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Multipotent skin-derived precursors: adult neural crest-related precursors with therapeutic potential

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We previously made the surprising finding that cultures of multipotent precursors can be grown from the dermis of neonatal and adult mammalian skin. These skin-derived precursors (SKPs) display multi-lineage differentiation potential, producing both neural and mesodermal progeny *in vitro*, and are an apparently novel precursor cell type that is distinct from other known precursors within the skin. In this review, we begin by placing these findings within the context of the rapidly evolving stem cell field. We then describe our recent efforts focused on understanding the developmental biology of SKPs, discussing the idea that SKPs are neural crest-related precursors that (i) migrate into the skin during embryogenesis, (ii) persist within a specific dermal niche, and (iii) play a key role in the normal physiology, and potentially pathology, of the skin. We conclude by highlighting some of the therapeutic implications and unresolved questions raised by these studies.

Keywords: stem cells; dermal papilla; hair follicles; spinal cord injury; cancer; plasticity

1. INTRODUCTION

A number of studies published in the past 7 years have challenged the traditional view that stem cells present in somatic tissues are restricted to producing that tissue's cell types. Neural stem cells isolated from the embryonic or adult central nervous system (CNS) have been reported to differentiate into muscle (Galli *et al.* 2000; Tsai & McKay 2000; Bani-Yaghoub *et al.* 2004), blood (Bjornson *et al.* 1999) and endothelial cells (Wurmser *et al.* 2004), and to contribute to all three primary germ layers when injected into blastocysts (Clarke *et al.* 2000). Transplanted bone marrow-derived cells have shown evidence of contributing to the liver (Petersen *et al.* 1999; Theise *et al.* 2000; Grompe 2003), muscle (LaBarge & Blau 2002; Brazelton *et al.* 2003; Corbel *et al.* 2003; Doyonnas *et al.* 2004; Palermo *et al.* 2005) and brain (Brazelton *et al.* 2000; Mezey *et al.* 2000, 2003; Priller *et al.* 2001; Cogle *et al.* 2004), and also to the three primary germ layers following blastocyst injection (Jiang *et al.* 2002*b*). These and other studies suggested that 'tissue-specific' stem cells can express a wider differentiation potential if exposed to appropriate foreign environments, as occurs *in vitro* or following heterotopic transplantation. Such findings raise the possibility that tissue-derived stem cells are either more 'plastic' than previously thought or that the stem cells present in adult tissues are more similar to embryonic precursors than previously appreciated.

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One contribution of 14 to a Theme Issue 'Stem cells and brain repair'.

2. ISOLATION AND DIFFERENTIATION OF NOVEL MULTIPOTENT PRECURSORS FROM SKIN

Skin represents a complex and highly regenerative tissue containing a relatively broad stem cell repertoire, including resident epidermal and mesenchymal stem cells (MSCs), migratory melanocytic stem cells derived from the embryonic neural crest (NC) and blood vessel-associated haematopoietic and endothelial precursors. The idea that skin might provide an accessible, potentially autologous source of neural precursors (NPCs) was first suggested by the finding that Merkel cells, a type of sensory receptor with characteristics of neurons, could be generated within the skin of rodents (Nurse *et al.* 1984). On the basis of these findings, we hypothesized that skin contained a precursor cell with neurogenic capacity and used methods similar to those used to culture CNS neural stem cells (Reynolds & Weiss 1992; Reynolds *et al.* 1992) in an attempt to isolate these precursors. When skin cells of neonatal and adult rodents were dissociated to single cells and grown in suspension culture in the presence of the mitogens fibroblast growth factor-2 (FGF2) and epidermal growth factor (EGF), floating spheres of proliferating cells were generated (Toma *et al.* 2001). These spheres were positive for nestin, an intermediate filament expressed in neural and skeletal muscle precursors. Moreover, differentiation of skin-derived spheres *in vitro* resulted in the de novo generation of separate subpopulations of cells expressing neuronal, glial, smooth muscle and adipocyte markers (Toma *et al.* 2001). Importantly, clonally derived colonies were able to produce this same complement of cell types, confirming the multipotency of these cultured precursors from skin.

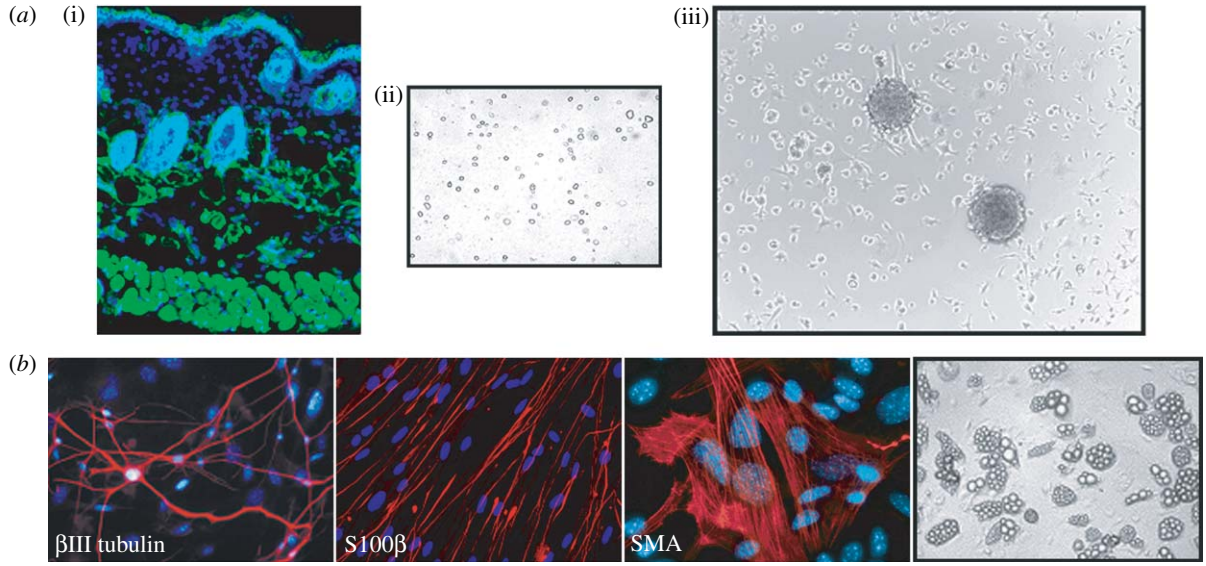

Figure 1. Isolation and differentiation of skin-derived precursors. (*a*) Isolation of SKPs. (i) Fluorescence micrograph of post-natal mouse skin, with nuclei labelled by Hoechst (blue) and epithelial cells of the epidermis and hair follicles labelled with anti-cytokeratin (green). Non-specific cytokeratin staining also illuminates the subcutaneous muscle layer. (ii) Enzymatically and mechanically dissociated mouse skin. (iii) Growth of SKP spheres in suspension cultures when dissociated skin cells are exposed to the mitogens FGF2 and EGF. (*b*) Differentiation of SKP spheres. Under appropriate conditions, SKP spheres differentiate into neurons that express βIII tubulin, glial cells that express S100β, smooth muscle cells that express smooth muscle SMA and adipocytes containing characteristic intracellular lipid droplets. FGF2, fibroblast growth factor-2; EGF, epidermal growth factor; SMA, smooth muscle actin.

A direct comparison of these precursors with other types of stem cells and precursors known to be resident in skin indicated that skin-derived precursors (SKPs) were a distinct population (Toma *et al.* 2001; Fernandes *et al.* 2004). In particular, they were antigenically distinct from MSCs and, when grown under the same conditions, the MSCs grew adherently while SKPs grew as floating spheres (Toma *et al.* 2001). SKPs were derived from the dermis and did not apparently produce keratinocytes, distinguishing them from epidermal stem cells (Toma *et al.* 2001). Finally, SKPs neither express c-kit, a marker of melanocytic stem cells and haematopoietic stem cells, nor other melanocyte stem cell markers such as dct (Fernandes *et al.* 2004). We also compared SKPs directly to CNS neural stem cells cultured as neurospheres and again found significant differences; while both CNS stem cells and SKPs expressed nestin, SKPs also expressed a number of proteins, including fibronectin, that were not expressed by CNS neural stem cells (Fernandes *et al.* 2004). In addition, while both CNS stem cells and SKPs could generate one mesodermal cell type, smooth muscle cells, only SKPs were able to generate adipocytes (Toma *et al.* 2001). Thus, on the basis of these findings, we proposed that SKPs were novel multipotent precursors resident in the mammalian dermis (figure 1).

3. SKPs: WHAT, WHERE AND WHY?

The notion of multi-lineage adult stem cell plasticity has been challenged by a number of findings in recent years, including demonstrations of cell fusion by bone marrow-derived cells (Alvarez-Dolado *et al.* 2003; Vassilopoulos *et al.* 2003; Wang *et al.* 2003; Nygren *et al.* 2004), an inability to repeat earlier reports of plasticity (Morshead *et al.* 2002; Vallieres & Sawchenko 2003; Wehner *et al.* 2003) and the production of non-functional cell types (Lu *et al.* 2004; Neuhuber *et al.* 2004). These findings raised several fundamental questions regarding the biological properties and *in vivo* relevance of cultured adult stem cells, which we have attempted to address with regards to SKPs. What is their developmental origin? Are SKPs an endogenous precursor cell? Where are SKPs located *in vivo* and what are their biological roles?

(a) *SKPs exhibit properties of embryonic neural crest precursors*

The capacity of SKPs to generate both neural and mesodermal progeny provided our first clue to their potential developmental origin. In particular, one embryonic precursor population with a similar developmental potential is NC stem cells. The embryonic NC is a subpopulation of neuroectodermally derived cells that undergoes an epithelial–mesenchymal transformation and subsequently exits from the dorsal neural tube. These embryonic NC cells consist of a transient population of multipotent and fate-biased precursors that ultimately generate a wide variety of non-CNS cell types. Among the clearly identified derivatives of NC cells are the entire peripheral (sensory, autonomic and enteric) nervous system, catecholaminergic adrenal cells, melanocytes, Merkel cells, and, at the cranial levels, the facial dermis, cartilage, bone, adipocytes and meninges (Halata *et al.* 1990; Couly *et al.* 1992; Le Douarin & Kalcheim 1999; Etchevers *et al.* 2001; Jiang *et al.* 2002*a*; Le Douarin & Dupin 2003). The NC also contributes a variety of mesodermal cell types outside the cranial regions, including adipocytes within parasympathetic ganglia of the gut (Le Lievre & Le Douarin 1975), peripheral nerve fibroblasts (Halata *et al.* 1990; Joseph *et al.* 2004)

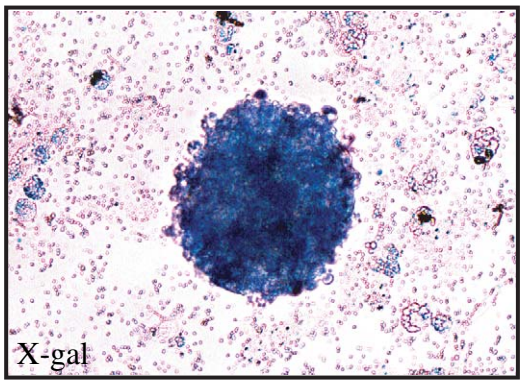

Figure 2. SKPs from the facial skin of Wnt1-cre;R26R compound transgenic mice. Spheres generated from the facial skin of Wnt1-cre;R26R transgenic mice, which indelibly express β-galactosidase in neural crest derivatives, react positively with the β-galactosidase substrate X-gal.

and the outflow tract of the heart (Fukiishi & Morriss-Kay 1992; Serbedzija & McMahon 1997; Jiang *et al.* 2000; Chan *et al.* 2004). Clonal analysis of embryonic NC cells showed that migrating multipotent NC precursors ultimately reach a variety of different target tissues and, while they were traditionally thought to terminally differentiate within these tissues, recent evidence has raised the possibility that these NC precursors persist in at least some adult tissues such as the gut (Kruger *et al.* 2002). We therefore examined the possibility that SKPs may be NC-derived precursors that are maintained into adulthood within the dermis of the skin.

These studies revealed four lines of evidence supporting this possibility (Fernandes *et al.* 2004). First, a side-by-side comparison of gene expression in SKPs and NC-containing embryonic neural tube revealed that SKPs express a variety of NC-associated transcription factors including Slug, Snail, Twist, Pax3 and Sox9. Moreover, SKPs also expressed SHOX2 and Dermo-1, the transcription factors associated with the mesenchymal capability of cranial NC cells. In contrast, few of these transcription factors were expressed by CNS neural stem cells. Second, phenotypic analysis of the neural cells differentiated from SKPs revealed that they were largely peripheral nervous system cell types including Schwann cells and catecholaminergic neurons, cells that are derived from the NC during normal development. Third, when genetically tagged SKPs were transplanted into NC migratory pathways in the embryonic chick, they were able to specifically follow the host NC migratory streams, ultimately ending up in peripheral sensory and autonomic ganglia, peripheral nerves and the skin. Fourth, when SKPs were derived from the facial skin of Wnt1-cre;R26R compound transgenic mice, which selectively and indelibly express β-galactosidase in peripheral NC derivatives, SKP spheres were β-galactosidase positive (Fernandes *et al.* 2004; figure 2).

Together these data strongly support the hypothesis that SKPs are NC-related precursors in the skin that retain multipotency into adulthood. These data also indicate that SKPs generated from facial skin derive from the NC embryonically. Do SKPs generated from trunk skin share this embryonic origin? Unfortunately, attempts to use the same lineage tracing approach to answer this question were confounded by the partial penetrance of the Wnt1-cre;R26R β-galactosidase transgene in back skin (Fernandes *et al.* 2004). In particular, while only a fraction of SKPs isolated from the back skin of these mice were β-galactosidase positive, many known NC progeny, including some melanocytes and Schwann cells, were also not β-galactosidase positive in the back skin (Fernandes *et al.* 2004), making such studies inconclusive. Thus, it is formally possible that trunk-derived SKPs have all of the properties of NC precursors, but derive from a different, as-yet-uncharacterized population of NC-like precursors in the embryo, and/or that they derive from NC stem cells that do not express Wnt1.

(b) *SKPs are endogenous precursors that arise in skin during embryogenesis and persist into adulthood*

A key question that has emerged in the stem cell field over the past several years is to what extent cultured precursor cells reflect the properties of the endogenous cells from which they are derived (Fernandes *et al.* 2004). This issue has arisen as a consequence of studies showing (i) that biased progenitors can dedifferentiate in culture to become multipotent (Kondo & Raff 2000), (ii) that cells with similarities to embryonic stem cells can be derived upon long-term culture of, for example, bone marrow cells (Jiang *et al.* 2002*b*,*c*), and (iii) in the most remarkable example, that nuclei isolated from even terminally differentiated cells like olfactory receptor neurons can be used to clonally generate an entire mouse (Eggan *et al.* 2004; Li *et al.* 2004). Thus, while it has always been thought that embryonic precursor cells like NC stem cells exhibit broader potential in culture than in their more restrictive *in vivo* environments, these new studies raise the possibility that much more profound alterations can occur as a consequence of long-term culture.

Is the multipotential nature of SKPs a consequence of such culture-induced alterations? Two lines of evidence argue against this possibility. In one series of studies, we asked whether skin contained a precursor cell capable of generating neurons, a SKP-derived cell type never found in skin, without any passaging and expansion as spheres. In these experiments, skin cells that were plated under conditions used to analyse the neurogenic potential of primary embryonic NC stem cells (Stemple & Anderson 1992; Shah *et al.* 1996; Morrison *et al.* 1999) were capable of generating cells with the morphological and antigenic characteristics of differentiated peripheral catecholaminergic neurons (Fernandes *et al.* 2004). Thus, skin contains a NPC cell that normally does not generate catecholaminergic neurons *in vivo*, but that can do so when placed in the appropriate environmental conditions in culture. In a second series of studies, we cultured skin cells under conditions where single cells gave rise to single, clonal spheres. These primary clones had all of the characteristics of SKPs, and when differentiated were multipotent. Thus, SKPs that had never been passaged had the same properties as SKPs that had been expanded in culture (Fernandes *et al.* 2004). Moreover, this clonal sphere assay allowed us to determine when the

endogenous SKPs first arrive in skin. These studies indicated that mouse SKPs could first be detected in the back skin at embryonic day 15 (E15), that their numbers are then relatively high during late embryogenesis, decrease to lower levels at birth and then are maintained into adulthood (Fernandes *et al.* 2004). Together, these studies support the idea that SKPs represent an endogenous embryonic NC-derived precursor cell that persists into adulthood, and whose full potential is normally restricted by its *in vivo* environment but is revealed in culture.

(c) *One niche for SKPs is the dermal papillae of hair and whisker follicles*

If SKPs are restricted by their skin environment from generating, for example, neurons, then what is their *in vivo* environment? Four lines of evidence indicate that one niche for SKPs is the dermal papillae of hair and whisker follicles (Fernandes *et al.* 2004). First, *in situ* hybridization for transcription factors expressed by SKPs (as well as embryonic NC stem cells) such as Slug, Snail and Twist revealed that the most robust expression of these markers in adult mouse skin occurred in dermal papillae of follicles during the anagen growth phase. Thus, follicle papillae cells express SKP markers. Second, SKPs express markers that are enriched in follicle dermal papillae, including nexin, Wnt5a and versican. Third, when dermal papillae are dissected from whisker follicles and cultured under SKP conditions, they generate floating spheres of cells with the characteristics of SKPs. Finally, lineage tracing with the Wnt1-Cre;R26R mice revealed that whisker follicle dermal papillae are entirely NC derived, and that even dermal papillae of the back skin hair follicles contain NC-derived cells. Thus, these findings support the idea that follicle dermal papillae are a niche for the endogenous NC-derived SKPs. However, this is probably not the only niche for SKPs in skin, since SKPs can be routinely derived from human foreskin (Toma *et al.* 2005; described below), a tissue that contains no hair follicles.

Interestingly, follicular dermal cells, including dermal papillae cells, have long been thought to be multipotent precursors that potentially play an important role in normal skin turnover and in wound healing (Jahoda & Reynolds 2001; Gharzi *et al.* 2003). In this regard, cultured follicular dermal papilla cells have been shown to generate mesodermal cell types, including adipocytes and osteoblasts (Jahoda *et al.* 2003), and to even contribute to the haematopoietic system (Lako *et al.* 2002). They are also known to be the key regulators of hair follicle initiation and the follicle growth cycle, something that is discussed below. Like SKPs, follicle papillary cells proliferate in response to FGF2 and have been described as growing in clusters (Jahoda & Oliver 1984). Finally, follicle dermal papillae first condense at about E15 in mouse back skin, at the same time-point when SKPs can first be isolated, and the cells that form these embryonic papillae are positive for both TrkC and the p75 neurotrophin receptor (Botchkarev *et al.* 1998, 1999), markers of embryonic NC precursors (Stemple & Anderson 1992; Luo *et al.* 2003). Thus, the convergence of findings for follicular papillary cells and SKPs strongly support the idea that NC-derived SKPs comprise at least a fraction of the cells within the follicle dermal papillae.

4. PROSPECTS FOR THERAPEUTIC APPLICATIONS OF SKPs

One of the most exciting aspects of the ongoing work on SKPs is the prospect that they will provide an accessible, multipotent human precursor cell for a variety of purposes. While the most obvious of these is their potential use as an accessible source of cells for transplantation, human SKPs could also provide accessible human precursors for discovery research, something discussed in more detail below. As a requisite to such studies, however, it was essential to derive methods for isolating and expanding human SKPs, and to demonstrate that, like the rodent SKPs, they represented an accessible, multipotent precursor cell population.

(a) *Human SKPs are similar to rodent SKPs*

The initial work indicating that isolation of human SKPs was feasible was performed using 'scalp tags' from the placement of the stereotaxic neurosurgical apparatus on patients ranging from 40 to 70 years of age; small clusters of nestin-positive, fibronectin-positive cells could be derived from these very small scalp biopsies (Toma *et al.* 2001), suggesting that human skin also contained a SKP-like cell. More recent work using other human skin sources has now verified that human SKPs can be isolated and expanded, and are very similar to their rodent counterparts in terms of their potential. In our own work, we have focused upon human foreskins from 0–2-year-old males, and using a protocol very similar to that used for the rodent cells, we can routinely isolate and expand human SKPs. These human SKPs express markers typical of rodent SKPs, including NC transcription factors, and can differentiate into both neural and mesodermal progeny. Importantly, clonal analysis demonstrated that a single human cell can give rise to cells of both embryonic lineages. In addition, analysis of expanded human SKPs demonstrated that they maintain their differentiative abilities for at least 1 year in culture, and that these long-term passaged human SKPs maintained a normal karyotype, at least as analysed by G-banding. With regard to their embryonic origin, it is difficult to definitively ascertain whether human SKPs represent NC-derived precursors that are laid down in skin during embryogenesis and persist post-natally, as we believe to be the case for rodent SKPs. However, a number of lines of evidence indicate that they may well be. First, human SKPs express transcription factors expressed by NC stem cells. Second, human foreskin contains a population of NPCs that can differentiate into neurons without any isolation, passaging or expansion. Third, human SKPs have a potential similar to NC precursors, generating mesodermal progeny such as adipocytes, and neural cell types that are peripheral in nature, such as Schwann cells and catecholaminergic neurons (Toma *et al.* 2005).

Two recent papers have provided independent evidence in support of the existence of SKP-like NPCs in human skin (Belicchi *et al.* 2004; Joannides *et al.* 2004). In one study, the dermis of skin biopsy samples from 41 to 77-year-old healthy adults were grown into SKP-like spheres using FGF2 and EGF, and then the isolated spheres were adherently expanded using a serum-based protocol. These expanded adherent cultures retained the ability to generate spheres expressing the NPC markers nestin and musashi when placed in SKP-like serum-free conditions, and clonal analysis revealed a neural/mesodermal bipotentiality. Spheres generated following serum-based expansion were able to produce neurons upon differentiation, and neurogenesis was enhanced in the presence of hippocampal astrocyte-conditioned medium (Joannides *et al.* 2004), as previously reported for CNS NPCs (Song *et al.* 2002).

In a second study, skin samples were obtained from 14-week aborted foetuses and from 12 to 65-year-old healthy volunteers. In this study, skin cells were first fluorescent activated cell sorted for the human AC133(+) fraction, which has previously been shown to contain the sphere-forming cells from multiple stem cell populations, including human neural stem cells (Uchida *et al.* 2000). Consistent with this notion, no sphere-forming activity was found in the AC133-negative population. Single AC133(+) cells grew into spheres that could be clonally passaged, expressed nestin, and could differentiate into neuronal cells. Following transplantation into the lateral ventricles of neonatal immune-compromised SCID mice, cells from the AC133(+)-derived spheres displayed robust migration and some evidence of neuronal and glial differentiation (Belicchi *et al.* 2004). Thus, cells with the properties of SKPs have been isolated from human skin of differing ages and origins, supporting the notion that SKPs provide an accessible, autologous human stem cell population.

(b) *Functionality of SKP-derived neural and mesodermal cells*

For any precursor cell population to be used therapeutically, it is essential that rigorous functional assays be performed to definitively establish that they actually generate bona fide differentiated cell types. This is particularly true as there are experimental limitations to many of the assays that are commonly used. For example, some groups have found that bone marrow-derived cells can, under some conditions, undergo a rapid apparent neuronal transdifferentiation, but recent studies indicate that such 'neurons' are actually non-functional (Lu *et al.* 2004; Neuhuber *et al.* 2004). In addition, the apparent transition of bone marrow to neural, cardiomyocyte and hepatic lineages *in vivo* may be largely, if not completely, due to cell fusion (Alvarez-Dolado *et al.* 2003; Vassilopoulos *et al.* 2003; Wang *et al.* 2003; Nygren *et al.* 2004). Thus, it is essential to ensure the functionality of cell types generated from stem cells via a variety of assays with complementary strengths. In this regard, our approach has been to first pursue culture studies, looking for (i) appropriate morphological changes, (ii) selective induction and expression of cell type-specific genes, and (iii) stability of differentiated cellular phenotypes, and to then use both *ex vivo* and *in vivo* transplantation to assay functionality. We are currently pursuing such an approach to ascertain the validity and functionality of SKP-derived neural and mesodermal cell types, and will discuss our neural work in more detail here.

As mentioned previously, SKPs apparently generate peripheral neural progeny, consistent with a NC origin. Schwann cells are a peripheral glial cell type of therapeutic interest, owing to their ability to myelinate axons and to provide a conducive growth and regeneration environment for CNS axons (Richardson *et al.* 1980, 1984; David & Aguayo 1981; Xu 1995*b*, 1997, 1999; Pearse *et al.* 2004). To ask whether SKPs generate bona fide Schwann cells, we performed a series of culture and *in vivo* studies, all of which strongly support the conclusion that SKP-derived Schwann cells are 'real' (McKenzie *et al.* 2006). First, SKPs responded to extrinsic cues that have been shown to enhance the differentiation and the proliferation of Schwann cells from NC stem cells to produce cultures highly enriched in cells with the morphological and biochemical phenotype of Schwann cells; they generated parallel arrays of bipolar cells that expressed the Schwann cell markers S100β, glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), protein 0 (P0), peripheral myelin protein-22 (PMP22) and p75NTR (Fernandes *et al.* 2004). Importantly, they do not express the proteins characteristic of neurons or other mesodermal cell types. Second, when these SKP-derived Schwann cells were cultured in the presence of peripheral neural explants, they associated with axons, divided and expressed myelin proteins, while at the same time undergoing a morphological differentiation consistent with myelination. Third, when SKPs were transplanted into either the peripheral nerve or the CNS of *shiverer* mutant mice, which are genetically deficient in a key myelin protein, MBP (Rosenbluth 1980*a*,*b*), they myelinated axons, as shown by electron microscopy. Significantly, this myelination was also seen with human SKPs. Thus, by all criteria, including production of functional myelin, SKPs generate bona fide Schwann cells (McKenzie *et al.* 2006).

What about SKP-derived neurons? Differentiation of SKPs under neurogenic conditions has resulted in the production of cells that fulfil most, but not yet all, criteria for neuronal differentiation (Fernandes 2006). In culture, differentiating rodent SKPs undergo an appropriate sequence and time-course of changes, progressing from proliferating cells that express the NPC marker nestin to non-proliferating cells that adopt complex neuron-like morphologies and express pan-neuronal genes (βIII tubulin, neurofilaments, GAP-43 and MAP2). These SKP-derived neurons are probably peripheral in nature, since they express peripherin and proteins characteristic of a catecholaminergic phenotype, and almost all of them are positive for p75NTR, a hallmark of peripheral neurons. These putative neurons do not express any non-neuronal protein that we have examined. These SKP-derived neurons further mature when they are transplanted into slice cultures derived from the hippocampus, as they express and maintain expression of the catecholaminergic neurotransmitter enzyme,

tyrosine hydroxylase, for at least five weeks, and begin to express neuronal voltage-gated calcium channels. Interestingly, the neurons differentiated from SKPs maintain their peripheral phenotype, even in this CNS environment. However, the gold standard for demonstrating functional neuronal differentiation is appropriate electrophysiological characteristics, including the generation of depolarization-induced action potentials, something that we are starting to assay now. In general, reaching this standard using multipotent stem cells has proven to be difficult, requiring long and somewhat complex differentiation protocols and the presence of astrocyte-associated factors. Nonetheless, this is a necessary demonstration that will provide the ultimate proof that a given stem cell-derived cell is a bona fide neuron.

(c) *Potential clinical applications for human SKPs*

We are currently exploring several potential therapeutically relevant uses for SKPs, including autologous cell-replacement strategies, the effects of genetic alterations on stem cell function and differentiation and the potential involvement of transformed SKP-like precursors in tumours of NC-derived tissues.

(i) *Cell-replacement strategies*

The ability to differentiate SKPs into functional cell types has potential therapeutic implications for autologous cell-replacement strategies. As described above, a particularly promising avenue is cell therapy using SKP-derived glial cells, as we have generated considerable data indicating that SKPs can be used to produce functional myelinating Schwann cells which have potent growth-promoting effects on injured CNS neurons, and have extensively been used to promote regeneration of CNS axons in animal spinal cord injury models (Richardson *et al.* 1980; Paino *et al.* 1994; Xu 1995*a*,*b*, 1997, 1999; Guest *et al.* 1997; Menei *et al.* 1998; Oudega *et al.* 2001; Bunge & Pearse 2003; Pearse *et al.* 2004; Fouad *et al.* 2005). Moreover, the ability of Schwann cells to myelinate may make them useful replacement cells for oligodendrocytes that degenerate following spinal cord injury (Crowe *et al.* 1997; Shuman *et al.* 1997; Li *et al.* 1999; Casha *et al.* 2001; Warden *et al.* 2001; Beattie *et al.* 2002) or as a consequence of multiple sclerosis (Brierley *et al.* 2001; Halfpenny *et al.* 2002; Bachelin *et al.* 2005).

Besides neural cell types, we have also found that SKP cultures can be directed to generate skeletogenic derivatives normally associated with cranial NC cells. Specifically, SKPs reproducibly generate characteristic nodules that express chondrocyte oligomeric matrix protein and type II collagen, synthesize chondrocytic proteoglycans and ultimately produce mineralized calcium deposits associated with osteoblast activity (K. Fernandes, J. Biernaskie, J.-F. Lavoie and F. Miller 2002, unpublished data). Thus, SKPs may be useful as a source of certain mesenchymal derivatives.

(ii) *Mechanisms of disease development in genetic disorders*

In addition to transplantation-based therapies, the availability of accessible, autologous NPCs also opens novel doors to exploring the mechanisms and potential treatments of genetically based neural disorders. SKPs derived from humans having neurodegenerative conditions may serve as useful tools for investigating the effects of specific genetic alterations on the essential properties of NPCs (i.e. survival, proliferation and differentiation) or, alternatively, on the characteristics and functions of cells differentiated from mutant NPCs. Such studies on the cell biological properties of genetically compromised precursors, neurons or glial cells could provide novel information on the mechanisms of neurodegeneration. A similar approach has recently proved useful in defining the role of gut-derived NC stem cells in Hirschprung's disease (Iwashita *et al.* 2003).

(iii) *Involvement of SKP-like cells in tumours of neural crest-derived tissues*

One particularly exciting idea emerges from recent work documenting putative 'cancer stem cells' in paediatric neural tumours (Singh *et al.* 2003, 2004*a*,*b*). Such studies raise the possibility that transformed SKP-like 'neural crest cancer stem cells' may be important for the genesis of peripheral tumours of the NC. If this were the case, then cultures of transformed SKP-like cells isolated from biopsies could be expanded *in vitro* for drug screening and optimization of anti-cancer treatments. Conveniently, in at least some instances, tumorigenic and non-tumorigenic precursors could even be isolated from the same individuals, providing an ideal control tissue. In this regard, one candidate disorder is neuroblastoma (NB), which is the most common children's tumour and the most common extracranial paediatric solid tumour. NB affects migrating early NC precursors of the sympathoadrenal lineage and results in tumours whose primary sites are the adrenal glands and paraspinal sympathetic ganglia, with metastases to bone, lymph nodes, liver and even skin (Kushner 2004; Henry *et al.* 2005). A second candidate is neurofibromatosis type 1 (NF1; Lakkis & Tennekoon 2000; De Schepper *et al.* 2005). NF1 is a highly prevalent (1 : 3500) autosomal dominant disorder that results from mutations in the gene encoding neurofibromin. Clinical symptoms of NF1 are primarily observed in NC-derived cells and tissues, and include nerve sheath tumours, melanocytic hamartomas, Café au Lait spots, freckling in sun-protected areas and bone lesions. The presence of Schwann cell, fibroblastic and melanocytic tumour types in NF1 is particularly intriguing in light of the phenotypic instability between these NC-derived cells (Rizvi *et al.* 2002; Dupin *et al.* 2003; Real *et al.* 2005). A third class of disorders that may contain transformed SKP-like cells are tumours of the melanocyte NC lineage. The common dermal dendritic melanocytic proliferations contain embryonic-like spindled and dendritic melanocytes, and the classifications include dermal melanocytic hamartomas, several variants of benign blue naevi, malignant melanocytic tumours (malignant blue naevi) as well as borderline melanocytic tumours that appear to be of mixed neuroectodermal origin (Zembowicz & Mihm 2004).

5. IMPLICATIONS AND UNRESOLVED QUESTIONS

(a) *Relationship between SKPs and other skin stem cells*

Our findings that SKPs are a multipotent NC-related precursor that is laid down in the dermis during embryogenesis and persists into adulthood in a follicle dermal papilla niche raise a number of intriguing questions concerning possible physiological roles for SKPs.

One potential role for SKPs is in the regulation of the hair and whisker follicle growth cycle, given their niche in the follicle dermal papillae. The dermal cells that reside in these papillae are essential for the hair follicle growth cycle and have therefore been studied extensively. Interestingly, follicle dermal papillae are known to contain a resident precursor cell population: cultured papillary cells can differentiate into adipocytes and osteoblasts (Jahoda *et al.* 2003), and can even contribute to a small degree to the haematopoietic system in reconstitution experiments (Lako *et al.* 2002). *In vivo*, the cells within the follicle dermal papillae undergo marked changes during the hair cycle, increasing in number during anagen stages of hair follicle growth and decreasing during catagen stages of follicle decline. These changes may be partially due to the exchange of cells between the papilla and the nearby follicular connective tissue sheath (Tobin 2003*a*,*b*). The papilla cells are essential for hair follicle formation initially during embryogenesis and then on an ongoing basis in mature animals. During the normal hair cycle, potent papilla-derived signals such as Noggin and perhaps Wnts instruct the downward migration of epidermal stem cells residing within the hair follicle bulge, which then differentiate into new hair follicle cells and consequently generate a new hair shaft (Lavker *et al.* 2003). As a reflection of this essential regulatory role, transplanted dermal papillae are capable of inducing de novo hair follicle formation within the interfollicular epidermis (Reynolds & Jahoda 1992) and play a role in the conversion of adult corneal epithelium to follicular epithelium in response to embryonic dermis (Pearton *et al.* 2005). Our recent findings, therefore, raise a number of key questions in this regard. First, what proportion of the cells within the follicle dermal papillae are SKPs? Second, are SKPs the same cells as the previously characterized precursor cell population resident in this niche? Finally, are the precursor cells within the follicle papillae the same cells that regulate the hair follicle growth cycle?

SKPs may also provide a resident adult stem cell source for NC-derived cell types in the skin. In the face, the entire dermis is comprised of NC-derived progeny (Le Lievre & Le Douarin 1975; Nakamura 1982), while even in the thoracic regions, the dermis contains many different NC-derived cell types, including Schwann cells, Merkel cells and melanocytes. Intriguingly, Merkel cells are now known to be NC derived (Szeder *et al.* 2003), and previous work has demonstrated that the dermis contains a precursor cell capable of giving rise to Merkel cells *in vivo* (Nurse *et al.* 1984). Could SKPs be this resident dermal NC precursor cell? Given the multipotent nature of SKPs, they might also contribute to the mesodermal components of the dermis, including dermal fibroblasts, either during normal physiological turnover or during wound healing. In this regard, cells of the follicle dermal papillae and sheath have previously been proposed to participate in wound healing in the skin (Jahoda & Reynolds 2001).

In addition to questions regarding their potential physiological role, the elucidation of a novel precursor cell in skin also leads to questions about their interactions with other resident precursor cell populations. For example, MSCs are widespread throughout a variety of tissues (Young *et al.* 1995), including the dermal layer of the skin, where they are considered the source of the dermal connective tissue. Since MSCs themselves are embryonically derived from either the NC (cranially) or the mesoderm (sub-cranially), further work will be required to clarify the relationship between MSCs and SKPs. One possibility is that SKPs can differentiate in the dermis into a more mesodermally restricted precursor cell with the characteristics of MSCs. A second possibility is that these two populations of cells are independently generated within the dermis, with the MSCs contributing to mesodermal cell types, and SKPs representing a more multipotent precursor that also generates other NC-derived progeny found in the dermis.

Similar issues arise regarding SKPs and other known NC cells resident in the hair follicle. A number of NC-derived cell types are present within the hair follicle bulge and sub-bulge regions, including melanocyte stem cells (Nishimura *et al.* 2002), recently described epidermal NC stem cells (eNCSCs; Sieber-Blum *et al.* 2004), and non-innervated Merkel-like cells of unknown function (Narisawa *et al.* 1994). Melanocyte stem cells are clearly labelled in the bulge/sub-bulge area in dct–lacZ transgenic mice, and these generate transit-amplifying cells that differentiate into mature melanocytes in the hair follicle matrix immediately above the dermal papilla (Nishimura *et al.* 2002). Little is known regarding the functions of eNCSCs and non-innervated Merkel-like cells, though it has been hypothesized that they may serve a key regulatory function within this critical epidermal stem cell niche (Christiano 2004). Although SKPs and bulge NC-derived cells are present in different layers of the skin, it is possible that they are developmentally related and/or functionally interact, particularly given the regular emigration of cells from the bulge into the lower follicle and from the dermal papillae into the overlying connective tissue sheath (Taylor *et al.* 2000; Lavker *et al.* 2003; Tobin 2003*a*,*b*; Blanpain *et al.* 2004; Tumbar *et al.* 2004). It is even possible that under some conditions SKPs might give rise to more restricted NC precursors within the follicle such as melanocyte stem cells and/or might exchange with the eNCSCs in the bulge region. It also remains to be established whether any of these populations of NC precursors are related to recently described follicular cells that express a nestin–GFP transgene and that are reported to have the ability to differentiate into blood vessels, neurons and myelinating Schwann cells (Li *et al.* 2003; Amoh *et al.* 2004, 2005*a*–*c*).

(b) *Potential origins of SKPs*

Our recent data indicate that the first cells with SKP-forming ability arrive in murine back skin at E15, and that their numbers are high during late embryogenesis, decline somewhat into the early post-natal period, and then again in the adult (Fernandes *et al.* 2004). Given their probable NC origin, the most parsimonious explanation for this time-course is that the relevant NC precursors migrate into skin at E14/E15. In this regard, NC cells could arrive in the skin via a number of migratory routes. In mice, a first wave of trunk NC migration occurs at approximately E9–9.5, when crest cells migrate ventromedially from the neural tube (Serbedzija *et al.* 1990, 1994; Wilson *et al.* 2004). These cells generate diverse peripheral nervous system derivatives, including the peripheral nerves that eventually innervate targets that include the skin. Interestingly, multipotent NC stem cells (NCSCs) are present in the rodent peripheral sciatic nerve at E15 (Morrison *et al.* 1999), the time-point when (i) mouse back skin is first innervated (Peters *et al.* 2002), (ii) SKPs are first detected in mouse back skin (Fernandes *et al.* 2004), and (iii) the follicle dermal papillae first form from a population of cells expressing TrkC and p75NTR (Botchkarev *et al.* 1998, 1999), both of which are expressed on NC precursors (Stemple & Anderson 1992; Luo *et al.* 2003). Within the peripheral nerve, these NCSCs then apparently disappear by birth (Morrison *et al.* 1999), something that has been attributed to their terminal differentiation within the nerve itself. However, we propose that a subpopulation of these nerve NCSCs migrate into the skin, participate in the formation of the follicle dermal papillae, and then are maintained as SKPs into adulthood. Indeed, some evidence for nerve-derived NC cells populating the skin has been described in quail–chick chimera experiments (Halata *et al.* 1990). One prediction of this model is that other peripheral tissues might also contain SKP-like cells that are laid down during embryogenesis when they are first innervated.

Two other NC pathways could contribute NC precursors to skin. First, a second wave of trunk NC migration begins at approximately E10.5 (Serbedzija *et al.* 1990, 1994; Wilson *et al.* 2004), when crest cells delaminating from the neural tube shift into a dorsolateral migration pathway, taking them through the developing dermis, from where they ultimately migrate directly into the epidermis of the skin. Cells migrating along this pathway are predominantly of the melanocyte lineage (Wakamatsu *et al.* 1998). This migration reaches the dermis well before E15, and thus is unlikely to represent the predominant route for SKPs. Another potential source of NC-derived cells in skin is NC boundary cap cells. Boundary cap cells are NC cells that cluster at the sites of spinal and cranial nerve root entry/exit points into the CNS, i.e. the dorsal root entry zone and the ventral motor exit points. Embryonically, boundary cap cells help to regulate the passage of cells and axonal projections between the spinal roots and the CNS (Golding & Cohen 1997; Vermeren *et al.* 2003), but following the end of NC cell migration from the neural tube, they subsequently migrate distally down peripheral nerves, generating Schwann cells within the spinal roots and nociceptive neurons and satellite cells within the sensory ganglia (Maro *et al.* 2004; Hjerling-Leffler *et al.* 2005). Owing to the lack of specific boundary cap markers, it remains unclear how far distally these cells migrate, and whether they enter peripheral target tissues. However, it is possible that boundary cap cells correspond to previously identified cell populations that migrate along peripheral nerves to colonize sensory ganglia and skin (Sharma *et al.* 1995) and various peripheral target tissues (ventrally emigrating neural tube cells; Ali *et al.* 1999, 2003*a*,*b*; Sohal 1999*a*,*b*, 2002) such as trigeminal ganglia, facial cartilage, the gut, heart and vestibular system.

While we have focused here upon potential NC sources for SKPs, we have only formally shown that cranial SKPs are NC derived. Is it possible that non-cranial (thoracic) SKPs express NC-like characteristics without actually being embryologically derived from the NC? In this respect, it is worth noting the following points. First, there are a number of cell types and tissues that are produced developmentally by both the NC and the mesoderm, depending upon their location. For example, bone, cartilage and the entire dermis are produced by NC-derived mesenchymal cells in the craniofacial area (Le Lievre & Le Douarin 1975) and by mesoderm-derived mesenchymal cells in most of the rest of the body. Consistent with the notion that cells of different embryonic lineages can express similar genetic programmes, a recent study has shown that whisker follicle dermal papilla cells, which are NC derived (Fernandes *et al.* 2004), behave virtually indistinguishably from bone marrow-derived MSCs in their ability to expand and differentiate into bone, cartilage, fat and muscle (Hoogduijn *et al.* 2006). Thus, even though back skin dermal papilla express considerable NC-like properties, as further illustrated in a recent gene profiling study (Rendl *et al.* 2005), it is possible that they are derived from the mesodermal lineage.

Second, consider the examples of the vertebrate neurogenic placodes, particularly the olfactory, trigeminal, otic and epibranchial placodes (Graham & Begbie 2000). Developmentally, the neurogenic placodes are identified as focal regions of thickened cranial ectoderm located outside the neural plate (which is the source of the CNS and NC). These placodes give rise to a large percentage of cranial sensory neurons (including olfactory sensory neurons, trigeminal neurons and vestibular neurons), as well as a number of more specialized cell types (such as glial cells, olfactory epithelium, neuroendocrine cells and sensory hair cells). Thus, during normal development, neurons and glial cells can be generated from areas besides the CNS and NC. Can neurogenic placodes give rise to SKP-like cells? We have previously asked this question focusing upon the olfactory placode-derived olfactory epithelium. Using SKP culture conditions, we have isolated a population of cells from the P14 olfactory epithelium that (i) grows and can be expanded as spheres in response to FGF2 and EGF, (ii) expresses a marker profile similar to SKPs, including expression of nestin and fibronectin, and (iii) differentiates into neurons, glia and smooth muscle cells (J.G. Toma,

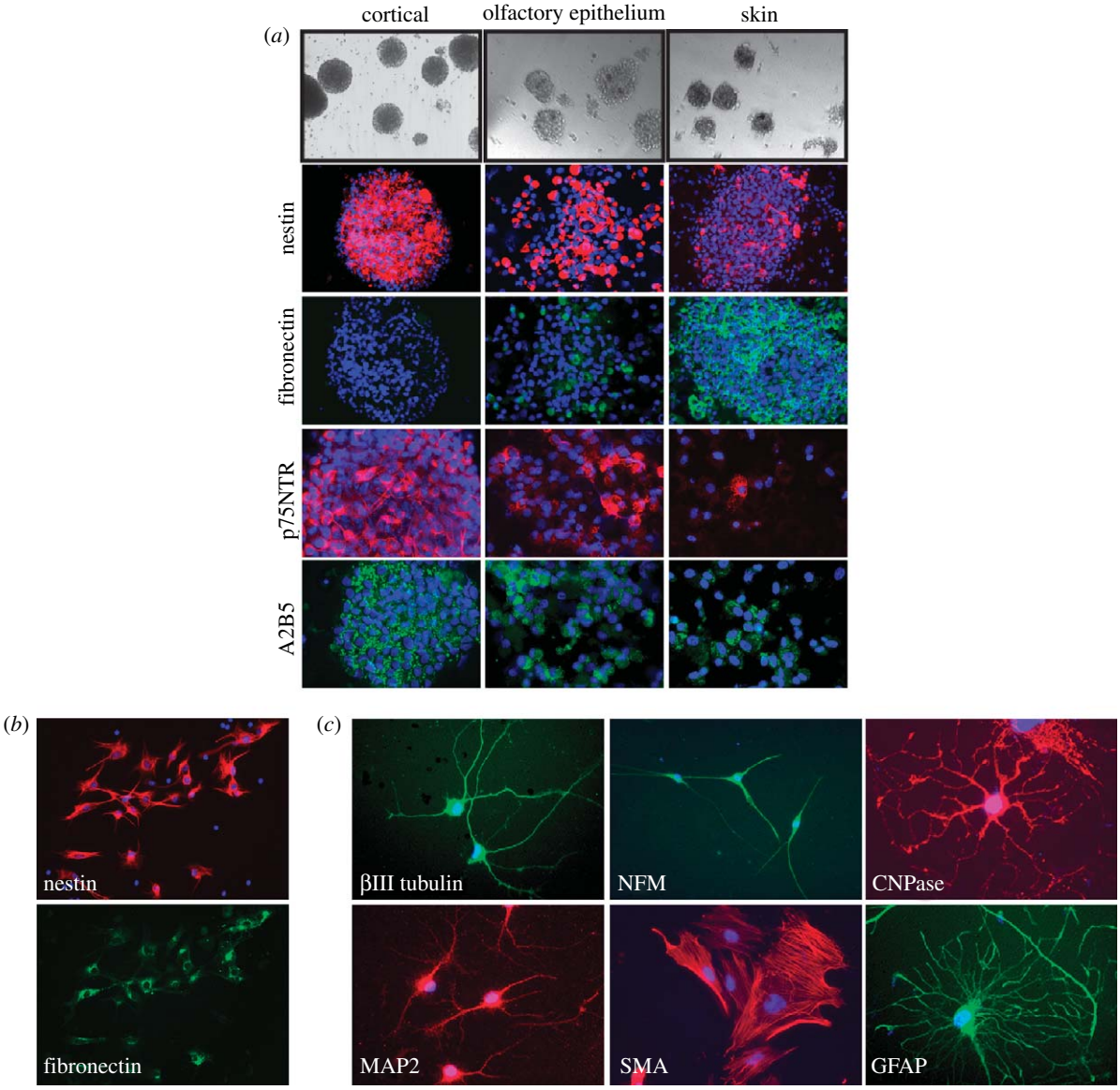

Figure 3. Characterization and differentiation of precursors isolated from the rodent olfactory epithelium. (*a*) Immunocytochemical comparison of spheres grown from embryonic rodent cortex, post-natal rodent olfactory epithelium and post-natal rodent skin. Note that olfactory epithelium-derived spheres have an immunocytochemical phenotype intermediate to cortical and skin-derived spheres. For example, while only very rare cells from SKP spheres were positive for the p75 neurotrophin receptor (p75NTR) and many cells from CNS cortical neurospheres were positive, a significant fraction of cells from the olfactory epithelium also expressed this receptor. (*b*) Cells differentiating for 1 day from olfactory epithelium-derived spheres co-express nestin and fibronectin, similar to SKPs. (*c*) After 2–3 weeks of differentiation, olfactory epithelium-derived spheres generate neurons that express βIII tubulin, MAP2 and NFM, smooth muscle cells that express SMA, and glial cells that express CNPase and GFAP. MAP2, microtubule-associated protein-2; NFM, medium neurofilaments; SMA, smooth muscle actin; CNPase, cyclic nucleotide phosphohydrolase; GFAP, glial fibrillary acidic protein.

A. Gloster, and F.D. Miller 1994, unpublished data; figure 3). Similar results have been obtained by others with the human olfactory epithelium (Zhang *et al.* 2004; Murrell *et al.* 2005). Thus, placodally derived structures contain a SKP-like cell, raising the possibility that SKPs themselves could potentially derive from an embryonic source other than the NC.

6. CONCLUSIONS

Regardless of embryonic origin, the finding that SKP-like cells reside in the adult olfactory epithelium, together with the possibility that SKPs migrate into peripheral tissues via embryonic peripheral nerves, raises the possibility that SKP-like cells may be present in many peripheral tissues. Further support for this idea derives from a recent report where SKP culture conditions were used to identify a multipotent cell from human dental pulp, a NC-derived tissue (Miura *et al.* 2003). Whether the SKP-like cells that have been isolated from these different tissues are similar in terms of their potency and/or origin is still an open question, but these findings do raise the provocative possibility that many adult tissues contain multipotent NC-related precursors that were laid down in those tissues during embryogenesis, and that have persisted into the adult.

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