

# Dermal CD271<sup>+</sup> Cells are Closely Associated with Regeneration of the Dermis in the Wound Healing Process

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**Stem cells have recently been shown to play important roles in wound healing. The aim of this study was to investigate the role of dermal CD271<sup>+</sup> cells in wound healing. Full-thickness wounds were produced on the backs of 5-year-old and 24-week-old mice, and time-course of wound closure, CD271<sup>+</sup> cell counts, and gene expression levels were compared. Delayed wound healing was observed in 24-week-old mice. The peak of CD271<sup>+</sup> cell increase was delayed in 24-week-old mice, and gene expression levels of growth factors in wounded tissue were significantly increased in 5-year-old mice. Dermal CD271<sup>+</sup> cells purified by fluorescence-activated cell sorting (FACS) expressed higher growth factors than CD271<sup>-</sup> cells, suggesting that CD271<sup>+</sup> cells play important roles by producing growth factors. This study also investigated dermal CD271<sup>+</sup> cells in patients with chronic skin ulcers. Dermal CD271<sup>+</sup> cells in patients were significantly reduced compared with in healthy controls. Thus, dermal CD271<sup>+</sup> cells are closely associated with wound healing.**

**Key words:** CD271; p75NTR; stem cell; wound healing; collagen type V.

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Healing of cutaneous wounds is a complex biological event involving interplay of a large number of resident and infiltrating inflammation cells. Although new therapeutic methods have been developed, the available treatments for chronic skin ulcers remain unsatisfactory, and more-effective treatment strategies are needed. Recently, it has been reported that clinical application of stem cells can improve wound healing in the skin (1, 2). In addition, adipose tissues serve as a source of adipokines and cytokines, with both local and systemic effects on health and disease (3), suggesting that stem cells could be a new therapeutic option for chronic skin ulcers. Stem cells are categorized into 3 types: embryonic, adult, and induced pluripotent stem cells. The most common types of adult stem cells are haematopoietic stem cells, bone marrow-derived mesenchymal stem cells (MSCs) and adipose-derived stem cells (ASCs). Since it is necessary to easily, safely, and efficiently collect a sufficient number of

cells for clinical applications, ASCs are more suitable for clinical applications compared with bone marrow-derived MSCs. Although many stem cell-associated markers for ASCs have been reported, including Lin, Integrin beta-1, CD34, Sca-1, and CD24 (4), a definite marker for ASCs remains unclear. Recently, we confirmed that p75 neurotrophin receptor (p75NTR; CD271)-positive cells in subcutaneous adipose tissues possessed multipotency (5). In addition, a previous study demonstrated that CD271 is a common stem cell marker for the epidermis and dermis, and that CD271<sup>+</sup> cells are characterized by excellent proliferation and multipotency (6). Since the CD271<sup>+</sup> subset consists of heterogeneous cell populations and contains a large population of stem cells, CD271<sup>+</sup> cells are a “CD271<sup>+</sup> stem cell-enriched population”.

Recently, we clarified that epidermal CD271<sup>+</sup> stem cells play an important role in the wound healing process in mice (7). Furthermore, we demonstrated that epidermal CD271<sup>+</sup> cells were decreased in chronic ulcers in humans (7). In addition, we have previously confirmed that dermal CD271<sup>+</sup> cells have a high proliferation potential and are able to differentiate into adipocytes, osteoblasts and chondrocytes (6). Therefore, we hypothesize that dermal CD271<sup>+</sup> cells are increased and play important roles in wound healing by producing various growth factors or cytokines. The aim of this study was therefore to investigate the dynamic movement time-course of dermal CD271<sup>+</sup> cells, accumulation of several kind of collagen, and gene expression levels of various cytokines and growth factors during the wound healing process in mice. A further aim was to investigate dermal CD271<sup>+</sup> cell numbers in patients with chronic skin ulcers. The results indicate that dermal CD271<sup>+</sup> cells are closely associated with regeneration of the dermis in wound healing by producing various growth factors. Furthermore, dermal CD271<sup>+</sup> cells in patients with chronic skin ulcers were significantly reduced compared with healthy controls. These results suggest that dermal CD271<sup>+</sup> cells are closely associated with regeneration of the dermis in wound healing.

## METHODS

### Mice

HR-1 mice were purchased from Hoshino Laboratory Animals (Ibaraki, Japan). All mice were healthy and fertile, and did not

display any evidence of infection or disease. Mice were 5-week-old (5W) and 24-week-old (24W) for all experiments. All mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. The animals were cared for in accordance with the International Guiding Principles for Biomedical Research Involving Animals (published by The Council for International Organization of Medical Science).

#### Macroscopic and histological assessment of wound healing in mice

Mice were anaesthetized with diethyl ether and their backs wiped with 70% alcohol. Longitudinal incisional full-thickness cutaneous wounds (10 mm) were generated laterally in the dorsum to each mouse using a disposable sterile scalpel. The wounds were covered with occlusive dressings (Tegaderm, 3M Canada, London, ON, Canada), and mice were caged individually. The areas of the open wounds were measured by multiplying the lengths of the major and minor axes of the wound, and relative size compared with the wound area on day 0 was calculated. For macroscopic analysis of wound closure, 5 mice were used in each group.

After the mice were sacrificed, wounds were harvested with a 2-mm border of unwounded skin tissue. The wounds were fixed in 4% paraformaldehyde and were then embedded in paraffin. Five- $\mu$ m paraffin sections were stained with haematoxylin and eosin (H&E). The epithelial gap, which is the distance between the migrating edges of keratinocytes, was measured under a light microscope.

#### Immunohistochemical staining

Paraffin-embedded tissues were cut into 5  $\mu$ m sections, deparaffinized in xylene, and rehydrated in PBS. Deparaffinized sections were treated with endogenous peroxidase-blocking reagent (DAKO Cytomation A/S, Copenhagen, Denmark) and proteinase K (DAKO) for 6 min at room temperature. Sections were then incubated with rabbit monoclonal antibody (mAb) specific for Collagen-1 (LSL, Tokyo, Japan), Collagen-3 (LSL) or Collagen-5 (BIOSS, Woburn, MA, USA), p75NTR/CD271 (Chemicon International, Carlsbad, CA, USA), and DAPI (VECTASHIELD H-1200; Vector Laboratories, Burlingame, CA, USA) was used for nuclear staining. In some experiments, non-wounded and wounded skin sections were double-stained using a mAb specific for CD45 (Merck Millipore Corporation, Darmstadt, Germany) and rabbit mAb specific for CD271. Sections were sequentially incubated with goat anti-Rabbit IgG (H+L) or goat anti-Mouse IgG (H+L) secondary Ab, Alexa Fluor<sup>®</sup> 488 conjugate (Invitrogen Corp., CA, USA). Sections were washed 3 times with PBS between incubations. For the analysis of accumulation of collagen-1, -3, and -5, fluorescence intensity/100  $\mu$ m<sup>2</sup> of dermis was measured using a NIH-image software (USA National Institutes of Health). For analysis of dermal CD271<sup>+</sup> cells, stained cells were counted in 200  $\mu$ m<sup>2</sup> of the wound bed per section.

#### Dermal cell isolation and fluorescence-activated cell sorting analysis of dermal CD271<sup>+</sup> cells

Mouse skin was obtained, and the dermis was separated after digesting with Dispase II (200 u/ml, 4°C; Wako Pure Chemical Industries, Ltd, Osaka, Japan) overnight. After fragmentation of dermis tissue using scissors or a scalpel, dermis tissue was treated with 0.2% collagenase (Sigma, St Louis, MO, USA) for 1 h. After centrifugation, cell suspensions were transferred through a sterile 100- $\mu$ m nylon mesh (BD) and a single-cell suspension was prepared. After removal of red blood cells and washing, single-cell suspensions were stained with a rabbit

mAb specific for p75NTR/CD271 (Chemicon International) or isotype-matched control mAb on ice. Single-cell suspensions were sequentially incubated with goat anti-rabbit IgG (H+L) secondary Ab, Alexa Fluor<sup>®</sup> 488 conjugate (Invitrogen Corp.) for 30 min on ice. Expression levels of p75NTR/CD271 were analysed using fluorescence-activated cell sorting (FACS; FACS Aria<sup>™</sup>, BD, USA).

#### Relative gene expression levels in wounded dermis by real-time RT-PCR analysis

Relative gene expression levels of collagen-1, -3, -5, CD271, Mki67, F4-80, CD11b, hepatocyte growth factor (HGF), fibroblast growth factor 2 (FGF2), insulin-like growth factor 1 (IGF1), platelet-derived growth factor (PDGF) b1, transforming growth factor- $\beta$  (TGF- $\beta$ ), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), epidermal growth factor (EGF), and interleukin-1 $\alpha$  (IL-1 $\alpha$ ) in wounded dermis was analysed by real-time RT-PCR. Total RNA was isolated from dermis using the RNAiso<sup>™</sup> (Takara, Shiga, Japan). cDNA was synthesized from total RNA by reverse transcription. Real-time semi-quantitative RT-PCR was performed using a SuperScript<sup>™</sup> III Platinum<sup>®</sup> Two-Step qRT-PCR kit (Invitrogen). The forward and reverse primer sequences are indicated in **Table I**. Amplification was normalized to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and differences between samples were quantified based on the  $\Delta\Delta$ CT method. Briefly, each set of samples was normalized using the difference in threshold cycle (CT) between the target gene and housekeeping gene (*GAPDH*):  $\Delta$ CT=(CT target gene – CT GAPDH). Relative mRNA levels were calculated using  $2^{-\Delta\Delta$ CT}, where  $\Delta\Delta$ CT= $\Delta$ CT sample –  $\Delta$ CT calibrator.

#### Dermis-derived CD271<sup>+</sup> and CD271<sup>-</sup> cell isolation and relative gene expression levels by real-time RT-PCR analysis

Mouse skin was obtained from the 5W HR-1 mice, and the dermal single-cell suspension was prepared as described above. Received cells were subjected to fluorescence activated cell sorting (BD FACS Aria<sup>™</sup>) to collect CD271-positive and negative cells. In brief, the cells were harvested and incubated in 1% BSA-PBS containing anti-p75NTR/CD271 mAb (Chemicon International) for 30 min on ice, then anti-rabbit IgG mAb labelled with Alexa Fluor 488 (Invitrogen Corp.) was added and the cells were incubated for another 30 min on ice. The cells were subsequently washed twice before sorting CD271-positive or -negative cells using FACS for use in gene expression analysis. Relative gene expression levels of FGF2, EGF, PDGFB, IGF-1, TGF- $\beta$ 1 and HGF by CD271<sup>+</sup> and CD271<sup>-</sup> cells were analysed by real-time

**Table I. Primers used in real-time PCR (RT-PCR) analysis**

Gene	Forward	Reverse
<i>GAPDH</i>	5'- CCGTGTTCTACCCCAAT-3'	5'- TGCTGCTTCACCACCTTCT-3'
<i>Collagen-1</i>	5'-GGAAACCCGAGGTATGCTTGA-3'	5'- CACTCGCCCTCCCGTCTT-3'
<i>Collagen-3</i>	5'- TTCTGAAGATGCTGTGATGTG-3'	5'- TGTTTTGTCAGTGGTATGTAATGTC-3'
<i>Collagen-5</i>	5'- GGACTCGGGGAACATTG -3'	5'- AGTGGTCCGTTTCATCTGTTCAA -3'
<i>Cd271</i>	5'-ATCTTGGCTGCTGTGGTTGTG-3'	5'-TAITTTTGCTTCAGCTGTTCCA-3'
<i>Mki67</i>	5'-CCATCATTGACCGCTCCTTAG-3'	5'- CTATCTCTGAGGCTCGCCTTGA-3'
<i>F4-80</i>	5'- GGCTGCCTCCCTGACTTTC-3'	5'- GGATCCTTTTGTCAGTTGAAGTTTC-3'
<i>Cd11b</i>	5'- GCAGCTGAATGGGAGGACAA-3'	5'- CAATAGGATCTTCGAGCATTCT-3'
<i>Hgf</i>	5'- TGGTGGCCCACTATTGT-3'	5'- GACGATTTGGGATGGCACAT-3'
<i>Egf</i>	5'- AGCTGTGCTTCTCACT-3'	5'- TGGGGTCACTGCTTTAAC-3'
<i>Fgf2</i>	5'- GGAGTTGTGTATCAAGGGAGTGT-3'	5'- AGCAGCCGCTCATCTTCT-3'
<i>Igf1</i>	5'- GTTGCTTCGGAGCTGTGAT-3'	5'- GATAGAGCGGGCTGCTTTTG-3'
<i>Pdgfb</i>	5'- TCAAGCTCGGGTGACCAATC-3'	5'- ACTTTCGGTGCTTGCCTTTG
<i>Tgfb1</i>	5'- CCGAGAGCCCTGGATACCA-3'	5'- GCCGCACACAGCAGTCTT-3'
<i>Tnfa</i>	5'- GCCAGCCGATGGGTTGA-3'	5'- GGCAGCCTTGCCTTGA-3'
<i>Il1a</i>	5'- TCGGGAGGAGACGACTCTAAATA-3'	5'- AGCGCTCACGAACAGTTGTG-3'

**Table II. Clinical features of patients with chronic skin ulcers**

Age, years/ Sex	Site of ulcer	Cause of ulcer	Disease duration (months)
68/F	Left lower leg	Venous ulcers	12
38/M	Left lower leg	Venous ulcers	120
55/F	Sacral region	Decubitus	180
63/M	Back	Venous ulcers	12
66/F	Left medial malleolus	Venous ulcers	60
81/M	Right lower leg	Venous ulcers + arteriosclerosis	24
93/M	Left lower leg	Burn	18

RT-PCR, as above described. The forward and reverse primer sequences are indicated in Table I.

*Human skin samples*

Skin specimen was obtained from 7 patients (4 men and 3 women) with chronic skin ulcers (Table II) and 10 age- and sex-matched healthy individuals (7 men and 3 women) were used as normal controls. A skin ulcer that did not change in size despite standard ointment treatment for more than 4 months was defined as a chronic ulcer in this study. Written informed consent was obtained from all patients and tissue donors in accordance with the Declaration of Helsinki. Informed consent for skin samples was obtained for each collection in accordance with the protocols approved by the Institutional Review Board of Fujita Health University. The mean ± SD age of normal controls were 48 ± 20 years, and all skin specimens were taken from the lower legs. The skin specimen was stained for CD271, as earlier described (7). Stained skin specimen was counted for dermal CD271+ cells in 5 high-power fields (0.07 mm<sup>2</sup>, magnification, ×400) from non-wound sites, wound edge and wound bed per section. Each section was examined independently by 2 researchers in a blinded fashion.

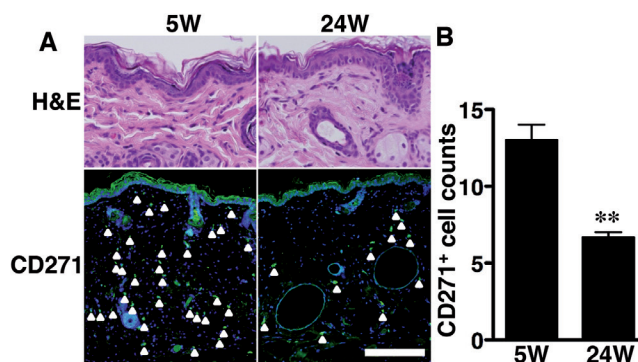
*Statistical analysis*

Mann-Whitney *U* test was used to determine the level of significance of differences between samples. *Ap*-value < 0.05 was considered statistically significant.

**RESULTS**

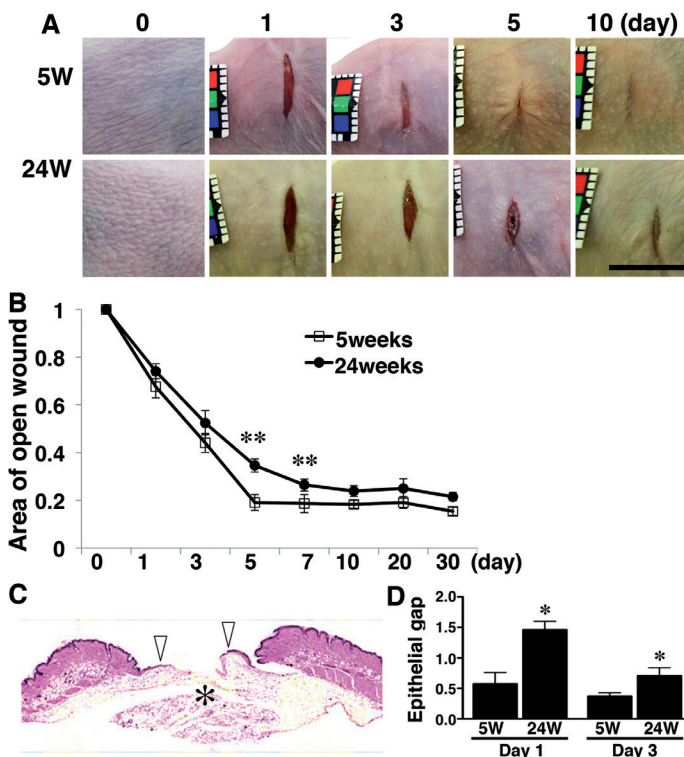
*Comparison of dermal CD271+ cell counts and wound healing process in 5W and 24W mice*

First, we examined the baseline number of dermal CD271+ cells in 5W and 24W mice. Dermal CD271+ cell counts (mean ± SD) were 13.0 ± 1.7 in 5W mice, and 6.7 ± 0.58 in 24W mice from 10 mice in each group (Fig. 1). Thus, the baseline number of dermal CD271+ cells was significantly decreased in 24W mice compared with 5W mice (*p* < 0.01). Next, we compared the macroscopic wound healing process in 5W and 24W mice. The time-course of the change in open wound area was measured to assess the normal macroscopic healing process (Fig. 2A, B). The open wound area was significantly larger in 24W mice than 5W mice from day 5 to day 7 post-wounding. To confirm the delayed wound healing in 24W mice, re-epithelialization was also assessed by microscopically measuring

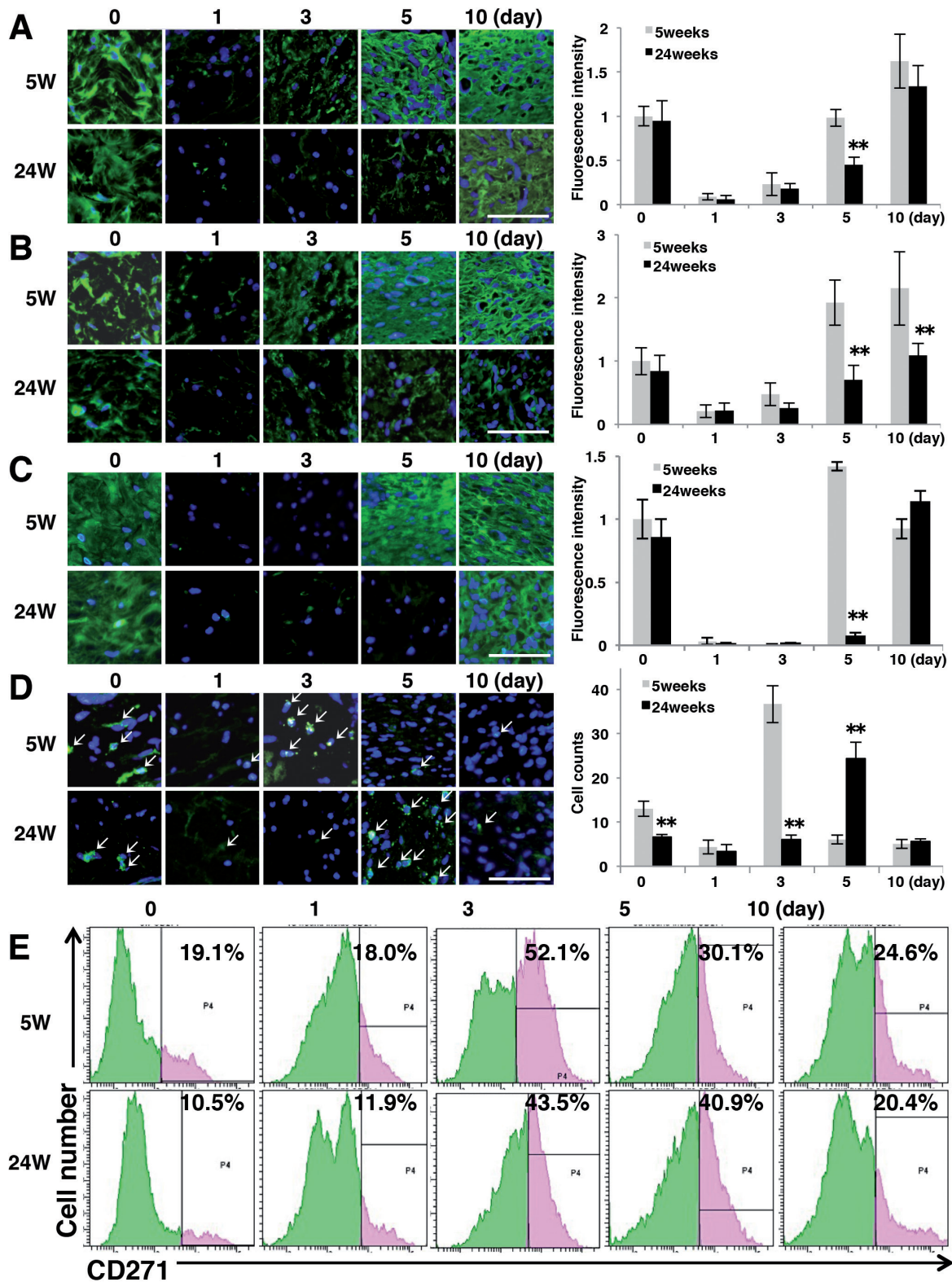


**Fig. 1. Skin histology in dermal CD271+ cell counts in 5-week-old (5W) and 24-week-old (24W) mice.** (A) Representative histological skin sections of haematoxylin and eosin (H&E) staining and CD271 staining 5W and 24W. Arrowheads indicate CD271+ cells in dermis. Bar indicates 100 µm. (B) Histograms show the mean ± SD values of CD271+ cell counts obtained from 10 mice. CD271+ cells/200 µm<sup>2</sup> in dermis were counted. \*\**p* < 0.01.

the epithelial gap (i.e. the distance between the migrating edges of keratinocytes; the distance between open triangles in Fig. 2C). Re-epithelialization finished



**Fig. 2. Wound healing process in 5-week-old (5W) and in 24-week-old (24W) mice.** (A) Representative photographs of intact skin, and wounded skin on days 1, 3, 5 and 10 post-wounding in 5W and 24W mice. Bar indicates 10 mm. (B) The areas of the open wounds were measured by multiplying the lengths of the major and minor axes of the wound. The ratio to initial wound size (day 0) was calculated. Each point shows the mean ± standard deviation (SD) values obtained from 5 mice. Significant differences between means of 5W and 24W are indicated: \*\**p* < 0.01. (C) Representative histology in 5W mice at day 1 after wounding (original magnification ×40). Open triangle shows wound edge, and asterisk shows wound bed. (D) The epithelial gap at day 1 and 3 after wounding, which is the distance between the migrating edges of keratinocytes (mm), was measured under a light microscope. Histograms show the mean ± SD values obtained from 5 mice. Significant differences between means of 5W and 24W are indicated: \**p* < 0.05.



**Fig. 3. Accumulation of collagen-1, -3, and -5 (A–C), and dermal CD271<sup>+</sup> cell counts (D) and CD271 expression in dermal cells (E) in 5-week-old (5W) and 24-week-old (24W) mice post-wounding.** Representative photographs of immunohistochemical staining and histograms showing relative fluorescence intensity of (A) collagen-1, (B) collagen-3, and (C) collagen-5 at wound bed. Each histogram shows the mean  $\pm$  standard deviation (SD) values of fluorescence intensity (per 100  $\mu\text{m}^2$ ) obtained from 10 mice. (D) Representative photographs of CD271 staining at wound bed. CD271<sup>+</sup> cells are indicated by arrows. Histograms show the mean  $\pm$  SD values of CD271<sup>+</sup> cell counts (per 200  $\mu\text{m}^2$ ) obtained from 10 mice. Among the 9 fields counted, 6 were from wound edges, and 3 from the middle of the wound bed. Each section was examined independently by 2 researchers in a blinded fashion. Significant differences between means of 5W and 24W are indicated: \*\* $p < 0.01$ . (E) CD271 expression in dermal cells assessed by flow cytometry. Dermal single-cell suspensions were stained with a monoclonal antibody (mAb) specific for CD271 or isotype-matched control mAb. CD271<sup>+</sup> (pink) and CD271<sup>-</sup> (green) were divided based on the histogram of isotype-matched control mAb. Results represent those obtained from  $\geq 3$  mice.

by 5 days after wounding in both 5W and 24W mice. However, the epithelial gap was longer in 24W mice relative to 5W mice both 1 and 3 days after wounding (Fig. 2D). Thus, wound healing was delayed in 24W mice compared with 5W mice.

*Time-course of changes in collagen-1, -3, and -5 accumulation, and dermal CD271+ cell counts in 5W and 24W mice during wound healing*

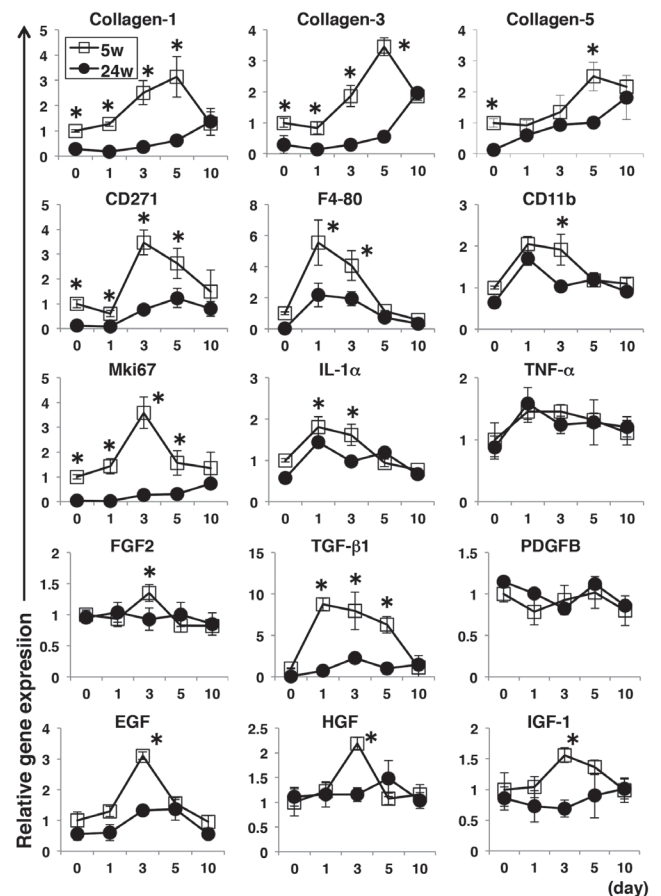
The accumulation of collagen is important for the wound healing process. Therefore, we next examined the accumulation of several types of collagen and dermal CD271+ cells number during wound healing. Compared with 5W mice, the accumulation of collagen-1 and -5 was significantly decreased on day 5 after wounding. Similarly, accumulation of collagen-3 in 24W mice was decreased on both day 5 and 10 after wounding. Thus, the accumulation levels of collagen-1, -3, and -5 were decreased in 24W mice during the wound healing process (Fig. 3A–C).

After wounding, dermal CD271+ cells were increased in both 5W and 24W mice, and total dermal CD271+ cell counts during wound healing seemed to decrease in 24W compared with 5W mice. The peak of dermal CD271+ cell increase was at 3 days after wounding in 5W mice. By contrast, the peak was at 5 days after wounding in 24W mice (Fig. 3D). We have confirmed these increased CD271+ cells were CD45- (Fig. S1<sup>1</sup>). Thus, the timing of increase in dermal CD271+ cells during wound healing was delayed in 24W mice compared with that in 5W mice. To confirm this delayed peak of dermal CD271+ cell counts in 24W mice, single-cell suspensions of dermis were stained with a mAb specific for CD271, and assessed by FACS (Fig. 3E). CD271- (green) and CD271+ (pink) cells were divided based on histograms from isotype-matched control mAb. The percentages of dermal CD271+ cells at day 3 and 5 were 52.1% and 30.1% in 5W mice, respectively. By contrast, these were 43.5% and 40.9% in 24W mice. Thus, FACS analysis confirmed that the timing of increase in dermal CD271+ cells was delayed in 24W mice compared with 5W mice.

*Comparison of relative gene expression levels in wounded dermis*

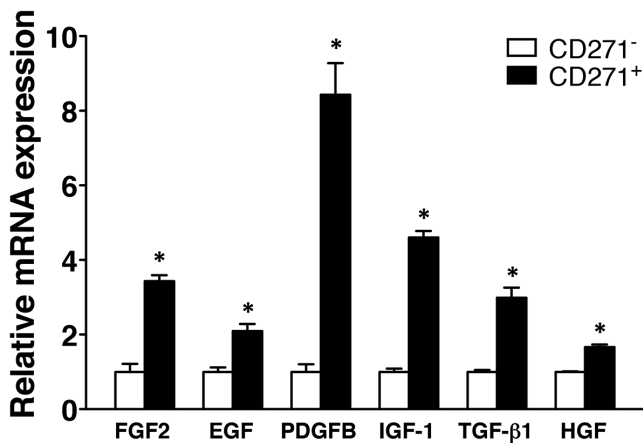
We next compared various gene expression levels in the dermis between 5W and 24W mice (Fig. 4). Relative gene expression levels in the dermis during the wound healing process were significantly elevated for collagen-1, -3, and -5 almost throughout the wound healing process. In addition, gene expression levels of

stem cell marker CD271 and cell surface markers for macrophage F4-80 and CD11b were also elevated in 5W mice. Furthermore, as reflected in the increase in various cells in the dermis during the wound healing process, the expression level of Mki67 was also significantly elevated in 5W mice throughout the wound healing process. Regarding the gene expression levels of cytokine and growth factors in the dermis, IL-1 $\alpha$ , FGF2, TGF- $\beta$ 1, EGF-1, HGF, and IGF-1 were elevated in 5W mice during the wound healing process. In contrast, there was no difference in gene expression levels of TNF- $\alpha$  and PDGF b1 between 5W and 24W mice. Collectively, the gene expression levels of dermal collagen-1, -3, -5, F4-80, CD11b, Mki67, HGF, EGF, FGF2, IGF-1, TGF $\beta$ -1, and IL-1 $\alpha$ , which are important for wound healing, in 5W mice, were significantly increased compared with 24W mice during wound healing.



**Fig. 4. Relative dermal mRNA expression levels of various cell surface markers, cytokines, and growth factors during wound healing in 5-week-old (5W) and 24-week-old (24W) mice.** Relative mRNA expression levels of dermal collagen-1, -3, -5, CD271, F4-80, CD11b, Mki67, IL-1 $\alpha$ , TNF- $\alpha$ , FGF2, IGF-1, TGF $\beta$ -1, PDGF b1, EGF, HGF, and IGF-1 were quantified by real-time PCR (RT-PCR) and normalized relative to endogenous glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) levels. Each point shows the mean  $\pm$  standard deviation (SD) values obtained from 5 mice in each group. Significant differences between 5W and 24W are indicated: \* $p < 0.05$ .

<sup>1</sup><https://doi.org/10.2340/00015555-2624>



**Fig. 5. Relative gene expression of various growth factors by purified dermal CD271<sup>+</sup> cells and CD271<sup>-</sup> cells.** Relative mRNA expression levels of FGF2, EGF, PDGFB, IGF-1, TGF-β1, and HGF were quantified by real-time PCR (RT-PCR) and normalized relative to endogenous glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) levels. Each bar shows the mean ± standard deviation (SD) values obtained from 4 mice in each group and mean value of CD271<sup>+</sup> were normalized to CD271<sup>-</sup>. Significant differences between means of CD271<sup>-</sup> and CD271<sup>+</sup> are indicated: \**p* < 0.05.

#### Relative gene expression of various growth factors by purified dermal CD271<sup>+</sup> cells

We hypothesized that dermal CD271<sup>+</sup> cells affect the wound healing process by producing growth factors. Therefore, we examined the gene expression levels of various growth factors by dermal CD271<sup>+</sup> and CD271<sup>-</sup> cells. Mouse dermal CD271<sup>+</sup> cells expressed 3.4-, 2.1-, 8.4-, 4.6-, 3.0-, and 1.7-fold higher FGF2, EGF, PDGFB, IGF-1, TGF-β1, and HGF transcripts than CD271<sup>-</sup> cells, respectively (Fig. 5). Thus, purified dermal CD271<sup>+</sup> cells expressed various growth factor gene transcripts at significantly higher levels than did CD271<sup>-</sup> cells.

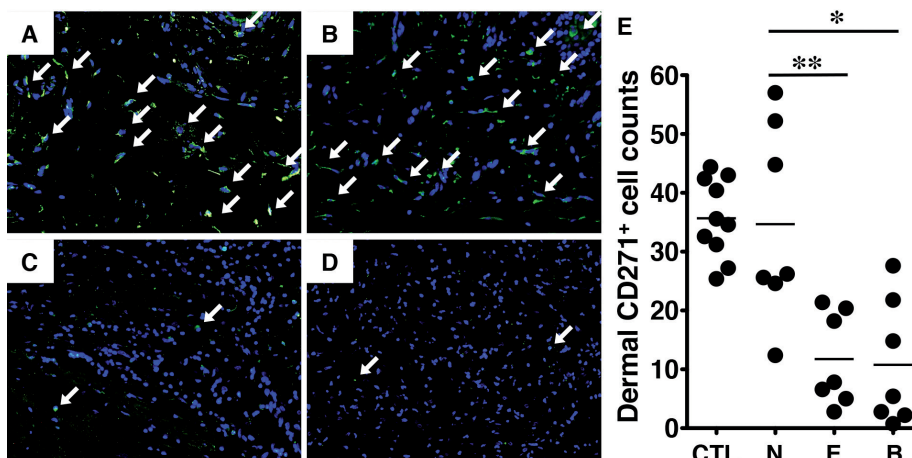
#### Dermal CD271<sup>+</sup> cells were decreased in patients with chronic ulcers

The mice wound healing model using 5W and 24W mice suggested that dermal CD271<sup>+</sup> cells play an important

role in the wound healing process. Therefore, we examined dermal CD271<sup>+</sup> cell counts in healthy individuals and patients with chronic skin ulcers (Table II). Skin biopsy specimens were obtained from non-wounded sites, the edge of the ulcer, and the wound bed in patients with chronic ulcers, and they were stained for both DAPI and CD271 as described in the Methods section. Skin samples were also obtained from age- and sex-matched healthy individuals as a control, and the site of skin samples was almost identical to that in patients with chronic ulcers. In healthy individuals, dermal CD271<sup>+</sup> cell counts (mean ± SD) were 35.7 ± 6.7 (Fig. 6A and E). In patients with chronic skin ulcers, dermal CD271<sup>+</sup> cell counts for non-wounded sites, the edge of the ulcer, and the wound bed were 34.7 ± 16.3, 11.7 ± 7.9, and 10.8 ± 10.7, respectively (Fig. 6B–E). Thus, dermal CD271<sup>+</sup> cell counts were similar between healthy individuals and non-wounded sites in patients with chronic skin ulcers (Fig. 6A, B and E). Surprisingly, dermal CD271<sup>+</sup> cells at wound edge and bed were significantly decreased in patients with chronic ulcers (Fig. 6B, C and E).

## DISCUSSION

Dermal CD271<sup>+</sup> cell numbers were decreased in 24W mice, and these mice showed delayed wound healing (see Figs 1 and 2). After wounding, the accumulation levels of collagen-1, -3, and -5 were decreased in 24W mice compared with 5W mice (see Fig. 3A–C), and the peak of increase in dermal CD271<sup>+</sup> cells was delayed in 24W mice (Fig. 3D, E). We stained both intact and wounded dermis with anti-CD45 and anti-CD271 antibody, and confirmed that dermal CD271<sup>+</sup> cells were negative for CD45 (Fig. S1<sup>1</sup>). When we assessed mRNA expression levels in the dermis, IL-1α, FGF2, TGF-β1, EGF-1, HGF, and IGF-1 levels were all elevated in 5W mice at some time during the wound healing process (Fig. 4). In addition, purified dermal CD271<sup>+</sup> cells expressed significantly higher FGF2, EGF, PDGFB, IGF-1, TGF-β1, and HGF mRNA expression levels than did CD271<sup>-</sup> cells (Fig. 5).



**Fig. 6. Dermal CD271<sup>+</sup> cell counts in healthy individuals and patients with chronic ulcers.** Representative photographs of (A) CD271 staining in healthy individuals, and (B) non-wounded site, (C) wound edge, and (D) wound bed in patients with chronic ulcers. Arrows indicate dermal CD271<sup>+</sup> cells. (E) Dermal CD271<sup>+</sup> cell counts in healthy individuals (CTL), non-wounded skin (N), wound edge (E), and wound bed (B) in patients with chronic skin ulcers. Stained skin specimen was counted for dermal CD271<sup>+</sup> cells in 5 high-power fields (0.07 mm<sup>2</sup>; ×400) per section. Significant differences between means of non-wounded site and wound edge or wound bed are indicated: \**p* < 0.05, \*\**p* < 0.005.

These results suggest that the timing of increase in dermal CD271+ cells is important for prompt wound closure by producing various cytokines and growth factors, which are important for wound healing and collagen accumulation. Surprisingly, in patients with chronic ulcers, the numbers of dermal CD271+ cells at the wound edge and in the wound bed were significantly decreased compared with non-wounded sites (Fig. 6). Taken together, these results suggest that dermal CD271+ cells are important for the proper wound healing process.

In the current study, collagen-5 accumulation and mRNA expression levels were significantly increased in 5W mice after wounding (Figs 3C and 4), suggesting that accumulation of collagen-5 has relevance for wound healing in addition to collagen-1 and -3. Fetal skin has been known to contain a greater proportion of type III collagen (8, 9), whereas adult skin consists predominantly of type I collagen (10). Thus, collagen-5 is a minor component, and the roles of collagen-5 in wound healing remain unclear. According to a previous study, collagen-5 may be involved in the migration of endothelial and pericytic cells during angiogenesis (11). Furthermore, it has been suggested that collagen-5 controls the diameter of collagen-1 fibres by hybrid-fibril formation (12). Consistent with this, Wenstrup et al. (13) revealed that type V collagen is required for collagen fibril nucleation and controls the initiation of collagen fibril assembly. Furthermore, patients with classic Ehlers–Danlos syndrome, in which mutations affecting type V collagen genes result in a non-functional COL5A1 and COL5A2 allele (14, 15), is characterized by impaired wound healing with abnormal scars (15, 16). Therefore, these previous and our results suggest that collagen-5 may be a key molecule in proper wound healing by regulating fibrillogenesis.

In this study, the gene expression of TGF- $\beta$ 1 was significantly higher in 5W mice. It has been reported that cultured MSCs produce various growth factors, such as vascular endothelial growth factor, HGF, IGF-1, TGF- $\beta$ , and granulocyte-macrophage colony-stimulating factor (17). Our previous study also confirmed the production of these growth factors by purified epidermal CD271+ cells (7). TGF- $\beta$  is involved in a number of processes in wound healing, including inflammation, angiogenesis, fibroblast proliferation, collagen synthesis, and remodelling of the new extracellular matrix (18–20). Furthermore, it has been reported that CD271+ cells also produce neurotrophins (21). Neurotrophins induce differentiation of dermal fibroblasts into myofibroblasts, which are the key effector in injury/repair processes and fibrosis, because they control extracellular matrix component deposition, tissue contraction, and wound resolution (21–23). Taken together, increased dermal CD271+ cells in injured skin may play important roles by producing various growth factors and neurotrophins, recruiting macrophages and fibroblasts, and inducing

differentiation of dermal fibroblasts into myofibroblasts, resulting in proper wound healing.

Dermal CD271+ cells increased earlier after wounding in 5W mice than in 24W mice (Fig. 3D and 3E). In addition, the accumulation of collagen-1, -3, and -5 occurred in parallel with an increase in CD271+ cells (Fig. 3A–C). Recently, it has been reported that the number of epidermal and dermal CD271+ cells decreases significantly with ageing (24), and studies on the functional impairments of stem cells by ageing have also been reported (25, 26). Duscher et al. (25) identified an age-related depletion of a subpopulation of MSCs characterized by a pro-vascular transcriptional profile. In addition, they also demonstrated that aged MSCs were significantly compromised in their ability to support vascular network formation *in vitro* and *in vivo*. Bustos et al. (26) compared the global gene expression of aged and young bone-marrow-derived MSCs using mouse model of lipopolysaccharide-induced lung injury, and demonstrated that the expression levels of inflammatory response genes depended on the age of the bone marrow-derived MSCs. In addition, they also demonstrated an age-dependent decrease in the expression of several cytokines and chemokine receptors. Collectively, our results and previous reports suggest that a decrease in dermal stem cells, delayed increase in dermal CD271+ cells, and decreased expression of various cytokines and chemokine receptors by dermal CD271+ cells may result in delayed wound healing in 24W mice.

Recently, Akamatsu et al. (24) demonstrated that the numbers of epidermal and dermal CD271+ cells decreased with ageing in humans. Although the numbers of dermal CD271+ cells at a non-wounded site in patients with chronic ulcers were similar to those in healthy individuals (Fig. 6A, B, and E), the numbers of dermal CD271+ cells in the wound bed and around its edges were dramatically decreased in patients with chronic ulcers (Fig. 6C–E). In addition, our previous study demonstrated that epidermal CD271+ cells were also dramatically decreased in patients with chronic skin ulcers compared with normal controls (7). Taken together, decreased dermal CD271+ cells at wound sites in patients with chronic ulcers may reflect the functional impairment of proliferative capacity or impaired migration of CD271+ cells by unknown factors other than ageing. Further studies are needed to clarify this issue. Nevertheless, these results suggest that both epidermal and dermal CD271+ stem cells play important roles in wound healing.

In conclusion, this is the first study to demonstrate that dermal CD271+ cells play important roles in wound healing. A possible mechanism for effects in wound healing is growth factor production by dermal CD271+ cells. Consistent with this, dermal CD271+ cells were significantly decreased in patients with chronic skin ulcers. Further studies are needed to address the role of dermal CD271+ cells in the pathogenesis of chronic skin ulcers.

## REFERENCES

1. Kim CH, Lee JH, Won JH, Cho MK. Mesenchymal stem cells improve wound healing in vivo via early activation of matrix metalloproteinase-9 and vascular endothelial growth factor. *J Korean Med Sci* 2011; 26: 726–733.
2. Lee SH, Lee JH, Cho KH. Effects of human adipose-derived stem cells on cutaneous wound healing in nude mice. *Ann Dermatol* 2011; 23: 150–155.
3. Kilroy GE, Foster SJ, Wu X, Ruiz J, Sherwood S, Heifetz A, et al. Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors. *J Cell Physiol* 2007; 212: 702–709.
4. Rodeheffer MS, Birsoy K, Friedman JM. Identification of white adipocyte progenitor cells in vivo. *Cell* 2008; 135: 240–249.
5. Yamada T, Akamatsu H, Hasegawa S, Yamamoto N, Yoshimura T, Hasebe Y, et al. Age-related changes of p75 neurotrophin receptor-positive adipose-derived stem cells. *J Dermatol Sci* 2010; 58: 36–42.
6. Hasebe Y, Hasegawa S, Hashimoto N, Toyoda M, Matsumoto K, Umezawa A, et al. Analysis of cell characterization using cell surface markers in the dermis. *J Dermatol Sci* 2011; 62: 98–106.
7. Iwata Y, Akamatsu H, Hasegawa S, Takahashi M, Yagami A, Nakata S, et al. The epidermal Integrin beta-1 and p75NTR positive cells proliferating and migrating during wound healing produce various growth factors, while the expression of p75NTR is decreased in patients with chronic skin ulcers. *J Dermatol Sci* 2013; 71: 122–129.
8. Hallock GG, Rice DC, Merkel JR, DiPaolo BR. Analysis of collagen content in the fetal wound. *Ann Plast Surg* 1988; 21: 310–315.
9. Merkel JR, DiPaolo BR, Hallock GG, Rice DC. Type I and type III collagen content of healing wounds in fetal and adult rats. *Proc Soc Exp Biol Med* 1988; 187: 493–497.
10. Epstein EH, Jr. (Alpha1(3))3 human skin collagen. Release by pepsin digestion and preponderance in fetal life. *J Biol Chem* 1974; 249: 3225–3231.
11. Hering TM, Marchant RE, Anderson JM. Type V collagen during granulation tissue development. *Exp Mol Pathol* 1983; 39: 219–229.
12. Adachi E, Hayashi T. In vitro formation of hybrid fibrils of type V collagen and type I collagen. Limited growth of type I collagen into thick fibrils by type V collagen. *Connect Tissue Res* 1986; 14: 257–266.
13. Wenstrup RJ, Florer JB, Brunskill EW, Bell SM, Chervoneva I, Birk DE. Type V collagen controls the initiation of collagen fibril assembly. *J Biol Chem* 2004; 279: 53331–53337.
14. Symoens S, Syx D, Malfait F, Callewaert B, De Backer J, Vanakker O, et al. Comprehensive molecular analysis demonstrates type V collagen mutations in over 90% of patients with classic EDS and allows to refine diagnostic criteria. *Hum Mutat* 2012; 33: 1485–1493.
15. Ritelli M, Dordoni C, Venturini M, Chiarelli N, Quinzani S, Traversa M, et al. Clinical and molecular characterization of 40 patients with classic Ehlers-Danlos syndrome: identification of 18 COL5A1 and 2 COL5A2 novel mutations. *Orphanet J Rare Dis* 2013; 8: 58.
16. Viglio S, Zoppi N, Sangalli A, Gallanti A, Barlati S, Mottes M, et al. Wound repair capability in EDS fibroblasts can be retrieved by exogenous type V collagen. *ScientificWorldJournal* 2008; 8: 956–958.
17. Ennis WJ, Sui A, Bartholomew A. Stem cells and healing: impact on inflammation. *Adv Wound Care (New Rochelle)* 2013; 2: 369–378.
18. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, et al. Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc Natl Acad Sci U S A* 1986; 83: 4167–4171.
19. Nall AV, Brownlee RE, Colvin CP, Schultz G, Fein D, Cassisi NJ, et al. Transforming growth factor beta 1 improves wound healing and random flap survival in normal and irradiated rats. *Arch Otolaryngol Head Neck Surg* 1996; 122: 171–177.
20. Penn JW, Grobbelaar AO, Rolfe KJ. The role of the TGF-beta family in wound healing, burns and scarring: a review. *Int J Burns Trauma* 2012; 2: 18–28.
21. Palazzo E, Marconi A, Truzzi F, Dallaglio K, Petrachi T, Humbert P, et al. Role of neurotrophins on dermal fibroblast survival and differentiation. *J Cell Physiol* 2012; 227: 1017–1025.
22. Hinz B. Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 2007; 127: 526–537.
23. Ashrafi M, Baguneid M, Bayat A. The role of neuromediators and innervation in cutaneous wound healing. *Acta Derm Venereol* 2016; 96: 587–594.
24. Akamatsu H, Hasegawa S, Yamada T, Mizutani H, Nakata S, Yagami A, et al. Age-related decrease in CD271(+) cells in human skin. *J Dermatol* 2016; 43: 311–313.
25. Duscher D, Rennert RC, Januszyk M, Anghel E, Maan ZN, Whittam AJ, et al. Aging disrupts cell subpopulation dynamics and diminishes the function of mesenchymal stem cells. *Sci Rep* 2014; 4: 7144.
26. Bustos ML, Huleihel L, Kapetanaki MG, Lino-Cardenas CL, Mroz L, Ellis BM, et al. Aging mesenchymal stem cells fail to protect because of impaired migration and antiinflammatory response. *Am J Respir Crit Care Med* 2014; 189: 787–798.