Nestin is a marker of unipotent embryonic and adult progenitors differentiating into an epithelial cell lineage of the hair follicles

Yuta Baba¹, Saki Onishi-Sakamoto¹, Kaori Ide¹,2 & Koji Nishifuji¹,2*

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 determined the exact stage when nestin-expressing ORS stem/precursor cells or their descendants appear during HF morphogenesis, and whether they are present 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 14 (K14) and K5, 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 K15, an HF multipotent stem cell marker. However, when hairs were depilated in Nes-CreERT2/CAG-CAT-EGFP mice 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 HF primordia and adult HFs.

The hair follicle (HF) is a complex structure consisting of several layers of keratinocytes arranged in concentric circles. The bulge region of the outer root sheath (ORS), the outermost layer of the HF where the arrector pili muscle attaches, is a stem cell niche for HF components. 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.

Nestin, a class VI intermediate filament protein, is a specific marker of neural stem/progenitor cells. 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, pancreatic islets, testes, and the heart. Moreover, studies using nestin-driven green fluorescent protein (ND-GFP) transgenic mice revealed that ND-GFP cells in the upper HF are multipotent because they can differentiate into cell lineages with characteristics of neural cells, glial cells, muscle cells, melanocytes, and keratinocytes in vitro. In addition, recent genomic analysis during follicle morphogenesis suggested that a small amount of nestin gene is expressed in placodes and epidermal cells. Previously, we reported that ORS keratinocytes in adult mice were the descendants of nestin-expressing cells, despite being an epithelial cell lineage. 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 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, with Nes-Cre mice (courtesy of Ryoichiro Kageyama, Kyoto University, Japan); both mouse strains were on a C57BL/6 background. The Nes-CreERT2/CAG-CAT-EGFP mouse line was generated by...
crossing CAG-CAT-EGFP mice with Nes-CreERT2 mice (courtesy of Itaru Imayoshi and Ryoichiro Kageyama, Kyoto University, Japan), which harbor the CreERT2 gene downstream of the nestin promoter and which are on a C57BL/6 background. CAG-CAT-EGFP and Nes-CreERT2 mice were provided by the Center for Animal Resources and Development at Kumamoto University. CAG-CAT-EGFP and Nes-CreERT2 mice were also 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 #25-70 and #29-76, respectively. All animal experiments were carried out in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, and all methods were carried out in accordance with relevant guidelines and regulations. Mice were kept in cages with standard woodchip bedding in a conventional mouse room under constant room temperature (25 °C) 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. 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). In addition, the dorsal skin of 4-week-old Nes-CreERT2/CAG-CAT-EGFP mice (n = 3) administered OHT for 5 consecutive days was sampled on the sixth day after the initiation of OHT administration (Fig. 3a). Finally, 7-week-old Nes-CreERT2/CAG-CAT-EGFP mice (n = 3) were administered OHT for 5 consecutive days, their dorsal hairs were depilated to induce anagen HF on the sixth day after the 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 (2 μm), 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 (1:200; clone 6AT316; Abcam, Cambridge, UK), polyclonal rabbit anti-GFP (1:200; Medical & Biological Laboratories, Nagoya, Japan), polyclonal rabbit anti-laminin (1:200; Abcam), monoclonal rabbit anti-K5 (1:200; Abcam), monoclonal mouse anti-K14 (1:200; clone LL002; Abcam), monoclonal mouse anti-K15 (1:100; clone LHK15; Abcam), monoclonal rabbit anti-vimentin (VIM) (1:200; clone EPR3776; Abcam), monoclonal rabbit anti-SOX2 (1:50; clone SP76; Abcam), monoclonal rabbit anti-SOX10 (1:50; clone EPR4007-104; Abcam), monoclonal rabbit anti-S100 alpha 6 (S100A6) (1:200; clone EPR13084-69; Abcam), and monoclonal mouse anti-trichohyalin (1:200; clone AE15). Slides were then labeled with either Alexa Fluor 488- or 546-conjugated secondary antibodies (1:400; Life Technologies, Carlsbad, CA, USA) or combinations thereof for double immunofluorescence. Nuclei were counterstained with Hoechst 33258 (1:400; Life Technologies). Slides were examined using 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 Student’s t-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 the ratios of EGFP+K14+ cells to K14+ cells between the first anagen HFs and depilation-induced anagen HFs in OHT-administered Nes-CreERT2/CAG-CAT-EGFP mice using Graph Pad Prism8.2.1 software (GraphPad Software, San Diego, CA, USA). A p-value 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) by monoclonal and polyclonal anti-GFP antibodies (Fig. 1 and Supplementary Fig. S1). In addition, most EGFP+ cells were double-positive for K14 and K5, an ORS keratinocyte marker (Fig. 1), suggesting that the EGFP+ cells in ORS of lanugo HFs are the descendants of nestin-expressing cells. In contrast, the fluorescent intensity of EGFP in follicular keratinocytes expressing trichohyalin, an inner root sheath keratinocyte marker, was much lower than those in outer HF epithelial cell layer (Fig. 1). The ratio of EGFP+K14+ cells to K14+ cells in neonatal mouse HFs was 79.4% ± 9.7% (n = 6). Conversely, 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 present in spinous and granular layers, but not in the basal layer of the 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). HF morphogenesis in an embryo begins with placode formation and then progresses through germ, advanced germ, peg, and bulbous peg stages. Although we did not observe EGFP+ cells in the epithelial cell layer during germ or advanced germ stages, they were present in the 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 the bulbous peg stage, the majority of HF cells expressed EGFP. Furthermore, the frequency of EGFP+ cells in the epithelial cell layer during the bulbous peg stage (95.1% ± 4.5%, n = 6, Fig. 2a) was significantly higher than in the early hair peg stage (8.5% ± 2.3%, n = 6; Student’s t-test, p < 0.0001). Moreover, some dermal spindle cells were immunolabeled for EGFP, suggesting that these were nestin-expressing cells that eventually differentiate into papilla cells present in neonatal hair follicles or perivascular cells that make up the capillaries around hair follicles.

EGFP+ cells in Nes-Cre/CAG-CAT-EGFP mouse embryos exhibit characteristics of epithelial cells during the early hair peg stage. Next, we investigated whether EGFP+ cells in Nes-Cre/CAG-CAT-EGFP mouse embryos resembled 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. In contrast, most of these cells were not immunolabeled for VIM, although a few EGFP+ cells expressed VIM in a small area of cytoplasm (Fig. 2b). These findings indicate that EGFP+ cells in the early peg stage were of an epithelial cell lineage, although we could not exclude the possibility that they were derived from mesenchymal cells at an earlier stage of HF development. We could not determine whether EGFP+ cells were immunolabeled for K14 because hair peg epithelial cells were only faintly stained by the K14 antibody used in this study (data not shown). During the early hair peg stage, EGFP+ cells in HF epithelia in Nes-Cre/CAG-CAT-EGFP mouse embryos were not immunolabeled for the neural stem cell markers SOX2 or S100A6, or the neural crest cell marker SOX10 (Fig. 2b). Dermal papilla cells in the early hair peg stage were immunolabeled for VIM, SOX2, and S100A6, indicating the specificities of the antibodies used in this study. Moreover, EGFP+ cells in hair bulb epithelia of postnatal Nes-Cre/CAG-CAT-EGFP mice were immunolabeled for SOX10, demonstrating the specificity of the anti-SOX10 antibody to melanocytes. Our study also revealed that some dermal spindle cells were immunolabeled for EGFP and SOX2, suggesting they were skin-derived precursors with multipotent differentiation potential.
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 EGFP expression in epithelial cells of embryonic HFs in Nes-Cre/CAG-CAT-EGFP mice. Skin collected at germ (A), advanced germ (B), peg (C, D), and bulbous peg (E) stages was subjected to immunolabeling for EGFP (green) and laminin (white). Scale bars, 10 μm (A, B, C, E), 5 μm (D). (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 promoter, depilation of truncal hairs, and sample collection. (b) Immunolabeling of EGFP (A, B, F, J, N), K14 (C, K), and K15 (G, O) in first-anagen HFs (A–I) and depilation-induced anagen HFs (J–Q) 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), but EGFP+ cells in the isthmus region were not immunolabeled with K15 (O) as in the first-anagen HFs. Nuclei were counterstained by Hoechst 33258 (D, H, L, P). Scale bars, 20 μm (A, M), 10 μm (E, I, Q). (c) Comparison of frequencies of EGFP+K14+ cells in K14 positive cells between first-anagen and depilation-induced anagen HFs of OHT-administered Nes-CreERT2/CAG-CAT-EGFP mice (Student's t-test). ****, p < 0.0001.
OHT-induced EGFP+ cells capable of differentiating into ORS keratinocytes are present in postnatal 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 were present in adult HFs. To achieve this, we performed double-immunofluorescence analysis of EGFP and K14 expression in OHT-administered Nes-CreERT2/CAG-CAT-EGFP mice at 5 weeks of age, when HFs uniformly undergo their first anagen (n = 3) (Fig. 3a)33. 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 were not immunolabeled for K15. We further examined whether EGFP+ cells were 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 frequency of EGFP+K14+ cells in K14+ cells of depilation-induced anagen HFs was 92.1% ± 4.6% (n = 6), which was significantly higher than the frequency observed in the first anagen (6.5% ± 1.8%, n = 6) (Student’s t-test, p < 0.0001; Fig. 3c). Moreover, double-immunofluorescence analysis of EGFP and K15 revealed that EGFP+ cells in the depilation-induced anagen HFs of OHT-administered Nes-CreERT2/CAG-CAT-EGFP mice were not immunolabeled for K15, as in the first anagen (Fig. 3b). These findings suggest that most of the ORS keratinocytes in depilation-induced anagen HFs are the descendants of nestin-expressing adult progenitor cells. Keratinocytes in the 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 cells32. In this study, we found that nestin-expressing progenitor cells of ORS keratinocytes appeared in the HF primordium as early as the hair peg stage. Moreover, these cells were immunolabeled for K15, suggesting that they have characteristics of epithelial progenitor cells. Co-expression of nestin and cytokeratins was previously observed in progenitor cells for lens epithelial cells33 and Sertoli cells34 in mouse embryos. However, these epithelial cells were not immunolabeled for the neural stem cell markers SOX2 or S100A6. Progenitor cells of mouse ORS keratinocytes were reported to not be derived from stem/progenitor cells expressing the neural crest cell markers Wnt1 or plasminogen activator35. 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 in the early peg stage. A previous study reported weak nestin gene expression in E14.5 placodes and epidermis33. Conversely, our study did not observe EGFP+ epithelial cells in HFs before the peg stage. A possible reason for this discrepancy is that nestin expression in HF epithelia in early HF development was too weak to be detected by the immunofluorescence staining method used in this study.

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 epithelia37. Conversely, S100A6+ cells have been identified in the bulge region of adult mouse HFs38. 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-expressing cells or their descendants in mice after 4 weeks of age. A previous study demonstrated the presence of nestin-positive, K15-negative cells in the anagen HFs from human scalp39. The same study also indicated that GFP+ cells located immediately below sebaceous glands expressed 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 observed in the ORS of whisker HFs in ND-GFP mice41. We postulate that nestin was transiently expressed in K15+ cells before 4 weeks of age, or that nestin expression in K15+ cells of truncal 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. On the basis of a comparison of the differentiation potencies between EGFP+ cells and K15+ HF bulge cells37, we suggest nestin-expressing cells in adult HFs are downstream of K15+ epithelial pluripotent stem cells. In contrast, previous studies revealed that GFP+ cells in ND-GFP mice exhibited multipotency in vitro34. Moreover, GFP+ cells in ND-GFP mice transdifferentiated into neural cells when subcutaneously transplanted into nude mice44. It is possible that some stimuli related to cell culture or transplantation triggered the 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 cells39. 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 marker of unipotent progenitor cells for the outermost HF cell layer in postnatal HFs. Future studies to elucidate molecular interactions necessary for the differentiation of these multipotent stem cells into downstream cells are expected.

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

1. Schneider, M. R., Schmidt-Ullrich, R. & Paus, R. The hair follicle as a dynamic miniorgan. *Curr. Biol.* **19**, R132–R142 (2009).
2. Ohryama, M. Hair follicle bulge: A fascinating reservoir of epithelial stem cells. *J. Dermatol. Sci.* **46**, 81–89 (2007).
3. Morris, R. J. et al. Capturing and profiling adult hair follicle stem cells. *Nat. Biotechnol.* **22**, 411–417 (2004).
4. Ito, M., Cotsarelis, G., Kizawa, K. & Hamada, K. Hair follicle stem cells in the lower bulge form the secondary germ, a biochemically distinct but functionally equivalent progenitor cell population, at the termination of catagen. *Differentiation* **72**, 548–557 (2004).
5. Lendahl, U., Zimmerman, L. B. & McKay, R. D. CNS stem cells express a new class of intermediate filament protein. *Cell* **60**, 385–395 (1996).
6. Day, K., Shefer, G., Richardson, J. B., Enikolopov, G. & Yablonka-Reuveni, Z. Nestin-GFP reporter expression defines the quiescent state of skeletal muscle satellite cells. *Dev. Biol.* **304**, 246–259 (2007).
7. Zulewski, H. et al. Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* **50**, 521–533 (2001).
8. Jiang, M. H. et al. Characterization of Nestin-positive stem Leydig cells as a potential source for the treatment of testicular Leydig cell dysfunction. *Cell Res.* **24**, 1466–1485 (2014).
9. Kachinsky, A. M., Dominov, J. A. & Miller, J. B. Intermediate filaments in cardiac myogenesis: Nestin in the developing mouse heart. *J. Histochem. Cytochem.* **43**, 843–847 (1995).
10. Liu, F. et al. The bulge area is the major hair follicle source of nestin-expressing pluripotent stem cells which can repair the spinal cord compared to the dermal papilla. *Cell Cycle* **10**, 830–839 (2011).
11. Amoh, Y., Li, L., Katsuoka, K., Penman, S. & Hoffman, R. M. Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. *Proc. Natl. Acad. Sci. USA* **102**, 5530–5534 (2005).
12. Amoh, Y. et al. Nascent blood vessels in the skin arise from nestin-expressing hair-follicle cells. *Proc. Natl. Acad. Sci. USA* **101**, 13291–13295 (2004).
13. Amoh, Y., Li, L., Katsuoka, K. & Hoffman, R. M. Chemotherapy targets the hair-follicle vascular network but not the stem cells. *J. Invest. Dermatol.* **127**, 11–15 (2007).
14. Amoh, Y. et al. Multipotent nestin-expressing stem cells capable of forming neurons are located in the upper, middle and lower part of the vibrissa hair follicle. *Cell Cycle* **11**, 3515–3517 (2012).
15. Sennett, R. et al. An integrated transcriptome atlas of embryonic hair follicle progenitors, their niche, and the developing skin. *Dev. Cell* **34**, 577–591 (2015).
16. Onishi, S. et al. Progenitor cells expressing nestin, a neural crest stem cell marker, differentiate into outer root sheath keratinocytes. *Vet. Dermatol.* **30**, 365 (2019).
17. Kawamoto, S. et al. A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. *FEBS Lett.* **470**, 263–268 (2000).
18. Isaka, F. et al. Ectopic expression of the bHLH gene Math1 disturbs neural development. *Eur. J. Neurosci.* **11**, 2582–2588 (1999).
19. Kilkenney, C., Browne, W. J., Cuthill, I. C., Emerson, M. & Altman, D. G. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *PLoS Biol.* **8**, e1000412 (2010).
20. Scheitz, C. J. F. & Tumbar, T. New insights into the role of Runx1 in epithelial stem cell biology and pathology. *J. Cell. Biochem.* **114**, 985–993 (2013).
21. Müller-Röver, S. et al. A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. *J. Invest. Dermatol.* **117**, 3–15 (2001).
22. Alliot, F., Rutin, J., Leenen, P. J. & Pessac, B. Pericytes and periendothelial cells of brain parenchyma vessels co-express aminopeptidase N, aminopeptidase A, and nestin. *J. Neurosci. Res.* **58**, 367–378 (1999).
23. Toma, J. G. et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat. Cell Biol.* **3**, 778–784 (2001).
24. Perdigoto, C. N. et al. Polycomb-mediated repression and sonic hedgehog signaling interact to regulate Merkel cell specification during skin development. *PLoS Genet.* **12**, e1006151 (2016).
25. Yamada, J. & Jinno, S. S100A6 (calcyclin) is a novel marker of neural stem cells and astrocyte precursors in the subgranular zone of the adult mouse hippocampus: S100A6 (Calcyclin) is a novel marker for adult neurogenesis. *Hippocampus* **24**, 89–101 (2014).
26. Kelsh, R. N. Sorting out Sox10 functions in neural crest development. *Cell Cycle* **28**, 788–798 (2006).
27. Iahoda, C. A., Reynolds, A. J., Chaponnier, C., Forestes, J. C. & Gabbiani, G. Smooth muscle alpha-actin is a marker for hair follicle dermis in vivo and in vitro. *J. Cell Sci.* **99**, 637–636 (1991).
28. Driskell, R. R., Giangreco, A., Jensen, K. B., Mulder, K. W. & Watt, F. M. Sox2-positive dermal papilla cells specify hair follicle type in mammalian epidermis. *Development* **136**, 2815–2823 (2009).
29. Ito, M. & Kizawa, K. Expression of calcium-binding S100P proteins A4 and A6 in regions of the epidermal sac associated with the onset of hair follicle regeneration. *J. Invest. Dermatol.* **116**, 956–963 (2001).
30. Nonaka, D., Chiriboga, L. & Rubin, B. P. Sox2: A pan-schwannian and melanocytic marker. *Am. J. Surg. Pathol.* **32**, 1291–1298 (2008).
31. Fernandes, K. J. L. et al. A dermal niche for multipotent adult skin-derived precursor cells. *Nat. Cell Biol.* **6**, 1082–1093 (2004).
32. Frederiksen, K. & McKay, R. Proliferation and differentiation of rat neuroepithelial precursor cells in vivo. *J. Neurosci.* **8**, 1144–1151 (1988).
33. Yang, J., Bian, W., Gao, X., Chen, L. & Jing, N. Nestin expression during mouse eye and lens development. *Mech. Dev.* **94**, 287–291 (2000).
34. Froydman, K., Pellinemi, L. J., Lendahl, U., Virtanen, I. & Eriksson, J. E. The intermediate filament protein nestin occurs transiently in differentiating testis of rat and mouse. *Differentiation* **61**, 243–249 (1997).
35. Wong, C. et al. Neural crest–derived cells with stem cell features can be traced back to multiple lineages in the adult skin. *J. Cell Biol.* **175**, 1003–1015 (2006).
36. Amoh, Y. et al. Human and mouse hair follicles contain both multipotent and monopotent stem cells. *Cell Cycle* **8**, 176–177 (2009).
37. Liu, Y., Lyle, S., Yang, Z. & Cotsarelis, G. Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. *J. Invest. Dermatol.** **121**, 963–968 (2003).
38. Sieber-Blum, M. et al. Characterization of epidermal neural crest stem cell (EPI-NCSC) grafts in the lesioned spinal cord. *Mol. Cell Neurosci.* **32**, 67–81 (2006).
39. Mistriotis, P. & Andreadis, S. T. Hair follicle: A novel source of multipotent stem cells for tissue engineering and regenerative medicine. *Tissue Eng. Part B Rev.* **19**, 265–278 (2013).

Acknowledgements

The authors are grateful to Drs. Junichi Miyazaki, Itaru Imayoshi and Ryoichiro Kageyama for providing CAG-CAT-EGFP, Nes-CreERT2, and Nes-Cre mice. This study was supported by JSPS KAKENHI Grant Number 15K14866.
Author contributions
Y.B. acquisition of data, analysis and interpretation of data, drafting the manuscript. S.O. interpretation of data and reviewing. K.I. reviewing and editing. K.N. conception and design of the study, resource acquisition, interpretation of data and edit the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-22427-2.

Correspondence and requests for materials should be addressed to K.N.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022