Runx1 modulates developmental, but not injury-driven, hair follicle stem cell activation

Karen M. Osorio, Song Eun Lee, David J. McDermitt, Sanjeev K. Waghmare, Ying V. Zhang, Hyun Nyun Woo and Tudorita Tumbar*

Aml1/Runx1 controls developmental aspects of several tissues, is a master regulator of blood stem cells, and plays a role in leukemia. However, it is unclear whether it functions in tissue stem cells other than blood. Here, we have investigated the role of Runx1 in mouse hair follicle stem cells by conditional ablation in epithelial cells. Runx1 disruption affects hair follicle stem cell activation, but not their maintenance, proliferation or differentiation potential. Adult mutant mice exhibit impaired de novo production of hair shafts and all temporary hair cell lineages, owing to a prolonged quiescent phase of the first hair cycle. The lag of stem cell activity is reversed by skin injury. Our work suggests a degree of functional overlap in Runx1 regulation of blood and hair follicle stem cells at an equivalent time point in the development of these two tissues.

KEY WORDS: Runx1/Aml1, Hair follicle, Keratinocyte proliferation, Skin, Stem cell activation, Stemness

INTRODUCTION

Adult stem cells (SCs) of regenerative tissue, such as blood, hair and epidermis are essential for homeostasis and injury repair. They may be kept quiescent in specialized micro-environments called niches, which are crucial in providing control of proliferation and preventing disease (Fuchs et al., 2004; Moore and Lemischka, 2006; Watt and Hogan, 2000; Ma et al., 2005). Major developmental pathways are shared by many tissue SCs, but a common core of specialized ‘stemness’ genes remains largely unknown (Fuchs et al., 2004; Mikkers and Frisen, 2005). In this study, we test the role of a master regulator of hematopoietic stem cells (HSCs) and blood development, the transcription factor Runx1 (Speck and Gilliland, 2002), in hair follicle stem cells (HFSCs).

Runx1 is required for definitive blood formation (Speck and Gilliland, 2002; Speck et al., 2002), while its disruption in adulthood leads to an apparent increase of the HSC pool, as defined by cell surface markers (Growney et al., 2005; Ichikawa et al., 2004). Runx1 is mutated in 20-30% of individuals with acute myeloid leukemia and myelodysplastic syndrome (Coffman, 2003; Wang et al., 2006), and affects cell survival, proliferation and differentiation (Blyth et al., 2005; Mikhail et al., 2006). Runx1 also plays roles in muscle (Wang et al., 2005), nervous system (Theriault et al., 2005; Chen et al., 2006) and skin, where it affects hair follicle (HF) shaft structure (Raveh et al., 2006). The role of Runx1 in HFSCs is unknown.

The HF is an epidermal appendage embedded deep into the dermis (Cotsarelis, 2006). It is composed of concentric layers or sheaths of mainly epithelial cells (keratinocytes) surrounding the hair shaft. The outer root sheath contains the HFSCs in the bulge region below the sebaceous gland. Bulge cells regenerate the rapidly proliferating matrix progenitor cells that further differentiate into the inner layers of the HF and the hair shaft (Fig. 1A). As with blood development, the life of a HF can also be divided into primitive and definitive waves, known as morphogenesis and adult hair cycling, respectively. Morphogenesis is the initial temporary phase of hair shaft production, which provides the cellular architecture that will eventually enclose a powerful SC niche: the bulge (Cotsarelis, 2006; Cotsarelis et al., 1990; Oshima et al., 2001). At the end of morphogenesis, adult HFSCs complete maturation and enter quiescence. The transition from morphogenesis into the adult stage of hair regeneration is initiated by activation and proliferation of bulge HFSCs.

The adult HF undergoes periodic phases of growth and proliferation (anagen), regression and apoptosis (catagen), and quiescence (telogen) that are synchronously orchestrated in mouse skin during youth and take ~3 weeks to complete (Mueller-Rover et al., 2001) (Fig. 1B). A mesenchymal structure (dermal papillae) functions as a signaling center and contacts the hair germ structure right beneath the bulge SC niche. The dermal papillae sends signals that are thought to synergize with those from the bulge environment, to activate bulge HFSC proliferation and hair growth (anagen) (Cotsarelis, 2006; Fuchs et al., 2004; Pantaleev et al., 2001). These activating signals antagonize the inhibitory micro-environment of the bulge, thought to be set up in part by the outer root sheath cells including the bulge and germ themselves (Fuchs et al., 2004; Spradling et al., 2001; Watt and Hogan, 2000), and in part by other cell types surrounding the bulge. Single cell assays and transplantations suggest that bulge SCs contribute to making de novo functional niches (Blanpain et al., 2004). However, it is currently unclear whether all bulge and germ cells are stem and/or early progenitor cells, or whether some perform specialized niche cell roles.

To address the role of Runx1 in adult HFSCs, we targeted its gene locus in skin epithelial cells (keratinocytes). We show that Runx1 modulates HFSC activation and suggest an overlap in the transcriptional control of SC function at an analogous developmental stage for hair and blood.

MATERIALS AND METHODS

Mice
To generate K14-Cre/Runx1<sup>Fl/Fl</sup> mice, we mated hemizygous K14-Cre (CD1) and homozygous Runx1<sup>Fl/Fl</sup> (C57Bl6) mice; F1 K14-Cre/Runx1<sup>Fl/Fl</sup>C57Bl6 progeny were bred subsequently with Runx1<sup>Fl/Fl</sup>C57Bl6 mice. Runx1<sup>F<sub>1</sub>lacZ</sup> mice were maintained on C57Bl6 background. Genotyping was as described (Growney et al., 2005; North et al., 1999;
Vasioukhin et al., 1999). We used wild-type littermate controls housed in cages with knockouts of the same sex post weaning at PD (postnatal day) 21. Skin color of animals at PD28 was assessed by visual inspection of the entire back skin, on over 24 litters and over 129 mice. Mice with any gray patches on the back were scored in anagen.

**BrdU labeling**

BrdU (5-bromo-3-deoxy-uridine) (Sigma-Aldrich) was injected intraperitoneally at 25 μg/g body weight in saline buffer (PBS) at PD20. This was followed by administration of 0.3 mg/ml BrdU in the drinking water. Animals were sacrificed after 3-4 days (11 Runx1+/+ mutants and six wild-type mice). Staining of skin sections was described previously (Tumbar, 2006).

**Skin injury**

Mouse work was approved by the Cornell University IACUC, and has been described previously (Tumbar et al., 2004). Close shaving of Runx1+/– skin could result in hair growth, but using scissors avoided this problem. Hair pluck was carried out with human facial hair removing wax. All wounds were performed lateral of the midline using a dissection scalpel, and control skin was from the opposite equivalent side of the torso.

**Histology, immunofluorescence and X-Gal staining**

Staining of skin tissue for immunofluorescence and for Hematoxylin and Eosin (H&E) were as described previously (Tumbar, 2006; Tumbar et al., 2004). MVM Basic Kit (Vector Laboratories) was used for mouse antibodies. Nuclei were labeled by 4’6’-diamidino-2-phenylindole (DAPI). For 5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside (X-Gal) staining, 10 μm skin sections were fixed for 1 minute in 0.1% glutaraldehyde and washed in PBS. Incubation in X-gal solution (North et al., 1999) was at 37°C for 12-16 hours. Antibodies were from: (1) rat [α6 and β4 integrins (1:100), CD34 (1:150) (BD Pharmingen) and BrdU (1:300, Abcam)]; (2) rabbit [β-Gal (1:2000, Cappel), K5&K14 (1:1000, Covance), K6 (1:1000), LEF1 (1:250; E. Fuchs, Rockefeller University), RUNX1 (1:800); T. Jessel, Columbia University], Sox9 (rabbit, 1:100; M. Wegner, Erlangen-Nuernberg University, Germany) (Stolt et al., 2003), active capase 3 (1:500; R&D Systems), K67 (1:100; Novocastra), S100A6 (1:100, Lab Vision) and Tenasin C (1:500, Chemicon); (3) guinea pig [K15 (1:5000, E. Fuchs)]; and (4) mouse [AE13 (1:50, Immunoqueast), AE15 (1:10; T. T. Sun, NYU) and GATA3 (1:100, Santa Cruz)]. Secondary antibodies were coupled to the following fluorophores: FITC, Texas-Red or Cy5 (Jackson Laboratories).

**Microscopy and image processing**

Images were acquired using the IP-Lab software (MV1) on a light fluorescence microscope (Nikon) equipped with a CCD 12-bit digital camera (Rettiga EXi, QImaging) and motorized z-stage. To eliminate the out of focus blur, we deconvolved z-stacks (AutoQuant X software, MV1). Single images and projections through stacks were assembled and enhanced for brightness, contrast and levels using Adobe Photoshop and Illustrator.
expression patterns in skin development in hypothesized that it might also play a regulatory role in HFSCs. We repressed. As Runx1 was known to be a master regulator of blood stem cells (Speck and Gilliland, 2002; Speck et al., 2002), we hypothesized that it might also play a regulatory role in HFSCs.

To begin to examine its role in HFSCs we first determined Runx1 expression patterns in skin development in Runx1lacZ/+ reporter mice previously generated (North et al., 1999). Newborn skin showed Runx1 expression at the epidermal-dermal junction (see Fig. S1 in the supplementary material). We also observed Runx1 expression in the bulge, outer root sheath, matrix and cortex during anagen, and in the lower outer root sheath during catagen (see Fig. S1 in the supplementary material), as reported (Raveh et al., 2006). The upper HF area (infundibulum) showed variable levels of Runx1, but we found no expression in the interfollicular epidermis. During telogen to anagen transition, we found Runx1 expressed in the bulge, as expected from our mRNA analyses of sorted cells. Runx1 levels increased from top to bottom of the hair bulge with maximal expression in the hair germ (Fig. 2C, part b). Moreover, we examined the localization of endogenous Runx1 protein by immunofluorescence with specific antibodies (Chen et al., 2006), at different SC activation stages. Nuclear Runx1 protein overlapped CD34 bulge expression in only a few lower bulge cells during telogen-anagen transition (PD21) (Fig. 2C, parts c,d). Furthermore, during anagen (PD24 and P29) more nuclear Runx1+ cells were present throughout the bulge (see Fig. S2B in the supplementary material). These differences of Runx1 expression in bulge cells underscore the topological heterogeneity of cells within this area. In particular, the germ and lower bulge, which mark the hair region that proliferates first at the telogen-anagen transition, expressed the highest levels of Runx1.

To determine whether Runx1 expression accompanied or preceded the onset of bulge SC proliferation, we stained serial skin sections with antibodies to Runx1 and Ki67, a marker of proliferation. Nuclear Runx1 was present in approximately six to eight cells of hair germ and base of bulge segments, in 50-90% of follicles within each skin section. Ki67 staining was found in only one or two cells/follicle (Fig. 2C, part e), in ~40% of the follicles (over 150 total follicles from two back skin regions were examined). Co-staining for Runx1 and Ki67 during different anagen stages revealed that some but not all Runx1+ cells were Ki67+. Conversely, we found Ki67+ cells that were Runx1− (see Fig. S2B in the supplementary material). Moreover, prominent β-gal staining of Runx1lacZ/+ skin showed Runx1 expression in fully quiescent (Ki67−) hair germs at PD21 (see Fig. S2A in the supplementary material). Together, these data demonstrate that Runx1 expression precedes the bulge proliferation stage, and suggests a more complex and potentially non-cell autonomous role in keratinocyte proliferation.

Runx1 disruption prolongs the hair cycle quiescent phase and impairs HFSC colony formation

To study Runx1 role in HFs, we deleted its function in epithelial cells using keratin 14 (K14) promoter-driven Cre mice (Vasioukhin et al., 1999). Under this promoter, Cre expression turns on during embryonic hair morphogenesis, and remains active in the basal layer of the epidermis and the outer root sheath of the HF, including the HFSCs. We documented the efficiency of K14-Cre recombination in Rosa26R reporter mice by X-gal staining (Soriano, 1999), which showed over 90% of follicles are targeted (Fig. 3A). To study Runx1 role in HFs, we deleted its function in epithelial cells using keratin 14 (K14) promoter-driven Cre mice (Vasioukhin et al., 1999). Under this promoter, Cre expression turns on during embryonic hair morphogenesis, and remains active in the basal layer of the epidermis and the outer root sheath of the HF, including the HFSCs. We documented the efficiency of K14-Cre recombination in Rosa26R reporter mice by X-gal staining (Soriano, 1999), which showed over 90% of follicles are targeted (Fig. 3A). To study Runx1 role in HFs, we deleted its function in epithelial cells using keratin 14 (K14) promoter-driven Cre mice (Vasioukhin et al., 1999). Under this promoter, Cre expression turns on during embryonic hair morphogenesis, and remains active in the basal layer of the epidermis and the outer root sheath of the HF, including the HFSCs. We documented the efficiency of K14-Cre recombination in Rosa26R reporter mice by X-gal staining (Soriano, 1999), which showed over 90% of follicles are targeted (Fig. 3A). To study Runx1 role in HFs, we deleted its function in epithelial cells using keratin 14 (K14) promoter-driven Cre mice (Vasioukhin et al., 1999). Under this promoter, Cre expression turns on during embryonic hair morphogenesis, and remains active in the basal layer of the epidermis and the outer root sheath of the HF, including the HFSCs. We documented the efficiency of K14-Cre recombination in Rosa26R reporter mice by X-gal staining (Soriano, 1999), which showed over 90% of follicles are targeted (Fig. 3A). To study Runx1 role in HFs, we deleted its function in epithelial cells using keratin 14 (K14) promoter-driven Cre mice (Vasioukhin et al., 1999). Under this promoter, Cre expression turns on during embryonic hair morphogenesis, and remains active in the basal layer of the epidermis and the outer root sheath of the HF, including the HFSCs. We documented the efficiency of K14-Cre recombination in Rosa26R reporter mice by X-gal staining (Soriano, 1999), which showed over 90% of follicles are targeted (Fig. 3A). To study Runx1 role in HFs, we deleted its function in epithelial cells using keratin 14 (K14) promoter-driven Cre mice (Vasioukhin et al., 1999). Under this promoter, Cre expression turns on during embryonic hair morphogenesis, and remains active in the basal layer of the epidermis and the outer root sheath of the HF, including the HFSCs. We documented the efficiency of K14-Cre recombination in Rosa26R reporter mice by X-gal staining (Soriano, 1999), which showed over 90% of follicles are targeted (Fig. 3A). To study Runx1 role in HFs, we deleted its function in epithelial cells using keratin 14 (K14) promoter-driven Cre mice (Vasioukhin et al., 1999). Under this promoter, Cre expression turns on during embryonic hair morphogenesis, and remains active in the basal layer of the epidermis and the outer root sheath of the HF, including the HFSCs. We documented the efficiency of K14-Cre recombination in Rosa26R reporter mice by X-gal staining (Soriano, 1999), which showed over 90% of follicles are targeted (Fig. 3A).
shown). However, Runx14/4 showed no premature HF anagen cessation, hair loss or hair thinning, phenotypes that are commonly associated with severe malnutrition (Rushon, 2002; Paus et al., 1999).

The hair shafts began to appear on wild-type and Runx14/4 animals skin at ~PD5. Mild structural defects of the hair coat were apparent as described in detail elsewhere in another epithelial (K5-Cre) Runx1 knockout mouse (Raveh et al., 2006), and was consistent with Runx1 expression in the hair cortex. To look for effects of Runx14/4 mutation on HF development, we analyzed the histology of sections from a skin region of the mouse upper back during morphogenesis and the first adult hair cycle (Fig. 3F,G). Skin morphology and expression of Ki67 in differentiated hair cell lineage markers appeared normal in morphogenesis (data not shown). At PD21, both mutant and wild-type follicles were in catagen VIII (Muller-Rover et al., 2001; Paus et al., 1999) or telogen (Fig. 3F, see Fig. S3B in the supplementary material). Thus, HF morphogenesis appeared largely unperturbed by Runx1 deletion.

Starting with PD21, HFs of the Runx14/4 mice showed a noticeable phenotype. Wild-type follicles reached full anagen and produced new hair shafts by PD29 (Fig. 3F-H). By contrast, Runx14/4 HFs were quiescent (catagen VIII or telogen) at all time points analyzed beyond PD21 (Fig. 3F, see Fig. S3B in the supplementary material). The telogen stage in mutant mice encompassed the entire back skin, and, unlike wild-type mice, Runx14/4 mice were unable to re-grow hair within 2 weeks of gentle hair removal with scissors (Fig. 3H). To quantify this effect, we used skin color of PD28-29 mice (Fig. 3I, see Fig. S7A in the supplementary material). The telogen stage in mutant mice was demonstrated prolonged telogen in 4 mice. (G) Summary of hair cycle stage determined by microscopy of Hematoxylin and Eosin stained skin sections. In brackets are numbers of mice analyzed. At PD21, telogen or catagen VIII were designated Tel. (H) Wild-type but not 4 mouse skin at PD25 produces new hair during first hair cycle following morphogenesis. After gently shaving far from the skin the hair was carefully clipped with scissors to avoid injury produced by close shaving. (I) One hundred and one wild-type and 4 mice analyzed by skin color at PD29 show 4 mice in telogen (pink skin) while virtually all wild-type mice are in anagen (black skin color) (P<0.001). (J) Bright-field images of Hematoxylin and Eosin stained keratinocytes on feeder cells, 2 weeks post-plating. Wild-type keratinocyte colony is outlined. (K) Growth curve from 100,000 live keratinocytes plated on feeders. Runx14/4 keratinocyte proliferation is impaired (P<0.0001) after ~3 weeks in culture. (L) Arrow indicates an example of colony imaged by phase contrast (L). (M) Quantification of primary keratinocyte colonies obtained from equal numbers of wild type and 4 plated cells. 4 mutant show impaired colony formation (P<0.019).
staining. In addition, we ruled out the possibility that anagen onset in mutant mice was influenced by their lower weight, by comparing skin color of Runx114/44 animals at PD29 with small wild-type littermates of similar weight (see Fig. S5 in the supplementary material).

At PD21 Runx114/44 HFs displayed a slight increase in the number of outer root sheath cells below the bulge (see Fig. S3A in the supplementary material), suggesting increased survival of these cells normally destined to die. Apoptotic (caspase positive) cells indicating end of catagen were detectable in the germ cells below the bulge at PD21 in both Runx114/44 and wild type (data not shown). Progressive reduction in number of cells and narrowing of the germ-like structure below the bulge became apparent in Runx114/44 follicles at PD24, PD25, PD29 and PD38 (see Fig. S3B in the supplementary material). Moreover, the shrinking ‘hair germ’ displayed one or two apoptotic cells in over 40% mutant HFs at PD24, whereas growing wild-type follicles showed no caspase staining at this stage (see Fig. S4B,D in the supplementary material). Thus, cells shown to normally express Runx1 at high levels display increased survival in Runx1 mutant follicles, suggesting a role of Runx1 in apoptosis of keratinocytes during catagen.

The telogen-like morphology of mutant follicles suggested lack of differentiated hair lineage in the absence of functional Runx1. To determine whether Runx114/44 mutant follicles showed any differentiated cells, we performed immunofluorescence staining with specific hair lineage markers characteristic of anagen phase at PD21 and PD29 (see Fig. S6A in the supplementary material). We detected none of these markers, including that of progenitor matrix cells (Ephrin B1), in any of the Runx114/44 follicles. This was consistent with a true telogen block as assessed by hair morphology (Fig. 3F), and suggested that Runx1 works upstream, at the SC level, in skin keratinocytes. To further analyze this possibility, we examined SC behavior by clonogenicity assays. It has been established that generation of large keratinocyte colonies is initiated by independent SC populations of interfollicular epidermis and HFs (Barrandon and Green, 1987; Gambardella and Barrandon, 2003). Cultured keratinocytes from PD2 mice showed 80% fewer colonies in Runx114/44 versus wild-type cells (Fig. 3J,L,M) and a drastic proliferation defect over time (Fig. 3K). Most mutant-forming colonies were small and eventually stopped growing, and the few that expanded over time amplified from the rare Runx1 untreated cells (owing to ~90% Cre efficiency, data not shown). As Runx1 is not in interfollicular epidermis, we expected to obtain some normal-growing Runx114/44 keratinocyte colonies derived from this SC compartment, but our culture results did not fit this expectation. The result might be explained by the finding that all cultured keratinocytes, regardless of their HF or interfollicular origin, expressed Runx1 (not shown). This result suggested that all skin keratinocytes use Runx1 for their proliferation in culture.

In summary, the phenotypes observed in vitro and in vivo in the epithelial Runx1 knockout suggests that Runx1 acts in hair follicles at the stem cell level (see Fig. S6B in the supplementary material). Specifically, Runx1 deletion affected the ability of HFSCs to proliferate in vitro and to produce in vivo all differentiated hair lineages, including the progenitor-matrix cells at the onset of the adult hair cycling stage. Based on these phenotypes, we hypothesized four possible developmental mechanisms by which Runx114/44 could impair adult HFSC function to initiate hair cycling: (1) lack of adult HFSCs; (2) lack of activation/proliferation of quiescent HFSCs; (3) impairment of HFSC differentiation; (4) loss of HFSCs because of lack of maintenance/self-renewal. We next proceeded to test each mechanism.

**HFSCs are present in the Runx114/44 niche but show deregulation of hair cycle gene effectors** To test the first mechanism, we asked whether bulge SCs were either missing or in reduced numbers in Runx114/44 versus wild-type skin at PD21 during telogen-anagen transition. A significant fraction of bulge cells behaved as SCs in previous functional assays (Gambardella and Barrandon, 2003). Loss of bulge SCs can be accompanied by aberrant expression of known bulge and outer root sheath markers such as CD34, α6- and β4-integrins, keratin 15 (K15) and keratin 14 (K14), Sox9, S100A6 and Tenascin C. In immunostaining assays at PD21, we detected depletion of Runx1 in mutant follicles, but no change in expression level of these markers (Fig. 4A). Moreover, this expression was maintained in the arrested Runx114/44 mutant HFs at PD24 and PD29 (data not shown). The qualitative immunofluorescence results were supported by quantitative FACS analyses (Fig. 4B) of PD20 wild-type and mutant skin cells, which showed no significant difference (P=0.2) in the frequency of bulge SC population (defined by CD34+/α6-integrin+) (Fig. 4C). These results suggest that the HFSCs were present at normal numbers in the mutant follicles.

We next examined whether the mutant bulge cells displayed perturbation in expression of genes with known hair functions that might contribute to the Runx1 hair phenotype (Nakamura et al., 2001; Otto et al., 2003; Topley et al., 1999). We analyzed the following specific factors by RT-PCR of bulge and outside the bulge basal sorted cells: Bcl2, Bdnf, Dkk1, Dvl2, Stat3, Tgfb1, Noggin, Bmp4, Fzd2, Sfrp1, Fyn, Dab2 and p21. As expected, Fzd2, Sfrp1 and Dab2 were increased in the wild-type bulge fraction, as documented by our previous microarray analyses (Tumbar et al., 2004), and this pattern was maintained in the Runx114/44 cells (not shown). Whereas some of the tested genes were unchanged or showed sample-to-sample variation in expression levels in both mutant and WT bulge cells, several were consistently increased in Runx114/44 bulges (Fig. 4D,E). This change in expression agrees with the role of these factors as catagologen effectors, or negative regulators of proliferation or hair growth. The exception was a slight but statistically significant increase in Stat3 expression (also see qRT-PCR, Fig. S4E in the supplementary material). This disagreed with the prolonged telogen of Stat3 knockout mice, but might possibly be due to a compensatory effect of mutant bulge cells. Gapdh served as a loading control. These results demonstrate the misregulation of some known hair cycle effector genes (Nakamura et al., 2001), in the Runx114/44 bulge cells.

Taken together, these data suggest that Runx114/44 HFSCs probably contained the SCs, but these cells may have failed to timely exit the quiescent phase and sustain hair growth, possibly owing to changes in gene expression known to affect normal hair cycling. This conclusion is supported by functional assays described later in the paper.

**Runx114/44 bulge stem cells fail to proliferate during telogen-anagen transition** A second possible mechanism for explaining the Runx114/44 phenotypes in vivo and in vitro was a failure to proliferate by either the HFSCs or the early progenitor cells. In the former possibility, Runx114/44 bulge SCs do not divide, and do not give rise to early progenitor cells. In the latter, Runx114/44 bulge SCs divide and make progenitor cells, which in turn fail to proliferate.
To distinguish between these scenarios, we BrdU labeled skin cells continuously for 4 days at the anagen onset (PD20-PD24), in order to track cells that divided during this time. We then determined the localization of BrdU+ cells in the hair germ or the bulge. If bulge cells divided but their early progeny cells failed to proliferate further, we expected to see some BrdU+ cells in the CD34+/α6-integrin+ bulge cells. Inspection of skin sections co-stained for BrdU and CD34 at PD23 and 24 revealed that 100% of wild-type follicles were in anagen, and 67% of these follicles displayed variable numbers of BrdU+ bulge cells. Conversely, Runx1+/−/− follicles (5/5 mice) were in telogen and showed complete lack (100% follicles) of BrdU in the bulge (Fig. 5A,B). Furthermore, all wild-type follicles displayed bright BrdU+ germ cells, while 90% of Runx1+/−/− hair germs had no BrdU+ cells. The remaining 10% contained only one or two dim BrdU+ cells (see Fig. S4A in the supplementary material), which were probably the result of to incomplete Runx1 targeting. These BrdU+ germ cells found in the mutant follicles were caspase negative but positive for K5, which is normally expressed by epithelial hair germ cells (see Fig. S4C in the supplementary material). To determine whether we failed to detect activated (BrdU+) bulge cells because of possible apoptosis of these cells, we looked for the expression of caspase in bulge cells at PD24. Although we detected one or two apoptotic cells in ~40% Runx1+/−/− germs (see Fig. S4B in the supplementary material), the frequency of apoptotic cells in the bulge was below detection. The wild-type follicles were in early anagen and contained no apoptotic caspase-positive cells (see Fig. S4D). These data supported the first possibility discussed above, in which the bulge SCs remained quiescent in the Runx1+/−/− mutant.

To further examine the failure of bulge SCs to proliferate at their normal activation stage, we counted BrdU-positive cells in sorted CD34+/α6-integrin+ bulge cells isolated from mice continuously labeled with BrdU during anagen onset (PD20-PD24). These cells stained for undifferentiated keratinocyte markers K5 and α6-integrin, documenting at least 90% homogeneity of our sorted cells (Fig. 5C). Staining for BrdU revealed 10-30% positive wild-type cells and 0% BrdU-positive Runx1+/−/− cells (Fig. 5E). In conclusion, these data ruled out the possibility that Runx1+/−/− mutation allowed SC activation from quiescence, but simply blocked the proliferation of the early progenitor matrix cells. Instead, we showed that Runx1+/−/− stem cells remained quiescent at a stage when wild-type stem cells undergo developmentally controlled activation.
Proliferation and differentiation of Runx1<sup>14/14</sup> HFSCs in response to skin injury

Our experiments suggested that Runx1<sup>14/14</sup> SCs failed to respond to normal growth activation signals during the initiation of adult hair cycling phase. If Runx1<sup>14/14</sup> SCs were functional, one might expect that in response to a different activation signal they would be able to proliferate, differentiate and generate new hairs (Fig. 6A). To test this hypothesis, we employed skin injury as the source of activation signal (Fuchs et al., 2004). We used a total of 38 days post-wounding (performed at PD21 or PD29) to test whether the injury-triggered hair growth in Runx1<sup>14/14</sup> mutants resulted in normal proliferation and differentiation of bulge cells. Four to 18 days post-wounding (performed at PD21) we detected Ki67+ proliferating bulge cells, and new hair shaft growth in the wounded area (Fig. 6B.C.D). The HF had essentially normal morphology and cycled normally (Fig. 6C). Furthermore, we found all differentiated lineage markers correctly expressed in the newly grown Runx1<sup>14/14</sup> hair bulbs by immunofluorescence staining (Fig. 6E). This indicated that Runx1<sup>14/14</sup> did not affect the differentiation potential (multipotency) and fate decision of progenitors and HFSCs, a step upstream of the previously shown Runx1 effect on aspects of terminal differentiation (Raveh et al., 2006).

**Runx1<sup>14/14</sup> effect on long-term regenerative potential of HFSCs**

Finally, to test a fourth possible mechanism for Runx1 action, we examined the long-term regeneration potential of Runx1<sup>14/14</sup> HFSCs population, a definitive hallmark of self-renewing SCs. During a time period of more than 1 year, we induced four or five rounds of back skin injury at these stages reversed the Runx1<sup>14/14</sup> quiescence block.

The prolonged telogen described here could be consistent with a role of Runx1 in regulating early stem/progenitor cell fate choice and differentiation to hair cell lineages. Thus, we asked whether the injury-triggered hair growth in Runx1<sup>14/14</sup> mutants resulted in normal proliferation and differentiation of bulge cells. Four to 18 days post-wounding (performed at PD21) we detected Ki67+ proliferating bulge cells, and new hair shaft growth in the wounded area (Fig. 6B.C.D). The HF had essentially normal morphology and cycled normally (Fig. 6C). Furthermore, we found all differentiated lineage markers correctly expressed in the newly grown Runx1<sup>14/14</sup> hair bulbs by immunofluorescence staining (Fig. 6E). This indicated that Runx1<sup>14/14</sup> did not affect the differentiation potential (multipotency) and fate decision of progenitors and HFSCs, a step upstream of the previously shown Runx1 effect on aspects of terminal differentiation (Raveh et al., 2006).
morphogen released from the growing follicles, which triggered new growth in the surrounding dormant follicles. Follicles eventually re-entered the quiescent phase, as shown by the pink skin color. At this point, we repeated the skin wounding in a different region of the skin to reinitiate another cycle of SC activation and hair growth (Fig. 6F). Occasionally, upon a new injury cycle we found a gray or black patch of anagen skin at the site of a previous wound (see Fig. S7C in the supplementary material). This suggested initiation of a new hair cycle in the absence of immediate injury in a skin area that was previously activated by injury to grow hair. An important issue is whether HFs would begin cycling spontaneously at later developmental stages in the complete absence of injury. Suggestively, out of 10 uninjured mutant mice analyzed between PD42-PD48, five were in early anagen while five remained in telogen. It is difficult, however, to rule out the role of spontaneous injury in this delayed anagen initiation (bites, scratching, scraping) as even shaving can trigger hair growth in mutant animals. Addressing unambiguously the role of Runx1 in spontaneous hair cycles in older mice will require further investigation.

Taken together, these results suggested that during later developmental stages beyond the initiation of the adult phase: (1) Runxl1^{−/−}/H9004^{−/−} HFSCs maintained their long-term potential and repeated stimulation did not exhaust the mutant SC pool; and (2) Runxl1^{−/−}/H9004^{−/−} HFSC activation could occur in the absence of injury, at least in follicles that had already been previously directly initiated via injury, and in follicles found in the vicinity of actively growing hairs.

**DISCUSSION**

**Runx1 modulates hair cycling**

In this work, we examined the function of Runx1, a hematopoietic SC factor, in the hair follicle. We found that Runx1 is important for normal hair cycling at the transition into adult skin homeostasis. Mice that lack functional Runx1 in skin epithelial cells are able to produce normal hair follicles during morphogenesis, but these follicles displayed a prolonged first telogen. The hair follicle quiescence is rapidly overcome by injury, which triggers proliferation and differentiation of the HFSCs. Importantly, the hair growth can spread far into unwounded areas, and can also resume once again spontaneously in follicles that had been already removed from quiescence by previous injury. It remains unclear whether at later developmental time points hair follicles might be capable to cycle spontaneously, in the absence of any injury. The Runx1 mutant phenotype underscores differences in developmental versus injury triggered hair growth, a phenotype also displayed by the Stat3 knockout mouse (Sano et al., 1999; Sano et al., 2000). The relationship between these transcription factors in HFs remains to
be elucidated. Finally, the skin phenotype of the Runx1 mutant mice is accompanied by a severe impairment of keratinocyte proliferation in vitro, and by changes of gene expression levels in the SC compartment in vivo of factors known to regulate the quiescent phase of the hair cycle.

**Runx1 regulates HFSC activation**

Here, we show that \( \text{Runx1}^{+/+} \) mutation results in complete lack of newly differentiated hair lineages in the first hair cycle. Our data suggests that in \( \text{Runx1}^{+/+} \) follicles the bulge HFSCs: (1) were present and functional at the time of phenotype onset; (2) together with progenitor cells remained quiescent at a key developmental activation time point; (3) retained intrinsic ability to proliferate and differentiate, and produce essentially normal hairs; and (4) were maintained in the \( \text{Runx1}^{+/+} \) bulge over prolonged periods of time and repeated stimulation.

The injury response of Runx1 mutant mice might be explained by alternative but less likely models that we formally acknowledge here. Although not yet demonstrated experimentally, it is possible that the bulge contains SC populations specialized to perform either normal homeostasis or injury repair. The first SC population is Runx1 dependent, whereas the second one is not. Another possibility is that injury conditions of stressed/ischemic skin trigger the lineage conversion of a non-hair to a hair SC type. This possibility is hard to reconcile with our data showing spreading of the hair growth at uninjured areas far from the wound, a phenomenon present in both wild-type and mutant follicles.

Runx1 is expressed in a broad area that includes hair germ and bulge cells preceding SC activation. It is unclear whether the protein acts intrinsically in the SCs or acts on SCs through the niche. Its germ expression prior to activation correlated with the apparent effect of Runx1 disruption on increased outer root sheath survival during the catagen/telogen transition. We detected Bcl2, an apoptosis regulator at increased levels in the bulge, and overexpression of Bcl2 (Nakamura et al., 2001) had a similar effect on the hair cycle as disruption of Runx1.

Although a role of Runx1 in the SC environment through secreted protein downstream targets is an attractive model, we cannot eliminate the possibility that Runx1 also functions within SCs to set the intrinsic rate of HFSC proliferation. This possibility is suggested by our in vitro cell culture assays, in which wild-type but not \( \text{Runx1}^{+/+} \) HFSCs could generate large keratinocyte colonies in the time frame of our experiments. The regulation of epithelial skin cell culture growth by Runx1 warrants further investigation. In a clinical setting, achieving rapid expansion of keratinocytes in amounts useful for engineering artificial skin is extremely difficult, although it proves crucial for individuals with severe burns (Rochat and Barrandon, 2004). As we understand more how control of epithelial SC proliferation is achieved in the tissue and how cell growth conditions perturb this balance, we will be able to apply more systematic approaches to in vitro SC manipulation for epidermal and hair engineering.

**Is Runx1 a ‘stemness’ gene?**

Hematopoietic and hair SCs exist in tissues with distinct physiological roles and origins, that arise from different cell types of the early embryo (mesoderm and ectoderm). However, these two tissues share a fundamental functional characteristic: they regenerate continuously throughout life, and rely on adult SC activity to sustain extensive cellular turnover of their differentiated progeny cells. It is already known that blood and HF cells share common transcription factors that can regulate fate and differentiation of committed progenitor cells (DasGupta and Fuchs, 1999; Kaufman et al., 2003).

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/6/1059/DC1

**References**


