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LETTERS

Wnt-dependent *de novo* hair follicle regeneration in adult mouse skin after wounding

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The mammalian hair follicle is a complex 'mini-organ' thought to form only during development[1]; loss of an adult follicle is considered permanent. However, the possibility that hair follicles develop *de novo* following wounding was raised in studies on rabbits[2,3], mice[4] and even humans fifty years ago[5]. Subsequently, these observations were generally discounted because definitive evidence for follicular neogenesis was not presented[6]. Here we show that, after wounding, hair follicles form *de novo* in genetically normal adult mice. The regenerated hair follicles establish a stem cell population, express known molecular markers of follicle differentiation, produce a hair shaft and progress through all stages of the hair follicle cycle. Lineage analysis demonstrated that the nascent follicles arise from epithelial cells outside of the hair follicle stem cell niche, suggesting that epidermal cells in the wound assume a hair follicle stem cell phenotype. Inhibition of Wnt signalling after re-epithelialization completely abrogates this wounding-induced folliculogenesis, whereas overexpression of Wnt ligand in the epidermis increases the number of regenerated hair follicles. These remarkable regenerative capabilities of the adult support the notion that wounding induces an embryonic phenotype in skin, and that this provides a window for manipulation of hair follicle neogenesis by Wnt proteins. These findings suggest treatments for wounds, hair loss and other degenerative skin disorders.

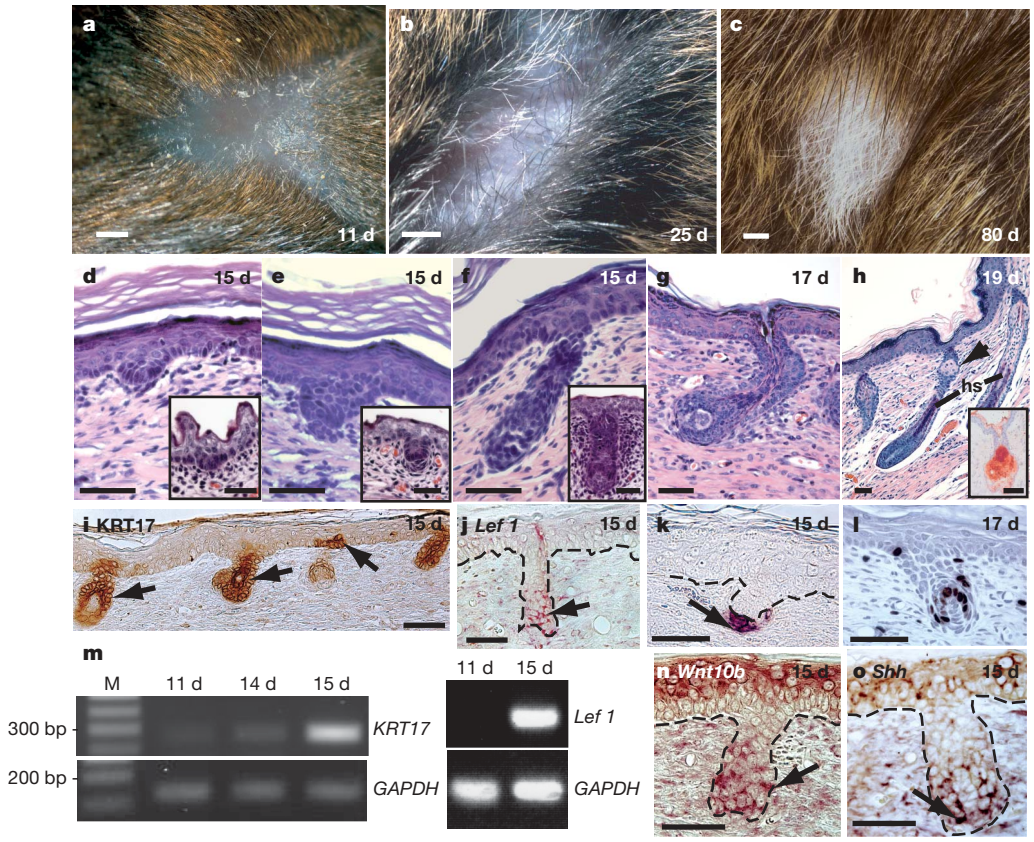

Figure 1 | Hair follicle regeneration recapitulates embryonic development. **a**–**c**, Hair appears in the re-epithelialized wound after several weeks. Scale bar, 1 mm. **d**–**h**, Histology of skin after wounding shows regenerating hair follicles mimicking stages of normal embryonic hair follicle development (examples shown in insets **d**–**f**). Sebaceous gland (arrowhead, **h**) also regenerates from the new follicle (**h**, inset, sebaceous gland stained with oil red O). hs, hair shaft. **i**–**o**, Nascent hair follicles proliferate and express genes and proteins (as indicated) that are associated with hair follicle differentiation, as detected by immunostaining (**i**), BrdU detection (**l**), *in situ* hybridization (**j**, **n**, **o**), alkaline phosphatase activity (**k**) and RT–PCR (**m**). Arrows point to signal. Scale bars, **d**–**l**, **n**, **o**, 50 μm. Time after wounding is in days (d). M, DNA size marker.

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During our studies on wound healing in mice, we noticed structures within the centre of large healing wounds that resembled early developing hair follicles. To characterize these structures, we performed timed experiments in which a 1 cm^2 square of full-thickness back skin was excised from 3-week-old mice, at least 2 weeks after the last hair follicles had formed. By 10–11 days after wounding, contracture and re-epithelialization resulted in wound closure and an approximately 0.25 cm^2 area composed of an epidermis and a dermis with no evidence of hair follicles (Fig. 1a). At 14–19 days after wounding, small epidermal downgrowths that resembled developing embryonic hair follicles were present (Fig. 1d–h). Older (7–8-week-old to 10-month-old) mice also showed hair follicle neogenesis after wounding, but larger wounds (2.25 cm^2) were required to trigger follicle formation (Supplementary Table 1). The final rather than initial size of the wound seemed to correlate with hair follicle neogenesis because the larger wound in older mice also yielded a 0.25 cm^2 area immediately after re-epithelialization. (Further characterization of hair follicle neogenesis with respect to density, type and orientation of the hair follicles is presented in Supplementary Table 2, Supplementary Figs 1–3 and Supplementary Notes 1, 2.)

Hair follicles consist of at least ten different epithelial and mesenchymal cell types geared towards the production of hair[7]. In the embryo, hair follicle development begins with the formation of a small cluster of epidermal cells (epithelial placode) that can be detected by expression of cytokeratin 17 (KRT17), an intermediate filament protein[8]. Placodes overlie a dermal condensate, which is identified by alkaline phosphatase activity[9]. Through a series of mesenchymal–epithelial interactions initiated by activation of Wnt and requiring downstream Shh signalling, placode cells proliferate, move downward and engulf the dermal condensate, eventually forming a mature follicle that cyclically produces hair throughout life[10].

We discovered that hair follicle neogenesis following wounding paralleled embryonic follicle development at the molecular level. The regenerated hair follicles expressed KRT17, *Lef1*, alkaline phosphatase, *Wnt10b* and *Shh* (Fig. 1i–o), which is analogous to embryonic follicles[10]. The truly nascent nature of these follicles was demonstrated by absence of expression (by PCR with reverse transcription; RT–PCR) of hair follicle differentiation markers KRT17 (ref. 8) and *Lef1* (refs 10, 11) in the epidermis for several days after wound closure (Fig. lm), and their subsequent appearance coinciding with the development of hair germs and pegs (Fig. li, j, m). The newly formed hair follicles also proliferated normally (Fig. 1l) and generated hair as well as sebaceous glands (Fig. 1c, h).

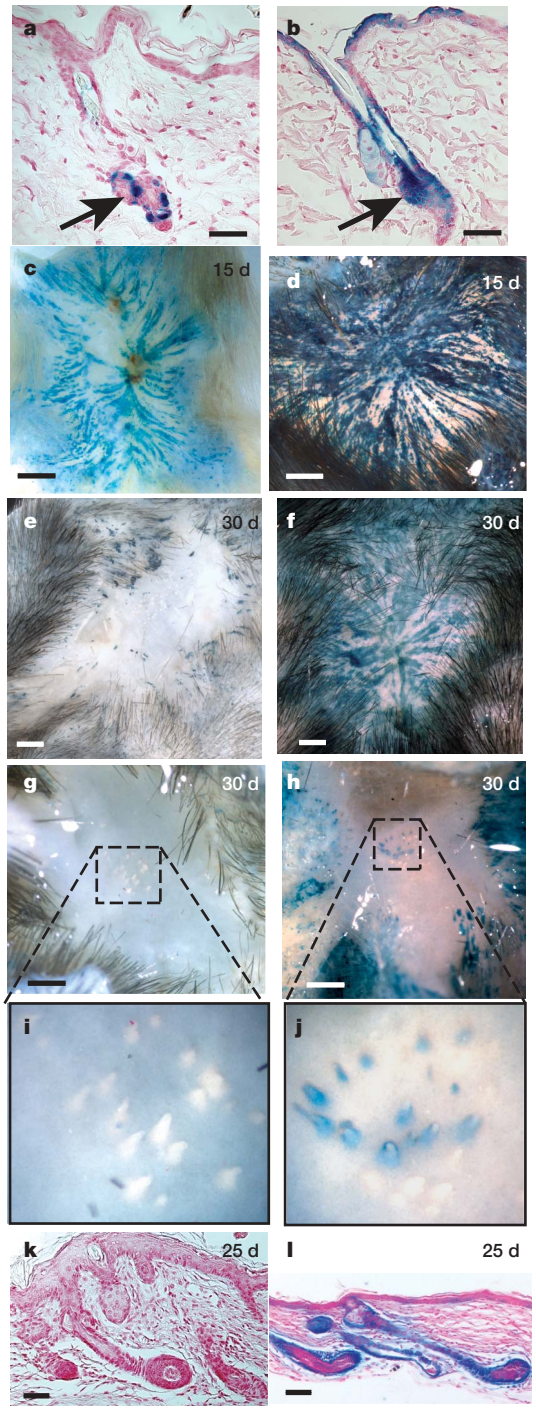

Figure 2 | Regenerated hair follicles originate from non-hair-follicle stem cells. Whole-mounted and sectioned skin, treated to detect *lacZ* expression (blue). **a**, Before wounding, hair follicle stem cells in the bulge (arrow) are labelled by treatment with RU486 in *Tg(Krt1-15-cre/PGR)22Cot;R26R* mice (continued in left panels). **b**, In contrast, both hair follicle stem cells in the bulge (arrow) and ~50% of epidermal cells are labelled in *Krt1-15-CrePR*/R26R* mice (continued in right panels). After wounding, the progeny of the labelled cells move towards the centre of the wound (**c**–**j**), but labelled hair follicles are found predominantly in *Krt1-15-CrePR*/R26R* mice (**g**–**l**). Epidermis was removed in **g**–**j** to visualize the hair follicles (**i**, **j**) of close-ups of **g** and **h**, respectively. Scale bars, 1 mm (**c**–**h**), 50 μm (**a**, **b**, **k**, **l**).

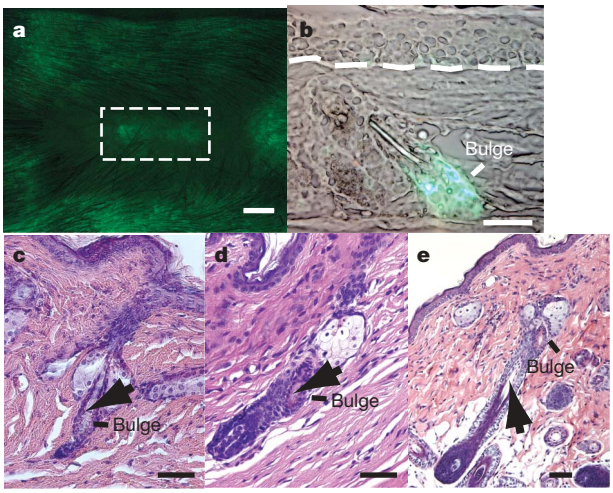

Figure 3 | Functional hair follicle stem cells are re-established in regenerated hair follicles. **a**, **b**, Green fluorescence from the enhanced green fluorescent protein (EGFP) indicates *Krt1-15* promoter activity, a marker of hair follicle stem cells, in *Tg(Krt1-15-EGFP)2Cot* transgenic mouse skin. Thirty-five days after wounding, a cluster of fluorescence is visible in the centre of the wound in whole-mounted skin (**a**). **b**, Histology of this area localizes *Krt1-15* promoter activity to the newly formed bulge in regenerated follicles (dashed line, dermal–epidermal junction). Regenerated hair follicles cycled through stages of rest (35 days, **c**) then growth (40 days, **d**; 45 days, **e**), indicating functional stem cells. (arrows, hair shafts). Scale bars, 1 mm (**a**), 50 μm (**b**–**e**).

We asked next whether the *de novo* follicles arise from hair follicle stem cells in skin bordering the wound. Hair follicle stem cells have been localized to the bulge area[12,13], and bulge cells in follicles surrounding a 4 mm wound send progeny towards the centre of the wound during re-epithelialization[14,15]. However, the majority of bulge cell progeny contribute transiently to the new epidermis and do not persist beyond three weeks[14]. To investigate whether the nascent follicles that developed following larger wounds originated from hair follicle stem cells, we performed genetic lineage analysis using inducible *Tg(Krt1-15-cre/PGR)22Cot;R26R* transgenic mice (Fig. 2, Supplementary Fig. 4 and Supplementary Table 3)[13,14]. These mice express CrePR1, a fusion protein consisting of Cre-recombinase and a truncated progesterone receptor that binds the progesterone antagonist RU486 under the control of the *Krt1-15* promoter, which is active predominantly in bulge cells of adult mouse skin[13,16]. Treatment of adult *Tg(Krt1-15-cre/PGR)22Cot;R26R* mice with RU486 resulted in permanent expression of *lacZ* in bulge cells and in all progeny of labelled bulge cells[13,14]. We discovered that although bulge cell progeny migrated to the centre of the larger wounds, they did not persist. Less than 3% of the new hair follicles were labelled, suggesting that non-hair-follicle bulge cells were the primary source of regenerated follicles (Fig. 2, Supplementary Fig. 4 and Supplementary Table 3).

To investigate further the cells of origin of the regenerated follicles, we examined *Krt1-15-CrePR*;R26R* transgenic mice. These mice possess a mutated progesterone receptor, PR*, containing a larger portion of the progesterone binding domain compared with the PR1 clone[17]. The *Krt1-15-CrePR** mice exhibit Cre recombinase activity during development when the *Krt1-15* promoter is active in the epidermis[16]. In these mice, approximately 70% of bulge and 50% of non-bulge epidermal cells are labelled before wounding (Fig. 2b, Supplemental Table 3; ref. 14). After wounding, approximately half of the regenerated follicles possessed cells expressing *lacZ* (Fig. 2h, j, l). Regenerated follicles were chimaeric for *lacZ* expression (Fig. 2l), indicating that multiple progenitor cells were required for new hair follicle formation, as in development[18]. Thus, together with near absence of *lacZ* expression in regenerated follicles of induced *Tg(Krt1-15-cre/PGR)22Cot;R26R* skin, these data indicate that new follicles originated from cells outside of the hair follicle stem cell niche. The new follicles arose from cells in the epidermis and/or

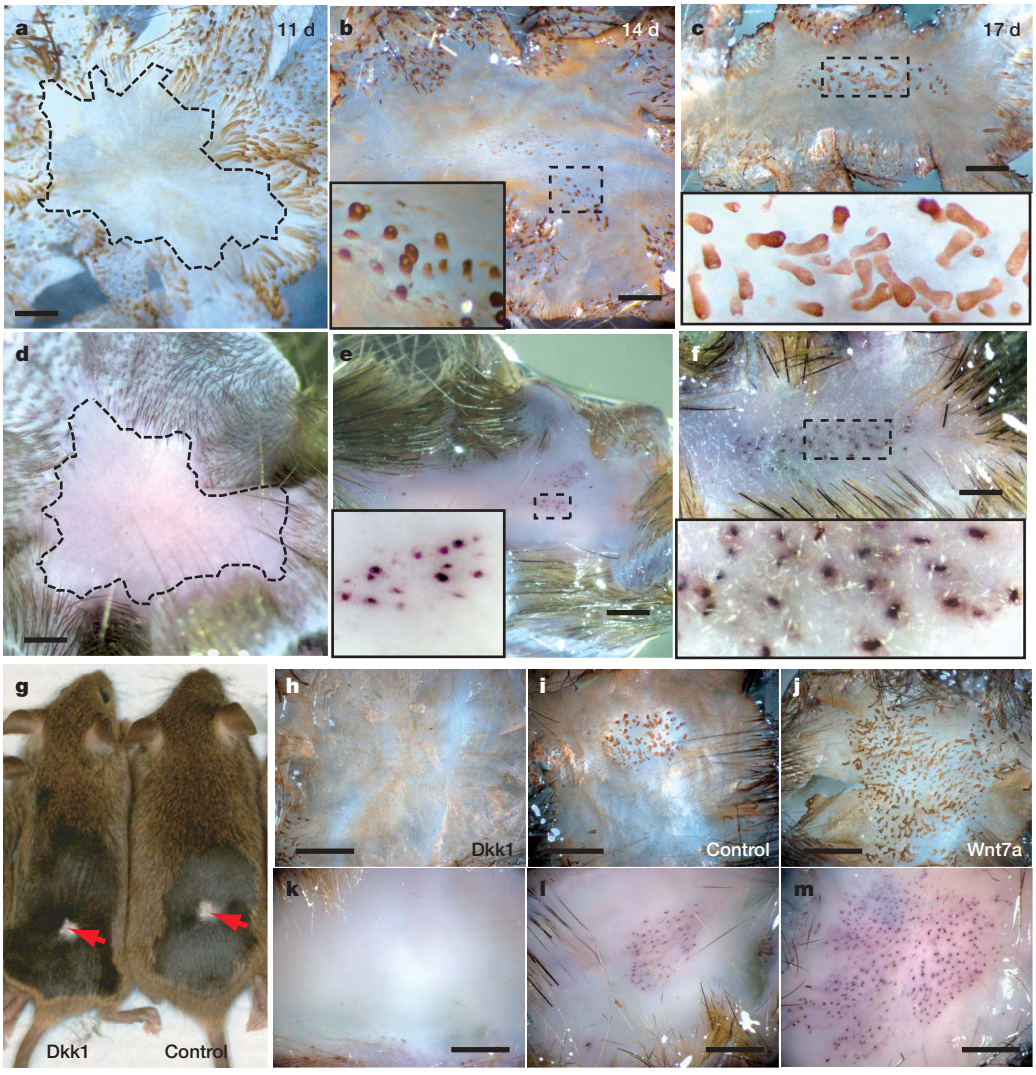

Figure 4 | Hair follicle neogenesis requires Wnt signalling, and overexpression of Wnt potentiates *de novo* follicle formation. Newly regenerating hair follicles in skin stained for KRT17 protein (**a**–**c**, **h**–**j**) and alkaline phosphatase activity (**d**–**f**, **k**–**m**). No hair follicles are visible at 11 days (**a**, **d**), but new follicles form in the centre of the wound at 14 (**b**, **e**) and 17 (**c**, **f**) days post wounding. **a**, **d**, Dashed line marks wound border. **b**, **c**, **e**, **f**, Insets show close-up views of dashed areas of same panel. Dkk1 expression induced in *tetO-Dkk1*;*K5rtTA* mice from 0 to 17 days after wounding does not affect wound closure (**g**), but blocks hair follicle regeneration (**h**, **k**). **j**, **m**, Overexpression of *Wnt7a* in *Krt1-14-Wnt7a* transgenic mice results in increased numbers of hair follicles in a larger area compared with non-transgenic control littermates (**i**, **l**). **h**–**m**, Seventeen days after wounding. Scale bars, 1 mm.

upper portion of the follicle (infundibulum). Both of theses areas are considered to possess stem cells that normally undergo epidermal rather than follicular differentiation[19,20]. Our findings are the first to indicate that non-hair-follicle stem cells in genetically normal adult mice acquire competence to form hair follicles in response to wounding. Whether epidermal or infundibular cells give rise to the new follicles awaits elucidation of reliable markers for these areas. Similarly, identification of the origin of the new hair follicle dermal papilla, which could arise from mesenchymal stem cells, is dependent on a better understanding of skin precursor cells[21].

Because hair follicle stem cells are necessary for follicle survival and cycling[14], we determined whether the regenerated follicles possessed a functional stem cell population. As with normal follicles, we found label-retaining cells and *Krt1-15* promoter activity (Fig. 3, Supplementary Fig. 5), consistent with the re-establishment of a hair follicle stem cell population. The regenerated follicles produced hairs and cycled up to three times within 90 days after wounding (Fig. 3c–e, Supplementary Note 2), indicating the presence of functional stem cells.

The new hairs lacked pigment (Fig. 1c) and associated melanocytes (data not shown), suggesting that the melanocyte stem cell niche was not re-established or that it could not be repopulated[22]. These findings parallel the lack of bulge-derived epithelial cells in the regenerated hair follicles, because, in mice, melanocyte precursors localize to the bulge. Furthermore, melanocytes are not normally present in the interfollicular epidermis of adult mouse back skin[22], precluding repopulation from an epidermal population.

Because Wnt proteins have a key role in normal hair follicle development and cycling[11,23,24], we tested whether Wnt blockade inhibits hair follicle neogenesis following wounding. We induced expression of secreted Dkk1, a Wnt inhibitor, in *tetO-Dkk1*;*K5rtTA* mice at the time of wounding until 17 days later (Fig. 4g–i, k, l). Although the time to re-epithelialization was normal, hair follicles did not form (Supplementary Table 1). Induction of Dkk1 between days 0 and 10 after wounding did not prevent hair follicle neogenesis. However, transient induction of Dkk1 after wound closure inhibited neogenesis and indicated the necessity of Wnt signalling for hair follicle neogenesis (Supplementary Table 1).

We then asked whether overexpression of the secreted ligand, Wnt7a, in *KRT14-Wnt7a* (which targets the epidermis) transgenic mouse epidermis (Supplementary Fig. 6), would enhance hair follicle neogenesis following wounding. Wnt7a has been shown to maintain the hair-follicle-inducing capacity of cultured dermal papilla cells[25]. The overexpression of activated β-catenin, an intracellular Wnt effector, in epidermis induces new hair follicles[26,27], and exogenous Wnt promotes formation of cysts with hair follicle differentiation[28]; however, to date, there has been no evidence that extracellular Wnt ligands can promote actual hair follicle neogenesis in adult skin. Wound closure (time to re-epithelialization) was normal in *KRT14-Wnt7a* mice. However, the transgenic mice developed over twice the number of hair follicles within the wounded area compared with controls (Supplementary Table 1, Fig. 4i, j, l, m). The increased hair follicle number was due to a larger area within the wound that developed follicles ($18 \pm 4\%$ in controls versus $40 \pm 15\%$ in *KRT14-Wnt7a* mice, $P = 0.05$) at the same density as controls (Supplementary Table 2). Thus, excess Wnt in combination with wound healing potentiates regeneration of hair follicles, perhaps by altering cell fate and increasing the number of cells competent to produce hair.

To define better which cell population is responding to Wnt and Dkk during hair follicle regeneration, we examined transgenic mice in which the β-catenin gene *Ctnnb1* had been deleted in the epidermis before wounding using *KRT14-CreER Ctnnb1*$^{flx/flx}$ mice (Supplementary Table 1)[11]. After application of tamoxifen, the *Ctnnb1* gene is deleted in the epidermis of these mice. This resulted in complete inhibition of new hair follicle formation, indicating that Wnt signalling in epidermal keratinocytes is required for hair follicle regeneration (Supplementary Figs 7 and 8).

A major therapeutic goal for those studying stem cells is the ability to coax these cells to differentiate into different cell types with the hope of eventually forming organs through tissue engineering[29]. By taking advantage of existing regenerative programmes, we demonstrated that a wound stimulus is sufficient to trigger regeneration of hair follicles from epithelial cells that do not normally form hair. This *de novo* formation of hair follicles in adult animals recapitulates embryogenesis at the molecular level, and provides a potential window for manipulating the number of hair follicles that form, by exposure to Wnts. This raises the possibility of treating acute wounds with modulators of the Wnt pathway to decrease scar formation, and treating hair loss by regenerating follicles through wounding and Wnt pathway activation.

METHODS SUMMARY

Wounding. C57BL/6J, SJL or mixed strains of C57BL/6J and SJL mice (Jackson laboratory) were anaesthetized with ketamine/xylazine, and a $1\,cm^2$ or $2.25\,cm^2$ cm full thickness excision of skin was made on the mid back of 3×7–8-week-old or 10-month-old mice, respectively. Three-week-old mice were used for all experiments, except as indicated. All animal protocols were approved by the University of Pennsylvania IACUC.

Whole-mount hair follicle neogenesis assay. To detect newly developing hair follicles in the wound, we incubated the skin in 20 mM EDTA in PBS at 37 °C overnight. Then, we gently peeled the epidermal portion off under a Leica MZFLIII dissecting microscope, fixed the epidermis in formalin for 1 h, rinsed with PBS, blocked with 0.6% H_2O_2 in methanol, and performed immunostaining for KRT17 in 1.5 ml eppendorf tubes. We fixed the dermis in acetone at 4 °C overnight, rinsed it with PBS, and then incubated with NBT/BCIP (Roche) for 30 min at 37 °C. We stopped the reaction with 20 mM EDTA in PBS.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Immunohistochemistry. Immunohistochemistry using antibodies against KRT17 (1:5,000 dilution, a gift from P. Coulombe), BrdU (1:500; Harlan-Seralab), β-catenin (1:2,000, Sigma) was done as previously described[14,30]. Detection of alkaline phosphatase and β-galactosidase activity was done as described[31]. Immunohistochemical studies were repeated at least three times.

***In situ* hybridization.** *In situ* hybridization for Wnt10b, Shh or Lef1 was done using previously described riboprobes and methods[16,23]. Studies were repeated twice.

RT–PCR. Healed skin was excised at 11, 14 and 15 days after wounding. The epidermis was separated from the dermis by incubation with 20 mM EDTA for 2 h at 37 °C. RNA was isolated from the epidermis using RNeasy (Qiagen) according to the manufacturer's instructions. We used 1 μg of total RNA to synthesize cDNA with a superscript cDNA synthesis kit (Invitrogen). PCR was carried out at 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, for 30 cycles to detect the expression of KRT17 and Lef1 and GAPDH using described primer sequences[23]. These experiments were repeated three times.

Lineage analyses of hair follicle neogenesis. *Tg(Krt1-15-cre/PGR)22Cot;R26R* and *Krt1-15-CrePR*/R26R* transgenic mice were treated with RU486 for 5 days during the first telogen stage[14] and then wounded. Skin samples were collected at 1 and 15 days after wound closure, and processed for β-galactosidase activity, which appeared blue[14]. The β-galactosidase-expressing cells in the skin were observed before and after peeling off the epidermis after EDTA (20 mM in PBS) treatment at 37 °C. 2–3 mice were used at each time point and experiments were repeated twice.

Detection of hair follicle stem cells in the regenerated follicles. We wounded *Tg(Krt1-15-EGFP)2Cot* mice[13] and then observed the fluorescence of the skin under a dissecting microscope 30 days later. We then prepared frozen sections from the centre of the wound encompassing the fluorescence, fixed the tissue in formalin for 10 min, stained the nuclei with Hoechst dye and detected fluorescence with an upright Leica DM4000B microscope equipped with a Leica DC500 digital camera[14]. For detection of label retaining cells, we injected BrdU (50 mg per g body weight; Sigma) intraperitoneally into mice twice daily for 3 days from 20 days after wounding and then fixed the tissues for BrdU detection at 23 and 40 days after wounding[32]. 2–3 mice were used at each time point and experiments were repeated twice.

***tetO-Dkk1;K5rtTA* transgenic mice.** Production of these mice was previously described[33]. Mice were placed on chow formulated with 1 g kg^{-1} doxycycline (BioServ) for either 0–17, 11–14, 12–15 or 0–10 days after wounding.

***KRT14-Wnt7a* transgenic mice.** A mouse Wnt7a cDNA fragment was generated by RT–PCR from mouse embryonic day E14.5 skin RNA using primers 7aF*Bam*HI cgcggatccgcgaacatrgacccggaaagcgcgg and 7aR*Bam*HI tcgcggatccgcgtcacttgcacgtatacatc (accession number M89801, nucleotides 1–18 and nucleotides 1,032–1,050, respectively). The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of a *KRT14-hGH* transgenic vector expression cassette[34] resulting in *KRT14-Wnt7a-hGH*. Sequencing revealed three differences to accession number M89801: nucleotides 817 and 818 were altered resulting in a leucine to lysine amino acid substitution. In addition, nucleotide 427 was altered and resulted in a tryptophan to arginine change. These changes are consistent with the Wnt7a sequence NM_009527. The same nucleotide alterations also could be found in clones amplified from the mouse keratinocyte cell line PAM212. For producing transgenic mice a 5 kb *Eco*RI fragment of the *KRT14-Wnt7a-hGH* vector was gel purified and micro-injected into fertilized oocytes (B6SJLF1/J x B6SJLF1/J cross, Jackson Laboratories). Transgenic founder animals were identified by PCR (primers 7aF*Bam*HI and 7aSeqR ccagcttcatgttctcctccagg, nucleotides 573–595 of M89801) and Southern blotting (cDNA used as probe). Detailed phenotypic analysis of *KRT14-Wnt7a* mice will be published elsewhere, but notably the hair follicle dermal papilla of these mice contain ~25–38% more cells compared with normal. It is possible that the larger number of dermal papilla cells contributes to the greater number of hair follicles that form after wounding. Control mice in *KRT14-Wnt7a* experiments refer to littermates not carrying the transgene.

***KRT14CreER;Ctnnb1$^{flx/flx}$*.** To generate these conditional β-catenin knockout mice, *Ctnnb1$^{flx/flx}$* mice (on a mixed background of 129×C57Bl6, a gift from W. Birchmeier) were crossed with *KRT14CreER* mice (on a background of CD1, The Jackson Laboratory). *Ctnnb1$^{flx/+}$*;*KRT14CreER* males were crossed with *Ctnnb1$^{flx/flx}$* females. Tamoxifen (1 mg per 10 g body weight; Sigma) was given via intraperitoneal injection from 9 to 14 days after wounding.

Statistics. Hair follicle numbers are expressed as mean ± s.d. The student's two-tailed *t*-test function in Excel was used to calculate *P* values. Details of statistics from experiments are presented in Supplementary Table 1.

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