Stimulation of the Follicular Bulge LGR5+ and LGR6+ Stem Cells with the Gut-Derived Human Alpha Defensin 5 Results in Decreased Bacterial Presence, Enhanced Wound Healing, and Hair Growth from Tissues Devoid of Adnexal Structures

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Background: Discovery of leucine-rich repeat-containing G-protein–coupled receptors 5 and 6 (LGR5 and LGR6) as markers of adult epithelial stem cells of the skin and intestine permits researchers to draw on the intrinsic cellular fundamentals of wound healing and proliferation dynamics of epithelial surfaces. In this study, the authors use the intestine-derived human alpha defensin 5 to stimulate epithelial proliferation, bacterial reduction, and hair production in burn wound beds to provide the field with initial insight on augmenting wound healing in tissues devoid of adnexal stem cells.
Methods: Murine third-degree burn wound beds were treated with (1) intestine-derived human alpha defensin 5, (2) skin-derived human beta defensin 1, and (3) sulfadiazine to determine their roles in wound healing, bacterial reduction, and hair growth.
Results: The human alpha defensin 5 peptide significantly enhanced wound healing and reduced basal bacterial load compared with human beta defensin 1 and sulfadiazine. Human alpha defensin 5 was the only therapy to induce LGR stem cell migration into the wound bed. In addition, gene heat mapping showed significant mRNA up-regulation of key wound healing and Wnt pathway transcripts such as Wnt1 and Wisp1. Ex vivo studies showed enhanced cell migration in human alpha defensin 5–treated wounds compared with controls.
Conclusions: Application of human alpha defensin 5 increases LGR stem cell migration into wound beds, leading to enhanced healing, bacterial reduction, and hair production through the augmentation of key Wnt and wound healing transcripts. These findings can be used to derive gut protein–based therapeutics in wound healing. (*Plast. Reconstr. Surg.* 132: 1159, 2013.)

Leucine-rich repeat-containing G-protein–coupled receptor (LGR) is a seven-pass transmembrane protein receptor with significant sequence and structural homology to the follicle-stimulating hormone, thyroid-stimulating hormone, and luteinizing hormone receptor families. Recent discovery of LGR5 and LGR6 as markers of both intestinal and epidermal stem cells in mammals has led researchers to explore the clinical applicability of these cells in wound healing, tissue engineering, and transplantation.[1,2] LGR6+ cells in the skin are located at the follicular bulge in both mice and humans and

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appear to be capable of producing all cell lineages of the skin, including the sebaceous gland and interfollicular epidermis.[3] The LGR5$^+$ stem cell, also found in the human intestine at the +4 position within the crypt, when localized in the skin, is initially found in the same region as the LGR6$^+$ stem cell.[4] However, following prenatal embryologic development, this cell appears to migrate toward the dermal papilla and establish itself in hair shaft production.[2,3] This upward migration of the LGR6$^+$ stem cell and alternative downward migration of the LGR5$^+$ stem cell along the hair follicle is considerably similar to the migration pattern of the LGR stem cell, which produces epithelial lineages of the intestine.

Similarly, the gut, skin, and other mucosal surfaces produce antimicrobial peptides, in particular, a group referred to as defensins, which are differentiated based on secondary protein structure and divided into alpha and beta defensin families.[5] Defensins constitute a structural class of small cationic peptides that exert broad-spectrum antimicrobial activities through microorganism permeabilization.[6] However, not only do these peptides carry intrinsic antibacterial properties by disrupting membrane integrity, they also appear to enhance the proliferation and migration of local epithelial cells within their regional niche and improve wound healing.[7–9] It is here, within epithelial wound beds, that local defensins are thought to elicit intracellular Ca^{2+} mobilization and increase epithelial cell migration and proliferation to quickly recreate a competent barrier over subepithelial structures.[10] Although the skin typically produces beta defensins, the intestine produces both alpha and beta defensins. Alpha defensins are expressed primarily in the Paneth cell of the intestine, neutrophils, and certain macrophage populations, whereas beta defensins are primarily epithelial based.

Sterile injury to epithelial surfaces has been shown to increase the expression of beta defensins locally to initiate an innate immune response that increases resistance to overt infection and microbial colonization.[11] However, following deep burns, researchers have found diminished levels of certain beta defensins within the burn wound bed and surrounding tissues, suggesting that reduced levels may facilitate invasion of the tissue and deeper structures.[12,13] Moreover, some have reported that there may be direct communication between the beta defensin peptides and known cationic nuclear localization signal sequences within the genome, suggesting a role for this peptide in nuclear translocalization and feed-forward augmentation of gene expression during bacterial challenge and wound healing.[14]

Complete destruction of the epidermis, dermis, and hypodermal structures within the context of third-degree burn wound beds not only obliterates epithelium and its ability to produce defensins, but also subsequently removes the follicular bulge and LGR stem cell niche, which may rely on defensin peptides for epithelial status communication. Elimination of these structures presents a difficult defect for local tissue to overcome in that the barrier has been breached and the innate immunomodulatory defensin system is incapacitated. Subsequently, the epithelial stem cell population at the bulge is no longer present to repopulate the wound and develop cells capable of antimicrobial peptide production. It is here, within this cutaneous third-degree burn wound bed, that we suggest a potential role for local application of the gut-derived human alpha defensin 5, within a murine model system, to determine whether the addition of these peptides can enhance wound healing, reduce bacterial invasion, and promote hair shaft development through the stimulation of local LGR5$^+$ and LGR6$^+$ stem cells.

MATERIALS AND METHODS

Murine Burn Model Design

Seventeen age- and sex-matched C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Me.) were shaved and labeled on the dorsum skin to develop a 2 × 3 grid of 3-mm-diameter wound beds spaced 1 cm apart. Burns were created using a disposable Aaron high-temperature cautery (AA01; Bovie Medical Corp., Clearwater, Fla.) by placing the preheated tip to the raised dorsal skin for 5 seconds until all epidermal, dermal, and hypodermal tissues were eliminated and the panniculus carnosus was visible under gross observation within the predetermined wound bed area. Both hematoxylin and eosin and antibody immunofluorescent labeling of LGR5, LGR6, and 4′,6-diamidino-2-phenylindole were used to validate the thermal destruction of the follicular bulge and adnexa, indicating that a full-thickness wound had been created. Mice were then subsequently treated, using a sterile pipette, with topical agents at the wound bed. The agents applied to the wound beds included the following: 1 μl of either molecular grade water control (MilliQ water; Millipore Corp., Billerica, Mass.), Cell-Gro (Mediatech, Inc., Manassas, Va.), human beta defensin 1 (Innovagen, Lund, Sweden) at 100 ng/μl, human alpha defensin 5 (Innovagen)

at 100 ng/μl, or sulfadiazine (Sigma-Aldrich, St. Louis, Mo.) at 1 mg/μl. All wound bed procedures and data collection were performed in triplicate for intravariable validation. On days 0, 5, 10, and 15 of the study, a subpopulation of three mice were killed and their burn wound beds were harvested for immunofluorescent labeling, fluorescent in situ hybridization, and mRNA expression studies. Wound beds were placed in Allprotect (Qiagen, Venlo, The Netherlands), Carnoy's solution (Sigma-Aldrich), and 10% neutral buffered formalin (Sigma-Aldrich) for RNA extraction, fluorescent in-situ hybridization, and immunofluorescence, respectively. All animals involved in this study were approved for use under Laboratory Animal Care and Use Committee protocol no. 158-11-009 and complied with the volume entitled *Recognition and Alleviation of Distress in Laboratory Animals*.[15] Researchers were blinded to the identity of the substance applied to wound beds, the identity of each mouse, the experimental group of both images, and specimen taken during the study. Each mouse was identified solely by an individual protocol identifier number.

Bacterial Adhesion

C57BL/6J murine wound bed samples acquired at days 0, 5, 10, and 15 were placed directly into Carnoy's solution fixative for paraffin embedment and 3-μm sectioning. Fluorescent in situ hybridization examination was performed by methods published previously by Lough et al.[16] for microbial adherence on epithelial surfaces. 16S rRNA fluorescent hybridization used the universal bacterial probe EUB338 with a 5′-Cy3 conjugate (*Cy3*5′-GCT GCC TCC CGT AGGAGT-3′; MWG Biotech, Ebersberg, Germany).

Immunofluorescence

Wound beds acquired at days 0, 5, 10, and 15 were placed directly into 10% neutral buffered formalin fixative for paraffin embedment and 3-μm sectioning. Dako (Carpinteria, Calif.) ancillary reagents were used for staining according to the manufacturer's protocol. Antibody dilutions were applied according to the manufacturer's insert: LGR5 (primary rabbit anti-mouse sc-135238, secondary goat anti-rabbit-FITC), LGR6 (primary goat anti-mouse scbt-48236 secondary donkey anti-goat-rhodamine) (Santa Cruz Biotechnology, Dallas, Texas), and Prolong Gold with 4′,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, Calif.) were used to stain for DNA and preserve fluorescence. Images were captured on an Olympus BX41 epifluorescence and Olympus FluoView laser scanning confocal scope (Olympus, Tokyo, Japan). Relative fluorescent and chromogenic intensities were acquired using ImageJ (National Institutes of Health, Bethesda, Md.) and SigmaGel (SPSS, Inc., Chicago, Ill.) software programs as described previously by Lough et al.[16]

Reverse-Transcriptase Polymerase Chain Reaction and Heat Mapping

RNA from wound bed tissues were stabilized in Allprotect, extracted, and verified using the Allprep DNA/RNA/Protein Kit (Qiagen) and NanoDrop (Thermo Fisher Scientific, Inc., Waltham, Mass.). RNA expression was assayed using the RT^2-PCR Array system (SABiosciences Corp., Frederick, Md.) according to the manufacturer's protocol. Data acquisition was performed on a StepOne Plus RT-PCR machine (Applied Biosystems, Foster City, Calif.). Heat mapping, gene tables, and p values were developed using the SABiosciences reverse-transcriptase polymerase chain reaction Web-based application software. LGR5 and LGR6 reverse-transcriptase polymerase chain reaction expression values were determined using the following National Center for Biotechnology Information RefSeq mRNA accession numbers: NM_010195.2 for LGR5 (forward primer, CCTACTCGAAGACTTACCCAGT; reverse primer, GCATTGGGGTGAATGATAGCA) and NM_001033409.3 for LGR6 (forward primer, GGAGGTCTCACAGAGCATGA; reverse primer, AGCACTTTGGCACAGCATAG). Qiagen First Strand cDNA kit and Qiagen SYBR Green Master Mix were used according to the manufacturer's protocol.

LGR6$^+$ Stem Cell Isolation

Following euthanasia, C57BL/6(UBC-GFP) mice were shaved, cleansed, and skinned. Circumferential full-thickness skin was harvested and underlying fat was removed. The skin was cut into small 1- to 2-mm pieces for enzyme digestion. LGR6$^+$ stem cell isolation was conducted using the Snippert et al. protocol published in their supplemental information document.[3]

Wound Assay

Fluorescence-activated cell sorting–isolated LGR6$^+$, CD34$^+$, and CD73$^+$ C57BL/6(UBC-GFP) murine cells were grown to confluence in Dulbecco's Modified Eagle Medium/F12 (Sigma-Aldrich), 10% fetal bovine serum (Atlas Biologicals, Fort Collins, Col.), 10 ng/ml endothelial growth factor (Invitrogen), 20 ng/ml basic fibroblast growth factor (Invitrogen), 20 nM hydrocortisone (Sigma-Aldrich), 5 μg/ml insulin

(Sigma-Aldrich), and 100 U/ml penicillin/streptomycin (HyClone Laboratories, Inc., Logan, Utah) at 37°C in 5% carbon dioxide. Using a sterile pipette tip, the confluent layer of cells was disrupted or wounded linearly and the appropriate agent (human alpha defensin 5 or sulfadiazine) was added at 100 ng and 1 mg, respectively. The distance of wound edges was captured at 0, 6, and 12 hours using an inverted Olympus IX71 scope.

RESULTS

Application of the Human Alpha Defensin 5 Peptide to Wound Beds Results in Reduced Bacterial Adhesion

Prior studies have indicated that the presence of antimicrobial peptides at epithelial surfaces reduces the basal level of bacterial adhesion and invasion into deeper structures.[16–18] Following induction of the third-degree burn wound bed grid, we validated elimination of the epithelium, adnexa, and LGR stem cell structures using hematoxylin and eosin staining and immunofluorescent antigen labeling when compared with nonburn controls. In addition, co-labeling of sections with fluorescent-conjugated LGR5 and LGR6 antibodies was used to confirm the elimination of $LGR5^+$ and $LGR6^+$ in tissues.

Following burn grid induction, burn wound beds were exposed topically to 1 μl of either (1) MilliQ water; (2) the epithelium-derived human beta defensin 1 at 100 ng/μl; (3) the Paneth cell–derived human alpha defensin 5 at 100 ng/μl; or (4) sulfadiazine, 1 mg/μl daily for 15 days during wound healing by sterile pipette (Fig. 1). At days 0, 5, 10, and 15, a subset of the study population (three C57BL/6J mice) were killed and their wound beds were collected and placed into Carnoy's solution fixative. Subsequently, fluorescence in situ hybridization was performed using the 16S rRNA bacterial oligonucleotide probe EUB338 with a 5′-Cy3 conjugate: *Cy3*5′-GCT GCC TCC CGT AGGAGT-3′ (MWG Biotech) and epifluorescent microscopy was used to evaluate bacterial presence within the wound bed.

Human alpha defensin 5 was the only agent to visibly reduce bacterial load when compared with the antibiotic sulfadiazine on day 5 as depicted by 16S rRNA Cy3-EUB338 oligonucleotide probe hybridization to microorganisms (Fig. 2, *above*). To validate and quantify these findings, images were converted to gray-scale tagged image file formats, and white intensity was graphed per pixel unit (Fig. 2, *center* and *below*, *left*) and quantified. Day-5 averaged wound bed relative intensity was 1 ± 0.14 for sulfadiazine versus 0.071 ± 0.012 for human alpha defensin 5 ($p < 0.05$). Further image analysis quantification, using National Center for Biotechnology Information openware ImageJ software targeting the fluorescence of Cy3-EUB338 binding expression, found those wound beds treated with sulfadiazine to be significantly more bacteria-bound than those treated with the human alpha defensin 5 peptide: 1.0 ± 0.16 for day-5 sulfadiazine-treated and 0.19 ± 0.020 for human alpha defensin 5–treated ($p < 0.05$) (Fig. 2, *below*, *right*). These results suggest that the topical application of the gut-derived defensin human alpha defensin 5 can reduce bacterial adhesion and infiltration into cutaneous burn wound bed tissues when evaluated using epifluorescent microscopy and quantitative imaging software.

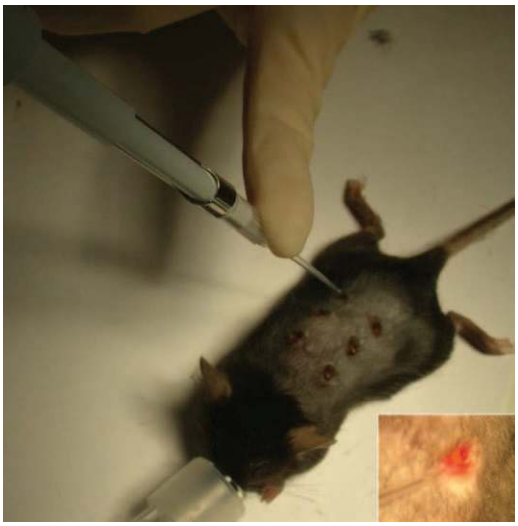

Fig. 1. Third-degree wound bed induction and application of topical therapies. Example wound bed grid of 3-mm-diameter third-degree burns. (*Inset*) Topical application of the resuspended peptide at the wound site.

Application of Human Alpha Defensin 5 Results in Augmented Wound Healing and New Hair Growth

Previous research by others has shown that the expression of defensins, primarily beta defensins, has been associated with an augmented rate in cutaneous and mucosal wound healing.[8,19] In addition to reducing the bacterial load of the wound, defensins provide a local proliferative and migratory effect on regional epithelial cells by stimulating an immunomodulatory circuit that recruits inflammatory cells to damaged areas and facilitates Ca^{2+} modulation, leading to epithelial cell migration.[20]

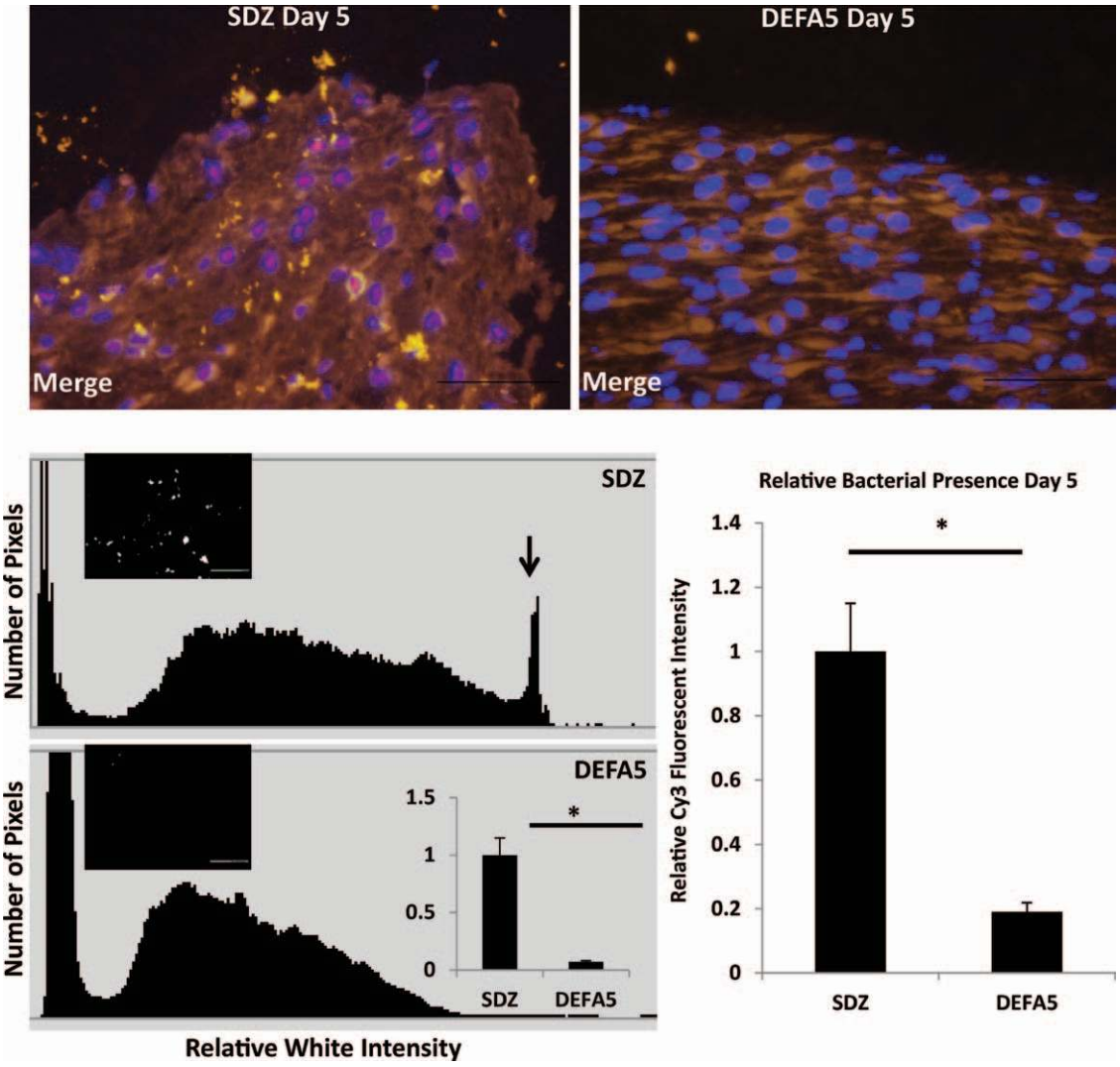

Fig. 2. 16S rRNA fluorescent in situ hybridization indicating the presence of bacterial adhesion at the third-burn wound bed. (*Above*) Merged images of 5′-Cy3-EUB338 16S rRNA oligonucleotide and 4′,6-diamidino-2-phenylindole–co-labeled third-degree burn wound bed specimen (bacterial organisms are depicted as *yellow grains*) at day 5 after burn induction treated daily with sulfadiazine and human alpha defensin 5. (*Center* and *below*, *left*) Example quantification of white pixel intensity of Cy3 fluorescence gray-scale converted image of (*inset*) a wound bed treated with sulfadiazine and containing more 16S rRNA labeling per unit area than human alpha defensin 5–treated burns. (*Inset graph*) Averaged white pixel intensity of 16S rRNA expressed in both sulfadiazine-treated and human alpha defensin 5–treated burn wound beds at day 5 using gray-scale imaging software. (*Below*, *right*) Averaged red channel fluorescence of 16S rRNA expressed in both sulfadiazine-treated and human alpha defensin 5–treated burn wound beds at day 5. *Scale bar* = 100 µm (*$p < 0.05$).

Within this stage, we monitored the progression of healing over the course of 15 days following the daily application of (1) MilliQ water control; (2) human alpha defensin 5 (100 ng/µl); (3) human beta defensin 1 (100 ng/µl); or (4) sulfadiazine (1 mg/µl). Comparing the first 10 days, we observed enhanced healing in those beds receiving human alpha defensin 5, with almost complete wound closure by day 10 (Fig. 3, *above* and *second row*). In addition to observing an increased rate of healing with human alpha defensin 5, there was also an unexpected appearance and continued growth of nascent hair in and around the healing tissue, something not observed in wound beds treated with other agents (Fig. 3, *third* and *fourth rows*).

To validate and quantify the ability of human alpha defensin 5 to augment healing, wound beds were also evaluated for wound area reduction (healing rate). Here, TIFF images were first

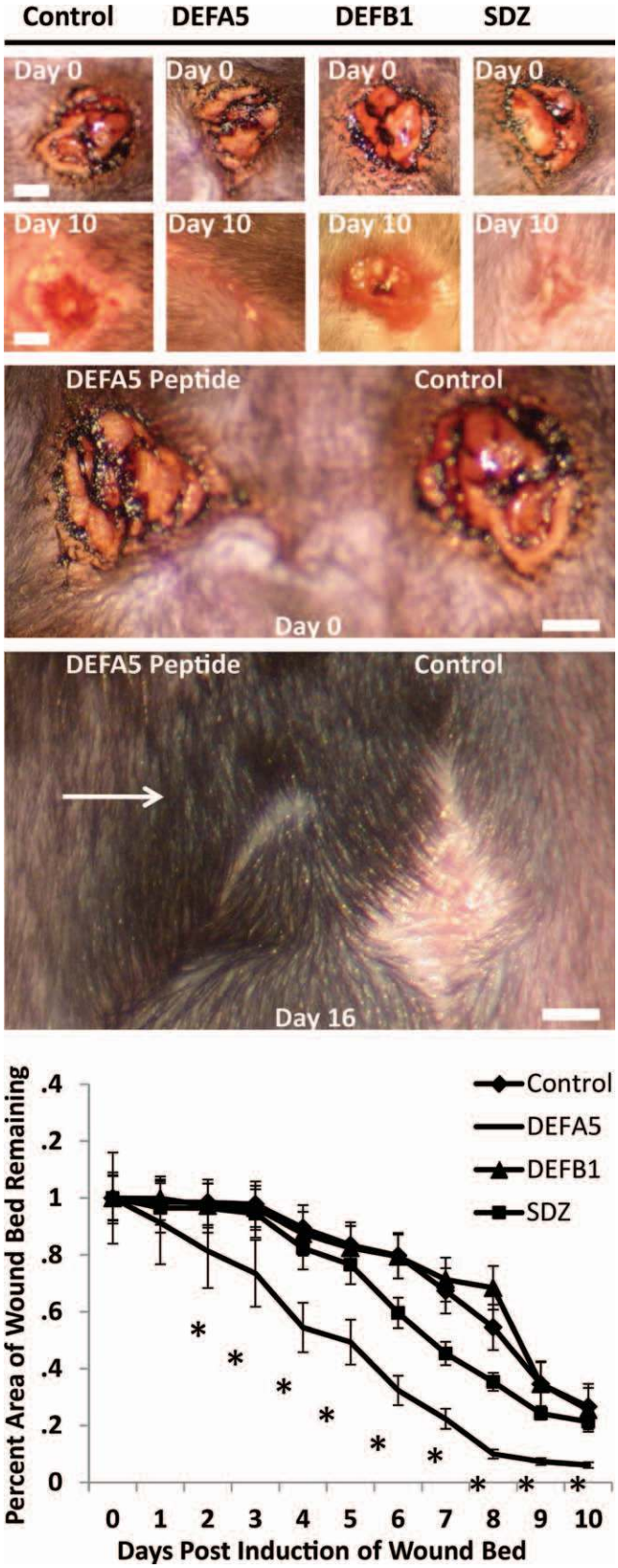

Fig. 3. Third-degree burn wound healing kinetics and nascent hair growth in treated burn wounds devoid of adnexal structures. (*Above* and *second row*) Gross imaging using a Leica Wild M680 to

standardized based on external measurement at the wound site and then placed into an image database. Once standardized, the wound areas were converted to digital voids (black area), and regression of the void was tracked over the first 10 days of healing. Each wound bed void was assigned a wound bed calculated area and correlated with time from wound induction. Wound reduction was plotted as a percentage of initial wound area (Fig. 3, *below*). Our results indicate that wound beds treated with the gut-derived human alpha defensin 5 deviate significantly from those beds treated with MilliQ water, human beta defensin 1, and sulfadiazine by day 2 and continue to plot at significantly reduced wound size over the next 8 days ($p < 0.05$).

Application of Human Alpha Defensin 5 to Wound Beds Results in Increased LGR5 and LGR6 Expression

Understanding that defensins exhibit chemotactic capabilities, we evaluated whether those cells migrating into the wound beds treated with human alpha defensin 5 therapy exhibited LGR5 and LGR6 expression during healing, despite having had the adnexal follicular bulge thermally obliterated. Using 17 C57BL/6J mice, we monitored for LGR5 and LGR6 expression within the healing tissues over the course of 15 days.

Our results indicate that those wound beds treated with the gut-derived human alpha defensin 5 showed increased LGR5 and LGR6 expression within wound tissue. Confocal fluorescent imaging on day 5 of the study shows that human alpha defensin 5–treated tissues contain cells expressing both LGR5 (green) and LGR6 (red), suggesting migration into tissues when compared with those wounds treated with sulfadiazine, which did not (Fig. 4, *left*). Immunofluorescent green and red channel intensity quantification of the average LGR5 and LGR6 conjugate expression validates confocal findings: LGR5 and LGR6 expression at sulfadiazine-treated LGR5 (0.0.9 ± 0.02) and LGR6 (0.11 ± 0.03) versus human alpha defensin 5–treated LGR5 (0.48 ± 0.13) and LGR6 (1 ± 0.19) ($p < 0.05$) (Fig. 4, *below*, *left*).

To confirm the quantitative confocal microscopic intensity patterns from imaging LGR5 and LGR6, we also performed reverse-transcriptase polymerase chain reaction on burn wound tissues. Averaged LGR5 and LGR6 mRNA expression within human alpha defensin 5 wound beds was found to be 95.8 ± 10.6 and 259.2 ± 20.2, respectively, compared with undetectable levels of LGR5 and LGR6 in sulfadiazine-treated wounds at day 5 (Fig. 4, *right*). The magnitudes of these fold-level comparisons within human alpha defensin 5–treated tissues and those specimens treated with sulfadiazine suggest that it is the absolute presence or void of cells expressing LGR5 and LGR6 migrating into the wound that defines these fold values. A wound bed receiving migratory cells that are actively transcribing LGR mRNA transcripts compared with a tissue void of these cells leads to log-fold differences in levels of expression, rather than what is typically seen in direct transcript up-regulation of similar wound beds containing the same cell subset population. As such, these data support a migratory response of cells expressing LGR5 and LGR6 into the wound bed rather than the conversion of cells presently in the wound bed into epithelial stem cells or progenitors.

Application of Human Alpha Defensin 5 Results in Up-Regulation of Key Wound Healing and Wnt Pathway mRNA Transcripts

A fraction of the total RNA extracted for LGR reverse-transcriptase polymerase chain reaction expression was also used for gene array pathway analysis and heat mapping. Using the RT^2-PCR Wound Healing Pathway Array and Wnt Pathway Array (SABiosciences), we investigated the role of human alpha defensin 5 versus sulfadiazine in augmenting key transcript expression within the wound. Our findings indicate that several gene subsets are significantly up-regulated within the wound beds receiving human alpha defensin 5 when compared with sulfadiazine therapy (Fig. 5, *left*). Understanding that the LGR stem cell system is responsive to Wnt ligands in both the gut and skin, we also evaluated RNA extracted from the wound bed for Wnt pathway transcripts. We discovered that certain Wnt pathway gene subsets are significantly up-regulated (Fig. 5, *right*).

Fig. 3. (*Continued*) image healing of third-degree burn wound beds over 10 days while being treated with indicated agents MilliQ water, human alpha defensin 5, human beta defensin 1, and sulfadiazine. *Scale bar* = 1 mm. (*Third* and *fourth rows*) Gross imaging using a Leica Wild M680 to tracking nascent hair growth of third-degree burn wound beds over 16 days in a side-by-side comparison of human alpha defensin 5–treated versus control-treated wound beds. *Arrow* indicates the growth of new hair. *Scale bar* = 1 mm. (*Below*) Quantification of wound healing over time following treatment (*$p < 0.05$). *DEFA5*, human alpha defensin 5; *DEFB1*, human beta defensin 1; *SDZ*, sulfadiazine.

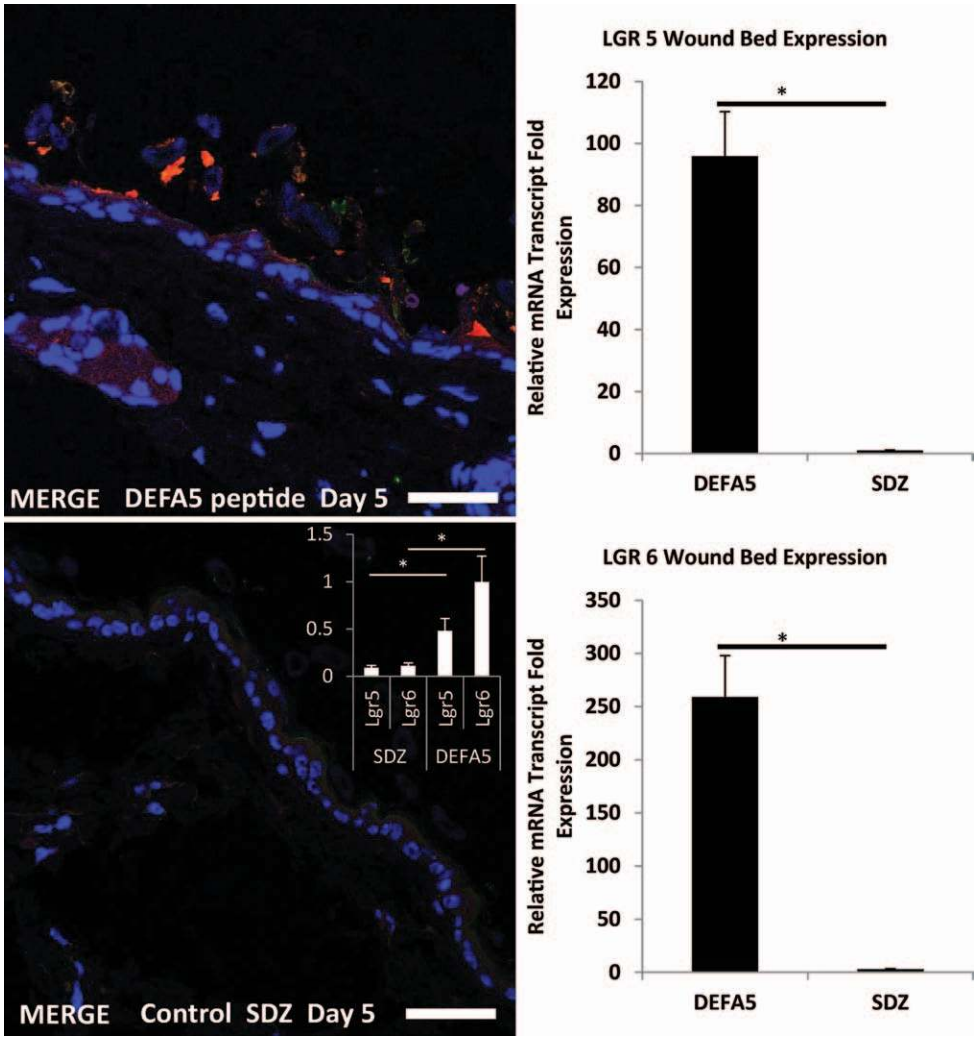

Fig. 4. LGR5 and LGR6 stem cell migration into burn tissue following treatment with topical focal agents. (*Left*) LGR5 (green) and LGR6 (red) immunofluorescent antibody labeling of sulfadiazine and human alpha defensin 5–treated wound beds at day 5. *Inset graph* represents averaged LGR5 and LGR6 expression within confocal field using green and red fluorescent intensity per wound bed at day 5. (*Right*) Reverse-transcriptase polymerase chain reaction quantification of fold increase in RNA extracted from replicate wound beds treated with human alpha defensin 5 and sulfadiazine. *Scale bar* = 50 μm (*$p < 0.05$).

Of interest, the expression of the *Wnt1* gene has been shown to cause rapid growth of hair follicles in addition to being a marker for the development of multipotent dermal stem cells that reside within a the follicular bulge.[21–23] In addition, *Wnt1* inducible signaling pathway protein (*Wisp1*) is also upregulated in both heat mapping studies indicating that high levels of Wnt1 are being produced within the human alpha defensin 5–treated burn wound. *Wisp1* expression is associated with proliferation, cytoprotection, and extracellular matrix production in wounds, further suggesting human alpha defensin 5's role as a peptide capable of inducing multiple factors beyond microorganism reduction.

Application of Human Alpha Defensin 5 Peptide to an Ex Vivo Wound Assay Results in Increased Migration Kinetics of LGR6$^+$ Stem Cells

To test the increased rate of healing by LGR6$^+$ stem cell populations seen in vivo, a simple ex vivo wound healing assay was performed using fluorescence-activated cell sorting isolated LGR6$^+$, CD34$^+$, and CD73$^+$ C57BL/6(UBC-GFP) murine cells (Fig. 6). Following isolation, cells were plated for culture. At confluence, the cells were treated with either (1) human alpha defensin 5 or (2) control media, and the cells were divided by sterile pipette tip to induce a wound model. At 0, 6, and 12 hours, fluorescent images of the

green fluorescent protein–expressing cells were captured on an inverted cell culture scope and the percentage distance of the initial wound edges was calculated (Fig. 7, *above*, and *second* and *third rows*) and plotted (Fig. 7, *below*). The ex vivo data presented here show that the addition of the human alpha defensin 5 peptide does lead to augmented closure (cell migration) across an artificial cell culture wound.

DISCUSSION

Over the years, clinicians and researchers have continued to search for antimicrobial agents that not only reduce microorganism wound burden but also possess less cytotoxic side effects. From burns to chronic wounds, there is the potential for manipulation of naturally occurring self-derived antimicrobial peptides, in that these agents typically function through membrane permeabilization, a mechanism less likely to lead to microbial resistance. With the continued risk of infections in burn wounds and the advancing epidemic of bacterial resistance to current antibiotic therapies, there is a desperate need for the development of a new class of topical antimicrobial agents for use in cutaneous burns and wounds.

Over the past few decades, the role of antimicrobial peptides has been evaluated within the setting of wound healing and has led some researchers to the development of synthetic peptides in gel-based forms for clinical and therapeutic use.[24–26] Although these agents bring promise for further development, we must not forget the potential that exists elsewhere in the human body. For example, the intestinal mucosa, which coexists in harmony with 10^9 microorganisms per milliliter of effluent, uses other families of antimicrobial agents. One of these peptides, human alpha defensin 5, is thought to maintain the basal bacterial level of the bowel wall and defend the LGR stem cell niche of the intestinal epithelium.

In this report, we apply our knowledge of the gut-derived human alpha defensin 5 antimicrobial peptides to cutaneous wound healing. Here, we provide additional supportive evidence that the alpha defensin class of antimicrobial peptides reduces bacterial adhesion and invasion within a cutaneous third-degree burn wound model. From these data, it appears that, relevant to previously published literature, epithelium-derived beta defensins are not only reduced in burn wound beds but are also less effective in treating wounds devoid of adnexal structures. This is thought to occur subsequent to the destruction of the native epithelium, which is responsible for expression of the beta defensin peptides. Without functional keratinocytes in or around the wound, not only is the barrier disrupted but beta defensins are not translated into useful peptides. Furthermore, the destruction of the adnexa and follicular bulge cell mass subsequently depletes the burn region of the LGR stem cell niche, preventing focal proliferative expansion of epithelial progenitors. Without these epithelial progenitors migrating up from the hair shaft and onto the damaged epithelial surface, the healing trajectory of the local tissue is left to fibroblast infiltration, scar formation, and contracture.

Here, we suggest a novel role for application of the naturally occurring gut-derived human alpha defensin 5. This antimicrobial peptide appears to significantly reduce bacterial load within burned skin so as to prevent bacterial invasion of healing tissues. One reason for these results may be because the cutaneous commensal flora, present on undamaged skin (e.g., *Streptococcus* and *Staphylococcus*), is altered significantly by burned tissue and allows for other species (e.g., *Pseudomonas*, *Acinetobacter*, *Klebsiella*, and *Proteus* groups) to thrive in the burned tissue. Human alpha defensin 5 has a broad spectrum of activity against both gram-positive and gram-negative microorganisms and against those using either aerobic or anaerobic metabolisms. It may be this alteration in wound flora and the broader antimicrobial activity of human alpha defensin 5 that permits a more readily reduced bacterial load within a burn wound following topical application, as seen in our fluorescent in situ hybridization studies.

Beyond the antimicrobial effect of human alpha defensin 5, previous 5-bromo-2′-deoxyuridine studies have shown that alpha defensins do have the capacity to increase cellular proliferation, promote Ca^{2+} modulation-dependent migration of local cells into wounds, and promote collagen synthesis.[27] Furthermore, of these defensin-induced response genes discussed in the literature, some have been localized to the Wnt/beta catenin pathway, further advocating a Wnt-associated mechanism following the delivery of the peptide. These known processes may explain why the application of human alpha defensin 5 appears to have increased the rate of burn wound bed healing, within our model, when compared with the epithelium-derived defensin human beta defensin 1 and sulfadiazine antibiotic. These data suggest a possible role in local cellular proliferation and cell migration of nearby epithelial cells expressing LGR5 and LGR6 into the burn wound

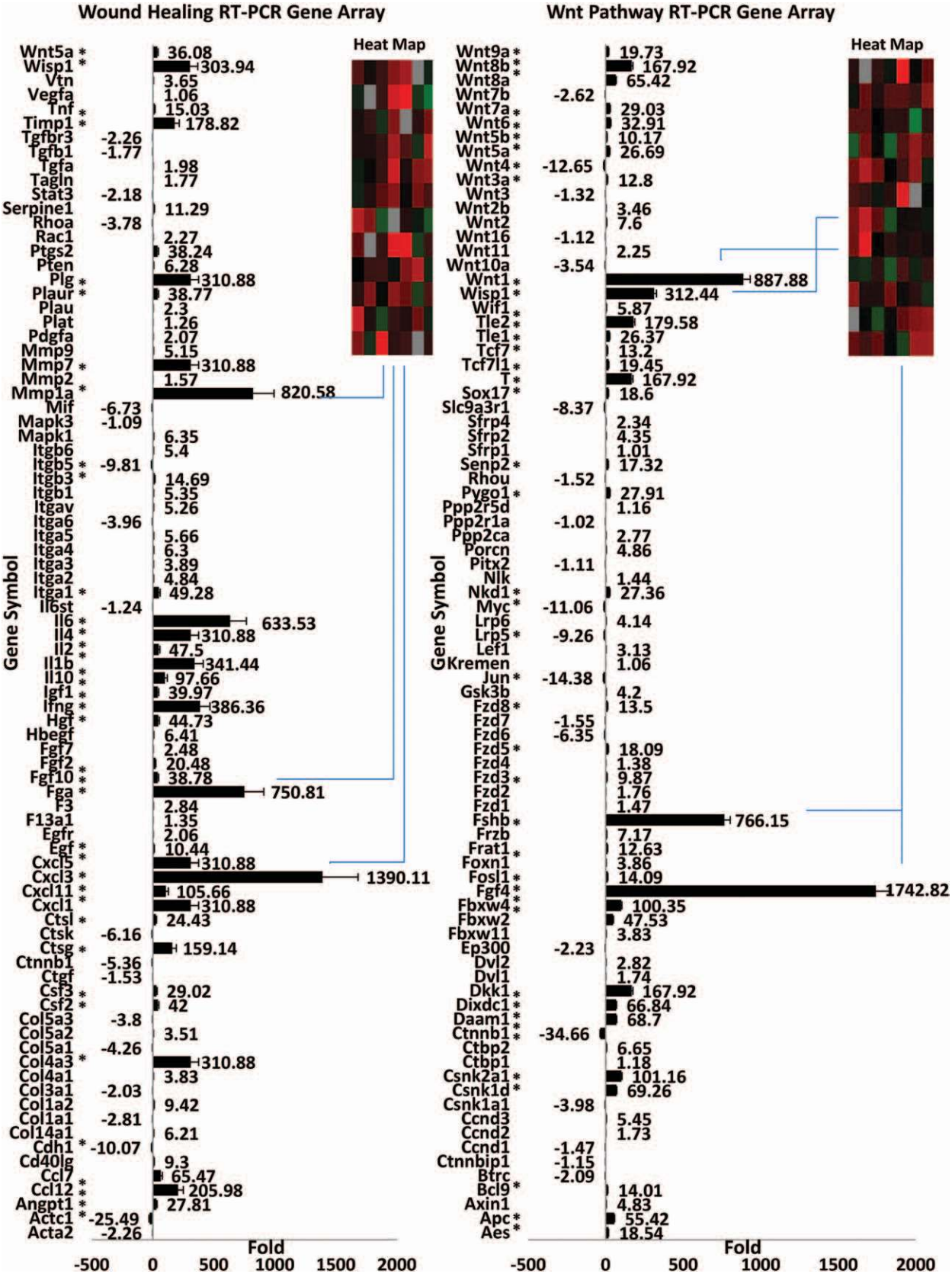

Fig. 5. Reverse-transcriptase polymerase chain reaction quantification and gene heat mapping comparison of wound beds treated with human alpha defensin 5 and wound beds treated with sulfadiazine. (*Left*) Averaged wound healing RT²-PCR Array pathway fold regulation and heat map for wound beds comparing human alpha defensin 5–treated

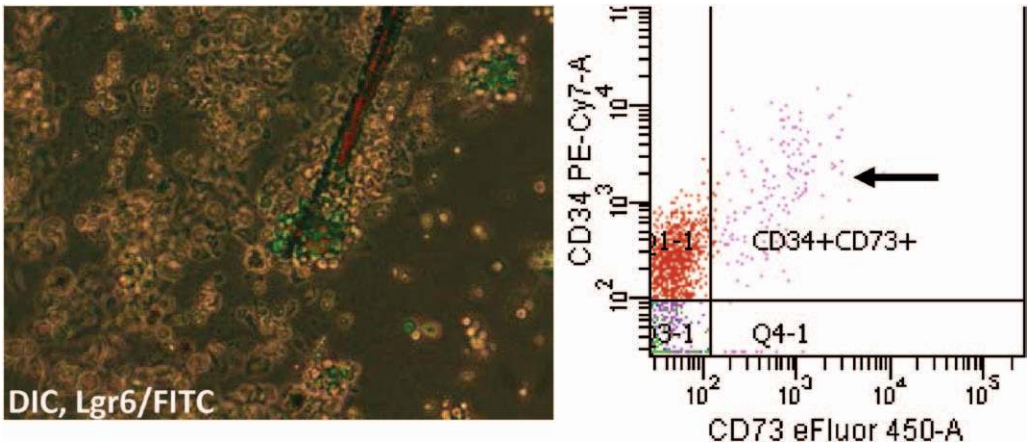

Fig. 6. Location of LGR6 expressing cells of the hair follicle and fluorescence-activated cell sorting of co-expressing LGR6$^+$, CD34$^+$CD73$^+$ green-fluorescent protein–labeled cells for cell culture expansion. (*Left*) LGR6 fluorescent antibody (green) expression of cells on the hair follicle following partial epidermal 10 units/μl Dispase (Worthington Biochemical Corp., Lakewood, N.J.) digestion for 30 minutes at 37°C on a slow rocker. (*Right*) LGR6$^+$ cells expressing additional CD34 and CD73 markers. *Arrow* indicates population isolated (approximately 1 to 3 percent of all cells).

bed. Not only did the treatment of the wound bed with human alpha defensin 5 augment an array of wound healing transcripts, as seen in a subset of heat-mapped genes, but the intestinal peptide appears to have also induced the up-regulation of key hair follicular bulge and telogen phase Wnt pathway genes, such as *Wnt1* and *Wisp1*.[21]

In the literature, these two genes, *Wnt1* and *Wisp1*, have been shown to be responsible for hair growth, epithelial stem cell differentiation, cellular proliferation, cytoprotection, and extracellular matrix production within mammalian skin. Perhaps crosstalk between Wnt/beta-catenin–dependent pathways involving defensin induced up-regulation of Wnt1 ligands, surrounding the wound bed, could be used for the development of future synthetic antimicrobial peptides and wound healing and hair restoration therapy.

Finally, LGR6$^+$ cells, once isolated from the mesenchymal support structure of the subepithelial compartment, were shown to react quickly to human alpha defensin 5 exposure in an ex vivo wound assay model. These cells appeared to heal the "in vitro wound" and return to confluence faster than did the sulfadiazine-treated control cells. This isolated migratory phenomenon could be further developed for therapeutic application in stem cell transplantation. Within this setting, LGR epithelial stem cells could be isolated from a small donor site and expanded in culture for implantation into burn wound beds to build a more appropriate and functional epithelium than our standard split-thickness skin graft used in burn wound coverage. Here, the LGR stem cell could potentially produce both sufficient cellular mass within the wound and derive a new hair shaft and sweat gland, something the split-thickness skin graft is incapable of intrinsically developing because of the adnexal structures remaining at the donor site following harvest.

In addition, with the recent advancement in composite tissue allografting as a means of defect repair, mechanisms of epithelial rejection could be circumvented through the development of epithelial chimerism.[28] By using both donor and recipient LGR stem cell populations, the dermis of a graft could be co-colonized by both stem cell systems in an attempt to promote progressive tolerance while the chimeric epithelium proliferates on composite tissues.

The functions of antimicrobial peptides have long been associated with health, disease, and immunomodulation of epithelium-derived innate immunity.[5,29] Our findings suggest an additional role for the intestine-derived human alpha defensin 5 in treatment of cutaneous wound beds that

Fig. 5. (*Continued*) systems to sulfadiazine-treated systems. (*Right*) Averaged Wnt RT^2-PCR array healing pathway fold regulation and heat map for wound beds comparing human alpha defensin 5–treated systems to sulfadiazine-treated systems. (*Bar graph*) Positive values indicate fold up-regulation favoring human alpha defensin 5–treated wounds; negative values indicate fold up-regulation favoring sulfadiazine-treated wounds. Heat map colors are indicated as (*red*) more expressed in human alpha defensin 5–treated burns to (*green*) more expressed in sulfadiazine-treated burns. Color intensity correlates with fold expression (*$p < 0.05$).

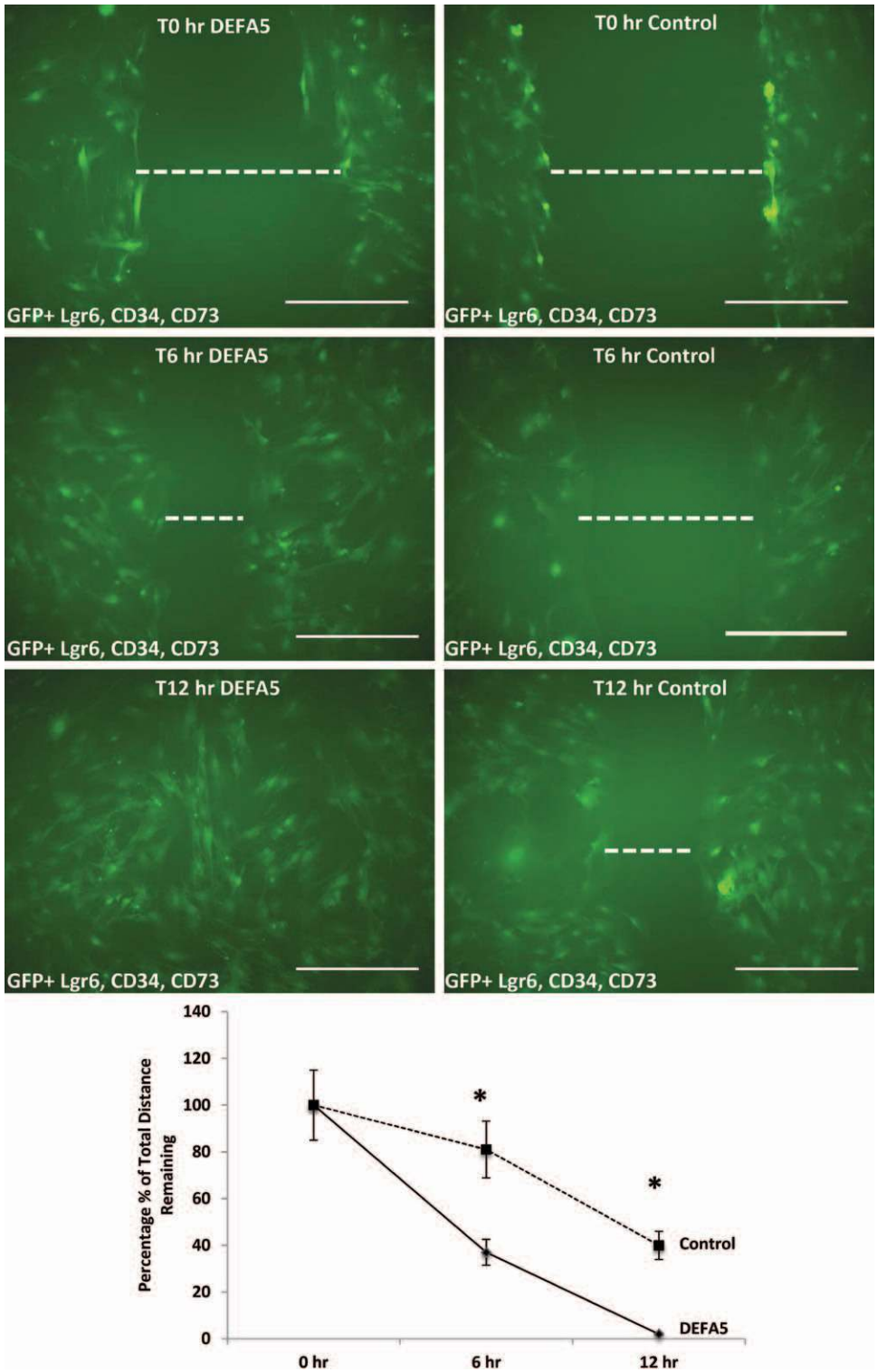

Fig. 7. In vitro LGR6$^+$ stem cell wound assay. (*Above* and *second* and *third rows*) Closure of induced wound within cell culture system of fluorescence-activated cell sorting–isolated LGR6$^+$ stem cells following the application of human alpha defensin 5 or sulfadiazine to the media. *Dotted line* indicates the distance of separation at 0, 6, and 12 hours following disruption of the cell layer. (*Below*) The averaged reduction in the distance line over time expressed as a percentage of initial distance. *Scale bar* = 50 μm (*$p < 0.05$).

are devoid of their stem cell–containing follicular bulge. Here, human alpha defensin 5 not only appears to protect the tissue during healing, by reducing the level of bacterial presence, but also increases local LGR5$^+$ and LGR6$^+$ stem cell migration into the wound. This migration leads to enhanced healing rates through the up-regulation of key wound healing markers and Wnt pathway ligands, which are known to promote epithelial proliferation, healing, and hair follicle growth. Here, we propose a unique therapeutic role of the gut-derived human antimicrobial peptide, human alpha defensin 5, in the treatment of full-thickness cutaneous wounds through mechanisms that reduce bacterial load, augment healing, and increase nascent hair growth.

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