Isolation of multipotent adult stem cells from the dermis of mammalian skin

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We describe here the isolation of stem cells from juvenile and adult rodent skin. These cells derive from the dermis, and clones of individual cells can proliferate and differentiate in culture to produce neurons, glia, smooth muscle cells and adipocytes. Similar precursors that produce neuron-specific proteins upon differentiation can be isolated from adult human scalp. Because these cells (termed SKPs for skin-derived precursors) generate both neural and mesodermal progeny, we propose that they represent a novel multipotent adult stem cell and suggest that skin may provide an accessible, autologous source of stem cells for transplantation.

Adult stem cells have been isolated from several tissue sources, including the central nervous system[1,2], bone marrow[3–5], retina[6] and skeletal muscle[7,8]. All of these stem cells preferentially generate differentiated cells of the same lineage as their tissue of origin: neural stem cells are biased to generate neurons and glia; bone marrow mesenchymal stem cells to generate mesodermal cell types; and haematopoietic stem cells to generate blood cells. However, several recent transplant studies indicate that at least a fraction of stem cells in these populations can generate cells of a different embryonic lineage *in vivo*. For example, neural stem cells can generate blood[9] and skeletal muscle cells[10], and can contribute to many embryonic tissues when transplanted into blastocysts[11]. Similarly, transplanted bone marrow stem cells can contribute to skeletal muscle[8,12] and liver[13], and can generate cells producing neuronal markers in the brain[14,15].

These findings indicate that adult tissues contain tissue-biased stem cells, and that even such biased stem cells may be much more 'plastic' than previously appreciated. Such findings provide new insights into cellular differentiation, and have therapeutic implications, particularly for the nervous system; both neural and embryonic stem cells can promote functional recovery upon transplantation into the injured nervous system[16,17] . However, although the therapeutic potential of neural stem cells is clear, a number of important problems remain[18]. Fetal tissue is the current tissue source for human neural stem cells, raising important ethical issues. Moreover, the use of human fetal tissue, even if acceptable by society, involves heterologous transplantation. To circumvent these two problems, we have attempted to isolate adult stem cells from mammalian skin, a highly accessible tissue source. The possibility that skin contains a precursor capable of generating neural cell types was indicated by the finding that Merkel cells, neural sensory receptors found in the dermis, can be generated in adult skin[19]. With this in mind, we describe here the isolation of stem cells (termed SKPs for skin-derived precursors) from adult rodent skin.

Results

Isolation of neural precursors from skin. To isolate neural precursors from skin, we adapted a technique devised to generate neural stem cells from the brain[1,20]. Skin from juvenile (3–21 postnatal days) or adult mice was dissociated and cultured in uncoated flasks with epidermal growth factor (EGF) and fibroblast growth factor (FGF). Many cells adhered to the plastic, and many died, but by 3–7 days, small spheres of floating cells formed. These cells were transferred to a new flask 7–10 days after initial culturing; again, many cells adhered, but cells in the floating spheres proliferated to generate larger spheres (Fig. 1a), which were then isolated, dissociated and cultured in fresh medium plus growth factors ('passaged'). Purified populations of floating spheres were obtained after 3–4 weeks using this process of selective adhesion. The spheres could be dissociated to single cells when passaged, and would subsequently proliferate to generate new spheres (Fig. 1b).

To determine whether juvenile or adult spheres contained cells with characteristics of neural stem cells, we dissociated and plated them onto poly-D-lysine/laminin without growth factors, and 12–24 h later immunostained them for nestin, a marker for neural precursors[21]. After three passages, ≥ 60% of the cells produced nestin (Fig. 1c), a property they maintained over subsequent passages. These nestin-positive cells have been passaged in this manner for over 14 months (over 50 passages). In a typical dissection, 12×10^6 cells were isolated from 8 cm^2 of adult skin, and after 10 days these cells then generated about 8,000 spheres of 5 to 30 cells each. After three additional weeks of passaging, the number of SKPs in spheres totalled 5×10^5 and $2–3 \times 10^6$ from adult and juvenile skin, respectively. The SKPs then doubled every 2 to 3 days.

To determine whether SKPs could generate neural cell types, we differentiated juvenile SKPs at passages 3 and greater by plating them on poly-D-lysine/laminin in the absence of growth factors. Immunostaining (Fig. 1d,e) and western blot analysis (Fig. 2d) revealed that SKPs generated cells expressing neuronal marker genes. At 7 days of differentiation, a subpopulation of morphologically complex cells coexpressed nestin and neuron-specific βIII-tubulin, a profile typical of newly born neurons (Fig. 1d). At later timepoints of 4–21 days, cells also expressed neurofilament-M (NFM) (Figs 1e, 2d), neuron-specific enolase (data not shown), NeuN (Fig. 1e) and a neuron-specific Tα1 α-tubulin:nlacZ transgene (Fig. 1e)[22,23]. Finally, some cells produced GAD (Fig. 1e), a marker for GABAergic neurons, which are not found in the peripheral nervous system. Similar results were obtained for adult SKPs (Figs 1e, 2d), although at early passages some of the βIII-tubulin

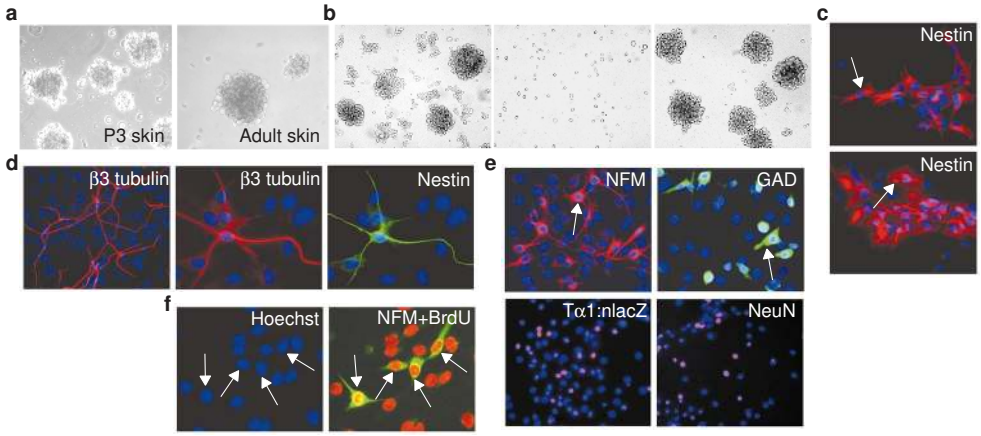

Figure 1 **SKPs are dividing, nestin-positive cells that can differentiate into neurons. a**, Phase micrographs of spheres of cells isolated from P3 skin and cultured for 3 weeks (left) or from adult skin, cultured for 2 months (right). **b**, Following the initial purification, SKPs grow as spheres in suspension (left), can be dissociated to single cells (middle) and then will proliferate to generate spheres again over the course of one week (right). **c**, Nestin-immunoreactive cells (arrows, red) derived from P3 skin after 3 weeks of culture (top), or adult skin after 5 weeks (bottom). In this and all subsequent panels, cells are counterstained with Hoechst (blue) to show nuclei. **d**, Cells immunoreactive for neuron-specific βIII-tubulin (left), or double-labelled for βIII-tubulin (middle, red) and nestin (right, green). In both cases, juvenile SKPs were passaged for 3 weeks before differentiation. **e**, Neurofilament M-positive cells (top left, arrow) and GAD-positive cells (top right, arrow) can still be generated from adult SKPs after 20 weeks and 34 weeks of passaging, respectively. Adult (bottom left) and juvenile (bottom right) SKPs also differentiate into cells expressing a nuclear, neuron-specific Tα1:nlacZ transgene (bottom left) and the neuron-specific antigen NeuN (bottom right). Adult SKPs were passaged for 18 weeks and differentiated for 8 days. Juvenile SKPs were passaged for 4 weeks and differentiated for 2 weeks. **f**, Double-labelling of adult SKPs that were cultured in the presence of BrdU for the first two days of differentiation and then immunostained 5 days later for NFM. Note that all SKPs are BrdU-positive (red nuclei), and that all NFM-positive cells (green, arrows), are positive for BrdU.

Table 1 Quantification of the percentage of differentiated progeny generated by juvenile and adult SKPs

	Juvenile		Adults	Clones
	Short passage (≤ 3 months)	Long Passage (≥ 6 months)	(≥ 6 months)	
Neurons	~6%	~3%	~7%	~5%
CNPase-positive glial cells	~9%	~11%	~10%	~7%
Smooth muscle cells	Rare	Rare	Rare	Rare
Adipocytes	Highly variable, ranging from 1% to greater than 25%			

Neurons are defined as cells that produce NFM or βIII-tubulin. For neurons, glia and smooth muscle cells, SKPs were differentiated by withdrawing EGF and FGF and adding either 3% rat serum or 1–3% FBS. For adipocytes, SKPs were differentiated in the presence of 10% FBS. The variability in the number of adipocytes generated may be a consequence of differences in the FBS preparations. The percentages indicated for clones derive from both the short-term and long-term clonal analysis (see text).

and neurofilament-positive cells were less typically neuronal in morphology. Quantification of cells differentiated at short (≤ 3 months) and long (≥ 6 months) passage times revealed that about 6% of the cells produced neuronal markers at both timepoints (Table 1).

Immunostaining and western blots revealed that neonatal and adult SKPs also generated cells producing the glial markers[24] GFAP and CNPase at 7–21 days post-plating (Fig. 2a–d). Double-labelling for these proteins (Fig. 2a) demonstrated there were: cells producing GFAP but not CNPase (potentially astrocytes); cells producing CNPase but not GFAP (potentially oligodendrocytes or their precursors); and bipolar cells producing both markers (potentially Schwann cells). A subpopulation of GFAP-positive cells also coexpressed nestin (Fig. 2c), a profile typical of developing astrocytes and Schwann cells. Moreover, some cells were positive for A2B5, a marker for oligodendrocyte precursors[24] (Fig. 2a). Quantification revealed that about 9% of differentiated cells produced CNPase and 10–20% produced GFAP, at both long and short passage times (Table 1).

Immunocytochemical analysis supported two additional conclusions. First, glial versus neuronal markers were produced in distinct subpopulations of SKP progeny (Fig. 2b). Second, the differentiated neurons observed in these cultures were generated from dividing precursor cells. In particular, bromodeoxyuridine (BrdU) labelling demonstrated that, in the first two days after plating, all SKPs divided at least once (Fig. 1f), and did not produce neuronal or glial proteins (data not shown). By 7 days of differentiation, neurofilament-positive cells were observed that had incorporated BrdU in the first two days of culture (Fig. 1f), and the number of nestin-positive cells was greatly decreased (Fig. 2c) from ≥ 60% at 24 h to about 5–10% at 7 days.

SKPs derive from dermis and generate mesodermal cell types. To characterize SKPs better, we analysed them for production of markers associated with other stem cells, and determined their origin

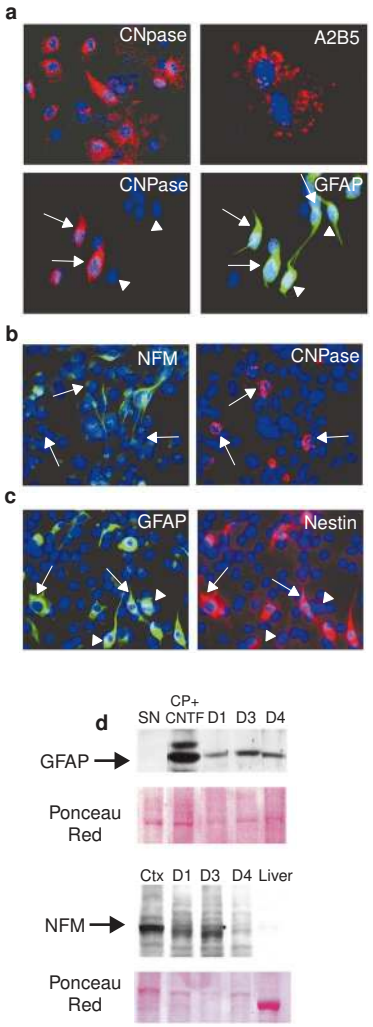

Figure 2 **SKPS can generate glial cells**. **a**, Immunocytochemical analysis revealed that CNPase-positive cells differentiated from juvenile SKPs after 5 weeks of passaging (top left) and A2B5-positive cells differentiated from juvenile SKPs after 29 weeks (top right). Double-labelling of differentiated adult SKPs that were passaged for 20 weeks and immunostained for CNPase (bottom left, red) and GFAP (bottom right, green). Note that some of the cells (arrows) produce both proteins, whereas others produce only GFAP (arrowheads). **b**, Double-labelling of juvenile SKPs cultured for 3 weeks and then differentiated indicates that neurofilament M-positive cells (left, green) are distinct from the cells that produce the glial marker CNPase (right, red, arrows in both panels). **c**, A subpopulation of GFAP-positive cells (left, green, arrows) also produce nestin (right, red, arrows) after 11 days of differentiation. Note that some cells produce only GFAP (arrowheads). Cells were differentiated from adult SKPs that were passaged for 33 weeks. **d**, Western blot analysis for neurofilament M (NFM) and GFAP in differentiated SKPs from juveniles and adults. Cells were derived from adult (D1) or juvenile (D3 and D4, two different cultures) SKPs that had been passaged from 12 to 30 times and then differentiated for 14 days. Positive controls are adult mouse cortex (Ctx) and cultured cortical progenitor cells with CNTF (CP + CNTF), whereas cultured sympathetic neurons (SN) and liver are negative controls. The Ponceau Red staining indicates the rough relative protein levels in each lane. The GFAP band migrates at relative molecular mass 52,000 (M_r 52K) and NFM at 145K.

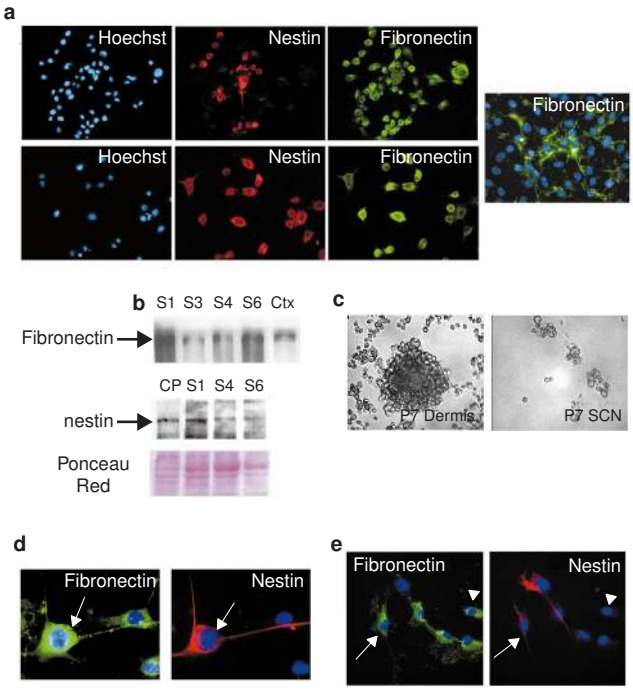

Figure 3 **Production of nestin and fibronectin by SKPs. a**, **b**, SKPs co-produce nestin and fibronectin. **a**, Double-labelling of SKPs for fibronectin and nestin. Top, the same field of adult SKPs passaged for 13 months; bottom, P6 SKPs passaged for 4 months. SKPs were plated overnight before immunocytochemistry. Far right, fibronectin immunocytochemistry on a culture of SKPs differentiated for one week. **b**, Western blot analysis for fibronectin and nestin in spheres of adult (S1) and juvenile SKPs (S3, S4 and S6 were passaged from 7 to 40 times). The Ponceau Red is as described in Fig. 2d. Ctx indicates cortex and CP cortical progenitors. Nestin and fibronectin bands migrate at M_r 200K and 220K, respectively. **c**, **d**, Spheres of nestin-positive, fibronectin-positive cells can be derived from dermis but not epidermis or sciatic nerve. **c**, Phase-contrast micrograph of cells derived from P7 dermis (left) or sciatic nerve (right, P7 SCN) and cultured in suspension for 4 (dermis) or 6 (sciatic nerve) weeks in the presence of EGF and FGF. **d**, Co-production of fibronectin and nestin (arrows) in cells from dermis spheres that were plated down overnight. **e**, Nestin-positive, fibronectin-positive cells can be immediately dissociated from the dermis. Double-label immunocytochemical analysis for fibronectin and nestin on cells dissociated from the dermis and cultured on poly-D-lysine/laminin for 5 days in DMEM/F12 plus B27 and 3% rat serum. Arrow, cell co-producing nestin and fibronectin; arrowhead, cell producing fibronectin only.

within skin. Immunocytochemistry and western blot analysis revealed that, although SKPs produced the neural marker nestin, both when in spheres (Fig. 3b) and when plated on an adhesive substratum (Fig. 1c), they did not produce two markers for neural crest stem cells[25], the p75 neurotrophin receptor and PSA-NCAM (data not shown). In contrast, juvenile and adult SKPs produced fibronectin, a protein that is produced by bone marrow mesenchymal stem cells[26], both when in spheres (Fig. 3b) and when plated onto poly-D-lysine/laminin (Fig. 3a).

Three compartments in skin that might contain SKPs are the epidermis, dermis and nerve terminals. To determine the origin of SKPs, we cultured juvenile (P7 and P18) and adult epidermis, dermis and sciatic nerve under the same conditions used to culture SKPs. Only the dermis generated spheres of proliferating cells similar to those seen with whole skin (Fig. 3c). No viable cells were obtained from the epidermis, and the sciatic nerve yielded a small, heterogeneous population of cells that did not proliferate well (Fig. 3c). To characterize dermis-derived spheres, they were passaged for four weeks, and then plated on poly-D-lysine/laminin for 24 h. Immunocytochemistry revealed that, like SKPs, they co-produced nestin and fibronectin (Fig. 3d).

To determine whether nestin- and fibronectin-positive cells are present within adult dermis before culture, we plated immediately isolated dermal cells on poly-D-lysine/laminin for 15 h or 5 days in

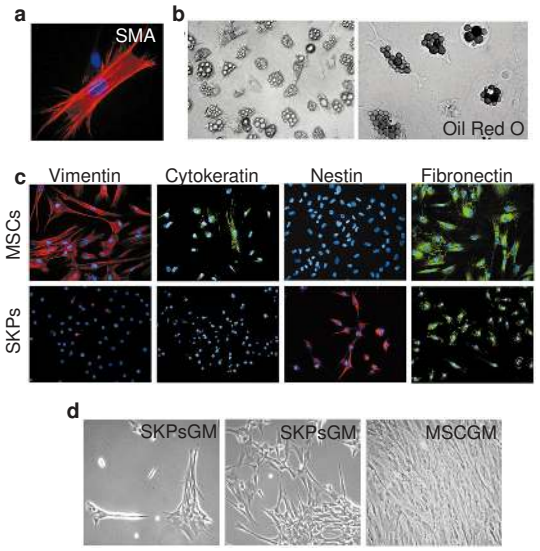

Figure 4 **SKPs can differentiate into smooth muscle cells and adipocytes.** **a**, An SMA-positive smooth muscle cell differentiated from juvenile SKPs that were passaged for 3 months. **b**, In high serum (10% FBS) with no added growth factors, adult SKPs differentiate into cells with the morphological characteristics of adipocytes. Left, phase-contrast micrograph; right, brightfield picture of a culture stained with Oil Red O, which stains lipid droplets. **c**, SKPs differ from mesenchymal stem cells in their production of the intermediate filament proteins vimentin and nestin. Immunocytochemical analysis of mesenchymal stem cells (MSCs; top) and SKPs (bottom) for vimentin, cytokeratin, nestin and fibronectin. In both cases, cells were dissociated and plated on to poly-D-lysine/laminin-coated slides overnight before immunocytochemistry. Mesenchymal stem cells produce high levels of vimentin and no nestin, whereas SKPs produce nestin but not vimentin. A subpopulation of mesenchymal stem cells also produce cytokeratin. Note the difference in morphology of the two cell types: SKPs are clearly much smaller and less flattened than mesenchymal stem cells. **d**, Mesenchymal stem cells do not proliferate in suspension when grown under SKPs conditions. Phase micrographs of mesenchymal stem cells grown on uncoated tissue-culture plastic for 2 weeks in SKPs medium and growth factors (SKPsGM; left and centre) or in mesenchymal stem cell medium (MSCGM; right). Note that the mesenchymal stem cells adhere to the uncoated plastic and survive but do not proliferate in the SKPs medium, whereas they rapidly proliferate to reach confluence in the mesenchymal stem cell medium. Similar results were obtained in SKPs medium containing FGF and EGF or FGF, EGF and LIF.

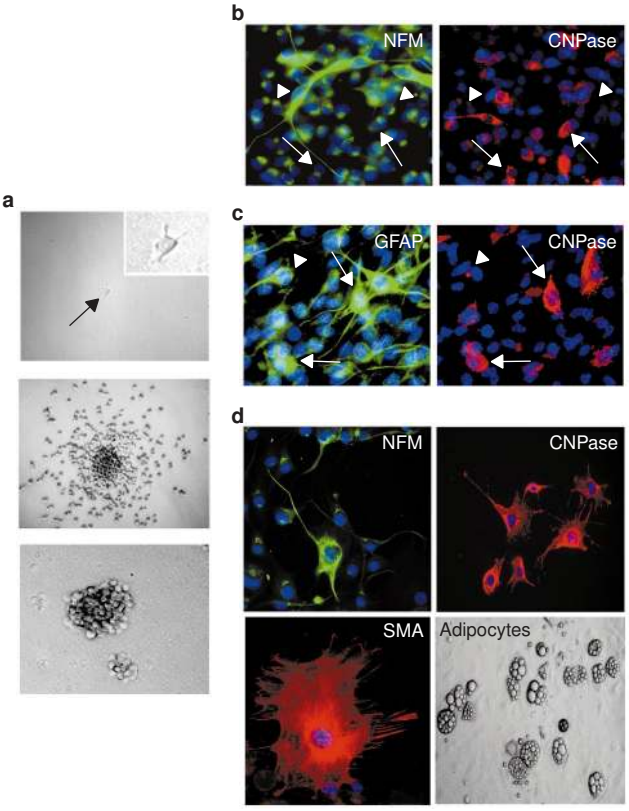

Figure 5 **Clonal analysis indicates that individual SKPs can give rise to neural and mesodermal cell types.** **a**, A single cell (top, arrow; inset is a 3-fold enlargement of the same cell) was isolated by limiting dilution, and over the course of 3 weeks proliferated on poly-D-lysine/laminin to generate a clone of cells (middle). Alternatively, single cells were passaged and expanded over a period of 5 months to generate flasks of spheres that could be differentiated under different conditions (bottom). **b**, **c**, Single clones generated on poly-D-lysine/laminin (similar to that shown in the top and middle panels in **a**) were immunostained for NFM and CNPase (**b**) or GFAP and CNPase (**c**). In **b**, arrowheads indicate the NFM- positive cells and arrows the CNPase-positive cells. In **c**, arrowheads indicate cells that only produce GFAP, and arrows indicate cells producing both GFAP and CNPase. Clones were generated from juvenile SKPs that were passaged for 4 months. **d**, Immunocytochemical and morphological analysis of progeny differentiated from one representative SKPs clone that was expanded for 5 months. The cell types detected after differentiation included NFM-positive neurons (NFM), CNPase-positive glial cells (CNPase), smooth muscle actin-positive cells (SMA) and adipocytes.

medium lacking growth factors. Immunocytochemistry revealed a subpopulation of cells that co-produced nestin and fibronectin at both timepoints (Fig. 3e). We used immunocytochemistry on sections of adult skin to determine the location of these cells *in situ*; although the occasional nestin-positive cell was observed in the dermis, these cells did not detectably co-produce fibronectin (data not shown). Thus, dermis contains cells that co-produce nestin and fibronectin immediately upon dissociation, but such co-production was not obvious *in situ*; whether these cells generate the proliferating spheres of SKPs is unknown.

Because SKPs produce markers of neural and mesenchymal stem cells, we determined whether they generated mesodermal as well as neural cell types. When SKPs were differentiated in 3% rat serum, they generated a small subpopulation of smooth muscle cells, as judged by expression of smooth muscle actin (SMA) and morphology (Fig. 4a, Table 1). Moreover, when SKPs were differentiated in 10% fetal bovine serum, they differentiated into cells with the morphology and lipid droplet inclusions characteristic of adipocytes (Fig. 4b, Table 1). Both of these cell types are of mesodermal origin.

We next asked how similar SKPs were to bone marrow mesenchymal stem cells, which also produce fibronectin and differentiate into mesodermal cell types but which, unlike SKPs, are selectively adherent. Immunocytochemistry revealed that mesenchymal stem cells, which are relatively large, flattened cells, produced high levels of vimentin and fibronectin but no nestin, and that the occasional cell produced detectable levels of cytokeratin (Fig. 4c), a marker for epithelial cells and some peripheral neural precursors. In contrast, SKPs produced nestin and fibronectin, but not vimentin or cytokeratin (Fig. 4c). We then determined whether mesenchymal stem cells would proliferate as non-adherent cells when cultured under conditions used to culture SKPs. This analysis revealed that, even after 3 weeks, mesenchymal stem cells survived but did not proliferate, and remained adherent to the uncoated tissue-culture plastic (Fig. 4d). In contrast, sister cultures grown in mesenchymal stem cell medium proliferated and reached confluence within about 1 week (Fig. 4d). Thus, SKPs are distinct from mesenchymal

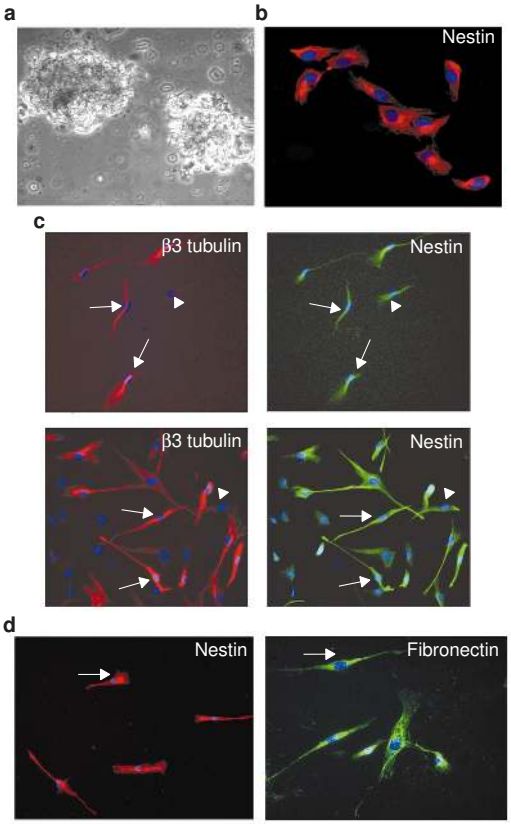

Figure 6 **Isolation and differentiation of SKPs from human skin**. **a**, Phase micrograph of clusters of floating cells derived from adult human scalp. **b**, Immunocytochemical analysis revealed that many of these cells were immunoreactive for nestin (red). Nuclei were stained blue with Hoechst. **c**, Double-labelling of differentiated human SKPs revealed that a subpopulation of nestin-positive cells (right, green, arrows) also produced neuron-specific βIII-tubulin (left, red, arrows). Arrowheads indicate nestin-positive cells that produce low or undetectable levels of βIII-tubulin. Nuclei were stained blue with Hoechst. The top versus bottom panels were derived from SKPs isolated from two different human samples. **d**, Immunocytochemistry revealed that although many of the human SKPs are nestin-positive (left, arrow), almost all of them are fibronectin-positive (right, arrow).

stem cells with regard to their growth factor requirements, their adherence, their morphology and their selective production of the intermediate filament proteins nestin and vimentin.

Individual SKPs are multipotent. To determine whether individual SKPs could generate both neurons and glia, juvenile spheres were dissociated and individual cells were isolated by limiting dilution into multiwell plates coated with poly-D-lysine/laminin; microscopy revealed that many wells contained no cells, but that the occasional well contained a single cell (Fig. 5a). These individual cells were cultured for 5 weeks in conditioned medium plus FGF and EGF; over that time, over 50% of these single cells proliferated to generate clones (Fig. 5a). Cells in these clones were then differentiated by withdrawing growth factors and adding 3% rat serum for 2 weeks. Of these differentiated clones, the six that were analysed for NFM and CNPase all generated neurons and CNPase-positive glial cells (Fig. 5b). Moreover, about 5% of the differentiated cells produced NFM and 8% produced CNPase, numbers similar to those obtained in mass cultures (Table 1). Analysis of eight additional clones for CNPase and GFAP revealed that each of these clones generated glial cells that produced GFAP only, CNPase only, and both GFAP and CNPase (Fig. 5c). In these clones, about 10% of cells were positive for CNPase and 15% for GFAP, numbers that were also similar to mass cultures.

To determine whether individual SKPs could generate both neural and mesodermal progeny, single cells were again isolated by limiting dilution into uncoated wells containing conditioned medium and growth factors. These cells were passaged as non-adherent spheres (Fig. 5a) over a period of 5 months. Cells from individual clones were then differentiated by plating onto poly-D-lysine/laminin in the absence of growth factors (neural conditions) for 2 weeks, or plating in the presence of 10% fetal bovine serum (adipocyte conditions) for 2 weeks. Immunocytochemistry revealed that, of the six clones analysed, all of them generated neurons, CNPase- and GFAP-positive glial cells, smooth muscle cells and adipocytes (Fig. 5d). Thus, individual SKPs can self-renew for at least 5 months, and then differentiate into cells of both neural and mesodermal origin.

Isolation of nestin-positive neural precursors from human skin. To determine whether SKPs could also be generated from human skin, we analysed tags of scalp tissue generated by placement of a stereotactic neurosurgical apparatus. Scalp tags totalling 1 cm^2 or less from each of nine individuals were dissociated and cultured in EGF, FGF and leukaemia inhibitory factor (LIF), conditions optimized for human central nervous system stem cells[27]. As for rodent skin, most cells adhered to the plastic or died, but after 7 days, small floating clusters were observed. These clusters were then partially dissociated and transferred to new wells, where they slowly increased in size (Fig. 6a). After additional passaging, at 4–6 weeks spheres were plated on poly-D-lysine/laminin in 3% FBS with no growth factors, and analysed. Immunolabelling at 2–3 days revealed that many of the cells in the clusters were nestin-positive (Fig. 6b) with the percentage varying from around 50% to around 80% in the six cases that were analysed, and that virtually all of the cells were fibronectin-positive (Fig. 6d). Double-label immunocytochemistry at the same or longer timepoints revealed that, in all cultures, some nestin-positive cells also expressed βIII-tubulin and displayed elongated neurites (Fig. 6c). Thus, adult human skin can also generate nestin-positive precursor cells that, when differentiated, will produce neuronal proteins.

Discussion

Together, the data presented here support four major conclusions. First, they demonstrate that multipotent, nestin-positive, fibronectin-positive stem cells (SKPs) can be generated from juvenile and adult skin, that these precursors derive from the dermis, and that they are distinct from mesenchymal stem cells. Second, they indicate that individual clones of SKPs can differentiate into cells of both neuroectodermal and mesodermal lineage, including (but perhaps not limited to) neurons, glia, smooth muscle cells and adipocytes. Third, these studies show that SKPs can be passaged for at least 1 year without losing the capacity to generate these diverse cell types. Finally, the human studies indicate that similar precursors may be present within adult human skin. Thus, SKPs apparently represent a novel multipotent adult stem cell that is perhaps less 'biased' than other adult stem cells. The ability to isolate and expand such a stem cell from an accessible, potentially autologous tissue source such as mammalian skin has important therapeutic implications.

What is the cell(s) in the skin that gives rise to SKPs, and what is its normal role *in vivo*? Our data indicate that SKPs come from the dermis and that a subpopulation of cells immediately isolated from the dermis co-produce nestin and fibronectin. Although we do not know if these cells are SKPs, this observation raises the intriguing possibility that SKPs are a previously unidentified dermal stem cell that, in response to environmental cues, generates diverse cell types during renewal or repair.

What is the relationship between SKPs and other, previously identified adult stem cells? Although SKPs were isolated using techniques similar to those used to isolate neural stem cells, they clearly

differ. Adult neural stem cells are strongly biased to generate neural cells[1,2], do not produce fibronectin, and have never been demonstrated to generate adipocytes. With regard to neural crest stem cells[25], SKPs do not produce the markers PSA-NCAM or the p75 neurotrophin receptor, and they do not differentiate into tyrosine hydroxylase-positive neurons (data not shown). SKPs also clearly differ from adherent bone marrow mesenchymal stem cells with regard to the growth factors they require to proliferate, their selective expression of the intermediate proteins nestin and vimentin, their morphology and their ability to grow as spheres in suspension. Moreover, mesenchymal stem cells are strongly biased to generate cells of mesodermal origin[3,5] and are not easily induced to produce neural proteins[14,15,26,28]. Thus, we suggest that SKPs represent a new, multipotent adult stem cell that easily generates cells of more than one embryonic lineage. Whether SKPs are this multipotent *in vivo*, and/or whether similar stem cells are present in other adult tissues remains to be determined.

An additional implication of the findings presented here is a therapeutic one. Cell-replacement therapies show particular promise in the nervous system, where transplanted embryonic stem cells have been shown to promote functional recovery in animal models of, for example, spinal-cord injury[16]. However, although the therapeutic potential of such transplants is clear, a number of problems remain[18]. In particular, fetal tissue is the current tissue source for human neural and embryonic stem cells, raising important ethical issues. Moreover, the use of human fetal tissue involves heterologous transplantation, and the requisite accompanying immunosuppression is particularly problematic in individuals with long-term neural problems, such as spinal-cord injury or Parkinson's disease. In this regard, the fact that SKPs are generated from a potentially autologous, accessible adult tissue source, skin, and that they can readily generate neural cell types provides a potential solution to these problems. □

Methods

Cell culture.

Tissue for these studies was derived from previously characterized homozygous Tα1:nlacZ, line K6 transgenic mice[22,23] in a CD1 background; these mice express a neuron-specific β-galactosidase transgene from the Tα1 α-tubulin promoter. For juvenile (3–21 days) and adult (2 months to 1 year) mice, skin from abdomen and back was carefully dissected free of other tissue, cut into 2–3 mm^3 pieces, washed 3 times in Hanks balanced salt solution (HBSS), and then digested with 0.1% trypsin for 40 min at 37 °C, followed by 0.1% DNAase for 1 min at room temperature. Tissue pieces were then washed twice with HBSS, once with medium (DMEM-F12, 3:1, 1 μg ml^{-1} fungizone [Gibco-BRL], 1% penicillin/streptomycin [BioWhittaker]) containing 10% rat serum (Harlan Bioproducts), and twice with serum-free medium. Skin pieces were then mechanically dissociated in medium, and the suspension poured through a 40 μM cell strainer (Falcon). Dissociated cells were centrifuged at 168*g* and resuspended in 10 ml medium containing B-27 (Gibco-BRL), 20 ng ml^{-1} EGF and 40 ng ml^{-1} bFGF (both Collaborative Research). Cells were cultured in 25-cm^2 tissue culture flasks (Corning) in a 37 °C, 5% CO_2 tissue-culture incubator.

To passage floating clusters of cells, medium containing spheres was centrifuged, the pellet mechanically dissociated with a fire-polished Pasteur pipette, and cells were re-seeded in fresh medium containing B-27 and growth factors as above. Cells were passaged every 6–7 days. For differentiation, spheres were centrifuged, the growth factor-containing supernatant removed, and spheres were resuspended in fresh medium containing B-27 and either 3% rat serum or 1–3% fetal bovine serum (FBS; Gibco-BRL). In some cases (where indicated), cells were plated in the presence of 10% FBS. Spheres or single cells dissociated from spheres were plated onto 4-well Nunclon culture slides coated with poly-D-lysine/laminin, and maintained in the same medium, which was changed every 3–7 days. In total, 18 cultures of juvenile SKPs and 6 cultures of adult SKPs have been passaged, differentiated and analysed.

For clonal analysis, juvenile SKPs that had been passaged for 4 months were serially diluted into medium containing B27, FGF and EGF in multiwell chambers, and each individual chamber then assessed microscopically for the presence or absence of a single cell. Many wells contained no cells, whereas a few contained one cell. Wells containing more than one cell were discarded from the experiment. The medium was then supplemented to 50% with filtered SKPs-conditioned medium containing EGF, FGF and B27. In the short-term clonal analysis experiment, these single cells were plated in 8-well chamber slides (Nunc) coated with poly-D-lysine/laminin, and monitored every second day. After 5 weeks of expansion, cells were switched into medium containing B-27 and 3% rat serum, but no growth factors, and differentiated for an additional 2 weeks. For the long-term clonal analysis experiment, single cells were plated in 48-well uncoated tissue-culture dishes (Falcon). At six weeks, clones from a single cell were passaged into 6 wells of a 48-well plate, and then 2 weeks later were passaged into 6 wells of a 12-well plate. One to two weeks later, the cells were passaged into two 25-cm^2 tissue-culture flasks, and were maintained as for other SKPs cultures.

For the dissected sciatic nerve, dermis and epidermis, cells were initially dissociated and cultured as described for whole skin. BrdU-labelling of SKPs was performed as previously described[29].

To culture human SKPs, 2 to 3 pieces of scalp tissue ranging between 4 mm^2 and 9 mm^2 generated by placement of the stereotaxic apparatus for neurosurgery were washed 2–3 times with HBSS, any subcutaneous tissue was removed, and the skin was cut into small 1–2 mm^3 pieces. Tissue pieces were transferred to 15 ml tubes (Falcon), washed 3 times with HBSS, and enzymatically digested in 0.1% trypsin for 40 min at 37 °C, and then washed as for mouse tissue. Dissociated cells were suspended in 5 ml of the same medium used for mouse cultures, with the addition of 20 ng ml^{-1} LIF (R&D Systems Inc.). The cell suspension was placed in Falcon 6-well tissue culture plates and maintained in a tissue-culture incubator at 37 °C, 5% CO_2. Cells were subcultured by partial dissociation of the clusters that formed every 7–10 days.

Human mesenchymal stem cells were isolated as previously described[5] and purchased from Poietics/Biowhittaker. Cells were thawed and cultured at a density of 5,000 cells cm^{-2} using mesenchymal stem cell growth medium and supplement (Poietics/Biowhittaker). Once cells reached confluence, they were passaged once and then used for experiments after the second passage. To compare the growth of mesenchymal stem cells in mesenchymal stem cell medium versus SKPs medium, cells were plated at 5,000 cells cm^{-2} in uncoated tissue-culture flasks containing either mesenchymal stem cell growth medium and supplements or DMEM/F12 plus B27, 40 ng ml^{-1} FGF, 20 ng ml^{-1} EGF with or without 10 ng ml^{-1} LIF. Similar results were obtained in the presence or absence of LIF.

Immunocytochemistry and quantification.

Immunocytochemical analysis of cultured SKPs and mesenchymal stem cells was performed as previously described[30]. The primary antibodies that were used were: anti-nestin polyclonal (1:250, the kind gift of R. McKay, NINDS), anti-nestin monoclonal (1:400; Pharmingen), anti-βIII-tubulin monoclonal (1:500, Tuj1 clone; BABCO), anti-neurofilament-M polyclonal (1:200; Chemicon), anti-GAD polyclonal (1:800; Chemicon), anti-NSE polyclonal (1:2,000; Polysciences), anti-NeuN monoclonal (1:50; Chemicon), anti-β-galactosidase monoclonal (1:800; Sigma-Aldrich), anti-GFAP polyclonal (1:200; DAKO), anti-CNPase monoclonal (1:400; Promega), anti-p75NTR polyclonal (1:500; Promega), anti-SMA monoclonal (1:400; Sigma-Aldrich), anti-A2B5 monoclonal (the kind gift of J. Snipes and M. N. I.), anti-fibronectin polyclonal (1:400; Sigma-Aldrich), anti-vimentin monoclonal (1:200; Chemicon), anti-cytokeratin polyclonal, wide spectrum (1:200; DAKO), and anti-BrdU monoclonal (1:100; Chemicon). Secondary antibodies were cy3-conjugated goat anti-mouse (1:200), cy3-conjugated goat anti-rabbit (1:400), FITC-conjugated goat anti-mouse (1:50–1:100), and FITC-conjugated goat anti-rabbit (1:200) (all from Jackson Immunoresearch Laboratories). For BrdU immunocytochemistry, cells were treated essentially as described[29].

For quantification of the percentage of cells producing a given marker protein, in any given experiment at least three fields were photographed, and the number of positive cells determined relative to the total number of Hoechst-labelled nuclei. In a typical experiment, each field contained 100 to 500 cells, and a total of 500 to 1,500 cells were counted per marker.

Western blot analysis.

For biochemical analysis of SKPs, four cultures of SKPs (one adult and three juvenile; referred to as 1, 3, 4 and 6 with S denoting spheres and D differentiated cells) that had been passaged from 7 to 40 times were analysed either as spheres, or following differentiation by plating in medium containing 1% FBS, B-27 and fungizone for 14 days on 60-mm dishes coated with poly-D-lysine/laminin. For biochemical analysis of undifferentiated SKPs growing in spheres, cell lysates were prepared and western blot analysis performed as previously described for cortical progenitor cells[30]. Briefly, equal amounts (50–100 μg) of protein from each culture were analysed on 7.5% or 10% polyacrylamide gels. The primary antibodies used were: anti-nestin (1:1,000; Chemicon), anti-neurofilament medium (1:1,000; Sigma-Aldrich), anti-glial fibrillary acidic protein (1:1,000; DAKO) and anti-fibronectin (1:1,000; Sigma-Aldrich). As positive controls, we used purified fibronectin, and protein from mouse cortical progenitor cells[29,30] cultured in the absence or presence of CNTF[31], conditions which lead to neuronal or astrocytic differentiation, respectively.

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