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Topology of Feather Melanocyte Progenitor Niche Allows Complex Pigment Patterns to Emerge

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Color patterns of bird plumage affect animal behavior and speciation. Diverse patterns are present in different species and within the individual. Here, we study the cellular and molecular basis of feather pigment pattern formation. Melanocyte progenitors are distributed as a horizontal ring in the proximal follicle, sending melanocytes vertically up into the epithelial cylinder which gradually emerges as feathers grow. Different pigment patterns form by modulating the presence, arrangement, or differentiation of melanocytes. A layer of peripheral pulp further regulates pigmentation via patterned agouti expression. Lifetime feather cyclic regeneration resets pigment patterns for physiological needs. Thus, the evolution of stem cell niche topology allows complex pigment patterning via combinatorial co-option of simple regulatory mechanisms.

The color of avian plumage can be patterned within feathers and along the body axes. Pigmentation adapts to seasonal changes and physiological needs (*1*–*4*). Complex pigment patterns have co-evolved with feather shapes to generate spectacular plumage, as seen in male peacocks which inspired Darwin to propose sexual selection (*5*). Fossil evidence shows feathered dinosaurs exhibited feather pigment patterns (*6*, *7*). Compared with their reptile ancestors endowed with multiple types of chromatophores, birds have only pigment cell type, melanocyte (*8*). Feathers and hairs are ectodermal organs derived from convergent evolution (*4*, *9*), and melanocytes in both display color variation and share basic molecular pathways (*4*, *8*). In hair, melanocyte stem cells (McSCs) reside in the follicle bulge (*10*). Upon activation, they give rise to progeny in the matrix to color hair fibers (*10*, *11*). Feather colors are complex (fig. S1) and the mechanisms for pigment patterning is poorly understood (*2*, *12*). Here we explore the cellular and molecular basis of pigment pattern formation.

First we mapped McSCs in regenerating feather follicles. In growing phase, melanocytes (pigmented or apigmented) were found in the epithelium above papilla ectoderm (Fig. 1, A and B, and fig. S2). Among different chicken breeds and non-Gallus birds, we found small apigmented $Mitf^{+}$ $MART\text{-}1^{+}$ melanocytes in the lower to middle collar bulge region (Fig. 1B and fig. S3). Melanocytes in the lower bulge were negative for differentiation markers TRP1 and TYR (Fig. 1A and fig. S2B) and thus represented melanocyte progenitors. From the middle bulge and above, there were progressively increasing levels of Mitf, MART-1, TRP1, TYR and melanin, as well as increases in melanocyte cell size and dendricity (Fig. 1, B and B″, and fig. S2B).

To gauge their cell proliferative behavior, we employed BrdU labeling. Immature melanocytes in the lower bulge retained label longer than those in the middle bulge or above (fig. S4A). Lower bulge melanocytes were of low density (fig. S5) and exhibited low levels of proliferating cell nuclear antigen (PCNA) (17.4 ± 6.5%). These slow-cycling apigmented melanocytes retained BrdU for 8 days, much shorter than hair McSCs that retain label for about 70 days (*10*). The results indicate melanocyte progenitors cycle more actively in feathers than in hairs. To track movement of their progeny, DiI labeling revealed a vertically upward melanocyte flow from the lower bulge to barb ridges (Fig. 1C). In vitro experiments also showed that these cells could proliferate and differentiate (fig. S4B). Hence, the immature melanocytes in the lower bulge are melanocyte progenitors, possibly including McSCs.

We then examined dynamic changes during feather cycling (*9*). When feathers entered resting phase, melanocyte progenitors descended to the papilla ectoderm and became negative for PCNA (Fig. 1, A′ and B′, and fig. S6), consistent with a quiescent state. Transplantation of melanocyte progenitors from adult quail pigmented feathers to chicken embryos showed quail cells can be incorporated into developing feather buds and produce pigment (Fig. 1D). As feather follicle epithelium is cylindrical in configuration, melanocyte progenitors are distributed in a ring in 3D with distinctive dynamic behavior in different feather cycle phases (Fig. 1, B and B′, and figs. S3C and S6C).

To explore pigment patterning, we first determined whether feather whiteness is achieved by one or multiple cellular mechanisms. White feathers from White Leghorn, Speckled Sussex, and Delaware chickens were examined. Apigmentation in barbs could be caused by either absence of melanocytes or by suppressed melanocyte differentiation (figs. S7 and S15A). When melanocytes were absent from barb ridges, progenitors could be either present or absent in the stem cell niche (figs. S7 and S15A). Thus one color pattern can be achieved using different cellular mechanisms.

Feathers are made from the distal to proximal end, so we explored the roles of temporal control in pigment patterning. Flight feathers from Taiwan Country chickens were studied because the feather changed from a distal black vane to a proximal white calamus (fig. S8A), suggesting a temporal transition of melanocyte behavior. When the apigmented calamus was forming, melanocytes were absent above the collar, but melanocyte progenitors were still present in the lower collar. Only limited numbers of pigmented melanocytes were found in the middle collar (0-3 cells/section). Although keratinocytes still proliferated to form the calamus, melanocyte progenitors were negative for PCNA (fig. S8B). Hence,

pigment patterns can form by temporal regulation of melanocyte progenitor activity. Some progenitors are preserved for the next regenerative cycle and patterning potential is conserved.

We then explored how periodically striped patterns can be created. The horizontal barring of Barred Plymouth Rock feathers (Fig. 2A) is a classical model for pigment patterning (*13*, *14*). This fascinating sex-linked trait is semi-dominant; homozygous males have wider white bands than heterozygous males, and previous work has implicated both coding and noncoding variation at the CDKN2A/B tumor suppressor locus as a potential cause (*14*). When the growing feather follicle cylinder was cut open vertically from the posterior side, a chevron-shaped barring pattern was revealed (fig.S9). Serial sections showed the bars traveled toward the anterior side (Fig. 2B). The melanocyte lineage was absent in and below the apigmented regions (Fig. 2A), but melanocyte progenitors were still preserved in posterior collar bulge. Thus black and white barbs can be divided into four phases (early black, late black, early white, late white) with a fluctuating presence of melanocyte progenitors (Fig. 2B′ and fig. S10, A to B′). Loss of melanocyte progenitors is caused by premature differentiation, not apoptosis (fig. S10C). The cyclic presence and absence of melanocyte progenitors implies a feedback mechanism (figs. S10D and S11). The results are consistent with a gain-of-function alteration in the CDKN2A/B tumor suppressor locus that causes premature melanocyte progenitor differentiation in a temporally and topologically restricted manner. The genetic defect in Barred Plymouth Rock chickens may cause aberrant feedback control that displays as repetitive stripes (*14*) (fig. S11). Chevron patterning and the circular McSC niche topology make the periodicity possible by allowing deletion of progenitors in the anterior while preserving progenitors in the posterior niche. There are certainly other striped feathers that are not based on this genetic defect (*3*), but we expect them to follow similar principles (figs. S11 and S15B).

The two-dimensional feather vane allows more complex pigment patterns to form. Feathers from guineafowls, Silver Spangled Hamburg and Silver Laced Wyandotte chickens show multiple spots, a single distal spot and a laced edge, respectively. Melanocytes were present in apigmented regions but remained undifferentiated (Fig. 3, A and B, and figs. S12 and S13, A to D). To test whether these cells could still differentiate, melanocytes from apigmented barbs were cultured. Under α-MSH stimulation, they were induced to express tyrosinase activity (fig. S13, E and F).

Surprisingly, this rescue failed when they were co-cultured with adjacent pulp (fig. S13G), suggesting pigment modulators might be secreted from adjacent mesenchyme. Agouti signaling protein (ASIP), an antagonist of α–MSH (*15*, *16*), is an important mammalian pigment modifier capable of switching eumelanogenesis to pheomelanogenesis and regulating melanocyte precursor behavior in hair follicles (*17–20*). Its role in feather pigmentation has been proposed but not well-defined (*21*). We searched for sources of pigment modulators by quantitative PCR and whole-mount in situ hybridization, and found ASIP was consistently up-regulated in peripheral pulp adjacent to apigmented barbs (Fig. 3, C and C′, and fig. S13H). When we inserted ASIP-coated beads into the pulp adjacent to regenerating black feather barbs, a white zone was produced from the distal toward the proximal, reflecting inhibition of melanocyte differentiation during feather growth (Fig. 3D and fig. S13I). Thus activators and inhibitors can work together to modulate pigment pathways and produce a spectrum of complex patterns (*22–24*).

A unique feature of plumage regeneration is that feathers can change from a downy to sexually dimorphic appearance under physiological conditions (*1*) (fig. S14A). Feathers with different pigment patterns grew from the same follicle and had similar melanocyte precursor distributions (fig. S14B). Altered ASIP expression in the pulp of sexually dimorphic feathers corresponded to apigmented regions with suppressed melanocyte differentiation (Fig. 4A). Classical transplantation experiments which swapped feather germs between different follicles suggested that dermal papillae with attached papilla ectoderm serve as the signaling center which stores color patterning information (*25*). Thus properties of the stem cell niche can change in response to systemic factors and this effect can be mediated by mechanisms including altered ASIP expression in the pulp. Though inhibition of melanocyte differentiation by the mesenchyme occurs in human palmoplantar skin (*26*), the pattern is fixed for life. In birds, an additional dimension of control has evolved to pattern ASIP expression in the pulp dynamically in different regenerating cycles, permitting melanocyte progenitor activity to be modulated by systemic hormones.

In summary, we found several principles behind the formation of complex pigment patterns in avian plumage (*27*). We learned that complex patterns are not encoded directly in DNA but result from interaction between a DNA-encoded cellular property and physical-chemical principles (*27*). We propose a generic model to represent the progression of melanocyte lineage in regenerating feathers (fig. S15B). The evolution of the multidimensional niche topology increases patterning possibilities, allowing various regulations to be choreographed with greater temporospatial freedom in the new morphogenetic space (Fig. 4B). I. Pigmentation can be patterned along the proximal-distal feather axis, which is also a timing axis (*28*). II. The cylindrical follicle configuration (*24*) creates a novel medial-lateral dimension once the feather vane opens, providing more patterning possibilities. III. Interactions with peripheral pulp provide a third dimension of regulation. IV. Systemic factors, such as hormonal status or seasonal changes, add a fourth dimension of regulation (*1*). With variation in each dimension, the combinatorial potential of pattern complexity is enormous. In addition, variation in pigment chemistry and structural colors contributes to richer color patterns (*2*, *3*). While these were not studied here, we expect the patterning principles to be similar. Thus the complexity of avian pigment patterns is enriched by co-opting mechanisms regulating simple melanocyte behavior (fig. S15).

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Supporting Materials
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Materials and Methods
Figs. S1 to S15
References (*29*–*38*)

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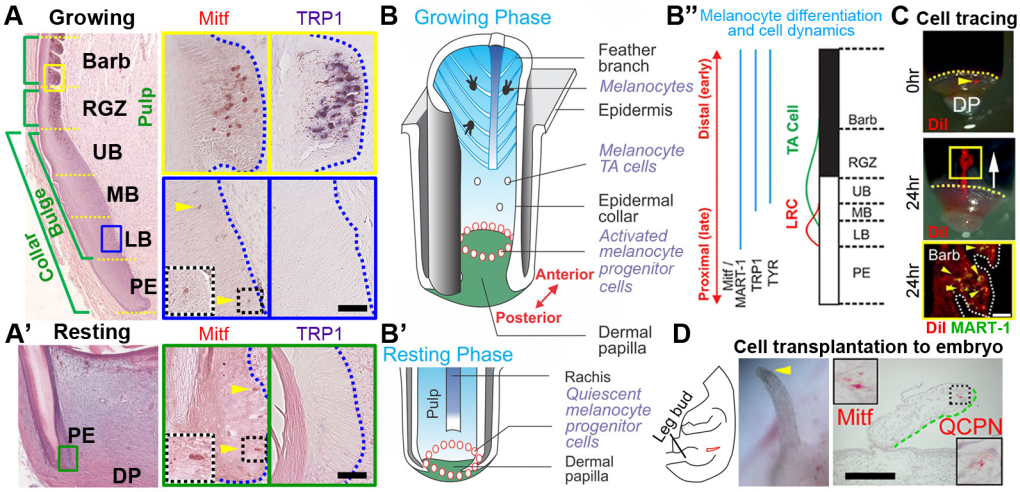
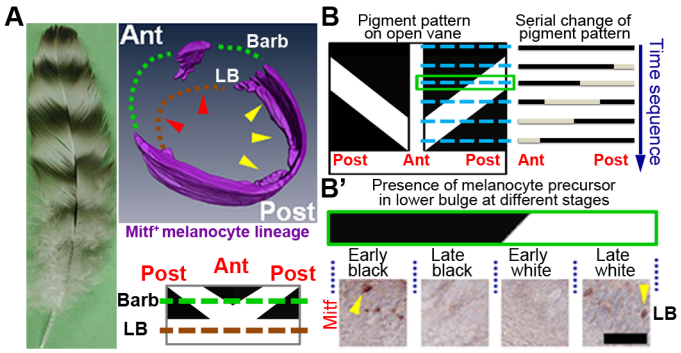


Fig. 1. Identification of feather melanocyte progenitor and its niche. (**A**) In growing phase, undifferentiated melanocytes were in the lower bulge (blue box) and differentiated melanocytes in the barbs (yellow box). (**A′**) In resting phase, undifferentiated melanocytes were in the papilla ectoderm (green box). Progenitors are indicated by yellow arrowheads. (**B** and **B′**) Schematic drawing showing feather follicle and cells within. (**B″**) Summary of melanocyte progenitor markers (fig. S2). (**C**) DiI labeling at LB (yellow arrowhead, 0 hour) and tracing showed labeled cells moved upwards to barb ridges (yellow box, 24 hours; white arrow indicates direction of cell movement). Some DiI positive cells expressed melanocyte marker, MART-1, in the barb ridges at 24 hours of tracing (arrowheads, lower panel). (**D**) Cell transplantation from quail resting papilla ectoderm (from black feather) to White Leghorn chicken embryo (left). The developing feather bud became pigmented (yellow arrowhead, middle). QCPN staining confirmed that Mitf^{+} pigment cells were derived from quails (right). LB: lower bulge; LRC: label retaining cell; MB: middle bulge; PE: papilla ectoderm; RGZ: ramogenic zone; TA: transit amplifying; UB: upper bulge. Scale bars: 50 μm in (A), (A′), and (C); 150 μm in (D).

Fig. 2. Melanocyte progenitor niche topology allows periodic stripe formation in feathers from Barred Plymouth Rock chickens. (**A**) Three-dimensional reconstruction of Mitf^{+} melanocyte lineage (purple) showed presence/absence (yellow/red arrowheads, respectively) of progenitors in lower bulge (LB). Lower panel depicts the pigment pattern when the specimen was sampled. Green/brown dashed lines indicate the level of barbs/LB, respectively. (**B**) Pigment patterns formed at different time points showed a shift of alternating black and white bands toward the anterior. (**B′**). The boxed pigment pattern in (B) was divided into four phases. Melanocyte progenitors were present only in late white and early black (yellow arrowheads). Ant: anterior; Post: posterior; LB: lower bulge. Scale bar: 50 μm.



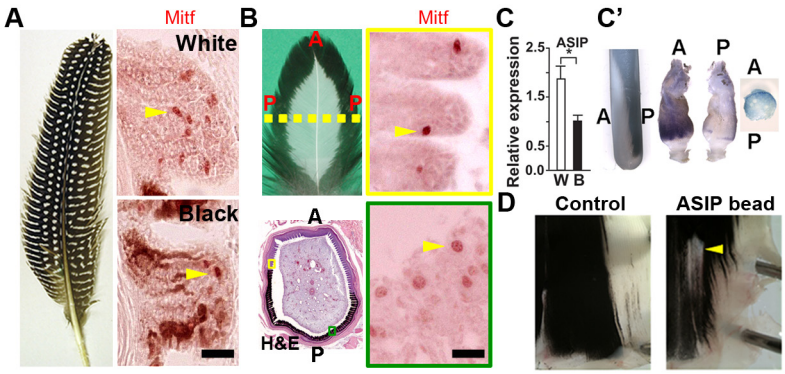


Fig. 3. Peripheral pulp inhibits melanocyte differentiation. (**A**) Feather from guineafowl. Mitf^{+} melanocytes were present in both pigmented and apigmented barb ridges (yellow arrowheads). (**B**) Breast feather from Silver Laced Wyandotte chicken. The dashed line indicates the sampling time point. Mitf^{+} melanocytes (yellow arrowheads) were present in both white and black barb ridges. (**C**) Quantitative PCR showed higher ASIP expression in the white region. * $P < 0.05$ ($N = 3$). (**C'**). Whole-mount in situ hybridization demonstrated higher ASIP expression in peripheral pulp adjacent to apigmented epithelium. (**D**) Insertion of ASIP-coated beads into growing follicles in vivo induced a white stripe (yellow arrowhead). A: anterior; P: posterior; B: black; W: white. Scale bar: 25 μm.

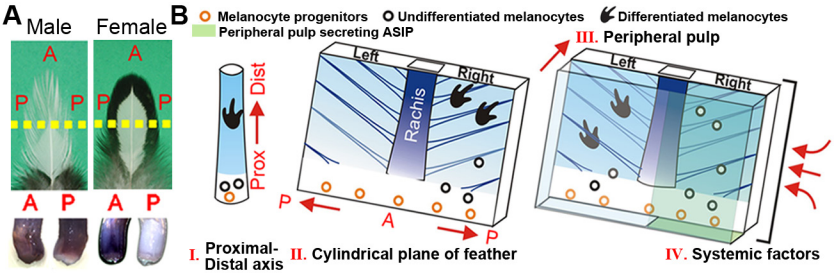


Fig. 4. Physiological regulation and principles of feather pigment pattern formation. (**A**) Wing covert feathers in adult Silver Laced Wyandotte chickens showed sexually dimorphic pigment patterns (upper panel). Whole-mount in situ hybridization revealed higher ASIP expression corresponding to apigmented regions (lower panel). Dashed line indicates the sampling time. A: anterior; P: posterior. (**B**) Multidimensional regulation of simple melanocyte behavior (fig. S15) allows temporospatial regulatory freedom.