Notch signaling in bulge stem cells is not required for selection of hair follicle fate

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Notch signaling plays an important role in hair follicle maintenance, and it has been suggested that Notch is also required for follicular fate selection by adult hair follicle stem cells in the bulge. Here we demonstrate that, on the contrary, Notch signaling in bi-potential bulge stem cells or their uncommitted descendents acts to suppress the epidermal fate choice, thus ensuring follicular fate selection. To examine the role of Notch signaling in adult hair follicle stem cells, we used a Krt1-15-CrePR1 transgenic mouse line to delete Rbpj or all Notch proteins specifically in the bulge stem cells. We conclusively determined that in the absence of Notch signaling, bulge stem cell descendents retain their capacity to execute the follicular differentiation program but fail to maintain it owing to their genetic deficiency. The defect in terminal differentiation caused the diversion of Notch-deficient hair follicles to epidermal cysts, and the presence of wild-type cells could not prevent this conversion. Importantly, our analysis revealed that a functional Notch signaling pathway was required to block bulge stem cells from migrating into, and assuming the fate of, interfollicular epidermis. Taken together, our findings yield detailed insight into the function of Notch signaling in hair follicle stem cells and reveal the mechanism of the replacement of Notch-deficient adult hair follicles by epidermal cysts.

KEY WORDS: Notch, Bulge, Fate selection, Stem cells, Mouse

INTRODUCTION

The mammalian hair follicle continuously cycles through three distinct phases: (1) anagen of variable length (regenerative/proliferative phase); (2) catagen, a short destructive phase during which the lower part of the hair follicle containing the proliferating and keratogenous zones is removed; and (3) telogen (resting phase) (Muller-Rover et al., 2001). The quiescent hair follicle stem cells reside in the bulge located in the permanent part of the hair follicle near the arrector pili muscle attachment site (Blanpain and Fuchs, 2006; Cotsarelis et al., 1990). During each hair cycle, a few bulge stem cells respond to signals from dermal papillae and give rise to new progenitor cells that reside in the hair matrix. This highly proliferative structure surrounds the dermal papilla at the base of the bulb and generates the new anagen hair shaft (Hardy, 1992; Millar, 2002). Matrix cells divide and differentiate into the outer root sheet (ORS), inner root sheet (IRS), cuticle, cortex and medulla of the hair (Legue and Nicolas, 2005). An elaborate network of signaling pathways regulates hair follicle morphogenesis and maintenance (Fuchs and Horsley, 2008; Millar, 2002). The Notch signaling pathway contributes to the maintenance of the follicular structure but not to cell fate selection during hair follicle morphogenesis (Pan et al., 2004). In addition, Notch signaling ensures an optimal proliferative environment in the matrix during first anagen by suppressing Tgfβ and activating Kit ligand (Lee et al., 2007).

Notch regulates keratinocyte proliferation, commitment and differentiation decisions in intact skin and culture (Blanpain et al., 2006; Lee et al., 2007; Pan et al., 2004; Rangarajan et al., 2001). In response to ligand binding, Notch receptors undergo sequential proteolysis by two enzymes (ADAM metalloprotease followed by γ-secretase) to release the active Notch intracellular domain (NICD), which translocates into the nucleus, binds to Rbpj and nucleates the recruitment of a transcription-activating complex (Lubman et al., 2004). This overall scheme is termed ‘canonical’ Notch signaling. Part of Notch function in epidermal keratinocytes is mediated by a poorly defined, Rbpj-independent signal (Demehri et al., 2008; Rangarajan et al., 2001).

Although Notch receptors do not function during hair germ cell induction or cell fate acquisition within the embryonic hair follicle, they are required to complete terminal differentiation in IRS cells. In the anagen hair follicle bulb, three Notch receptors are expressed in partially overlapping domains (Pan et al., 2004). Each follicle is derived from two to four multi-potent bulge stem cells (Jaks et al., 2008; Kopan et al., 2002), which give rise to oligo-lineage hair follicular progenitors (Legue and Nicolas, 2005) located adjacent to the dermal papilla in the matrix. Notch proteins are not expressed in these oligo-lineage progenitors (Kopan and Weintraub, 1993; Pan et al., 2004; Powell et al., 1998). Notch1 is expressed and activated in mitotically active IRS and cortex precursors (Cai et al., 2009; Pan et al., 2004), whereas Notch2 and Notch3 are expressed in their postmitotic descendents, respectively (Pan et al., 2004). In the absence of Notch signaling, a hair shaft still forms and contains properly positioned cells expressing markers for each fate (Pan et al., 2004). However, because IRS cells fail to properly adhere to each other, follicular architecture cannot be maintained, leading to the transformation of these aberrant hair follicles into epidermal cysts during the first anagen by overproliferating IRS cells (Pan et al., 2004).

Partial reduction in Notch signaling has also been associated with the conversion of hair follicles to epidermal cysts in adults (see Fig. S1 in the supplementary material) (Vauclair et al., 2005; Yamamoto et al., 2003). However, it is unclear whether the switch from a hair follicle to an epidermal unit during the hair cycle in adult Notch-deficient animals reflects (1) epidermal fate selection by Notch-deficient hair follicle stem cells in the bulge or (2) terminal differentiation defects caused by loss of Notch proteins in committed hair follicle progenitors that lead to aberrant hair shaft formation and to conversion of the hair follicle into an epidermal cyst. Previous studies have supported the former possibility by demonstrating that mosaic loss of Notch signaling by Rbpj removal in hair follicles

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RESULTS AND DISCUSSION

Notch signaling is not required for follicular fate determination of bulge stem cells

To directly examine the possibility that Notch is required for follicular fate selection during hair regeneration, we used Krt1-15-CrePR1 transgenic mice (Ito et al., 2005) to remove canonical Notch signaling specifically in bulge stem cells in adulthood and determine whether bulge stem cells lacking Notch signaling could produce daughters that choose the hair follicle fate. Rbpj (Yamamoto et al., 2003) deletion was induced by topical application of RU486 onto the skin of postnatal day 50 (P50) Krt1-15-CrePR1; Rbpj^fl/fl; Rosa26R (K15-RBP-jCKO) mice at the beginning of the second telogen. Fourteen days later, bulge cells were activated by depilation, and 14 days after that we harvested the skin to examine the extent of labeling within the regenerating hair follicles (Fig. 1A). If Notch signaling were required to select/execute the follicular fate, Rbpj-deficient lacZ-positive cells would not be able to enter the follicular program. Contrary to this prediction, X-Gal and antibody stainings conclusively showed that blue Rbpj-deficient stem cells contributed descendents to hair matrix progenitors as well as to hair follicle keratin (AE13)-expressing cortex and cuticle cells (Fig. 1B-D). To rule out the possibility that depilation overcame the Notch deficiency, we repeated the experiment by topically treating skin with RU486 and waited for the next spontaneous anagen before harvesting the skin (see Fig. S2A in the supplementary material). As seen with follicles regenerating after depilation, hair follicles spontaneously entering anagen contained blue Rbpj-deficient cells (see Fig. S2C in the supplementary material). Therefore, canonical Notch signaling is not required for hair follicle fate selection by descendents of bulge stem cells.

The analysis of a complete allelic series of mice lacking Notch signaling components in their hair follicles revealed a tight inverse correlation between Notch dosage in follicular keratinocytes and the level of hair follicle distortion (Fig. 2A,B). Importantly, we noticed that Msx2-Cre^+/+; Rbpj^fl/fl hair follicles displayed a milder disruption at P9 than that seen with total loss of Notch receptors or γ-secretase, which led to epidermal keratin cyst formation during the first anagen (Fig. 2A,B) (Pan et al., 2004). Similar to hair follicles retaining some Notch activity (Msx2-Cre^+/+; Notch1^fl/fl; Notch2^fl/fl; Notch3^+/–; N12N3hCKO) that formed epidermal cysts only in the second anagen (Fig. 2C and see Fig. S1 in the supplementary material), Msx2-Cre^+/+; Rbpj^fl/fl follicles did not form epidermal cysts in the first anagen and retained a recognizable follicular morphology at P9 (Fig. 2). As shown previously (Demehri et al., 2008; Rangarajan et al., 2001), Rbpj-independent Notch signals contribute to hair follicle maintenance (see Fig. S3 in the supplementary material). Thus, to address the concern that the demonstrated ability of Rbpj-deficient bulge stem cells to choose a follicular fate was preserved by Rbpj-independent Notch signal(s), we generated mice lacking all Notch proteins in bulge stem cells (Krt1-15-CrePR1; Notch1^fl/fl; Notch2^fl/fl; Notch3^−/−; Rosa26R, or K15-N12N3CKO). Following the Cre induction/hair depletion protocol (Fig. 1A), we showed that Notch-deficient stem cell descendents were also fully capable of contributing daughters to hair follicle structures (Fig. 1E). This finding demonstrates that neither arm of Notch signaling is required for stem cells to choose the follicular fate.

Although Notch-deficient bulge stem cells (blue) migrated down and formed anagen hair follicle progenitors and differentiated hair keratinocytes, the defect in maintaining terminal differentiation resulted nonetheless in the formation of
Fig. 1. Hair follicle stem cells lacking total Notch signaling are capable of choosing a follicular fate. (A) Diagram of the timeline/cohorts used to delete Rbpj (or Notch genes) in adult hair follicle stem cells, to induce the hair cycle, and to examine the behavior of anagen hair follicles 14 days after hair cycle induction. Note that the topical application of RU486 started when the mice were at ~P50 and the following analyses are performed at the end of the experimental period (i.e. 28 days after the last RU486 treatment). (B) X-Gal staining shows Rbpj-deleted bulge cells (blue) in Krt1-15-CrePR1; Rbpjlox/lox; Rosa26R (K15-RBP-jCKO) and control hair follicles that are not depilated and hence are still in the telogen phase of the hair cycle. Depilated hairs, however, have entered anagen. Blue Rbpj-deleted cells in K15-RBP-jCKO hair follicles have migrated down to the bulb and are detectable in all layers of the anagen hair follicle. (C) AE13 (cortex and cuticle marker) staining confirms that blue Rbpj-deleted keratinocytes in K15-RBP-jCKO undergo the follicular differentiation program. The inset shows the normal pattern of AE13 staining in an anagen hair follicle (counterstained with Hematoxylin). (D) Immunohistochemistry for Rbpj demonstrates that blue cells in K15-RBP-jCKO anagen hair follicles are depleted of Rbpj protein. (E) Bulge stem cells deleted for all Notch receptors can differentiate into hair follicle keratinocytes and are detectable in anagen hair by X-Gal staining on Krt1-15-CrePR1; Notch1flox/flox; Notch2flox/flox; Notch3–/–; Rosa26R (K15-RBP-jCKO) skin after Cre/hair induction as outlined in A. (F) Back skin of K15-RBP-jCKO and control mice highlighting the aberrant hair shafts produced by keratinocytes derived from Rbpj-deficient stem cells. The genotype of control mice is shown in A. Scale bars: 25 μm.

aberrant hair shafts (Fig. 1B,E,F, Fig. 2 and see Fig. S2C in the supplementary material). To study the long-term consequences of Notch loss in bulge stem cells, we deleted Rbpj (K15-RBP-jCKO) or Notch receptors (K15-N1N2N3CKO) by topical application of RU486 onto the skin during the second telogen, and monitored the hair phenotype over time by histological analyses (Fig. 3A). In the absence of Notch signaling, hair follicles eventually transformed into keratin 10- and loricrin-positive epidermal cysts (Fig. 3B,C) following the destruction of anagen hair follicles in K15-RBP-jCKO and K15-N1N2N3CKO skin. Such cysts were predominantly located deep in the dermis (Fig. 3B). Hair follicles are polyclonal, being derived from two to four stem cells (Jaks et al., 2008; Kopan et al., 2002), and not all bulge cells undergo Notch gene/Rbpj deletion with our topical application paradigm. Thus, most follicles are chimeric, containing descendents of Notch/Rbpj-deficient stem cells as well as wild-type descendents of stem cells that are Notch signaling competent. Importantly, a significant number of keratin cyst-forming cells contained intact Notch signaling, demonstrating that the ‘field effect’ of aberrant hair shafts formed by Notch/Rbpj-deficient keratinocytes was sufficient to set them on the path to become epidermal cells (Fig. 3B). Accordingly, inactivation of Notch signaling in most bulges resulted in complete alopecia within 10 weeks (Fig. 3D), despite the presence of many stem cells that did not experience Cre activation. Importantly, K15-N1N2N3CKO hair loss was much more severe than that of RU486-treated Krt1-15-CrePR1; Notch1flox/flox; Notch2flox/flox; Notch3–/–; Rosa26R (K15-N1CKO) mice (Fig. 3D and see Fig. S4 in the supplementary material). Considering that Notch3 is a null allele and that Notch1/3-deficient hair follicles are indistinguishable from Notch1-deficient hair follicles (Pan et al., 2004), the severity of the K15-N1N2N3CKO hair phenotype confirms that deletion of Notch2 and Notch1 occurred in these Notch3-deficient bulge cells. In conclusion, replacement of Notch-deficient hair follicles by epidermal cysts is a secondary by-product of terminal differentiation defects that cannot be rescued by normal cells, and does not reflect a defective hair follicle fate-selection process. These findings show that the function of Notch signaling during hair regeneration is similar to its role during hair morphogenesis.

Removal of the Notch signaling pathway exposes the bi-potentiality of bulge stem cells

To examine the developmental potential of Notch signaling-deficient bulge stem cells, we mapped the cell lineages derived from these cells in K15-RBP-jCKO skin following the induction of Cre-mediated gene deletion (Fig. 4A). Importantly, a significant number of bulge-derived Rbpj-deficient blue cells migrated upward and generated keratin 10+, keratin 6– interfollicular epidermal cells in the isthmus and epidermis (Fig. 4B-D). Of the K15-RBP-jCKO hair follicles counted on X-Gal-stained 5 μm skin cross-sections, 39% (78 of 200) contained blue Rbpj-deficient cells above the bulge region (see Fig. S5 in the supplementary material). Similar
evidence for epidermal fate selection by K15-N1N2N3CKO bulge stem cells was also observed after RU486 treatment (data not shown). The migration of Notch/Rbpj-deficient bulge cells into the epidermis preceded the secondary effects of aberrant hair shaft formation: bulge-derived Notch/Rbpj-deficient cells were already present in the interfollicular epidermis as hair follicles spontaneously entered anagen (Fig. 4). At this point, epidermal thickness, hair follicle structure and dermal cellularity were normal. In addition, staining the skin sections for CD45 (Ptprc – Mouse Genome Informatics), a pan-leukocyte marker, confirmed that there

Fig. 2. Stepwise deletion of Notch pathway components in hair follicle keratinocytes results in progressive hair follicle deterioration culminating in transformation into epidermal keratin cysts. (A) H&E-stained skin sections from a comprehensive allelic series of Notch-deficient mice at P9 demonstrate progressive hair follicle destruction, which is most evident in mice lacking total Notch signaling (N1N2N3CKO and PSDCKO), which show densely packed keratin cysts beneath the epidermis. Note that Rbpj-deficient hair follicles have a milder phenotype than those lacking total Notch signaling. (B) A scoring system devised to permit a quantitative measure of hair follicle destruction (e.g., 0, intact hair follicle; 10, keratin cyst) confirms the inverse correlation between Notch dosage and the level of hair follicle deterioration. The destruction level of RBP-jCKO hair follicles (asterisk) is similar to that of N1N2N3CKO. Hair follicles in three randomly selected 100X microscope fields are scored and the average score ± s.d. is presented in the bar chart. (C) Immunofluorescence staining for keratin 14 and loricrin (an epidermis-specific marker) shows the transformation of Msx2-Cre+; Notch1fl/fl; Notch2fl/fl; Notch3–/– (N1N2N3hCKO) hair follicles to fully formed epidermal keratin cysts (asterisks) in the second anagen (P35). Msx2-Cre+, Notch1fl/fl; Notch2fl/fl; Notch3fl/fl (N12hN3hCKO), Msx2-Cre+; Notch1fl/fl; Notch3fl/fl (N1N2hN3CKO), Msx2-Cre+; Notch1fl/fl; Notch3–/– (N1N2N3CKO), Msx2-Cre+; Notch1fl/fl; Notch2–/– (N12N3CKO), Msx2-Cre+; Notch1–/– (N12hN3CKO), Msx2-Cre+; Notch1–/– (N1N2N3CKO), Msx2-Cre+; Notch1–/–; Notch2–/– (N12hN3CKO), Msx2-Cre+; Notch1–/–; Notch2–/– (N1N2N3CKO), Msx2-Cre+; Notch1–/–; Notch2–/– (PSDCKO). Scale bars: 100 μm in A; 50 μm in C.
was no inflammation present at this early stage when epidermal migration of Notch/Rbpj-deficient bulge cells was well underway (Fig. 4E). Furthermore, the occasional presence of bulge-derived Rbpj-deficient cells in the isthmus of otherwise normal-looking hair follicles (i.e. club hairs of telogen hair follicles; see Fig. S2B and Fig. S6 in the supplementary material) demonstrated that the upward migration of Notch/Rbpj-deficient bulge cells started before the hair follicles entered anagen. Considering that under similar circumstances, wild-type bulge stem cells do not choose an epidermal fate (Fig. 4 and see Fig. S5 in the supplementary material).
material) (Ito et al., 2005; Levy et al., 2005), these observations demonstrate that Notch signaling acts to prevent hair follicle stem cells or their uncommitted descendents from randomly adopting the epidermal fate. How wounds reverse this block (Ito et al., 2005) remains an interesting question to be investigated.

Conclusions

In summary, and in contrast to Wnt signaling (Huelsken et al., 2001), neither arm of Notch signaling is required for follicular fate selection by bulge stem cells. Instead, Rbpj-dependent Notch signals restrict bulge cells (or their uncommitted, migratory descendents) to the follicular fate. In addition to cells selecting the follicular fate, a substantial fraction of Notch/Rbpj-deficient stem cells produce descendents that spontaneously choose the epidermal fate and migrate upwards, joining the interfollicular epidermis and producing epidermal cells deficient in terminal differentiation. The hair follicles formed by Notch-deficient stem cells properly associate with dermal papillae, produce a bulb expressing hair keratins, but fail to maintain the identity of IRS cells and medulla (Pan et al., 2004). Thus, both during hair follicle development and regeneration, Notch contributes to terminal differentiation. The progressive transformation of hair follicles to epidermal cysts caused by a stepwise reduction in Notch dosage is the direct result of hair shaft disintegration due to increasingly defective terminal differentiation.

This report identifies Notch signaling as a novel regulator of bulge stem cell fate selection, acting to constrain this bi-potential cell to the hair follicle fate. The ability of Notch/Rbpj-deficient stem cells to enter both hair follicle and epidermal fates under the normal homeostatic state with similar probability indicates a stochastic fate-selection process. This is in contrast to the classical role for Notch in fate selection as seen in the fly neuroectoderm, where a default homeostatic state with similar probability indicates a stochastic fate-to-enter both hair follicle and epidermal fates under the normal circumstances. R.K. and S.D. were supported by NIH grant GM55479-10 from NIH/NIGMS. Deposited in PMC for release after 12 months.

Supplementary material

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References