transfected into HUVEC using lipofectamin 2000 reagent following the manufacturer's instructions. After 48 h, the transfectants were collected for further analysis.

Flow cytometry

hUC-MSCs were cultured in 6-well plates and treated with or without Ang-II (100 ng/ml, 500 ng/ml or 1000 ng/ml) for 24 h, 48 h, and 72 h, respectively. HUVEC or HUVEC transfectants were pretreated with 1000 ng/ml Ang-II for 24 h, and then the medium was changed with normal medium, MSCs-CM or Ang-II-MSC-CM for indicated times, respectively. After treatment, cells were harvested using trypsin-EDTA, washed with PBS, resuspended in PBS at 1 × 10^6/mL, and stained with Annexin V and prop-

idium iodide solution (PI; from the BD Apoptosis Detection kit) for 20 min. Analyses were performed by flow cytometry (FC500; Beckman Coulter, Brea, CA, USA) using FC500MPLCXP-2.1 software.

Pig model and hUC-MSCs transplantation

Male pigs (8 to 10 weeks old) with an average weight of 18 to 20 kg were used in this study. The pigs were purchased from the First Affiliated Hospital of PLA General Hospital and were housed in the animal center of the First Affiliated Hospital of PLA General Hospital. All animal experimental procedures were consistent with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the First Affiliated Hospital of PLA General Hospital. Hair was removed from the dorsal surface and the animals were anesthetized. Three 5 cm^2 full-thickness excisional skin wounds were created on each side of the midline. The skin wounds were divided randomly into the following groups: a blank sample, 1 mL DMEM injected into the wound at six injection as a negative control, MSCs (treatment with 1×10^7 hUC-MSCs, 1×10^7 in 1 mL DMEM injected into the wound at six injection), Ang-II-MSCs (treatment with 1×10^7 Ang-II-MSC, 1×10^7 in 1 mL DMEM injected into the wound at six injection). The wounds incision was closed with acellular pig skin, and all skin wounds were closely monitored during the postoperative period.

Evaluation of wound closure

Wound areas were measured photographically every week after hUC-MSCs transplantation, A less than 1% residual wound area was considered as complete wound healing. The percentage of wound closure was calculated as follows:

[(Area of original wound-area of actual wound)/area of original wound] × 100. Quantitative measurements of the wound area were assessed using Image Pro Plus 5.1 image analysis software (Media Cybernetics, Silver Spring, MD).

Histochemistry and immunohistochemistry

Histological analysis of the skin was performed by taking 1 cm^2 biopsy punches from areas of interest (including the wound and the surrounding skin) at indicated times after the different treatments.

Freshly harvested samples were fixed and embedded for histochemical analysis. The sections were stained with hematoxylin and eosin. To analyze the mechanism through which the injected Ang-II-MSCs promoted angiogenesis, serial frozen section were tested with factor VIII. For Masson's trichrome staining, the tissue sections were deparaffinized and rehydrated, and were then stained according to the Masson's Trichrome Staining Kit (Yike Biotech, Guangzhou, China) instructions. The ESCs in the epidermis was located by CK19.

Tube formation assay

To evaluate the angiogenesis of HUVECs under different treatment. The tube formation assay of HUVEC or HUVEC transfectants after indicated treatment was performed. Matrigel (BD Biosciences Pharminger, San Diego, USA) was added to 96-wellplates in a volume of 50 μL per well. Then incubating the plates at 37°C for 30 min to form a gel layer [34]. After gel formation, 2.5×10^4 cells of HUVECs, shRNA-VEGFR2 HUVECs, and shRNA-control HUVECs were applied to each well, along with the addition of 150 μL of supernatant from different treatment conditions including control (fresh DMEM), MSC supernatant, and Ang II pretreated-MSC supernatant. The plates were then incubated at 37°C for 12 h. After incubation, tube formation was examined under an inverted phase-contrast microscope.

Statistical analysis

All results are presented as mean ± SD. Statistics were performed using GraphPad Prism 5 Software. The data were collected from at least three independent experiments. A value of $P < 0.05$ was considered to be statistically significant.

Results

Effect of Ang-II on apoptosis of hUC-MSCs

In order to elucidate the effect of different doses of Ang-II on apoptosis of hUC-MSCs, hUC-MSCs were treated by 0 ng/mL (control group), 100 ng/mL (100 ng/mL group), 500 ng/mL (500 ng/mL group) and 1000 ng/mL

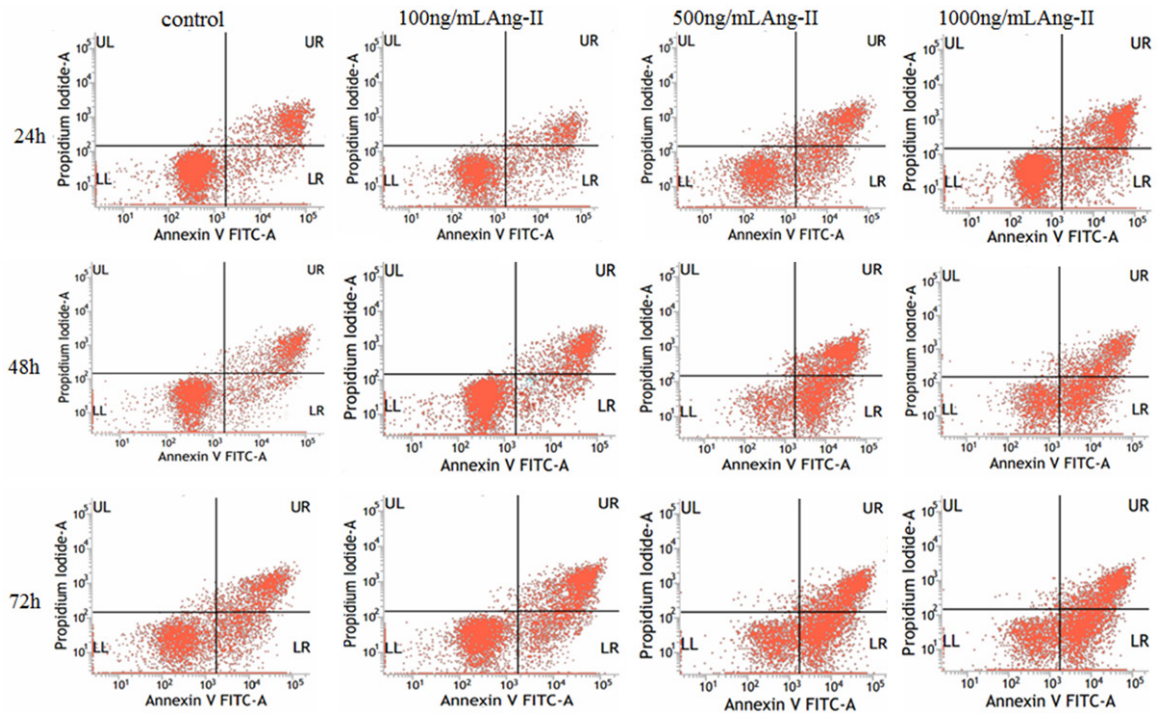

Figure 1. Effect of Ang-II on apoptosis of hUC-MSCs. hUC-MSCs were treated with 0, 100, 500, 1000 ng/mL Ang-II for 24, 48, 72 h. The cells were stained with Annexin V/ PI and apoptosis levels were determined using flow cytometry.

(2000 ng/mL group) Ang-II for indicated times, and cell apoptosis was analyzed using flow cytometry assay. After 48 h treatment, the apoptosis rate of hUC-MSCs in the control group, 100 ng/mL group, 500 ng/mL group and 1000 ng/mL group was 10.48%, 10.88%, 21.8%, 24.67%, respectively (**Figure 1**). However, at 72 h, compared with that in the control group (14.53%) and 100 ng/mL group (14.86%), the apoptotic rate of hUC-MSCs in 500 ng/mL (30.81%) and 1000 ng/mL (35.71%) groups was significantly increased (**Figure 1**). The results indicated that 1000 ng/mL Ang-II treatment promoted the apoptosis of hUC-MSCs.

Ang-II-MSCs exhibited better therapeutic effects in wound healing

Previous study showed that low dose Ang-II treatment enhanced the function of MSCs on angiogenesis, which resulted in promoting wound healing [32, 35, 36]. In this study, 5-cm^2 full-thickness excisional skin wounds of pig was done, and essential medium (control), hUC-MSCs (MSCs group), and hUC-MSCs pretreated by 100 ng/mL Ang-II (Ang-II-MSCs) were transplanted to the wounds, respectively. Wound closure percentages of the three groups at different times are shown in **Figure 2A**. The percentage of wound closure in the groups Ang-II-MSCs treated group was significantly greater than other at different days (**Figure 2B**), suggesting that Ang-II-MSCs accelerated wound healing. H&E staining was used to assess the features of the wounds. And we found more sle features in the granular and spinous layers and displayed a flatter epithelium with a more clearly developed stratum corneum in the Ang-II-MSCs group compared with that in other groups (**Figure 2C**). In addition, the result of factor-VIII showed the number of new blood vessel in the cutaneous wounds of the Ang-II-MSCs group was significantly increased than that in the other two groups (**Figure 2D**). The results of Masson's trichrome staining showed that Ang-II-MSCs treated wounds displayed an arranged collagen network and smooth collagen deposition (**Figure 2E**), both of which were absent in the other groups, suggesting that Ang-II-MSCs treated wounds had more mature collagen development than that in the other groups. CK19 was expressed in both epidermis and skin appendages. The expression of CK19 in the Ang-II-MSCs group was much higher than that in other two groups (**Figure 2F**), suggesting that Ang-II-MSCs have better curative effect.

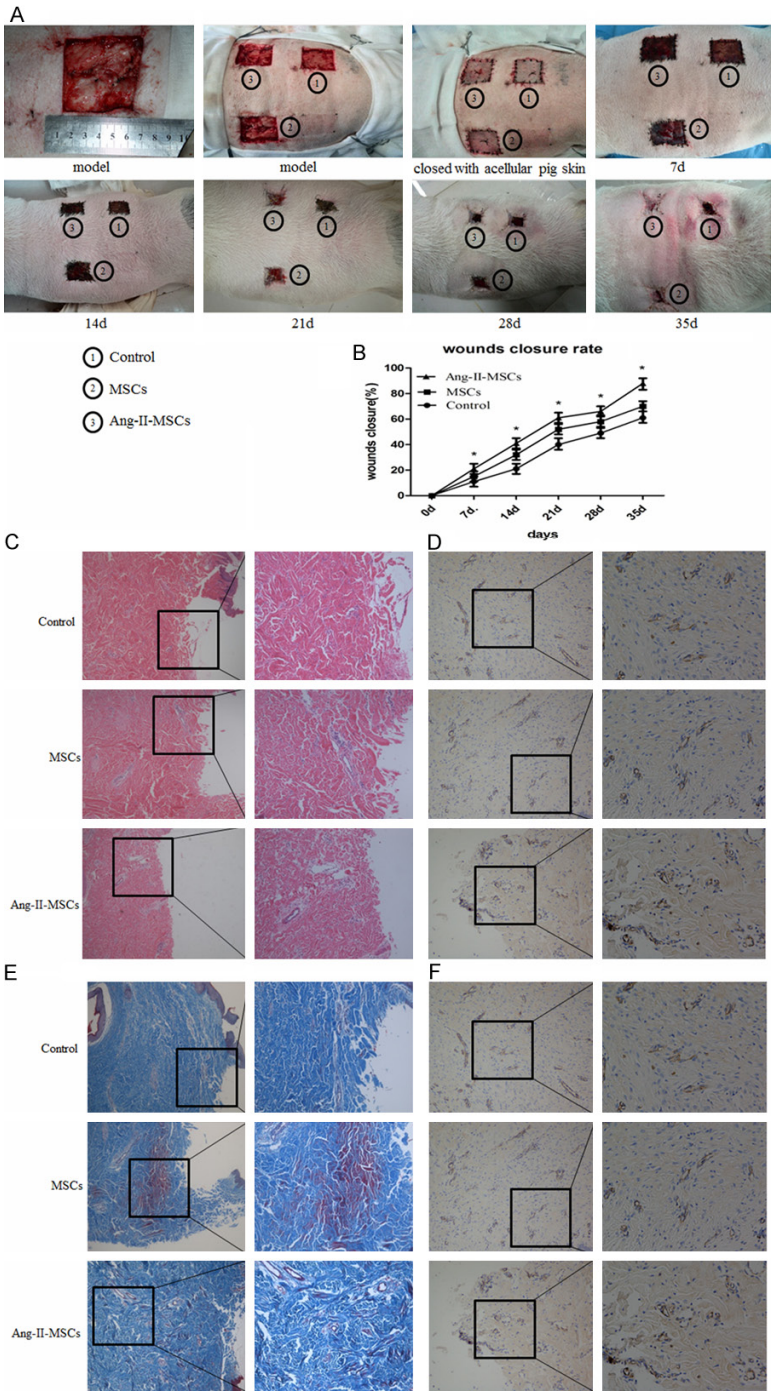

Figure 2. Ang-II pretreatment of hUC-MSCs promote wound closure in vivo. After pig model of 5cm^2 full-thickness excisional skin wounds was established, and wounds were closed with acellular pig skin after transplanting essential medium (control), MSCs or Ang-II-MSCs, respectively. (A) The images were taken at indicated times. (B) The wound closure rate was measured, Ang-II pretreatment of hUC-MSCs (Ang-II-MSC) treated wounds healed faster compared with those in the other groups, *P < 0.05. HE staining (C), factor VIII staining (D), Masson's trichrome staining (E), and immunohistochemical analysis for CK19 (F) of tissues from three groups at day 21 were done, respectively.

Ang-II-MSCs conditional medium treatment improved the function of HUVECs

Increasing the anti-apoptotic function of ECs is very important for angiogenesis. In order to evaluate the paracrine effect of hUC-MSCs on ECs, we observed the effects of essential medium (control), the hUC-MSCs (MSCs-CM) or Ang-II-MSCs (Ang-II-MSCs-CM) on biological characteristics of HUVECs in vitro. We investigated the effects of CM on HUVECs apoptosis by flow cytometry and Western blot. The results confirmed that HUVECs exposed to Ang-II-MSCs-CM produced little cytotoxicity in HUVECs (**Figure 3**), indicated that Ang-II-MSCs-CM significantly increased the capacity of anti-apoptosis on HUVECs in comparison with that in the other two groups. Furthermore, HUVECs were seeded onto a Matrigel matrix and incubated with control medium, MSCs-CM or Ang-II-MSCs-CM for 12 h, the results indicated that HUVECs cultured with Ang-II-MSCs-CM dramatically formed more tube-like structures (**Figure 4**).

VEGFR2 knockdown in HUVECs resulted in cell apoptosis

Vascular endothelial growth factor (VEGF) served critical roles in regulating angiogenesis, which could bind with its receptor (VEGFR) and initial related signal transduction (reference). We used small interference RNA specific to VEGFR2 (siVEGFR2) to block the function of VEGF/VEGFR2 signaling pathway of HUVECs,

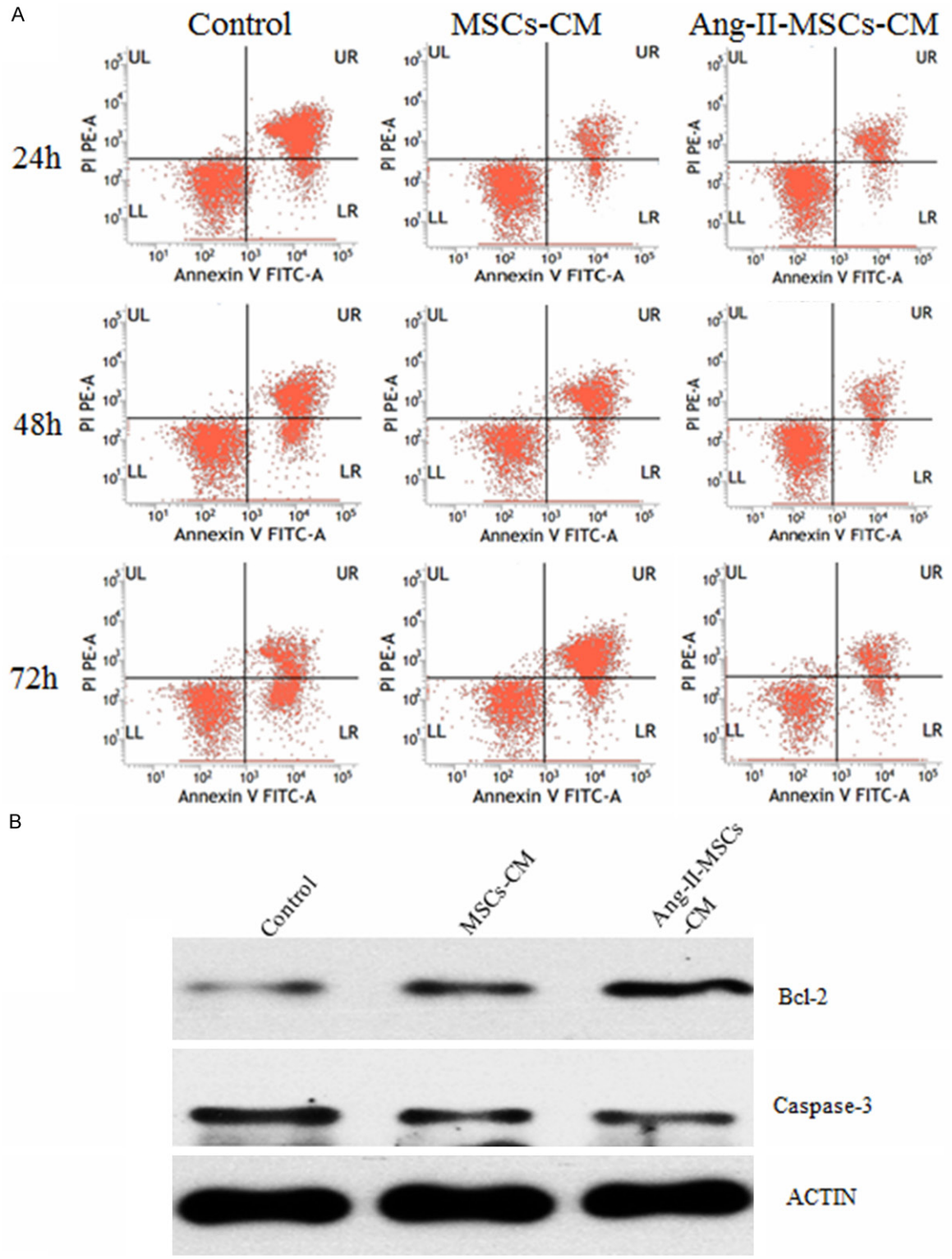

Figure 3. CM from Ang-II-MSCs improved the anti-apoptotic capacity of ECs. HUVECs were first treated with 1000 ng/ml Ang-II for 24 h, then the medium was changed with essential medium (control), conditioned medium of MSCs (MSC-CM), and conditioned medium of Ang-II treated MSCs (Ang-II-MSC-CM), respectively. After treatment with control, MSC-CM, Ang-II-MSC-CM for the indicated times. A. The cells were stained with Annexin V/ PI and apoptosis levels were determined using flow cytometry. B. Cell apoptosis related proteins, caspase 3 and Bcl-2 were detected using Western blot.

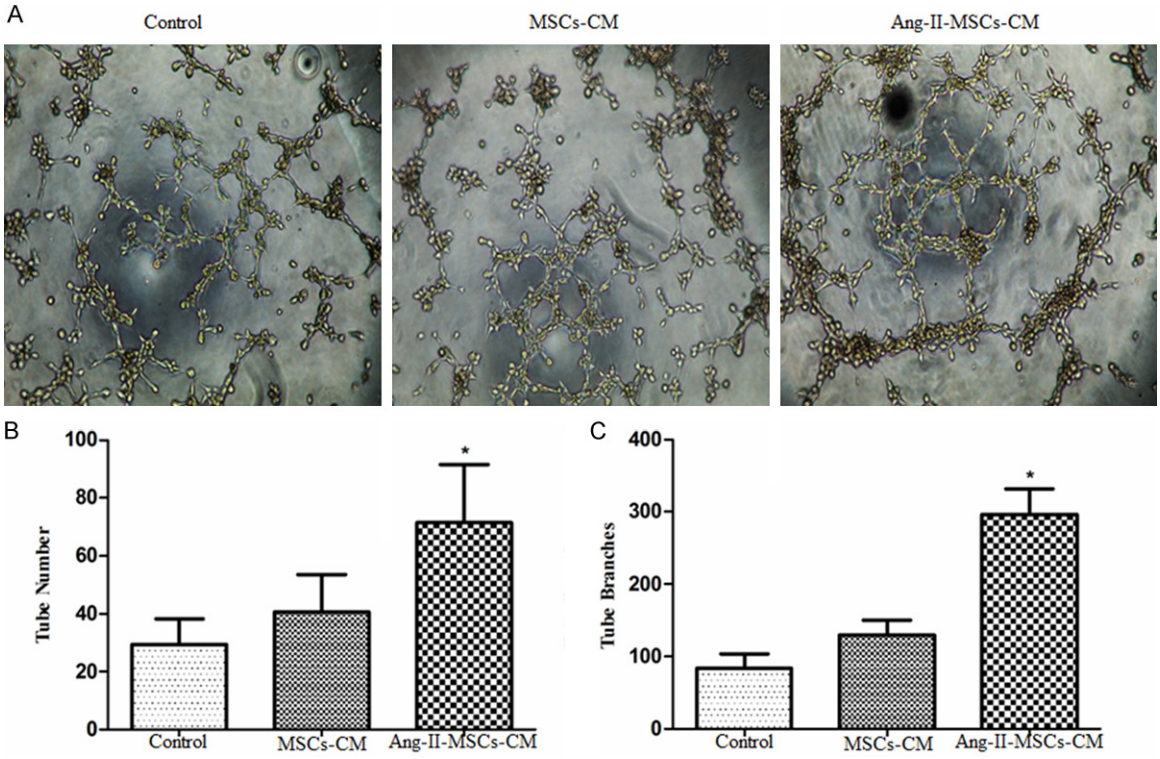

Figure 4. Ang-II-MSC-CM increased the ability for tubular formation of ECs. HUVECs were cultured in Matrigel and treated with essential medium (control), MSC-CM, Ang-II-MSC-CM for 12 h. (A) Representative photographs of HUVECs formed tube-like structures were taken. Statistical analysis for the number of tube formations (B) and branches (C) of HUVECs was done. At least five wells were viewed, and experiments were repeated for three times. All values were expressed as mean ± SEM, *P < 0.05 (n = 5 in each group).

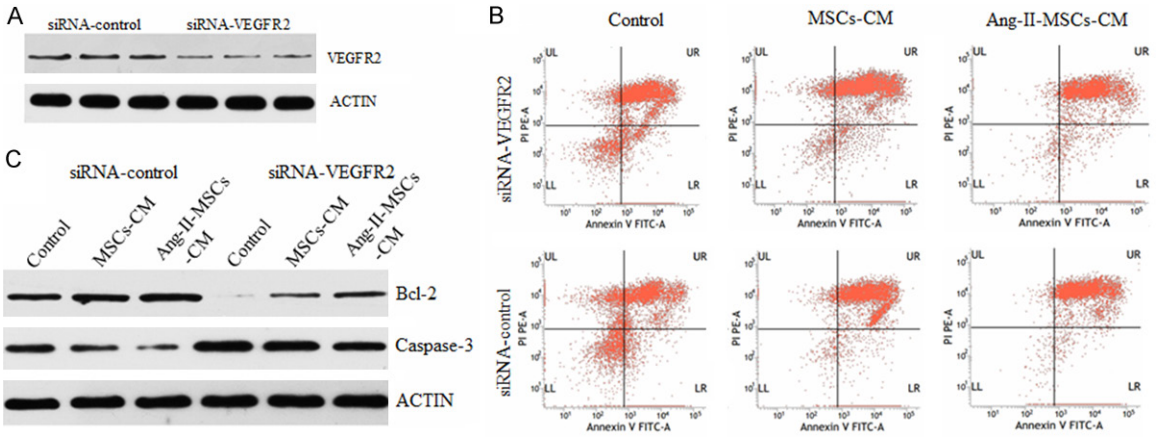

Figure 5. Effect of VEGFR2 on apoptosis of HUVECs. HUVECs were transfected with siRNA-control or SiVEGFR2, respectively. A. After 24 h transfection, the expression of VEGFR2 was measured using western blot. B. The apoptotic percentage of HUVECs transfectants was detected using flow cytometry after indicated treatment. C. And apoptosis related proteins, capsase 3 and Bcl-2, were detected by Western blot.

the expression of VEGFR2 was successfully silenced in HUVECs after transfection (**Figure 5A**). The result of flow cytometry showed the more apoptotic cells in the siVEGFR2 group was measured compared with that in Control group (**Figure 5B**). However, the anti-apoptotic function of Ang-II-MSCs-CM on HUVECs was disappeared after VEGFR2 knockdown (**Figure**

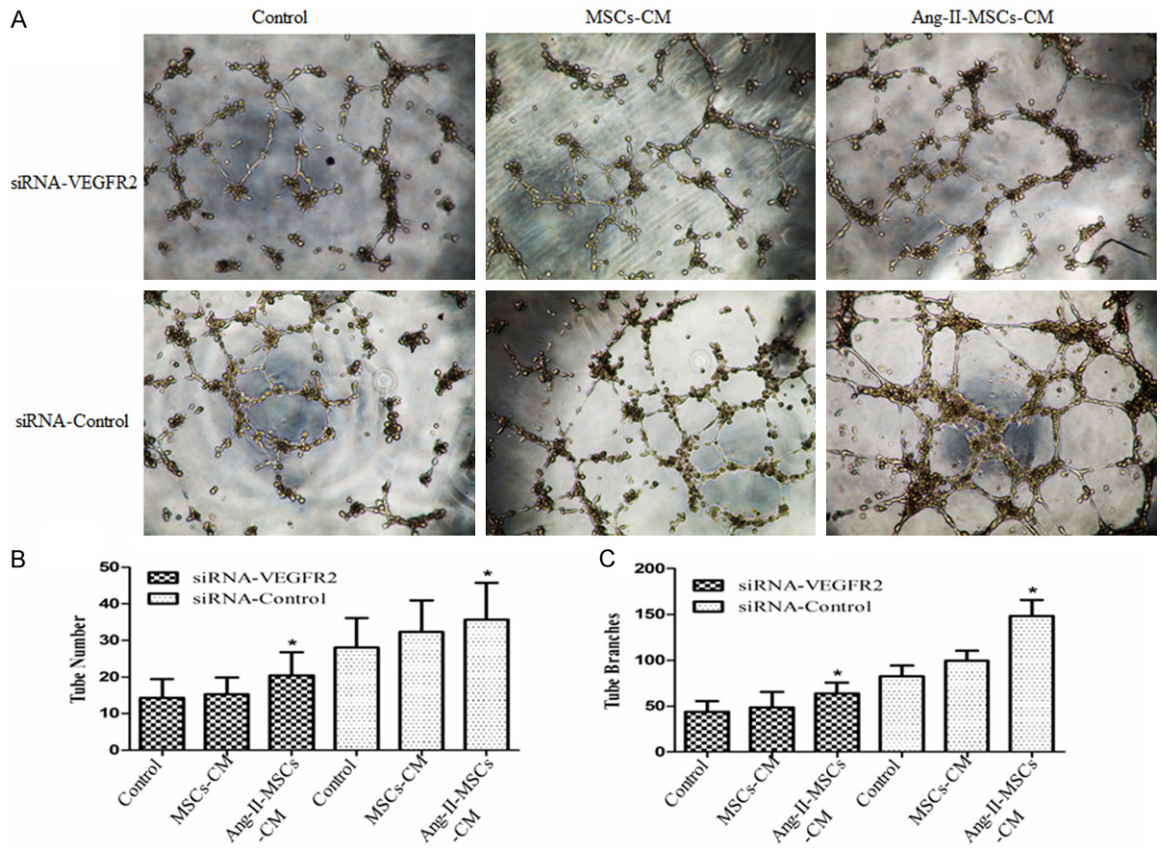

Figure 6. Effect of VEGFR2 on tubular formation of HUVECs. siRNA-control HUVECs and siRNA-VEGFR2 HUVECs were cultured in Matrigel and treated with essential medium (control), MSC-CM, Ang-II-MSC-CM for 12 h. (A) Representative photographs of HUVECs formed tube-like structures were taken. Statistical analysis of the number of tube formations (B) and branches (C) was done. At least five wells were viewed, and experiments were repeated for three times. All values were expressed as mean ± SEM, $^{**}P < 0.01$, $^{*}P < 0.05$ (n = 5 in each group).

5B). After indicated treatment, the expression of apoptosis related proteins was detected using Western blot assay. The expression of caspase-3 was upregulated and the expression of Bcl-2 was downregulated in the siVEGFR2 group compared with those in control group (**Figure 5C**), it meant that VEGFR2 knockdown might lead to promote HUVECs apoptosis.

Tube formation of HUVECS was in a VEGFR2 dependent manner

To further evaluate the angiogenesis ability of HUVECs knockdown VEGFR2, tube formation assay was performed in HUVECs of siVEGFR2 and control groups. We found that the number of tube in group of siVEGFR2 was decreased significantly compared with that in control group (**Figure 6A**). However, after Ang-II-MSCs-CM treatment, more tube-like structures were observed in control group in comparison with that in siVEGFR2 group (**Figure 6B** and **6C**). It suggested that tube-like structure formation of HUVECs was in a VEGFR2 dependent manner.

Discussion

Optimal wound healing requires a well-orchestrated integration of numerous molecular and cellular events that are mediated by cytokines, growth factors, and chemokines. the blood supply is the key to enhance normal wound healing. Several studies have demonstrated that MSCs secreted some nutrition factors such as VEGF, basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF), which functioned in promoting neovascularization of injured tissues [37-40]. In this study, we confirmed that Ang-II-MSCs-CM improved the anti-apoptosis capacity of endothelial cells and increased angiogenesis. Ang-II-MSCs transplantation markedly enhanced angiogenesis and wound healing.

Currently, Endothelial dysfunction can be broadly defined as an imbalance between vasodilating and vasoconstricting substances [41]. Damaged tissue generates a large number of inflammatory factors due to the normal human body reaction [42]. Over time, the excess levels of inflammatory factors in the bloodstream can lead to the impaired function of the vessel endothelial cells [43]. However, owing to endothelial dysfunction, the de novo endothelial cells cannot expand, leading to difficulties in neovascularization or angiogenesis, which primarily refers to the developmental formation of vascular structures. A previous study confirmed that MSCs ameliorate wounds by promoting angiogenesis of ischemic tissue via improving the function of endothelial cells [44, 45]. In our study, we proved that conditioned medium from Ang-II-MSCs dramatically enhanced the anti-apoptotic activity of endothelial cells exposure to 1000 ng/mL Ang-II (**Figure 3**), which contributed to the improvement of angiogenesis in skin wounds.

With the development of technology related to the treatment of skin wounds, stem cell transplantation is performed to enhance therapeutic efficacy. For instance, MSC transplantation improves wound healing after tissue damage in both pre-clinical and clinical studies [46, 47]. However, the therapeutic benefit of MSCs remains to be improved since the viability of the transplanted MSCs is poor [48]. Thus, many efforts, including genetic modification [19] and pretreatment [49], have been made to improve the other functions of MSCs, such as paracrine action, which has recently been considered a major mechanism of stem cell based therapy.

VEGF is one of the most important players that regulates vessel formation during embryonic development, wound healing and maintaining vessel homeostasis in adult organisms [50]. Its enhancement is the critical therapeutic strategy of tissue ischemia. In endothelial cells, binding of VEGF to the VEGFR2 activates multiple signaling pathways that trigger process of angiogenesis. We showed that the VEGFR2 mediated VEGF signaling and promoted angiogenesis. Knockdown of VEGFR2 impaired tube-like formation of HUVECs, this demonstrates that VEGFR2 plays an important role in the anti-apoptosis of HUVEC and significantly contribute to angiogenesis.

In conclusion, the present results show that ECs and VEGFR2 are involved in Ang-II induced hUC-MSCs promoting wound healing. The cytoprotection of Ang-II-MSCs on HUVECs might be mediated by VEGFR2, which leads to enhance cell proliferation, anti-apoptosis, and tube formation.

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Disclosure of conflict of interest

None.

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