Nestin as a marker of unipotent epithelial progenitor cells differentiate into outer root sheath keratinocytes in embryonic and adult hair follicles

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Article

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Abstract

Nestin is an intermediate filament protein transiently expressed in neural stem/progenitor cells. We previously demonstrated that outer root sheath (ORS) keratinocytes of adult hair follicles (HFs) in mice descend from nestin-expressing cells, despite being an epithelial cell lineage. This study aimed to define the exact stage when nestin-expressing ORS stem/precursor cells or their descendants appear during HF morphogenesis, and whether they are also recognized in adult HFs. Using *Nes-Cre/CAG-CAT-EGFP* mice, in which enhanced green fluorescent protein (EGFP) is expressed following Cre-based recombination driven by the nestin promoter, we found that $EGFP^+$ cells appeared in the epithelial layer of embryonic HFs as early as the peg stage. $EGFP^+$ cells in hair pegs were positive for keratin 5, but not vimentin, Sox2, Sox10, or S100 alpha 6. Tracing of tamoxifen-induced $EGFP^+$ cells in postnatal *Nes-CreERT2/CAG-CAT-EGFP* mice revealed labeling of some isthmus HF epithelial cells in the first anagen stage. $EGFP^+$ cells in adult HFs were not immunolabeled for keratin 15, an HF multipotent stem cell marker. However, when hairs were depilated to induce the anagen stage after tamoxifen injection, the majority of ORS keratinocytes in depilation-induced anagen HFs were labeled for EGFP. Our findings indicate that nestin-expressing unipotent progenitor cells capable of differentiating into ORS keratinocytes are present in both the HF primordia and adult HFs.

Introduction

The hair follicle (HF) is a complex structure consisting of several layers of keratinocytes arranged in concentric circles [26]. The bulge region of the outer root sheath (ORS), the outermost layer of the HF where the arrector pili muscle attaches, is the stem cell niche for HF components [23]. Previous studies demonstrated that keratin 15 (K15)-expressing HF bulge cells are multipotent epithelial stem cells capable of differentiating into keratinocytes and sebocytes in the HF epithelia and interfollicular epidermis [10, 20].

Nestin, a class VI intermediate filament protein, is a specific marker of neural stem/progenitor cells [17]. Embryonic nestin-positive cells can differentiate into neurons and glial cells. In addition, nestin expression occurs in multiple cell types in adult tissues, such as skeletal muscle satellite cells [6], pancreatic islets [32], testis [12], and the heart [13]. Moreover, studies using nestin-driven green fluorescent protein (ND-GFP) transgenic mice revealed that ND-GFP cells in the upper HF are multipotent, as they can differentiate into cell lineages with characteristics of neural cells, glial cells, muscle cells, melanocytes, and keratinocytes *in vitro* [2–5, 18]. Previously, we reported that ORS keratinocytes of adult mice are the descendants of nestin-expressing cells, despite being an epithelial cell lineage [24]. This study aimed to define the exact stage when nestin-expression ORS stem/precursor cells appear during HF morphogenesis. We also investigated whether such stem/precursor cells are present in adult HFs.

Materials And Methods

Mice

The *Nes-Cre/CAG-CAT-EGFP* mouse line [24] was generated by crossing *CAG-CAT-EGFP* mice (courtesy of Junichi Miyazaki, Osaka University, Japan), in which the chloramphenicol acetyltransferase (*CAT*) gene is flanked by two loxP sites [14], with *Nes-Cre* mice (courtesy of Ryoichiro Kageyama, Kyoto University, Japan) [9]; both mouse strains have a C57BL/6 background. The *Nes-CreERT2/CAG-CAT-EGFP* mouse line was generated by crossing *CAG-CAT-EGFP* mice with *Nes-CreERT2* mice (courtesies of Itaru Imayoshi and Ryoichiro Kageyama, Kyoto University, Japan), which harbor the *CreERT2* gene downstream of the nestin promoter and also have a C57BL/6 background. *CAG-CAT-EGFP* and *Nes-CreERT2* mice were provided by the Center for Animal Resources and Development at Kumamoto University. *Nes-CreERT2* mice were provided by RIKEN BioResource Research Center through the National Bio-Resource Project of MEXT (Japan).

The Animal Research Committee and Specific Biosecurity Management Subcommittee of Tokyo University of Agriculture and Technology approved all experiments using genetically-arranged mice with approval numbers of #25–70 and #29–76, respectively. All animal experiments were carried out in accordance with Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines [16]. Mice were kept in cages with standard woodchip bedding in a conventional mouse room under constant room temperature (25ºC) and humidity (40%) and a 12 h light/dark cycle with ad libitum feeding/drinking.

Administration of 4-hydroxy-tamoxifen (OHT) to *Nes-CreERT2/CAG-CAT-EGFP* mice

OHT (10 mg; Cayman Chemical; Ann Arbor, MI, USA) was dissolved in ethanol to obtain a 100 mg/mL OHT suspension, which was further dissolved in corn oil (Merck, Darmstadt, Germany) to obtain a 10 mg/mL OHT solution. Finally, a volume of solution containing 1 mg of OHT was injected intraperitoneally for 5 consecutive days into *Nes-CreERT2/CAG-CAT-EGFP* mice aged 4 weeks or 7 weeks (n = 6, 20.1 ± 1.1 g body weight).

Tissue collection

Embryonic skin was harvested from *Nes-Cre/CAG-CAT-EGFP* mice (n = 6) at various gestational ages (embryonic days 15.5, 16.5, and 18.5) when early or advanced hair germs and hair pegs were recognized [26]. Trunk skin was also sampled from this mouse line at postnatal days 0 and 33 (P0 and P33), when lanugo and anagen HFs were recognized (n = 6) [22]. In addition, the dorsal skin of 4-week-old *Nes-CreERT2/CAG-CAT-EGFP* mice (n = 3) administered OHT for 5 consective days was sampled on the sixth day after initiation of OHT administration (Fig. 3a). Finally, 7-week-old *Nes-CreERT2/CAG-CAT-EGFP* mice (n = 3) were administered for OHT for 5 consective days, their dorsal hairs were depilated to introduce anagen HF on the sixth day after initiation of OHT administration, and their dorsal skin was sampled on the seventh day after depilation (Fig. 3a). Skin samples were fixed with 10% neutral buffered formalin, embedded in paraffin, and subjected to immunofluorescence analysis.

Immunofluorescence analysis

Paraffin-embedded formalin-fixed skin samples were sectioned, deparaffinized, and pretreated with 10 mM citrate buffer (pH 6.8) for antigen retrieval. Sections were then incubated with blocking buffer (5% goat serum, 3% skim milk, and 0.2% Tween 20 in phosphate-buffered saline) before incubation with the following primary antibodies: monoclonal mouse anti-GFP (clone 6AT316; Abcam, Cambridge, UK), polyclonal rabbit anti-GFP (Medical & Biological Laboratories, Nagoya, Japan), polyclonal rabbit anti-laminin (Abcam), monoclonal rabbit anti-K5 (Abcam), monoclonal mouse anti-K14 (clone LL002; Abcam), monoclonal mouse anti-K15 (clone LHK15; Abcam), monoclonal rabbit anti-vimentin (VIM) (clone EPR3776; Abcam), monoclonal rabbit anti-SOX2 (clone SP76; Abcam), monoclonal rabbit anti-SOX10 (clone EPR4007-104; Abcam), monoclonal rabbit anti-S100 alpha 6 (S100A6) (clone EPR13084-69; Abcam), and monoclonal mouse anti-trichohyalin (clone AE15). Slides were then labeled with either Alexa Fluor™ 488- or 546-conjugated secondary antibodies (Life Technologies, Carlsbad, CA, USA) or combinations thereof for double immunofluorescence. Nuclei were counterstained with Hoechst 33258 (Life Technologies). Slides were examined under a confocal laser-scanning microscope (LSM710NLO 2 photon, Carl Zeiss, Jena, Germany; Nikon AX, Nikon, Tokyo, Japan) and image data were captured using imaging software (ZEN, Carl Zeiss; Nikon AX R, Nikon).

Statistical analysis

A Mann-Whitney U test was performed to compare frequencies of $EGFP^+$ cells in epithelial cells at peg and bulbous peg stages. Furthermore, the same test was performed to compare ratios of $EGFP^+K14^+$ cells to $K14^+$ cells of anagen HFs between *Nes-Cre/CAG-CAT-EGFP* and OHT-administered *Nes-CreERT2/CAG-CAT-EGFP* mice using Graph Pad Prism8 software (GraphPad Software, San Diego, CA, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

$EGFP^+$ cells are present in the HF epithelium from the early hair peg stage in *Nes-Cre/CAG-CAT-EGFP* mouse embryos

We first examined the tissue distribution of $EGFP^+$ cells in lanugo HFs of *Nes-Cre/CAG-CAT-EGFP* mice at the neonatal (P0) time point. Immunofluorescence analysis revealed that outer HF epithelial cells from the upper isthmus to the inferior regions were uniformly immunolabeled for EGFP (n = 3) (Fig. 1). In addition, most $EGFP^+$ cells were double-positive for K14, an ORS keratinocyte marker (Fig. 1), but negative for trichohyalin, an inner root sheath keratinocyte marker (Fig. 1). The ratio of $EGFP^+K14^+$ cells to $K14^+$ cells in neonatal mouse HFs was 79.4% ± 9.7% (n = 6). In contrast, no $EGFP^+$ cells were found in the follicular epithelium of neonatal *CAG-CAT-EGFP* mice (n = 3) (Fig. 1), indicating the specificity of EGFP immunolabeling after Cre-based recombination driven by the nestin promoter. $EGFP^+$ cells were also recognized in spinous and granular layers, but not in basal layer of inter-follicular epidermis in neonatal *Nes-Cre/CAG-CAT-EGFP* mice (data not shown).

Based on the above findings, we hypothesized that progenitor cells of ORS keratinocytes start expressing nestin during the embryonic stage. Therefore, we performed double-immunofluorescence analysis for EGFP and laminin to investigate whether $EGFP^+$ cells are present in the epithelial layer during HF morphogenesis in *Nes-Cre/CAG-CAT-EGFP* mouse embryos (n = 6) ; laminin is expressed in the epidermal basement membrane. HF morphogenesis in an embryo begins with placode formation and then progresses through germ, advanced germ, peg, and bulbous peg stages [27]. Although we did not observe $EGFP^+$ cells in the epithelial cell layer during germ or advanced germ stages, they were present in dermis surrounding germs in *Nes-Cre/CAG-CAT-EGFP* mice (Fig. 2a). In addition, a small subset of $EGFP^+$ cells was observed in the epithelial cell layer of early hair pegs of this mouse line (Fig. 2a). During bulbous peg, the majority of HF cells expressed EGFP. Furthermore, the frequency of $EGFP^+$ cells in the epithelial cell layer during bulbous peg (95.1% ± 4.5%, n = 6, Fig. 2a) was significantly higher than in early hair peg (8.5% ± 2.3%, n = 6; Mann-Whitney U test, $p = 0.0022$).

$EGFP^+$ cells of *Nes-Cre/CAG-CAT-EGFP* mouse embryos exhibit characteristics of epithelial cells during the early hair peg stage

Next, we investigated whether $EGFP^+$ cells of *Nes-Cre/CAG-CAT-EGFP* mouse embryos resemble epithelial or mesenchymal cell lineages during the early hair peg stage. Double-immunofluorescence analysis revealed that $EGFP^+$ cells in hair peg epithelia were immunolabeled for K5 but not VIM (Fig. 2b), indicating that $EGFP^+$ cells were of an epithelial cell lineage. We could not detect whether $EGFP^+$ cells were immunolabeled for K14 because hair peg epithelial cells were only faintly stained by the K14 antibody used in this study. During the early hair peg stage, $EGFP^+$ cells in *Nes-Cre/CAG-CAT-EGFP* mouse embryos were not immunolabeled for neural stem cell markers SOX2 [25] or S100A6 [30], or the neural crest cell marker SOX10 [15] (Fig. 2b).

OHT-induced $EGFP^+$ cells capable of differentiating into ORS keratinocytes are present in HF isthmus epithelia of *Nes-CreERT2/CAG-CAT-EGFP* mice

We next investigated whether nestin-expressing stem/progenitor cells capable of differentiating into ORS keratinocytes are present in adult HFs. To achieve this, we performed double-immunofluorescence analysis of EGFP and K14 expression in OHT-administrered *Nes-CreERT2/CAG-CAT-EGFP* mice at 5 weeks of age, when HFs uniformly undergo their first anagen (n = 3) (Fig. 3a) [22]. We found that a small subset of $K14^+$ cells in the isthmic region was immunolabeled for EGFP in OHT-administered *Nes-CreERT2/CAG-CAT-EGFP* mice (Fig. 3b). Moreover, double-immunofluorescence analysis of EGFP and K15 revealed that $EGFP^+$ cells in HFs (Fig. 3b) were not immunolabeled for K15. We further examined whether $EGFP^+$ cells are present in depilation-induced anagen HFs of *Nes-CreERT2/CAG-CAT-EGFP* mice (n = 3) (Fig. 3a). Our results revealed that the majority of $K14^+$ cells were immunolabeled for EGFP in depilation-induced anagen HFs of OHT-administered *Nes-CreERT2/CAG-CAT-EGFP* mice. The median [range] frequency of GFP^+K14^+ cells in $K14^+$ cells of depilation-induced anagen HFs was 92.1% [85.9–96.8%] (n = 6), which was significantly higher than the frequency observed in first anagen (6.5% [4.5–10.0%], n = 6) (Mann-

Whitney U test, $p = 0.0022$; Fig. 3c). Keratinocytes in inter-follicular epidermis were not immunolabeled for EGFP in *Nes-Cre/CAG-CAT-EGFP* mice after 5 weeks of age (data not shown).

Discussion

During the embryonic stage, nestin is temporarily expressed in neuroepithelial stem/progenitor cells [7]. In this study, we found that nestin-expressing progenitor cells of ORS keratinocytes appear in the HF primordium as early as the hair peg stage. Moreover, these cells were immunolabeled for K5, suggesting that they have characteristics of epithelial progenitor cells. Co-expression of nestin and cytokeratins was previously recognized in progenitor cells for lens epithelial cells [31] and Sertoli cells [8] in mouse embryos. However, these cells were not immunolabeled for neural stem cell markers SOX2 or S100A6. Progenitor cells of mouse ORS keratinocytes are reportedly not derived from stem/progenitor cells expressing neural crest cell markers Wnt1 or plasminogen activator [29]. Taken together, nestin-expressing progenitor cells of ORS keratinocytes in HF primordia are postulated to be epithelial cells that are not derived from neural crest cells. However, our results do not exclude the possibility that progenitor cells for ORS keratinocytes are derived from nestin-expressing mesenchymal cells that trans-differentiate into epithelial cells during a brief period of the early peg stage.

This study failed to demonstrate $S100A6^+$ cells in epithelia during the hair peg stage, consistent with a previous report showing an absence of $S100A6^+$ cells in hair germ epithelia [11]. Conversely, $S100A6^+$ cells have been identified in the bulge region of adult mouse HFs [11]. Therefore, $S100A6^+$ cells may appear in HF epithelia after the peg stage. Further studies to define the exact time $S100A6^+$ cells appear in HF epithelia are expected.

Our findings demonstrate that OHT-driven EGFP-expressing epithelial cells in the isthmic region of anagen HFs in 5-week-old *Nes-CreERT2/CAG-CAT-EGFP* mice are distinct from K15-expressing cells. $EGFP^+$ cells are either nestin-expresing cells or the descendants of progenitor cells expressing nestin in mice after 4 weeks of age. A previous study demonstrated the presence of nestin-positive, K15-negative cells in anagen HFs of human scalp [1]. The same study also indicated that GFP^+ cells located immediately below sebaceous glands express the stem cell marker CD34 but not K15 in ND-GFP mice. The stemness of $EGFP^+$ cells in the isthmus of *Nes-CreERT2/CAG-CAT-EGFP* mouse HFs remains to be elucidated. Notably, an epithelial cell population co-expressing GFP and K15 was previously recognized in the ORS of whisker HFs in ND-GFP mice [5]. We postulate that nestin was transiently expressed in murine anagen HFs before 4 weeks of age, or that nestin expression in $K15^+$ cells of trancal HFs was too weak to be detected in the mouse line used in this study.

Our findings further imply that ORS keratinocytes are descendants of postnatal nestin-expressing progenitor cells in mouse skin. Therefore, we postulate that nestin-expressing unipotent epithelial progenitor cells for ORS keratinocytes exist in first-anagen HFs. Based on a comparison of differentiation potencies between $EGFP^+$ cells and $K15^+$ HF bulge cells [19], nestin-expressing cells in adult HFs are

suggested to be downstream of $K15^+$ epithelial pluripotent stem cells. In contrast, previous studies revealed that GFP^+ cells in ND-GFP mice exhibit multipotency *in vitro* [5]. Moreover, GFP^+ cells in ND-GFP mice transdifferentiated into neural cells when subcutaneously transplanted into nude mice [28]. It is possible that some stimuli caused by cell culture or transplantation triggered pluripotency of those cells. Accordingly, the transdifferentiation capacity of nestin-expressing HF epithelial cells into distinct cell lineages *in vivo* remains to be elucidated.

Attempts have been made to regenerate complete HFs using totipotent or pluripotent stem cells [20]. However, to achieve more efficient HF regeneration, the detailed molecular mechanisms involved in differentiation of distinct cell lineages for each cell layer of HFs must be identified. Our study reveals a molecular signature of unipotent progenitor cells for the outermost HF cell layer in postnatal HFs. Future studies to elucidate molecular interactions necessary for differentiation of these multipotent stem cells into downstream cells are expected.

Declarations

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Footnote

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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Figures

Figure 1

Presence of $EGFP^+$ cells in HFs of neonatal *Nes-Cre/CAG-CAT-EGFP* mice. Truncal skin of *Nes-Cre/CAG-CAT-EGFP* (A) and *CAG-CAT-EGFP* (B) mice was collected at P0 and subjected to immunolabeling for EGFP (green). (C,D) Double-immunolabeling for EGFP (green) and (C) K14 (red) or (D) trichohyalin (red) in the outer layers of lanugo HFs of *Nes-Cre/CAG-CAT-EGFP* mice at P0. Nuclei were counterstained by Hoechst 33258 (blue). Scale bars, 20 μm.

Figure 2

$EGFP^+$ cells with characteristics of epithelial cells in *Nes-Cre/CAG-CAT-EGFP* mouse embryos during the hair peg stage. (a) Time-course analysis of protein expression in $EGFP^+$ cells of embryonic HFs in *Nes-Cre/CAG-CAT-EGFP* mice. Skin collected at germ (A), advanced germ (B), peg (C and D), and bulbous peg (E) stages was subjected to immunolabeling for EGFP (green) and laminin (white). (b) Immunolabeling for EGFP (A, E, I, M, R), K5 (B), VIM (F), SOX2 (J), SOX10 (N), and S100A6 (S) in hair peg epithelia. Nuclei were counterstained by Hoechst 33258 (C, G, K, O, T). A subset of cells in anagen hair bulbs, presumably melanocytes, were immunolabeled for SOX10 (Q). Scale bars, 10 μm (D, H, L, P, U), 20 μm (Q).

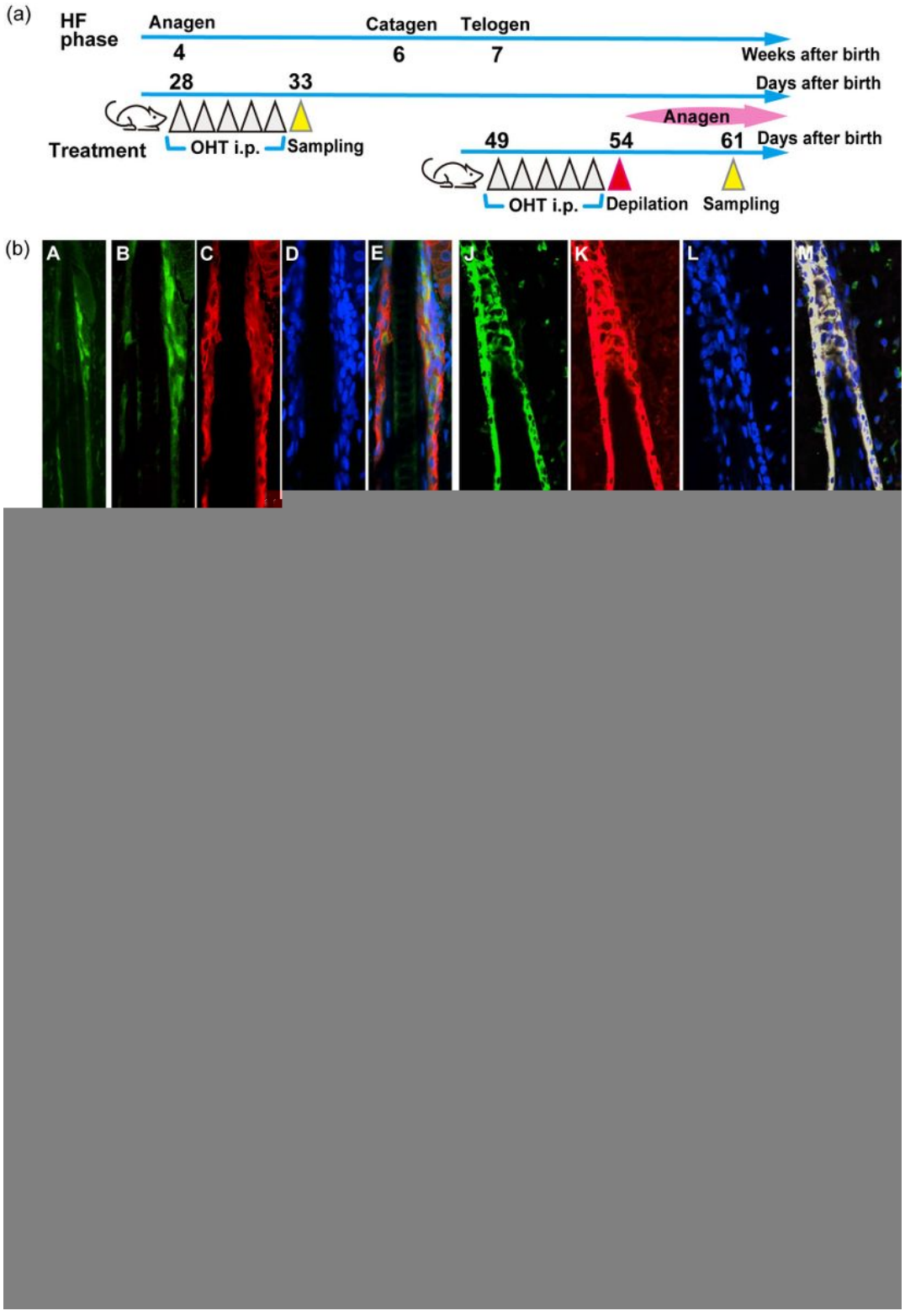

Figure 3

EGFP^{+} cells are present in postnatal HF epithelia and differentiate into ORS keratinocytes.

(a) Scheme of OHT induction to express EGFP under the nestin promotor, depilation of truncal hairs, and sample collection. (b) Immunolabeling of EGFP (A, B, F, J), K14 (C) and K15 (G) in first-anagen HFs (A–I) and depilation-induced anagen HFs (J–M) of *Nes-CreERT2/CAG-CAT-EGFP* mice. EGFP^{+} cells in the

isthmus region of first-anagen HFs (A, B, F) were immunolabeled for K14 (C) but not K15 (G). The majority of $EGFP^+$ cells in depilation-induced anagen HFs were immunolabeled for K14 (K). Nuclei were counterstained by Hoechst 33258 (D, H, and L). Scale bars, 20 μm (A, M), 10 μm (E, I). (c) Comparison of frequencies of $EGFP^+K14^+$ cells in $K14^+$ cells in first-anagen HFs with depilation-induced anagen HFs of OHT-administered *Nes-CreERT2/CAG-CAT-EGFP* mice (Mann-Whitney U test) (F). **, $p = 0.0022$.