hucMSC-derived exosomes ameliorate pressure ulcers by inhibiting HMGB1.

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Research Article

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Abstract

Background:Pressure ulcers (PUs) are a type of chronic wound in the elderly population. Previous studies have shown that exosomes derived from stem cells contain cytokines and growth factors that affect tissue repair and can represent a therapeutic strategy for wound healing. Thus, fully understanding how to extract exosomes and their mechanism of action can help promote the management of chronic refractory wound healing as a new cell-free treatment model.

Methods:In this study, we isolated exosomes from human umbilical cord mesenchymal stem cells (hucMSC-Exos) and examined their effects on wound healing. A total of 15 mice that were randomly divided into three groups, subjected to three ischemia–reperfusion (I-R) cycles and treated with different doses of hucMSC-Exos for different times. Quantitative real-time polymerase chain reaction (qRT–PCR) was used to analyze collagen mRNA levels in tissue samples. HMGB1 levels were examined by Western blotting and immunohistochemistry. α-SMA, CD34, and HMGB1 expression levels were compared to investigate the potential mechanisms.

Results:We found that hucMSC-Exos could be taken up by fibroblasts and significantly regulated and improved fibroblast fibrosis and in PU wound healing in vivo. Furthermore, we observed that hucMSC-Exo treatment of PU wounds downregulated the expression of HMGB1, which was previously shown to have a deleterious effect on the wound healing process.

Conclusions: Our findings indicate that hucMSC-Exos regulate the repair of PU wounds in part by inhibiting HMGB1 expression . Exosome treatment has provided new perspectives in regenerative medicine and trauma management.

1. Introduction

Pressure ulcers (PUs) are local tissue injuries caused by long-term pressure on the skin and/or underlying tissues in bony protrusion body areas, blood circulation disorders, continuous ischemia/hypoxia, and malnutrition. Pressure or shear force damage the integrity of the epidermis, dermis, adipose tissue, muscle and bone and can lead to ischemia–reperfusion (I-R) injury and induce chronic refractory skin ulcers [1-2]. Repeated I-R cycles have been shown to be more deleterious to skin tissue than simple long-term ischemia, and the reperfusion period impacts tissue damage [3]. During repeated I-R events, leukocyte infiltration, oxidative stress, infection, and inflammation create an imbalance between pro- and anti-inflammatory factors, leading to a reduction in tissue perfusion and necrosis [4]. Repetitive I-R events create unfavorable conditions for the delivery of growth factors that are necessary for the repair and homeostasis of damaged tissues [5]. PU afflicts particularly elderly populations, and treating PU remains a challenge, suggesting a real need the development of new therapies. Recent studies have shown the importance of paracrine signaling from tissue stem cells in promoting wound healing and damaged tissue regeneration [6]. Exosomes are secreted vesicles (50-150 nm) containing proteins, lipids or nucleic acids that can deliver paracrine signals between cells and may represent a therapeutic opportunity for

tissue repair and regeneration. Interestingly, exosomes derived from human umbilical cord mesenchymal stem cells (hucMSC-Exos) are an accepted source and feasible delivery system for a large number of cytokines and growth factors [6] and can affect the tissue response to injury, promoting wound healing [7]. The use of exosomes as therapeutic molecule delivery systems is if great interest because of their high biodegradability, low toxicity, immunogenicity and ability to protect their internalized cargo [8-10].

Our previous work demonstrated that hucMSC-Exos decreased the number of inflammatory cells and the levels of high mobility group box 1 (HMGB1) [11], which activates the release of proinflammatory cytokines in PUs. To our knowledge, this study was the first to investigate the effect of hucMSC-Exos on the PU microenvironment and the levels of HMGB1. However, the mechanisms underlying the therapeutic effects of exosomes in excessive inflammation of PU are unclear. The immunomodulatory effects of HMGB1 have been linked to tissue repair, fibrosis [12], chronic inflammation and increased I-R severity [13-15]. Thus, the effect of hucMSC-Exos on HMGB1 levels could be of interest for improving PU wound healing. In this study, we evaluated the effect of hucMSC-Exos on PU wound healing in vitro and in vivo and the potential mechanism of HMGB1 regulation by hucMSC-Exos. We showed that hucMSC-Exos could be taken up by fibroblasts and alter their activation status, leading to increased wound healing in vivo while decreasing the expression of HMGB1. The results presented in this study reinforce the interest in the use of in hucMSC-Exos for PU wound healing treatment and indicate HMGB1 as a candidate target.

2. Materials And Methods

2.1 Animals

Fifteen 6-week-old male BALB/c mice were purchased from the Guangdong Medical Laboratory Animal Center. All experimental and animal care procedures were approved by the Animal Care and Use Committee of Shenzhen People's Hospital. The animals were randomly divided into three groups and underwent three I-R cycles (Fig. 2A). The mice were evaluated at 1, 7 and 14 days. Before all procedures, the mice were allowed to acclimatize to their environment for 1 week.

2.2 Mouse skin wound model and treatments

The mice were anesthetized with isoflurane inhalation (1.8%). To create the pressure ulcer wound, the dorsal back was shaved and cleaned with 75% alcohol and subsequently pinched between two circular magnets (Dongguan JinKun Magnet products, CO) that were 10 mm in diameter, 5 mm thick, and had a strength of 4000 G. The full-thickness skin was pulled up and placed between a pair of magnetic disks. The dorsal skin was exposed to a 12 h of magnetic pressure and 12 h of rest (one I-R cycle). A total of 3 rounds of I-R were performed. The compressed area was left uncovered for the rest of the procedure after the PU wound induction protocol was performed.

Mice were randomly assigned to 3 different treatment groups and were left untreated (control) or injected at 4 sites with 200 µl of PBS or 200 µg of purified hucMSC-Exos resuspended in 200 µl of PBS around the wounds. All wounds were studied by histopathological analysis every day postwounding. The reduction

in wound size was calculated using the equation: wound-size reduction (%) = (AO – At)/AO × 100, where AO is the initial wound area, and At is the wound area at each day postwounding. On days 1, 2, and 3 after injection of the DIR-labeled exosomes into the wounds, the fluorescence was measured every 12 h by bioluminescence imaging.

2.3 Cell Culture

hucMSCs were purchased from Cyagen (stock number: HUXUC-01001 batch number: 160117I31). MSCs were cultured immediately upon receipt using umbilical cord mesenchymal stem cell culture medium (Cyagen, Basal Medium, For Human: Umbilical Cord MSCs). Primary cultures of MSCs and dermal fibroblasts were established using standard procedures. MSCs and fibroblasts were cultured in an incubator at 37 °C with 5% CO2. MSCs were subcultured at a ratio of 1:3 every 2 or 3 days until passage 5. The medium was then replaced with TM-ACF PLUS Medium Human (MesenCult) supplemented with a cell growth supplement (MesenCult TM-ACF PLUS 500X Supplement). MSCs were passaged at a ratio of 1:2 in T175 flasks, and the culture supernatant was collected for exosome purification when the cell confluence reached 90%.

2.4 Exosome purification and identification

At the end of the incubation period, the media was collected and centrifuged at 400× g for 10 minutes, 2000× g for 10 minutes, and 10,000× g for 30 minutes to remove cell fragments and protein aggregates. Then, the supernatant was centrifuged at 100,000× g for 120 minutes to pellet the exosomes. To purify the exosomes, the supernatant was discarded, and the exosome pellet was washed with ice-cold PBS followed by centrifugation at 100,000× g for 120 minutes. The washed exosome pellet was resuspended in PBS and stored at -80 °C or directly added to sample loading buffer for purity analysis by Western blotting. The protein levels of the exosomes were measured using a BCA protein assay kit (Pierce Protein Biology; Thermo Fisher Scientific Life Sciences). Exosome purification was confirmed by Western blot analysis of the exosomal surface markers CD9/CD63/CD81. Exosome shape and size were analyzed by transmission electron microscopy (TEM) (Tecnai G2 Spirit Biotwin).

2.5 In vitro exosome internalization and effects on fibroblasts.

To examine the effect of hucMSC-Exos on fibrosis in vitro, primary fibroblasts were treated with purified and DIR-labeled hucMSC-Exos (100 μl, 1 mg/ml) for 12 h. Human fibroblasts and DIR-labeled hucMSCs-exos were seeded on coverslips in 96-well plates at 37 °C and 5% CO2 in a humidified atmosphere and washed three times before being counted with Hoechst nuclear stain (Hoechst 33342, Germany) for immunofluorescence microscopy imaging. To analyze the effect of DIR-labeled hucMSC-Exos on fibroblast activation, fibroblasts were seeded on 24-well plates (5 x 105 cells/well) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 ng/ml fibroblast growth factor 2, 20% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Fibroblasts were cultured for 3 days in the presence of DIR-labeled hucMSC-Exos (0.5 mg/ml) or PBS or were left

untreated (control). Treated fibroblasts were collected for mRNA expression analysis by quantitative real-time polymerase chain reaction (RT–qPCR).

2.6 Histology

Cutaneous wound bed tissues were dissected on day 14, fixed in 4% phosphate-buffered formalin (pH 7.4), embedded in paraffin, sectioned at a thickness of 5 μm, and mounted on glass slides. The sections were stained with hematoxylin and eosin (H&E) using standard procedures and observed under a light microscope (Olympus BX53).

2.7 Assessment of collagen

Masson's trichrome staining, which differentially stains collagen blue, was used to evaluate collagen deposition in the wound bed and assess healing. The mRNA levels of Type I and III collagen and transforming growth factor-β(TGF-β) were examined by RT–qPCR. Expression levels were normalized to the housekeeping gene GAPDH and converted to relative values. The primer sequences used in this study were as follows: Type I collagen: forward, 5'- TGACCGATGGATTCCCGTTC -3', reverse, 5'-GCAGGGCCTTCTTGAGGTTG -3'; Type III collagen: forward, 5'- AAGCACTGGTGGACAGATTC-3', reverse, 5'-CGGCTGGAAAGAAGTCTGAG -3'; GAPDH: forward, 5'- AGCTTGTCATC AACGGGAAG -3', reverse, 5'-TTTGATGTTAGTGGGGTCTCG -3'; and TGF-β: forward, 5'-ACAACCCACACCTGATCCTC-3', reverse, 5'-GTTCGTGGACCC ATTTCCAG-3'.

2.8 Immunohistochemistry

To evaluate inflammation and angiogenesis, biotinylated antibodies against alpha-smooth muscle actin (α-SMA) (Abcam), the endothelial marker CD34 (Abcam) and HMGB1 (R&D Systems) were used to label PU wound sections. To assess the number of vessels, the entire area of the wound on the slide was observed under a microscope, the number of vessels was counted in each of the four quadrants of the wound, and the average was calculated. Staining was scored blindly by 4 independent experimenters.

2.9 HMGB1 measurement

HMGB1 expression was examined by RT–qPCR using the following primer set: forward, 5'-CTTCCTCATCCTCTTCATCC-3' and reverse, 5'-GCCCTATGAGAA GAA AGC TG-3'. GAPDH was used as an internal standard. For Western blot analysis, proteins were isolated from skin wound tissue using RIPA lysis buffer. Protein concentrations were measured with a BCA protein assay kit. Anti-HMGB1 (Sigma–Aldrich) antibodies were used for Western blot analysis. The blots were developed with Western Lightning Plus ECL reagent.

2.10 Statistical analysis

Group data are expressed as the mean and SD. The data were analyzed using GraphPad Prism version 6 (GraphPad Software). Statistical differences were determined by Student's t test or analysis of variance,

and p values < 0.05 were considered statistically significant.

3. Results

3.1 Isolation and characterization of hucMSC-Exos

To study the effects of exosomes on PUs, hucMSC-Exos were isolated and characterized (Fig. 1). TEM and nanoparticle tracking analysis (NTA) showed that exosomes purified from the hucMSC culture supernatants were round membrane-bound vesicles ranging 30–150 nm in diameter (Fig. 1A and 1C). Western blot analysis showed enrichment of the exosomal markers CD9, CD63, and CD81 in hucMSC-Exos compared to hucMSC extracts (Fig. 1B). Fig. 1D shows the NTA data of exosomes in real time.

3.2 hucMSC-Exos promote cutaneous wound healing in mice

The experimental flow chart of the BALB/C wound healing model and representative images of wounds are shown in Fig. 2A and 2B. We showed that the skin wound shrinkage rate was increased when wounds were treated with hucMSC-Exos in comparison to PBS or no treatment (Fig. 2C). An increased wound healing rate was observed from 7 days after treatment. Thus, treatment of mouse PU wounds with hucMSC-Exos could improve wound healing in vivo.

3.3 In vivo bioluminescence imaging

To evaluate whether exosomes were taken up and localized in the wound after treatment, mice with PU wounds were treated with DIR-labeled hucMSC-Exos. As shown in Fig. 3, the signal was detectable immediately after hucMSC-Exo administration and remained detectable at least 48 h posttreatment, indicating that hucMSC-Exos could be taken up by cells in the wound and might exert a continuous effect.

3.4 Effects of exosome treatment on scar formation

Reductions in scar width, changes in collagen levels, and tissue re-epithelialization are commonly used to assess wound healing. To evaluate the effect of hucMSC-Exos on PU wound healing, we analyzed trichrome-stained tissue sections of wounds 14 days after exposure to hucMSC-Exos, PBS or no treatment (Fig. 4A). Wound re-epithelialization analysis showed that the hucMSC-Exo group had smaller wound scar areas and regular epidermal and dermal structures than the other groups. The dermal layer was densely packed with collagen fibers, and the epidermis was arranged in parallel. No appendages, such as sebaceous glands, sweat glands or hair follicles, were observed in the three groups (Fig. 4A). The collagen fibers in the hucMSC-Exo group were arranged neatly and densely, while the collagen fibers in the PBS group were disorganized (Fig. 4B). These results confirm the beneficial effects of hucMSC-Exo treatment on PU wound healing and proper skin tissue regeneration.

3.5 hucMSC-Exos affect wound fibrosis

Because fibroblasts play a crucial role in wound healing and fibrosis, we evaluated whether hucMSC-Exos could be taken up and affect fibroblast activation. We tracked the fate of DIR-labeled exosomes in fibroblast cultures and observed the presence of numerous labeled particles inside fibroblasts. Internalized exosomes were mainly localized to the perinuclear zone (Fig. 5A). The expression of α-SMA and CD34, which indicate fibroblast activation and vasculature, respectively, was evaluated by immunochemistry (Fig. 5B) in vivo and RT–qPCR (Fig. 5C) in vitro after the administration of hucMSC-Exos, PBS or no treatment. As expected, α-SMA expression was significantly downregulated in exosome-treated PU wounds. The expression of CD34 and vasculature structures were increased in wounds treated with hucMSC-Exos compared to PBS-treated or untreated wounds. Taken together, our findings suggest that hucMSC-Exos can be internalized by fibroblasts and accelerate PU wound healing and tissue regeneration in part by impacting fibrosis and fibroblast activation.

3.6 HMGB1 localization in wounds

To assess fibrosis and inflammation in relation to HMGB1, the expression of collagen I (Col I), collagen III(Col III), TGF-β, and HMGB1 was examined in wounds. We observed a significant decrease in Col I, Col III and TGF-β mRNA expression after treatment with hucMSC-Exos (Fig. 6A). Interestingly, the mRNA and protein expression of HMGB1, which was previously linked to poor prognosis of PU wound healing, was also decreased in PU wounds after hucMSC-Exo treatment (Fig. 6A-C). We also confirmed the decrease in the level of α-SMA in hucMSC-Exo-treated wound tissue by Western blotting (Fig. 6B). Thus, the results of this proof-of-concept study suggest that the use of hucMSC-Exos to treat PUs may be an interesting and feasible approach. The effect of hucMSC-Exo treatment on HMGB1 is of particular interest and needs to be further investigated.

4. Discussion

PUs are a type of skin lesion that has been identified as the primary cause of I-R, which is associated with an anti-inflammatory phase characterized by severe defects in cell-mediated immunity [16]. Many cellular elements and complex molecular interactions are involved in this inflammatory reaction. hucMSC-Exos, which contain various bioactive molecules, including mRNAs and microRNAs, can mediate intracellular communication [16, 17]. Exosomes have been shown to have important paracrine effects on wound repair. Exosomes can promote cell migration and metastasis and reduce the negative effects of wound healing inflammation [18]. In the present study, we successfully isolated and purified exosomes from hucMSCs. In addition to demonstrating that DIR-labeled exosomes could be internalized by fibroblasts, we further showed that hucMSC-Exos decreased α-SMA expression in vitro and in vivo. These results indicate that hucMSC-Exos can directly act on fibroblasts and modulate their fibrotic activity.

HMGB1 is a chromatin-bound nuclear protein that can also act as a cytokine under stress conditions or during cell death [11]. Extracellular HMGB1 is a key factor in the induction of sterile immunity and activates the inflammatory pathway when it is released by ischemic cells. HMGB1 is released in large quantities from the cell and plays a cytokine-like role during the injury process [19,20]. HMGB1 can also

stimulate fibrosis and scar formation [21-23], and HMGB1 inhibition inhibits fibroblast migration and collagen synthesis, which can sharply reduce scarring. In the present study, we found that the expression levels of HMGB1 were decreased in PU wounds after hucMSC-Exo treatment, which paralleled the decreased expression of the fibrosis markers Col I, Col II and TGF-β. Based on previous works showing the deleterious effects of HMGB1 on wound healing and chronic inflammation, and our present results suggest that the downregulation of HMGB1 expression by hucMSC-Exos can directly accelerate PU wound healing. How hucMSC-Exos impact HMGB1 remains to be determined. Because exosomes have been shown to transport microRNAs, direct inhibition through RNA interference is one possible mechanism. Previous studies have shown the presence of miRNA221 in hucMSC-Exos [24]. Interestingly, miRNA221 might regulate the FAK pathway [25], which is involved in controlling HMGB1 expression. Thus, understanding the effect of miRNA221 on the regulation of HMGB1 expression and PU wound healing is of great interest and may be a potential therapeutic strategy. The beneficial effect of hucMSC-Exos on decreasing fibrosis marker expression was confirmed in vivo in our PU wound model. Treatment of PU wounds in mice with hucMSC-Exos accelerated wound shrinkage and tissue organization during the wound healing process. Notably, the organization of collagen and revascularization of repaired tissue were expedited by hucMSC-Exo treatment. Thus, hucMSC-Exo treatment can improve fibrosis, which can lead to improved tissue regeneration. It is also possible that hucMSC-Exo treatment can directly affect tissue re-epithelialization, which remains to be investigated in our model.

5. Conclusions

In this study, we first confirmed that exosomes can promote the healing of PUs by inhibiting the secretion of HMGB1 and scar hyperplasia while promoting tissue re-epithelialization and vascularization. Compared with stem cell therapy, exosomes have unique advantages in alleviating many safety concerns and are becoming a promising strategy for the treatment of PUs.

Abbreviations

HMGB1 high mobility group box 1

hucMSC-Exo exosomes from human umbilical cord mesenchymal stem cells (hucMSC-Exos)

Pus Pressure ulcers

I-R cycles ischemia–reperfusion cycles

TEM transmission electron microscopy

RT–qPCR quantitative real-time polymerase chain reaction

α-SMA alpha-smooth muscle actin

TEM transmission electron microscopy

Col I collagen I

Col III collagen III

TGF-β transforming growth factor-β

Declarations

Authors' contributions

FY, MHG, and LY contributed to data analysis and interpretation, data collection, literature review, and drafted the manuscript. FRL and LY contributed to the conception and design of the study. FY, and MHG contributed to the MSC production and characterization. FY, MHG, FRL and LY participated in the data collection and interpretation. All authors critically reviewed and approved the final manuscript.

Acknowledgments

All authors have made substantial contributions to the conception or design of the work and to revising and drafting the manuscript.

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Ethics approval and consent to participate

This work was approved by the Ethics Committee of the Medical Ethics Committee of Shenzhen People's Hospital.

Availability of data and materials

All materials are available from the corresponding author.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Footnotes

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The authors have declared no conflicts of interest.

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Figures

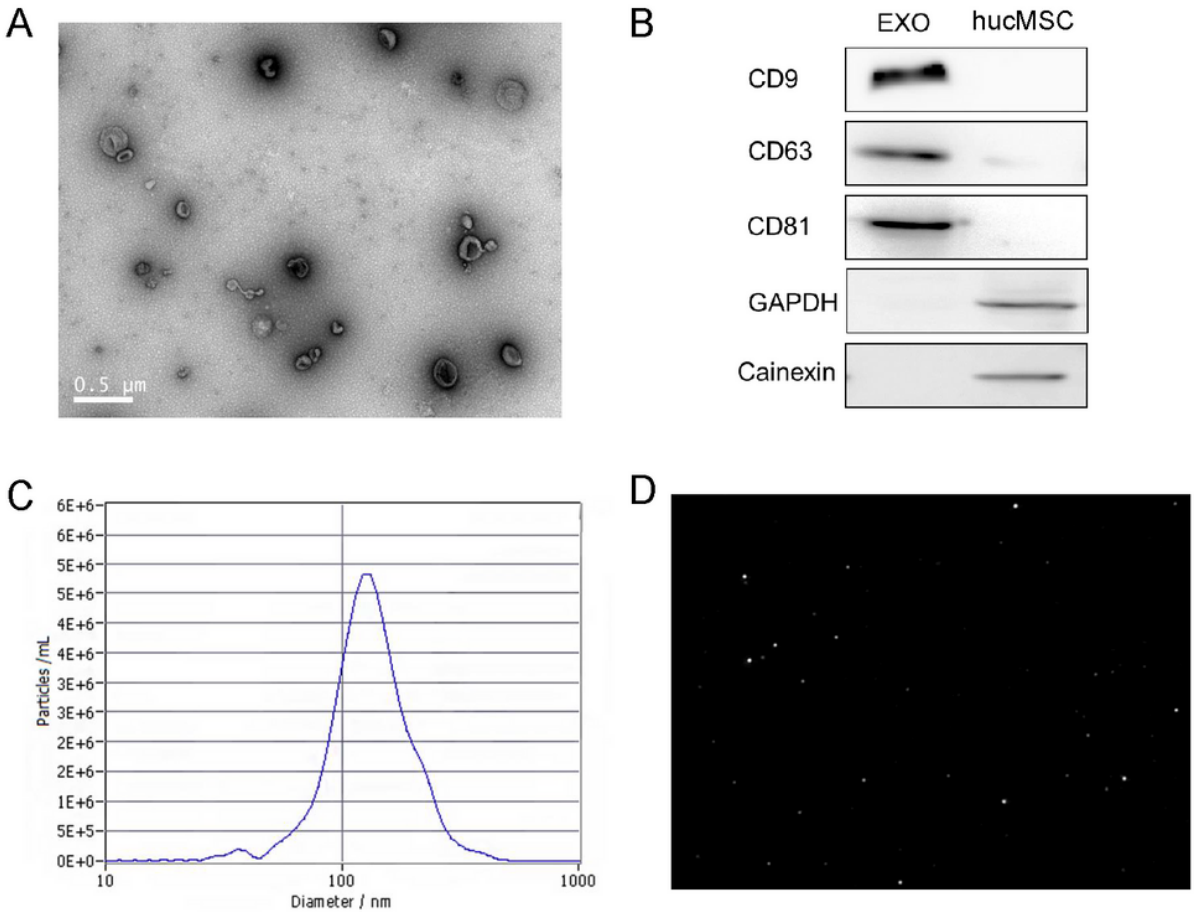

Figure 1

Exosome identification. (a) TEM analysis of exosomes isolated from hucMSC-conditioned medium. Arrowheads indicate exosomes. Scale bar: 500 nm. (b) Western blotting was performed on hucMSC-Exos and hucMSCs. CD9, CD63, and CD81 expression in exosomes was measured. (c) NTA of exosomes isolated from MSC-conditioned medium. The particle size and concentration distribution map shows that the MSC exosome particle size range was 30-150 nm. (d) The exosome image tracked by NTA in real time is a screenshot of the data .

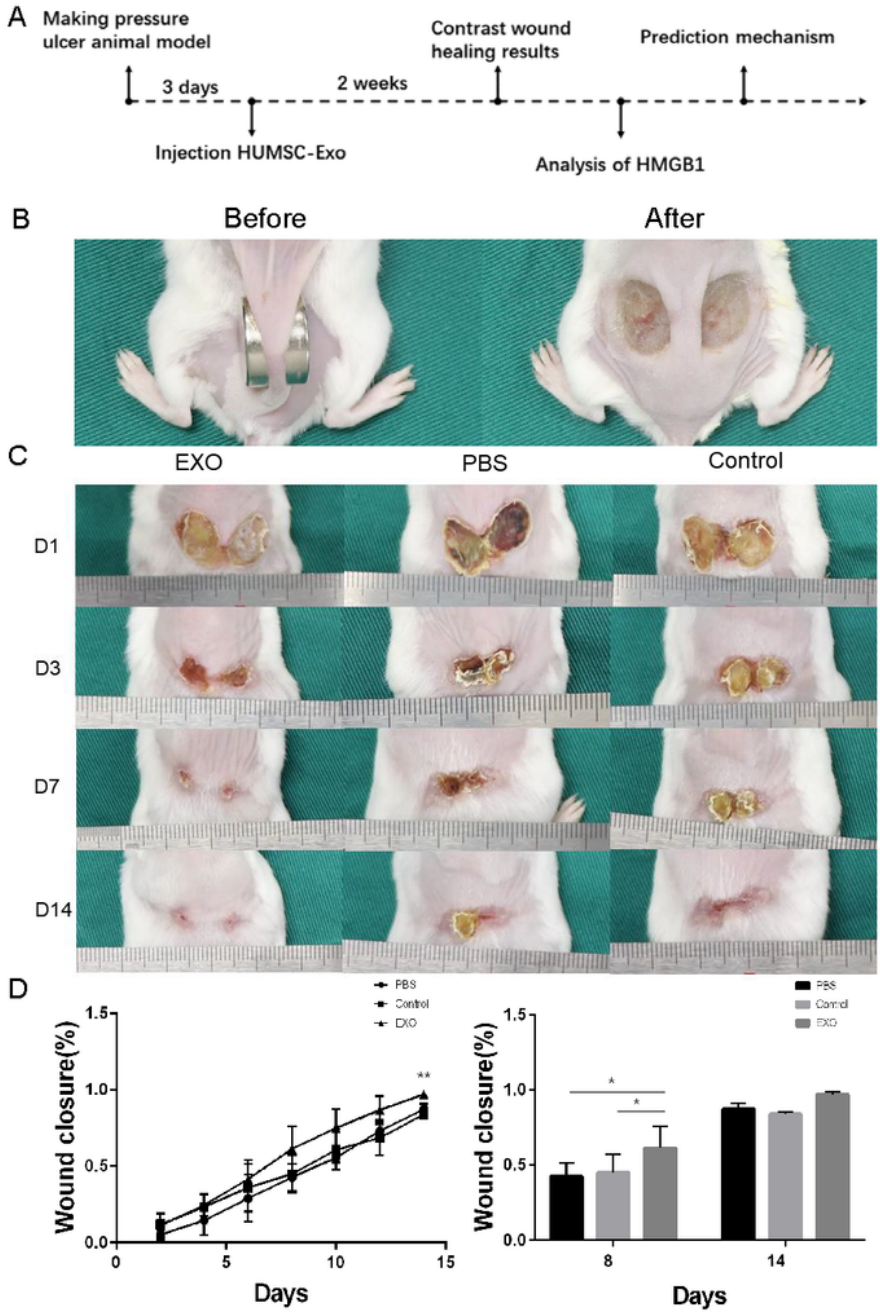

Figure 2

Macroscopic appearance of cutaneous wounds treated with hucMSC-Exos. (a) The experimental flow chart. (b) The PUs were induced with a pair of magnet disks. (c) Gross view of wounds treated with PBS or hucMSC-Exos at 1, 3, 7, and 14 days. (d) The effects of treatment with PBS or hucMSC-Exos on wound closure at 4, 7, and 14 days. *P < 0.05.

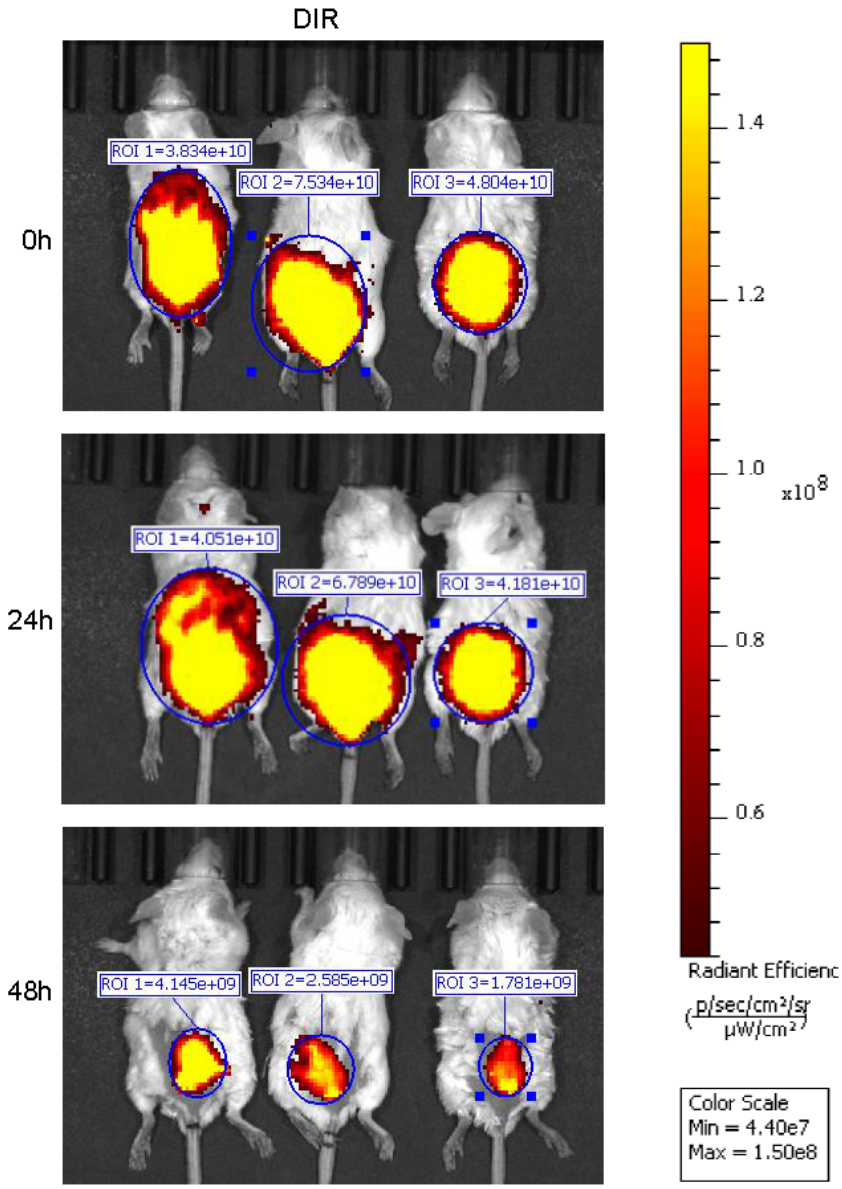

Figure 3

In vivo imaging of exosomes. Intradermal metabolism rate of exosomes was analyzed by live imaging of representative mice every 24 h.

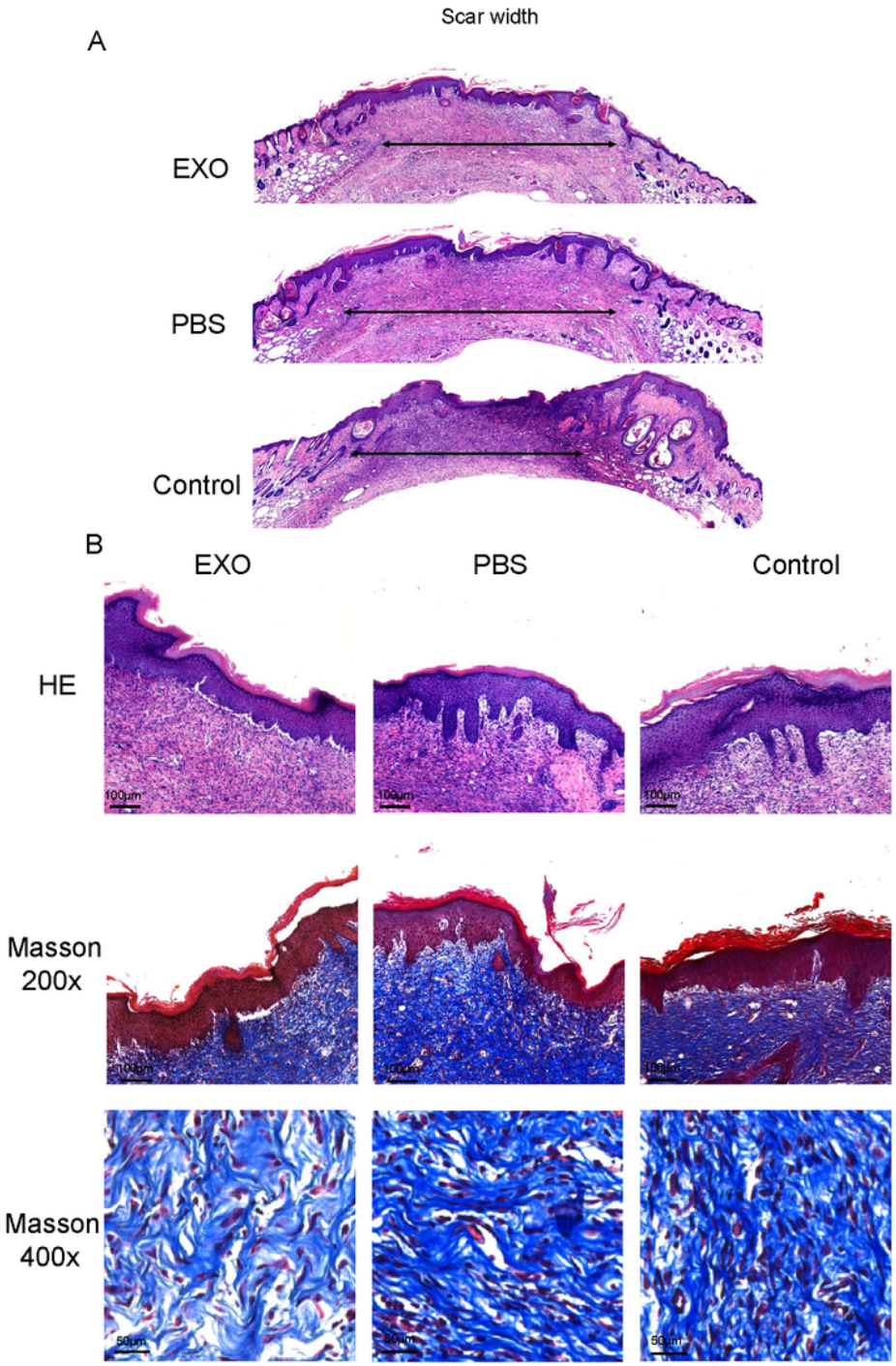

Figure 4

Histological analyses of cutaneous wounds treated with hucMSC-Exos, PBS and no treatment at 14 days postwounding. (a) The double-headed arrows indicate the edges of the scar in H&E-stained wound sections, which shows the effects of PBS and hucMSC-Exos on wound re-epithelialization. (b) Evaluation of collagen maturity by Masson's trichrome staining of wounds following treatment with PBS or hucMSC-Exos. Scale bar =100 μm.

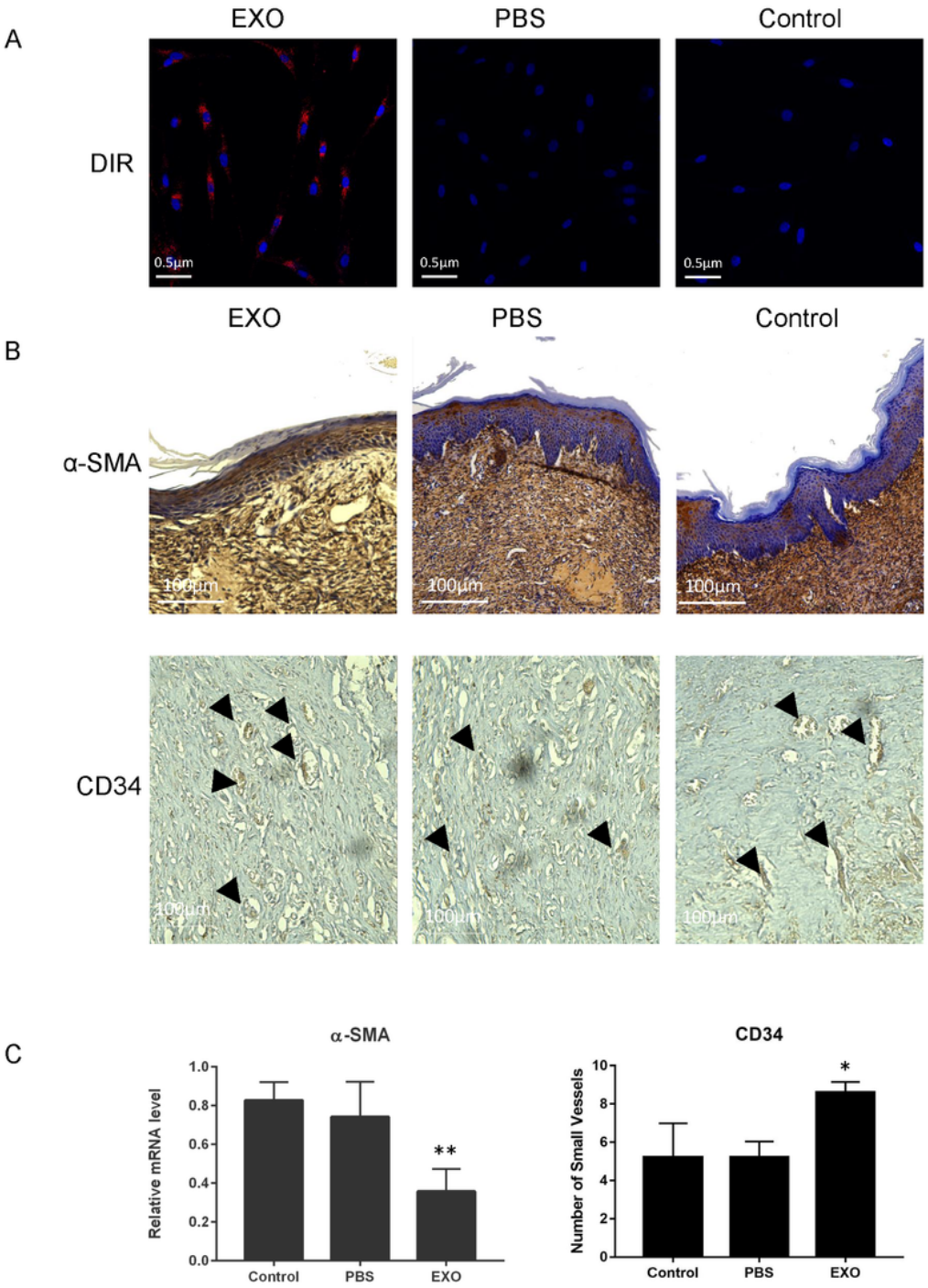

Figure 5

Effects of hucMSC-Exo treatment on wounds. (a) In vitro wound healing properties of exosomes and cellular uptake analysis revealed that exosomes were internalized (red), and they mainly localized to the perinuclear zone. Scale bar = 100 μm. (b) Immunohistochemical analysis of α-SMA levels in wounds at 14 days postwounding. Immunohistochemical analysis of CD34 levels in wounds at 14 days

postwounding. Scale bar = 100 μm. (c) mRNA expression of α-SMA in fibroblasts treated with hucMSC-Exos, PBS, and control for 24 h. Average number of small vessels in four quadrants. *p < 0.05, **p < 0.01

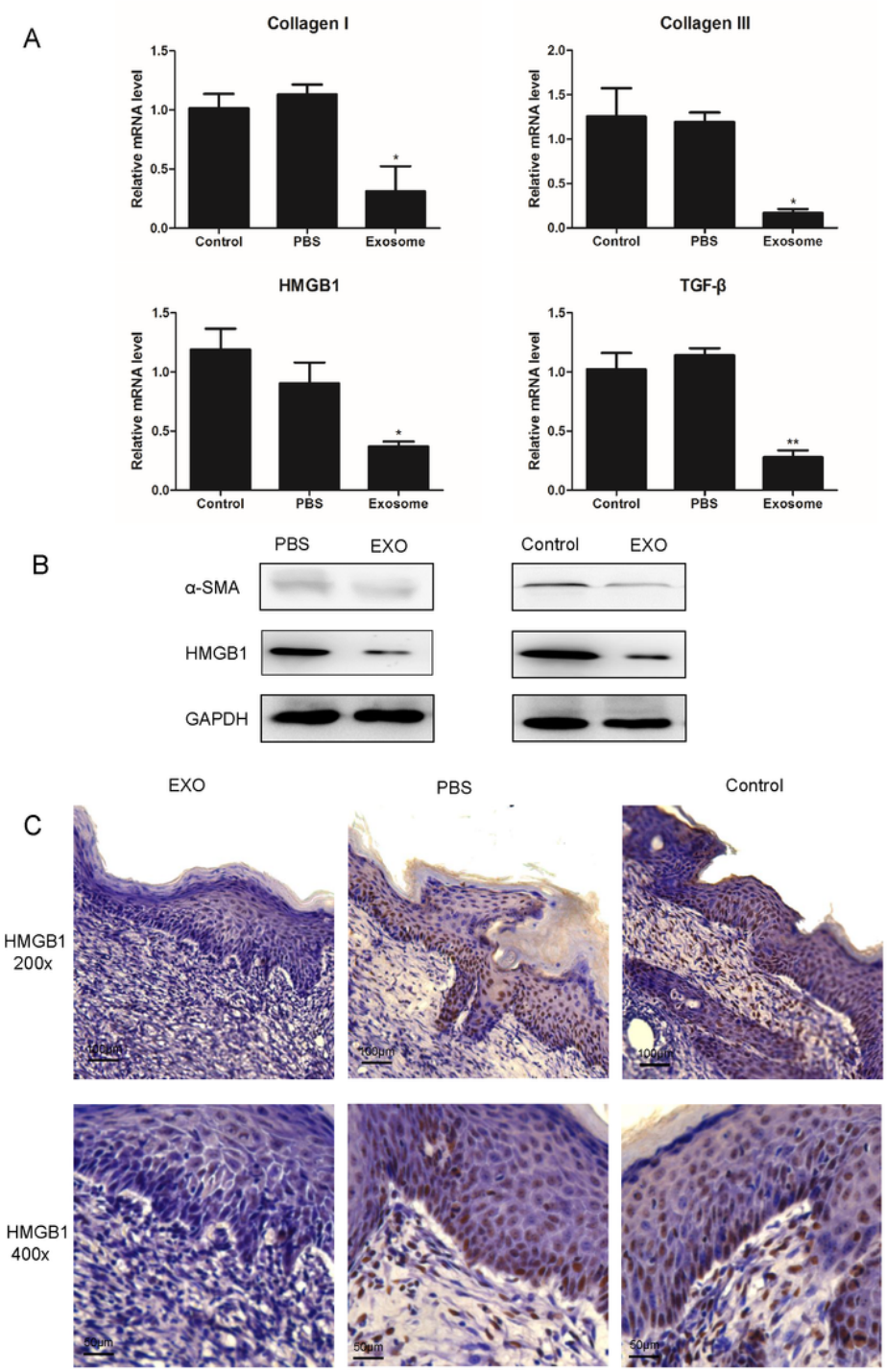

Figure 6

HMGB1 localization in wounds. (a) mRNA expression of HMGB1, COLI, COLIII, and TGF-β in wounds treated with 1 mg/ml hucMSC-Exos and PBS at 14 days postwounding. (b) Western blot analysis of the

expression of HMGB1 and α-SMA in wounds treated with 1 mg/ml hucMSC-Exos and PBS at 14 days postwounding. *P < 0.05, **P < 0.01. (c) Immunohistochemical analysis of HMGB1 levels in wounds at 14 days postwounding. Scale bar = 100 μm.