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The Bulge Area Is the Origin of Nestin-Expressing Pluripotent Stem Cells of the Hair Follicle

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ABSTRACT

Nestin-expressing pluripotent stem cells have been found both in the bulge area (BA) as well as the dermal papilla (DP). Nestin-expressing stem cells of both the BA and DP have been previously shown to be able to form neurons and other non-follicle cell types. The nestin-expressing stem cells from the DP have been termed skin precursor or SKP cells. Both nestin-expressing DP and BA cells have been previously shown to effect repair of the injured spinal cord and peripheral nerve, with the BA being the greater and more constant source of the stem cells. The BA contains nestin-expressing stem cells throughout the hair cycle, whereas nestin-expressing dermal papillae stem cells were found in early and mid-anagen only. Our previous studies have shown that the nestin-expressing stem cells in the BA and DP have similar morphological features. The cells from both regions have a small body diameter of approximately 7 μm with long extrusions, as shown by 2-photon imaging. In the present study, using 2-photon imaging of whisker follicles from transgenic mice expressing nestin-driven green fluorescent protein (ND-GFP), we demonstrate that the BA is the source of the nestin-expressing stem cells of the hair follicle. The nestin-expressing stem cells migrate from the BA to the DP as well as into the surrounding skin tissues including the epidermis, and during wound healing, suggesting that the BA may be the source of the stem cells of the skin itself. J. Cell. Biochem. 112: 2046–2050, 2011.

KEY WORDS: STEM CELLS; HAIR FOLLICLE; VIBRISSA; NESTIN; GFP; BULGE AREA; DERMAL PAPILLA; MIGRATION; SKIN; TWO-PHOTON IMAGING

The hair follicle is a complex skin appendage consisting of six concentric cylinders with several distinct cell types that produce highly specialized proteins. Follicle function is regulated at least in part by the adjacent mesenchymal dermal papilla (DP) [Hardy, 1992] (Fig. 1). The hair follicle continuously cycles through three major stages: anagen is the hair growth phase, catogen the involution phase, and telogen the resting phase [Li et al., 1991]. All three are regulated by specific molecular mechanisms [Hardy, 1992; Paus and Cotsarelis, 1999; Hoffman, 2000].

A major paradigm change in our understanding of the biology of the hair follicle, as well as stem cells themselves, occurred when nestin-expressing stem cells were discovered in the hair follicle bulge area (BA) [Li et al., 2003]. Nestin is a neural stem cell marker, and its discovery in the hair follicle suggested that the nestin-expressing hair follicle stem cells are multipotent or pluripotent [Li et al., 2003]. The nestin-expressing stem cells in the hair follicle were subsequently shown to be able to differentiate into neurons and other non-follicular cell types [Sieber-Blum et al., 2004, 2006; Amoh et al., 2005a, 2009; Hoffman, 2006; Biernaskie et al., 2009], thereby confirming the observation of Li et al. [2003]. Nestin-expressing pluripotent stem cells were also found in the DP [Biernaskie et al., 2009; Liu et al., 2011].

Transgenic mice, with green fluorescent protein (GFP) expression driven by the nestin regulatory element [nestin-driven GFP (ND-GFP)] [Li et al., 2003; Mignone et al., 2004], were originally used to identify nestin-expressing cells in the hair follicle BA [Li et al., 2003].

Nestin-expressing stem cells of both the BA and DP have been previously shown to repair peripheral nerves and spinal cord injury [Amoh et al., 2005b, 2008; Biernaskie et al., 2009]. The nestin-expressing pluripotent stem cells from the DP have been termed skin precursor or SKP cells [Biernaskie et al., 2009]. In a previous study, we showed that the major source of nestin-expressing pluripotent stem cells in the hair follicle is the BA. Nestin-expressing DP cells were found only in early and middle anagen. In contrast, the BA contained nestin-expressing stem cells throughout the hair cycle and to a greater extent than the DP. Both nestin-expressing DP and BA stem cells differentiated into neuronal and glial cells after

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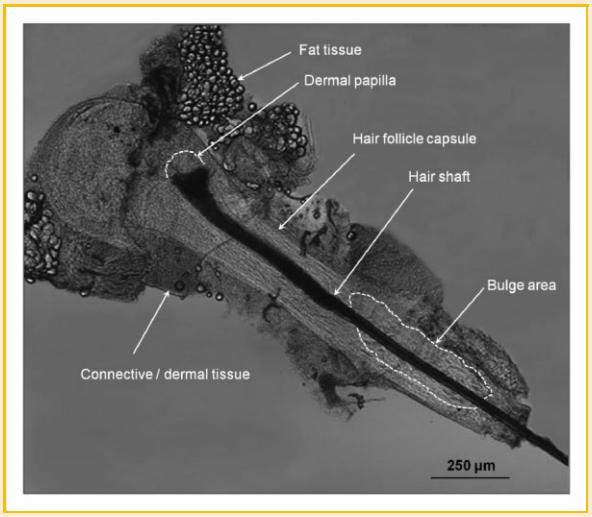

Fig. 1. Transmission image of isolated single vibrissae follicle from the whisker pad of transgenic mice. The hair shaft is surrounded by a capsule. Fat, connective, and dermal tissues are found around the hair follicle capsule. Dermal papilla and bulge area are depicted by white dotted lines.

transplantation to the injured spinal cord and enhanced injury repair and locomotor recovery, with the BA being the greater and more constant source of the stem cells [Liu et al., 2011].

Nestin-expressing stem cells have been found as well in the upper outer root sheath [Li et al., 2003] and in the inner structure of the outer root sheath [Hoang et al., 2009]. It was hypothesized that nestin-expressing stem cells of the bulge participate in the generation of the hair follicle during anagen. The bulge may also contain a population of putative keratinocyte stem cells which gives rise not only to hair follicles, but also to the epidermis [Lavker and Sun, 2000]. Melanocyte stem cells may also reside in the bulge of the hair follicle [Lin and Fisher, 2007]. Differentiated melanocytes may migrate from the bulge to the bulb to export pigments to hair-producing keratinocytes. Thymosin β4-expressing cells emanating from the bulge, and migrating downward, may give rise to a subpopulation of matrix cells that subsequently generate the hair shaft [Philp et al., 2004].

The present study, using time-lapse, 2-photon confocal imaging of whiskers from ND-GFP mice, suggests that the BA is the origin of nestin-expressing stem cells of the hair follicle and possibly of the skin itself.

MATERIALS AND METHODS

ANIMALS

Transgenic mice, with GFP expression driven by the nestin regulatory element (nestin-driven GFP [ND-GFP]) [Li et al., 2003; Mignone et al., 2004], at different ages [4 weeks up to 4 months], were used to image nestin-expressing stem cells of the hair follicle and the skin. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

ISOLATION OF VIBRISSA FOLLICLES (WHISKERS) FROM ND-GFP TRANSGENIC MICE

The mice were anesthetized with a ketamine solution. Whisker pads ($2 \times 2\,mm^2$), from ND-GFP transgenic mice, were sterilized with a 70% isopropyl alcohol and excised under sterile conditions. Using a binocular microscope (MZ6, Leica), the vibrissa pad was dissected to single vibrissae follicles with fine needles (Fig. 1). The isolated vibrissae follicles were washed in PBS three times and incubated in culture medium at 37°C, 5% CO_2, 95% humidity. Vibrissa hair follicles, with and without hair follicle capsules, were used for imaging (please see below).

CELL CULTURE AND GROWTH MEDIUM

For in vitro culture, the rigid capsules from the follicles were removed. The BA and DP areas were separated with fine needles and incubated in DMEM-F12 medium containing B-12, N2, 1% penicillin/streptomycin (Gibco/BRL) on Gelfoam® (Pharmasia & Upjohn Co., Kalamazoo, MI) at 37°C, 5% CO_2, 95% humidity. The medium was changed every other day.

CONFOCAL 2-PHOTON MICROSCOPY

Confocal 2-photon microscopy (Fluoview FV1000, Olympus Corp., Tokyo, Japan) was used for two- (*x,y*) and three-dimensional (3D, *x,y,z*) high-resolution imaging of vibrissa follicles from the ND-GFP transgenic mice. A cw semiconductor laser at 473 nm for GFP excitation and a tunable Mai Tai HP femtosecond laser emitting at 700–1,020 nm (Newport-Spectra Physics, Irvine, CA) were used for deep tissue imaging of autofluorescence and GFP. Fluorescence images were obtained using the 20×/0.50 UPlan FLN and 40×/1.3 Oil Olympus UPLAN FLN objectives.

HISTOLOGY

Skin and whisker pads of ND-GFP transgenic mice were excised under anesthesia. Tissues were embedded in tissue-freezing medium (Triangle Biomedical Science, Durham, NC) and frozen in liquid nitrogen for 10 min and at −80°C overnight. Frozen sections, with 7 μm thickness, were prepared with a Leica CM1850 cryostat. The sections were air-dried for 5–10 min and stained with hemotoxylin–eosin. The samples were imaged with an Olympus model IX71 microscope with a CCD camera (MACROFIRE, Optonics).

RESULTS

Single whisker pads, isolated from ND-GFP transgenic mice, contained hair follicles in different stages of the hair cycle. The BA of the hair follicles contained variable numbers of nestin-expressing stem cells during anagen, catagen, and telogen. The DP had nestin-expressing stem cells in anagen with similar morphological features as nestin-GFP expressing cells in the BA. Figure 2 demonstrates typical nestin-GFP expressing stem cells of the hair follicle BA. It was found that the cells have a round/oval shaped body with a typical diameter of 7 μm and with elongated extrusions.

Round/oval shaped nestin-expressing stem cells were also observed outside of the BA. They migrated towards the DP along

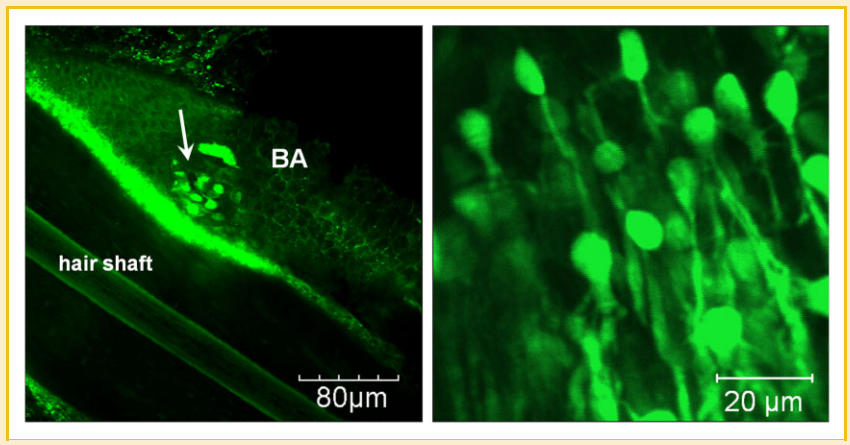

Fig. 2. Typical nestin-GFP expressing stem cells within the hair follicle bulge (white arrow). The cells have an oval-shaped body with a typical size of 7 μm and dendritic-like arms. Images were obtained from fresh isolated hair follicles by confocal 3D optical slicing.

the hair shaft (Fig. 3). Cell migration was monitored by 3D time-lapse confocal 2-photon imaging for several days. Some of the nestin-expressing stem cells moved into the DP. Cells moved in a non-synchronized way at different speeds. We observed movement of 5–40 μm over 2 days at velocities of 100–800 nm per hour, respectively (Fig. 3).

3D optical sectioning was also performed in excised skin containing hair follicles. Single nestin-expressing stem cells were observed in the dermis as well as the epidermis and around small newly-growing hairs. These observations suggest that the nestin-expressing stem cells migrate to support regeneration of the skin as well as new hair follicle generation (Fig. 4).

When the skin was wounded (3 days after biopsy) clusters of nestin-expressing stem cells were found outside the bulge moving toward the epidermis. Figure 5 shows one optical section with three clusters of the stem cells (A,B,C). Using optical zoom, cluster A was observed to have the highest concentration of nestin-expressing stem cells, containing 16 cells within the field of view ($120 \times 120\ \mu m^2$) and approximately 1,100 cells/mm^2. The cells in the clusters had exactly the same morphology as the stem cells in the BA, indicating the stem cells moved from the BA toward the epidermis.

DISCUSSION

Nestin-expressing stem cells were previously found both in the BA and DP of the mouse whisker follicle [Liu et al., 2011]. However, nestin-expressing BA cells and DP cells co-existed in the mouse vibrissal follicle only during part of the hair follicle cycle. Nestin-expressing stem cells were present in the DP only in the early and middle anagen stage, independent of age, while the BA contained the stem cells in all phases of the hair cycle [Liu et al., 2011].

Skin-derived precursor cells (SKPs) were shown to be multipotent neural crest-related stem cells that grow as self-renewing spheres and are capable of generating neurons and myelinating glial cells.

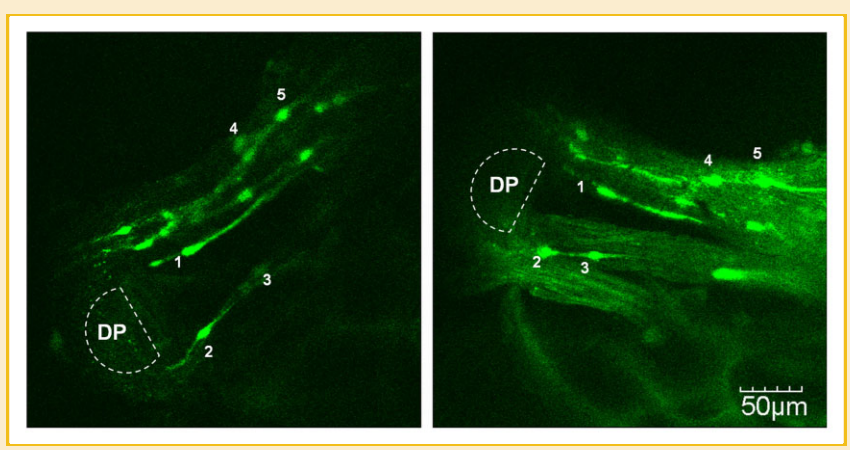

Fig. 3. A single optical plane image out of 3D optical stack images, taken during time-lapse imaging. Nestin-expressing stem cells moved out from the bulge area in the direction of the dermal papilla (DP). The left image is at time 0 and the right image is after 2 days. Some cells moved approximately 40 μm in the direction of the dermal papilla.

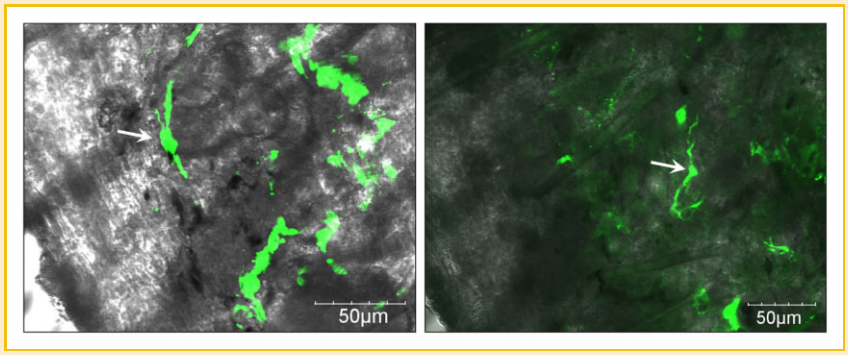

Fig. 4. Nestin-expressing stem cells of the hair follicles were found in the epidermis and dermis at different depths. Optical en-face sections are shown, demonstrating the presence of nestin-GFP expressing stem cells. Arrows: elongated nestin-GFP-expressing stem cells with round/oval bodies.

Hunt et al. [2008] observed that the DP of the rodent vibrissal follicle is 1,000-fold enriched for sphere-forming neural crest-derived cells compared with whole facial skin. Biernaskie et al. [2007] used SKPs to repair the contused rat spinal cord. SKPs reduced the size of the contusion cavity, myelinated endogenous host axons and recruited endogenous Schwann cells into the injured cord. McKenzie et al. [2006] showed that SKPs could participate in nerve repair. Our present and previous study [Liu et al., 2011] suggest the origin of SKPs is the BA nestin-expressing stem cells.

Cells from the BA of the mouse and human express nestin and can differentiate into many cell types, including neurons and glial cells [Li et al., 2003; Sieber-Blum et al., 2004; Amoh et al., 2005a,b; Yu et al., 2006, 2010] and effect nerve [Amoh et al., 2005b] and spinal cord [Amoh et al., 2008] repair in mouse models. Yu et al. [2006, 2010] demonstrated that nestin-expressing stem cells, with neural crest characteristics from the BA of cultured human hair follicles, can form spheroid structures and differentiate into neurons and other cell types, similar to the mouse nestin-expressing stem cells.

In a comparative study, we previously showed that nestin-expressing BA cells and DP cells differentiated into neuronal cells which appeared to accelerate spinal cord injury repair. Nestin-expressing pluripotent stem cells from both regions had similar effects on locomotor recovery. The greater and more constant expression of the nestin-expressing stem cells in the BA than the DP suggested the BA is be the major source of the pluripotent stem cells

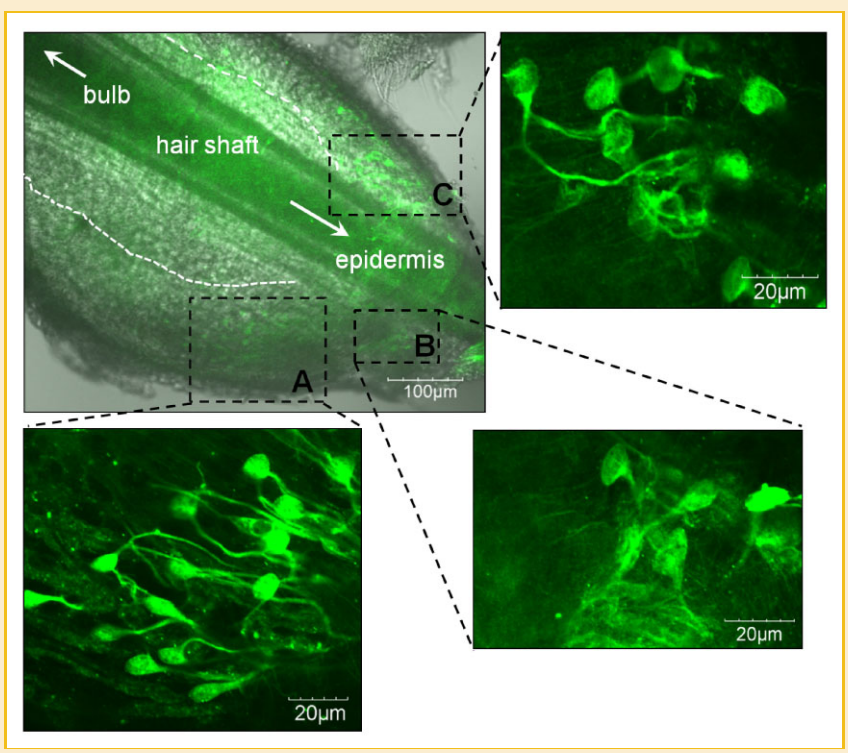

Fig. 5. In response to skin wounding, nestin-GFP-expressing stem cells were found outside the bulge. The bulge area is depicted by white dotted lines.

of the hair follicle [Liu et al., 2011]. The present study suggests the BA is the origin of the DP nestin-expressing stem cells (SKPs).

In conclusion, the present study indicates that the nestin-expressing cells from the BA of the hair follicle are the origin of the nestin-expressing stem cells of the hair follicle. The nestin-expressing stem cells were observed migrating toward the DP and also the epidermis where they may take part in new hair follicle generation as well as skin regeneration. For example, we observed the nestin-expressing stem cells migrating from the BA to wounded skin, which suggests the participation of the nestin-expressing cells in the wound-healing processes.

The nestin-expressing cells moved in a non-synchronized way with different speeds along the hair follicle. We observed movement of 5–40 μm during 2 days at velocities of 100–800 nm per hour (Fig. 4). The velocities may be related with in vitro culture conditions of the whiskers which are different from their natural environment.

The data presented in this report thus suggest that the BA is the origin of the nestin-expressing stem cells. The results of the present study have profound implications regarding pluripotent stem cells of the hair follicle and the skin itself and their application to regenerative medicine.

REFERENCES

Amoh Y, Li L, Katsuoka K, Penman S, Hoffman RM. 2005a. Multipotent nestin-positive, keratin-negative hair-follicle-bulge stem cells can form neurons. Proc Natl Acad Sci USA 102:5530–5534.

Amoh Y, Li L, Campillo R, Kawahara K, Katsuoka K, Penman S, Hoffman RM. 2005b. Implanted hair follicle stem cells form Schwann cells that support repair of severed peripheral nerves. Proc Natl Acad Sci USA 102:17734–17738.

Amoh Y, Li L, Katsuoka K, Hoffman RM. 2008. Multipotent hair follicle stem cells promote repair of spinal cord injury and recovery of walking function. Cell Cycle 7:1865–1869.

Amoh Y, Kanoh M, Niiyama S, Kawahara K, Satoh Y, Katsuoka K, Hoffman RM. 2009. Human and mouse hair follicles contain both multipotent and monopotent stem cells. Cell Cycle 8:176–177.

Biernaskie J, Sparling JS, Liu J, Shannon CP, Plemel JR, Xie Y, Miller FD, Tetzlaff W. 2007. Skin-derived precursors generate myelinating Schwann cells that promote remyelination and functional recovery after contusion spinal cord injury. J Neurosci 27:9545–9559.

Biernaskie J, Paris M, Morozova O, Fagan BM, Marra M, Pevny L, Miller FD. 2009. SKPs derive from hair follicle precursors and exhibit properties of adult dermal stem cells. Cell Stem Cell 5:610–623.

Hardy MH. 1992. The secret life of the hair follicle. Trends Genet 8: 55–61.

Hoang MP, Keady M, Mahalingam M. 2009. Stem cell markers (cytokeratin 15, CD34, and nestin) in primary scarring and nonscarring alopecia. Br J Dermatol 160:609–615.

Hoffman RM. 2000. The hair follicle as a gene therapy target. Nat Biotechnol 18:20–21.

Hoffman RM. 2006. The pluripotency of hair follicle stem cells. Cell Cycle 5:232–233.

Hunt DP, Morris PN, Sterling J, Anderson J, Joannides A, Jahoda C, Compston A, Chandran S. 2008. A highly enriched niche of precursor cells with neuronal and glial potential within the hair follicle dermal papilla of adult skin. Stem Cells 26:163–172.

Lavker RM, Sun TT. 2000. Epidermal stem cells: Properties, markers, and location. Proc Natl Acad Sci USA 97:13473–13475.

Li L, Margolis LB, Hoffman RM. 1991. Skin toxicity determined in vitro by three-dimensional, native-state histoculture. Proc Natl Acad Sci USA 88:1908–1912.

Li L, Mignone J, Yang M, Matic M, Penman S, Enikolopov G, Hoffman RM. 2003. Nestin expression in hair follicle sheath progenitor cells. Proc Natl Acad Sci USA 100:9958–9961.

Lin JY, Fisher DE. 2007. Melanocyte biology and skin pigmentation. Nature 445:843–850.

Liu F, Uchugonova A, Kimura H, Zhang C, Zhao M, Zhang L, Koenig K, Duong J, Aki R, Saito N, Mii S, Amoh Y, Katsuoka K, Hoffman RM. 2011. The bulge area is the major hair follicle source of nestin-expressing pluripotent stem cells which can repair the spinal cord compared to the dermal papilla. Cell Cycle 10:830–839.

McKenzie IA, Biernaskie J, Toma JG, Midha R, Miller FD. 2006. Skin-derived precursors generate myelinating Schwann cells for the injured and dysmyelinated nervous system. J Neurosci 26:6651–6660.

Mignone JL, Kukekov V, Chiang AS, Steindler D, Enikolopov G. 2004. Neural stem and progenitor cells in nestin-GFP transgenic mice. J Comp Neurol 469:311–324.

Paus R, Cotsarelis GN. 1999. The biology of hair follicles. Engl J Med 341: 491–497.

Philp D, Nguyen M, Scheremeta B, St-Surin S, Villa AM, Orgel A, Kleinman HK, Elkin M. 2004. Thymosin beta4 increases hair growth by activation of hair follicle stem cells. FASEB J 18:385–387.

Sieber-Blum M, Grim M, Hu YF, Szeder V. 2004. Pluripotent neural crest stem cells in the adult hair follicle. Dev Dyn 231:258–269.

Sieber-Blum M, Schnell L, Grim M, Hu YF, Schneider R, Schwab ME. 2006. Characterization of epidermal neural crest stem cell (EPI-NCSC) grafts in the lesioned spinal cord. Mol Cell Neurosci 32:67–81.

Yu H, Fang D, Kumar SM, Li L, Nguyen TK, Acs G, Herlyn M, Xu X. 2006. Isolation of a novel population of multipotent adult stem cells from human hair follicles. Am J Pathol 168:1879–1888.

Yu H, Kumar SM, Kossenkov AV, Showe L, Xu X. 2010. Stem cells with neural crest characteristics derived from the bulge region of cultured human hair follicles. J Invest Dermatol 130:1227–1236.