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Author manuscript

Nature. Author manuscript; available in PMC 2015 April 10.

Published in final edited form as:

Nature. 2005 December 15; 438(7070): 1026–1029. doi:10.1038/nature04222.

## Mapping stem cell activities in the feather follicle

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### Abstract

It is important to know how different organs ‘manage’ their stem cells. Both hair and feather follicles show robust regenerative powers that episodically renew the epithelial organ. However, the evolution of feathers (from reptiles to birds) and hairs (from reptiles to mammals) are independent events and their follicular structures result from convergent evolution. Because feathers do not have the anatomical equivalent of a hair follicle bulge, we are interested in determining where their stem cells are localized. By applying long-term label retention<sup>1</sup>, transplantation<sup>2</sup> and DiI tracing to map stem cell activities, here we show that feather follicles contain slow-cycling long-term label-retaining cells (LRCs), transient amplifying cells and differentiating keratinocytes. Each population, located in anatomically distinct regions, undergoes dynamic homeostasis during the feather cycle. In the growing follicle, LRCs are enriched in a ‘collar bulge’ niche. In the moulting follicle, LRCs shift to populate a papillar ectoderm niche near the dermal papilla. On transplantation, LRCs show multi-potentiality. In a three-dimensional view, LRCs are configured as a ring that is horizontally placed in radially symmetric feathers but tilted in bilaterally symmetric feathers. The changing topology of stem cell activities may contribute to the construction of complex feather forms.

Feathers are of great interest because their robust regenerative ability can produce different feather morphologies from common feather precursor cells. In the past decade, much was learned about hair stem cells<sup>1–3</sup>. In comparison, little is known about feather stem cells. On the basis of surgery and dermal papilla transplantation, it was proposed<sup>4, 5</sup> that cells in the follicle collar<sup>6</sup> (corresponding to the hair matrix; Fig. 1a and Supplementary Fig. 1a) have the enduring capacity to produce subsequent feather generations, but it was not known which region was essential. Papillar ectoderm, a population of keratinocytes tightly associated with the dermal papilla, was considered to have regenerative capacity; but after stripping off the papillar ectoderm, feathers could still regenerate<sup>7, 8</sup>. How the epidermal collar generates the feather is another long-standing unresolved issue. One view is that the collar is an anlage of a pre-patterned feather<sup>8</sup>. Another is that the collar cells sustain proliferation potential to generate epithelial cells that are then specified<sup>4</sup>.

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

The authors declare no competing financial interests.

Here we apply modern stem cell biology concepts and current technologies to revisit these fundamental biological issues. Three criteria were used to map stem and progenitor cell activities in the feather follicles during the growth and moulting phases: long-term label retention for slow-cycling cells<sup>9, 10</sup>, grafting for multi-potentiality<sup>1</sup>, and DiI tracing for epidermal cell migration<sup>11</sup>. More than 60 yr ago, a region was identified in the collar as a “columnar epithelium of collar with elongated nuclei”, but the function of this region was not known<sup>4</sup> (Supplementary Fig. 1b). Here we show that this region, which we name the ‘collar bulge’, is a niche enriched with LRCs that show stem cell characteristics. We further analyse the dynamics of LRCs and stem or progenitor cell activities during physiological feather cycling.

We began by identifying LRCs in growing feather follicles. One-month-old chickens were given drinking water containing 5-bromo-deoxyuridine (BrdU) for 1 week, which labelled all epithelial cells (Fig. 1b, left, and Supplementary Fig. 1c). During the chase period, cells undergoing active proliferation lost their label, but the collar bulge was enriched in label-retaining cells (Fig. 1b, middle, arrow). Some randomly scattered epidermal cells also retained label (arrow-head). By contrast, a 1-h pulse of BrdU labelled transient amplifying cells in the basal layer, excluding the collar bulge region (Fig. 1b, right).

We mapped the multipotency of these cells by measuring how they are incorporated into hosts. The first assay involved quail–chicken grafting<sup>12</sup>. We microdissected adult quail follicle epithelium into several fragments (Fig. 1c, left, and Supplementary Fig. 2a) and transplanted them into chicken limb buds at embryonic day 5 (E5). From the explanted bulge, quail keratinocytes were incorporated into the feather bud, interbud epidermis (Fig. 1c, middle), basal layer of barb ridges and barbule plates, among others (Supplementary Fig. 2b). By contrast, transplants from the ramogenic zone (the region where transient amplifying cells start to differentiate and form branches; see Fig. 1a), intermediate layer (suprabasal layers in the collar), or follicle sheaths did not participate in the formation of host skin (Fig. 1c, right), and the papillar ectoderm showed a low incorporation rate (Table 1). We further verified these results by pre-labelling cells with BrdU before grafting. LRCs migrated out and participated in skin morphogenesis, whereas transient amplifying cells stayed at the site of transplantation (Supplementary Fig. 2c). The second assay used adult follicles as hosts. To avoid immunological rejection, we used DiI to label microdissected follicular fragments, which were autologously transplanted to a different follicle. After 5 d, the bulge-derived cells were incorporated into different structures in the feather filaments, whereas fragments from the ramogenic zone or feather sheath were not (Supplementary Fig. 2d).

We then traced the lineage and fate of feather precursor cells in the collar region with BrdU pulse–chase and DiI labelling (Supplementary Fig. 3). In both, we observed that transient amplifying cells in the lower feather follicle generate cells that became feather filaments, feather sheaths, follicle sheaths and interfollicular epidermis (Fig. 1d). This is consistent with the notion that the collar region contains the source of stem and transient amplifying cells, favouring the previous view that new cells are generated and then specified<sup>4</sup>.

Feather follicles go through cycles of tissue remodelling to moult and to regenerate<sup>6, 13</sup>. We next considered what happens to the stem cells during this process. In the resting phase, the

calamus (the feather shaft beneath the vane) keratinizes and the collar is reduced in size. Towards the base, the calamus tapers and eventually the wedge-shaped terminus is dislodged from the rest (Fig. 2a–c). Owing to shrinkage of the collar, the bulge morphology is lost, but LRCs remain and shift towards the lower collar, eventually coming in direct contact with the dermal papilla (Fig. 2a). In the moulting phase, LRCs become nested by the dermal papilla (Fig. 2b and Supplementary Fig. 4), taking the position of the papillar ectoderm in the growth phase follicles (yet the papillar ectoderm in the growth phase is not populated with LRCs). Dislodging of the old feather is followed by regeneration of a plump collar and initiation of new feather growth. The bulge reforms and gradually ascends back to the higher position. LRCs are diffuse in the beginning (Fig. 2c) and then become restricted, reassuming the typical collar bulge configuration in growth phase (see Fig. 1b, middle). Proliferating-cell nuclear antigen staining was used to locate transient amplifying cells in these remodelling follicles. Not much proliferation was found in the moulting phase follicles (Fig. 2, right panels). In the initiation phase, cells, excluding those in the LRC region, started to proliferate.

We then considered how cells in the moulting phase behave in the graft assay. From the resting follicle, we can only isolate the calamus-forming zone, the remaining collar (containing the papillar ectoderm region next to the dermal papilla, populated by LRCs at this stage) and the follicle sheath. Transplantation from quails to chicken hosts showed that cells from the calamogenic zone and the follicle sheath were not incorporated into the host. However, the remaining collar epithelium showed a high efficiency of incorporation into developing skin (Table 1 and Supplementary Fig. 2e). Transplantation of DiI-labelled fragments into adult follicles gave similar results (data not shown). Cells in the follicle sheath labelled with DiI showed no movement in the resting phase follicle and a little bit of movement in the moulting phase follicle, but resumed an upward movement when follicles entered the initial growth phase (Supplementary Fig. 3e).

Because there are different forms of feathers, we considered how LRCs are configured in the growth phase of downy (radially symmetric) and flight (bilaterally symmetric) feather follicles (Fig. 3a). Marginal plates stained for Sonic hedgehog (Shh) were used to help to delineate the orientation of barb ridges in opened adult feather follicle preparations<sup>14, 15</sup> (Fig. 3b). Through helical barb ridge organization, barbs reach the rachis (the feather shaft in the vane) with an angle  $\theta$  (angle of helical growth)<sup>16</sup>. This angle is big in flight feathers, but nearly  $0^\circ$  in downy feathers (Fig. 3b). When feather follicles at the growth phase were sectioned along the anterior–posterior plane, we found that LRC-positive bulges were at the same level along the anterior–posterior axis in downy feathers, but formed a slant (lower on the rachis side) in flight feathers (Fig. 3c). This implies that the LRC ring is tilted towards the rachis side in these feathers. A three-dimensional reconstruction of serial sections<sup>17</sup> indeed shows that LRCs are configured as a horizontally placed ring in adult downy feathers, but a tilted ring in flight feather follicles (Fig. 3d, e).

The LRC tilting angle  $\alpha$  approaches  $0^\circ$  in downy feathers, but increases in flight feathers, reaching a maximum angle of about  $45^\circ$ . We analysed cellular and molecular asymmetries at the ramogenic plane. In embryonic downy feathers, barb ridges form around the same time<sup>18</sup>. In flight feathers, new barb ridges are generated sequentially from the barb

generative zone in the posterior feather follicle<sup>19</sup>. With helical organization towards the anterior follicle, the barb ridges slant to create the rachis<sup>16</sup>. This anterior–posterior maturation gradient is demonstrated by the expression of feather keratin A (Fig. 3f). Previous work has shown that bone morphogenetic protein (BMP) and Shh are involved in the formation of the barbs and rachis<sup>13, 15</sup>. We now can investigate further whether the tilting LRCs are related to the BMP–Shh pathway, the maturation gradient and positioning of the rachis.

Our studies show that although feather and hair follicles arose separately around 155–225 million years ago, they both evolved to have LRCs, transient amplifying cells and differentiating keratinocyte populations. Both evolved a follicular structure with localized growth zones in the proximal end. In both, the dermal papilla, sitting in the follicle base, is essential for the cyclic regeneration, and both share morphogenesis signalling pathways. There are also differences. Feather LRCs are located in the proximal feather follicle and bulge into the mesenchymal pulp. The hair follicle bulge is contiguous with the outer layer of the follicle—the outer root sheath<sup>10</sup>. The different topology implies that feathers and hairs use different strategies to preserve their stem cells during moulting. As a consequence, cell migration patterns are also different. In hair follicles, a downward flux of bulge to outer root sheath to hair matrix<sup>1</sup> is followed by the upward flux to form hair filaments<sup>20</sup>. In feathers, two upstream migrations are observed: collar to ramogenic zone to feather filament, and collar to follicle sheath to interfollicular epithelium. Another difference is that feather LRCs are not as quiescent as hair follicle bulge cells, and the label was diluted after 1 month. This may be due to the faster growth rate of feathers. In other organs, the duration of long-term retention has been shown to depend on different local proliferation dynamics<sup>9</sup>. However, the comparison of hairs and feathers renders support to the concept that LRCs and transient amplifying cells are in transient homeostasis and can populate different niches (such as the bulge or papillar ectoderm) as regulated by the state of the microenvironment through different phases of their growth cycles<sup>21–23</sup>, and that the bulge stem cell model<sup>1</sup> is of paramount importance to the maintenance and regeneration of episodically renewing organs.

Another feature of the feather follicles is that the topology allows unusual evolutionary possibilities. Early feathers were radially symmetric, lacked a rachis and were composed of equivalent barb branches, similar to the downy feathers found today. A rachis emerged later, making feathers bilaterally symmetric, and set up the basis for further evolution towards flight<sup>24–26</sup>. It is compelling to speculate that the tilting LRCs may help to break the radial symmetry in the ramogenic plane, setting up the anterior–posterior maturation gradient and hence the bilateral symmetry (Fig. 3g). However, this hypothesis remains to be tested. So, what makes the LRC ring tilt? Swapping dermal papilla between downy and flight feathers showed that both feather symmetry and LRC configuration are determined by the dermal papilla (Z.Y., T.-X.J., R.B.W. and C.-M.C., unpublished data). This implies that feather symmetry is not irreversibly molecularly encoded in specific feather follicles. Rather, feather stem cells are true stem cells that can be moulded into different feather forms, depending on the niches created by the dermal papilla. Thus, here we have answered several decade-old questions about feather stem cells, but have exposed new areas to be explored concerning how different complex forms can be built from epidermal stem cells from one cycle to the next.

## METHODS

### BrdU labelling

For pulse labelling, chickens were injected intraperitoneally with BrdU (Sigma) at 50mg per kg (body weight). Feathers were collected 1 h later. For label-retaining studies of growth phase follicles, 1-month-old chickens were fed BrdU in their drinking water ( $1 \text{ mg ml}^{-1}$ ) for 1 week, and ‘chased’ (left to metabolize the BrdU in their system) for 1 week, 2 weeks, and so on. Samples were fixed in 4% paraformaldehyde, mounted in paraffin, sectioned at  $8 \mu\text{m}$ , stained with antibody against BrdU (Chemicon) and counterstained with haematoxylin and eosin.

In chickens, neighbouring flight feathers on the wing moult in sequential order. The order helps us to predict the feather cycle stage of a given feather and the timing was verified by vascularity in the calamus region. In our colony, flight feathers moult in chicken aged about 2 months. At 3–4 weeks before the expected events, we fed chickens BrdU for 1 week and chased for 2 weeks. The birds were then killed so that flight feathers at different cycling stages coexisted from the proximal to the distal wing.

### DiI labelling

DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecule Probes) was prepared as described<sup>11</sup>. We anaesthetized 3-month-old chickens with 10 mg per kg (body weight) of ketamine and xylocaine (2:1). DiI was injected through the follicle wall. Follicles were collected at desired times and processed.

### Immunostaining and *in situ* hybridization

Immunostaining and *in situ* hybridizations were processed as described<sup>27</sup> with an automated Discovery system (Ventana Medical Systems). RNA probes for feather keratin A (nucleotides 571–1099; X17511) have been described<sup>28</sup>.

### Multipotentiality grafting assay

For quail–chicken grafting, different parts of quail feather follicles were dissected (Fig. 1c and Supplementary Fig. 2a) and transplanted to E5 chicken embryo limb buds. At various stages of development, embryos were collected, fixed and sectioned. Samples were stained for quail-cell-specific antigen (QCPN antibody, Hybridoma Bank).

For adult follicle transplantation, similar dissections were done, labelled with DiI and then transplanted back to another feather follicle in the same chicken. Follicles were collected after 4–5 d.

### Three-dimensional reconstruction

We used ten sections ( $42 \mu\text{m}$  between each) for reconstruction<sup>29</sup>. Sections were digitized using IGL Trace software (<http://synapses.bu.edu/tools/trace/trace.htm>). These images were aligned and rendered as a three-dimensional view of LRC using Rhinoceros NURBS modelling for Windows (Robert McNeel and Associates).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

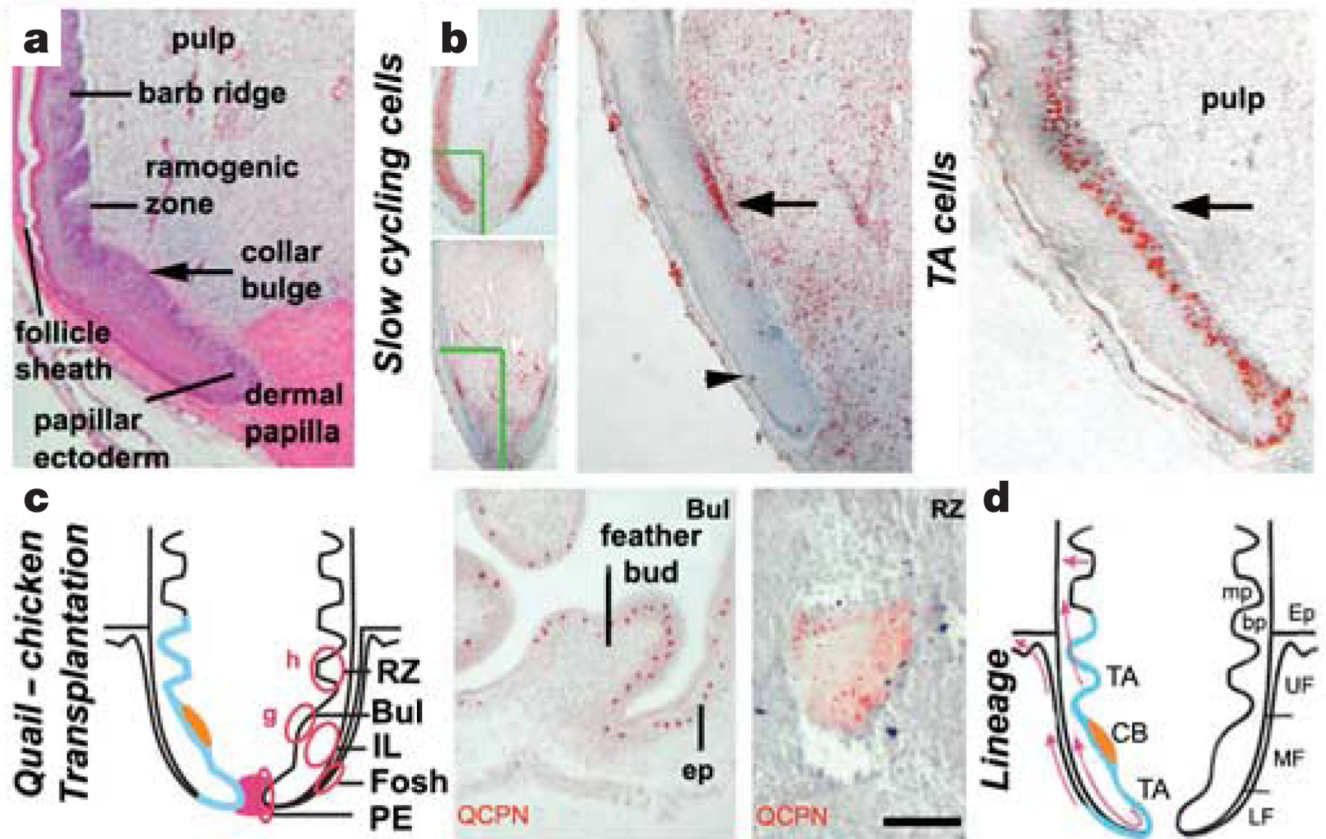
We thank G. Cotsarelis and R. Prum for helpful input; and all members of the Chuong laboratory for discussion. This work is supported by grants from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (to C.M.C) and from the National Cancer Institute (to R.B.W.).

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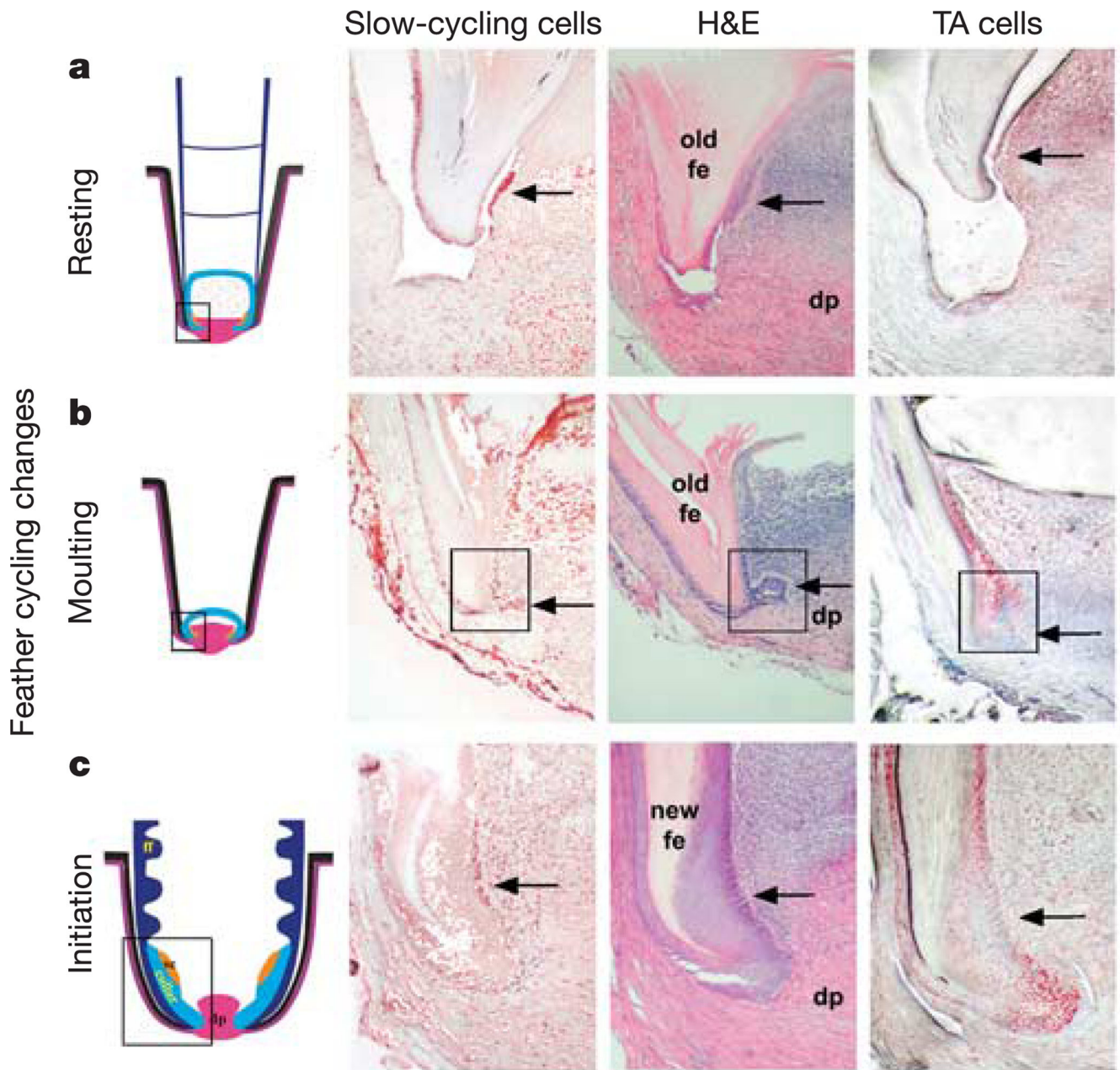




**Figure 1. Identification of feather epithelial stem cells**

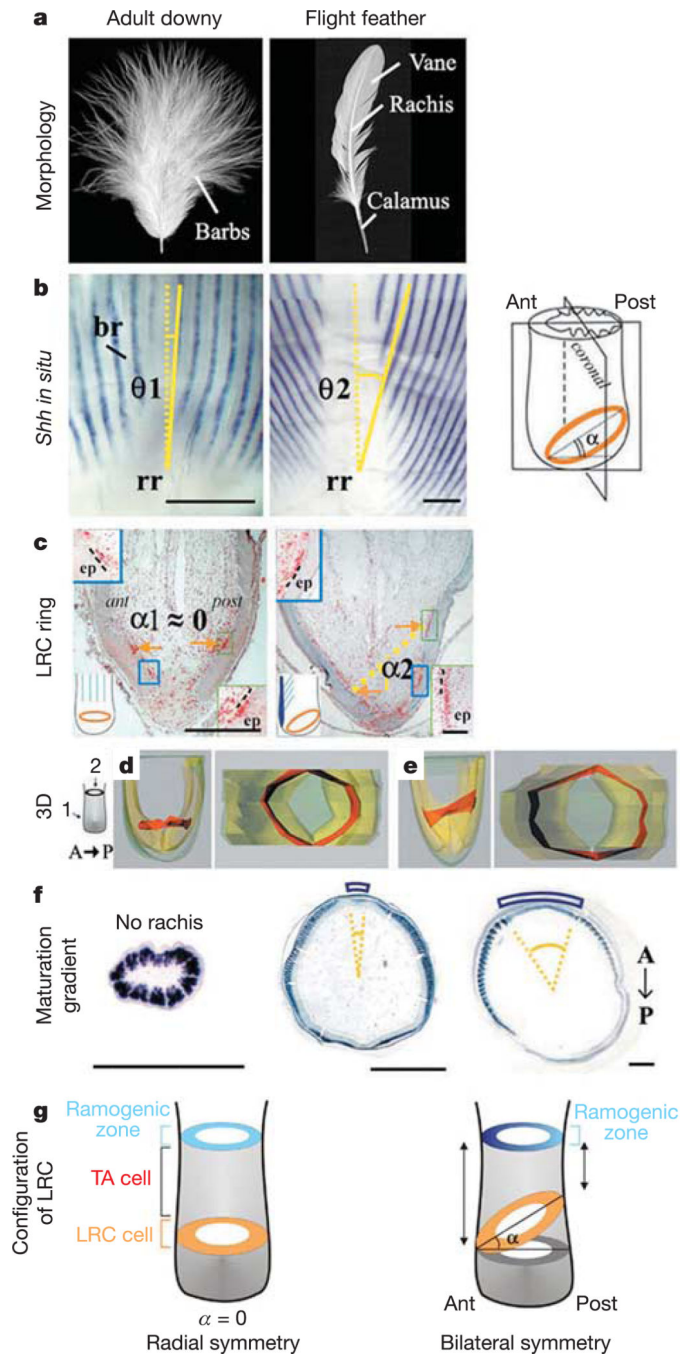
**a**, Feather follicle. Half a feather follicle is shown with haematoxylin and eosin staining. See Supplementary Fig. 1a, b for a more complete illustration. **b**, Left, BrdU labelling before (top) and 1 week after (bottom) chase in the whole follicle and collar region. Middle, enlargement of boxed area in bottom left image. Enlargement of boxed area in top left image is shown in Supplementary Information. Right, a 1 h pulse of BrdU leaves the collar bulge unlabelled. Arrows indicate LRC; arrowhead indicates scattered LRCs. **c**, Left, regions dissected from adult quail feather follicles for grafting to chicken embryos (see Supplementary Fig. 2 for microdissection and Table 1 for results). Collar bulge (Bul) transplant after 5 d (middle) shows that quail cells (red; stained with QCPN antibody) are incorporated into bud, interbud and barb ridges. Transplants from the ramogenic zone (RZ; right), papilla ectoderm (PE), intermediate layer (IL) and follicle sheath (Fosh) do not show incorporation (Table 1). Dark grains in right image are carbon particle markers. **d**, Summary of epithelial cell migration in regenerating feather follicles. bp, barb plate; CB, collar bulge; LF, lower follicle sheath; MF, middle follicle sheath; mp, marginal plate; TA, transient amplifying cells; UF, upper follicle sheath. Scale bar, 100  $\mu$ m.





**Figure 2. Stem cells during moulting**

LRC and transient amplifying cells were identified in resting (a), moulting (b) and initiation phase (c) follicles. Specimens (right panels) represent boxed regions in the schematic drawings (left panel). Slow-cycling cells (arrow), haematoxylin and eosin stained (H&E) cells, and transient amplifying (TA) cells are shown. Boxed regions from moulting phase follicles are enlarged in Supplementary Fig. 4. Note the changing positions of the LRC niche to accommodate the remodelling of feather follicles. Because stem cells in the feather cycle faster than hairs, LRCs in different stages have to be labelled at different times; therefore, these LRCs may represent the original stem cell population or cells that move into the niche under homeostasis and show stem cell activities. dp, dermal papilla; fe, feather.



**Figure 3. Configurations of LRC cells in different types of feather**

**a**, Gross morphology. **b**, Whole-mount *in situ* hybridization for *Shh* stains marginal plate (regions between barb ridges) to reveal barb ridges (br) and their angle  $\theta$  with the rachidial ridge (rr). **c**, LRC cells (orange) form a horizontal ring (downy feathers) or an angled ring with a tilt angle  $\alpha$  (flight feathers). Lower left insets show drawings for orientation. Also shown are higher magnification views of LRCs in the epithelium (ep, green box) and mesenchyme (blue box). Broken line delineates the basement membrane. Light blue indicates barbs; dark blue indicates rachis. **d**, **e**, Three-dimensional reconstruction of serial

sections showing that the LRC ring (red) is horizontal in radially symmetric downy feathers (**d**) but tilted in bilaterally symmetric flight feathers (**e**). The images show side (left) and top (right) views. **f**, Cross-sections at the level of the ramogenic zone. *In situ* hybridization of feather keratin A shows homogenous staining in natal (left) and adult downy (middle) feathers, but an anterior–posterior (A–P) maturation gradient in flight feathers (right). The blue arc and yellow lines delineate the rachis. **g**, Hypothetical model. In bilaterally symmetric flight feathers, the LRC ring forms a tilting angle  $\alpha$ . Asymmetry in the LRC region may break the radial symmetry at the ramogenic zone, leading to the formation of a maturation gradient and bilateral symmetry. Scale bars: 100  $\mu\text{m}$  (**b**); 0.5 mm (**c**, **f**).

**Table 1**

Multipotency of follicular components in the quail–chicken graft assay

	<b>Transplantation donor</b>	<b>LRC</b>	<b>% incorporation</b>	<b><i>n</i></b>
Growth phase	Intermediate layer	–	0	10
	Ramogenic zone	–	0	10
	Collar bulge	+	57	14
	Papillar ectoderm	±	12.5	8
	Follicle sheath	–	0	4
Moulting phase	Calamogenic zone	–	0	4
	Remaining collar (including PE)	+	75	8
	Follicle sheath	–	0	4

Different feather follicular components from growth and moulting phase adult quail feather follicles were dissected (Fig. 1c) and grafted to chicken embryonic limb buds. *n* indicates number of experiments. Quail feather fragment incorporation into chicken skin is shown as a percentage. The presence of a LRC population is based on data from Figs 1 and 2. In one out of eight experiments, papillar ectoderm (PE) from growth phase follicles shows incorporation.