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Thymic stromal lymphopoietin controls hair growth

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SUMMARY

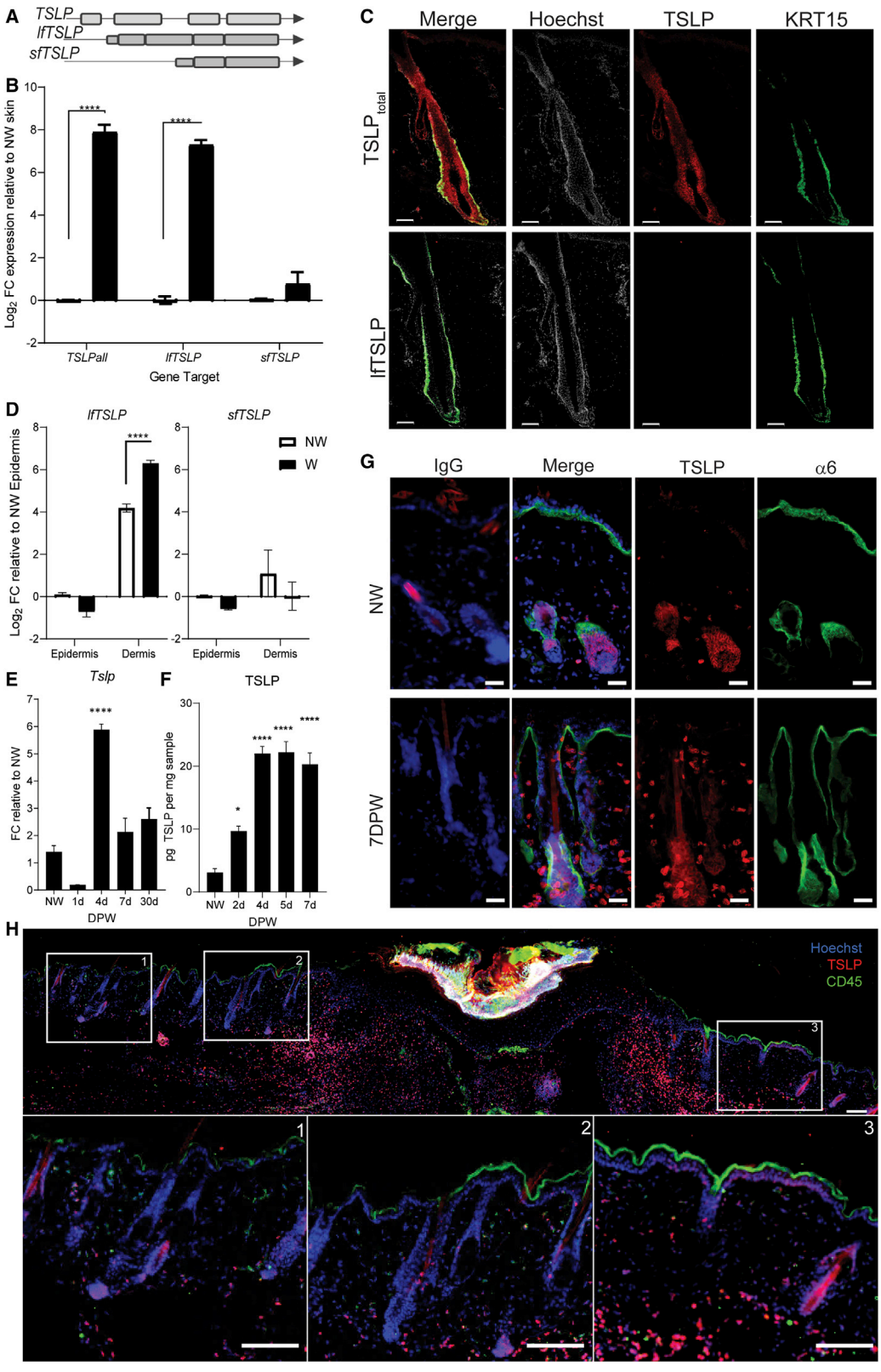
Skin tissue regeneration after injury involves the production and integration of signals by stem cells residing in hair follicles (HFSCs). Much remains unknown about how specific wound-derived factors modulate stem cell contribution to hair growth. We demonstrate that thymic stromal lymphopoietin (TSLP) is produced in response to skin injury and during the anagen phase of the hair cycle. Intradermal injection of TSLP promoted wound-induced hair growth (WIHG), whereas neutralizing TSLP receptor (TSLPR) inhibited WIHG. Using flow cytometry and fluorescent immunostaining, we found that TSLP promoted proliferation of transit-amplifying cells. *Lgr5*^{CreER}-mediated deletion of *Tslpr* in HFSCs inhibited both wound-induced and exogenous TSLP-induced hair growth. Our data highlight a novel function for TSLP in regulation of hair follicle activity during homeostasis and wound healing.

INTRODUCTION

Hair follicles (HFs) are a defining feature of mammals and function as self-renewing miniature organs. Specifically, HFs harbor stem cells that control cyclic growth of hair follicles under homeostatic conditions and contribute to re-epithelialization of the interfollicular epidermis upon wounding (Blanpain and Fuchs, 2014; Gay et al., 2013; Ge et al., 2017; Greco et al., 2009; Ito et al., 2007; Jaks et al., 2008; Joost et al., 2018; Wang et al., 2017). The HF microenvironment controls the activation state of hair follicle stem cells (HFSCs) and supports continuous renewal of hair by cycling through phases of anagen (active hair growth), catagen (regression), and telogen (resting) (Abbasi and Biernaskie, 2019; Blanpain et al., 2004; Cotsarelis et al., 1990; Handjiski et al., 1994; Stenn and Paus, 2001). Loss of HFSC function results in the loss and/or inability to grow hair because of a variety of etiologies, such as chemotherapy and radiotherapy for cancer treatment, autoimmune-mediated destruction of HFSCs in alopecia areata, and stunted anagen cycles in androgenic alopecia (AGA) (Garza et al., 2011). Symptoms of hair disorders are multidimensional and can negatively affect psychological health, psycho-social relationships, and other factors that influence quality of life (Hunt and McHale, 2005; Saed et al., 2017). Factors released after wounding activate HFSC and epidermal progenitors to support hair cycling and epidermal repair (Abbasi and Biernaskie, 2019; Abbasi et al., 2020; Blanpain and Fuchs, 2014; Nelson et al., 2013). In fact, microneedle infliction of micro-injury is a common treatment to ameliorate hair loss in AGA, though the specific mechanisms responsible for microneedle-induced hair growth are not well understood (Fertig et al., 2018; Hou et al., 2017). Untangling how HFSCs mobilize during homeostatic hair cycling and wound regeneration will provide insights for regenerative approaches for these disease states.

Advances in epithelial stem cell biology have defined the contribution of stem cells expressing leucine-rich G protein-coupled receptor 5 (LGR5) in HF cycling under homeostatic conditions and after wounding (Jaks et al., 2008; Joost et al., 2018; Snippert et al., 2010; Wang et al., 2017). Furthermore, proliferation of a progenitor-like, intermediate population of transit-amplifying cells (TACs) are essential to form the inner HF lineages of the hair follicle during hair cycling and HF regeneration after injury (Hsu et al., 2011, 2014). However, it is unclear which specific factors are responsible for HFSC activation and TAC expansion. Prior research has focused largely on signaling networks involved in HFSC activation, such as the Sonic Hedgehog (SHH) and Wnt/β-catenin signaling pathways (Choi et al., 2013; Ito et al., 2007; Lim et al., 2018). Despite these efforts, it remains unclear how wound-derived factors regulate hair growth.

Physical abrasion and exposure to allergens or microbial products result in the production of "alarmins," such as interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP) by epithelial cells (Tao et al., 2017). Production of these cytokines triggers the activation of specific type 2 immune responses to promote tissue repair (Tao et al., 2017). Although they may work in synergy, "alarmins" have apparent pleiotropic roles in modifying the function of



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T cells, B cells, mast cells, neutrophils, and eosinophils in barrier tissues (Al-Shami et al., 2005; Kim et al., 2013; Rochman and Leonard, 2008; Salimi et al., 2013; Shane and Klonowski, 2014). TSLP and TSLP receptor (TSLPR) have been heavily studied in myeloid and lymphocyte compartments related to inflammatory conditions such as atopic dermatitis (Corren and Ziegler, 2019; Leyva-Castillo et al., 2013). The role of TSLP in the hair follicle microenvironment and its effect on epithelial cells remain largely unexplored.

In this study, we identified TSLP as a potent inducer of hair growth in response to skin injury. We show that local delivery of exogenous TSLP promotes hair growth both in the presence and absence of skin injury. Using *Lgr5*^{CreER}.*Tslpr*$^{fl/fl}$ mice, we demonstrate that TSLP acts through TSLPR on LGR5^{+} keratinocytes to promote expansion of TACs both during wound healing and normal tissue homeostasis. Furthermore, we found that TSLP increased expression of the cell cycle regulator cyclin D1 and the progenitor factor DDX6 in a TSLPR-dependent manner. Our findings delineate TSLP as a novel and locally produced cytokine that directly stimulates hair follicle cell proliferation in the skin.

RESULTS

TSLP is produced in the skin in response to injury

There are two variants of human TSLP whose expression is dictated by two putative promoter regions with different open reading frames that share a C-terminal region (Figure 1A) (Fornasa et al., 2015). Long-form TSLP (*lfTSLP*) is linked to type 2 immune responses and is highly induced in pathological conditions such as allergic diseases (Fornasa et al., 2015; Kim et al., 2013). Short-form TSLP (*sfTSLP*) is absent in mice but constitutively expressed in human epidermis (Bjerkan et al., 2015). Using quantitative reverse transcription PCR (qRT-PCR) with primers designed to discriminate between the two variants, we found that only *lfTSLP* was consistently upregulated in human skin 24 h after scratch wounding *ex vivo* (Figure 1B). To distinguish lfTSLP from sfTSLP at the protein level, we generated lfTSLP-specific polyclonal antibodies targeting the N-terminal region unique to lfTSLP. We validated the specificity of the lfTSLP antibody and confirmed upregulation of lfTSLP in atopic dermatitis lesions using immunofluorescence (Figure S1A), which is in agreement with previous findings (Imai, 2019; Islam and Luster, 2012; Kim et al., 2013; Salimi et al., 2013; Tao et al., 2017). lfTSLP was undetectable in healthy human skin, but TSLP$_{total,}$ as detected by the antibody that recognizes both variants, was abundantly expressed in the hair follicle (Figure 1C) and epidermis (Figures S1B and S1C), indicating that sfTSLP, but not lfTSLP, is expressed in the normal skin. qRT-PCR analysis of enzymatically separated epidermal and dermal parts of wounded skin revealed that lfTSLP was most significantly upregulated in the dermal part that contained dermis and hair follicles of both human (Figures 1D, S1D, and S1E) and mouse skin (Figure S2A). Interestingly, TSLP expression was largely restricted to hair follicles, sebaceous glands, and sweat glands in normal human skin, as shown by immunostaining (Figures S1B–S1E). Analysis of TSLP expression across different murine tissues revealed that TSLP expression was highest in the skin compared with other tissues, including lymphoid organs (Figures S2B and S2C).

TSLP functions via its heterodimeric receptor, composed of TSLPR (encoded by *Crlf2*) and IL7Ra; the receptor complex is highly conserved between human and mouse (Al-Shami et al., 2005; Fornasa et al., 2015; Leyva-Castillo et al., 2013; Verstraete et al., 2017; Wilson et al., 2013). Through analysis of existing transcriptomic datasets of full-thickness healing mouse wounds (Chen et al., 2010), we noted that *Tslp*, *Il7ra*, and *Crlf2* were all increased after wounding (Figure S2D). Consistently, qRT-PCR-based time course analysis revealed that *Tslp* mRNA peaked at about

Figure 1. TSLP is produced in the skin in response to injury

(A) Schematic illustration representing locus for human *TSLP*.

(B) qRT-PCR analysis of *TSLP* variants in human skin 24 h after wounding. Data presented are from 4 independent experiments using 4 different human donors in technical duplicates.

(C) Immunostaining human skin of TSLP$_{total}$ or long-form TSLP (lfTSLP; red) and KRT15 (green). Images presented are representative of 3 independent experiments using 3 different human donors. Scale bar: 100 μm.

(D) qRT-PCR analysis of *lfTSLP* and *sfTSLP* of human skin 24 h after *ex vivo* wounding. Data are from technical duplicates or triplicates of 4 experiments using 4 different human donors.

(E) qRT-PCR analysis of *Tslp* in RNA isolated from healing back skin wounds normalized to NW. Data are from 3 independent experiments, 4 pooled wounds per animal, n = 3 mice (NW, 4 DPW, 30 DPW) or n = 6 mice (1 DPW, 7 DPW) in technical triplicates.

(F) ELISA of TSLP in whole-tissue lysates of skin wounds (2 independent experiments, n = 2 or 3 mice per group).

(G) Immunostaining for TSLP (red) and ITGα6 (green) in non-wounded skin (top) or 7 DPW (bottom) skin from WT C57BL6 female mice. Scale bar: 20 μm.

(H) Immunostaining for TSLP (red) and CD45 (green) 7 DPW. All scale bars: 100 μm.

**p < 0.01, ***p < 0.001, and ****p < 0.0001. Error bars represent ±SEM. FC, fold change; NW, non-wounded; W, wounded; DPW, days post-wounding. See also Figures S1 and S2.

4 days after wounding (Figure 1E). Analysis by ELISA verified that TSLP protein level remained elevated for at least 7 days after wounding (Figure 1F). We detected TSLP in telogen and early anagen hair follicles of non-wounded skin and in hair follicles of skin 7 days post-wounding (Figures 1G and 1H). As expected, TSLP is highly expressed the dermal cells that likely included fibroblasts and immune cells, as indicated by the positive immunostaining of TSLP in both $CD45^+$ and $CD45^-$ cell populations (Figures S2E and S2F). In addition, we found that TSLP is readily detected in hair follicle bulge and hair germs (Figure 1G). The notable expression pattern of TSLP in HF keratinocytes and surrounding stromal cells coinciding with anagen entry suggests that TSLP plays an important role in anagen induction during tissue regeneration.

TSLP is expressed throughout the hair cycle and accelerates the onset of wound-induced hair growth

Stem cells in hair follicles mobilize after injury and aid regeneration of hair follicles, sebaceous glands, and the epidermis (Blanpain and Fuchs, 2014; Blanpain et al., 2004; Ge et al., 2017; Hsu et al., 2014; Joost et al., 2018). In particular, skin injury triggers activation of stem cells in surrounding telogen hair follicles to enter into hair cycling, a phenomenon understood as wound-induced hair growth (WIHG) that begins 7 days post-wounding (Abbasi and Biernaskie, 2019; Chen et al., 2015; Ito et al., 2005; Rahmani et al., 2018). As wound-induced TSLP upregulation preceded WIHG initiation and persisted locally in the skin, we hypothesized that TSLP has an important role in hair cycle activation. We first profiled TSLP expression throughout the hair cycle following depilation-induced entry of anagen phase. We monitored hair cycling on the basis of skin pigmentation as a defined temporal criterion (Chase, 1954; Müller-Röver et al., 2001; Stenn and Paus, 2001) (Figure S3A). qRT-PCR and ELISA revealed that TSLP was upregulated throughout the hair cycle, reaching peak expression during mid-anagen, when hair follicles extend deeper into the tissue, and then downregulated to lowest expression during catagen (Figures 2A and 2B). TSLP immunostaining was most prominent in cells of the outer root sheath and in the hair follicle bulge during mid-anagen (Figure 2C). In contrast, *Crlf2* and *Il7ra* expressions did not exhibit significant changes throughout the hair cycle (Figures 2A andS3B). To further determine *Tslp* transcriptional changes in wound-induced hair cycles, we wounded mouse skin in synchronized telogen and anagen phases and collected tissues 5 days after wounding. As expected, *Tslp* expression increased in response to injury and peaked in anagen skin; however, wounding anagen skin did not exhibit added effects to the already elevated *Tslp* expression (Figure S3C). These data suggest that TSLP functions locally in the hair follicle microenvironments to promote tissue regeneration.

The dynamic pattern of TSLP expression in hair cycling and response to skin injury led us to reason that TSLP may have a functional role in WIHG onset. We predicted that TSLP accelerates hair follicle cell proliferation, leading to expedited hair growth. To test this idea, we administered recombinant TSLP directly to the wound bed of small (4 mm diameter) and large (12 mm diameter) excisional punch wounds at the time of wounding. We found that wounds treated with TSLP consistently showed accelerated WIHG, as measured by the area of skin that entered anagen (Figures 2D, 2E, and S3D–S3F). Next, we interrupted TSLPR signaling using neutralizing antibodies (nAbs) administered directly to the wound bed immediately after wounding and again 4 days after wounding. TSLPR nAb-treated mice showed reduced hair growth compared with the IgG control (Figures S3G and S3H). Together, these data indicate that exogenous TSLP is sufficient and required for driving WIHG in large and small full-thickness wounds.

We next examined tissue sections from 13-day-old wound beds treated with 100 ng TSLP or 0.01% BSA in PBS to determine how TSLP altered behavior of hair follicle and other skin cells. TSLP-treated tissue showed morphological changes indicative of anagen entry 13 days after wounding and increased expression of the cell proliferation marker Ki67 compared with that of the control group (Figures 2F–2H). Similarly, TSLP treatment resulted in increased hair follicle length, epidermal thickness, and diameter of the hair follicle bulge (Figures 2H and S3I). Thus, TSLP delivery to wounds increased cellular proliferation and accelerated hair cycle entry into anagen and growth.

TSLP expands the $CD34^+ITG\alpha6^{lo}$ transit-amplifying cell population

Next, we sought to determine whether TSLP is sufficient to drive hair cycling in the absence of injury. Mice were given subcutaneous (s.c.) injections of recombinant TSLP or vehicle control at second telogen (Figure 3A). TSLP treatment alone was sufficient to drive hair cycling in wild-type (WT) mice (Figures 3B and 3C); interestingly, s.c. TSLP treatment resulted in hair growth temporally consistent with normal wound-induced hair growth. These findings lead us to postulate that hair follicle stem cells were directly responsive to local TSLP. To this end, we treated mice with TSLP and, 7 days later, pulsed animals with 5-ethynyl-2′-eoxyuridine (EdU) for 2 h to label proliferating cells. We then collected tissues and analyzed the frequency of cells expressing stem cell markers of the hair germ (LGR5), hair bulge (CD34), and EdU incorporation (Figure 3D). Fluorescence-activated cell sorting (FACS) analysis confirmed that TSLP treatment resulted in the expansion of $LGR5^+$ HFSCs, $CD34^+$ progenitors, and total EdU^+ cells

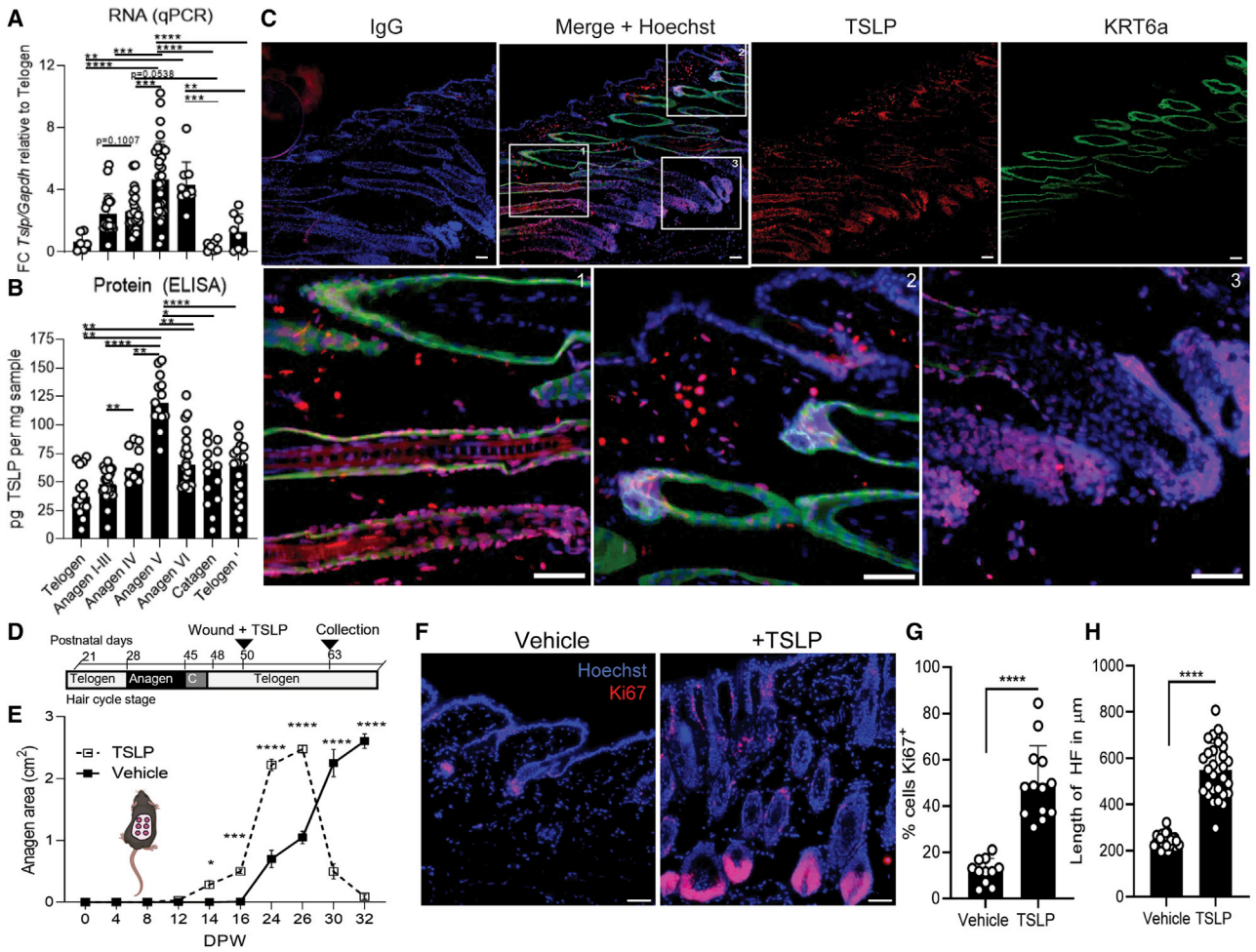

Figure 2. TSLP is expressed throughout the hair cycle and accelerates onset of wound-induced hair growth

(A) *Tslp* expression by qRT-PCR. Data represent 10–31 mice per group from 4 independent experiments. "Telogen" denotes telogen following a complete hair cycle.

(B) TSLP concentration in skin tissue by ELISA. Data represent 9–24 mice per group from 4 independent experiments.

(C) Immunostaining of skin samples collected from mice in anagen 8 days after depilation. Red, TSLP; green, KRT6a; blue, nuclei. Scale bars: 100 μm.

(D) Experimental timeline indicating hair follicle stages and treatments of vehicle (0.01% BSA) or recombinant mouse TSLP (100 ng/wound) following 4 mm punch biopsy.

(E) Quantification of skin area that entered anagen; represents data from 3 experiments using n = 8 mice per group.

(F–H) Immunostaining for Ki67 (F), (G) Ki67 quantification, and (H) hair follicle length measurement from mouse skin 13 days after wound and treatment with vehicle or TSLP. Data are from 2 experiments using n = 3 mice per group; 11–30 images per group were analyzed. Scale bars: 50 μm.

Error bars represent ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. See also Figure S3.

(Figures 3E–3G and S4A–S4E). Interestingly, EdU^{+} cells were enriched in the CD34^{+}ITGα6^{lo} population (Figures 3H–3K), previously characterized as TAC cells (Hsu et al., 2014).

Two distinct CD34^{+} stem cell populations exist within the hair follicle, distinguished by expression intensity of integrin α6 (ITGα6); lower levels of ITGα6 indicate suprabasal positioning of cells derived from basal progenitors and higher levels of ITGα6 mark cells attached to the basement membrane (Barker et al., 2010; Blanpain et al., 2004). CD34^{+}ITGα6^{lo} cells are early progeny of ITGα6^{hi} basal bulge stem cells (Blanpain et al., 2004). Both CD34^{+}ITGα6^{hi} and CD34^{+}ITGα6^{lo} cells manifest self-renewal properties of stem cells: they withstand multiple passages in tissue culture *ex vivo* and can give rise to interfollicular epidermis and hair (Blanpain et al., 2004; Morris et al., 2004). By stratifying CD34^{+} cells by ITGα6 expression, we observed that

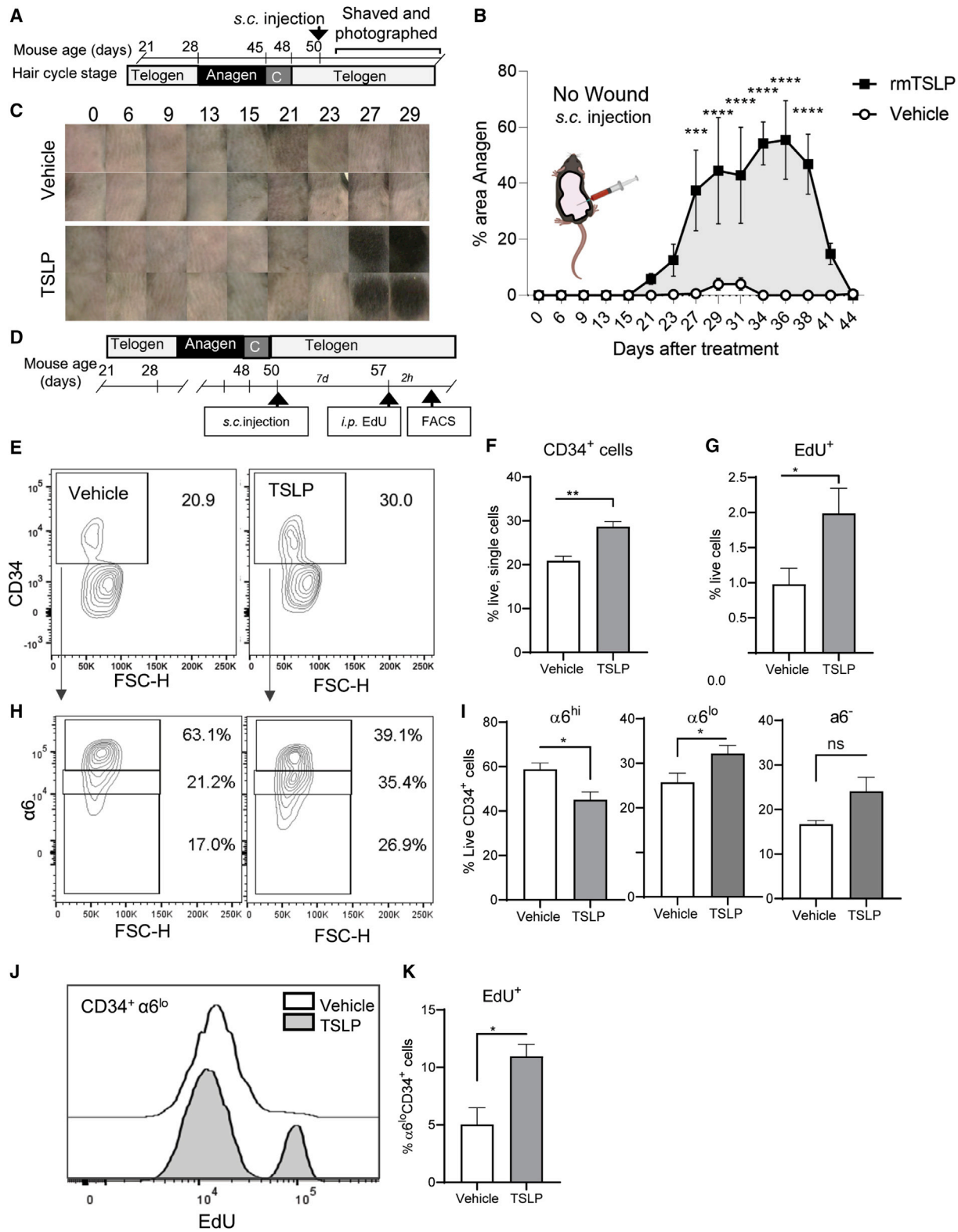

Figure 3. TSLP expands CD34$^+$ITGα6^{lo} transit amplifying cells (TACs)

(A) Experimental timeline for hair growth analysis following s.c. TSLP (250 ng).

(B) Quantification of skin area that has entered anagen. Data are from n = 3 mice per group.

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TSLP-treated skin contained a unique CD34^{+}ITGα6^{lo} cell population that was not present in the vehicle-treated skin (Figures 3H, 3I, and S4F). Further analysis of this ITGα6^{lo} cell population revealed increased EdU incorporation, indicating that the CD34^{+}ITGα6^{lo} cells are highly proliferative compared with CD34^{+}ITGα6^{hi} and CD34^{-} cells (Figures 3J, 3K, and S4E–S4L). Together, these results indicate that TSLP drives the amplification of TACs, highlighting TSLP as a core growth factor of HF niche.

Murine epithelial cells express TSLPR

We next sought to identify cell types that mediate accelerated hair growth in response to TSLP. Flow cytometry analysis of tissues revealed that hair follicles were enriched with TSLPR^{+} cells compared with whole skin (Figures 4A–4C). Immunostaining of 7 day skin wound beds revealed that cells in wound-adjacent hair follicles expressed TSLPR (Figures 4D and 4E). As TSLP is a cytokine with immunomodulatory function, we asked whether TSLPR expression changes occur in immune cells and keratinocytes during wound healing. We used flow cytometry to quantify TSLPR expression in CD45^{+} immune and CD45^{-} non-immune cell compartments. Unexpectedly, we found that TSLPR expression was greatest on CD45^{-} cells in hair follicles of non-wounded tissue and that the CD45^{-}TSLPR^{+} cell population expanded during wound healing (Figures 4F and S5). We then performed immunostaining to determine the spatial orientation of TSLPR^{+} cells around the wound bed and in non-wounded telogen skin. TSLPR expression was localized largely to the outer root sheath of the hair follicle in both non-wounded and wounded skin, though wounded skin also exhibited strong TSLPR staining in the leading edge of the epidermis in comparison with non-wounded epidermis (Figures 4G and 4H). Notably, LGR5^{+} progenitors in the hair follicle contribute to the leading wound edge during tissue regeneration (Joost et al., 2018). The strong TSLPR signal on epithelial cells in and around the hair follicle bulge area suggests a role for TSLPR in hair follicle responses to injury.

TSLPR expression in LGR5^{+} HFSC is essential for WHIG

Given the positive expression of TSLP and TSLPR in epidermal stem cell compartments, we hypothesized that TSLP acts directly on HFSC to expand the TAC compartment. To test this hypothesis, we crossed *Lgr5^{CreER}* mice with *Tslpr$^{fl/fl}$* mice. Epithelial cell-targeted ablation of *Tslpr* was achieved by 4 consecutive daily topical treatments of 4-hydroxytamoxifen (4OHT) and confirmed by flow cytometry, which showed significant knockdown of TSLPR expression in LGR5^{+} cells (Figures 5A–5E and S6A), but not in CD45^{+} hematopoietic cells (Figures 5F and 5G). We did not detect a difference in the frequency of LGR5^{+} cells in 4OHT-induced mutant mouse skin compared with littermate controls, indicating that TSLPR loss did not deplete LGR5^{+} stem cell population. To address whether LGR5^{+} cells require TSLPR for WIHG, we treated *Lgr5^{CreER}.Tslpr$^{fl/fl}$* mice and their littermate controls with topical 4OHT to induce *Tslpr* ablation prior to wounding at second telogen (Figure 5H, top). As expected, control mice showed hair growth by day 23; in sharp contrast, *Lgr5^{CreER}.Tslpr$^{fl/fl}$* mice did not exhibit signs of WIHG for at least 32 days after wounding (Figures 5H and 5I). We next sought to address whether TSLPR was essential for accelerated WIHG in response to exogenous TSLP (Figure 5J). To do this, we treated animals with three tropical doses of 4OHT and then wounded telogen skin along with TSLP treatment (Figure 5J). As expected, littermate controls exhibited WIHG 17 days after wounding (Figures 5J and S6B–S6D). In contrast, mutant skin wounds did not show WIHG until 23 days after TSLP treatment (Figures 5J and S6B–S6D). In agreement with the hair growth phenotype, cyclin D1, a key cell cycle regulator (Tetsu and McCormick, 1999), was increased in control wounds or non-wounded tissues treated with TSLP; deletion of *Tslpr* in HFSC markedly diminished cyclin D1 expression in TSLP-treated wounds (Figures S6E–S6F). Together, these data underscore that TSLP acts through TSLPR in HFSCs and TACs to promote WIHG and homeostatic hair growth in the absence of wound injury.

TSLP promotes accumulation of keratinocyte progenitor factor DDX6

After wounding, HFSCs and epidermal stem cells are activated to expand cell populations and migrate to regenerate skin appendages and fill the wound gaps (Ge et al., 2017;

(C) Representative photos of mouse back skin after s.c. TSLP treatment.
(D) Experimental timeline for analysis of TSLP-driven cell proliferation.
(E) Representative flow cytometry plots of CD34^{+} cells.
(F) Quantification of CD34^{+} cells pre-gated on live, single cells.
(G) Quantification of total, live, EdU^{+} cells.
(H) IFlow cytometry plots representing ITGα6 expression pre-gated on CD34^{+} cells.
(I) Quantification of CD34^{+}ITGα6 subpopulations.
(J) Flow cytometry histogram overlay of CD34^{+}ITGα6^{lo} cells from TSLP- and vehicle-treated groups.
(K) Quantification of EdU^{+}CD34^{+}ITGα6^{lo}. Graphs represent means of 2 experiments using 6–8 mice per group ± SEM.
*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. See also Figure S4.

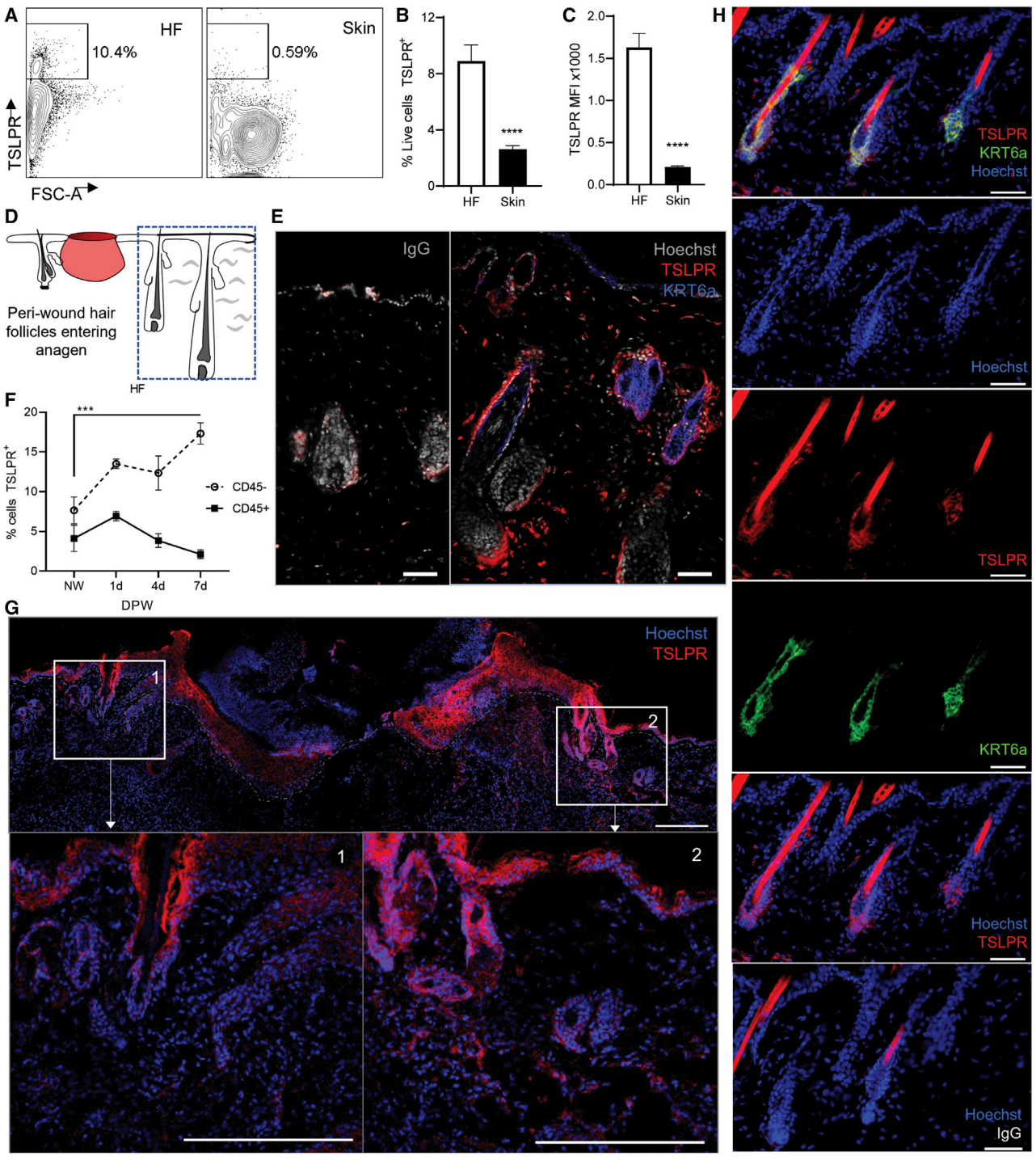

Figure 4. Murine epithelial cells express TSLPR

(A) Representative flow cytometry plots pre-gated on live, single cells.

(B) Quantification of TSLPR$^+$ relative cell abundance.

(C) Mean fluorescence intensity of surface expression of TSLPR on cells from hair follicle (HF) and skin from non-wounded tissue. Data presented represent averages ± SEM from 4 experiments using n = 7 mice (HF) and n = 20 mice (skin).

(D) Schematic showing location of tissue section portrayed in (E).

(E) Immunostaining of hair follicles neighboring 7-day-old wound bed. Red, TSLPR; blue, KRT6a. Scale bars: 50 μm.

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Ito et al., 2007; Jensen et al., 2008; Levy et al., 2007). To determine additional molecular mechanisms mediating TSLP signaling in wound beds, we used a bioinformatics approach to identify differentially expressed genes (DEGs) that were upregulated in mouse skin treated with 3 μg s.c. TSLP continuously for 7 days using osmotic pumps (Christmann et al., 2013). We cross-referenced those genes with upregulated genes from small biopsy-induced normal healing wounds inflicted in mouse (Chen et al., 2010) or human (Iglesias-Bartolome et al., 2018) (Figure S8A; Table S1). Among the top shared DEGs was the upregulation of *DDX6*, which encodes an RNA helicase. Notably, DDX6 maintains epidermal stem and progenitor cell identities by suppressing translation of transcripts associated with keratinocyte differentiation programs such as KLF4 (Di Stefano et al., 2019; Wang et al., 2015).

We hypothesized that lfTSLP has a direct role on DDX6 transcription in human epidermal keratinocytes. We used qRT-PCR to measure transcriptional levels of DDX6 in primary normal human epidermal keratinocytes (NHEKs) treated with the recombinant human lfTSLP and sfTSLP. We found that lfTSLP treatment significantly increased expression of DDX6 by three-fold, whereas sfTSLP did not (Figure 6A). Consistently, KLF4 and Filaggrin (FLG), both of which are differentiation markers subject to downregulation by DDX6 (Wang et al., 2015), were decreased in cells treated by lfTSLP (Figure 6B). Furthermore, we confirmed that DDX6 was detectable at the protein level on immunofluorescence staining and observed increased formation of distinct DDX6 foci in response to lfTSLP (Di Stefano et al., 2019; Ostareck et al., 2014; Wang et al., 2015) (Figures 6C and 6D). To verify that TSLP directly alters proliferation of epidermal keratinocytes, we assessed phenotypic changes of cell proliferation marker Ki67 in epidermal keratinocytes following TSLP treatment. By flow cytometry, we found that treatment with lfTSLP but not sfTSLP resulted in keratinocyte proliferation, as evidenced by the increases in abundance and mean fluorescence intensity (MFI) of Ki67 (Figures 6E–6G). These data indicate that lfTSLP plays a dominant role in preserving keratinocyte stemness and promoting keratinocyte proliferation.

DISCUSSION

Our studies demonstrate that TSLP is sufficient to initiate hair cycle activation in quiescent hair follicles and accelerate hair growth after wounding. We report that TSLP is upregulated during the anagen phase of the hair cycle and following skin injury and peaks at about 4 days after wounding. We defined a novel function for TSLP acting on LGR5$^+$ cells and/or their progeny to promote generation of new hair follicles following skin injury. *Lgr5*^{CreER}-mediated genetic deletion of TSLPR or antibody-mediated biological blockade of TSLPR prior to wounding resulted in significantly delayed hair growth. We showed that TSLP drove generation of the TAC compartment *in vivo* and promoted keratinocyte proliferation *in vitro*. To our knowledge, a mechanism of TSLP regulation of hair growth by acting directly on hair follicle keratinocytes has not been described previously.

It has been well documented that TSLP is upregulated after injury in response to microbial products, skin distress signals, or inflammatory cytokines (Allakhverdi et al., 2007). TSLP is upregulated by airway epithelial cells and dendritic cells in response to TLR3 ligands, including double-stranded RNA, which may be released locally following physical trauma (Kato et al., 2007; Tanaka et al., 2009). Interestingly, TLR3$^{-/-}$ mice lack regenerative capacity compared with WT mice, indicating that TLR3 is crucial for wound-induced hair neogenesis (Bhoopalam et al., 2020; Garza et al., 2011; Kim et al., 2016, 2019; Nelson et al., 2013, 2015; Wier and Garza, 2020). However, it remains unknown whether TSLP-TSLPR signaling in skin wounds is coupled with or downstream of TLR3-depenent regeneration.

TSLP is expressed by various immune cells, including dendritic cells, mast cells, macrophages, eosinophils, and T cells (Al-Shami et al., 2005; He et al., 2008; Kim et al., 2013; Kubo et al., 2014; Oyoshi et al., 2010; Pandey et al., 2000; Reche et al., 2001). Our immunostaining suggests that keratinocytes are not the sole source of TSLP during homeostatic hair cycle and wounding healing and that additional immune and non-immune cells in the dermis contribute to local TSLP production. Emerging evidence has delineated TSLP functions in airway epithelial cell migration (Kabata et al., 2020), T_{Reg} expansion (Leichner et al., 2017), innate lymphoid cell activation (Kim et al., 2013), and sebaceous gland activity (Choa et al., 2021). We expect that TSLP exhibits pleiotropic activity beyond the known functions in the skin and other tissues. Although we did not observe significant changes in TSLPR$^+$CD45$^+$ cell populations in response to injury, we do not exclude that TSLP modulates immune cell function in the local tissue environment to favor hair growth.

(F) Flow cytometry for TSLPR$^+$ cells after wounding. Data are from 2 experiments using n = 2–4 mice per group in technical duplicates.
(G) Immunostaining TSLPR (red) of 7-day-old wound beds. Images 1 and 2 (insets) were enlarged to show detail. Scale bars: 200 μm.
(H) TSLPR immunostaining in non-wounded mouse back skin in telogen. Scale bars: 50 μm. Green, keratin 6a (KRT6a); blue, nuclei.
See also Figure S5.

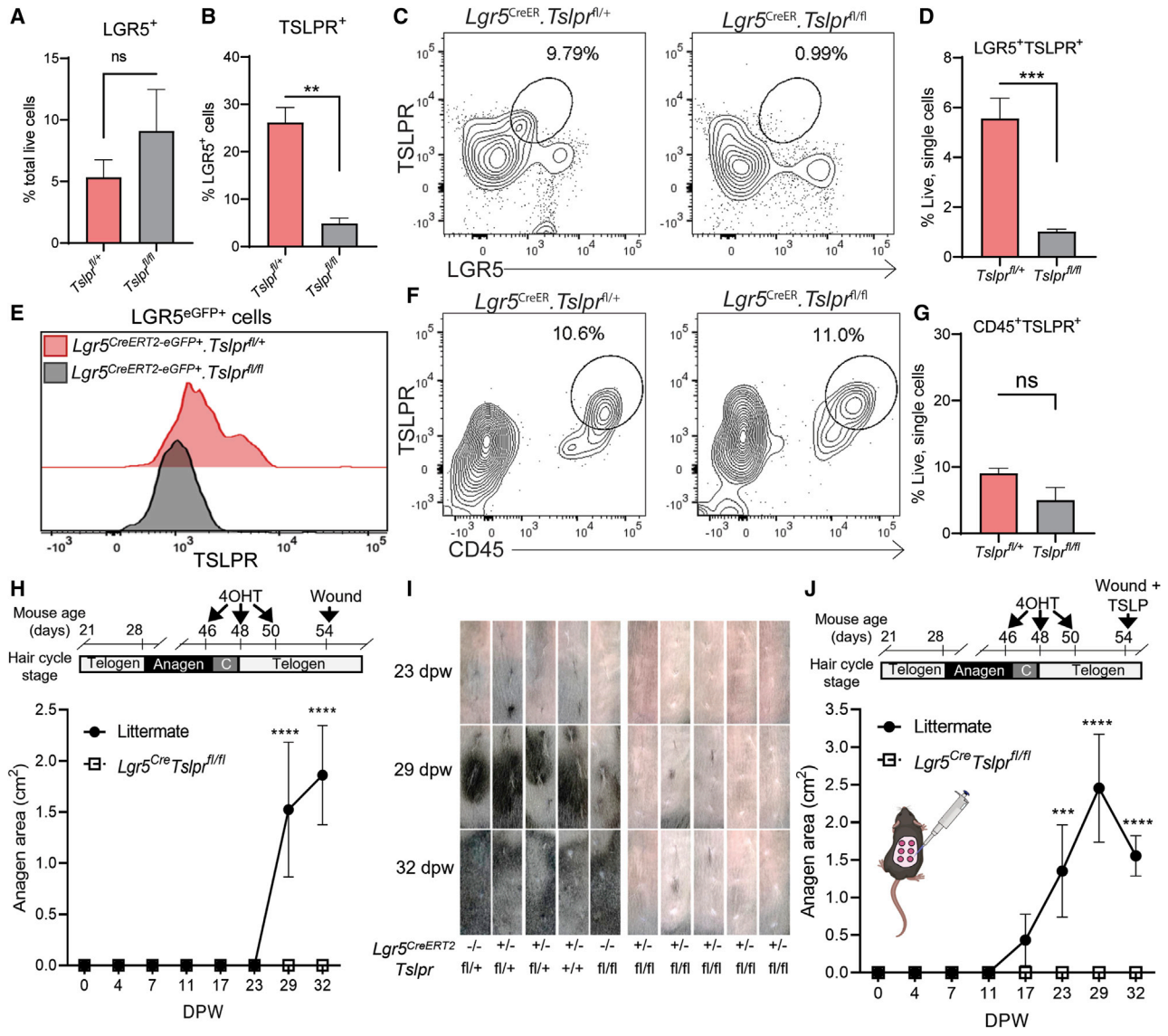

Figure 5. TSLPR expression by LGR5^{+} HFSCs is essential for WHIG

(A–G) Quantification of TSLPR^{+} cells by flow cytometry from *Lgr5^{CreER}.Tslpr$^{fl/fl}$* mice and *Lgr5^{CreER}.Tslpr$^{fl/+}$* mice treated daily for 3 consecutive days with 4-hydroxytamoxifen (4OHT). (A) LGR5-$^{eGFP+}$ cells pre-gated on total live cells. (B) TSLPR^{+} cells pre-gated on LGR5^{eGFP+} cells. (C) Flow plot and (D) quantification of TSLPR^{+}LGR5^{+} cells, pre-gated on live, single cells. (E) Histogram for TSLPR expression from total LGR5^{eGFP+} cells. (F) Flow cytometry plot and (G) quantification of TSLPR^{+}CD45^{+} cells, pre-gated on live, single cells. Data are from 2 independent experiments using 5–7 mice per group.

(H) Experimental timeline of quantification of skin that has entered anagen following wounding.

(I) Representative photos of mouse back skin after 4OHT treatment of *Lgr5^{CreER}.Tsfpr$^{fl/fl}$* and *Lgr5^{CreER}.Tslpr$^{fl/+}$* mice.

(J) Quantification of anagen skin area of mice treated with or without TSLP. Graph represents averages ± SEM of n = 4–6 mice per group across 3 experiments.

*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. See also Figure S6.

It is reported that TSLP is negatively regulated by VDR and RXR signaling pathways and loss of RXR in epidermal cells results in type 2 skin inflammation in mice (Ganti et al., 2017; Li et al., 2005). K14-driven overexpression of TSLP in embryonic epidermal cells leads to skin inflammation in adult mice (Li et al., 2005). To our surprise, one dose of 100 or 250 ng TSLP into wounded or naive telogen skin was associated with neither inflammatory skin erosions nor delayed wound healing. Animals treated with this amount of TSLP did not exhibit

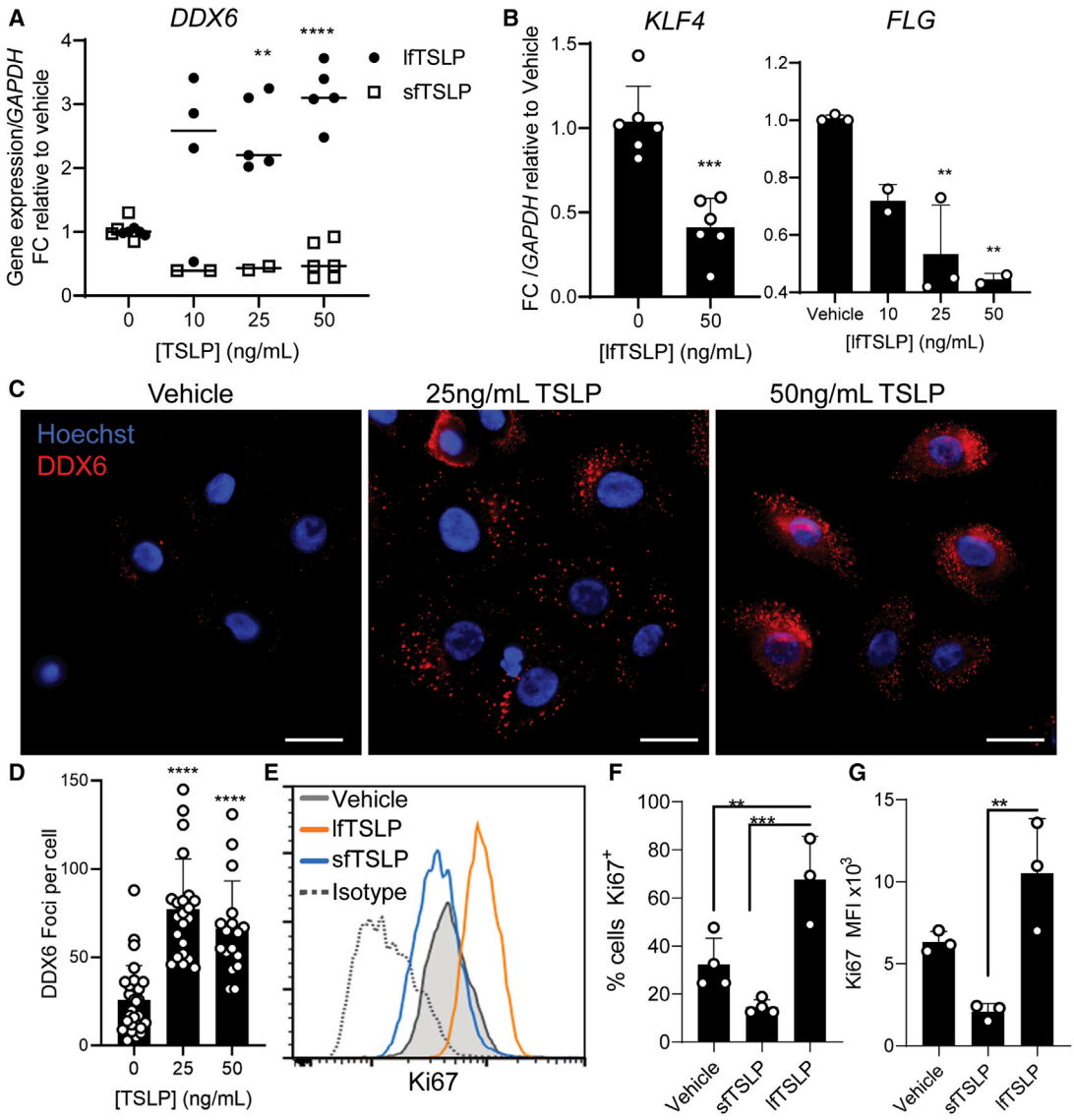

Figure 6. TSLP promotes accumulation of progenitor factor DDX6

(A and B) qRT-PCR analysis of *DDX6*, *KLF4*, and *FLG* of RNA isolated from primary human keratinocytes treated with sfTSLP or lfTSLP for (A) 16 h or (B) 24 h.

(C) Immunostaining of DDX6 (red) in primary human keratinocytes stimulated with human TSLP (100 ng/mL) for 20 h. Scale bars: 20 μm.

(D) Quantification of DDX6. Graph represents average ± SEM DDX6 intensity from n = 17–28 images/group; ****p < 0.0001.

(E) Representative histogram of Ki67 staining of primary human keratinocytes stimulated with vehicle control, 3 nM lfTSLP, or sfTSLP for 40 h. Isotype IgG used for control.

(F and G) Quantification of $Ki67^+$ (F) cell abundance and (G) Ki67 MFI in primary human keratinocytes stimulated with 3 nM lfTSLP and sfTSLP for 24 h. Data represent averages ± SEM of 3 independent experiments using 2 or 3 different human donors in technical triplicates. **p < 0.01, ***p < 0.001, and ****p < 0.0001.

See also Table S1.

changes in scratching behavior compared with vehicle-treated animals (data not shown). In contrast to our findings, s.c. injection of TSLP (2.5 μg, 10 times higher than the dose we used) into the cheek induced robust scratching behavior (Wilson et al., 2013). Thus, the observed phenotypic differences between our study and the earlier reports are likely due to differences in dose, timing, and location of TSLP delivery. One may predict that a short period of exposure of low-dose TSLP is beneficial under specific circumstances, such as hair growth, whereas

prolonged exposure of high-level TSLP induces skin and systemic atopic-like symptoms.

Recent studies have defined essential cues for hair cycle regulation that require heterologous cell populations and intricate crosstalk between stem cells and their progeny (Blanpain and Fuchs, 2014; Hsu and Fuchs, 2012; Hsu et al., 2011; Joost et al., 2018; Yang et al., 2017). TACs arise from the hair germ stem cells in response to cues from the underlying dermal papilla, which then confer signals to the bulge stem cells to complete hair growth (Greco et al., 2009; Hsu et al., 2011, 2014; Lien et al., 2011; Rompolas et al., 2013; Yang et al., 2017). We found that TSLP treatment drove the expansion of $CD34^{+}ITG\alpha6^{lo}$ cells, and this was correlated with keratinocyte TSLPR-dependent upregulation of cyclin D1. Additional studies will be required to dissect whether TSLP initiates activation of stem cells in the hair germ to initiate TAC expansion.

$CD34^{+}ITG\alpha6^{lo}$ and $CD34^{+}ITG\alpha6^{hi}$ cells are defined to retain multipotent function with capacity to regenerate epidermis, hair follicle, and sebaceous glands in mice (Blanpain et al., 2004; Morris et al., 2004). Interestingly, $CD34^{+}ITG\alpha6^{hi}CD200^{+}$ cells are lost in AGA patients, whereas $K15^{hi}ITG\alpha6^{hi}$ HFSCs are retained in the hair bulge, but they lack growth cues in AGA and are therefore unable to generate hair (Garza et al., 2011). TSLP was also found markedly reduced in patients with alopecia areata, and treatment with diphenylcyclopropenone elevated TSLP in patients that showed signs of hair growth (Gong et al., 2021). Microneedle treatments induce micro-injuries to activate hair cycling and constitute a common treatment of non-scarring alopecia types, achieving an impressive efficacy of more than 80% (Fertig et al., 2018; Hou et al., 2017). Our study provides functional insights for how wound-derived factors might drive hair growth. Additional studies are necessary to determine whether the findings obtained from animal model studies phenocopy human conditions and whether TSLP-TSLPR signaling can override signals inhibiting proliferation of HFSC in alopecia. Lasty, it will be interesting to see whether TSLP expression levels determine the probability of a patient's response to microneedle treatment.

In summary, this study demonstrates that TSLP promotes proliferation of HFSCs, driving wound-induced hair growth in mice. Further work is necessary to define the precise keratinocyte-intrinsic molecular mechanisms mediating TSLP/TSLPR signaling in HFSC. Additionally, it remains to be explored if and how TSLP modulates the immune microenvironment that may indirectly affect hair phenotypes. By identifying and dissecting the specific roles of TSLP in HFSCs and healing skin, our findings reveal a novel mechanism of hair growth and regeneration.

EXPERIMENTAL PROCEDURES

For further details, see supplemental experimental procedures.

Animal studies

C57BL6/J and $Lgr5^{CreER}$ (Jackson Laboratory, Bar Harbor, ME) and $Tslpr^{fl/fl}$ mice were maintained under specific pathogen-free conditions. Skin wounds (4 or 12 mm diameter) were induced on the back of anesthetized mice during 2nd telogen unless otherwise stated. Recombinant TSLP (555-TS-010; R&D Systems) (100 ng in 5 μL PBS) was delivered directly to the wound site immediately after wounding or delivered subcutaneously (250 ng in 100 μL 0.01% BSA in PBS) using a 31G needle. $Lgr5^{CreER}.Tslpr^{fl/fl}$ mice were treated with 50 μg 4-hydroxytamoxifen (H7904; Sigma-Aldrich) dissolved in 100% ethanol at postnatal day (p) 46, p48, and p50. 4OHT was delivered directly to the center back skin and wounds at p54.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.01.017.

AUTHOR CONTRIBUTIONS

J.L.S., J.Y.Z., and A.S.M. designed the studies. J.L.S. wrote the manuscript draft. J.L.S., J.Y.Z., and A.S.M. provided revisions. J.L.S. performed and designed the experiments and analyzed and interpreted the data. D.L.C. performed biocomputational data analyses. J.C.M. collected patient biopsy samples. J.Y.Z. and A.S.M. supervised the work and conceptualized and helped interpret the experimental results. S.F.Z. provided $Tslpr^{fl/fl}$ mice and insightful comments. All authors reviewed the manuscript.

CONFLICTS OF INTERESTS

A.S.M. consulted and received funds from Silab, but this funding was not directly used for this study. A.S.M. is currently employed by Janssen Pharmaceuticals. All other authors declare no competing interests.

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