

# Adipose-derived stem cells-induced burn wound healing and regeneration of skin appendages in a novel skin island rat model

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## Abstract

**Background:** The study of effectiveness of adipose-derived stem cells (ASCs) in treating burn wounds is still a developing field. The process of wound contraction in areas of loose skin is a major confounding factor in the evaluation and study of burn wound healing in animal models.

**Methods:** To evaluate the effect of local ASCs administration, deep partial thickness burn wounds were induced by 30s application of hot copper plates in a novel skin island burn wound rat model to avoid interference from primary wound contraction. Skin islands were divided into two treatment groups—control group (n = 9) injected with PBS and ASCs-treated group (n = 9) injected with  $5 \times 10^5$  ASCs intradermally. Progress in wound healing was checked at regular intervals after injury (on 1st, 2nd, 3rd, and 4th week) by measuring the mean wound area and analyzing the wound histologically and immunohistochemically, after unstitching the overlying skin to expose the skin island.

**Results:** It was found that local intradermal injection of ASCs improved burn wound healing at all given time points when compared with control groups, especially in the first 2 weeks ( $p < 0.05$ ). The percentage of live follicles increased gradually in the ASCs-treated groups compared with control groups between the 3rd and 4th weeks ( $p < 0.05$ ). The vascular density and proliferating cell nuclear antigen index were also significantly increased in the ASCs-treated groups.

**Conclusion:** Thus, in this study, a novel burn wound rat model with reduced interference from wound contraction has been put forth to investigate the therapeutic effects of local administration of ASCs on burn wound healing. Local injection of ASCs not only improved burn wound recovery but also enhanced angiogenesis and skin appendage regeneration.

**Keywords:** Adipose-derived stem cell; Angiogenesis; Burn; Rat; Skin appendage; Wound healing

## 1. INTRODUCTION

Despite the known risk of mortality associated with severe burn injury, improved resuscitation and treatment of inhalation injury, appropriate infection control, and standardized critical care have improved the clinical outcome of severely burned patients in recent years. However, severe burn remains a devastating injury affecting nearly every organ and leading to significant morbidity and mortality.

Stem cell therapy has recently emerged as an innovative treatment for burn injuries, hypertrophic scar, or sepsis.<sup>1-3</sup> Literature survey reveals that mesenchymal stem cells (MSCs) contribute not only to local wound healing but also participate in systemic inflammatory responses. The severe burn-induced persistent inflammatory response increases susceptibility to infections and

sepsis, potentially leading to multiorgan failure and death. During major burn injury-induced inflammatory response, proinflammatory cytokines such as interleukin (IL)-1 $\beta$ , IL-6, IL-8, tumor necrosis factor (TNF)- $\alpha$  or interferon (IFN)- $\gamma$ , and anti-inflammatory cytokines such as IL-4, IL-10, and granulocyte-colony stimulating factor are released.<sup>4-6</sup> Several studies have reported that the levels of various cytokines are high in burn patients; increased cytokine levels are also related to sepsis and mortality in these patients.<sup>6</sup> MSCs are known to function at several levels of the inflammatory response, especially in the early stage of sepsis, to regulate a wide panel of inflammatory cytokines and inhibit leukocyte infiltration into several target organs.<sup>7,8</sup> MSC also contribute to skin appendage regeneration and wound healing through MAPK/ERK signal pathway.<sup>9</sup> Significant evidences from basic and clinical research supporting MSCs angiogenic role seen in the treatment of ischemic limbs, myocardial infarction, and retinopathy are emerging.<sup>10-12</sup> Recently, the use of adipose-derived stem cells (ASCs) has emerged in cell therapy. ASCs have the advantage of being easily and abundantly available from fat tissue extracted by liposuction. ASCs can differentiate into different lineages and stimulate the release of multiple growth factors.<sup>13</sup> Their therapeutic effects are being investigated for conditions such as degenerative knee, renal failure, hepatic failure, and myocardial infarction.<sup>14-17</sup> ASCs have found application in the engineering of various kinds of tissue, such as bone, auricular tissue, periodontal tissue, and tendon.<sup>18-21</sup>

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However, there are few studies reporting the application of ASCs in treating acute burn injuries and its effectiveness is still uncertain. In a systematic review, Conde-Green et al revealed that fat grafts and ASCs have beneficial effects on acute burn wound healing, but also pointed out the lack of decisive evidence.<sup>22</sup> Using a mouse model, Bliley et al showed that treatment with human ASCs improved healing of full-thickness burn wound observed as a reduction in wound area; ASCs treatment improved wound healing by enhancing vascularity, collagen deposition, and adipogenesis.<sup>3</sup> However, the effect of ASCs on skin appendage regeneration in burn injury has not been studied. Furthermore, efficient and cost-effective treatments that can accelerate burn wound healing, decrease the size of burn wound excision area, and reduce the tissue necrosis by preventing conversion of deep partial thickness to full-thickness burns do not exist.

Thus, the aim of our study is to investigate the effect of locally administered ASCs on deep partial thickness burn wound healing in rat. To alleviate interference from primary wound contraction in rodents, which is limited in the humans but could confound quantitative and qualitative evaluation of wound repair in these animal models,<sup>23</sup> we designed an innovative skin island burn wound model in rat and further used it to evaluate the wound healing and skin appendage regeneration after local injection of ASCs.

## 2. METHODS

### 2.1. Cell isolation and culture

Rat ASCs were derived from inguinal fat pad, which was isolated and cultured from Sprague Dawley (SD) rat (250–300 g, BioLASCO Taiwan Co., Ltd) as previously described.<sup>15,19</sup> The cell surface markers CD11B, CD29, CD31, CD45, and CD90 were analyzed by flow-cytometry (FACS Canto II; Becton Dickinson, Franklin Lakes, NJ, USA). The differentiation of ASCs into bone and adipose in the presence of the induction medium<sup>15</sup> was checked with von Kossa/Oil red O staining (Sigma-Aldrich, Taiwan) under light microscope (Olympus IX51, OLYMPUS Corporation, Tokyo, Japan). ASCs from passages three to six were used for local injection.

### 2.2. Skin island burn wound rat model

The study was approved by the Animal Care/Use Committee of Taipei Veterans General Hospital (IACUC2014-163). We created a contact burn injury model in SD rats. For this, the SD rat was anesthetized with Zoletil (0.1 mL/100 mg) by intraperitoneal injection. Fig. 1 illustrates the model nonlethal contact burn wound on the skin island on the back of the rat. A copper plate, 1 cm × 1 cm in size, heated to 90°C (by placing in boiling water), was applied for 30 s over the assigned depilated skin area on the rat's back. Each skin island had three such contact burn wounds, area of each of these wound units corresponded to the area of copper plate (1 cm × 1 cm). Post injury and after application of corresponding treatments the skin islands were embedded into the subcutaneous pockets by suturing the skin around circumferential incision, Fig. 1D. This model was used for the following two reasons—(1) reduction in number of experimental animals used and (2) interference by wound contraction and epithelium migration from the adjacent uninjured skin could be avoided by isolating the injured skin from adjacent skin by the circumferential incision.

### 2.3. Localized injection of ASCs into the rat burn wound

To evaluate the effect of locally injected ASCs on the healing of burn wounds, progress in healing were compared between control wounds and ASCs-treated burn wounds in the same animal. Two skin islands, each had three contact burn wounds, were designed

and divided into two groups—Control group (n = 3) and ASCs-treated (n = 3) wound groups (Fig. 1A). Thirty minutes after the application of contact burn protocol, the wounds in control group were intradermally injected with 0.2 mL of PBS, and the wounds in ASCs-treated group received intradermal injections of  $5 \times 10^5$  ASCs resuspended in 0.2 mL of PBS. Progress in wound healing was checked at different time intervals after treatment, ie, at 1st, 2nd, 3rd, and 4th weeks (three rats at each time-point; total 12 rats); the sutured skin island was opened and its gross appearance was imaged using a standardized digital photograph. The mean wound area was calculated using ImageJ (National Institutes of Health, Bethesda, MD, USA). At each time-point, nine whole-layer skin samples of contact burn wounds per group were obtained for histological (H&E) and immunocytochemical studies (CD31 and PNCA staining; Abcam). The total number of follicles and live follicles were measured under  $\times 200$  magnification. Vascular density and expression of proliferating cell nuclear antigen (PCNA) were evaluated.

### 2.4. Statistical analysis

The group differences were analyzed in SPSS 12.0 by using two-tailed paired Student's *t* test and one-way analysis of variance. The quantitative results are presented as mean  $\pm$  SD. *p* value  $< 0.05$  indicated statistical significance.

## 3. RESULTS

### 3.1. Characterization and differentiation of rat ASCs

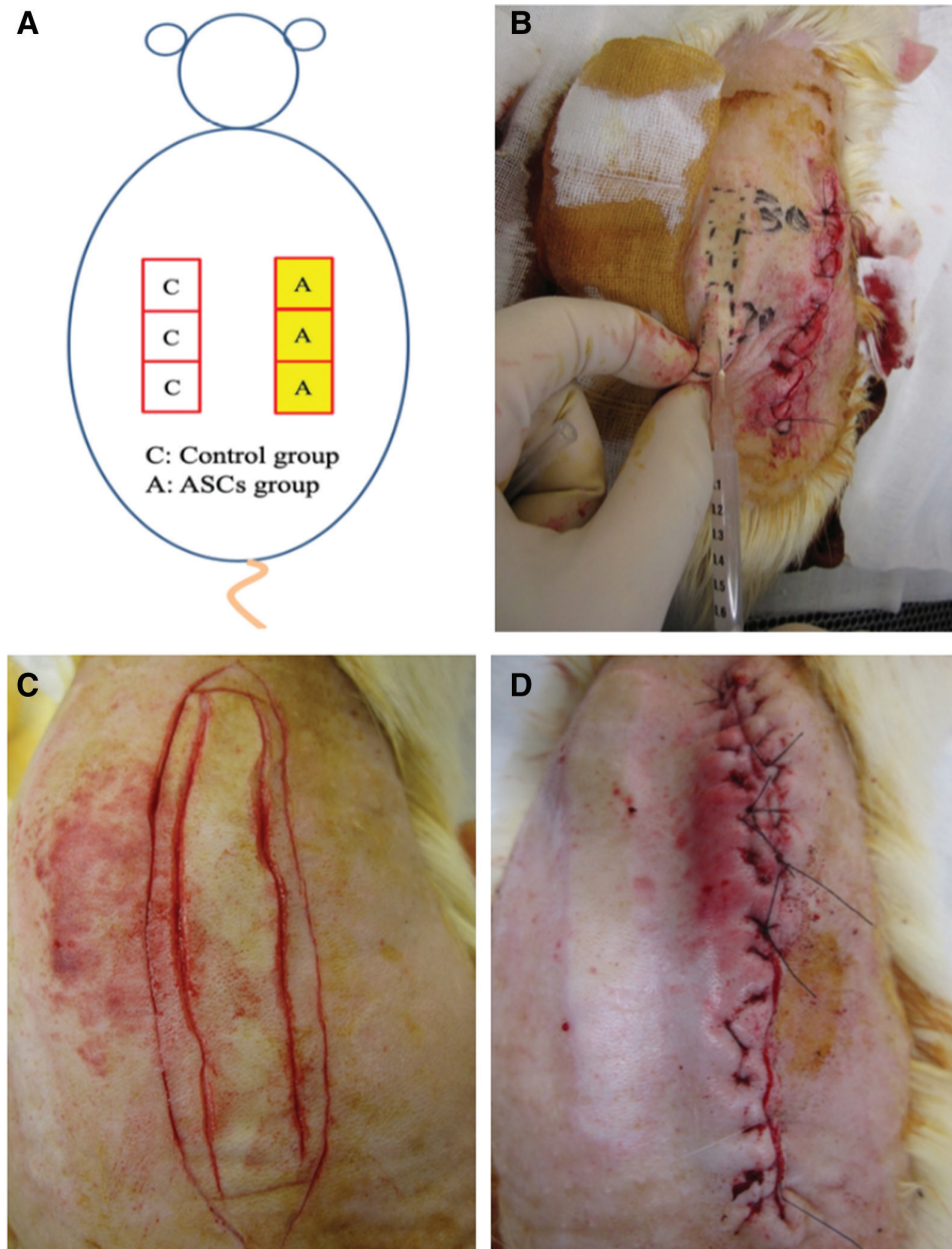
Harvested rat ASCs was passaged thrice and before being injecting into the rats, they were subjected to flow cytometric analysis and differentiation assays. We found that purified rat ASCs expressed high levels of CD29 and CD90, whereas CD11B, CD31, and CD45 (Fig. 2A) were not highly expressed. After induction of differentiation, rat ASCs were found to differentiate via adipogenesis and osteogenesis (Fig. 2B).

### 3.2. Confirmation of burn injury

In this study, in each of the 12 animals used for the experiments, a total of six deep partial thickness burn wounds were induced and were divided equally into control and ASCs-treated groups (ie, each rat had three control burn wounds and three ASCs-treated burn wounds); and observations on recovery were made at four different time-points (on 1st, 2nd, 3rd, and 4th week). During the entire period of the experiments, no wound infections or systemic adverse events were observed in any of the experimental animals. The depth of burn injury was confirmed by histopathological studies, which showed that necrotic tissue extended to reticular dermis indicating that partial-thickness burns were indeed induced by the protocol used in this study (Fig. 3).

### 3.3. Wound healing and skin appendage regeneration

To chart the course of burn wound healing, the sutured skin was opened at different intervals of time (at 1st, 2nd, 3rd, and 4th week) and gross appearance of the embedded skin islands was recorded. For comparison, the mean areas of the deep partial thickness contact burn wounds at four different time-points were measured. It was found that at all given time points the wound healing in ASCs-treated groups was improved as indicated by improved hair growth in ASCs-treated wounds as compared with control groups. This finding was true especially in the first 2 weeks ( $p < 0.05$ ) (Fig. 4A, B). To evaluate the effect of ASCs on wound healing, the follicular density was measured under  $\times 200$  magnification. It was found that between the 3rd and 4th weeks, the percentage of live follicles increased gradually in the ASCs-treated groups compared with control groups ( $p < 0.05$ ) (Fig. 4C).



**Fig. 1** A, The assignment and treatment protocols for nonlethal contact burn wounds on two strips of skin islands on the depilated rat back; each skin island had three burn wounds induced by 30 s application of heated copper plate, with each wound area corresponding to copper plate area (1 cm × 1 cm); B, Adipose-derived stem cells (ASCs)-treated group—three wounds in a skin island was locally intradermally injected with  $5 \times 10^5$  ASCs; Control group—the three wounds in another skin island in the same animal were injected with PBS; Progress in healing was checked regularly at four different time-points; experiments were carried out in triplicates, ie, observations were carried out on three rats (total  $n = 9$  for each group); C, After injection, the skin around the skin island was incised circumferentially to avoid wound contraction; D, Finally, the skin around the skin island was sutured with nylon thread thus in effect embedding the skin island in subcutaneous pocket.

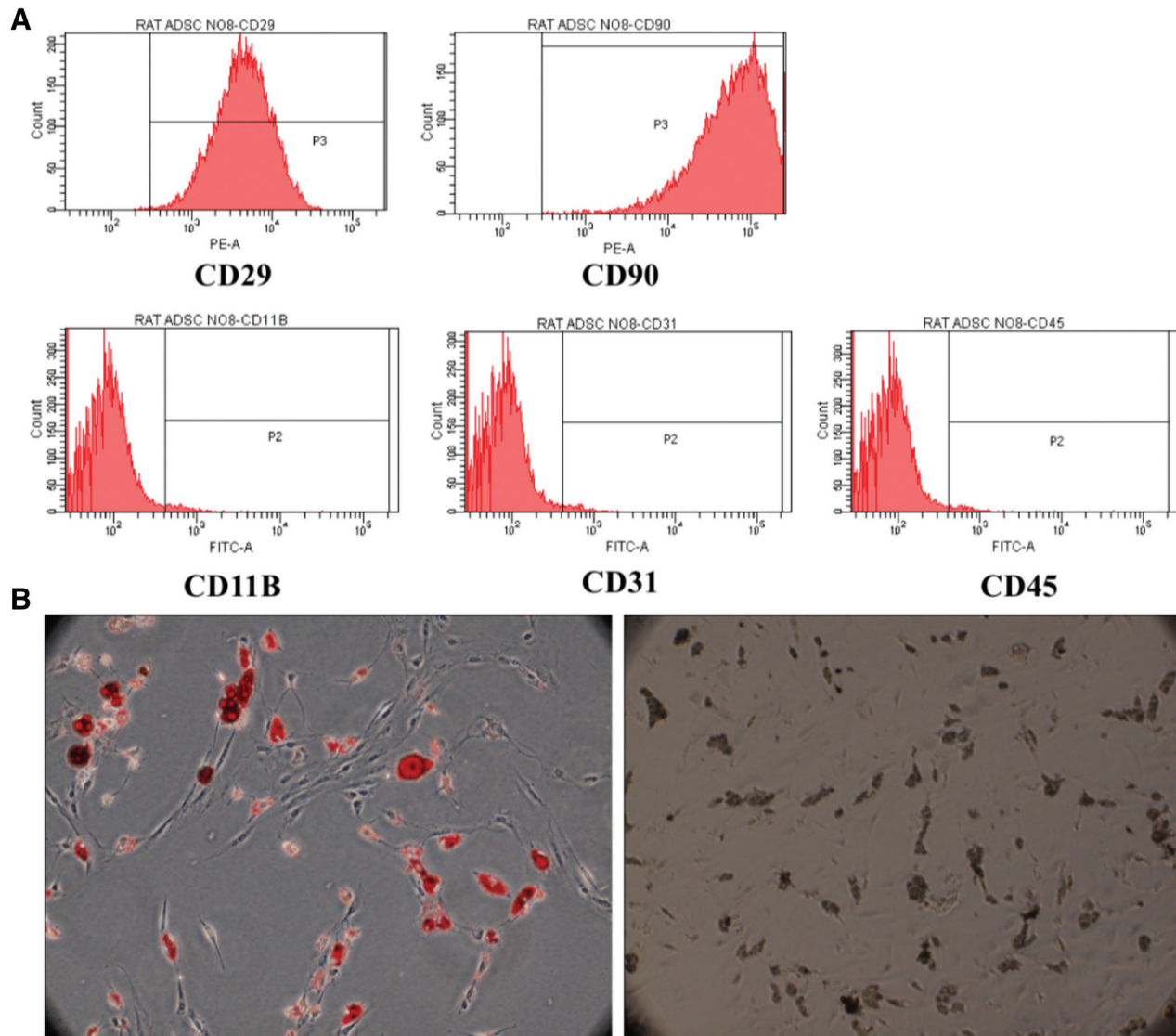
### 3.4. Mechanism of ASCs-mediated burn wound healing

The mechanism of ASCs-mediated wound healing was also investigated through CD31 and PCNA staining of the skin biopsy samples. Angiogenesis was observed in ASCs-treated group during the 3rd and 4th weeks post injury as indicated by the significantly higher number of CD31 positive cells and increased vascular density as compared with the control group ( $p < 0.001$ ). Significant increase in vascular density was observed in ASCs-treated groups beginning from 1st week of recovery (Fig. 5A). The nucleus of stromal cells including endothelial cells and fibroblasts were PCNA positive; PCNA positive cells were

counted to estimate PCNA index. PCNA index was significantly increased by the 3rd week in the ASCs-treated group compared with control group ( $p < 0.05$ ) (Fig. 5B).

## 4. DISCUSSION

In severe burn injuries, burn patients suffer from not only massive skin loss but also excessive systemic inflammatory responses. Delay in wound healing enhances risks of burn wound sepsis or infection; uninterrupted vicious inflammatory cycle increases mortality. Although considerable progress



**Fig. 2** A, The adipose-derived stem cells (ASCs) characterized by flow cytometry were positive for CD29 and CD90 surface staining (above) but not for CD11B, CD31, and CD45 surface staining (below); B, When induced, the ASCs exhibited adipogenic and osteogenic differentiation; Adipogenic differentiation forming lipid vesicles was confirmed with oil red O staining (left); Calcium deposits stained with von Kossa confirmed osteogenesis (right).

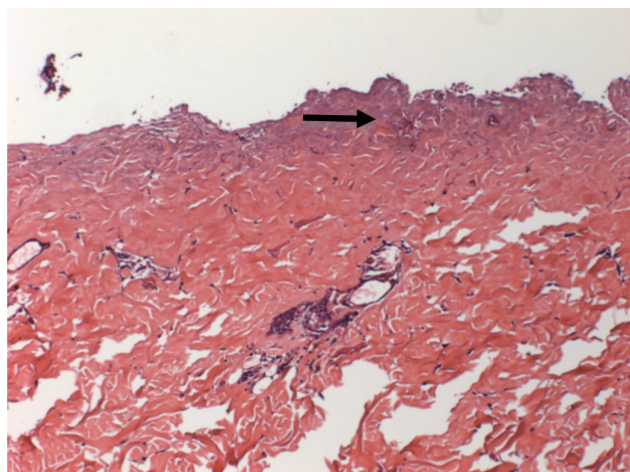
has been accomplished in burn treatment techniques such as synthetic dressing, debridement and skin grafting techniques, tissue-engineered skin substitutes, and topical growth factors application, methods to appropriately regenerate skin appendages and effectively prevent hypertrophic scarring are lacking.

Our skin island burn wound model was designed to alleviate interference from wound contraction in rodents. In previously studied models, mechanical fixation devices or splints have been employed to avoid wound contraction.<sup>24,25</sup> Although these methods were useful in keeping the wound volume relatively constant and thus allowing morphological or biomolecular quantification of wound response, many limitations remain. Using such mechanical devices is very complicated and due consideration must be given to application procedure and durability of the application. Moreover, these mechanical procedures are limited in stopping the normal influx of local cells to the wound site.<sup>25</sup> The skin island method designed here was found to be convenient and easily implementable. Furthermore, the circumferential incision prevented migration of cells and appendages

from adjacent healthy skin and also protected the wounds in the island from infection. This skin island wound model method for evaluating wound healing is novel and not mentioned in literature to date.

This study demonstrated that local injection of ASCs into burn wounds can be performed with ease in rat models; ASCs injection promoted appendage regeneration and wound healing. This indicates that local application could be an optional method as a less invasive cell therapy in future. From the observations that ASCs injection stimulated neoangiogenesis at the site of injury, we hypothesize that the grafted ASCs accumulate at the target site and subsequently either differentiate into endothelial cells or enhance angiogenesis through paracrine effect, leading to wound healing. It has been shown that ASCs can differentiate into endothelial cell and improve angiogenesis in critical limb ischemia model.<sup>26</sup> Further study might be helpful to elucidate the observed ASCs-mediated angiogenesis.

Three weeks after ASCs injection, a significant increase in the number of hair follicles was seen. Although there are reports



30 s

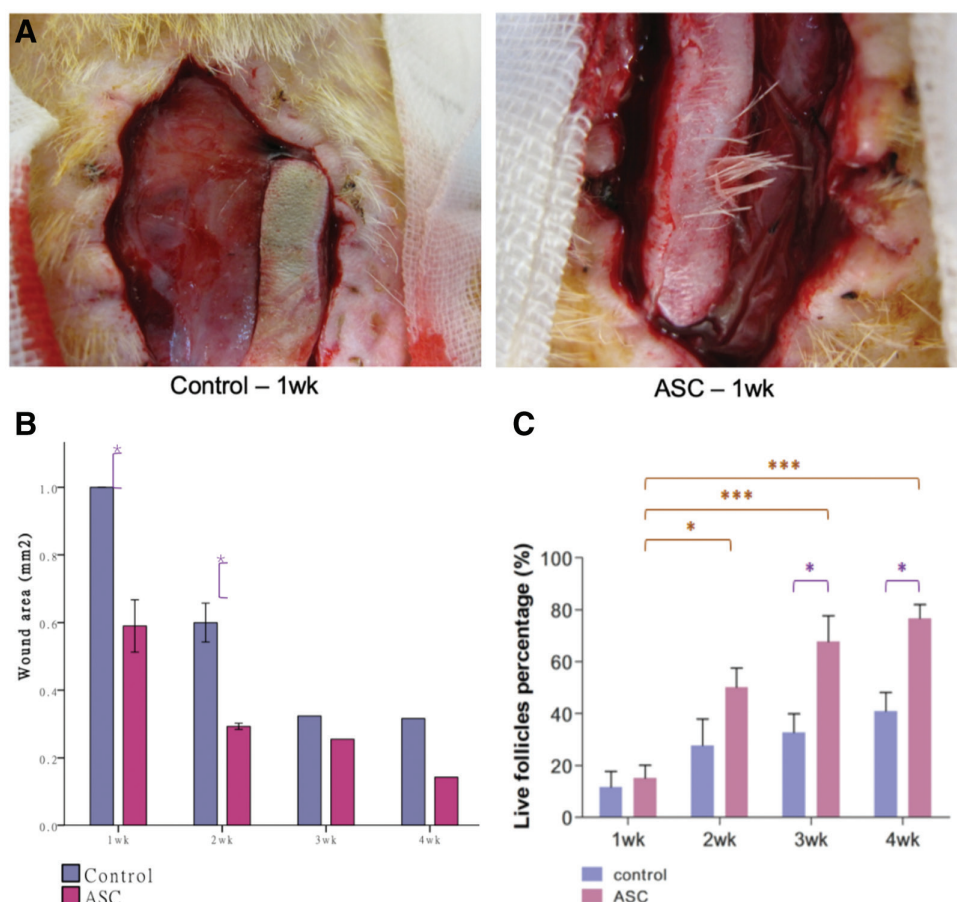
**Fig. 3** The depth of burn injury was confirmed by histopathological studies, which showed that necrotic levels involved the reticular dermis (arrows), indicating deep partial thickness burn wound.

indicating potential differentiation of stem cells into appendage progenitor cells during skin regeneration, the regenerative

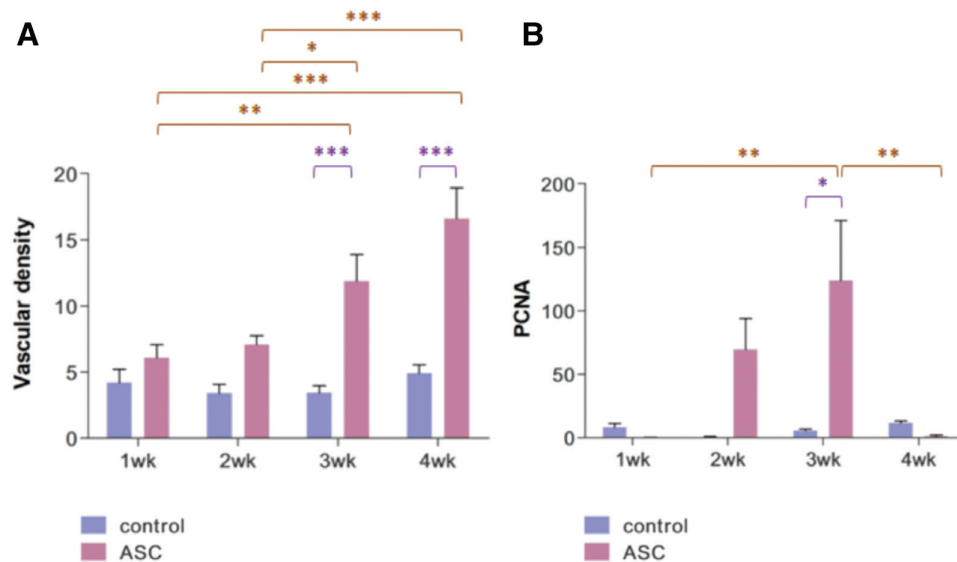
outcome of ASCs observed in this study was more likely due to their paracrine signaling.<sup>27,28</sup> The results of PCNA staining experiments indirectly confirm that ASCs may act by accelerating proliferation and differentiation of appendage progenitor cells. ASCs are known to secrete angiogenic growth factors such as HGF and VEGF, which in turn contribute to tissue repair through angiogenic and antiapoptotic activities.<sup>29,30</sup> When pre-incubated with bFGF in vitro, ASCs secrete other growth factors in abundance.<sup>30</sup> Taken together with the results from PCNA staining experiments, these reports led us to hypothesize that growth factors secreted from transplanted ASCs is responsible for the skin appendage regeneration seen here.

The mechanism of ASCs in promoting tissue repair is still insufficiently investigated. It is known that ASCs can modulate adverse inflammatory reactions to give positive outcomes. For example, ASCs facilitate tissue regeneration by suppression of TNF- $\alpha$ , IL-6R, and IL-12b.<sup>31</sup> But TNF- $\alpha$  plays a role on ASCs proliferation, increased expression of proangiogenic factors (FGF-2, VEGF, IL-8, and MCP-1) and inflammatory cytokines (IL-1 $\beta$ , IL-6), and activation of angiogenic and regenerative potential of ASCs.<sup>32</sup> In tissue injuries occurring under acute hypoxic stress conditions, ASCs could upregulate HIF-1, stimulate vascular endothelial growth factor and resistance to significant oxygen deprivation.<sup>33</sup>

Depending on the anatomy and the extent of damage in the given tissue and organ, the commonly used ASCs application



**Fig. 4** A, As early as the first week after burn injury, wound healing was improved in adipose-derived stem cells (ASCs)-treated group with more hair growth when compared to control group; B, A greater decrease in the mean wound area of deep partial thickness burn wounds and better healing was observed in ASCs-treated group when compared to control group, especially in the first 2 weeks after injury; C, The percentage of live follicles, observed under light microscope, increased gradually in ASCs-treated group, compatible with gross findings (statistical significance is indicated as follows: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).



**Fig. 5** A, Local injection of adipose-derived stem cells (ASCs) increases vascular density and angiogenesis in deep partial thickness contact burn wound; B, Proliferating cell nuclear antigen (PCNA) index showed significant increase in the third week in the ASCs-treated group compared with control group (statistical significance is indicated as follows: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

routes are either local injections or systemic administration. Evidence is building for the role of paracrine effects of ASCs in its therapeutic relief, rather than for its effectiveness through local implantation and direct differentiation.<sup>34,35</sup> Our previous studies have shown that ASCs also exhibit antioxidative and antiapoptotic effects in rescuing tissue from acute ischemic injury.<sup>15</sup> In addition, ASCs may secrete soluble factors such as CINC-1, which activate ERK1/2 and Akt phosphorylation, leading to improved cell survival, tissue repair, and appendage recovery.<sup>9</sup> Thus, local injection of ASCs has potential in skin appendage recovery and wound repair.

Delivery of ASCs via various routes is dependent on the “native” homing of ASCs to the site of disease or injury.<sup>34,36</sup> Venous injections of ASCs have been used in treating bladder dysfunction, erectile dysfunction, chronic kidney disease, and so on.<sup>34,37,38</sup> Intraarterial injection has also proved to be an effective route of systemic administration. ASCs can be implanted through renal arteries to suppress acute rejection after kidney transplantation.<sup>39</sup> Cerebral artery occlusion could also be ameliorated by injection of ASCs via carotid artery.<sup>36</sup> However, regardless of administration route, the therapeutic efficacy depends on ensuring that sufficient ASCs reside in the damaged area.

Here,  $5 \times 10^5$  ASCs transplanted into the subcutaneous space survived in the injection sites throughout the follow-up period, with a slow transition to deeper subcutaneous adipose tissue layers.<sup>40</sup> There have been conflicting reports on the number of ASCs used and the results obtained with regards to local ASCs injection. Bliley et al found one-time application of  $6.8 \times 10^6$  ASCs to be effective in subeschar injection; increased vascularity, collagen deposition, and dermal adipogenesis were noted following ASCs treatment.<sup>3</sup> In contrast, Loder et al showed that local injection of  $1 \times 10^6$  ASCs improved burn wound healing in terms of wound area, wound depth, and apoptotic activity in a mouse model.<sup>41</sup> Chang et al demonstrated the therapeutic efficacy of autologous ASCs in treating acute burn wounds in rats by local injection of  $5 \times 10^6$  ASCs.<sup>42</sup> Further, Kuo et al injected  $1 \times 10^7$  ASCs subcutaneously in full-thickness wound of diabetic rats and found significant decrease in wound healing time.<sup>43</sup> Furthermore, Hanson et al showed that  $1 \times 10^6$  ASCs, intradermally injected, could improve partial-thickness cutaneous wound

healing in porcine model.<sup>44</sup> However, another study by Karimi et al reported no significant improvement utilizing  $1 \times 10^6$  ASCs treatment in acute burn wound healing ( $p > 0.05$ ).<sup>45</sup> Poor results in the above reports may be due to disruption of delicate micro-environment and inflammation and multifocality of many disorders.<sup>46</sup> Therefore, optimizing cell delivery is a critical component of a successful cell-based therapy. Hydrogel and collagen scaffold delivery of stem cells have been shown to significantly enhance wound healing.<sup>47–50</sup> Further investigations into various routes of cell-delivery including cell-seeded skin substitution needs to be conducted with a view to improve treatment outcomes.

There are several limitations in the present study. Except route of cell delivery and cell engraftment rate, another limitation is the size and depth of the burn wounds. Since the immune environment may substantially differ between major and minor burn wounds, future studies of larger and deeper burn wounds that are better representations of the typical clinical scenario are needed to further evaluate the therapeutic efficacy of ASCs in clinical practice. Our experiments indicated that ASCs could improve the wound healing process by angiogenesis and skin appendage regeneration. However, the detailed mechanism of ASCs is beyond the scope of this study and to be elucidated. In particular, it is unclear whether these ASCs differentiate into endothelial cells, for example, and become part of the vasculature, or if they play a different supportive role at the wound site in promoting neovascularization and wound healing. Currently, our laboratory is conducting further work that addresses this question.

In conclusion, using the new skin island burn wound rat model, our study demonstrates that local injection of ASCs could have beneficial therapeutic effects in treating burn wounds. Local injection of ASCs not only improved burn wound recovery but also enhanced skin appendage regeneration. The healing mechanism may involve ASCs-mediated paracrine stimulation of neoangiogenesis and inhibition of apoptosis.

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