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Epithelial and Mesenchymal Cell Biology

Isolation of a Novel Population of Multipotent Adult Stem Cells from Human Hair Follicles

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Hair follicles are known to contain a well-characterized niche for adult stem cells: the bulge, which contains epithelial and melanocytic stem cells. Using human embryonic stem cell culture conditions, we isolated a population of adult stem cells from human hair follicles that are distinctively different from known epithelial or melanocytic stem cells. These cells do not express squamous or melanocytic markers but express neural crest and neuron stem cell markers as well as the embryonic stem cell transcription factors Nanog and Oct4. These precursor cells proliferate as spheres, are capable of self-renewal, and can differentiate into multiple lineages. Differentiated cells not only acquire lineage-specific markers but also demonstrate appropriate functions in *ex vivo* conditions. Most of the Oct4-positive cells in human skin were located in the area highlighted by cytokeratin 15 staining *in vivo*. Our data suggest that human embryonic stem cell medium can be used to isolate and expand human adult stem cells. Using this method, we isolated a novel population of multipotent adult stem cells from human hair follicles, and these cells appear to be located in the bulge area. Human hair follicles may provide an accessible, autologous source of adult stem cells for therapeutic application. *(Am J Pathol 2006, 168:1879–1888; DOI: 10.2353/ajpath.2006.051170)*

Adult mammalian stem cells were previously thought to differentiate exclusively into cells of their tissue of origin. A number of recent reports have shown that tissue-based adult stem cells may be much more "plastic" than previously appreciated,[1–3] and the multipotency of adult stem cells is probably not due to cellular fusion *in vivo*.[2,4] Recent studies also showed that adult stem cells can improve functions of injured tissues in animal models.[5–7]

Each hair follicle perpetually goes through three stages: growth (anagen), involution (catagen), and rest (telogen). Hair follicles not only serve as an appendage specialized for hair shaft production but also as a "niche" for tissue regeneration. Stem cells in the hair bulge, a clearly demarcated structure within the lower permanent portion of hair follicles,[8–11] can generate the interfollicular epidermis, hair follicle structures, and sebaceous glands. The bulge epithelial stem cells can also reconstitute in an artificial *in vivo* system to a new hair follicle.[12,13] Stem cells that give rise to the melanocyte lineage have been recently identified in the bulge and sub-bulge outer root sheath regions in adult mice.[14,15] Nestin-positive cells are present in the bulge area in mouse and can give rise to neurons, smooth muscle cells, and melanocytes.[16] Neural-crest-like stem cells have been identified in mouse whisker hair follicles, and bulge cells from mouse whisker hairs grow as adherent monolayer cells and appear to be multipotent.[17,18] The hair bulge has also been found to serve as a local reservoir for mast cell precursors.[19] These data suggest that the hair bulge is a unique differentiation-restricted area for adult stem cells.[10,11,13] Other studies have demonstrated the potential of rodent and human dermis-derived precursors to generate neural, glial cells and smooth muscle cell derivatives.[19,20] In addition, tagged skin cells have been found in multiple tissues after transplantation to blastocysts,[21,22] indicating that there are cells in the skin that could be used to reconstitute tissues beyond those of the skin.

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Most of the prior hair stem cell functional studies were performed using rodent tissues. In this study, we have developed a new method to isolate human adult stem cells by culturing cells from human hair follicles in a human embryonic stem cell (hESC) culture condition. We have shown that the isolated cells are capable of differentiating into neurons, smooth muscle cells, and melanocytes in specific induction medium. The differentiated cells not only acquire lineage-specific markers but also demonstrate appropriate functions in *ex vivo* conditions. These cells appear to be located in the bulge area of human hair follicles.

Materials and Methods

Cell Culture

Human hair follicles were isolated as described previously.[23–25] Human scalp tissues (0.5 × 2 cm^2 or less) from 13 patients (50 to 65 years old) and 3 autopsies were obtained through the Cooperative Human Tissue Network with approval from the Institutional Review Board of the University of Pennsylvania. Tissues were rinsed, trimmed to remove excess adipose tissues, cut into small pieces, and subjected to enzymatic dissociation in 12.5 mg/ml dispase (Invitrogen, Carlsbad, CA) in Dulbecco's modified Eagle's medium (DMEM) for 24 hours at 4°C. After treatment, the epidermis was peeled off from the dermis, and hair follicles were plucked from the dermis. Hair follicles were rinsed thoroughly with phosphate-buffered saline to prevent contaminating epidermal or dermal cells and examined under an inverted microscope. To confirm the integrity of plucked hair follicles, we fixed some hair follicles in 10% buffered formalin, embedded them in paraffin, and sectioned and stained them with hematoxylin and eosin (H&E). To obtain viable single cells from follicular epithelium, hair follicles were treated twice with 0.25% trypsin/ethylenediamine tetraacetic acid (Invitrogen) for 30 minutes at 37°C. The cell suspension was filtered through a 40-μm cell strainer (BD Falcon, Bedford, MA), and cell numbers were counted. Single cells were cultured in noncoated flasks in media designated for hESCs. Human ESC medium consisted of 80% knockout DMEM/F-12 medium (Invitrogen), 20% knockout serum replacer (Invitrogen), 200 mmol/L L-glutamine (Invitrogen), 0.1 mmol/L β-mercaptoethanol (Sigma, St. Louis, MO), 1% nonessential amino acids (Invitrogen), and 4 ng/ml basic fibroblast growth factor (bFGF) (Research Diagnostics, Concord, MA).[26,27] This medium was conditioned by using it for 48 hours as growth medium for mouse embryonic fibroblasts (MEFs) as described previously.[28] Human ESC medium conditioned by MEFs was mixed with fresh hESC medium at a 3:1 ratio, sterilized by filtration, and supplemented with additional bFGF at 4 ng/ml before use.

For self-renewal, dissociated individual cells from hair spheres were serially diluted in hESC medium in uncoated 96-well plates. Each well was assessed microscopically for the presence of a single cell. The wells containing no cells or more than one cell were excluded. The human ESC line H9 was obtained from the WiCell Research Institute, Inc., (Madison, WI). Cells were cultured on mitotically inactivated MEF feeder layers as described previously.[26,27] Embryoid bodies were derived from hESCs as described previously.[28]

Differentiation Assays

Hair spheres and attached cells were enzymatically dissociated into single cells before plating onto tissue culture-grade plastic coated with 10 ng/ml fibronectin (melanogenic differentiation) or 0.1% Matrigel (BD Biosciences, San Jose, CA; neuronal and smooth muscle differentiation) in differentiation medium. Melanogenic differentiation medium exclusively differentiated hESCs into melanocytic lineages.[29] It contains dexamethasone (0.05 μmol/L; Sigma), insulin-transferrin-selenium (1×; Sigma), linoleic acid-bovine serum albumin (1 mg/ml; Sigma), low-glucose DMEM (30%; Invitrogen), MCDB 201 (20%; Sigma), L-ascorbic acid (10^{-4} mol/L; Sigma), conditioned media of mouse L-Wnt3a cells (American Type Culture Collection, Manassas, VA) (50%), stem cell factor (100 ng/ml; R&D Systems, Minneapolis, MN), endothelin-3 (100 nmol/L; American Peptide, Sunnyvale, CA), cholera toxin (20 pmol/L; Sigma), the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (50 nmol/L; Sigma), and bFGF (4 ng/ml; R&D Systems). For smooth muscle differentiation, dissociated cells were cultured in a medium containing 90% knockout DMEM/F-12 medium, 1% nonessential amino acids solution, 10% fetal calf serum, and 10 ng/ml transforming growth factor-β1 (R&D Systems). Neuron differentiation medium and steps were used as previously described.[20]

Immunocytochemistry and Immunohistochemistry

Cells were fixed with 4% paraformaldehyde and stained with primary antibodies specific for Oct4 (mouse monoclonal, 1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA), Nanog (goat polyclonal, 1:100; R&D Systems), microphthalmia-associated transcription factor (MITF) (mouse monoclonal, 1:200; NeoMarkers), HMB45/SILV (mouse monoclonal, 1:500; DakoCytomation, Carpinteria, CA), microtubule-associated protein-2 (MAP2, mouse monoclonal, 1:1000; Sigma), chromogranin (rabbit polyclonal, 1:100; Zymed), Neurofilament-M (NFM) (mouse monoclonal, 1:100; a gift from Dr. Virginia Lee), glutamate (rabbit polyclonal, 1:1000; Sigma), pan-cytokeratin (mouse monoclonal, 1:500; Sigma), and smooth muscle actin (SMA) (mouse monoclonal, 1:100; Chemicon, Temecula, CA). Monoclonal antibodies against the surface markers of hESCs—stage-specific embryonic antigen stage-specific embryonic antigen (SSEA)-3 and SSEA-4—were obtained from The Wistar Institute (where they were originally developed). Isotype-matched mouse antibodies or normal rabbit IgG was used as control. After washings, primary antibody binding was detected via corresponding goat anti-mouse or anti-rabbit Alexa Fluor 488-conjugated, or donkey anti-goat Alexa Fluor 568 sec-

ondary antibodies (1:600 to 1:800; Invitrogen). Cells were counterstained with either Hoechst 33258 (Invitrogen) or TO-PRO-3 (Invitrogen) to show nuclei. Staining was observed by a Nikon E600 upright fluorescence microscope or Leica TCS SP2 confocal microscope. Hematoxylin and eosin, Fontana-Mason (melanin), and tyrosinase (TYR) (monoclonal, 1:75; Novocasta Lab Ltd., Newcastle On Tyne, UK) staining was performed on 5-μm sections following standard protocols. Human vascular smooth muscle cells (HUVSs), human primary neurons, and epidermal keratinocytes served as positive or negative controls. Oct4 (goat polyclonal, 1:50; Santa Cruz Biotechnology) and cytokeratin 15 (C8/144B, mouse, 1:50; Dako) immunohistochemical stains were performed on consecutive normal human scalp sections as previously described.[30,31]

Collagen Gel Contraction Assay

After 14 days of differentiation into smooth muscle cells, dissociated single cells were embedded at 4.5×10^4 cells/well in 3 ml of a buffered collagen solution and added to tissue reconstruct trays (Organogenesis, Canton, MA). The buffered collagen solution consisted of bovine collagen type I (Organogenesis) at a final concentration of 0.83 mg/ml in minimal essential medium with Earle's Salts (BioWhittaker), 200 mmol/L L-glutamine, 10% fetal calf serum, and 7.5% sodium bicarbonate (BioWhittaker). Human epidermal keratinocytes and HUVSs (American Type Culture Collection) were used as negative and positive controls, respectively. The diameters of collagen gels were measured every 24 hours after seeding.

Human Skin Reconstructs

Human skin reconstructs were generated as described previously.[32] Briefly, dermal reconstructs consisted of bovine collagen type I with embedded dermal fibroblasts. Cells treated in melanocyte differentiation medium for 14 days were seeded into tissue reconstruct trays together with keratinocytes onto dermal reconstructs at a 1:5 ratio of melanocytic cells to keratinocytes. Reconstructs containing undifferentiated cells were used as a control. Two weeks later, reconstructs were harvested and fixed in 10% neutral buffered formalin for 2 to 3 hours, processed by routine histological methods, and embedded in paraffin.

RNA Isolation and Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using a Cells-to-cDNAII kit (Ambion, Austin, TX). Per kit protocol, 20 ng of total RNA was used for the first-strand cDNA synthesis. Real-time PCR was performed on an iCycler (Bio-Rad, Hercules, CA) using 1 μl of cDNA and iQ SYBR Green reagents (Bio-Rad). Standard real-time PCR protocol was used with melting curve analysis to ensure amplification specificity. Human epidermal keratinocytes and embryonic bodies at day 4, derived from human embryonic stem cells (H9), were used as negative and positive controls, respectively, to amplify stem cell markers. Human epidermal keratinocytes and melanocytes were used as negative and positive controls respectively, to amplify melanocytic markers MITF and TYRP1. Epidermal keratinocytes and human neurons were used as negative and positive controls, respectively, for neuronal markers. Keratinocytes and HUVSs were used as negative and positive controls, respectively, for smooth muscle cell markers. Relative expression levels were calculated using 18S rRNA expression levels for comparison. The primer sequences are listed in Table 1.

Results

Isolation and Expansion of Sphere-Forming Cells from Human Hair Follicles

We cultured human hair follicle-derived cells in hESC medium, which is sufficient to maintain hESCs in an undifferentiated state without the need for feeder cells.[28] Sixteen fresh human scalp tissues were used in this study. Differential interference contrast and histological examinations showed that plucked human hair follicles were intact with little dermal contamination (Figure 1, A–D). The follicular epithelium was trypsinized and filtered to obtain a single-cell suspension containing cells of varying size and shape. Dissociated single cells were pelleted and cultured in conditioned hESC medium. Most of the single cells did not proliferate and gradually died. A few cells attached to the plate and continued to grow slowly as monolayer cells. Some small round cells proliferated and became 2- to 3-cell aggregates in suspension after 3 to 5 days. These aggregates slowly increased in size, gradually attached to the plate, and after 40 to 50 days, generated three-dimensional sphere-like structures, which we termed hair spheres (Figure 1, E–H). A hair sphere normally contains a sphere at its core and sprouting adherent fibroblastic cells at the periphery. We were able to isolate sphere-forming cells from 8 of 13 surgical specimens and 0 of 3 autopsy specimens. The number of spheres formed was directly proportional to the amount of scalp tissue used. Approximately in 10^4 to 10^5 seeded follicular cells were capable of forming a hair sphere. We termed these sphere-forming cells human hair follicle stem cells (hHFSCs). Therefore, hESC culture medium can be used to isolate and expand a subpopulation of sphere-forming cells from human hair follicles.

Human Hair Sphere Cells Are Capable of Self-Renewal

We then performed limiting dilution assays to test whether single cells derived from hair spheres were able to reform spheres. Hair spheres were enzymatically or mechanically dissociated into single cells and seeded into 96-well plates at 0.5 to 1 cell/well. Only wells containing a

Table 1. Primer Sequences

Gene	Symbol	Primer sequence
Nanog	NANOG	S: 5′-TGCTTATTCAGGACAGCCCT-3′ AS: 5′-TCTGGTCTTCTGTTTCTTGACT-3′
Snail	SNAI1	S: 5′-GACTAGAGTCTGAGATGCCC-3′ AS: 5′-CAGACATTGTTAAATTGCCCG-3′
Twist	TWIST	S: 5′-TCGAGAGATGATGCAGGACGT-3′ AS: 5′-TCTGGCTCTTCCTCGCTGTT-3′
Slug	SLUG	S: 5′-CATACAGCCCCATCACTGTG-3′ AS: 5′-CTTGGAGGAGGTGTCAGATG-3′
Sex-determining region Y-box 9	SOX9	S: 5′-AATGGAGCAGCGAAATCAAC-3′ AS: 5′-CAGAGAGATTTAGCACACTGATC-3′
Bone morphogenetic protein-4	BMP4	S: 5′-AGTGTGGCATCCGAGCTGAG-3′ AS: 5′-CGAGATAGCTTGGACGGGAATC-3′
Nestin	NES	S: 5′-CCCTGACCACTCCAGTTTAG-3′ AS: 5′-CCTCTATGGCTGTTTCTTTCTC-3′
β3-tubulin	TUBB3	S: 5′-AAGCCAGCAGTGTCTAAACCC-3′ AS: 5′-GGGAGGACGAGGCCATAAATAC-3′
Microtubule-associated protein 2	MAP2	S: 5′-GTGACAAGGAGTTTCAAACAGGAA-3′ AS: 5′-CTGATGGATAACTCTGTGCGAGA-3′
Tyrosinase-related protein 1	TYRP1	S: 5′-CATGTCACTGCAACGGCAATT-3′ AS: 5′-AAGTCCAGAAGATTTCTCCTGACT-3′
Microphthalmia-associated transcription factor	MITF	S: 5′-GAACTCAAAAGTCAACCGCTGAA-3′ AS: 5′-GCGTGATGTCATACTGGAGGAG-3′
Calponin	CNN3	S: 5′-GACTCCCGAGTACCCAGAGC-3′ AS: 5′-GCCCTAGGCGGAATTGTAGTAG-3′
Desmin	DES	S: 5′-CAGTTGAAGGAAGAAGCAGAGAAC-3′ AS: 5′-CTCCAGGTCAATGCGAGCTAG-3′
Cytokeratin 20	CK20	S: 5′-CTGAATAAAGACCTAGCTCTCCTCAAA-3′ AS: 5′-TGTTGCCCAGATGCTTGTGT-3′
18S	18S	S: 5′-CGGAGGTTCGAAGACGATCAGATA-3′ AS: 5′-TTGGTTTCCCGGAAGCTGCC-3′

S, sense strand; AS, anti-sense strand.

single cell were observed daily for proliferation. Some wells contained large flattened cells that proliferated into monolayer cultures with fibroblastic morphology, but they were unable to reform spheres. Small round cells in some wells were able to regenerate adherent spheres. They started to proliferate 2 to 4 days after seeding, and a small aggregate developed after 20 to 30 days. A typical hair sphere appeared after approximately 8 weeks, indicating their capacity for self-renewal (Figure 1, I–L). Approximately 1% of the seeded disassociated hair sphere cells were capable of forming a new hair sphere. Human hair follicle-derived stem cells maintained sphere-forming capacity within a period of 8 months in the hESC medium, and then they gradually became flattened and stopped proliferating, suggesting that these cells have a finite life span in the hESC medium *in vitro*.

Hair Spheres Express Neural Stem Cell and Neural Crest Stem Cell Markers

To characterize cells in the hair spheres, we studied their gene and protein expression. Neural crest stem cells and nestin-positive neural stem cells have been identified in the adult gut, dermis, and mouse hair follicles.[16,17,20,33] We examined hair spheres for the expression of nestin (*NES*) as well as genes transcribed in embryonic neural crest stem cells.[34] Real-time RT-PCR analyses showed that hair spheres expressed *NES* as well as the neural crest stem cell markers *SLUG*, *SNAIL*, *TWIST*, *SOX9*, and *BMP4* (Figure 2C). We performed nestin immunocytochemical staining on hair spheres and disassociated hair sphere cells and demonstrated that nestin-positive cells were present in the hair sphere (Figure 2, A and B) with approximately 10% of the cells being nestin positive. The results demonstrate that cells in the hair spheres express both neural and neural crest stem cell markers.

Human Hair Sphere-Forming Cells Are Distinctively Different from Known Hair Stem Cells

Unlike epithelial and melanocytic stem cells, hHFSCs cultured in hESC medium did not express any lineage-specific markers such as MITF and tyrosinase-related protein 1 (TYRP1) for melanocytes, MAP2 and β3-tubulin for neurons, calponin and desmin for smooth muscle cells, cytokeratin for keratinocytes, or CK20 for Merkel cells (data not shown).

Because hHFSCs can survive and expand in hESC medium, we tested whether these cells express Nanog and Oct4. Nanog and Oct4 are transcription factors involved in stem cell self-renewal and are thought to be the intrinsic factors that mediate pluripotency in embryonic stem cells.[35–39] However, their expressions might not be as restricted as previously thought. Oct4 expression has been demonstrated in adult stem cells, and rare positive cells can be identified in skin.[40] We found that a few cells within the hair spheres were positive for these transcriptional factors by immunocytochemistry and confocal microscopy (Figure

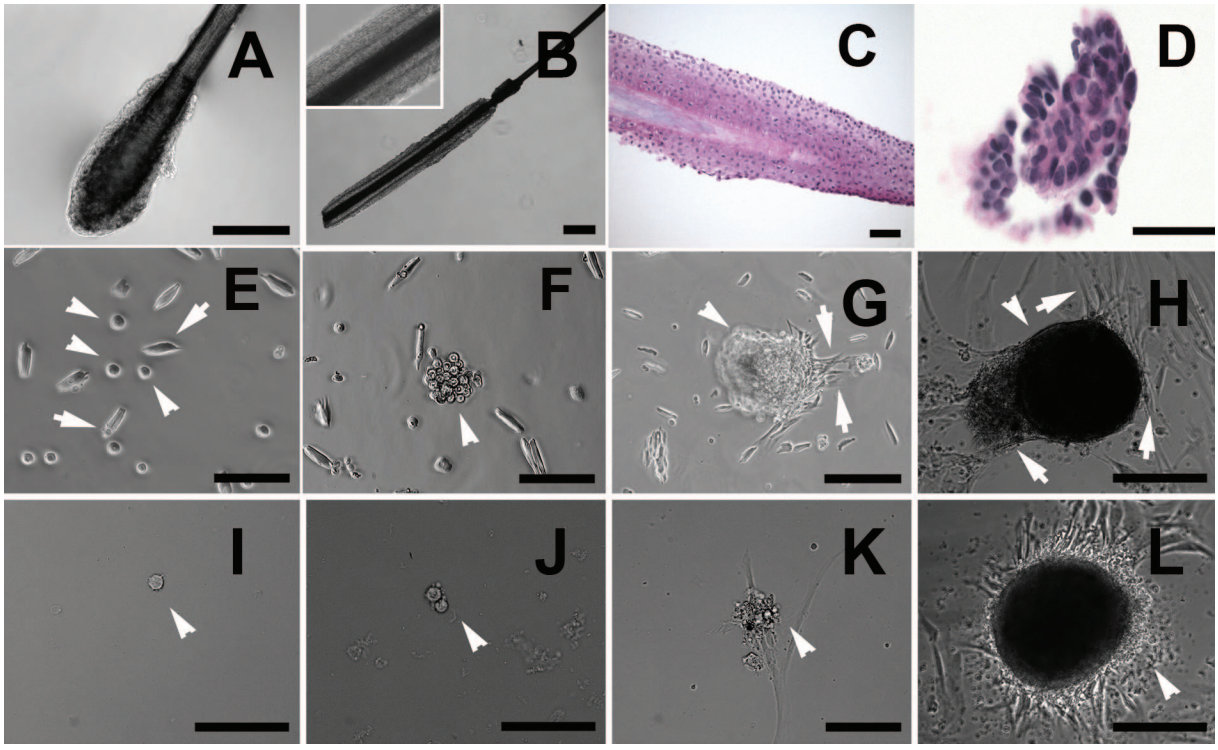

Figure 1. Isolation and expansion of hair spheres in hESC medium. **A:** Morphology of a plucked catagen/telogen hair follicle. **B:** Morphology of a plucked anagen hair follicle. **C:** Histology of a plucked anagen hair follicle (H&E stain). **D:** Histology of a plucked catagen/telogen hair follicle (H&E). **E:** A suspension of single cells, obtained from trypsinized follicular epithelium, contains a heterogeneous cell population. Some cells display relatively large spindle morphology (**arrows**), whereas others are small round cells (**arrowheads**). **F:** Small round cells proliferate to form a cell cluster in suspension in hESC medium (**arrowheads**). **G** and **H:** Cell clusters become adherent to the culture plates and form sphere-like structures (**arrowheads**) with sprouting, adherent fibroblastic cells at the periphery (**arrows**). **I–L:** A single cell derived from a hair sphere recapitulates the similar process and re-forms a sphere with adherent fibroblastic cells at the periphery (**arrowheads**). Representative images were taken from at least three independent experiments. Scale bars in **A**, **B**, **C**, **D**, and **G** = 200 μm; in **E**, **F**, **H**, **I**, **J**, **K**, and **L** = 100 μm.

2A). To confirm the observation, we dissociated hair spheres into single cells and plated them onto coated glass chamber slides. The resulting cultures contained heterogeneous populations of small round cells (3 to 5%), intermediate cells (15 to 20%), and large flattened cells (75 to 82%). Confocal microscopy revealed that most of the small round cells were positive for Nanog or Oct4 (3 to 5%). Oct4 staining showed a characteristic punctate nuclear staining pattern as previously described,[40] and Nanog, a homogenous nuclear staining pattern.[35,37] Rare small round cells were positive for both Nanog and Oct4 (data not shown). A few intermediate and large flattened cells also expressed Oct4 (Figure 2B). Unlike hESCs, hHFSCs do not express SSEA-3 and -4 (data not shown). Thus, hHFSCs are distinctively different from previously identified lineage-specific epithelial or melanocytic stem cells from hair follicles.

Melanocytic Differentiation of Human Hair Sphere-Forming Cells

We proceeded to induce differentiation of hHFSCs into melanocytes. Dissociated hair spheres were cultured in a melanogenic differentiation medium[29] for 14 days, when 20 to 40% of cells in medium adopted a dendritic morphology typical of melanocytes (Figure 3A). Real-time RT-PCR revealed that the differentiated cells gained the expression of melanocyte markers *MITF* and *TYRP1*, whereas they lost expression of the stemness gene *NANOG* (Figure 3B). Melanin pigment could be detected in differentiated cells by Fontana-Mason stain (Figure 3C). Expression of melanocyte markers MITF and silver (HMB45) proteins was detected by immunocytochemistry (Figure 3D). To study whether hHFSC-derived melanocytes have acquired functions similar to normal melanocytes, we introduced the differentiated melanocytes into human skin reconstructs that mimic human skin architecture. We have previously used these organotypic cultures to study interactions among melanocytes, epidermal keratinocytes, and dermal fibroblasts[32] and have shown that normal melanocytes migrated to the dermal epidermal junction. In this study, similar to normal melanocytes, hHFSC-derived melanocytes, identified by immunoreactivity for tyrosinase and melanin pigment, homed to the dermal-epidermal junction. Undifferentiated cells did not show any melanocytic phenotypes in the reconstructs (Figure 3E). These results suggest that, in the human skin environment, hHFSC-derived melanocytes not only produce melanin pigment but also respond to skin patterning cues in ways similar to those of normal epidermal melanocytes.

Neural Differentiation of Human Hair Sphere-Forming Cells

Next, we examined whether hHFSCs could differentiate into neurons, a type of cells not present in hair or skin. Fourteen days after culture in neuronal differentiation me-

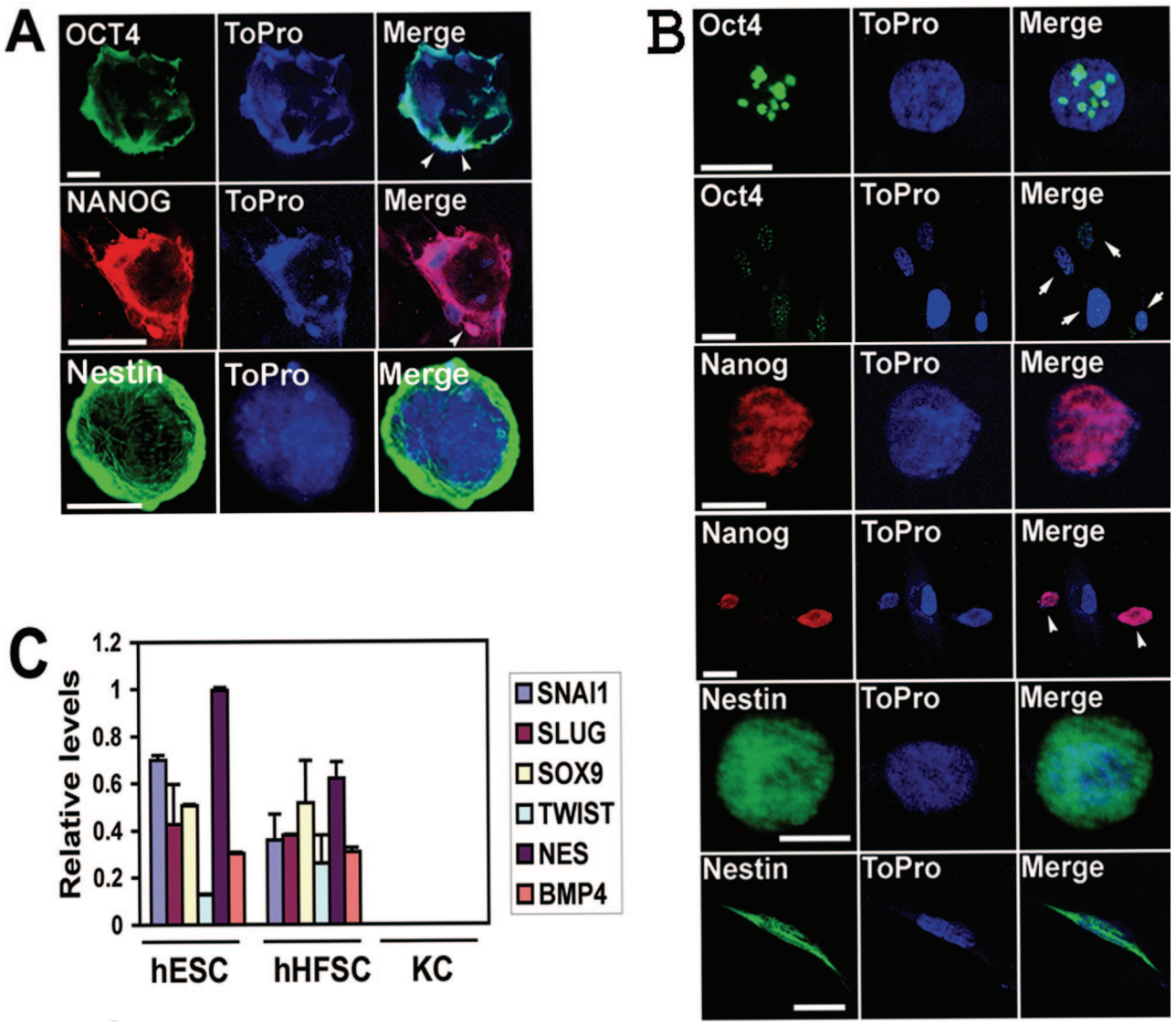

Figure 2. Human hair follicle-derived stem cells express various stem cell markers. **A:** Immunocytochemistry and confocal microscopy reveals expression of Nanog (red) and Oct4 (green) in a portion of heterogeneous populations within hair spheres. Their expression was typically localized to nuclei (stained blue with To Pro 3) (**arrowheads**). Nestin shows a cytoplasmic staining pattern. **B:** Spheres were dissociated into single cells and plated onto Matrigel-coated chamber slides for staining. Confocal images show that individual cells display nuclear staining pattern for Nanog (red) and Oct4 (green); cytoplasmic staining for Nestin. A characteristic punctate pattern of Oct4 staining is observed in both small round cells (**top**) and intermediate to large cells (**bottom**, **arrows**). The latter show a relatively weak staining intensity. Nanog immunoreactivity is observed in small round cells (**bottom**) and a portion of intermediate sized cells (**top**, **arrows**). Nestin also stains spindled cells (**bottom**). **C:** Real-time RT-PCR analysis shows that hHFSCs expressed *SNAIL*, *SLUG*, *SOX9*, *TWIST*, nestin (*NES*), and *BMP4* genes. We used epidermal keratinocytes (KC) and embryonic bodies derived from hESCs as negative and positive controls, respectively. Representative images were taken from at least three independent experiments. Data shown are mean ± SD from three independent experiments. Scale bars in **A** for Oct4 = 18.75 μm and for Nanog and Nestin = 75 μm; and in **B** for Oct4: top = 3.75 μm, bottom = 30 μm; for Nanog: top = 7.5 μm, bottom = 30 μm; and for Nestin: top = 7.5 μm, bottom = 30 μm.

dium, approximately 10% of cells showed long dendritic processes and expressed MAP2, NFM, and chromogranin A (CGA) proteins (as assayed by immunocytochemistry) (Figure 4A). MAP2 antibody predominantly stained the dendritic processes but not the cell bodies of differentiated cells, a staining pattern characteristic of neurons. Examination of neurotransmitters showed the presence of glutamate-positive cells; however, dopamine-positive cells were not identified. We detected the expression of *MAP2* and β3-tubulin (*TUBB3*) genes by real-time RT-PCR. The gain in neural markers was accompanied by a loss of *NANOG* gene expression after neuronal differentiation (Figure 4B).

Smooth Muscle Cell Differentiation of Human Hair Sphere-Forming Cells

To test whether hHFSCs could differentiate into mesoderm-derived lineages, we induced smooth muscle differentiation. In smooth muscle differentiation medium, approximately 80% of hHFSCs were induced into smooth

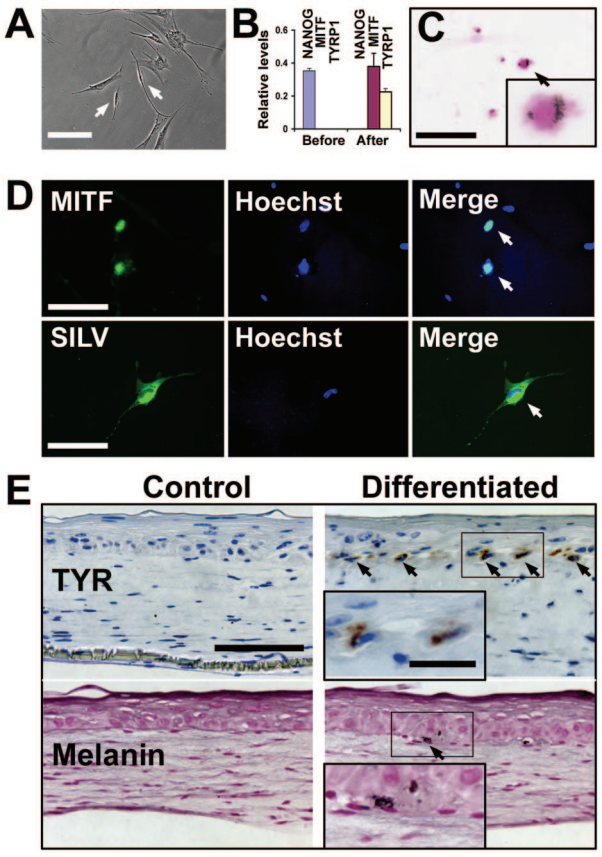

Figure 3. Melanocytic differentiation of hHFSCs. **A:** Dissociated, hair sphere-derived cells were treated in melanocyte differentiation medium for 14 days. We observed dendritic cells with typical melanocyte morphology (**arrows**). **B:** *MITF* and *TYRP1* gene expression is detected after differentiation; however, expression of stemness gene *NANOG* is undetectable after differentiation. **C:** Fontana-Mason staining shows melanin pigment in a portion of differentiated cells (**arrow** and **inset**). **D:** Differentiated cells exhibit nuclear and cytoplasmic immunoreactivity for MITF and SILV/HMB45, respectively. **E:** After embedding in three-dimensional human skin reconstructs, differentiated cells homed to the dermal-epidermal junction (**arrows** and **insets**). They are readily identifiable by TYR immunoreactivity or by Fontana-Mason staining. Reconstructs embedded with undifferentiated hFSCs do not show TYR and melanin staining. Representative images were taken from two independent experiments. Scale bars in **A** and **D** (SILV) = 200 μm; in **B**, **D** (MITF), and **E** = 100 μm; and in **inset** in **E** = 30 μm.

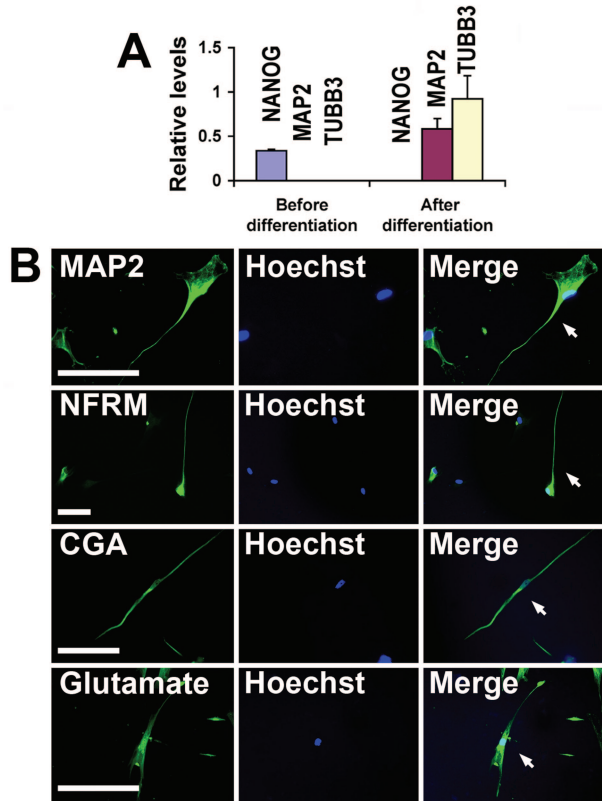

Figure 4. Neural differentiation of hHFSCs. **A:** Real-time RT-PCR demonstrates expression of neuronal markers *MAP2* and β3-tubulin (*TUBB3*) and loss of *NANOG* after neuronal differentiation. **B:** Differentiated cells become immunoreactive for MAP2, NFM, CGA, and neurotransmitter glutamate. MAP2 preferentially stains dendritic processes versus cell bodies (**arrow**). NFM, CGA, and glutamate stain cytoplasm of neuron-differentiated cells (**arrows**). Representative images were taken from at least three independent experiments. Scale bars in **A** = 100 μm.

muscle cells that acquired abundant cytoplasm and displayed immunoreactivity for SMA (Figure 5A). After differentiation, additional smooth muscle markers *CNN3* and *DES* were also detected by real-time RT-PCR, accompanied by the loss of *NANOG* gene expression (Figure 5B). To examine whether hHFSC-derived smooth muscle cells have acquired appropriate functions, we mixed differentiated cells into a collagen gel. Cultured HUVSs were used as a positive control. Human HFSC-derived smooth muscle cells contracted collagen gels; the contraction rate was identical to that of HUVSs (Figure 5, C and D), suggesting that they had acquired contracting function similar to those of HUVSs.

Oct4-Positive Cells Are Located in the Bulge

We extended our studies to identify a potential stem cell niche for hHFSCs. We performed Oct4 and cytokeratin 15 (K15) immunohistochemistry on consecutive normal human scalp tissues. K15 staining has been shown to highlight the bulge area.[31,41] Rare Oct4-positive cells can be seen in the epidermis as previously described[40]; however, most of the Oct4-positive cells in skin were located in the hair follicles within the area highlighted by K15 staining in both anagen and catagen/telogen hair follicles (Figure 6A). We then performed immunostaining on plucked human hair follicles after they were cultured in hESC medium for 6 days. Nanog- and Oct4-positive cells were readily identified in the bulge area of catagen/telogen follicles (Figure 6B). Expression of Nanog and Oct4 in bulge cells was further confirmed by confocal microscopy (Figure 6C). The results suggest that hHFSCs reside in the bulge.

Discussion

We have discovered that hESC medium can be used to isolate and expand adult stem cells from human hair follicles. We recently also showed that hESC medium can be used to isolate a subpopulation of tumor cells with stem cell characteristics from human melanomas[42] as well as sphere-forming cells from human foreskin (data

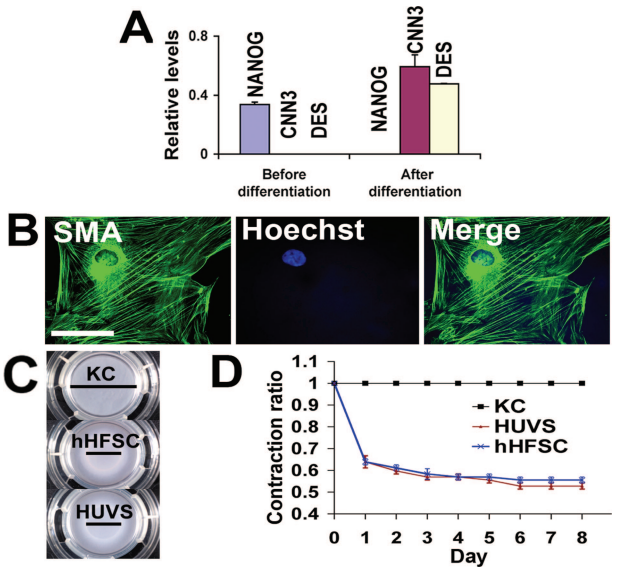

Figure 5. Smooth muscle cell differentiation of hHFSCs. **A:** Real-time RT-PCR demonstrates expression of calponin (*CNN3*) and desmin (*DES*) and loss of *NANOG* after smooth muscle differentiation. **B:** Differentiated cells become immunoreactive for SMA. SMA stains cytoplasm of differentiated smooth muscle cells. **C:** Contraction assays show hHFSC contracted collagen gels. Epidermal keratinocytes (KC) and HUVSs serve as negative and positive controls, respectively. **Black bars** indicate collagen gel diameters. **D:** Quantification of collagen gel shrinkage shows that differentiated cells contract collagen gel similarly to HUVS control. Data shown are mean ± SD of contraction ratio relative to keratinocyte control from three independent experiments. Scale bars in **A** = 100 μm.

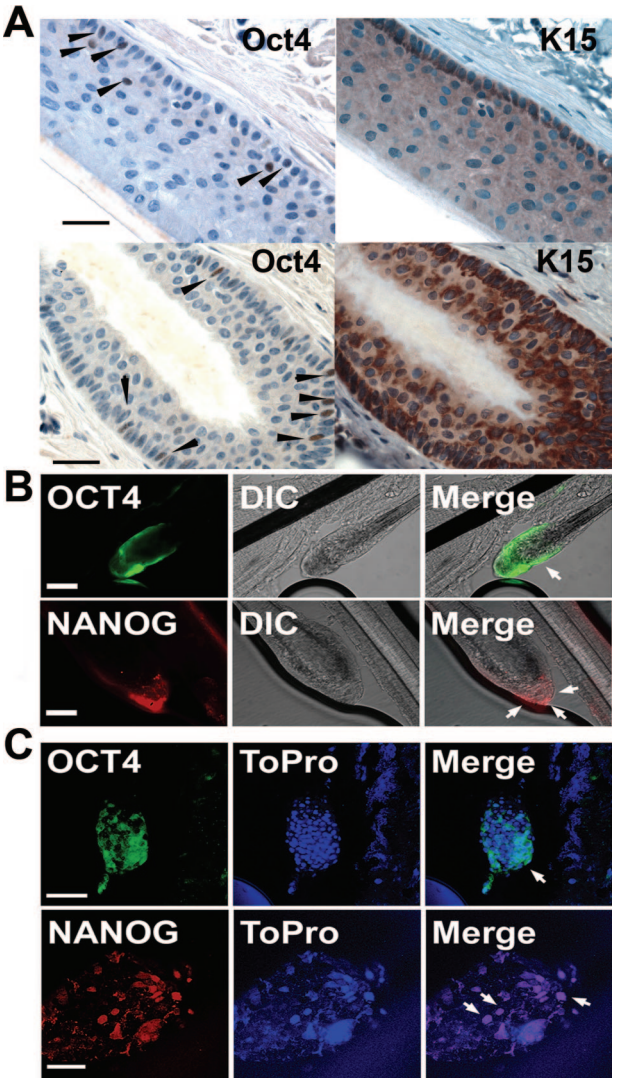

Figure 6. Oct4-positive cells are resided in the hair bulge. **A:** Oct4 and K15 immunohistochemical stains on consecutive normal human scalp tissues. **Top:** An anagen hair follicle; **bottom:** a catagen/telogen hair follicle. K15 highlights the bulge area, and a few Oct4-positive cells are present within K15-highlighted areas. **B:** Individual hair follicles were cultured in hESC medium for 6 days and then stained with Oct4 and Nanog. Immunostaining and differential interference contrast (DIC) images show that catagen/telogen bulge contains OCT4-positive cells (green, **arrows**) or NANOG-positive cells (red, **arrows**). **C:** Confocal images of catagen/telogen bulges reveal nuclear localization of OCT4 (green, **arrow**) and Nanog (red, **arrow**) in the bulge cells. Nuclei are counterstained with TO-PRO-3 (blue). Representative images were taken from at least three independent experiments. Scale bars in **A** = 30 μm; in **B** = 200 μm; and in **C** = 18.75 μm.

not shown), suggesting that this condition may be applied to isolate adult stem cells from other tissues as well.

Skin appears to be a stem cell-rich organ. Shih et al[43] isolated dermal stem cells from human adult scalp, Toma et al[44] isolated dermal stem cells from foreskin, and Belicchi et al[45] isolated dermal stem cells from fetal skin.[45] Neural-crest-like stem cells have been isolated from mouse whisker hair using a medium containing chick embryo extract and fetal calf serum[17,18]; these mouse hair stem cells grew as adherent monolayer cells. However, human hair follicle-derived stem cells were not able to proliferate using their medium condition (data not shown), suggesting different biological behavior of mouse and human stem cells. Using Nestin-driven green fluorescent protein (GFP) transgenic mice (Nestin is a marker for neural progenitor cells), Hoffman and colleagues[16] showed that GFP-positive cells are present in the hair bulge. These cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes *in vitro*. More recently, they implanted the GFP-expressing stem cells into the gap region of a severed sciatic nerve; these cells greatly enhanced the rate of nerve regeneration and the restoration of nerve function. These cells transdifferentiated largely into Schwann cells, which are known to support neuron regrowth.[46] Our study showed for the first time that human hair follicles also contain a stem cell population that can be differentiated into neuron, smooth muscle cell, and melanocyte lineages in induction medium.

Nanog and Oct4 are transcription factors that mediate hESC self-renewal and pluripotency.[36–39] Oct4 belongs to the family of POU-domain transcription factors that are normally expressed in pluripotent cells of the developing embryo and mediate pluripotency.[36,47,48] Consistent with its role in maintaining pluripotency, the reactivation of Oct4 has been correlated with efficient reprogramming of somatic cells after nuclear transfer into oocytes[49,50] or cell fusion of somatic cells with ES cells.[51] Moreover, ectopic expression of Oct4 in certain somatic cells has been associated with active dedifferentiation.[52] The reactivation of pluripotency-associated genes has been observed in various somatic cancer cells, such as breast and colon cancers,[53,54] although the relevance of this finding in adult cells is unclear. Our data demonstrate

that Oct4-positive cells are present in human skin, and most of them are located in the hair follicles *in vivo*. It has been suggested that a subpopulation of pluripotent stem cells present in the inner cell mass of the pre-implantation embryo may never differentiate; instead, they might persist and seed adult tissues. Such rare pluripotent cells may be responsible for recent observations of unexpected adult somatic tissue plasticity.[55] It is possible that these Oct4-positive cells in the hair follicles are related to these pluripotent stem cells that can perceivably give rise to follicular melanoblasts, Merkel cells, and other cells. These stem cells might generate diverse cell types during tissue renewal or repair in response to environmental cues. More research is warranted to further characterize these stem cells in the hair follicles.

The hair bulge is a stem cell niche, which can be highlighted by K15 staining. We have demonstrated that most of the Oct4-positive cells in human skin are located in the areas highlighted by K15 staining *in vivo*, suggesting that these stem cells are located in the bulge area, an area that provides an unique differentiation-restricted environment for adult stem cells.[10,11,13] Oct4- or Nanog-positive cells are present in the bulge area when individual hair follicles are cultured in hESC medium, further suggesting that these newly identified human follicular stem cells are located in the bulge.

In conclusion, our data indicate that human hair follicles contain multipotent stem cells other than epithelial and melanocytic stem cells, and these cells are located in the bulge area. These cells show promising plasticity in *ex vivo* and *in vitro* conditions, making them potential candidates for cell engineering and cell replacement therapies. Human scalp tissues are easily accessible, and the fact that hair spheres can be generated from autologous adult tissue makes it an attractive source for individualized cell-based therapies.

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