DEVELOPMENTAL DYNAMICS 231:258–269, 2004

RESEARCH ARTICLE

Pluripotent Neural Crest Stem Cells in the Adult Hair Follicle

M. Sieber-Blum,[1]* M. Grim,[2] Y.F. Hu,[1] and V. Szeder[1,2]

We report the presence of pluripotent neural crest stem cells in the adult mammalian hair follicle. Numerous neural crest cells reside in the outer root sheath from the bulge to the matrix at the base of the follicle. Bulge explants from adult mouse whisker follicles yield migratory neural crest cells, which in clonal culture form colonies consisting of over a thousand cells. Clones contain neurons, smooth muscle cells, rare Schwann cells and melanocytes, demonstrating pluripotency of the clone-forming cell. Targeted differentiation into Schwann cells and chondrocytes was achieved with neuregulin-1 and bone morphogenetic protein-2, respectively. Serial cloning in vitro demonstrated self-renewal capability. Together, the data show that the adult mouse whisker follicle contains pluripotent neural crest stem cells, termed epidermal neural crest cells (eNCSC). eNCSC are promising candidates for diverse cell therapy paradigms because of their high degree of inherent plasticity and due to their easy accessibility in the skin. ***Developmental Dynamics 231:258–269, 2004.*** **© 2004 Wiley-Liss, Inc.**

Key words: neural crest; stem cell; adult stem cell; neuron; Schwann cell; smooth muscle cell; melanocyte; chondrocyte; epidermis; hair follicle; bulge

Received 21 January 2004; Revised 13 April 2004; Accepted 19 April 2004

INTRODUCTION

The neural crest is a transient embryonic tissue that originates in the neural folds, invades the embryo, and differentiates in distinct locations into a wide array of adult cell types and tissues. Neural crest derivatives include neurons, Schwann cells, and glia of the autonomic and enteric nervous systems, most primary sensory neurons, endocrine cells (e.g., the adrenal medulla and C-cells of the thyroid), the smooth musculature of the cardiac outflow tract and great vessels, pigment cells of the skin and internal organs, as well as bone, cartilage, and connective tissue of the face and ventral neck (Le Douarin and Kalcheim, 1999).

Wnt-1 is expressed transiently by premigratory and early migratory neural crest cells, and by dorsal neural tube cells (Wilkinson et al., 1987; Davis et al., 1988; Molven et al., 1991; McMahon et al., 1992; Wolda et al., 1993; Echelard et al., 1994). R26R/+ mice express β-galactosidase in a cre-inducible manner (Soriano, 1999), whereas Wnt1-cre mice express cre under the control of the Wnt1- promoter (Danielian et al., 1998). In Wnt1-cre/R26R compound transgenic mice; therefore, neural crest cells and their progeny permanently express β-galactosidase (Friederich and Soriano, 1991; Echelard et al., 1994; Danielian et al., 1998; Soriano, 1999; Chai et al., 2000; Jiang et al., 2002; Szeder et al., 2003), providing a genetic approach to neural crest cell tracing.

At the onset of migration, the neural crest is a mixed population of cells that consists of pluripotent stem cells, fate-restricted cells, and cells that are committed to a particular cell lineage (Sieber-Blum and Cohen, 1980; Sieber-Blum and Sieber, 1984; Bronner-Fraser and Fraser, 1988; Stemple et al., 1988; Baroffio et al., 1988; Sieber-Blum, 1989; Gershon et al., 1993; Ito et al., 1993; Henion and Weston, 1997). Microenvironmental cues as well as cell-autonomous mechanisms affect neural crest stem cell survival, proliferation, and lineage commitment. Some

[1]Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin
[2]Institute of Anatomy, First Medical Faculty, Charles University Prague, Prague, Czech Republic
Grant sponsor: National Institute of Neurological Disorders and Stroke, NIH; USPHS; Grant number: NS38500; Rockefeller Brothers Fund, New York; Charles E. Culpeper Biomedical Pilot Initiative, Grant number: 03-128; Ministry of Education of the Czech Republic; Grant number: VZ 1111 00003-3G; Grant number: LN 00A065.
*Correspondence to: Maya Sieber-Blum, Ph.D., Department of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, Milwaukee, WI 53226. E-mail: sieberbl@mcw.edu

DOI 10.1002/dvdy.20129
Published online 28 July 2004 in Wiley InterScience (www.interscience.wiley.com).

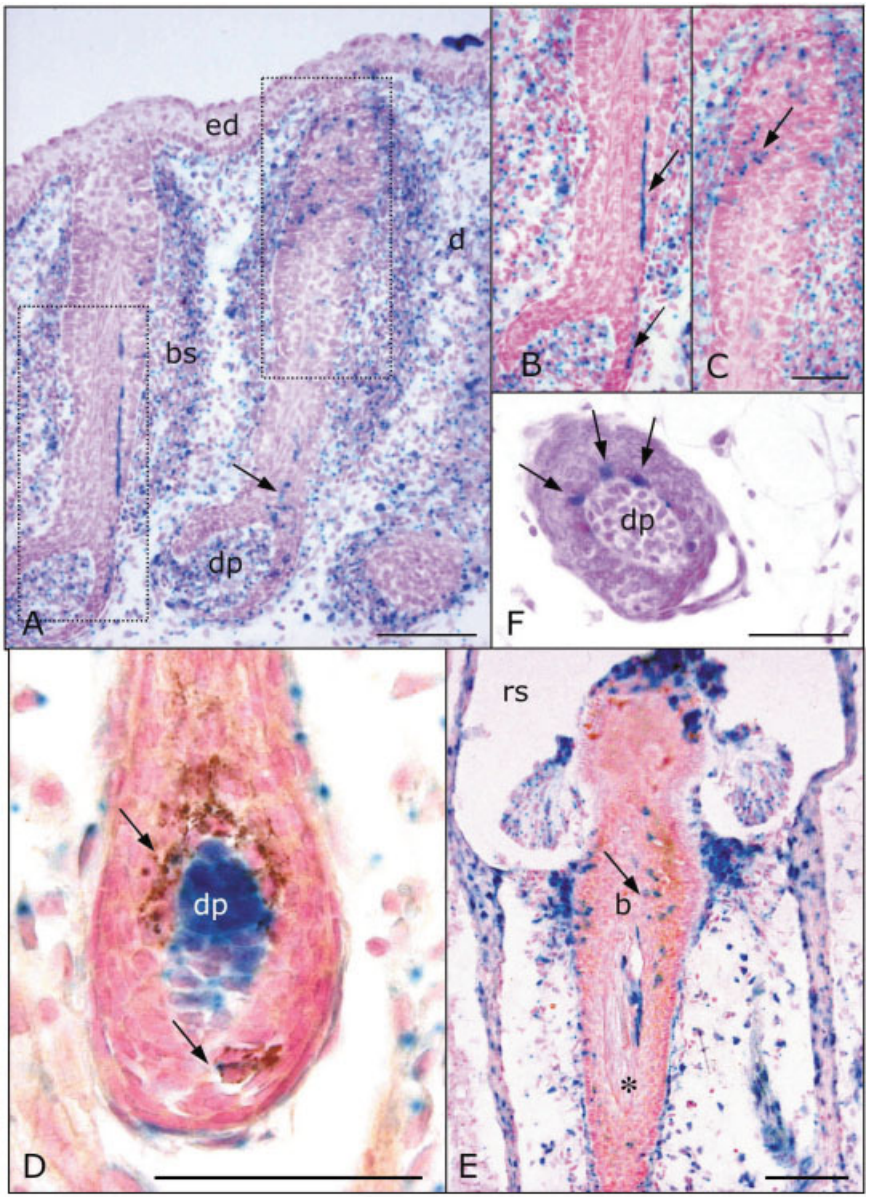

Fig. 1. X-gal–positive cells in the Wnt1-cre/R26R prenatal and postnatal mouse whisker follicle. Blue, X-gal reaction product; red, nuclear red counterstain. **A:** Tangential section through whiskers of a gestational day 16.5 mouse embryo. X-gal–positive neural crest-derived cells are present in the dermis (d) between whiskers, in the dermal papilla (dp), and in the blood sinuses (bs) that surround the whiskers. In addition, neural crest cells are localized in the outer root sheath within the follicle. ed, epidermis. **B:** Higher magnification of the area marked at the left in A. A stream of neural crest cells extends from the bulge region to the matrix near the dermal papilla (arrows), suggesting migratory behavior within the follicle. **C:** Higher magnification of area marked at the right in A. This section shows the bulge area. Many neural crest cells are present (e.g., arrow). **D:** Section through the base of a whisker follicle of a newborn double-transgenic mouse. Several X-gal–positive melanocyte are visible (e.g., arrows). **E:** Tangential section through a postnatal day 24 whisker follicle. The bulge region (b) contains several X-gal–positive neural crest cells (e.g., arrow). The hair is marked by an asterisk. rs; ring sinus. **F:** Cross-section through a whisker from an adult double-transgenic mouse at the level of the dermal papilla (dp). Several neural crest-derived X-gal–positive cells (e.g., arrows) are present in the matrix, which surrounds the dermal papilla. Scale bars = 100 μm in A, 50 μm in C (applies to B,C), 50 μm in D, 100 μm in E, 50 μm in F.

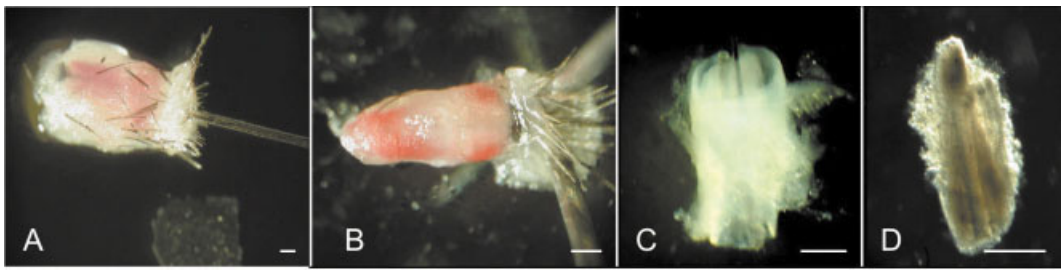

Fig. 2. Dissection of the bulge from adult whisker follicles. **A:** Dissected follicle surrounded by dermis and fat tissue. On the left, the dark matrix and, on the right, the hair are visible. **B:** Dissected whisker follicle devoid of dermis. On the right, below the skin, the ring sinus, and on the left, the cavernous sinus are visible. **C:** Bulge within the connective tissue capsule. **D:** Isolated bulge. Scale bars = 100 μm in A–D.

neural crest stem cells elude these signals and persist within crest-derived tissues. Neural crest stem cells have been identified in the embryonic dorsal root ganglia (Duff et al., 1991), sympathetic ganglia (Duff et al., 1991), somatic ectoderm (Richardson and Sieber-Blum, 1993), cardiac outflow tract (Ito and Sieber-Blum, 1993), and in the embryonic sciatic nerve (Bixby et al., 2002). Recently, Kruger et al. (2002) have iso-

lated neural crest-derived stem cells also from adult intestine. Here, we describe an additional adult tissue that harbors pluripotent neural crest stem cells, the epidermis of the hair follicle.

RESULTS

Neural Crest-Derived Cells in the Embryonic and Postnatal Facial Skin

Figure 1 shows sections through embryonic and postnatal Wnt1-cre/R26R facial skin of an embryonic day (E) 16.5 double transgenic embryo. Neural crest-derived cells are identified by their blue 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) reaction product. In the face, many structures are of neural crest origin. These cells include the facial dermis (Fig. 1A, d) and the dermal papilla of hair follicles (Fig. 1A, dp) but not the epidermis (Fig. 1A, ed). The whisker follicle is surrounded by blood sinuses (Fig. 1A, bs), the connective tissue of which is also of neural crest origin. The inner layers of the outer root sheath, which constitutes the epidermal invagination of the hair follicle, contain neural crest cells as well. Some neural crest cells within hair follicles form streams that extend from the bulge region to the base of the follicle, which contains the matrix that forms the new hair (Fig. 1B, arrows; higher magnification of area marked at the left in A), suggesting that neural crest cells migrate within the hair follicle. A large number of neural crest cells reside in the bulge region both of embryonic (Fig. 1C, arrow; E16.5) and postnatal whiskers (Fig. 1D, arrows; postnatal day 24). Because melanocytes within hair follicles provide melanin to the growing hair, the matrix is expected to contain neural crest cells. X-gal–positive neural crest cells are indeed present in the matrix of whiskers both from newborn (Fig. 1E, arrows) and adult (Fig. 1F, arrows; cross-section) mice. Many of them contain pigment granules (Fig. 1E, e.g., arrow). In summary, there are neural crest cells localized in the epidermis of the whisker follicle along the entire length of the follicle, both prenatally and postnatally.

Neural Crest Cells Emigrate From Bulge Explants

The objective of this study was to determine, by in vitro clonal analysis, the developmental potentials of the neural crest cells that reside in the bulge area, which is a known niche for keratinocyte stem cells (Oshima et al., 2001) and melanocyte stem cells (Nishimura et al., 2002). To this end, we microdissected the bulge area. Whiskers were dissected from the skin (Fig. 2A), and the dermis and fat tissue were then removed mechanically and with buffer rinses (Fig. 2B), exposing the ring sinus below the skin and the cavernous sinus near the base of the follicle. The capsule of the whisker was cut longitudinally, and subsequently the follicle was transected both above the cavernous sinus and below the skin, which yielded the bulge area within the collagen capsule (Fig. 2C). Finally, the bulge region was rolled out of the capsule (Fig. 2C) and placed into a collagen-coated culture plate, where it adhered to the substratum within 1 hr.

Figure 3 shows a bulge explant 4 days postexplantation. In Figure 3A, the explant and the emigrated cells are shown with phase contrast optics. Figure 3B shows a higher magnification with focus on the explant. Numerous X-gal–positive neural crest cells are present within the bulge. Figure 3C shows at higher magnification the area to the right marked in A. Figure 3D is the corresponding brightfield image, showing that all cells are X-gal–positive. Figure 3E shows with phase contrast optics the area to the left marked in (A). Figure 3F is the corresponding brightfield image, which shows that all cells are X-gal–positive. At 48 hr postexplantation, 0–15 cells and, at 72 hr, 126.8 ± 41.5 cells per explant are present on average. The increase in cell numbers over time is due to emigration of new cells and concomitant rapid proliferation (initial doubling time approximately 6 hr). Bulge explants from wild type and single-transgenic littermates are X-gal–negative. Emigrated cells also express Sox10, a marker for neural crest cells (Fig. 4; Kuhlbrodt et al., 1998; Rehberg et al., 2002), which confirms the neural crest origin of the cells that emerge from bulge explants.

Bulge Explant-Derived Neural Crest Cells Are Pluripotent

In vitro clonal analysis established that neural crest cells from bulge explants are pluripotent. Figure 5A–D shows the time course of a clone as it grows during the first 3 days: (Fig. 5A) 6 hr, (Fig. 5B) 18 hr, (Fig. 5C) 48 hr, and (Fig. 5D) 72 hr after cloning. Six hours after cloning, cells have a stellate morphology (Fig. 5A) and an initial doubling time of approximately 6 hr, which in subsequent days increases to 12–24 hr. At 24 hr of clonal culture, clones contain 4–16 cells. The continuously changing shape of the clone illustrates the high cell motility (Fig. 5). By 2 weeks of culture, this type of colony consists of thousands of cells and constitutes 83.0 ± 2.7% of all colonies (Table 1). The remaining 17% of colonies are small, consisting of four to eight cells and contain one of two types cells: either flattened cells that resemble smooth muscle cells or unidentified small elongated cells.

At 2 weeks of culture, many cells in clones exhibit long processes (Fig. 6A–C). Others resemble morphologically mature smooth muscle cells (Fig. 6B, arrowhead). Many proliferating cells are still present at 2 weeks in clonal culture (see doublet of late anaphase cells in Fig. 6B, arrow) and all cells are X-gal–positive (Fig. 6C). To identify different cell types, clones were first processed for X-gal reaction and subsequently for immunocytochemistry with cell type-specific antibodies. Cells with long processes are intensely immunoreactive for neuron-specific β-III tubulin (Fig. 7A). Figure 7B shows X-gal reactivity in the areas marked in Figure 7A. The X-gal reaction product is most intense in and often limited to inclusion bodies, as has been described previously by Rico et al. (2002) and Szeder et al. (2003). Large flattened cells are immunoreactive for smooth muscle actin (Fig. 7A, fluorescein; Fig. 7C, Texas Red). Rarely, clones also contain Schwann cell progenitors as shown by SCIP immunoreactivity (Fig. 7D,E; Zorick et al., 1996)

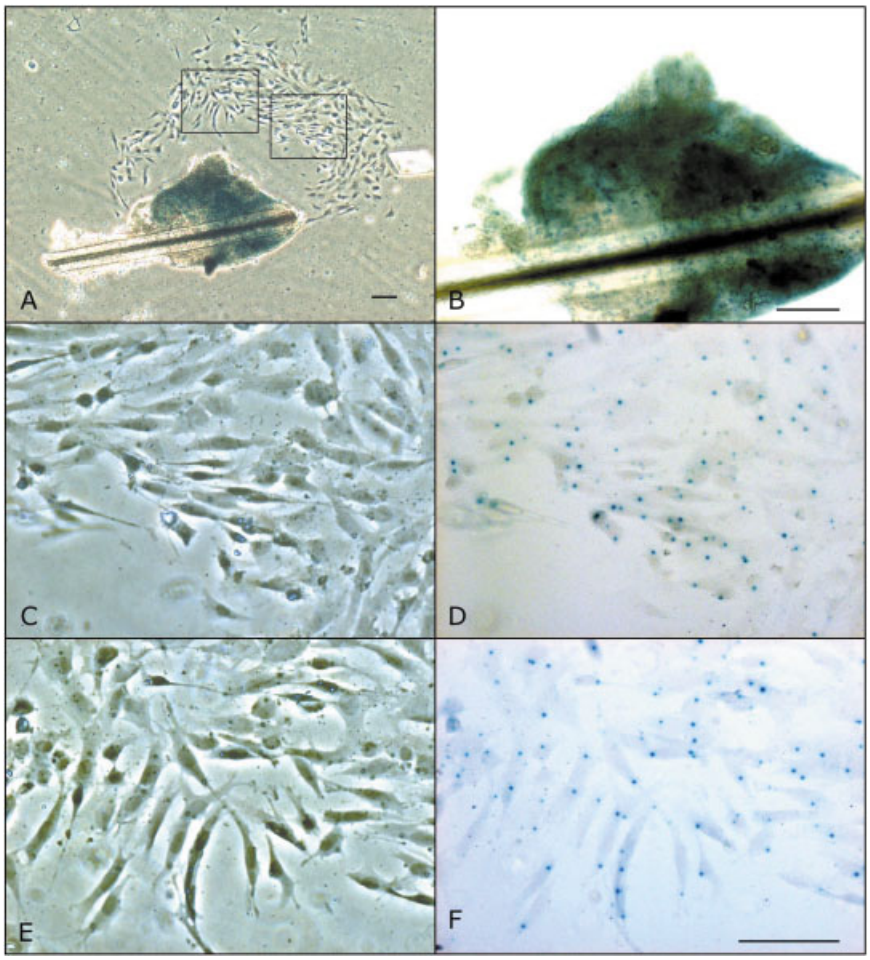

Fig. 3. Bulge explant. X-gal–reacted explant 4 days postexplantation. **A:** Phase contrast image of a bulge explant. The explant with the hair still inside and emigrated cells are visible. The explant got displaced away from the cells during mounting of the cover slip. **B:** The same bulge explant with focus on the bulge. Numerous X-gal–positive neural crest cells are present within the bulge. **C:** Area marked at the right in A, shown with phase contrast optics. **D:** Corresponding brightfield image. All emigrated cells are X-gal–positive. **E:** Area marked on the left in A shown with phase contrast optics. **F:** Corresponding image with brightfield optics. All cells are X-gal–positive. Scale bars = 100 μm in A, 100 μm in B, 100 μm in F (applies to C–F).

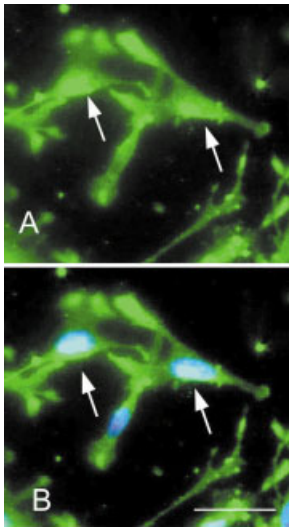

Fig. 4. Sox10 expression in bulge explants. Sox10 is a marker for early migrating neural crest cells. All cells that have emigrated from bulge explants express Sox10 at high levels, confirming that they are neural crest cells (arrows). Scale bar = 50 μm in B (applies to A,B).

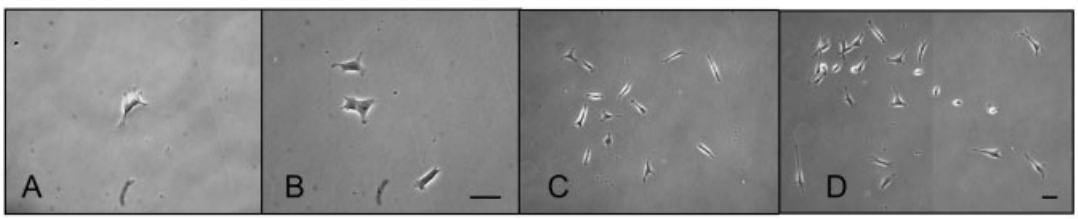

Fig. 5. Bulge-derived cells in clonal culture. **A:** One cell 6 hr after cloning. **B:** Four daughter cells from the same clone-forming cell as in A at 18 hr. **C:** The same clone at 48 hr consists of approximately 13 cells. **D:** The same clone at 72 hr. The changing shape of the clone reflects the high motility of the cells. Scale bars = 50 μm in B (applies to A,B), 50 μm in D (applies to C,D).

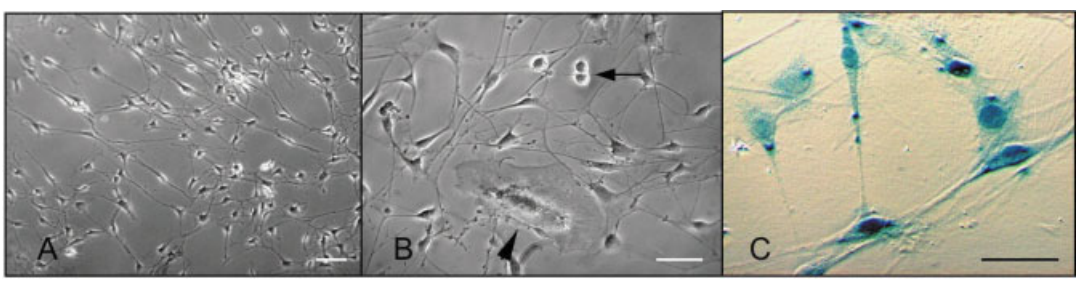

Fig. 6. Clones at 2 weeks in clonal cultures. At 2 weeks in clonal culture, colonies consist of thousands of cells. **A:** A small part of a 2-week-old clone. Most cells are elongated. **B:** Higher magnification of a different area. Some cells are large and flattened (arrowhead). Proliferating cells are still present (see postmitotic doublet marked by the arrow). **C:** All cells in clones are X-gal–positive (Hoffmann contrast optics), confirming that they are neural crest-derived cells. Scale bars = 50 μm (applies to A–C).

TABLE 1. Percentage of Primary and Secondary Clones Formed[a]

Clone type	Clones formed by stem cells (% of total ± SEM)
Primary (from day 4 bulge explants)	83.0 ± 2.7*
Secondary (from day 3 primary clones	73.5 ± 6.7**
Secondary (from day 5 primary clones)	66.2 ± 4.4***

[a]Average number of cells per primary clone at the time of subcloning, 28.5 ± 3 (day 3) and 52.4 ± 5.5 (day 5).
*$P = 0.13$.
**$P = 0.015$.
***$P = 0.31$.

and S100 immunoreactivity (Fig. 7F,G; Parkinson et al., 2001). The presence of pigment cells in clones is documented by MelEM immunoreactivity (Fig. 7H,I; Nataf et al., 1993; Alexanian and Sieber-Blum, 2003). The differentiation of multiple cell types within clones demonstrates that bulge-derived neural crest cells are pluripotent.

Targeted Differentiation Into Schwann Cell Progenitors

Shah et al. (1994) have reported the neuregulin promotes differentiation of neural crest cells into Schwann cells. In the presence of neuregulin-1 (10 nM), clones contain large numbers of Schwann cell progenitors, and neurons are present as well. Figure 8 shows a quadruple stain that combines glial fibrillary acidic protein (GFAP; Schwann cell marker) immunoreactivity (Texas Red) with β-III immunoreactivity (fluorescein), DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride) nuclear stain, and X-gal reaction. Figure 8A–D focuses on a group of GFAP-positive Schwann cell progenitors: GFAP, Texas Red, immunoreactivity (Fig. 8A), DAPI (Fig. 8B), β-III tubulin (fluorescein; absent, Fig. 8C). Figure 8D shows merged images in pseudocolor. Figure 8E–I shows a group of neurons in a different area of the same clone: β-III tubulin immunoreactivity (Fig. 8E), merged brightfield and fluorescein images to better visualize the X-gal reaction product within neurons (e.g., Fig. 8F, arrows), corresponding DAPI stain (Fig. 8G), and GFAP immunofluorescence (Fig. 8H, absent). Figure 8I shows merged images in pseudocolor. Taken together, the data show that, while Schwann cell progenitors rarely develop in our regular culture medium, their number increases greatly in the presence of neuregulin-1, which suggests that neuregulin-1 directs stem cells to differentiate along the Schwann cell lineage. Furthermore, the data in Figure 8 show that neurons do not express a glia marker and Schwann cell progenitors do not express a neuronal marker.

Targeted Differentiation Into Chondrocytes

To determine whether bulge-derived neural crest cells can generate the full spectrum of cranial neural crest derivatives, we sought to differentiate them into chondrocytes. Sox9 is required for the commitment of neural crest cells to the chondrogenic lineage (Mori-Akiyama et al., 2003) as it is a potent activator of type II collagen expression in chondrocytes (Kypriotou et al., 2003). BMP-2 causes robust up-regulation

Fig. 7. Differentiated cell types expressed in clones. **A,B:** Quadruple stain combining anti–neuron-specific β-III tubulin antibodies (Texas Red) with anti-smooth muscle actin monoclonal antibody (fluorescein), DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride) nuclear stain (blue), and X-gal reactivity (shown in B). Several neurons and one smooth muscle cell are visible (focus is on neurons). B: X-gal reaction of two areas marked in A. **C:** Smooth muscle cell (anti-smooth muscle actin antibody; Texas Red). **D:** Rare SCIP-immunoreactive Schwann cell. **E:** Corresponding phase contrast image. **F:** Rare S100-immunoreactive Schwann cell progenitor. **G:** Corresponding phase contrast image. **H:** Three MelEM-immunoreactive melanocyte progenitors. **I:** Corresponding phase contrast image. Scale bars = 50 μm in A (applies to A,B), in C, in D (applies to D–G), in H (applies to H,I).

Fig. 8. Targeted differentiation of epidermal neural crest cells into Schwann cell progenitors. To obtain larger numbers of Schwann cell progenitors, clones were grown in the presence of neuregulin-1. Quadruple stain combining β-III tubulin (fluorescein), glial fibrillary acidic protein (GFAP, Texas Red), DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride) nuclear stain (blue), and X-gal reaction (black). **A:** GFAP stain with X-gal reaction product (e.g., arrow). Several Schwann cell progenitors are visible. **B:** DAPI nuclear stain of same area as in A. **C:** β-III tubulin immunoreactivity (Texas Red) in the same area is absent. Arrow depicts same cell and X-gal reaction product as in A and B. **D:** Merged images of GFAP (Texas Red), β-III tubulin (fluorescein, absent), DAPI nuclear stain, and X-gal reaction. This series of images shows that Schwann cell progenitors are present in large numbers and that they do not express a neuronal marker. In a different area in the same clone, neurons are present (arrow). **E:** β-III tubulin stain; several multipolar neurons are present. **F:** Merged brightfield and β-III tubulin images of the same area as in E to show that neurons contain X-gal reaction product (arrows in E, F, G, and I). **G:** Corresponding DAPI nuclear stain. **H:** GFAP immunoreactivity in the same area is absent (Texas Red). **I:** Merged images in pseudocolor of β-III tubulin (fluorescein), GFAP (Texas Red, absent), and DAPI nuclear stain (blue). The data show that neurons do not express a Schwann cell marker. Scale bar = 10 μm in I (applies to A–I).

Fig. 9. Targeted differentiation of epidermal neural crest cells (eNCSC) into chondrocytes. To determine whether eNCSC are able to generate chondrocytes, we grew clones in the presence of BMP-2. After 2 weeks in culture, most cells in clones were immunoreactive for collagen type II, a marker for chondrocytes. Triple stain combining collagen type II immunoreactivity (Texas Red), X-gal reaction (black), and DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride) nuclear stain (blue). **A:** Collagen type II immunoreactivity. Six cells are visible, and they contain X-gal reaction product (e.g., arrows). **B:** Merged images in pseudocolor of collagen type II immunoreactivity (Texas Red), DAPI (blue), and X-gal (black). **C:** Brightfield image of the same area as in A and B to better visualize the X-gal reaction product (e.g., arrows). Scale bar = 50 μm in C (applies to A–C).

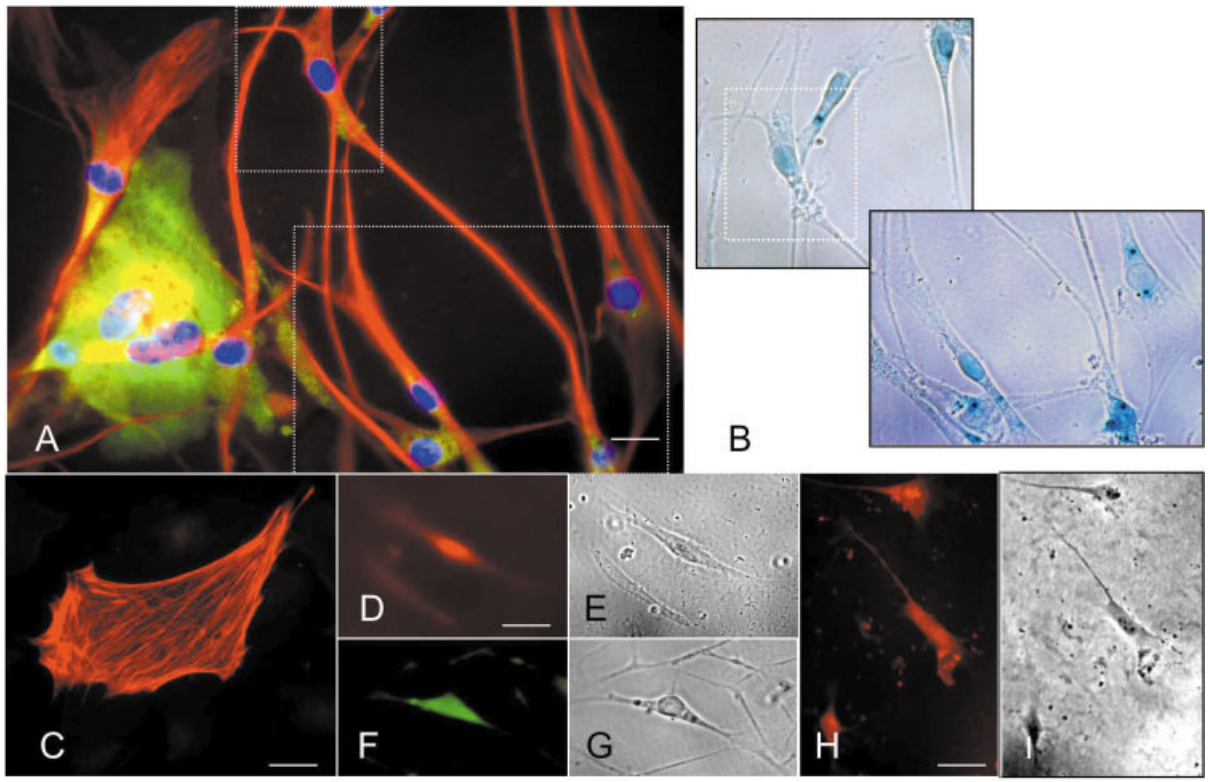

Fig. 7.

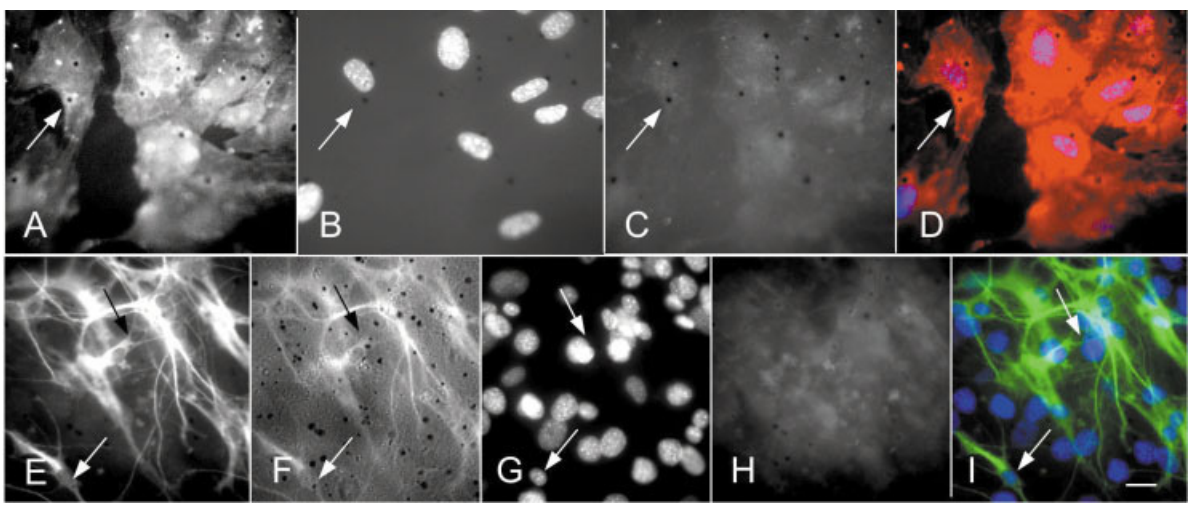

Fig. 8.

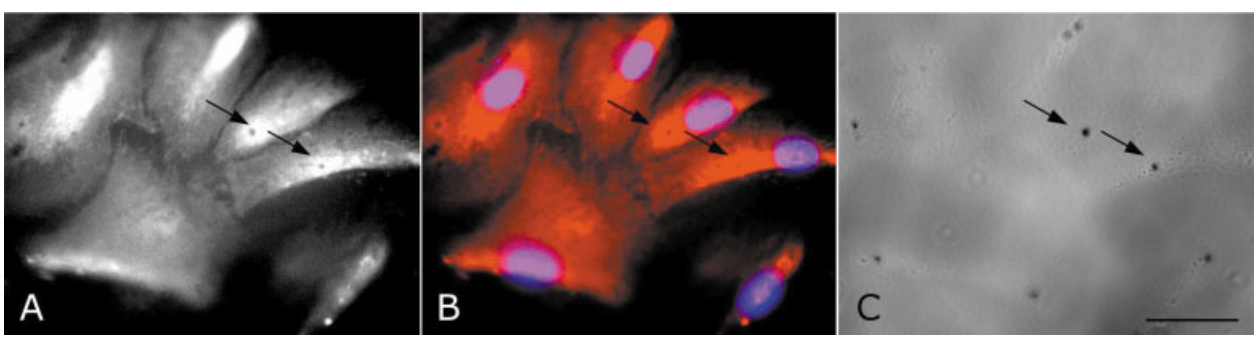

Fig. 9.

of Sox9 (Zehentner et al., 2002). We, therefore, cultured bulge-derived neural crest cells in the presence of BMP-2 (10 ng/ml) for 2 weeks in clonal culture. Under these conditions, most cells in clones become collagen type II immunoreactive, indicating that they have differentiated into chondrocytes. Figure 9A shows a group of collagen type II-immunoreactive cells with X-gal reaction product (arrows). Figure 9B shows merged images of collagen type II immunoreactivity (Texas Red), DAPI (blue), and X-gal (black, arrows). Figure 9C shows the same area with brightfield optics to better visualize the X-gal reaction product. The data show that BMP-2 directs bulge-derived neural crest cells in clonal culture to differentiate along the chondrogenic cell lineage.

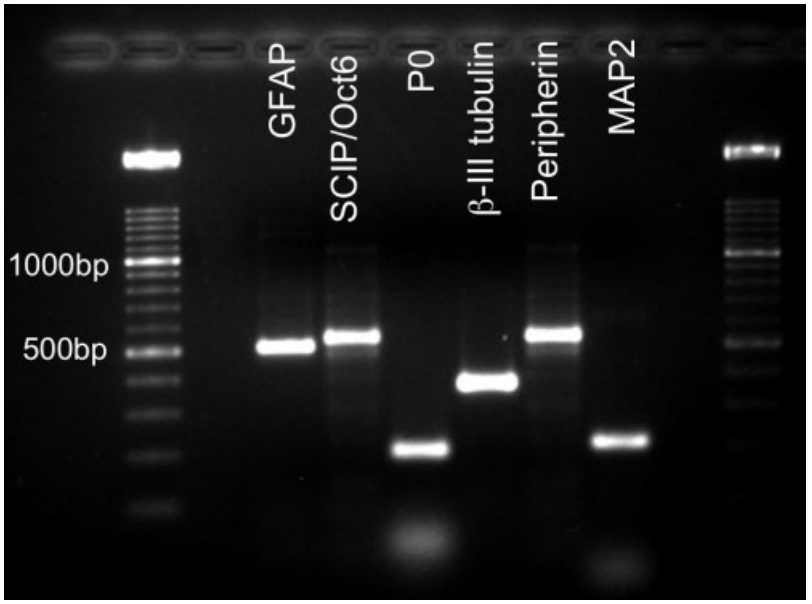

Fig. 10. Reverse transcriptase-polymerase chain reaction to detect Schwann cell- and neuron-specific gene expression. In cells grown in the presence of neuregulin (10 nM), glial fibrillary acidic protein (GFAP), SCIP/Oct6, and protein zero (P0) were abundantly expressed. Likewise, in cultures that were grown in nonsupplemented culture medium, the neuronal genes β-III tubulin, peripherin, and microtubule-associated protein (MAP2) were expressed.

Verification of Neuronal and Schwann Cell Differentiation by Reverse Transcriptase-Polymerase Chain Reaction

To establish the specificity of the antibody stains and to test additional neuronal markers by different means, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) for three neuronal and three Schwann cell markers. Bulge-derived cells grown for 2 weeks in culture medium supplemented with neuregulin-1 express the Schwann cell markers GFAP, SCIP/Oct6, and protein zero (P0) abundantly (Fig. 10). Cells that were grown in regular culture medium in the absence of neuregulin-1 express β-III tubulin, peripherin, and microtubule-associated protein (MAP2; Fig. 10). The authenticity of the RT-PCR products were verified by sequencing.

Bulge-Derived Neural Crest Cells Can Undergo Self-Renewal

We determined whether bulge-derived neural crest cells can undergo self-renewal by serial cloning in vitro. To this end, primary clones were prepared. Either 3 or 5 days later, primary clones were resuspended by trypsinization with the aid of a glass cloning ring. The resuspended clone was seeded again at clonal density (20–50 cells per 35-mm culture plate) and incubated for 2 weeks (Fig. 11A). Figure 11B shows a 5-day-old secondary clone. The insert shows the secondary clone-forming cell shortly after plating at higher magnification. Figure 11C shows a higher magnification of the area marked in Figure 11B. The morphology of cells in secondary clones is similar to that in primary clones and in primary explants. At the end of the culture period, secondary clones were analyzed with cell type-specific antibodies. Figure 11D–I shows a triple stain that combines X-gal (Fig. 11F,I, black dots, arrows) with β-III tubulin (Fig. 11E, Texas Red) and smooth muscle actin (Fig. 11G, fluorescein) antibodies. A β-III tubulin-immunoreactive neuronal cell is shown in (Fig. 11E); it does not express smooth muscle actin (Fig. 11D). In a different area of the same secondary clone, a group of smooth muscle cells (Fig. 11G) is present, which do not express β-III tubulin (Fig. 11H). The data indicate that the primary clone contained stem cells, which were able to generate at least two distinct differentiated cells types, which fulfills the criterion for self-renewal.

Primary clones from day 4 bulge explants comprise 83.0 ± 2.7% of all colonies. The percentage of secondary clones formed by pluripotent cells is 73.5 ± 6.7% when taken from day 3 primary clones and 66.2 ± 4.4% when prepared from day 5 primary clones (Table 1). Thus, the portion of stem cells is maintained at relatively high levels over an estimated total of 18 doublings in primary and clonal culture, despite that our culture medium was developed to support the differentiation of neural crest cells (Ito and Takeuchi, 1984; Ito et al., 1993). Taken together, we have shown that bulge-derived neural crest cells are pluripotent and that they can undergo self-renewal. Thus, bulge-derived neural crest cells fulfill the criteria for pluripotent stem cells (epidermal neural crest stem cells, eNCSC).

eNCSC Are Distinctly Different From Schwann Cell Progenitors of the Adult Sciatic Nerve

Because whiskers follicles are innervated by myelinated nerves, we sought to determine whether eNCSC are, in fact, Schwann cell

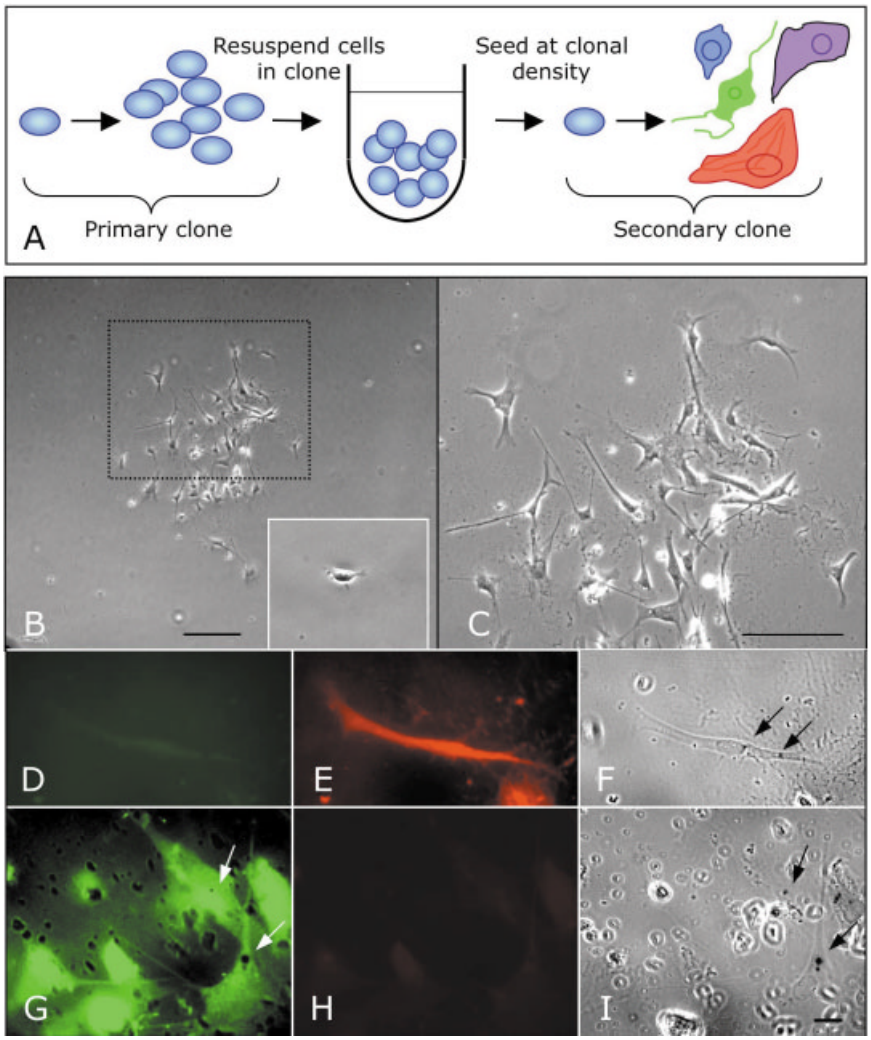

Fig. 11. Serial cloning. We have determined that bulge-derived neural crest cells can undergo self-renewal. **A:** Cells from primary bulge explants were put into clonal culture and let grow into a colony (primary clone). Clones were subsequently isolated by putting a glass cloning ring around them, and they were then resuspended by adding two drops of 0.025% trypsin in 0.02% ethylenediaminetetraacetic acid in phosphate-buffered saline. When the cells detached, trypsinization was stopped by 1 mg/ml of trypsin inhibitor in culture medium. The cells were aspirated, diluted with 1.5 ml of culture medium, and placed into a new collagen-coated 35-mm plate (secondary clones). Plates with secondary clones were grown for 2 weeks and then analyzed with cell type-specific antibodies. **B:** Five-day-old secondary clone. Insert: The secondary clone-forming cell. **C:** Higher magnification of area marked in B. Cell morphology in secondary clones resembles that in primary clones. After 2 weeks, secondary clones were fixed and processed for immunocytochemistry. D–I: Triple stain combining β-III tubulin (Texas Red) with smooth muscle actin (fluorescein) and X-gal reaction (black). **D:** Smooth muscle actin (fluorescein) immunoreactivity is absent. **E:** β-III tubulin stain in the same area as in D shows one neuronal cell. **F:** The corresponding phase contrast image shows X-gal reaction product (arrows). **G:** Smooth muscle actin immunoreactivity (fluorescein) shows several smooth muscle cells (arrows) in a different area of the same secondary clone. **H:** Same area as in G, but with Texas Red illumination. Smooth muscle actin-immunoreactive cells do not express the neuronal marker. **I:** Corresponding phase contrast image with X-gal reaction product (arrows). Scale bars = 100 μm in B, 100 μm in C, 10 μm in I (applies to D–I).

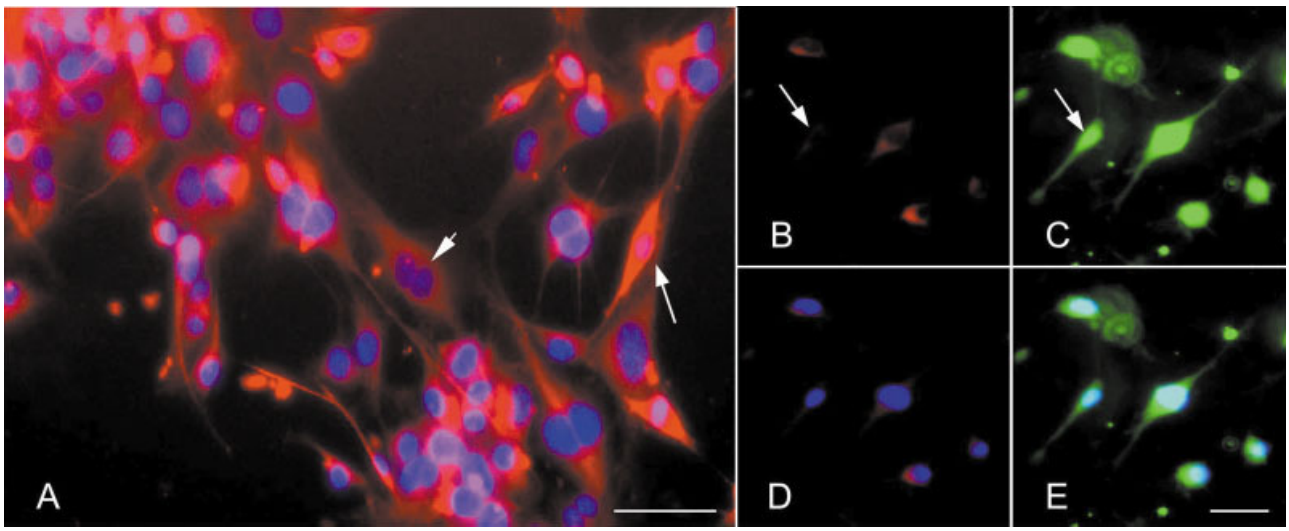

Fig. 12. Are epidermal neural crest cells (eNCSC) identical with adult Schwann cell progenitors? Whisker follicles are innervated by myelinated nerves. Thus, our preparation could be contaminated by nerve endings. To determine whether eNCSC are adult Schwann cell progenitors, we compared eNCSC with Schwann cell precursors from adult sciatic nerve explants. **A:** Nestin/DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride) double labeling in day 5 bulge explant. Virtually all cells, in particular stellate cells (e.g., long arrow), are intensely nestin immunoreactive (Texas Red). Cells with the morphology of differentiating smooth muscle cells (short arrow) express lower levels of nestin. **B:** By contrast, cells at culture day 5 that had emigrated from adult sciatic nerve explants express nestin at low levels only or not at all (e.g., arrow; Texas Red). **C:** The same cells, however, were intensely immunoreactive for SCIP (fluorescein fluorescence, arrow), a marker for Schwann cell progenitors (Zorick et al., 1996), whereas eNCSC differentiate only rarely into SCIP-positive cells under these culture conditions (Fig. 7D). **D:** Nestin/DAPI merged images of area shown in B and C. **E:** SCIP/DAPI merged images of area shown in B, C, and D. It can be concluded, thus, that eNCSC and adult Schwann cell progenitors are distinctly different types of cell. Scale bar = 50 μm in A, 50 μm in E (applies to B–E).

progenitors derived from contaminating nerve endings. We therefore determined similarities and differences between cells that emigrate from whisker bulge explants and from sciatic nerve explants from adult mice. Both types of tissue were cultured under the same conditions in regular culture medium. Nestin is a marker for both neural stem cells (Lendahl et al., 1990) and neural crest stem cells (Lothian and Lendahl, 1997; Mujtaba et al., 1998; Josephson et al., 1998). As expected, bulge-derived cells express nestin at high levels (Fig. 12A; e.g., arrow). Differentiating cells with smooth muscle cell morphology expressed nestin at lower levels (Fig. 12A, arrowhead). By contrast, cells that have emigrated from adult sciatic nerve explants express nestin at low levels only or not at all (Fig. 12B, e.g., arrow). Whereas SCIP-positive cells are observed only very rarely in bulge-derived cultures (Fig. 7D,E) under these culture conditions, all sciatic nerve-derived cells express SCIP at high levels. Figure 12C,E shows SCIP expression in the same cells shown in Figure 12B,D.

At 24 hr of clonal culture, 73.7 ± 4.9% of sciatic nerve-derived colonies consist of 1–2 cells with Schwann cell-like morphology. These cells die within the second 24 hr in clonal culture, suggesting that they were dependent on axonal contact. The remaining 26.3% of colonies contain 9.3 ± 0.9 cells per clone at 48 hr and 12.8 ± 1.6 cells at 72 hr in clonal culture, suggesting a low rate of proliferation that is possibly combined with cell death.

Thus, while eNCSC have a high rate of proliferation and express nestin at high levels but not SCIP, sciatic nerve-derived cells have a lower rate of proliferation under the same culture conditions, most of them die within the first 48 hr, and they are intensely immunoreactive for SCIP, but not for nestin. These observations demonstrate that adult bulge-derived neural crest cells and adult sciatic nerve-derived Schwann cell progenitors are distinctly different types of cell.

DISCUSSION

In this study, we have documented the existence of pluripotent neural crest stem cells, termed eNCSC, in the bulge region of the adult mouse whisker follicle. The presence of neural crest-derived cells in the hair follicle is not surprising, as neural crest-derived melanocyte progenitors have been described in this location by Peters et al. (2002) and Nishimura et al. (2002). Moreover, Merkel cells, which are also of neural crest origin (Szeder et al., 2003), are present in large numbers in the basal layer of the outer root sheath in whisker follicles. However, the persistence in the adult organism of pluripotent stem cells that are derived from a transient embryonic tissue, the neural crest, is surprising.

In the mouse and in all other vertebrate embryos studied to date, Wnt1 expression during embryogenesis is transient and limited to neural crest cells and dorsal neural tube cells (Wilkinson et al., 1987; Davis et al., 1988; Molven et al., 1991; McMahon et al., 1992; Wolda et al., 1993; Echelard et al., 1994). In E9.5 double-transgenic Wnt1-cre/R26R mice, dorsal neural tube cells, premigratory neural crest cells, and migratory neural crest cells, but no other cell types, express cre as detected with an anti-cre antibody (Sieber-Blum et al., 2003). At E16.5, epidermal cells express the Wnt-3 and Wnt-5a genes (St-Jacques et al., 1998; Millar et al., 1999; Reddy et al., 2001; Fuchs et al., 2001) but not Wnt-1. As an important negative control, we have nevertheless confirmed the absence of Wnt-1 expression in epidermal cells of hair follicles and surface skin (Sieber-Blum et al., 2003). The expression by bulge-derived cells of Sox-10, a neural crest marker, and of nestin, a neural crest stem cell marker (among other stem cells), support the notion that X-gal–positive cells are indeed neural crest cells, as do their differentiated progeny in clones (neurons, Schwann cells, smooth muscle cells, melanocytes, and chondrocytes), all of which are physiological neural crest derivatives.

eNCSC are located in the epidermis of the hair follicle. Stem cells with neurogenic and gliogenic potential have been isolated also from the dermis (SKP, skin-derived precursors; Toma et al., 2001). Comparisons between the two studies are difficult, because there are fundamental differences in the techniques applied to obtain eNCSC and SKP cells. We have isolated eNCSC from a discrete location, the epidermal follicular bulge. By contrast, SKP cells are observed only when dermis is present and neural crest cell markers are not expressed (Toma et al., 2001). We have cloned minimally expanded eNCSC with the aim to preserve their innate characteristics. In contrast, Toma et al. (2001) start with cultures of high cell density that are expanded for several weeks. Currently available data thus suggest that eNCSC and SKP cells are unlikely to be of the same origin.

Our observation of neural crest stem cells in the follicular epidermis has implications for the interpretation of the work by Kobayashi et al. (1993), Oshima et al. (2001), Li et al. (2003), and Nishimura et al. (2002). By in vitro clonal analysis, Kobayashi et al. (1993) have identified keratinocyte precursors with stem cell properties in the bulge region of the rat whisker follicle. During hair growth, these stem cells migrate from the bulge region to the matrix at the base of the follicle to participate in hair regeneration (Kobayashi et al., 1993; Oshima et al., 2001; Li et al., 2003). Combined, the different studies suggest that the bulge cell population is of mixed origin, containing keratinocyte stem cells, pluripotent neural crest stem cells, and stem cells that are committed to the melanogenic lineage, all of which may undergo hair cycle-related migration within the follicle.

Nishimura et al. (2002) used the Dct-lacZ mouse, in which β-galactosidase is expressed under the control of the dopachrome tautomerase promoter. Because this enzyme is considered to be specific for melanocytes, lacZ-positive cells in the Nishimura study identify cells that are committed to the melanogenic lineage. It is likely that a subset of X-gal–positive cells observed in Wnt1-cre/R26R whisker follicles are identical to the X-gal–positive cells in

Dct-lacZ follicles, in particular as 83.0 ± 2.7%, rather than 100%, of clones in our cultures were formed by pluripotent stem cells (Table 1). If committed pigment cell progenitors are present in the bulge, why did we not observe pigmented colonies in our clonal cultures? There are two possible explanations. First, the majority of our Wnt1-cre/R26R mice are white and, therefore, do not contain recognizable melanized pigment cells. The second reason concerns our culture medium. As formulated originally by Ito et al. (Ito and Takeuchi, 1984; Ito et al., 1993), the culture medium contained the tumor promoter TPA and cholera toxin to promote melanogenesis. Neurons do not develop in these culture conditions (M.S.-B., unpublished observation). In contrast, our primary interest lies in neurogenesis. Therefore, we did not supplement the culture medium with TPA and cholera toxin, which makes it conducive to neuronal differentiation but does not support melanogenesis.

eNCSC are intriguing for several reasons. First, like embryonic neural crest stem cells, eNCSC have an innate high degree of plasticity. They can give rise to the entire array of cranial neural crest cells, including neurons, Schwann cells, smooth muscle cells, chondrocytes, melanocytes, and possibly other cell types that originate from the cranial neural crest. Second, they are abundant and easily accessible. Thus, eNCSC are attractive candidates for diverse cell therapy applications. Because they are located in an accessible tissue, only minimally invasive surgery will be necessary to harvest them. Furthermore, because they can be obtained from the living organism, they are good candidates for autologous transplantation, which will avoid both transplant rejection and graft-versus-host disease.

EXPERIMENTAL PROCEDURES

Animals and Genotyping

Heterozygous Wnt1-cre mice were mated with R26R heterozygotes. Genotyping was performed exactly as described (Szeder et al., 2003).

Bulge Explants

Whiskers were dissected from the whisker pad of 8- to 10-week-old Wnt1-cre/R26R double-transgenic mice according to Baumann et al. (1996). The connective tissue was scraped from the follicle with a bent electrolytically sharpened tungsten needle and rinsed several times, thus exposing the ring sinus and cavernous sinus (Fig. 1B). The capsule was then cut longitudinally with a small scalpel, the blood flushed with a stream of buffer, the follicle cross-sectioned first at the level above the cavernous sinus and then below the skin, yielding the bulge region within the capsule (Fig. 1C). The bulge was rolled out of the capsule, rinsed three times, and plated in an empty collagen-coated 35-mm culture plate that had been preincubated for 3 hr with culture medium. The bulge explants adhered to the substratum within 1 hr, at which time 1.5 ml of culture medium was added. The culture medium was designed to accommodate the survival and proliferation of neural crest stem cells, as well as their differentiation into multiple phenotypes, including neurons, smooth muscle cells, glia, chondrocytes, and melanocytes (Ito et al., 1993; Sieber-Blum, 1999). It consisted of 75% alpha-modified MEM medium, 5% day 11 chick embryo extract, and 10% of fetal calf serum (HyClone), and it was supplemented with 1 μg/ml gentamicin as described previously (Sieber-Blum, 1999). Fifty percent of the culture medium was exchanged every other day.

Clonal Cultures

Cells started to emigrate from explants 48–72 hr postexplantation. At 4 days postexplantation, the bulge explant was removed, leaving the emigrated cells on the collagen substratum. The emigrated cells were then resuspended by trypsin digestion exactly as described for mouse neural crest cells (Sieber-Blum, 1999). The percentage of single cells was 100, due to the sparse arrangement within early primary explants (Fig. 3A,C,E). Cells were plated at 20 cells per cm^2, which at a plating efficiency of approximately 10% yielded 8–10 clones per 35-mm culture plate. The cells adhered within 30 min. One hour after plating, single cells were marked by circling the underside of the plate with a diamond marker (4 mm diameter). Fifty percent of the culture medium was exchanged every other day.

Subclones were prepared by removing the culture medium and placing a glass cloning ring around the clone. The clones within the rings were then rinsed with phosphate-buffered saline (PBS) and subsequently detached by trypsinization as described for primary explants. The clonal cell suspension consisting of 20–50 cells was subsequently placed into a new 35-mm culture dish.

X-gal Reaction and Indirect Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde for 5 min at room temperature, and X-gal histochemistry was performed according to Galileo et al. (1990) with the modification that potassium ferricyanide and potassium ferrocyanide were used at 5 mM and incubation was overnight at 30°C. Subsequently, the cultures were processed for indirect immunocytochemistry as follows. Cells were post-fixed with 4% paraformaldehyde for 30 min on ice, rinsed 3 × 10 min with PBS, blocked with 2% normal goat serum for 20 min and then incubated with pooled primary antibodies overnight in the cold. Subsequently, the plates were rinsed 3 × 10 min with PBS, incubated with pooled secondary antibodies for 2 hr at room temperature, rinsed 4 × 20 min with PBS, stained with DAPI nuclear stain (3 μM; Molecular Probes, Eugene, OR), rinsed again, and finally mounted with ProLong Antifade (Molecular Probes) and cover-slipped. The following primary antibodies were used: mouse monoclonal antibody against smooth muscle actin (1:800; Sigma, St. Louis, MO); mouse monoclonal antibody against neuron-specific β-III tubulin (1:200; Chemicon, Temecula, CA); polyclonal rabbit anti–β-III tubulin antibodies (1-400; gift of A. Frankfurter; Lee et al., 1990); rabbit polyclonal antibodies against S100 protein (1:200; Novocastra Laboratories, Newcastle upon Tyne, UK); rabbit anti-

SCIP antibodies (1:300; gift of G. Lemke; Zorick et al., 1996); MelEM (melanocyte marker; 1:1; Hybridoma Bank; Nataf et al., 1993); anti-Sox10 rabbit serum (1:100; Chemicon), nestin mouse monoclonal antibodies (1:400; BD Biosciences); and mouse anti-GFAP ascites fluid (1:500; Chemicon). The following secondary antibodies were used at a dilution of 1:200: Texas Red-conjugated and fluorescein-conjugated goat-anti-mouse immunoglobulin G (IgG) and goat anti-rabbit IgG designated for multiple labeling (Jackson ImmunoResearch, West Grove, PA). The X-gal reaction in tissue sections was performed exactly as described (Szeder et al., 2003).

RT-PCR

By using Trizol reagent (Invitrogen), total RNA was prepared from cells that were grown for 2 weeks either in the presence of neuregulin1 (10 nM; for Schwann cell markers) or in its absence (for neuronal markers). Reverse transcription was performed with 3 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). PCR amplification was carried out with 50 ng of reverse transcribed DNA template, 10 pmol of primers, and 0.2 mM dNTP. The PCR reaction consisted of denaturation at 94°C for 45 sec, annealing for 45 sec (temperature dependent on the primer pair), and extension at 72°C for 1 min. Annealing temperatures were as follows: P0, 59°C; MAP2 and GFAP, 58°C; beta-III tubulin and peripherin, 55°C; SCIP/Oct6, 52°C. PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. The following primers were used. P0: forward, 5′-ACTATGCCAAGGGACAACCTTACATC-3′; reverse, 5′-ACATAGAGCGTGACCTGAGAGGTC-3′; product size, 196 bp. MAP2: forward, GGCCCAAGCTAAAGTTGG-3′; reverse, 5′-CAAGCCAGACCTCACAGCG-3′; product size, 215 bp. Neuron-specific β-III tubulin: forward, 5′-CCCGTGGGCTCAAAATGT-; reverse, 5′-TGGGGGCAGTGTCAGTAGC-3′; product size, 380 bp. SCIP/Oct6: forward, 5′-AAGAACATGTGCAAGCTCAA-3′; reverse, 5′-ACAACAAAAAGAGTCCAGGC-3′; product size 528 bp. GFAP; forward, 5′-CAAGCCAGACCTCACAGCG-3′; reverse, 5′-GGTGTCCAGGCTGGTTTCTC-3′; product size 508 bp. Peripherin: forward, 5′-ACAGCTGAAGGAAGAGATGG-3′; reverse, 5′-GATTTGCTGTCCTGGGTATC-3′; product size 538 bp. The RT-PCR products were sequenced for verification.

ACKNOWLEDGMENTS

We thank A.P. McMahon, P. Soriano, and H. Sucov for providing the Wnt1-cre and R26R mouse lines and G. Lemke for the SCIP antibody. We also thank Solomon Senok for his advice on follicle dissection and Stephen Duncan for his comments on the manuscript. Additionally, we thank Eva Kluzáková and Joan Ward for excellent technical assistance. M.S.-B. was funded by the National Institute of Neurological Disorders and Stroke, NIH; USPHS; and the Rockefeller Brothers Fund, New York; Charles E. Culpeper Biomedical Pilot Initiative. M.G. was funded by the Ministry of Education of the Czech Republic.

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