The Neural Crest Origins of Skin-derived Precursors: An Accessible Source of Myelinating Schwann Cells

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August 15, 2005

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 978-0-494-25207-9
Our file Notre référence
ISBN: 978-0-494-25207-9

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Abstract

Skin-derived precursors are multipotent stem cells capable of differentiation into neural and mesodermal progeny. Described here is evidence that SKPs are of a neural crest origin; SKPs neural progeny are peripheral in subtype including peripheral catecholaminergic neurons and Schwann cells, SKPs express embryonic neural crest transcription factors, SKPs migrate via neural crest migratory pathways when transplanted into chick embryos and SKPs respond to factors know to influence neural crest stem cell differentiation in a similar manner. The expression of the neural crest transcription factors in situ identified the dermal papillae of hair follicles as a potential niche for SKPs in the skin. Cells isolated from microdissected vibrissae papillae generated spheres with SKPs properties when cultured under SKPs conditions and like SKPs, could be differentiated into neurons and smooth muscle. In agreement with the hypothesis that SKPs are of a neural crest origin, transgenic mice expressing β-galactosidase in all neural crest progeny are characterized by β-galactosidase expression in the dermal papillae, a potential SKPs niche and SKPs cultured from the whisker pads of these mice were also β-galactosidase-positive. In order to demonstrate the functionality of SKP progeny as a proof of principle of their utility for potential therapeutic cell transplantation therapies, SKPs and SKP-derived Schwann cells were transplanted into various injury and disease

models to assess their ability to myelinate. Cell culture protocols based on glial differentiation from the neural crest stem cell literature were adapted to selectively promote and expand Schwann cell differentiation from SKPs. SKP-derived Schwann cells transplanted into injured nerve were able to myelinate regenerating axons. SKPs and SKP-derived Schwann cells transplanted into congenitally dysmyelinated neonatal brains were able to form normal compact myelin. These data demonstrate the SKPs are a neural crest precursor that persists in adult skin that represents an accessible, autologous source of precursors capable of generating functional myelinating cells that could be used to treat disease and injury of the nervous system.

Resumé

Les cellules précurseurs dérivées de la peau (SKPs) sont des cellules souches au potentiel multiple. Nous décrivons ici des évidences selon lesquelles les SKPs originent de la crête neurale; la progéniture neurale des SKPs est de nature périphérique incluant des neurones cathécholaminergiques et des cellules de Schwann, les SKPs expriment des factors de transcription associés à la crête neurale, les SKPs migrent via le chemin migratoire de la crête neurale lorsqu'elles sont transplantées dans des embryons de poussins et les SKPs répondent à des factors qui influencent la différenciation des cellules souches de façon similaire. L'expression in situ de factors de transcription a établie la papillae dermale du follicule pilaire comme étant la niche des SKPs dans la peau. Des cellules isolées d'une vibrissae papillae ayant été micro-dissectée a généré des sphères ayant les propriétés des SKPs lorsqu'elles sont cultivées dans les mêmes conditions que les SKPs et comme les SKPs, elles peuvent se différencier en neurones et en muscle lisse. En accord avec l'hypothèse que les SKPs originent de la crête neurale, des souris transgéniques qui expriment l'enzyme β-galactosidase dans toute la progéniture de la crête neurale sont charactérisées par l'expression de β-galactosidase dans la papillae dermale, une niche potentielle pour les SKPs, et les SKPs cultivées à partir de la pulpe des moustaches de ces souris sont aussi positives pour

l'enzyme β-galactosidase. Dans le but de démontrer que la progéniture des SKPs est fonctionelle afin de prouver que par principle elles ont une utilité potentielle dans les thérapies de transplantation cellulaire, des SKPs et des cellules de Schwann derivées de SKPs ont été transplantées dans des modèles variés de blessures et de maladies afin de determiner leur abilité à myéliniser. Des protocoles de culture cellulaire tirés de la littérature traitant de la différenciation de la glia à partir de cellules souches de la crête neurale ont été adaptés afin de promouvoir et d'accroître la différenciation des cellules de Schwann à partir des SKPs. Des cellules de Schwanns derivées de SKPs ont été transplantées dans un nerf blessé et ont été capables de myéliniser les axones en regénérescence. Des SKPs et des cellules de Schwann derivées de SKPs ont été transplantées dans des cerveaux néonataux démyélinisés de façon congénitale et ont été capables de former de la myéline compacte normale. Ces données démontrent que les SKPs sont des précurseurs de la crête neurale qui persistent dans la peau de l'adulte et qui représentent une source accessible et autologue de précurseurs capables de générer des cellules myélinisées fonctionelles qui pourront peut-être être utilisées pour traiter certaines maladies et blessures du système nerveux.

Acknowledgements

I would first and foremost like to thank Freda Miller for taking me on as a student at a time when my masters project wasn't going well and my lab at the time was closing down. It was a time of uncertainty for me and I will always be grateful for the generosity that she extended to me. I'd also like to thank Freda for all her guidance and support over the years, and for providing an environment in the lab that allowed me to grow both as a student and a person.

I would also like to thank David Kaplan, who when needed took an active role in my time at the lab. He always treated me in a fair and professional manner and though I suspect that he thinks his career advice may have fallen on deaf ears, it was much appreciated. Thanks go also to the SKPs team, especially Karl Fernandes and Jean Toma for all their help along the way.

I would also thank the members of my advisory committee Tim Kennedy and Stefano Stifani as well as my mentor Andrea Leblanc, who helped with all matters official. I'd also like to say a special thanks to Tim for his continuous support, by both writing an endless supply of reference letters for all the fellowships I didn't get, and for his undying enthusiasm for science which can't help but be infectious. Meeting and working with Tim was one of my first interactions with a "Boss", and that he clearly loved what he was doing was a great inspiration to me.

During my time in the lab I also made some great friends who helped along the way, be it by helping to polish off pitchers of Griffon Rousse at the Thompson house or by otherwise distracting me when experiments went horribly wrong.

Greg Walsh, G-money, my first friend and partner in crime in Montreal. Already a veteran grad student by the time we met, Greg showed me the ins and outs of grad student life; Tuesday night is just a good a night as any to go drinking, especially when its hip-hop night at Angel's; keep them waiting a for a while for the next house party, that way it'll be even more hype; and the "sidle" were just a few of Greg's sagely pearls of wisdom. I remember one of the first conversations I had with Greg was something along the lines of: "Do you play vids?" "Yep." "Wanna come over, play some N64 and drink some beers?" "Word." I knew at that moment that we'd become fast friends. Moving into 300 Carré St. Louis was the best decision I ever made and those parties will go down in infamy.

Karun Singh, fellow baller and Steel-town homie. Karun and I played ball at the gym in Montreal nearly everyday, best ball of my life. Special-K was quick to join the Montreal posse and was an invaluable addition. We tore it up man.

Jeff Biernaksie, a future leader of tomorrow, whose other nick-name I can't write here but I'm sure he knows which one I'm thinking of. I owe Jeff a lot of thanks and probably more beers for all his help on

various projects. I wouldn't have been able to get those damn cells to myelinate without him. Jeff also became a great friend of mine, him and Karun made the Toronto years a blast. Now that I'm out of the lab I'm sure Jeff and K's bowels are recovering from all the chilli-chung-pow I dragged them to at 4 in the morning.

Brad, with whom the lunch-time posse was complete. I owe Brad a special thanks for taking care of me at the St. Mike's emergency room when my hand was in danger of amputation from a cat bite. Needless to say the doctor's lounge is much nicer than the ER hallway, and has cable.

I'd also like to send a shout out to Nina Orike, who became the big sister that I never had. Nina had one of the sweetest apartments ever on St. Laurent, and chillin' on the hammock on the balcony while chowin' down on some jerk chicken was a favourite pastime of mine. Much love sis.

Thanks also go out to the Quebecois contingent in the lab; Anne, who both helped with my project, didn't laugh at my French and is possibly the nicest person you'll ever meet, Annie who started in the lab as my summer student and also tried to "learn me" some French over Friday night beers at the Thompson house; Fannie, someone from whom one can't help but absorb some French speaking ability and who also exposed us Tête-Carré's in the lab to the occasional mechoui, and francophone nightlife in Montreal and finally Mathieu Fortier who taught me the art of swearing in French.

My family definitely deserves special thanks. My sister Amy who came to visit Montreal a few times, thanks for all your love and support. I'd also like to thank my Aunt Joanne, who kept me in touch with a steady supply of emails from home and also bailed me out of a nasty Vet bill back in undergrad.

Alan, my father and friend. His pride in me for what I was doing in school always made me feel special. He was always behind me, being a parent despite the fact that I'm now in my late 20's, and is the best Dad a guy could ask for. Janet, my mother, where do I begin? Without her constant cash infusions I'd have been eating Kraft dinner for the past 6 years and probably been living in a box over a sewer after I moved back to TO. I'll always be your 'tiger' Mom (a nick-name that the boyz now enjoy at my expense). Her love and support always keep me going. How can I fail with such wonderful people holding me up?

Last and certainly not least, a special thanks and much love go out to my Baby, Karine. Karine put up with me (and my cat Zeus, yes the same cat that caused a near amputation) while writing my thesis. She's also doing her best to make me bilingual, but until then, was more than willing to translate my abstract for me. Karine provides me with endless unconditional support and praise, and her capacity to love is a constant source of amazement to me. Karine, Je t'aime fort avec tout mon coeur.

Contributions of Authors

Chapter 2: A dermal niche for multipotent adult Skin-derived Precursor cells

I participated in the writing and preparation of the manuscript in collaboration with Dr. Freda Miller and Dr. Karl Fernandes and performed many of the experiments. See below for a specific explanation of my contributions by figure:

Figure 1: I performed the cultures and sample preparation and the RT-PCR shown in panels a, b and d. I also performed the differentiation and immunostaining shown in panel f.

Figure 3, panel d: I performed the initial embryonic SKPs cultures, designed the experiment investigating the number of SKPs spheres generated as a function of age through discussion with Dr. Karl Fernandes and Dr. Nao Kobayashi and subsequently performed the first experiments which were later repeated by K.F and N.K.

Figure 4: I generated some of the *in situ* probes used in panels a-t. The *in situ* hybridizations were performed by Dr. Pleasantine Mill. I performed the cultures and sample collection for the RT-PCR shown in panel u.

Figure 5: I generated some of the *in situ* probes used in panels a-u. The *in situ* hybridizations were performed by P.M.

Figure 6: I performed the cultures and sample collection and the RT-PCR shown in panel a. I performed the cultures, differentiations and immunostaining for panels b-f.

Figure 7: I generated some of the *in situ* probes used in panel b. The *in situ* hybridizations and staining were performed by Dr. Pleasantine Mill.

Figure 8: I performed the cultures, differentiations, immunostaining and β-galactosidase staining for all panels.

Supplementary Figures: I performed the RT-PCR in figure S1, panel b. I performed the hair follicle cell, SKPs mixing experiments shown in figure S2, panel f. I performed all the cultures, differentiations and immunostaining shown in figure S3, panels a-h.

Chapter 3: Skin-derived Precursors generate myelinating Schwann cells for the injured and dysmyelinated nervous system

I wrote the manuscript in collaboration with Dr. Freda Miller and performed all of the experiments with the following exceptions; All surgeries were performed by Dr. Jeff Biernaskie, who also performed some immunostaining on sectioned nerves. Robert Temkin was involved in sample preparation for electron microscopy and operation of the electron microscope. Dr. Jean Toma cultured human SKPs and provided them for other experiments. Dr. Rajiv Midha provided training and guidance with regards to the sciatic nerve surgeries.

General Introduction

Stem cells have been intensively investigated over the last decade due to their potential clinical application for the treatment of disease and injury. Perhaps most promising with respect to therapeutic potential are embryonic stem cells, cells derived from early embryos that are capable of generating all the cell types of the embryo proper. The derivation and use of these cells however is surrounded by ethical debate. Stem cells have also been discovered in a variety of adult tissues that persist for the lifespan of the organism and may provide an alternative to embryonic stem cell sources. Although initially thought to have a differentiation potential restricted to that of their tissue of origin, some adult stem cell populations have recently been shown to exhibit unexpected potency. The use of adult stem cells as a source of cells for various clinical applications would bypass the ethical issues surrounding the derivation and use of embryonic stem cells. In order for stem cell therapies to be realized, a tissue source should be plentiful and accessible and the stem cells themselves should ideally be expandable *in vitro* to allow for generation of material for autologous transplantation therapies that would not require the immunosuppression of the patient. The skin is one such accessible source of adult stem cells, termed Skin-derived precursor cells (SKPs), that were first described by Toma *et al.*, in 2001. These stem cells were able to generate neural and mesodermal progeny, including cell types not normally observed in skin. The general aim of this thesis is

to further characterize SKPs and their differentiated progeny as a first step in their realization in stem cell-based therapies.

Rationale and Specific Aims of Research

Specific Aim 1: What is the developmental origin of SKPs? Given that SKPs generate neural and mesodermal cell types, we hypothesize that they are derived from the neural crest, a transient embryonic structure in vertebrate development that also gives rise to neural and mesodermal progeny. To test this hypothesis we evaluated the gene expression, differentiation and migratory potential of SKPs as compared to neural crest cells. We also examined a transgenic mouse line in which the neural crest lineage is marked to assess whether SKPs derived from these animals express the transgene and are therefore neural crest-derived.

Specific Aim 2: Do SKPs have an endogenous niche within the Skin? It has been previously shown that when dermis and epidermis are isolated, SKPs could be generated only from the dermis, demonstrating that the cells in the skin that give rise to SKPs are located there. Can we more specifically identify a structure in the dermis that serves as a niche for SKPs and are there cells endogenous to the skin with SKPs-like properties? In order to answer these questions we examined the skin for

expression of genes characteristic to SKPs identified under specific aim 1. If the expression of SKPs markers identifies a specific structure within the skin, can this structure be isolated and demonstrated to contain cells with SKPs-like activity? Namely, can they generate SKPs spheres and differentiate into cell types normally generated by SKPs.

Specific Aim 3: Are SKPs-derived Schwann cells functional and capable of myelination in models of injury and disease? There is much interest in the clinical application of stem cells. In order to realize the therapeutic potential of SKPs, our specific aim was to assess the functionality of one therapeutically relevant SKP-derived cell type, Schwann cells. The transplantation of Schwann cells has been shown to promote regeneration of the injured spinal cord and peripheral nerve as well as remyelinate the demyelinated CNS. We propose to identify cell culture protocols to maximize Schwann cell yield from cultures of differentiating SKPs and to demonstrate their functionality *in vivo* after transplantation into the injured peripheral nerve and dysmyelinated brain.

Chapter 1: Introductory Literature Review

Introduction to Stem Cells

Stem cells can be defined as cells that divide such that they are capable of self-renewal, the generation of at least one daughter cell with the same properties as the original cell, and the generation of a number of more differentiated progeny (Weissman et al., 2001). Different types of stem cells vary in their potentiality, ranging from embryonic stem (ES) cells that are termed pluripotent due to their ability to generate all the cell types of the embryo proper, to stem cells with more restricted potentiality such as neural stem cells that differentiate only into the cell types of the nervous system, neurons, astrocytes and oligodendrocytes. Adult stem cells (also referred to as tissue-specific stem cells) are those that persist potentially throughout the lifetime of the organism, and are thought to be important for the maintenance and/or regeneration of the tissues in which they reside. There has been much recent interest in adult stem cells, as their potential clinical application would circumvent the ethical concerns raised by the use of embryonic stem cells and the sources thereof.

Some of the best-characterized examples of adult stem cells are derived from tissues that normally exhibit a high cellular turnover and/or a high capacity for regeneration. Hematopoietic stem cells for example, have been prospectively isolated from the bone marrow and are responsible for the generation of all the cell types of the hematopoietic

system throughout the lifespan of the organism. As evidence to their regenerative capacity, a single hematopoietic stem cell is capable of the reconstitution of the entire hematopoietic system of lethally-irradiated animals (McCulloch and Till, 1960; Osawa et al., 1996). Epithelial tissues, such as the skin and the gut, which undergo constant turnover and as such require the constant generation of new cells, also contain stem cells that are thought to be responsible for the maintenance of those tissues [reviewed in (Leedham et al., 2005; Morasso and Tomic-Canic, 2005)].

The study of the stem cells in the hematopoietic system has helped to define some of the key concepts in stem cell biology. Through the use of a panel of cell markers and fluorescent activated cell sorting (FACS) analysis, the various lineages of the hematopoietic system have been identified and more importantly, the existence of intermediate precursors that are characterized by a reduced capacity for renewal and a more restricted differentiation potential was demonstrated [Reviewed in (Weissman et al., 2001)]. The stepwise progression of differentiation and generation of precursors with increasingly restricted differentiation potential, combined with the finding that these more restricted precursors were incapable of reconstituting the entire hematopoietic system after transplantation into lethally-irradiated animals led to the proposition that stem cell progeny became increasingly committed over time and that the process of differentiation was irreversible. This notion of stem cell differentiation proceeding through a number of irreversible steps holds

true in most instances, although cases of dedifferentiation, the process by which a differentiated cell or restricted precursor reverts to a state of greater potentiality, have been described.

One example of dedifferentiation is the reversion of oligodendrocyte precursor cells (OPCs) to a cell with properties of multipotent neural stem cells that can be differentiated into neurons and astrocytes as well as oligodendrocytes (Kondo and Raff, 2000). OPCs, which normally differentiate from multipotent neural stem cells and can be considered a restricted precursor, typically divide only a few times before terminally differentiating into mature oligodendrocytes. While *in vivo* their potential is restricted to that of an oligodendrocyte fate, *in vitro*, the treatment of OPCs with fetal calf serum can also induce astrocyte differentiation (Raff et al., 1983). Kondo and Raff demonstrated that after a 3 day pulse of purified OPC cultures with 15% serum followed by culture in fibroblast growth factor 2 (FGF2), cells were generated that could be grown as neurospheres and differentiate into not only astrocytes and oligodendrocytes, but also neurons. These experiments demonstrated that a restricted unipotent precursor cell, in this case OPCs, were able to regress to a more primitive multipotent neural stem cell and provided an example of dedifferentiation or reprogramming.

While it was generally believed that tissue-specific stem cells were restricted to differentiate into the cell types of the tissue from which they were derived, a number of studies have brought this dogma into question

and suggested that stem cells may have a greater plasticity than previously appreciated and be able to transdifferentiate, generating cell types from tissues other than their tissue of origin. Bone marrow cells for example, were shown to migrate into the brain after intravenous injection and to express neuronal markers (Brazelton et al., 2000; Mezey et al., 2000). In addition to repopulating the hematopoietic system of lethally-irradiated hosts, single purified hematopoietic stem cells (HSCs) were shown to differentiate into liver, lung, gut, and skin (Krause et al., 2001). Though the initial interpretation of these data suggested that HSCs were capable of a broader differentiation potential than previously thought, the demonstration that bone marrow cells could fuse with other cells and acquire their properties raised doubts as to the validity of these conclusions (Terada et al., 2002; Alvarez-Dolado et al., 2003; Wang et al., 2003). Similarly, while neural stem cells have been claimed to reconstitute the hematopoietic system (Bjornson et al., 1999) and more impressively, to contribute to all germ layers in chick and mouse chimeras (Clarke et al., 2000), the reproducibility of hematopoietic reconstitution by neural stem cells has been questioned (Morshead et al., 2002) and the possibility of cell fusion can still not be entirely excluded (Joshi and Enver, 2002).

Perhaps one of the most striking examples of adult stem cell plasticity is that of multipotent adult progenitor cells (MAPCs) (Jiang et al., 2002). By selecting for cells negative for the hematopoietic markers

CD45 and GlyA, rare cells from a variety of tissues including blood, brain and muscle, were identified that could give rise to clonally-derived colonies when grown in the presence of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) [and leukemia inhibitory factor (LIF) in the case of rodent cells] (Jiang et al., 2002). These cells could then be expanded for 120 population doublings without transformation, differentiate into all three germ layers *in vitro* and contribute extensively to a variety of tissues after injection into blastocysts (Jiang et al., 2002). While the possibility of fusion cannot be completely discounted, the *in vitro* differentiation potential and robust frequency and contribution in chimeras make fusion an unlikely explanation for these observations (Jiang et al., 2002). It has been suggested that the unexpected potentiality of MAPCs could be a result of reprogramming *in vitro* after extensive proliferation. Alternatively, MAPCs, which express the ES cell markers Oct-4 and Rex-1, may represent ES cell-like stem cells that persist in adult tissues throughout life (Jiang et al., 2002).

Neural Stem Cells

It was long held in the field of developmental neuroscience that neurogenesis, the creation of new neurons, occurred only during development and that once neurons had made their appropriate connections throughout the brain, no new neurons were added. Anecdotally, I can recall hearing as a child that I would have all the

neurons I would ever have by the age of 12, and that after that, all I had to look forward to was a slow and steady decline as my neurons began to wear out and die. This notion had become dogma in the scientific community despite early studies that demonstrated newly born cells with neuronal morphology in the hippocampus and olfactory bulb by radioactively labeling mitotic cells with tritiated thymidine in the adult brain (Altman and Das, 1965; Altman, 1969). It was difficult to make irrefutable conclusions about adult neurogenesis at the time however, due to a lack of means to definitively identify the newly born cells as neurons with cell markers etc (Emsley et al., 2005).

It wasn't until decades later, when it was shown that cells from the adult brain could differentiate into neurons *in vitro* (Reynolds and Weiss, 1992) that interest in the possibility of adult neurogenesis was rekindled. Both adult and embryonic neural stem cells were shown to form floating clusters of cells termed 'neurospheres' when cultured in defined media containing EGF and/or FGF2 (Reynolds et al., 1992; Tropepe et al., 1999). The formation of neurospheres *in vitro* proved to serve as an important functional assay for stem cells. Self-renewal can be easily assayed by the capacity of single cells in a neurosphere to clonally generate secondary neurospheres. Neural stem cells will clonally form neurospheres at cell densities of 10000 cells/ml or less in liquid culture as determined by cell mixing experiments (Tropepe et al., 2000). Multipotentiality can be assessed by differentiating clonally derived

neurospheres and observing the presence of neurons, astrocytes and oligodendrocytes by immunocytochemistry for markers specific to those cell types. With robust assays to examine self-renewal and multipotentiality, it becomes possible to examine factors that regulate these processes. For example, the role of the transcriptional repressor Bmi-1 in stem cell biology was examined through the use of *in vitro* neurosphere assays (Molofsky et al., 2003). Neurospheres were formed from both brain and gut tissue derived from Bmi-1 knockout mice, it was found that neurospheres cultured from the knockout mice generated fewer secondary neurospheres as compared to wild type animals, indicative of decreased self-renewal. Although stem cell self renewal was affected, the proliferation of restricted neuronal or glial precursors was unaffected, as the frequency of clones generating neurons or glia only remained unchanged (Molofsky et al., 2003).

The demonstration that neurogenesis occurs throughout adult life and not just during a small defined period during development raised the possibility that the adult central nervous system may have a greater capacity for regeneration than originally anticipated. Furthermore, a greater understanding of the properties and normal behaviour of adult stem and precursor cells in the brain, may allow for the development of strategies to augment its natural regenerative capacity for the treatment of injury and disease. The adult dentate gyrus and subventricular zone are two brain structures known to generate neurons throughout the life

of the organism. In the case of the dentate gyrus, progenitors from the subgranular zone migrate to the dentate granule cell layer (Cameron et al., 1993) where they integrate into hippocampal circuitry and may play a role in learning and memory (Shors et al., 2001). Similarly, neural stem cells that reside in the subventricular zone, differentiate into neuroblasts that migrate via the rostral migratory stream to the olfactory bulb (Altman, 1969; Lois and Alvarez-Buylla, 1994), where they terminally differentiate into neurons and may play a role in olfactory memory (Rochefort et al., 2002).

It was subsequently shown that although under normal conditions, neurogenesis was restricted to discrete regions of the adult brain, stem cells could be isolated from a variety of other regions of the adult brain such as the cortex, and even from areas of white matter such as optic nerve and the corpus callosum, that were capable of neuronal differentiation *in vitro* (Palmer et al., 1999). Furthermore, despite an apparent lack of adult neurogenesis in the cortex, a targeted induction of neuronal death by apoptosis in the cortex is sufficient to induce neurogenesis in this region (Magavi et al., 2000). In this study, apoptosis was induced in a specific population of neurons by the injection of a photosensitive toxin in a region to which the neurons of interest project their axons. The toxin was retrogradely transported to the cell body where it was later activated by exposure to lights. This non-invasive means of inducing apoptosis in the neurons ensured that

no collateral damage was done to other cell types in the region. The authors observed the appearance of new neurons within the damaged area of the cortex, as visualized by cells exhibiting BrdU incorporation that also expressed neuronal markers. In addition, these neurons received innervation and also projected axons to the targets that the original neurons had innervated. This study demonstrates that even regions of the brain without any apparent constitutive neurogenesis may have an unappreciated capacity to respond to injury, perhaps one that can be further augmented, and generate new neurons that functionally integrate into the brain (Magavi and Macklis, 2002).

Neural Crest Development

The neural crest is a transient embryonic structure unique to vertebrates, contributing to greatly varied tissues including the peripheral nervous system, adrenal gland, skin, teeth, heart and head. The term neural crest refers to the cells at the most dorsal aspect of the neural tube around the time of tube closure and was first observed by Swiss anatomist Wilhelm His (His, 1868). The presumptive neural crest is specified even before the formation of the neural tube, at the time of neural induction, a process by which neural tissue is first induced in the ectodermal layer of the developing embryo. In this process, the Spemann organizer (Spemann and Mangold, 1924), consisting of dorsal mesoderm, releases bone morphogenic protein (BMP) antagonists

including noggin (Smith and Harland, 1992), chordin (Piccolo et al., 1996) and follistatin (Hemmati-Brivanlou et al., 1994), which inhibit the activity of ectodermally-derived BMP that has an inhibitory effect on neuralization. To summarize, the default fate of ectoderm is neural and neural induction can be described as an inhibition of an inhibitory signal. The tissue at the border of the ectoderm and the newly formed neural plate forms the presumptive neural crest. Embryonic grafting experiments in which ectoderm was transplanted into the developing neural plate demonstrated that neural crest was induced at the ectopically formed borders of ectoderm and neural tissue (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995). Similarly, when neural plate tissue is co-cultured with epidermal ectoderm, neural crest is generated *in vitro* (Liem et al., 1995).

While strong inhibition of BMP signaling results in the formation of the neural plate, intermediate or low levels of inhibition may specify a neural crest fate. This has been demonstrated in developing xenopus embryos, in which injection of low levels of dominant-negative BMP receptor resulted in the induction of neural crest markers while injection of high levels induced markers of neural plate but not neural crest (Marchant et al., 1998). It has been proposed that a gradient of the neuralizing factors secreted by mesoderm results in the induction of the neural plate at high concentrations and induction of the neural crest at low concentrations (Marchant et al., 1998).

The transcription factors slug and snail are amoungst the earliest known markers of the neural crest and are expressed as early as the formation of the neural plate (Essex et al., 1993; Nieto et al., 1994). These transcription factors appear to play an active role in the induction of neural crest as their ectopic expression leads to an expansion of the neural crest in the embryo (del Barrio and Nieto, 2002). Other transcription factors are expressed later in neural crest development and are involved in their proper migration and differentiation. Twist is a basic helix-loop-helix transcription factor that is important for early mesodermal development during gastrulation and then later is involved in the migration and differentiation of the cranial neural crest (O'Rourke and Tam, 2002; Soo et al., 2002). The transcription factor Pax3 was identified as the gene responsible for the *splotch* mutation in mice and has been shown to have multiple roles in neural crest development. Pax3 has been shown to be responsible for the migration and expansion of neural crest precursors that contribute to melanocytes and cardiac development (Conway et al., 2000; Hornyak et al., 2001) and to be required for the development of the enteric nervous system (Lang et al., 2000).

After neural induction has occurred, the neural plate invaginates bringing together the neural folds in a process called neurulation. At the time of tube closure (in Chick and Xenopus embryos) or just prior (in Mouse embryos), cells begin to delaminate from the dorsal neural tube and migrate into the embryo. Some of the early migrating cells from the

dorsal neural tube are not yet entirely committed to a neural crest fate, and if these cells are transplanted back into the ventral neural tube they are capable of generating ventral neural tube cell types, including motor neurons and floor plate cells (Ruffins et al., 1998). Furthermore, when single cells within the dorsal neural tube are labeled, it can be observed that their progeny can contribute to both the spinal cord and neural crest tissues demonstrating that neural tube cells and neural crest cells can share a common progenitor (Serbedzija et al., 1994).

The contributions to the organism by the neural crest has been studied most extensively in the avian system, thanks to the amenability of avian embryos to the generation of chimeric animals. Through the use of quail neural tube donors transplanted into chick embryos, the contribution of quail neural crest cells to the host embryo could be tracked on the basis of characteristic differences between quail and chick heterochromatin and nuclear structure (Le Douarin et al., 1975). By transplanting small segments of neural tube from specific levels of the anteroposterior axis, it was observed that neural crest cells made contributions to various tissues in a manner specific to their level of origin. While the pigmented cells of the skin, melanocytes, are derived from the entire length of the neural crest, the various ganglia of the peripheral nervous system are derived from specific regions. For example, the enteric ganglia are derived specifically from the anterior cervical spinal regions as well as from the levels of the lumbrosacral spinal cord (Institut

d'Embryologie du Cnrs Nogent-sur-Marne, 1977), while the cardiac outflow tract of the heart is populated by neural crest cells primarily from the axial levels of somite 1 and 2 (Chan et al., 2004). As in the trunk, the cranial neural crest cells from different levels along the neural tube axis make specific contributions to the bones, cartilage, dermis and connective tissue of the head, face and neck, as well as to meninges, pericytes and the endothelium of the blood vessels of the forebrain (Bronner-Fraser, 1995; Etchevers et al., 2001).

The avian system also allowed for the cross-transplantation of segments of neural crest from specific levels along the anteroposterior axis to different levels, in order to examine the commitment and plasticity of the neural crest cells. For example, if neural crest removed from levels that colonize the gut and normally generate the enteric nervous system were transplanted to a region involved in the development of the adrenal glands and sympathetic ganglia, would the neural crest cells migrate and differentiate in a manner appropriate to their new location on the anteroposterior axis, or would they migrate abnormally and/or generate progeny according to their origin? Precisely this experiment was performed using a chick/quail chimera system and it was found that cells were able to respond to the environmental cues of their new environment by migrating and differentiating in a manner appropriate to their newfound location on the anteroposterior axis (Le Douarin et al., 1975; Fontaine-Perus et al., 1982). Similar results were observed in the analysis of glial

subtypes. Specifically, the generation of Schwann cells or enteric glial cells depends on the differentiation environment and not on the origin of the neural crest cells on the neural axis from which they were derived (Dulac and Le Douarin, 1991).

A collection of *in vitro* studies compliments these *in vivo* studies of plasticity and demonstrates the heterogeneous nature of the neural crest cell population. By clonal analysis it has been shown that in both the avian and rodent systems, that neural crest migratory cells are a mixed population of restricted precursors that give rise to single cell types as well as multipotent precursors that give rise to two or more cell types (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988; Stemple and Anderson, 1992; Ito et al., 1993). Furthermore, the *in vitro* cell biology appears to mirror what has been observed *in vivo* with respect to the timing of migration of particular precursor types. The earliest migrating trunk neural crest cells migrate ventrolaterally and give rise to the neurons and glia of the peripheral nervous system while later migrating neural crest cells follow the dorsolateral migratory stream and generate the melanocytes of the skin (Serbedzija et al., 1989; Erickson et al., 1992). This is recapitulated *in vitro* as few melanocyte producing colonies are observed from the initial wave of cells migrating from neural tube explants, whereas later migrating cells readily generate melanocytes (Henion and Weston, 1997). Neural crest cells *in vitro* also show considerable plasticity with respect to their anteroposterior identity.

Cranial neural crest cells are the only neural crest cells that normally contribute to bone and cartilage during development, but when trunk neural crest cells are cultured in conditions known to support the differentiation and survival of cartilage and bone, they are capable of differentiating into chondrocytes and osteoblasts (McGonnell and Graham, 2002). Complimentary *in vivo* studies demonstrated that trunk neural crest cells transplanted into the head were also able to respond to local cues and differentiate into cartilage-forming chondrocytes (McGonnell and Graham, 2002).

While these works demonstrated that the fate of neural crest cells is largely determined after they migrate from the neural tube, it raised questions as to whether uncommitted neural crest cells persist throughout migration and in target tissues. When fragments of dorsal root ganglia from an early stage of quail development (Day 4-5) were transplanted back into the neural crest migratory streams of younger chicks (Day 2), a subpopulation of cells was able to migrate normally and generate cell types beyond those normally found in the DRG (Le Lievre et al., 1980; Schweizer et al., 1983). All post-mitotic neurons did not survive the transplant, but a population of precursors that remained undifferentiated, were able to migrate to various targets and generate appropriate progeny including autonomic catecholaminergic neurons, adrenomedullary cells, sensory neurons in the dorsal root ganglion (DRG) (Schweizer et al., 1983; Dupin, 1984) and Schwann cells (Fontaine-Perus et al., 1988). The

presence of cycling precursors in the donor ganglion tissue correlates with the ability of donor cells to generate varied cell types, as after all the neurons in the ganglia have entered mitotic arrest, the plasticity is no longer observed (Dupin, 1984). Similarly in the rodent system, neural crest stem cells capable of generating neurons, glia and myofibroblasts can be prospectively isolated from the developing peripheral nerve by flow-cytometry, but only up until E16 (Morrison et al., 1999). Based on these findings it was largely thought that neural crest stem cells were indeed a transient cell population that became increasingly committed through time and ceased to exist during embryonic development.

More recently however, studies have demonstrated the persistence of neural crest stem cells through adulthood in a variety of neural crest derived tissues. Multipotent neural crest cells capable of generating neurons, glia and myofibroblasts can be isolated from post-natal and adult gut by FACS (Kruger et al., 2002). While stem cells could still be isolated, they do however lose the capacity to generate some types of neurons that are normally generated embryonically during the development of the enteric nervous system (Kruger et al., 2002). It is thought that this occurs due to a reduction in responsiveness to neurogenic factors such as BMP-4 and to an increase in responsiveness to gliogenic factors such as the notch-ligand delta (Kruger et al., 2002).

Another neural crest-derived tissue from which cells with neural crest potentiality can be derived is the dental pulp. Much of the dental

tissue and surrounding supportive tissues are neural crest-derived (Miletich and Sharpe, 2004) and several studies have demonstrated the differentiation of neurons and glia from cultured dental papilla cells (Gronthos et al., 2002; Miura et al., 2003; Nosrat et al., 2004).

Evidence of Neural Crest Cells in Skin

It has been demonstrated that a subpopulation of neural crest precursors remains multipotent after migration to the skin. Clonally-derived colonies generated from embryonic quail skin *in vitro* contain both melanocytes and neurons, though the percentage of colonies containing neurons was reported to decrease as a function of age (Richardson and Sieber-Blum, 1993).

There is however evidence in the literature that suggests that multipotent neural crest-derived precursors may persist in the skin throughout adulthood. Histological studies have shown that malignant skin tumours can contain cells with characteristics of melanocytic, neural and chondrocytic differentiation (Wahlstrom and Saxen, 1976) and it has been postulated that this presence of these cell types in the tumour may be explained by a neural crest tumour origin (Webb, 1982). It has also been shown that melanoma metastasis correlates with expression of HNK-1 (Thies et al., 2004), a marker of migrating neural crest cells (Rickmann et al., 1985; Bronner-Fraser, 1986), suggesting that reactivation of gene expression present transiently during embryonic

neural crest development may underlie the process of metastasis by this type of tumour (Thies et al., 2004). Furthermore, *in vitro* studies using a cat melanoma cell line, demonstrates that melanoma cells can be differentiated into neuronal and glial cells concomitant with neural gene expression programs (Rasheed et al., 2005). The fact that melanoma cells can be differentiated into other neural crest progeny, provides further evidence that multipotent neural crest cells may be resident within the skin that may also be the source of neural crest-related cancers common to the tissue.

Neural crest stem cells

Neural crest stem cells can be isolated from neural tube explants such that the migrating neural crest cells migrate from the explanted tubes onto the culture dish (Stemple and Anderson, 1992). Single neural crest cells were able to generate adherent colonies containing neurons, glia and smooth muscle. If these primary clones were trypsinized and re-plated at a clonal density, a subpopulation of those cells would go on to form secondary clones with the same multipotentiality, indicative of self-renewal (Stemple and Anderson, 1992; Shah et al., 1996). Similar to work in the avian system, not all colonies were multipotent, but instead generated neurons or glia only, providing further evidence that the neural crest is a mixed population of stem cells and more committed precursors (Stemple and Anderson, 1992). Prospective isolation of neural crest stem

cells has subsequently been achieved by FACS, selecting for cells expressing the low affinity neurotrophin receptor, p75 and negative for peripheral myelin protein zero (P0) and has identified multipotent neural crest stem cells from neural tube, embryonic sciatic nerve and gut (Morrison et al., 1999; Bixby et al., 2002). Neural crest cells isolated from either the nerve or gut, appear to have intrinsically different properties such as differential responsiveness to neuralizing or gliogenic factors. Although neural crest cells isolated from embryonic sciatic nerve are multipotent *in vitro* or when transplanted back into embryonic chick neural crest migratory streams (Morrison et al., 1999), they primarily differentiate into Schwann cells when transplanted back into the peripheral nerve. In contrast, neural crest cells isolated from the gut of the same embryonic age, generate primarily neurons when transplanted into the peripheral nerve (Bixby et al., 2002).

The isolation of neural crest stem cells *in vitro* has allowed for the identification of factors that affect their differentiation. Glial growth factor (GGF), a member of the neuregulin family of growth factors as well as notch signaling, promote Schwann cell differentiation from neural crest stem cells (Shah et al., 1994; Morrison et al., 2000). Endothelin-3 has also been shown to influence neural crest differentiation, by selectively promoting the proliferation of glial and melanocytic precursors *in vitro*. (Lahav et al., 1996; Lahav et al., 1998). BMP2 and BMP4 have been shown to promote the differentiation of autonomic neurons and to a lesser

extent the differentiation of smooth muscle from neural crest stem cells, while TGF-β promotes smooth muscle differentiation exclusively (Shah et al., 1996).

While BMPs promote autonomic neuronal differentiation, activation of wnt/beta-catenin signaling promotes sensory neuron differentiation from neural crest stem cells (Lee et al., 2004). The BMP signal appears to block the wnt signaling pathway, as even in the presence of activated wnt signaling due to the expression of a constitutively active form of beta-catenin, the addition of BMP prevents the adoption of a sensory neuronal fate (Kleber et al., 2005). The simultaneous activation of both pathways maintains the neural crest cells in an undifferentiated state, as explant cultures treated with both BMP2 and wnt-1 did not yield neurofilament positive neurons, instead many *Sox10* positive progenitors were observed (Kleber et al., 2005). When neural crest stem cells are simultaneously stimulated with a neurogenic signal in BMP2 and a gliogenic signal in GGF, BMP signaling is dominant such that the cells differentiate primarily into neurons and not Schwann cells (Shah and Anderson, 1997). The commitment of a multipotent neural crest cell to a Schwann cell fate requires at least 48 hours exposure to a gliogenic factor such as GGF. This delay is consistent with the fact that neurogenesis precedes that of gliogenesis in the developing ganglia of the peripheral nervous system (Shah and Anderson, 1997) and is discussed in greater detail in the 'Schwann cell Lineage' section that follows.

The study of neural crest stem cells has proven to have direct relevance to human disease, including Digeorge syndrome (Epstein, 2001), characterized by congenital cardiac and craniofacial defects and Hirschsprung disease (Newgreen and Young, 2002), a developmental disorder in which the enteric ganglia fail to form. Genes involved in glial cell line-derived neurotrophic factor (GDNF) and endothelin signaling as well as *Sox10* have been implicated in the development of the enteric nervous system and their mutation has been associated with Hirschsprung disease (Herbarth et al., 1998; Newgreen and Young, 2002). The expression of GDNF receptor, endothelin receptor type B and *Sox10* have all been shown to be enriched in enteric neural crest stem cells as compared to the entire embryo at the same embryonic age (Iwashita et al., 2003). GDNF has been shown to promote migration of gut neural crest cells from explant cultures and GDNF receptor knockout mice demonstrate defects in enteric neural crest migration, such that neural crest cells fail to colonize the gut below the esophagus (Iwashita et al., 2003). Similarly, endothelin receptor type B knockouts also demonstrate aganglionosis of the lower gut. The function of the receptor in these animals appears to be intrinsic to the neural crest cells themselves, as the transplantation of wild type neural crest cells into the knockout mice resulted in normal gut colonization (Kruger et al., 2003). The proliferation and differentiation properties of the neural crest cells in these mice were unaffected (Iwashita et al., 2003). Whereas the role of

GDNF and endothelin signaling in the development of the enteric nervous system seems to relate largely to the migration of neural crest cells, the major role of *Sox10* appears to lie in the regulation of survival and proliferation of the neural crest progenitors. In *Sox10* heterozygous mice, there is a decrease in the size of the progenitor pool and progenitors are observed to prematurely adopt a neural-restricted differentiation potential which may ultimately prevent their normal colonization of the lower gut (Paratore et al., 2002). In animals that completely lack *Sox10* expression, there is dramatic apoptosis of neural crest cells early in their migration (Kapur, 1999). *Sox10* may also play an indirect role in the migration of enteric neural crest precursors by regulating the expression of endothelin receptor which is required for their normal migration as described above (Zhu et al., 2004b).

The importance of *Sox10* extends beyond that of the enteric neural crest and has been shown to be an important player in neural crest development in general. *Sox10* deficiency also causes defects in melanocytes and the peripheral nervous system (Southard-Smith et al., 1999; Britsch et al., 2001). Its expression is induced shortly after neural crest induction and is maintained in cells that have delaminated from the neural tube and began migration (Southard-Smith et al., 1999). Neural crest stem cells also express *Sox10* in culture, and its expression is maintained in the Schwann cell lineage (Paratore et al., 2001; Kim et al., 2003a). Cultures of neural crest stem cells from *Sox10* knockout mice

exhibited increased apoptosis and a loss of Schwann cell differentiation, indicating that *Sox10* promotes the survival of multipotent neural crest cells and plays a role in the acquisition of glial fate (Paratore et al., 2001). Ectopic expression of *Sox10* in neural crest stem cells inhibits neuronal differentiation, even if stimulated by neurogenic factors like BMP2, and by inhibiting neuronal differentiation, *Sox10* may be maintaining multipotency in these cells (Kim et al., 2003a).

A certain degree of plasticity has been demonstrated even amoungst terminally differentiated neural crest progeny. Melanocytes stimulated with endothelin-3 *in vitro*, proliferate and can generate colonies containing both melanocytes and Schwann cells (Dupin et al., 2000). Conversely, Schwann cells expressing myelin proteins isolated form embryonic nerve can when treated with endolthelin-3, generate colonies containing both melanocytes and Schwann cells (Dupin et al., 2003). The transdifferentiation of one neural crest progeny into another proceeds through a dedifferentiation step to a bipotent glial-melanocytic precursor as evidenced by the presence of cells in the colonies expressing both glial and melanocyte lineage markers (Dupin et al., 2003). The plasticity of the glial-melanocytic lineage may explain the observation of pigmented cells upon injury of the adult nerve (Rizvi et al., 2002), and the presence of pigmented cells in neurofibromas, peripheral nerve tumours consisting of mainly Schwann cells (Fetsch et al., 2000).

Although it is generally thought that neural stem cells from the peripheral nervous system generate peripheral neurons and Schwann cells and that stem cells derived from the central nervous system generate neuronal sub-types specific to the central nervous system, astrocytes and oligodendrocytes, there are examples of differentiation that cross this apparent boundary. Populations of FGF-dependent neural stem cells from embryonic spinal cord (Mujtaba et al., 1998) or cortex (Gajavelli et al., 2004) have been shown to be capable of generating neural crest cells types including peripherin expressing peripheral neurons and Schwann cells, when differentiated in the presence of BMPs. The ability of cortical neural stem cells to generate neural crest progeny is somewhat of an *in vitro* cell culture artifact, as it is only revealed after the stem cells had been passaged several times in FGF (Gajavelli et al., 2004). That caveat aside, the process does involve in the induction of embryonic neural crest transcription factors such as slug (Gajavelli et al., 2004), and hence appears to recapitulate bona fide neural crest induction.

The Schwann Cell Lineage

Schwann cells are the major glial subtype of the peripheral nervous system. Myelinating Schwann cells generate the protective and insulating myelin layer that wraps large caliber axons in peripheral nerves. Non-myelinating Schwann cells associate with and engulf bundles of small caliber axons but do not form the multi-layered membranous

specialization that is myelin. A third type of peripheral nervous system cell are satellite cells, found surrounding neuronal cell bodies in the dorsal root ganglia and are also neural crest-derived. Schwann cell precursors can arrive at the developing nerves from the neural crest in one of two ways. In the case of sensory nerves extending from the dorsal root ganglia, neural crest precursors that have migrated to the DRG are thought to exit the ganglia and migrate along the developing nerve as it extends to its target. In support of this notion is the observation that Schwann cell precursors never precede the growing nerve and instead are most often observed behind the advancing growth cones (Carpenter and Hollyday, 1992). Neural crest cells are also able to migrate ventromedially from the dorsal neural tube directly to the nerves that they will eventually myelinate as is the case for the spinal cord ventral roots (Bhattacharyya et al., 1994).

The differentiation of neural crest cells into Schwann cells is thought to be directed by cells found along their migration pathways. Sensory neurons are the first cell type formed in the developing dorsal root ganglia, and their presence precedes that of Schwann cells (Lawson and Biscoe, 1979). In the zebrafish model, ablation of early-migrating neural crest cells permits neuronal differentiation from later-migrating neural crest cells that would normally only generate non-neuronal cell types including Schwann cells (Raible and Eisen, 1996). At this stage, the newly born neurons of the ganglia express various isoforms of the

neuregulin family of growth factors (Meyer and Birchmeier, 1994) that consists of multiple soluble and cell-associated isoforms resulting from the alternative splicing of a single gene (Lemke, 1996). Taken together with the demonstration that neuregulins act as Schwann cell mitogens (Brockes et al., 1980; Morrissey et al., 1991) and survival factors (Syroid et al., 1996) *in vitro*, it was postulated that the family of growth factors may be involved in Schwann cell development (Marchionni et al., 1993). This hypothesis was confirmed when knockout mice generated for erbB3, a tyrosine kinase receptor to the neuregulin family, were characterized to have a complete absence of Schwann cell and Schwann cell precursors during development (Riethmacher et al., 1997). Knockout mice for the erbB2, also a receptor for the neuregulin family die *in utero* at E11, but even at this early stage a deficiency of Schwann cell precursors was observed (Lee et al., 1995). When the cardiac defects in these mice were repaired by re-expressing erbB2 in cardiac tissue, later development could be studied which confirmed the loss of the Schwann cell lineage in these animals (Woldeyesus et al., 1999). Interestingly, subsequent to the loss of Schwann cells, there was a loss of motor and sensory neurons which suggested that these neuronal populations are in fact dependent on Schwann cells for survival in addition to their targets of innervation (Lobsiger et al., 2002).

In support of the idea that early-migrating neural crest cells that had differentiated into sensory neurons somehow directed later-migrating

neural crest cells to differentiate into Schwann cells, it has been shown that some neuregulin isoforms are axon-derived and bound, and are expressed during the differentiation of Schwann cells from neural crest precursors (Morrissey et al., 1995). The treatment of neural crest stem cells *in vitro* with GGF2, a soluble member of the neuregulin family promotes the differentiation of Schwann cells *in vitro* (Shah et al., 1994). In addition to the neuregulin signal, notch signaling also appears to be involved in the development of peripheral ganglia. The notch ligand *Delta1* is expressed by neuronal cells in the developing ganglia while migrating neural crest cells express the receptor notch (Weinmaster et al., 1991; Williams et al., 1995). The activation of notch signaling in neural crest cells *in vitro* through the addition of soluble ligand, delta, or the expression of constitutively active notch, blocks neuronal differentiation while promoting the differentiation of glia (Morrison et al., 2000; Wakamatsu et al., 2000). Thus by neuregulin and notch signaling, the early-migrating neural crest cells that differentiate into neurons in the peripheral ganglia can instruct the differentiation of late-migrating neural crest cells into glia.

The Schwann cell lineage can be described by several distinct stages that can be defined by marker expression and by cell survival requirements. While both Schwann cell precursors and neural crest precursors express p75NTR and nestin, Schwann cell precursors can be distinguished by their expression of low levels of the peripheral myelin

protein, PMP22 and the transcription factor Oct-6 (Blanchard et al., 1996). It should be noted however that low levels of PMP22 and P0 expression have also been reported in cells that retain neuronal differentiation potential in addition to Schwann cell potential (Hagedorn et al., 1999) and as such should not be considered specific to committed Schwann cell precursors. While Schwann cell precursors are dependent on growth factors or serum for survival *in vitro*, mature Schwann cells are independent of exogenous growth factors due to an autocrine survival loop involving PDGF, NT3 and IGF-1 (Eccleston et al., 1990; Gavrilovic et al., 1995; Meier et al., 1999; Lobsiger et al., 2000). As Schwann cell precursors continue to differentiate into more mature Schwann cells they begin to express glial fibrillary acidic protein (GFAP) and O4, a sulfatide antigen also associated with immature oligodendrocytes (Jessen et al., 1990; Mirsky et al., 1990). The two types of mature Schwann cells can also be distinguished on the basis of their gene expression. As Schwann cells begin to myelinate axons, they down-regulate the expression of Krox-24, GFAP and P75 and up-regulate the expression of Krox-20 and myelin proteins. Non-myelinating Schwann cells however, maintain their expression of p75 and Krox-24 [Reviewed in (Lobsiger et al., 2002)].

Schwann cell Transplantation for Injury and Disease

Injury to the nervous system can be particularly intractable, in part due to the distances involved. In some instances, an injured axon may

have to grow a meter or more to re-innervate its target and restore function. In the PNS, the degeneration of a nerve after injury proceeds in a very characteristic manner termed Wallerian degeneration, after the British physiologist Augustus Waller who first observed that the break down of myelin in the portion of a nerve distal to the transection (Waller, 1850). Within 24-48 hours of injury, distal myelin begins to fragment and is eventually cleared by macrophages, which invade the nerve. Schwann cells in the nerve begin to proliferate and these Schwann cells along with a collagen matrix serve as a growth promoting substrate for axons regenerating past the injury site [Reviewed in (Koeppen, 2004)].

Though the PNS does exhibit considerable regenerative potential, functional recovery can be incomplete and in cases where the proximal and distal ends of the damaged nerve are not or cannot be re-opposed, axon regeneration past the injury site is not possible. In these cases nerve grafts, or artificial grafts can be used to bridge the gap in the nerve. Current sources of nerve tissue for grafting are biopsy of one of the patient's own nerves which can result in morbidity and/or neuroma (Vanderhooft, 2000). Alternatively, by seeding artificial conduits with Schwann cells, regeneration across the gap can be enhanced (Guenard et al., 1992; Rodriguez et al., 2000). Human Schwann cells can be expanded *in vitro*, facilitating their use for transplantation (Rutkowski et al., 1995). Like nerve grafts, the current sources of Schwann cells for transplantation are from nerve biopsy, and survival of transplanted

Schwann cells and regeneration of the nerve is improved when autologous sources of cells are used (Rodriguez et al., 2000). Special considerations may also be necessary when contemplating autologous sources of Schwann cells in older patients as it has been shown that the proliferative capacity of Schwann cells harvested from human nerves decreases dramatically with age (Fansa et al., 2000).

There is also considerable interest in the use of Schwann cell transplantation to treat disease and injury of the CNS. Unlike the PNS, the CNS demonstrates very little regeneration after injury, due to signals that are inhibitory to axon growth in the myelin of the CNS (Cadelli et al., 1992; Schwab and Bartholdi, 1996) and an inability of regenerating axons to penetrate the glial scar (Fawcett and Asher, 1999). CNS neurons are capable of regeneration when provided a permissive substrate however, as demonstrated by the regeneration of transected CNS axons into peripheral nerve grafts containing Schwann cells (Richardson et al., 1980). Transplanted Schwann cells can serve two different functions in the injured CNS. First, when transplanted into demyelinated lesions of the spinal cord, Schwann cells are able to myelinate host axons (Blakemore and Crang, 1985) and restore nerve conduction (Kohama et al., 2001). The remyelination of the injured CNS by autologous Schwann cells has even been demonstrated using a demyelination model of monkey spinal cord (Bachelin et al., 2005).

Second, when Schwann cells are transplanted into injured spinal cords, they can promote axonal regeneration and improve functional recovery (Martin et al., 1991; Takami et al., 2002). How do Schwann cells promote regeneration in the CNS? Myelin-associated glycoprotein (MAG) is a major part of the inhibitory signal that prevents growth of regenerating CNS neurons (McKerracher et al., 1994). In the PNS, Schwann cells also express MAG, but down-regulate MAG expression after injury and during regeneration (Willison et al., 1988). Schwann cells in the injured peripheral nerve also upregulate the expression of cell adhesion molecules that promote growth, including L1, neural cell adhesion molecule (NCAM) and laminin (Daniloff et al., 1986; Grumet, 1992; Doyu et al., 1993). In addition to providing a substrate that both permits and promotes growth, Schwann cells express a variety of soluble trophic factors including neurotrophins, GDNF and FGF2, that may support the survival of regenerating neurons and promote their growth past the site of injury (Heumann et al., 1987; Meyer et al., 1992; Hammarberg et al., 1996; Grothe et al., 1997).

In addition to their ability to promote regeneration, Schwann cells may also be utilized as cell based vehicles of growth factor delivery. Schwann cells can be engineered to express various growth factors by transfection with expression plasmids or infection with retroviral vectors and then transplanted to deliver the growth factors to a targeted area (Lawrence et al., 2004; Timmer et al., 2004).

Stem cells represent an alternative source of Schwann cells for transplantation that may be generated in a less invasive manner. It has been suggested that bone marrow stromal cells (MSCs), which have been demonstrated to generate adipocytes, chondrocytes and osteoblasts (Friedenstein et al., 1976; Piersma et al., 1985; Owen and Friedenstein, 1988), are also capable of generating neural cell types (Sanchez-Ramos et al., 2000). More specifically, it has been suggested that MSCs may serve as a source of Schwann cells (Dezawa et al., 2001). The differentiation of Schwann cells from MSCs however requires their sequential treatment with reducing agents, retinoic acid and a cocktail of growth factors. Recently, the neural differentiation capacity of MSCs has been questioned, with the demonstration that some of the culture conditions used may have caused morphological changes and non-specific antigenic changes in the cells that were mistaken for neuronal differentiation (Lu et al., 2004; Neuhuber et al., 2004). These reservations aside, there are several studies demonstrating that transplanted MSCs can remyelinate demyelinated lesions in the spinal cord (Sasaki et al., 2001; Akiyama et al., 2002a) even when administered intravenously (Akiyama et al., 2002b). One caveat to these myelination studies is that although remyelination of host axons does occur, the evidence that the transplanted cells were directly responsible for the remyelination is correlational. Further studies are required to determine if the remyelination observed is directly attributable to the transplanted bone

marrow cells, or if the stromal cells somehow recruit and induce endogenous cells to remyelinate the lesion.

Skin and Hair follicle development

The skin is the human body's largest organ and serves important roles in protection and homeostasis. What begins as a single layer of ectodermal cells at the time of gastrulation becomes a complex and differentiated structure with both ectodermal and mesodermal components. Mature skin is characterized by two main layers, epidermis and dermis and it is through the interaction of these two layers that epidermal appendages develop, including hair and whisker follicles, feathers, scales, sebaceous glands, teeth and mammary glands. These appendages serve a wide variety of functions from species to species and can include protection, sensation, thermoregulation, decoration for mating purposes and the rearing of young. The hair follicle is perhaps the most widely studied of these appendages and a great deal is known about the signaling events that underlie the epidermal/mesenchymal interactions that drive both the initial embryonic development of the hair follicle and the hair follicle cycle as well. The development of hair follicles begins at approximately E13 in mice with the formation of hair placodes, regions of increased keratinocyte density, accompanied by dermal condensations that underlie the placodes (Schmidt-Ullrich and Paus, 2005). Signaling between these two epidermal and dermal structures drives the

development of a mature hair follicle and involves BMP, Wnt, FGF, sonic hedgehog (SHH) and notch signaling [reviewed in (Schmidt-Ullrich and Paus, 2005)]. Interestingly, many of these factors are the same factors involved in neural crest determination around the time of neural tube closure. The mature hair follicle undergoes a continuous cycle of growth (anagen), regression (catagen), rest (telogen) and shedding of the hair shaft (exogen or early anagen) and involves the same signaling pathways that drive initial hair follicle morphogenesis. The hair follicle consists of the following key features: the outer root sheath, the outer most layer of the hair follicle contiguous with the epidermal layer consisting of keratinocytes; the inner root sheath, a multilayered structure also consisting of keratinocytes that immediately surrounds the hair shaft; the bulge, part of the permanent portion of the hair follicle just distal to the insertion point of the arrector pili muscle and in the proximity of sebaceous glands and finally the dermal papilla, a dense region of mesenchymal cells at the base of the hair follicle that has important inductive functions in hair follicle development and cycle (see Figure 1).

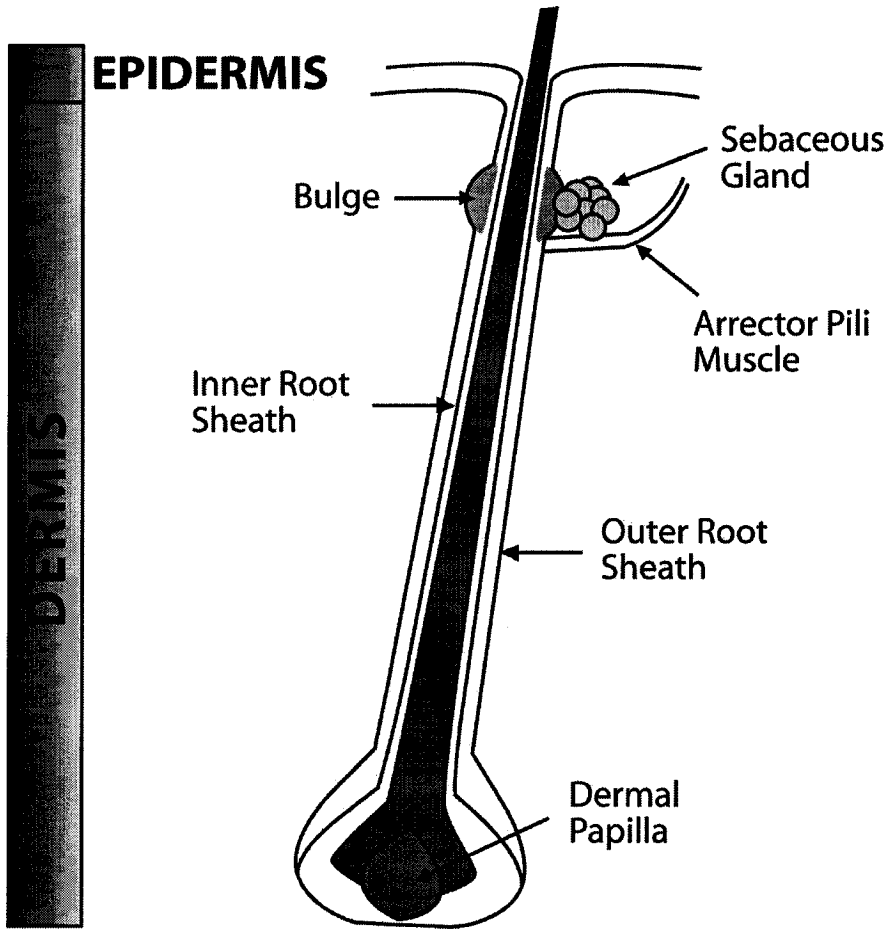

Figure 1. Diagram of hair follicle anatomy.

The continuous cycling requires the presence of stem cell populations to replenish the various cell types of the follicle. One stem cell population that has been well characterized is epidermal stem cells that reside in the bulge region of the hair follicle. The possibility that the bulge served as a niche for a stem cell population was first suggested after the observation of label-retaining cells specifically in this region

(Cotsarelis et al., 1990). The location of these label-retaining cells correlates with the finding that the bulge region contains cells with great proliferative potential *in vitro* lending further support to the hypothesis that the bulge is an epidermal stem cell niche (Rochat et al., 1994). By observing the fate of label retaining cells, cells from the bulge can be observed to contribute not only to the outer root sheath, inner root sheath and hair matrix, but in the case of growing neonatal skin or wounded adult skin, cells were able to migrate upwards and laterally to contribute to normal epidermis (Taylor et al., 2000). In addition, by transplantation of genetically tagged bulge regions of vibrissae follicles, cells from the bulge were demonstrated to not only be able to reconstitute the expected cell types in the lower regions of the hair follicle, but also were able to generate sebaceous glands indicating that cells in the bulge had unexpected multipotentiality (Oshima et al., 2001). Epidermal stem cells from the bulge have now been prospectively isolated by various techniques, including transgenic approaches that identify label retaining cells specifically in the hair follicle (Tumbar et al., 2004) or by keratin 15 expression (Morris et al., 2004). These isolated cells of which some are also positive for the hematopoietic stem cell marker CD34, contribute to hair follicle cell types during the hair cycle and also to the epidermis after injury (Morris et al., 2004; Tumbar et al., 2004). In addition, despite their extremely low *in vivo* proliferation rate, cells isolated from the bulge are enriched for cells with large *in vitro* proliferative capacity as compared to

hair follicle cells as a whole (Morris et al., 2004). The bulge has also very recently been identified as a niche for cell with neural crest potential (Amoh et al., 2005a) (Sieber-Blum et al., 2004) and will be discussed in greater detail in the discussion section of this thesis.

The follicular papillae represents a permanent but dynamic dermal component of the hair follicle. It is a key source of signals that drive hair morphogenesis and cycling as demonstrated by the ability of transplanted intact dermal papillae or cultured dermal papillae cells to induce de novo hair follicles in skin (Jahoda, 1992; Reynolds and Jahoda, 1992; Jahoda et al., 1993). In addition to its importance for hair follicle development and maintenance, the dermal papillae has also been proposed to be a source of mesodermal cell types for skin wound-healing and turnover (Gharzi et al., 2003). Using a punch biopsy models of skin wound-healing, it has been shown that new skin can be formed, comprised entirely of hair follicle cells, with dermal hair follicle cells contributing extensively to the dermis, generating large numbers of dermal fibroblasts (Gharzi et al., 2003).

The Stem Cell Niche

The stem cell niche can be defined as a specific location in a tissue in which a stem cell resides and that provides a protective environment that promotes the maintenance of stem cell properties. One of the best-understood examples of a stem cell niche comes from studies

on Drosophila germ stem cells (GSCs). It has been shown that GSCs are held in place by cap cells in the Drosophila ovary by cell-cell interactions via DE-cadherin (Song et al., 2002). The loss of this protein, results in the loss of GSCs in the adult ovary. Through a BMP-mediated signal, the niche also represses the expression of genes involved in differentiation, thereby promoting stem cell self renewal (Song et al., 2004). This mechanism would appear to be evolutionarily conserved, as another member of the cadherin family, N-cadherin as well as BMP signaling have been implicated in the maintenance of hematopoietic stem cells in their niche within the bone marrow of mice (Zhang et al., 2003). Osteoblastic cells lining the bone that express N-cadherin appear to physically bind the hematopoietic stem cells and when BMP signaling was eliminated through the use of an inducible Cre recombinase transgenic mouse, the resulting expansion in osteoblastic cells led to an consequent increase in the number of hematopoietic stem cells associated with them, demonstrating that the cells within the niche regulate stem cell number (Zhang et al., 2003).

If the function of the stem cell is to provide a source of replacement cells that can be mobilized after injury and during wound healing, the niche must also allow for signals from the surrounding tissue to reach the stem cell population so that it may respond appropriately (Whetton and Graham, 1999). The mechanisms by which stem cells within a niche may respond to external signals remain unclear, but there are clear examples

of stem cells within niches that become mobilized in response to a signal originating from outside the niche itself. Epidermal stem cells in the hair follicle bulge, which normally cycle extremely slowly, go through cyclic periods of activation in response to dermal papillae derived signals during the hair follicle cycle (Tumbar et al., 2004). The result is that some epidermal stem cells leave the niche, proliferate, and contribute to the epidermally-derived portions of the hair follicle during the growth of the anagen phase. The response to skin injury is even more pronounced with large numbers of epidermal stem cells becoming activated and migrating upwards to regenerate the injured interfollicular epidermis (Taylor et al., 2000). In many respects, the identification of non-stem cells or support cells of the niche is as important as identifying the stem cells themselves, as the interaction between stem cell and niche is essential for the maintenance of stem cells, and for stem cells to respond to tissue damage.

Skin-derived Precursors (SKPs)

Skin-derived precursors were first described by Toma *et al.* (Toma et al., 2001), and were isolated from neonatal or adult rodent skin, adapting protocols used to culture neural stem cells from the adult brain (Reynolds and Weiss, 1992; Vescovi et al., 1993). Briefly, dissociated skin cells from neonatal or adult animals were cultured in non-coated

culture flasks in the presence of the mitogens FGF2 and EGF. Spheres of floating clusters of cells reminiscent of neurospheres were formed.

Upon mitogen withdrawal and culture in serum on coated substrates, induction of neuronal markers including βIII-tubulin, neurofilament, neuron specific enolase and NeuN was observed, indicative of neuronal differentiation. The induction of the glial markers CNPase, a marker of myelinating glia as well as GFAP were also observed. In addition to neural cell types, mesodermal cell types were also generated including smooth muscle actin (SMA)-positive smooth muscle cells and adipocytes. In total, 3-7% of differentiated cells expressed neuronal markers, 7-11% expressed glial markers, SMA-positive cells were quite rare and the number of adipocytes varied greatly (1-25%) from preparation to preparation, regardless of the age of the source animal or the number of times the cells were passaged before differentiation. Another important finding from the initial work was that single SKPs cells could be expanded to yield clonally-derived lines that maintained the ability to generate neural and mesodermal progeny in proportions similar to population-derived lines indicating that single cells within SKPs cultures were indeed multipotent and able to generate all the cell types generated by the differentiation in mass culture.

Immunocytochemical characterization of SKPs spheres demonstrated that a subpopulation of the cells expressed nestin which has traditionally been used as a marker of neural precursors but is also

expressed in other precursor populations including muscle and hair follicle precursor cells (Sejersen and Lendahl, 1993; Li et al., 2003). SKPs can be antigenically distinguished from CNS-derived neurospheres on the basis of fibronectin expression, a mesenchymal marker (Azizi et al., 1998). The two stem cell populations can also be functionally distinguished on the basis of differentiation potential, as while SKPs differentiate into adipocytes, CNS-derived neurospheres do not. Although SKPs expressed the mesenchymal marker fibronectin, they did not behave like mesenchymal stem cells, which failed to grow when cultured non-adherently in SKPs proliferation media. SKPs also did not generate keratinocytes on the basis of cytokeratin expression by differentiated cells, and were therefore unlikely to be related to epidermal stem cells. SKPs therefore, appeared to be a novel stem cell population, with characteristics that distinguished them from neural and mesenchymal stem cells.

In order to more precisely determine the location of SKPs within the skin, cells were dissociated from either the epidermis or the dermis, which were physically separated after enzymatic digestion of intact skin. It was shown that SKPs cultures could be generated from the dermis preparation but not from that of the epidermis (Toma et al., 2001).

Human skin samples from scalp tissue obtained as a bi-product of stereotactic brain surgery were also able to generate nestin and fibronectin-positive SKPs spheres under similar conditions and could be

differentiated to yield βIII-tubulin-positive cells with neuronal morphology indicating that human skin also contained SKPs-like cells (Toma et al., 2001).

Since the initial description of SKPs, human SKPs have been further characterized (Toma et al., 2005). SKPs can also be cultured from human foreskin tissue from patients ranging from 4 weeks to 12 years of age. Foreskin-derived spheres also expressed nestin and fibronectin and could also generate neural and mesodermal cell types upon differentiation. Single human SKPs cells could also generate clonally derived lines that not only maintained normal growth characteristics and dependence on mitogens for proliferation, but also were karyotypically normal, as analyzed by G-banding, after 15 months in culture. Furthermore it was shown that differentiated human SKPs also express markers characteristic of more differentiated neuronal cells including MAP2 and GAP43, and co-expressed the Schwann cell markers s100β and p75, in cells with bipolar morphology typical of Schwann cells.

The presence of neural precursors in rodent (Kawase et al., 2004; Yang et al., 2004), porcine (Dyce et al., 2004) and human (Belicchi et al., 2004; Joannides et al., 2004) skin has subsequently been independently confirmed by other research groups and demonstrated that the presence of SKP-like cells skin across mammalian species. It is likely that SKPs-like cells can be found in the skin of non-mammalian vertebrates as well, which all share in common the neural crest as a defining embryonic

developmental feature. In this regard, the isolation of cells with neural crest differentiation potential from embryonic quail skin has been documented (Richardson and Sieber-Blum, 1993).

In the study by Belicchi et al., human skin was obtained from aborted fetuses as well as healthy volunteers aged 12-65 years old. In a modification to the protocol used in our lab to culture SKPs, cells liberated from human skin after enzymatic digestions were first subjected to magnetic activated cell sorting for AC133 to isolate the AC133-positive fraction. AC133 has been previously be shown to enrich for stem cell activity in neurosphere cultures and is also a marker of hematopoietic stem cells (Uchida et al., 2000). This enrichment also proved true in the case of the SKP-like cells as all of the sphere-forming activity was found in the AC133-positive fraction, which represented 6% and 1% of the fetal and adult skin respectively (Belicchi et al., 2004). The ACC-positive fraction yielded cells which could clonally generate spheres, that could be subsequently passaged and differentiated into βIII-tubulin-positive neuronal cells, and GFAP-positive glial cells *in vitro.* Spheres cultured in this manner were also transplanted into the lateral ventricle of the brains of adult severe combined immunodeficient (SCID) mice and cells were observed to migrate into the cortex with some evidence of neuronal and glial differentiation based on the expression of a single marker, MAP2ab and GFAP respectively. The authors also reported cells that had associated with and integrated into blood vessels that expressed the

marker Ve-cadherin, possibly indicative of endothelial differentiation (Belicchi et al., 2004).

In the study by Joannides et al., human skin was obtained from biopsy samples from seven volunteers aged 41-77 years. The dermis was separated from the epidermis and then enyzmatically digested to isolate single cells which were subsequently cultured in FGF2 and EGF. The authors reported sphere formation but with limited expansion, so in an effort to further expand the cells, spheres were then cultured adherently in high concentrations of serum and passaged/expanded several times (Joannides et al., 2004). Cells expanded in this manner, retained the ability to form floating clusters of cells when transferred back to serum-free media containing FGF2 and EGF. These spheres expressed nestin and musashi-1, markers of neural progenitors, that could clonally yield both neural and mesodermal progeny based on βIII-tubulin/NFM and fibronectin/SMA expression respectively. When differentiated in astrocyte-conditioned medium, cells co-expressing neurofilament and βIII-tubulin were observed, as well as rare GFAP-positive glial cells. Neuronal cells were also shown to be glutamate or GABA positive and 10 of 15 cells analyzed demonstrated depolarization-induced calcium transients indicative of voltage-gated calcium channels (Joannides et al., 2004). These studies are important not only because they independently confirm our initial findings, but also because they also

demonstrate that SKPs can be cultured from human skin, regardless of skin source or age of the volunteer.

In the following chapters I will present work that demonstrates that SKPs are a neural crest-related precursor that i) generates neural crest progeny including peripheral neurons and Schwann cells, ii) expresses embryonic neural crest transcription factors and iii) migrates via neural crest migratory pathways after *in vivo* transplantation. The expression of neural crest transcription factors was subsequently used to define a niche for SKPs in the dermal papillae of hair follicles. I will also present data that examines the functionality of SKP-derived Schwann cells and that demonstrates their capacity to myelinate axons after transplantation into brain and nerve.

This work demonstrates that SKPs represent an attractive adult stem cell source of neural crest derivatives, capable of expansion *in vitro* from small amounts of accessible tissue. The potential therefore exists for the use of SKPs to generate material for autologous cell transplantation therapies as well as to serve as a tool for the study of human neural crest-related diseases.

Chapter 2:

A DERMAL NICHE FOR MULTIPOTENT ADULT SKIN-DERIVED PRECURSOR CELLS

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ABSTRACT

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.

INTRODUCTION

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 (Joshi and Enver, 2002). Although some reported cases of multipotency are due to unanticipated cellular fusion events (Terada et al., 2002; Alvarez-Dolado et al., 2003; Wang et al., 2003), 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 (MAPCs) isolated from bone marrow cells (Jiang et al., 2002) and neural stem cells from the central nervous system (CNS) (Clarke et al., 2000) contribute to many different developing tissues. One caveat to these studies is that multipotency was demonstrated only after these stem cell populations were expanded in culture, raising the possibility that it was a consequence of culture-induced de-differentiation and/or reprogramming (Joshi and Enver, 2002).

We have previously described a multipotent precursor cell population from adult mammalian dermis (Toma et al., 2001). 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 (Toma et al., 2001; Joannides et al., 2004). One endogenous embryonic stem cell population that has a similar broad differentiation potential and that contributes to the dermis is neural crest stem cells (NCSCs) (Le Douarin, 1982). We therefore hypothesized that SKPs represent a multipotent neural crest-like precursor cell that colonizes mammalian tissues during embryogenesis, 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 can generate neural and/or mesodermal progeny. Because we previously demonstrated that SKPs are distinct from mesenchymal stem cells (Toma et al., 2001), 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 (Ito et al., 2003), (Asakura, 2003) whereas only neurospheres contained cells

expressing p75NTR (see Supplementary Information Fig. S1a). We then analyzed SKPs for expression of genes associated with embryonic NCSCs. RT-PCR analysis (Fig. 1a) showed that SKPs expressed the transcription factors genes *slug* (Nieto et al., 1994), *snail* (Smith et al., 1992), *twist* (Soo et al., 2002), *Pax3* (Conway et al., 1997), and *Sox9* (Cheung and Briscoe, 2003), expressed in various populations of embryonic NCSCs (Le Douarin and Dupin, 1993) *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 (Li et al., 1995) and SHOX2 (Clement-Jones et al., 2000) (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 or adult SKPs passaged from 1 – 15 times. Thus, SKPs express genes characteristic of embryonic NCSCs and/or their embryonic derivatives.

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 (Stemple and Anderson, 1992). Immunocytochemistry, RT-PCR and western blot analysis revealed a subpopulation of differentiated cells with neuronal morphology that co-

expressed the pan-neuronal markers βIII-tubulin and neurofilament M (NFM; Fig. 1e), as well as 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 S1c,d). These neurons were generated by differentiated embryonic and neonatal SKPs, and by SKPs at passaged 1 - 20 times.

We have previously reported that SKPs differentiate into bipolar cells co-expressing glial fibrillary acidic protein (GFAP) and CNPase, consistent with a Schwann cell phenotype (Toma et al., 2001). Further characterization demonstrated 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 (Monuki et al., 1989).

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) (Hadjantonakis et al., 1998), 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 (White and Anderson, 1999) (Fig. 2a,b). Analysis three days later (H&H stage 30)

revealed that approximately half the transplanted cells had migrated from the transplant site into peripheral neural crest targets, whereas very few 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 subpopulation expressed S100β, a marker for Schwann cells (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 then 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 differentiation of neurons from embryonic NCSCs (Stemple and Anderson, 1992). Immunocytochemistry (Fig. 3a, also see Supplementary Information, Fig. S2a) and Western blot analysis (Fig. 3b) revealed 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 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 SKPs-forming cell from dissociated skin gave rise to a single, clonal SKPs sphere. Three types of evidence supported this conclusion: first, at low densities (25,000 to 100,000 cells/ml 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 quantitated 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 ten fold 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 both 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 that expressed the melanoblast/melanocyte markers trp1, c-kit, or dct (see Supplementary Information, Fig. S2g) (Nocka et al., 1989; Nishimura et al., 2002) or the Schwann cell markers MBP, P0, p75NTR (Fig. 1d) or Sox10 (data not shown) (Britsch et al., 2001). Second, we have recently found that when primary mouse skin cells are FACS-sorted for Sca-1, all of the sphere-forming ability 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 the 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 (Lako et al., 2002). (Jahoda et al., 2003). At E18, hair follicle papillae were characterized by robust alkaline phosphatase activity (Paus et al., 1999), expression of versican, a cell surface proteoglycan (du Cros et al., 1995), (Kishimoto et al., 1999), and nexin, an anagen-specific matrix modifying factor (Jensen et al., 2000) (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 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 (Panteleyev et al., 1997) (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 vibrissae papillae, which are amenable to micro-dissection. Initially, we asked whether whisker papillae expressed snail, slug or twist mRNAs. 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 later stages (Reddy et al., 2001), 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), consistent with our dorsal skin data (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 the majority of the neonatal SKPs cells were positive for versican (Fig. 6b). We then obtained two lines of evidence that indicated 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); second, YFP-positive adult mouse vibrissal cells generated SKP-like spheres

when co-cultured with unlabelled E16 skin cells (see Supplementary Information, Fig. S2f).

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 (Moll et al., 1996)], 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 that are specific to follicle papillae in skin, and third, postnatal vibrissal papillae contain cells that can proliferate as nestin-positive SKP spheres, and that can differentiate into cells with attributes of neurons and smooth muscle cells.

Follicle dermal papillae contains 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 (Jiang et al., 2000). 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 analyzed for the β-galactosidase expression by in situ hybridization (Fig. 7a; 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 follicle sheath (see Supplementary Information, Fig. S4a). These latter β-galactosidase-positive cells are likely progeny of NCSC-derived melanoblast stem cells previously reported to inhabit these niches (Nishimura et al., 2002), and/or may represent other NCSC-derived progeny present in these compartments such as Merkel cells (Szeder et al., 2003) or Schwann cells. β-galactosidase-positive cells were also identified in the 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 the Wnt1Cre;R26R mice was analyzed next. At P9, a time when SKP-like spheres can be isolated from these 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 papillae cells also stained positive for β-galactosidase activity (Fig. 7d). Similarly, at E18.5, almost all cells in the newly formed papillae (Fig. 7c) and many of the dermal cells were β-galactosidase positive, consistent with the neural crest origin of the

facial dermis. Thus, vibrissal papillae cells are almost entirely of neural crest origin.

Next we determined whether SKPs are 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 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 papillae, a niche thought to contain precursor cells (Lako et al., 2002; Jahoda et al., 2003) that is a major locus for regulatory dermal-epidermal interactions (Fuchs and Raghavan, 2002).

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 (Botchkareva et al., 1999), at a timepoint when the first nerves, which contain p75NTR-positive NCSCs (Morrison et al., 1999), arrive in skin and innervate the developing follicles (Peters et al., 2002), 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 the less probable, face

and dorsal skin dermis are also very similar, yet 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 that are never found in their tissue of origin, in this case neurons. This ability likely reflects the potential of the cells 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, since neural crest is the developmental origin of these precursors, at least in the facial skin, then these findings imply that similar multipotent neural crest-derived precursors may be found elsewhere. Cells similar to SKPs have recently been isolated from other neural crest-derived tissues, such as human dental pulp (Miura et al., 2003), which is neural crest derived, and we have found similar SKP-like cells in a placodally-derived structure, the adult olfactory epithelium (Andrew 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, with regard to skin itself, 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 the niche itself that maintains these cells in a precursor state. In this regard, dysregulation of a niche containing such an endogenous multipotent precursor might provide and explanation for skin tumours of mixed mesodermal and neural lineages.

METHODS

Cell Culture

SKPs were cultured as described in Toma *et al.*, (Toma et al., 2001). 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). Dissociated cells were pelleted and plated in DMEM-F12, 3:1 (Invitrogen), containing 20 ng/ml EGF and 40 ng/ml FGF2 (both Collaborative Research), hereafter referred to as proliferation media. Cells were cultured in 25 cm^2 tissue culture flasks (Falcon) in a 37°C, 5% CO2 tissue culture incubator. SKPs were passaged by mechanically dissociating spheres and splitting 1:3 with 75% new media and 25% conditioned media from the initial flask. Neurospheres from the E13 embryonic telencephalon (Reynolds and Weiss, 1992) were cultured under the same conditions. For neuronal

differentiation, SKP spheres or primary dissociated skin cells were mechanically dissociated and plated on chamber slides (Nunc) coated with poly-D-lysine/laminin in DMEM-F12 3:1 supplemented with 40 ng/ml FGF2 and 10% FBS (BioWhittaker) for 5-7 days. Cells were then cultured an additional 5-7 days in the same media without FGF2 but with the addition of 10 ng/ml NGF (Cedar Lane), 10 ng/ml BDNF (Peprotech) and 10 ng/ml NT3 (Peprotech). For Schwann cell differentiation, dissociated spheres were cultured in DMEM-F12 3:1 supplemented with 10% FBS for 7 days, then switched to the same media supplemented with 4 μM forskolin (Sigma).

For the vibrissae experiments, rat vibrissal follicles were dissected from P6 to 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 papillae removed. Papillae were digested with trypsin for 15 min at room temperature and mechanically dissociated. Single cells were plated on 2-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 papillae cells, cells were plated on chamber slides coated with poly-D-lysine/laminin/fibronectin in SKPs proliferation medium supplemented with 5% chick embryo extract. After 7 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 in uncoated 24-well tissue culture plates (Falcon) in proliferation media 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), 20 ng/ml EGF and 40 ng/ml FGF2, 1 μg/ml 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 (Hadjantonakis et al., 1998) dissociated vibrissal follicle cells 100:1 and uncoated flasks in proliferation media with 25,000 cells/ml.

Immunocytochemistry, *in situ* hybridization, and X-gal staining

Immunocytochemical analysis for cells was performed either using coated slides and the cytospin system (Thermo Shandon) for SKP spheres, or on cells plated on chamber slides (Nunc) as previously described (Toma et al., 2001)(Barnabe-Heider and Miller, 2003). The following primary

antibodies were used: anti-nestin monoclonal, 1:400 (BD Biosciences), anti-βIII-tubulin monoclonal, 1:500 (Tuj1 clone; BABCO), anti-neurofilament-M polyclonal, 1:200 (Chemicon), anti-GFAP polyclonal, 1:200 (DAKO), anti-p75NTR polyclonal, 1:500 (Promega), anti-SMA monoclonal, 1:400 (Sigma), anti-fibronectin polyclonal, 1:400 (Sigma), anti-trp1 polyclonal, 1:200 (Chemicon), anti-c-kit polyclonal (Cell Signaling Technology), anti-S100β monoclonal, 1:1000 (Sigma), anti-MBP polyclonal, 1:100 (Chemicon), anti-TH monoclonal, 1:200 (Chemicon), and Sca-1, 1:100 (Becton Dickinson). Secondary antibodies used were: Alexa488-conjugated goat anti-mouse, 1:1000 and Alexa594-conjugated goat anti-rabbit, 1:1000 (both from Molecular Probes). Processing of skin samples for histological analysis and *in situ* hybridization was performed as described in Mo et al (Mo et al., 1997). The probes used in this study were as follows: β-galactosidase (Ambion); nexin and versican (kind gifts from Dr. Bruce Morgan); K17 (P. Coulombe); Wnt5a (A. McMahon); Snail (T. Gridley); 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 previously described (Mill et al., 2003). Briefly, staining for lacZ was carried out on tissues fixed in 2.7% formaldehyde, 0.02% Nonidet-P40, PBS overnight at 4°C, followed by overnight cryoprotection in 30% sucrose in PBS at 4°C prior to 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 (2 min), rinsing three times in PBS, rinsing twice in 0.1M sodium phosphate containing 2 mM MgCl2, 0.1% sodium desoxycholate, and 0.02% NP40, and then immersing in standard X-gal staining solution overnight at 37°C.

RT-PCR

RNA was prepared from samples using Trizol (Invitrogen), and cDNA was generated with Revertaid Reverse Transcriptase (Fermentas) as directed by manufacturer. For all cDNA synthesis a –RT control was performed. PCR reactions were carried out as follows: 92°C 2 min., 30-35 cycles of 94°C for 60 s., Gene-specific annealing temperature for 60 s. and 72°C for 60 s. PCR primers used were as follows:

Gene	Primer Sequence	Anneal Temp (°C)	Product (BP)
Pax3	ggaggcggatctagaaaggaagga	59	374
	cccccggaatgagatggttgaa		
Slug	cgtcggcagctccactccactctc	60	348
	tcttcagggcacccaggctcacat		
Twist	cgccccgctcctctgctctacc	67	352
	gccgccgccgccaccacctc		
Snail	cggcgccgtcgtccttct	61.5	398
	ggcctggcactggtatctcttcac		

Sox9	ccgcccatcacccgctcgcaatac	59.5	544
	gcccctcctcgctgatactggtg		
P75	gtgcggggtgggctcaggact	62	422
	ccacaaggcccacaaccacagc		
SHOX2	ccgccgccgccaagaccac	63	355
	tccccaaacccgctcctacaaa		
DβH	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		

Western blot analysis

Lysates were prepared and Western blot analysis performed as described previously (Barnabe-Heider and Miller, 2003). Equal amounts of protein (50-100 μg) were analyzed on 7.5% or 10.5% polyacrylamide gels. The primary antibodies used were anti-DβH monoclonal 1:1000 (Pharmingen), anti-peripherin polyclonal, 1:1000 (Chemicon), anti-p75NTR polyclonal, 1:1000 (Promega), anti-TH monoclonal, 1:800 (Chemicon), anti-βIII-tubulin monoclonal, 1:1000 (Tuj1 clone; BABCO), 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 (HH) stage 18 (Hamburger and Hamilton, 1951). 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 (~200-250 total 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 HH stage 30 (~ 3 more days) after which time the embryos were removed from the shell contents, quickly decapitated, eviscerated, fixed in 3.7% formaldehyde/PBS for 2 hours, and immersed in 30% sucrose/PBS overnight at 4°C. The embryos were embedded in OCT, frozen at -70°C, sectioned at 20μm and mounted onto tissue-adhering slides.

FIGURES AND LEGENDS

Figure 1.

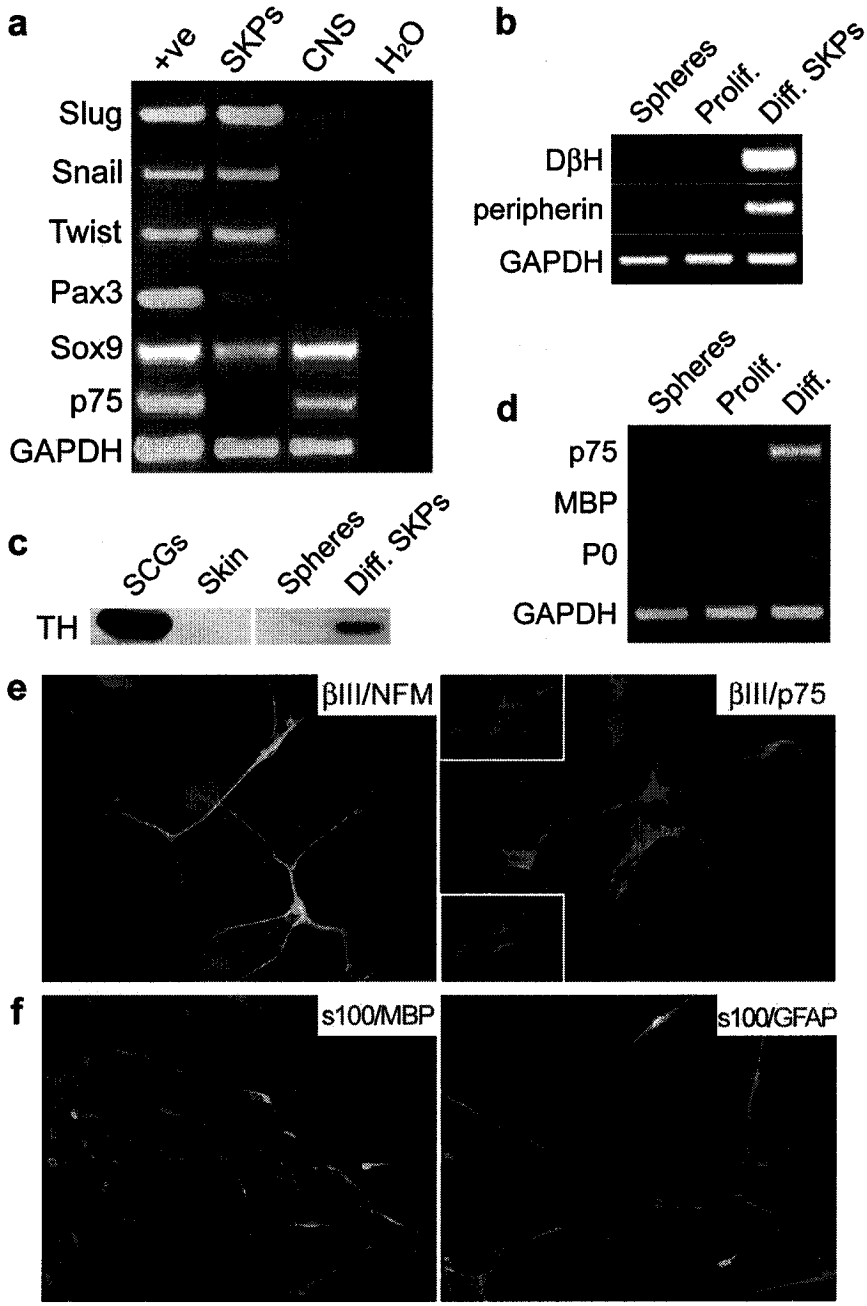

Figure 1. ***SKPs express markers of embryonic neural crest and differentiate into peripheral neurons and Schwann cells.*** **(a)** RT-PCR for genes involved in embryonic neural crest determination and migration in total RNA isolated from SKP spheres compared to embryonic telencephalic neurospheres (CNS), both of which were cultured in the presence of FGF2 and EGF. RNA from 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 mRNA (DβH) and peripherin mRNA in murine SKPs differentiated for 1 week in 10% serum (Diff. SKPs). SKP spheres and dissociated SKPs plated in proliferation medium (PM) do not express these mRNAs. **(c)** Western blot 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 peripheral sympathetic neurons from 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.

Figure 2.

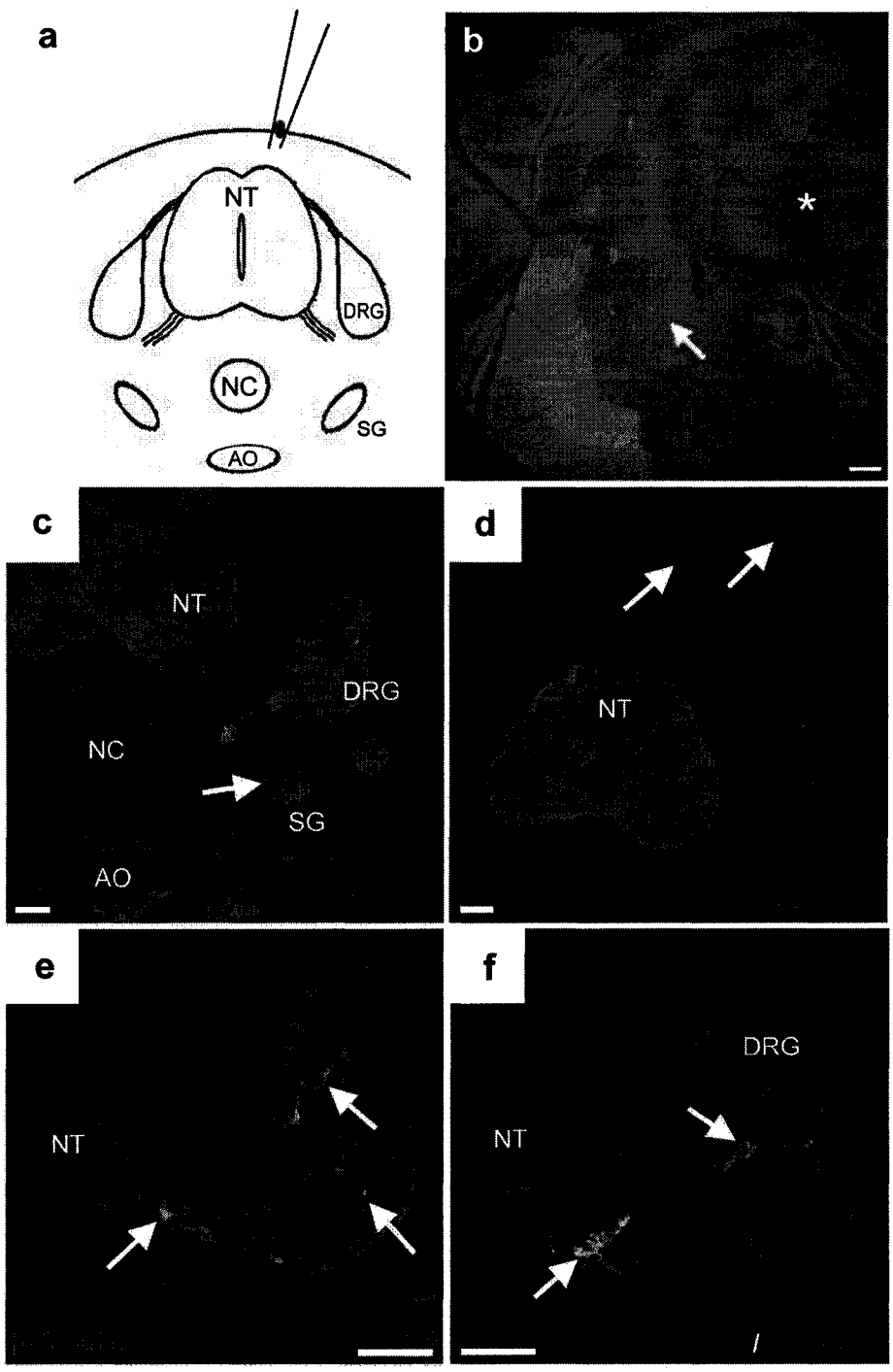

Figure 2. ***SKPs migrate like neural crest cells when transplanted in ovo.*** **(a)** Schematic representation of a cross-section through a stage-30 chick embryo showing some of the major anatomical landmarks near, or associated with, the neural crest migratory stream. **(b)** Photomicrograph of a single 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.

Figure 3.

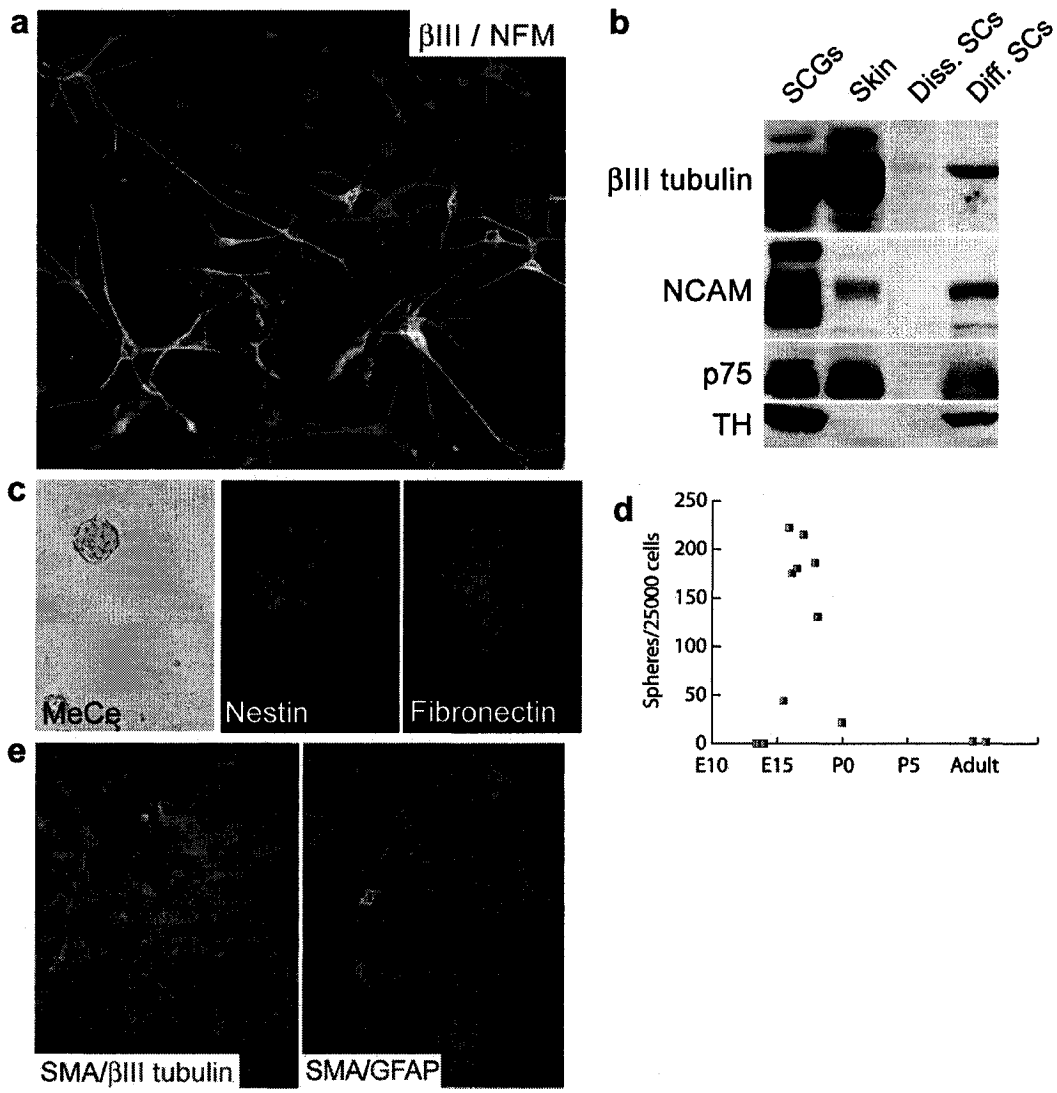
a
βIII / NFM
b
SCGs
Skin
Diss. SCs
Diff. SCs
βIII tubulin
NCAM
p75
TH
c
MeCε
Nestin
Fibronectin
d
Spheres/25000 cells
250
200
150
100
50
0
E10
E15
P0
P5
Adult
e
SMA/βIII tubulin
SMA/GFAP

Figure 3. ***Multipotent endogenous SKPs are abundant in skin during late embryogenesis and persist into adulthood.*** **(b)** 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 analyzed 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 (SCG). **(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 labeled with antibodies to nestin and fibronectin. **(d)** Quantitation 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. **(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.

Figure 4.

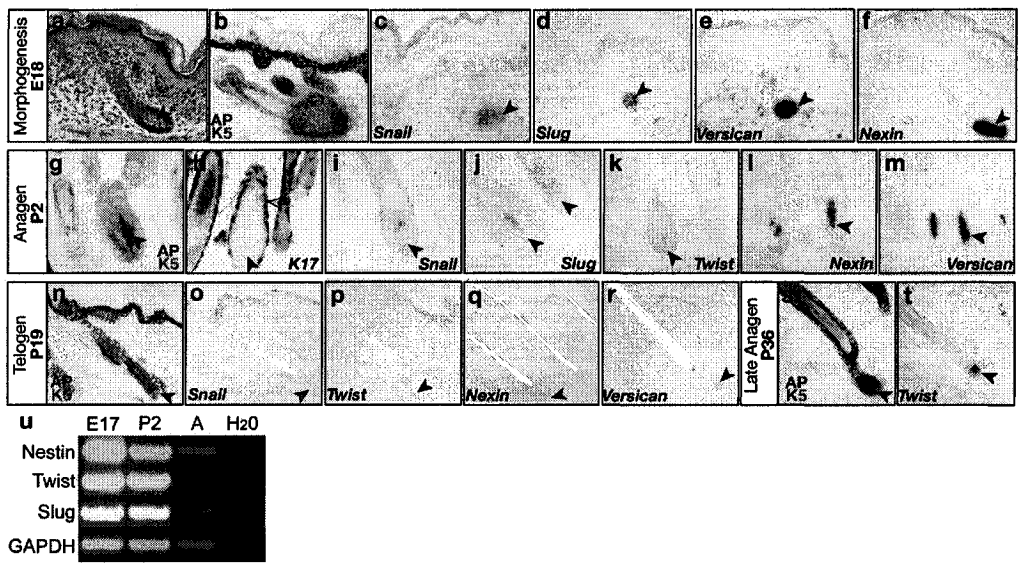

Figure 4. ***Expression of SKP transcription factors is localized to the follicle papillae of dorsal hair follicles and is hair-cycle dependent.*** Red arrowheads indicate follicle papillae. **(a-f)** At E18.5, a dorsal hair follicle in late morphogenesis has a well-developed dermal papilla ensheathed 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 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 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.

Figure 5.

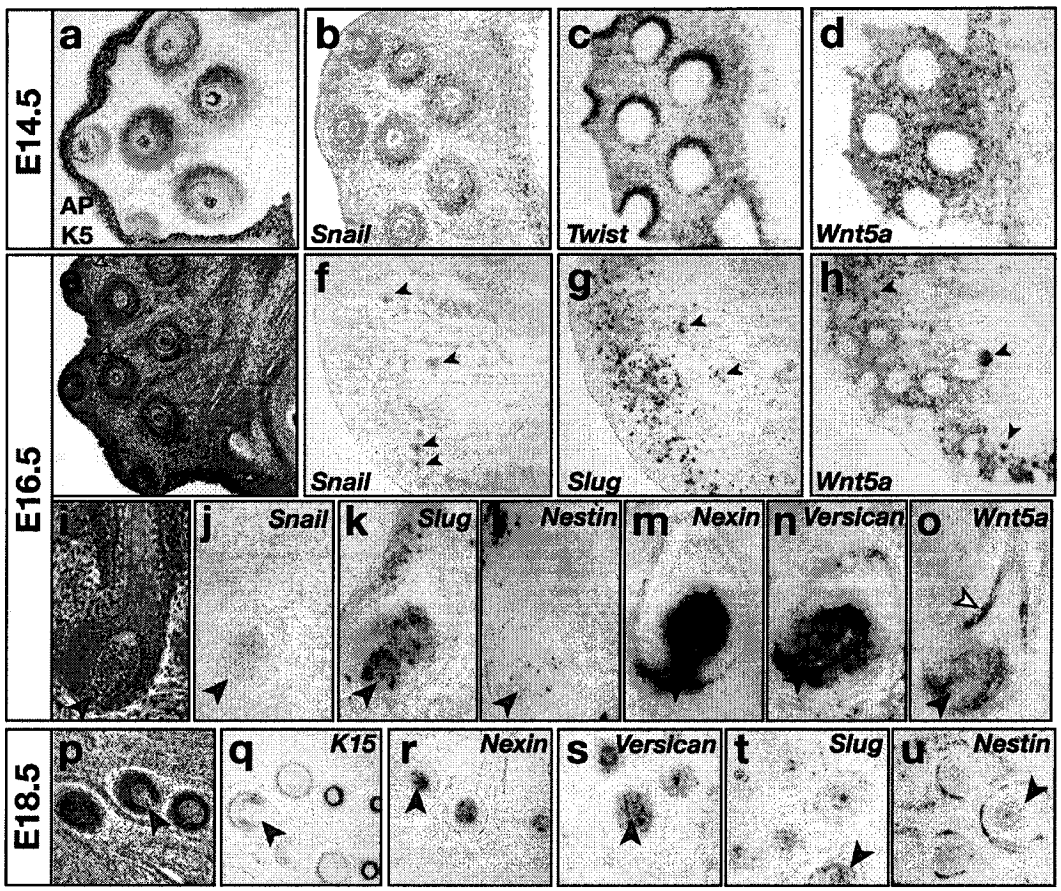

Figure 5. ***Neural crest-derived facial dermis dynamically expresses SKP markers, which become progressively restricted to follicle papillae of vibrissae and hair follicles.*** Red arrowheads indicate follicle papillae. **(a-d)**, Serial sections of E14.5 whisker pad. Concentric rings characteristic 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, smaller hair follicles are observed (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 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 SKP markers, *slug* **(t)** and *nestin* **(u)**. The epidermal stem cell marker *Keratin15* (Liu et al., 2003) is highly expressed in the outer root sheath and at low levels in the matrix adjacent to the papillae, but not in the papillae.

Figure 6.

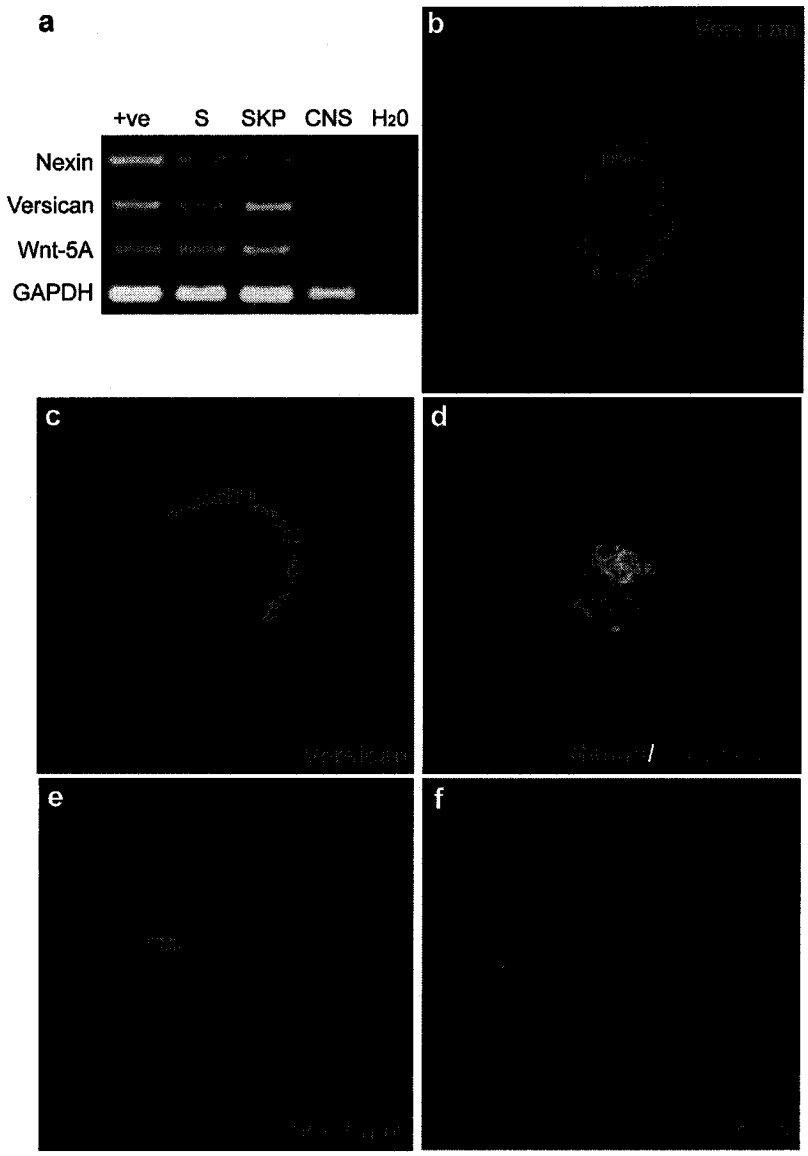

Figure 6. ***Adult whisker papillae contain SKP-like cells.*** **(a)** RT-PCR for the follicle papilla markers nexin, versican and Wnt-5a in total RNA isolated from E12 embryos (+), skin (S), SKP spheres (SKP) or CNS neurospheres (CNS). Note that SKPs but not neurospheres express all of

these markers. **(b)** Immunocytochemical analysis for the follicle papilla marker versican in a SKPs sphere. Note that most, if not all of the cells express versican. **(c, d)** Immunostaining of papillae spheres for versican **(c)**, or for nestin and fibronectin **(d)**. **(e, f)** Fluorescence photomicrographs of whisker papilla spheres that was differentiated for three days and then immunostained for βIII-tubulin **(e)** or for SMA **(f)**.

Figure 7.

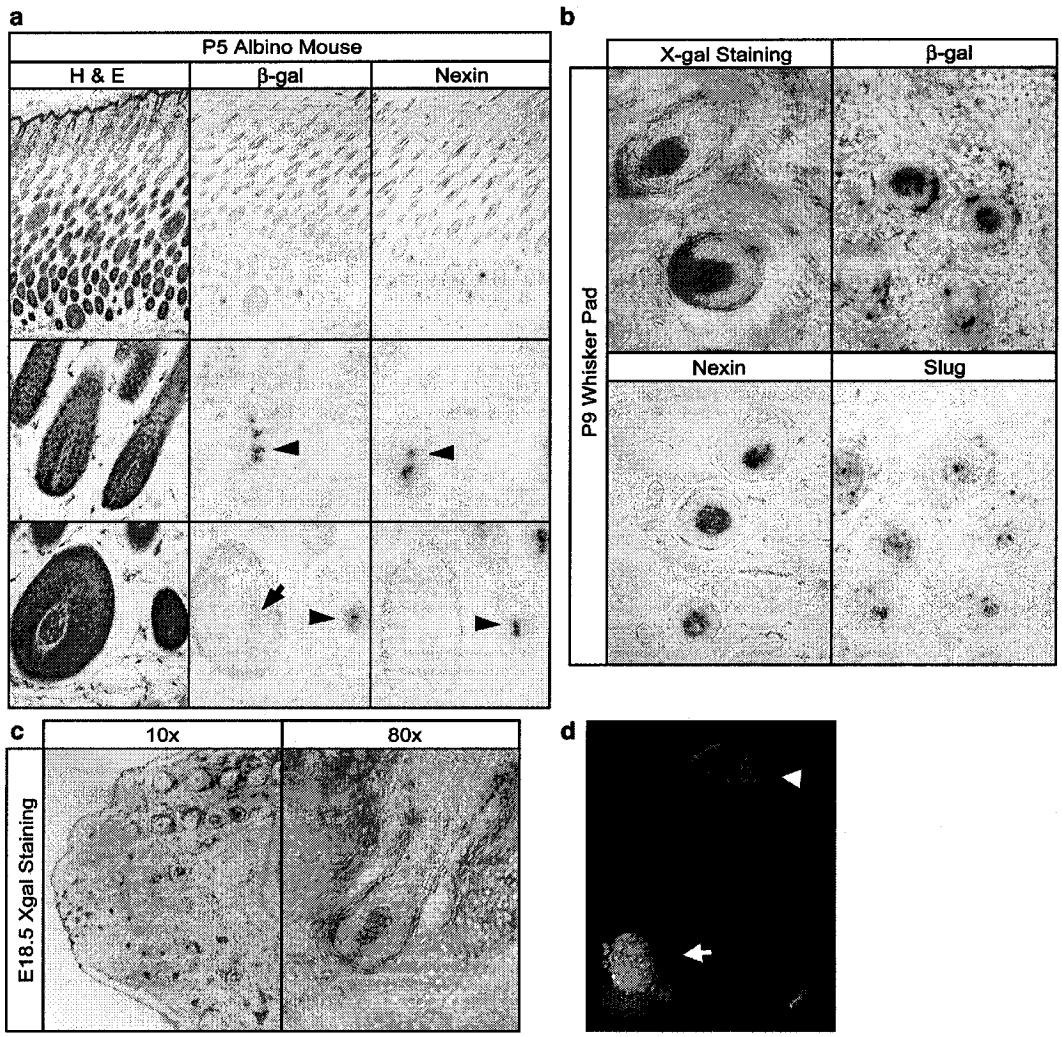

Figure 7. Hair and whisker papillae contain neural crest-derived cells. (a) Neural-crest-derived cells are found in hair follicle dermal papillae of Wnt1Cre;R26R mice. Overlapping expression of β-galactosidase (β-gal) and the dermal papilla marker nexin, in postnatal day 5 (P5) neonatal Wnt1Cre;R26R 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 section 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 wisker 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, 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.

Figure 8.

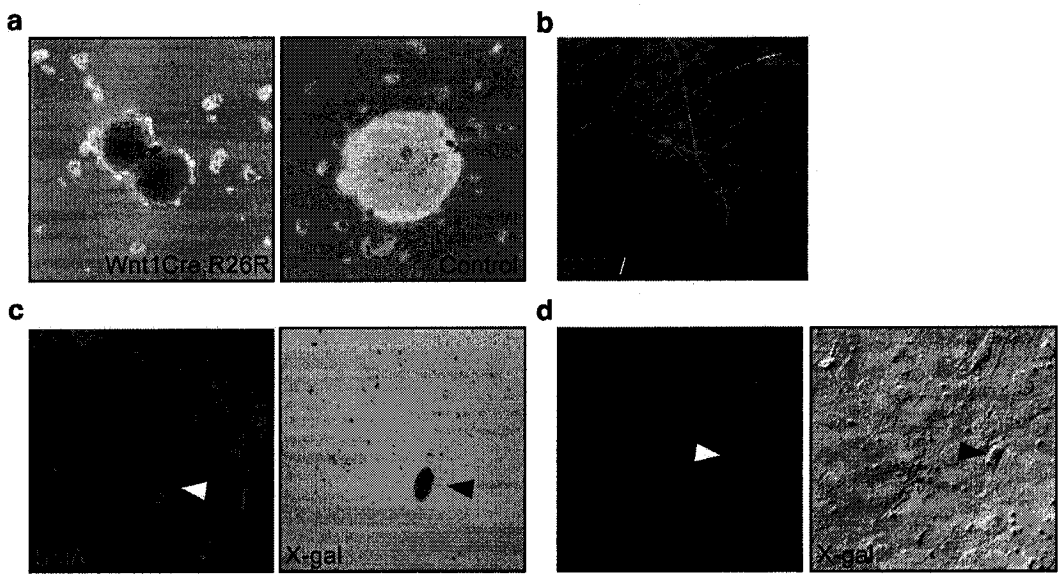

Figure 8. ***SKPs derived from facial skin are of neural crest origin.*** **(a)** X-gal stained SKPs spheres generated from the whisker pad skin of a litter of neonatal Wnt1Cre;R26R mice (left) or, as a control, from dorsal skin of a neonatal rat. Because the transgene is not penetrant in facial skin 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 are trangene-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 SKPs 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 while 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.

Figure S1.

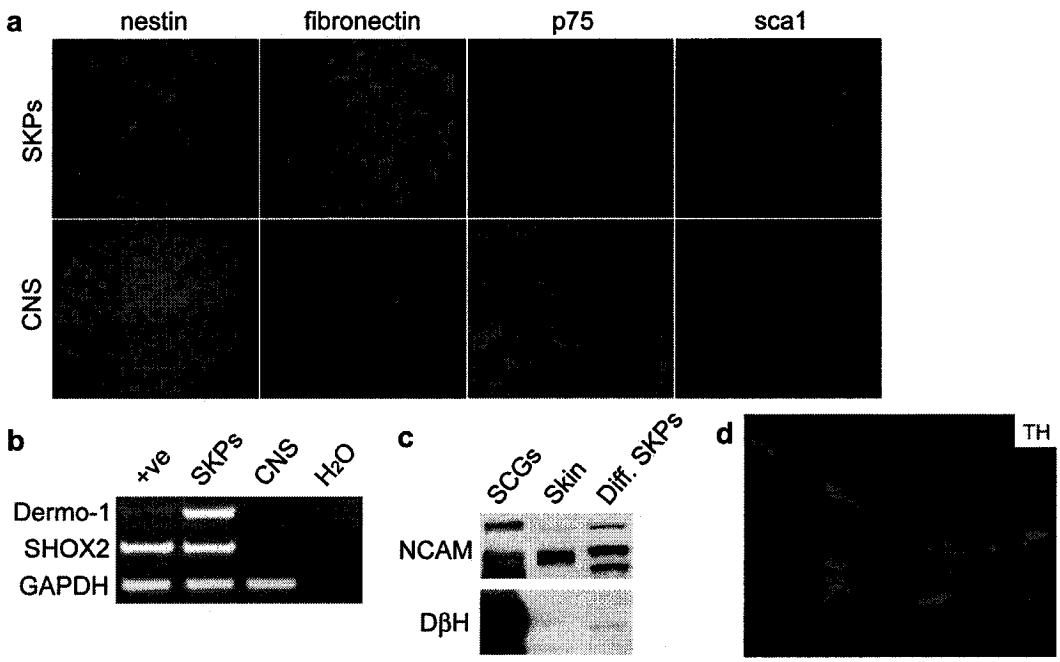

Figure S1. (a) Immunocytochemical analysis of SKP spheres culture from neonatal mouse (nestin, fibronectin, Sca-1) or rat (p75NTR) back skin, and passaged two or three times (top panels) as compared to neurospheres cultured from the embryonic telencephalon and passaged a similar number of times (bottom panels). In all panels, the immunostained cells are red and the blue is from Heochst staining of the nuclei to show all cells. Note that SKPs express fibronectin and 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 describe 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.

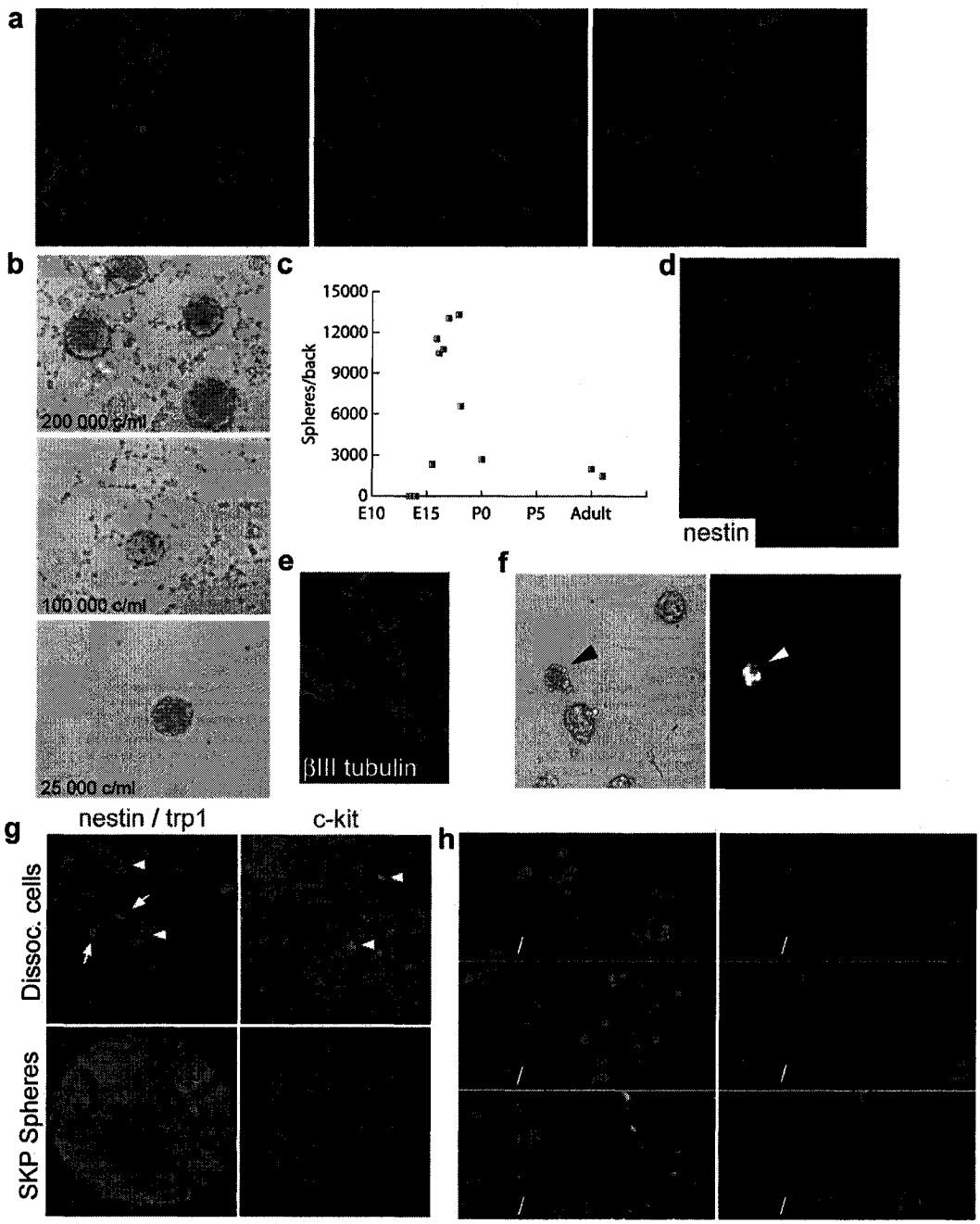

Figure S2. (a) Immunocytochemical analysis for βIII-tubulin in primary E18 (middle panel) or adult (right panel) murine skin cells versus E18

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 poy-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 vibrisall cells and E18 primary skin cells and culture 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 of a primary SKPs sphere. In all panels, the blue derives from Hoechst staining of cell nuclei. **(h)** Fluorescence micrographs of primary dissociated dorsal skin cells double-labeled 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.

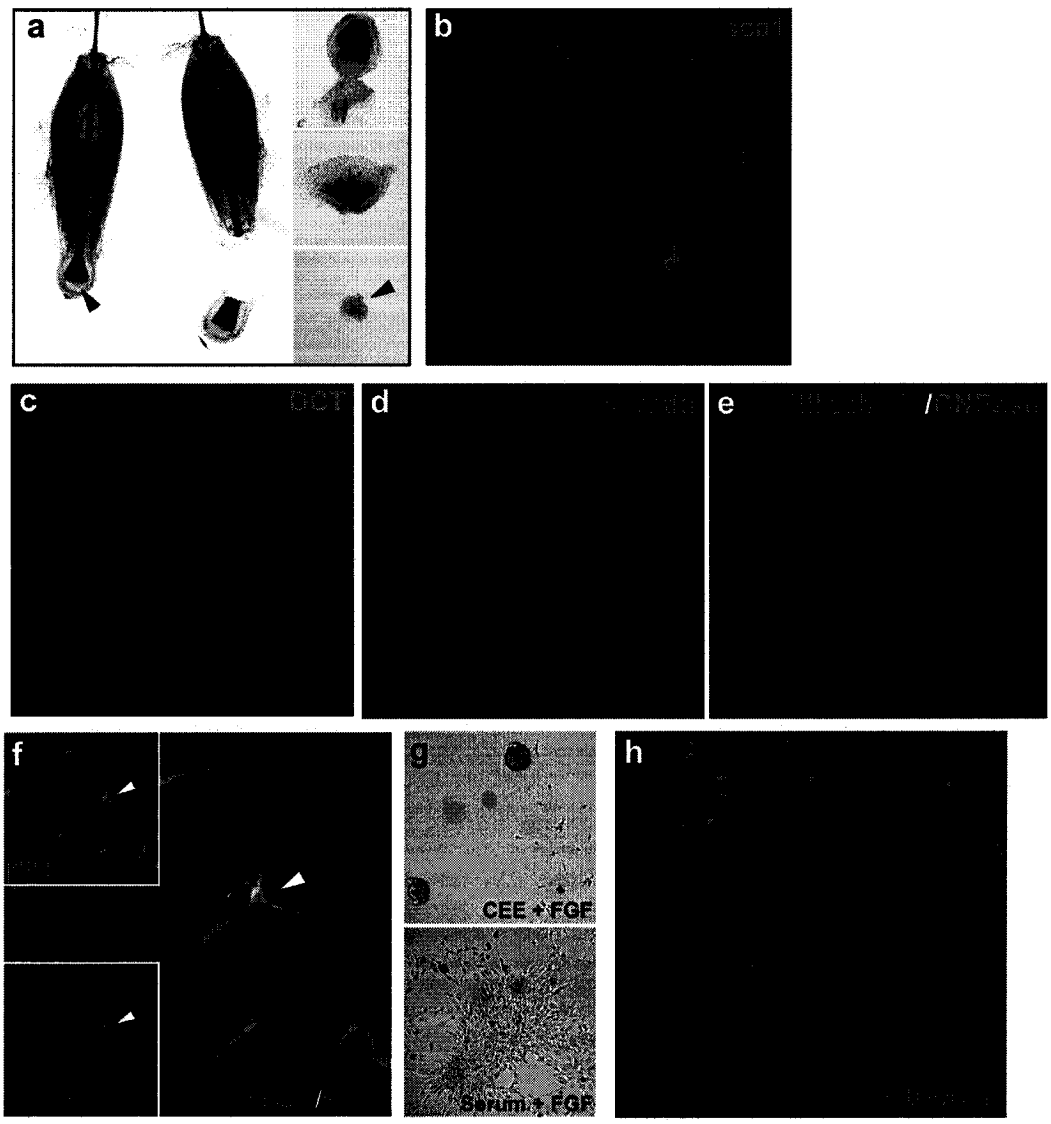

Figure S3. **(a)** Photomicrographs of a vibrissal papilla dissection from an adult pigmented mouse. The left picture shows the intact vibrissa, while other pictures show the dissection. The arrowhead indicates the papilla. **(b, c)** Immunocytochemical analysis of dissected whisker papilla cells that 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 dissected papilla cells 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 immunstained for βIII-tubulin. In panels **b-e, f** and **h,** nuclei were stained blue with Hoechst.

Figure S4.

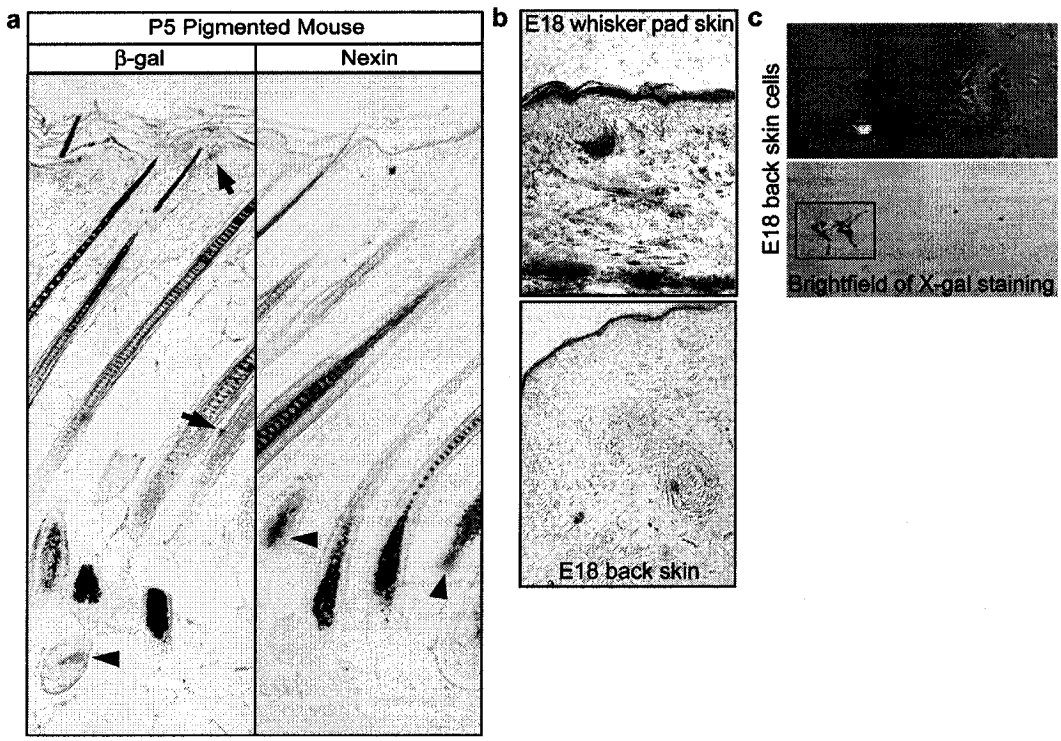

Figure S4. (a) Overlapping expression of β-galactosidase and the dermal papilla marker, nexin, in postnatal day 5 (P5) Wnt1Cre;R262R dorsal skin from pigmented mice. Pigmented melanin granules are deposited in the developing hair shaft by neural crest-derived melanoblasts resident and interspersed in 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;R26R 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 the 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.

ACKNOWLEDGMENTS

We thank Anne Aumont, Yephat Wang-Ninio, Amélie Rioux-Taché for excellent technical support, the Miller and Kaplan laboratories for valuable discussion of this work, and Andras Nagy for providing mice. This work was funded by The Canadian Stem Cell Network and CIHR to FDM, CIHR to CCH, NIH to PAL, and NSERC to VR. KJLK was funded by fellowships from the CIHR/Canadian Neurotrauma Research Program and Restracomp, KMS from CIHR and Restracomp, FBH from CIHR, JB from the Parkinsons Foundation of Canada, and NRK from the Christopher Reeves Paralysis Foundation.

Introduction to Chapter 3

In the previous chapter we showed evidence that SKPs are a neural crest precursor that persists in the skin through adulthood. While neural crest precursors have been isolated from other adult tissues, such as gut and tooth, SKPs are the first example of neural crest precursors in an accessible tissue. The accessibility of the skin lends SKPs to clinical applications in which a small skin biopsy could be used to generate large numbers of cells for autologous transplantation. Several issues need to be addressed before SKPs-based therapies can be realized. Cell culture protocols need to be designed that promote differentiation of SKPs into desirable cell types and the functionality of SKPs progeny must be tested by *in vivo* transplantation experiments.

In the next chapter we focus on one SKP-derived cell type, Schwann cells. Schwann cells are an attractive cell type to study for several reasons. First, there is abundant literature on the conditions for the culture and proliferation of primary Schwann cells and differentiation of Schwann cells derived from neural crest stem cells which we can use to devise cell culture protocols to maximize Schwann cell yield from SKPs. Second, Schwann cells have been shown to be useful in the treatment of a variety of diseases and injuries including spinal cord injury and multiple sclerosis. Finally, assays for the functionality of Schwann cells are unambiguous, with the demonstration of myelination at the ultrastructural level being the gold standard.

Chapter 3:

SKIN-DERIVED PRECURSORS GENERATE MYELINATING SCHWANN CELLS FOR THE INJURED AND DYSMYELINATED NERVOUS SYSTEM

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Nonstandard abbreviations:

SKP - skin-derived precursor

SC - Schwann cell

EM - electron microscopy

MBP - myelin basic protein

ABSTRACT

While neural stem cells hold considerable promise for treatment of the injured or degenerating nervous system, their current human sources are embryonic stem cells and embryonic neural tissue. Here, we demonstrate that rodent and human SKin-derived Precursors (SKPs), neural crest-related precursors found in postnatal dermis, represent an adult source of functional, myelinating Schwann cells. Specifically, cultured SKPs responded to neural crest cues to generate Schwann cells, and these Schwann cells proliferated and induced myelin proteins when in contact with axons in culture. When either naive or differentiated SKPs were transplanted into the injured peripheral nerve or the dysmyelinated brain of the *shiverer* mutant mouse, SKP-derived Schwann cells associated with and myelinated axons. Thus, SKPs generate functional neural progeny in response to appropriate neural crest cues, and in so doing provide a highly accessible, potentially autologous source of myelinating cells for treatment of nervous system injury, congenital leukodystrophies and dysmyelinating disorders.

INTRODUCTION

There is considerable interest in the use of stem cells for treatment of the traumatized or diseased nervous system. While embryonic stem cells and embryonically-derived neural stem cells are candidate sources of transplantable neural precursors, their use is associated with both ethical and clinical issues, such as the requirement for immune suppression. The recent discovery of precursor cells with neural potential in peripheral adult tissues such as skin (Toma et al., 2001), gut (Kruger et al., 2002) and pancreas (Seaberg et al., 2004) provides a potential adult stem cell source for neural transplantation. The skin, the most accessible of these tissues, contains a neural crest related precursor cell (termed SKPs for SKin-derived Precursors) capable of differentiation into peripheral neurons and Schwann cells (SCs) (Toma et al., 2001; Fernandes et al., 2004). Since SKPs can be isolated and expanded from human skin (Joannides et al., 2004; Toma et al., 2005), they may well provide an accessible, autologous source of neural crest-derived cell types for transplantation.

SCs are one neural crest-derived cell type that have been proposed for a number of clinical applications based upon their ability to remyelinate demyelinated lesions (Blakemore and Crang, 1985; Kohama et al., 2001) and to promote regeneration and remyelination in the injured spinal cord (Takami et al., 2002; Pearse et al., 2004). Artificial grafts seeded with

SCs have also been shown to promote regeneration after peripheral nerve injury (Guenard et al., 1992). However, human SCs are derived from invasive nerve biopsies, and their expansion is limited, making it desirable to identify a renewable, accessible SC source. In this regard, bone marrow stromal cells have been shown to generate SCs by some groups (Dezawa et al., 2001; Akiyama et al., 2002a), but others have reported that these same cells do not generate bona fide neural cell types (Vitry et al., 2003; Neuhuber et al., 2004). Here, we have asked whether SKPs generate functional neural progeny and, if so, whether they represent an accessible, alternative SC precursor source. Our findings indicate that SKPs do indeed differentiate into SCs in response to appropriate neural crest cues and that these SCs are capable of myelinating the injured peripheral nerve and dysmyelinated CNS.

MATERIALS AND METHODS

Cell Culture. Rodent and human SKPs were cultured as previously described (Toma et al., 2001; Fernandes et al., 2004; Toma et al., 2005). Briefly, for rodent SKPs, skin from mouse embryos (E15-19), mouse or rat neonates (P2-P6) was cut into 2-3 mm^2 pieces. Tissue was digested with 0.1% trypsin or 1 mg/ml collagenase for 10-45 min at 37°C, mechanically dissociated and filtered through a 40 μm cell strainer (Falcon). Cells were plated in DMEM-F12, 3:1 (Invitrogen), with 20 ng/ml EGF and 40 ng/ml

FGF2 (both Collaborative Research), hereafter referred to as proliferation media. SKPs were passaged by mechanically dissociating spheres and splitting 1 to 3 with 75% new medium and 25% conditioned medium. Human SKPs were generated from infant circumcisions as described (Toma et al., 2005). For SC differentiation, primary or secondary spheres were dissociated and plated on poly-D-lysine and laminin (BD Biosciences), cultured in DMEM-F12 3:1 with 10% FBS for 7 days, and then switched to the same medium plus 4 μM forskolin (Sigma). For some experiments, SKPs were differentiated in DMEM-F12 3:1 with 1% N2 supplement, 10 ng/ml heregulin-β1 (R&D Systems) and 4 μM forskolin, referred to as SC differentiation medium or SC conditions. Medium was changed every 3-4 days. Proliferating colonies of SCs were isolated with cloning cylinders (Corning), trypsinized from the dish and replated in the same medium. Cultures of increasing purity were obtained by sequential passaging. Pure cultures (> 95% purity) were passaged once a week, up to 5 times with cells being frozen at early passages for later use. Clonal spheres were prepared as described (Fernandes et al., 2004) and were differentiated similarly with the addition of 1% serum for the first 3 days. Human SCs were differentiated as for rodent SKPs with the addition of 1-5% serum.

DRG cocultures. Cocultures were prepared basically as described (Eldridge et al., 1987), with some modifications. Briefly, dorsal root

ganglia were dissected from E15 Sprague Dawley rat embryos, suspended in drops of DMEM:F12 medium (3:1) with N2 and 50 ng/ml NGF (Cedarlane), and plated on chamber slides coated in poly-D-lysine and laminin. 24 hrs later YFP-tagged SKPs differentiated for 7 days in SC differentiation medium were injected into the ganglia with a nanoinjector (Drummond). In some experiments SKPs were plated at the same time as the ganglia. Ascorbic acid (10 μM) and 10 ng/ml IGF-1 (R&D Systems) were added after 7 days for an additional 2 weeks.

Cerebellar slice cultures. Organotypic slice cultures (300 μm) were generated from the cerebellum of P12-P14 *shiverer* mutant mice (Jackson Laboratories) with a McIlwain tissue chopper (Mickle Laboratory Engineering Co.), and maintained as described (Stoppini et al., 1991). Briefly, slices were grown on Millicell-CM membranes (Millipore) over MEM (Gibco), HBSS (Gibco) and heat inactivated horse serum (Gibco) in a ratio of 2:1:1, supplemented with 1% Penicillin-Streptomycin, glucose and sodium bicarbonate. Approximately 10^5 SKPs differentiated for 1 week in SC differentiation medium were transplanted into white matter tracts using a nanoinjector. Medium was changed every three days and immunocytochemistry performed at two weeks.

Transplantation experiments. For the peripheral nerve experiments, 6-8 week old Sv129 (Charles River) or homozygous *shiverer* (Jackson

laboratories) mice were used. Animals were anaesthetized with isofluorane (in 40% O_2 and 60% N_2O), the sciatic nerve was exposed and crushed with #5 forceps for 1 minute approximately 5 mm proximal to the nerve bifurcation. Naive or differentiated SKPs, or purified SKP-SCs (10^5 cells in 1-2 ml of media) were immediately transplanted into the distal nerve by injection with a 10 µl Hamilton syringe fitted with a 33-gauge needle. All animals received daily injections of cyclosporine A (30 mg/kg). Animals were anesthetized with a lethal dose of Somnotol, and perfused with saline, followed by 4% paraformaldehyde with or without 1% glutaraldehyde for EM and immunocytochemistry, respectively.

For the CNS experiments, P1 homozygous *shiverer* mice were cryoanesthetized, and dissociated murine or human SKPs (1×10^5 in 1 µl of F12 medium) were injected through the skull into the cerebellum and midbrain via a 33-gauge Hamilton syringe. To identify the transplant regions, murine SKPs were generated from YFP-transgenic mice (Hadjantonakis et al., 1998) and human SKPs were labeled by adding Cell Tracker CM DiI (1:1000, Molecular Probes) to the donor cell suspension 30 minutes prior. Mice were sacrificed at 4 weeks, transplanted regions were identified by fluorescence illumination, isolated and trimmed, and then these tissue pieces were processed for EM. As controls, the homotopic regions that did not contain fluorescence label were isolated. All procedures were approved by the Hospital for Sick

Children Research Institute, in accordance with guidelines of the Canadian Council on Animal Care.

Immunocytochemistry. Immunocytochemical analysis for cells was performed on plated cells in chamber slides (Nunc) as previously described (Toma et al., 2001). The following primary antibodies were used: anti-βIII-tubulin monoclonal (1:500, Covance), rabbit anti-neurofilament-M (1:500, Chemicon), polyclonal chicken anti-green fluorescent protein (1:1000, Chemicon), anti-GFAP polyclonal (1:500, DAKO), anti-$p75^{NTR}$ polyclonal (1:500, Promega), anti-S100β monoclonal (1:1000, Sigma), anti-pmp22 monoclonal (1:200, Neomarkers), anti-MBP monoclonal (1:200, Chemicon), anti-MBP monoclonal (1:200, Serotec) or anti-Ki67 monoclonal (1:200, BD Biosciences Pharmingen). Secondary antibodies used were: Alexa488-conjugated goat anti-mouse, Alexa555 goat anti-mouse IgM, Alexa555 goat anti-rabbit and Alexa350 goat anti-mouse (1:1000; all from Molecular Probes). Alternatively, semithin nerve sections were incubated with rat anti-MBP (1:20, Serotec) followed by a peroxidase conjugated anti-rat secondary (Jackson Immunoresearch) and visualized with nickel-enhanced diaminobenzidine (Vector Laboratories).

Confocal and electron microscopy. Confocal imaging was done using a Zeiss LSM 5 Pascal confocal microscope and image processing software (Zeiss). Colocalization was verified by imaging adjacent 1 or 2

μm optical slices. Samples for EM were post-fixed in 4% PFA/1% glutaraldehyde for 1 hour, washed in PBS, fixed for 1.5 hour in 1% osmium tetroxide, washed in phosphate buffer, and dehydrated through a graded ethanol series. Samples were then washed 3 x 10 minutes in propylene oxide, incubated 1.5 hours in 1:1 Spurr resin/ethanol, 2 x 2 hours in 100% Spurr resin and polymerized overnight at 70° C. Thin sections were collected on copper grids and stained with uranyl acetate and lead citrate. Samples for immuno-EM were post-fixed in 4%PFA/1% glutaraldehyde for 1 hour, washed in PBS, cryoprotected in 30% glycerol for 4 hours and immersed in liquid nitrogen. Freeze-substitution was performed over 48 hours at –90°C in 1% uranyl acetate in methanol using an automatic freeze-substitution system (Leica). The temperature was raised to -35° C over 18 hours, samples were washed in methanol, and embedded in Lowicryl HM20 as follows: 1:1 HM20 methanol for 2 hours, 3:1 HM20/methanol for 2 hours followed by 100% HM20 for 18 hours all at –35°C. The plastic was polymerized with UV for 48 hours at –35°C. Sections on nickel grids were incubated with rat-anti MBP (Serotec, 1:20) for 4 hours, washed in PBS, and stained with 10nm colloidal gold conjugated anti-rat secondary (1:20, Sigma) for two hours, all at room temperature. Sections were then fixed for 5 minutes in 2% glutaraldehyde in PBS and stained with uranyl acetate and lead citrate. Sections were examined using an FEI Tecnai 20 transmission electron microscope.

RESULTS

Rodent and human SKPs generate myelinating Schwann cells. We have previously shown that both rodent and human SKPs can generate, under basal differentiation conditions, a low number of cells with characteristics of SCs (Fernandes et al., 2004; Toma et al., 2005). To enhance the generation and expansion of SCs from SKPs, we utilized two extrinsic cues, forskolin (which increases intracellular cAMP) and heregulin-β, that promote the differentiation and proliferation of SCs from embryonic neural crest precursors (Morgan et al., 1991; Shah et al., 1994). Neonatal mouse or rat SKPs differentiated in serum and forskolin for 7 days generated parallel arrays of S100β positive cells, characteristic of SCs (Fig. 1A). In contrast, few bipolar S100β positive cells were observed in cultures differentiated in serum alone (Fig. 1B). Further characterization revealed that many S100β-positive cells co-expressed myelin basic protein (MBP) or peripheral myelin protein (pmp22) (Fig. 1C,D), as well as GFAP and p75NTR (data not shown). Approximately 20-30% of the differentiated cells expressed SC markers.

While this represented an increase in SC differentiation, the presence of serum supported the differentiation and proliferation of other cell types, including SMA-positive myofibroblasts. We therefore hypothesized that we could further enhance SC yield by culturing

neonatal rat SKPs with both forskolin and heregulin-βin the absence of serum. When single, clonal rat SKP spheres, generated as we have previously described (Fernandes et al., 2004), were differentiated for 10-12 days in forskolin and heregulin-β, 83% of the clones contained GFAP-positive SCs (Fig. 1E), compared to only 3% when cultured in the absence of these cues (Fernandes et al., 2004). Thus, heregulin-β and forskolin instruct SKPs to generate SCs, as they do for embryonic neural crest precursors (Shah et al., 1994). When mass cultures of rodent SKPs were differentiated with forskolin and heregulin-β for 7 days, small colonies of SKP-derived SCs (SKP-SCs) were readily observed, and could be isolated with cloning cylinders and replated in the same conditions for 1-2 weeks to ultimately yield cultures of greater than 95% purity (data not shown).

Similar conditions could be used to expand human SKP-SCs. Under basal differentiation conditions, neonatal human foreskin SKPs generate few identifiable SCs (Toma et al., 2005). In contrast, when human SKPs were differentiated in 1% serum with forskolin and heregulin-β, parallel arrays of many bipolar S100β-positive cells were observed (Fig. 1F), and immunocytochemistry revealed that many of these cells co-expressed GFAP or p75NTR (data not shown) and a subpopulation expressed pmp22 or MBP (Fig. 1G,H). Thus, human SKPs also respond to neural crest cues to generate SCs.

Rodent SKPs myelinate peripheral axons in culture and in the injured nerve. To assess the functionality of SKP-SCs, we cocultured neonatal, YFP-expressing mouse SKPs with dorsal root ganglion (DRG) explants under conditions where primary SCs will myelinate sensory neuron axons (Eldridge et al., 1987). SKPs were differentiated with forskolin and heregulin-β for 7 days, and were then cocultured in the absence of these cues with freshly prepared E15 rat DRG explants. Within 1-3 days, many of the YFP-tagged SKPs associated with sensory axons (Fig. 2A). At 1 week, the number of YFP-positive cells populating axons had increased (Fig. 2B), and many of these were dividing, as monitored by expression of the proliferation marker Ki67 (Fig. 2D). Since SC mitogens were absent from these cultures, we conclude that SKP-SCs proliferated in response to axon-derived signals, as do bona fide SCs. We then added ascorbic acid (Eldridge et al., 1987) and insulin-like growth factor (Cheng et al., 1999), factors known to induce myelination *in vitro*. Triple-labelling two weeks later revealed YFP-positive, pmp22-positive myelinating fibers surrounding bIII-tubulin-positive axons (Fig. 2C,E). Thus, SKP-SCs are indistinguishable from primary SC when cocultured with peripheral neurons.

We next assessed the ability of rodent SKP-SCs to myelinate the peripheral nervous system *in vivo*. Neonatal YFP-tagged murine SKPs were differentiated in heregulin-β and forskolin for 5-7 days, and were transplanted into the uninjured sciatic nerve of immunosuppressed adult

mice. After one to two weeks, transplanted YFP-positive cells had migrated proximally and distally from the transplantation site and were aligned longitudinally in the nerve (Fig. 3A). Immunocytochemistry for neurofilament-M (NFM) and MBP revealed that although transplanted cells were apparently closely aligned with axons, few expressed myelin proteins (Fig. 3A).

In the uninjured nerve, all axons are associated with endogenous SCs. To increase the ability of transplanted cells to associate with and myelinate axons, we performed a sciatic nerve crush prior to transplantation. Neonatal YFP-positive SKPs were differentiated for one week under SC conditions, and were injected distal to the crush injury. Immunocytochemical analysis of nerve cross-sections by confocal microscopy two weeks later revealed that many of the YFP-tagged cells engulfed neurofilament-positive axons over a series of adjacent optical slices (Fig. 3B). These YFP-positive cells co-expressed MBP or pmp22 and exhibited a morphology consistent with that of myelinating SCs (Fig. 3C,D). Similar profiles were observed up to 4 weeks post-injury, the longest timepoint examined. Interestingly, similar results were obtained following transplantation of naive SKPs, although the percentage of YFP-positive cells expressing myelin-specific proteins was lower (data not shown).

To definitively demonstrate myelination by SKP-SCs, we utilized *shiverer* mice which carry a mutant MBP gene, and which have been

used extensively to study myelination by transplanted cells (Cheng et al., 1999; Liu et al., 2000). While the *shiverer* PNS exhibits grossly normal compact myelin, myelination by wildtype transplanted cells can be visualized by MBP immunostaining. To perform these experiments, the sciatic nerve of adult *shiverer* mice was crushed, and rat SKP-SCs that had been expanded and purified were transplanted distal to the crush. Four weeks later, semithin sections of *shiverer* nerves were stained for MBP. This analysis revealed many MBP-positive myelin sheaths as far as 3-4 mm from the transplant site in the transplanted but not control nerves (Fig. 4A,B). Immuno-EM for MBP confirmed the presence of MBP-positive myelin, and demonstrated that transplant-derived myelin was compact with repeating major dense lines (Fig. 4C,D). Thus, SKP-SCs can myelinate regenerating axons in the peripheral nerve.

Rodent and human SKPs myelinate CNS axons in *shiverer* mice. Nerve-derived SCs can myelinate CNS axons, and their transplantation has therefore been suggested as a potential treatment for multiple sclerosis (Blakemore and Crang, 1985) and spinal cord injury (Takami et al., 2002; Pearse et al., 2004). To ask whether SKP-SCs could repair the dysmyelinated CNS, we transplanted neonatal YFP-tagged SKPs differentiated for 7 days in SC conditions into white matter tracts of organotypic cerebellar slice cultures derived from *shiverer* mice (Fig. 5A). As predicted, MBP expression was limited to the transplanted cells (Fig.

5A,B). Immunocytochemical analysis two weeks post-transplantation revealed that YFP-positive cells co-expressed MBP and formed fibrous tracts within the cerebellar white matter (Fig. 5C). These MBP-positive cells also co-expressed S100β, confirming their identity as SCs (Fig. 5D). Thus, SKP-SCs can associate with and potentially myelinate CNS axons.

We next asked whether SKP-derived cells could myelinate the CNS *in vivo*, by transplanting undifferentiated neonatal, YFP-tagged SKPs into the brains of newborn *shiverer* mice, at a timepoint when myelination is commencing *in vivo*. Unlike the PNS, the *shiverer* CNS is characterized by extensive dysmyelination, such that any compact myelin can only be attributed to transplanted cells (Cheng et al., 1999; Liu et al., 2000; Vitry et al., 2003). Four weeks post-transplantation, we identified areas containing transplanted YFP-positive cells, and processed these regions for EM. This analysis revealed that compact myelin was present in transplanted regions of *shiverer* brains (Fig. 6A) but not in adjacent regions that did not contain labeled cells (data not shown) or in control shiverer brains (Fig. 6B). At higher magnification, compact myelin with repeating major dense lines was evident in transplanted brains (Fig. 6C) while non-transplanted brains exhibited loosely wrapped axons characteristic of *shiverer* myelin (Cheng et al., 1999; Liu et al., 2000) (Fig. 6D). Thus, even naive SKPs will differentiate into myelinating SCs and myelinate axons, presumably in response to axonal cues within the developing postnatal CNS.

Finally, we asked whether human SKPs could generate myelinating SCs *in vivo*, as they could *in vitro* (Fig. 1F-H). Undifferentiated neonatal human foreskin SKPs were labeled with DiI and transplanted into the neonatal *shiverer* mouse brain. After 6 weeks, transplant sites were visualized by DiI and processed for EM. Compact myelin similar to that seen in the rodent SKPs transplants was observed in transplanted regions (Fig. 6E,F) but not in adjacent DiI-negative regions. Similar results were obtained in 3 shiverer mice transplanted with human SKPs and analyzed by EM. Since compact myelin was never observed in the CNS of 6 control *shiverer* mice, and since it has never been previously reported (Cheng et al., 1999; Liu et al., 2000; Vitry et al., 2003), we conclude that, like their rodent counterparts, human SKPs were able to myelinate the dysmyelinated CNS.

DISCUSSION

In summary, data reported here indicate that both human and rodent SKPs respond to environmental cues that regulate neural crest stem cells to generate proliferating SC precursors, and that these SC precursors can differentiate into mature SCs that myelinate axons in the injured peripheral nerve and the dysmyelinated CNS. Moreover, our data demonstrate that these *in vivo* environments are themselves sufficient to direct naive SKPs to differentiate into myelinating SCs. Thus, SKPs provide a highly accessible, potentially autologous source of human SC

precursors for cell therapy. These data, together with our previous work (Toma et al., 2001; Fernandes et al., 2004; Toma et al., 2005) indicate that SKPs represent a bona fide adult neural crest precursor cell that can generate functional neural progeny, a finding of importance from both basic research and clinical perspectives.

The demonstration that adult human skin contains a multipotent precursor that can generate functional SCs has a number of implications. First, our findings indicate that SKPs could be used as a source of human, potentially autologous, SCs for transplantation in disorders ranging from spinal cord injury to dysmyelinating disorders. In this regard, while neonatal foreskin was the source of human SKPs used here, SKPs can also be isolated and expanded from adult human skin biopsies (Toma et al., 2001; Joannides et al., 2004; Toma et al., 2005). In contrast, human SCs are currently generated from peripheral nerves, and any autologous approach requires nerve biopsies, an invasive procedure. Second, these data suggest that SKPS represent a candidate initiating cell for tumors such as those seen in neurofibromatosis-1, which partially manifests in skin, and are thought to involve a transformed SC or SC precursor (Suter and Scherer, 2003). Third, a number of serious human peripheral neuropathies involve SCs, including genetic disorders such as Charcot-Marie-Tooth syndrome (Suter and Scherer, 2003). We propose that SKPs isolated from skin biopsies of patient populations would provide a source of compromised SCs and their precursors and could potentially

provide novel insights into disease pathogenesis. Finally, since SKPs clonally generate both neural and mesodermal cells types in culture (Toma et al., 2001; Fernandes et al., 2004; Toma et al., 2005), the finding that at least some of these progeny are functional *in vivo*, as shown here, provides strong support for the concept that SKPs represent an expandable, clinically-useful source of human neural crest-derived cell types for a variety of purposes.

ACKNOWLEDGEMENTS

We thank Nao Kobayashi and Anne Aumont for experimental help, and members of the Miller/Kaplan laboratories for valuable input. This work was funded by grants to FDM from the Canadian Stroke and Stem Cell Networks, Canadian Heart and Stroke Foundation, Neurosciences Canada and the CIHR. IAM was funded by the Canadian Stem Cell Network and Hospital for Sick Children Foundation, and JB by the Parkinsons Foundation of Canada. FDM holds a Canada Research Chair.

FIGURES AND LEGENDS

Figure 1.

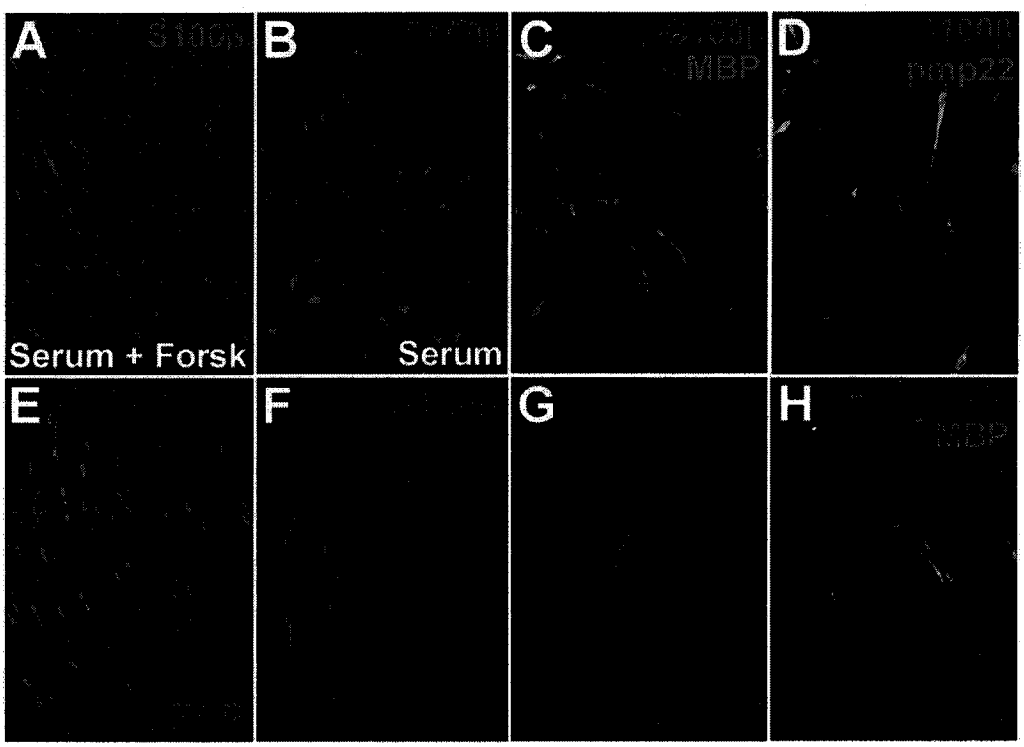

Figure 1. Rodent and human SKPs differentiate into Schwann cells in response to neural crest cues. (A) Neonatal rat SKPs differentiated in serum and forskolin or (B) in serum alone and immunostained for S100β (red). (C,D) Neonatal rat SKPs differentiated in serum and forskolin for 10 days and then immunostained for S100β (red) and (C) MBP or (D) pmp22 (green). Panels show the merged images. (E) A representative clonal rat SKPs sphere differentiated for 10 days in forskolin and heregulin-β and then immunostained for GFAP (red). (F-H)

Human neonatal foreskin SKPs passaged 3 times, differentiated in serum, forskolin and heregulin-β and immunostained for (F) S100β (red), (G) pmp22 (red) or (H) S100β (red) and MBP (green). Panels F-H each show differentiation of a different line of human SKPs. In all panels, nuclei were visualized by hoechst staining (blue).

Figure 2.

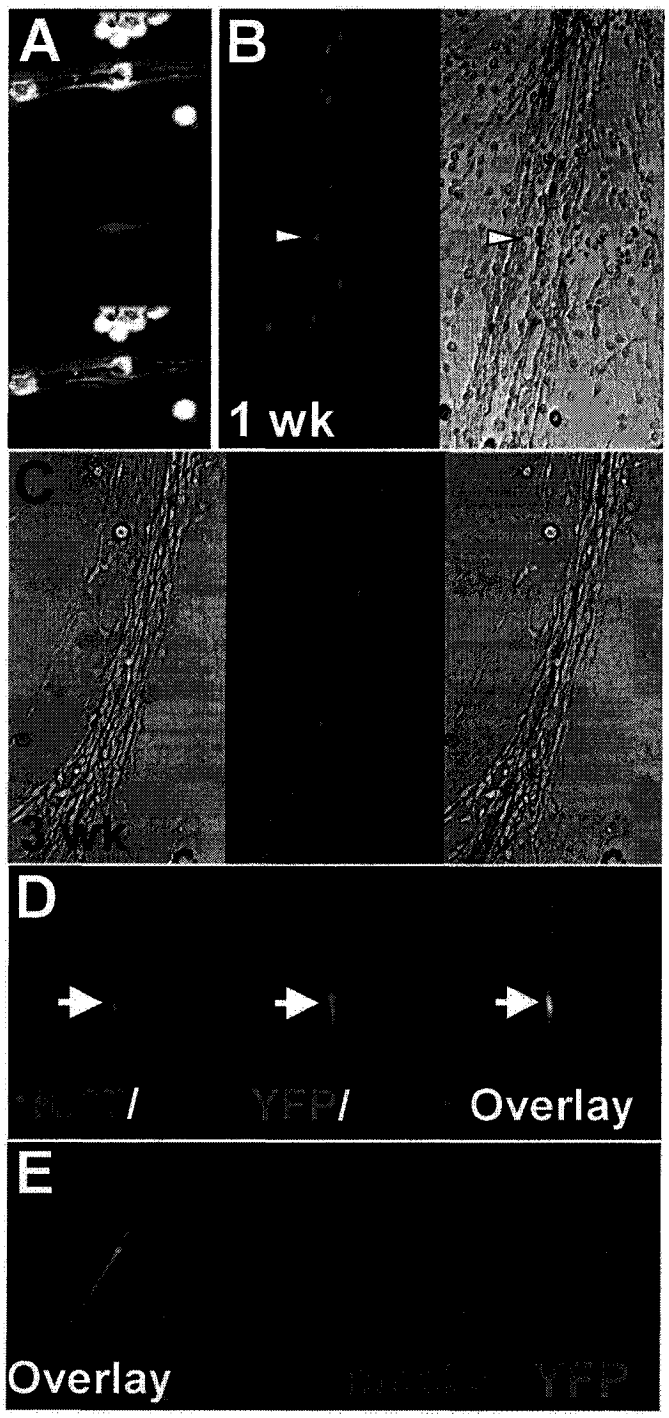

Figure 2. SKP-derived Schwann cells associate with axons, proliferate and express myelin proteins when cocultured with sensory neurons. (A) A YFP-positive SKP-SC associated with a neuronal process within 1 day of coculture as visualized by phase (top) and fluorescence microscopy (middle, bottom is the merge) to visualize axons and YFP, respectively. (B,C) Fluorescence microscopy combined with differential interference contrast microscopy to visualize YFP-tagged cells and axons, respectively in cocultures. Differentiated, YFP-tagged SKPs with DRG explants were cocultured for 1 week in the presence of N2 and NGF (B), or for 2 additional weeks (3 in total) with N2, ascorbic acid and IGF-1 to promote myelination *in vitro* (C). (D) Cocultures maintained as in panel B, and immunostained for the neuronal marker βIII-tubulin (blue, all panels), the proliferation marker Ki67 (red, left panel), and YFP (green, middle panel; merged image on right). Arrow indicates a proliferating transplanted cell associated with axons. (E) Cocultures maintained as in panel C, and immunostained for βIII-tubulin (blue, all panels),YFP (green, right panel), and pmp22 (red, middle right panel; merged image on left).

Figure 3.

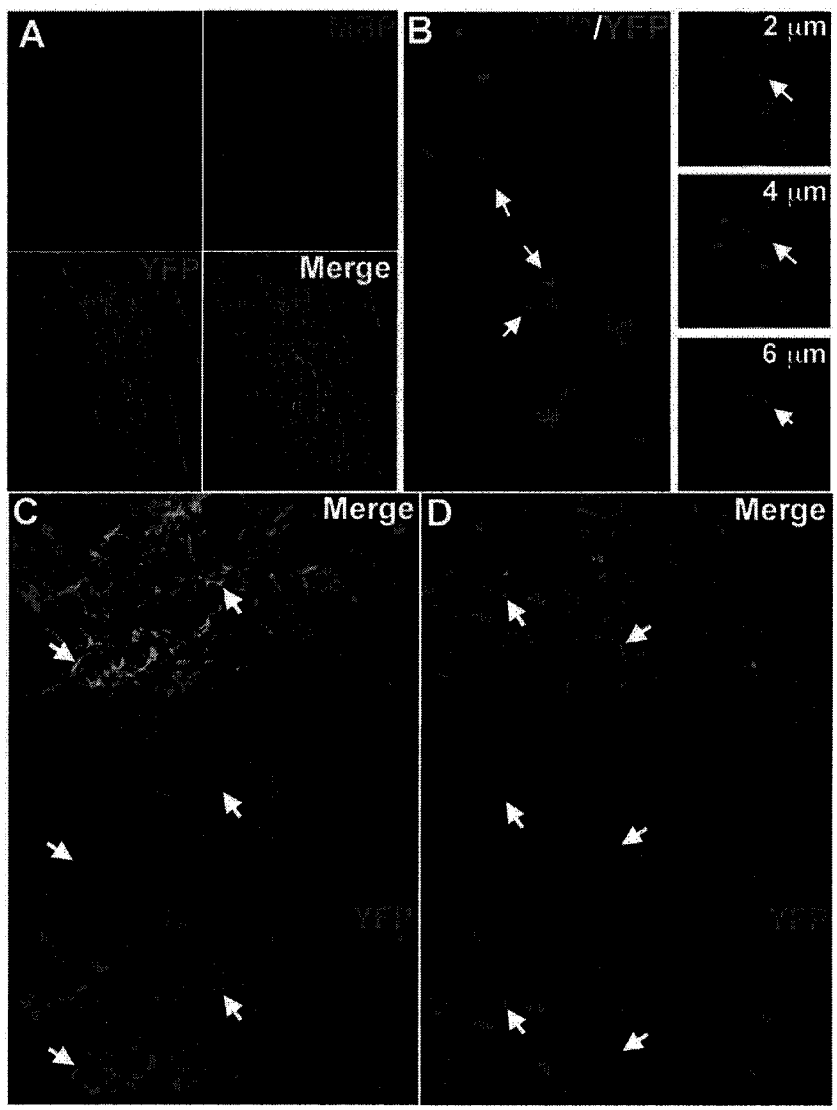

Figure 3. SKP-derived Schwann cells associate with axons and express myelin proteins when transplanted into the injured peripheral nerve. (A) Confocal microscopy of longitudinal sections of uninjured sciatic nerve 7 days after transplantation of YFP-tagged SKPs differentiated under SC conditions. Sections were immunostained for

neurofilament medium (NFM, blue, top left panel), myelin basic protein (MBP, red, top right panel), YFP (green, bottom left panel). The merged image is shown in the bottom right panel. Similar results were obtained with 5 transplanted animals. (B) Confocal microscopy of cross-sections of injured sciatic nerve 2 weeks after distal transplantation of YFP-tagged SKPs differentiated for 7 days under SC conditions. Sections were immunostained for NFM (red) and YFP (green). A series of 2 mm optical slices at high magnification (right row) demonstrated NFM-positive axons engulfed by transplanted cells (arrows). (C,D) Confocal microscopy of cross-sections of injured sciatic nerve 2 weeks after transplantation of differentiated, YFP-tagged SKPs. Sections were immunostained for YFP (green, bottom panels) and (C) MBP or (D) pmp22 (red, middle panels) with arrowheads indicating colocalization. Similar results were obtained with 7 transplanted animals.

Figure 4.

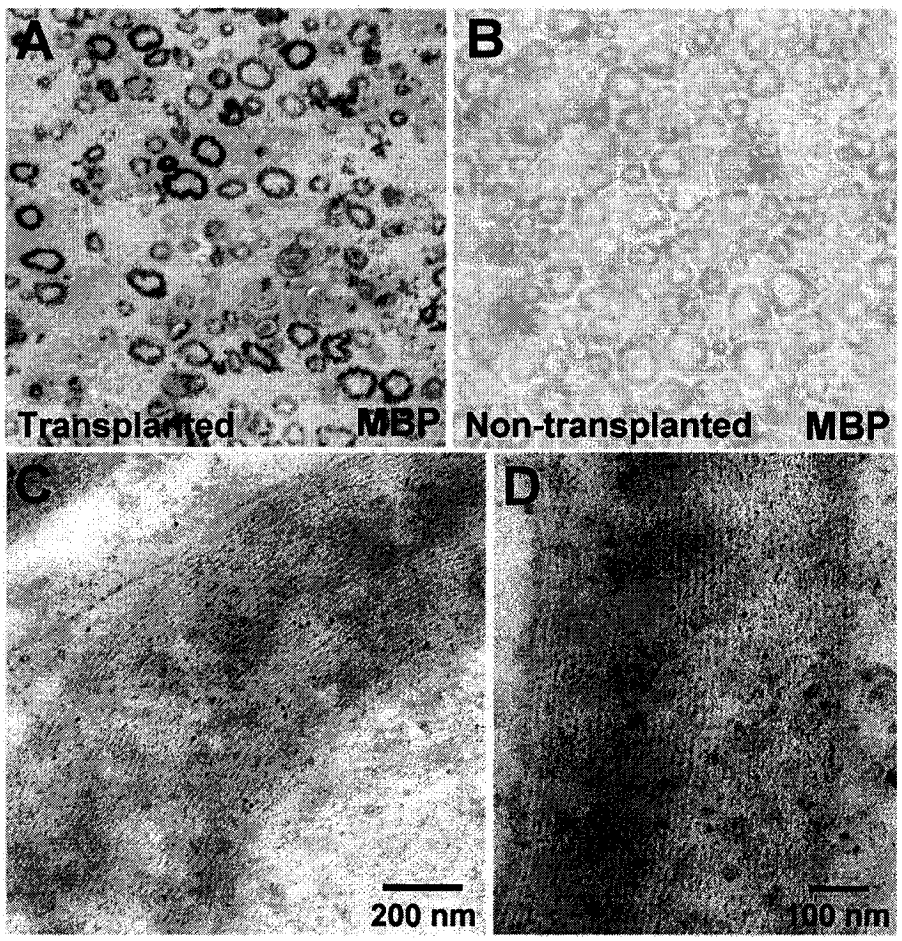

Figure 4. Structural evidence of myelination in the injured peripheral *shiverer* nerve by SKP-derived Schwann cells. (A,B) High magnification (100x) micrographs of semithin cross-sections of (A) injured *shiverer* sciatic nerve 4 weeks after transplantation of purified mouse SKP-SCs or, for comparison, (B) of the non-transplanted and uninjured contralateral *shiverer* nerve. Sections were immunostained for MBP and visualized with DAB. (C,D) Electron micrographs of ultrathin cross-

sections of injured *shiverer* sciatic nerve 4 weeks after transplantation of purified SKP-SCs. Sections were immunostained for MBP and visualized with gold-labeled secondary antibody.

Figure 5.

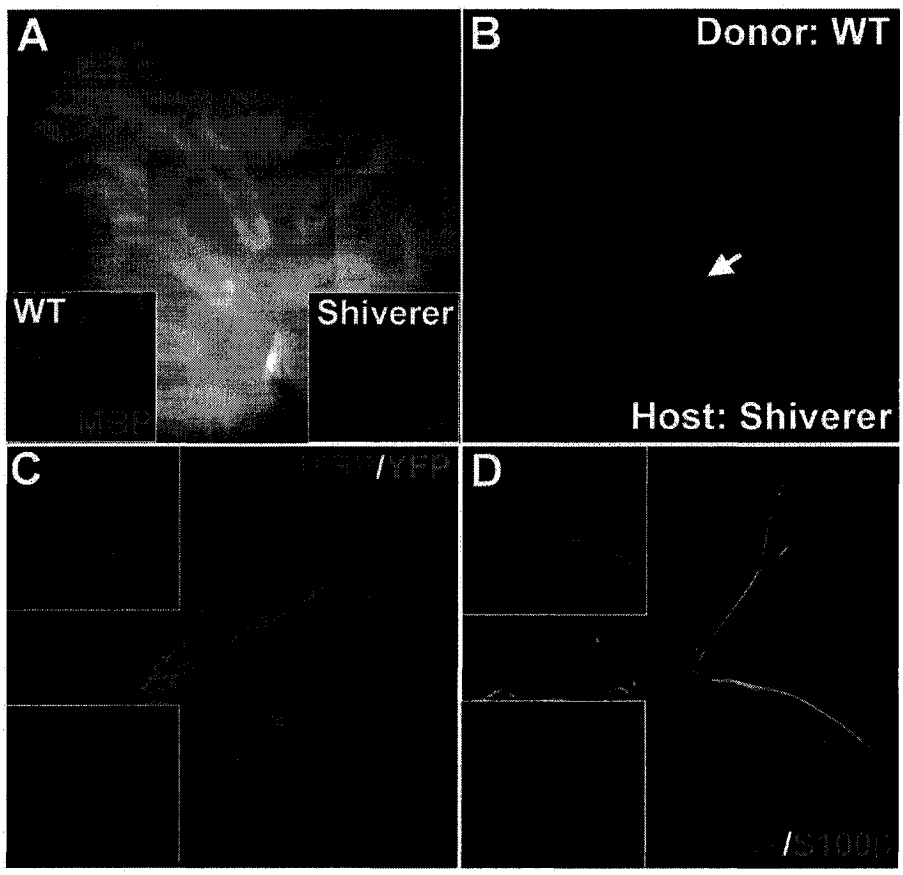

Figure 5. Transplanted SKP-derived Schwann cells associate with axons and express a myelination program in the dysmyelinated *shiverer* cerebellum ex vivo. (A) Representative two week-old cerebellar slice culture as visualized by autofluorescence.

Immunostaining confirmed that slice cultures from wild type (left inset) but not shiverer (right inset) cultures expressed MBP (red). (B) Fluorescence micrograph of differentiated SKPs transplanted into slice cultures and immunostained for MBP (red) after 3 days. Arrow indicates transplant site. In A and B, nuclei were visualized with hoechst (blue). (C,D) Fluorescence micrographs of SKP-derived cells on cerebellar slices 2 weeks after transplantation of differentiated, YFP-tagged SKPs, as visualized by immunostaining for (C) MBP (red) and YFP (green) or (D) MBP (red) and S100β (green). Similar results were obtained in 3 independent slice culture experiments.

Figure 6.

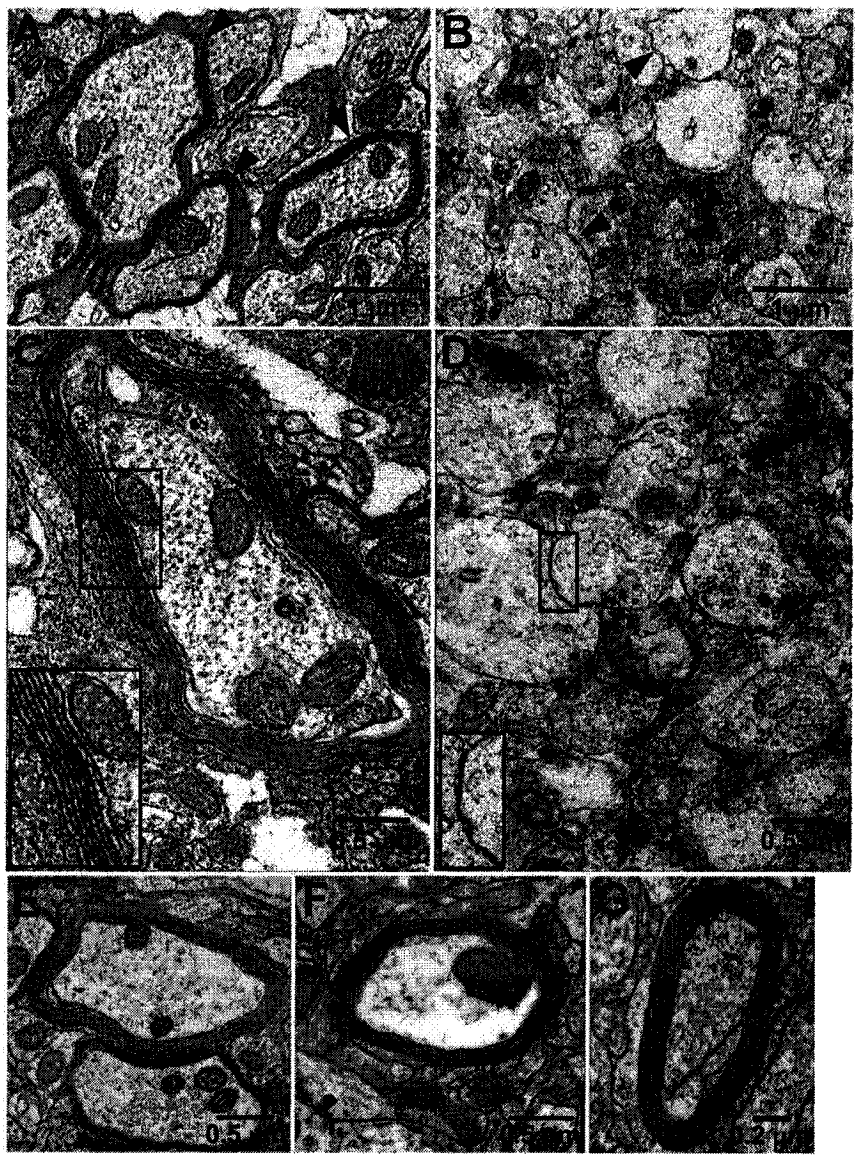

Figure 6. Transplanted, naive rodent and human SKPs generate Schwann cells to myelinate axons in the *shiverer* brain. (A-D) Electron micrographs of ultrathin sections of the *shiverer* midbrain 4 weeks after transplantation at birth with undifferentiated murine SKPs.

Arrowheads indicate myelin sheaths surrounding axons in regions of the *shiverer* midbrain that had been transplanted with SKPs (A,C) (regions identified by YFP expression), but not in control brains (B,D). Compact myelin with major dense lines was observed only in transplanted regions (insets in C,D). Similar results were obtained in 3 transplanted *shiverer* animals. (E,F,G) Electron micrographs of compact myelin generated by human SKPs transplanted into the brains of three different neonatal *shiverer* mice, as analyzed at 6 weeks post-transplantation.

Chapter 4: General Discussion

This thesis follows the characterization of SKPs from their developmental origins and localization in the skin, to the assessment of functionality of one type of differentiated SKPs progeny, Schwann cells. One of our first clues that SKPs may be related in some way to the neural crest came from a pilot DNA microarray study. From this experiment, in which we compared the gene expression of SKPs to CNS-derived neurospheres and an embryonic fibroblast cell line, the expression of several transcription factors involved in various aspects of neural crest development including slug, shox2 and dermo-1 were found to be enriched in SKPs. We subsequently confirmed these findings by RT-PCR and expanded our investigation to examine the expression of other neural crest transcription factors.

The identification of these factors not only provided us with evidence as to the developmental origins of SKPs but also provided potential markers with which we could attempt to identify a niche for SKPs within the skin. When we examined the expression of slug, snail and twist, we found that their expression was largely restricted to the dermal papillae of hair follicles. We subsequently demonstrated that SKPs spheres also express the dermal papillae markers nexin, versican and wnt-5a, an indication of their origin in the skin. To confirm that the dermal papillae is a SKPs niche, we cultured cells from microdissected papillae

and demonstrated that SKPs spheres could be generated that expressed SKPs markers and differentiated into normal SKPs progeny.

The expression of SKPs/neural crest markers in the dermal papillae was dynamic and occurred specifically during the anagen or growth phase of hair and whisker follicles. It will be interesting to determine if there is a hair follicle cycle stage dependence on the ability to culture SKPs, which could be easily tested by synchronizing the hair cycle in adult animals by depilation. The dermal papillae has already been reported as a source of cells that can generate osteoblasts and adipocytes (Jahoda et al., 2003) and contribute to the hematopoietic system (Lako et al., 2002). SKPs can differentiate into adipocytes and we also have evidence that when cultured under conditions that promote the differentiation of bone and cartilage, SKPs express chondrocyte and osteoblasts markers and generate deposits of collagen and mineralized calcium, indicative of chondrocyte and osteoblast activity (K. Fernandes, J. Biernaskie I. McKenzie, F. Miller, unpublished data). It is possible that the stem cell activity previously reported in cells of the dermal papillae represents the mesodermal potential of SKPs. The relationship between SKPs and the dermal papillae-derived cells that contribute to the hematopoietic system remains to be determined. The neural crest does not contribute to the hematopoietic system, and as such, I find it unlikely that this cell population is related in some way to SKPs. Moreover, if it is a SKP cell responsible for this activity, it is likely a rare event similar to

that found in the case of neural stem cells repopulating the hematopoietic system (Bjornson et al., 1999).

The data presented in this thesis also demonstrates that a multipotent neural crest precursor is maintained in a tissue that is the target of neural crest migration during development. While neural crest precursors have been found in other adult tissues as well, including gut (Kruger et al., 2002) and tooth, SKPs are by far more accessible making them a more attractive option for possible clinical applications and also demonstrate the greatest potentiality. Other adult neural crest populations isolated thus far are restricted to neural differentiation whereas SKPs demonstrate neural and mesodermal potential. This not only increases the number of potential clinical applications, but also raises interesting questions as to the relationship of various neural crest stem cell populations and perhaps suggests that SKPs possess characteristics of a primordial neural crest cell that has maintained a greater potentiality.

Other groups have also recently demonstrated that the hair follicle is a reservoir of neural precursors (Sieber-Blum et al., 2004; Amoh et al., 2005a). While there are many similarities between SKPs and the cells described in these studies, one key difference is the location in the hair follicle from which they are derived. Both groups identify the niche of their cells as the hair follicle bulge, also described as the niche for epidermal stem cells. In Sieber-Blum et al., after microdissection and explant culture of the bulge area of vibrissae follicles, the cells that migrated out

of the explants were expanded adherently *in vitro* and then differentiated into neurons, Schwann cells, melanocytes and smooth muscle. Wnt1cre;R26R mice were used in this study and it was subsequently shown that the cells migrating from the explanted bulge regions expressed the β-galactosidase transgene and were therefore neural crest derived. Amoh *et al.*, also isolate cells from the bulge region of vibrissae hair follicles, but do so from nestin-GFP transgenic mice which have been previously shown to have GFP-positive cells in that region (Li et al., 2003). Despite the ability of these nestin-expressing bulge cells to differentiate into some neural crest derivatives, the authors do not raise the possibility that they are related in some way to the neural crest. They claim that these nestin-positive bulge cells differentiate into astrocytes and oligodendrocytes based solely on the expression of a single antigen, GFAP and CNPase respectively. The morphology of the cells expressing these antigens is not consistent with their proposed identity and a more likely interpretation is that the glia generated are in fact Schwann cells, which also express these markers and that the precursors involved have properties of neural crest precursors. Another interesting finding by Amoh *et al.*, is that the nestin-positive bulge cells are capable of differentiating into keratin 5/8 (K5/8)-positive cells, with K5/8 being a marker of keratinocytes. Presuming that the cells they describe are in fact the same cells described by Sieber-Blum *et al.*, and that they are neural crest precursors, this would be the first evidence that a neural crest precursor

could generate ectodermal progeny. Although to date SKPs have not been shown to differentiate into keratin-expressing cells, it would be interesting to revisit the issue using the keratinocyte optimized culture conditions used by Amoh *et al.*, to determine if SKPs also share this capacity.

The ability of the nestin-expressing bulge cells to generate keratinocytes is somewhat surprising, especially when one considers that despite the fact that keratinocytes represent 10-18% of the *in vitro* differentiated progeny, the authors do not report the generation of any keratinocytes after transplantation of the cells back into skin, something that has been shown in bulge transplant experiments by other groups. Furthermore, the authors do report extensive neuronal differentiation from transplanted bulge cells, which is unexpected when one considers that neuronal differentiation does not normally take place in the skin.

In a separate study the same group has suggested that the same nestin-positive bulge cells were able to contribute to the formation of new blood vessels when transplanted into the skin (Amoh et al., 2005b). Neural crest cells also contribute to blood vessels during development, in both the brain and to the cardiac outflow tract of the heart. The hair follicle has already been shown to be a source of cells to repair injury to the dermis and epidermis, perhaps another endogenous function of neural crest stem cells in the hair follicle is to provide a source of cells for angiogenesis after injury to the skin. In support of this hypothesis, in a

study that transplanted human SKP-like cells into the lateral ventricles of the brains of mice reported that a sub-population of cells that migrated into the forebrain, expressed the endothelial marker Ve-cadherin and integrated into the walls of blood vessels.

Are SKPs and bulge-localized neural cell precursors related or do they represent distinct cell populations? It is difficult to make direct comparisons of the two studies mentioned above and ours, given the different culture conditions used and the different cell markers used to examine differentiation. This caveat aside, one potential difference between the neural precursors derived from the bulge and SKPs, is the ability of the former to generate melanocytes. It has been proposed that the bulge may also be a niche for melanocyte stem cells, as DCT-positive melanoblasts located there are capable of repopulating melanocytes throughout the hair follicle (Nishimura et al., 2002). While this may suggest that neural crest precursors in the bulge are distinct from SKPs, another possibility is that SKPs are capable of melanocyte differentiation and that it has as of yet not been observed. The vast majority of SKP differentiation experiments were conducted with SKPs generated from albino mice and as a result melanocytes could not be easily identified.

Given the dynamic changes in hair follicle structure during the hair cycle, it is perhaps not surprising that there is considerable cellular movement throughout the hair follicle. Cells from the bulge migrate extensively contributing to the outer root sheath over a large portion of the

vertical axis of the hair follicle including the matrix near the base (Cotsarelis et al., 1990). Likewise, cells from within the dermal papillae can contribute to dermal sheath cells and *vice versa* (McElwee et al., 2003; Tobin et al., 2003). The exchange of cells between different hair follicle compartments could allow for the two related populations of neural crest precursors to contribute to the various neural crest progeny of the hair follicle and skin during normal maintenance or after injury.

Human SKPs appear to be very similar to their rodent counterparts with a few exceptions. The cell cycle time of human SKPs is approximately 10-fold slower than that of rodent SKPs. Human SKPs spheres also express p75NTR as determined by RT-PCR while rodent SKPs do not. Aside from these differences, both human and rodent SKPs possess the same differentiation repertoire and express markers associated with the neural crest. The vast majority of human SKPs cultures in our lab were generated from foreskin tissue derived from circumcisions, although we have also cultured SKPs successfully from scalp tags resulting from stereotactic neurosurgeries and other groups have cultured SKPs from skin sources other than scalp and foreskin (Belicchi et al., 2004; Joannides et al., 2004). Given the data demonstrating a SKPs niche within hair follicles of dorsal skin, it raises questions as to the niche of SKPs within the foreskin, which is a hairless structure. It would be interesting to perform histological studies examining the expression of neural crest markers in the tissue to

determine if a niche within the foreskin can be identified. While the foreskin lacks hair follicles, it does have a papillar structure, not to be confused with the dermal papillae of hair follicles but instead referring to the ridged nature of the epidermis, in a similar sense as to the skin of the finger tips. Within this ridged structure there are concentrated areas of mechanoreceptors (Taylor et al., 1996), so there are discreet regions within the skin of the foreskin that could possible serve a SKPs niche.

The foreskin is highly innervated, containing many nerve terminals, and a high concentration mechanoreceptors including Merkel cells and Meissner corpuscles (Cold and Taylor, 1999). The foreskin is also characterized by a dartos smooth muscle layer, the same type of muscle found in the scrotum that controls its tone in response to temperature change (Cold and Taylor, 1999). The endogenous function of SKPs in this tissue may be to maintain neural crest cell types, including Merkel and smooth muscle cells, or provide cells to regenerate the tissue after injury. It is known for example, that Merkel cells can regenerate in adult skin (Nurse et al., 1984) and as such it would follow that a neural crest precursor be present in the skin as a source for these cells. Some experiments to examine the endogenous role of SKPs in the skin are already underway by others in the lab. By transplanting SKPs cultured from GFP transgenic mice back into injured skin and observing how they differentiate, we hope to determine what contributions SKPs make, if any to the skin.

The differentiation of SKPs into Schwann cells follows a predictable and well-established path. Some of the earliest markers of Schwann cell differentiation, s100β and GFAP, are observed soon after SKPs are differentiated. The expression of GFAP by the SKP-derived Schwann cells is maintained, for as long as the cells are maintained in Schwann cell mitogens such as heregulin-β. When SKPs are subsequently differentiated in serum and the Schwann cell mitogens are removed, the expression of GFAP is extinguished and the expression of myelin proteins is induced. This expression profile has been previously observed of Schwann cells *in vitro* (Bachelin et al., 2005), and provides further support to the notion that SKPs do in fact differentiate into *bona fide* Schwann cells that follow a normal course of differentiation *in vitro* and recapitulates the progression of Schwann cell differentiation *in vivo*. The observation that heregulin-β, which promotes Schwann cell differentiation from neural crest stem cells also promotes Schwann cell differentiation from SKPs is further evidence that SKPs are a neural crest precursor.

Heregulin has been shown to instructively promote Schwann cell differentiation from neural crest stem cells, meaning that more stem cells adopt a Schwann cell fate at the expense of other possible cell fates (Shah et al., 1994; Shah et al., 1996). It would appear that heregulin affects SKPs differentiation in a similar manner as the number of clonally-derived SKPs spheres that yield Schwann cells increases from 15% to

83% when the spheres are differentiated in serum with neurotrophins or serum-free media supplemented with heregulin respectively.

Aside from instructing multipotent stem or precursor cells to adopt a Schwann cell fate, heregulin and forskolin also act on the committed Schwann cell precursors and immature Schwann cells by promoting their proliferation. Unlike experiments examining the differentiation of neurons from skin-derived cells, one cannot discount the possibility that Schwann cells present in the cultures of differentiating SKPs are derived from Schwann cells present in the skin that proliferate under the culture conditions used. There are several reasons this is an unlikely scenario. First, when dissociated skin cells are cultured under Schwann cell differentiation conditions, one can readily identify distinct colonies of Schwann cells that presumably arise from single cells. In contrast, when SKPs are differentiated under the same conditions at a similar density, the distribution of Schwann cells in the culture is more even, indicative of an event that occurs with higher frequency than the proliferation of rare Schwann cells from the Skin contaminating the culture. Second, proliferating SKPs do not express markers of Schwann cells as determined by immunocytochemistry and RT-PCR, suggesting that Schwann cells do not contaminate SKPs cultures. Finally, if Schwann cells present in differentiated SKPs cultures were a result of contamination from Schwann cells from skin, one would not expect to

observe Schwann cell differentiation from clonally-derived SKPs spheres which in fact does occur.

It is perhaps fortunate that the same factors that promote the differentiation of Schwann cells from SKPs, also maintain them in a proliferative state such that they can be expanded many times over. This allows us to expand and purify SKP-derived Schwann cells over time, obtaining cultures that are greater than 95% Schwann cells. With an increased Schwann cell yield we can transplant greater numbers of Schwann cells and also transplant fewer other cell types, decreasing the chances of undesirable effects, but is a pure Schwann cell population required?

The results from our transplantation experiments suggest that the answer to this question may be dependent on the context. We transplanted both undifferentiated SKPs and SKP-derived Schwann cells into the injured peripheral nerve and although both cell populations survived and expressed myelin proteins, the efficiency was approximately four fold higher with differentiated Schwann cells as measured by the degree of colocalization of myelin proteins with GFP, which marked the transplanted cells. It is important to note however, that the undifferentiated SKPs were able to differentiate within the nerve into myelinating Schwann cells. It is also possible that the decreased efficiency observed when using undifferentiated SKPs represents a delay, caused by the additional time it takes for the cells to differentiate *in vivo*,

and that if given enough time, may ultimately achieve a similar level of myelination to that of the pre-differentiated cells.

We also transplanted undifferentiated SKPs into the brains of shiverer mice and found that they were able to differentiate and myelinate host axons. This is an interesting finding in that it demonstrates that naïve SKPs are capable of Schwann cell differentiation in a completely foreign environment, the CNS. There is however considerable similarity in the growth factors involved in the development and survival of glia in the peripheral and central nervous systems. Neuregulins are expressed in the CNS (Meyer and Birchmeier, 1994), including the cerebellum, the location of SKPs transplants and are involved in oligodendrocyte differentiation and myelination (Kim et al., 2003b). It is possible that neuregulins expressed in the developing mouse brain at the time of transplantation promote the differentiation of SKPs into Schwann cells.

In this regard, it may be important to consider growth factor expression in the context of specific diseases in order to determine whether SKPs or SKPs-derived Schwann cells should be transplanted. For example, in the case of multiple sclerosis, it has been reported that the expression of neuregulins is dramatically reduced within lesions and is hypothesized to be the cause of poor remyelination (Viehover et al., 2001). As a result, transplantation of undifferentiated SKPs would likely be unsuccessful without the addition of exogenous growth factors to promote their differentiation *in vivo*.

Another group has independently reported glial differentiation of SKP-like cells after transplantation into the lateral ventricles of brains of adult mice (Belicchi et al., 2004). They report the expression of GFAP by cells that had migrated into the forebrain and note that cells migrated extensively to white matter regions including the corpus callosum. The authors also state that the cells do not express GalC, a marker of myelinating glia and conclude that the transplanted cells had differentiated into astrocytes (Belicchi et al., 2004). An alternative interpretation would be that the cells they observed had actually differentiated into Schwann cells, which would also express GFAP, but remain in a non-myelinating state, possibly due to lack of axonal contact. In the intact and disease free adult brain, transplanted cells would not have access to axons that had already been myelinated by endogenous oligodendrocytes and therefore the induction of myelin protein expression and initiation of myelination would not occur. In our studies, cells were transplanted both at a time when normal myelination was in process, but also into a mutant mouse characterized by dysmyelination which may have both granted the transplanted cells access to endogenous axons as well as provided them with a competitive advantage over the endogenous cells with defective myelination capability.

These transplantations studies have also demonstrated that SKPs are safe with respect to tumourgenic potential. SKPs are an attractive stem cell population for the development of cell-based transplantation

therapies. They are accessible and can be readily expanded, making them an ideal source for patient-specific autologous material. Unlike embryonic stem cells which form teratomas after transplantation (Martin, 1981), SKPs are not tumourgenic. As an adult neural crest stem cell population, they also have a broad differentiation potential including neural and mesodermal progeny, allowing for the generation of a variety of cell types. In addition to their potential utility for transplantation-based therapies, SKPs may also be realized as a source of donor cells for nuclear transfer for the process of therapeutic cloning. In a recent study it was shown that porcine SKP-like cells had several characteristics that made them ideal candidates as a source of nuclei for nuclear transfer including: a high proportion of cells in the culture in G1/G0 cell cycle stage; high genome stability and low frequency of karyotypic abnormalities after long term culture as compared to fetal fibroblasts; yielded embryos more similar to naturally-derived embryos than those derived from fetal fibroblast nuclear transfer with respect to gene expression and pre-implantation development (Zhu et al., 2004a).

The ability to culture an accessible neural crest precursor from adult tissue also has significance to the study of human disease. A number of diseases that present with symptoms in the skin and involve neural crest cell types, including melanoma, Neurofibromatosis Type 1 and Neuroblastoma. In some instances, such as neurofibromas and melanomas, a transformed SKP cell could in fact be the initiating cell and

would explain some of the multi-lineage differentiation observed in these lesions. Culturing SKPs from patients with these diseases to study the differentiation and transformation of skin-derived neural crest progenitors may help our understanding of the diseases progression and provide a source of cells for potential drug testing.

Summary and Conclusion

In summary, I have presented data that demonstrate SKPs are a neural crest precursor that reside within the skin and are maintained there throughout adulthood. Several lines of evidence support this hypothesis; SKPs exhibit neural crest differentiation potential, migrate via neural crest migratory pathways in chick embryos, and they express neural crest transcription factors. Data from analysis of Wnt1Cre transgenic mice in which the neural crest lineage is marked by β-galactosidase expression, also demonstrate that SKPs from the whisker pads are neural crest-derived and suggest that SKPs from body skin may be as well.

By characterizing the expression of the neural crest transcription factors expressed by SKPs in skin, we identified the dermal papillae of hair follicles as a potential niche for SKPs and subsequently demonstrated that SKPs likewise express dermal papillae specific markers and that cells with SKPs-like properties can be cultured from microdissected papillae.

The ability to culture a neural crest precursor cell from an accessible adult tissue, makes it possible to generate neural crest cell types for autologous transplantation therapies. One cell type of particular interest is the Schwann cell, which has been proposed as an ideal cell type for a variety of conditions including multiple sclerosis and spinal cord injury. We identified cell culture protocols that enhance Schwann cell differentiation from SKPs and subsequently demonstrated that these were

bona fide Schwann cells that could myelinate both the peripheral and central nervous system.

This thesis follows SKPs from their very beginning as a neural crest progenitors colonizing the skin, to the demonstration of the functional differentiation of myelinating Schwann cells. Perhaps one day SKPs will be realized as an adult source of neural crest progenitors for the treatment of disease and injury. SKPs may also prove to be invaluable research and biotechnology tools, for the study of neural crest-related developmental disorders and for development of therapeutic cloning and/or gene therapy vectors.

REFERENCE LIST

Akiyama Y, Radtke C, Kocsis JD (2002a) Remyelination of the rat spinal cord by transplantation of identified bone marrow stromal cells. J Neurosci 22:6623-6630.

Akiyama Y, Radtke C, Honmou O, Kocsis JD (2002b) Remyelination of the spinal cord following intravenous delivery of bone marrow cells. Glia 39:229-236.

Altman J (1969) Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J Comp Neurol 137:433-457.

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319-335.

Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425:968-973.

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

Amoh Y, Li L, Yang M, Jiang P, Moossa AR, Katsuoka K, Hoffman RM (2005b) Hair follicle-derived blood vessels vascularize tumors in skin and are inhibited by Doxorubicin. Cancer Res 65:2337-2343.

Asakura A (2003) Stem cells in adult skeletal muscle. Trends Cardiovasc Med 13:123-128.

Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ (1998) Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats--similarities to astrocyte grafts. Proc Natl Acad Sci U S A 95:3908-3913.

Bachelin C, Lachapelle F, Girard C, Moissonnier P, Serguera-Lagache C, Mallet J, Fontaine D, Chojnowski A, Le Guern E, Nait-Oumesmar B, Baron-Van Evercooren A (2005) Efficient myelin repair in the macaque spinal cord by autologous grafts of Schwann cells. Brain 128:540-549.

Barnabe-Heider F, Miller FD (2003) Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. J Neurosci 23:5149-5160.

Baroffio A, Dupin E, Le Douarin NM (1988) Clone-forming ability and differentiation potential of migratory neural crest cells. Proc Natl Acad Sci U S A 85:5325-5329.

Belicchi M, Pisati F, Lopa R, Porretti L, Fortunato F, Sironi M, Scalamogna M, Parati EA, Bresolin N, Torrente Y (2004) Human skin-derived stem cells migrate throughout forebrain and differentiate into astrocytes after injection into adult mouse brain. J Neurosci Res 77:475-486.

Bhattacharyya A, Brackenbury R, Ratner N (1994) Axons arrest the migration of Schwann cell precursors. Development 120:1411-1420.

Bixby S, Kruger GM, Mosher JT, Joseph NM, Morrison SJ (2002) Cell-intrinsic differences between stem cells from different regions of the peripheral nervous system regulate the generation of neural diversity. Neuron 35:643-656.

Bjornson CR, Rietze RL, Reynolds BA, Magli MC, Vescovi AL (1999) Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. Science 283:534-537.

Blakemore WF, Crang AJ (1985) The use of cultured autologous Schwann cells to remyelinate areas of persistent demyelination in the central nervous system. J Neurol Sci 70:207-223.

Blanchard AD, Sinanan A, Parmantier E, Zwart R, Broos L, Meijer D, Meier C, Jessen KR, Mirsky R (1996) Oct-6 (SCIP/Tst-1) is expressed in Schwann cell precursors, embryonic Schwann cells, and postnatal myelinating Schwann cells: comparison with Oct-1, Krox-20, and Pax-3. J Neurosci Res 46:630-640.

Botchkareva NV, Botchkarev VA, Chen LH, Lindner G, Paus R (1999) A role for p75 neurotrophin receptor in the control of hair follicle morphogenesis. Dev Biol 216:135-153.

Brazelton TR, Rossi FM, Keshet GI, Blau HM (2000) From marrow to brain: expression of neuronal phenotypes in adult mice. Science 290:1775-1779.

Britsch S, Goerich DE, Riethmacher D, Peirano RI, Rossner M, Nave KA, Birchmeier C, Wegner M (2001) The transcription factor Sox10 is a key regulator of peripheral glial development. Genes Dev 15:66-78.

Brockes JP, Lemke GE, Balzer DR, Jr. (1980) Purification and preliminary characterization of a glial growth factor from the bovine pituitary. J Biol Chem 255:8374-8377.

Bronner-Fraser M (1986) Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. Dev Biol 115:44-55.

Bronner-Fraser M (1995) Origins and developmental potential of the neural crest. Exp Cell Res 218:405-417.

Cadelli DS, Bandtlow CE, Schwab ME (1992) Oligodendrocyte- and myelin-associated inhibitors of neurite outgrowth: their involvement in the lack of CNS regeneration. Exp Neurol 115:189-192.

Cameron HA, Woolley CS, McEwen BS, Gould E (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56:337-344.

Carpenter EM, Hollyday M (1992) The location and distribution of neural crest-derived Schwann cells in developing peripheral nerves in the chick forelimb. Dev Biol 150:144-159.
Chan WY, Cheung CS, Yung KM, Copp AJ (2004) Cardiac neural crest of the mouse embryo: axial level of origin, migratory pathway and cell autonomy of the splotch (Sp2H) mutant effect. Development 131:3367-3379.
Cheng HL, Russell JW, Feldman EL (1999) IGF-I promotes peripheral nervous system myelination. Ann N Y Acad Sci 883:124-130.
Cheung M, Briscoe J (2003) Neural crest development is regulated by the transcription factor Sox9. Development 130:5681-5693.
Clarke DL, Johansson CB, Wilbertz J, Veress B, Nilsson E, Karlstrom H, Lendahl U, Frisen J (2000) Generalized potential of adult neural stem cells. Science 288:1660-1663.
Clement-Jones M, Schiller S, Rao E, Blaschke RJ, Zuniga A, Zeller R, Robson SC, Binder G, Glass I, Strachan T, Lindsay S, Rappold GA (2000) The short stature homeobox gene SHOX is involved in skeletal abnormalities in Turner syndrome. Hum Mol Genet 9:695-702.
Cold CJ, Taylor JR (1999) The prepuce. Br J Urol 83:34-44.
Conway SJ, Henderson DJ, Copp AJ (1997) Pax3 is required for cardiac neural crest migration in the mouse: evidence from the splotch (Sp2H) mutant. Development 124:505-514.
Conway SJ, Bundy J, Chen J, Dickman E, Rogers R, Will BM (2000) Decreased neural crest stem cell expansion is responsible for the conotruncal heart defects within the splotch (Sp(2H))/Pax3 mouse mutant. Cardiovasc Res 47:314-328.
Cotsarelis G, Sun TT, Lavker RM (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61:1329-1337.
Daniloff JK, Levi G, Grumet M, Rieger F, Edelman GM (1986) Altered expression of neuronal cell adhesion molecules induced by nerve injury and repair. J Cell Biol 103:929-945.
del Barrio MG, Nieto MA (2002) Overexpression of Snail family members highlights their ability to promote chick neural crest formation. Development 129:1583-1593.
Dezawa M, Takahashi I, Esaki M, Takano M, Sawada H (2001) Sciatic nerve regeneration in rats induced by transplantation of in vitro differentiated bone-marrow stromal cells. Eur J Neurosci 14:1771-1776.
Doyu M, Sobue G, Ken E, Kimata K, Shinomura T, Yamada Y, Mitsuma T, Takahashi A (1993) Laminin A, B1, and B2 chain gene expression in transected and regenerating nerves: regulation by axonal signals. J Neurochem 60:543-551.

du Cros DL, LeBaron RG, Couchman JR (1995) Association of versican with dermal matrices and its potential role in hair follicle development and cycling. J Invest Dermatol 105:426-431.
Dulac C, Le Douarin NM (1991) Phenotypic plasticity of Schwann cells and enteric glial cells in response to the microenvironment. Proc Natl Acad Sci U S A 88:6358-6362.
Dupin E (1984) Cell division in the ciliary ganglion of quail embryos in situ and after back-transplantation into the neural crest migration pathways of chick embryos. Dev Biol 105:288-299.
Dupin E, Glavieux C, Vaigot P, Le Douarin NM (2000) Endothelin 3 induces the reversion of melanocytes to glia through a neural crest-derived glial-melanocytic progenitor. Proc Natl Acad Sci U S A 97:7882-7887.
Dupin E, Real C, Glavieux-Pardanaud C, Vaigot P, Le Douarin NM (2003) Reversal of developmental restrictions in neural crest lineages: transition from Schwann cells to glial-melanocytic precursors in vitro. Proc Natl Acad Sci U S A 100:5229-5233.
Dyce PW, Zhu H, Craig J, Li J (2004) Stem cells with multilineage potential derived from porcine skin. Biochem Biophys Res Commun 316:651-658.
Eccleston PA, Collarini EJ, Jessen KR, Mirsky R, Richardson WD (1990) Schwann Cells Secrete a PDGF-like Factor: Evidence for an Autocrine Growth Mechanism involving PDGF. Eur J Neurosci 2:985-992.
Eldridge CF, Bunge MB, Bunge RP, Wood PM (1987) Differentiation of axon-related Schwann cells in vitro. I. Ascorbic acid regulates basal lamina assembly and myelin formation. J Cell Biol 105:1023-1034.
Emsley JG, Mitchell BD, Kempermann G, Macklis JD (2005) Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. Prog Neurobiol 75:321-341.
Epstein JA (2001) Developing models of DiGeorge syndrome. Trends Genet 17:S13-17.
Erickson CA, Duong TD, Tosney KW (1992) Descriptive and experimental analysis of the dispersion of neural crest cells along the dorsolateral path and their entry into ectoderm in the chick embryo. Dev Biol 151:251-272.
Essex LJ, Mayor R, Sargent MG (1993) Expression of Xenopus snail in mesoderm and prospective neural fold ectoderm. Dev Dyn 198:108-122.
Etchevers HC, Vincent C, Le Douarin NM, Couly GF (2001) The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. Development 128:1059-1068.

Fansa H, Keilhoff G, Wolf G, Schneider W (2000) [Cultivating human Schwann cells for tissue engineering of peripheral nerves]. Handchir Mikrochir Plast Chir 32:181-186.
Fawcett JW, Asher RA (1999) The glial scar and central nervous system repair. Brain Res Bull 49:377-391.
Fernandes KJ, McKenzie IA, Mill P, Smith KM, Akhavan M, Barnabe-Heider F, Biernaskie J, Junek A, Kobayashi NR, Toma JG, Kaplan DR, Labosky PA, Rafuse V, Hui CC, Miller FD (2004) A dermal niche for multipotent adult skin-derived precursor cells. Nat Cell Biol 6:1082-1093.
Fetsch JF, Michal M, Miettinen M (2000) Pigmented (melanotic) neurofibroma: a clinicopathologic and immunohistochemical analysis of 19 lesions from 17 patients. Am J Surg Pathol 24:331-343.
Fontaine-Perus J, Chanconie M, Le Douarin NM (1988) Developmental potentialities in the nonneuronal population of quail sensory ganglia. Dev Biol 128:359-375.
Fontaine-Perus JC, Chanconie M, Le Douarin NM (1982) Differentiation of peptidergic neurones in quail-chick chimaeric embryos. Cell Differ 11:183-193.
Friedenstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267-274.
Fuchs E, Raghavan S (2002) Getting under the skin of epidermal morphogenesis. Nat Rev Genet 3:199-209.
Gajavelli S, Wood PM, Pennica D, Whittemore SR, Tsoulfas P (2004) BMP signaling initiates a neural crest differentiation program in embryonic rat CNS stem cells. Exp Neurol 188:205-223.
Gavrilovic J, Brennan A, Mirsky R, Jessen KR (1995) Fibroblast growth factors and insulin growth factors combine to promote survival of rat Schwann cell precursors without induction of DNA synthesis. Eur J Neurosci 7:77-85.
Gharzi A, Reynolds AJ, Jahoda CA (2003) Plasticity of hair follicle dermal cells in wound healing and induction. Exp Dermatol 12:126-136.
Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S (2002) Stem cell properties of human dental pulp stem cells. J Dent Res 81:531-535.
Grothe C, Meisinger C, Hertenstein A, Kurz H, Wewetzer K (1997) Expression of fibroblast growth factor-2 and fibroblast growth factor receptor 1 messenger RNAs in spinal ganglia and sciatic nerve: regulation after peripheral nerve lesion. Neuroscience 76:123-135.
Grumet M (1992) Structure, expression, and function of Ng-CAM, a member of the immunoglobulin superfamily involved in neuron-neuron and neuron-glia adhesion. J Neurosci Res 31:1-13.
Guenard V, Kleitman N, Morrissey TK, Bunge RP, Aebischer P (1992) Syngeneic Schwann cells derived from adult nerves seeded in

semipermeable guidance channels enhance peripheral nerve regeneration. J Neurosci 12:3310-3320.
Hadjantonakis AK, Gertsenstein M, Ikawa M, Okabe M, Nagy A (1998) Generating green fluorescent mice by germline transmission of green fluorescent ES cells. Mech Dev 76:79-90.
Hagedorn L, Suter U, Sommer L (1999) P0 and PMP22 mark a multipotent neural crest-derived cell type that displays community effects in response to TGF-beta family factors. Development 126:3781-3794.
Hammarberg H, Piehl F, Cullheim S, Fjell J, Hokfelt T, Fried K (1996) GDNF mRNA in Schwann cells and DRG satellite cells after chronic sciatic nerve injury. Neuroreport 7:857-860.
Hemmati-Brivanlou A, Kelly OG, Melton DA (1994) Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. Cell 77:283-295.
Henion PD, Weston JA (1997) Timing and pattern of cell fate restrictions in the neural crest lineage. Development 124:4351-4359.
Herbarth B, Pingault V, Bondurand N, Kuhlbrodt K, Hermans-Borgmeyer I, Puliti A, Lemort N, Goossens M, Wegner M (1998) Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease. Proc Natl Acad Sci U S A 95:5161-5165.
Heumann R, Lindholm D, Bandtlow C, Meyer M, Radeke MJ, Misko TP, Shooter E, Thoenen H (1987) Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: role of macrophages. Proc Natl Acad Sci U S A 84:8735-8739.
His W (1868) Untersuchungen uber die erste Anlage des Wirbeltierleibes. Die erste Entwicklung des Hunchen im Ei. Leipzig: F.C.W. Vogel.
Hornyak TJ, Hayes DJ, Chiu LY, Ziff EB (2001) Transcription factors in melanocyte development: distinct roles for Pax-3 and Mitf. Mech Dev 101:47-59.
Institut d'Embryologie du Cnrs Nogent-sur-Marne F (1977) Development of choline acetyltransferase and cholinesterase activities in enteric ganglia derives from presumptive adrenergic and cholinergic levels of the neural crest. Cell Differ 6:199-216.
Ito CY, Li CY, Bernstein A, Dick JE, Stanford WL (2003) Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. Blood 101:517-523.
Ito K, Morita T, Sieber-Blum M (1993) In vitro clonal analysis of mouse neural crest development. Dev Biol 157:517-525.
Iwashita T, Kruger GM, Pardal R, Kiel MJ, Morrison SJ (2003) Hirschsprung disease is linked to defects in neural crest stem cell function. Science 301:972-976.

Jahoda CA (1992) Induction of follicle formation and hair growth by vibrissa dermal papillae implanted into rat ear wounds: vibrissa-type fibres are specified. Development 115:1103-1109.
Jahoda CA, Reynolds AJ, Oliver RF (1993) Induction of hair growth in ear wounds by cultured dermal papilla cells. J Invest Dermatol 101:584-590.
Jahoda CA, Whitehouse J, Reynolds AJ, Hole N (2003) Hair follicle dermal cells differentiate into adipogenic and osteogenic lineages. Exp Dermatol 12:849-859.
Jensen PJ, Yang T, Yu DW, Baker MS, Risse B, Sun TT, Lavker RM (2000) Serpins in the human hair follicle. J Invest Dermatol 114:917-922.
Jessen KR, Morgan L, Stewart HJ, Mirsky R (1990) Three markers of adult non-myelin-forming Schwann cells, 217c(Ran-1), A5E3 and GFAP: development and regulation by neuron-Schwann cell interactions. Development 109:91-103.
Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM (2000) Fate of the mammalian cardiac neural crest. Development 127:1607-1616.
Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41-49.
Joannides A, Gaughwin P, Schwiening C, Majed H, Sterling J, Compston A, Chandran S (2004) Efficient generation of neural precursors from adult human skin: astrocytes promote neurogenesis from skin-derived stem cells. Lancet 364:172-178.
Joshi CV, Enver T (2002) Plasticity revisited. Curr Opin Cell Biol 14:749-755.
Kapur RP (1999) Early death of neural crest cells is responsible for total enteric aganglionosis in Sox10(Dom)/Sox10(Dom) mouse embryos. Pediatr Dev Pathol 2:559-569.
Kawase Y, Yanagi Y, Takato T, Fujimoto M, Okochi H (2004) Characterization of multipotent adult stem cells from the skin: transforming growth factor-beta (TGF-beta) facilitates cell growth. Exp Cell Res 295:194-203.
Kim J, Lo L, Dormand E, Anderson DJ (2003a) SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. Neuron 38:17-31.
Kim JY, Sun Q, Oglesbee M, Yoon SO (2003b) The role of ErbB2 signaling in the onset of terminal differentiation of oligodendrocytes in vivo. J Neurosci 23:5561-5571.
Kishimoto J, Ehama R, Wu L, Jiang S, Jiang N, Burgeson RE (1999) Selective activation of the versican promoter by epithelial-mesenchymal interactions during hair follicle development. Proc Natl Acad Sci U S A 96:7336-7341.

Kleber M, Lee HY, Wurdak H, Buchstaller J, Riccomagno MM, Ittner LM, Suter U, Epstein DJ, Sommer L (2005) Neural crest stem cell maintenance by combinatorial Wnt and BMP signaling. J Cell Biol 169:309-320.

Koeppen AH (2004) Wallerian degeneration: history and clinical significance. J Neurol Sci 220:115-117.

Kohama I, Lankford KL, Preiningerova J, White FA, Vollmer TL, Kocsis JD (2001) Transplantation of cryopreserved adult human Schwann cells enhances axonal conduction in demyelinated spinal cord. J Neurosci 21:944-950.

Kondo T, Raff M (2000) Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. Science 289:1754-1757.

Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105:369-377.

Kruger GM, Mosher JT, Bixby S, Joseph N, Iwashita T, Morrison SJ (2002) Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. Neuron 35:657-669.

Kruger GM, Mosher JT, Tsai YH, Yeager KJ, Iwashita T, Gariepy CE, Morrison SJ (2003) Temporally distinct requirements for endothelin receptor B in the generation and migration of gut neural crest stem cells. Neuron 40:917-929.

Lahav R, Ziller C, Dupin E, Le Douarin NM (1996) Endothelin 3 promotes neural crest cell proliferation and mediates a vast increase in melanocyte number in culture. Proc Natl Acad Sci U S A 93:3892-3897.

Lahav R, Dupin E, Lecoin L, Glavieux C, Champeval D, Ziller C, Le Douarin NM (1998) Endothelin 3 selectively promotes survival and proliferation of neural crest-derived glial and melanocytic precursors in vitro. Proc Natl Acad Sci U S A 95:14214-14219.

Lako M, Armstrong L, Cairns PM, Harris S, Hole N, Jahoda CA (2002) Hair follicle dermal cells repopulate the mouse haematopoietic system. J Cell Sci 115:3967-3974.

Lang D, Chen F, Milewski R, Li J, Lu MM, Epstein JA (2000) Pax3 is required for enteric ganglia formation and functions with Sox10 to modulate expression of c-ret. J Clin Invest 106:963-971.

Lawrence JM, Keegan DJ, Muir EM, Coffey PJ, Rogers JH, Wilby MJ, Fawcett JW, Lund RD (2004) Transplantation of Schwann cell line clones secreting GDNF or BDNF into the retinas of dystrophic Royal College of Surgeons rats. Invest Ophthalmol Vis Sci 45:267-274.

Lawson SN, Biscoe TJ (1979) Development of mouse dorsal root ganglia: an autoradiographic and quantitative study. J Neurocytol 8:265-274.

Le Douarin NM (1982) The Neural Crest. Cambridge, UK: Cambridge University Press.
Le Douarin NM, Dupin E (1993) Cell lineage analysis in neural crest ontogeny. J Neurobiol 24:146-161.
Le Douarin NM, Renaud D, Teillet MA, Le Douarin GH (1975) Cholinergic differentiation of presumptive adrenergic neuroblasts in interspecific chimeras after heterotopic transplantations. Proc Natl Acad Sci U S A 72:728-732.
Le Lievre CS, Schweizer GG, Ziller CM, Le Douarin NM (1980) Restrictions of developmental capabilities in neural crest cell derivatives as tested by in vivo transplantation experiments. Dev Biol 77:362-378.
Lee HY, Kleber M, Hari L, Brault V, Suter U, Taketo MM, Kemler R, Sommer L (2004) Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. Science 303:1020-1023.
Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature 378:394-398.
Leedham SJ, Brittan M, McDonald SA, Wright NA (2005) Intestinal stem cells. J Cell Mol Med 9:11-24.
Lemke G (1996) Neuregulins in development. Mol Cell Neurosci 7:247-262.
Li L, Cserjesi P, Olson EN (1995) Dermo-1: a novel twist-related bHLH protein expressed in the developing dermis. Dev Biol 172:280-292.
Li L, Mignone J, Yang M, Matic M, Penman S, Enikolopov G, Hoffman RM (2003) Nestin expression in hair follicle sheath progenitor cells. Proc Natl Acad Sci U S A 100:9958-9961.
Liem KF, Jr., Tremml G, Roelink H, Jessell TM (1995) Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell 82:969-979.
Liu S, Qu Y, Stewart TJ, Howard MJ, Chakrabortty S, Holekamp TF, McDonald JW (2000) Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc Natl Acad Sci U S A 97:6126-6131.
Liu Y, Lyle S, Yang Z, Cotsarelis G (2003) Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. J Invest Dermatol 121:963-968.
Lobsiger CS, Taylor V, Suter U (2002) The early life of a Schwann cell. Biol Chem 383:245-253.
Lobsiger CS, Schweitzer B, Taylor V, Suter U (2000) Platelet-derived growth factor-BB supports the survival of cultured rat Schwann cell precursors in synergy with neurotrophin-3. Glia 30:290-300.
Lois C, Alvarez-Buylla A (1994) Long-distance neuronal migration in the adult mammalian brain. Science 264:1145-1148.

Lu P, Blesch A, Tuszynski MH (2004) Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? J Neurosci Res 77:174-191.
Magavi SS, Macklis JD (2002) Induction of neuronal type-specific neurogenesis in the cerebral cortex of adult mice: manipulation of neural precursors in situ. Brain Res Dev Brain Res 134:57-76.
Magavi SS, Leavitt BR, Macklis JD (2000) Induction of neurogenesis in the neocortex of adult mice. Nature 405:951-955.
Marchant L, Linker C, Ruiz P, Guerrero N, Mayor R (1998) The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient. Dev Biol 198:319-329.
Marchionni MA, Goodearl AD, Chen MS, Bermingham-McDonogh O, Kirk C, Hendricks M, Danehy F, Misumi D, Sudhalter J, Kobayashi K, et al. (1993) Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature 362:312-318.
Martin D, Schoenen J, Delree P, Leprince P, Rogister B, Moonen G (1991) Grafts of syngenic cultured, adult dorsal root ganglion-derived Schwann cells to the injured spinal cord of adult rats: preliminary morphological studies. Neurosci Lett 124:44-48.
Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78:7634-7638.
McCulloch EA, Till JE (1960) The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Radiat Res 13:115-125.
McElwee KJ, Kissling S, Wenzel E, Huth A, Hoffmann R (2003) Cultured peribulbar dermal sheath cells can induce hair follicle development and contribute to the dermal sheath and dermal papilla. J Invest Dermatol 121:1267-1275.
McGonnell IM, Graham A (2002) Trunk neural crest has skeletogenic potential. Curr Biol 12:767-771.
McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13:805-811.
Meier C, Parmantier E, Brennan A, Mirsky R, Jessen KR (1999) Developing Schwann cells acquire the ability to survive without axons by establishing an autocrine circuit involving insulin-like growth factor, neurotrophin-3, and platelet-derived growth factor-BB. J Neurosci 19:3847-3859.
Meyer D, Birchmeier C (1994) Distinct isoforms of neuregulin are expressed in mesenchymal and neuronal cells during mouse development. Proc Natl Acad Sci U S A 91:1064-1068.
Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol 119:45-54.

Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR (2000) Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 290:1779-1782.
Miletich I, Sharpe PT (2004) Neural crest contribution to mammalian tooth formation. Birth Defects Res C Embryo Today 72:200-212.
Mill P, Mo R, Fu H, Grachtchouk M, Kim PC, Dlugosz AA, Hui CC (2003) Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development. Genes Dev 17:282-294.
Mirsky R, Dubois C, Morgan L, Jessen KR (1990) 04 and A007-sulfatide antibodies bind to embryonic Schwann cells prior to the appearance of galactocerebroside; regulation of the antigen by axon-Schwann cell signals and cyclic AMP. Development 109:105-116.
Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S (2003) SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 100:5807-5812.
Mo R, Freer AM, Zinyk DL, Crackower MA, Michaud J, Heng HH, Chik KW, Shi XM, Tsui LC, Cheng SH, Joyner AL, Hui C (1997) Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. Development 124:113-123.
Moll I, Paus R, Moll R (1996) Merkel cells in mouse skin: intermediate filament pattern, localization, and hair cycle-dependent density. J Invest Dermatol 106:281-286.
Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, Morrison SJ (2003) Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature 425:962-967.
Monuki ES, Weinmaster G, Kuhn R, Lemke G (1989) SCIP: a glial POU domain gene regulated by cyclic AMP. Neuron 3:783-793.
Morasso MI, Tomic-Canic M (2005) Epidermal stem cells: the cradle of epidermal determination, differentiation and wound healing. Biol Cell 97:173-183.
Morgan L, Jessen KR, Mirsky R (1991) The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (04+) to a myelin phenotype (P0+, GFAP-, N-CAM-, NGF-receptor-) depends on growth inhibition. J Cell Biol 112:457-467.
Morris RJ, Liu Y, Marles L, Yang Z, Trempus C, Li S, Lin JS, Sawicki JA, Cotsarelis G (2004) Capturing and profiling adult hair follicle stem cells. Nat Biotechnol 22:411-417.
Morrison SJ, White PM, Zock C, Anderson DJ (1999) Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. Cell 96:737-749.
Morrison SJ, Perez SE, Qiao Z, Verdi JM, Hicks C, Weinmaster G, Anderson DJ (2000) Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. Cell 101:499-510.

Morrissey TK, Kleitman N, Bunge RP (1991) Isolation and functional characterization of Schwann cells derived from adult peripheral nerve. J Neurosci 11:2433-2442.
Morrissey TK, Levi AD, Nuijens A, Sliwkowski MX, Bunge RP (1995) Axon-induced mitogenesis of human Schwann cells involves heregulin and p185erbB2. Proc Natl Acad Sci U S A 92:1431-1435.
Morshead CM, Benveniste P, Iscove NN, van der Kooy D (2002) Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations. Nat Med 8:268-273.
Moury JD, Jacobson AG (1990) The origins of neural crest cells in the axolotl. Dev Biol 141:243-253.
Mujtaba T, Mayer-Proschel M, Rao MS (1998) A common neural progenitor for the CNS and PNS. Dev Biol 200:1-15.
Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I (2004) Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. J Neurosci Res 77:192-204.
Newgreen D, Young HM (2002) Enteric nervous system: development and developmental disturbances--part 1. Pediatr Dev Pathol 5:224-247.
Nieto MA, Sargent MG, Wilkinson DG, Cooke J (1994) Control of cell behavior during vertebrate development by Slug, a zinc finger gene. Science 264:835-839.
Nishimura EK, Jordan SA, Oshima H, Yoshida H, Osawa M, Moriyama M, Jackson IJ, Barrandon Y, Miyachi Y, Nishikawa S (2002) Dominant role of the niche in melanocyte stem-cell fate determination. Nature 416:854-860.
Nocka K, Majumder S, Chabot B, Ray P, Cervone M, Bernstein A, Besmer P (1989) Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice--evidence for an impaired c-kit kinase in mutant mice. Genes Dev 3:816-826.
Nosrat IV, Smith CA, Mullally P, Olson L, Nosrat CA (2004) Dental pulp cells provide neurotrophic support for dopaminergic neurons and differentiate into neurons in vitro; implications for tissue engineering and repair in the nervous system. Eur J Neurosci 19:2388-2398.
Nurse CA, Macintyre L, Diamond J (1984) Reinnervation of the rat touch dome restores the Merkel cell population reduced after denervation. Neuroscience 13:563-571.
O'Rourke MP, Tam PP (2002) Twist functions in mouse development. Int J Dev Biol 46:401-413.

Osawa M, Hanada K, Hamada H, Nakauchi H (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273:242-245.
Oshima H, Rochat A, Kedzia C, Kobayashi K, Barrandon Y (2001) Morphogenesis and renewal of hair follicles from adult multipotent stem cells. Cell 104:233-245.
Owen M, Friedenstein AJ (1988) Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp 136:42-60.
Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH (1999) Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J Neurosci 19:8487-8497.
Panteleyev AA, Paus R, Wanner R, Nurnberg W, Eichmuller S, Thiel R, Zhang J, Henz BM, Rosenbach T (1997) Keratin 17 gene expression during the murine hair cycle. J Invest Dermatol 108:324-329.
Paratore C, Eichenberger C, Suter U, Sommer L (2002) Sox10 haploinsufficiency affects maintenance of progenitor cells in a mouse model of Hirschsprung disease. Hum Mol Genet 11:3075-3085.
Paratore C, Goerich DE, Suter U, Wegner M, Sommer L (2001) Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. Development 128:3949-3961.
Paus R, Muller-Rover S, Van Der Veen C, Maurer M, Eichmuller S, Ling G, Hofmann U, Foitzik K, Mecklenburg L, Handjiski B (1999) A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. J Invest Dermatol 113:523-532.
Pearse DD, Pereira FC, Marcillo AE, Bates ML, Berrocal YA, Filbin MT, Bunge MB (2004) cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat Med 10:610-616.
Peters EM, Botchkarev VA, Muller-Rover S, Moll I, Rice FL, Paus R (2002) Developmental timing of hair follicle and dorsal skin innervation in mice. J Comp Neurol 448:28-52.
Piccolo S, Sasai Y, Lu B, De Robertis EM (1996) Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell 86:589-598.
Piersma AH, Brockbank KG, Ploemacher RE, van Vliet E, Brakel-van Peer KM, Visser PJ (1985) Characterization of fibroblastic stromal cells from murine bone marrow. Exp Hematol 13:237-243.
Raff MC, Miller RH, Noble M (1983) A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. Nature 303:390-396.

Raible DW, Eisen JS (1996) Regulative interactions in zebrafish neural crest. Development 122:501-507.
Rasheed S, Mao Z, Chan JM, Chan LS (2005) Is Melanoma a Stem Cell Tumor? Identification of Neurogenic Proteins in Trans-differentiated Cells. J Transl Med 3:14.
Reddy S, Andl T, Bagasra A, Lu MM, Epstein DJ, Morrisey EE, Millar SE (2001) Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis. Mech Dev 107:69-82.
Reynolds AJ, Jahoda CA (1992) Cultured dermal papilla cells induce follicle formation and hair growth by transdifferentiation of an adult epidermis. Development 115:587-593.
Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707-1710.
Reynolds BA, Tetzlaff W, Weiss S (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12:4565-4574.
Richardson MK, Sieber-Blum M (1993) Pluripotent neural crest cells in the developing skin of the quail embryo. Dev Biol 157:348-358.
Richardson PM, McGuinness UM, Aguayo AJ (1980) Axons from CNS neurons regenerate into PNS grafts. Nature 284:264-265.
Rickmann M, Fawcett JW, Keynes RJ (1985) The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. J Embryol Exp Morphol 90:437-455.
Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C (1997) Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature 389:725-730.
Rizvi TA, Huang Y, Sidani A, Atit R, Largaespada DA, Boissy RE, Ratner N (2002) A novel cytokine pathway suppresses glial cell melanogenesis after injury to adult nerve. J Neurosci 22:9831-9840.
Rochat A, Kobayashi K, Barrandon Y (1994) Location of stem cells of human hair follicles by clonal analysis. Cell 76:1063-1073.
Rochefort C, Gheusi G, Vincent JD, Lledo PM (2002) Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. J Neurosci 22:2679-2689.
Rodriguez FJ, Verdu E, Ceballos D, Navarro X (2000) Nerve guides seeded with autologous schwann cells improve nerve regeneration. Exp Neurol 161:571-584.
Ruffins S, Artinger KB, Bronner-Fraser M (1998) Early migrating neural crest cells can form ventral neural tube derivatives when challenged by transplantation. Dev Biol 203:295-304.

Rutkowski JL, Kirk CJ, Lerner MA, Tennekoon GI (1995) Purification and expansion of human Schwann cells in vitro. Nat Med 1:80-83.
Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR (2000) Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164:247-256.
Sasaki M, Honmou O, Akiyama Y, Uede T, Hashi K, Kocsis JD (2001) Transplantation of an acutely isolated bone marrow fraction repairs demyelinated adult rat spinal cord axons. Glia 35:26-34.
Schmidt-Ullrich R, Paus R (2005) Molecular principles of hair follicle induction and morphogenesis. Bioessays 27:247-261.
Schwab ME, Bartholdi D (1996) Degeneration and regeneration of axons in the lesioned spinal cord. Physiol Rev 76:319-370.
Schweizer G, Ayer-Le Lievre C, Le Douarin NM (1983) Restrictions of developmental capacities in the dorsal root ganglia during the course of development. Cell Differ 13:191-200.
Seaberg RM, Smukler SR, Kieffer TJ, Enikolopov G, Asghar Z, Wheeler MB, Korbutt G, van der Kooy D (2004) Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotechnol 22:1115-1124.
Sejersen T, Lendahl U (1993) Transient expression of the intermediate filament nestin during skeletal muscle development. J Cell Sci 106 (Pt 4):1291-1300.
Selleck MA, Bronner-Fraser M (1995) Origins of the avian neural crest: the role of neural plate-epidermal interactions. Development 121:525-538.
Serbedzija GN, Bronner-Fraser M, Fraser SE (1989) A vital dye analysis of the timing and pathways of avian trunk neural crest cell migration. Development 106:809-816.
Serbedzija GN, Bronner-Fraser M, Fraser SE (1994) Developmental potential of trunk neural crest cells in the mouse. Development 120:1709-1718.
Shah NM, Anderson DJ (1997) Integration of multiple instructive cues by neural crest stem cells reveals cell-intrinsic biases in relative growth factor responsiveness. Proc Natl Acad Sci U S A 94:11369-11374.
Shah NM, Groves AK, Anderson DJ (1996) Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. Cell 85:331-343.
Shah NM, Marchionni MA, Isaacs I, Stroobant P, Anderson DJ (1994) Glial growth factor restricts mammalian neural crest stem cells to a glial fate. Cell 77:349-360.
Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E (2001) Neurogenesis in the adult is involved in the formation of trace memories. Nature 410:372-376.

Sieber-Blum M, Cohen AM (1980) Clonal analysis of quail neural crest cells: they are pluripotent and differentiate in vitro in the absence of noncrest cells. Dev Biol 80:96-106.
Sieber-Blum M, Grim M, Hu YF, Szeder V (2004) Pluripotent neural crest stem cells in the adult hair follicle. Dev Dyn 231:258-269.
Smith DE, Franco del Amo F, Gridley T (1992) Isolation of Sna, a mouse gene homologous to the Drosophila genes snail and escargot: its expression pattern suggests multiple roles during postimplantation development. Development 116:1033-1039.
Smith WC, Harland RM (1992) Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. Cell 70:829-840.
Song X, Zhu CH, Doan C, Xie T (2002) Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. Science 296:1855-1857.
Song X, Wong MD, Kawase E, Xi R, Ding BC, McCarthy JJ, Xie T (2004) Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. Development 131:1353-1364.
Soo K, O'Rourke MP, Khoo PL, Steiner KA, Wong N, Behringer RR, Tam PP (2002) Twist function is required for the morphogenesis of the cephalic neural tube and the differentiation of the cranial neural crest cells in the mouse embryo. Dev Biol 247:251-270.
Southard-Smith EM, Angrist M, Ellison JS, Agarwala R, Baxevanis AD, Chakravarti A, Pavan WJ (1999) The Sox10(Dom) mouse: modeling the genetic variation of Waardenburg-Shah (WS4) syndrome. Genome Res 9:215-225.
Spemann H, Mangold H (1924) Uber Induktion von Embryonanlagen durch Implantation artfremder Organisatoren. Arch Mikr Anat EntwMech 100:599-638.
Stemple DL, Anderson DJ (1992) Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell 71:973-985.
Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37:173-182.
Suter U, Scherer SS (2003) Disease mechanisms in inherited neuropathies. Nat Rev Neurosci 4:714-726.
Syroid DE, Maycox PR, Burrola PG, Liu N, Wen D, Lee KF, Lemke G, Kilpatrick TJ (1996) Cell death in the Schwann cell lineage and its regulation by neuregulin. Proc Natl Acad Sci U S A 93:9229-9234.
Szeder V, Grim M, Halata Z, Sieber-Blum M (2003) Neural crest origin of mammalian Merkel cells. Dev Biol 253:258-263.
Takami T, Oudega M, Bates ML, Wood PM, Kleitman N, Bunge MB (2002) Schwann cell but not olfactory ensheathing glia transplants improve hindlimb locomotor performance in the moderately contused adult rat thoracic spinal cord. J Neurosci 22:6670-6681.

Taylor G, Lehrer MS, Jensen PJ, Sun TT, Lavker RM (2000) Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell 102:451-461.

Taylor JR, Lockwood AP, Taylor AJ (1996) The prepuce: specialized mucosa of the penis and its loss to circumcision. Br J Urol 77:291-295.

Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW (2002) Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542-545.

Thies A, Schachner M, Berger J, Moll I, Schulze HJ, Brunner G, Schumacher U (2004) The developmentally regulated neural crest-associated glycotope HNK-1 predicts metastasis in cutaneous malignant melanoma. J Pathol 203:933-939.

Timmer M, Muller-Ostermeyer F, Kloth V, Winkler C, Grothe C, Nikkhah G (2004) Enhanced survival, reinnervation, and functional recovery of intrastriatal dopamine grafts co-transplanted with Schwann cells overexpressing high molecular weight FGF-2 isoforms. Exp Neurol 187:118-136.

Tobin DJ, Gunin A, Magerl M, Paus R (2003) Plasticity and cytokinetic dynamics of the hair follicle mesenchyme during the hair growth cycle: implications for growth control and hair follicle transformations. J Investig Dermatol Symp Proc 8:80-86.

Toma JG, McKenzie IA, Bagli D, Miller FD (2005) Isolation and characterization of multipotent skin-derived precursors from human skin. Stem Cells 23:727-737.

Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, Miller FD (2001) Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 3:778-784.

Tropepe V, Sibilia M, Ciruna BG, Rossant J, Wagner EF, van der Kooy D (1999) Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev Biol 208:166-188.

Tropepe V, Coles BL, Chiasson BJ, Horsford DJ, Elia AJ, McInnes RR, van der Kooy D (2000) Retinal stem cells in the adult mammalian eye. Science 287:2032-2036.

Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E (2004) Defining the epithelial stem cell niche in skin. Science 303:359-363.

Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH, Weissman IL (2000) Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci U S A 97:14720-14725.

Vanderhooft E (2000) Functional outcomes of nerve grafts for the upper and lower extremities. Hand Clin 16:93-104, ix.

Vescovi AL, Reynolds BA, Fraser DD, Weiss S (1993) bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. Neuron 11:951-966.
Viehover A, Miller RH, Park SK, Fischbach G, Vartanian T (2001) Neuregulin: an oligodendrocyte growth factor absent in active multiple sclerosis lesions. Dev Neurosci 23:377-386.
Vitry S, Bertrand JY, Cumano A, Dubois-Dalcq M (2003) Primordial hematopoietic stem cells generate microglia but not myelin-forming cells in a neural environment. J Neurosci 23:10724-10731.
Wahlstrom T, Saxen L (1976) Malignant skin tumors of neural crest origin. Cancer 38:2022-2026.
Wakamatsu Y, Maynard TM, Weston JA (2000) Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. Development 127:2811-2821.
Waller A (1850) Experiments on the section of the glossopharyngeal and hypoglossal nerves of the frog, and observations on the alterations produced thereby in the structure of their primitive fibers. Phil Trans R Soc Lond 140:423-429.
Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M (2003) Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 422:897-901.
Webb JN (1982) The ultra-structure of a melanotic schwannoma of the skin. J Pathol 137:25-36.
Weinmaster G, Roberts VJ, Lemke G (1991) A homolog of Drosophila Notch expressed during mammalian development. Development 113:199-205.
Weissman IL, Anderson DJ, Gage F (2001) Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Annu Rev Cell Dev Biol 17:387-403.
Whetton AD, Graham GJ (1999) Homing and mobilization in the stem cell niche. Trends Cell Biol 9:233-238.
White PM, Anderson DJ (1999) In vivo transplantation of mammalian neural crest cells into chick hosts reveals a new autonomic sublineage restriction. Development 126:4351-4363.
Williams R, Lendahl U, Lardelli M (1995) Complementary and combinatorial patterns of Notch gene family expression during early mouse development. Mech Dev 53:357-368.
Willison HJ, Trapp BD, Bacher JD, Quarles RH (1988) The expression of myelin-associated glycoprotein in regenerating cat sciatic nerve. Brain Res 444:10-16.
Woldeyesus MT, Britsch S, Riethmacher D, Xu L, Sonnenberg-Riethmacher E, Abou-Rebyeh F, Harvey R, Caroni P, Birchmeier C (1999) Peripheral nervous system defects in erbB2 mutants

following genetic rescue of heart development. Genes Dev 13:2538-2548.
Yang LY, Zheng JK, Liu XM, Hui GZ, Guo LH (2004) Culture of skin-derived precursors and their differentiation into neurons. Chin J Traumatol 7:91-95.
Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li L (2003) Identification of the haematopoietic stem cell niche and control of the niche size. Nature 425:836-841.
Zhu H, Craig JA, Dyce PW, Sunnen N, Li J (2004a) Embryos derived from porcine skin-derived stem cells exhibit enhanced preimplantation development. Biol Reprod 71:1890-1897.
Zhu L, Lee HO, Jordan CS, Cantrell VA, Southard-Smith EM, Shin MK (2004b) Spatiotemporal regulation of endothelin receptor-B by SOX10 in neural crest-derived enteric neuron precursors. Nat Genet 36:732-737.

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Dr Freda Miller	BTRC	X				
Anne Aumont	BTRC		X			
Annie Sylvestre	BTRC		X			
Greg Walsh	BTRC				X	
Anna Lee	BTRC				X	
Fanie Barnabe-Heider	BTRC				X	
Catherine Menard	BTRC				X	
Mahnaz Akhvan	BTRC		X			
Jean Toma	BTRC					X
Mathieu Fortier	BTRC				X	
Paul Hein	BTRC				X	
Yephat Wang	BTRC		X			
Amelie Rioux-Tache	BTRC		X			
Laura Craig	BTRC				X	
Karun Singh	BTRC				X	
Patrizia Zanassi	BTRC					X
Nina Orike	BTRC					X
Nao Kobayashi	BTRC					X
Karl Fernandes	BTRC					X
Annie Paquin	BTRC			X		
Ian Mckenzie	BTRC				X	

5. EMERGENCY: Person(s) designated to handle emergencies

Name:	Dr Freda Miller	Phone No: work: 398-4261	home: 482-2186
Name:	Greg Walsh	Phone No: work: 398-3334	home: 288-6271

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

The biohazard material involved is the non-replicating *adenovirus*; the risk group is #2.

ii) the procedures involving biohazards:

The adenovirus is used in 2 different types of experiments:

1- microinjections of the virus into transgenic mice

2- infection with the virus in primary cultures and cell line

In both types of experiments the virus is used as a vector to insert certain specific genetic material, of a primarily neural origin into neurons. The virus is attenuated, and the genetic material they carry may cause a phenotypic effect but is not contagious. All work is done in Class II laminar flow hoods.

iii) the protocol for decontaminating spills

- **For a small spill, the area is covered with and wiped with paper towels that have been soaked in bleach (1% bleach, for at least 10 minutes.)**
- **The work area is wiped down before and after work with 70% ethanol.**
- **If a large spill (>500ml) occurs then it is allowed to settle for 30 minutes, before being decontaminated, as above.**
- **If the virus gets on skin, then the area is disinfected at once (medical held sought if necessary)**
- **Any contaminated clothing or materials are autoclaved.**

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

NO

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

YES

9. What precautions are being taken to reduce production of infectious droplets and aerosols?

- **The animals and cell culture experiments will be performed in class II laminar flow hoods.**
- **Whenever work is done, there will always be an autoclaved beaker of 1% bleach at hand to decontaminate small items (e.g. pipette tips, Pasteur pipettes); and spray containers of 1% bleach and 70% ethanol are in close proximity to the work.**
- **Personnel working with the virus will wear lab coats and gloves.**

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
BTRC	BT-106	Forma Scientific	1286	17719-312	April, 2001
BTRC	BT-106	Forma Scientific	1286	17719-311	April, 2001
BTRC	BT-106	Forma Scientific	1286	19727-301	April, 2001