Effect of Flightless I Expression on Epidermal Stem Cell Niche During Wound Repair

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Objective: Activation of epidermal stem cells (EpSCs) from their quiescent niche is an integral component of wound reepithelialization and involves Wnt/ β -catenin (β -Cat) signaling and remodeling of the actin cytoskeleton. The aim of this study was to investigate the effect of Flightless I (Flii), a cytoskeletal protein and inhibitor of wound healing, on EpSC activation during wound repair.

Approach: Genetically modified Flii mice (Flii knockdown: *Flii*^{+/-}, wild type: WT, *Flii* overexpressing: *Flii*^{Tg/Tg}) received two incisional wounds along the lateral axis of the dorsal skin. Indicators of EpSC activation (epidermal growth factor receptor 1 [EGFR1], leucine-rich repeats and immunoglobulin-like domains-1 [Lrig1], K14), Wnt/ β -Cat signaling (Lgr6, Flap2, β -Cat, and axis inhibition protein 2 [Axin2]), and cell proliferation (proliferating cell nuclear antigen [PCNA]) were assessed using immunohistochemistry. β -Cat stabilization was examined using western blotting with cell cycling and differentiation of isolated CD34⁺ITGA6^{high} EpSCs examined using real time-quantitative polymerase chain reaction after treatment with wound-conditioned media.

Results: *Flii*^{+/-} led to increased numbers of activated EpSCs expressing PCNA, elevated EGFR1, and decreased Lrig1. EpSCs in *Flii*^{+/-} hair follicle niches adjacent to the wounds also showed expression of Wnt-activation markers including increased β -Cat and Lgr6, and decreased Axin2. EpSCs (CD34⁺ITGA6^{high}) isolated from *Flii*^{+/-} unwounded skin showed elevated expression of cell-cycling genes including Δ Np63, filaggrin (*Fila*), involucrin (*Invo*), cyclin D1 (*Ccnd1*), and cell-division cycle protein-20 (*Cdc20*); and elevated Δ Np63 and *Invo* after treatment with wound-conditioned media compared with WT and *Flii*^{Tg/Tg} counterparts.

Innovation: Flii was identified as an inhibitor of EpSC activation that may explain its negative effects on wound reepithelialization.

Conclusion: Flii may inhibit EpSC activation by interrupting Wnt/ β -Cat signaling. Strategies that reduce Flii may increase activation of EpSCs and promote reepithelialization of wounds.

Keywords: wound repair, skin, stem cells, epidermis

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INTRODUCTION

HEALING OF ACUTE INJURIES requires a rapid and efficient reepithelialization process to restore skin integrity and minimize the risk of secondary infections.¹ To get a

glimpse into the mechanisms involved in this process, murine skin wound models offer a relatively quick and easily accessible approach as the skin layers and structures including stratified epidermis and stem cell

niches are similar between mice and humans.² Similar to humans, healing of murine skin requires the expansion of the neoepidermis across the wound bed, a process sustained by epidermal stem cells (EpSCs) that arise from niches found in the inter-follicular epidermis, hair follicles (HFs), and sebaceous glands.³ In response to injury, EpSCs are transiently activated to proliferate and differentiate into migratory cells from their otherwise habitually quiescent niches⁴ and play a pivotal role in the reepithelialization process.^{5–8} Activated EpSCs show upregulation of proliferating cell nuclear antigen (PCNA) and epidermal growth factor receptor 1 (EGFR1), and downregulation of the quiescence keeper leucine-rich repeats and immunoglobulin-like domains-1 (Lrig1), also an EGFR1 antagonist.^{9,10}

Wnt-signaling is also known to be a positive stimulus for EpSCs activation during cutaneous wound repair.¹¹ The expression of β -catenin (β -Cat) and its negative regulator, axis inhibition protein 2 (Axin2), are required to regulate canonical-Wnt signaling in adult stem cells.¹² Agonists of the Wnt-signaling pathway, including R-spondin leucine-rich repeat-containing G protein-coupled receptor 6 and 5 (Lgr6 and Lgr5) mark specific populations of epithelial stem cells, and Lgr6 is found to amplify canonical Wnt signaling in adult stem cells.¹³

In concert with Wnt-signaling pathways, actin-remodeling proteins have been found to modulate stem cell lineage, commitment, and activation.^{14–16} Flightless I (Flii), a distinctive actin-remodeling protein, and negative regulator of wound healing,¹⁷ can inhibit Wnt-signaling by binding to negative regulators of the Wnt signaling pathway through Disheveled (Dvl) protein interactions.¹⁸ Flii-associated protein 2 (Flap2, also known as LRRFIP2), a binding partner to Flii, also interacts with Dvl, resulting in elevated levels of cytoplasmic β -Cat and increased lymphoid enhancer-binding factor 1 (Lef1)/T cell factor-mediated transcription in the nucleus.^{19,20} As a negative regulator of wound healing, mice with decreased *Flii* expression (*Flii*^{+/-}) have improved wound repair and enhanced reepithelialization, whereas mice overexpressing *Flii* (*Flii*^{Tg/Tg}) show impaired wound healing responses.^{17,21} Flii has also been shown to be a positive regulator of tissue regeneration, with delayed or impaired regeneration of HFs, with reduced epithelial encapsulation, being observed in *Flii*^{+/-} mice.²² Moreover, digit regeneration is observed in *Flii*^{Tg/Tg} mice after severe proximal amputation of murine claw, with sustained expression of β -Cat and cyclin D1 (*Ccnd1*) in the germinal matrix, suggesting a role for Flii in the canonical Wnt-mediated pathway.²³ Here, the effect of Flii on the EpSC niche, its activa-

tion, and differentiation and the effect on Wnt-signaling was investigated using *in vitro* and *in vivo* wound models. Dissecting the effect of cytoskeletal involvement in the EpSC niche could further our understanding of the reepithelialization process.

CLINICAL PROBLEM ADDRESSED

Wound reepithelialization is required to re-establish the skins barrier after injury. The signals that activate EpSCs from their resting, quiescent state to a proliferative, migratory state that enables the reestablishment of the epidermis is largely unknown. Using a murine model of acute wound injury the fundamental role of Flii in EpSC activation during reepithelialization has been investigated. Although mouse wound models do not completely replicate the human situation, they do allow interrogation of the molecular and cellular signaling pathways that regulate these activities and provide an important first step toward the development of new approaches to improve wound healing.

MATERIALS AND METHODS

Animal studies

All experiments and maintenance of mice were conducted according to Australian Standards for Animal Care under protocols approved by the Child Youth and Women's Health Service Animal Ethics Committee (WCHN) and carried out in accordance with the Australian code of practice for the care and use of animals for scientific purpose (AE1019/10/18). All mouse strains were congenic on the BALB/c background and BALB/c mice were used as wild-type (WT) control animals. *Flii*-deficient mice were generated by switching a null allele (*Flii*^{tm1Hdc}) with an endogenous allele (*Flii*⁺) locus, with animals heterozygous for this mutation designated *Flii*^{+/-}.²⁴ Transgenic *Flii* overexpressing mice (strain name: [Tg1FLII] 2Hdc) were generated by incorporating a 17.8-kb fragment of a human cosmid clone that spans the entire *FLII* locus, with animals homozygous for the transgene in addition to the endogenous *Flii* allele designated *Flii*^{Tg/Tg}. Details regarding the generation of the transgenic mice strains were described previously, showing elevated levels of Flii protein in various tissues including skin.²⁵ Wounds collected at the time of surgery were fixed in 10% formalin overnight, followed by dehydration through ethanol and xylene, and then embedded in paraffin.

Synchronization of the hair follicle cycle

Mouse skin samples with differential HF cycle stages were obtained from previous experiments

conducted by Dr. James Waters.²² In brief, HF's were depilated in the second telogen phase of the hair cycle to induce anagen phase. Mice were killed at the time points of days 6, 12, and 19 corresponding to the various induced hair-cycle stages of anagen, catagen, and telogen phases, respectively.

Wound healing mouse model

A murine incisional wound model was used to investigate the effect of *Flii* on EpSCs. Given that murine excisional wounds heal mainly by contraction rather than reepithelialization, which is the fundamental healing process in human cutaneous repair, murine incisional wounds were used in this study.^{2,26} Two full thickness, 1 cm incisions, were created along the lateral axis of the dorsal skin on each animal of *Flii*^{+/-}, WT, and *Flii*^{Tg/Tg} (female mice 8–10 weeks old) as described previously.¹⁷ Mice were killed postwounding on days 3, 7, and 14 corresponding to the wound healing stages of inflammation, proliferation, and remodeling with stem cell activation and differentiation occurring between days 3 and 7. Power studies for the incisional mouse model showed that a sample size of 6 would give 90% power using a 5% test level and a one-tailed test. Six mice per genotype per time point were used for the experiments. Harvested dorsal skin was fixed in 10% formalin overnight, followed by dehydration through ethanol and xylene, and embedding in paraffin wax.

Immunohistochemistry

Paraffin-embedded skin samples were sectioned (4 μ m) before antigen retrieval using citrate buffer and trypsin as described previously.²² After antigen retrieval, 3% normal goat serum diluted in phosphate-buffered saline was used for blocking for 30 min. Primary antibodies including *Flii* (1:200; Santa Cruz Biotechnology), PCNA (1:200; Santa Cruz Biotechnology), CD34 (1:400; Abcam), keratin 15 (K15) (1:400; Abcam), Involucrin (Invo) (1:200; Lab Vision), keratin 1 (K1) (1:200; Abcam), keratin 14 (K14) (1:100; Abcam), EGFR1 (1:400; Abcam), Lrig1 (1:400; Abcam), Axin2 (1:200; Abcam), Lgr6 (1:200; Abcam), β -Cat (1:200; Santa Cruz Biotechnology), and Flap2 (1:400; MyBioSource) were diluted in the blocking buffer and applied. Species-specific Alexa Fluor 488, 568 or 633-conjugated secondary antibodies (1:400; Invitrogen) were diluted in phosphate-buffered saline and applied for detection. Nuclear counterstain 4,6-diamidino-2-phenylindole was applied last. Fluorescence-activated cell sorting (FACS) isolated CD34⁺ITGA6^{high} EpSCs were first fixed in 10% formalin solution overnight, and followed by blocking and antibody staining as described previously.²⁷ Stained samples were imaged, followed

by measurement of gray intensity using Olympus CellSens Dimension software.

Protein isolation and western blot

Protein was extracted from *Flii*^{+/-}, WT, and *Flii*^{Tg/Tg} mouse incisional wounds with normal unwounded tissue removed as described previously.²⁷ After brief homogenization in lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, 50 mM NaCl, 0.5% Triton X-100) containing protease inhibitor tablet (1 per 10 mL; Complete, Mini; Roche). Samples were centrifuged and supernatants collected. Five milligrams of protein was run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels at 100 V for 1 h and transferred to nitrocellulose membrane using standard Towbins buffer with 20% methanol at 100 V for 1 h. After blocking in 15% milk-blocking buffer for 10 min, primary antibodies including *Flii* (1:200; Santa Cruz Biotechnology), Axin2 (1:1,000; Abcam), and β -Cat (1:200; Santa Cruz Biotechnology) were diluted in buffer and applied to the membrane at 4°C overnight. Species-specific secondary horseradish peroxidase-conjugated antibodies were diluted in 5% milk-blocking buffer and applied to the membrane at room temperature for 1 h. Protein bands were detected using Super Signal West Femto (Pierce Biotechnology) and visualized with GeneSys analysis software (Syngene).

Cell isolation and flow cytometry

Dorsal skin was harvested from mice that were killed (age 8–10 weeks) and sterilized in iodine 10% antiseptic saline solution. Skin was cut into 1 cm wide squares and digested in serum-free Dulbecco's Modified Eagle Medium (DMEM) (Sigma)-buffered Dispase II (0.05 g/mL) solution overnight at 4°C, followed by removal of dermis the next day. The epidermal tissue was then digested in 0.25% trypsin/Hank's Balanced Salt Solution (HBSS) (Sigma) for 20 min at 37°C, followed by addition of fetal bovine serum (FBS; 1:10) and filtering through a 70 and 40 μ m nylon strainer. For flow cytometry, cell suspension was first pelleted down and resuspended in blocking buffer (2% FBS and 2% HEPES in phosphate-buffered saline) to incubate on ice for 30 min. Cells were then stained with PE-Cy5-conjugated ITGA6 (1:30; BD Pharmingen) and biotinylated-CD34 (1:50; eBioscience) primary antibodies, followed by streptavidin APC-Cy7 (1:300) secondary antibody, and subsequently stained with Live/Dead violet 405 nm (1:2,000; ThermoFisher). Stained and isolated cells were immediately analyzed and positive CD34⁺ITGA6^{high} EpSCs were collected into low calcium Epilife medium (20% chelated FBS in Epilife; ThermoFisher)

using FACS Aria III sorter and FACSDiva 8.0 analysis system (BD Bioscience).

Collection of wound-conditioned media and epidermal stem cells *in vitro* assay

Primary mouse skin fibroblasts were cultured in low-calcium Epilife medium (20% chelated FBS in Epilife; ThermoFisher) in a T75 flask until a monolayer was formed. Fibroblast-conditioned media was collected after 4 h of cell culturing in fresh media and stored for future use. In parallel cultures, confluent primary mouse skin fibroblasts were scratch-wounded using a sterile cell scraper, and the scratched surface was allowed to recover for 4 h. The media was then collected as the wound-conditioned media. Isolated CD34⁺ITGA6^{high} EpSCs from unwounded skin of *Flii*^{+/-}, WT, and *Flii*^{Tg/Tg} mice were subsequently cultured in control fibroblast-conditioned media or wound-conditioned media and allowed to incubate for 24 h at 37°C/5% CO₂. The EpSCs were then collected for real-time quantitative polymerase chain reaction (RT-qPCR) analysis.

Real-time quantitative polymerase chain reaction

Total RNA was isolated from 2×10^5 CD34⁺ITGA6^{high} EpSCs per experimental group using Ultraclean tissue and cells RNA isolation kit (MoBio Laboratories) according to the manufacturer's protocol. Total cDNA was reverse-transcribed from equal amount of RNA (600 ng) per sample using iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's protocol. Quantitative PCR was performed using SYBR Green Supermix (Bio-Rad) in CFX connect real-time PCR system and analyzed by CFX Maestro software (Bio-Rad). Reactions were performed in triplicate. Expression of CyPA and Rps16 was used to normalize samples. Primer sequences are given in Supplementary Table S1.

Data collection and statistical analysis

To ensure the EpSCs investigated in the *in vivo* study were under the influence of wounding, gray intensity values used for statistical analysis were measured only from the two most adjacent HF's on each side of an incisional wound and normalized to the values from negative controls. PCNA-positive cells were counted from two most adjacent HF's on each side of an incisional wound, and the percent of positive cells was then calculated as the number of positive PCNA cells over the total number of cells in those two specific HF's. Data were analyzed using the Student's *t*-test to compare between two groups. A value of $p < 0.05$ was considered significant.

RESULTS

Flii is dynamically expressed by EpSCs during wound repair

Quiescent EpSCs express distinctive markers including CD34, K15, and high levels of integrin $\alpha 6$ (ITGA6).^{28,29} Altering *Flii* expression did not cause a statistically significant change in the number of quiescent EpSCs at any HF cycling stages (Supplementary Fig. S1). *Flii* was predominantly expressed by quiescent EpSCs in the lower HF (Fig. 1a). CD34⁺ITGA6^{high} EpSCs subsequently isolated by FACS from WT mouse skin (Fig. 1b) were found to express both K15 and *Flii* (predominately in the cytoplasm) (Fig. 1c). *Flii* levels were significantly higher in the HF's and surrounding tissue of *Flii*^{Tg/Tg} mice compared with WT and *Flii*^{+/-} counterparts in unwounded (day 0) skin (Fig. 1d, e). In response to wounding, decreased, intermediate, and increased wound area and gape were observed in *Flii*^{+/-}, WT, and *Flii*^{Tg/Tg} skin, respectively (Supplementary Fig. S2). *Flii* was measured in the two most adjacent HF's to the wound (Fig. 1f) and was found to be increased in both *Flii*^{+/-} and WT HF's, and yet remained relatively constant in *Flii*^{Tg/Tg} at day 3 postwounding (Fig. 1d, e). By day 7, *Flii* levels returned to unwounded skin levels in *Flii*^{+/-} HF's, whereas high *Flii* expression remained in WT and *Flii*^{Tg/Tg} counterparts. By day 14 all *Flii* expression in the HF's returned to baseline levels.

Flii decreases proliferation of cells within HF's adjacent to the wounds

The effect of *Flii* on cell proliferation in HF's adjacent to the wound site was assessed by measuring the levels of PCNA and EGFR1 (Fig. 2a, b). In unwounded skin, few PCNA-positive cells were observed within HF's of *Flii*^{Tg/Tg} skin compared with *Flii*^{+/-} and WT counterparts (Fig. 2b). In response to wounding, the percentage of PCNA-positive cells increased significantly in all mouse strains (day 3 and 7) with the highest number of PCNA-positive cells being in the *Flii*^{+/-} mice (Fig. 2b). In unwounded skin, EGFR1 expression was significantly higher than WT and *Flii*^{Tg/Tg} mice. Upon wounding, EGFR1, within the HF's, decreased in the *Flii*^{+/-} and WT mice, whereas no significant change in expression was observed in *Flii*^{Tg/Tg} mice that remained relatively constant through the entire healing process (Fig. 2c). By day 14, EGFR1 levels returned to unwounded levels in all three genotypes.

Flii reduces activation of EpSCs adjacent to the wound

To address whether *Flii* expression affected the activation of quiescent EpSCs adjacent to a wound,

Figure 1. Flii is expressed by isolated EpSC and by EpSCs adjacent to the wound in murine skin. **(a)** Immunofluorescence detection of Flii in CD34⁺K15⁺ EpSCs. The epidermal-dermal junction is shown by white dash lines; e, epidermis; d, dermis. Arrowheads denote positive cells. Scale bars, 50 μ m. **(b)** FACS of CD34⁺ITGA6^{high} EpSCs from WT murine skin. **(c)** Flii and K15 expressed in single CD34⁺ITGA6^{high} EpSC (Δ) adjacent to a 3T3 fibroblast. Scale bars, 5 μ m. **(d)** Flii expression measurement in HF bulge EpSCs adjacent to the wound. $n=6$. Mean $\pm$ SEM. $**p < 0.005$. $Flii^{+/-}$, Flii knockdown; WT, wild type; $Flii^{Tg/Tg}$, Flii overexpressing. **(e)** Flii expression in HF bulge EpSCs adjacent to the wound as shown from the inset region in (f) at day 7. **(f)** H&E image denotes an example of an assessed region specific to the inset. Scale bars, 20 μ m. EpSC, epidermal stem cell; FACS, fluorescence-activated cell sorting; Flii, Flightless I; H&E, hematoxylin and eosin; HF, hair follicle; K15, keratin 15; SEM, standard error of the mean.

the activation antagonist, Lrig1 was assessed in the HF bulge EpSCs in $Flii^{+/-}$, WT, and $Flii^{Tg/Tg}$ mice (Fig. 2a, d). In unwounded skin, Lrig1 expression was significantly higher in $Flii^{Tg/Tg}$ EpSCs compared with $Flii^{+/-}$ counterparts. In WT mice, wounding decreased the expression of Lrig1, which remained decreased even up to 14 days postinjury. In contrast, high levels of Flii led to a delay in the reduction in Lrig1 expression with the lowest level occurring at day 7 (Fig. 2a, d). Low levels of Flii led to a decrease in Lrig1 expression in response to wounding and this remained significantly decreased even at 14 days postwounding (Fig. 2d). To assess the population of undifferentiated EpSCs, K14 expression was quantified in the HF bulge EpSCs. K14 was

significantly elevated in $Flii^{Tg/Tg}$ HF bulge EpSCs compared with $Flii^{+/-}$ and WT in unwounded skin (Fig. 2a, e). No significant change in K14 expression was observed in response to wounding in $Flii^{+/-}$ and WT mice. However, in $Flii^{Tg/Tg}$ mice, wounding reduced K14 expression in the HF bulge EpSCs at day 3 but returned to high levels by day 14 and was significantly elevated compared with $Flii^{+/-}$ and WT mice at both days 7 and 14 (Fig. 2a, e). In addition, Involucrin expression was significantly elevated in $Flii^{+/-}$ HF bulge EpSCs compared with WT and $Flii^{Tg/Tg}$ counterparts and K1 expression was significantly decreased in $Flii^{Tg/Tg}$ HF bulge EpSCs compared with $Flii^{+/-}$ and WT counterparts at both days 3 and 7 in response to wounding (Supplementary Fig. S3).

Figure 2. Effect of *Flii* on activation of EpSCs adjacent to the wound during healing (a) Immunofluorescence images of PCNA, Lrig1 and K14 in HF EpSCs at day 7, and EGFR1 at day 3. PCNA-positive cells shown as pseudo-stained purple, EGFR1 shown as pseudo-stained yellow, Lrig1 shown as pseudo-stained green and K14 staining shown as pseudo-stained yellow. The epidermal–dermal junction shown by white dash lines; e, epidermis; d, dermis. Scale bars, 20 μm. *Flii*^{+/-}, *Flii* knockdown; WT; *Flii*^{Tg/Tg}, *Flii* overexpressing. (b) PCNA expression quantified as percent of positive cells in HFs adjacent to the wound, (c–e) EGFR1, Lrig1, and K14 expression measured as gray intensity in HFs adjacent to the wound. *n* = 6. Mean ± SEM. **p* < 0.05. ***p* < 0.005. EGFR1, epidermal growth factor receptor 1; Lrig1, leucine-rich repeats and immunoglobulin-like domains-1; PCNA, proliferating cell nuclear antigen.

Wnt/β-Cat signaling is affected by *Flii* expression in EpSCs adjacent to the wound

The effect of altered *Flii* expression on key regulators involved in Wnt/β-Cat signaling was investigated. *Lgr6* was highly elevated in *Flii*^{Tg/Tg} HFs in unwounded skin compared with WT and *Flii*^{+/-} mice (Fig. 3a, b). This trend quickly shifted to the opposite direction upon wounding, with *Lgr6* being significantly increased in *Flii*^{+/-} mice and

decreased in *Flii*^{Tg/Tg}. Noticeably, although *Lgr6* expression peaked in *Flii*^{+/-} HFs adjacent to the wound at day 7 (Fig. 3a) and at day 14 in WT, *Lgr6* expression remained reduced in *Flii*^{Tg/Tg} HFs during the entire acute phase of wound healing, only starting to return toward baseline levels at day 14 (Fig. 3b).

Flap2 was highly expressed in *Flii*^{Tg/Tg} HF compared with *Flii*^{+/-} counterpart in unwounded

Figure 3. Effect of Flii on Wnt/β-Cat during wound healing (a) Immunofluorescence of Lgr6, Flap2, β-Cat, and Axin2 in HF of *Flii*^{+/-}, WT and *Flii*^{Tg/Tg} murine skin at day 7. Lgr6 shown as pseudo-stained yellow, Flap2 shown as pseudo-stained red, β-Cat shown as pseudo-stained yellow, and Axin2 shown as pseudo-stained red. The epidermal-dermal junction shown by white dash lines; e, epidermis; d, dermis. Scale bars, 20 μm. *Flii*^{+/-}, Flii knockdown; WT; *Flii*^{Tg/Tg}, Flii overexpressing. (b–e) Lgr6, Flap2, β-Cat, and Axin2 expressions were measured as gray intensity in HF adjacent to the wound at days 0, 3, 7, and 14 postwounding. *n*=6. Mean ± SEM. **p*<0.05. ***p*<0.005. β-Cat, β-catenin; Axin2, axis inhibition protein 2.

skin (Fig. 3c). An increase in Flap2 expression was observed at day 3 in *Flii*^{+/-} and WT HF with expression levels similar to those observed in *Flii*^{Tg/Tg} mice. Although a significant decline in Flap2 expression was observed at day 7 in *Flii*^{Tg/Tg} HF, Flap2 expression in *Flii*^{+/-} and WT was maintained to day 7 and returning to baseline at day 14 (Fig. 3a, c).

Cytoplasmic β -Cat was significantly elevated in *Flii*^{Tg/Tg} HF in unwounded skin compared with *Flii*^{+/-} and WT counterparts (Fig. 3d). This trend quickly reversed in response to wounding with β -Cat expression being significantly elevated in *Flii*^{+/-} HF adjacent to the wound (at days 3 and 7) and returning to baseline levels at day 14 (Fig. 3a, d). In contrast the opposite was observed in *Flii*^{Tg/Tg} HF with β -Cat levels decreasing in response to injury and returning to baseline at day 14 (Fig. 3d). Axin2 was significantly lower in *Flii*^{Tg/Tg} HF in unwounded skin comparatively (Fig. 3e). In response to injury, Axin2 decreased in HF in *Flii*^{+/-} and WT mice with levels starting to return to normal levels at day 14. In contrast, high Flii led to increased Axin2 expression with levels still rising at day 14 (Fig. 3a, e).

To further investigate the role of Flii in Wnt/ β -Cat signaling at a protein level, the level of β -Cat and Axin2 in *Flii*^{+/-}, WT, and *Flii*^{Tg/Tg} wounds ($n=6$) was characterized using western blotting (Fig. 4). Consistent with previous findings, the le-

vel of Flii protein was less than half in *Flii*^{+/-} wounds, compared with WT and there was a two-fold increase in *Flii*^{Tg/Tg} wounds (Fig. 4a, b). β -Cat was significantly higher in the wounds of *Flii*^{+/-} mice compared with WT and *Flii*^{Tg/Tg} counterparts (Fig. 4a, c), whereas the relative expression of Axin2 was significantly lower in *Flii*^{+/-} wounds compared with WT and *Flii*^{Tg/Tg} wounds (Fig. 4a, d).

In vitro profiling of EpSCs with differential Flii expression in response to wound-conditioned media

To investigate the effect of wound-conditioned media on EpSCs with different levels of Flii, isolated CD34⁺ITGA6^{high} EpSCs from *Flii*^{+/-}, WT, and *Flii*^{Tg/Tg} unwounded epidermis were incubated in either fibroblast-conditioned media (termed control) or wound-conditioned-media (termed treated) for 24 h (Fig. 5). mRNA was then extracted from the CD34⁺ITGA6^{high} EpSCs and RT-qPCR performed for genes required for EpSCs activation and Wnt-responsiveness. *Flii* expression was confirmed in the untreated CD34⁺ITGA6^{high} EpSCs and was significantly lower in *Flii*^{+/-} EpSCs than WT and *Flii*^{Tg/Tg} (Fig. 5c).

Before treatment with wound conditioned media, expression of the cell proliferation associated gene, Δ Np63, was significantly higher in the *Flii*^{+/-} EpSCs than WT and *Flii*^{Tg/Tg} counterparts (Fig. 5a).

Figure 4. Flii deficiency results in β -Cat stabilization in the wound. **(a)** Western blot analysis of Flii, β -Cat, Axin2, and β -tubulin protein levels in the day 7 incisional wounds. **(b)** Band densitometry analysis of relative expression of Flii protein as fold change of Flii to β -tubulin protein from each genotype normalized to the mean WT value. **(c)** Relative expression of β -Cat protein normalized to the mean WT value. **(d)** Relative expression of Axin2 protein normalized to the mean WT value. $n=6$ per group. Mean $\pm$ SEM. * $p<0.05$.

Figure 5. mRNA expression of genes involved in cell proliferation, differentiation, and Wnt/ β -Cat responsiveness in isolated EpSCs at 24 h postincubation in fibroblast-conditioned media or wound-conditioned media. **(a)** RT-qPCR results of *ΔNp63*, *Fila*, *Invo*, *Ccnd1*, and *Cdc20* in EpSCs (termed control) treated with fibroblast-conditioned media and **(b)** EpSCs (termed treated) treated with wound-conditioned media for 24 h. **(c)** RT-qPCR results of *Axin2*, *Lgr6*, and *Flii* in control EpSCs and **(d)** treated EpSCs. Values of relative mRNA expression are normalized to ribosomal protein s16 and cyclophilin A mRNAs. RNA was isolated from 4×10^5 isolated *Flii*^{+/-}, WT and *Flii*^{Tg/Tg} EpSCs. Data are reported as average + standard deviation. $n=3$; $*p<0.05$. Error bars for qPCR calculated from technical triplicates. *Ccnd1*, cyclin D1; *Fila*, filaggrin; *Cdc20*, cell-division cycle protein-20; *Invo*, involucrin; RT-qPCR, real-time quantitative polymerase chain reaction.

Consistently, two of the known Wnt-responsive cell cycle genes, *Ccnd1* and cell-division cycle protein-20 (*Cdc20*) showed upregulation in *Flii*^{+/-} EpSCs compared with WT and *Flii*^{Tg/Tg} EpSCs. Epidermal differentiation markers filaggrin (*Fila*) and *Invo* were both significantly elevated in *Flii*^{+/-} EpSCs compared with WT and *Flii*^{Tg/Tg} EpSCs (Fig. 5a). After treatment with wound-conditioned media, although the expression of *ΔNp63* and *Invo* remained consistently higher in the *Flii*^{+/-} EpSCs than WT and *Flii*^{Tg/Tg} counterparts (Fig. 5b), *Ccnd1*, *Cdc20*, and *Fila* were now reduced in *Flii*^{+/-} EpSCs to levels of expression comparable with the other genotypes (Fig. 5b). Before treatment with wound-conditioned media, *Axin2* expression was

lower in *Flii*^{Tg/Tg} EpSCs than *Flii*^{+/-} and WT counterparts (Fig. 5c). Only *Invo* expression pattern remained significantly higher in *Flii*^{+/-} EpSCs than WT and *Flii*^{Tg/Tg} counterparts (Fig. 5b). No difference was observed in *Lgr6* expression among the genotypes before treatment with wound-conditioned media; however, subsequently downregulation of *Lgr6* expression in *Flii*^{Tg/Tg} EpSCs when compared with *Flii*^{+/-} and WT counterparts was observed (Fig. 5d).

DISCUSSION

Human and murine skin share many similarities during wound repair including homeostasis,

inflammation, proliferation, and remodeling.² Compared with murine skin, human skin has a thicker epidermis and lacks *panniculus carnosus* in the subcutaneous tissue that contributes to less wound contraction during repair. The cutaneous healing process in humans largely depends on deposition of granulation tissue and successful reepithelialization.² The reepithelialization process that occurs during the proliferation phase of wound repair requires the activation of quiescent EpSCs, their proliferation, and migration over the site of injury and their differentiation into a stratified epidermis. This process is facilitated by the activation of the Wnt/ β -Cat signaling pathway that is regulated by Flii.^{4,7,30} Flii is a known cytoskeletal regulator of cell proliferation and migration and is dynamically expressed within the EpSC niche in the lower region of HFs during HF regeneration.^{17,22} Flii inhibition of Wnt-signaling may hence play a potential role during wound-induced EpSC activation.

Activation and proliferation of EpSCs was investigated using EGFR1, Lrig1, and PCNA. As a receptor for epidermal growth factor, EGFR1 activation triggers a variety of pathways that ultimately contribute to cell proliferation.³¹ In response to these cellular processes, progenitor-like cells such as EpSCs innately reduce EGFR1 expression through its degradation mechanisms to minimize potential carcinogenic development.^{32,33} In agreement with this, declining expression of EGFR1 was observed in WT EpSC niche during initial wound repair at days 3 and 7. Before wounding, EGFR1 expression in HFs was higher in *Flii*^{+/-} than in WT and *Flii*^{Tg/Tg} mice. Given that wounding leads to decreased expression of EGFR1 in *Flii*^{+/-} and WT HFs, whereas an unchanged low baseline level in *Flii*^{Tg/Tg} mice, the initially high EGFR1 level in *Flii*^{+/-} may have reduced the effect of the degradation process and this could be observed in the higher EGFR1 expression in *Flii*^{+/-} HFs at day 3 than WT and *Flii*^{Tg/Tg} mice. Taken together this higher EGFR1 expression may assist with activation of EpSC and their migration toward the wound site.

The amelioration of EGFR1 activation could also be achieved by reducing the level of its antagonist Lrig1, which is a stem cell quiescent keeper. Consistent with previous studies where Lrig1 was found to be continuously suppressed in the basal epidermal cells and appendages at the wound edge during most of the wound healing period,³⁴ our data from WT group also showed the same result. Of interest, Flii deficiency further reduced Lrig1 in the HFs during the entire acute period of wound

repair compared with *Flii*^{Tg/Tg} and WT counterparts. Because reducing Flii is known to enhance cell migration and Lrig1⁺ EpSCs are known to migrate to the wound and contribute to wound repair,^{9,17} this result potentially suggests that Flii deficiency may promote migration of Lrig1⁺ EpSCs from the niche toward the wound to facilitate wound repair.

Downregulation of the cell proliferation-associated gene *Δ Np63* in the HF has been found to decrease the potential for cell proliferation.³⁵ In this study, *Δ Np63* was found to be consistently elevated in *Flii*^{+/-} EpSCs before and after treatment with wound-conditioned media suggesting Flii may have a role in EpSC activation independent of wounding. In addition, the findings of elevated cell cycling genes *Ccnd1* and *Cdc20* expression in control *Flii*^{+/-} EpSCs indicates that *Flii* deficiency may enable EpSC niches to become more metabolically active, resulting in a quicker and earlier activation of EpSCs adjacent to a wound. Consequently, the percentage of PCNA-positive proliferative cells in the HF niche adjacent to the wound could have increased significantly because of the reduced presence of Flii in the *Flii*^{+/-} skin.

Successful stratification and differentiation of the neoepidermis is essential for regaining the protective skin barrier after wound closure.³⁶ Flii deficiency has previously been shown to increase K1 and Invo expression in mice skin during embryonic development and promote reestablishment of skin barrier.³⁷ In agreement with this, increased expression of Invo was observed in *Flii*^{+/-} HFs at days 3 and 7 postwounding. Increased expression of differentiation markers filaggrin (*Fila*) and Invo in *Flii*^{+/-} EpSCs under control conditions was found together with sustained Invo expression after treatment with wound-conditioned media. Significantly higher K14 expression was also found in *Flii*^{Tg/Tg} niche before wounding and from day 7 onward. The upregulation of this nondifferentiated keratin marker together with lower K1 expression at days 3 and 7 indicates that Flii overexpression may suppress epidermal stratification and differentiation. Together these data suggest that reducing Flii may promote the early onset of stratification of newly migrated cells across the wound bed.

Activated Wnt/ β -Cat signaling is known to be a positive stimulus during wound repair and an optimal level of the signaling is required to maintain the homeostasis of stratified epidermis by modulating epithelial TA cell differentiation.⁸ Consistent with previous findings that *Flii*^{Tg/Tg} may facilitate tissue regeneration through canonical

Wnt-signaling,²³ high levels of β -Cat were observed with low levels of Axin2 in *Flii*^{Tg/Tg} niche in unwounded skin, suggesting that the level of Flii being expressed in *Flii*^{Tg/Tg} niche has a positive impact on β -Cat and potential activation of Wnt/ β -Cat signaling during tissue homeostasis. When Flii levels were increased in response to wounding, β -Cat/Axin pattern from differential *Flii* mice was quickly reversed. The high β -Cat and low Axin2 was observed in the *Flii*^{+/-} EpSC niche rather than *Flii*^{Tg/Tg} counterpart. This indicates that an optimal Flii level may be required to keep the Wnt/ β -Cat signaling in its activated states. In addition, a consistent *Axin2* expression pattern observed in isolated EpSCs before and after wound-conditioned media treatment also indicates that Flii may impact β -Cat by Axin2 at a transcriptional level.

The expression of *Lgr6*, an R-spondin Wnt-signaling promoter at the receptor level, was observed to coincide with the expression of β -Cat before wounding and during the acute stage of repair until day 7 under all three levels of Flii expression. This suggests that Wnt-signaling may be modulated by Flii expression during homeostasis and wound repair. The level of Flii in unwounded *Flii*^{Tg/Tg} and wounded *Flii*^{+/-} niches may enable a more structurally stable R-spondin Wnt coreceptor, which could be beneficial for transmitting Wnt signals. In addition, the downregulation of *Lgr6* in *Flii*^{Tg/Tg} EpSCs upon wound-conditioned media treatment *in vitro* suggests that this promotional pathway of Wnt-signaling may be modu-

lated by Flii at a transcriptional level during wound repair.

Previous findings that a potential negative feedback loop between Flii and Lef1 mediated transcription suggest a mechanism dependent on the expression of all interacting partners including Flii, its binding partner Flap2, and β -Cat.¹⁹ The coincided expression pattern of Flap2 with Flii across the three genotypes before wounding and at day 3 may hence be beneficial to rescue cells affected by the negative regulatory effect brought about by high Flii at the very early stage of wound repair. However, lower levels of Flap2 observed in *Flii*^{Tg/Tg} EpSCs comparatively at day 7 may have ameliorated this effect and permitted baseline Wnt-activation to take place.

In human chronic wounds, Flii levels do not resolve but remain elevated and potentially contribute to reduced reepithelialization.³⁸ Our results collectively suggest Flii is a negative regulator of EpSC activation and Flii deficiency may facilitate Wnt/ β -Cat signaling during wound repair (Fig. 6). Together with the positive role played by Flii in HF regeneration, it is possible that Flii may modulate the differentiation and lineage preference of somatic stem cells including EpSCs based on their physiological context. Future investigations including identifying the specific molecular partners cooperating with either Flii or Flii-interacting proteins in canonical Wnt-signaling may provide in-depth mechanistic understanding of stem cell quiescence and activation during wound repair.

Figure 6. Model summarizing results. Flii is increased in response to wounding, where proliferation and activation of EpSCs is required, the negative effect of Flii is overcome by a reduction in the levels of Axin2, sustained Flap2, and stabilized β -Cat during the cascade of canonical WNT signaling. Although overexpression of Flii exacerbates its negative effects, reducing the level of Flii ameliorates this, leading to improved healing responses. At the receptor level, high levels of Flii contribute to destabilization of the LGR6-WNT receptor while lowered EGFR1 results in elevated Lig1 antagonistic effects. In the cytoplasm, high Flii and lowered Flap2 expose β -Cat to degradation by Axin2. In the nucleus, high Flii prevents β -Cat from increasing transcription of cell cycling genes, which also contributes to decreased proliferation and differentiation.

INNOVATION

The results of this study demonstrate that the cytoskeletal protein Flii is a negative regulator of EpSC activation, proliferation, and differentiation. Reducing the expression of Flii increases the activation and proliferation of EpSCs in the HF niche through processes involving Wnt/ β -Cat signaling. Modulating EpSCs in response to injury could potentially have an impact on the reepithelialization process.

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KEY FINDINGS

- EpSCs express Flii dynamically within the HF niche.
- Reducing Flii increases activation and proliferation of EpSCs in the HF niche during wound repair.
- Reducing the expression of Flii increases the activation and proliferation of EpSCs in the HF niche through processes involving Wnt/ β -Cat signaling.

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SUPPLEMENTARY MATERIAL

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Table S1

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Abbreviations and Acronyms

β -Cat	= β -catenin
Axin2	= axis inhibition protein 2
Ccnd1	= cyclin D1
Cdc20	= cell-division cycle protein-20
Dvl	= disheveled
EGFR1	= epidermal growth factor receptor 1
EpSC	= epidermal stem cell
FACS	= fluorescence-activated cell sorting
FBS	= fetal bovine serum
Fila	= filaggrin
Flii	= Flightless I
HF	= hair follicle
Invo	= involucrin
K1	= keratin 1
K14	= keratin 14
K15	= keratin 15
Lef1	= lymphoid enhancer-binding factor 1
Lrig1	= leucine-rich repeats and immunoglobulin-like domains-1
PCNA	= proliferating cell nuclear antigen
RT-qPCR	= real-time quantitative polymerase chain reaction
SEM	= standard error of the mean
WCHN	= Women's Health Service Animal Ethics Committee
WT	= wild type