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# A dermal niche for multipotent adult skin-derived precursor cells

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**A fundamental question in stem cell research is whether cultured multipotent adult stem cells represent endogenous multipotent precursor cells. Here we address this question, focusing on SKPs, a cultured adult stem cell from the dermis that generates both neural and mesodermal progeny. We show that SKPs derive from endogenous adult dermal precursors that exhibit properties similar to embryonic neural-crest stem cells. We demonstrate that these endogenous SKPs can first be isolated from skin during embryogenesis and that they persist into adulthood, with a niche in the papillae of hair and whisker follicles. Furthermore, lineage analysis indicates that both hair and whisker follicle dermal papillae contain neural-crest-derived cells, and that SKPs from the whisker pad are of neural-crest origin. We propose that SKPs represent an endogenous embryonic precursor cell that arises in peripheral tissues such as skin during development and maintains multipotency into adulthood.**

Although adult mammalian stem cells were previously thought to differentiate solely into cells of their tissue of origin, a number of recent reports have identified cultured adult stem cells that show a surprisingly diverse differentiation repertoire[1]. Although some reported cases of multipotency are due to unanticipated cellular fusion events[2–4], compelling evidence still exists for the multipotency of a number of cultured adult stem cell populations. For example, blastocyst injection studies show that both multipotent adult progenitor cells (MAPC) bone marrow cells[5] and neural stem cells from the central nervous system (CNS)[6] contribute to many different developing tissues. One caveat to these studies is that multipotency was demonstrated only after these stem cells were expanded in culture, raising the possibility that it was a consequence of culture-induced de-differentiation and/or reprogramming[1].

We have previously described a multipotent precursor cell population from adult mammalian dermis[7]. These cells — termed SKPs, for skin-derived precursors — were isolated and expanded from rodent and human skin and differentiated into both neural and mesodermal progeny, including cell types never found in skin, such as neurons[7,8]. One endogenous embryonic stem cell population that contributes to dermis and has a similar broad differentiation potential is neural-crest stem cells (NCSCs)[9]. We therefore proposed that SKPs represent a multipotent neural-crest-like precursor that arises in embryonic mammalian tissues, and is maintained into adulthood. Here we provide evidence supporting this hypothesis and identify a dermal niche for these precursors.

## RESULTS

### SKPs share characteristics with, and have multipotentiality similar to, embryonic NCSCs

To characterize the origin of SKPs, we first compared them to stem cell populations that generate neural and/or mesodermal progeny. Because we previously demonstrated that SKPs are distinct from mesenchymal stem cells[7], we focused on CNS neural stem cells and embryonic NCSCs. Immunocytochemical comparison of SKPs and embryonic CNS neurospheres revealed that the two populations were distinct: both expressed nestin and vimentin, but only SKPs expressed fibronectin and the precursor cell marker Sca-1 (refs 10,11), whereas only neurospheres contained cells expressing p75NTR (see Supplementary Information, Fig. S1a). We then analysed SKPs for expression of genes associated with embryonic NCSCs. RT–PCR analysis (Fig. 1a) showed that SKPs expressed the transcription factor genes *slug*[12], *snail*[13], *twist*[14], *Pax3* (ref. 15) and *Sox9* (ref. 16), expressed in various populations of embryonic NCSCs[17] *in vivo*. Except for *Sox9*, all of these genes were expressed at lower or undetectable levels in embryonic CNS neurospheres (Fig. 1a). SKPs also express the transcription factors Dermo-1 (ref. 18) and SHOX2 (ref. 19; see Supplementary Information, Fig. S1b), which are expressed in embryonic dermis and craniofacial regions. A similar pattern of gene expression was observed in embryonic, neonatal and adult SKPs passaged from 1–15 times. Thus, SKPs express genes characteristic of embryonic NCSCs and/or their embryonic derivatives.

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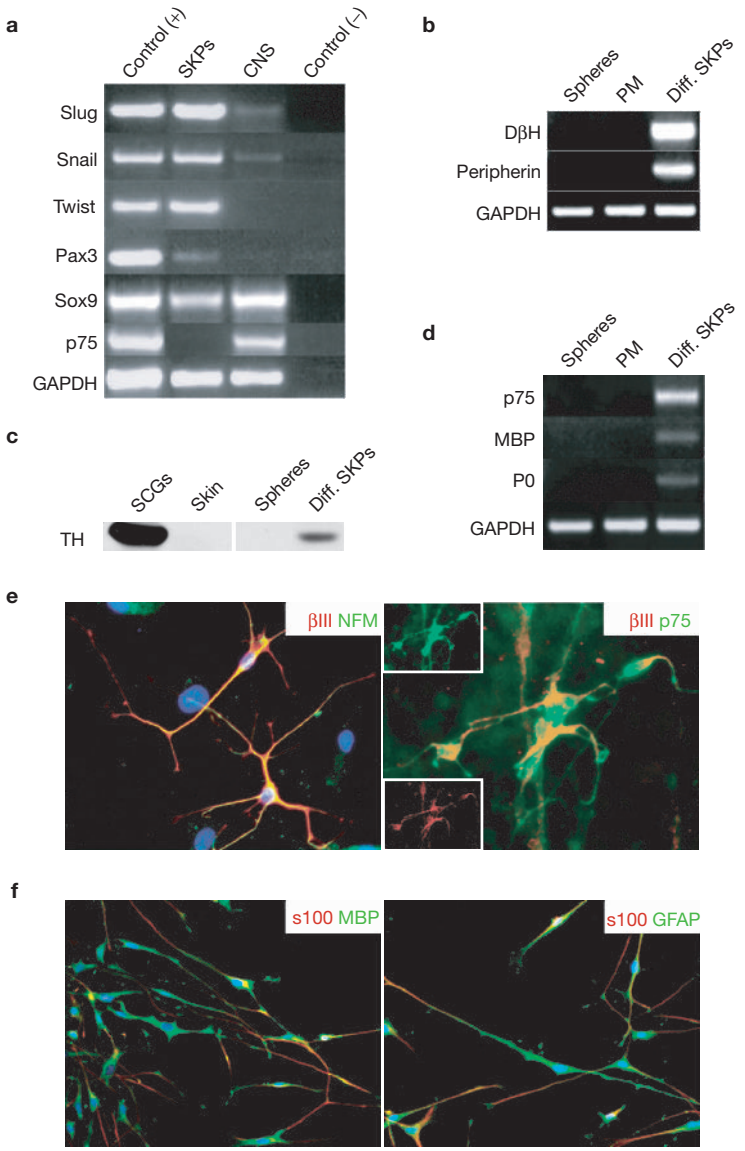


**Figure 1** SKPs express markers of embryonic neural crest and differentiate into peripheral neurons and Schwann cells. (**a**) RT–PCR for genes involved in embryonic neural crest determination and migration in total RNA isolated from SKP spheres compared with embryonic telencephalic neurospheres (CNS), both of which were cultured in the presence of FGF2 and EGF. RNA from an E12 neural tube functioned as a positive control (+), RT–PCR for GAPDH was used as a loading control and reaction with no input nucleic acid was run in the last lane as a negative control (–). (**b**) RT–PCR for two markers of peripheral catecholaminergic neurons, dopamine-β-hydroxylase (DβH) and peripherin, in murine SKPs differentiated for one week in 10% serum (Diff. SKPs). SKP spheres and dissociated SKPs plated in proliferation medium (PM) do not express these mRNAs. (**c**) Western blot analysis for tyrosine hydroxylase (TH) in murine SKP spheres compared with SKPs differentiated for 14 days in 10% serum supplemented with neurotrophins. The positive control was protein isolated from cultured sympathetic neurons of the superior cervical ganglion (SCGs). (**d**) RT–PCR for three markers of peripheral Schwann cells, p75NTR, myelin basic protein (MBP) and P0 peripheral myelin protein (P0), in total RNA from undifferentiated and differentiated rat SKPs. (**e**) Immunocytochemical analysis of differentiated murine SKPs for markers of peripheral neurons. Left, morphologically complex differentiated cells co-express the neuronal markers βIII-tubulin and NFM (yellow cells in the merged image). Right, differentiated cells co-express βIII-tubulin (red; bottom inset) and p75NTR (green; top inset), proteins expressed by virtually all peripheral neurons. (**f**) Immunocytochemical analysis of differentiated SKPs, showing that a subset of bipolar cells co-express: left, S100β (red) and MBP (green); or right, S100β (red) and GFAP (green). In panels **e** and **f**, the blue derives from Hoechst-stained nuclei.

We next tested whether SKPs differentiated into cell types that are exclusively neural-crest-derived during embryogenesis, such as peripheral catecholaminergic neurons and Schwann cells. For catecholaminergic neurons, SKPs were differentiated for one to three weeks under conditions used to differentiate embryonic NCSCs into peripheral neurons[20]. Immunocytochemistry, RT–PCR and western blot analysis revealed a sub-population of differentiated cells with neuronal morphology that co-expressed the pan-neuronal markers βIII-tubulin and neurofilament M (NFM; Fig. 1e), and proteins typical of peripheral neurons, including p75NTR (Fig. 1e), peripherin (Fig. 1b), NCAM (see Supplementary Information, Fig. S1c) and the catecholaminergic markers tyrosine hydroxylase and dopamine-β-hydroxylase (Fig. 1b,c; also see Supplementary Information, Fig. S1c,d). These neurons were generated by differentiated embryonic and neonatal SKPs, and by SKPs passaged from 1–20 times.

We previously reported that SKPs differentiate into bipolar cells co-expressing glial fibrillary acidic protein (GFAP) and CNPase, consistent with a Schwann-cell phenotype[7]. Further characterization demonstrated

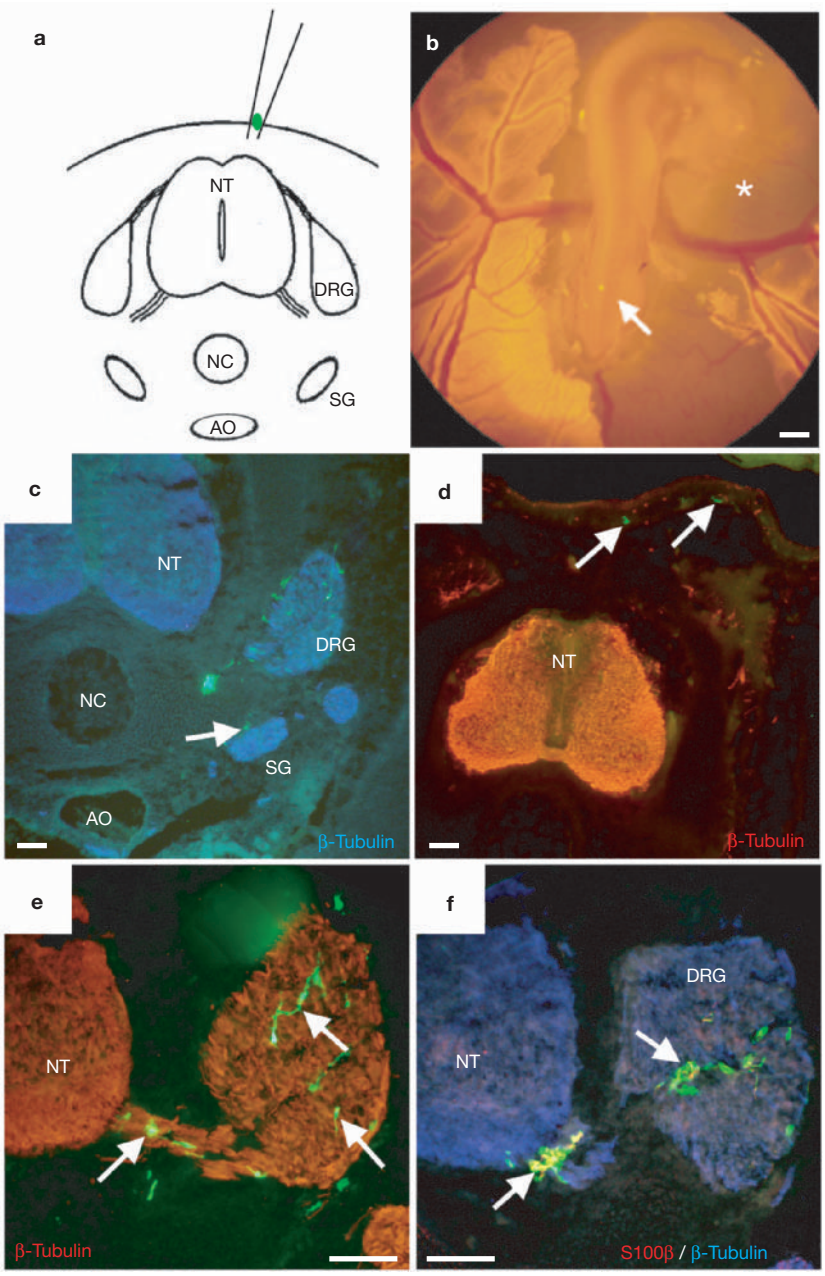


**Figure 2** SKPs migrate like neural crest cells when transplanted *in ovo*. (**a**) Schematic representation of a cross-section through a stage-30 chick embryo showing some of the major anatomical landmarks near, or associated with, the neural-crest migratory stream. (**b**) Photomicrograph of a single YFP-positive sphere (arrow) transplanted into a chick embryo *in ovo*. Asterisk indicates the head of the embryo. (**c**–**e**) Cross-sections of stage-30 chick embryos, immunolabeled for βIII-tubulin, show that the transplanted SKPs had migrated to the sympathetic ganglia (**c**; arrow), skin (**d**; arrows), spinal nerve and DRG (**e**; arrows). (**f**) Immunohistochemical analysis for βIII-tubulin (blue) and S100β (red) shows that many of the transplanted SKPs in both the spinal nerve and DRG (arrows) express S100β at stage 30. NT, neural tube; NC, notochord; DRG, dorsal root ganglia; SG, sympathetic ganglia; AO, aorta; and g, gut. Scale bars represent 100 μm.

that these cells also expressed S100β and p75NTR (Fig. 1d,f), and, when differentiated in forskolin to elevate intracellular cAMP, myelin basic protein (MBP) and P0 peripheral myelin protein (Fig. 1d,f), as reported for cultured Schwann cells[21].

To determine whether SKPs exhibited neural crest potential *in vivo* as well as *in vitro*, we generated SKPs from back skin of neonatal mice expressing yellow fluorescent protein from the actin promoter (actin–YFP)[22], and after one or no passages, transplanted single spheres of 200–250 cells into the chick neural crest migratory stream *in ovo* at Hamburger-and-Hamilton stage 18 (ref. 23) (Fig. 2a,b). Analysis three days later (H&H stage 30) revealed that about half of the transplanted cells had migrated into peripheral neural crest targets, whereas very few had migrated into the neural tube. Many YFP-positive cells were present in the spinal nerve and dorsal root ganglia, and some migrated to peripheral nerves or to the vicinity of the sympathetic ganglia (Fig. 2c,e). Interestingly, some YFP-positive cells were detected in the dermal layer of the skin (Fig. 2d). Of the SKPs in the DRG and spinal nerve, a sub-population expressed S100β, a marker for Schwann cells

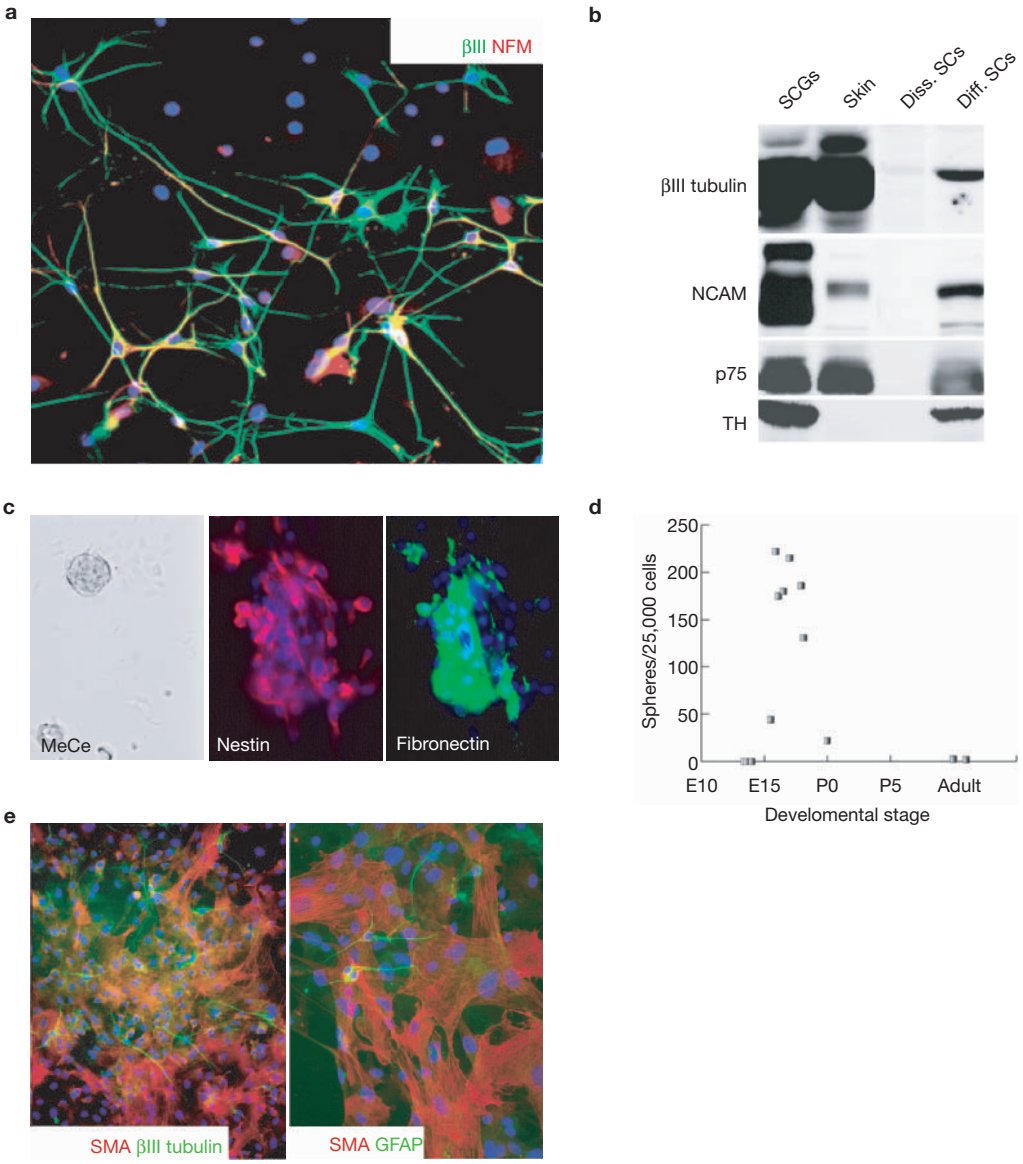


**Figure 3** Multipotent endogenous SKPs are abundant in skin during late embryogenesis and persist into adulthood. (**a**) Double-labelling of differentiated primary E18 murine skin cells with antibodies against βIII-tubulin (green) and NFM (red). Cells are yellow in the merged image. Note that more cells express βIII-tubulin than NFM, because these are early and late neuronal markers, respectively. (**b**) Western blot analysis for proteins expressed in peripheral neurons in E18 skin cells differentiated under conditions similar to those in **a** (Diff. SCs). For comparison, equal amounts of protein were analysed from lysates of whole skin at the same age (skin), dissociated E18 skin cells that were not differentiated (Diss. SCs) and from primary cultures of peripheral sympathetic neurons (SCGs). (**c**) Phase-contrast micrograph of primary SKP spheres grown immobilized in methylcellulose (left). The two other panels are fluorescent photomicrographs of a primary sphere grown in methylcellulose that was double-labelled with antibodies to nestin and fibronectin. (**d**) Quantification of the number of cells that give rise to primary SKP spheres in murine back skin isolated at various developmental ages ranging from E13 to adulthood. Cell numbers are expressed relative to a given number of primary skin cells. (**e**) Immunocytochemical analysis of primary murine SKP clones, one for βIII-tubulin (green) and smooth-muscle actin (SMA; red), and one for SMA (red) and the glial cell marker GFAP (green), demonstrating that these primary SKP clones can generate both neural and mesodermal progeny.

(Fig. 2f). Thus, transplanted SKPs migrated along neural crest migratory pathways into neural-crest-derived structures.

## SKPs arise during embryogenesis and are maintained into adulthood

These data suggested that SKPs are embryonic neural-crest-related precursors that arise in the dermis during embryogenesis and persist into adulthood. To test this hypothesis, we asked whether skin contained an endogenous precursor cell that could differentiate into neurons, a cell type never found in skin. Skin cells from embryonic day 18 (E18) or adult mice were dissociated and immediately differentiated under conditions that promote neuronal differentiation from embryonic NCSCs[20]. Immunocytochemistry (Fig. 3a; also see Supplementary Information, Fig. S2a) and western blot analysis (Fig. 3b) revealed that some differentiated primary skin cells exhibited neuronal morphology and co-expressed βIII-tubulin, NFM and p75NTR, a phenotype comparable to SKP-derived peripheral neurons. Moreover, these differentiated skin cells expressed tyrosine hydroxylase, a marker for peripheral

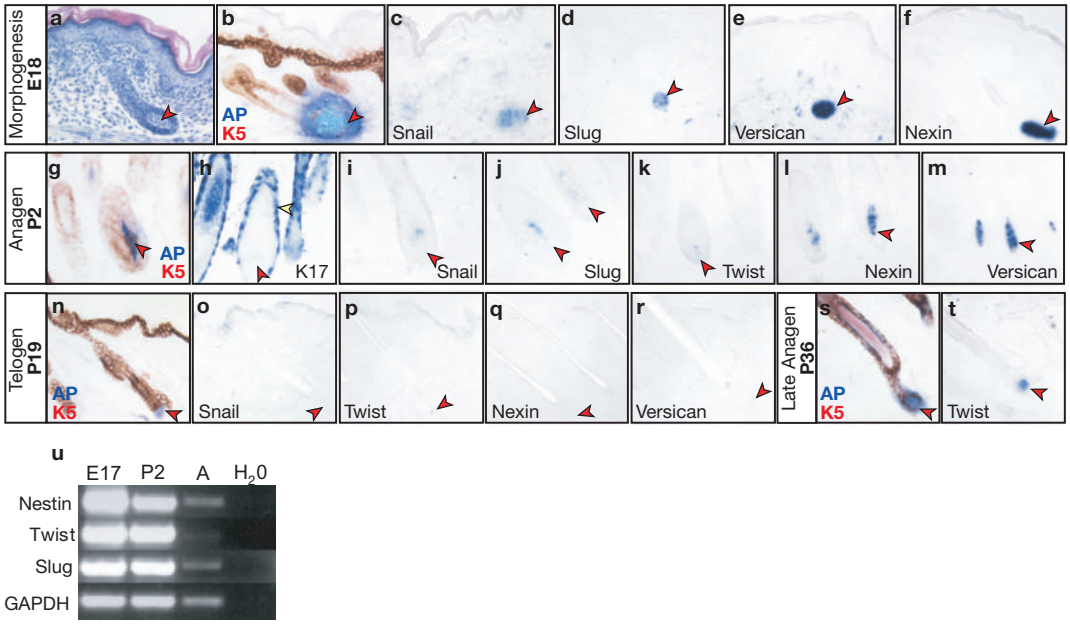


**Figure 4** Expression of SKP transcription factors is localized to the follicle papillae of dorsal hair follicles and is hair-cycle dependent. Red arrowheads indicate follicle papillae. (**a**–**f**) At E18.5, a dorsal hair follicle in late morphogenesis has a well-developed dermal papilla ensheathed by epidermal progenitor (matrix) cells (**a**). These follicle papillae express high levels of endogenous alkaline phosphatase (AP; **b**), as well as elevated versican (**e**) and nexin (**f**) expression. Overlapping localized expression of the SKP markers snail (**c**) and slug (**d**) is observed in follicle papillae. (**g**–**m**) In P2 postnatal skin, hair follicles continue to mature as they approach the first synchronized anagen phase. Expression of the SKP markers snail (**i**), slug (**j**) and twist (**k**) continues to localize to the follicle papillae, marked with high levels of alkaline phosphatase (**g**), nexin (**l**) and versican (**m**) expression, but do not overlap with Keratin 17 (K17; **h**), a marker for the outer root sheath (yellow arrowhead) and the bulge epidermal stem cell niche, or with Keratin 5 (K5; **g**, red signal), a basal epidermal layer marker. (**n**–**r**) Serial sections of P19 dorsal skin. At this age, hair follicles are in the first synchronized telogen phase and associated follicle papillae are small structures at the distal tip of resting follicles. Although these structures express low levels of alkaline phosphatase (**n**), telogen follicle papillae do not express the anagen markers nexin or versican (**q**, **r**), or the SKP markers snail (**o**) or slug (data not shown), although very low levels of twist (**p**) are detected. (**s**, **t**) Serial sections of P36 skin. In the next wave of anagen, SKP markers are re-expressed with anagen markers in the follicle papillae of growing follicles. (**u**) RT–PCR for SKP markers nestin, twist, and slug in developing (late gestation E17 and early postnatal P2) and adult skin.

catecholaminergic neurons that is not detectable in embryonic skin (Fig. 3b). The number of neurons generated in these experiments was much higher from embryonic than adult skin.

We then asked when SKPs could first be isolated from skin. Initially, we confirmed that at low cell densities, one primary SKP-forming cell gave rise to a single, clonal SKP sphere. Three types of evidence supported this conclusion: first, at low densities (25,000–100,000 cells $ml^{-1}$ with E18 skin cells), increasing the primary cell number increased the number of SKP spheres linearly (see Supplementary Information, Fig. S2b); second, when YFP-positive and unlabelled skin cells were mixed, the YFP-positive and negative spheres were mutually exclusive (see Supplementary Information, Fig. 2f); third, when immobilized as individual cells in methylcellulose, primary skin cells generated fibroblast growth factor 2 (FGF2)- and epidermal growth factor (EGF)-dependent spheres (Fig. 3c) expressing the SKP markers nestin, fibronectin (Fig. 3c) and Sca-1 (data not shown). When re-plated in methylcellulose, single cells from primary spheres generated secondary spheres expressing the same SKP markers, indicative of self-renewal. Importantly, similar numbers of SKP spheres were obtained using methylcellulose and low-density liquid cultures, confirming the clonality of spheres obtained in liquid cultures.

Using these assays, we quantified the number of SKP-like precursors in skin (Fig. 3d; also see Supplementary Information, Fig. S2c), and demonstrated that SKP spheres were never generated from mouse skin before E14. From E15 to E19 there was a burst of SKP-forming cells in skin which peaked at 0.5–1% of skin cells, and then decreased approximately tenfold by postnatal day 0. Although the frequency of sphere-forming cells seemingly decreased further into adulthood, their actual number was similar in back skin at postnatal day 0 and in adulthood (see Supplementary Information, Fig. S2c).

We then differentiated clonally derived primary embryonic spheres (see Supplementary Information, Fig. 2d) and determined whether they, like passaged SKPs, differentiated into neural and mesodermal progeny. Double-label immunocytochemistry for SMA and βIII-tubulin revealed that all of these clones generated SMA-positive cells, and 75% also generated βIII-tubulin-positive, SMA-negative neurons (Fig. 3e). Analysis for SMA and GFAP revealed that, of these clones, 100% generated SMA-positive cells, and 15% also generated GFAP-positive, SMA-negative glial cells (Fig. 3e). Similar results were obtained with primary spheres from methylcellulose cultures (see Supplementary Information, Fig. S2e). Thus, after E14.5, embryonic mouse skin contains precursor cells that can generate neurons, and embryonic primary clonal spheres exhibit a differentiation potential similar to passaged SKPs.

We also performed two sets of experiments that showed that SKPs did not arise by transdifferentiation or de-differentiation of Schwann cells or melanoblasts, both neural-crest-derived cell types that are abundant in skin. First, analysis of primary, unpassaged SKP spheres from neonatal skin revealed that they did not contain cells expressing the melanoblast/melanocyte markers trp1, c-kit or dct (see Supplementary Information, Fig. S2g)[24,25], or the Schwann-cell markers MBP, P0, p75NTR (Fig. 1d) or Sox10 (data not shown)[26]. Second, we have recently found that when primary mouse skin cells are FACs-

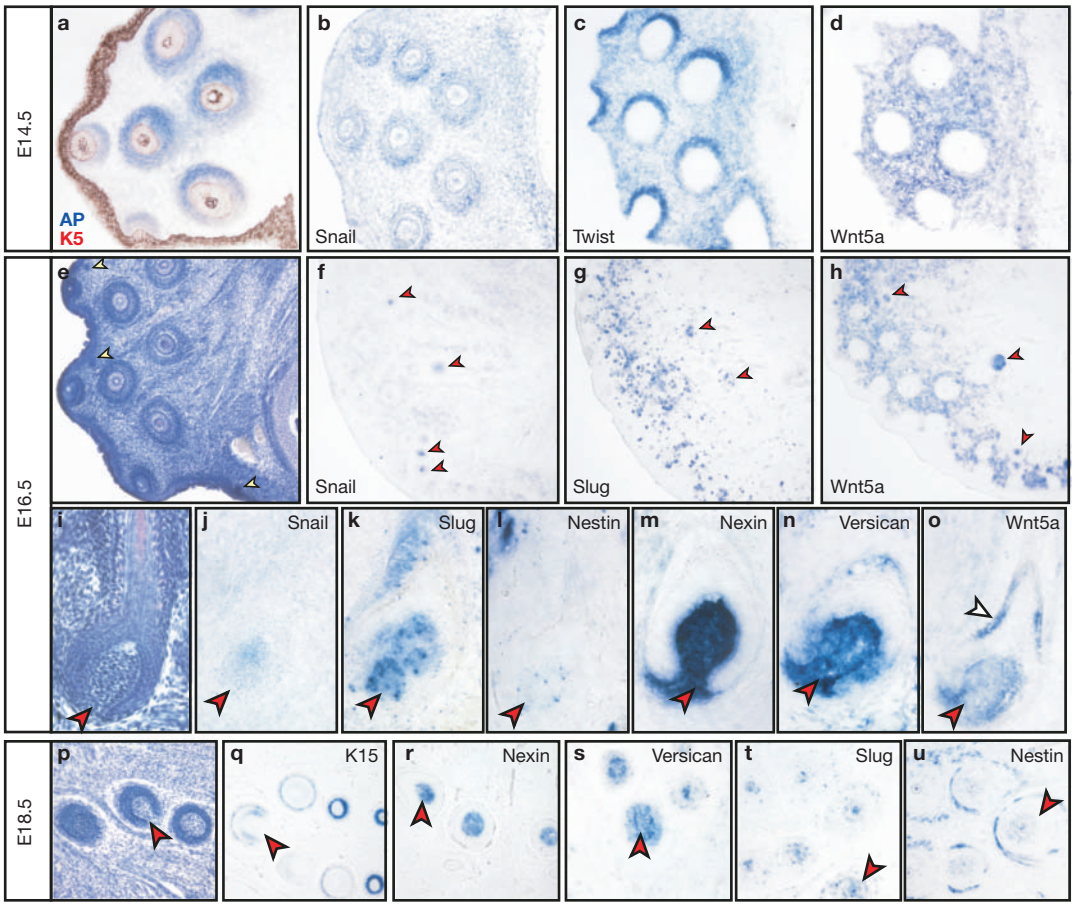


**Figure 5** Neural-crest-derived facial dermis dynamically expresses SKP markers, which become progressively restricted to follicle papillae of vibrissae and hair follicles. Red arrowheads indicate follicle papillae. (**a**–**d**) Serial sections of E14.5 whisker pad. Concentric rings characteristic of cross-sectioned developing vibrissae follicles illustrate epidermal downgrowths (**a**; Keratin5, K5; red) and associated dermal condensates (alkaline phosphatase, AP; blue). These dermal condensates express higher levels of SKP markers snail (**b**), slug (data not shown), and twist (**c**) than surrounding dermis, as well as anagen papilla markers nexin and versican (data not shown). Wnt5a expression, another marker of papilla development, is expressed throughout the vibrissae pad dermis (**d**). (**e**–**o**) Vibrissae pad cross-section at low magnification (**e**–**h**, serial sections) and high magnification photomicrographs of adjacent sections through a single vibrissae follicle longitudinally sectioned from an E16.5 embryo (**i**–**o**, serial sections). (**e**) Interspersed with deeply-penetrating concentric rings of vibrissae follicles which have engulfed associated follicle papillae, are smaller hair follicles (yellow arrowheads). Localized expression of SKP markers snail (**f**, **j**), slug (**g**, **k**), nestin (**l**), and twist (data not shown) is detected in papillae of vibrissae and hair follicles, along with high levels of the papilla markers nexin (**m**) and versican (**n**). In addition to follicle papilla expression (**h**, **o**), Wnt5a is also diffusely detected in the upper dermis and in the outer root and inner root sheath (**o**; white arrowhead) of vibrissae. (**p**–**u**) Serial sections of E18.5 whisker pad, where **p**–**s** are serial sections, and **t**–**u** are serial sections. By E18.5, vibrissae papillae (**p**) continue to express high levels of nexin (**r**) and versican (**s**), but reduced expression of the SKP markers, slug (**t**) and nestin (**u**). The epidermal stem cell marker Keratin15 (ref. 47) is expressed strongly in the outer root sheath and at low levels in the matrix adjacent to the papillae, but not in the papillae.

sorted for Sca-1, all of the sphere-forming ability of SKPs is found within the Sca-1-positive population (K.M.S., D.R.K. and F.D.M., unpublished observation). However, immunocytochemical analysis of primary dissociated skin cells for Sca-1 and the melanoblast/melanocyte markers dct, c-kit and trp1, or the Schwann cell markers MBP, GFAP and CNPase (see Supplementary Information, Fig. S2h) demonstrated that neither melanoblast/melanocytes nor Schwann cells were Sca-1-positive.

### Dermal papillae of hair and whisker follicles are one niche for endogenous SKPs

As SKPs express a distinctive panel of embryonic transcription factors, we reasoned that it might be possible to use them to identify a niche for endogenous SKPs *in vivo*. To test this, we first confirmed that mouse skin contained cells expressing nestin, snail and twist from embryogenesis to adulthood (Fig. 4u). We then performed *in situ* hybridization on back skin sections from E18 to adulthood postnatal day 36 (P36) (Fig. 4a–t). This study revealed that *slug*, *snail* and *twist* mRNAs were all expressed in the hair follicle papilla, a dermally derived structure located at the base of the follicle (Fig. 4a) that is thought to contain multipotent precursor cells[27,28]. At E18, hair follicle papillae were characterized by robust alkaline phosphatase activity[29], expression of versican, a cell surface proteoglycan[30,31], and nexin, an anagen-specific matrix-modifying factor[32] (Fig. 4a,b,e,f). Low-level expression of twist (data not shown), slug and snail (Fig. 4c,d) was also clearly detected in these developing structures. Two days postnatal, most of the maturing follicles were approaching the first synchronized anagen growth phase of the hair cycle, and low-level expression of snail, slug and twist could be detected in the

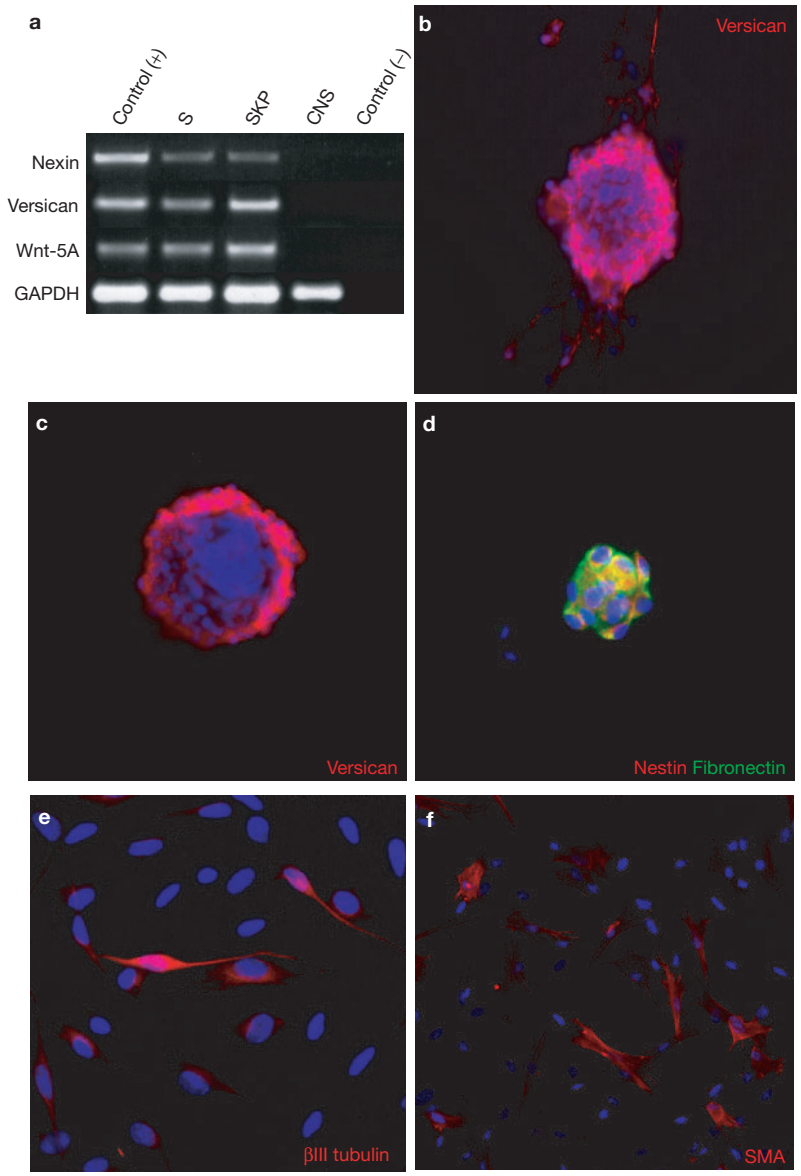


**Figure 6** Adult whisker papillae contain SKP-like cells. (**a**) RT–PCR for the follicle papilla markers nexin, versican and Wnt-5a in total RNA isolated from E12 embryos (+), skin (S), SKP spheres (SKP) or CNS neurospheres (CNS). Note that SKPs but not neurospheres express all of these markers. The negative control (–) was a reaction without added nucleic acid. (**b**) Immunocytochemical analysis for the follicle papilla marker versican in a SKP sphere. Note that most, if not all of the cells express versican. (**c**, **d**) Immunostaining of whisker papilla spheres for versican (**c**), or for nestin and fibronectin (**d**). (**e**, **f**) Fluorescence photomicrographs of whisker papilla spheres differentiated for three days and then immunostained for βIII-tubulin (**e**) or for SMA (**f**).

papillae (Fig.4g,i–k) in addition to alkaline phosphatase, nexin and versican (Fig. 4g,l,m). Expression of these markers did not overlap with the expression of keratin 17, a marker for the bulge epidermal stem cell niche and the outer root sheath[33] (Fig. 4h), or with keratin 5, a marker for the basal epidermis (Fig. 4g). By P19, hair follicles were in the first synchronous telogen rest phase, and their associated papillae were small structures at the distal tip of resting follicles (Fig. 4n). These structures still expressed alkaline phosphatase (Fig. 4n), but did not express the anagen markers nexin or versican (Fig. 4q,r), or the transcription factors snail (Fig. 4o) or slug (data not shown); very low levels of twist were still detected (Fig. 4p). At P36, during the next wave of anagen, twist (Fig. 4s,t), snail and slug (data not shown) were all re-expressed, along with nexin and versican (data not shown). Thus, the embryonic transcription factors slug, snail and twist are coordinately expressed in papillae during the anagen growth phase of the hair cycle.

To determine whether follicle papillae represented an endogenous niche for SKPs, as these data suggested, we examined the larger whisker vibrissal papillae, which are amenable to micro-dissection. At E14.5, when whisker follicles first form, the epidermal downgrowths are surrounded by dermal condensates that express alkaline phosphatase (Fig. 5a). Expression of snail and twist was widespread at this stage (consistent with the neural crest origin of the facial dermis), but was highest in these dermal condensates (Fig. 5a–c), as was expression of slug, versican, and nexin (data not shown). In contrast, *Wnt5a* mRNA, which is enriched in papillae at

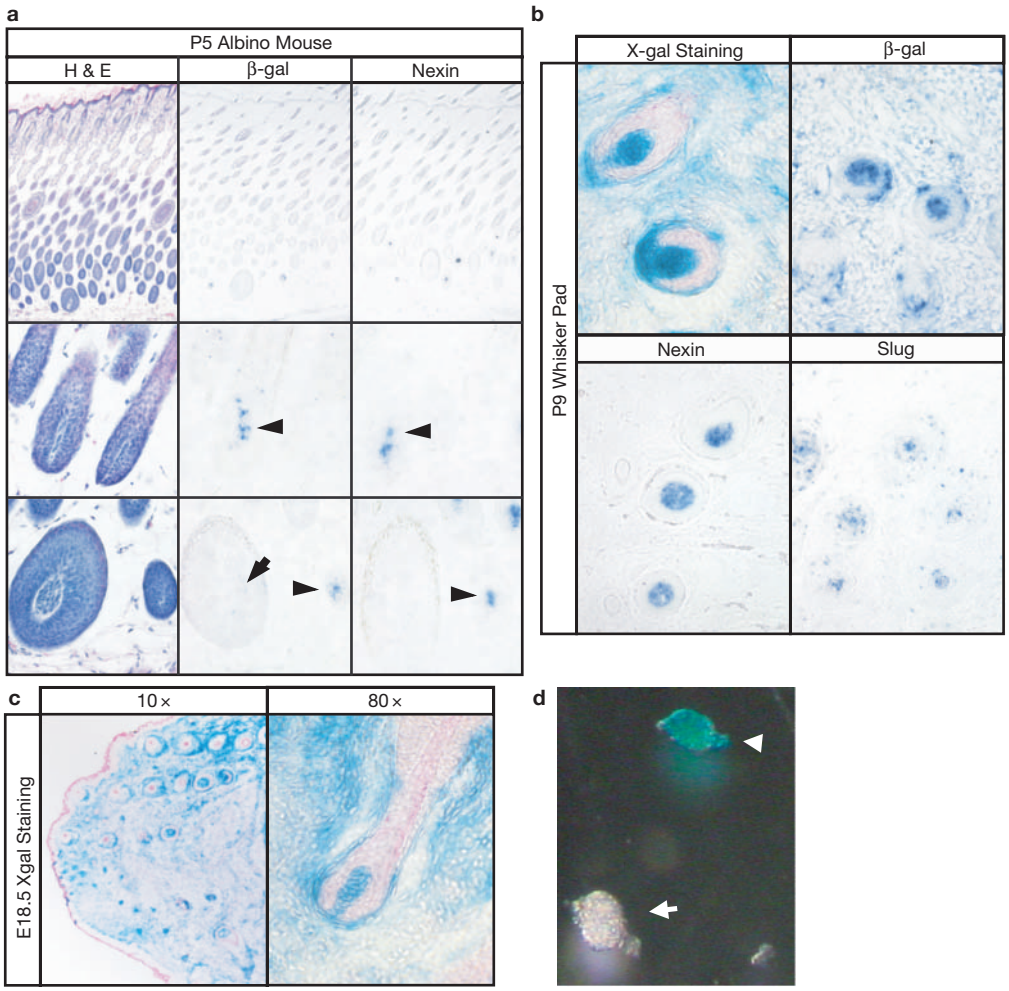


**Figure 7** Hair and whisker follicle papillae contain neural crest-derived cells. (**a**) Neural-crest-derived cells are found in hair follicle dermal papillae of Wnt1Cre;R26R mice. Overlapping expression of β-galactosidase (β-gal) and the dermal papilla marker nexin, in postnatal day 5 (P5) neonatal Wnt1Cre;Rosa26R dorsal skin of an albino mouse. The faint β-galactosidase-positive cells observed in the lower matrix (arrow) are most probably melanoblasts, whereas strong β-galactosidase-positive cells that co-express nexin in the adjacent serial sections are found in the follicle papillae (arrowheads), and potentially represent NCSC-derived cells that are the *in vivo* source of SKPs. In all cases, the left, middle and right panels are photomicrographs of adjacent serial sections, with the left panel being haematoxylin and eosin-stained (H & E), and the middle and right panels being hybridized with probes specific to β-galactosidase and nexin mRNAs, respectively. (**b**–**d**) Whisker papillae are of neural-crest origin. (**b**) Sections through the whisker pad of a P9 Wnt1Cre;R26R mouse showing cross-sections of vibrissae at the level of the papillae that were either stained for β-galactosidase activity using X-gal, or were analysed for expression of β-galactosidase, nexin and slug mRNAs by *in situ* hybridization. Note that all of these genes were expressed in the whisker papillae, and that many cells outside of the follicle in the dermis were also β-galactosidase positive, consistent with the neural-crest origin of facial dermis. (**c**) X-gal-stained sections (blue) of the whisker pad from an E18.5 Wnt1Cre;R26R mouse, and photomicrographs taken at different magnifications. Note that at both developmental stages, the whisker papillae are completely blue, indicating that most of the cells in these papillae are neural-crest-derived. (**d**) Dissected papillae from a Wnt1Cre;R26R mouse (arrow) and from its wild-type littermate (arrowhead) that were stained with X-gal to detect β-galactosidase activity.

later stages[34], was uniformly expressed in the dermis (Fig. 5d). By E16.5, the vibrissal papillae were distinct structures, and contained cells that expressed snail (Fig. 5f,j), slug (Fig. 5g,k), twist (data not shown), nexin (Fig. 5m), versican (Fig. 5n) and Wnt5a (Fig. 5h,o). At this stage, the papillae also contained nestin-expressing cells (Fig. 5l). At E18.5, vibrissal papillae continued to express slug, nexin and versican (Fig. 5r–t) in a pattern distinct from that of keratin 15 (Fig. 5q). The expression of slug, snail and twist persisted in the whisker papillae postnatally. Slug, snail and twist were also expressed in newly formed hair follicle papillae in the E16.5 whisker pad (data not shown), as seen in dorsal skin (Fig. 4).

Consistent with the idea that follicle papillae are an endogenous niche for SKPs, RT–PCR demonstrated that neonatal SKPs, but not CNS neurospheres, expressed the papilla markers nexin, versican and Wnt5a (Fig. 6a). Moreover, immunostaining confirmed that most neonatal SKP cells were versican-positive (Fig. 6b). We then obtained two lines of evidence indicating that whisker follicles contained SKP-like cells: first, immunostaining of dissociated, differentiated whisker cells revealed a sub-population of nestin- and βIII-tubulin-positive cells with neuronal morphology (data not shown); and second, YFP-positive adult mouse vibrissal cells generated SKP-like spheres when co-cultured with unlabelled E16 skin cells (see Supplementary Information, Fig. S2f).

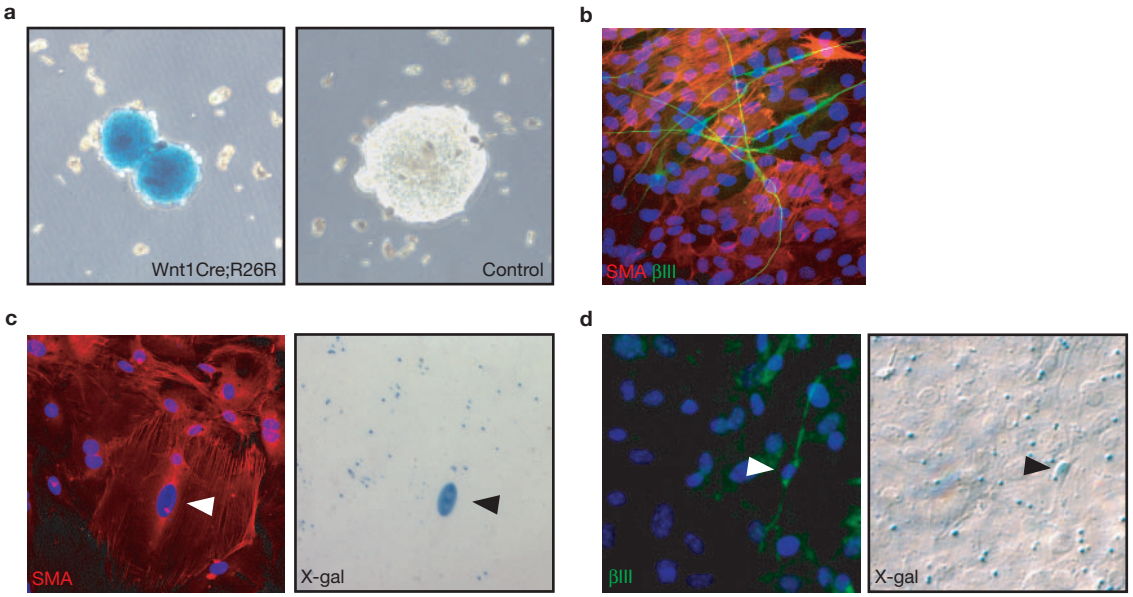


**Figure 8** SKPs derived from facial skin are of neural-crest origin. (**a**) X-gal-stained SKP spheres generated from the whisker pad skin of a litter of neonatal Wnt1Cre;R26R mice (left) or, as a control, from dorsal skin of a neonatal rat. Because the transgene is not penetrant in facial skin of all Wnt1Cre;R26R mice, whisker pads were initially stained with X-gal to assay transgene expression, and then SKPs were generated from the contralateral whisker pad of animals where penetrance was high. Note that all of the sphere cells were transgene positive. The spheres depicted were passaged twice. (**b**) Double-label immunocytochemistry for SMA and βIII-tubulin on a single isolated Wnt1Cre;R26R-positive whisker pad SKP sphere differentiated for two weeks. Note that, like dorsal skin SKPs, both SMA-positive smooth muscle cells and βIII-tubulin-positive neurons were generated. (**c**, **d**) Photomicrographs of transgene-positive isolated Wnt1Cre;R26R whisker pad spheres that were differentiated for two weeks, stained for X-gal, and then immunostained for either SMA or βIII-tubulin. In each pair, the left panel is the fluorescence photomicrograph, and the right a brightfield photomicrograph showing the same field. Arrows denote the same cell in both panels. Note that although the β-galactosidase transgene was downregulated in most cells during differentiation (all of the parent sphere cells were transgene positive), some smooth muscle cells and neurons still expressed the nuclear transgene.

We therefore reasoned that dissected vibrissal papillae (see Supplementary Information, Fig. S3a) may contain SKP-like cells. Initially, we confirmed that they did not contain melanoblasts/melanocytes, Schwann cells or βIII-tubulin-positive neurons, as indicated by the following evidence: first, dissected papillae contained Sca-1-positive cells, but not dct-positive cells (see Supplementary Information, Fig. S3b,c); and second, dissociated papillae cells did not express keratin 18 (a marker for Merkel cells[35]), CNPase or βIII-tubulin (see Supplementary Information, Fig. S3d,e). We then asked whether dissected papillae contained cells with SKPs-like potential. As seen for total vibrissal cells, differentiation of dissociated papilla cells generated a sub-population of nestin- and βIII-tubulin-positive cells with the morphology of immature neurons (see Supplementary Information, Fig. S3f). Moreover, when cultured in EGF and FGF-2, dissociated papilla cells generated spheres (see Supplementary Information, Fig. S3g) that expressed versican (Fig. 6c), nestin and fibronectin (Fig. 6d). Finally, differentiated papillae spheres generated cells that expressed βIII-tubulin or SMA with morphologies similar to the young neurons and smooth muscle cells produced by SKPs (Fig. 6e,f; also see Supplementary Information, Fig. S3h). Thus, three lines of evidence argue that hair and whisker follicle papillae are endogenous niches for SKPs: first, follicle papillae contain cells that express the same embryonic transcription factors as do SKPs; second, SKPs express markers specific for follicle papillae in skin; and third, postnatal vibrissal papillae contain cells that proliferate as nestin-positive SKP spheres and differentiate into cells with attributes of neurons and smooth muscle cells.

## Follicle dermal papillae contain neural-crest-derived cells, and SKPs from facial skin are neural crest-derived

The above findings argue that SKPs are neural-crest-related precursors that arise in dermis during embryogenesis and persist into adulthood, and that follicle papillae are niches for these precursors. These findings predict that follicle papillae, whose embryonic origin is currently unknown, would contain neural-crest-derived cells, and that SKPs themselves would be neural-crest-derived. To test these predictions, we used a genetic method previously used to 'tag' neural-crest-derived cells *in vivo*. Specifically, mice expressing a *Wnt1–Cre* transgene were crossed to those expressing a 'floxed' RosaR26R reporter allele, thereby marking the progeny of NCSCs with β-galactosidase[36]. Using this approach, we first attempted to determine whether hair follicle papillae were neural-crest-derived; serial sections of dorsal skin from Wnt1Cre;RosaR26R animals were analysed for β-galactosidase expression by *in situ* hybridization (Fig. 7a; also see Supplementary Information, Fig. S4a). At P5, when all pelage follicles enter the first synchronized anagen phase, we observed β-galactosidase-positive cells in the follicle papillae (Fig. 7a) and occasional cells in the outer dermal follicle sheath (see Supplementary Information, Fig. S4a). These β-galactosidase-positive cells did not express keratin 15 (data not shown). Faintly β-galactosidase-positive cells were also occasionally found in the epithelial compartment of the hair follicle unit, including a few cells in the matrix (Fig. 7a), the bulge region (data not shown) and exiting to the interfollicular epidermis (see Supplementary Information, Fig. S4a). These latter β-galactosidase-positive cells are most probably progeny of NCSC-derived melanoblast stem cells reported to inhabit

these niches[24], and/or may represent other NCSC-derived progeny, such as Merkel cells[37] or Schwann cells. β-galactosidase-positive cells were also identified in follicle papillae of adult mice (data not shown). Thus, hair follicle dermal papillae contain neural-crest-derived cells. However, low transgene penetrance in dorsal skin (see Supplementary Information, Fig. S4b,c) made it difficult to assess the precise contribution made by neural crest to this follicle compartment.

The developing whisker pad of Wnt1Cre;R26R mice was analysed next. At P9, a time when SKP-like spheres can be isolated from papillae (Fig. 6), almost all whisker papillae cells expressed the β-galactosidase transgene, as they did nexin and slug (Fig. 7b). The vast majority of dissected neonatal whisker papilla cells also stained positive for β-galactosidase activity (Fig. 7d). Similarly, at E18.5, almost all cells in newly formed papillae (Fig. 7c) and many of the dermal cells were β-galactosidase positive, consistent with the neural-crest origin of facial dermis. Thus, vibrissal papillae are almost entirely of neural crest origin.

Next, we determined whether SKPs were neural-crest-derived. SKP spheres generated from neonatal Wnt1Cre;Rosa26R whisker pads and stained with X-gal after 0–2 passages were all β-galactosidase-positive (Fig. 8a). When individual transgene-positive spheres from whisker pads were differentiated for two weeks, they differentiated into SMA-positive smooth muscle cells and βIII-tubulin-positive neurons (Fig. 8b), demonstrating SKP-like potential. Although the transgene was downregulated in most cells during differentiation, occasional β-galactosidase-positive smooth muscle cells and neurons were still observed (Fig. 8c,d). Thus, SKPs generated from facial skin are neural-crest-derived. These findings, combined with our data, showing that whisker follicle papillae are neural-crest-derived, that follicle papillae express many genes in common with SKPs, and that isolated vibrissal papillae generate SKP-like spheres, strongly support the idea that hair and whisker follicle papillae represent endogenous niches for these adult neural-crest-related precursor cells.

## DISCUSSION

The data presented here support two major conclusions. First, they indicate that mammalian skin contains a multipotent adult precursor cell that shares characteristics with embryonic NCSCs, including neural-crest-like differentiation potential *in vitro* and *in vivo* and, for facial skin, a neural-crest origin. These endogenous precursors first arise during mid-embryogenesis and persist into adulthood, and can be isolated, cultured and expanded as SKPs, during which time they maintain their multipotentiality. Second, our findings demonstrate that one niche for these endogenous precursor cells is the follicle papilla, a niche thought to contain precursor cells[27,28] that is a major locus for regulatory dermal–epidermal interactions[38].

We demonstrate here that whisker follicle dermal papillae, which contain SKP-like cells, are neural-crest-derived. Moreover, we show that dorsal skin hair follicle papillae contain neural-crest-derived cells, and that SKPs generated from dorsal skin have neural-crest potential. In this regard, dorsal skin hair follicle papillae derive from p75NTR-positive cells during embryogenesis[39] at a time when the first nerves, which contain p75NTR-positive NCSCs[40], arrive in skin and innervate the developing follicles[41], consistent with a potential neural-crest origin. Nonetheless, we have not definitively established whether dorsal skin SKPs are neural-crest precursors that reside in a hair follicle niche, or whether they derive from a different embryonic origin, but share a similar differentiation potential. Although the latter possibility may seem less probable, face and dorsal skin dermis are also very similar, but derive from different embryonic origins.

The implications of these findings are broad. First, they indicate that at least one adult tissue contains endogenous, surprisingly multipotent precursor cells that can differentiate into cell types never found in their tissue of origin, in this case neurons. This ability probably reflects their potential when they migrate into skin during embryogenesis, as there was no evidence of transdifferentiation or de-differentiation. These cells are maintained in the adult, with their environment presumably restricting their potential *in vivo*. Second, because neural crest is the developmental origin of these precursors, at least in facial skin, these findings imply that similar multipotent neural-crest-derived precursors may be found elsewhere. Cells similar to SKPs have been isolated from human dental pulp[42], which is neural-crest-derived, and we have found SKP-like cells in a placodally derived structure, the adult olfactory epithelium (A. Gloster, J.G.T., M.A. and F.D.M., unpublished observations). It will be interesting to determine whether neural-crest-derived mesenchymal tissues, such as the frontal bones of the skull, also contain cells with SKP-like properties. Finally, our findings identify a precursor-cell niche for a neural-crest-related cell that could potentially generate neural-crest progeny found in skin, such as Merkel cells, Schwann cells, mesodermal cell types, and potentially even melanoblasts. We propose that it is their niche that maintains these cells in a precursor state. In this regard, dysregulation of a niche containing such an endogenous multipotent precursor cell might provide an explanation for skin tumours of mixed mesodermal and neural lineages.

## METHODS

**Cell culture.** SKPs were cultured as described[7]. Briefly, dorsal or facial skin from mouse embryos (E15–19), mouse or rat neonates (P2–P6), or adults (3 weeks and older) was dissected from the animal and cut into 2–3-mm$^2$ pieces. Tissue was digested with 0.1% trypsin for 10–45 min at 37 °C, mechanically dissociated and filtered through a 40-μm cell strainer (Falcon, BD Biosciences, San Diego, CA). Dissociated cells were pelleted and plated in DMEM-F12 (3:1; Invitrogen, Carlsbad, CA), containing 20 ng ml$^{-1}$ EGF and 40 ng ml$^{-1}$ FGF2 (both from Collaborative Research, Bedford, MA), hereafter referred to as proliferation medium. Cells were cultured in 25-cm$^2$ tissue culture flasks (Falcon) in a 37 °C, 5% $CO_2$ tissue-culture incubator. SKPs were passaged by mechanically dissociating spheres and splitting 1:3 with 75% new medium and 25% conditioned medium from the initial flask. Neurospheres from the E13 embryonic telencephalon[43] were cultured under the same conditions. For neuronal differentiation, SKP spheres or primary dissociated skin cells were mechanically dissociated and plated on chamber slides (Nalge Nunc, Rochester, NY) coated with poly-D-lysine–laminin in DMEM-F12 (3:1) supplemented with 40 ng ml$^{-1}$ FGF2 and 10% FBS (Biowhittaker, Walkersville, MD) for 5–7 days. Cells were then cultured for an additional 5–7 days in the same medium without FGF2, but with the addition of 10 ng ml$^{-1}$ nerve growth factor (Cedar Lane, Hornby, Canada), 10 ng ml$^{-1}$ brain-derived neurotrophic factor (BDNF; Peprotech, Rocky Hill, NJ) and 10 ng ml$^{-1}$ NT3 (Peprotech). For Schwann-cell differentiation, dissociated spheres were cultured in DMEM-F12 (3:1) supplemented with 10% FBS for seven days, then switched to the same medium supplemented with 4 μM forskolin (Sigma, St Louis, MO).

For the vibrissae experiments, rat vibrissal follicles were dissected from P6–P21 whisker pads and excess tissue was carefully removed. In some experiments, such as Sca-1 immunostaining, papillae were dissected from mice of a similar age. The inner root sheath was opened with tungsten needles and the papilla removed. Papillae were digested with trypsin for 15 min at room temperature and mechanically dissociated. Single cells were plated on two-well chamber slides coated with poly-D-lysine–laminin–fibronectin and cultured using the neuronal differentiation protocol described above. Alternatively, total vibrissal cells were dissociated and treated in the same way. To generate spheres from isolated papilla cells, cells were plated on chamber slides coated with poly-D-lysin–laminin–fibronectin in SKP-proliferation medium supplemented with 5% chick embryo extract. After seven days, adhered spheres were removed from the slide and a single sphere was then re-plated in a new chamber slide for differentiation using the neuronal differentiation protocol described above.

Sphere counts in solution were performed after seeding 25,000–200,000 cells ml$^{-1}$ in uncoated 24-well tissue-culture plates (Falcon) in prolif-

eration medium for 4–7 days. Methylcellulose sphere counts were performed by plating dissociated, individual skin cells (100,000) in DMEM-F12 (3:1), 1.5% methylcellulose (Sigma), 2% B27 (Gibco-BRL, Carlsbad, CA), 20 ng $ml^{-1}$ EGF, 40 ng $ml^{-1}$ FGF2, 1 μg $ml^{-1}$ fungizone (Invitrogen) and 1% penicillin–streptomycin. Cells were cultured in 3.5-cm plates in a 37 °C, 5% $CO_2$ incubator and sphere formation was scored after 10–14 days. For passaging, individual spheres were picked from the methylcellulose, dissociated to single cells and re-plated again in methylcellulose. Cell mixing experiments were performed by mixing dissociated E16 or E18 skin cells with YFP-tagged[22], dissociated vibrissal follicle cells 100:1 in uncoated flasks with proliferation medium at 25,000 cells $ml^{-1}$.

**Immunocytochemistry, *in situ* hybridization and X-gal staining.** Immunocytochemical analysis for cells was performed either using coated slides and the cytospin system (Thermo Shandon, Pittsburgh, PA) for SKP spheres, or on cells plated on chamber slides (Nalge Nunc) as described[7,44]. The following primary antibodies were used: anti-nestin monoclonal (1:400; BD Biosciences), anti-βIII-tubulin monoclonal (1:500; Tuj1 clone; BABCO, Evanston, IL), anti-neurofilament-M polyclonal (1:200; Chemicon, Temecula, CA), anti-GFAP polyclonal (1:200; DAKO, Copenhagen, Denmark), anti-p75NTR polyclonal (1:500; Promega, Madison, WI), anti-SMA monoclonal (1:400; Sigma), anti-fibronectin polyclonal (1:400; Sigma), anti-trp1 polyclonal (1:200; Chemicon), anti-c-kit polyclonal (1:400; Cell Signaling Technology, Beverly, MA), anti-S100β monoclonal (1:1,000; Sigma), anti-MBP polyclonal (1:100; Chemicon), anti-TH monoclonal (1:200; Chemicon) and Sca-1 (1:100; Becton Dickinson). The following secondary antibodies were used: Alexa488-conjugated goat anti-mouse (1:1,000) and Alexa594-conjugated goat anti-rabbit (1:1,000); both were from Molecular Probes (Eugene, OR). Processing of skin samples for histological analysis and *in situ* hybridization was performed as described[45]. The probes used in this study were as follows: β-galactosidase (Ambion, Austin, TX); nexin and versican (gifts from B. Morgan); K17 (P. Coulombe); Wnt5a (A. McMahon); Snail (T. Gridley); and Slug, twist and nestin probes were all generated using the RT–PCR primers detailed below.

Immunohistochemistry and alkaline phosphatase staining on skin sections was performed as described[46]. Briefly, staining for lacZ was performed on tissues fixed in 2.7% formaldehyde, 0.02% Nonidet-P40 and PBS overnight at 4 °C, followed by overnight cryoprotection in 30% sucrose in PBS at 4 °C before mounting. Cryosections were cut at 12-μm thickness and stained overnight at 37 °C in X-gal staining solution. Sections were counterstained in eosin and mounted. Plated cells and spheres were X-gal stained by briefly fixing in 4% paraformaldehyde (for 2 min), rinsing three times in PBS, rinsing twice in 0.1 M sodium phosphate containing 2 mM $MgCl_2$, 0.1% sodium desoxycholate and 0.02% NP40, and then immersing in standard X-gal staining solution overnight.

**RT–PCR.** RNA was prepared from samples using Trizol (Invitrogen) and cDNA was generated with Revertaid Reverse Transcriptase (Fermentas, Vilnius, Lithuania) as directed by the manufacturer. For all cDNA synthesis, a 'minus reverse transcriptase' control was performed. PCR reactions were performed as follows: 92 °C, 2 min; 30–35 cycles of 94 °C, 60 s; gene-specific annealing temperature for 60 s; and 72 °C for 60 s. See Supplementary Information for primer details.

**Western blot analysis.** Lysates were prepared and western blot analysis performed as described[44]. Equal amounts of protein (50–100 μg) were resolved on 7.5% or 10.5% polyacrylamide gels. The primary antibodies used were: anti-DβH monoclonal (1:1,000; Pharmingen, San Diego, CA), anti-peripherin polyclonal (1:1,000; Chemicon), anti-p75NTR polyclonal (1:1,000; Promega), anti-TH monoclonal (1:800; Chemicon), anti-βIII-tubulin monoclonal (1:1,000; Tuj1 clone; BABCO) and anti-NCAM monoclonal (1:800; Chemicon).

***In ovo* transplantations.** Fertile White Leghorn chicken eggs (Cox Brothers Poultry Farm, Truro, NS) were incubated at 37 C in a humidified chamber until Hamburger-and-Hamilton stage 18. A small opening was made in the side of the shell and a small bolus of neutral-red/water solution (1% w/v) was applied to the chorioallantoic membrane to visualize the underlying embryo. An incision was made into the anterior, medial corner of two somites in the lumbar region for each embryo using needles made from flame-sharpened tungsten wire. One or two SKP neurospheres (totalling ~200–250 cells) were transplanted into the incision site that corresponds to the dorsal-most region of the neural-crest migratory pathway. The shells were subsequently sealed and the embryos incubated until Hamburger-and-Hamilton stage 30 (about three more days) after which time the embryos were removed from the shell contents, quickly decapitated, eviscerated, fixed in 3.7% formaldehyde/PBS for 2 h and immersed in 30% sucrose/PBS overnight at 4 °C. The embryos were embedded in OCT embedding medium, frozen at –70 °C, sectioned at 20-μm thickness and mounted onto tissue-adhering slides.

*Note: Supplementary Information is available on the Nature Cell Biology website.*

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COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: see *Nature Cell Biology* website for details.

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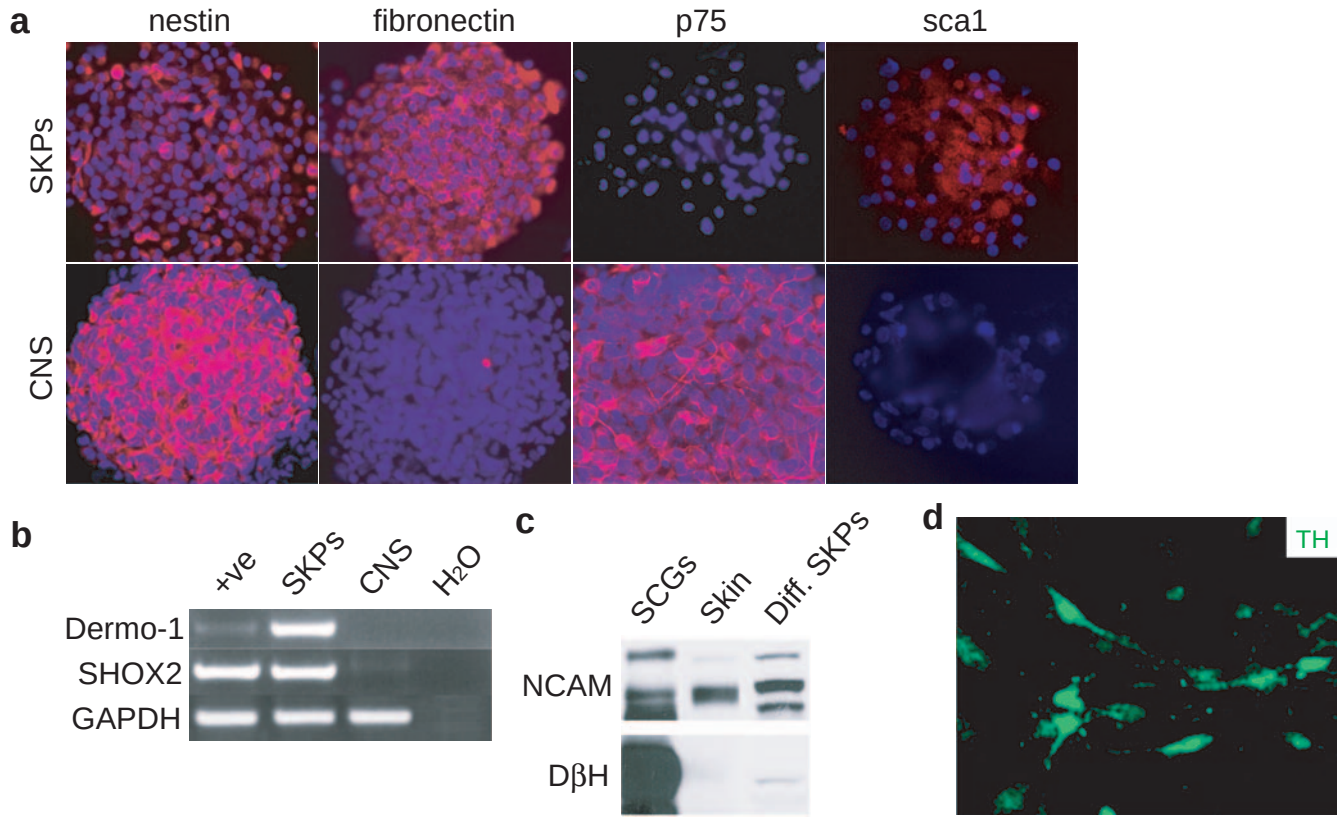


**Figure S1** a, Immunocytochemical analysis of SKP spheres cultured from neonatal mouse (nestin, fibronectin, Sca-1) or rat (p75NTR) back skin, and passaged two or three times (top panels) as compared to neurospheres cultured from the embryonic telencephalon and passaged a similar number of times (bottom panels). In all panels, the immunostained cells are red, and the blue is from Hoechst staining of the nuclei to show all cells. Note that SKPs express fibronectin and Sca-1 but not p75NTR, whereas neurospheres do not express fibronectin or Sca-1, but do express p75NTR. b, RT-PCR for Dermo-1 and SHOX2, transcription factors involved in dermal and craniofacial development, in RNA from SKPs and CNS neurospheres as described in Fig. 1. The positive control (+ve) was RNA from E16 forelimb. c, Western blot analysis for NCAM and dopamine-β-hydroxylase in murine SKP spheres versus SKPs differentiated for 14 days in 10% serum supplemented with neurotrophins. The positive control was protein isolated from cultured peripheral sympathetic neurons from the superior cervical ganglion (SCGs). d, Immunostaining of differentiated SKPs shows that a subset of differentiated cells express tyrosine hydroxylase (TH).

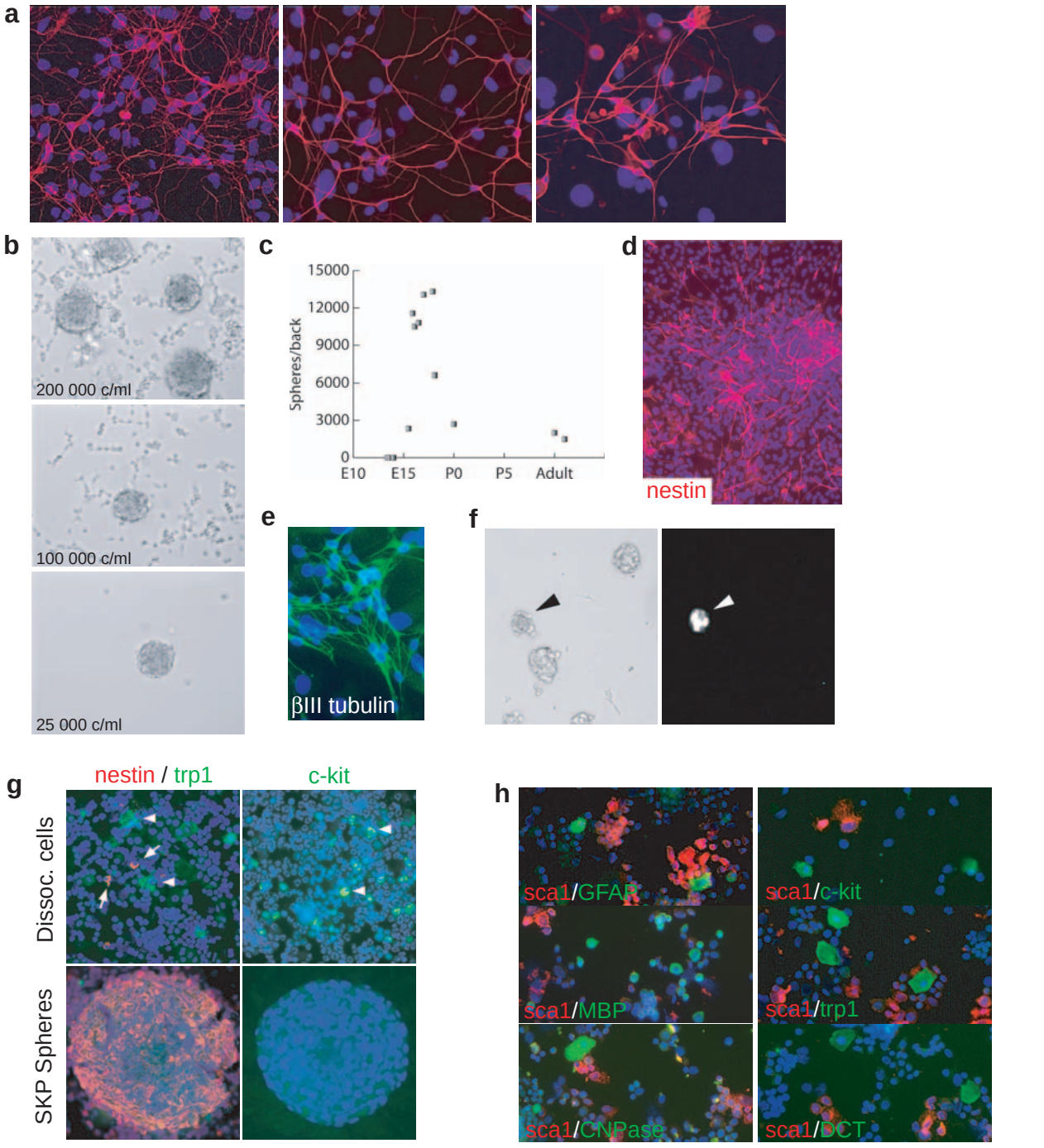


**Figure S2** a, Immunocytochemical analysis for βIII-tubulin in primary E18 (middle panel) or adult (right panel) murine skin cells versus E18 murine CNS telencephalic cells (left panel). All cells were plated in FGF2 and 10% serum for 7 days and differentiated in medium containing 5% serum supplemented with neurotrophins. b, Phase contrast micrographs of primary murine SKP spheres grown for 1 week at varying concentrations of starting cells (c/ml). c, Quantitation of the number of cells that give rise to primary SKP spheres in murine back skin isolated at various developmental ages ranging from embryonic day 13 to adulthood. Cell numbers were normalized to the total number of cells present in a back skin isolation. d, Immunocytochemical analysis for nestin in a primary murine SKP clone plated onto poly-d-lysine/laminin. e, Immunocytochemical analysis for βIII-tubulin in a primary murine SKPs clone grown in methylcellulose and then differentiated. f, Primary SKP spheres generated by mixing limiting numbers of YFP-positive adult vibrissal cells and E18 primary skin cells and culturing in the presence of FGF2 and EGF for one week. The left panel is a phase micrograph and the right panel a fluorescence micrograph of the same field. The arrowhead indicates a YFP-positive sphere. g, Immunocytochemical analysis of primary SKP spheres for markers of melanoblasts and hematopoietic stem cells. The two left panels were immunostained for nestin (red, arrows) and for trp1 (green, arrowheads), with the top panel being total dissociated skin cells, and the bottom a primary SKP sphere. The two right panels were immunostained for c-kit (green), with the top panel being a cytospin of a bone marrow aspirate (positive cells are marked with arrowheads), and the bottom panel a primary SKPs sphere. In all panels, the blue derives from the Hoechst staining of cell nuclei. h, Fluorescence micrographs of primary dissociated dorsal skin cells double-labelled for the precursor cell marker Sca-1 (red) and for the Schwann cell markers GFAP, myelin basic protein (MBP), and CNPase, or the melanoblast/melanocyte markers c-kit, trp1 and dct (all in green). The blue is Hoechst staining of the nuclei.

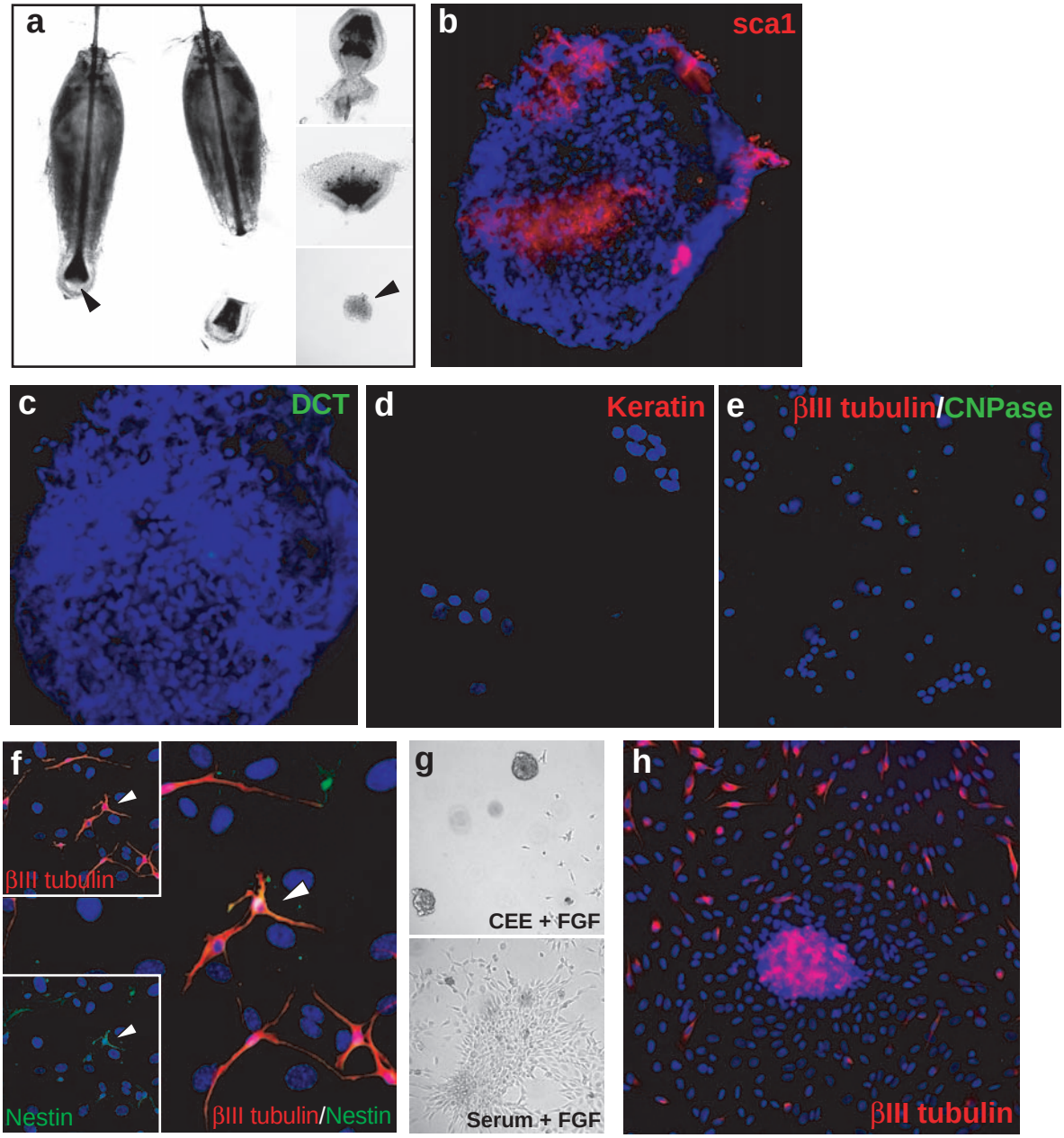


**Figure S3** a, Photomicrographs of a vibrissal papilla dissection from an adult pigmented mouse. The left picture shows the intact vibrissa, while the other pictures show the dissection. The arrowhead indicates the papilla. b,c, Immunocytochemical analysis of dissected whisker papillae for Sca-1 and dct. d,e, Fluorescence photomicrographs of dissociated papilla cells that were immunostained for keratin 18 (d) or βIII-tubulin and CNPase (e). f, Immunocytochemical analysis for nestin and βIII-tubulin in adult vibrissal papilla cells directly differentiated for 7 days in FGF2, 10% serum and neurotrophins. g, Phase photomicrographs of dissociated papilla cells that were cultured for one week in the presence of FGF2 plus serum or chick embryo extract (CEE). Note that in the presence of CEE, floating spheres of cells were generated. h, Fluorescence photomicrograph of a whisker papilla sphere that was differentiated on an adherent substratum for 3 days and then immunostained for βIII-tubulin. In panels b-e, f and h, nuclei were stained blue with Hoechst.

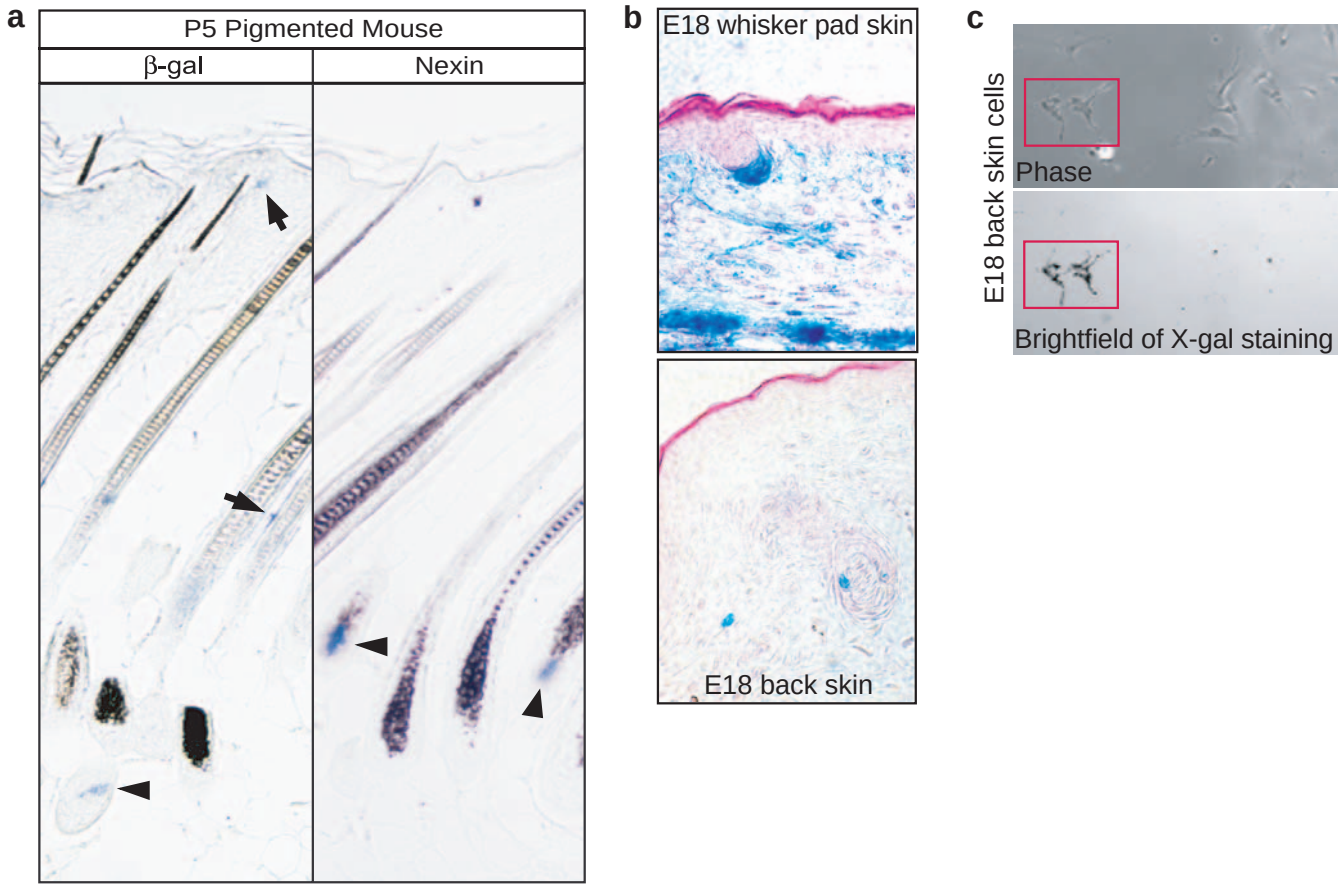


**Figure S4** a, Overlapping expression of β-galactosidase and the dermal papilla marker, nexin, in postnatal day 5 (P5) Wnt1Cre;Rosa26R dorsal skin from pigmented mice. Pigmented melanin granules are deposited in the developing hair shaft by neural crest-derived melanoblasts resident and interspersed in the matrix. A subpopulation of follicle papilla cells express mRNAs for both β-galactosidase (arrowhead) and nexin (arrowhead). Beyond the outer root sheath, occasional β-galactosidase-positive cells can also be seen in the dermal sheath (lower arrow), a structure closely associated with follicle papillae. Single β-galactosidase-positive cells are seen emerging from the upper portions of the follicle into the interfollicle epidermis and are likely melanoblasts (upper arrow). b, Expression of the β-galactosidase transgene in E18 whisker pad versus E18 back skin from Wnt1Cre;Rosa26R embryos, as detected by X-gal staining of cryostat sections. Similar results were obtained at a variety of developmental ages using both X-gal staining and *in situ* hybridization for the β-galactosidase transgene. Note that very few transgene-positive cells were detected in dorsal skin, in spite of the fact that there are abundant melanoblasts and Schwann cells at this point, as demonstrated for neonatal skin in Supplementary Fig. 2h. c, Photomicrographs of dissociated back skin cells from neonatal Wnt1Cre;R26R mice that were stained with X-gal one day after plating. The upper panel is a phase micrograph while the lower is a brightfield micrograph of the same field showing that neural crest-derived pigmented melanocytes (boxed in red) were β-galactosidase negative.

**Table S1** Primer Sequences

| Gene | Primer Sequence | Anneal Temp (°C) | Product (BP) |
|---|---|---|---|
| Pax3 | Ggaggcggatctagaaaggaagga | 59 | 374 |
| | Cccccggaatgagatggttgaa | | |
| Slug | Cgtcggcagctccactccactctc | 60 | 348 |
| | Tcttcagggcacccaggctcacat | | |
| Twist | Ctttccgcccacccacttcctctt | 57 | 334 |
| | Gtccacgggcctgtctcgctttct | | |
| Snail | Cggcgccgtcgtccttct | 61.5 | 398 |
| | Ggcctggcactggtatctcttcac | | |
| Sox9 | Ccgcccatcacccgctcgcaatac | 59.5 | 544 |
| | Gcccctcctcgctgatactggtg | | |
| P75 | Gtgcggggtgggctcaggact | 62 | 422 |
| | Ccacaaggcccacaaccacagc | | |
| SHOX2 | Ccgccgccgccaagaccac | 63 | 355 |
| | Tccccaaacccgctcctacaaa | | |
| DBH | Acccgggggacgtactcatcac | 59 | 353 |
| | Cgggaagcggacagcagaag | | |
| Peripherin | Gccgccaaccgcaaccat | 61.5 | 333 |
| | Gatccggctctcctcccttcc | | |
| MBP | Tggccccggggacacttc | 61.1 | 332 |
| | Gccgctgaccaccccaccat | | |
| P0 | Cctggctgccctgctcttctcttc | 60.1 | 452 |
| | Ccccgatcactgctcccaacac | | |
| Dermo-1 | Gcggcgctacagcaagaaatc | 61.4 | 356 |
| | Ccatgcgccacacggagaagg | | |
| Nexin | Ccacgcaaagccaagacgac | 57.4 | 289 |
| | Gaaaccggcctgctcatcct | | |
| Versican | Tggaaggcacagcagtttacc | 56.3 | 427 |
| | Tcatggcccacacgattcac | | |
| Wnt-5a | Ccccctcgccatgaagaagc | 60.1 | 552 |
| | Cagccgccccacaaccagt | | |
| GAPDH | Gtcttcaccaccatggagaag | 56 | 281 |
| | Gtgatggcatggactgtggtc | | |