

## Skin-Derived Precursor Cells Promote Wound Healing in Diabetic Mice

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**Background:** Impaired wound healing as one of the complications arising from diabetes mellitus is a serious clinical issue. Recently, various cell therapies have been reported for promotion of wound healing. Skin-derived precursor cells (SKPs) are multipotent adult stem cells with the tendency to differentiate into neurons. We investigated the potency of promoting diabetic wound healing by the application of SKPs.

**Methods:** Skin-derived precursor cells isolated from diabetic murine skin were cultured in sphere formation medium. At passage 2, they were suspended in phosphate-buffered saline (PBS), and applied topically to full-thickness excisional cutaneous wounds in diabetic mice. Application of PBS served as controls (n = 21 for each group; n = 42 total).

Time to closure and percentage closure were calculated by morphometry. Wounds were harvested at 10 and 28 days and then processed, sectioned, and stained (CD31,  $\alpha$ -smooth muscle actin, and neurofilament heavy chain) to quantify vascularity and neurofilaments.

**Results:** Wounds treated with SKPs demonstrated a significantly decreased time to closure (18.63 days) compared with PBS-control wounds (21.72 days,  $P < 0.01$ ), and a significant improvement in percentage closure at 7, 10, 14, and 18 days compared with PBS-control wounds ( $P < 0.01$ ). Histological analysis showed that the *Capillary Score* (the number of vessels/mm<sup>2</sup>) was significantly higher in SKP-treated wounds at day 10 but not at day 28. *Nerve Density* (the number of neurofilaments/mm<sup>2</sup>) had increased significantly in SKP-treated wounds at day 28 compared with control group. Some applied SKPs were stained by neurofilament heavy chain, which demonstrates that SKPs directly differentiated into neurons.

**Conclusions:** Skin-derived precursor cells promoted diabetic wound healings through vasculogenesis at the early stage of wound healing. Skin-derived precursor cells are a possible therapeutic tool for diabetic impaired wound healing.

**Key Words:** skin-derived precursor cells, SKPs, wound healing, diabetes mellitus, diabetic foot ulcer

(*Ann Plast Surg* 2014;73: 00–00)

Complications arising from diabetes mellitus have become serious public health issues, of which one such complication is impaired wound healing.<sup>1</sup> Chronic wounds, particularly diabetic foot ulcers, respond poorly to conventional treatment, making them very difficult to manage. Also in terms of economic burden, diabetic foot ulcers are serious issues all over the world.<sup>2</sup> Direct cost estimates (in 2010-

adjusted to US dollars) range from US\$3096<sup>3</sup> for a Wagner grade 1 lesion to US\$107,900 for an ulcer resulting in amputation.<sup>4</sup>

The pathophysiologic relationship between diabetes and impaired wound healing is complex. Vascular, neurologic, immunologic, and biochemical abnormalities contribute to impairment of tissue restoration. Severely impaired activities of key cells, growth factors, and cytokines for wound healing are other important factors in diabetic ulcers.<sup>5–7</sup>

To treat such a disruption of the wound-healing mechanism, various stem cell therapies have been studied recently. Adult stem cells derived from various tissues have a broader potential or plasticity, and could be used in autologous cell-replacement therapies. Cell sources such as mesenchymal stem cells (MSCs),<sup>8–11</sup> endothelial progenitor cells,<sup>12</sup> and adipose-derived stromal cells<sup>13</sup> have been reported for the cell therapies of diabetic delayed wounds in vivo.

As a practical therapeutic approach, the skin has many advantages as a potential stem cell source, including easy accessibility and high self-renewal ability. Rheinwald and Green<sup>14,15</sup> and O'Connor et al<sup>16</sup> first reported autologous cultured epithelial sheets using human keratinocytes and demonstrated their use in the clinical treatment of burns.

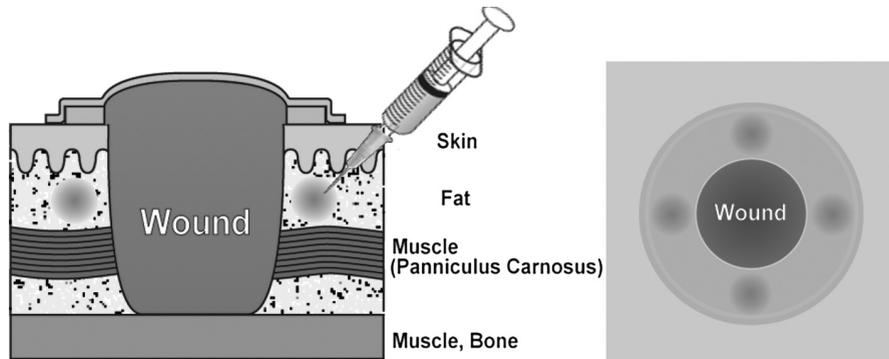
Toma et al<sup>17</sup> demonstrated that stem cells can also be isolated from the dermis and expanded in vitro, which were designated as



**FIGURE 1.** Excisional wound model. Two 8-mm full-thickness excisional wounds were created bilaterally in the dorsal skin of diabetic mice. A 16-mm silicon splint was placed concentric with the wound and affixed using cyanoacrylate glue and 6–0 nylon sutures. The stents prevented wound contraction.

Received April 2, 2014, and accepted for publication, after revision, July 23, 2014. From the Departments of \*Plastic and Reconstructive Surgery, and †Cardiac Surgery, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan. Conflicts of interest and sources of funding: Supported by Japan Society for the Promotion of Science KAKENHI [Grant-in-Aid for Scientific Research (C)] Grant 23592645.

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**FIGURE 2.** Left, Topical application of SKPs to wound bed. SKPs suspended in PBS were injected into the subcutaneous layer. Right, SKP-suspended PBS and control PBS were injected at 4 points around the wound as shown in the figure.

skin-derived precursor cells (SKPs). Skin-derived precursor cells have similarities to stem cells of the embryonic neural crest, and can differentiate into a variety of neural and mesodermal cell phenotypes, including peripheral neurons, glial cells, smooth muscle cells, osteoblasts, chondrocytes, and adipocytes.<sup>18,19</sup> Several reports mentioned the characteristic tendency of differentiation into neurons and tried to use the regenerative therapy for peripheral and central nervous dysfunctions.<sup>20–22</sup>

Skin-derived precursor cells have never been reported as a stem cell source for the treatment of diabetic wounds. We hypothesize that SKPs can promote diabetic wound healing through their multipotency, just as other adult stem cells. Diabetic peripheral neuropathy is known to be one important factor to cause diabetic foot ulcer. Therefore, the tendency of SKPs differentiating into neurons might contribute to the treatment of diabetic neuropathy and lead to better wound healing compared with other cell sources.

In this study, we used the diabetic murine model of excisional wound healing which revealed significantly delayed wound healing compared to normoglycemic mice,<sup>23</sup> and investigated whether SKPs promoted wound healing.

## MATERIALS AND METHOD

### Cell Isolation

All experimental procedures were approved by the Division of Experimental Animals of Nagoya University.

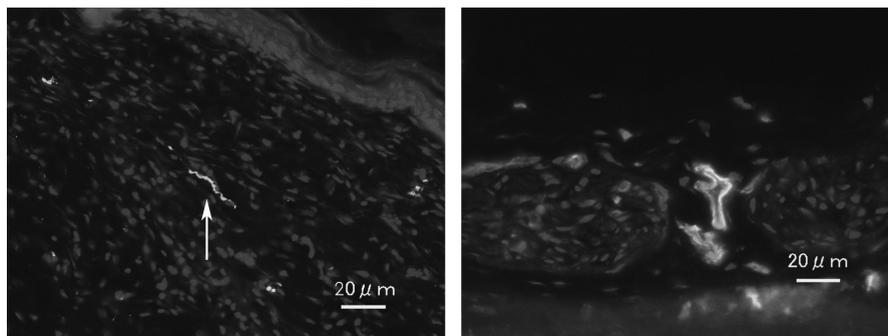
Skin-derived precursor cell culture was previously described by Toma et al.<sup>17</sup> Tissues for these studies were derived from diabetic mice. Adult diabetic mice (C57BLKS/J Iar- + Lepr<sup>db</sup>/+ Lepr<sup>db</sup>) aged

10 weeks were obtained from Chubu Kagaku Shizai (Nagoya, Japan). The mice had symptoms of obesity and impairment in wound healing.<sup>24,25</sup> Skin from the abdomen and back of adult mice was harvested, cut into 2 to 3 mm<sup>3</sup> pieces and digested with 0.1% trypsin for 40 minutes at 37°C. Tissue pieces were washed with medium [Dulbecco modified Eagle medium-F12 3:1, 1 µg/mL fungizone (both from Gibco-BRL, Grand Island, NY), 1% penicillin/streptomycin (BioWhittaker, Walkersville, Md)] containing 10% fetal bovine serum (Sigma-Aldrich, St Louis, Mo), and twice with serum-free medium. Skin pieces were then mechanically dissociated in medium, and the suspension poured through a 40-µm cell strainer (BD Biosciences, San Diego, Calif). Dissociated cells were centrifuged at 168g and resuspended in 10-mL medium containing B-27 (Gibco-BRL), 20 ng/mL epidermal growth factor (both from Collaborative Research, Bedford, Mass), and 40 ng/mL basic fibroblast growth factor (Kaken Pharmaceutical Co, Ltd, Tokyo, Japan). Cells were cultured in 25-cm<sup>2</sup> tissue-culture floating flasks (Corning, Lowell, Mass) in a 37°C, 5% CO<sub>2</sub> tissue-culture incubator.<sup>17</sup> Under the cultural condition, these dermal cells proliferate to form “spheres” in suspension. Skin-derived precursor cells were passaged by mechanically dissociating spheres and splitting 1:3 with 75% new medium and 25% conditioned medium from the initial flask. Cells were passaged every 6 to 7 days.

Before the application, cultured SKPs were mechanically dissociated and labeled with PKH26 (Sigma-Aldrich).

### Study Design

All experiments used a wound-healing model developed and described previously (Fig. 1).<sup>26,27</sup> Animals were anesthetized, shaved,



**FIGURE 3.** Left, High-power field of SKP-treated wound section stained by anti-NF-H antibody. Filamentous structures (the white arrow) were confirmed and counted as *neurofilaments* in wound area. Right, High-power field of SKP-treated section immunofluorescently stained by anti-CD31 and anti- $\alpha$ -SMA antibody. Some vessels showed double-stained appearance by CD31 and  $\alpha$ -SMA. Tubular structures were counted as the *Capillary Score*.

and prepared according to standard sterile procedure. An 8-mm punch biopsy tool was used to create 2 circular, full-thickness cutaneous wounds (extending through panniculus carnosus) bilaterally on the shaved dorsal skin of diabetic mice. A donut-shaped silicone splint (Tokyu Hands, Inc, Tokyo, Japan), with an external diameter of 16 mm and an internal diameter of 8 mm, was centered on the wound and affixed using cyanoacrylate adhesive (Toagosei Co Ltd, Tokyo, Japan) and interrupted 6-0 nylon sutures (Ethicon, Inc, Somerville, NJ). A semiocclusive dressing (Tegaderm; 3M, St Paul, Minn) was applied to cover the wound.

The wounds were randomly divided into 2 groups: 100  $\mu$ L of phosphate-buffered saline (PBS) application as control group and  $1.0 \times 10^6$  cells of SKPs suspended with 100  $\mu$ L of PBS application as SKP-treated group.

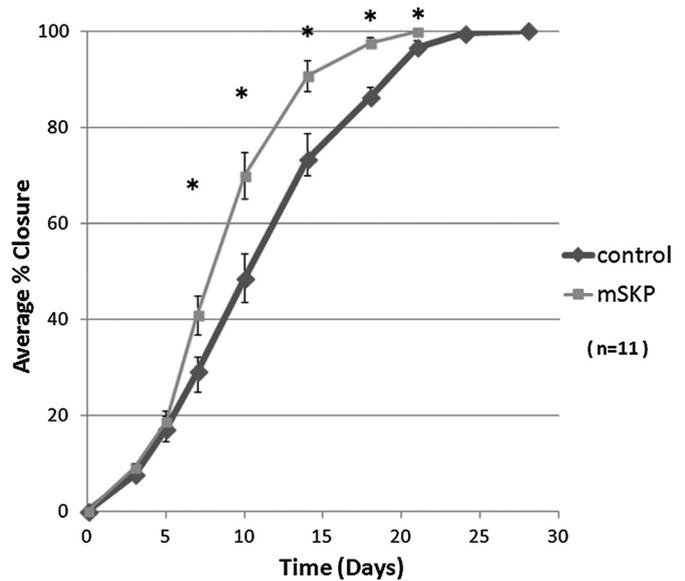
Cultured SKPs were injected into the subcutaneous layer around the dorsal wounds (Fig. 2). Ten wounds of each group (20 wounds) were harvested at postoperative day 10, and 11 wounds of each group (22 wounds) were harvested at postoperative day 28, by which time all the wounds had already closed. Wounds were excised with 2-mm margin beyond the wound edge. Each sample was placed in optimal cutting temperature medium and processed for frozen sections.

### Analysis

Digital photographs were taken at days 0, 3, 5, 7, 10, 14, 18, 21, 28, and beginning on the day of wounding. Photographs were acquired with a 10-megapixel digital camera (Canon Inc, Tokyo, Japan) from a distance of 5.0 cm, with the lens parallel to the wound. Time-to-closure was defined as the number of days for complete reepithelialization. Wound area was measured using digital selection by the public domain software Image J ver.1.47 (NIH, Bethesda, Md). Percentage wound closure was calculated as  $[1 - (\text{wound area})/(\text{original wound area})] \times 100\%$ .

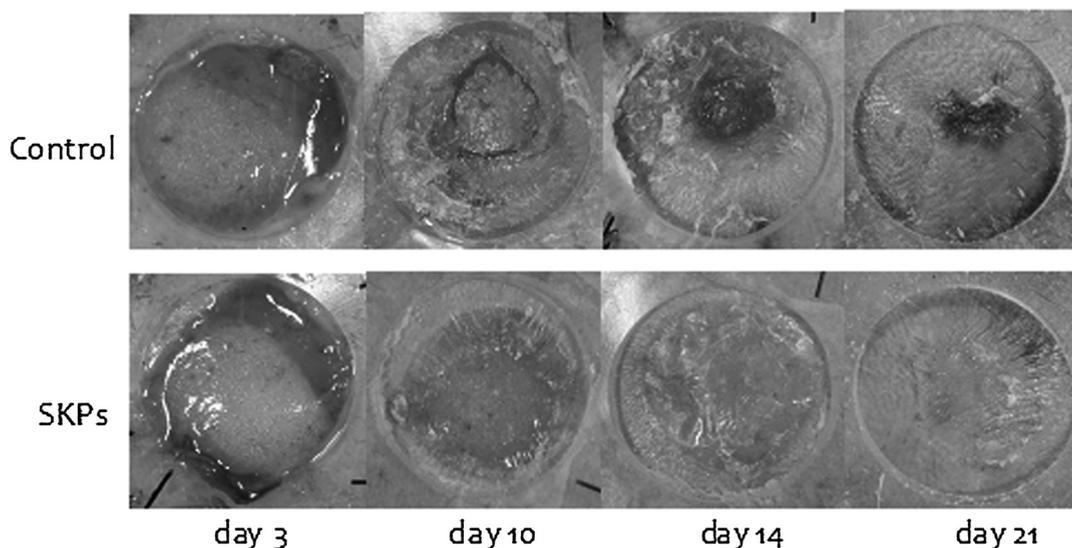
Serial 8- $\mu$ m frozen sections were stained with DAPI (4', 6-diamidino-2-phenylindole) (Vector Laboratories Inc, Burlingame, Calif) and analyzed by immunohistochemistry.

To identify endothelium and vascular smooth muscle cells, we used rat antimouse CD31 antibody (eBioscience, San Diego, Calif) and rabbit antimouse  $\alpha$ -smooth muscle cell actin ( $\alpha$ -SMA) antibody (Millipore, Billerica, Mass) as the primary antibodies. As secondary



**FIGURE 5.** Percentage wound closure. Wound healing is expressed as percentage closure (mean  $\pm$  standard error) relative to original size  $[1 - (\text{wound area})/(\text{original wound area}) \times 100]$ . SKP-treated wounds featured greater percentage closure at all time points (with significance at 7, 10, 14, and 18 days compared with PBS controls). By day 21, almost 100% of SKP-treated wounds were completely closed, whereas PBS controls took more than 28 days to completely close all the wounds.

antibodies, we used Alexa Fluor 647 conjugate rabbit anti-rat IgG antibody (Invitrogen, Carlsbad, Calif) and Alexa Fluor 488 conjugate donkey anti-rabbit IgG (Invitrogen). Vessels showed double-stained appearance by anti-CD31 and  $\alpha$ -SMA antibody. Tubular structures stained by anti-CD31 and  $\alpha$ -SMA antibody in each wound area were counted, and we calculated the *Capillary Score* (the number of vessels/ $\text{mm}^2$ ) (Fig. 3).



**FIGURE 4.** Time course of wound closure. Serial photographs allowed longitudinal assessment of percentage closure and time to closure for each group. Differences between SKP-treated wounds and PBS controls were significant at all time points from day 7 until day 21.

In addition, to identify neurofilaments, we used rabbit anti-mouse neurofilament heavy chain (NF-H) antibody (Novus Biologicals, Littleton, Colo) as the primary antibody and Alexa Fluor 488 conjugate donkey anti-rabbit IgG (Invitrogen) as a secondary antibody. We counted filamentous structures stained as neurofilaments in each wound area and calculated the *Nerve Density* (the number of neurofilaments/mm<sup>2</sup>) (Fig. 3).

Immunofluorescence staining of frozen sections was viewed on a BIOREVO BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). A blinded investigator recorded findings by at 200× magnification.

**Statistics**

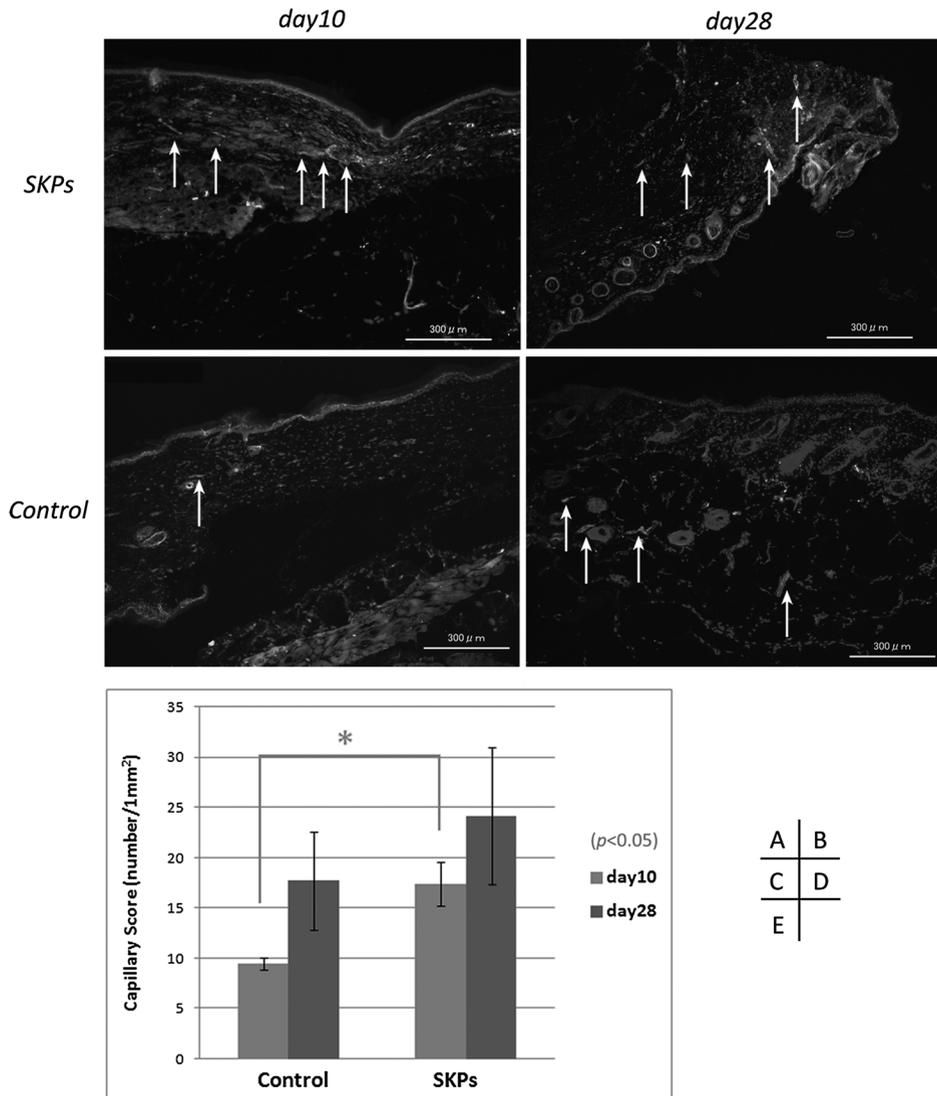
All statistical data are expressed as mean ± SEM. Data were analyzed by Student *t* test or Mann-Whitney *U* test using SigmaStat

(SPSS, Inc, Chicago, Ill). Values of *P* < 0.05 were considered significant. No correction was made for multiple testing.

**RESULTS**

**Topical SKP Application Promotes Wound Healing**

Each skin sample was harvested at days 10 and 28. At day 10, all samples were filled with the effusion, and the surface was still raw under macroscopic observation. On the other hand, at day 28, all the wounds were completely closed (Fig. 4). Diabetic wounds treated with PBS controls had 29.06% ± 2.98% closure at day 7, 48.44% ± 5.14% closure at day 10, 73.20% ± 5.50% closure at day 14, 86.27% ± 2.02% closure at day 18, and 96.54% ± 2.53% closure at day 21. Diabetic wounds treated with SKPs exhibited improved wound



**FIGURE 6.** Fluorescent microscopic view of wound sections at 200× magnification; Nuclei were stained by DAPI. Vascular endothelial cells were stained by anti-CD31 antibody. α smooth muscle cells were stained by anti-α-SMA antibody. A, SKP-treated wounds section at day 10; microvessels stained by CD31 revealed tubular structures as shown by the arrows. B, SKP-treated wounds section at day 28. C, PBS-control wounds section at day 10. D, PBS-control wounds section at day 28. E, The *Capillary Score* was expressed as the average number of vessels per 1 mm<sup>2</sup> of wound image. The *Capillary Score* was significantly greater (*P* = 0.019) for SKP-treated wounds over PBS controls at day 10. At day 28, no significant difference between the 2 groups was identified (*P* = 0.385).

healing compared with PBS control treatments at all the time points with statistical significance at days 7, 10, 14, and 18, respectively (Fig. 5). Specifically, SKPs treatment resulted in 40.81% ± 4.12% closure at day 7 ( $P < 0.01$ ), 69.93% ± 4.83% closure at day 10 ( $P < 0.01$ ), 90.69% ± 3.28% closure at day 14 ( $P < 0.01$ ), and 97.56% ± 1.12% closure at day 18 ( $P < 0.01$ ); 99.82% ± 0.83% closure at day 21 was not statistically greater than 96.54% ± 2.53% closure seen in control treatment ( $P = 0.05$ ).

Median time to closure for SKP-treated wounds (18.63 ± 0.67 days) was significantly improved relative to control (21.72 ± 0.64 days,  $P < 0.01$ ).

### SKPs Therapy Group Significantly Increases Capillary Score Compared With Control Group

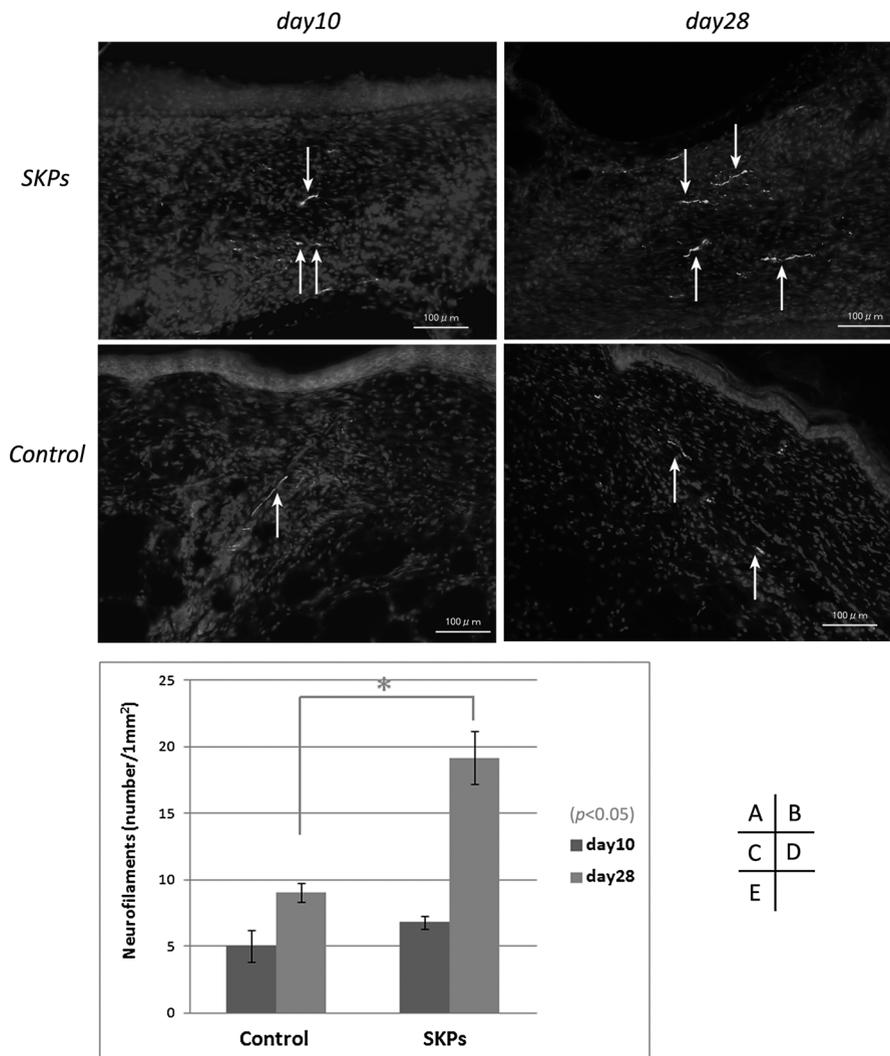
Image-analysis segmentation of CD31-stained sections was performed. At day 28, the *Capillary Score* for SKP-treated wounds was

24.19 ± 6.80. Although SKP-treated wounds did not have a significantly increased *Capillary Score* compared with 17.70 ± 4.83 of PBS-control wounds ( $P = 0.385$ ), the *Capillary Score* of SKP-treated wounds at day 10 was 17.41 ± 2.17, which showed significant increase compared with 9.47 ± 0.63 of PBS-control wounds ( $P = 0.01$ ) (Fig. 6).

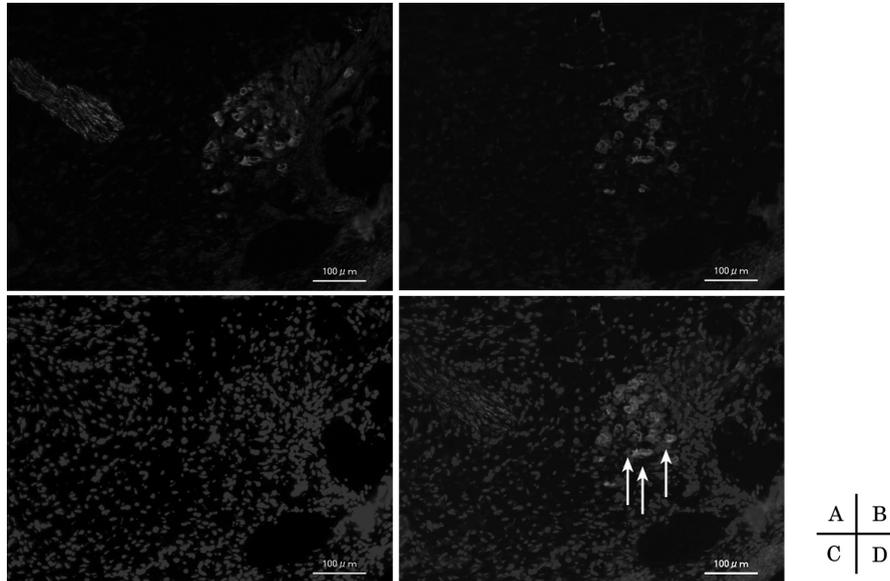
Immunofluorescence staining of frozen sections showed no double-stained region by PKH26 and CD31 antibody.

### SKPs Can Differentiate into Peripheral Nerve Fibers

*Nerve Density* of SKP-treated wounds at day 10 was 6.78 ± 0.50, whereas it was 5.01 ± 1.19 for PBS-control wounds. A significant difference between both groups was not identified ( $P = 0.291$ ) (Fig. 7). However, *Nerve Density* at day 28 had significantly increased in SKP-treated wounds (19.16 ± 1.96) compared with PBS-control wounds (9.04 ± 0.73,  $P < 0.01$ ). In addition, double-stained regions by PKH26 labeling SKPs and anti-NF-H antibody were



**FIGURE 7.** Fluorescent microscopic views of wound sections at 200× magnification. Nuclei are stained by DAPI and appear blue. Neurofilaments were stained by anti-NF-H antibody (white arrow). A, SKP-treated wounds section at day 10; arrows show stained neurofilaments in wound area. B, SKP-treated wounds section at day 28. C, PBS-control wounds section at day 10. D, PBS-control wounds section at day 28. E, Neurofilaments were expressed as the average number of filamentous structure stained by anti-NF-H antibody per 1 mm<sup>2</sup> of wound image. There were no significant differences between SKP-treated wounds and PBS-control wounds at day 10. However, SKP-treated wounds showed a significant increase compared with PBS-control wounds at day 28.



**FIGURE 8.** Fluorescent microscopic view of double-stained region in wound section, which shows the possibility of differentiation to neurons for applied SKPs. A, NF-H. B, SKPs labeled by PKH26. C, DAPI. D, Arrows show the double-stained region which localizes at the edge of panniculus carnosus in wound area (merge).

confirmed in SKP-treated wounds section, which were supposed to be the regenerations of neurofilaments directly derived from applied SKPs (Fig. 8).

## DISCUSSION

In this study, we demonstrated for the first time that SKPs enhanced wound healing of diabetic mice. Excisional wounds in diabetic mice have been reported to show significant delay in wound closure, decreased granular tissue formation, decreased wound bed vascularity, and diminished proliferation.<sup>23</sup>

Promoting wound healing by a variety of adult progenitor cells including MSCs,<sup>8–11</sup> endothelial progenitor cells,<sup>12</sup> and adipose-derived stromal cells<sup>13</sup> have been reported in vivo. Although each study design has been different in animal strain, age, sex, and excisional wound size, the median time to wound closure ranged approximately 14 to 28 days in studies using murine excisional wound models. We considered our result of 18 days could stand favorably compared with previous studies using other stem cells. Lin et al reported diabetic murine excisional wound model treated by MSCs similar to our study design. Despite the larger excisional wound in our study, more than 40% wound closure was achieved at day 7 compared with 37% in their study. This result suggested that SKPs can be considered a good option for the treatment compared with other stem cells.

For the promotion of wound healing, a well-vascularized wound bed is one important factor. In this study, the *Capillary Score* of SKP-treated wounds at day 10 was significantly higher than PBS-control wounds. No significant difference was identified at day 28. These results indicate that SKPs contribute to the early stage of wound healing through vasculogenesis.

A previous study<sup>28</sup> reported that SKPs directly differentiate vascular smooth muscle cells with the addition of transforming growth factor  $\beta$  in vivo. However, in our histological analysis of immunofluorescent stained region by PKH26 and anti-CD31 antibody, direct differentiation to vessels of applied SKPs was not confirmed (data not shown). Therefore, we suggest that paracrine signaling effects increased the *Capillary Score*. However, details surrounding the paracrine effects of SKPs are still unclear.

Diabetic foot ulcer is related with peripheral neuropathy.<sup>29–31</sup> Peripheral nervous dysfunction leads to the unawareness of repeated physical damage to the feet. In addition, small fiber neuropathy causes reduced axon-related vasodilation that leads to impaired peripheral blood circulation and the incurable wounds.<sup>29</sup> Illigens and Gibbons<sup>32</sup> made an excisional wound to injure small sensory and autonomic nerves in otherwise healthy individuals and showed delayed wound healing. The mechanism by which dysfunction of small sensory nerve fibers contributes to reduced wound-healing capacity is not fully understood, and indicates that peripheral neuropathy may directly influence wound healing.<sup>33</sup> Significant increase of *Nerve Density* was observed at day 28, but not at day 10. Wound healing had already enhanced by day 10, thus the relation between wound healing and nerve regeneration was not clear in this study. However, we consider that the ability of SKPs regarding nerve regeneration could clinically contribute to the prevention of recurrence in diabetic foot ulcer.

Double-stained region with PKH26 and NF-H were confirmed in our study. These results indicate that the microenvironment surrounding wounds affect some SKPs, and SKPs themselves differentiate into neurons despite no additional induction procedure. As previous reports claimed that SKPs differentiated into peripheral nerves in vivo,<sup>20,21</sup> we also consider that the finding suggests the tendency of SKPs to differentiate into neurons.

Our final aim is to demonstrate the utility of SKPs therapy for diabetic foot ulcers, which needs additional investigations by use of a clinically similar model of diabetic foot ulcer.

## CONCLUSIONS

Skin-derived precursor cells promote diabetic wound healing through neovascularization at the early stage of wound healing. Direct differentiations to peripheral neurofilament are also confirmed in this study. This leads to the conclusion that it is possible that SKPs are a novel therapeutic tool for diabetic foot ulcer.

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