

Rat epidermal stem cells promote the angiogenesis of full-thickness wound

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Research

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Abstract

Background: Full-thickness wounds are a serious problem which badly affects patients' life quality and also become the difficult problem for clinicians. Stem cells have great prospects in the treatment of wounds. Our previous experiments proved that autologous basal cell suspension can promote wound healing, and there are epidermal stem cells (ESCs) in basal cell suspension. We then conducted experiments to explore the effect of ESCs on full-thickness wound.

Methods: In our study, the rat ESCs were isolated and expanded, and transfected with lentivirus to stably express EGFP. Experimental rats were randomly divided into 2 groups, in the ESCs group, the rat ESCs were sprayed on the Full-thickness wounds of the rats, while in control group, sprayed the PBS on the wound. Wound healing and neovascularization were then evaluated. Colonization, division and differentiation of ESCs on the wound were discovered by immunofluorescence.

Results: The result suggested that rat ESCs can colonize, divide and proliferate in the wound. What's more, the rat ESCs around blood vessels can differentiate into vascular endothelial cells and form a lumen-like structure. Compared with the control group, spraying the rat ESCs on the wound bed can promote angiogenesis and accelerate wound healing.

Conclusions: Our study proved that rat ESCs were safe and effective for treating full-thickness wounds, and under certain conditions, ESCs can differentiate into vascular endothelial cells to promote angiogenesis and wound healing.

Background

The skin is the largest organ of the human body. It has many important functions such as metabolism, absorption, protection, body temperature regulation, secretion, sensation and so on. At the same time, it is easy to be damaged when stimulated by external chemical and physical factors. Full-thickness skin wounds caused by burns, car accident injuries, war injuries, avulsion injuries and other traumas are the most common diseases in clinical emergency departments, has always been the difficult problems for clinicians. Patients with full-thickness skin defects, the wounds are healed mainly by the migration of stem cells adjacent to the skin epithelial cells and the regeneration of the remaining skin appendages. If the area of the patient's skin defect is too large, it may take much long time to heal and cause a lot of follow-up problems like scars and wound contraction which affected normal skin function, aesthetics and psychology, so accelerating the speed of repair after skin injury have become the focus of attention of clinicians. At present, its treatment mainly relies on surgical debridement, flap covering skin grafting, wound dressing, application of epidermal factor and hyperbaric oxygen therapy and so on, but lack simple and effective methods [1–4]. Therefore, how to increase the effectiveness of wound healing and regain the skin function are the problems faced by burns and plastic surgeons.

Effective wound repair requires the formation of a large number of new blood vessels in the granulation tissue to maintain the nutrition of the wound bed and promote the deposition of extracellular matrix.

Therefore, the growth of new blood vessels in the wound tissue plays a very important role to form the granulation tissue, improve the microcirculation of the wound, reduce the incidence of infection and then promote the healing of chronic refractory wounds or deep burn wound. Impaired neovascularization will directly lead to delayed wound healing or poor wound healing [5–8].

Stem cells are considered to be ideal seed cells for promoting the wound healing due to their strong self-renewal capacity and multi-directional differentiation potential [9–10]. Our previous researches demonstrated that autologous basal cell suspension can promote skin re-epithelialization and promote wound healing. Moreover, in the treatment of chronic wounds, blade thick skin transplantation combined with autologous basal cell suspension can improve the survival rate of the skin and increase the quality of skin healing. The number of epidermal stem cells (ESCs) is very small (accounting for 1–10% of epidermal basal cells) [11–13]. We speculated that the epidermal stem cells might play an important role in wound healing, so we next studied the effects of epidermal stem cells on wound healing.

Methods

Isolation of rat ESCs

One six-week-old rat was sacrificed by cervical dislocation. The back skin of the rat was taken and placed in a 15 ml centrifuge tube with 1% phosphate buffered saline (PBS; 10010023; Gibco), and then transported in an ice box to the ultra-clean bench in the laboratory. Remove the muscle layer in the ultra-clean table, cut the skin to a size of $1 \times 1 \text{ cm}^2$ and place it in a sterile 15 ml centrifuge tube, add 2 ml 10xTryple (A1217702; Gibco), digest in a constant temperature water bath at 37 °C for 15–30 minutes, shaking every 3 minutes. Take several T25 culture flasks, evenly coat the culture flask (about $5 \mu\text{g}/\text{cm}^2$) with fibronectin (FN; Shanghai Fibronectin Biotechnology, Shanghai, China) solution 1 ml (0.5 mg/ml) before planting the basal cell suspension, and leave it in a 37 °C incubator for 20 minutes. After the skin is completely digested, rinse the skin with 1% PBS to stop the digestion, scrape the basal cells with a sterile scalpel, rinse and collect the cells with keratinocyte serum-free medium (K-SFM; 17005042; Gibco), filter the cell suspension in a 50 ml centrifuge tube with a 200-mesh filter. Transfer the cell suspension to a 15 ml centrifuge tube, centrifuge at 1000r/min for 10 minutes, discard the supernatant, and resuspend in 4 ml complete medium by pipetting. Add the above basal cell suspension to the coated culture flask and let it stand in a 37 °C incubator for 20 minutes. It can be seen that about 10% of the cells adhere to the wall first. These cells are regarded as ESCs. ESCs were cultured in K-SFM medium at 37 °C incubator, and changed the medium every 2 days.

Immunofluorescence and confocal microscopy

The third-generation rat ESCs were digested, then resuspended and cultured in confocal glass bottom petri dishes. After the cells adhered, they were washed 3 times with PBS, 5 minutes each time, fixed with 4% paraformaldehyde (P0099-500 ml; Beyotime Biotechnology, Shanghai, China) for 20 minutes, and washed with PBS 3 times, then 0.5% Triton X-100 (P0096-500 ml; Beyotime Biotechnology, Shanghai,

China) permeated at room temperature for 20 minutes. Dip in PBS three times, add 5% goat serum (C0265; Beyotime Biotechnology, Shanghai, China) onto the glass bottom of the petri dish, and block at room temperature for 40 minutes. Aspirate the 5% goat serum blocking solution, add 100 μ l of diluted primary antibodies: p63 (1:200, ab735; Abcam), α 6-integrin (1:200, ab235905; Abcam), CD31 (1:200, ab24590; Abcam) and CD34 (1:200, ab81289; Abcam), and incubate at 4 °C overnight. Wash the petri dish three times with PBS for 5 minutes each time. After absorbing excess liquid from the petri dish, add diluted fluorescent secondary antibodies: goat anti-rabbit IgG labeled with Alex Fluor 488 (1:200, ab150077; Abcam) and goat anti-mouse IgG labeled with Alexa Fluor 594 (1:200, ab150116; Abcam), and incubate for 1 hour at room temperature in the dark. DAPI (D9542; Sigma-Aldrich) was added dropwise and incubated for 5 minutes in the dark, and rinsed 4 times with PBS for 5 minutes each time. The liquid on the dish was sucked off, mounted with anti-fluorescence quencher, observed under a fluorescence microscope and collected images.

Flow Cytometry

Collect the third-generation rat ESCs by centrifugation and aspirate the supernatant. Resuspend cells briefly in 0.5 ml PBS. To fix and permeabilize cells by BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit. Adding 2–3 ml of incubation buffer to each tube, then rinse by centrifugation. Resuspending cells in 100 μ l of incubation buffer per test tube. Blocking in incubation buffer for 10 minutes at room temperature. Adding the primary antibodies (p63, Abcam, ab124762, 1:200; α 6-integrin, Abcam, ab77906, 1:200) to the test tube at the appropriate dilution. Incubating at room temperature for 60 minutes. Following the instructions and rinsing cells in incubation buffer by centrifugation. Resuspending the cells in the fluorescent-labeled secondary antibodies, and diluting in incubation buffer as recommended by the manufacturer. Incubating at room temperature for 30 minutes, then rinsing in incubation buffer by centrifugation. Resuspending the cells in 0.5 ml PBS and analyzing by flow cytometry.

Lentivirus and transfection

To generate ESCs with stable enhanced green fluorescent protein (EGFP) expression, cells were infected with a lentiviral vector encoding the full-length human EGFP gene or empty lentiviral vector as the control (OBiO Technology, Shanghai, China). Stable clones were selected after 2 weeks using 1 μ g/ml puromycin, and the expression level of EGFP was determined by Immunofluorescence.

Animal experiment

To explore the function of ESCs on full-thickness wound bed in vivo, the rats' dorsal wound model was adopted. Twenty rats were anesthetized by inhaling isoflurane (INH), and a diameter of 2-cm full-thickness wound was made on the dorsal skin of each rat. The wounds were divided into 2 groups randomly: control group and ESCs group. The ESCs can stably express EGFP and evenly spray to the wound bed by a 2-ml syringe. For the ESCs group, 1 ml cell suspension with a cell density of 1×10^5 /ml were evenly sprayed to the wound bed, while in control group, sprayed 1 ml PBS. The rats and wounds were observed, photographed, and measured daily until the rats were sacrificed. Wound healing time was recorded, and the residual wound area rate was calculated as $[(\text{day } n \text{ area})/(\text{day } 0 \text{ area})] \times 100\%$ ($n = 0, 3,$

7, 14 or 21). Six rats of each group were sacrificed at days 0, 3, 7, 14, and 21, respectively, and the wound tissues were harvested and separated into two halves across the center: one half was processed for histological and immunohistochemistry analysis, and the other was rapidly frozen in liquid nitrogen for western blots analysis.

Immunohistochemistry staining analysis

The paraffin-embedded fixed tissue sections of each group were deparaffinized and rehydrated in xylene and graded ethanol. Antigen retrieval was performed by using Proteinase K solution (20 µg/ml) at 37 °C for 15 minutes. After Bloxall blocking, the sections were blocked with goat serum for 30 minutes and then incubated with primary antibodies: anti-CD31 (1:100, ab24590; Abcam) overnight at 4 °C. After washing in PBST, the sections were then incubated with an HRP conjugated secondary antibody (1:2000, ab97051; Abcam) for 1 hour at room temperature. The sections were further incubated with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin and observed by microscope.

western blot analysis

Western blotting was performed using antibodies directed against CD31 (1:1000, ab24590; Abcam) and GAPDH (Sigma-Aldrich; SAB1405848; 1:6000). GAPDH served as an internal control. For western blotting, cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Cell Signaling Technology) containing PMSF (100:1, v/v) (Cell Signaling Technology) for 30 minutes. A BCA Protein Assay Kit (Pierce, Thermo Scientific) was used to measure the total protein concentrations. Aliquots (40 µg) of total cellular protein were resolved by SDS-PAGE (10 ~ 12%), electrotransferred to PVDF membranes, and blocked with 5% skim milk (w/v) at room temperature for 1 hour. The membranes were then incubated with primary antibodies on an orbital shaker at 4 °C overnight, and secondary antibodies (HRP-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit) were added and incubated for 1 hour at room temperature. Protein-antibody complexes were then detected by chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo, USA).

Tissues immunofluorescence analysis

Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through graded ethanol. Antigen retrieval was performed using citrate buffer in a pressure cooker at 95 °C for 30 minutes. The 4-µm sections of each group were blocked in 10% goat serum (16210064; Gibco) for 30 minutes at 37 °C, then adding primary anti-rats antibody (CD31, Abcam, ab24590, 1:200). After incubating at 4 °C overnight, the sections were washed with PBST and incubated with the following secondary antibody for 1 hour: goat anti-mouse IgG labeled with Alexa Fluor 594 (1:200, ab150116; Abcam). DAPI was added dropwise and incubated for 5 minutes in the dark, and rinsed 4 times with PBS for 5 minutes each time. The liquid on the Petri dish was sucked off, mounted with anti-fluorescence quencher. Sections were documented with a fluorescence microscope (OLYMPUS, Japan).

Statistical analysis

Values were expressed as the mean \pm standard deviation (SD) unless otherwise indicated. Comparisons of expression difference between control and experimental groups were conducted by Student's t test. All statistical analyses were performed by SPSS 20.0 software (SPSS, Chicago, IL, USA), and $P < 0.05$ indicates that the difference was statistically significant.

Results

Morphology and identification of rat ESCs

The number of epidermal stem cells is very small and the number of epidermal stem cells remaining after injury is further reduced. Therefore, the insufficient source of seed cells has become a bottleneck that restricting the application of cell therapy in wound. Our previous study found that FN precoating culture dishes can promote the adhesion and proliferation of ESCs [14]. In our study, we used FN to harvest and expand rat ESCs. The isolated original representative skin stem cells are round in shape, small in size and strong in refraction. After overnight cultivation, some cells adhered to the wall, the cells were polygonal, and the nuclei were larger. After culturing rat ESCs for 3 days and changing the medium, it can be seen that the cells have formed a clonal colony of cells and adhere firmly. After 7 days of culture, the cells proliferated significantly, and the cells were connected in a sheet-like shape, paving stones (Fig. 1A). Rat ESCs were passaged and expanded, and third-generation cells were taken for immunofluorescence identification. The cells can express p63 and 6 α integrin, while CD31 and CD34 were negative (Fig. 1B), that suggested the cells we isolated were ESCs but not vascular endothelial cells. The results of cell flow cytometry analysis indicated that the p63 and 6 α integrin positive cells accounted for approximately 98.53% of the third-generation cells (Fig. 1C).

Rat ESCs improves wound closure and healing quality of SD rats

The third-generation rat ESCs were transfected with lentivirus to make the ESCs express EGFP (Fig. 1D). We used the ESCs that stably express EGFP for the following animal experiments. To investigate whether ESCs can influence the healing of wound bed in vivo, we implemented our experiments using the rats' dorsal wound model. As the results showed, compared with the control group, the ESCs group displayed a dramatically higher healing quality (Fig. 2A), lower residual wound area (Fig. 2B), and shorter healing time (Fig. 2C). These results suggested that ESCs spray could promote wound healing and improve the healing quality significantly.

Rat ESCs promotes the angiogenesis of wound

To assess the angiogenesis, the wound area sections on day 7 and day 14 were stained with CD31 for immunohistochemistry. Microvessel density (MVD) was assessed through CD31-positive cells at five areas randomly. As the results showed, the ESCs group displayed significantly higher MVD than the control group at both time point, and the CD31 expression was strong positive (Fig. 3A and B). Similarly,

the western blots of the wound snap-frozen samples on day 7 and day 14 also showed a markedly higher CD31 expression in ESCs group compared with the control group (Fig. 3C). All the results above demonstrated that ESCs could improve wound healing by accelerating angiogenesis.

Rat ESCs could differentiate into vascular endothelial cells

In order to explore the role of rat ESCs that sprayed on the wound surface in the process of wound healing, we have transfected rat ESCs with lentivirus in our previous experiments to make the ESCs express EGFP stably. We took tissue samples from the wounds of rats for immunofluorescence staining. The results suggested that on the 7th day, 14th day and even 21st day, ESCs that stably expressed EGFP can still be seen on the wounds of rats (Fig. 4A). The ESCs are mainly colonized in the subcutaneous tissue layer, and the ESCs can be seen to divide and proliferate (Fig. 4B). Immunofluorescence staining of blood vessels with CD31 can be seen that vascular endothelial cells are stained red, that is, CD31 expression is positive, and ESCs can be seen in CD31 positive cells (Fig. 4C). The above results show that the ESCs that sprayed on the wound surface can be colonized on the wound to divide and proliferate, and can differentiate toward the vascular endothelial cells to form a lumen structure.

Discussion

Various factors can cause the skin wounds, and the types and locations of wounds caused by different causes may also be different, so the treatment of wounds is various and difficult. Moreover, wound may affect the life quality of patients to varying degrees and increase the social medical burden. Among the factors that cause the wound non-healing, vascular regeneration disorder is one of the key factors. Therefore, a large amount of research is currently focused on vascular regeneration, hoping to promote wound healing by promoting the angiogenesis. A large number of studies have reported that increasing wound angiogenesis can significantly promote wound repair [15–19]. However, because wound repair is a pathophysiological process involving multiple factors, any unbalanced factors will cause abnormal wound healing. Therefore, wound repair is still a common problem in the world.

In recent years, the stem cell therapy has brought a new dawn for wound repair, because stem cells have the promising characteristics, like the ability of multi-directional differentiation and secreting paracrine growth factors. Adult stem cells are favored by scholars because of their immunocompatibility and ethical constraints. ESCs play an important role in skin repairment, for ESCs have the ability of self-proliferation and differentiation, and thus can promote wound healing and restore the normal epidermal structure and skin function [20–22]. A research found that Rhesus putative ESCs can trans-differentiate into corneal epithelium-like cells when cocultured with human corneal limbal stroma and corneal epithelial cells [23]. This meant that ESCs may have the potential for multi-directional differentiation under certain conditions.

In our research, We successfully isolated and expanded rat ESCs in vitro for treatment of wound. The results showed that rat ESCs can promote the healing of full-thickness skin defects in rats. Moreover, we found that rat ESCs can promote the angiogenesis in wound bed of the rats. In this experiment, we

transfected the rat ESCs with lentivirus to make the ESCs which we expanded in vitro to express EGFP stably. In this way, we can track the ESCs which sprayed on the wound bed. Even on the 21st day after the operation, ESCs that stably expressed EGFP can still be seen on the wounds of the rats. The ESCs are mainly colonized in the subcutaneous tissue layer, moreover, the ESCs proliferate in the wound bed. Furthermore, the ESCs that near the blood vessels can express CD31, which is the marker of the vascular endothelial cells. That meant ESCs can differentiate into vascular endothelial cells. Some ESCs can be seen around the vessels to form the vascular tubes. We therefore speculated that rat ESCs in the vicinity of blood vessels can differentiate into vascular endothelial cells, and can participate in the formation of lumen-like structures, which meant that rat ESCs might have partial functions of vascular endothelial cells.

Conclusion

To sum up, our study showed that rat ESCs can promote the formation of new blood vessels and accelerate wound healing in full-thickness skin defects in rats. The mechanism within is that rat ESCs can colonize and proliferate in the wound bed, and the rat ESCs near the blood vessels can differentiate into vascular endothelial cells to promote the angiogenesis. Our research provided a certain theoretical basis for ESCs to treat full-thickness skin defect wounds, and proved that ESCs are safe and effective to treat wounds even after in vitro expansion, and we found that ESCs can differentiate into vascular endothelium cells and even have the ability to form the vessel tubes under certain conditions. The mechanism of the effect of ESCs on wound healing and the multi-directional differentiation potential of ESCs are worthy of further study to further understand the therapeutic effects of ESCs and expand their therapeutic scope for diseases.

Abbreviations

ESCs: Epidermal stem cells; FN:Fibronectin; PBS:Phosphate buffered saline; K-SFM:Keratinocyte serum-free medium; EGFP:Enhanced green fluorescent protein; BCA:Bicinchoninic acid assay kit; CD31:Cluster of differentiation 31; CD34:Cluster of differentiation 34; GAPDH:Glyceraldehyde-3-phosphate dehydrogenase; IF:Immunofluorescence; IHC:Immunohistochemistry; WB:Western blot; MVD:Microvessel density.

Declarations

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No.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

JYZ and BT designed the research and acquired funding; SBH and ZCH isolated and cultured the ESCs, performed the animal experiments and drafted the manuscript; PW and XLC participated in the animal study; YXD participated in data analysis; PC and HLX performed the western blot analysis; and YZ participated in reviewing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures involving animals were approved by the Institutional Animal Care and Use Committees at Sun Yat-Sen University and conducted in accordance with the national guidelines on animal care.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

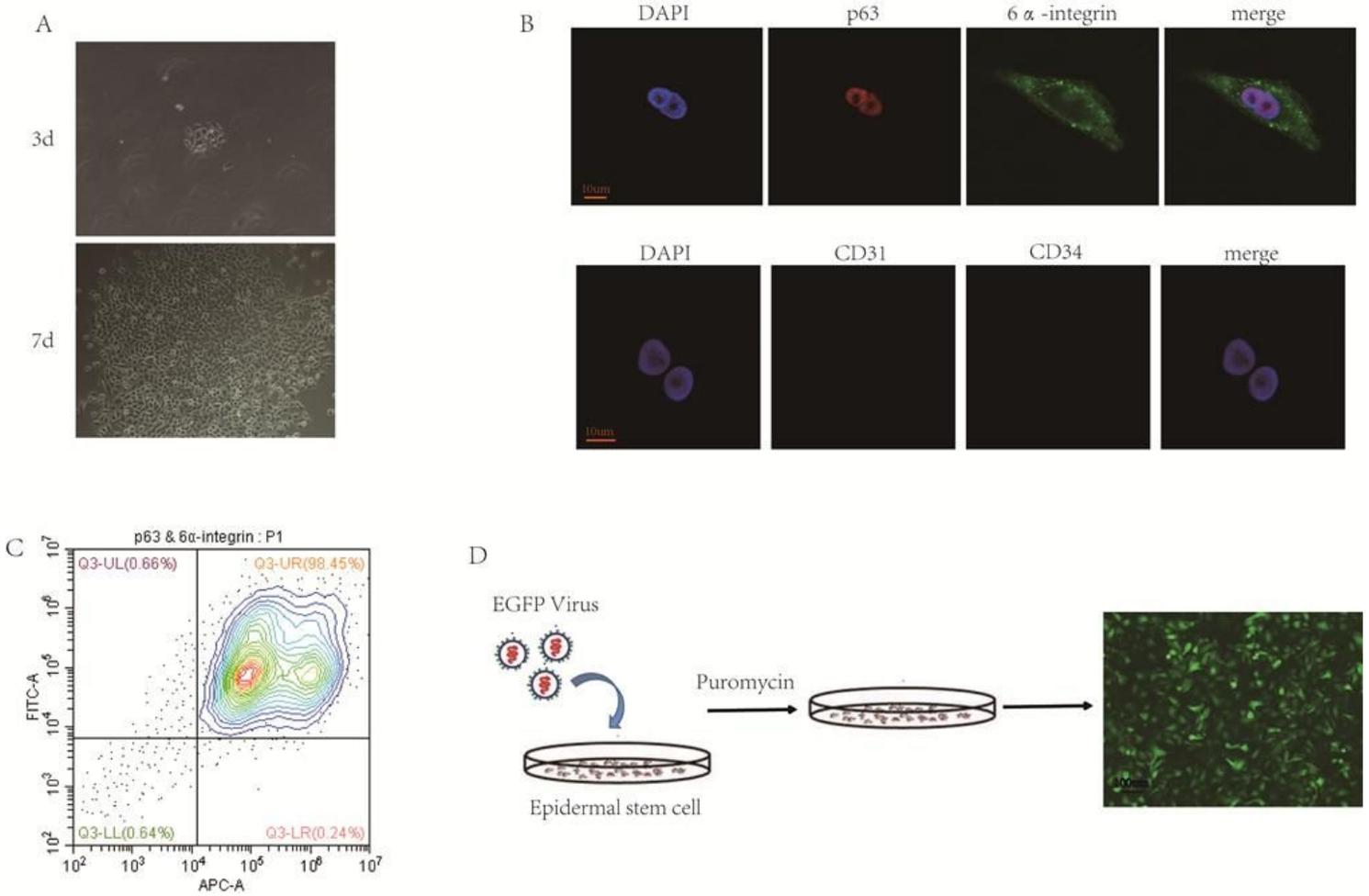


Figure 1

The isolation, identification and lentivirus transfection of rat ESCs. A) Rat ESCs morphology on 3rd and 7th day (10x microscope). B) p63, 6α integrin, CD31 and CD34 to identify the third-generation cells. C) Flow cytometry to detect the proportion of p63 and 6α integrin positive cells. D) The rat ESCs were transfected with lentivirus and stably express EGFP.

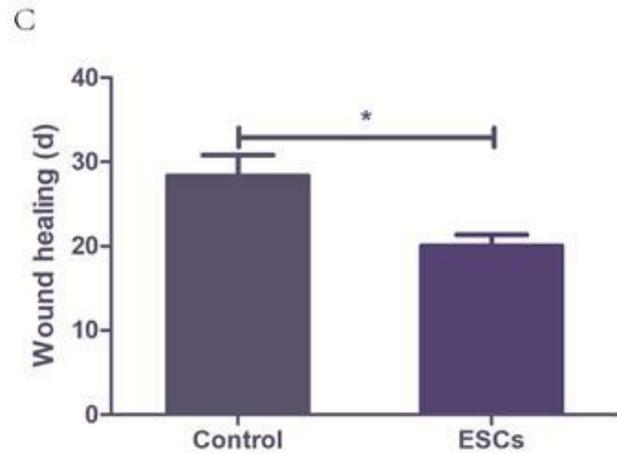
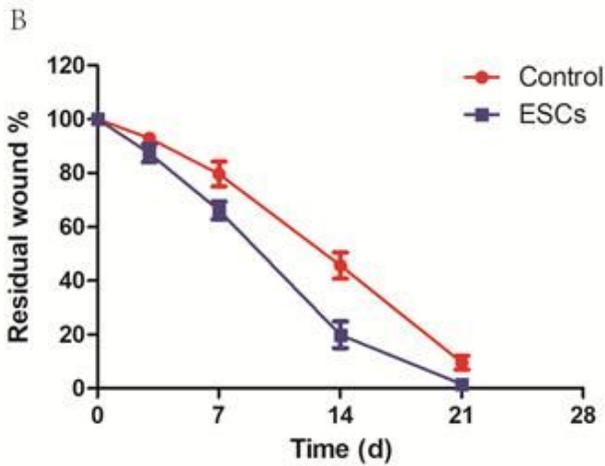
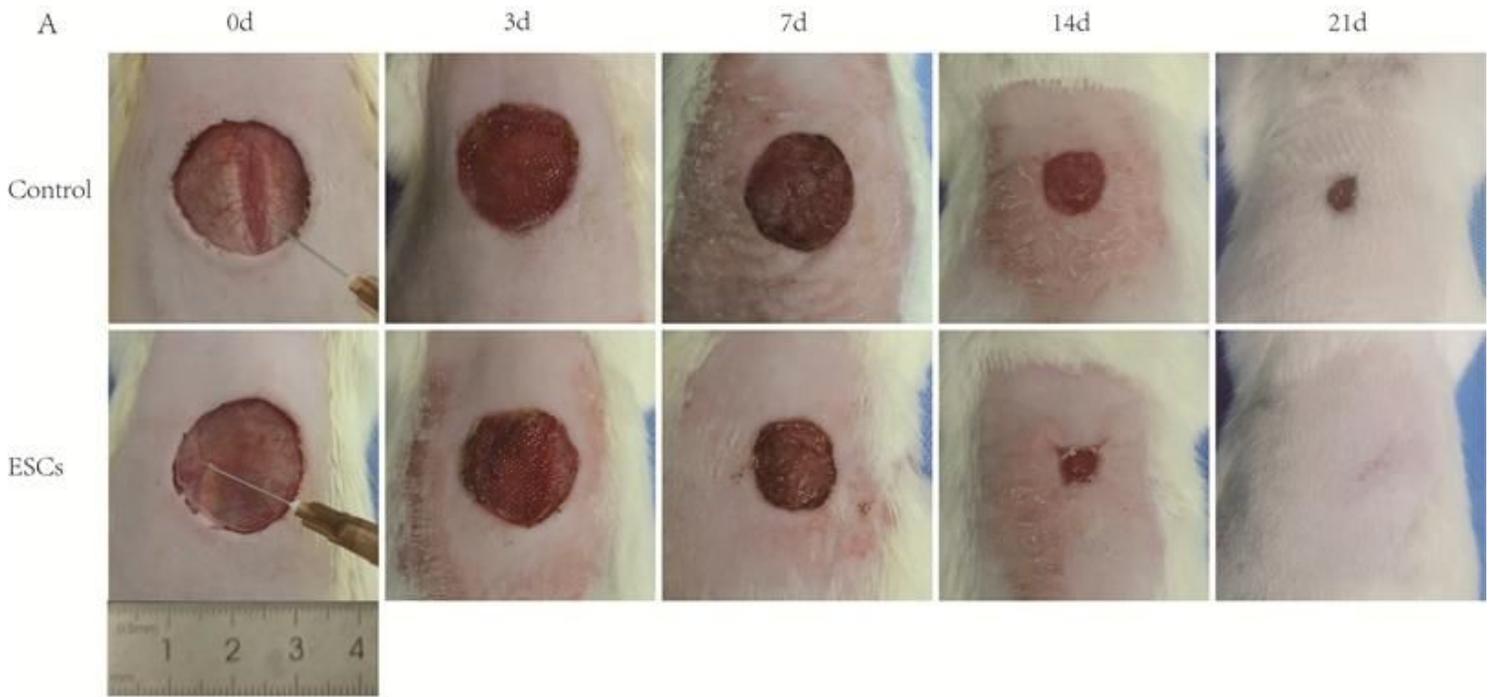


Figure 2

The rat ESCs accelerated wound closure and improved healing quality of rats. A) The wound pictures of rats' dorsal from negative control group and ESCs group were taken on post-injury days 0, 3, 7, 14, and 21. B) Residual wound rates of negative control group and ESCs group on post-injury days 0, 3, 7, 14, and 21. C) The completed wound healing time of negative control group and ESCs group. * $P < 0.05$.

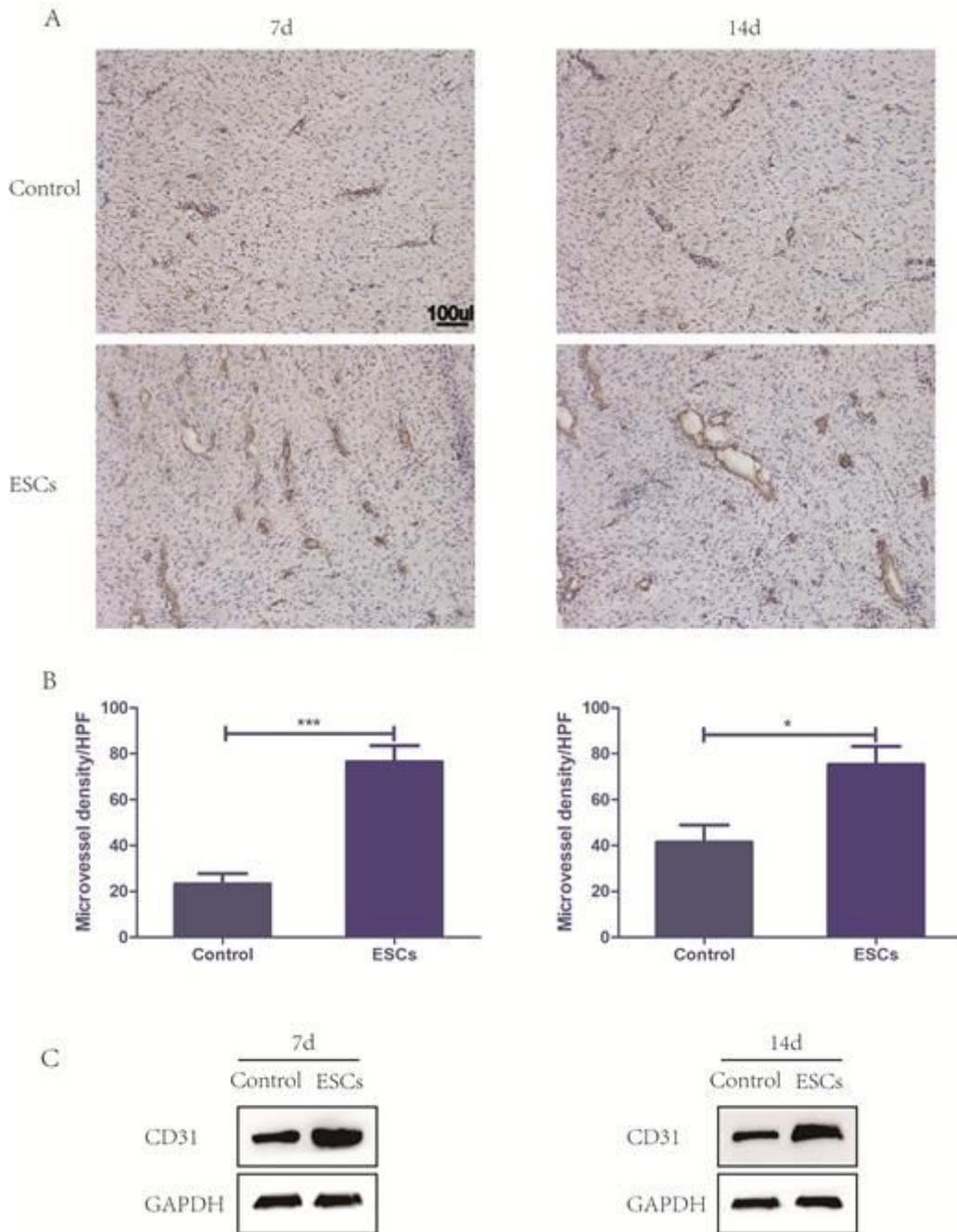


Figure 3

The expression of angiogenesis factors of the rats wounds on day 7 and day 14. A and B) Represent area and analysis of wound tissue sections stained with CD31 on post-injury day 7 and day 14 showing the microvascular regeneration in rats wound in negative control group and ESCs group. C) Representative western blot analysis showing relative protein levels of CD31 for each group at day 7 and day 14. *** $P < 0.0001$, * $P < 0.05$.

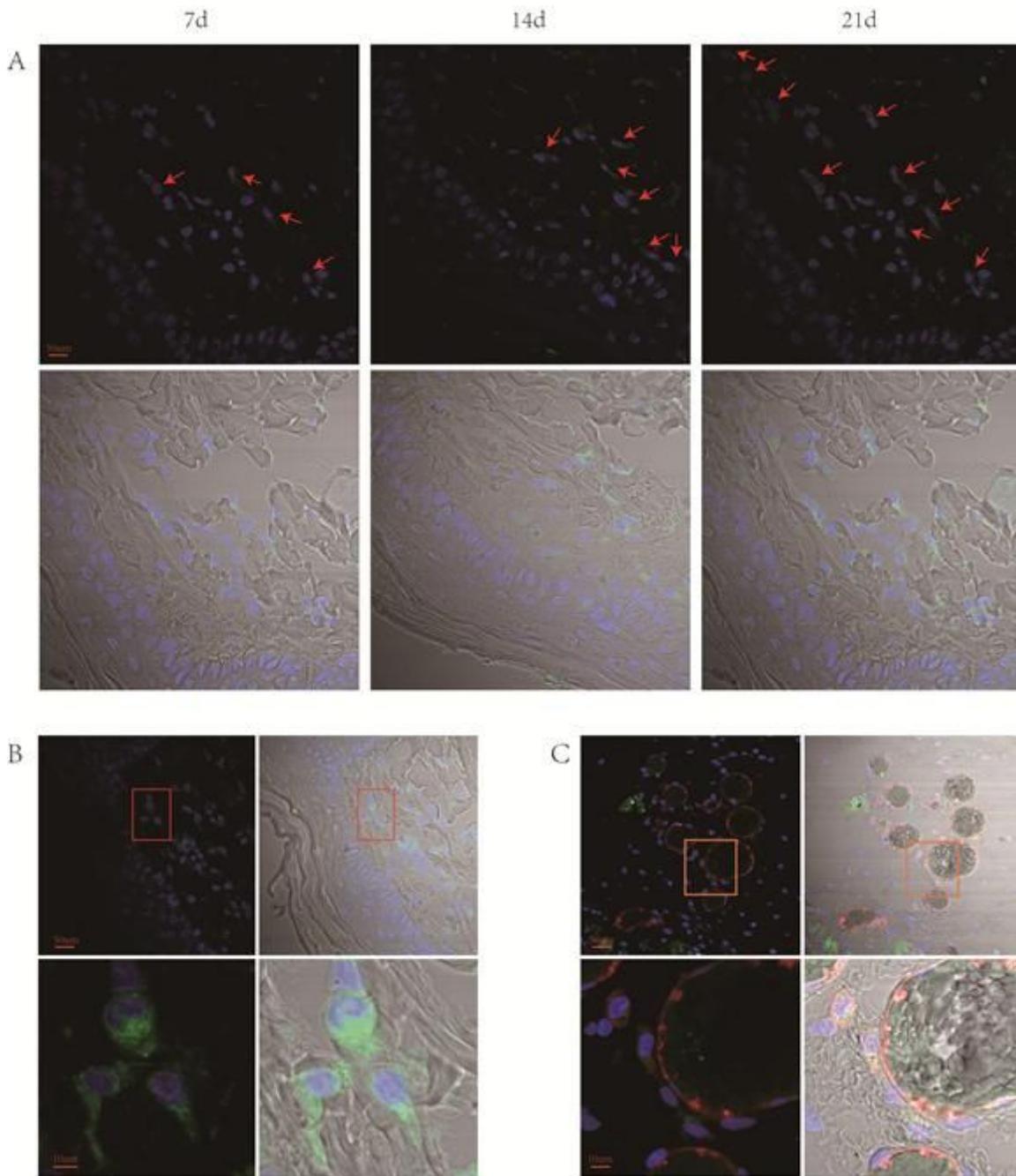


Figure 4

Tracking rat ESCs in the wound on post-injury day 14. A) The rat ESCs which expressed EGFP can be seen in the wound on the 7th, 14th and 21st day after the operation. B) The rat ESCs have a dividing and proliferating phase. C) The rat ESCs near the blood vessels can express CD31 and participate in the formation of lumen-like structures.