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Earlier- born secondary hair follicles exhibit phenotypic plasticity

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Abstract

The mouse pelage is composed of four distinct hair types. The fact that the follicles that generate these hair types form in successive waves during late embryonic development suggested the model that distinct epigenetic states of the inductive mesenchyme fixed when the follicles are formed specify the distinctive hair morphologies. This model is inconsistent with the observation that many follicles produce different hair types in successive hair cycles. In this study, the characteristics of the hair follicles that switch between the production of different hair types were examined. These follicles were born earlier than those that do not switch between hair types and made longer hairs. They also expressed a higher level of Sox2 in the dermal papilla and had more DP cells per follicle. These observations are consistent with the hypothesis that different birthdates specify the potential of different follicles. However, rather than directly specifying hair type, birthdate correlates with three types, guard hairs, a plastic population that can make awl, auchene or zigzag hairs, and a population that normally makes only zigzag hairs. Although Sox2 expression levels in the DP identify this subset during the morphogenetic cycle, Sox2 expression is not a fixed epigenetic state specified when the follicle is first formed.

Keywords

Hair type conversion; Sox2; dermal papilla

Introduction

The mouse pelage is composed of four hair types with distinct morphologies: Guard, awl, auchene and zigzag. Pelage hair follicles form in three temporally distinct waves during late embryonic/peri-natal development. The first wave forms guard hairs, while the second and third waves preferentially form awl or auchene hairs and zigzag hairs respectively in the first

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hair coat. It has been proposed that these different follicle types arise because the dermal papillae of follicles that form at different times during development are intrinsically different, perhaps reflecting some epigenetic restriction imposed as development progresses that alters their inductive properties (1). The fact that there are genetic regulators such as *Eda/Edar*, *BMP*, *Igfbp5*, *FGF*, and *Sox2/Sox18* (2–8) that differentially affect follicles formed at different times supports this idea, as does the fact that follicles induced in the adult form zigzag hairs (9). However, the type of hair produced by an individual follicle is not fixed over the lifetime of the animal for a significant fraction of follicles. During normal development, roughly 30% of the follicles switch from production of zigzag or auchene hairs to the production of larger hair types. Furthermore, experimental manipulation of DP cell number in existing follicles can change the hair type produced by a follicle as well (10). Thus while the idea that the follicles that produce four hair morphs maintain unique properties that are intrinsically different by virtue of their birthdate is proven incorrect, the data reported to date remains consistent with the hypothesis that there are 3 intrinsically distinct populations. Instead of different DP populations corresponding to the different hair types, this data identifies three follicle types; 1. guard hair follicles 2. a developmentally plastic population that makes awl, auchene or zigzag hairs, and 3. a static population that makes zigzag hairs. To investigate the phenomenon of follicle type conversion, we asked what distinguishes the follicles that switch between hair types during normal development from those that do not.

Methods

Hair dye

Post natal day (p)11 (follicles in mid anagen stage) or p20 (follicles in telogen stage) mice were anesthetized and their back hair was dyed. Dyes were then washed with warm water and the mice were dried with kimwipes. Skin was harvested after the completion of 2nd cycle for subsequent analysis.

Follicle dissection

Follicles from P8 skin were carefully pulled away with a fine forceps from the dermal side of the skin. For telogen skin, individual follicles were carefully dissected with scalpels from a strip of skin. Dissected follicles were segregated into sub-groups based on the type of hair formed in the first hair coat and hair type conversion between the 1st and 2nd hair coats was scored. 1st cycle hair was identified by the persistence of hair dye.

Sox2:GFP intensity analysis

Sox2:GFP/+ mice at P8 were sacrificed to harvest mid-anagen skin. Collected skin was fixed with 4% paraformaldehyde overnight at 4 C. The next day, follicles were dissected and segregated based on the hair type and mounted on a glass slide in 50% glycerol. A 0.6 micron thick optical section through a mid-sagittal plane was collected for each follicle with a Nikon T1 confocal microscope using a 40X objective. For each session, the detector was set just below saturation for the GFP signal in the DP of guard hair follicles and kept fixed for the measurement of all follicle types. The average intensity value for GFP was calculated within a region of interest corresponding to the DP using NIS Elements software. All cells in

the DP region are GFP positive. Background levels were determined by averaging fluorescence intensity over a GFP negative region of the slide and this value was subtracted from the DP-ROI average fluorescence intensity. *Sox2:GFP*^{lo} zigzag follicles consistently score significantly higher than background levels ($P < 0.001$).

DP number counts

Skin biopsies were fixed with 4% paraformaldehyde overnight at 4 C, and rinsed in PBS. Follicles were dissected and segregated into *Sox2:GFP*^{hi} and *Sox2:GFP*^{lo} zigzag classes under a dissecting microscope with fluorescence optics. Follicles were then incubated overnight in methanol, stained with TOPRO-3 (Molecular Probes, 1:1000 in PBS). Data in Fig. 3C was collected from unpigmented follicles (*SoxGFP*⁺, *tyr*^{c/c}) to prevent melanin from obscuring cells in the upper DP. A Z-stack of 0.6 micrometer optical sections through each follicle was collected with a Nikon T1 confocal microscope. Individual nuclei within the boundaries of the DP, defined by GFP fluorescence, were counted.

Results

Earlier born follicles are more likely to undergo hair type conversion

The population that exhibits phenotypic plasticity during normal development comprises roughly 30% of the hair coat. Guard hair follicles appear to make guard hairs throughout life. Follicles that make auchene hairs in the first hair coat predominantly make awl hairs in the second. However, roughly 20% of the follicles that make zigzag hairs in the first hair coat make awl or auchene hairs in the second while the remaining 80% continue to make zigzag hairs. While there is some minor conversion in hair type between the second and third hair coats, it amounts to a few percent of follicles. Thus, our analysis focused on the first and second hair coats. With the exception of guard hairs, hairs formed in the previous hair cycles remain anchored in the hair follicle. This allows the history of hair production to be evaluated by dissecting individual follicles. To gain insight into the properties of zigzag follicles that convert to production of different hair types, the length of the zigzag hair that was produced in the first hair cycle was measured in follicles that either continued to make a zigzag hair or went on to the production of a larger hair type in the second cycle. In all animals examined, the average length of the first zigzag hair was about 13% longer in follicles that produced an awl or auchene hair in the second cycle than in those that continued to produce a zigzag hair ($n=6$ mice, minimum of 20 follicles each type per mouse, $p < 0.001$) (Fig. 1). This greater length could reflect either a faster growth rate of hair in follicles born at the same time, or a similar growth rate in follicles born earlier in development. To distinguish between the two possibilities, hair was dyed at p11 roughly midway through the first growth cycle, and hair follicles were harvested after the second cycle ($n=2$ mice) for comparison between follicles that converted to production of larger hair types and those that did not. Consistent with previous data, the first zigzag hairs in follicles that underwent conversion were significantly longer than those in follicles that did not (5.55 mm vs. 4.94 mm, $p < 0.001$). Notably, the dyed segment is significantly longer in the zigzag hairs in follicles that converted to the production of larger hair types, but the undyed proximal segment was not (Fig. 2). Furthermore, dye was found extending into the third segment in converting follicles, while it was restricted to the mid point of the second

segment in those that did not convert. The observations that growth rate subsequent to dye application is similar, yet a larger fraction of the hair was produced prior to dye application leads us to conclude that the follicles that ultimately convert to production of different hair types began hair production earlier in development.

Sox2 expression in DP marks the follicles that convert

Sox2 has been implicated in the specification of hair type (8, 12, 14). Several studies reported that Sox2 is preferentially expressed in the DP of guard/awl/auchene follicles and absent in zigzag follicles (8, 13). In vivo Sox2 expression is faithfully recapitulated by a GFP reporter inserted in the Sox2 locus (15). Using this Sox2 reporter line, endogenous Sox2 expression was inferred by measuring the intensity of GFP fluorescence in DP cells. Among follicles of the first hair coat, DP cells in guard hair follicles express the highest levels of *Sox2:GFP*, while awl, auchene and zigzag follicle DP express progressively lower levels (Fig. 3A and 3B). In contrast to previous reports, virtually all the follicles producing zigzag hair exhibited low but detectable GFP expression during anagen. Notably, a subset of zigzag follicles in the first hair coat express *Sox2:GFP* at higher levels than the remaining zigzag follicle population ($18.8 \pm 2.1\%$ $n=1681$ zigzag follicles from 8 mice) (Fig. 3A). This subset is quantitatively similar to the fraction that converts from the production of zigzag to larger hair types between the first and second cycles ($18.7 \pm 5.1\%$ in wild type $n=1560$ zigzag follicles in 5 mice). To address whether this *Sox2:GFP* zigzag population is the earlier born zigzag population that converts to production of larger hair types, zigzag follicles from p8-p10 mice were harvested and segregated by GFP expression. Over 90% of high *Sox2:GFP* expressing follicles ($n=140/154$ *Sox2:GFP* zigzag follicles in 5 mice) had produced longer zigzag hairs defined by hair that had completed at least two segments at this stage. These data suggest that preferential expression of Sox2 in the DP in the first hair cycle marks a phenotypically plastic subset of follicles that includes both auchene follicles and the subset of zigzag follicles likely to switch to the production of larger hair types.

DP cell number is higher in the follicles with higher Sox2 expression

Our previous study reported that the number of DP cells per follicle is critical to determining the hair type produced (10). To investigate whether the difference of *Sox2:GFP* expression in zigzag follicles correlates with a difference in DP cell number, zigzag follicles from p11 *Sox2:GFP* mice ($n=2$) were dissected and segregated into *Sox2:GFP* and *Sox2:GFP* subgroups. The average number of DP cells was significantly higher in *Sox2:GFP* (26.8 ± 2.0 DP cells, $n=12$ follicles) vs *Sox2:GFP* zigzags (21.2 ± 2.3 DP cells, 13 follicles, $p < 0.001$) (Fig 3C). Thus, higher Sox2 expression also marks the zigzag follicles with a larger number of DP cells.

Sox2 expression in DP changes between hair cycles

Higher *Sox2:GFP* in the first cycle is a marker of birthdate, but the question remains whether *Sox2:GFP* expression is a marker of a fixed epigenetic state of the DP cell or a reflection of the follicle environment that can be reprogramed. If the former, then the fraction of total follicles that express *Sox2:GFP* at higher levels should be fixed despite the conversion towards production of larger hair types in the *Sox2:GFP* population, while the

fraction of follicles producing zigzag hairs that express high levels of GFP should decrease. But if the latter, the fraction of follicles expressing higher levels of *Sox2:GFP* will change as the pelage composition changes between hair cycles. To distinguish between the two possibilities, the percentage of follicles with higher levels of *Sox2:GFP* was scored and compared between the 1st and 2nd hair cycle. *Sox2:GFP* expression during the 1st hair cycle was scored by two independent methods: evaluation of randomly dissected follicles in which hair type was identified, and skin sections in which it was not. Whether scored as the fraction of total hair follicles expressing higher GFP in the DP in skin sections or in dissected follicles, roughly 31% express *Sox2:GFP* above the threshold defined as “Hi” in first cycle zigzag hair follicles. (256 *Sox2:GFP*hi/856 total dissected hair, (n=3 mice). Avg. = $30.1 \pm 0.9\%$; 165 *Sox2:GFP*hi/514 total follicles in skin section (n=3 mice). Avg. = $31.7 \pm 3.4\%$). We then asked if the fraction of *Sox2:GFP*hi follicles changed in the 2nd cycle. While the percentage of follicles producing zigzag hairs that expressed high levels of GFP (n=8 mice, 1st cycle: $18.8 \pm 2.1\%$ n=318/1681 to 2nd cycle: $9.6 \pm 3.6\%$ n=44/454 $p < 0.001$) was reduced, this reduction did not account for all of the follicles that had converted to the production of larger hair types (Fig 3E). Instead, there was a net increase in the proportion of total follicles that expressed high levels of GFP to $39 \pm 1.4\%$ (n=3 mice, $p < 0.001$ (Fig. 3D). These data indicate that a new set of follicles in 2nd hair cycle expressed *Sox2:GFP* at a higher level than that found in the previous cycle. Hence *Sox2* expression is not a strict lineage marker, but rather a reflection of the signaling environment in the DP.

DISCUSSION

This work demonstrates that the follicles that show plasticity with respect to hair type produced during normal development of the mouse are born earlier than the subset of zigzag follicles that do not normally convert to the production of larger hair types. Although this is consistent with a developmental plasticity based on the birthdate of the follicle, the follicles that undergo conversion also tend to have more DP cells and express higher levels of *Sox2*. DP cell number correlates with the type of hair produced and a change in DP cell number can cause a change in the type of hair produced. *Sox2* expression level in the DP also correlates with the type of hair produced and functional studies suggest that it plays a role, as yet ill-defined in the formation of awl and auchene hairs (14). Any or all of these three correlated factors may contribute to the probability that a follicle converts to the production of a larger hair type.

The switch to production of different hair types is most frequent between the first and second hair coats when the mouse has grown dramatically and the density of follicles is correspondingly reduced. Adjacent follicles have been shown to affect the behavior of their neighbors during anagen re-entry (16), and may also influence the type of hair made by neighboring follicles. If we posit that competitive interactions between follicles contribute to the size of follicle and type of hair produced, then it could follow that these earlier born follicles enjoy competitive advantage not because of their birthdate or other intrinsic difference of their DP population, but because they form under the influence of fewer neighbors. To resolve this question, we must decipher whether the follicles that don't normally exhibit phenotypic plasticity do so under more permissive conditions, and whether

either increased Sox2 activity or augmented DP cell number is sufficient to drive phenotypic conversion in this population.

The higher expression of *Sox2:GFP* in some zigzag follicles has not been reported previously. Instead, it has been reported that zigzag follicles do not express Sox2 in the DP (1, 8). This difference may result from the different *Sox2:GFP* lines, or the sensitivity of GFP detection. An analysis of *Sox2* function in the dermal papilla suggested that Sox2 controls BMP signals that regulate hair growth rate (13). It is noteworthy that in our analysis comparing follicles producing the same hair type, stronger *Sox2* gene expression does not correlate with a faster growth rate. While it is possible that the level of Sox2 in *Sox2:GFP^{hi}* zigzag follicles is nonetheless below biologically significant levels, Lesko et al. also reported that within a hair type, deletion of *Sox2* in the DP did not affect overall hair length or girth (14). This contrast warrants further study.

The switch between production of different hair types in a single follicle has proven to be a sensitive indicator of hair “quality” that can be used to explore the mechanism of hair decline and methods to reverse it in an accessible model system. Here we show that the capacity to express *Sox2* at different levels is not a property fixed at birth. Identification of signals that maintain or augment expression of Sox2 in DP cells may provide a strategy for the maintenance or restoration of hair growth.

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Fig. 1.

Follicles that convert to production of awl or auchene hairs make longer zigzag hairs than those that continue to make zigzag hairs. (A) Examples of zigzag follicle types. z-z indicates a follicle that made zigzag hairs in the first and second cycles. z-au and z-aw are zigzag follicle that made an auchene or awl hair in the second cycle, respectively. (B) Graph of the first zigzag hair length difference between z-z and z-au/aw in 6 mice. The length of the first zigzag hair in z-z was normalized to 1 for comparison. At least 20 hairs were measured per mouse per category. Error bars = s.d. *indicates $p < 0.001$

Fig. 2.

Dye at mid-cycle reveals follicles that convert between hair types start hair production earlier. (A) Example of hair dyed at P11. The follicle on the left made another zigzag while the follicle on the right made an awl hair in the second cycle. The blue line indicates where the dyed or undyed hair length was scored. (B) Graphic illustration of the length difference between dyed area (orange) vs undyed area (black). z-z, n=63 follicles and z- auch/awl, n=83 follicles from 2 mice. Error bars = s.d. * indicates $p < 0.001$. (Mouse 1 z-z = 4.95 ± 0.3 mm n=33, n=41 z-auch/awl = 5.56 ± 0.15 mm n= 41; $p < 0.001$; Mouse 2 z-z 4.94 ± 0.27 , n=30 z-auch/awl 5.54 ± 0.30 n= 44, $p < 0.001$).

Fig. 3.

Analysis of *Sox2:GFP* in follicles. (A) Example of *Sox2:GFP* expression by different hair type (from left to right: guard, awl, auchene, zigzag high and zigzag low). Note that the zigzag population was divided into *Sox2:GFP^{hi}* and *Sox2:GFP^{lo}* as shown in the lower panel. (B) Relative *Sox2:GFP* intensity in a mid-sagittal optical plane from mid anagen follicles (n=3 mice). GFP fluorescence in guard hair DP was normalized to 1. (C) Difference in the number of DP cells per follicle between *Sox2:GFP^{hi}* and *Sox2:GFP^{lo}* zigzag follicles during the anagen phase (P11). (D) Change in % of total follicles scored as *Sox2:GFP^{hi}* between 2 hair cycles. (E) Change in the fraction of zigzag follicles scored as *Sox2:GFP^{hi}*. Error bars = s.d. *indicates $p < 0.001$.