Hedgehog Signaling in Papillary Fibroblasts Is Essential for Hair Follicle Regeneration during Wound Healing

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Patients suffering from large scars such as burn victims not only encounter aesthetic challenges but also ongoing itching or pain that substantially deteriorates their quality of life. Skin appendages such as hair follicles rarely regenerate within the healing wound. Because they are crucial for skin homeostasis and the lack thereof constitutes one of the main limitations to scarless wound healing, their regeneration represents a major objective for regenerative medicine. Fibroblasts, the main resident cell type of the skin dermis, mediate embryonic hair follicle morphogenesis and are particularly involved in wound healing because they orchestrate extracellular matrix remodeling and collagen deposition in the wound bed. Importantly, dermal fibroblasts originate from two distinct developmental lineages with unique functions that differently mediate the response to epidermal signals such as Hedgehog signaling. In this study, we show that Hedgehog signaling in the reticular fibroblast lineage promotes the initial phase of wound repair, possibly by modulating angiogenesis and fibroblast proliferation, whereas Hedgehog signaling in papillary fibroblasts is essential to induce de novo hair follicle formation within the healing wound.


INTRODUCTION

Embryonic and early fetal skin heal by regeneration, whereas postnatal wounds typically form a scar lacking skin appendages such as hair follicles (HFs) (Bullard et al., 2003; Leavitt et al., 2016). In murine skin, de novo HFs form only under particular circumstances such as in large wounds (wounding-induced hair follicle neogenesis [WHN]) (Ito et al., 2007; Lim et al., 2018; Phan et al., 2020; Rognoni et al., 2016) or upon modulation of embryonic signaling pathways, including epidermal Wnt/β-catenin activation (Lo Celso et al., 2004), dermal Wnt/β-catenin inhibition (Rognoni et al., 2016), or epidermal or dermal Hedgehog (Hh) activation (Lim et al., 2018). Intriguingly, concomitant Hh pathway activation in adjacent epithelial and stromal cells is sufficient to induce new HFs in intact hairless skin (Sun et al., 2020). Fibroblasts are pivotal for embryonic HF morphogenesis (Mok et al., 2019; Schmidt-Ullrich and Paus, 2005) and during wound closure (Eming et al., 2014) but lose their capability to induce new HFs thereafter unless embryonic programs are reactivated. Dermal fibroblasts originate from two distinct lineages with unique functions in homeostasis and disease (Driskell et al., 2013; Lichtenberger et al., 2016; Mastrogiannaki et al., 2016; Rinkevich et al., 2015). Embryonic HF development is initiated by self-organizing dermal cells of the papillary fibroblast lineage (Fpap) that form the dermal condensate (DC). DC signals instigate epidermal HF placode formation and subsequent HF maturation (Gupta et al., 2019). Fibroblasts of the DC remain at the lower pole of the mature HF as the dermal papilla (DP), which orchestrates HF cycling (Driskell et al., 2009). Fpap are indispensable for HF formation (Driskell et al., 2013), and human Fpap are able to induce HFs in bioengineered skin (Higgins et al., 2017). Conversely, fibroblasts of the reticular lineage (Fret) but not Fpap are capable of differentiating into adipocytes (Driskell et al., 2013; Festa et al., 2011; Korosec et al., 2019; Mastrogiannaki et al., 2016). Fret mediate the initial phase of wound healing by depositing and reorganizing collagens and other extracellular matrix molecules, whereas Fpap populate the wound only after re-epithelialization has occurred (Driskell et al., 2013), which is likely why healed wounds lack HFs. Both fibroblast lineages respond differently to epidermal signals. For instance, sustained epidermal Wnt/β-catenin signaling leads to the formation of ectopic HFs and changes in the underlying dermis mediated by TGFβ and Hh signaling, selectively targeting Fret or Fpap (Lichtenberger et al., 2016). Among other signaling pathways (Fu and Hsu, 2013; Rendl et al., 2008; Schmidt-Ullrich and Paus, 2005), Hh signaling is indispensable for HF morphogenesis and cycling (Chiang et al., 1999).

In this study, we show that modulation of dermal Hh signaling in distinct fibroblast lineages differently affects...
wound repair and WIHN. By genetically deleting or activating Smo (Smo), one of the main activating protagonists of the Hh pathway, either in all dermal fibroblasts or exclusively in Fpap, we show that Hh signaling in Fret promotes wound closure. In contrast, activation of the Hh pathway in Fpap is essential for HF regeneration after injury. We hereby provide, to our knowledge, previously unreported insights on fibroblast lineage-specific signaling and functionality during wound healing in the light of potential targets for regenerative medicine.

**RESULTS**

**Modulation of stromal Hh signaling affects HF density and HF cycling**

Dermal fibroblast lineages respond differently to epidermal signals under homeostatic conditions. Epidermal Shh was shown to activate downstream Hh signaling primarily in Fpap, whereas Fret were less responsive, albeit functionally dependent (Lichtenberger et al., 2016; Zhang et al., 2016). To assess the implication of the Hh pathway in fibroblasts of distinct origin, we genetically deleted one Smo allele in all fibroblasts by Dermo1Cre;Smo+/ mice (Dermo1SmoΔ/+ (Figure 1a) or in Fpap only by Blimp1Cre;Smo+/ mice (Blimp1SmoΔ/+ (Figure 1b). Of note, homozygous Smo knockout led to embryonic lethality (Lichtenberger et al., 2016). In postnatal and adult skin, Sca1 acts as a marker for Fret (Donati et al., 2014; Festa et al., 2011; Lichtenberger et al., 2016; Mastrogiannaki et al., 2016; Rognoni et al., 2016; Telerman et al., 2017). Hh target gene expression is generally higher in Fpap (Lichtenberger et al., 2016). Heterozygous Smo deletion led to significantly decreased expression of Smo and its downstream effector Gli1 in FACS-isolated Sca1-kinetic Fpap of Dermo1SmoΔ/+ and Blimp1SmoΔ/+ telogen skin (Figure 1d and e), whereas Gli2 expression was not significantly affected (Supplementary Figure S1a and b).

To achieve lineage-specific overexpression of Smo, we crossed R26-SmoM2 (Jeong et al., 2004) to the congenitally active Cre lines mentioned earlier; however, we were unable to generate viable Smo-overexpressing offspring. We therefore employed the tamoxifen-inducible PDGFRαCreER line. PDGFRαCreER;SmoM2 mice (PDGFRαSmoKI/+ and PDGFRαSmoKI/KI) (Figure 1c and Supplementary Figure S1d) show significant papdermal Hh pathway overactivation at postnatal day 3 on intraperitoneal administration of tamoxifen (Supplementary Figure S1d). In adult telogen PDGFRαSmoKI/+ skin, Smo and Gli1 expressions are increased in SCA1-negative Fpap (Figure 1f), whereas SCA1-positive Fret exhibit significant induction of Gli1 and Ptc1 (Figure 1f and Supplementary Figure S1c).

Importantly, HF density was significantly decreased in Dermo1SmoΔ/+ and Blimp1SmoΔ/+ telogen skin in comparison with that in littermate controls (Figure 1g, h, j, and k), suggesting that embryonic HF morphogenesis requires Hh signaling in Fpap. As expected, postnatal Hh overactivation did not modify HF density (Figure 1i and l) in PDGFRαSmoKI/+ telogen skin.

Papdermal Hh modulation in Dermo1SmoΔ/+ and PDGFRαSmoKI/KI leads to significantly altered HF length on postnatal day 10 (Figure 1g, i, j, and l), hinting at an altered first hair cycle. Intriguingly, this was not detected on Smo deletion in f pap (Figure 1h and k), suggesting that reticular Hh signaling influences HF cycling, possibly by affecting adipogenesis (Zhang et al., 2016). Of note, Smo modulation had no significant effect on HF matrix cell proliferation and Wnt/β-catenin signaling in postnatal day 10 HFs (data not shown).

In addition, Smo modulation did not significantly affect in vitro fibroblast proliferation, migration, and adipogenesis (Supplementary Figure S2a–f) or dermal white adipose tissue layer thickness in telogen skin (Supplementary Figure S2g).

**Hh signaling in Fret affects wound closure**

To elucidate the role of dermal Hh signaling in wound healing, full-thickness 8-mm diameter adult dorsal skin wounds were monitored for 14 days. Although there was no difference in the healing ability between Blimp1SmoΔ/+ and Blimp1SmoWT wounds (Figure 2b), the initial phase of wound closure was significantly delayed in Dermo1SmoΔ/+ mice (Figure 2a) and conversely, was significantly accelerated in PDGFRαSmoKI/+ mice in comparison with that in littermate controls (Figure 2c).

Moreover, Smo deletion in papillary or all fibroblasts led to a significant decrease of CD31+ blood vessels, whereas Smo overexpression significantly increased wound bed vascularization (Figures 2d–i). In line, Vegfa expression was significantly increased in PDGFRαSmoKI/KI wound beds, suggesting that Hh signaling in fibroblasts promotes neoangiogenesis (Figure 2i). Hh modulation had no significant effect on total cell density (Supplementary Figure S3a), epidermal proliferation (Supplementary Figure S3b), collagen content (Supplementary Figure S2c–e), and the number of α-smooth muscle actin–positive fibroblasts in the wound bed (Figure 2f and g). However, Smo overexpression led to a significant increase of proliferating cells within the granulation tissue and of PDGFRα+ fibroblasts in the wound bed at postwound day 14 (Figure 2j–n). Altogether, these data indicate an important role for stromal Hh signaling during the initial phase of wound closure, possibly by promoting vascularization and fibroblast proliferation within the healing wound.

**Inhibition of Hh signaling in Fpap blocks WIHN after injury**

To assess the implication of stromal Hh signaling in WIHN, we first assessed the number of de novo HFs in 2-mm diameter neonatal full-thickness wounds 7 days after wounding. Dermo1SmoΔ/+ and Blimp1SmoΔ/+ neonatal wounds exhibited a significant decrease of de novo HFs per section, whereas homozygous overexpression of Smo significantly increased WIHN (Figure 3a–f). In contrast to previous reports (Harn et al., 2021; Ito et al., 2007; Lim et al., 2018), we not only observed WIHN at the wound center but also at the wound periphery.

Because de novo HFs are rare in adult wounds (Rognoni et al., 2016) and because their formation is therefore less evident to objectify in histological sections, we employed a different method to evaluate WIHN in adult skin. Whole-mount 1-cm² wound beds were stained for alkaline phosphatase (AP) to detect de novo DPs (AP+ DPs) on postwound day 30. Adult Dermo1SmoΔ/+ mice harbored significantly fewer AP+ DPs in the wound bed than littermate controls.
Figure 1. Modulation of stromal Hh signaling affects HF density and hair cycling. (a–c) Breeding strategy for the generation of (a) Dermo1SmoΔ/+, (b) Blimp1SmoΔ/+ and (c) PDGFRαSmoKI/+ and PDGFRαSmoKI/KI mice. (d–f) qPCR analysis of Hh pathway effectors in FACS-sorted SCA-1-neg Fpap and SCA-1-pos Fret of adult telogen skin. Data represent FC normalized to Gapdh. (g–i) Representative H&E stainings from adult telogen or P10 skin. (j–l) Quantification of HFs per mm skin and HF length in telogen or P10 skin, respectively. (d–f, l) One-way ANOVA with Holm-Sidak’s multiple comparison test and (j–l) unpaired students t-test were performed. Bars = 100 μm. *P < 0.05; **P < 0.01; error bars indicate mean ± SD. FC, fold change; Fpap, fibroblasts of the papillary lineage; Fret, fibroblasts of the reticular lineage; HF, hair follicle; Hh, Hedgehog; neg, negative; pos, positive; P10, postnatal day 10; WT, wild-type.
Figure 2. Stromal Hh signaling affects wound closure by altering vascularization, fibroblast proliferation, and PDGFRα expression. (a–c) Wound area (mm²) over time of adult 8 mm diameter wounds (two or three independent experiments). (a) n = 5,5; (b) n = 8,3; (c) n = 11,8,9. (d–f) IHC for CD31 of PW14 adult wounds. (g–i) Quantification of CD31+ BV/mm² and of Vegfa gene expression from bulk wound RNA. (j–l) IF of PW14 adult wounds and quantification of (m) Ki-67+ and (n) PDGFRα+ cells. Dashed lines demarcate (d–f) wound border or (j–l) epidermis. (a–c) Two-way ANOVA with Tukey’s multiple comparison test, (g, h, m, n) unpaired students t-test, and (i, m, n) one-way ANOVA with Holm-Sidak’s multiple comparison test were performed. Bars = 100 μm. *P < 0.05; **P < 0.01. Error bars indicate mean ± SD. Hh, Hedgehog; IF, immunofluorescence; IHC, immunohistochemistry; ns, non-significant; PW14, postwound day 14; WT, wild-type.
Figure 3. Hh signaling in Fpap is essential for WIHN. (a–c) IF of P2 2 mm diameter wounds and (d–f) quantification of de novo HFs (white arrowheads) at PW7. (a) White dot and (b) star indicate the unclassifiable epidermal structure and HFs, respectively. (c) The red star indicates aberrant fluorescence. (g, i, k) Adult 10 mm² wounds at PW30 stained for AP⁺ DP⁺s (red arrows) and (h, j, l) quantification thereof. (a–c, g, i, k) White and red dashed lines demarcate wound bed border and area with high WIHN, respectively. (d, e, h, j) Unpaired t-tests and (f, l) one-way ANOVA with Sidak-Holms multiple comparison test were performed. Bars = 200 µm for a, b, and c or 500 µm for h, i, and k. *P < 0.05, **P < 0.01, ****P < 0.0001. Error bars represent mean ± SD. AP, alkaline phosphatase; DP, dermal papilla; Fpap, fibroblasts of the papillary lineage; HF, hair follicle; Hh, Hedgehog; IF, immunofluorescence; ns, non-significant; P2, postnatal day 2; PW30, postwound day 30; PW7, postwound day 7; WIHN, wounding-induced hair follicle neogenesis; WT, wild-type.
Hh signaling in Fpap entails DC gene expression

Next, we reanalyzed a published single-cell RNA sequencing dataset of Sma-overexpressing SM22-positive wound myofibroblasts (SM22-SmoM2) and control myofibroblasts (Lim et al., 2018) to further examine the implication of papillary Hh signaling in WHN. Importantly, classification of cells into Fpap and Fret according to their expression of reticular marker Sca-1 or papillary marker Blimp1 revealed that the majority of cells within the Blimp1-positive and Sca-1-negative cell clusters overlap in control myofibroblasts as well as in SM22-SmoM2, and therefore both indeed represent Fpap (Figure 4a). We next analyzed differentially expressed genes between Sma-overexpressing Fpap and Fret (Sca-1 negative vs. Sca-1 positive; Blimp1 positive vs. Blimp1 negative) (Figure 4b) and performed gene ontology analyses thereof. Importantly, genes associated with regulation of epithelium and stem cell proliferation; wound healing; tissue remodeling; organ growth; and development of epithelium, vasculature, and skin were enriched in Fpap (Figure 4c), thus supporting the findings elaborated earlier.

Moreover, genes associated with DC formation and HF morphogenesis such as Bmp7, Lams3, and Trps1 (Lim et al., 2018; Sennett et al., 2015) were enriched in SM22-SmoM2 Sca-1-negative and Blimp1-positive Fpap in comparison with those in SM22-SmoM2 Sca-1-positive or Blimp1-negative Fret and control myofibroblasts (Figure 5a and b). We next analyzed the expression of DC genes in Blimp1SmoΔ/+, Dermo1SmoΔ/+, PDGFRαSmoKI/+, and PDGFRαSmoKI/KI dermis and littermate control wound dermis (total tissue). Importantly, Bmp7, Lamc3, and Trps1 as well as DC-associated genes Prom1 and Dcn were downregulated in Blimp1SmoΔ/+ wound beds in comparison with those in Blimp1SmoWT (Figure 5d), whereas these genes were upregulated on Sma overexpression in PDGFRαSmoKI/+ and PDGFRαSmoKI/KI wound beds (Figure 5e), confirming the quantification of WHN. Paradoxically, Dermo1SmoΔ/+ wound beds displayed decreased expression of Trps1, Dcn, and Prom1 and concomitant upregulation of Gli1, Lamc3, and Bmp7 (Figure 5c). Nevertheless, DC genes Lamc3 and Trps1 expression significantly correlated with Gli1 expression in Dermo1SmoΔ/+ as much as in Blimp1SmoΔ/+ and control wound beds (Supplementary Figure S4a and b), indicating their dependency on Hh signaling.

Lim et al. (2018) have defined two Hh-active cell populations among SM22-SmoM2 fibroblasts that differentially express DC-associated genes. Hh-active II was described as an SM22-SmoM2 population that additionally coexpresses Alpl and Lef1. Our reanalysis revealed that SM22-SmoM2 Sca-1-negative Blimp1-positive Fpap and Alpl+Lef1+ Hh-active II cells cluster correspondingly (Supplementary Figure S5a) and share a similar DC gene expression pattern as the Sca-1-negative or Blimp1-positive (Figure 5a and b) papillary SM22-SmoM2 population that we defined with common enrichment of genes such as Lams3, Bmp7, CD24a, or Cxcr4. Hence, the Hh-active II population might in fact constitute Fpap.

Altogether, these data provide fundamental evidence for altered DC gene transcription on modulation of the Hh pathway in Fpap.

DISCUSSION

In this study, we show that modulation of Hh signaling in distinct dermal fibroblast lineages differently affects wound healing and WHN. Although epidermal Shh primarily induces target gene expression in the Fpap, Fret express Hh target genes as well, even though to a lesser extent (Lichtenberger et al., 2016). In line, Zhang et al. (2016) showed that SCA-1-positive/CD24-positive Fret act as preadipocytes and require Hh-derived Shh during adipogenesis (Zhang et al., 2016).

Pan dermal deletion or overactivation of Smo led to a significant delay (Figure 2a) or acceleration (Figure 2c) of wound closure, respectively. However, healing velocity remained unaffected on papillary Smo deletion in Blimp1SmoΔ/+ (Figure 2b), which is not surprising because Fpap increase in number at late stages of wound healing. Furthermore, it suggests that Hh signaling in Fret, which mediate extracellular matrix deposition and remodeling at early stages of wound healing (Driskell et al., 2013; Korosec et al., 2019; Lichtenberger et al., 2016; Rinkevich et al., 2015), plays a role.

Moreover, Hh signaling is pivotal for murine embryonic and adult angiogenesis (Pola et al., 2003, 2001). We observed that Smo modulation in all fibroblasts as well as in Fpap exclusively led to altered wound bed vascularization (Figure 2d–i), which might influence wound bed closure. Importantly, fibroblasts cultured from human papillary but not from reticular dermis promote the formation of vessel-like structures in vitro (Sorrell et al., 2008), supporting that Fpap might modulate angiogenesis during wounding as well. Nevertheless, although angiogenesis is initiated quickly after injury by proangiogenic factors secreted by infiltrating immune cells (DiPietro, 2016), Fpap appear in the wound bed at later stages of wound healing only (Driskell et al., 2013). Endothelial cells express Blimp1 but not Dermo1 (Driskell et al., 2013; Joost et al., 2020). It is therefore possible that downregulation of Hh signaling in Blimp1-expressing endothelial cells contributes to the observed phenotype in Blimp1SmoΔ/+, whereas in Dermo1SmoΔ/+, Hh inhibition in Fpap and Fret led to decreased angiogenesis (Figure 2d, e, g, and h).

Importantly, cell proliferation and density of PDGFRα+/ fibroblasts in wound beds after 14 days are significantly increased on Smo overexpression (Figure 2j–m). Intriguingly, also the expression of PDGFRα was strongly increased on Smo overexpression (Figure 2j). Hh signaling has been shown to activate PDGFRα expression and signaling (Sabbatino et al., 2014; Xie et al., 2001), which might indirectly aggravate fibroblast proliferation. In line, PDGFRα is involved in
Figure 4. Gene ontology analysis of top DEGs in SM22-SmoM2 papillary versus reticular fibroblasts reveals enrichment of genes associated with epithelial development, wound healing, and vasculature development. (a) UMAPs of fibroblast subsets from Lim et al. (2018) showing SM22-CTRL and SM22-SmoM2, Sca-1-pos and Sca-1-neg, Blimp1-pos and Blimp1-neg, and Sca-1-neg Blimp1-pos fibroblast populations. (b) Top DEG in Smo-overexpressing SM22-SmoM2 Sca-1-neg papillary versus Sca-1-pos reticular or Blimp1-pos papillary and Blimp1-neg reticular wound bed fibroblasts generated from scRNA transcriptomics. Reanalysis of Lim et al. (2018) (GSE112671) data set using R. (c) GO analysis of top 30 DEGs was performed with PANTHER, version 7 (Mi et al., 2019). DEG, differentially expressed gene; GO, gene ontology; neg, negative; pos, positive; scRNA, single-cell RNA; SM22-CTRL, control myofibroblast; SM22-SmoM2, Smo-overexpressing SM22-positive wound myofibroblast; UMAP, Uniform Manifold Approximation and Projection.
**Figure 5.** Hh activation during wound healing promotes DC gene expression in fibroblasts of papillary origin. (a, b) Heatmap of differential expression of DC signature genes in (a) Sca-1-neg versus Sca-1-pos and (b) Blimp1-pos versus Blimp1-neg fibroblasts generated from scRNA data set (GSE112671) published by Frech et al. Papillary Hh Signaling Promotes HF Regeneration.
dermal remodeling and promotion of angiogenesis (Horikawa et al., 2015) and might therefore promote wound closure on Smo overexpression. Although adult wounds of less than 6 mm² primarily heal by contraction, bigger wounds only contract to a certain degree before forming a significant scar. This entails the activation of regenerative programs and concomitant WIHN within the re-epithelialized wound (Ito et al., 2007; Takeo et al., 2015). Interestingly, WIHN also depends on tissue mechanics and the spatial organization of collagen fibrils (Ham et al., 2021). Modulation of developmental pathways such as epidermal or dermal Wnt signaling (Ito et al., 2007; Rognoni et al., 2016), dermal LEF1 (Phan et al., 2020), and epidermal and dermal Hh signaling (Lim et al., 2018) enhances the skin’s regenerative capacities. We show that monoallelic deletion of Smo in Fpap impairs WIHN in neonatal wounds, whereas Smo overexpression leads to a significant increase of de novo HF formation and thus a more regenerative phenotype (Figure 3). Of note, WIHN was not significantly higher in neonatal PDGFRαSmoKI/+ than in neonatal PDGFRαSmoWT, possibly because Hh signaling is already highly active in neonatal skin, and only homozygous overexpression provides the necessary overactivating stimulus (Figure 3f). WIHN was significantly decreased in neonatal but not in adult Blimp1SmoΔ/+ wounds (Figure 3e, i, and j), although the same tendencies were noticeable. That the phenotype is less evident on Blimp1Cre-mediated Smo deletion in adults is likely due to much lower frequencies in de novo HF formation within adult wounds. Importantly, by reanalyzing single-cell RNA sequencing data from Smo-overexpressing wound myofibroblasts, we found that genes associated with epithelial development and wound healing were significantly enriched in Fpap compared with those in Fret (Figure 4). Furthermore, DC-associated genes (Rendl et al., 2005; Sennett et al., 2015) were enriched in the papillary compartment of SM22-SmoM2 cells (Figures 5a and b). In line, DC-associated genes Bmp7, Lamc3, Trps1, and Prom1 were upregulated or downregulated in wound beds on Smo overexpression (Figure 5e) or Smo deletion in Fpap (Figure 5d), respectively, further emphasizing the relevance of papillary Hh signaling in WIHN.

Lim et al. (2018) have identified two Hh-active II populations among the SM22-SmoM2 cells, which differentially express DC-associated genes. Importantly, we show that their Hh-active II population shares a similar DC gene expression pattern as the SM22-SmoM2 Fpap population we defined (Figure 5a and b) and that both populations cocluster (Supplementary Figure S5a). Thus, the Hh-active II population likely constitutes Fpap.

Of note, Dermo1SmoΔ/+ wound bed gene expression analysis led to paradoxical upregulation of Gli1, Lamc3, and Bmp7 expression (Figure 5c). This might be in part due to nonmesenchymal cells lacking Smo deletion contributing to these data from bulk wound beds. In fact, Dermo1Cre is expressed in mesenchymal cells only (Lichtenberger et al., 2016; Sošič et al., 2003), whereas Blimp1Cre is expressed in Fpap (Driskell et al., 2013) and in cells of the innate (Severa et al., 2014) and adaptive (Xin et al., 2011) immune system, both of which play crucial roles during wound healing (Strbo et al., 2014). Moreover, recent reports have implicated Hh signaling in innate immune cell activation and functions (Benson et al., 2004). It is therefore possible that Hh activation in immune cells contributes to the paradoxical gene expression pattern that we observed in Dermo1SmoΔ/+ but not in Blimp1SmoΔ/+ wound beds.

Altogether, we provide fundamental evidence that Hh signaling in Fret modulates the initial phase of wound closure and that increased angiogenesis, fibroblast proliferation, and PDGFRα signaling might contribute to the observed phenotype (Figure 5f). In contrast, Hh signaling in Fpap is essential for the formation of de novo HFs within the healing wound (Figure 5f). Hence, we report a dual role of the Hh signaling pathway in dermal fibroblasts of different lineages during wound healing and accentuate that reactivating developmental programs/signals such as the Hh pathway in the wound dermis may achieve regenerative healing in mammals.

MATERIALS AND METHODS

**Mice**

All animal experiments have been carried out in accordance with protocols authorized by the Austrian Ministry of Science and Research after approval by the National Ethical Committee for Animal Experimentation. The following mouse strains were maintained on a C57Bl/6CBA background: Smo+/ (Long et al., 2001), R26-SmoM2 (Jeong et al., 2004), Dermo1Cre (Sošič et al., 2003), Blimp1Cre (Ohinata et al., 2005), PdgfraH2BeGFP (Hamilton et al., 2003), and PdgfraCreER (Rivers et al., 2008). To activate CreER, tamoxifen (Sigma-Aldrich, St. Louis, MO) was administered intraperitoneally at a dosage of 75 μg/ml at postnatal day 0 for neonatal or to mice aged 3 weeks. Male and female sex-matched neonatal (postnatal days 2–10) mice, mice aged 4 weeks, or mice aged 6–8 weeks were used for experiments at the smallest number predicted to generate statistically relevant data.

**Wounding experiments**

A total of 8 mm diameter full-thickness punch wounds and 1 cm² excisional full-thickness wounds (adult) or 2 mm diameter full-thickness punch wounds (neonatal) were inflicted on shaved dorsal skin after intraperitoneal anesthesia. Wounds were harvested after 14 (postwound day 14), 30 (postwoundday 30), or 7 (postwound day 7) days and were analyzed following published protocols (Ito et al., 2007; Rognoni et al., 2016).

**Whole-mount DP detection assay**

To assess WIHN, postwound day 30 wounds were incubated in 20 mM EDTA in PBS for 2 hours at 37 °C. Epidermis and dermis were separated with forceps under a dissection microscope (SZ51, Olympus, Tokyo, Japan), and the dermis was subjected to AP
staining as previously published (Ito et al., 2007). AP+ de novo DPs were quantified under microscopical view, and representative images were imaged with a SteREO microscope Lumar, version 12 (Carl Zeiss, Jena, Germany).

**Histology**

Skin samples were processed to generate paraffin (formalin-fixed, paraffin-embedded, 5 μm) or cryopreserved (50 μm) sections. H&E and Masson Trichrome stainings were performed following standard protocols. For immunohistochemistry and immunofluorescence stainings, 5 μm formalin-fixed, paraffin-embedded sections were stained following standard immunohistochemistry and immunofluorescence protocols using antibodies listed in Supplementary Table S1. Streptavidin-HRP complex (RE7104-CE, Leica, Wetzlar, Germany) and AEC solution (K3469, Dako, Glostrup, Denmark) were used for signal development. Immunofluorescence staining of 50 μm sections was performed as previously described (Mastrogiannaki et al., 2016; Salz and Driskell, 2017) using antibodies listed in Supplementary Table S1. Nuclei were stained with DAPI (Sigma-Aldrich), and glycerol was used for mounting slides.

**Imaging and image analysis**

Immunostained sections and cells were imaged with an LSM980 confocal microscope (Zeiss), and brightfield images were imaged with an Aperio slide scanner (Leica). Adobe Photoshop CS6 was used to conduct background corrections. Neonatal de novo HF s were quantified manually in five sections per replicate according to morphological criteria (Müller-Röver et al., 2001; Rognoni et al., 2016). H&E stainings were analyzed with Aperio ImageScope 12.1 Software (Leica), collagen content was quantified in Masson Trichrome stainings with Fiji Software (Schindelin et al., 2012) following published protocols (Chen et al., 2017). CD31+ vessels were quantified using StrataQuest (TissueGnostics, Wien, Austria). Cell quantifications in postwound day 14 wound bed immunofluorescence stainings were carried out using Halo Image Analysis Software (Indica Labs, Albuquerque, NM).

**Cell isolation and flow cytometry**

Adult telogen or neonatal dorsal skin was used to isolate PDGFRα+/EGFP+ (Derma1Cre; Smo+/ or Blimp1Cre; Smo+/+) or CD140a+ (PDGFRαCreER; SmoM2) fibroblasts. Cells were isolated following published protocols for the isolation of neonatal or adult dermal fibroblasts (Collins et al., 2011) using enzymes listed in Supplementary Table S2 for digestion. Cells were blocked with TruStain FcX (anti-mouse CD16/32) antibody (101320, 1:500, BioLegend) and antibodies listed in Supplementary Table S1. Streptavidin-HRP complex (RE7104-CE, Leica, Wetzlar, Germany) and AEC solution (K3469, Dako, Glostrup, Denmark) were used for signal development. Immunofluorescence staining of 50 μm sections was performed as previously described (Mastrogiannaki et al., 2016; Salz and Driskell, 2017) using antibodies listed in Supplementary Table S1. Nuclei were stained with DAPI (Sigma-Aldrich), and glycerol was used for mounting slides.

**RNA isolation and RT-qPCR**

FACS-sorted cells were lysed in RNeasy Mini kit lysis buffer (74106, Qiagen, Hilden, Germany) with 0.1% β-mercaptoethanol. For the extraction of dermal wound bed RNA, wounds were excised and incubated in 20 mM EDTA in PBS for 2 hours at 37 °C, and epidermis and dermis were separated. The dermis was lysed using the Qiagen Tissue Lyser LT (69980, 30 Hz for 4 minutes).

Total RNA was isolated with RNeasy Mini kit (74106, Qiagen) with on-column DNase I digestion. cDNA synthesis was performed with Revert Aid H Minus First Strand cDNA synthesis Kit (K1631, Thermo Fisher Scientific). TaqMan 2x Universal PCR Master Mix (4324018, Applied Biosystems, Waltham, MA) was used for PCR reactions, along with murine Taqman Probes purchased from Applied Biosystems: Smo, Pch1, Gli1, Gli2, Col1a1, Col3a1, Lamc3, Bmp7, Enpp2, Trps1, Dcn, Prom1, and Vegfa.

**Analysis of publicly available dataset GSE112671**

We used Seurat, version 3.1.5 (Butler et al., 2018), to generate a Seurat object from GSE112671 data tables (Lim et al., 2018). Data were normalized and scaled according to default parameters. Cells were clustered with the FindClusters function (res = 0.2) and Run-UMAP (dims = 10). Fibroblasts formed a separate cluster, characterized by the expression of PDGFRA, LOX, and DPT as previously described in Lim et al. (2018), and were extracted for second-level clustering (res = 0.2, dim = 10). Fibroblasts from SM22-Tomato (control myofibroblasts) and SM22-SmoM2/Tomato (SM22-SmoM2) mice were categorized into Sca-1-positive (expression greater 0) and Sca-1-negative (expression = 0) or Blimp1-positive (expression >0) and Blimp1-negative (expression = 0) cells. The top 30 differential gene expression was calculated using FindMarkers function (min.pct = 0.1, logfc.threshold = 0.25) and used for gene ontology term analysis. Heat map, version 1.0.12, function was used for graphical representation of mean expression (Log-normalized data from Seurat object) of genes of interest between the groups. FeaturePlot function was used to show gene expression on Uniform Manifold Approximation and Projections (slot = data).

**Gene ontology analysis**

Gene ontology analysis of the top 30 differentially expressed genes (biological process) was performed with PANTHER Tool, version 14, following published protocols. Fisher’s exact test and false discovery rate were used to test for statistical significance (Mi et al., 2019).

**Statistical analysis**

GraphPad Prism 8 software (GraphPad Software, La Jolla, CA) was used for analysis of numerical data, generation of graphs, and statistical tests, including one-way ANOVA with Sidak-Holms multiple comparison test, two-way ANOVA with Tukey’s multiple comparison test, or unpaired student’s t-test and linear regression and Pearson’s correlation coefficient. Error bars represent mean ± SD.
Data availability statement
The dataset GSE112671 related to this article can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112671, hosted at Gene Expression Omnibus Repository (Lim et al., 2018).

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CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
We gratefully acknowledge critical input from Maria Kasper and Peter Petzelbauer, excellent support from the MUV core facilities, and assistance with histological stainings from Nina Zila and Barbara Sternitzky. This research was supported by grants to BML from the Austrian Science Fund (FWF, V 523-B28) and the Federation of European Biochemical Societies (Follow-Up Research Fund) and by a Doctoral Fellowship granted to SF by the Austrian Academy of Sciences.

AUTHOR CONTRIBUTIONS
Original Draft Preparation: SF, AF, BML; Writing - Review and Editing: SF, AF, BML; Resources: BML; Supervision: BML; Visualization: SF, AF; Writing - Review and Editing: SF, AF, AK, BML; Funding Acquisition: SF, BML; Investigation: SF, BML; Adipocyte lineage cells contribute to the skin stem cell niche to drive hair cycling. Cell 2011;146:761–71.

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.2021.11.026.

REFERENCES
Lo Celso C, Prowse DM, Watt FM. Transient activation of beta-catenin signalling in adult mouse epidermis is sufficient to induce new hair follicles but continuous activation is required to maintain hair follicle tumours. Development 2004;131:1787–99.

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Supplementary Figure S1. Knockout or overexpression of Smo in stromal cells does not significantly affect Gli2 expression levels. (a–d) qPCR analysis of Hh pathway effectors in FACS-sorted SCA-1-neg papillary and SCA-1-pos reticular fibroblasts of (a–c) adult telogen skin and (d) P3 neonatal skin. Data represent FC normalized to Gapdh. Data points represent biological replicates. One-way ANOVA with Holm-Sidaks multiple comparison test was performed. *P < 0.05; **P < 0.01; ***P < 0.001. Error bars indicate mean ± SD. FC, fold change; Hh, Hedgehog; neg, negative; ns, non-significant; P3, postnatal day 3; WT, wild-type.
Supplementary Figure S2. Modulation of stromal Hh signaling has no significant impact on fibroblast proliferation, migration, or adipogenesis. (a) Representative fluorescent stainings of in vitro EdU incorporation assays of neonatal FACS-sorted SCA-1-neg Fpap and SCA-1-pos Fret and (b) statistical analysis thereof. (c) Representative images of in vitro migration assays at 24 h time point of neonatal FACS-sorted SCA-1-neg Fpap and SCA-1-pos Fret and (d) statistical analysis thereof. (e) Representative fluorescent stainings of adipogenesis assay and NileRed staining of neonatal FACS-sorted SCA-1-neg Fpap and SCA-1-pos Fret and (f) statistical analysis thereof. (g) Quantification of DWAT thickness (μm) in adult telogen skin. Bars = 50 μm. Unpaired student’s t-test and one-way ANOVA with Holm-Sidak’s multiple comparison test were performed. Error bars indicate mean ± SD. DWAT, dermal white adipose tissue; EdU, 5-ethyl-2’-deoxyuridine; Fpap, fibroblasts of the papillary lineage; Fret, fibroblasts of the reticular lineage; h, hour; Hh, Hedgehog; neg, negative; ns, non-significant; pos, positive; WT, wild-type.

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Supplementary Figure S3. Modulation of stromal Hh signaling has no significant impact on epidermal proliferation, collagen expression, or fibroblast activation within the wound bed. (a, b) Quantification of (a) total dermal cells/mm² and Ki-67⁺ epidermis cells/mm² of PW14 adult wounds. (c) Masson Trichrome stainings and IHC for CD31 of PW14 adult wound beds and (d) quantification thereof. White dashed lines demarcate wound bed border. (e) Quantification of bulk RNA from adult dermal PW14 wound beds. Data are represented as FC normalized to GAPDH. (f) Representative immunofluorescent stainings of PW14 adult wound beds and (g) quantification of nonvessel (CD31⁺) associated αSMA⁺ fibroblasts/mm². Bars = 100 μm for c or 50 μm for f. Unpaired student’s t-test and one-way ANOVA with Holm-Sidak’s multiple comparison test were performed. Error bars indicate mean ± SD. αSMA, α—smooth muscle actin; FC, fold change; Hh, Hedgehog; IHC, immunohistochemistry; ns, non-significant; PW14, postwound day 14; WT, wild-type.
Supplementary Figure S3. Continued.

Supplementary Figure S4. DC gene expression in wound beds significantly correlates with Hh signaling. (a, b) Correlation and linear regression of DC gene (a) LAMC3 and (b) TRPS1 with GLI1 expressed in adult dermal PW14 wound beds. Data represent FC normalized to GAPDH. Data points represent biological replicates pooled from three independent experiments. Pearson’s correlation coefficient and linear regression were performed. DC, dermal condensate; FC, fold change; PW14, postwound day 14; WT, wild-type.
Supplementary Figure S5. Sca-1-neg Blimp1-pos Fpap correspond to Hh-active II cluster from scRNA transcriptomics. (a) Features lots of Alpl and Lef1 define the Hh-active II population (red circle) in SM22-CTRL and SM22-SmoM2 subsets as described in Lim et al. (2018) and UMAP of fibroblast subsets highlighting Sca-1-neg Blimp11-pos Fpap population. Fpap, fibroblasts of the papillary lineage; Hh, Hedgehog; neg, negative; pos, positive; scRNA, single-cell RNA; SM22-CTRL, control myofibroblast; SM22-SmoM2, SM22-positive wound myofibroblast; UMAP, Uniform Manifold Approximation and Projection.

Supplementary Table S1. Antibodies with Dilutions

<table>
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<th>Antibody</th>
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<th>Dilution</th>
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<tr>
<td>rat anti-mouse CD31</td>
<td>Dianova/DIA310</td>
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<td>biotinylated rabbit anti-rat IgG</td>
<td>Vector/BA-4001</td>
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<tr>
<td>rat anti-mouse Vimentin</td>
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<tr>
<td>chicken anti-mouse Keratin 14</td>
<td>Biolegend/906004</td>
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<td>donkey anti-rat AF488</td>
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<td>TruStain FcX</td>
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<td>Donkey anti-chicken AF633</td>
<td>Invitrogen/A21103</td>
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<tr>
<td>CD31-Pacific Blue</td>
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<tr>
<td>Sca1-AF700</td>
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<td>1:200</td>
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Abbreviations: Cat, catalog; No, number.

Supplementary Table S2. Enzymes for Cell Isolation

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<tr>
<td>Thermolysine</td>
<td>Sigma-Aldrich/T7902</td>
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<tr>
<td>Collagenase I</td>
<td>Thermo Fisher Scientific/17100017</td>
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<td>Collagenase II</td>
<td>Gibco, 17101015()</td>
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<tr>
<td>Collagenase IV</td>
<td>Sigma-Aldrich, C5138</td>
<td>0.5 mg/ml</td>
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<tr>
<td>Hyaluronidase</td>
<td>Sigma-Aldrich, H4272</td>
<td>0.1 mg/ml</td>
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</table>

Abbreviations: Cat, catalog; No, number.