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Report

Neural Potential of a Stem Cell Population in the Hair Follicle

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ABSTRACT

The bulge region of the hair follicle serves as a repository for epithelial stem cells that can regenerate the follicle in each hair growth cycle and contribute to epidermis regeneration upon injury. Here we describe a population of multipotential stem cells in the hair follicle bulge region; these cells can be identified by fluorescence in transgenic nestin-GFP mice. The morphological features of these cells suggest that they maintain close associations with each other and with the surrounding niche. Upon explantation, these cells can give rise to neurosphere-like structures in vitro. When these cells are permitted to differentiate, they produce several cell types, including cells with neuronal, astrocytic, oligodendrocytic, smooth muscle, adipocytic, and other phenotypes. Furthermore, upon implantation into the developing nervous system of chick, these cells generate neuronal cells in vivo. We used transcriptional profiling to assess the relationship between these cells and embryonic and postnatal neural stem cells and to compare them with other stem cell populations of the bulge. Our results show that nestin-expressing cells in the bulge region of the hair follicle have stem cell-like properties, are multipotent, and can effectively generate cells of neural lineage in vitro and in vivo.

INTRODUCTION

Stem cells from a number of adult tissues have demonstrated unexpectedly broad differentiation potential upon cultivation in vitro or transplantation.[1-5] However, this notion of plasticity of adult stem cells has been challenged and, at least for some reported cases, it has been explained by the presence of other types of stem cells in the population or by fusion with differentiated cells or committed lineage-specific progenitors upon transplantation.[3,5-7] Nevertheless, recent evidence provides further support to the idea of the inherent plasticity of at least some types of adult tissue-specific stem cells.[8-12]

The bulge region of the hair follicle serves as a repository of stem cells for hair and skin. The bulge contains cells contributing to the cyclical regeneration of hair and, in response to injury, to the epidermis.[13-18] There are at least two distinct populations of cells in the bulge that fit the definition for epithelial stem cells.[13,19-21] The first population is in contact with the basal lamina at birth and maintains this contact at later stages; the second is suprabasal and appears during the postnatal hair cycles. Although both populations possess stem properties, they can be discerned by their localization and distinct transcriptional profiles.[19] Furthermore, the bulge region of the whisker follicle also contains cells of neural crest origin which can self-renew and, upon explantation, produce neurons, Schwann cells, smooth muscle cells and melanocytes.[22,23] The bulge region also contains a population of cells expressing nestin, an intermediate filament and a marker of neural stem and early progenitor cells;[24-26] these cells can reconstitute much of the outer root sheath of the hair follicle with each hair cycle and behave as stem cells for the follicle.[25] Importantly, these different populations in the bulge region have been identified using different approaches and it is plausible that there is an overlap or even identity between these groups of cells. Moreover, stem cells in the bulge region might be related to a population of multipotential cells residing in the dermis, within the papillae of the follicle.[8,27]

We now report that nestin-expressing cells in the bulge region have broad differentiation potential and can generate cells of various lineages, including neuronal cells, when cultivated under clonal conditions. We have further confirmed the neural potential of these cells by transplanting them into chick embryo. We applied transcriptional profiling to demonstrate that these cells have a unique gene expression signature and to deduce their relation to embryonic or adult neural stem cells. Our results suggest that nestin-expressing

cells of the bulge region represent a unique population of multipotent stem cells with a broad differentiation potential.

MATERIALS AND METHODS

Transgenic animals and cell growth. Generation and characterization of transgenic nestin-GFP mice has been described.[26] After transcardial perfusion of the animals with Hank's balanced salt solutions (HBSS), the skin from the back was removed and placed into a solution of 2 mg/ml of collagenase type 1 and 2 mg/ml collagenase type 2 in HBSS for two hours at 37°C. After digestion, the fatty subepidermal layer was removed by gently wringing the tissue with forceps and the tissue was washed three times with DMEM/F12 (Gibco). Individual hair follicles were removed under a dissecting microscope, cells were isolated by trituration, checked under fluorescent microscope, and placed in neural growth media as described for neurospheres.[26] The media was supplemented every two to three days with 20 ng/ml epidermal growth factor (EGF; Sigma, St. Louis, MO), 20 ng/ml fibroblast growth factor (FGF; Sigma), and 10 ng/ml leukemia inhibitory factor (LIF; Chemicon, Temecula, CA). Spheres of cell were apparent after three-four weeks. For differentiation, these cells were transferred onto laminin- and poly-ornithine-(Sigma) coated slides and incubated in DMEM/F12 with 5% fetal bovine serum (FBS; Gibco) or growth factors as described in the text. To grow the cells under clonal conditions, single cell suspension was plated to low density (<3,000 cells/ml) in 1.2% methylcellulose to prevent their movement and aggregation during cultivation. Individual nestin-GFP-expressing cells were then identified by fluorescence and additionally checked under phase-contrast microscope to ensure that they indeed represent single cells. The position of such cells was marked and they were further monitored upon cultivation. Small neurosphere-like colonies were apparent after several days; they were individually transferred to new plates with the same medium but without methylcellulose and cultivated further for two to three weeks. For differentiation, they were plated and cultivated with FBS or growth factors as described above. Production of neurospheres from embryonic and adult brain of nestin-GFP mice was performed as described.[26]

Primary keratinocyte cultures were established from one to two day old newborn mice, essentially as described.[28] Briefly, pelts were floated on dispase II (Roche, Indianapolis, IN) overnight at 4°C, the epidermis was removed from the dermis, and keratinocytes were dissociated by stirring in DMEM, 10% FBS, 1% penicillin/streptomycin. Cells were filtered to remove debris, collected and resuspended in keratinocyte medium (EMEM, Cambrex, East Rutherford, NJ; 8% chelated FBS, 1% PS, 0.05M Ca2+ and 10 ng/ml epidermal growth factor). Keratinocytes were plated in 60 mm plates. Differentiation was induced by addition of 1.5 mM Ca^{2+} to the medium for 96 hours.

Immunohistochemical staining. Immunocytochemical analysis of GFP and other markers expression was performed as described[26] using conventional techniques or the FocusClear technology[29] with FocusClear and MountClear from Pacgen (Vancouver, Canada). Following primary antibodies were used: anti-nestin (R401, Chemicon, 1:100 dilution), anti-βIII-tubulin (TuJ1, Promega, Madison, WI, 1:1000 dilution), anti-GFAP (Sigma, 1:400 dilution), anti-Rip (Iowa University Hybridoma Bank maintained by the University of Iowa, Iowa City, IA, 1:10 dilution), S100a (Chemicon, 1:200), peripherin (Chemicon, 1:200), GABA (Chemicon, 1:100), keratin (Chemicon, 1:200), Sma (Sigma, 1:500). Biotinylated secondary antibodies (Vector, Burlingame, CA, 1:200 dilution) were used in combination with streptavidin-bound Alexa-594 or Alexa-633 dye (Molecular Probes, Eugene, OR) at 1:400 dilution.

Chick embryo transplantation. Fertilized chick eggs were incubated at 38°C and 80% humidity for approximately 44 hours, until they reached the 19–21 somite stage. A portion of the upper eggshell was opened and drawing ink was injected under the blasto-derm to visualize the embryo. For transplantation, mechanically dissected fragments of hairspheres or neurospheres generated from actin-GFP transgenic mice (from Jackson Laboratory) were inserted either into the otic placode or inside the neural tube (both ventrally and dorsally) at the level of the last pair of somites. After sealing and incubation for two to four days, the embryos were removed from the eggs and fixed in 4% paraformaldehyde for one hour. After incubation in 15% sucrose and 30% sucrose for 24 hours, embryos were embedded in OCT cryomedium and subjected to cryosectioning. 16 μm thick cross-sections were mounted on Superfrost/Plus slides, rinsed in PBS and incubated for several minutes in blocking solution (10% serum and 0.3% Triton X100 in PBS). Sections were incubated with primary mouse monoclonal antibodies (Tuj from Covance at 1:500 dilution, and Isl1 from Developmental Studies Hybridoma Bank maintained by the University of Iowa, Iowa City, IA, at 1:500 dilution) in blocking solution for one to two hours at room temperature. After washing several times with PBS during 15 minutes, sections were incubated for one hour with donkey anti-mouse secondary antibody conjugated with TRITC (1:200, Jackson ImmunoResearch Laboratories). After washing with PBS, sections were covered with glass coverslips using Vectashield Hard Set mounting medium with DAPI (Vector), and pictures were collected with a Zeiss Axioplan2 Imaging fluorescent microscope equipped with Hamamatsu ORCA-ER digital camera using AxioVision 3.1 software.

Transcription profiling and Q-PCR. Total RNA was isolated using TRIZOL (Invitrogen, Carlsbad, CA) and was reverse transcribed into cDNA using the Taqman Probe kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Q-PCR was performed as described.[30] Transcriptional profiling was performed using Affymetrix Mouse 430A microarrays at CSHL Genomics facility. The data were hierarchically clustered[31] using the Cluster software package (rana.lbl.gov/EisenSoftware.htm). The QPCR response was measured for each of the markers and the fold change with respect to the embryonic brain was calculated. To quantify the relationships between the tissue samples, the correlation between their expression profiles was calculated as follows. The raw expression values were first averaged over the replicates for each samples to create expression profile vectors for each type of tissue sample. These values were then log transformed and for each pairwise comparison of tissues, the Eisen correlation coefficient[31] was calculated. The distance between two tissues was then presented as 1-S(X,Y).

RESULTS

The nestin gene encodes an intermediate filament protein which is selectively expressed in the neuroepithelium of the developing embryo and in the neurogenic areas of the adult brain.[32,33] We have generated transgenic animals in which expression of the green fluorescent protein (GFP) is driven by regulatory elements that direct nestin expression in the neuroepithelium of the embryo and have shown that in these nestin-GFP animals, GFP expression faithfully reproduces the pattern of nestin expression in the developing

and the adult brain.[26] GFP-expressing cells are also seen in the bulge region of the hair follicles of nestin-GFP transgenic animals; these cells form the outer root sheath of the hair follicle throughout the hair cycle, compatible with them being stem cells of the follicle.[24,25] We examined the distribution of GFP-expressing cells in the follicle, using the FocusClear approach[26,29] to visualize fine details of this cell population and its niche. In the hair follicle of the dorsal skin of nestin-GFP mice, these cells are present in the bulge region as a compact group consisting of two-four rows of relatively small oval shape cells (appearing in a semicircular array in Fig. 1A). The cells are interconnected by thin filaments which run both along and between the rows; the latter type of filaments is particularly thin (less than 1 μm in diameter) and resembles cytonemes and cytoplasmic bridges that can connect individual cells.[34,35] Furthermore, individual follicles are connected through a network of nestin-GFP-positive vascular processes located closer to the apical surface (Fig. 1B), perhaps reflecting active angiogenesis in the perifollicular space.[25,36]

GFP-expressing cells in the bulge region were also positive for nestin and S100a, but not for the neural markers βIII-tubulin, glial fibrillary acidic protein (GFAP), or peripherin (although peripherin-positive processes seemed to surround the nestin-GFP bulge region) (Fig. 1C), confirming that they do not correspond to differentiated cells of the neural lineage.

Expression of nestin is a hallmark of stem and progenitor cells of the nervous system; therefore, we sought to determine whether nestin-GFP cells from the bulge region of the hair follicle can generate cells of the neural lineage. Stem cells of the nervous system are capable in vitro of forming neurospheres, clonally derived groups of cells which grow in suspension and can generate differentiated neurons and glia upon plating onto appropriate substrates. To examine the potential of nestin-GFP cells of the hair follicle to generate neural progeny, we isolated the bulge area of the hair follicles from the dorsal skin samples of the nestin-GFP transgenics, using fluorescent signal as a guide, and cultivated them in the growth media designed for growing neurospheres from brain-derived stem cells. After 14–20 days in vitro a large number of neurosphere-like colonies (designated as hairspheres) became apparent. When these hairspheres were plated onto plates coated with polyornithine and laminin, they attached to the substrate and gradually spread out such that individual cells started to migrate away from the hairsphere (Fig. 2a–h); the center of the attached hairsphere retained bright GFP fluorescence, whereas cells migrating from the center to the periphery started to lose the fluorescent signal, reflecting a decrease in nestin expression. After seven to ten days, cells with neuronal morphology appeared on the plate (Fig. 2i). Their neuronal phenotype was confirmed by immunostaining for neuronal βIII-tubulin. Moreover, a subpopulation of these cells was immunostained positive for the neurotransmitter GABA, thus identifying them as GABAergic neurons (Fig. 2j) (note that GABAergic neurons are not produced in the peripheral nervous system). Hairspheres also gave rise to cells with astrocyte-like morphology, which were positive upon immuno-staining for the astrocytic marker GFAP (Fig. 2k). Together, these results suggest that cells from the bulge region are capable of producing cells of neuronal and glial lineages, as observed for the neurospheres isolated from the neural tissues.

Moreover, we found that hairspheres are also capable of generating cells of nonneural lineages. We found that, after plating, hairspheres can also generate smooth muscle-like cells, as determined by morphological appearance and expression of smooth muscle actin (SMA) (Fig. 2m), and adipocyte-or sebocyte-like cells, as judged by the presence of characteristic large vacuole-like inclusions (Fig. 2l). Thus, bulge-derived hairspheres can give rise to the differentiated cell types characteristic of the hair follicle as well as neuronal and glial cells (note that neurospheres derived from the central nervous system have not been reported to give rise to such a range of cell types). Together, these observations indicate that nestin-GFP cells from the hair follicle are capable of giving rise to a range of cell types;

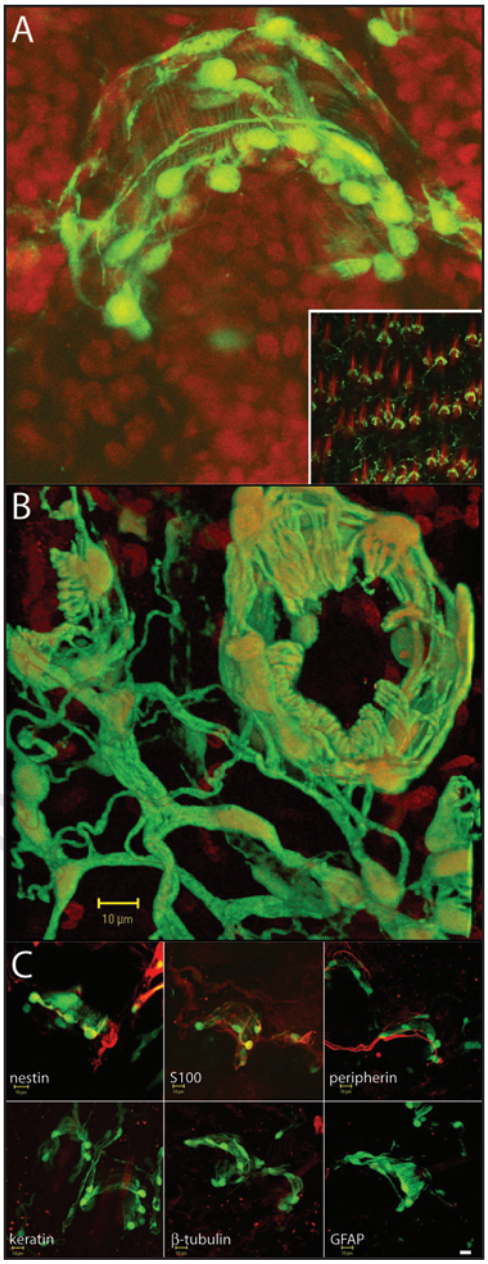

Figure 1. Nestin-GFP cells in the hair follicle. (A) GFP-expressing cells in the bulge region of the telogen hair follicle in the dorsal skin of nestin-GFP transgenic mice. Note fine processes running along and across the rows of cells. Inset shows a lower magnification view of the tissue. (B) GFP expressing cells in the follicle are connected to a network of GFP-expressing vessels located closer to the skin surface. (C) Colabeling of nestin-GFP cells in the bulge with antibodies (red) to (a) nestin, (b) S100a, (c) peripherin, (d) keratin, (e) β-tubulin and (f) GFAP. Scale bar is 15 μm in (A), 10 μm in (B), and 10 μm in all six images in (C).

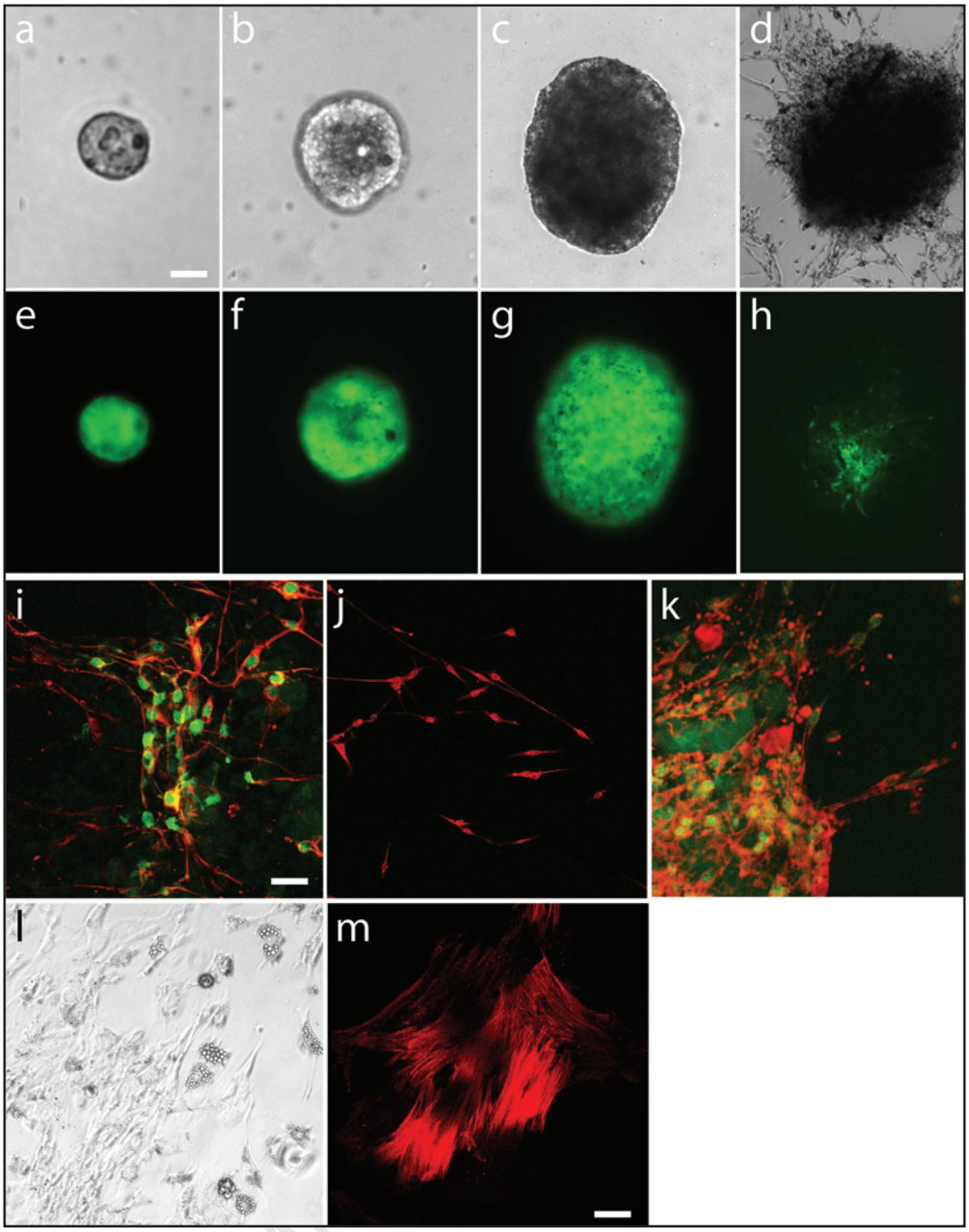

Figure 2. Nestin-GFP cells from the hair follicle generate multiple cell types. The bulge area was microdissected from the hair follicle and cells were plated into media containing FGF and EGF under conditions for growing neurospheres. After the neurosphere-like colonies were formed, they were plated onto laminin- and polyornithine-coated plates in the presence of FBS and later examined by immunochemistry. (a–c) and (e–g) neurosphere-like colonies grown from the hair follicle bulge explants (hairspheres); (a–c) phase contrast, (e–g) fluorescence. (a and e) colonies 12 days after cultivation, (b and f) 20 days, (c and g) 24 days. (d and h) two days after plating onto coated plates, hairspheres adhere to the surface; cells start to migrate away from the sphere and lose their GFP fluorescence; (d) phase contrast, (h) fluorescence. (i) one week after plating cells start to express neuronal marker β-tubulin (red); some cells still express low levels of GFP (green). (j) two weeks after plating GABA-positive neuronal cells are evident. (k) one week after plating cells start to express astrocytic marker GFAP (red); some cells still express low levels of GFP (green). (l) two weeks after plating adipocyte-like cells with large vacuole-like inclusions are evident. (m) two weeks after plating some cells express smooth muscle actin. Scale bar is 50 μm in (a–h), 25 μm in (i–l) and 10 μm in (m).

they also indicate that these cells can generate cells not only of the ectodermal but also of the mesodermal origin.

To determine whether cells of different lineages can be produced by individual nestin-GFP cells of the hair follicle, we initiated the growth of hairspheres under clonal conditions which ensure that they are derived from single cells. Nestin-GFP cells from the hair follicles were plated as a single cell suspension at clonal density (<3,000 cells/ml) in 1.2% methylcellulose to prevent their aggregation during cultivation. Single nestin-GFP-expressing cells were identified based on their fluorescence and their location was marked; they were further analyzed using phase contrast microscopy to ensure that they represented single cells. When small hairspheres in the media became apparent, they were individually transferred to a new plate and cultivated further without methylcellulose. They were then either dissociated to produce secondary hairspheres or grown on substrate-coated plates in the presence of either fetal bovine serum (FBS), brain-derived neurotrophic factor (BDNF), platelet-derived growth factor (PDGF), or ciliary neurotrophic factor (CNTF). After two to three weeks the phenotype of the hairspherederived cells was determined by morphological observations and immunostaining.

We found that clonally derived hairspheres can generate multiple cell types, including neurons, astrocytes, smooth muscle-like cells, and keratinocytes (Fig. 3), and melanocytes (judged by the appearance of pigmented cells in the sphere). Moreover, the results of differentiation was dependent on the presence of specific growth factors: hairspheres plated and cultivated in the presence of BDNF produced only βIII-tubulin-positive neuronal cells, in the presence of PDGF produced βIII-tubulin-positive and GFAP-positive (astrocytic) cells, and in the presence of CNTF gave rise to βIII-tubulin-

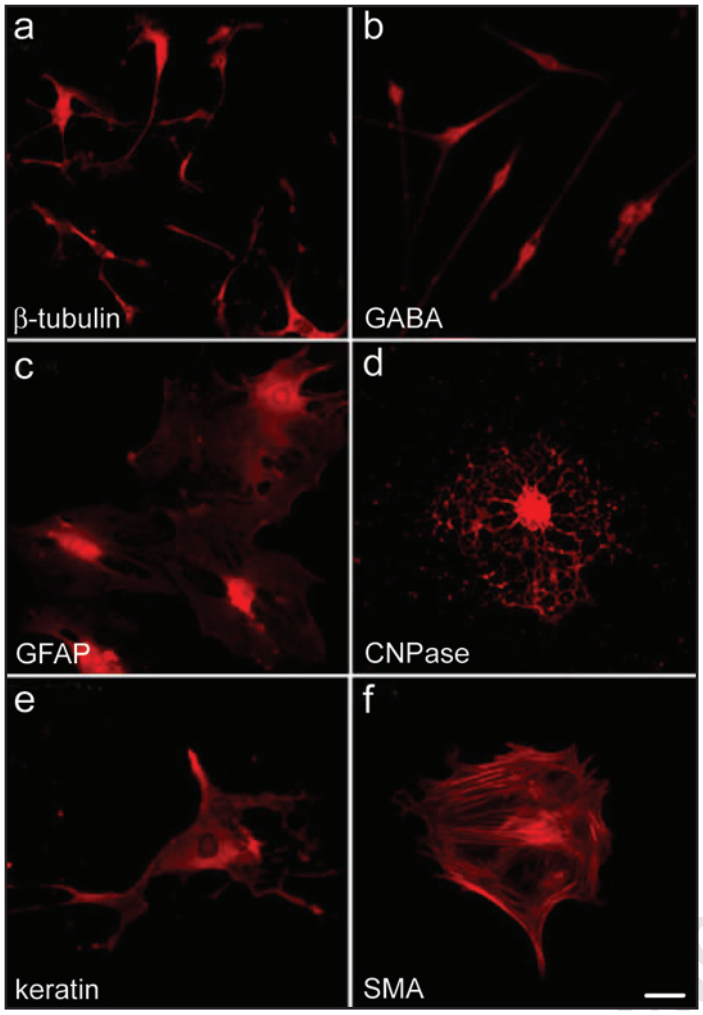

Figure 3. Clonally derived hairspheres generate multiple cell types. Nestin-GFP cells were isolated from the bulge of the hair follicle, grown under clonal conditions in 1.2% methylcellulose, transferred to regular media for further growth, and then plated onto laminin- and polyornithine-coated plates in the presence of FBS with or without added growth factors and later examined by immunochemistry with antibodies to β-tubulin (a), GABA (b), GFAP (c), CNPase (d), keratin (e), and smooth muscle actin (f). Scale bar is 20 μm in (a–d), and 10 μm in (e and f).

positive, GFAP-positive, and CNPase-positive (oligodendrocytic) cells. No keratin- or SMA-positive cells were detected. In contrast, in the presence of FBS only, hairspheres generated keratin-positive and SMA-positive cells, but did not produce neurons. Together, these experiments demonstrate that individual nestin-GFP positive cells in the bulge region of the hair follicle are multipotential and, under clonal conditions, can generate cells of the ectodermal and the neural origin.

We next addressed whether the cells of the bulge region that demonstrate their neuronal potential in vitro, can also manifest these properties in vivo. We generated hairspheres from the bulge cells of mice with ubiquitous expression of GFP, transplanted them into the neural tube and the otic placode of the two-day old chick embryo and examined them two and four days after transplantation; in parallel, similar experiments were performed with neurospheres. When cells of the hairsphere were implanted into the ventral part of the neural tube, they mainly remained inside the developing neural tube (Fig. 4a–e). Although many of them started to express neuronal marker βIII-tubulin, they also did not incorporate into the developing neural tissue. When hairpshere cells were implanted into the dorsal part of the neural tube, they did not incorporate into the neural tube (Fig. 4f–j). Most of them migrated dorsolaterally in a manner similar to the neural crest cells, although a small number of cells was detected in the dermal layer. The majority of cells migrated towards the targets of neural crest cells and were found in close association with the dorsal root ganglia, spinal nerve, and the developing meninges of the neural tube. Most of the migrating cells expressed βIII-tubulin two days after transplantation (Fig. 4b–e) and extended long processes four days after transplantation (Fig. 4i and j) (however, most of the cells detected in close contact with βIII-tubulin-positive cells of the dorsal root ganglia and peripheral nerves did not express βIII-tubulin). Finally, when hairsphere cells were implanted into the otic placode, they were found in association with the cranial ganglia two days after transplantation (Fig. 4k–m); very few of these cells expressed βIII-tubulin and none expressed Islet-1 which marks the cells of the ganglia. Cells of the neurospheres, used as a control population, migrated less actively than the hairphere cells when implanted into the dorsal part of the neural tube (Fig. 4n and o) or into the otic placode (Fig. 4p); two days after implantation most of the cells near the neural tube (but few of the cells near the developing otic vesicle) expressed βIII-tubulin (Fig. 4o). Thus, hairsphere cells were able, when injected into the dorsal part of the neural tube, to migrate along the pathways of migration of the neural crest cells, and to differentiate into neuronal cells, thus demonstrating their neuronal potential in vivo.

Together, our results show that bulge region of the hair follicle contains a small population of cells that can serve as stem cells for the hair follicle but can also generate cells of other lineages including neuronal, thus displaying properties of neural stem/progenitor cells of the developing and adult nervous system. We therefore examined the relation between the nestin-GFP cells of the bulge region and the neural stem cells of the brain using transcriptional profiling. We generated hairspheres from dorsal skin of early postnatal (p4) and adult (p49) mice, as well as neurospheres from embryonic (e14) and postnatal (p4) brain. We used Affymetrix gene arrays to determine the global gene expression patterns in these cell populations and then compared the transcriptional profiles of each population, using correlation coefficient[31] as a measure of their relatedness (Fig. 5A). As expected, the RNA expression profiles from p4 and p49 hairspheres were much closer to each other than to the profiles of other cells types (0.004 compared to 0.0079–0.0145). Furthermore, they were equidistant from the profiles of p4 neurosphere cells (0.0140 and 0.0145 respectively), and were close in their similarity to neurosphere cells from the embryonic brain (0.0079 and 0.0097, respectively). Interestingly, both p4 and p49 hairsphere RNAs were significantly closer to RNAs from the embryo-derived- than the p4-derived neurospheres, compatible with the notion of greater plasticity of the embryonic, compared to adult, neural stem cells. Together, the results of the microarray transcriptional profiling underscore the distinct nature of the nestin-expressing cells in the hair follicle.

We next used quantitative real time RT-PCR (Q-PCR) to verify the gene array data and to probe selected genes that have been reported to be expressed in hair follicle stem cells and epithelial stem cells or dermis-derived multipotent stem cells,[8,19-21] or have been described as markers of neural stem or neural crest cells. In addition to the cell populations described above, we examined two populations corresponding to differentiated and undifferentiated keratinocytes cultivated in vitro.[28] The results of Q-PCR show a

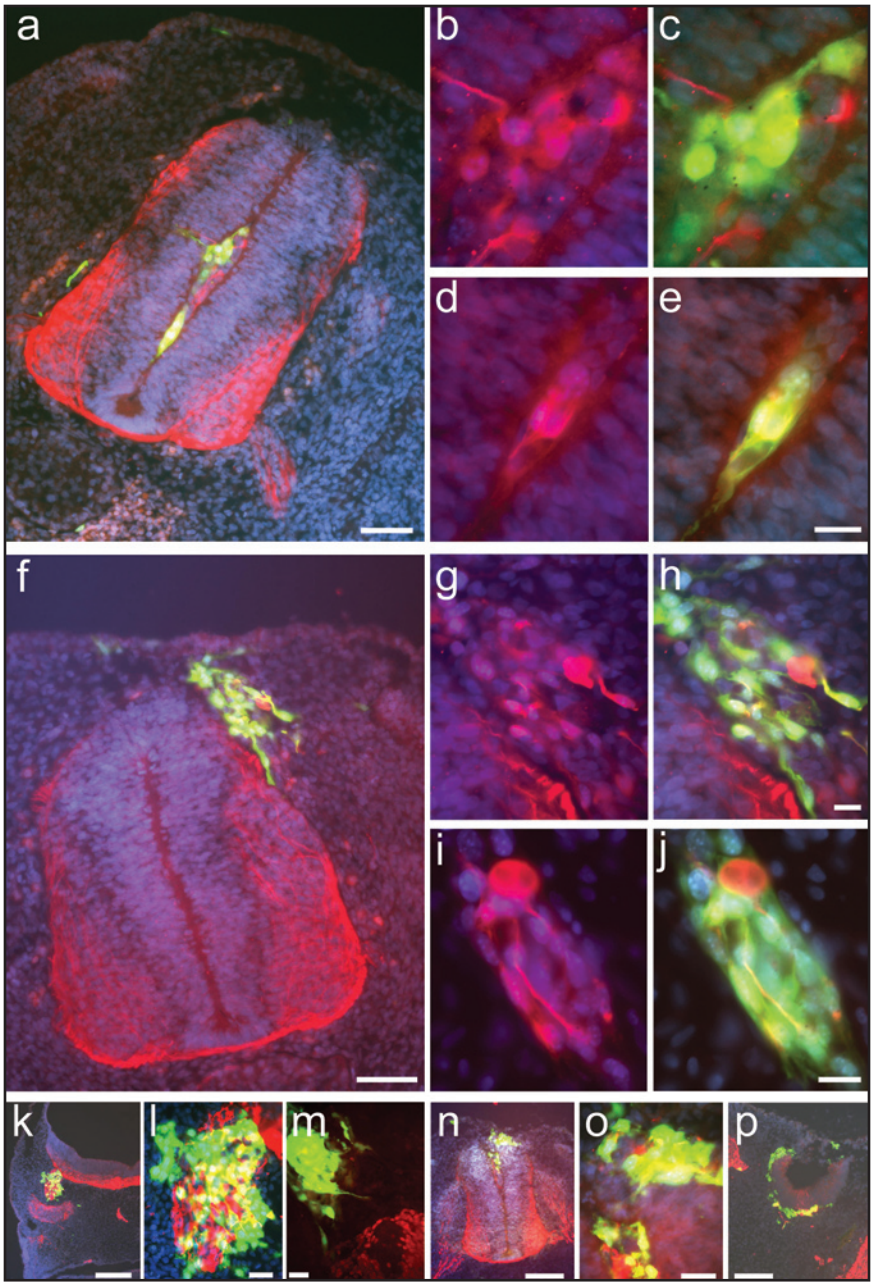

Figure 4. Hairspheres generate neuronal cells upon transplantation into developing chick embryo. Hairspheres and neurospheres generated from actin-GFP mice were transplanted into developing two day chick embryo. (a) hairsphere cells implanted into the ventral part of the chick neural tube (two days) and analyzed 4 days later. Here and in all other images (except for m) - green - GFP, red - TuJ1, blue - DAPI. (b–e) many transplanted cells start to express β-tubulin. (f) hairsphere cells implanted into the dorsal part of the chick neural tube (two days) and analyzed two days later. (g and h) higher magnification of implanted cells shown in (f); cells start to express β-tubulin. (i and j) four days after implantation cells express higher levels of β-tubulin and extend long processes. (k–m) hairsphere cells implanted into the otic placode of the chick embryo and analyzed two days later. Cells are found in association with cranial ganglia; note, however, that although they mingle with β-tubulin-expressing ganglia cells, very few of them express β-tubulin (l), and none of the cells expressed Islet-1 (red) (m). (n and o) neurosphere cells implanted into the dorsal part of the chick neural tube and analyzed two days later. Many cells start to incorporate into the neural tube and many of them express β-tubulin (higher magnification in l). (p) neurosphere cells implanted into the otic placode migrate towards the perimeter of the placode. Scale bar is 100 μm in (a), 20 μm in (b–e), 100 μm in (f), 20 μm in (g and h), 20 μm in (i and j), 400 μm in (k), 50 μm in (l), 50 μm in (m), 200 μm in (n), 50 μm in (o), 200 μm in (p).

specific pattern of marker genes expression in hairspheres and confirm our microarray results (Fig. 5B; the results are presented with the expression level in embryonic neurospheres taken as a baseline). When the data from this limited gene set were used to determine the relatedness of the populations, again p4 and p49 hairsphere cells were much closer to each other than to other tested cell types (Fig. 5B). They were also closer to the population of embryo- or early postnatal-derived neurospheres than to undifferentiated or differentiated keratinocytes, further underscoring their stem/progenitor status.

We compared the lists of genes analyzed in microarray experiments to identify genes which are differentially expressed in hairspheres and neurospheres. Most of the genes that have been previously described as strongly expressed in the bulge (e.g., CD34, Tcf3, S100a4, S100a6, Col18a1, Peg3, Crip1, Gas1, Igfbp5, Ltbp2, Ppap2a, Sparc, Tpst1[19-21] are up-regulated in hair- vs. neuro-spheres (Table 1; see also below Table 2). Several other categories of genes that are upregulated in hairspheres are presented in (Table 1). Up-regulated genes include those involved in Wnt signaling, a pathway critical for differentiation of stem cells towards hair follicle lineages; importantly, these genes include inhibitors of the Wnt pathway Srfp1, Dab2, Dkk3, and Tcf3, compatible with the requirement to prevent differentiation of cells in the hairsphere. Furthermore, hairspheres were enriched in transcripts of genes related to TGFβ signaling which is required to suppress the growth of keratinocytes (Ltbp1-3, Idb1-3, Igfbp5, Igfbp7, Timp2, Tnc, Efn1). Hairspheres were also enriched in transcripts encoding members of the IGFBP family of proteins (Igfbp3, 5, 6, and 7) which suppress the action of the insulin growth factor (IGF), a stimulator of epidermal proliferation, by binding and sequestering it. Furthermore, hairspheres had low levels of expression of many regulators of the cell cycle (cyclins A2, B1, and D1, Cdc2a, Cdc7, Cdkn1b, Chek2, Wee1, Mki67 and PCNA) consistent with their slow-cycling properties (note that hairspheres grow much slower than neurospheres). Together, the list of genes up or downregulated in hairspheres is consistent with the stem/progenitor like properties, slow rate of division, and suppression of the differentiation program in these cells. Furthermore, the list includes many genes that have been associated with embryonic or neural stem cells (e.g., Sox1, Fut4, Hesx1, Pou3f3). At the same time, the list reflects the epidermal origin of these cells, and many of the genes which determine specific neural lineages were down-regulated in hairspheres compared to neurospheres (e.g., Olig1, Olig2, Sox2, Ascl1, Dcx, Lhx2, Hes5, Dscam; see Table 1). Of note, although several well defined markers of neural crest cells (Snail1, Dct, Mitf, End, Twist2) were up-regulated in hairspheres, a larger number of neural crest markers is expressed at lower levels (Endrb, Sox9, Sox10, Snail2, Kitl, c-Kit, p75, Hand2, Ret), arguing against a purely neural crest origin of the hairsphere cells.

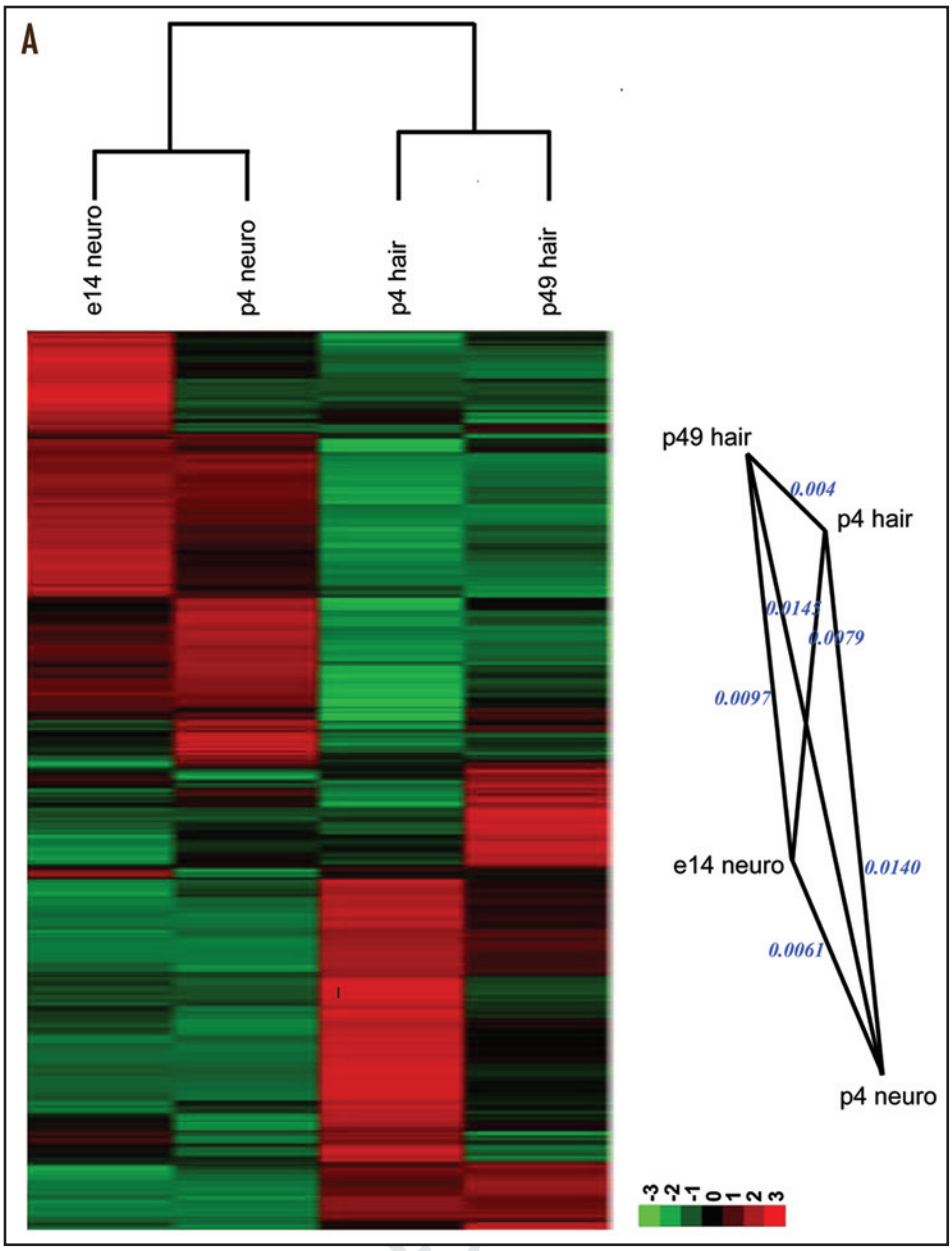

Figure 5A. For legend, see page 2168.

We have further focused on a subset of genes whose expression in the bulge is upregulated according to all published bulge expression profile reports[19-21] and which have been proposed to represent a molecular signature of bulge cells.[19] Remarkably, we found that 89% of the genes listed as comprising the molecular signature are also expressed in hairspheres (Table 2). Moreover, 64% of those genes are upregulated in hair- compared to neurospheres; note that the molecular signature was determined by comparing bulge cells with differentiated keratinocytes or total epidermis, and the remaining 36% of the genes in the set may correspond to genes that are still preferentially expressed in hairspheres but encode products related to the more general stem/progenitor traits of hair- and neuro-sphere cells.

We next compared the gene sets expressed in the hairspheres isolated from p4 and p49 skin. Since the fully formed bulge appears after the first hair cycle and its formation involves the appearance of the subrabasal bulge cells,[19] it is conceivable that this comparison may point to genes that are preferentially expressed in the subrabasal layer (i.e., assuming that p4 bulge cells include the basal, whereas p49 cells include both basal and suprabasal cells). Indeed, we found that several of the markers that define the suprabasal population (Pmp22, Ramp1, Dcamkl1, Col3a, Sema4g) were expressed at higher levels in p49 hairspheres than in p4 hairspheres. Remarkably, most of the genes controlling the cell cycle were expressed at lower levels in p49- than in p4-derived cells (cyclins A2, B1, and B2, Cdca1, Cdc2a, Cdc7, Cdkn2b, Chek2, Wee1, Mki67, and PCNA), echoing the lower level of division of subrabasal cells.[19] A list of genes differentially expressed between p4- and p49-derived hairsphere cells (e.g., Tnnt2, Ddx3y, Mylf1, Igf2, Dkk1, Eif2s3y, Epha3, Nnat, Pxip1, Diap3 expressed at higher levels in p4 cells and C1qa, Laptm5, Tlr4, Ccl9, Ptprc, Spon2, H2Aa, Chrd, Enpep, Ccl21a expressed at higher levels in p49 cells) will be useful in characterizing the developmental changes in the bulge stem cell populations.

Together, the RNA profiling data support the notion of the neural potential of nestin-GFP cells from the hair follicle bulge region and suggest that these cells represent a distinct population, similar and over-lapping but not identical to the previously described cell types.

DISCUSSION

We here describe a population of cells in the bulge region of the hair follicle that is multipotential and, after clonal propagation, can generate cells of neural, epidermal, and mesodermal lineage. The neural potential of these cells is supported by transcriptional profiling, which shows that they also express many markers of neural stem cells. It is further confirmed by transplantation experiments which show that these cells can generate neuronal cells in vivo. Together, these data clearly demonstrate the neural potential of a unique population of nestin-expressing cells residing in the hair follicle.

The morphological features of these bulge region cells (similar phenotype, tight grouping, connection through thin filaments and cytoplasmic bridges, connection to the vascular network) support the view that they represent a defined cell population which preserves close contacts within the group and with surrounding tissues. Such contacts may be a part of the arrangement that defines and preserves the stem cells niche in the bulge.[16,37,38] At the same time, the ability of these cells to maintain their progenitor properties when isolated and grown in vitro and the transcriptional signatures which show clear differences both with other types of stem cells and between cells isolated at different stages of hair follicle development indicate that many features of these cells are preserved upon cultivation in

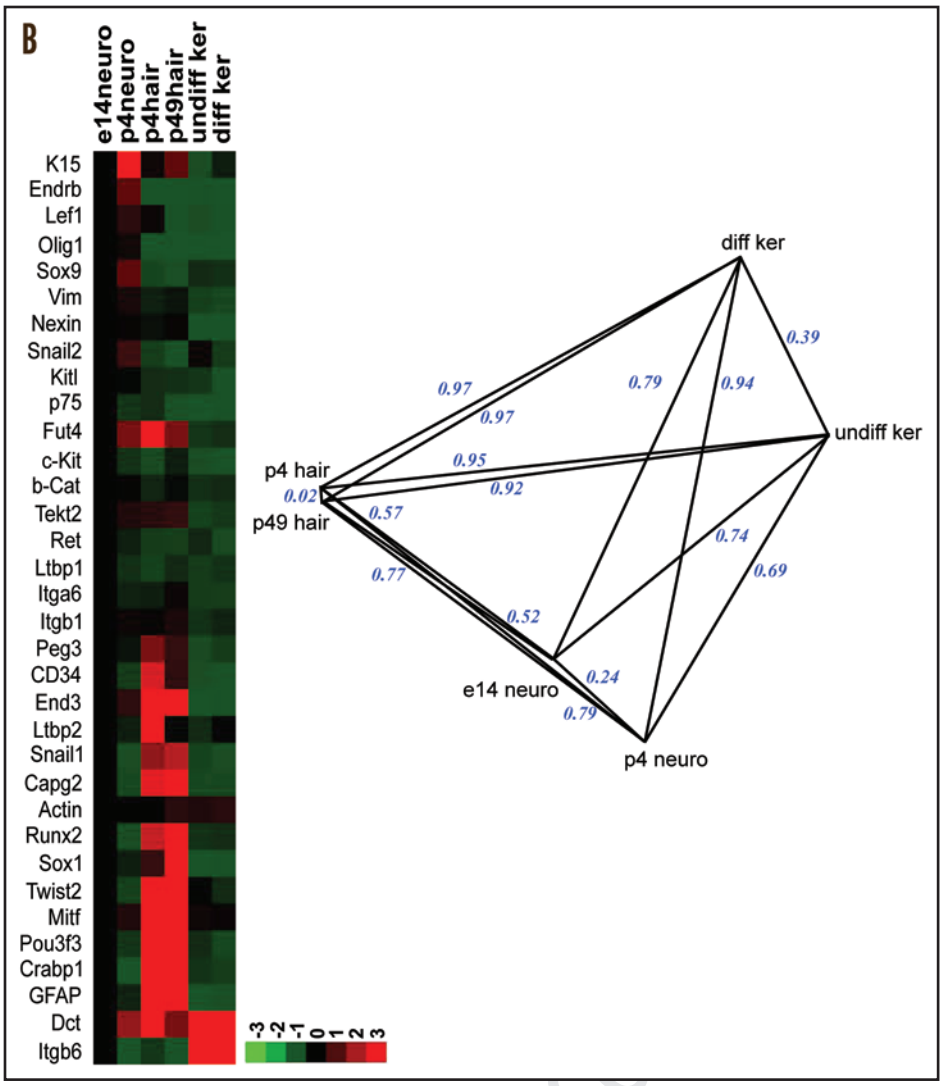

Figure 5B. Transcription profiling of hairsphere and neurosphere derived cells. (A) Clustering of the genes expressed in hairspheres isolated from p4 and p49 animals and neurospheres isolated from 14 day embryo (e14) or postnatal brain (p4). The relations between the samples are presented as correlation coefficients. The data were generated using Affymetrix gene arrays. (B) Expression of selected markers, determined by Q-PCR; the samples include, in addition to those analyzed in (A), undifferentiated and differentiated keratinocytes. The relations between the samples are presented as correlation coefficients.

vitro and may be an intrinsic property of these cell populations. Our results provide further support to the notion that a unique combination of intrinsic and extrinsic properties defines the maintenance and self-renewal of the stem cells in their niche.

Transcriptional profiling of nestin-expressing cells provides additional clues to their identity and supports the conclusion that they represent epithelial stem cells of the bulge region. First, there is a remarkable overlap between the genes expressed in this cell population and stem cells isolated from the bulge using alternative approaches;[19-21] moreover, this overlap is near 90% for the small subset of genes that comprise the molecular signature of stem cells from the bulge.[19] Second, many of these genes may be related to the constraints that prevent these cells from differentiating along their normal lineage: e.g., among upregulated genes are inhibitors of the Wnt pathway, mediators of the TGFβ signaling, and inhibitors of IGF signaling; these three pathways are known to induce epidermal proliferation and differentiation. Third, certain differences between hairspheres isolated from p4 and p49 skin (when hair is actively engaged in cycling) resemble the differences between the basal and suprabasal populations of bulge stem cells[19] (RNA profiles, lower expression of cell cycle-related genes). Fourth, while there is much similarity between brain-derived and skin-derived stem/progenitor cells (i.e., hairspheres and neurospheres), there are also profound differences which underscore the unique profile of the bulge region-derived cells. Transcriptional profiling also argues against these nestin-GFP cells being neural crest cells of the bulge region since many of the reliable markers of the neural crest lineage are expressed in hairspheres at lower levels than in neurospheres (note however, that the migration and differentiation patterns of these cells upon transplantation into the developing nervous system of the chick resemble those of neural crest cells).

Several reports describe cells with stem cell-like properties in the bulge region of the hair follicles of the skin;[19-21,39] in addition, a population of neural crest-derived cells has been isolated from the bulge of the vibrissal hair.[22,23,40,41] Furthermore, a multipotent cell population with properties of neural crest cells has been discovered in the adult dermis.[8,27] Clearly, the described populations, which were identified and isolated using different approaches, can overlap with each other to different degree or even represent the same population of cells. However, our data argue against the equivalence between the population of nestin-GFP expressing cells we describe and at least some of the above populations. For instance, although some properties of the dermis-derived multipotent cells[8,27] resemble the nestin-GFP cells we describe here, they are different in that they reside in the dermis, do not seem to appear in the bulge region, and are of neural crest origin (note that expression of a large part of the neural crest markers is decreased in our nestin-GFP cells, Fig. 5). Similarly, although some of the features of our nestin-GFP cells (e.g., their ability to migrate dorsolaterally and then follow the direction of migration of neural crest cells) resemble those of neural crest-derived stem cells from the bulge of the whiskers,[22,23,40] however, transcriptional profiling would again argue against the equivalence between these cells populations, since there is no apparent overlap between their transcriptional signatures. A caveat of such comparisons relates to the possible changes during cultivation in vitro; note, however, that most of the intrinsic differences with respect to self renewal and neuronal differentiation potential are preserved when neuronal precursors from different areas of the developing and adult CNS are cultivated as neurospheres in vitro, thus arguing against a homogenizing effect of in vitro cultivation on the profile of neuronal precursor cells.[42] Overall, the location of nestin-GFP cells, their behavior in vitro and in vivo, and the

Table 1 **Transcriptional profiling of hairspheres relative to neurospheres**

	Genes upregulated in hairsphere cells compared to neurosphere cells
Known bulge proteins	S100a6 (13x), S100a4 (12.5x), CD34 (12x), b1-intg (4.8x), Tcf3 (4.6x)
TGFb-induced proteins	Igfbp7 (7.6x), Timp2 (7.2x), Ltbp2 (5.6x), Ltbp1 (5x), Igfbp5 (3.8x), Tnc (3.2x), EfnB1 (2.8x), Idb2 (2.6x), Idb3 (2.2x), Ltbp3 (2.2x)
Wnt signaling	Wisp1 (13.8x), Wnt2 (9x), Dkk3 (7.6x), Fzd2 (6.6x), Tcf3 (4.6x), Fzd7 (4.6x), Dab2 (4.2x), Sfrp1 (3.8x)
Signaling	Crabp1 (19x), Agtr2 (18.4x), Pdgfrb (15.6x), Ednra (15.2x), Edg2 (15x), Cxcl12 (14.4x), Ccl12 (14.2x), Ccl8 (12.6x), Ppap2a (6x), Tpst1 (4.2x), Igfbp5 (3.8x), EfnB2 (2.8x), EfnB1 (2x)
Cytoskeleton, ECM, adhesion	Col1a1 (22x), Dpt (15.8x), Bgn (13.8x), Sdc1 (12.2x), Col6a1 (12x), Itga8 (10.2x), Icam1 (8.8x), Timp2 (7.2x), Col18a1 (3.6x), Tnc (3.2x), Tekt2 (1.7x)
Cell cycle, cell growth	S100a6 (13x), S100a4 (12.5x), Anxa1 (11x), Emp1 (10.2x), Emp3 (8.2x), Cktsfb1 (6.4x), Ltbp2 (5.6x), Ltbp1 (5x), Inhbb (3.6x), Gas1 (3.4x), Ltbp3 (2.2x),
Transcription	Hoxc8 (13x), Rinx2 (11,4x), Hoxa9 (10.8x), Shox2 (10.8x), Pitx2 (10.6x), Tcfap2c (9.4x), Vax2 (9.4), Ebf2 (9x), Ebf1 (8.8x), Hoxd8 (8.8x), Idb2 (2.6x), Idb3 (2.2x)
	Genes upregulated in neurosphere cells compared to hairsphere cells
Signaling	Gabrb3 (19.6x), Slc6a1 (17x), Ptprz1 (12.2x), Ntrk2 (9x), Ednrb (8x), Fzd9 (7.8x), Adcyap1r1 (5x)
Cytoskeleton, ECM, adhesion	Catnd2 (9.4x), Dscam (8x), Dcx (6x), Enah(5.8x), Tro (5.8x), Dmd (5.8x), Cdh4 (4.4x), Celsr2 (4x), Chl1 (3.8x), Fmn2 (3.8x), Gphn (3.8x)
Cell cycle, cell growth	Igfbp2 (13.8x), Gadd45g (6.4x), Ccnd1 (4.4x), Ptn (4x)
Transcription	Olig1 (20.4x), Olig2 (14,2x), Ascl1 (13.4x), Nkx2-2 (11.6x), Foxg1 (11x), Sox2 (11x), Sox9 (9x), Lmo1 (7.8x), Lhx2 (7x), Sox6 (5.6x)
Other	Fabp7 (22x), Mt3 (12.2x), Nnat (9.8x), Qk (9.2x), Csen (7x), Fhl1 (6.8x), Dpysl3 (5x), Gpd2 (4.6x)

The changes (times-fold) for selected genes are indicated in parenthesis.

Table 2 **Bulge molecular signature genes expressed in hairspheres**

Cytoskeleton	Flnb, Sdcbp, Tekt2, *Dmd, Enah, Macf1*
Cell adhesion/ECM	CD34, Col6a1, Col18a1, Itm2a, Prlr, Tnc, *Igsf4*
Transcription	Dbp, Idb2, Idb3, Peg3, *Fhl1, Foxp1, Idb1, Ndn*
Cell cycle/Growth	Gas1, *Ptn, Fgf1*
Signaling	Cktsfb1, Dab2, Dkk3, Fstl1, Fzd2, Ltbp2, Ppap2a, *Plxna, Ptprk*
Transport	Kcnk2, Slc29a1, Slc39a8, *Txn1*
Other	Crip1, Lrrfip1, Sardh, *Eps8, Pole4*

The list of the bulge "molecular signature" genes and their assignment to categories is taken from Blanpain et al., 2005. Genes that are downregulated in hairspheres as compared to neurospheres are shown in italics.

transcription profiles of these cells resemble most the properties of cells isolated directly from the hair follicle bulge region.[19-21] This supports their identity as stem cells for the hair follicle and extends the repertoire of such cells by showing their broad differentiation potential.

Expression of nestin in these bulge cells with stem-like properties parallels findings from our group and others that this intermediate filament marks stem/progenitor cells in other tissues as well; examples now include neural stem cells, liver oval cells, satellite cells in the muscle, stem cells in the pancreas, and progenitors of Leydig cells.[10,26,39,43-46] This raises an intriguing question of whether expression of nestin simply marks the stem/progenitor state of the cells or contributes actively to this state.

Our study concerns a more general issue of the plasticity of stem cells in adult organisms. Our data indicate that a single nestin-positive cells can give rise to a variety of cell lineages, including neuronal, glial, smooth muscle, and sebaceous gland cells as well as melanocytes and keratinocytes. This may reflect the true range of lineages that nestin-GFP cells give rise to in vivo, when residing in the hair follicle, or may reflect the changes that these cells undergo during cultivation in vitro. Finally, these results may reflect the true potential of these cells which is not realized to its full degree in vivo either due to the strict genetic/epigenetic program established in these cells or due to the interactions of the stem cells with their niche, whose signals restrict the range of potential fates that stem cells acquire in the tissue.

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