STEM CELLS

REGENERATIVE MEDICINE

HucMSC-Exosome Mediated-Wnt4 Signaling Is Required for Cutaneous Wound Healing

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ABSTRACT

Mesenchymal stem cell-derived exosomes (MSC-Ex) play important roles in tissue injury repair, however, the roles of MSC-Ex in skin damage repair and its mechanisms are largely unknown. Herein, we examined the benefit of human umbilical cord MSC-derived exosome (hucMSC-Ex) in cutaneous wound healing using a rat skin burn model. We found that hucMSC-Ex-treated wounds exhibited significantly accelerated re-epithelialization, with increased expression of CK19, PCNA, collagen I (compared to collagen III) in vivo. HucMSC-Ex promoted proliferation and inhibited apoptosis of skin cells after heat-stress in vitro. We also discovered that Wnt4 was contained in hucMSC-Ex, and hucMSC-Ex-derived Wnt4 promoted β-catenin nuclear translocation and activity to enhance proliferation and migration of skin cells, which could be reversed by β-catenin inhibitor ICG001. *In vivo* studies confirmed that the activation of Wnt/β-catenin by hucMSC-Ex played a key role in wound re-epithelialization and cell proliferation. Furthermore, knockdown of Wnt4 in hucMSC-Ex abrogated β-catenin activation and skin cell proliferation and migration in vitro. The in vivo therapeutic effects were also inhibited when the expression of Wnt4 in hucMSC-Ex was interfered. In addition, the activation of AKT pathway by hucMSC-Ex was associated with the reduction of heat stress-induced apoptosis in rat skin burn model. Collectively, our findings indicate that exosome-delivered Wnt4 provides new aspects for the therapeutic strategy of MSCs in cutaneous wound healing. STEM CELLS *2015;33:2158–2168*

INTRODUCTION

Cutaneous wound requires a well-orchestrated integration of the differentiation, migration, proliferation, and apoptosis of skin cells to create the multilayered tissue that constitutes the skin [1–3]. Skin burn is very common and has a consistently high rate of mortality and morbidity [4], which not only destroy the barrier function of the skin but also alter the perceptions of pain, temperature and touch [5]. Thus, there is an urgent need to find an alternative approach to prompt wound healing.

Mesenchymal stem cells (MSCs) have a significant promise for regenerative medicine due to convenient isolation, low immunogenicity, the ability to transdifferentiate, and create a favorable environment for tissue regeneration [6, 7]. Human umbilical cord MSCs (HucMSCs) are inexhaustible and can be harvested at a low cost without an invasive procedure and they are promising cells in the formation of tissue regenerative during inflammation and tissue injuries [8, 9]. We have previously found that hucMSCs can ameliorate acute kidney injury and mouse hepatic injury [10–13]. Interestingly, Bruno et al. confirmed that microvesicles derived from MSCs could protect against acute renal tubular injury [14, 15]. Similarly, MSC-derived exosomes (MSC-Ex) have been proved to contribute to neurite outgrowth [16], which has been described as a new mechanism of cell-to-cell communication [17]. Recently, we also found that hucMSC-Ex alleviated liver fibrosis and promoted renal injury repair [18, 19]. Therefore, it is suggested that MSC-Ex might be a main mechanism of MSCs effects on wound healing. Since exosomes delivered components are complicated, exosomes-mediated signaling might have more differences in various injury models. Wnt/β-catenin signaling plays an important role in skin development [3, 20–22] and wound healing [23]. The active Wnt proteins can be delivered by exosomes and affect gene expression of target cells [24, 25]. However, whether hucMSC-Ex-mediated delivery of Wnts and its function in wound healing are not clear.

In this study, we investigated the role of hucMSC-Ex in wound healing using deep second-degree burn injury. HucMSC-Ex-derived Wnt4 protein activates β-catenin signaling in skin cells and promotes their proliferation and migration, leading to the enhancement of wound healing. Moreover, our findings indicate that heat stress inhibits AKT signaling in skin cells, while treatment with hucMSC-Ex reverses this inhibition and improves the survival of skin cells.

MATERIALS AND METHODS

The study was approved by the ethical committee of Jiangsu University (2012258).

Cell Culture

HucMSCs were isolated and identified as previously described [26]. HucMSCs and human lung fibroblasts (HFL1) were cultured in serum-free Dulbecco's modified Eagle's medium (DMEM) medium (Life Technologies, Carlsbad, CA). Keratinocytes HaCAT cells were purchased from ATCC and maintained in DMEM containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NE) at 37°C with 5% CO_2. Dermal fibroblasts (DFL) were isolated and sorted from rat back skin as previously described [27, 28] and cultured in DMEM containing 10% FBS at 37°C with 5% CO_2.

Isolation and Characterization of Exosomes

Exosomes were extracted and purified as previously described [18, 19]. Cell supernatants were centrifuged to remove cell debris and then passed through a 0.22-µm filter. Final exosomes were obtained and stored at −70°C. The protein content, as the quantification of exosomes, was determined using a BCA protein assay kit (CWBIO, Beijing, China). The final concentration of exosomes used for treating skin cells in vitro was 160 µg/ml and total 200 µg exosomes were applied to treat each animal. The morphology of the extracted exosomes was observed using transmission electron microscopy (FEI Tecnai 12, Philips, The Netherlands). Size distribution within exosome preparations was analyzed by measuring the rate of Brownian motion using a NanoSight LM10 system which is equipped with a fast video capture and particle-tracking software (NanoSight, Amesbury, U.K.). The CD9, CD63, and CD81 molecules which frequently located on the surface of exosomes were analyzed using Western blot.

Rat Skin Wound Model and Treatment

Adult female Sprague-Dawley rats (weighing 220 ± 20 g) were purchased from the Animal Centre of Chinese Academy of Sciences (Shanghai, China). Rat model of skin deep second-degree burn wound was established as described previously with added modifications [29]. Rats were anesthetized with sodium thiopental at a dose of 40 mg/kg b.wt. After the hair on their upper back was shaved, the back skin of rats was injured with 80°C water for 8 seconds to create a 16 mm diameter wound, then covered with gauze soaking saline for 6 minutes on the wound. Meanwhile, 1×10^6 cells (hucMSC and HFL1) suspended in 200 µl phosphatic buffer solution (PBS), 200 µg exosome (hucMSC-Ex and HFL1-Ex) suspended in 200 µl PBS, or 200 µl PBS were injected subcutaneously at three sites. The normal group had no treatment. The animals were housed individually. At 1 week and 2 weeks after treatment, the rats were sacrificed and the wound area was cut for further analysis.

H&E Staining

The wound skin and surrounding skin (4 mm^2) were fixed in 4% paraformaldehyde (pH 7.4), and gradually dehydrated, embedded in paraffin, cut into 4-µm sections, and stained with H&E stain for light microscopy. Each slide was given a histological score ranging from 1 to 10 according to the following parameters [30]. The criteria used for histological scores of wound healing are summarized in Supporting Information Table S1.

Wnt Reporter Activity Assay

For the luciferase reporter assay, HEK293T cells were cotransfected with TOP-Flash or FOP-Flash luciferase reporter. Transfection efficiency was normalized by cotransfection with a β-actin-Renilla reporter containing a Renilla luciferase gene under the control of a human β-actin promoter. The activities of firefly luciferase and Renilla luciferase were quantified using the dual-luciferase reporter assay system (Promega, Madison, Wisconsin). For quantification of Wnt activity, after transfection for 6 hours, HEK293T cells were treated with equal amounts of exosomes or PBS from different samples. Wnt reporter activity was determined by TOP/FOP luciferase.

Lentiviral Knockdown of Wnt4 in hucMSCs

The lentiviral expression vector containing the Wnt4 shRNA sequence (Sigma, Saint Louis, Missouri) was selected for specifically targeting Wnt4 silence, which was classified as Lenti-wnt4-shRNA, and Lenti-GFP-shRNA as negative control vector. The Wnt4 shRNA lentivirus vectors were generated by ligating the vector Tet-pLKO-puro. Wnt4 shRNA oligonucleotide sequences are: Forward, 5′-CCGGCCCAAGAGATACTGGTTGTATCTCGAGATACAACCAGTATCTCTTGGGTTTTTG-3′; Reverse, 5′-AATTCAAAAACCCAAGAGATACTGGTTGTATCTCGAGATACAACCAGTATCTCTTGGG-3′. The sequences of control shRNA are: Forward, 5-CCGGGCAAGCTGACCCTGAAGTTCATCTCGAGATGAACTTCAGGGTCACGTTGCTTTTTG-3′ Reverse, 5′-AATTCAAAAAGCAAGCTGACCCTGAAGTTCATCTCGAGATGAACTTCAGGGTCACGTTGC-3′. The recombinant lentivirus was produced by cotransfecting HEK293T cells with PLKO-GFP-shRNA or PLKO-Wnt4-shRNA, PU1562, and PU1563 plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The virus-containing supernatant was harvested at 48 hours and 72 hours post-transfection. HucMSCs were transduced with the prepared lentivirus (Lenti-wnt4-shRNA or Lenti-GFP-shRNA). Stable cell lines were obtained after selection with 1 µg/ml of puromycin (Sigma, St. Louis, MO) for 15 days. The expression of shRNA was induced by addition of 80 µg/ml doxycycline for 2 days. The efficiency of wnt4 knockdown was evaluated using real-time quantitative RT-PCR and Western blot. The stable cell lines were cultured in serum-free medium for 48 hours, then the supernatants were collected and exosomes isolated for further study.

TUNEL Assay

The apoptotic skin cells in tissue slides were measured using an in situ cell apoptosis detection kit according to the manufacturer's instruction (Boster, Wuhan, China). The number of positive cells was calculated in three or more random fields.

Statistical Analysis

All data were shown as means ± SD. The statistically significant differences between groups were assessed by analysis of variance or *t* test using Prism software (GraphPad, San Diego, CA). *p* value $<.05$ was considered significant.

RESULTS

HucMSC-Ex Promotes Cell Proliferation and Re-epithelialization in Rat Deep Second-Degree Burn Injury Model

To investigate the roles of hucMSC-Ex in wound healing, hucMSC-Ex was first extracted and identified as previously described [18, 19]. The morphology of hucMSC-Ex was observed under transmission electron microscopy, and its size was measured using NanoSight analysis. The results showed that hucMSC-Ex was about 100 nm spherical vesicles (Supporting Information Fig. S1A, S1B). The results of Western blot showed that exosomal markers, including CD63, CD81, and CD9, were expressed in both hucMSC-Ex and control exosomes from human lung fibroblasts HFL1 (HFL1-Ex) (Supporting Information Fig. S1C).

We established a rat deep second-degree burn injury model and infused exosomes and their derived cells into the injured rats, separately. The results of histological evaluation of wounds at 1 week postinfusion showed that the number of epidermal and dermal cells significantly increased in hucMSCs or hucMSC-Ex-treated wounds (Fig. 1A), while wounds that were treated with PBS, HFL1 cells, or HFL1-Ex were still in second-degree burn injury state (Fig. 1A). The results of PCNA immunochemical staining showed that hucMSCs and hucMSC-Ex groups had more PCNA-positive cells in wound area than that in PBS, HFL1, and HFL1-Ex groups at both 1 and 2 weeks postinfusion (Fig. 1B).

Strikingly, at 2 weeks postinfusion, HE staining results showed that wounds treated with hucMSCs or hucMSC-Ex significantly enhanced re-epithelialization (complete re-epithelialization in all 6 wounds; $n = 6$) compared to the PBS group (complete re-epithelialization in one of six wounds; $n = 6$) or HFL1 group (complete re-epithelialization in two of six wounds; $n = 6$) or HFL1-Ex (complete re-epithelialization in two of six wounds; $n = 6$) (Fig. 1A). In consistent with the above results, the histological scores were significantly higher in hucMSC and hucMSC-Ex groups than that in PBS, HFL1, and HFL1-Ex groups (Fig. 1C). We determined the relative expression of collagen I to collagen III using quantitative RT-PCR and found that the ratio of collagen I to collagen III was higher in hucMSC and hucMSC-Ex groups than that in PBS, HFL1, and HFL1-Ex groups, suggesting that hucMSCs and hucMSC-Ex are able to reduce the formation of scar in wound area (Fig. 1D).

To confirm the promoting role of hucMSC-Ex in re-epithelialization, we determined the expression of CK19, a epithelial marker, using immunofluorescent staining. The results revealed that CK19 expression was remarkably higher in hucMSC and hucMSC-Ex groups at 1 week postinfusion than that in PBS, HFL1, and HFL1-Ex groups (Fig. 1E). At 2 weeks postinfusion, there formed a complete epidermal structure in the CK19-positive area of the wounds in hucMSC and hucMSC-Ex groups but not in PBS, HFL1, and HFL1-Ex groups (Fig. 1E). The increased levels of CK19 and PCNA in hucMSC and hucMSC-Ex groups were further confirmed using Western blot (Fig. 1F). Taken together, these results indicate that hucMSC-Ex prompts the same repair of skin second-degree burn injury as hucMSCs by enhancing proliferation of skin cells and re-epithelialization in wound area.

HucMSC-Ex Inhibits Heat Stress-Induced Apoptosis and Enhances Proliferation of Skin Cells In Vitro

In order to verify the above results and explore the mechanisms for hucMSC-Ex-induced repair, we treated the immortal human keratinocytes HaCAT and primary cultured DFL at 43°C for 40 minutes to mimic burn injury model in vivo. In comparison to PBS group, hucMSC-Ex significantly inhibited heat stress-induced apoptosis in HaCAT while HFL1-Ex had minimal effect. The similar effects were also observed in primary DFL (Fig. 2A). The results of cell proliferation assay showed that treatment with hucMSC-Ex but not HFL1-Ex promoted the proliferation of HaCAT and DFL cells in a time-dependent manner after heat stress (Fig. 2B, 2C). The expression of PCNA was enhanced by hucMSC-Ex but not HFL1-Ex in HaCAT and DFL cells (Fig. 2D, 2E). Western blot analyses of apoptosis-associated proteins in HaCAT and DFL cells showed that Bax level was lower in hucMSC-Ex group than that in PBS and HFL1-Ex groups, while the expression of Bcl-2 was higher in hucMSC-ex group than that in PBS and HFL1-Ex groups (Fig. 2D, 2E). These results suggest that hucMSC-Ex inhibits heat stress-induced apoptosis in skin cells and prompts their proliferation.

HucMSC-Ex Activates Wnt/β-Catenin Signaling to Prompt Wound Healing

Considering the significant role of β-catenin signaling in skin development [3, 20–22] and cutaneous wound healing [23], we hypothesized that wnt/β-catenin signaling might be involved in the biological effects of hucMSC-Ex on wound healing. We found that hucMSC-Ex enhanced TOP-flash reporter activity in 293T cells, while HFL1-Ex had no effect (Fig. 3A). HucMSC-Ex treatment induced more nuclear translocation of β-catenin in HaCAT and DFL cells than PBS and HFL1-Ex (Fig. 3B and Supporting Information Fig. S2A). The expression of β-catenin and its downstream genes (cyclin-D1, cyclin-D3, and N-cadherin) in DFL cells were significantly increased by hucMSC-Ex but not PBS and HFL1-Ex (Supporting Information Fig. S2B). The induction of β-catenin downstream genes (cyclin-D1, cyclin-D3, and N-cadherin) and the increase of TOP-flash reporter activity by hucMSC-Ex were almost completely abrogated by ICG001, which selectively inhibits β-catenin/CBP interaction (Fig. 3C, 3D), suggesting the specific activation of β-catenin signaling by hucMSC-Ex.

To demonstrate the functional role of β-catenin activation by hucMSC-Ex, we detected the cell cycle and migration of skin cells. The results showed that hucMSC-Ex significantly promoted wound closure of scratch, which could be reversed by simultaneous treatment with ICG001 (Fig. 3E and Supporting Information Fig. S2C). HucMSC-Ex prompted the transition of G1 to M phase during cell cycle (Fig. 3F). We next determined the role of β-catenin activation in hucMSC-Ex-mediated wound healing in vivo. Coinjection of ICG001 significantly inhibited the enhancement of wound healing and the increase of PCNA expression by hucMSC-Ex in vivo (Fig. 3G, 3H). In summary, hucMSC-Ex activates wnt/β-catenin signaling to enhance wound healing.

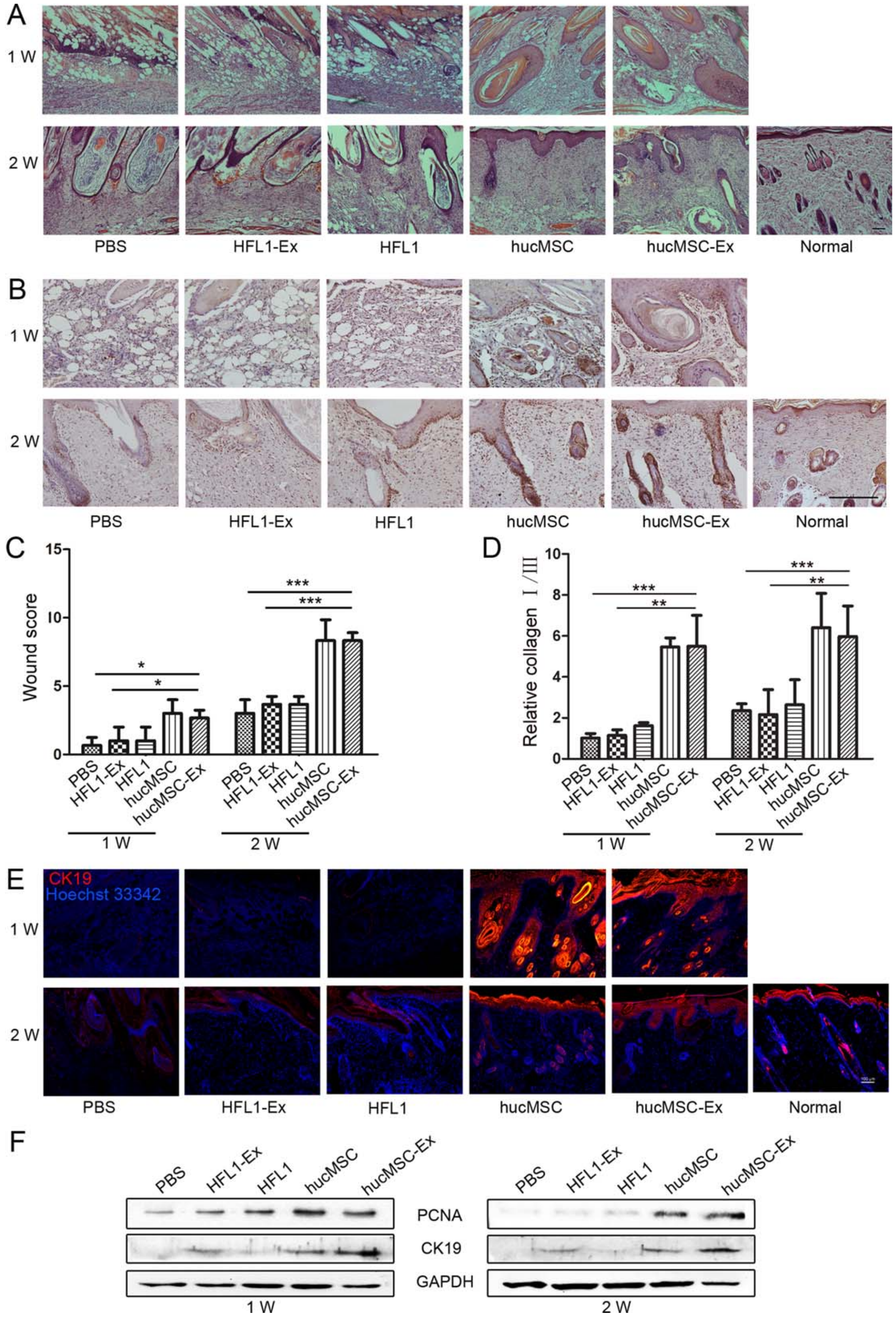

Figure 1. HucMSC-Ex accelerated the recovery of skin second-deep burn injury in rats. **(A):** Representative micrographs of wound histological images (H&E stain) at 1 week and 2 weeks after treatment. **(B):** Representative images of immunohistochemical staining of PCNA in each group. **(C):** Wound histological scores were calculated at 1 week and 2 weeks after treatment ($n = 6$; *, $p < .05$; ***, $p < .001$). **(D):** Quantitative analyses for relative mRNA level of type I and III collagen in wound tissue at 1 week and 2 weeks after treatment ($n = 6$; **, $p < .01$; ***, $p < .001$). **(E):** Representative immunofluorescence images of CK19 expression showed re-epithelialization in wound area. Scale bar = 100 μm. **(F):** Western blot assay for PCNA and CK19 expression in wounds at 1 week and 2 weeks after treatment. Abbreviations: HFL1, human lung fibroblasts; hucMSC-Ex, human umbilical cord mesenchymal stem cell-derived exosome.

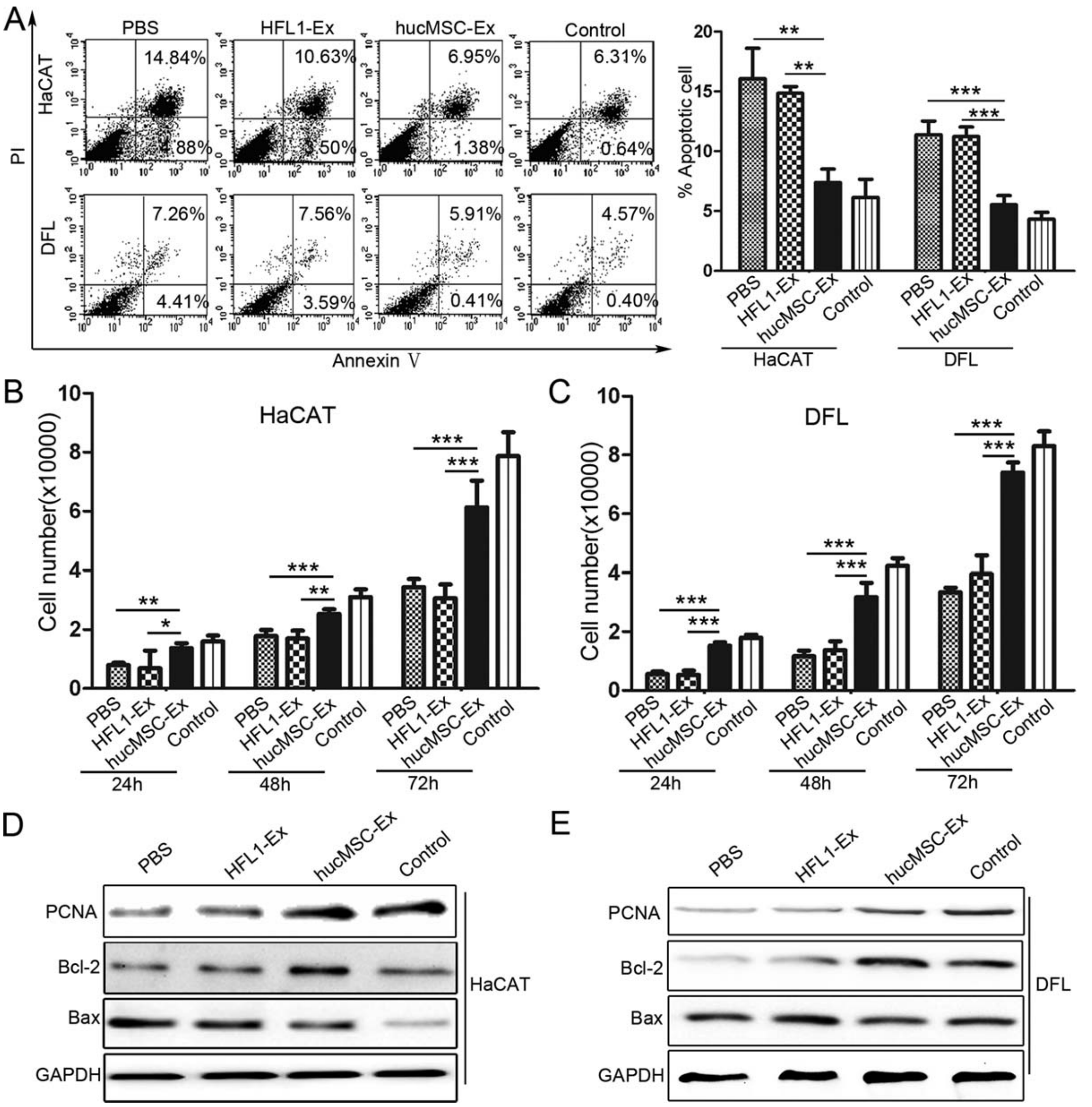

Figure 2. HucMSC-Ex inhibited heat stress-induced apoptosis of keratinocytes and promoted their proliferation. **(A):** HaCAT and DFL cells were cultured under treatment of PBS, HFL1-Ex, or hucMSC-Ex for 24 hours after 43°C 40 minutes heat stress and subjected to flow cytometric assay for apoptotic cells ($n = 3$; **, $p < .01$; ***, $p < .001$). **(B):** HaCAT and **(C)** DFL cells were cultured under treatment of PBS, HFL1-Ex, or hucMSC-Ex for 24 hours after 43°C 40 minutes heat stress. The number of cells at indicated time points was shown ($n = 3$; *, $p < .05$; **, $p < .01$; ***, $p < .001$). **(D):** Western blot assay for PCNA, Bcl-2, and Bax expression in HaCAT cells treated with PBS, HFL1-Ex, or hucMSC-Ex for 24 hours after 43°C 40 minutes heat stress. **(E):** Western blot assay for PCNA, Bcl-2, and Bax expression in DFL cells treated with PBS, HFL1-Ex, or hucMSC-Ex for 24 hours after 43°C 40 minutes heat stress. Abbreviations: DFL, dermal fibroblasts; HFL1, human lung fibroblasts; hucMSC-Ex, human umbilical cord mesenchymal stem cell-derived exosome.

HucMSC-Ex Delivered-Wnt4 Induces β-Catenin Activation

Since hucMSC-Ex activated Wnt/β-catenin signaling to prompt wound healing, we next focused on which component in hucMSC-Ex that mediated this effect. We screened the expression of Wnt family members including Wnt1, Wnt2, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt6, Wnt7b, Wnt10b, and Wnt11 in hucMSCs and HFL1 cells. We found that Wnt4 was significantly higher in hucMSCs than that in HFL1 cells (Fig. 4A). The higher expression of Wnt4 in hucMSC-Ex was further confirmed using Western blot (Fig. 4B). To investigate the role of Wnt4 in hucMSC-Ex-mediated β-catenin activation and wound healing, we knocked down Wnt4 in hucMSCs using shRNA (Fig. 4C). Knockdown of Wnt4 reduced the expression of Wnt4 in hucMSC-Ex and inhibited the transcriptional activity of β-catenin in 293T cells (Fig. 4D). The enhanced nuclear translocation of β-catenin and the increased expression of its downstream targets by hucMSC-Ex were also abrogated by Wnt4 knockdown (Fig. 4E, 4F; Supporting Information Fig. S3A, S3B). The phosphorylation of GSK3β, a classic negative regulator of Wnt signaling pathway, was promoted by hucMSC-Ex. However, knockdown of Wnt4 in hucMSC-Ex barely affected this phenomenon (Fig. 4F).

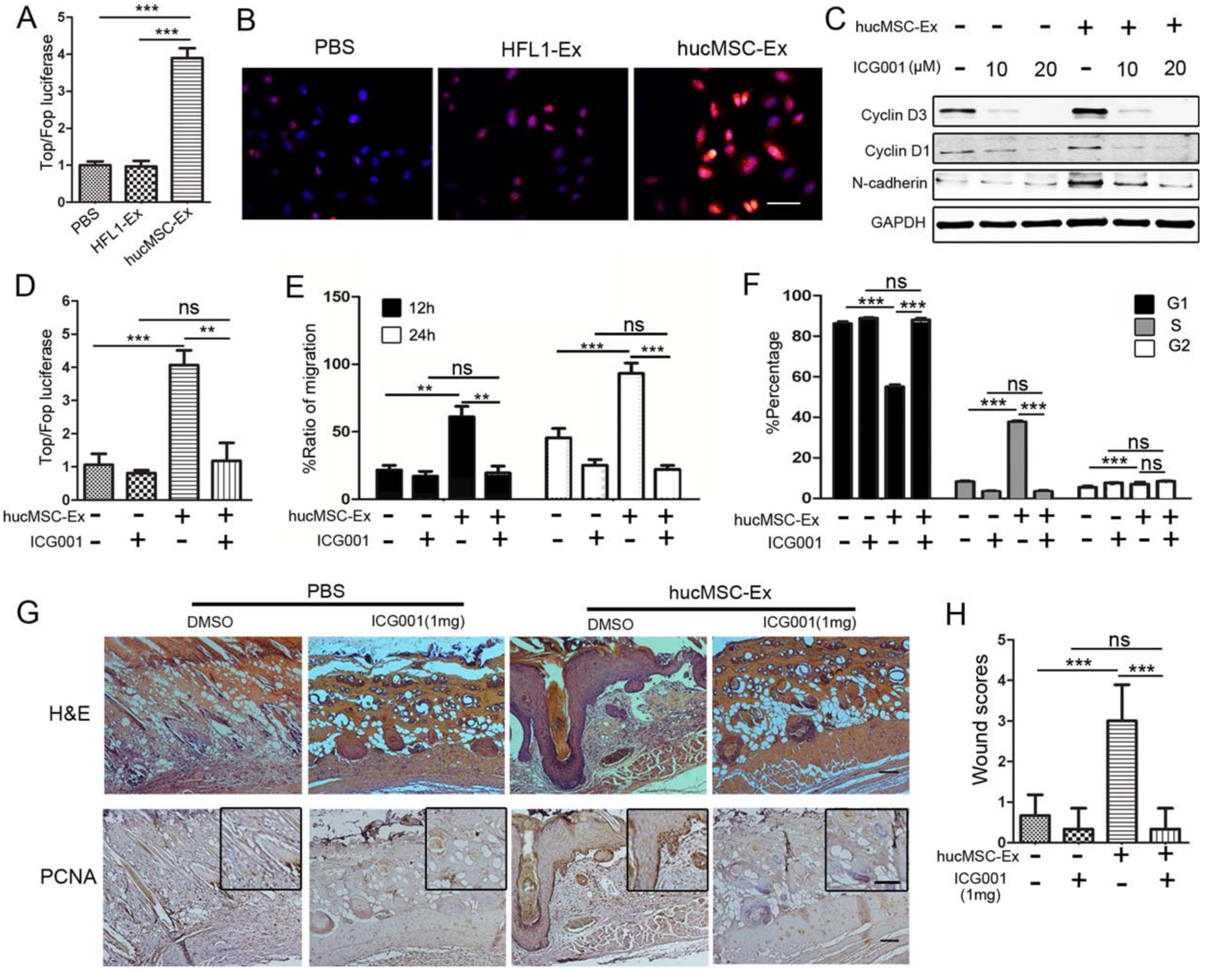

Figure 3. HucMSC-Ex activated β-catenin signaling to prompt wound healing. **(A):** 293T cells transfected with the TOP-Flash or FOP-Flash luciferase reporter were treated with PBS, HFL1-Ex, or hucMSC-Ex. The ratio between TOP-Flash and FOP-Flash luciferase activity was determined at 24 hours after treatment ($n = 3$, ***, $p < .001$). **(B):** HaCAT cells were treated with PBS, HFL1-Ex, or hucMSC-Ex for 24 hours. The nuclear translocation of β-catenin was determined by immunofluorescence staining. **(C):** Western blot assay for the expression of β-catenin downstream targets (cyclin D1, cyclin D3, and N-cadherin) in HaCAT cells treated with PBS or hucMSC-Ex in the presence or absence of ICG001(20 μM/ml). **(D):** The ratio between Top-Flash and Fop-Flash luciferase activity was determined at 24 hours after treatment with hucMSC-Ex in the presence or absence of ICG001 (20 μM/ml) ($n = 3$, **, $p < .01$; ***, $p < .001$). **(E):** The migration of HaCAT cells treated with hucMSC-Ex for 12 and 24 hours in the presence or absence of ICG001 (20 μM/ml) was determined by cell scratch assay ($n = 3$, **, $p < .01$; ***, $p < .001$). **(F):** Cell cycle analysis of HaCAT cells treated with hucMSC-Ex for 24 hours in the presence or absence of ICG001 (20 μM/ml) ($n = 3$, ***, $p < .001$). **(G):** The rat model was subcutaneously injected with hucMSC-Ex and ICG001 (1 mg/rat). The wound was subjected to H&E staining and immunohistochemical staining for PCNA expression at 1 week after treatment. Scale bar = 100 μm. **(H):** Wound histological scores ($n = 6$ at 1 week after treatment; ***, $p < .001$). Abbreviations: HFL1, human lung fibroblasts; hucMSC-Ex, human umbilical cord mesenchymal stem cell-derived exosome.

Furthermore, hucMSC-Ex-induced cell cycle progression and cell migration were also abolished by Wnt4 knockdown (Fig. 4G, 4H and Supporting Information Fig. S3C). Wnt4 knockdown delayed wound healing and reduced the expression of PCNA induced by hucMSC-Ex in vivo (Fig. 4I, 4J). These results indicate that Wnt4 plays an important role in hucMSC-Ex-mediated wound healing.

HucMSC-Ex Reverses Acute Thermal Injury-Induced Apoptosis in Skin Cells Through Activation of AKT Pathway

In order to explore the mechanism by which hucMSC-Ex reversed acute thermal injury-induced apoptosis in vitro and in vivo, we determined the status of AKT and MAPK signaling in HaCAT and DFL treated with or without hucMSC-Ex after heat stress. There was no significant change in MAPK pathway except a slight downregulation of phosphorylated p38 after hucMSC-Ex treatment (Supporting Information Fig. S4). However, the phosphorylated form of AKT was significantly increased in both HaCAT and DFL cells after hucMSC-Ex treatment (Fig. 5A). AKT inhibitor LY294002 suppressed the activation of AKT and the induction of Bcl-2 by hucMSC-Ex (Fig. 5B).

In consistent with the in vitro study, the activation of AKT and the increase of Bcl-2 protein level by hucMSC-Ex were also observed in skin tissues using Western blot and immunofluorescent staining (Fig. 5C, 5D). Moreover, hucMSC-Ex treatment reduced the number of apoptotic cells in wound area while the simultaneous treatment with LY294002 reversed this effect (Fig. 5E). We further tested the role of AKT signaling in hucMSC-Ex-mediated promotion of cell proliferation and migration. As

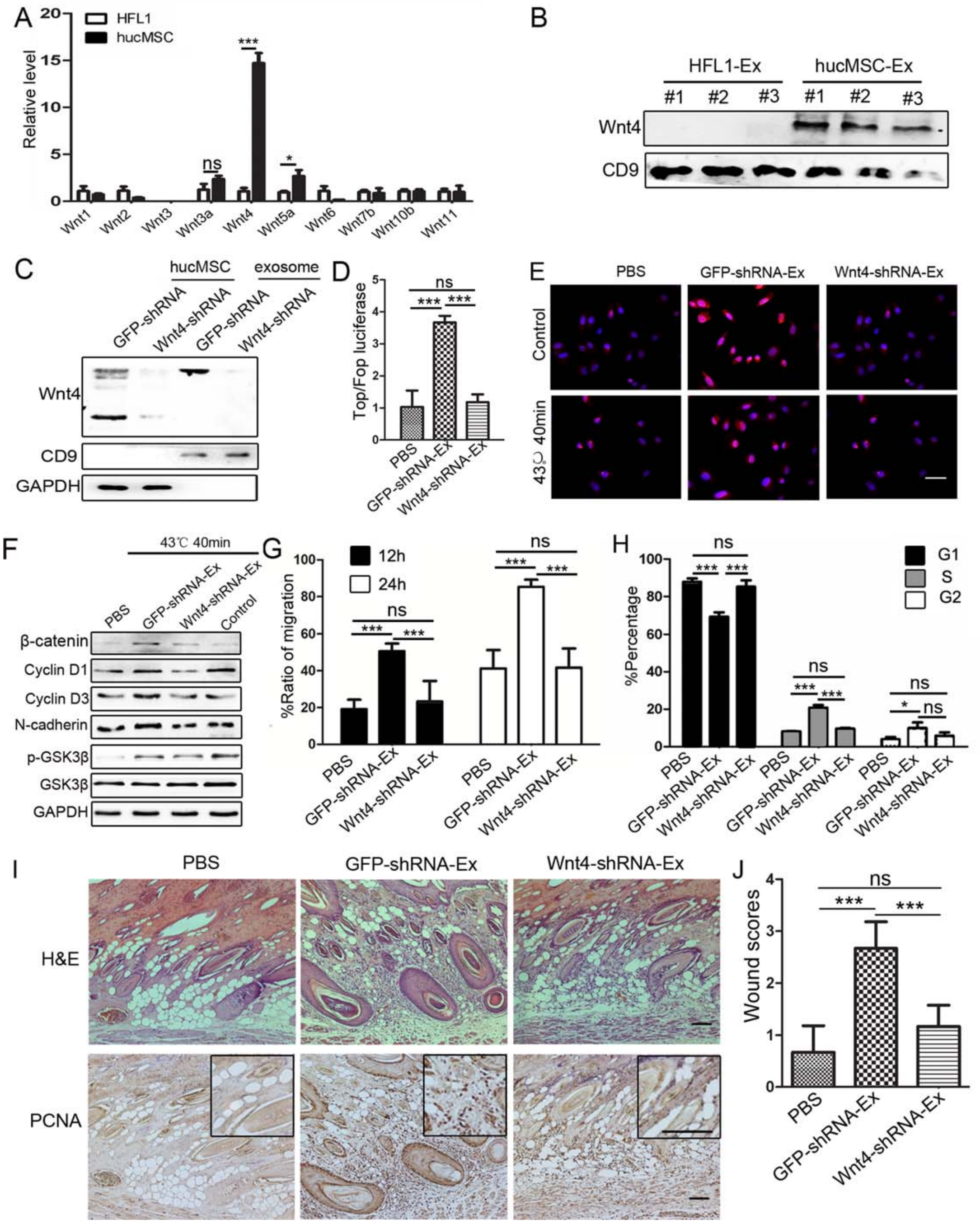

Figure 4. Wnt4 in hucMSC-Ex mediated the activation of β-catenin signaling and wound healing. **(A):** The mRNA levels of Wnt family members in hucMSC and HFL1 cell were detected using quantitative RT-PCR ($n = 3$, *, $p < .05$; ***, $p < .001$). **(B):** Western blot assay for the expression of Wnt4 in hucMSC-Ex and HFL1-Ex. **(C):** HucMSCs were transfected with Wnt4-shRNA or GFP-shRNA by lentivirus. The expression of Wnt4 in hucMSC and hucMSC-Ex was determined using Western blot assay. **(D):** The ratio between TOP-Flash and FOP-Flash luciferase activity was determined at 24 hours after treatment with exosomes from hucMSC transfected with Wnt4-shRNA (Wnt4-shRNA-Ex) or GFP-shRNA (GFP-shRNA-Ex) by lentivirus ($n = 3$, ***, $p < .001$). **(E):** HaCAT cells were treated with PBS, GFP-shRNA-Ex or Wnt4-shRNA-Ex under normal or heat stress conditions for 24 hours. The nuclear translocation of β-catenin was detected by immunofluorescence. **(F):** Western blot assay for the expression of β-catenin and its downstream targets (cyclin D1, cyclin D3, and N-cadherin) and the phosphorylation of GSK3β in HaCAT cells treated with PBS, GFP-shRNA-Ex, or Wnt4-shRNA-Ex under heat stress condition. **(G):** The migration of HaCAT cells treated with PBS, GFP-shRNA-Ex, or Wnt4-shRNA-Ex for 12 and 24 hours was detected using cell scratch assay ($n = 3$, ***, $p < .001$). **(H):** Cell cycle transition analysis of HaCAT cells treated with PBS, GFP-shRNA-Ex, or Wnt4-shRNA-Ex for 24 hours ($n = 3$, *, $p < .05$; ***, $p < .001$). **(I):** Rat wound models were treated with PBS, GFP-shRNA-Ex, or Wnt4-shRNA-Ex and then subjected to H&E staining and immunohistochemical staining of PCNA at 1 week after treatment. Scale bar = 100 μm. **(J):** Wound histological scores ($n = 6$ at 1 week after treatment; ***, $p < .001$). Abbreviations: HFL1, human lung fibroblasts; hucMSC-Ex, human umbilical cord mesenchymal stem cell-derived exosome.

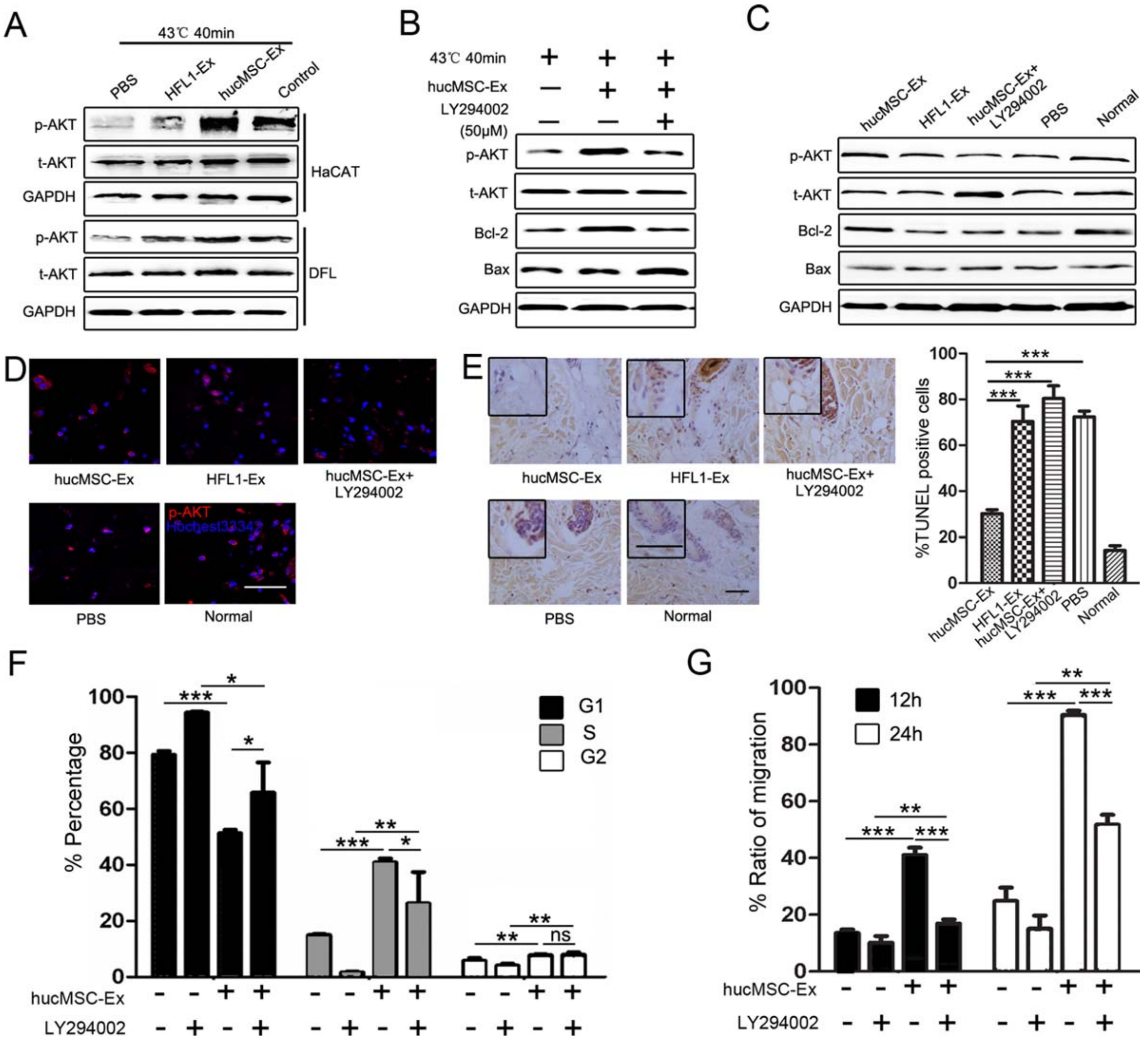

Figure 5. HucMSC-Ex inhibited heat stress-induced apoptosis by activating AKT signaling. **(A):** HaCAT and DFL cells were subjected to heat stress (43°C, 40 minutes) and treated with HFL1-Ex or hucMSC-Ex for 12 hours. The expression of total and phosphorylated AKT was determined by Western blot. **(B):** HaCAT cells were treated as described in (A) in the presence or absence of LY294002. The expression of total and phosphorylated AKT(p-AKT), Bcl-2, and Bax was determined by Western blot. **(C):** Western blot results examined the expression of p-AKT, Bcl-2, and Bax in vivo. **(D):** Representative immunofluorescence images of p-AKT expression in the injured skin tissues treated with hucMSC-Ex in the presence or absence of LY294002 (0.5 mg/rat), HFL1-Ex, and PBS. **(E):** TUNEL assay of apoptotic cells in the injured skin tissues treated with hucMSC-Ex in the presence or absence of LY294002 (0.5 mg/rat), HFL1-Ex, and PBS ($n = 6$, ***, $p < .001$). Scale bar = 50 μm. **(F):** Cell cycle distribution analysis of HaCAT cells treated with hucMSC-Ex in the presence or absence of LY294002 for 24 hours ($n = 3$, *, $p < .05$; **, $p < .01$; ***, $p < .001$). **(G):** The migratory ability of HaCAT cells treated with hucMSC-Ex in the presence or absence of LY294002 (50 μM/ml) for 12 hours and 24 hours was examined by cell scratch assay ($n = 3$, *, $p < .05$; **, $p < .01$; ***, $p < .001$). Abbreviations: DFL, dermal fibroblasts; HFL1, human lung fibroblasts; hucMSC-Ex, human umbilical cord mesenchymal stem cell-derived exosome.

shown in Figure 5F, hucMSC-Ex promoted cell cycle transition from G1 to S phase, which could be partially inhibited by LY294002, suggesting that other pathways may also participate in this process. The similar effect of LY294002 on hucMSC-Ex-induced cell migration was also observed (Fig. 5G). These results suggested that hucMSC-Ex reversed acute thermal injury-induced apoptosis in skin cells mainly through activation of AKT pathway. To explore input signal of AKT pathway via hucMSC-Ex, we analyzed the cytokines within huc-MSC exosomes that may activate AKT pathway by luminex assay. The data indicated that hucMSC-Ex could deliver many cytokines including PDGF-BB, G-CSF, VEGF, MCP-1, IL-6, and IL-8, which may activate AKT signaling (Supporting Information Table S5).

HucMSC-Ex Mediates Bifurcated Activation of Wnt4/β-Catenin and AKT Signaling to Promote Wound Healing

The Wnt/β-catenin signaling can be directly or indirectly regulated by AKT [31, 32]. Considering that AKT and β-catenin signaling were both activated by hucMSC-Ex, we next investigated the relationship between AKT and β-catenin signaling after hucMSC-Ex treatment. Inhibition of AKT by LY294002 had minimal effects on the increased TOP-flash reporter activity and the nuclear translocation of β-catenin induced by hucMSC-Ex (Fig. 6A, 6B), but reversed the phosphorylation of GSK3β induced by hucMSC-Ex (Fig. 6C), suggesting that exosomal Wnt4 mediates the activation of Wnt/β-catenin signaling independent of GSK3β. In consistent with the in vitro results, LY294002 almost

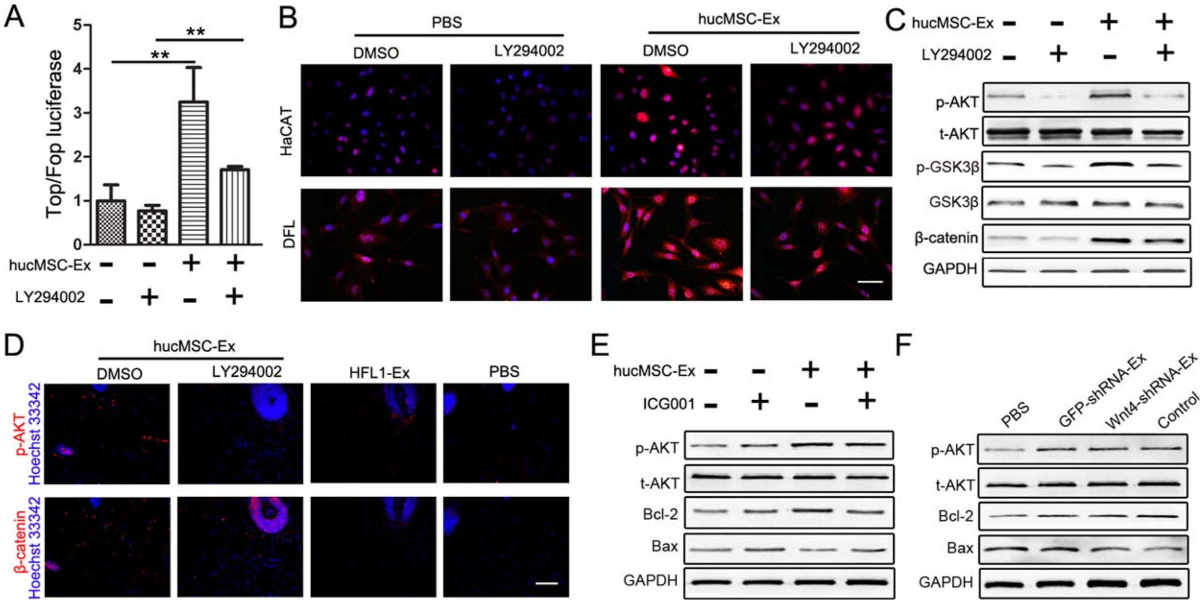

Figure 6. HucMSC-Ex-mediated bifurcated activation of AKT and Wnt4/β-catenin signaling to promote wound healing. **(A):** The ratio between TOP-Flash and FOP-Flash luciferase activity was determined at 24 hours after treatment with hucMSC-Ex in the presence or absence of LY294002 (50 μM/ml) ($n = 3$, **, $p < .01$). **(B):** HaCAT and DFL cells were treated with hucMSC-Ex in the presence or absence of LY294002 (50 μM/ml) for 24 hours. The nuclear translocation of β-catenin was detected by immunofluorescence. **(C):** Western blot assay for β-catenin and p-AKT and total AKT, p-GSK3β, and GSK3β in DFL cells treated with PBS or hucMSC-Ex in the presence or absence of 50 μM/ml of LY294002. **(D):** Immunofluorescence staining of p-AKT and β-catenin in consecutive sections of representative wounds that were treated with PBS, HFL1-Ex, or hucMSC-Ex in the presence or absence of LY294002 (0.5 mg/rat). Scale bar = 100 μm. **(E):** Western blot assay for p-AKT, Bcl-2, and Bax expression in DFL cells treated with PBS or hucMSC-Ex in the presence or absence of ICG001. **(F):** Western blot assay for p-AKT, Bcl-2, and Bax in DFL cells treated with PBS, GFP-shRNA-Ex, or Wnt4-shRNA-Ex under heat stress condition. Abbreviations: DFL, dermal fibroblasts; HFL1, human lung fibroblasts; hucMSC-Ex, human umbilical cord mesenchymal stem cell-derived exosome.

completely inhibited the activation of AKT but had minimal effect on the increase of β-catenin by hucMSC-Ex in vivo (Fig. 6D). In contrary, ICG001 treatment and knockdown of Wnt4 had no effect on the activation of p-AKT by hucMSC-Ex (Fig. 6E, 6F). The above results revealed that hucMSC-Ex enhanced wound healing by parallel activation of Wnt4/β-catenin and AKT signaling.

DISCUSSION

In this study, we investigated whether hucMSC-Ex had the same therapeutic effect as hucMSCs in skin injury models and explored the underlying mechanisms. We demonstrated that hucMSC-Ex significantly promoted wound healing in a rat deep second-degree burn injury rat model.

Exosomes are emerging as a new mechanism for cell-to-cell communication and is an important part of cells [14]. Exosomes are known to contain mRNAs, microRNAs, and proteins [33, 34]. The previous study shows MSC-Ex promote tissue injury repair through the horizontal transfer of mRNAs and microRNAs [15, 17, 35]. The active proteins can be delivered by exosomes resulting in biological effects of target cells [36, 37]. But few studies have defined the function of protein molecules transported by MSC-Ex in regenerative medicine.

Accumulating evidence indicates that Wnts require a particular lipid modification for proper secretion and function. Wnts are lipid-modified by the acyltransferase porcupine in the endoplasmic reticulum and acts on target cells in an autocrine or paracrine manner [38, 39]. The unmodified Wnts are tightly associated with the plasma membrane and are hard to spread over a distance to act on target cells [40, 41]. In tissue culture cells that are stably expressing Wingless (Wg; Drosophila ortholog of Wnt1), only a fraction of the Wnts is actually secreted into the media [42]. In mammalian and *Drosophila* cells, Wnts cannot be detected in exosome-free cell culture media [24]. Interfering with the lipid modification of Wnts results in a protein that is no longer active or hydrophobic [42–44]. In addition to essential lipid modification, proper secretory paths of Wnts are required for their function [45]. Several mechanisms have been proposed to explain how Wnts might function as long-range signaling molecules, including their lateral diffusion by association with heparan sulfate proteoglycans, solubilization by high-density lipoproteins and carrier proteins [40, 41]. Recently, several groups confirm that exosomes are important carriers for Wnt secretion and extracellular traveling [24, 25, 46]. Exosomes-mediated delivery of Wnts promotes the growth of Drosophila wings [24]. In this study, we find that hucMSC-Ex can deliver Wnt4 to enhance wound healing. Wnt4 knockdown in hucMSC abrogated β-catenin activation skin cells in vitro and the therapeutic effects of hucMSC-Ex in vivo, suggesting that hucMSC-exosomal Wnt4 is critical for the biological activities of hucMSC-Ex in wound healing.

Although our results showed that hucMSC exosomes promoted the phosphorylation of GSK3β and inhibited the activity of GSK3β. However, knockdown of Wnt4 in hucMSC-Ex barely affected the activity of GSK3β. Recent study has revealed that exosome-free Wnt signaling sequestrate GSK3β inside multivesicular endosomes to indirectly inhibit its activation [47]. It has been shown that AKT activation results in the phosphorylation of GSK3β [32]. Our data revealed that hucMSC exosome delivered many factors, such as G-CSF, PDGF-BB, VEGF, MCP-1, IL-6, IL-8, can affect the activation of AKT/GSK3β pathway. We further discovered that PI3K/AKT pathway by hucMSC-Ex also partly affected the activation of Wnt/β-catenin signaling through inhibiting the activity of GSK3β, which synergized with exosomal Wnt4. However, the interference experiments of Wnt4 confirmed that hucMSC-Ex-mediated Wnt4 was the key factor for activating β-catenin signaling. That may be why AKT pathway inhibitor LY294002 could slightly inhibit transcription activity of β-catenin and the promoting role of hucMSC-Ex in skin cells migration and cell cycle transition was not completely reversed by LY294002. Because the exosomal components are very complicated, it requires further studies to determine whether there are other molecules enhancing the activity of Wnt/β-catenin signaling in wound healing.

Previous studies have revealed that apoptosis plays an important role in regulating stem cell-dependent regeneration at the early stages of wound healing [48, 49]. Thus, it is necessary to investigate the mechanism of antiapoptotic effects by hucMSC-Ex. Further study showed the knockdown of Wnt4 and ICG001 treatment did not significantly affect the expression of apoptosis-related proteins Bcl-2 and Bax. This result indicated that hucMSC-Ex may inhibit apoptosis through other pathway(s). Long-term heat stress (4 weeks) can promote AKT activation and proliferation of fibroblasts [50]. Deregibus et al. reported that endothelial progenitor cell-derived microvesicles activate AKT signaling by a horizontal transfer of mRNAs [51]. Our results indicated that acute thermal injury inhibited AKT signaling and can be reversed by hucMSC-Ex. The results of Luminex assay showed that hucMSC-Ex contained many soluble factors, which have been reported to affect AKT pathway. However, due to the complexity of hucMSC exosomal components, it is hard to determine which factor(s) in hucMSC-Ex mediate the activation of AKT pathway.

In summary, we find the beneficial role of hucMSC-Ex in wound healing using rat burn injury. HucMSC-Ex enhances wound closure by delivering Wnt4 to activate Wnt/β-catenin in skin cells, and inhibits acute heat stress-induced skin cell apoptosis via activation of AKT pathway. Our findings suggest that administration of allogeneic hucMSC-derived exosomes is an alternative approach for stem cell-based therapy and may represent a novel therapeutic strategy for skin injury repair.

CONCLUSIONS

Our results have clearly demonstrated that hucMSC-Ex enhances skin second-degree burn injury repair and Wnt4 is the key mediator delivered by hucMSC-Ex in cutaneous wound healing.

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AUTHOR CONTRIBUTIONS

B.Z., M.W., and A.G.: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; X.Z., X.W., Y.Z., and H.S.: collection and/or assembly of data; L.W. and W.Z.: conception and design and data analysis; H.Q. and W.X.: conception and design, data analysis and interpretation, financial support, manuscript writing, and final approval of manuscript. B.Z., M.W., and A.G. contributed equally to this article.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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