Hair Follicle Grafting Therapy Promotes Re-Emergence of Critical Skin Components in Chronic Nonhealing Wounds

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An exploding public health crisis is the exponential growth in the incidence of chronic nonhealing ulcers associated with diseases such as diabetes. Various modalities have been developed to stimulate wound closure that is otherwise recalcitrant to standard clinical treatments. However, these approaches primarily focus on the process of re-epithelialization and are often deficient in regenerating the full spectrum of structures necessary for normal skin function. Autologous hair follicle grafting is a recent therapy to stimulate the closure of such nonhealing wounds, and we observed effects beyond the epidermis to other important components of the dermis. We found that hair follicle grafting facilitated the reappearance of various undifferentiated and differentiated layers of the epidermis with the restoration of epidermal junctions. In addition, other important structures that are critical for cutaneous health and function such as the blood and lymph vasculature, nerve fibers, and sweat gland structures were restored in postgrafted wounds. Interestingly, both immune cells and inflammatory signals were substantially decreased, indicating a reduction in the chronic inflammation that is a hallmark of nonhealing wounds. Our observation that punch wounds created on the postgrafted area likewise healed suggests that this is a self-sustaining long-term therapy for patients with chronic wounds.

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INTRODUCTION

Cutaneous wound healing is a complex process involving extensive crosstalk between multiple cell types that coordinates their activities to restore the barrier function of the skin. A growing public health problem is the increasing incidence of chronic, nonhealing wounds in conditions such as diabetic foot ulcers, venous leg ulcers, and pressure ulcers that are recalcitrant to standard treatment (Agale, 2013) and pose a huge socioeconomic burden worldwide (Sen et al., 2009). Although chronic wounds have multiple etiologies, they present common features such as excessive production of inflammatory cytokines, continuous infections and the formation of antimicrobial-resistant biofilms, compromised vasculature and innervation, and low numbers of stem cells (SCs), which are often dysfunctional (Frykberg and Banks, 2015).

Recent therapies to achieve wound healing after the failure of >6 weeks of standard care (Han and Ceilley, 2017) have utilized the application of SCs to the damaged tissue. Both mesenchymal SCs (MSCs) (Otero-Viñas and Falanga, 2016) and bone marrow–derived SCs (Badiavas and Falanga, 2003) have been employed for the management of cutaneous wounds. Autologous hair follicle (HF) grafting has recently been discovered as an efficacious therapy to stimulate closure of nonhealing wounds (Jiménez et al., 2012; Liu et al., 2015; Martínez et al., 2016; Martínez Martínez et al., 2017). The advantage of using HF grafts over epidermal grafts or cultured keratinocytes is also established in the field, in which HF grafting is a more effective and technically easier method to treat chronic wounds (Nuutila, 2021; Weyandt et al., 2009; Yang et al., 2015). In addition, these HF units contain multiple types of SCs, including both epithelial SCs and MSCs, neuronal progenitors, as well as muscle cell progenitors (Gentile et al., 2017; Mignone et al., 2007; Ou et al., 2020; Sieber-Blum et al., 2004; Tiede et al., 2007; Tohgi et al., 2017; Xu et al., 2013). Interestingly, MSCs have an additional value given their anti-inflammatory capacity (Chahal et al., 2019; Ghoryani et al., 2019; González et al., 2009; Pers et al., 2018; Prockop and Oh, 2012; Wang et al., 2016). We thus hypothesized that in addition to stimulating wound closure, this cocktail of diverse SCs may also stimulate the re-emergence of critical structures of the skin and dampen the chronic inflammation often associated with nonhealing wounds. Therefore, HF grafting may not only accelerate the closure of wounds but has the potential of providing more comprehensive healing over conventional therapeutic approaches.

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Abbreviations: HF, hair follicle; MSC, mesenchymal stem cell; SC, stem cell

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Table 1. Patient Details

Characteristics	Values
Age (y)	
Mean (SD)	40.20 (9.42)
Median (range)	38.50 (27–62)
Sex	
Male (%)	9 (90.00)
Female (%)	1 (10.00)
Duration of wound	6 wk – 40 y
Number of ulcers	14
Ulcer type	
Venous ulcers (%)	8 (57.14)
Venous and traumatic ulcers (%)	1 (7.14)
Venous and pyoderma gangrenosum ulcers (%)	3 (21.43)
Diabetic ulcers (%)	1 (7.14)
Leprosy ulcers (%)	1 (7.14)

RESULTS

HF grafting decreases the area and volume of chronic wounds

HF grafting was performed on 10 patients with a total of 14 leg ulcers of various etiologies (Table 1) using the method previously described (Budamakuntla et al., 2017) at a tertiary care center with Institutional Ethics Committee approval. Punch biopsies (3 mm) of the ulcer region were obtained before the treatment regimen and 3 months after treatment (Figure 1a). Patients included in this study exhibited no response to a minimum of 6 weeks of standard treatment. A biopsy of the wound area was taken as the baseline before grafting (pretreated wound). Two to 3 months after grafting, a punch biopsy was taken from the same wound area (post-treated wound). As an unwounded control, a contralateral biopsy was taken from the uninjured leg. Consistent with our previous clinical data (Budamakuntla et al., 2017), this study recapitulated the closure of the wounds (Figure 1b). One week after grafting, there was a noticeable decrease in the wound size, and the wound closure was completed between 8 and 12 weeks. Quantification of the wound area and volume of the chronic leg ulcers revealed a significant reduction of both parameters after HF grafting (Figure 1c).

HF grafting promotes restoration of the stratified layers of the epidermis

Given that HF grafting accelerated the re-epithelialization of the ulcer, we next examined the quality of the repaired skin. Histological examination of the postgrafted skin revealed a regenerated full-thickness tissue with an expanded epidermal compartment (Figure 2a). The pretreated wound bed was composed primarily of granulation tissue. In contrast, the post-treated wound area exhibited an intact epidermal compartment with noticeable dermal structures. The post-treated tissue was comparable with the unwounded skin except for the substantial epidermal thickening. The stratified layers of the epidermis exhibited the proper differentiation markers, with keratin 5 marking the basal layer and loricrin marking the granular layer of the epidermis (Figure 2b; top and middle panels). Moreover, the epidermis exhibited appropriate intercellular adhesion as scored by E-cadherin staining (Figure 2b; bottom panel), consistent with restoration of the barrier function of the tissue. Interestingly, the heightened epidermal thickness of the post-treated wound observed in the histological analysis (Figure 2a) is due to an expansion of both the basal layer (marked by keratin 5) and the differentiated layers (marked by loricrin) (Figure 2b; top and middle panels). The thickening of the epidermis is a normal phenomenon of the wound healing process and epidermal regeneration, where it has been documented to occur as a result of an expansion of the basal and suprabasal layers owing to the hyperproliferation of epithelial stem/progenitor cells (Pastar et al., 2014; Yang et al., 2016).

HF grafting promotes the restoration of dermal components critical for normal cutaneous function

Although re-epithelialization of the wound is relatively straightforward, one of the major challenges faced by therapies aimed at healing chronic wounds is restoring functional structures in the dermal layer of the skin. Patients with chronic wounds have defective regeneration of blood and lymph vasculature leading to inadequate blood supply and drainage of tissue fluid, respectively, from the wound site (Bauer et al., 2005; Franzeck et al., 1993; Jünger et al., 2000). We observed re-emergence of blood vessels (PECAM-1) and lymph vascular structures (LYVE-1) (Figure 3a; top and middle panels) in the post-treated skin. Loss of perception to touch and other sensory inputs is another critical feature of chronic wounds. Interestingly, re-emergence of peripheral nerves was also observed after grafting as denoted by the presence of class III β-tubulin, a microtubule component found almost exclusively in neurons (Figure 3a; bottom panel). Quantification of the wound bed area in the post-treated wounds with the unwounded tissue demonstrated that these structures were restored to almost homeostatic levels. Moreover, other crucial components of the dermal compartment, including fat-producing cells important for insulation and sweat glands for thermal regulation, were restored after grafting (Figure 3b; top and middle panels). Quantification of these structures revealed that the fat content in the wound bed was comparable between the postgrafted and the unwounded tissue. However, the reappearance of sweat glands did not achieve the level found in unwounded skin. Extracellular matrix proteins in the dermis are integral for supporting the structural integrity of the organ. The largest percentage of these extracellular matrix proteins in the dermis are collagens. Masson Trichrome staining reveals that collagen content (indicated by blue staining) is increased in post-treated wounds compared with that in pretreated wounds (Figure 3b; bottom panel). The post-treated wound exhibited a more organized parallel arrangement of collagen fibrils, which was comparable with that of the unwounded skin. This was unlike the disorganized fibrillar arrangement seen in the pretreated wound. Altogether, the HF grafting led to the re-emergence of many important dermal structures, which suggests that HF grafts can potentially restore these crucial functions in the healed skin.

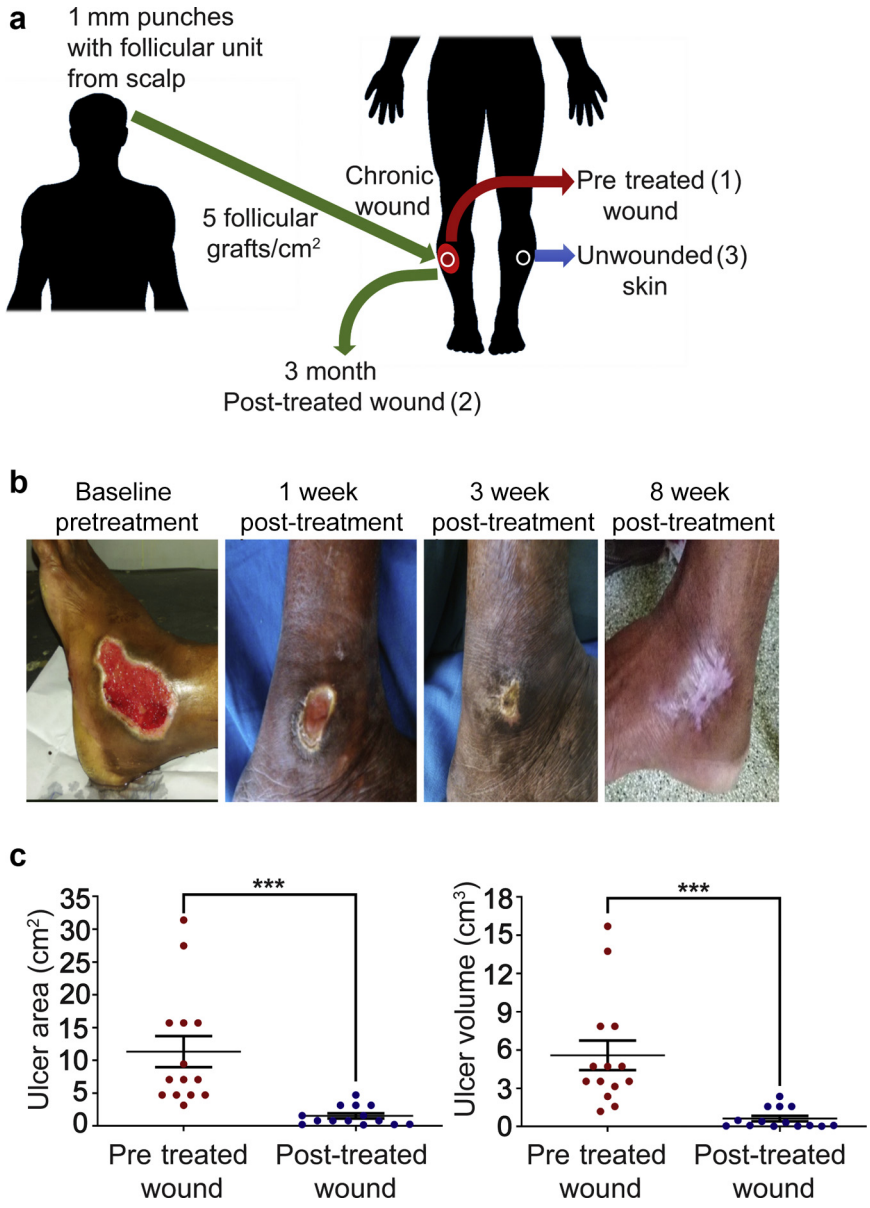

Figure 1. Hair follicle grafting decreases the area and volume of chronic wounds. (**a**) Schematic of treatment and tissues analyzed. Numbers in parentheses denote the order in which punch biopsies were taken. (**b**) Clinical photos of a patient with a venous ulcer at baseline pretreatment and 1 week, 3 weeks, or 8 weeks post-treatment showing a reduction in wound size after treatment. (**c**) Area (left panel) and volume (right panel) of ulcer post-treatment are significantly reduced compared with those in pretreated wounds. *P*-value is calculated by Wilcoxon matched-pairs signed-ranks test. $***P < 0.001$.

HF grafting dampens the inflammatory response of chronic wounds

A hallmark of chronic wounds is the presence of excessive inflammation leading to the notion that chronic wounds are trapped in the inflammatory phase of the wound healing program (Landén et al., 2016). One contributing factor to the persistent inflammatory response is the failure to inactivate and clear immune cells from the wound bed. Interestingly, MSCs, such as those associated with the HF, are known to have a potent anti-inflammatory role (Chahal et al., 2019; Ghoryani et al., 2019; González et al., 2009; Pers et al., 2018; Prockop and Oh, 2012; Wang et al., 2016). We thus investigated whether the grafting of HF units would have an impact on dampening the inflammatory profile of chronic wounds. After HF grafting, qPCR analysis revealed that the mRNA levels of the inflammatory cytokines *IL1α*, *IL6*, *TNFα*, and *IL8*, commonly upregulated in chronic wounds (Zhao et al., 2016), were significantly lowered compared with those in the pretreated wounds (Figure 4a). Consistent with the inflammatory gene expression analysis, the number of cells from both the adaptive (T cells, marked by CD3) and innate (macrophage, marked by CD11b) arms of the immune system were decreased in post-treatment skin samples as analyzed by immunofluorescence staining (Figure 4b).

DISCUSSION

Altogether, our study shows that HF graft therapy in chronic leg ulcers not only stimulates re-epithelialization of wounds but also results in the reappearance of epidermal and dermal components necessary for the proper functioning of the skin, which to our knowledge has not been reported previously. We attribute this effect to the presence of multiple SC pools contained in the grafted HF unit.

The mechanism of this reappearance remains unknown. However, it can be speculated that it can be due to at least two possibilities: (i) the grafted SCs associated with the HF

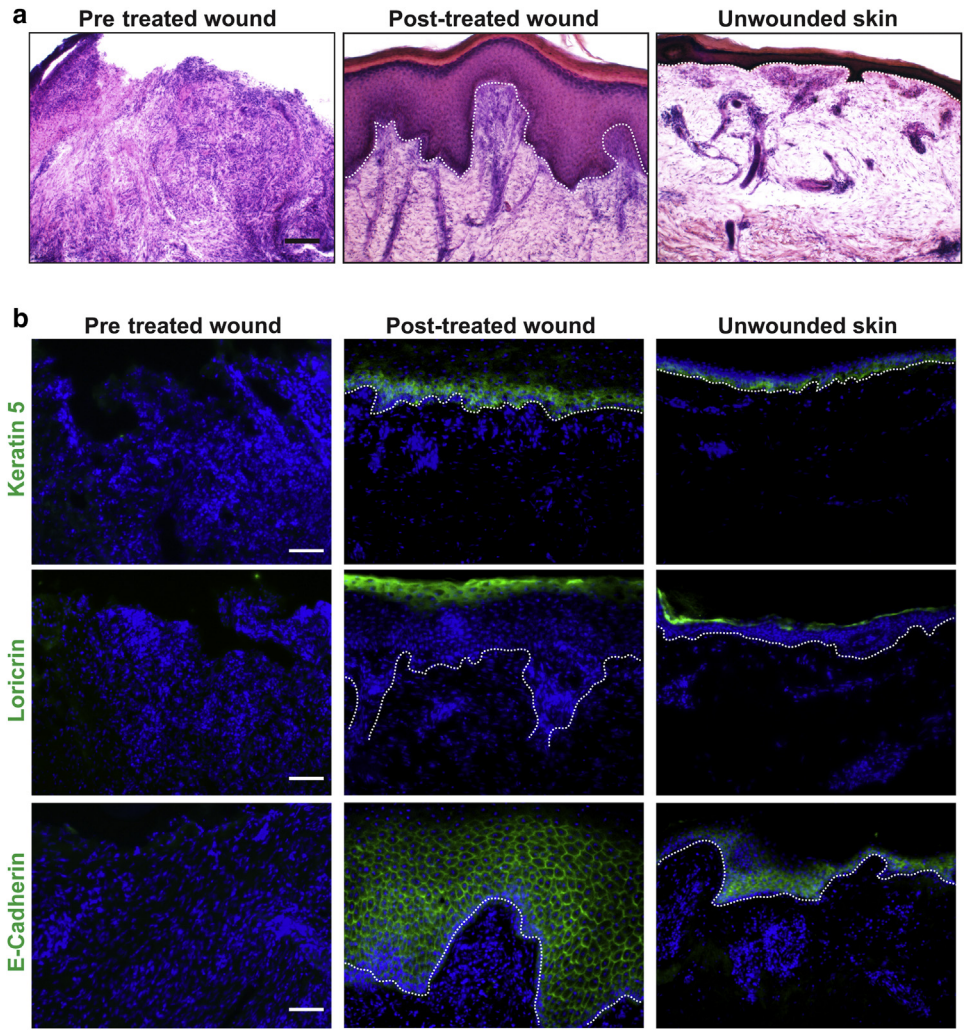

Figure 2. Hair follicle grafting promotes restoration of the stratified layers of the epidermis. (**a**) H&E staining showing the re-emergence of epidermal and dermal compartments after treatment. White dotted lines denote the basement membrane separating the epidermis from the dermis. Bar = 100 μm. (**b**) Immunofluorescence staining for the basal layer with an antibody recognizing keratin 5 (top panels), differentiated layers using an antiloricrin antibody (middle panels), and epidermal keratinocyte adherens junctions (E-cadherin; bottom panels) shows the restoration of these components after treatment. White dotted lines denote the basement membrane separating the epidermis from the dermis. Bar = 50 μm. Blue staining is Hoechst dye for nuclei.

themselves can differentiate into the dermal structures that are lost owing to the nonhealing nature of the wound and (ii) the grafted SCs can secrete factors capable of activating resident stem/progenitor cells from the proximal unwounded tissue to regenerate the dermal structures. Future mechanistic studies may utilize human HF grafts transplanted in cutaneous wounds made on nude mice or mice with diabetes. This can be a platform to conduct lineage tracing studies employing markers specific for the human chromosome to identify cells/structures arising directly from the grafted material. Alternatively, conditioned media prepared in vitro from human HF grafts can be used to treat wounds in these mice to investigate whether secreted factors play a major role in this healing process.

Another interesting observation made with the HF graft is the ability of this approach to dampen the inflammatory profile of the ulcers. It is interesting to speculate that the known anti-inflammatory capacity of MSCs may be the underlying factor in this observation. MSCs are being harnessed to control inflammation in several pathological scenarios (Wang et al., 2016). In fact, for arthritic joints, injection of MSCs has been used as a method to control tissue inflammation (Chahal et al., 2019; Ghoryani et al., 2019; González et al., 2009; Pers et al., 2018).

Finally, an important observation of our experimental protocol was the healing of punch biopsied tissue in the postgrafted wound. This result suggests that the HF grafting procedure is a self-sustaining, long-term therapy. It would therefore be interesting to determine whether the HF-derived SCs have repopulated the wound or whether they were able to recruit endogenous stems cells residing in re-established niches.

The value of this therapeutic intervention potentially applies to other large nonhealing wounds such as those in patients with a third-degree burn. The autologous nature of this approach would provide a source of SCs that is often lost in these patients as well as circumvent the need to administer antirejection drugs that have deleterious side effects. Moreover, the regeneration of skin in patients with burns is often lacking in sweat glands that are important for thermoregulation. The ability of HF grafts to induce the reappearance of sweat glands is to our knowledge a previously unreported aspect of this therapy.

MATERIALS AND METHODS

Human subjects

A total of 10 patients were included in the study with a total of 14 chronic nonhealing leg ulcers of various etiologies (venous, traumatic, diabetic, leprotic) of >6 weeks duration, who had been

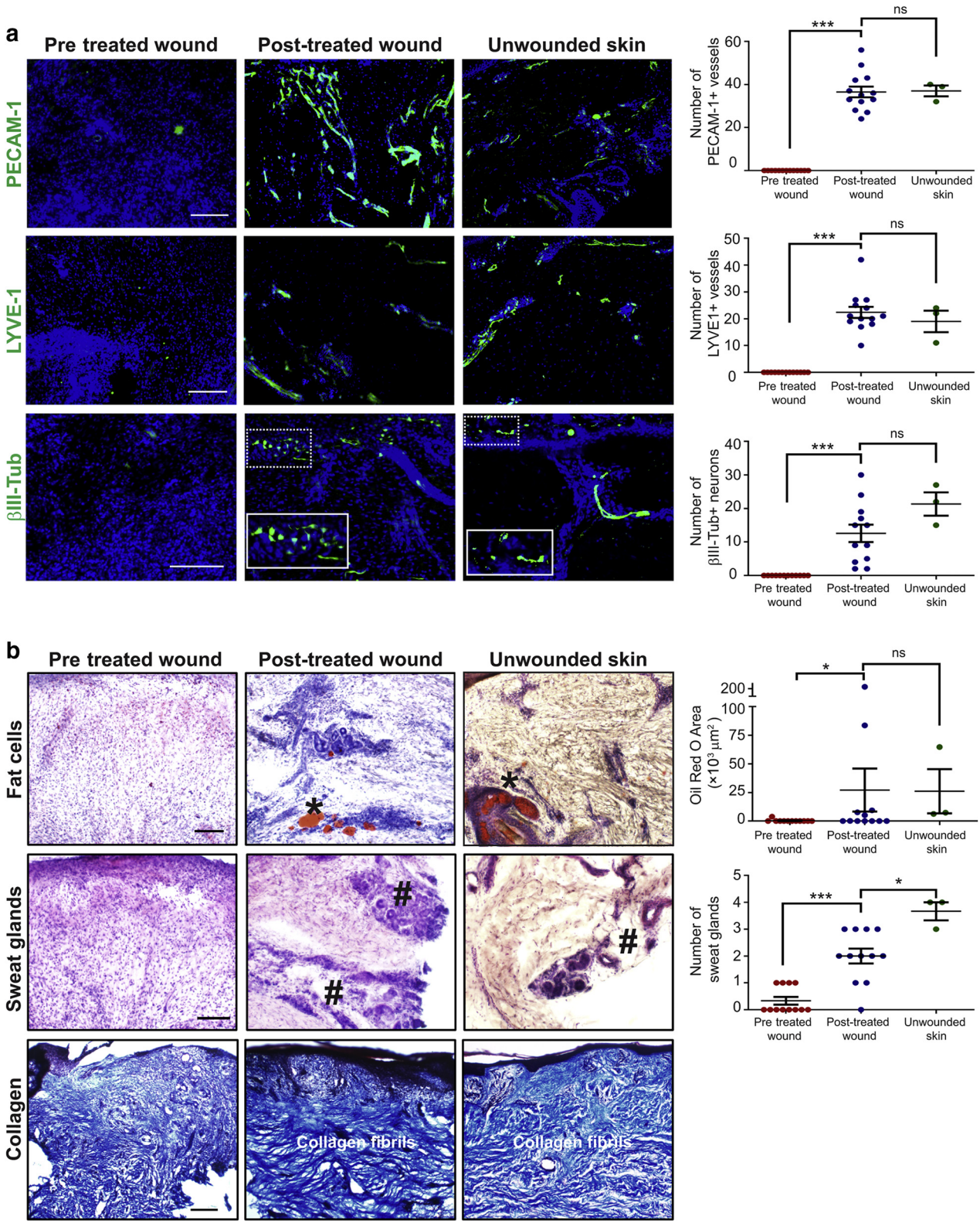

Figure 3. Hair follicle grafting promotes the restoration of dermal components critical for normal cutaneous function. (**a**) Immunofluorescence staining (green) for blood vessels (PECAM-1; top panel), lymph vessels (LYVE-1; middle panel), and nerve cells (βIII-Tub; bottom panel) shows the re-emergence of these functional dermal components after treatment (left panel). Bar = 100 µm. Blue staining is Hoechst dye for nuclei. Quantification of PECAM1-, LYVE1-, and βIII-Tub–stained structures in pretreated, post-treated, and unwounded skin. (**b**) Histological analysis of fat-containing cells using Oil Red O staining (top panel; asterisk [*] denotes lipid-containing cells) and sweat gland structures marked by hash (#) after H&E staining (middle panel) and collagen fibrils (Masson's trichrome staining; bottom panel) in the dermal compartment. Bar = 100 µm. Quantification was carried out by calculating the number of structures per field with biological replicates of $n \geq 12$ (for pretreated and post-treated wounds) and $n = 3$ (for unwounded skin). $^{***}P < 0.001$; $^{*}P < 0.05$; ns > 0.05. βIII-Tub, class III β-tubulin; ns, nonsignificant.

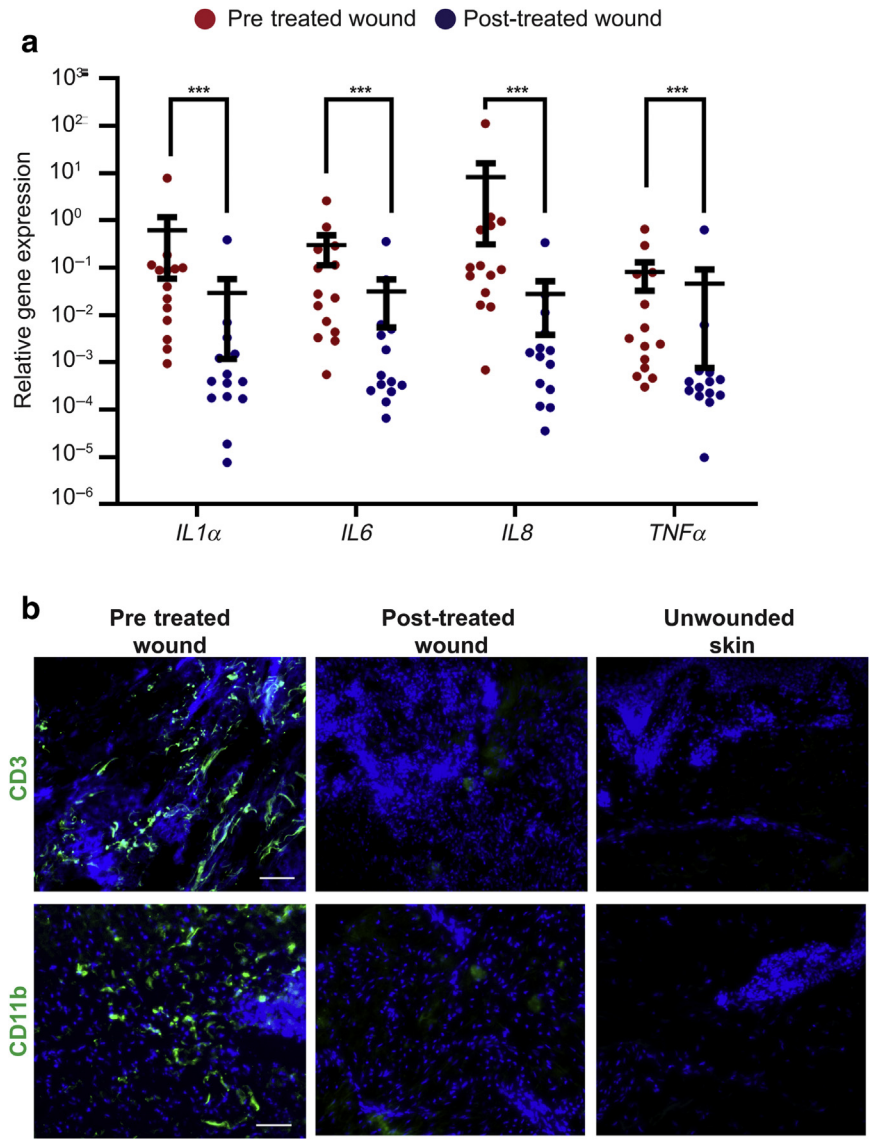

Figure 4. Hair follicle grafting dampens the inflammatory response of chronic wounds. (**a**) qPCR analysis of mRNA levels of major inflammatory cytokines significantly reduced post-treatment. ***$P < 0.001$; n = 14 ulcers. (**b**) Immunofluorescence staining (green) showing reduction of T cells (CD3; top panel) and macrophages/monocytes (CD11b; bottom panel) post-treatment. Bar = 50 μm. Blue staining is Hoechst dye for nuclei.

recalcitrant to standard treatment of care. Written informed consent for the procedure was obtained from the participants. Three of the patients consented to provide unwounded skin samples. Patients who had any bleeding disorders, uncontrolled diabetes, and infected ulcers were not included in the study. Demographic details of all patients, including age, onset and progression of the ulcers, and the treatment history, are summarized in Table 1. Approval to extract patient samples with informed consent was obtained from the Ethical Committee of Bangalore Medical College and Research Institute (Bangalore, India). Analysis of patient skin samples in the Jamora lab (Bangalore, India) was approved by the Institutional Ethics Committee of the Institute for Stem Cell Science and Regenerative Medicine (Bangalore, India) (Human Ethics Approval Certificate number inStem/IEC-8/003).

Surgical procedure and sample collection

The grafting procedure was described in Budamakuntla et al. (2017). Briefly, the procedure was performed in an outpatient setting after the application of local anesthesia. A micromotor device (Saeyang Microtech Co, Ltd, Daegu, South Korea) was used to extract single HFs units (1 mm circular punches) from the occipital area of the patient's scalp. Infected and necrotic tissue was initially removed from the chronic leg ulcers by debridement, and the follicular units were implanted into the wound bed with SAVA implanters (Vasa Surgiart Pvt, Ltd, Ahmedabad, India). Five follicular grafts per square centimeter were implanted in the wound area (on the basis of the minimum number of HFs required for regeneration determined in the pilot study of such therapy) (Jiménez et al., 2012). After completion of the grafting procedure, the ulcer area was covered with Vaseline gauze (Cuticell Classic, Adeshwar Meditex Pvt, Ltd, Palghar, India) and an elastic bandage (Leuokoband, Neptune Orthopaedics, Ahmedabad, India) for 24 hours. Patients were given a regimen of antibiotics and painkillers for 10 days after the procedure.

Wound area and volume measurement

The length, breadth, and depth of the chronic leg ulcers were measured by the clockface method (Sussman and Bates-Jensen, 1998) using a cotton-tip applicator and a disposable paper ruler. The area and volume of each ulcer before and 3 months after

Table 2. qPCR Primer Sequences

Gene Symbol	Forward Primer Sequence	Reverse Primer Sequence
IL1α	AGATGCCTGAGATACCCAAAACC	CCAAGCACACCCA GTAGTCT
IL6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTT CAGGTTG
IL8	CACACTGCGCCAACA	CATCTTCACTGATTCTTGGAT
TNFα	CTCCTTCCTGATCGTGGC	GTTCAGCCACTGGAGCT
β-Actin	TCCTTCCTGGGCATGGAGT	AGCACTGTGTTGGCGTACAG

treatment were calculated using the formula for an ellipse (wound resembled an elliptical shape) as follows: ulcer area = length × width × 0.7854 and ulcer volume = length × width × 0.7854 × depth as described previously (Blume et al., 2008; Suryanarayan et al., 2014).

RNA isolation, cDNA synthesis, and qPCR

Punch biopsies of the pretreated wound, post-treated wound, and unwounded skin were collected in RNA stabilization buffer. Each punch biopsy was homogenized in 1 ml RNAiso Plus reagent (DSS Takara Bio India Pvt, Ltd, New Delhi, India; catalog number 9109), and RNA was isolated according to the manufacturer's instructions. cDNA was prepared using PrimeScript cDNA Synthesis Kit (DSS Takara Bio India Pvt, Ltd; catalog number 2680A) according to the manufacturer's instructions. qPCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA; catalog number A25742) in CFX384 machine (Bio-Rad Laboratories, Hercules, CA). *β-Actin* expression was used for normalization. The primers used are listed in Table 2.

Tissue preparation and immunohistochemistry/immunofluorescence

Skin samples were fixed in 4% paraformaldehyde solution for 24 hours at 4 °C. Tissues were quenched in 125 mM glycine solution in Tris-buffered saline for 30 minutes and then incubated in 5%, 10%, and 20% sucrose solution for 6 hours, 12 hours, and 24 hours, respectively, before embedding in tissue freezing medium (Leica, Wetzlar, Germany). Sections that were 20 μm or 60 μm thick were sliced using a cryostat. H&E staining was used for viewing gross tissue histology. For immunofluorescence staining, the following primary antibodies and dilutions were used: keratin 5 (BioLegend, San Diego, CA; catalog number 905501, 1:200), loricrin (Abcam, Cambridge, United Kingdom; catalog number ab85679, 1:200), E-cadherin (Thermo Fisher Scientific, Waltham, MA; catalog number 13-1900, 1:500), PECAM-1 (Abcam; catalog number ab28364, 1:50), LYVE-1 (Abcam; catalog number ab14917, 1:25), class III β-tubulin (Promega, Madison, WI; catalog number G7121, 1:1,000), CD3 (eBioscience, San Diego, CA; catalog number 14-0032-85, 1:200), and CD11b (Developmental Studies Hybridoma Bank, Iowa City, IA; catalog number M1/70.15.11.5.2, 1:200). Alexa Fluor 488–labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a dilution of 1:300. Hoechst stain was used to mark the nuclei. Fat cells were stained by Oil Red O (Sigma-Aldrich, St. Louis, MO; catalog number O1391) solution for 3 minutes, followed by isopropanol washing and counterstain with hematoxylin. Collagen staining was done using Trichrome Stain Kit (Abcam; catalog number ab150686) according to the manufacturer's instructions.

Image collection and analysis

Imaging was performed with an Olympus IX73 microscope (Olympus, Tokyo, Japan) or FV1000 confocal microscope (Olympus). Images were analyzed on the Fiji (ImageJ) software (NIH, Bethesda, MD and LOCI, Madison, WI).

Statistical analysis

For the quantification of the vasculature, neuronal structures, fat content, and sweat gland structures, comparison of pretreated and post-treated samples was done using the nonparametric Wilcoxon matched-pairs signed-ranks test, and comparison of post-treated wound sample with unwounded skin samples was done using the Mann–Whitney test. Comparison of two groups was done using the nonparametric Wilcoxon matched-pairs signed-ranks test for the analysis of inflammatory cytokine expression. GraphPad Prism 5.02 (GraphPad Software, San Diego, CA) was used for all statistical analyses. $P < 0.05$ was considered significant.

Data availability statement

This paper does not include any next-generation sequencing datasets and has not used or made any particular codes for large-scale data analysis.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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