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Ultrastructural Characteristics of 5BrdU Labeling Retention Cells Including Stem Cells of Regenerating Feathers in Chicken

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Abstract

Feathers regenerate from stem cells localized in a region of the follicle indicated as the bulge of the collar. Stem cells are slow cycling cells and some of these cells can be identified after labeling experiments using 5-bromo-deoxyuridine to detect label retaining cells (5BrdU LRCs). The present electron microscopic analysis of 5BrdU LRCs has described the ultrastructural characteristics of small cells present in the bulge region of the follicle in regenerating feathers of chickens that include stem cells. Labeled feather stem cells are smaller than 10 μm in average diameter, possess large nuclei with high nuclear/cytoplasmic ratio, and contain evenly distributed ribosomes, sparse bundles of intermediate filaments, scarce or no endoplasmic reticulum, and few mitochondria. The nuclei are mainly euchromatic with a variable amount of heterochromatin clumps and the nucleoli show developed granular and fibrillar components. These features indicate that feather stem cells are transcriptionally active cells for ribosomal and proteins synthesis. The cell surface of feather stem cells often shows small and irregular folds resembling microvilli in contact with the surrounding cells. The latter characteristics may be related to the exchange of molecules and/or with the migration of stem cells among other epithelial cells of the collar epithelium.

Keywords

chick; feather regeneration; follicle; stem cells; ultrastructure; immunocytochemistry

INTRODUCTION

Feathers are regenerated through the life of birds from germinal cells localized inside the follicle (Chuong and Widelitz, 1999; Sawyer et al., 2003; Prum, 2005; Maderson et al., 2009). Birds can fully regenerate their feather either through seasonal molting or by plucking at both growth stage or resting stage. As laboratory conditions do not have seasonal change, current feather regenerating studies are conducted by plucking. In juvenile feathers, germinal cells are localized in the follicle after migrating from apical regions of the feather

filament in downfeathers into the forming follicle (Chodankar et al., 2002). After the first replacement of downfeathers with juvenile feathers, stem cells remain localized in the lower part of the collar inside the follicle (Fig. 1A, B). At any successive molting, germinal cells are activated and give rise to the anagen phase (Spearman and Hardy, 1985) of feather regeneration, but true stem cells are also activated following plucking (Yue et al., 2005).

Recent studies have shown that feather stem cells, in part identified by the detection of label retaining cells (LRCs), are mainly present in a ring-like region, the bulge, localized deep in the follicle between the lowermost collar in contact with the dermal papilla (the papillary collar) and the ramogenic zone where barb ridges are formed (Yue et al., 2005; Fig. 1B). In the ramogenic zone, cells become determined to form barb ridges as previously described (Spearman and Hardy, 1985; Chuong and Widelitz, 1999; Yu et al., 2004; Alibardi, 2005, 2007a, 2007b). Barb ridges merge into an axial rachis following a helical growth along the collar (Prum, 2005; Alibardi, 2008) but immature barbs initially remain curved inside the sheath (Fig. 1A). After the sheath is lost starting from the apical regions of the feather filament, barbs can distend into a planar vane (Maderson et al., 2009; Fig. 1B).

In hairs, a bulge region was previously identified, which represents a cell niche (Tumbar et al., 2004), and this also appears to be the case for feathers where stem cells tend to localize in a definite anatomical region, also indicated as the bulge region (Yue et al., 2005, 2006). Feather stem cells move from the bulge to the papillary collar in contact with the dermal papilla and give rise to transient amplifying cells for the sheath and other parts of the follicle. Other stem cells move toward the intermediate layer adjacent to the bulge and migrate upward in the ramogenic zone to give rise to barb ridges. While the plane of localization of stem cells is horizontal in downfeathers or in plumulaceous feathers (more or less parallel to the skin surface) in pennaceous feathers, this plane is tilted and this topological change appears responsible for the fusion of barb ridges into a rachis (Fig. 1A,B).

The cytological characteristics of stem cells in feathers are not known, and the present immunocytochemical study analyzes the ultrastructural features of 5-bromo-deoxy-uridine label retaining cells (5BrdU LRCs) present in the follicle of the regenerating feathers of the chick, in an attempt to detect whether stem cells have unique morphological features distinguishing them from the surrounding cells of the feather follicle.

MATERIALS AND METHODS

Five white leghorn chickens (two adults and three juveniles) (*Gallus gallus* Linnaeus, 1758) were utilized. Two chickens were sacrificed and the skin of the wing, pectoral and dorsal regions containing regenerating feathers, was immediately fixed for the ultrastructural study of the follicles. Fixation lasted for 8–12 h in 2.5% glutaraldehyde in 0.12 mol l⁻¹ phosphate buffer at pH 7.2–7.4. Tissues, represented by skin containing feather follicles, were rinsed in buffer for 20 min, and postfixed in 2% OsO₄ in buffer for 90 min, dehydrated in graded ethanol, infiltrated in propylene-oxide, and embedded in Durcupan resin. These tissues were sectioned at 1–3 µm thickness, in oblique or longitudinal sections with an ultramicrotome, and the sections were stained with 0.5% toluidine blue for the light microscopic study. From

selected areas, thin sections (60–90-nm thick) from the basal part of the follicle were also collected for the ultrastructural study. Thin sections were attached to copper grids, stained with uranyl acetate-lead citrate according to routine methods, washed in double-distilled water, dried, and then observed under a Philips CM-100 transmission electron microscope operating at 80 kV.

Three other chickens (1 month old) were treated with 5BrdU as previously indicated (Yue et al., 2005). Briefly, 10 wing cover feathers at resting phase were plucked from these chickens. After a week, these chickens incorporated 5BrdU diluted in drinking water (containing 1 mg/ml of 5BrdU) for a week (pulse period), and later received normal water for 2 weeks (to chase the tracer). The animals were sacrificed and the sample tissues from the regenerated region containing two to three feather follicles in their fourth week of regeneration, were fixed in 4% paraformaldehyde in 0.1 mol l⁻¹ phosphate buffer at pH 7.2 for 8–12 h, dehydrated in ethanol, infiltrated with Bioacryl or LR-White Resins for about 8 h (two samples), and embedded in these resins under UV at 4°C for 3 days (Scala et al., 1992). Another sample was instead embedded in paraffin for the detection of 5BrdU LRCs.

Semithin sections (2–4-µm thick) or paraffin sections (5–6-µm thick) containing feather follicles were collected with an ultramicrotome or a mictome and attached to gelatin-chromalum coated slides. The sections were pretreated with 0.2 mol l⁻¹ HCl for 2 h, rinsed in buffer, and incubated overnight at 0–4°C with monoclonal anti-5BrdU (Sigma or Chemicon), diluted 1:100 in 0.05 Tris buffer with 1% bovine serum albumine, at pH 7.2. Sections were rinsed in the buffer and incubated for 1 h at room temperature with secondary Biotinylated Goat anti Mouse IgG antibody (Vector Laboratories, 1:200 dilution) for 1 h and third antibody Streptavidin (Vector Laboratories, 1:200 dilution) for 30 min. AEC substrate kit (Vector Laboratories) was used to develop staining. Hematoxylin was used to perform a faint counterstaining.

From selected areas of the follicle, further sections were obtained with an ultramicrotome and collected on nickel grids. On these sections, the immunodetection of 5BrdU-labeled nuclei present in the tissue was done after incubating the grids in the primary antibody as above. After rinsing in buffer, the sections were incubated for 1 h at room temperature with an anti-mouse IgG conjugated with 10 nm colloidal gold (Sigma) diluted 1:50 in buffer. Finally, some of these sections underwent a silver-enhancement procedure using the Silver-Enhancement kit developed by (British Biocell International, Bristol). Sections were rinsed in buffer, then in double-distilled water, stained for 4 min in 2% aqueous uranyl acetate solution, dried, and observed using an electron microscope Zeiss 10C/CR.

RESULTS

Light Microscopic Immunolocalization of Label Retaining Cells

The longitudinal sections of the basal part of the follicle in juvenile and adult feathers showed the typical aspect of the collar surrounding the dermal papilla (Figs. 1 and 2). In numerous sections, the upper part of the collar showed a fold slightly protruding in the pulp, indicated as bulge and containing small immunolabeled cells (Fig. 2A,B). Sparse labeled cells were also seen in the dermal papilla and in the pulp.

The examination at higher magnification of the collar epithelium, including that of the bulge in adult feathers, showed that the basal or germinal epithelium contacting the papilla was made by cubic to columnar epithelial cells, often with a pseudostratified organization (Fig. 2B). In the bulge and in the nearby epithelium, small immunolabeled cells (less than 10 μm in diameter) were seen in the innermost layers, in contact with the pulp. In the middle part of the collar at the level of the bulge, indicated as intermediate layer (Lucas and Stettenheim, 1972; Alibardi, 2007b), larger and differentiating unlabeled or occasionally labeled cells were present (arrowheads in Fig. 2B). External cells of the collar tended to become spindle shaped and externally flat when these cells were incorporated in the forming sheath (Fig. 2B).

The immune-detection of 5BrdU-labeled nuclei of the feather follicle showed sparse labeled nuclei within the collar but more frequently labeled nuclei were seen in the upper collar in correspondence with the bulge region (arrows in Fig. 2). A few labeled nuclei were also present in the intermediate layers (arrowheads in Fig. 2B), in the pulp, and sparse cells were seen in the dermal papilla. Although these are static observations, the sparse labeled nuclei might indicate migrating stem cells from the bulge region into other areas of the collar. The main regions of the collar, dermal papilla and pulp, where labeled cells have been analyzed with the electron microscope are indicatively shown as numbers in Figure 2B: each number (3–5) represents a region corresponding to the following electron microscope images (indicated as Figs. 3–5).

Ultrastructural Features of Stem Cells Localized in the Collar

The ultrastructural preservation of details of cell organelles of tissues embedded in Bioacryl or LR-White Resins (Figs. 3 and 4) cannot be compared to that of tissues fixed in glutaraldehyde and embedded in Durcupan resin (Fig. 5). In fact, because of the extraction of cellular components in Bioacryl or LR White embedded tissues and to the limited staining needed to reveal the gold or gold-silver intensified particles over the cellular organelles, some enlarged vesicles in the cytoplasm of the small pale cells were sometimes present. Despite these technical problems, the overall cytoplasmic and nuclear characteristics of the small cells embedded in the hydrophilic resins were similar to the small cells observed in the hydrophobic Durcupan resin sections.

After immunogold-labeling using gold particles of 10 nm in diameter, sparse cells in the bulge area, previously seen with the light microscope (Fig. 2B, see areas indicated as 3 and 4), were also detected using the electron microscope (Fig. 3). The nuclear labeling in these prevalently euchromatic cells appeared mainly present over the euchromatin and the perinuclear chromatin but was less frequently observed or absent over heterochromatin clumps and was in general absent over the nucleolus (Fig. 3A,B).

The silver-enhancement method, producing large albeit irregular dark particles over the nuclei, allowed the rapid detection of LRCs in the bulge (Fig. 4) and also in the dermal papilla and pulp. Small cells of 6–10 μm in diameter localized in the upper collar and in the bulge region (areas indicated as 3 and 4 in Fig. 2B) often appeared electron-paler than dermal cells or other epithelial cells (Fig. 4A). Some of these small cells contacted the basement membrane, whereas other labeled cells apparently did not contact the basement

membrane (pseudostratified epithelium) and were contained within the collar epithelium. Their nuclei were ovoidal and occupied most of the cell (relative high nuclear/cytoplasmic ratio) so that their cytoplasm was not very extensive. The small cells possessed mainly euchromatic nuclei with one or two nucleoli while a low amount of clumps of heterochromatin were seen, especially associated with the nuclear membrane while inner chromatin clumps were generally sparse (Fig. 4B).

Despite the amount of heterochromatin present in these cells, the 5BrdU immunolabeling was generally seen over euchromatin and on the perinuclear areas while it was generally absent or occasional over the smaller areas occupied by the nucleolus (Figs. 3 and 4). The surrounding cells of the bulge area that showed similar characteristics of labeled cells but presented unlabeled nuclei were much more frequently observed than labeled cells. This confirmed the optical immunocytochemical observations indicating that stem cells or however 5BrdU LRCs were relatively few among the germinal epithelium of feathers.

The examination of cells localized in the bulge using better preserved sections (fixation in glutaraldehyde and embedding in Durcupan) showed that nucleoli presented a more or less equally developed granular and fibrillar components, although the latter in some sections appeared sometimes more extensive than the former (inset in Fig. 5). A specific and systematic study on the variations of these nucleolar components was however not done. Numerous free ribosomes, sparse mitochondria, and rare cisternae of endoplasmic reticulum, dictyosomes of the Golgi apparatus, or other cell organelles were present in these LRCs. Also keratin bundles, common in cells of the intermediate and especially of the differentiating outer layers, were absent or sparse in isolated filaments of the small pale cells (Fig. 4B). A characteristic feature of these small cells was the presence of short and irregular blebs or interdigitating microvilli on the cell surface (Fig. 5B). Few cell junctions (generally desmosomes) were seen along the perimeter or the interdigitating microfolds of these small cells. Some hemidesmosomes were also seen in contact with the basement membrane (double arrowheads in Fig. 5B), although they were less frequently seen than in epithelial cells of other regions of the collar. However, a quantitative study on the number of desmosomes or hemidesmosomes was not done because of the relatively limited sample available for these cells.

DISCUSSION

The identification of stem cells in the bulge derived in part from the detection of LRCs in regenerating follicle following a prolonged administration of 5BrdU, a cell proliferation marker (Yue et al., 2005). This is a characteristic of slow cycling cells such as stem cells while proliferating cells (transient amplifying cells) dilute the labeling as they divide numerous times to generate the high number of cells required for the formation of a new feather. In the lower part of the follicle epithelial cells present cytological characters of immaturity (Alibardi, 2010). Aside stem cells they may represent actively proliferating transient amplifying cells. Other cells, present in the “intermediate layer” of the collar epithelium (Lucas and Stettenheim, 1972), localized between the germinal layer and the sheath, are probably already committed to migrate in the ramogenic zone and differentiate

into barb/barbule cells, or to migrate externally to form the sheath (Alibardi, 2005, 2006, 2007a, 2007b).

Although LRCs include true stem cells, not all LRCs present in different tissues or organs are formed by stem cells. For example in isolated hematopoietic stem cells only 6% of these cells were LRCs (Kiel et al., 2007), but this percentage is not known for the stem cells present in other organs, including feathers. Overall, the ultrastructural characteristics of feather LRCs that include some true stem cells in the bulge region (Yue et al., 2005) are typical for immature cells, as they have previously been described for different tissues, including the epidermis and hairs (Costarelis et al., 1990; Akiyama et al., 2000). Feather LRCs and stem cells contain few cell organelles and keratin bundles, a cytoplasm almost devoid of endoplasmic reticulum and Golgi apparatus, free ribosomes indicating the production of endogenous proteins. Differentiating cells located near the bulge become more fusiform and richer in keratin bundles. Other 5BrdU negative cells have also shown a simple ultrastructure, and therefore not all cells with simplified cytoplasmic features in the collar or bulge represent stem cells.

Some LRCs observed in the present study were attached to the basement membrane through few hemidesmosomes, although their number was not quantified. The number of hemidesmosomes is lower in stem cells present in the bulge region of the hair follicle in comparison to other epithelial cells (Pasolli et al., 2007). The presence of few cell junctions may indicate that feather stem cells can migrate into other regions of the collar, as indicated by the Dil-fluorescent tracing studies conducted on feather follicles (Yue et al., 2005).

The irregular surface and the presence of cytoplasm blebs in small cells of the collar have been observed also in other species, such as the ostrich and zebrafish (Alibardi, unpublished observation). An irregular cell surface was also described for stem cells present in the hair bulge (Costarelis et al., 1990) and in other types of stem cells (Radley et al., 1999; Rieger et al., 1999; Bazan et al., 2004). The presence of small cytoplasmic elongations may indicate an increase of the cell surface for exchange of molecules with the surrounding cells or that these cells are migrating (Yue et al., 2005).

The amount of heterochromatin varies in different stem cells although euchromatin remains more abundant than heterochromatin. The broad variation in heterochromatin versus euchromatin reported for stem cells from different tissues suggests that the transcriptional activity is variable in these cells. Satellite cells of muscles and blood stem cells generally present extensive heterochromatin areas, which indicate transcriptionally poor active cells (Rubinstein and Trobaugh, 1973; Kahn and Simpson, 1974; Radley et al., 1999; Thoenen et al., 2004). The nucleus of germinal cell precursors and of neurons is instead prevalently euchromatic (Talbot and Garret, 2001; Bazan et al., 2004; Minniti et al., 2005). The nuclei of neoblasts (stem cells) for regeneration in the flatworm planaria indicated that heterochromatin increases parallel to the content in cell organelles (Rieger et al., 1999).

Stem cells in the hair bulge (Costarelis et al., 1990; Akiyama et al., 2000; Pasolli et al., 2007) show prevalence of euchromatin but larger areas of perinuclear heterochromatin than in feather stem cells. The latter appear to contain prevalent transcriptionally active

euchromatin probably for the biosynthesis of endogenous proteins. The presence of functional nucleoli in stem cells, as indicated by the presence of both fibrillar and granular components, is likely connected to a continuous production of ribosomes that are later utilized for the rapid synthesis of endogenous keratins. Some of these cells give origin to amplifying keratinocytes of the intermediate layer and the sheath or to keratinocytes of the ramogenic collar where cells begin to differentiate and give rise to barb and barbule cells.

In conclusion, the observations on a number of hematopoietic, mesenchymal, epithelial, and germinal stem cells have indicated that the amount of inactive chromatin is variable in different stem cells, that their cytoplasm is mainly occupied by ribosomes and that their cell surface is irregular. Although a variable percentage of LRCs represent true stem cells in different organs, in feathers these cells appear transcriptionally active as indicated by their prevalent euchromatin and developed nucleolus.

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Fig. 1.

Gallus gallus, schematic drawing of a regenerating pennaceous feather. In **A**, the bottom part of the follicle contains the dermal papilla and the papillary collar. The plane of distribution of stem cells in the bulge (green in Fig. 1A) is tilted. Stem cells give rise to transient amplifying cells (yellow region in Fig. 1A) that migrate toward the upper ramogenic collar. In the latter region, cells give rise to the growing barbs (1–1', 2–2', 3–3', . . . , 10–10', indicated in blue and red colors inside the sheath) and the rachis. The bottom part of the follicle (in parentheses) is enlarged in **B** where the same regions are shown in longitudinal section and the bulge is visible. Above the dermal papilla is present the pulp

while barb ridges are forming in the ramogenic collar. BR, barb ridges; BU, bulge; DP, dermal papilla; E, epidermis bordering the follicle; F, follicle; P, pulp; PC, papillary collar; R, rachis; RC, ramogenic collar; S, sheath; SC, stem cell; and TA, transient amplifying.

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Fig. 2.

Gallus gallus, light microscopic micrographs of feather follicle featuring the position of 5BrdU LRCs (brown stain). (A) A low magnification view shows the different parts of the feather follicle with the cluster of labeled cells in the bulge and the dermal papilla. Numerous labeled cells are also seen in the pulp along the bulge and decrease in the central pulp and in the ramogenic region where barb ridges are seen. Bar, 250 μ m. (B) Detail at higher magnification of the bulge containing numerous labeled cells. The numbers from 3 to 5 point to similar areas later analyzed with the electron microscope and indicated with the

same numbers in the following figures (3–5). Few labeled cells are sparse in the intermediate layer (arrowheads), and no labeled cells are seen in the lowermost (papillary) collar and the sheath. Sparse LRCs are present in the loose connective tissue forming the pulp and few are seen in the dermal papilla. Bar, 100 μm . b, bulge; br, barb ridges; co, collar; dp, dermal papilla; pu, pulp; and sh, sheath.

Fig. 3. *Gallus gallus*, detail on the nuclei of two 5BrdU LRCs, immunolabeled with gold particles. (A) Closeup of a nucleus showing perinuclear labeling (arrow) and a diffuse labeling in the euchromatin. The heterochromatin clump and the nucleolus appear unlabeled. Bar, 200 nm. (B) Detail of a nucleus containing a meshwork of most unlabeled heterochromatin among labeled euchromatin. Bar, 100 nm. eu, euchromatin; hc, heterochromatin; n, nuclei; and nu, nucleolous.

Fig. 4. *Gallus gallus*, ultrastructural details of 5BrdU LRCs in the bulge (areas 3–5 in Fig. 2B). (A) Two labeled nuclei mainly along the perinuclear heterochromatin (arrows) while their nucleoli are unlabeled (arrowheads). The pale cytoplasm appears vacuolated. Bar, 1.5 μ m. (B) Detail of LRC showing the labeled chromatin but not the nucleolous, sparse small keratin bundles, a desmosome. In the electron-pale and vacuolated cytoplasm sparse ribosomes are seen. Bar, 0.5 μ m. cy, cytoplasm; de, desmosome; k, sparse small keratin bundles; nl, nucleolous; and nu, nuclei.

Fig. 5. *Gallus gallus*, light microscopical (**A**) and ultrastructural (**B**) details of small pale cells localized in the upper collar (bulge region) of an adult feather. (**A**) Longitudinal section of the collar (arrowheads) containing sparse small cells, and surrounding the dermal papilla and the pulp. Arrows indicate the areas where small cells are present. Bar, 40 μ m. (**B**) Small cell connected to the basement membrane of the collar epithelium through hemidesmosomes (double arrowheads). The asterisks indicate the darker cytoplasm of another type of epithelial cell present in this area. Arrows show the folds of the plasma membrane while

double arrows indicate the few perinuclear heterochromatin clumps. Bar, 1 μm . The inset at the bottom right shows the dense fibrillar centers and the granular centers (arrows) of the nucleolus. Bar, 150 nm. de, cytoplasm of a dermal papilla cell; dp, dermal papilla; f, fibrillar centers of the nucleolus; n, nucleus; p, pulp; and pc, papillary collar.