SOX2 Epidermal Overexpression Promotes Cutaneous Wound Healing via Activation of EGFR/MEK/ERK Signaling Mediated by EGFR Ligands

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Oral mucosa contains a unique transcriptional network that primes oral wounds for rapid resolution in humans. Our previous work identified genes that were consistently upregulated in the oral mucosa and demonstrated that induction of one of the identified genes, transcription factor SOX2, promoted cutaneous wound healing in mice. In this study, we investigated the molecular and cellular mechanisms by which SOX2 accelerates wound healing in skin. RNA-sequencing analysis showed that SOX2 induced a proliferative and wound-activated phenotype in skin keratinocytes prior to wounding. During wound healing, SOX2 induced proliferation of epithelial and connective tissue cells and promoted angiogenesis. Chromatin immunoprecipitation assay revealed that SOX2 directly regulates expression of EGFR ligands, resulting in activation of EGFR. In vitro, skin keratinocytes overexpressing SOX2 promoted cell migration via the EGFR/MEK/ERK pathway. We conclude that induction of SOX2 in skin keratinocytes accelerates cutaneous wound healing by promoting keratinocyte migration and proliferation, and enhancement of angiogenesis via upregulation of EGFR ligands and activation of EGFR/MEK/ERK pathway. Through the identification of putative cutaneous SOX2 targets, such as HBEGF, this study opens venues to determine clinical targets for treatment of skin wounds.

INTRODUCTION

Wound healing has four overlapping phases in the repair process: hemostasis, inflammation, proliferation, and remodeling (Singer and Clark, 1999). In the proliferation phase, diverse types of cells, including keratinocytes, fibroblasts, endothelial cells, macrophages, and leukocytes, migrate into the wound area for re-epithelialization, angiogenesis, and production of the extracellular matrix. Growth factors derived from various cells localized in the wounded area are essential for proper and efficient wound healing (Eming et al., 2014). Activation of EGFR through EGFR ligands, regulates various phases of cutaneous wound healing (Reperinger et al., 2004; Tokumaru et al., 2000). Dysregulation of these interactive processes in diabetic wounds results in delayed wound healing (Brem and Tomic-Canic, 2007; Gurtner et al., 2008; Singer and Clark, 1999).

Oral wound healing has long been considered as an ideal system of wound resolution compared to skin because oral wounds resolve faster and without scar formation. Several studies determined the differences of the injury responses between oral mucosal and cutaneous wounds, focusing on the inflammation and migrating capacity of keratinocytes (Szpaderska et al., 2003; Turabelidze et al., 2014). We recently reported a longitudinal clinical wound healing study using healthy human subjects where paired comparative gene analysis was done between oral mucosa and skin tissue (Iglesias-Bartolome et al., 2018). This analysis revealed that wound-activated transcriptional networks are present at the basal state in the oral mucosa, priming the epithelium for wound repair. SOX2 and PITX1 were identified as being highly expressed in the oral mucosa compared to the skin, and we demonstrated that they play important functions during wound resolution (Iglesias-Bartolome et al., 2018).

SOX2, a member of the SoxB1 transcription factor family, is an important regulator of stem cell maintenance in epithelial tissues and has been found to promote tumor growth (Arnold et al., 2011; Boumahdi et al., 2014; Siegle et al., 2014). It has also been reported that SOX2 is associated with the development of immature tumors, including glioblastomas and teratomas (Garros-Regulez et al., 2016; Phi et al., 2007). In the skin, SOX2 is expressed in cells localized around the bulge region of hair follicles, dermal papilla, Merkel cells, and neural crest.
precursor cells, but has not been detected in epidermal keratinocytes (Driskell et al., 2009; Johnston et al., 2013). It has been reported that SOX2 has an important role in skin repair (Johnston et al., 2013), and we demonstrated that inducible epidermal-specific expression of SOX2 promoted cutaneous wound healing in mice (Iglesias-Bartolome et al., 2018). However, the detailed cellular and molecular mechanisms of rapid SOX2-mediated wound resolution in skin keratinocytes has not been determined. Herein, we performed in-depth analysis of the molecular and cellular changes induced by SOX2 in skin keratinocytes in the basal state and during wound healing. We identified SOX2-target genes and downstream EGFR/MEK/ERK pathway effectors as essential components in the promotion of cutaneous wound healing.

RESULTS
Epidermal-specific SOX2 expression accelerates cutaneous wound healing by promoting re-epithelialization and formation of granulation tissue

Utilizing a tamoxifen (Tam)-inducible K14CreERTM/LSLSOX2 (transgenic [TG]) mouse model (Supplementary Figure S1a online), we determined that mRNA and protein levels of SOX2 expression were significantly increased in Tam-treated TG mice compared with vehicle (Veh)-treated mice, using murine oral mucosa (buccal mucosa and hard palate) as positive controls (Figure 1a, 1b and Supplementary Figure S1b). Skin punch biopsies were obtained from SOX2-overexpressing (TG Tam) and age-matched control mice (TG Veh, wild-type [WT] Tam and WT Veh) to assess the wound healing process every 2 days up to 12 days, when there was almost complete wound closure in TG Tam mice. WT Tam- and WT Veh-treated mice confirmed that topical application of Tam had no effect on cutaneous wound healing. SOX2-overexpressing mice exhibited significantly accelerated wound closure kinetics compared with control groups from day 2 to day 12 (Figure 1c). Thickness of the epidermis was significantly increased in SOX2-overexpressing mice compared to control mice (TG Veh or WT Tam) (Supplementary Figure S1c). The re-epithelialization area was significantly larger in SOX2-overexpressing mice compared with control (TG Veh) mice at days 2 and 4 after wounding (Supplementary Figure S1d), corroborating our previous work (Iglesias-Bartolome et al., 2018). Furthermore, the granulation tissue area was significantly increased in SOX2-overexpressing mice compared with control (TG Veh) mice (Supplementary Figure S1e). These results indicate that SOX2 expression in skin keratinocytes accelerates cutaneous wound healing via the promotion of re-epithelialization and formation of granulation tissue.

Cutaneous SOX2 suppresses epidermal differentiation but induces wound-activated phenotype in basal state

To elucidate the molecular mechanisms underlying the accelerated wound healing in SOX2-overexpressing mice, we performed RNA sequencing (RNA-seq) analysis and compared gene expression between SOX2-overexpressing and control mice at unwounded (day 0) and wounded (day 4 after wounding) time points (Figure 2a). Clustering analysis showed that there were large differences in gene expression between SOX2-overexpressing and control mice in the unwounded time point. While these differences decreased after wounding, there was clear separation between Tam wounded and Veh wounded mice in principal component analysis (Supplementary Figure S2a online).

Next, we focused on differences in gene expression at basal state (day 0, unwounded) between SOX2-overexpressing and control mice (Figure 2b). Gene Ontology analysis of the genes upregulated in SOX2-overexpressing mice (unwounded Tam/Veh: reads per kilobase of transcript per million mapped reads [RPKM] >1; fold-change >2; q < 0.05; 1,226 genes) showed enrichment of genes related to skin development, epidermal cell differentiation, hair follicle development, and cell cycle (Figure 2c). Expression levels of epidermal differentiation genes (KRT1, KRT10, LOR, and SBSN) were suppressed in SOX2-overexpressing mice (Figure 2d, 2e, and Supplementary Figure S2b). However, SOX2-overexpressing skin did not result in expression of KRT13, a well-established oral keratinocyte marker (Supplementary Figure S2c). Keratinocytes at the wound edge switch their phenotype from differentiating to activated and start expressing KRT6, KRT16, and KRT17 (Freedberg et al., 2001; Wikramanayake et al., 2014). KRT17 is expressed in several skin disorders, including psoriasis and tumors (Markey et al., 1992; Yang et al., 2017) and promotes cell proliferation, migration, and wound healing (Mazzalupi et al., 2003). Interestingly, SOX2-overexpressing skin showed significantly increased expression of KRT17 in the unwounded time point (Figure 2d–2f and Supplementary Figure S2b). It has been reported that SOX2 directly regulates Shh in the development of neural stem cells (Favaro et al., 2009). Shh has important roles in the regulation of wound healing (Asai et al., 2006) and KRT17 expression (Callahan et al., 2004). RNA-seq analysis showed that Shh signaling–associated genes were upregulated at basal state in SOX2-overexpressing mice (Supplementary Figure S2d). Immunofluorescence staining showed that Shh was highly expressed in SOX2-overexpressing skin (Supplementary Figure S2e).

SOX2 promotes cell proliferation and tumor growth via regulation of cyclin expression (Boumahdi et al., 2014). Transcript levels of most cyclins were upregulated in SOX2-overexpressing mice (Figure 2g). We previously revealed that SOX2-overexpressing skin has a proliferative phenotype at basal state using proliferating cell nuclear antigen staining (Iglesias-Bartolome et al., 2018). We confirmed this result in transcriptome and protein levels with another proliferation maker, Ki-67. Ki-67 and proliferating cell nuclear antigen were also upregulated in SOX2-overexpressing mice (Figure 2g and Supplementary Figure S2f).

When using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood, CA) on the genes upregulated in SOX2-overexpressing mice in the unwounded time point, we found induction of networks associated with development and growth of epithelial tissue and cell cycle progression (Figure 2h) and genes associated with cellular movement (Supplementary Figure S2g).

Taken together, these results indicate that in epidermal keratinocytes, SOX2 suppresses epidermal differentiation while inducing a wound-activated phenotype. This is supported by the increased expression of KRT17 and
upregulation of genes associated with cell proliferation and migration in the basal state.

**SOX2 in skin keratinocytes promotes proliferation of keratinocytes and angiogenesis during wound healing**

To investigate gene networks regulated by SOX2 during wound healing, we compared the gene expression profiles of SOX2-overexpressing and control mice in the wounded state (day 4) (Figure 3a). Gene Ontology analysis of genes significantly upregulated in SOX2-overexpressing mice (wounded Tam/Veh, RPKM > 1, fold-change > 2, q < 0.05, 424 genes) showed enrichment for processes related to skin development, keratinocyte differentiation, intermediate filament cytoskeleton organization, mesenchymal cell development, and positive regulation of keratinocyte migration (Figure 3b).

Expression levels of genes described in wound-activated keratinocytes, including keratins KRT6, KRT16, and KRT17 were activated in SOX2-overexpressing mice particularly in the wounded timepoint, with the most notable change being for KRT17 (Figure 2d). Diseases and functions analysis via Ingenuity Pathway Analysis on the upregulated genes in SOX2-overexpressing mice showed that cutaneous SOX2 induced gene networks associated with proliferation of connective tissue and epithelial cells and cell movement during wound healing (Figure 3c and Supplementary Figure S3a online). We performed in-depth analysis of migrating tongue and granulation tissue to evaluate the correlation between SOX2 expression and the promotion of re-epithelialization and formation of granulation tissue. Immunofluorescence staining showed that SOX2+ cells were present in the migrating tongue, and a large number of proliferating cell nuclear antigen-positive keratinocytes were found in the wound area in SOX2-overexpressing mice (Figure 3d, 3e). Ki67, another marker of proliferation, showed upregulation in the RNA-seq analysis (Figure 2g). Furthermore, the numbers of Ki-67 positive cells in the wound area were significantly increased in SOX2-overexpressing mice (Figure 3f and Supplementary Figure S3c). Analysis via Ingenuity Pathway Analysis also showed induction of gene networks associated with vasculogenesis and angiogenesis in organism development (Figure 3c). It has been reported that SOX2 in tumors promotes angiogenesis through production of pro-angiogenic factors, including VEGF, TGFA, and EREG (Siegle et al., 2014). In addition to the upregulation of angiogenesis pathways (Figure 3c and Supplementary Figure S3b), we determined that the number of CD31+ endothelial cells and αSMA+ pericytes/myofibroblasts in the wound area were significantly increased in SOX2-overexpressing mice (Figure 3g, 3h and Supplementary Figure S3c). However, SOX2-overexpressing mice did not have increased mRNA levels of members of collagen family (Col1a1, Col1a2, and Col3a1) and FN1, and those expression...
**Figure 2.** SOX2-expressing skin keratinocytes present wound-activated phenotype in basal state.

(a) Unsupervised clustering analysis of RNA-sequencing gene expression data of the samples for each group in unwounded (day 0) and wounded (day 4) time points in transgenic mice treated with Veh and Tam. (b) Schematic representation of biopsy site and time course of treatment in the unwounded skin. (c) GO biological process terms enriched in data sets of genes differentially regulated by SOX2-overexpressing skin keratinocytes in transgenic mice in unwounded time point. (d) Relative mRNA expression levels of keratinization and epidermal cell differentiation markers throughout the wound healing process. (e) Immunoblot image showing KRT1, KRT10, and KRT17 expression in unwounded skin samples from transgenic mice treated with Veh and Tam. Actin was used as a loading control. n = 3. (f) Representative images of unwounded skin tissue from transgenic mice treated with Veh and Tam stained to detect expression of KRT17 (red), KRT5 (green), and DAPI (blue). Scale bar = 50 μm. (g) Relative mRNA expression levels of proliferation markers throughout the wound healing process. (h)IPA analysis of RNA-sequencing data from unwounded skin samples treated with Veh and Tam in transgenic mice: diseases and functions terms related to tissue development and cell cycle found in differentially regulated genes. Data is expressed as mean ± standard error of mean. **P < 0.01, *P < 0.05. B&H, Benjamini and Hochberg; FDR, false discovery rate; GO, Gene Ontology; IPA, Ingenuity Pathway Analysis; Tam, tamoxifen; Veh, vehicle.
Figure 3. SOX2-expressing skin keratinocytes promote proliferation of keratinocytes, connective tissue cells, and angiogenesis during wound healing. (a) Schematic representation of biopsy site in the skin at day 4 after wounding (wounded) and time course of treatment. (b) GO biological process terms enriched in data sets of genes differentially regulated in SOX2-overexpressing skin keratinocytes of transgenic mice during wound healing (wounded). (c) IPA analysis of RNA-sequencing data from wounded skin samples treated with Veh and Tam in transgenic mice: diseases and functions terms related to tissue development, cellular movement, and organismal development found in differentially regulated genes. (d, e) Representative images of migratory tongue in transgenic mice treated with Veh or Tam at day 4 to show expression of (d) SOX2 and (e) PCNA-positive keratinocytes. Scale bar = 50 μm. (f, h) Representative images of skin tissue from transgenic mice treated with Veh and Tam at day 4 (wounded) stained to show expression of (f) Ki-67⁺ cells, (g) endothelial cells (CD31⁺), and (h) pericytes/myofibroblasts (αSMA⁺). Scale bar = 50 μm. B&H, Benjamini and Hochberg; FC, fold-change; FDR, false discovery rate; GO, Gene Ontology; IPA, Ingenuity Pathway Analysis; PCNA, proliferating cell nuclear antigen; RPKM, reads per kilobase of transcript per million mapped reads; Tam, tamoxifen; Veh, vehicle.
Figure 4. SOX2-expressing skin keratinocytes induce upregulation of EGFR ligands expression and activation of EGFR.

(a) Venn diagrams summarizing comparison of upregulated genes. Left panel shows unwounded Tam/Veh (blue), wounded Tam/Veh (green) in mice and oral/skin in the basal state (Red) in human. Center panel shows Veh wounded/unwounded (blue) and Tam wounded/unwounded (green). Right panel shows relative mRNA expression levels of five genes determined as common genes between 47 genes and 378 genes (center) throughout the wound healing process. (b, c) Representative images of unwounded and wounded skin tissue from transgenic mice treated with Veh and Tam stained to show expression of the (b) HBEGF (green), (c) pEGFR (red), and DAPI (blue). Scale bar = 50 μm. (d) Immunoblots show EGFR and pEGFR expression in unwounded and wounded skin with Veh- or Tam-treated transgenic mice. Actin was used as a loading control. Bottom panels show quantification of each protein. n = 4 for unwounded, n = 8 for wounded. (e) Quantitative PCR analyses on chromatin samples from epidermis of Tam-treated transgenic mice after immunoprecipitation with anti-SOX2 and IgG control antibodies. n = 3. Data expressed as mean ± standard error of the mean. *P < 0.05. FC, fold-change; pEGFR, phosphorylated EGFR; RPKM, reads per kilobase of transcript per million mapped reads; Tam, tamoxifen; Veh, vehicle.
levels correlated with expression levels of TGF-β (Supplementary Figure S3d, S3e).

Neutrophils and macrophages infiltrate the wound area and promote re-epithelialization and angiogenesis via production of chemokines and growth factors (Lamagna et al., 2006). Therefore, we examined the numbers of CD68+ macrophages and myeloperoxidase+ neutrophils in the wound area by immunofluorescence staining. However, there was no significant difference between SOX2-overexpressing mice and control mice (Supplementary Figure S3f, S3g).

These results indicate that cutaneous SOX2 enhances cell proliferation in epidermis, as well as connective tissue formation and cell migration. The resulting promotion of re-epithelialization and angiogenesis is potentially directly involved in the accelerated wound healing process.

SOX2-overexpressing mice upregulate EGFR ligands genes

Next, we compared genes from unwounded Tam/Veh, wounded Tam/Veh in mice, and oral/skin (day 0) groups in human (Iglesias-Bartolome et al., 2018) to determine SOX2 target genes. This analysis identified 47 genes that were upregulated in all three groups (Figure 4a and Supplementary Figure S4a online). Consecutively, we performed another analysis comparing genes from Veh wounded/unwounded and Tam wounded/unwounded groups in mice and determined 378 genes commonly upregulated during the wound healing process (Figure 4a). Finally, we cross-referenced these two gene lists and identified five SOX2 target genes that are potentially instrumental to accelerated wound healing (Figure 4a). Among these genes, ALDH1A3 encodes an aldehyde dehydrogenase enzyme that is required for conversion of retinol to retinoic acids. It has been reported that topical application of retinoic acid improves cutaneous wound healing (Kitano et al., 2001). HAS3 is involved in the synthesis of the unbranched glycosaminoglycan hyaluronan, which is a major constituent of the extracellular matrix. Hyaluronan binds to its receptor CD44 present on endothelial cells, triggering the proliferation of endothelial cells and production of angiogenic cytokines (Pardue et al., 2008). Alcian blue staining revealed that hyaluronan was highly elevated in the wound area in SOX2-overexpressing mice (Supplementary Figure S4b).

AREG and HBEGF are EGFR ligands that regulate a number of cellular processes, such as proliferation, differentiation, migration, and survival via activation of EGFR signaling pathway (Singh et al., 2016). FAM83A activates downstream of EGFR signaling, independent of EGFR activation (Lee et al., 2012). Significantly heightened mRNA levels of AREG and HBEGF expression was confirmed by quantitative PCR in each group, corroborating the trend found in the RNA-seq analysis (Supplementary Figure S4c). Interestingly, other EGFR ligands were also upregulated in SOX2-overexpressing mice compared with control mice (Supplementary Figure S4d).

Immunofluorescence staining showed that HBEGF was highly expressed in keratinocytes in SOX2-overexpressing mice compared to control mice in the unwounded and wounded conditions (Figure 4b). Moreover, we found that phosphorylated EGFR was also significantly upregulated in SOX2-overexpressing mice in the unwounded and wounded time points (Figure 4c and 4d and Supplementary Figure S4e).

It has been reported that SOX2 increases the expression of EREG and TGFα in hair follicle stem cells and cancer cells (Siegle et al., 2014), which lead us to test whether SOX2 directly regulates transcription of EGF ligands in skin keratinocytes by chromatin immunoprecipitation assay. Integrative analysis using the RNA-seq data set and FIMO motif analysis revealed higher enrichment of SOX2 binding to the loci of AREG, HBEGF, EREG and TGFA. This was confirmed by chromatin immunoprecipitation quantitative PCR, where the targets showed approximately ninetofold-change compared with IgG control (Figure 4e). These results suggest that in skin keratinocytes, SOX2 potentially directly regulates the expression of EGFR ligands resulting in the activation of EGFR signaling.

SOX2 in skin keratinocytes promotes proliferation and migration in vitro

Next, we examined the effect of SOX2 expression in skin keratinocytes in vitro. We confirmed that mRNA and protein levels of SOX2 were significantly upregulated in Tam-treated primary keratinocytes derived from TG mice compared with those treated with Veh (Figure 5a–5c). Scratch assays demonstrated that SOX2 expression in skin primary keratinocytes leads to increased cell migration (Figure 5d), and increased the proliferative capacity (Figure 5e). We also showed that knockdown of SOX2 expression reverses the enhancement of cell migration in SOX2-expressing mouse keratinocytes (Supplementary Figure S5a, S5b online). Next, we examined whether SOX2 affects the EGFR signaling pathway in vitro. Western blot analysis shows that phosphorylated EGFR was significantly upregulated in SOX2-expressing keratinocytes in the basal state (Figure 5f). Moreover, pERK1/2 was significantly upregulated in SOX2-expressing keratinocytes at 15 and 30 minutes after scratching (Figure 5f).

We also examined SOX2-regulated transcription of EGFR ligands in primary keratinocytes by quantitative PCR. In the basal state, the expression of EGFR ligands was upregulated in SOX2-expressing keratinocytes compared to control keratinocytes. After scratching, expression of AREG, EREG, and HBEGF was significantly upregulated in both control and SOX2-expressing cells compared to the basal state. In particular, expression of HBEGF and EREG was significantly increased in SOX2-expressing skin keratinocytes compared with that in control after scratching (Figure 5g). These results indicate that SOX2 potentially modulates EGFR signaling pathway by regulating expression of EGFR ligands, resulting in promotion of cell migration.

Inhibition of EGFR signaling pathway suppresses enhancement of cell migration in SOX2-expressing skin keratinocytes

To determine the mechanism through which SOX2 enhances cell motility mediated by activation of the EGFR signaling pathway, we selectively blocked EGFR and ERK activation using an EGFR inhibitor (AG1478), an MEK inhibitor (U0126), and EGFR small interfering RNA (Supplementary Figure S6a online). AG1478 (Figure 6a), U0126 (Figure 6b), and EGFR small interfering RNA (Figure 6c) significantly
Figure 5. SOX2+ skin primary keratinocytes promote migration, proliferation and enhance pEGFR via EGFR ligands expression. (a) mRNA expression of SOX2 in transgenic mice keratinocytes treated by Veh and Tam for 48 hours. (b) Immunoblot images showing SOX2 expression in transgenic mice keratinocytes treated by Veh or Tam for 48 hours. RPS14 was used as a loading control. Right panel shows quantification of SOX2 protein. (c) Representative images of transgenic mice keratinocytes treated by Veh and Tam for 48 hours stained to show expression of SOX2 (red) and DAPI (blue). Scale bar = 20 μm. (d) Migrating transgenic mice keratinocytes treated with Veh and Tam after scratch. Images were taken at 0 and 24 hours after scratch. Values were determined by relative
SOX2 Activates EGFR Pathway during Wound Healing

A Uchiyama et al.

DISCUSSION

This study investigates the mechanistic role of SOX2 in epidermal keratinocytes during cutaneous wound healing. We previously reported that the transcription factor SOX2 establishes a network that is efficacious in promoting wound healing (Iglesias-Bartolome et al., 2018). The endogenous expression of SOX2 in epithelia of buccal mucosa and palate confers intrinsic features to these tissues, which translates to accelerated wound healing compared with skin.

In an effort to determine common shared mechanisms or pathways that accelerate wound healing in oral mucosa and SOX2-overexpressing skin, we utilized an animal model for conditional inducible cutaneous expression of SOX2 and performed gene expression analysis of unwounded and wounded skin, with and without SOX2 expression. We previously demonstrated that the oral mucosa has a unique transcriptional network that primes the epithelium for rapid wound healing in the basal state (Iglesias-Bartolome et al., 2018). This network is associated with psoriasis-enriched genes that establish a wound-activated phenotype in the oral mucosa at basal state. Herein, transcriptomic analysis showed that SOX2 in epidermal keratinocyte induced hyperproliferation, wound-activated keratin expression, and expression of genes associated with cell migration before wounding, suggesting that SOX2 function within the cutaneous keratinocyte coordinates the expression of a network of genes required for wound healing at basal state.

During the wound healing process, gene analysis revealed that cutaneous SOX2 resulted in increased expression of genes associated with proliferation of connective tissue and epithelium, cell movement, and angiogenesis. While it is known that the keratinocytes migrating into the wound area do not have high proliferative capacity, SOX2+ keratinocytes maintain this capacity during migration, therefore, potentially contributing to the promotion of re-epithelialization. It has been reported that keratinocytes and fibroblasts interact through cytokines or growth factors during wound healing (Werner et al., 2007), and that angiogenesis is regulated by various keratinocyte-derived growth factors, cytokines, and extracellular matrix environment (Tonnesen et al., 2000). It has been reported that in fibroblasts, SOX2 regulates the expression of type I collagen using a fibrosis model in lung has been reported that in fibroblasts, SOX2 regulates the extracellular matrix environment (Tonnesen et al., 2000). It various keratinocyte-derived growth factors, cytokines, and that angiogenesis is regulated by through cytokines or growth factors during wound healing. There are seven ligands that bind to the EGFR: EGF, TGFA, HBEGF, BTC, AREG, EREG, and EPGN (Singh et al., 2016). EGFR signaling plays an exceedingly important role in normal skin integrity and wound healing by regulating re-epithelialization, angiogenesis, migration, and proliferation of fibroblasts and inflammation (Bodnar, 2013; Repertinger et al., 2004). This points to these factors being key molecules produced by SOX2+ keratinocytes for rapid wound repair. We present evidence that SOX2+ keratinocytes promote wound healing by regulating both epidermis and dermis through production of growth factors and extracellular matrix that induce the promotion of re-epithelialization and formation of granulation tissue via enhancement of proliferation of keratinocytes and fibroblast, cell movement, and angiogenesis in the wounded region.

Recent work has demonstrated that impaired EGFR signaling has been implicated in diabetic wounds (Berlanga-Acosta et al., 2017; Xu and Yu, 2011), and exogenous administration of soluble EGFR ligands, especially HBEGF, accelerated cutaneous wound healing in normal and diabetic mouse models (Johnston et al., 2013; Shirakata et al., 2005). These studies indicate that activation of EGFR signaling by EGFR ligands has the capacity to accelerate wound healing. Through chromatin immunoprecipitation assays, we demonstrate that HBEGF, AREG, EREG, and TGFA are potential molecular targets of SOX2. We showed that the SOX2-dependent upregulation of EGFR ligands led to enhancement of activation of the EGFR. Utilizing selective inhibition, we also show that enhancement of cell migration in SOX2+ keratinocytes is dependent on EGFR/MEK/ERK signaling.

Taken together, we demonstrate that SOX2 in epidermal keratinocytes altered the cellular phenotype to a wound-activated condition before wounding, allowing skin tissue to respond more quickly to injury, which mimicked the rapid wound resolution in the oral mucosa. After wounding, SOX2 promoted EGFR/MEK/ERK kinase signaling and increased expression of angiogenesis-associated genes resulting in enhancement of re-epithelialization, angiogenesis, and wound healing.

MATERIALS AND METHODS

The detailed protocols and statistical analysis are described in Supplementary Materials and Methods online.

Mice

All animal studies were carried out according to the protocol approved by the Animal Use and Care Committee at the National Institute of Arthritis and Musculoskeletal and Skin Diseases. K14CreERTWLSL-SOX2 mice were generated by mating KRT14-cre/ ERT200Etu/K14Cre mice (The Jackson Laboratory, Bar Harbor, ME) and Rosa26CAG-loxp-stop-loxp-Sox2-IRES-Egfp mice (Lu et al., 2010).

Data expressed as mean ± standard error of the mean. ***P < 0.001, **P < 0.01, *P < 0.05, p, phosphorylated; RPKM, reads per kilobase of transcript per million mapped reads; Tam, tamoxifen; Veh, vehicle.
Figure 6. Inhibition of EGFR/MEK/ERK signaling pathway suppresses cell migration in SOX2-expressing skin keratinocyte. (a–c) Migrating primary keratinocytes treated with Veh and Tam in absence or presence of (a) 1 μM AG1478, (b) 10 μM U0126, and (c) siCtl and siEGFR. Images were taken at 0 and 24 hours after scratch. Values were determined by relative migrating area in three microscopic fields in n = 3–4 per group. Data expressed as mean ± standard error of the mean. **P < 0.01, *P < 0.05. (d) Schematic model summarizing the mechanistic roles of SOX2 in epithelial keratinocyte promote cutaneous wound healing. pEGFR, phosphorylated EGFR; siCtl, control small interfering RNA; siEGFR, EGFR small interfering RNA; Tam, tamoxifen, Veh, vehicle.
**Wound healing assay in vivo**
Full-thickness wounds were created and examined as described previously (Zheng et al., 2007). To confirm the effect of SOX2 overexpression on cutaneous wound healing in mice, 5 mg/ml of Tam dissolved in ethanol 200 µl or same volume of ethanol (as a control) was topically applied to K14CreERTM/LSL-SOX2 and WT mice dorsal skin for 5 consecutive days.

**RNA preparation and RNA-seq data analysis**
Total RNA from skin tissue and cells was extracted using RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. mRNA expression profiling and analysis were performed as described in **Supplementary Materials and Methods** at the National Institute of Arthritis and Musculoskeletal and Skin Diseases Genome Core Facility at the National Institutes of Health (Bethesda, MD).

**Accession numbers**
Raw and analyzed RNA-Seq data have been deposited in the Gene Expression Omnibus site, accession number GSE118859.

**Statistical analysis**
P-values were calculated using the Student t test (two-sided) or one-way analysis of variance. Data analysis was done with GraphPad Prism, version 7 (GraphPad Software, San Diego, CA). Error bars represent standard errors of the mean and numbers of experiments are as indicated.

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**CONFLICT OF INTEREST**
The authors state no conflict of interest.

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**AUTHOR CONTRIBUTIONS**
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**SUPPLEMENTARY MATERIAL**
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.02.004.

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SUPPLEMENTARY MATERIALS AND METHODS

Mice
All animal studies were carried out according to the protocol approved by the Animal Use and Care Committee at the National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health (Bethesda, MD). Two TG mouse lines (K14CreERTM and LSL-SOX2) were crossbred in order to generate the Tam-inducible epithelial-specific SOX2 TG mice (K14CreERTM/LSL-SOX2). Mice carrying a Tam-inducible Cre-mediated recombination system driven by the human keratin 14 promoter (K14CreERTM mice) were from The Jackson Laboratory (stock number 005107, STOCK Tg [KRT14-cre/ERT20Efu/K14Cre] and Rosa26CAG-loxp-stop-loxp-SOX2-IRES-Egfp [LSL-SOX2]) was reported previously (Lu et al., 2010). Both male and female mice between weeks 8 and 12 after birth were used in the studies. All experiments were conducted using littermate controls.

Wound healing assay in vivo
Full-thickness wounds were created and examined as described previously (Zheng et al., 2007). Briefly, mice were anesthetized and hair was shaved and cleaned with 70% ethanol. Six-millimeter full-thickness excisional dorsal skin wounds were created by a sterile disposable biopsy punch (Integra Miltex, York, PA). Wounds were covered by film dressings (PERME-ROLL; Nitto Denko, Osaka, Japan). For analysis, each wound site was digitally photographed at the indicated time points after wounding, and wound areas were measured on photograph using Image J, version 1.48, as reported previously (Uchiyama et al., 2014). Changes in wound area are expressed as percentages of the initial wound area in WT Veh-treated mice. To confirm the effect of SOX2 overexpression on cutaneous wound healing in mice, 5 mg/ml of Tam dissolved in ethanol 200 µl or same volume of ethanol (as a control) was topically applied to K14CreERTM/LSL-SOX2 and WT mice dorsal skin for 5 consecutive days.

Histological examination and immunofluorescence staining
Murine skins were removed, fixed in 4% paraformaldehyde overnight at 4°C. For frozen sections, murine skins and oral mucosa were excised, fixed in 4% paraformaldehyde in phosphate buffered saline for 30 minutes, and 4-µm frozen sections were prepared. Immunofluorescence staining were performed as described previously (Motegi et al., 2011). Mouse primary keratinocytes were fixed with 4% paraformaldehyde and treated with 0.1% Triton X-100 at room temperature for 30 minutes. After blocking with 3% dry milk—phosphate buffered saline supplemented with 5% normal donkey serum for 1 hour at room temperature, sections were stained with antibodies of interest followed by Alexa 488-, 555- and 568-conjugated secondary antibodies. Sections were counterstained with DAPI to visualize nuclei, mounted in ProLong Gold antifade reagent (Life Technologies, Carlsbad, CA). Confocal fluorescent images were taken with a Zeiss LSM780 microscope, coupled to Zen software (Carl Zeiss, Oberkochen, Germany). Final images were bright contrast adjusted with Zen 2012 (Carl Zeiss). Stained hematoxylin and eosiin slides were scanned at ×4, ×20, and ×40 using an Aperio CS Scanscope (Aperio, Buffalo Grove, IL).

Antibodies
Antibodies (Ab) used in this study were as follows: rat anti-mouse CD31 mAb (BD Bioscience, 550274), rat anti-mouse CD68 mAb (Bio-Rad Laboratories, Hercules, CA; MCA1957), rabbit anti-mouse myeloperoxidase polyclonal Ab (pAb) (Abcam, Cambridge, MA; ab 9535), rabbit anti-mouse keratin 1 pAb (Biolegend, San Diego, CA; 905601), guinea pig anti-mouse keratin 5 pAb (LSBio, Seattle, WA; LS-C2215), rabbit anti-mouse keratin 10 pAb (Biologend; 905404), rabbit anti-mouse keratin 6A pAb (Biologend; 905701), goat anti-mouse SOX2 pAb (R&D Systems, Minneapolis, MN; AF2018), goat anti-mouse Shh pAb (R&D Systems; AF464), rabbit anti-mouse K67 pAb (Abcam; ab15580), rabbit anti-mouse αSMA pAb (Abcam ab5694), rabbit anti-mouse proliferating cell nuclear antigen (Cell Signaling; 13110), sheep anti-mouse HB-EGF pAb (R&D Systems; AF8239), rabbit anti-mouse EGFR mAb (Abcam; ab 76153), rabbit anti-mouse phosphorylated EGFR (Tyr1068) mAb (Cell Signaling; 3777), rabbit anti-mouse MEK1/2 mAb (Cell Signaling: 8277), rabbit anti-mouse phosphorylated MEK1/2 (Ser217/221) mAb (Cell Signaling: 9154), rabbit anti-mouse p44/42 MAPK (Erk1/2) mAb (Cell Signaling; 4695), rabbit anti-mouse phosphorylated p44/42 MAPK (Erk1/2) (Thr202/Tyr204) mAb (Cell Signaling: 4370), rabbit anti-mouse p38 MAPK pAb (Sigma-Aldrich, St Louis, MO; M0800), rabbit anti-mouse phosphorylated p38 MAPK (Thr180/Tyr182) mAb (Cell Signaling: 9215), rabbit anti-mouse RPS14 pAb (Bethyl Laboratories, Montgomery, TX; A304-031A-T), rabbit anti-mouse actin pAb (Sigma-Aldrich; A2066), and goat IgG pAb (R&D Systems; AB-108-C).

Alexa 488, 555, and 647 conjugated secondary Abs and were obtained from Invitrogen.

Cell culture and treatments
Primary keratinocytes were isolated from K14CreERTM/LSL-SOX2 mice as previously described (Lichti et al., 2008), and were plated in low calcium medium (8% chelated fetal calf serum, 0.05 mM Ca2+) with 1 µM 4-hydroxytamoxifen (Sigma-Aldrich) dissolved in methanol or ethanol as vehicle. AG1478 and U0126 were purchased from Calbiochem (San Diego, CA). Cells were treated with AG1478 (1 µM) and U0126 (10 µM), siGENOME SMARTpool (Dharmacon, Lafayette, CO) for mouse SOX2 small interfering RNA (M-058489-01), EGFR small interfering RNA (D-040411-01), and non-targeting control small interfering RNA (M-040411-01) were obtained and transfected using RNAiMAX (Life Technologies) at final concentration of 20 nM. Small interfering RNA experiments were performed during 24–48 hours after small interfering RNA transfection.

Proliferation assay
Mouse primary keratinocytes were seeded onto a 24-well plate with vehicle and 4-hydroxytamoxifen and incubated in a standard CO2 incubator for 24 hours. Images cell confluence were automatically acquired with nine images per well every 6 hours for 48 hours within the CO2 incubator by the IncuCyteS3 instrument software (Essen BioScience, Ann Arbor, MI).
Scratch assay
Mouse primary keratinocytes were seeded on 24-well plates coated with type I collagen. When fully confluent, cells were treated with 10 μg/ml mitomycin C (Sigma-Aldrich) for 1 hour and washed three times with phosphate buffered saline. Cell monolayers were scratched with 200 μl pipets. At 24 hours after scratching, the cells migrating into cell-free area were photographed (Zeiss Axio Vert.A1), and relative migrating area was measured using Image J software.

Immunoblot analysis
Cells and tissues were disrupted in lysis buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylmethylsulfonylfluoride). After sonication, lysates were subjected to SDS-PAGE, followed by immunoblot analysis using primary antibodies. Anti-rabbit horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used with Clarity and Clarity Max Western ECL Blotting Substrates (Bio-Rad) to image immunoblots.

RNA preparation and RNA-seq data analysis
Total RNA from skin tissue and cells was extracted using RNaseasy kit (Qiagen) according to the manufacturer’s instructions. mRNA expression profiling was performed in the National Institute of Arthritis and Musculoskeletal and Skin Diseases Genome Core Facility at the National Institutes of Health. The Illumina TruSeq RNA sample preparation kit (Illumina, San Diego, CA) was used according to the manufacturer’s protocol. Expression values (RPKM) and fold-changes were calculated and analyzed with the Partek Genomics Suite (http://www.partek.com). Analysis of variance was performed to compare the wound healing process in the vehicle and Tam-treated skin samples by comparing each condition (unwounded/wounded; day 0/day 4). Gene Ontology terms (q < 0.05, fold-change >2 and RPKM >1) were obtained with TopGene (Chen et al., 2009). Ingenuity Pathway Analysis was used for identification of diseases and functions terms. For heatmaps of gene expression, genes were filtered based on a robust expression of MAX RPKM ≥1, heatmaps depict each gene maximum and minimum log(RPKM) and were created using GENE-E (Broad Institute; https://software.broadinstitute.org/GENE-E/).

Accession numbers
Raw and analyzed RNA-Seq data have been deposited in the Gene Expression Omnibus site, accession number GSE118859.

Real-time reverse transcriptase PCR
Total RNA was isolated by RNeasy Mini Kits (Qiagen) and was subjected to reverse transcription using a SuperScript III First-Strand Synthesis System for reverse transcriptase PCR (Invitrogen) according to manufacturer’s instructions. Quantitative real-time PCR was performed with Taqman or SYBR system (Applied Biosystems, Foster City, CA) using StepOnePlus real-time PCR system (Applied Biosystems) according to the manufacturer’s instructions. Taqman primer probe sets were obtained from Applied Biosystems; Sox2-Mm00488369_s1, Gusb- Mm00446953_m1. TaqMan Fast Advanced Master Mix and PowerUP SYBR Green Master Mix was from Bio-Rad, Hercules, CA, USA. SOX2, AREG, BTC, EREG, HBEFG, and TGFA were analyzed. Primer sequences used in quantitative real-time PCR are indicated in Supplementary Table S1 online. As an internal control, levels of β-actin (ACTB) or β-glucuronidase (Gusb) were quantified in parallel with target genes. Normalization and fold changes were calculated using the comparative Ct method.

Chromatin immunoprecipitation PCR
For chromatin immunoprecipitation, skin keratinocytes were harvested as described (Lichti et al., 2008). Chromatin immunoprecipitation was performed using Myers Lab ChIP Protocol (HAIB; ENCODE), with few changes. In brief, the cells were washed twice with ice-cold phosphate buffered saline and fixed with 1% formaldehyde at room temperature for 10 minutes, followed by quenching with 125 mM glycine for 3 minutes at room temperature. The cells were then lysed using Farnham lysis buffer followed by RIPA buffer, and the isolated nuclei preparation was sonicated for 12 minutes at 4°C (75W peak power; duty% factor of 5; 1,000 cycles/burst; and average power of 3.75W) using Covaris to achieve fragments with an average length of 200–500 bp. 100 ul of sonicated product was used as input control. Sonicated chromatin was immunoprecipitated with preincubated (4°C for 6 hours) 5 μg Sox2 or control IgG conjugated with Protein G Dynabeads (Invitrogen), at 4°C for overnight. Then beads were cleaned using a series of wash buffers (low, followed by high and LiCl wash buffer), and were resuspended with a mixture of 10 mM NaHCO3 and 1% SDS, followed by reverse-crosslinking at 65°C for overnight. The chromatin was purified using QIAquick kit (Qiagen), and eluted in 35 μl elution buffer, and subjected to quantification using PicoGreen dsDNA Quantification Kit (Invitrogen). Genomic sequence was refereed to UCSC Genome Browser with mouse assembly December 2011 (GRCm38/mm10). Genomic sequence was chosen from promoter/upstream by 10,000 bases. Mouse SOX2 motif was obtained from JASPAR (http://jaspar.genreg.net) and motif binding site (P < 0.0001) were searched with The MEME Suite (5.0.1) (http://meme-suite.org/index.html) and Find Individual Motif Occurences software (Bailey et al., 2009; Grant et al., 2011). Motif binding site was determined for each gene: HBEGF (−7,038 to 7,052), AREG (−5,580 to 5,594), EREG (−6,045 to 6,059), TGFA (−9,489 to 9,503) and primers were designed including motif binding sites. Relative binding was calculated with the 2ΔΔCt method and normalized by % input method (Lacazette, 2017).

Statistical analysis
For RNA-seq data, analysis of variance was performed by Partek Genomics Suite. A q < 0.05 was considered statistically significant (a q-value is an adjusted P-value, taking into account the false discovery rate). For all other data sets, data analysis was done with GraphPad Prism, version 7. P values were calculated using the Student t test (two-sided) or by analysis of one-way analysis of variance. P < 0.05 was considered statistically significant. Error bars represent standard errors of the mean and numbers of experiments are as indicated.
REFERENCES


Supplementary Figure S1. (a) Schematic representation of the TG animal model for inducible SOX2 expression in epidermal cells. (b) Representative images of unwounded skin tissue from TG mice treated with vehicle and Tam and murine oral (buccal mucosa and hard palate) stained to show expression of SOX2 (red) and DAPI (blue). Scale bar = 50 µm. (c) Representative hematoxylin and eosin images of skin sections from biopsies taken at day 0. Right panel shows quantification of thickness of the epidermis in six random microscopic fields from Tam-treated WT mice, vehicle- and Tam-treated TG mice. n = 4 mice per groups. Scale bar = 50 µm. (d) Representative hematoxylin and eosin images of skin sections from biopsies taken at day 2 and day 4 in TG mice treated with vehicle and Tam. Black arrows indicate re-epithelialization area. Bottom panel shows quantification of re-epithelialization area in mice at day 2 and day 4. n = 4 mice per groups. Scale bar = 100 µm (day 2) and 200 µm (day 4). (e) Representative hematoxylin and eosin images of skin sections from biopsies taken at day 4 in TG mice treated with vehicle and Tam. Black dotted line indicates granulation tissue area. Right panel shows quantification of granulation area in mice at day 4. n = 5 mice per groups. Scale bar = 200 µm. Data are expressed as mean ± standard error of the mean. **P < 0.01, *P < 0.05. Tam, tamoxifen; TG, transgenic; WT, wild-type.
Supplementary Figure S2. (a) Principal component analysis using RNA-sequencing data sets presented in Figure 2a. (b) Quantification of immunoblots of KRT1, KRT10, and KRT17 expression from unwounded skin samples in TG mice treated with Veh and Tam. Actin was used as a loading control. n = 3. (c) Representative images of unwounded skin tissue from TG mice treated with Veh and Tam and esophagus (positive control) stained to show expression of keratin 13 (KRT13, red) and DAPI (blue). Scale bar = 50 μm. (d) Relative mRNA expression levels of Shh signaling associated genes throughout the wound healing process. (e) Representative images of unwounded skin tissue from TG mice treated with Veh and Tam showing expression of Shh (green) and DAPI (blue). Scale bar = 50 μm. (f) Representative images of unwounded skin tissue from TG mice treated with Veh and Tam showing expression of Ki-67 (green) and DAPI (blue). Scale bar = 50 μm. (g) IPA analysis of RNA-sequencing data from unwounded skin samples treated with Veh and Tam in TG mice: diseases and functions terms related to cellular movement. q < 0.05. IPA, Ingenuity Pathway Analysis; Tam, tamoxifen; TG, transgenic; Veh, vehicle.
Supplementary Figure S3. Relative mRNA expression levels of genes they are on the list of (a) proliferation of connective tissue cells and (b) angiogenesis terms in Ingenuity Pathway Analysis (Figure 3c). (c) Panels show quantification of the Ki-67⁺, CD31⁺, and αSMA⁺ areas in six random microscopic fields from the center to periphery of wounded area in n = 4–5 mice per groups was performed using Image J. (d, e) Relative mRNA expression levels of genes associated with collagen and fibronectin and TGF-β. (f, g) Representative pictures of skin tissue from TG mice treated with Veh and Tam in wounded stained to show expression of the (f) macrophages (CD68, green), (g) neutrophils (myeloperoxidase, red), and DAPI (blue). Scale bar = 50 μm. Right panel shows quantification of the CD68⁺ and MPO⁺ areas in six random microscopic fields from the center to periphery of wounded area. n = 4 per groups. Date are expressed as mean ± standard error of the mean. **P < 0.01, *P < 0.05. MPO, myeloperoxidase; Tam, tamoxifen; Veh, vehicle.
Supplementary Figure S4. (a) mRNA expression levels of 47 SOX2-dependent genes throughout the wound healing process. (b) Representative pictures of Alcian blue staining of skin from transgenic mice treated with Veh and Tam in wounded show detection of hyaluronan in wound area. Scale bar = 200 μm. (c) mRNA expression of AREG and HBEGF in transgenic mice treated by Veh and Tam in unwounded and wounded samples by quantitative PCR. n = 3–4. (d) mRNA expression levels of EGFR ligands throughout the wound healing process. (e) Representative images of unwounded and wounded skin tissue from transgenic mice treated with Veh and Tam stained to show expression of EGFR (red) and DAPI (blue). Scale bar = 50 μm. Data are expressed as mean ± standard error of the mean. **P < 0.01, *P < 0.05. Tam, tamoxifen; Veh, vehicle.
Supplementary Table S1. Nucleotide sequences of primers used for PCR amplification

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<th>Variable</th>
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<td></td>
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ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR.

Supplementary Figure S5. (a) Immunoblot images showing SOX2 expression in transgenic mouse keratinocytes with siCtl and siSOX2. Cells were treated by Veh or Tam for 48 hours. RPS14 was used as a loading control. n = 3. (b) Migrating transgenic mice keratinocytes transfected with siCtl and siSOX2. Cells were treated with Veh and Tam. Images were taken at 0 and 24 hours after scratch. Values were determined by relative migrating area in three microscopic fields in n = 3 per group. Data are expressed as mean ± standard error of the mean. *P < 0.05. siCtl, control small interfering RNA; siSOX2, SOX2 small interfering RNA; Tam, tamoxifen; Veh, vehicle.

Supplementary Figure S6. (a) Immunoblot images showing EGFR expression in transgenic mice keratinocytes with siCtl and siEGFR. Cells were treated by Veh or Tam. Actin was used as a loading control. n = 3. siCtl, control small interfering RNA; siEGFR, EGFR small interfering RNA; Tam, tamoxifen; Veh, vehicle.