WOUND HEALING

Transcriptional signature primes human oral mucosa for rapid wound healing

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Oral mucosal wound healing has long been regarded as an ideal system of wound resolution. However, the intrinsic characteristics that mediate optimal healing at mucosal surfaces are poorly understood, particularly in humans. We present a unique comparative analysis between human oral and cutaneous wound healing using paired and sequential biopsies during the repair process. Using molecular profiling, we determined that wound-activated transcriptional networks are present at basal state in the oral mucosa, priming the epithelium for wound repair. We show that oral mucosal wound–related networks control epithelial cell differentiation and regulate inflammatory responses, highlighting fundamental global mechanisms of repair and inflammatory responses in humans. The paired comparative analysis allowed for the identification of differentially expressed SOX2 (sex-determining region Y-box 2) and PITX1 (paired-like homeodomain 1) transcriptional regulators in oral versus skin keratinocytes, conferring a unique identity to oral keratinocytes. We show that SOX2 and PITX1 transcriptional function has the potential to reprogram skin keratinocytes to increase cell migration and improve wound resolution in vivo. Our data provide insights into therapeutic targeting of chronic and nonhealing wounds based on greater understanding of the biology of healing in human mucosal and cutaneous environments.

INTRODUCTION

Improving wound healing resolution is a major medical and social priority due to the increase in incidence of traumatic injury, chronic wounds, and scarring (*1*). Although several studies characterize in detail the mechanisms and pathways altered in these deficient wounds, a different approach that defines factors involved in accelerated wound healing would allow for the identification of novel therapeutic targets to improve tissue repair. In this regard, oral wound healing and embryonic wound healing have long been considered models of optimal wound resolution characterized by rapid and scarless wound healing (*1*–*4*). Dissecting the different molecular events that drive wound healing resolution in oral mucosa compared with those of the skin will help us define why these oral lesions heal more efficiently and will provide a basis from which to translate those findings to treat deficient healing processes.

Cutaneous wound healing is well documented, with the overall classic interpretation for the repair pathway having four systematic phases: hemostasis, inflammation, proliferation, and remodeling (*5*). The molecular circuitries that drive these different phases of cutaneous repair have been characterized, whereas the unique environment of the oral cavity represents a different wound healing paradigm that remains poorly understood. Oral wounds heal at an accelerated rate compared to cutaneous wounds, and in vitro or animal model studies have attributed this to a variety of mechanisms including differential inflammatory response, distinct modulation of stem cell, proliferative and differentiation programs, and more efficient epithelial remodeling (*1*, *3*). Despite this progress, the lack of detailed clinical studies in humans comparing oral and cutaneous wound healing in vivo has limited the advancement of our knowledge on the mechanisms mediating accelerated wound healing. To close this gap, we characterized the molecular and histological aspects of wound healing in paired samples of oral mucosa and the skin in healthy human subjects. Wounds were induced simultaneously in the oral buccal mucosa and the skin and sequentially biopsied for comparison at progressive time points. We show that the oral cavity is primed for wound repair and that oral keratinocyte limited differentiation and proinflammatory responses may contribute to accelerated wound healing in oral mucosa. We also present evidence that transcriptional networks established by transcription factors such as SOX2 (sex-determining region Y-box 2) and PITX1 (paired-like homeodomain 1) mediate this phenotype and can be exploited to reprogram cutaneous keratinocytes to present oral keratinocyte features, including accelerated wound closure.

RESULTS

Wound-activated transcriptional networks present at basal state prime the oral mucosa for wound repair

Using paired and sequential biopsy samples, we contrasted oral mucosal healing with cutaneous healing to determine the differential regulation of these processes in the human setting (Fig. 1A). After clinical screening (day 0), baseline biopsies of paired, identically sized wounds in the oral buccal mucosa and skin were obtained

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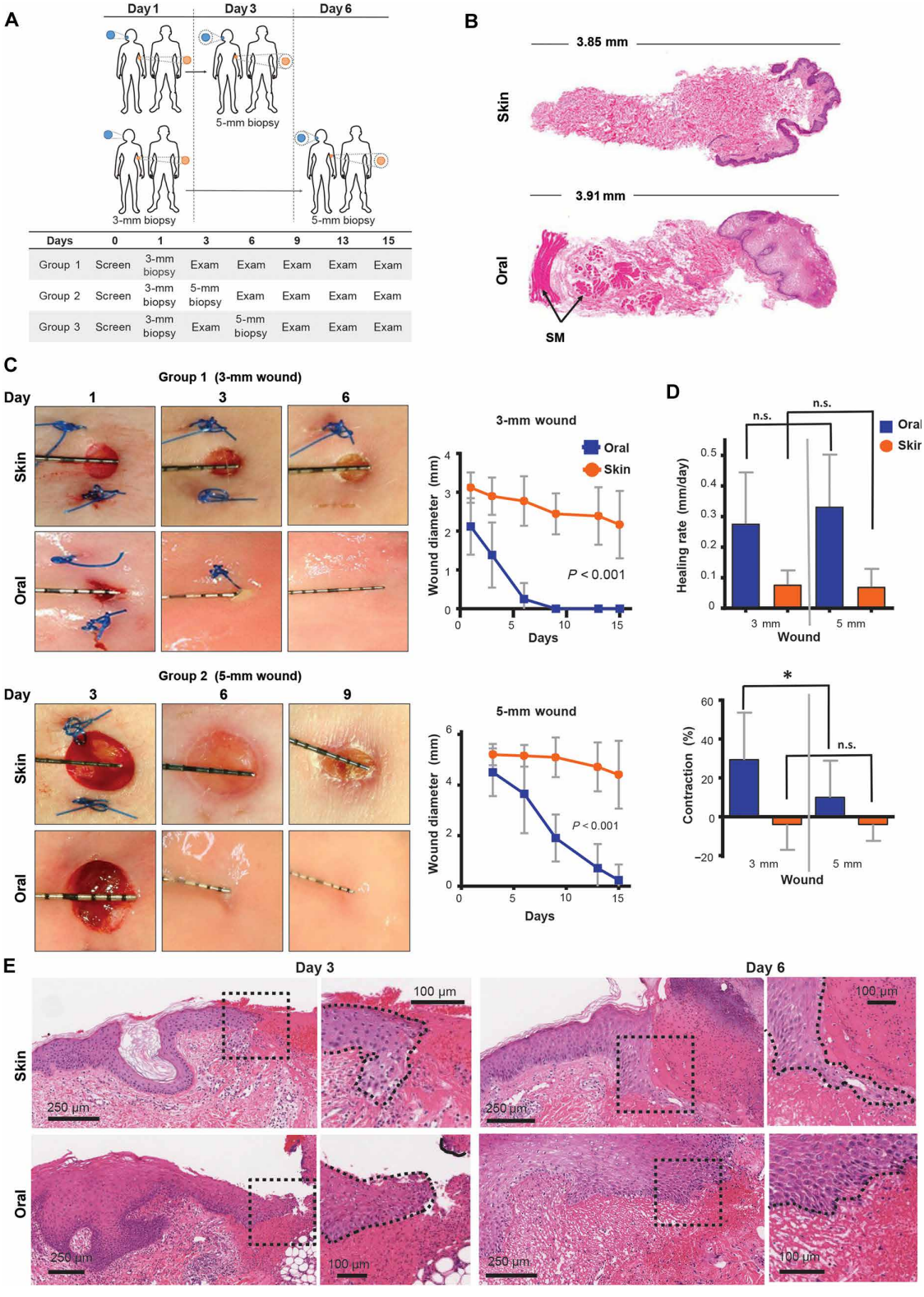

Fig. 1. Comparison of paired oral and skin wounds in human subjects. (**A**) Table and schematic of spatiotemporal human biopsy sample collection (ClinicalTrials.gov #NCT01078467). Baseline biopsies were performed to create paired identically sized wounds in the oral mucosa (blue) and skin (orange) on day 1. Follow-up biopsies of the wound areas were collected on days 3 and 6 of healing in two different groups. (**B**) Representative hematoxylin and eosin (H&E) pictures of longitudinal sections of biopsies taken at day 1. Thirty healthy subjects were randomized in three groups (with 10 subjects on each group). SM, smooth muscle. (**C**) Representative pictures and quantitation of healing time course of oral wounds and skin wounds in group 1 after a 3-mm primary biopsy and of group 2 after a 5-mm secondary biopsy. Markings on the dental periodontal probe are in millimeters. Biopsy sites were demarcated using blue polypropylene sutures. Data are mean values, and error bars represent SDs. Number of samples for each group are as follows: 3-mm wounds, day 1, $n = 29$; day 3, $n = 30$; day 6, $n = 20$; day 9, $n = 9$; day 13, $n = 9$; day 15, $n = 9$; 5-mm wounds, day 3, $n = 11$; day 6, $n = 20$; day 9, $n = 21$; day 13, $n = 21$; day 15, $n = 21$. Nonparametric two-sided t test. (**D**) Graphs show the healing rate of 3-mm wounds in group 1 and of 5-mm wounds in group 2 (top) and percentage of contraction immediately after the 3-mm biopsy in group 1 and after the 5-mm biopsy in group 2 (bottom). n.s., not significant. $^*P < 0.05$ by unpaired t test. Error bars represent SDs. (**E**) Representative H&E pictures of oral and skin wounds at days 3 and 6. Magnification of dotted boxes is shown on the right. In magnification, epithelium is marked with a dotted line.

(day 1) (Fig. 1B). Day 1 biopsies allowed for evaluation of homeostatic transcriptional profiles in human mucosal versus cutaneous surfaces. Follow-up biopsies of the wound areas were collected at day 3 (48 hours after the first biopsy) and day 6 (120 hours after the first biopsy). This approach evaluated distinct phases during the physiologic process of human wound healing.

Analysis of the healing time course revealed that oral wounds resolved significantly faster than skin wounds ($P < 0.001$; Fig. 1C).

This was observed after the first wound-inducing biopsy (3-mm wound; Fig. 1C, top) and after the secondary biopsy of the wound area (5-mm wound; Fig. 1C, bottom). There was no significant difference in healing rates between different sized wounds created in the same tissue bed; however, 5-mm oral wounds exhibited less contraction than 3-mm oral wounds (Fig. 1D). Histological analysis of the wound sections showed earlier re-epithelialization of the oral mucosa wounds compared to the skin (Fig. 1E). By day 3, oral wounds were almost completely covered by squamous epithelium, even in the absence of stromal healing.

RNA sequencing (RNA-seq) was used to characterize molecular mechanisms of the human wound repair process (Fig. 2A). Unsupervised clustering analysis of the gene expression data provided three major observations. First, oral and skin samples clustered separately, indicating distinct transcriptional identities consistent with unique tissue microenvironments. Although there is differential gene regulation between oral mucosa and skin during wound healing, most of these differences were already evident at starting basal conditions (day 1; Fig. 2, A and B, and fig. S1). Second, within the skin samples, there was separation between baseline biopsies and wound biopsies at days 3 and 6 with an overall up-regulation of gene activity in the skin evident during the healing process, indicating nonresolution of the skin wounds at the time points evaluated (Fig. 2A, skin). Third, and in contrast to the skin, the oral day 3 biopsies separated from days 1 and 6, indicating wound healing activity at day 3 that resolved by day 6 (return of gene expression to basal conditions; Fig. 2A, oral).

Consistent with these observations, analysis of variance (ANOVA) of the differential gene expression during wound healing in oral and skin revealed few significant gene expression changes during oral healing at day 3 (410) and none at day 6, whereas a large number of genes were differentially regulated during skin wound healing at both time points (skin day 3 versus day 1, 1473; skin day 6 versus day 1, 1836; Fig. 2B). These results demonstrate an enhanced transcriptional activity during skin wound healing but minimal differential transcriptional regulation in oral wounds, raising the possibility that the transcriptional regulatory networks responsible for the accelerated healing in oral mucosa are already present in the unwounded state.

To explore the intrinsic differences between oral mucosa and skin at baseline, we determined the significant differentially expressed genes between unwounded (day 1) oral mucosa and unwounded skin ($q < 0.05$ and fold change ≥ 2; table S1). Transcripts up-regulated in the oral environment were consistent with increased keratinocyte activation and with heightened antimicrobial defenses (day 1; Fig. 2C). Among the up-regulated transcripts in the oral samples, we found genes described in wound-activated keratinocytes (*6*), including keratins 6 (*KRT6*) and 16 (*KRT16*), small proline-rich (*SPRR*) and S100 proteins, defensins, serpins, and annexins among others (Fig. 2C). IPA showed that the top processes represented in the transcriptome of the oral mucosa were related to inflammatory skin disorders such as psoriasis, dermatitis, and skin hyperplasia (Fig. 2D), conditions in which transcriptional networks resemble those of the wound-activated skin (*7*–*10*). This indicated that gene networks related to increased proliferation, migration, and wound resolution were potentially already present in the oral mucosa at basal state.

Because psoriasis has been shown to present a particular expression signature of genes, inflammatory cytokines, and proteins related to wound healing, we used the gene list from the psoriasis gene signature in IPA to perform an unsupervised clustering of our samples (Fig. 2E). Results showed that skin wound samples (days 3 and 6) clustered more closely with oral samples at all days than unwounded skin (Fig. 2E). These results support our hypothesis that wound-activated networks are present in the oral epithelium at basal state. Psoriasis patients have accelerated wound healing with reduced scarring (*10*, *11*), suggesting that the presence of gene networks related to wound healing at basal state might be the key for the accelerated wound healing observed in psoriatic skin and oral mucosa.

Analysis of the transcriptome of the oral mucosa also revealed that gene networks related to cell movement and migration were highly activated in the unwounded oral mucosa (fig. S2A), showing up-regulation of genes linked to epithelial and immune cell migration (fig. S2B). Together, these results indicate that wound-activated transcriptional networks are present at basal state in the oral mucosa, priming the epithelium for wound repair.

Oral mucosa shows reduced differentiation and inflammatory response during wound healing

Consistent with the possibility that the transcriptional regulatory networks responsible for the accelerated healing in oral mucosa are already present in the unwounded state, we identified 250 genes ($q < 0.05$ and fold change ≥ 2; table S2) that are highly expressed in the unwounded oral mucosa but are only up-regulated in the skin during wound healing (Fig. 3A, black line in CIRCOS plot). Although there is ample evidence of the importance of the extracellular matrix and underlying stroma during re-epithelialization (*1*), the topmost significant gene ontologies (GOs) represented in this gene list were processes related to keratinization, epidermal cell differentiation, and responses to biotic stimulus and bacterium (Fig. 3A and table S3). Terms related to epithelial and immune cell migration were also represented (table S3). These results highlight specific gene networks, both intrinsic to keratinocyte biology and related to immune responses, as critical elements mediating the priming of the oral mucosa to wound repair.

Distinct keratin and structural protein expression profiles between oral mucosa and skin are indicative of inherent differences in the epithelial compartments of these tissues (Fig. 3B). Correspondingly, we found that characteristic signature keratins for each epithelium (*12*) were present: K4 in the oral mucosa and K10 in the cornified skin (Fig. 3C). We also examined reciprocal staining for K4 and K10 in skin and oral mucosa (fig. S3A) and confirmed the tissue-specific expression for each keratin.

Stress- and wound-activated keratins, including the keratin 6 family (*KRT6A*, *KRT6B*, and *KRT6C*) and *KRT16*, are uniquely active in unwounded oral epithelium and remain highly up-regulated during wound healing, whereas they are only expressed in the skin during the wound healing process (Fig. 3, B and D). These keratins are essential for keratinocyte migration and epithelial structure in vivo in murine oral epithelia at basal state and during wound healing (*13*, *14*).

Analyzing these human data sets identified distinct expression profiles for genes clustered in the epidermal differentiation complex (EDC) (*15*). Most of the EDC genes (*S100s*, *SPRRs*, and cell envelope precursors) that were up-regulated in skin during wounding (Fig. 3B) presented heightened expression at baseline day 1 in the oral mucosa.

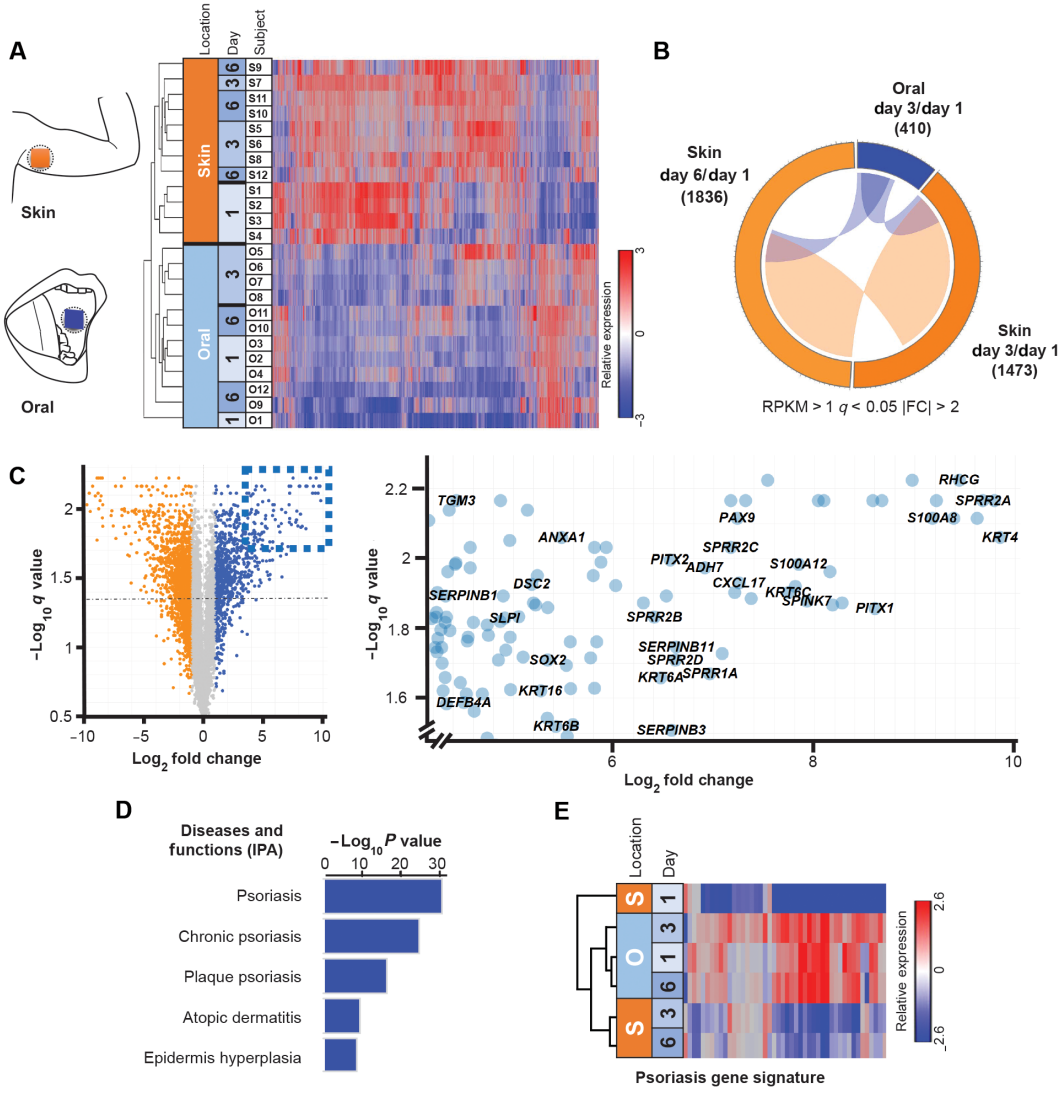

Fig. 2. Wound-activated transcriptional networks are present in the unwounded oral mucosa. (**A**) Schematic representation of biopsy site in the mucosa of the cheek and posterior axillary region of the arm (left) and unsupervised clustering analysis of RNA-seq gene expression data of the 24 paired samples at days 1, 3, and 6. O, oral; S, skin. Numbers indicate matching subjects. Paired oral and skin samples were chosen randomly from four subjects for each day (24 total samples from 12 individual subjects) and were a mix of males and females. (**B**) Circular ideogram plot (CIRCOS) of the differential gene expression during wound healing (ANOVA). No significant differences were found in oral biopsies taken at day 6 versus day 1 (day 6/day 1). Ribbon connectors indicate the same genes present in different data sets. Number of genes with differential expression in each comparison: oral day 3 versus day 1 (day 3/day 1), 410 genes; skin day 3 versus day 1 (day 3/day 1), 1473 genes; skin day 6 versus day 1 (day 6/day 1), 1836 genes. See fig. S1A for explanation on CIRCOS plot. RPKM, reads per kilobase million; |FC|, absolute fold change. (**C**) Volcano plot indicating differential gene expression between unwounded (day 1) oral mucosa and skin. Dotted line region is magnified on the right panel, highlighting some of the most significantly up-regulated genes in the oral mucosa compared to the skin. $P < 0.05$ by paired t test. (**D**) Ingenuity Pathway Analysis (IPA) showing diseases and functions terms in up-regulated genes in the oral mucosa compared to the skin. (**E**) Unsupervised hierarchical clustering using a psoriasis gene signature with the gene expression of oral mucosa and skin data at baseline (day 1) and during wound healing (days 3 and 6).

Differentiation markers such as involucrin (IVL), usually present in both epithelia, were down-regulated in the oral mucosa during healing but were present in the migratory tongue of the wounded skin (fig. S3B). Although the oral mucosa has a more extensive total area of proliferating cells during wound healing (Fig. 3E), there is no significant difference in the number of proliferating cells when this is corrected for surface area involved ($PCNA^+$ cells per square millimeter; Fig. 3E). In aggregate, these results indicate that oral wounds do not engage differentiation pathways during wound healing but, instead, are primed for wound repair by maintaining a larger pool of regenerative epithelial keratinocytes that aid in accelerated wound closure, as corroborated by activated keratin expression.

The differential expression of structural keratins observed between oral and cutaneous samples reflects the unique characteristics of these distinct epithelia and highlights the fact that the oral buccal mucosa is noncornified and therefore is more exposed to environmental signals (*16*). Hence, the oral epithelium is increasingly exposed to the commensal microbiota that inhabit barrier surfaces. The oral environment is home to some of the most rich and diverse microbial communities harbored on human body surfaces (*17*) and is an environment of constant mechanical stimulation during mastication shown to induce heightened immune responsiveness (*18*). Oral epithelia had minimally up-regulated inflammatory pathways during the healing process (Fig. 4A and fig. S4A) and increased antimicrobial defenses (fig. S4B). In contrast, in the cutaneous microenvironment, inflammatory responses were less active at steady state but became up-regulated throughout the healing process and did not resolve by day 6, suggestive of a chronic inflammatory response when compared to the oral mucosa (Fig. 4, A to C, and fig. S4A). Chronic inflammation is a hallmark of nonhealing wounds, and overactivation of immune processes during healing has detrimental effects on wound resolution, delaying closure and increasing fibrosis and scarring (*19*). Several immune mediators including proinflammatory cytokines, chemokines, and cyclooxygenases showed higher expression in the skin at basal state and were up-regulated in skin continuously through day 6 (Fig. 4C). Markers of fibrosis,

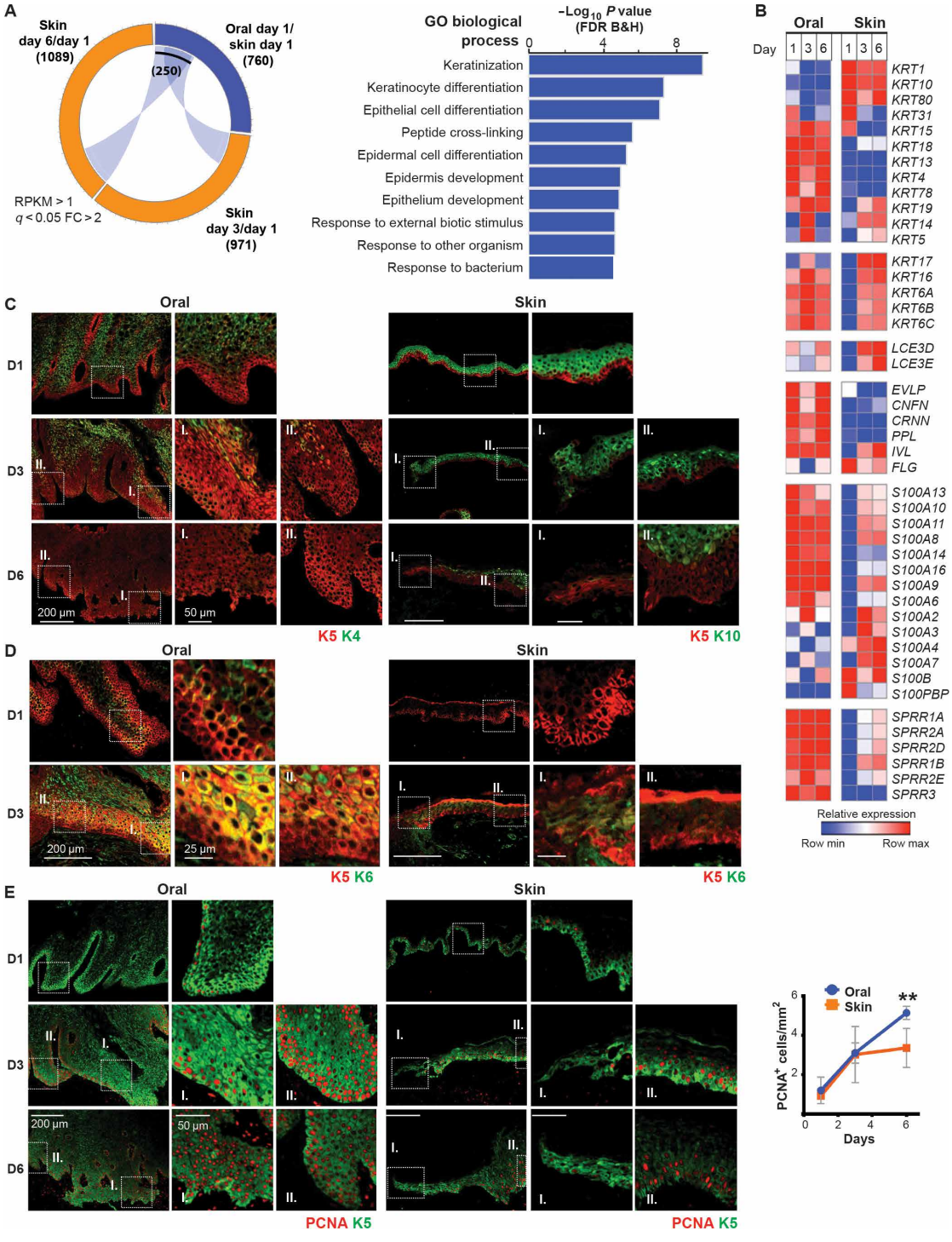

Fig. 3. Oral keratinocytes show reduced differentiation during wound healing. (**A**) CIRCOS plot summarizing cross-reference of transcripts up-regulated during skin wound healing (skin day 3/day 1 and skin day 6/day 1) with those up-regulated in the oral mucosa with respect to the skin at basal conditions (oral day 1/skin day 1). The black line indicates genes up-regulated in the unwounded oral mucosa that are up-regulated during skin wound healing (250 unique genes; see table S2). Ribbon connectors indicate that the same genes are present in different data sets. Right: Graph indicates GO biological process terms enriched in the oral day 1/skin day 1 data set. Oral day 1/skin day 1, 760 genes; skin day 3/ day 1, 971 genes; skin day 6/day 1, 1089 genes. B&H, Benjamini and Hochberg's; FDR, false discovery rate. (**B**) Relative mRNA expression of keratinization and epidermal cell differentiation markers throughout the wound healing process. (**C**) Representative pictures of unwounded (day 1) and wounded (days 3 and 6) oral mucosa and skin stained to show expression of the basal cell marker keratin 5 (K5, red) and differentiation markers keratin 4 (K4) for the oral mucosa and keratin 10 (K10) for the skin (green). Magnification of the dotted box is shown on the right of each picture. (**D**) Representative pictures of unwounded (day 1) and wounded (day 3) oral mucosa and skin stained to show expression of the basal marker cytokeratin 5 (K5, red) and activated epithelium keratin 6 (K6, green). (**E**) Representative pictures of unwounded (day 1) and wounded (days 3 and 6) oral mucosa and skin stained to show expression of the basal marker keratin 5 (K5, green) and proliferation marker proliferating cell nuclear antigen (PCNA; red). Quantification of number of cells positive for the proliferation marker PCNA per area (mm^2). $^{**}P < 0.01$ by unpaired *t* test, oral versus skin day 6. No asterisk means not statistically different between oral and skin. Data are mean values, and error bars represent SDs. $n = 3$ independent samples. For day 1, magnification shows the basal, unwounded expression of corresponding marker. For days 3 and 6, magnification shows the migratory tongue or wound area (I) or an adjacent epithelial area to the wound (II). D1, day 1; D3, day 3; D6, day 6.

including transforming growth factor–β targets (*20*), were up-regulated in skin wounds compared to oral wounds (fig. S4, A and C). Analysis of the gene expression changes in skin wound healing from day 3 to day 6 revealed that, although gene networks related to keratinocyte differentiation (peptide cross-linking and keratinization) were up-regulated by day 6, additional networks related to immune response were still active at this time (fig. S4D). This further corroborates the nonresolution of the inflammatory response in the skin wounds.

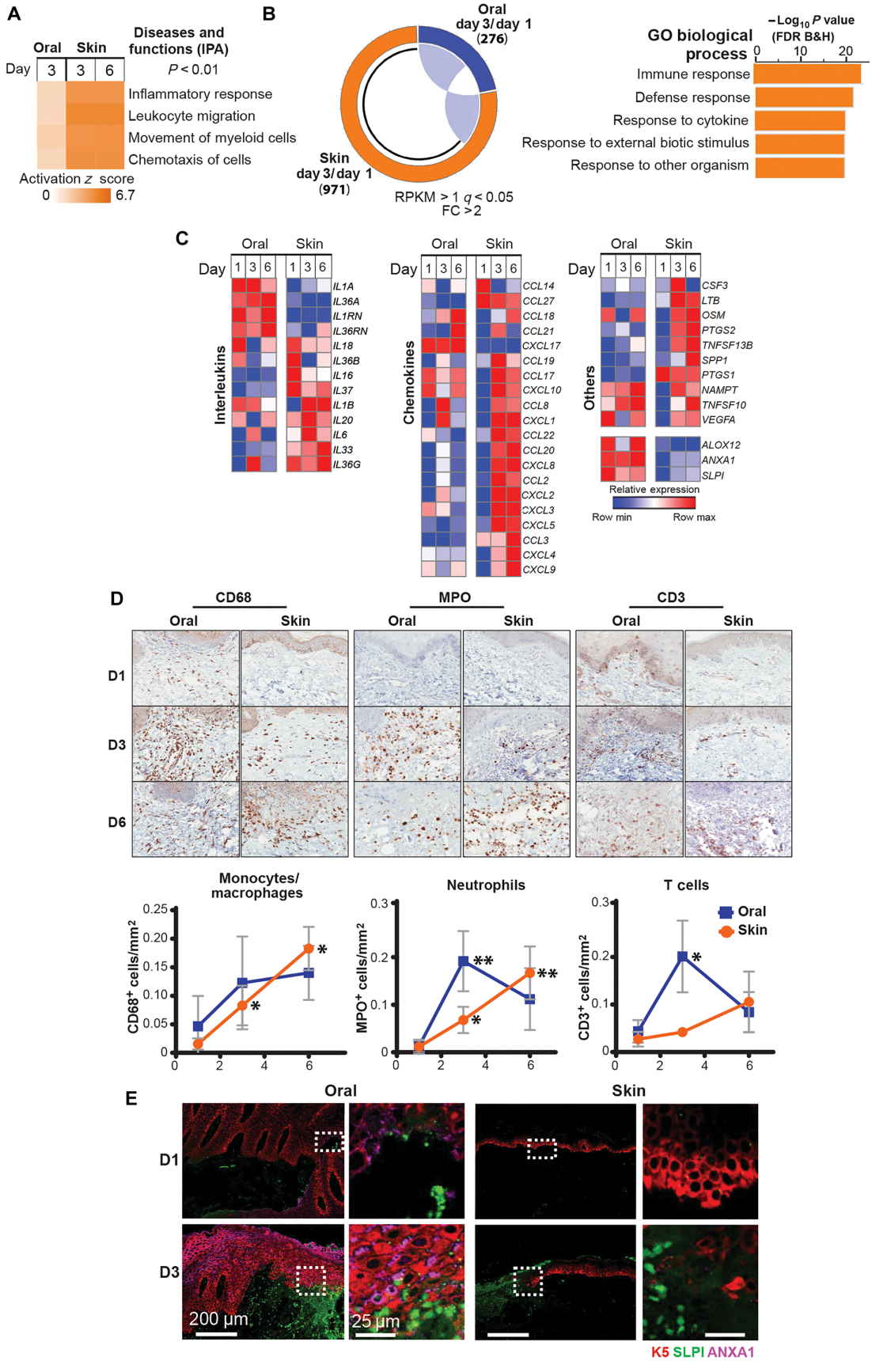

Fig. 4. Inflammatory pathways are more active and sustained in skin wounds than in oral wounds. (**A**) IPA analysis showing diseases and functions terms found in differentially regulated genes during the wound healing process relative to day 1, including terms related to inflammatory processes. (**B**) CIRCOS plot showing the genes exclusively up-regulated during skin wound healing (black line) and GO biological process terms enriched in this data set. Ribbon connectors indicate that the same genes are present in different data sets. Oral day 3/day 1, 276 genes; skin day 3/day 1, 971 genes. (**C**) Relative mRNA expression of interleukins, chemokines, and other inflammatory regulators during wound healing. (**D**) Representative pictures of recruitment of immune cells during the wound healing process in the oral mucosa and the skin. Bottom: Quantification of recruitment of specific immune cell types during the wound healing process in the oral mucosa and the skin. $*P < 0.05$, $**P < 0.01$ by unpaired *t* test. No asterisk means not statistically different, comparisons between days 3 and 6 versus day 1 (D1) oral or skin, respectively. Data are mean values, and error bars represent SDs. $n = 3$. MPO, myeloperoxidase. (**E**) Representative images of unwounded (day 1) and wounded (day 3) oral mucosa and skin stained to detect expression of the basal marker keratin 5 (K5, red) and the immune modulators secretory leukocyte peptidase inhibitor (SLPI) (green) and ANXA1 (magenta). Magnification of dotted boxes is shown on the right of each picture.

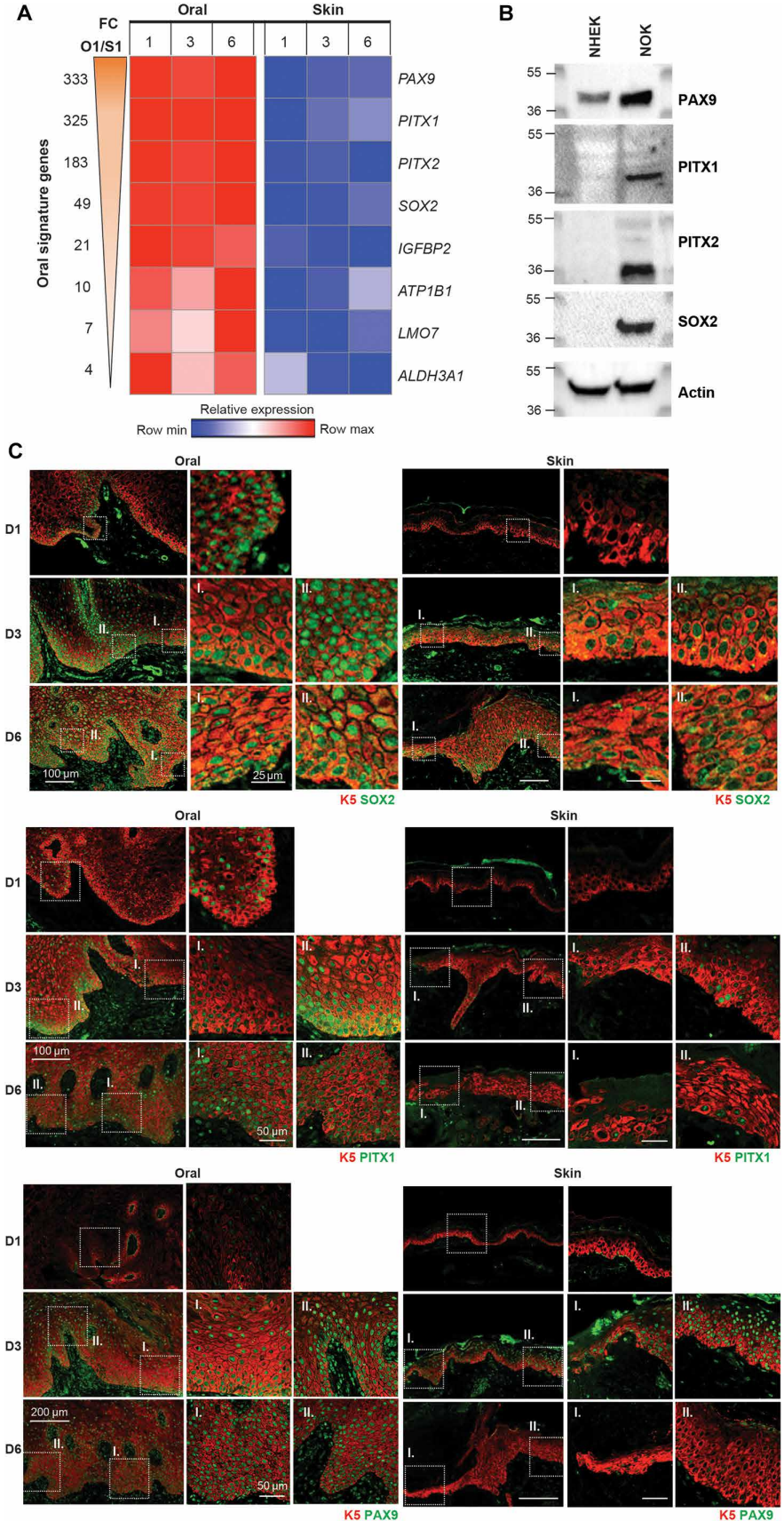

Fig. 5. Transcriptional networks in oral keratinocytes contribute to rapid wound resolution. (**A**) Relative mRNA expression of genes consistently up-regulated in oral mucosa and oral keratinocytes, presented by fold change of the unwounded oral mucosa with respect to the unwounded skin (O1/S1) in the wound healing data set. (**B**) Western blot of PAX9, PITX2, PITX1, and SOX2 in primary cultures of human oral (NOK) and skin (NHEK) keratinocytes. $n = 2$. (**C**) Representative pictures of unwounded (day 1) and wounded (days 3 and 6) oral mucosa and skin stained to show expression of the basal marker keratin 5 (K5, red) and the indicated transcription factor (SOX2, PITX1, or PAX9; green). Magnification of the dotted boxes is shown on the right of each picture. For day 1, magnification shows the basal, unwounded expression of corresponding marker. For days 3 and 6, magnification shows the migratory tongue or wound area (I) or an adjacent epithelial area to the wound (II).

Histological and quantitative evaluation of the cellular infiltrate during healing reflected an acute response in the oral environment with complete resolution by day 6, consistent with transcriptional responses that reverted to baseline by day 6 in the oral environment but continued to amplify in the skin (Fig. 4D). Associated with this rapid and controlled inflammatory response in the oral environment, we observed constitutive activation of several proresolution molecules [*ANXA1*, *SLPI*, *ALOX12*, and *IL1RN* (*21*)] in the oral mucosa (Fig. 4C). We confirmed that annexin A1 (ANXA1) and SLPI, two proteins that ameliorate wound healing by moderating chronic inflammation (*22*–*24*), showed increased expression in the oral mucosa and oral wounds compared to skin (Fig. 4E). These results show that oral mucosa is primed for wound repair by a series of wound-signature networks that help control epithelial cell differentiation and regulate proinflammatory responses.

Transcriptional networks in oral mucosa contribute to rapid wound resolution

Rapid oral versus skin wound healing has previously been identified in mouse and pig models (*3*, *25*, *26*) and replicated in vitro in oral keratinocyte models (*4*), suggesting a conserved mechanism in oral keratinocytes that allows for faster wound resolution. To identify conserved factors and signaling pathways in oral keratinocytes that may have physiological relevance for improved healing, we searched databases for overexpressed transcripts present in our human oral mucosa data set (Fig. 3A), which were also overexpressed in mouse oral mucosa in vivo (*25*) and in human (*27*) and mouse (*3*) oral keratinocytes (fig. S5). This analysis identified eight genes that were consistently up-regulated in oral mucosa and oral keratinocytes (Fig. 5A), four of which encode

for transcriptional regulators (*PAX9*, *PITX1*, *PITX2*, and *SOX2*), one that encodes the LIM domain–only 7 emerin-binding factor (*LMO7*) (*28*), and three that encode factors involved in various oral mucosa biological processes (*ALDH3A1*, *ATP1B1*, and *IGFBP2*) (*4*, *29*, *30*).

Transcription factors have the potential to reprogram cells to specific developmental states (*31*). Within the group of oral up-regulated transcription factors, we found the paired-like homeodomain PITX factors (*PITX1* and *PITX2*), the paired box homeodomain factor *PAX9*, and the HMG-domain *SOX2*, which is part of the SOX family of transcriptional drivers of somatic cell reprogramming (*32*–*34*).We confirmed the differential expression of the PAX9, PITX1, PITX2, and SOX2 transcriptional regulators in primary human oral [NOK (normal oral keratinocytes)], and skin [NHEK (normal human epidermal keratinocytes)], keratinocytes in vitro and in human biopsies, at baseline and during healing (Fig. 5, B and C). PITX1 and SOX2 were expressed in NOK cells and unwounded oral mucosa, whereas their expression was almost undetectable in NHEK cells and skin. Expression of these factors in the epithelial layer of the oral mucosa increased upon wounding. PAX9 showed increased expression in epithelia and dermis after wounding in both oral mucosa and skin and was less expressed in NHEK cells than in NOK cells.

To functionally validate the role of these factors in controlling a transcription-regulated wound healing program, we analyzed the gene expression changes in NOK cells treated with small interfering RNA (siRNAs) for *PAX9*, *PITX1*, *PITX2*, and *SOX2* (Fig. 6 and fig. S6A). Knockdown of these factors altered gene networks related to cell movement and migration (Fig. 6B and fig. S6, A to C). siRNA for PITX1 and SOX2 significantly reduced the migration capacity of NOK cells (**$P < 0.01$; Fig. 6C). siSOX2 affected gene networks related to the immune and defense responses, whereas siPITX1 altered epidermal developmental and differentiation and keratinization pathways (fig. S6, B and C).

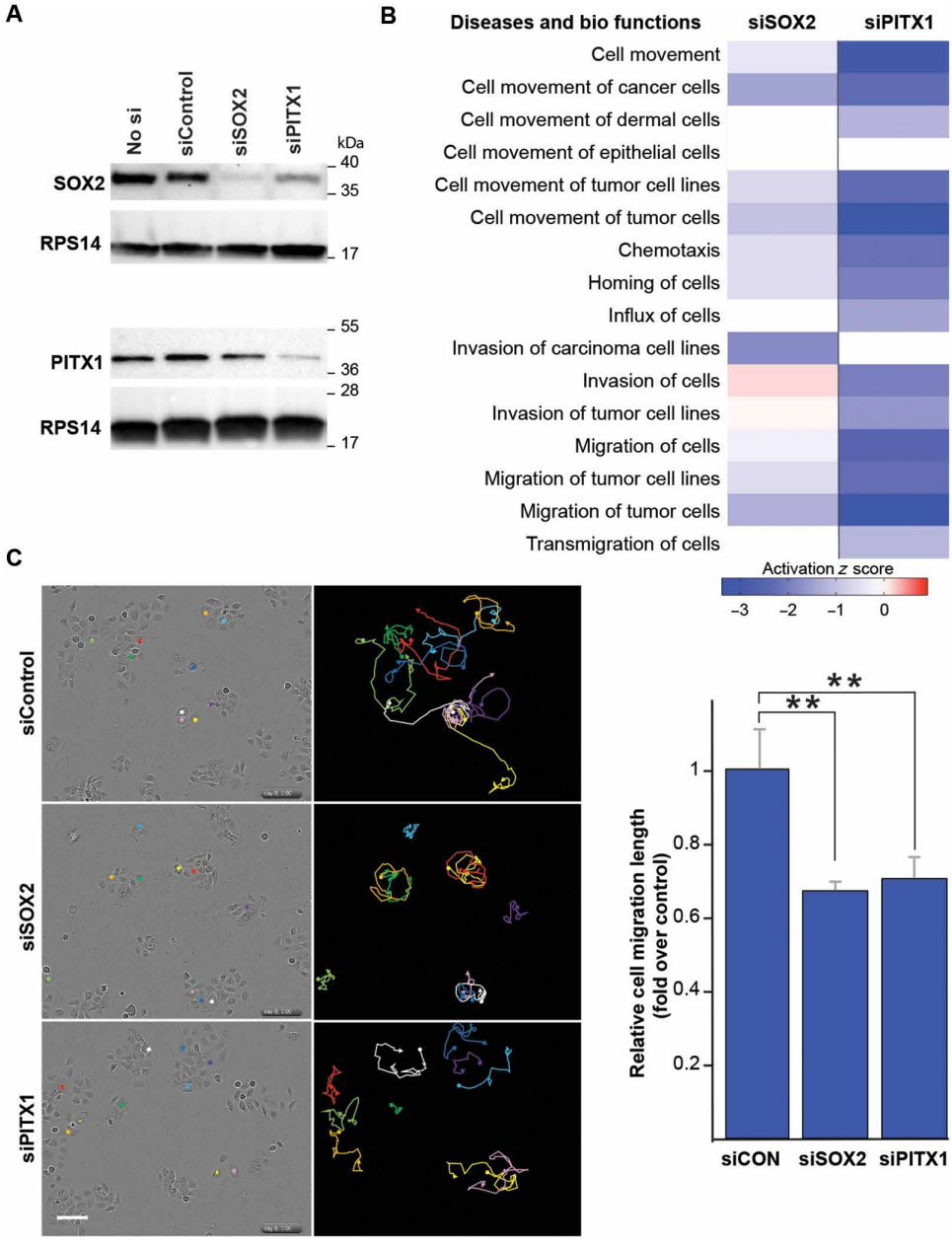

Fig. 6. Knockdown of oral signature genes reduces migratory capacity of primary oral keratinocytes. (**A**) Western blot of SOX2, PITX1, and total protein (RPS14) after transfection with respective siRNAs in NOK cells. (**B**) IPA analysis of RNA-seq data from NOK cells treated with siRNAs for SOX2 and PITX1: Diseases and functions terms related to migration and cell movement found in differentially regulated genes. $P < 0.05$ by IPA. (**C**) Relative cell migration distance of NOK cells transduced with siRNA for SOX2, PITX1, and siControl (siCON). Values were determined from three microscopic fields in $n = 3$ per group. **$P < 0.01$ by ANOVA, siSOX2 and siPITX1 versus control. Data are mean values, and error bars represent SDs. Scale bar, 100 μm.

We then evaluated the functional effects of overexpressing PITX1 and SOX2 in NHEK cells (Fig. 7 and fig. S7), which do not express these factors under normal conditions (Fig. 5, B and C). Transcriptomic analysis of NHEK cells transduced with PITX1 and SOX2 revealed a similar pattern of GO biological process as oral keratinocytes, with SOX2 affecting pathways related to the immune response including cytokines and antibacterial peptides and PITX1 expression resulting in the alteration of developmental and differentiation pathways (Fig. 7B). Both PITX1 and SOX2 expression in skin keratinocytes reduced expression of the skin differentiation marker *KRT1* and overexpression of the oral epithelial marker cornulin (*CRNN*; Fig. 7C). adPITX1 also increased expression of several markers enriched in the oral epithelium, including keratins (*KRT13* and *KRT78*), *SPRRs* (*SPRR2A*, *SPRR2E*, and *SPRR3*), *LCE3D*, *IGFBP2*, and *ALDH3A1*

(Fig. 7C and fig. S7B). Transduction with PITX1 and SOX2 significantly increased the migratory capacity of NHEK cells (**$P < 0.01$, *$P < 0.05$; Fig. 7D and fig. S7C). These results show that the differential expression of transcriptional regulators in oral versus skin keratinocytes confers a unique identity to oral keratinocytes and that skin keratinocytes can be reprogrammed to increase cell migration and improve wound resolution.

To investigate the effect of overexpressing SOX2 in epidermal keratinocyte on cutaneous wound healing in vivo, we generated epidermal-specific SOX2-overexpressing mice (*35*). Mice expressing a tamoxifen-inducible Cre driven by the keratin 14 promoter (K14CreERTM) were crossed with lox-stop-lox (LSL)–SOX2 mice to generate K14CreERTM/LSL-SOX2 mice. We compared the kinetics of cutaneous wound healing in K14CreERTM/LSL-SOX2 mice without and with tamoxifen-induced expression of SOX2 in basal keratinocytes (Fig. 8A and fig. S8). Immunohistochemical analysis showed that tamoxifen treatment induced SOX2 expression in epidermal keratinocytes in K14CreERTM/LSL-SOX2 mice (Fig. 8B), and short-term SOX2 expression in the skin amplified the K5 stem cell compartment (Fig. 8B).

Wound healing was significantly promoted in SOX2-overexpressing skin from 3 to 9 days after wounding compared to mice treated with vehicle (*$P < 0.05$; Fig. 8, C and D). SOX2 overexpression in epithelial keratinocytes led to skin acanthosis (Fig. 8E). Histological analysis in SOX2-overexpressing mice day 5 after wounding showed differences in the migratory tongue when compared to that of control mice (Fig. 8E). SOX2 overexpression significantly increased $PCNA^+$ proliferating epithelial cells (*$P < 0.05$; Fig. 8F). These findings demonstrate that genetic or pharmacological approaches to increase the expression or activity of the SOX2 transcription factor in the skin can improve cutaneous wound healing.

DISCUSSION

Wound healing is a major medical and social priority, and broadening our understanding of the mechanisms involved in wound repair is needed to improve wound care. Oral wounds are able to heal more rapidly and with fewer complications than cutaneous wounds; however, the lack of detailed comparative analysis in humans has limited the advancement of our knowledge in this area. Here, we identified the physiological and molecular determinants for this repair paradigm. Our findings could have widespread implications for the wound healing field. Pathways and molecules characterized in this study may facilitate rapid, scarless healing and could be considered for therapeutic application to non-oral mucosal sites.

To identify and explain the mechanisms that define accelerated oral wound healing, we analyzed the gene expression signature changes during oral mucosal and skin wound resolution in healthy human subjects. Oral mucosa samples obtained before wounding exhibited transcriptional networks that primed the epithelium for wound repair. Our data indicate that the major processes driving acute wound repair in healthy individuals were keratinocyte-driven. These networks were determined in part by the differential expression of a set of transcriptional regulators in oral versus skin keratinocytes, suggesting that pathways established during development are responsible for the differential wound resolution capacity of these cells.

Priming allows the oral mucosa to rapidly control and limit inflammatory responses, leading to fast inflammatory resolution. Oral keratinocyte activation and reduced differentiation allows for

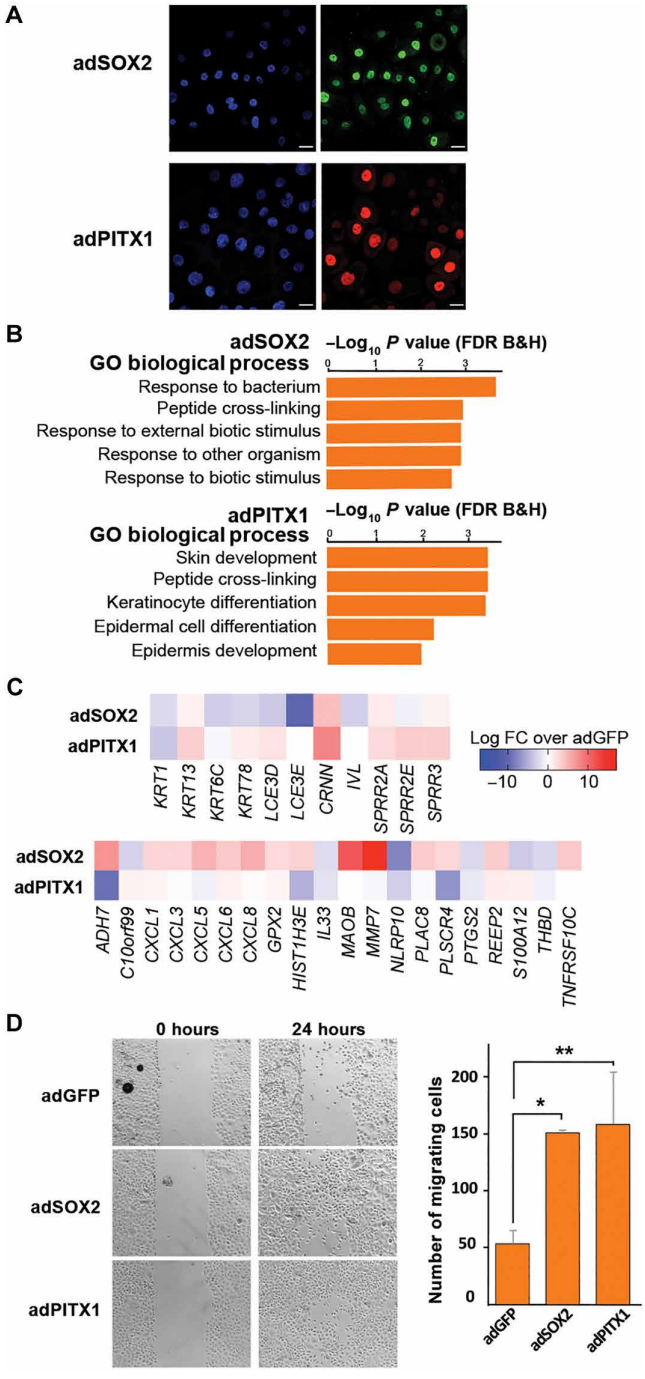

Fig. 7. Overexpression of SOX2 and PITX1 increases migratory capacity of primary skin keratinocytes. (**A**) Representative pictures of NHEK cells transduced with indicated adenoviruses and stained to show overexpression of corresponding proteins. Scale bars, 20 μm. (**B**) GO biological process terms enriched in data sets of genes differentially regulated by PITX1 and SOX2 overexpression in NHEK cells. (**C**) Fold change of the expression of genes related to differentiation and response to biotic stimulus in NHEK cells transduced with PITX1 and SOX2, presented as $\log_2$ fold change (log FC) over green fluorescent protein (GFP) expression. (**D**) Migrating NHEK cells transduced with SOX2, PITX1, and GFP (control) by adenoviral delivery. Images were taken at 0 and 24 hours after removal of silicone insert. Values were determined by counting the number of migrating cells at 24 hours in six microscopic fields in $n = 3$ per group. **$P < 0.01$, *$P < 0.05$ by ANOVA, adSOX2 and adPITX1 versus control. Data are mean values, and error bars represent SDs.

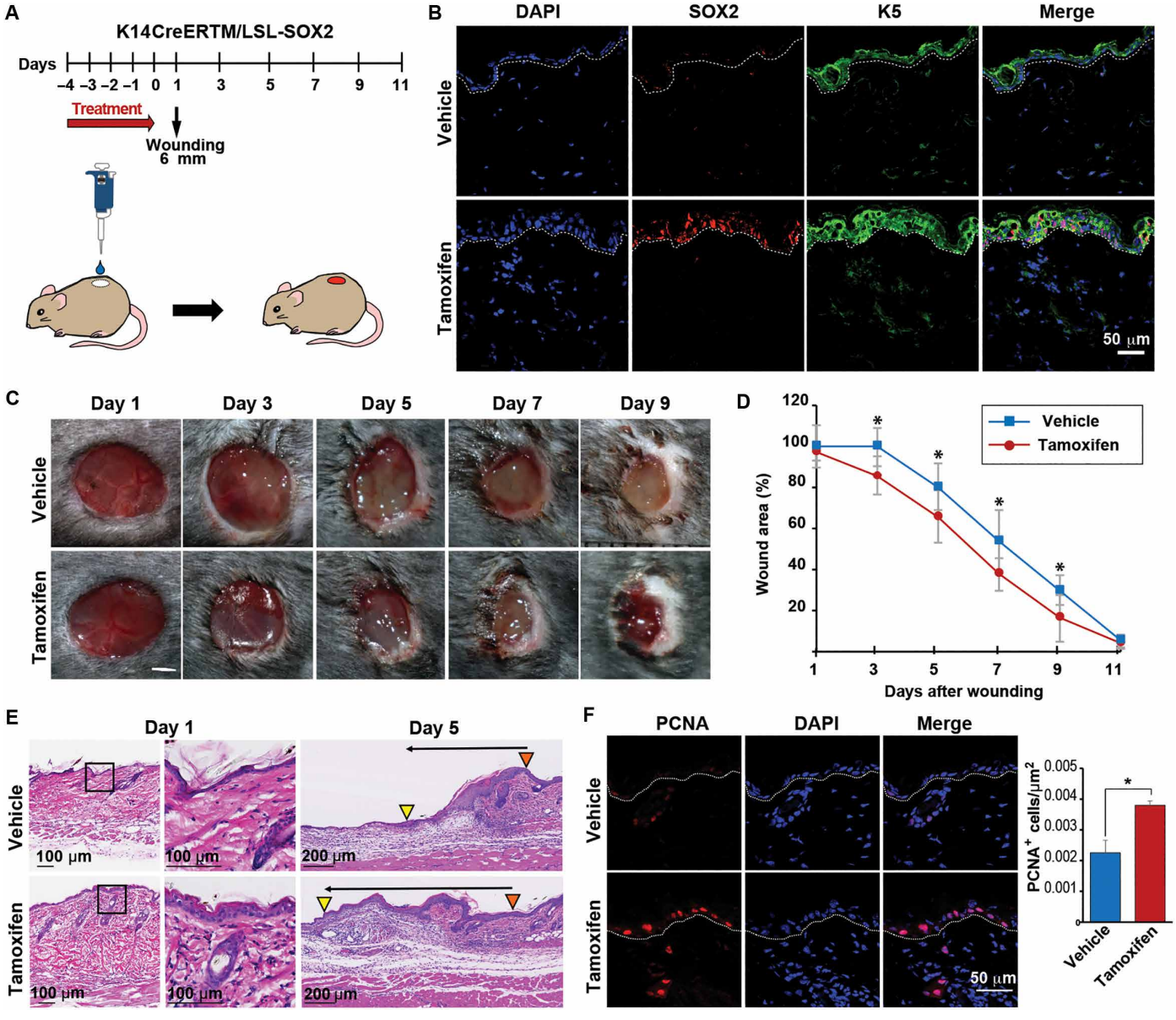

Fig. 8. Conditional overexpression of SOX2 contributes to cutaneous wound healing. (**A**) Schematic representation of the experimental design used for evaluating cutaneous wound healing in K14CreERTM/LSL-SOX2 mice. Mice were treated with vehicle (ethanol) or tamoxifen for five consecutive days by topical application on dorsal skin (day −4 to day 0, red arrow). Wounds were created as a 6-mm full-thickness excisional dorsal skin wound by biopsy punch (day 1). (**B**) Representative images of unwounded skin stained to show expression of SOX2 (red) and the basal marker keratin 5 (K5, green) in K14CreERTM/LSL-SOX2 mice treated with vehicle or tamoxifen. DAPI (4′,6-diamidino-2-phenylindole) in blue. Epithelium is marked with dotted lines. (**C**) Photographs of the wound areas after topical treatment with vehicle or tamoxifen in K14CreERTM/SOX2 mice at 1, 3, 5, 7, and 9 days after wounding. Scale bar, 1 mm. (**D**) Percent wound area at each time point relative to the original wound area in K14CreERTM/LSL-SOX2 mice treated with vehicle or tamoxifen. Quantification of the wound areas in $n = 7$ wounds per group was performed using ImageJ software. *$P <$ 0.05, tamoxifen versus vehicle at each day. Error bars represent SDs of seven wounds. (**E**) Representative H&E-stained sections of skin day 1 (unwounded) and day 5 (during wound healing, wound edges including epithelial tongue). Skin sections were from K14CreERTM/LSL-SOX2 mice after treatment with tamoxifen or vehicle. (**F**) Representative images of unwounded skin stained to show expression of $PCNA^+$ proliferating epithelial cells (red). DAPI in blue. *$P < 0.05$ by unpaired t test.

a rapid re-epithelialization of the wound area. It has been recently shown that re-epithelialization in mice is dependent on tissue-scale coordination of proliferation, differentiation, and migration (*36*), with acquisition of stem cell properties in de-differentiated epidermal cells (*37*). In human wound repair, the reduced expression of cytokines and fibrosis mediators such as annexin 1 and SLPI potentially contributes to the scarless wound healing observed in the oral mucosa.

Transcriptional regulators hold the key to the activation of the molecular events responsible for accelerated wound resolution in oral healing. Here, we identified factors that define oral keratinocytes and demonstrated that two of them, SOX2 and PITX1, regulate networks involved in wound closure. The SOX and PITX family of transcription factors have important roles in development, ranging from regulation of cell fate to axis and pattern formation (*32*, *34*).

Our results support that these transcription factors are responsible for establishing an oral mucosa–specific network that primes these epithelia for rapid and efficient wound healing. We further showed that SOX2 and PITX1 can be exploited to reprogram skin keratinocytes to present oral keratinocyte features, including accelerated wound resolution in vitro and in vivo. SOX2 is involved in adult stem cell maintenance for a myriad of epithelial tissues (*38*) and has been shown to regulate the functions of skin tumor–initiating cells (*39*, *40*). We showed that SOX2 induced an expansion of the $K5^+$ basal stem cell compartment of the skin in mice, indicating that the wound regenerative capacity of SOX2 might be linked to its stem cell regulatory functions. Our data also suggest that PITX1 regulates a separate set of processes related to the expression of structural proteins, including keratins, LCEs and SPRRs. PITX2 is an essential component of the genetic network activated by tissue damage during heart repair (*41*); however, more studies are needed to identify the specific pathways activated by these transcriptional regulators and their potential for wound repair and tissue regeneration.

Our analysis of wound resolution was limited by clinical concerns that restricted the location of the wound site and timing of the of biopsies. Although our study focused on buccal mucosa as a model for oral wound healing, the oral palate more closely resembles the histological features and differentiation profile of the skin. The location of the oral biopsy in the buccal mucosa was chosen on the basis of clinical parameters. Performing multiple wounds in the palate was not possible due to the extreme discomfort of unsutured or repeated wounds in that region and an increased risk of potential complications, including exposure of bone or tooth surfaces. Despite having different keratinization and terminal differentiation profiles, buccal mucosa, gingiva, and palate show similar accelerated wound healing when compared to cutaneous wounds (*3*, *20*). This indicates that there are mechanisms inherent within the oral cavity that increase wound resolution, beyond local differences in epithelial structure. Choosing buccal mucosa provided the most effective way to minimize discomfort and risk of clinical complications while still being able to study oral mucosa wound healing in subjects. In regard to the timing of the biopsies, our study focused on pairing oral mucosa and skin wounds, and this presented the challenge of comparing two tissue sites with differences in rate of healing that made it unfeasible to compare wounds at the same stage of closure. The number of time points was determined by taking into consideration the paired wounds in both locations, comfort of the subjects, and number of subjects in the clinical protocol.

Overall, we present human clinical data and histological and gene expression analysis that provide a comprehensive comparative analysis of the molecular and cellular mechanisms underlying the different wound healing processes in oral and skin epithelia. Our data indicate that the unique environment of the oral cavity represents a wound healing program geared toward rapid wound resolution (fig. S9). Ultimately, this human transcriptomic data set highlights fundamental global mechanisms of inflammation and repair in humans that will serve as an invaluable resource, providing insights into therapeutic targeting of chronic and nonhealing wounds.

MATERIALS AND METHODS

Study design

We performed a clinical study to obtain paired oral and skin baseline and wound samples and to analyze the healing profile of both locations. The clinical study was approved by the Institutional Review Board at National Institute of Dental and Craniofacial Research (NIDCR; ClinicalTrials.gov #NCT01078467). Inclusion criteria for enrollment consisted of participants being nonsmokers and having only occasional (social) alcohol consumption. Pregnancy was also an exclusion criterion. A sterile 3-mm punch biopsy (McKesson) was used to create a uniform, full-thickness biopsy in the mucosa of the cheek and posterior axillary region of the arm and followed up for up to 15 days. All subjects received an oral and skin biopsy at their day 1 time point (1 day after initial exam). The 30 healthy subjects were randomized in three groups with 10 subjects in each group: Group 1 subjects received one set of biopsies at their day 1 time point, group 2 subjects received a second set of biopsies at their day 3 time point, and group 3 subjects received a second set of biopsies at their day 6 time point. Day 3 and 6 biopsies were performed with a sterile 5-mm punch biopsy. For RNA-seq analysis, paired oral and skin samples were chosen randomly from four subjects for each day (24 total samples from 12 individual subjects). Wound healing was also analyzed in mice according to the protocol approved by the Animal Use and Care Committee at the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS). Full-thickness wounds were created in K14CreERTM/LSL-SOX2 mice and examined as described previously (*42*, *43*). Changes in wound area are expressed as percentages of the initial wound area in control mice. Supplementary Materials and Methods include full clinical study protocol and experimental details. Individual subject-level data are reported in table S4.

Statistical analysis

For RNA-seq data, statistical analysis was performed with the Partek Genomics Suite (www.partek.com): ANOVA was performed to compare the wound healing process in the oral and skin samples by comparing each wound day (days 3 and 6) with the nonwounded samples (day 1); paired *t* test was performed to compare oral with skin wound healing at each time point. $q < 0.05$ was considered statistically significant (q value is an adjusted P value taking into account the FDR). For all other data sets, data analysis was performed with GraphPad Prism version 5.01 for Windows (GraphPad Software); $P < 0.05$ was considered statistically significant. ANOVA followed by the Tukey's *t* test were used to analyze the differences in the groups for migration assays. Two-tailed, unpaired *t* tests were used to analyze the healing time and size differences between the groups, differences in PCNA expression between the groups, and differences in immune markers.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/10/451/eaap8798/DC1

Materials and Methods

Fig. S1. Visualization of up-regulated genes in oral mucosa compared to the skin by CIRCOS plot.

Fig. S2. Migration-related pathways in wound healing.

Fig. S3. Control stainings and IVL expression and proliferation in oral and skin wounds.

Fig. S4. Inflammatory pathways in wound healing.

Fig. S5. Expression of oral signature genes in wound healing and in primary human oral and skin keratinocytes.

Fig. S6. Knockdown of oral signature genes in primary oral keratinocytes.

Fig. S7. Overexpression of SOX2 and PITX1 in primary skin keratinocytes.

Fig. S8. Detection of GFP expression in K14CreER/LSL-SOX2 mice.

Fig. S9. Proposed model indicating the main characteristics that improve wound healing in the oral mucosa.

Table S1. Differentially regulated genes (oral versus skin day 1).

Table S2. Genes expressed in the unwounded oral mucosa at steady state that are up-regulated in the skin during wound healing.
Table S3. Significant GOs in wound-associated genes expressed in day 1 oral mucosa.
Table S4. Individual subject-level data.
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Science Translational Medicine

Transcriptional signature primes human oral mucosa for rapid wound healing

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Rapid repair

Wounds in the mouth heal faster and with less scarring than wounds in other locations on the body. To understand differences in healing, Iglesias-Bartolome *et al*. performed transcriptional analysis on sequential, paired oral and skin biopsies from healthy human subjects. Compared to baseline, skin samples showed a larger number of up-regulated genes on subsequent biopsies than oral samples, indicating that healing was unresolved. Oral wounds healed faster than skin wounds, and certain transcription factors were consistently up-regulated in the oral wounds but not in skin wounds. Overexpressing some of these transcription factors in a mouse model of skin wounding enhanced healing. The authors suggest that the molecular signature of the oral mucosa could be used to develop therapies for wound healing.