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Original Article

Isolation and Characterization of Multipotent Skin-Derived Precursors from Human Skin

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ABSTRACT

We have previously isolated, expanded, and characterized a multipotent precursor cell from mammalian dermis (termed skin-derived precursors [SKPs]) that can differentiate into both neural and mesodermal progeny. In this study, we report the isolation, expansion, and characterization of a similar precursor cell from neonatal human foreskin tissue. Like their rodent counterparts, human SKPs grew in suspension as spheres in the presence of the mitogens fibroblast growth factor 2 and epidermal growth factor and expressed nestin, fibronectin, vimentin, and characteristic embryonic transcription factors. Human SKPs could be maintained in culture for long periods of time and would still differentiate into neurons, glia, and smooth muscle cells, including cells with the phenotype of peripheral neurons and Schwann cells. Clonal analysis indicated that single SKP cells were multipotent and could give rise to all of these progeny. Moreover, human SKPs apparently derive from an endogenous precursor within human foreskin; a subpopulation of dissociated primary foreskin cells could differentiate into neurons, a cell type never seen in skin, and the initial spheres to develop from skin expressed the same markers and had the same potential as do passaged SKPs. Together, these data indicate that SKPs are an endogenous multipotent precursor cell present in human skin that can be isolated and expanded and differentiate into both neural and mesodermal cell types. STEM CELLS *2005;23:727–737*

INTRODUCTION

A significant amount of recent interest has focused on the possibility that adult human stem cells are a realistic therapeutic alternative to embryonic stem cells. This interest has arisen largely as a consequence of recent work demonstrating that several adult stem cells show a surprisingly diverse differentiation repertoire [1]. Although in some of these cases this multipotency was due to unanticipated cellular fusion events that occurred in vivo [2–4], compelling evidence still exists for the multipotency of several cultured adult stem cell populations, including multipotent adult progenitor cells isolated after long-term culture of bone marrow cells [5] and neural stem cells from the central nervous system (CNS) [6–9].

The most obvious therapeutic use of such multipotent adult human precursors is for cell transplantation and replacement. However, perhaps equally important is the possibility that expandable adult stem cell populations could be used to generate human cell types on an individual basis for screening or discovery research. In either case, the ideal human precursor cell population would be one that could be derived in an autologous fashion from small amounts of accessible human tissue biopsies. With this in mind, we previously isolated and characterized a multi-

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potent precursor cell population from a highly accessible tissue source, adult mammalian dermis [10], using an approach similar to that originally described by Reynolds and Weiss [6] to isolate adult stem cells from the CNS. These cells, termed skin-derived precursors (SKPs), were isolated and expanded from rodent skin and would differentiate into both neural and mesodermal progeny, including cell types that are never found in skin, such as neurons. One endogenous embryonic stem cell population that has a similar broad potential and that contributes to the dermis is neural crest stem cells [11], and, in this regard, our recent work indicates that SKPs represent an embryonic neural crest–related precursor cell that arises in skin during embryogenesis and that persists in lower numbers into adulthood [12].

We have previously provided evidence that a similar SKP-like precursor may reside in adult human skin [10]. Specifically, we demonstrated that small punch biopsies of human scalp contained cells that would proliferate in response to fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF), that a subpopulation of these cells expressed the SKP markers nestin and fibronectin, and that they could differentiate into βIII-tubulin–positive cells with the morphology of newly born neurons. In this study, we have built upon these initial findings and have isolated and expanded SKPs from human foreskin tissue. We demonstrate that these human SKPs are multipotent adult precursor cells that are capable of generating neural and mesodermal progeny even after long-term expansion and that they may well represent an endogenous neural crest–related precursor present in adult human dermis.

Materials and Methods

Establishment of Foreskin SKP Cultures

Pieces of human foreskin of 1–2 cm^2 deriving from voluntary circumcisions of children aged 4 weeks to 12 years of age were washed with Hanks' balanced salt solution (Invitrogen Corporation, Carlsbad, CA, http://www.invitrogen.com), cut into 4- to 6-mm pieces, washed again, and incubated in Liberase Blendzyme 1 (0.62 Wunsch U/ml; Roche Molecular Biochemicals, Laval, Quebec, Canada, http://www.roche-applied-science.com) overnight at 4°C. The epidermis was manually removed from each tissue piece, and the dermis was cut into 1-mm^3 pieces and incubated in Liberase Blendzyme 1 for 30–40 minutes at 37°C. DNase I was added for 1 minute, and 10% fetal bovine serum (FBS) (Cambrex, Walkersville, MD, http://www.cambrex.com) was added to inhibit the enzymes. The supernatant was removed, and tissue pieces were resuspended in medium (Dulbecco's modified Eagle's medium [DMEM]/F12, 3:1 [Invitrogen] containing 1% penicillin/streptomycin unless otherwise indicated) and manually dissociated by pipetting into a 2-ml pipette, a process that was repeated until the tissue could be broken down no further. The cell suspension was then centrifuged at 1,000 rpm for 5 minutes and the supernatant removed, leaving the pellet and 3 ml of medium behind. The pellet was resuspended in the remaining medium using a fire-polished Pasteur pipette, and the suspension passed through a 70-μm cell strainer (BD Biosciences, Mississauga, Ontario, Canada, http://www.bd.com). The strained cell suspension was then centrifuged, the medium removed, the pellet resuspended in 10 ml proliferation medium (DMEM-F12, 3:1 and 40 ng/ml FGF2, 20 ng/ml EGF [both from BD Biosciences], B27 [Invitrogen], and 1 μg/ml fungizone [Invitrogen]) and then transferred to a 25-cm^2 tissue culture flask (BD Biosciences).

For subculturing, medium containing SKPs growing in suspension was centrifuged at 1,000 rpm for 5 minutes and the supernatant was removed, leaving 6 ml of medium and the pellet behind. The pellet was resuspended in the remaining medium with a fire-polished Pasteur pipette, proliferation medium was added to a total of 20 ml, and the cell suspension was then split into two 25-cm^2 flasks. The cells were grown at 37°C for an additional 2–3 weeks and then split again as above.

For immunocytochemical analysis of SKP spheres, 100 μl of medium containing suspended spheres was removed from a flask and spun down onto coated slides using a ThermoShandon Cytospin 4 apparatus (Thermo Shandon Inc., Pittsburgh, PA, http://www.thermo.com). The slides were then air-dried for 5 minutes and analyzed. For quantitation of the size of SKP spheres grown in different growth factors, the diameter of spheres was measured along both the x and y axes, because spheres were not uniformly spherical. The average of these two measurements was then used as the diameter of the sphere. Within a given experiment, multiple spheres were measured in each well, the mean diameter and SD of all measured spheres in each individual well were determined, and then four wells per experimental manipulation were considered to obtain a statistical comparison between growth factor treatments.

Clonal Analysis

To obtain single cells, medium containing proliferating SKP spheres was centrifuged at 1,000 rpm for 5 minutes and the supernatant removed, leaving behind the pellet plus 2 ml medium. The supernatant was then filtered with a 0.2-μm syringe filter to provide conditioned medium free of cells. The cell pellet was resuspended in the remaining 2 ml medium with a fire-polished Pasteur pipette, and cell number was determined using a hemocytometer. Cells were diluted with medium to a calculated concentration of 1 cell per 100 μl, and 100 μl of this suspension was then added to individual wells of a 48-well culture plate. After several hours, each well was scored for the presence or absence of single cells. To each well containing a single cell, an additional 100 μl of conditioned medium plus EGF and FGF2 was added. Every 4–5 days, 50 μl of additional proliferation medium was added;

this medium was comprised of 50% fresh and 50% conditioned medium. Clones were monitored and photographed over a 10-week period, at the end of which the wells contained floating spheres. Each clone was dissociated and transferred to a single well of a 24-well plate, and after 7–10 days in proliferation medium, cells were again dissociated and transferred to 3 wells of a 12-well plate. After an additional 2 weeks in proliferation medium, all the cells from an individual clone were combined and transferred to a 25-cm^2 flask in a final volume of 10-ml proliferation medium. Cells were then maintained as for regular-passaged SKPs.

Differentiation of SKPs

Medium containing SKP cells in suspension was removed from a tissue culture flask and centrifuged, and the pellet was resuspended in 3 ml of remaining medium with a fire-polished Pasteur pipette. Cells were counted with a hemocytometer, and 10,000 cells were plated per well of a four-well chamber slide (Nunc, Rochester, NY, http://www.nuncbrand.com) coated with poly-D-lysine and laminin the previous day. For most experiments, cells were differentiated in medium containing 5% FBS. In some experiments, cells were plated in proliferation medium containing 10% FBS for 3 days, switched for 3 days to medium plus 5% FBS, and then switched into either (a) neuronal conditions comprised of Neurobasal medium (Invitrogen) containing 1%–5% FBS plus 50 ng/ml each of nerve growth factor (Cedar Lane, Hornby, Ontario, Canada, http://www.cedarlanelabs.com), brain-derived neurotrophic factor, and 10 ng/ml NT-3 (both from Peprotech, Rocky Hill, NJ, http://www.peprotech.com) or (b) Schwann cell conditions comprised of Neurobasal medium containing 1% FBS plus 1% N2 supplement, 4 μM forskolin, and 10 ng/ml heregulin β [12]. In all experiments, cells were differentiated for 2–3 weeks, with 50% of the medium changed every 3–4 days.

Immunocytochemistry

Differentiated cells on chamber slides were washed three times with HEPES-buffered saline (HBS), fixed with 4% paraformaldehyde in phosphate buffer for 15–20 minutes, and then washed three times with HBS. Cells were permeabilized with 0.2% NP40 for 5 minutes, washed three times for 5 minutes with HBS, and blocked for 1 hour at room temperature with 0.5% bovine serum albumin and 6% normal goat serum in HBS. Primary antibodies were then added in HBS containing 3% normal goat serum and left overnight at 4°C. Primary antibody was removed, cells were washed three times for 5 minutes with HBS, and the appropriate secondary antibody conjugated to CY3 or fluorescein isothiocyanate was added in HBS containing 3% normal goat serum for 1 hour at room temperature. Cells were washed three times for 5 minutes with HBS, stained with Hoechst 33258, and visualized using a Zeiss Axioplan upright microscope. Primary antibodies used were βIII-tubulin monoclonal (1:30, RDI, Flanders, NJ, http://www.researchd.com), neurofilament (NF-M) (1:200, Chemicon, Temecula, CA, http://www.chemicon.com), CNPase (1:100, Biocarta, San Diego, http://www.biocarta.com), GFAP monoclonal (1:25, Dako, Mississauga, Ontario, Canada, http://www.dakocytomation.com), GFAP polyclonal (1:200, Dako), p75NTR (1:400, Promega, Madison, WI, http://www.promega.com), S100β (1:1000, Sigma, Oakville, Ontario, Canada, http://www.sigmaaldrich.com), nestin (1:200, Chemicon), fibronectin (1:400, Sigma), vimentin (1:200, Chemicon), smooth muscle actin (SMA) (1:400, Sigma), GAP43 (Sigma), MAP2a,b,c (Sigma, clone HM-2, which recognizes all MAP2 isoforms), and versican (1:200, the kind gift of Dr. Richard LeBaron). Secondary antibodies were all from Jackson Immunoresearch Laboratories (West Grove, PA, http://www.jacksonimmuno.com).

To obtain an estimate of the percentage of cells adopting neuronal and glial phenotypes, random fields were selected and photographed, and for each field the total number of cells (as determined by counting Hoechst stained nuclei) and the total number of cells positive for neuronal or glial markers were determined. This analysis was performed for three different differentiation experiments, and the mean and standard deviation were determined.

Reverse Transcription–Polymerase Chain Reaction

RNA was prepared from samples using Trizol (Invitrogen), and cDNA was generated with Revertaid reverse transcription (RT) (Fermentas, Burlington, Ontario, Canada, http://www.fermentas.com) as directed by manufacturer. For all cDNA synthesis, a minus RT control was performed. Polymerase chain reaction (PCR) was carried out as follows: 92°C for 2 minutes, 30 to 35 cycles of 94°C for 60 seconds, gene-specific annealing temperature for 60 seconds, and 72°C for 60 seconds. PCR primers used were as follows: for p75NTR, tgggcccagaaggttgcgatgaa and aaaggggcccccagaaccaaacaca; for pax3, catccggccctgcgtcatctc and tggccttcttctcgctttcctctg; for snail, tggcctgtctgcgtgggtttttgt and cctgggctcggggcatctca; for slug, catctttggggcgagtgagtcc and cccccgtgtgagttctaatgtgtc.

Karyotyping

Karyotyping was performed by the Hospital for Sick Children Cytogenomic Services facility. SKP spheres were cultured as described above until near confluency. Colcemid (KaryoMAX, Invitrogen) was added to a final concentration of 0.05 μg/ml for 2 hours at 37°C. Cultures were centrifuged at 1,000 rpm for 10 minutes, after which they were washed with phosphate-buffered saline and treated with 0.05% trypsin/0.053 mM EDTA (Invitrogen) for 2–4 minutes. Trypsinization was arrested by the addition of α-MEM medium supplemented with 10% FBS (Sigma). Cells were then harvested according to standard cytogenetic protocol using 0.075 M hypotonic potassium chloride and 3:1 methanol

and acetic acid fixative. Metaphase preparations were made by dropping the fixed cell suspension onto precleaned slides in a Thermotron (Thermotron Industries, Holland, MI, http://www.thermotron.com), aged at 90°C for 90 minutes before G-banding using 2% trypsin and Giemsa/Leishman's stain. At least 20 metaphases were analyzed, and a minimum of 8 were karyotyped for each line.

Results

SKPs Can Be Routinely Isolated and Expanded from Neonatal Human Foreskin

Because rodent SKPs are more abundant in neonatal than adult skin [12], we used neonatal human foreskin tissue in our attempts to isolate and characterize human SKPs. Foreskin samples from circumcisions of children ranging from 4 weeks to 3 years of age were used for this study, with over 30 samples in all being analyzed. These samples were indistinguishable in terms of SKP isolation and properties. One sample was also obtained from a 12-year-old, and no overt differences in SKP growth or differentiation properties were noted relative to SKPs derived from younger children. Nonetheless, all data presented in this report derive from SKPs cultured from foreskins of children aged 3 years or younger.

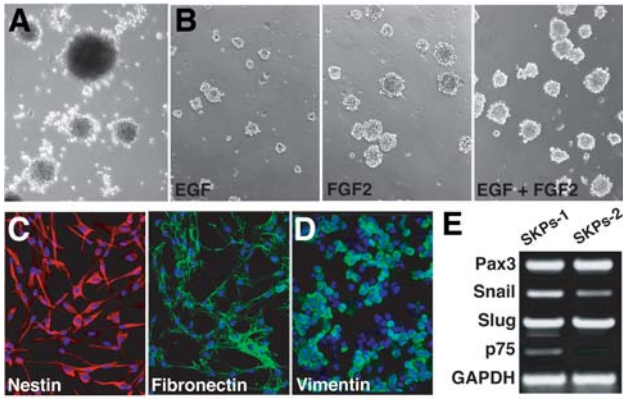

Figure 1. Isolation and characterization of human foreskin SKPs. **(A):** Phase photomicrograph of human foreskin SKP spheres immediately before passaging. Note the characteristic morphology, which is very similar to rodent SKP spheres. **(B):** Phase photomicrographs of human SKP spheres that were grown in EGF, FGF2, or both EGF and FGF2 for 2 weeks. **(C):** Fluorescence photomicrograph of human SKP cells that were adhered to a poly-D-lysine/laminin substratum overnight and then double-labeled with antibodies to nestin (left panel) and fibronectin (right panel). **(D):** Fluorescence photomicrograph of adherent SKP cells immunostained for vimentin. **(C, D):** The blue is Hoechst staining of nuclei to show all of the cells in the field. **(E):** Reverse transcription–polymerase chain reaction analysis for the expression of mRNAs for Pax3, Snail, Slug, p75NTR, and, as a control, GAPDH, in two human SKP preparations (SKPs-1 and SKPs-2) that had been passaged five and six times. Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor 2; SKP, skin-derived precursor.

To isolate SKPs, epidermis and dermis were dissociated from skin samples of approximately 1–2 cm^2, and dissociated dermal cells were cultured in defined medium containing EGF, FGF2, and B27. Over the first 2–3 weeks under these culture conditions, most cells adhered to the tissue culture plastic and/or died, but a small population of proliferating spheres of cells formed, with a morphology similar to that previously seen with rodent SKPs [10]. At approximately 3 weeks, the spheres were isolated, dissociated, and then split into medium containing the same growth factors and filtered SKP-conditioned medium. SKP cultures were then passaged and split every 2–3 weeks, so that at the end of 3 months, a 1-cm^2 piece of developing foreskin gave rise to approximately 1 to 2×10^7 cells present in culture as floating spheres (Fig. 1A).

To ask whether these proliferating spheres shared the same growth factor requirements as rodent SKPs, cells in spheres were triturated to single cells and were then passaged into defined medium containing B27, with or without EGF and FGF2. This experiment revealed that the human SKPs absolutely required EGF and FGF2 to proliferate, because no spheres were generated in their absence. Visual examination of the cultures also revealed that, although spheres could grow in EGF, their number and size were reduced relative to FGF2 alone or EGF plus FGF2 (Fig. 1B). Measurements of sphere number and size confirmed this conclusion; after 2.5 weeks of growth in EGF or FGF2 alone, the sphere numbers were approximately 20% and 80%, respectively, of those obtained with both growth factors, and spheres were approximately 50% and 90%, respectively, of the size of those generated in EGF plus FGF2 (mean sphere diameter of 58 ± 3.4 μm [SD] for EGF, 124 ± 4.9 μm for FGF2 versus 134 ± 5.9 μm for EGF plus FGF2 in one representative experiment; $p < .0001$ for the comparison between EGF and EGF plus FGF2; $p < .05$ for the comparison between FGF2 and EGF plus FGF2). The diameter of individual SKP cells was also measured, and cell size was in the range of 10–15 μm in diameter, thereby distinguishing SKPs from the very small spore stem cells previously isolated from skin [13].

To confirm that these human spheres were similar to rodent SKPs, we initially examined the cell-surface markers that they expressed. Human spheres of three passages or less were either plated onto poly-D-lysine and laminin overnight or cytospun as spheres onto slides and then were immunostained for nestin, fibronectin (Fig. 1C), and vimentin (Fig. 1D), all of which are expressed by rodent SKP cells [10, 12]. This analysis revealed that the human SKPs expressed all of these proteins but that they did not express tyrosinase or trp1, markers for melanoblasts/melanocytes (data not shown). Similar results were obtained with all samples whether the cells were analyzed as single cells, attached cells, or spheres.

We have recently found that rodent SKPs express several transcription factors that are associated with embryonic neural

crest stem cells and some of their developing embryonic derivatives [9]. We therefore used RT-PCR to analyze two independently isolated human SKP populations for their expression of a subset of these transcription factors, Pax3 [14], snail [15, 16], and slug [17, 18]. This analysis (Fig. 1E) revealed that, like rodent SKPs, human SKPs express these three transcription factors. In addition, human SKPs expressed low levels of the mRNA for p75 neurotrophin receptor (p75NTR) (Fig. 1E), another marker for embryonic neural crest stem cells. However, immunocytochemical analysis indicated that the p75NTR protein was expressed only at low or undetectable levels in SKP spheres (Fig. 2B). Thus, human SKPs share the same growth factor requirements as rodent SKPs and express similar genes.

SKPs Differentiate into Neurons, Glia, and Smooth Muscle Cells, Including Cells with Peripheral Neural Phenotypes

We have previously demonstrated that rodent SKPs will differentiate into both neural and mesodermal cell types, including neurons, glia, smooth muscle cells, and adipocytes [10]. Moreover, our recent studies indicate that the neural cell types are largely peripheral cells that derive from the neural crest during development, including catecholaminergic neurons and Schwann cells [12]. To ask whether human SKPs could generate the same differentiated cells, spheres at passages 3 through 9 were plated down on poly-D-lysine/laminin for 2–4 weeks in the presence of defined medium plus 5% FBS. Immunocytochemical analysis of these cultures revealed that the human SKPs generated many morphologically complex neurons that were positive for βIII-tubulin and/or NF-M (Figs. 3A, 3B). These cells always differentiated on top of a layer of nonneuronal cells. Almost all of the βIII-tubulin–positive neurons coexpressed p75NTR (Fig. 3C), a hallmark of peripheral neurons, and something that is also seen for the rodent SKP-derived neurons [12]. When cells were differentiated in the presence of neurotrophins to enhance the survival and differentiation of peripheral neurons (neuronal conditions), then these βIII-tubulin–positive cells also coexpressed two other neuron-specific proteins, GAP-43 (Fig. 3E) and MAP2 (Fig. 3D). These putative neurons did not express SMA, nor did they express markers of glial cells, including CNPase, GFAP, or S100β. To estimate the percentage of cells that differentiated into neurons, random fields of cells from three different experiments were quantitated to determine the total number of cells (as determined by counting Hoechst-positive nuclei) versus the total number of βIII-tubulin–positive, morphologically complex cells. This analysis revealed that 9.4% ± 0.2% of the cells in these experiments were differentiated neurons.

In addition to neurons, under the same differentiation conditions, human SKPs generated glial cells and potential smooth muscle cells (Fig. 4). With regard to glial cells, a subpopulation of bipolar cells coexpressed S100β and p75NTR (Fig. 4A), an expression profile typical of peripheral Schwann cells [12]. A similar

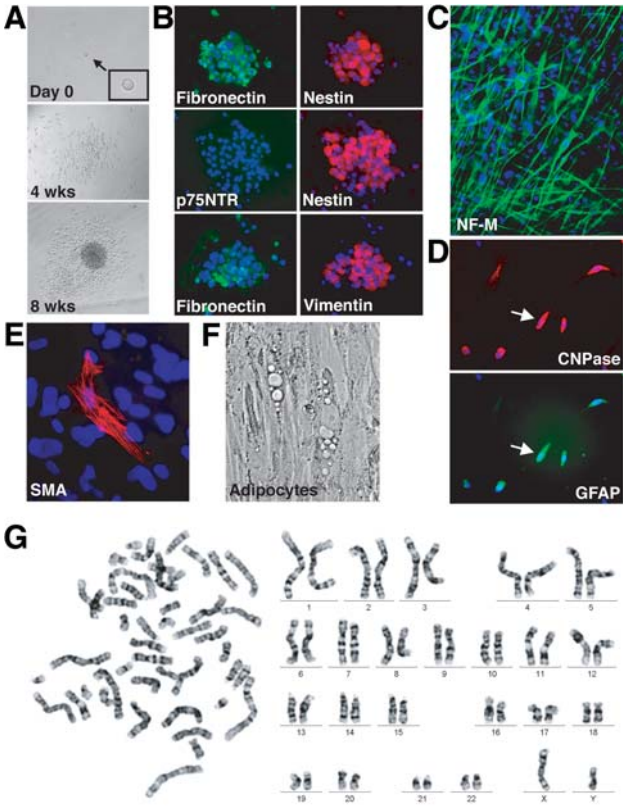

Figure 2. Single human SKP cells are multipotent. **(A):** Phase photomicrographs of a human SKP clone generated initially from a single, isolated cell on the day of plating (day 0, top panel) and after 4 (middle panel) and 8 (bottom panel) weeks of proliferation. Note that at 8 weeks, a nascent sphere is forming that will ultimately detach from the dish and grow in suspension. **(B):** Immunocytochemical analysis of single clonal SKP spheres that were double-labeled for nestin and fibronectin (top panels), nestin and p75NTR (middle panels), or vimentin and fibronectin (bottom panels). Nuclei of all the cells are blue as a consequence of the Hoechst counterstain. **(C):** Fluorescence photomicrograph of cells from a single SKP clone that were differentiated for 3 weeks in the presence of 5% FBS and then immunostained for NF-M. **(D):** Fluorescence photomicrographs of cells from a single SKP clone that were differentiated for 3 weeks in the presence of 5% FBS and then double-labeled for CNPase (top panels) and GFAP (bottom panels). The arrow indicates the same cell in both panels. **(E):** Fluorescence photomicrograph of cells from a single SKP clone differentiated for 3 weeks and then immunostained for SMA. **(F):** Phase photomicrograph of cells from a single SKP clone differentiated for 3 weeks in the presence of retinoic acid, showing cells with the characteristic morphology and lipid droplet inclusions of adipocytes. **(G):** G-banding of a metaphase chromosome spread obtained from one of the three SKP clones that were analyzed as shown in panels **(A–F)** after passaging for 15 months. The left panel shows the metaphase spread, and the right panel shows the ordered chromosomal pairs. Abbreviations: FBS, fetal bovine serum; NF-M, neurofilament; SKP, skin-derived precursor; SMA, smooth muscle actin.

subpopulation of bipolar cells also coexpressed CNPase, a marker of myelinating glia, including oligodendrocytes and Schwann cells, and GFAP, which is expressed in Schwann cells and astrocytes (data not shown; Figs. 2D, 5D). The coexpression of these four proteins and this bipolar morphology indicate that these cells are likely to be Schwann cells. Quantitation of the total number of CNPase-positive cells versus the total number of cells in three different experiments revealed that 4.3% ± 0.3% of the cells were differentiated glial cells. A subpopulation of SMA-positive cells with the morphology of smooth muscle cells or myofibroblasts was also observed in these experiments (Fig. 4B). These latter cells did not express any glial or neuronal proteins. These three different cell types, neurons, glial cells, and smooth muscle cells, were consistently seen upon differentiation of human SKP cultures.

To determine whether SKPs could be expanded long-term and still maintain their potential to generate these three cell types, we maintained and analyzed two human SKP cultures for 1 year, at the end of which they had been passaged 16 times. Over this entire time period, the SKPs maintained the same relatively slow growth rate, with the cells doubling in number every 2–3 weeks. In addition, G-banding of one of these lines revealed that their chromosomal karyotype was normal after 15 months of passaging (Fig. 5F). Immunocytochemical analysis of these long-passage SKP spheres demonstrated that they expressed the same markers as the lower-passage SKP spheres, including nestin, fibronectin, versican (Fig. 5A), and vimentin (data not shown). These cells were then differentiated for 3–4 weeks and examined immunocytochemically. This analysis revealed that these highly expanded SKPs were able to generate morphologically complex, βIII-tubulin and NF-M–positive neurons (Figs. 5B, 5C), as well as bipolar glial cells coexpressing CNPase and GFAP (Fig. 5D) and SMA-positive smooth muscle cells or myofibroblasts (Fig. 5E). Thus, SKPs retain their ability to differentiate appropriately, even when maintained and expanded for long periods of time in culture.

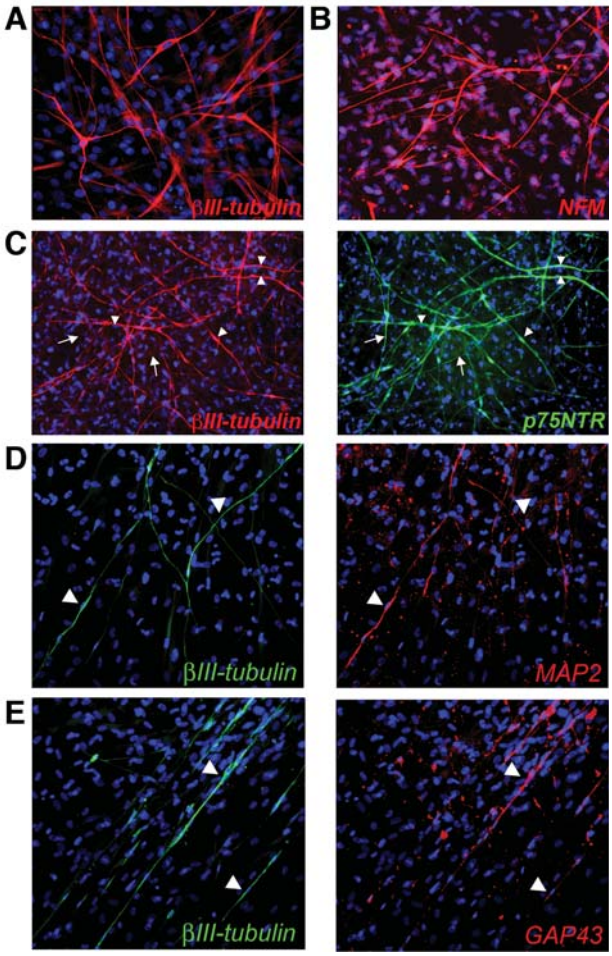

Figure 3. Human SKPs differentiate into neurons. **(A, B):** Fluorescence photomicrographs of two different human SKP preparations that were differentiated for 2–3 weeks in the presence of 5% fetal bovine serum and immunostained for **(A)** βIII-tubulin or **(B)** NFM. The blue is from the Hoechst staining of nuclei. Note that the positive cells are of typical neuronal morphology and have differentiated on top of a layer of nonneuronal cells. **(C–E):** Fluorescence photomicrographs of SKPs differentiated for 3 weeks in the presence of neurotrophins and then double-labeled for **(C)** βIII-tubulin (left panel) and p75NTR (right panel), **(D)** βIII-tubulin (left panel) and all isoforms of MAP2 (right panel), or **(E)** βIII-tubulin (left panel) and GAP43. In all cases, the arrowheads denote cells that are double-labeled for both markers, whereas in **(C)** the arrows denote cells that are positive for p75NTR but not for βIII-tubulin. Coexpression of these markers is characteristic of peripheral neurons, whereas the cells that express p75NTR but not βIII-tubulin are potentially p75NTR-positive Schwann cells. Note that the MAP2 antibody used in **(D)** detects MAP2a,b,c and is therefore not specific to dendrites in developing neurons. Abbreviations: NFM, neurofilament; SKP, skin-derived precursor.

Single SKPs Clonally Generate Neural and Mesodermal Progeny

To ask whether a single SKP cell was able to generate these different cell types, we performed clonal analysis. Human SKPs that had been passaged three times were dissociated to single cells;

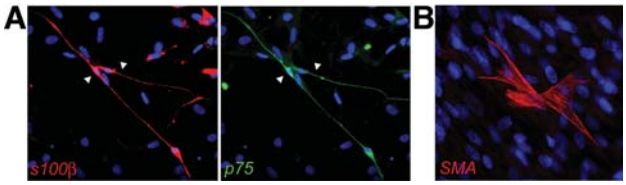

Figure 4. Human SKPs differentiate into glial and smooth muscle cells. **(A):** Fluorescence photomicrograph of human SKPs that were differentiated for 3 weeks in the presence of 1% serum, forskolin, and heregulin-β and then double-labeled for S100β (left panel) and p75NTR (right panel), a morphological and antigenic profile characteristic of Schwann cells. **(B):** Fluorescence photomicrograph of human SKPs differentiated in 5% fetal bovine serum for 3 weeks and then immunostained for SMA. Note the characteristic morphology of these cells, indicating that they are likely to be smooth muscle cells or myofibroblasts. Abbreviations: SKP, skin-derived precursor; SMA, smooth muscle actin.

these single cells were isolated by limiting dilution into 48-well plates (Fig. 2A) and then cultured in medium containing EGF, FGF2, B27, and 50% of the filtered medium conditioned by more densely growing SKP cultures. Initially, these cells were quiescent, but they slowly started to proliferate, so that by 4 weeks, small clones of dispersed cells were formed, whereas by 8 weeks, spheres were generated that would ultimately dissociate from the tissue culture plastic and grow in suspension (Fig. 2A). After 10 weeks, the clones were dissociated and moved to 24-well plates and then 12-well plates over a period of 3 weeks and in this manner were ultimately expanded into small flasks. Of the 92 wells containing single cells at the start of the experiment, 39 of these proliferated to generate clones, and 37 of those grew as spheres. The other two clones only ever grew adherently, even after expansion, and were not analyzed further. Thus, approximately 40% of the SKP cells were able to self-renew when isolated as clones.

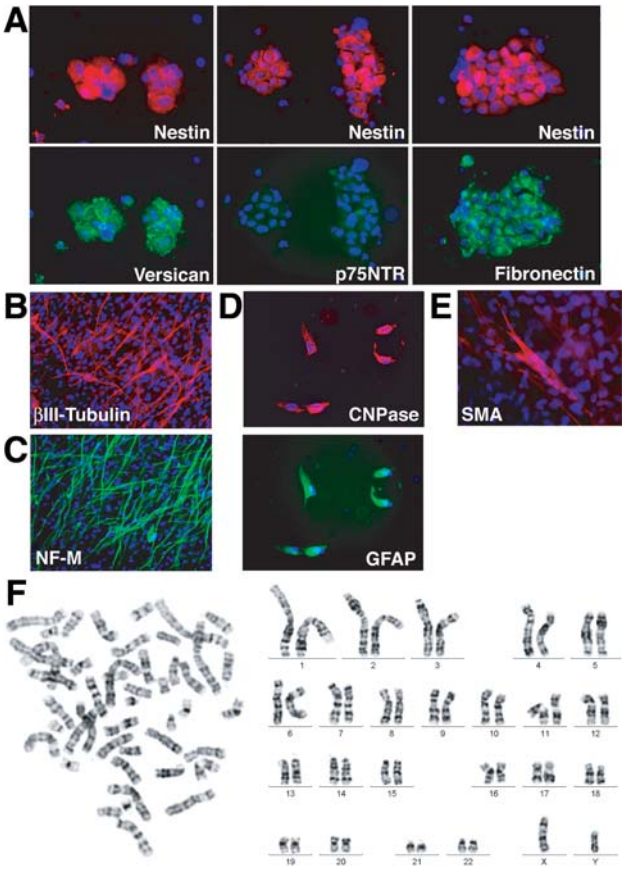

Figure 5. After 1 year in culture, human SKPs differentiate appropriately. **(A):** Fluorescence photomicrographs of human SKPs that had been expanded for 1 year in culture and that were double-labeled for nestin and versican (left panels), nestin and p75NTR (middle panels), or nestin and fibronectin (right panels). **(B–E):** Fluorescence photomicrographs of human SKPs that were expanded for 1 year in culture, differentiated for 3 weeks, and then immunostained for **(B)** βIII-tubulin and **(C)** NF-M to detect neurons, **(D)** CNPase and GFAP to detect Schwann cells, or **(E)** SMA to detect smooth muscle cells and myofibroblasts. In all panels, the nuclei are blue due to Hoechst counterstaining. Note that the morphology of the differentiated cell types is indistinguishable from that observed with shorter-term SKP cultures. **(F):** G-banding of a metaphase chromosome spread obtained from SKPs after passaging for 15 months. The left panel shows the metaphase spread, and the right panel shows the ordered chromosomal pairs. Abbreviations: NF-M, neurofilament; SKP, skin-derived precursor; SMA, smooth muscle actin.

Immunocytochemical analysis of these clonal spheres revealed that they were similar to the starting culture of SKPs with regard to marker protein expression. Immunocytochemical analysis of six of these clones revealed that all of the spheres coexpressed nestin, fibronectin, and vimentin, whereas only the occasional cell was detectably positive for p75NTR (Fig. 2B). We then asked whether these clonal SKP lines were able to generate both neural and mesodermal progeny by plating cells from three clones on poly-D-lysine/laminin, withdrawing their proliferation factors, and differentiating them either in the presence of 5% FBS plus or minus neurotrophins (neuronal differentiation conditions) or in 5 μm retinoic acid and 1% FBS to ask if they could generate adipocytes. G-banding of one of these clones demonstrated that the chromosomal karyotype was normal (Fig. 2G). Immunocytochemical analysis of these three clones revealed that all of them were able to generate neurons, glia, smooth muscle cells, and adipocytes. Under neuronal differentiation conditions, the clones generated a relatively dense network of βIII-tubulin and NF-M–positive, morphologically complex neurons on top of a bed of nonneuronal cells (Fig. 2C). Many of these βIII-tubulin–positive cells also coexpressed p75NTR (data not shown). Under these same conditions, a smaller subpopulation of potential bipolar Schwann cells was also observed, as indicated by their coexpression of CNPase and GFAP (Fig. 2D). Neuronal and glial proteins were never expressed in the same cells in these cultures. With regard to mesodermal cell types, in the neural differentiation conditions, we observed a subpopulation of SMA-positive cells with the morphology of smooth muscle cells or myofibroblasts (Fig. 2E) but never saw cells with the morphology and characteristic lipid droplet inclusions of adipocytes. In contrast, when the same clones were differentiated in the presence of retinoic acid, a small population of adipocytes was observed (Fig. 2F). Similar results were obtained with three independent clones, indicating that human SKPs are multipotent.

Human SKPs May Represent an Endogenous Dermal Precursor

We have recently demonstrated that rodent SKPs derive from an endogenous SKP-like precursor that is first present in skin during embryogenesis and that is then maintained at lower levels into adulthood [12]. We therefore asked whether human foreskin contained an endogenous SKP-like precursor that could generate neurons, a cell type that is never present in skin. To ask this question, four different human foreskin samples were dissociated into single cells, and these cells were plated adherently in the presence

of 5% FBS. Immunocytochemical analysis 2 weeks later revealed the presence, in all four samples, of a subpopulation of morphologically complex cells that expressed βIII-tubulin (Fig. 6A) and, in some cases, NF-M (data not shown), properties of newly born neurons. In addition, cells were observed that expressed CNPase and GFAP, consistent with the presence of Schwann cells in skin, and a separate population was observed that expressed SMA (data not shown). Because βIII-tubulin–positive neurons were never observed in freshly dissociated skin preparations (data not shown) and have never been reported in skin, we conclude that the neurons observed in these experiments were generated by differentiation of a precursor present in developing foreskin.

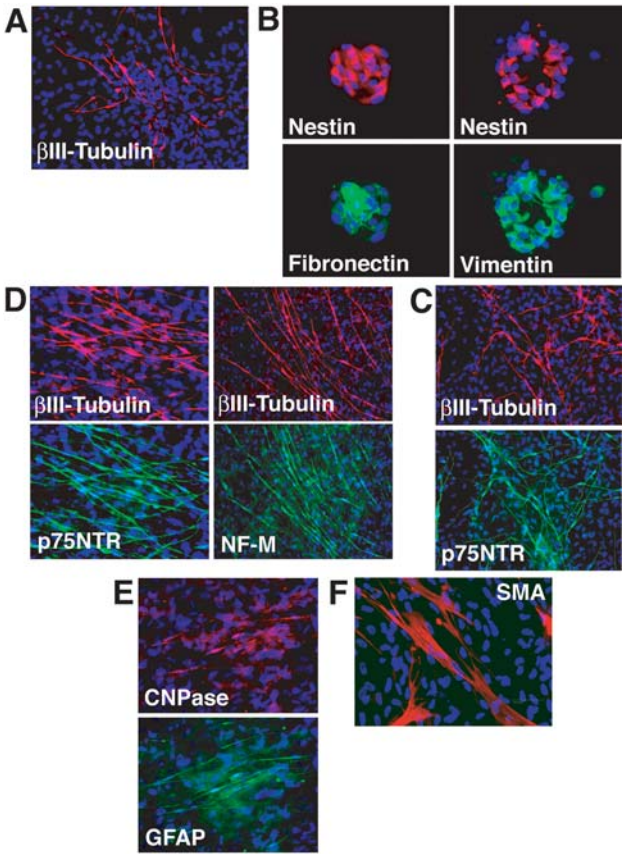

Figure 6. Primary human SKPs have the same properties as passaged SKPs. **(A):** Fluorescence photomicrograph of primary human foreskin cells that were differentiated for 2 weeks and then immunostained for βIII-tubulin (red). **(B):** Fluorescence micrographs of primary human SKP spheres that were double-labeled for either nestin and fibronectin (left panels) or nestin and vimentin (right panels). **(C):** Fluorescence micrographs of primary human SKPs that were differentiated for 3 weeks in the presence of 5% fetal bovine serum and then double-labeled for βIII-tubulin (top panel) and p75NTR (bottom panel). **(D):** Fluorescence micrographs of primary human SKPs that were differentiated for 3 weeks in the presence of neurotrophins and then double-labeled for βIII-tubulin and p75NTR (left panels) or βIII-tubulin and NF-M (right panels). Note that although all βIII-tubulin–positive neurons are p75NTR-positive, not all p75NTR-positive cells express βIII-tubulin. These latter p75NTR-positive cells may be Schwann cells. **(E):** Primary human SKPs that were differentiated for 3 weeks and then double-labeled for CNPase (top panel) and GFAP (bottom panel). Note the colocalization of these proteins in elongated, bipolar cells. **(F):** Primary human SKPs that were differentiated for 3 weeks and immunostained for SMA. In all panels, the blue nuclei are Hoechst stained to show all of the cells in the field. Abbreviations: NF-M, neurofilament; SKP, skin-derived precursor; SMA, smooth muscle actin.

We then asked whether the very first SKP spheres that were formed from skin (primary spheres) had the properties of the more long-term passaged human SKPs. Immunocytochemical analysis of nine different preparations of primary SKP spheres revealed that, like the passaged SKPs, they expressed nestin, fibronectin, and vimentin (Fig. 6B) but did not contain cells expressing tyrosinase or trp1 (data not shown), indicating that they did not contain melanoblasts/melanocytes. Similar results were obtained when these primary SKP spheres were plated down for 24 hours. We then differentiated primary SKP spheres under neuronal differentiation conditions to ask whether they could generate neurons. Immunocytochemical analysis demonstrated that all of the human samples analyzed in this way generated morphologically complex cells that coexpressed either βIII-tubulin and p75NTR (Figs. 6C, 6D) or βIII-tubulin and NF-M (Fig. 6D), a profile typical of peripheral neurons. In these same cultures, a significantly smaller population of cells had the bipolar morphology typical of Schwann cells and coexpressed CNPase and GFAP (Fig. 6E) or S100β and p75NTR (data not shown). Finally, some of the cells expressed SMA and had a morphology typical of smooth muscle cells or myofibroblasts (Fig. 6F). Thus, the initial, unpassaged spheres that are generated from dissociated skin cells are similar, if not identical, to passaged SKP spheres, supporting the idea that SKPs may arise from an endogenous precursor in human skin, as they do in rodent skin.

Discussion

The work presented here supports several conclusions. First, these data indicate that SKPs can be routinely isolated and expanded from small pieces of human skin and that they retain similar properties for up to 1 year in culture. Second, these data indicate that human SKPs can generate both neural and mesodermal cell types and that most of the neural cells generated by SKPs have characteristics of peripheral neurons and Schwann cells, consistent with a potential neural crest origin. Third, our clonal studies demonstrate that single SKP cells are truly multipotent and can generate neurons, glial cells, smooth muscle cells, and adipocytes. Fourth, experiments presented here indicate that human foreskin contains a population of cells that can generate neurons and that primary SKP spheres have the same properties as passaged SKPs, suggesting that human SKPs may represent an endogenous multipotent, neural crest–related precursor, as do rodent SKPs [12]. Together, these findings support the idea that SKPs represent an expandable, potentially autologous source of multipotent adult human

precursors.

The finding that similar mitogens (FGF2 and EGF) and culture protocols (sphere suspension cultures) can be used to isolate and expand populations of adult human CNS precursors [6, 18] and putative adult human neural crest precursors (as shown here) [10, 12] is intriguing and suggests that the similarities between these two populations of neural precursors may be greater than generally appreciated. In this regard, embryonic CNS neural precursors can, like SKPs and neural crest stem cells, differentiate into both Schwann cells and smooth muscle cells [19–21], and, conversely, both SKPs and embryonic neural crest stem cells can generate CNPase-positive, GFAP-negative cells with the morphology of oligodendrocytes under some conditions [10] (I.A.M., F.D.M., unpublished data). In light of the embryonic relationship between these two cell types [11], these findings suggest that the CNS/parasympathetic nervous system boundary may not be as immutable as previously thought, particularly when precursor cells are placed outside of their presumably restrictive in vivo environment.

The neural and mesodermal potential of SKPs suggests that they might be useful for several therapeutic purposes. For cell transplantation therapies, SKPs represent an accessible adult source of neural precursors for treating the damaged nervous system. In particular, although SKPs predominantly generate peripheral neural progeny, some of these have been shown to have therapeutic utility upon transplantation. For example, Schwann cells, which provide a conducive environment for CNS regeneration [22], are currently in clinical trials for treatment of multiple sclerosis [23], and Schwann cell transplantation has recently been shown to enhance functional recovery after spinal cord injury [24, 25]. We provide evidence here that human SKPs can generate Schwann cells, and we have recently demonstrated that the Schwann cells differentiated from rodent SKPs can myelinate axons both in culture and in vivo (I.A.M., Jeff Biernaskie, Nao R. Kobayashi, and F.D.M., unpublished data). In this regard, human Schwann cells can only be isolated by nerve biopsy and are difficult to expand in culture [26, 27], making SKPs a viable, potentially autologous alternative source of Schwann cells. A second, potentially useful cell type generated by rodent SKPs is catecholaminergic neurons. In particular, peripheral dopaminergic cell types have previously been suggested as potential donor cells for Parkinson's disease, but the source of such cells has been problematic. Although we have not yet demonstrated that human SKPs differentiate into catecholaminergic neurons, we have shown that rodent SKPs have this potential [12], raising the possibility that SKPs might provide a source of cells for treating Parkinson's disease.

Although cell transplantation is one possible therapeutic use for SKPs, particularly because they potentially can be isolated from small, autologous skin biopsies, we propose that of equal therapeutic interest is their use as a source of human cells for screening or discovery research. Human SKPs readily generate neurons, and because these postmitotic cells are impossible to obtain from living individuals, we propose that SKPs provide a viable, expandable source of primary human neurons for a wide variety of purposes. For example, SKPs may well provide the ability to isolate and analyze human neural cells from patient populations with genetic predispositions to diseases ranging from Alzheimer's disease to schizophrenia. Similar arguments could be made with regard to the other cell types that SKPs can generate. In the case of mesodermal cells, although human mesenchymal stem cells [28] share some of the same advantages as SKPs, they are not as accessible on an individual basis.

The dual neural/mesodermal potential of human SKPs has one final therapeutic implication. Certain skin tumors contain both neural and mesodermal components [29, 30]. Moreover, skin even occasionally manifests with neural tumors that are somewhat surprising, such as the neuroblastoma lesions that occur in the skin of infants [31]. We propose that an endogenous SKP-like cell could well provide the founder cell for some of these lesions, based on the recent literature indicating that stem cells provide founder cells for blood [32, 33], neural [34, 35], and breast cancers [36]. Moreover, the potential involvement of an SKP-like cell in cancer may not be limited to skin, because we have recently found similar cells in other rodent tissues with a neural crest contribution (unpublished data).

One of the major questions raised by our findings concerns the nature of the endogenous cells that generate SKP spheres and their potential physiological role. Although we are limited in our ability to experimentally address these questions with human SKPs, we have recently asked them for the more experimentally amenable rodent SKPs [10]. Our studies indicate that SKPs represent a neural crest–like precursor cell that arises in skin, likely via migration, during late embryogenesis and that persists in lower numbers in adult skin. Moreover, our studies indicate that one niche for the rodent SKPs is in the dermal papillae of hair and whisker follicles, a major follicle regulatory center [37] that has been previously proposed to contain multipotent precursor cells [38, 39]. What are the data suggesting that human SKPs are similar? First, we show here that, like rodent SKPs, human SKPs express embryonic transcription factors that are characteristic of embryonic neural crest precursor cells. Second, we show that human skin, like rodent skin, contains a precursor cell that can generate neurons immediately upon differentiation. Third, we show that primary human SKP spheres, like primary rodent spheres, are similar to passaged SKP spheres. There is, however, one major difference between our rodent study and the current work: Human foreskin does not contain hair follicles, implying that the niche for the endogenous SKP-like cells in at least this one type of human skin must be different.

If the human SKP parallels the rodent SKP, then the number of SKPs in adult human skin may well be lower than in neonatal skin [12]. Our previous work on scalp tissue isolated from

patients 40–70 years old [10], together with the data described here, indicate that, as in rodents, SKPs are present throughout the human lifetime. However, we have not systematically compared the numbers or differentiation potential of neonatal versus adult SKPs, nor have we compared the efficiency of SKP isolation from different adult tissues. This is obviously a key issue, particularly if we wish to use biopsies of adult human skin as a source of SKPs in the future, and is something that we are currently addressing.

In summary, we have demonstrated here that a multipotent adult precursor cell can be isolated and expanded from an accessible adult tissue source: skin. Although we still need to develop approaches for routinely isolating these cells from biopsies of adult patient populations and to demonstrate the functionality of their progeny in vivo and in vitro, the work described here provides the framework for our attempts to use SKPs as an autologous adult stem cell population for cell replacement and discovery research.

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