Multiple Origins of Multipotent Neural Crest-Derived Cells in the Adult Skin

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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>β-Gal</td>
<td>β-galactosidase</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>CEE</td>
<td>Chicken embryo extract</td>
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<tr>
<td>CNPase</td>
<td>2’, 3’-cyclic-nucleotide 3’-phosphodiesterase</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>Cre</td>
<td>Cre recombinase</td>
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<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-phenylindole dihydrochloride</td>
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<tr>
<td>Dct</td>
<td>Dopachrome tautomerase</td>
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<tr>
<td>Dhh</td>
<td>Desert hedgehog</td>
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<tr>
<td>D-MEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DP</td>
<td>Dermal papilla</td>
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<td>DS</td>
<td>Dermal sheath</td>
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<td>DNA</td>
<td>Desoxyribonucleic acid</td>
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<td>E</td>
<td>Embryonic day</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>Erb</td>
<td>Encodes EGF-receptor</td>
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<tr>
<td>ESCs</td>
<td>Embryonic stem cells</td>
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<td>ET-3</td>
<td>Endothelin-3</td>
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<td>EYFP</td>
<td>Enhanced yellow fluorescent protein</td>
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<tr>
<td>FA</td>
<td>Formaldehyde</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FN</td>
<td>Fibronectin</td>
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<tr>
<td>FNA/B</td>
<td>Follicular network A/B</td>
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<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
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<td>GDNF</td>
<td>Glial-derived neurotrophic factor</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillar acidic protein</td>
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<tr>
<td>GM</td>
<td>Growth medium</td>
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<td>HMG</td>
<td>High mobility group</td>
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<td>HSCs</td>
<td>Haematopoietic stem cells</td>
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<td>Ht-PA</td>
<td>Human tissue plasminogen activator</td>
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<td>ICM</td>
<td>Inner cell mass</td>
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<td>IRS</td>
<td>Inner root sheath</td>
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<tr>
<td>LRCs</td>
<td>Label-retaining cells</td>
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<tr>
<td>MACS</td>
<td>Magnetic cell sorting</td>
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<td>Map</td>
<td>Microtubule-associated protein</td>
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<td>Mitf</td>
<td>Microphthalmia-associated transcription factor</td>
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<td>NC</td>
<td>Neural crest</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
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<td>NCCs</td>
<td>Neural crest cells</td>
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<td>NCSCs</td>
<td>Neural crest stem cells</td>
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<tr>
<td>NF</td>
<td>Neurofilament</td>
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<tr>
<td>NG2</td>
<td>Chondroitin sulfate proteoglycan</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NRG</td>
<td>Neuregulin</td>
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<tr>
<td>ORS</td>
<td>Outer root sheath</td>
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<td>P</td>
<td>Postnatal day</td>
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<tr>
<td>P0</td>
<td>Myelin protein 0</td>
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<tr>
<td>p75</td>
<td>Low affinity neurotrophin receptor</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PDL</td>
<td>Poly-D-lysine</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PGP</td>
<td>Protein gene product</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<tr>
<td>P/S</td>
<td>Penicillin/Streptomycin</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>RIP</td>
<td>Regulation of phenobarbitol-inducible P450</td>
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<tr>
<td>SCF</td>
<td>Stem cell factor</td>
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<tr>
<td>SG</td>
<td>Sebaceous gland</td>
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<tr>
<td>SKPs</td>
<td>Skin-derived precursor cells</td>
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<tr>
<td>SMA</td>
<td>Smooth muscle actin</td>
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<tr>
<td>SM</td>
<td>Standard medium</td>
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<tr>
<td>Sox</td>
<td>Sry HMG box</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
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<tr>
<td>Trp</td>
<td>Tyrosinase-related protein</td>
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<tr>
<td>Wnt</td>
<td>Wingless and Integrated</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-Bromo-4-chloro-3-indolyl-D-galactopyranoside</td>
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1 SUMMARY

The goal of this project was to find a multipotent adult stem cell source suitable for use in transplantation therapies of degenerative diseases. The skin, a very accessible tissue, would be an ideal source that could be taken from a patient without overly invasive procedures and would eliminate the need for immunosuppression and the ethical issues associated with the use of embryonic stem cells. The work of my PhD focuses on the characterization of sphere-forming multipotent cells from the adult trunk skin of mice and humans, and on the description of their exact localization in vivo. Many publications report the isolation of multipotent cells from the skin, but in contrast to all of these cell populations, my cells express the neural crest stem cell (NCSC) markers p75 and Sox10, pointing to different origins of multipotent cells in the skin. To determine the nature of multipotent skin-derived cells, we genetically mapped the fate of neural crest cells in face and trunk skin of mice. In whisker follicles of the face, many mesenchymal structures are neural crest derived and, intriguingly, contain cells with sphere-forming potential. In the trunk skin, however, sphere-forming neural crest cells are restricted to the glial and melanocyte lineages. This result was confirmed by isolation of pure populations of these specific lineages using fluorescence activated cell sorting (FACS) and sphere formation assays. Thus, multipotent cells in the adult skin can be obtained from many neural crest derivatives, and these are of distinct nature in face and trunk skin. These results are very important, given the fact that until now, multipotent neural crest-derived cells in the skin had only been characterized clearly in vibrissal follicles of the mouse. These findings are relevant for the design of therapeutic strategies, because the potential of a multipotent cell in vivo likely depends on its nature and origin. The fact that our cells are derived from the neural crest, probably makes them better candidates for treatment of diseases of the peripheral nervous system (PNS) or lesions involving non-neural cell types such as chondrocytes or melanocytes. Injection of my cells into models of Parkinson’s disease or multiple sclerosis showed that their potential for giving rise to cell fates of the central nervous system (CNS) is very restricted or inexistent as the mice did not show any improvement. These cells were also not able to integrate into embryonic CNS tissue in either rat or chicken or into hippocampal brain slices. Skin-derived neural crest cells survived in the CNS environment, but were not
able to integrate into the tissue or to differentiate. These cells will need further characterization as to their potential *in vivo* and to their normal role in skin.
2 ZUSAMMENFASSUNG

Das Ziel dieses Projektes war es, eine geeignete Quelle für multipotente Stammzellen zu finden, um sie für zukünftige Transplantationstherapien einzusetzen. Vor allem Krankheiten, welche mit dem Verlust oder der Degeneration eines bestimmten Zelltyps zusammenhängen, könnten durch Zelltransplantationen behandelt werden.

Die Haut ist ein Organ, welches für eine Probeentnahme leicht zugänglich ist und somit eine ideale Quelle für die Gewinnung von Stammzellen darstellt. Der Patient könnte das Gewebe für seine Transplantation selber spenden, ohne dabei einen sehr invasiven Eingriff erleiden zu müssen. Eine immunsuppressive Behandlung wäre nicht nötig, und ethische Aspekte die mit der Verwendung von embryonalen Stammzellen zusammenhängen, könnten umgangen werden.

2. Zusammenfassung

3 INTRODUCTION

3.1 Stem cells and their therapeutic potential

Stem cells are the founder cells of every organ and tissue in the body. A true stem cell is defined by its ability to divide at the single cell level and generate an identical daughter cell (self-renewal) as well as another daughter cell that can differentiate into multiple distinct cell types (Crane and Trainor, 2006). These features allow stem cells to play a role in processes like organogenesis of the embryo, tissue homeostasis, regeneration and repair of certain tissues of the adult organism, and even diseases like cancer. In addition, self-renewal and multipotency make stem cells good candidates for the treatment of all types of degenerative diseases by cell-replacement therapy, an issue that has received a lot of attention by researchers. Different kinds of stem cells have been described to date, according to the time point and tissue of their isolation.

3.1.1 Embryonic stem cells (ESCs)

ESCs were first isolated from the mouse by *in vitro* culture of cells derived from the inner cell mass (ICM) of early embryos or blastocysts (Evans and Kaufman, 1981; Martin, 1981). Under the appropriate culture conditions, ESCs can proliferate indefinitely while retaining the ability to differentiate into all types of somatic cells (Guasch and Fuchs, 2005). Therefore, murine ESCs have been transplanted into rodent models of diseases associated with the loss of a particular cell type, such as myocardial infarction (Klug et al., 1996), neurodegenerative diseases (Lindvall and Kokaia, 2006; Lindvall et al., 2004), myelin disease (Brustle et al., 1999), and liver failure (Chinzei et al., 2002). The integration of ESC-derived cells into host tissues has been quite successful, and resulted in partial recovery of the animals in some cases. Nevertheless, problems like the formation of teratomas are associated with some ESC transplantations (Bjorklund et al., 2002; Chinzei et al., 2002). The reason could be that naïve, undifferentiated ESCs remain in the transplanted cell population that lead to tumor formation.

Pluripotent ESCs have also been isolated from human blastocysts (Reubinoff et al., 2000; Thomson et al., 1998), but the risk of developing cancer, as well as important ethical issues, have to be considered before the clinical application of human ESCs in patients is
feasible at a bigger scale.

3.1.2 Adult stem cells

Various tissue-specific stem cells have been found in the adult skin, intestine, muscle, blood, and nervous system of mammals. They are thought to give rise to cells of their tissue of origin and to be responsible for homeostatic cell replacement and tissue regeneration, because tissues like blood, skin, and gut must perpetually self-renew (Wagers and Weissman, 2004). Stem cells in adult tissues are thought to be localized in a special microenvironment called a “niche”. The niche shelters them from differentiation and apoptotic signals, maintaining a balance between stem cell quiescence and activity (Moore and Lemischka, 2006). Every time a stem cell exits the niche it must be replenished, but excessive stem cell production must be avoided because of cancer risk.

Recently, multipotent adult stem cell populations have been isolated from skin (Amoh et al., 2005; Belicchi et al., 2004; Dyce et al., 2004; Fernandes et al., 2004; Joannides et al., 2004; Shih et al., 2005; Sieber-Blum et al., 2004; Toma et al., 2001; Toma et al., 2005), bone marrow (Jiang et al., 2002a; Jiang et al., 2002b), muscle (Cao et al., 2003; Jiang et al., 2002b; Qu-Petersen et al., 2002) and brain (Jiang et al., 2002b; Rietze et al., 2001). These stem cells can give rise to cells not found in their tissues of origin. Therefore, several explanations have been proposed for their apparent plasticity: If a broad developmental potential is not inherent to an adult cell, it might be due to transdifferentiation, a process by which a cell differentiates from one lineage to an unrelated lineage by direct activation of an otherwise dormant differentiation program. Otherwise, this conversion could also happen via de-differentiation of a cell to a more primitive multipotent cell and subsequent redifferentiation along a new lineage pathway (Wagers and Weissman, 2004). Another possible explanation for apparent plasticity of adult stem cells especially after cell transplantations is cell fusion. Fusion events occur naturally in the generation of multinucleated skeletal myofibers from myoblasts or pathologically to mediate viral entry into a target cell. Cell fusion has recently been implicated in contributions of transplanted bone marrow cells to liver hepatocytes, cardiac myocytes and Purkinje neurons and is potentially associated with transfer of donor markers and nuclei (Wagers and Weissman, 2004).

The fact that some adult stem cells may be capable of differentiating across lineage
boundaries makes them interesting for clinical applications. If an accessible tissue could be used as an autologous stem cell source, there would be no need for immunosuppression and ethical issues could be avoided. As the plasticity of adult stem cells is thought to be more restricted than that of ESCs, the risk of developing cancer is lower. Few cases of successful transplantations using multipotent adult stem cells from another tissue or even the same tissue to be repaired have been reported until now. In one study, adult neural stem cells were injected intravenously and intracerebroventricularly into mice with Experimental Autoimmune Encephalomyelitis (EAE), a model for multiple sclerosis. The administered cells promoted multifocal remyelination and functional recovery (Pluchino et al., 2003).

3.2 Stem cells and their role in cancer

Cells with stem cell qualities have been identified in cancers of haematopoietic origin and in some solid tumors (Dean et al., 2005). This observation led to the hypothesis that stem cells might be at the origin of some cancers. The “cancer stem cell hypothesis” postulates that normal stem cells that give rise to multipotent progenitors and differentiated cells in a tissue can accumulate mutations. These changes in the DNA allow these cells to acquire aberrant proliferation properties and cause a pre-malignant lesion. Additional mutations would then lead to increased proliferation, decreased apoptosis, evasion of the immune system, and further expansion of the stem cell compartment. This implies that the stem cell represents the cell of origin of the tumor (Fig. 1).

Evidence for the existence of so-called cancer stem cells comes from studies of acute myelogeneous leukaemia, where it was shown that 0.1-1% of all isolated cells had leukaemia initiating activity (Lapidot et al., 1994). These leukaemia-initiating cells shared many markers and properties of normal haematopoietic stem cells (Bonnet and Dick, 1997; Hope et al., 2004). Therefore, it is now believed that leukaemia arises from a stem cell that becomes transformed and gives rise to a large population of clones. Stem cells with a capacity to self-renew and undergo pluripotent differentiation have also been isolated from human tumors of the central nervous system (CNS) (Hemmati et al., 2003; Singh et al., 2004a; Singh et al., 2004b) and breast cancer lesions (Al-Hajj et al., 2003; Dontu et al., 2003). There is also some evidence for the existence of stem cells in melanoma lesions.
3. Introduction

(Fang et al., 2005).

Figure 1. The cancer stem cell hypothesis
In healthy tissue multipotent stem cells give rise to transient amplifying progenitors and eventually to mature cells. Mutations in a stem cell can give rise to a stem cell with aberrant proliferation and result in a pre-malignant lesion. Additional mutations lead to further increased proliferation, decreased apoptosis, decreased responsiveness to differentiation-inducing cues, evasion of the immune system and further expansion of the stem cell compartment. Eventually all these processes lead to the formation of a tumor.

Tumor stem cells are thought to be only a small, multipotent fraction of the whole tumor cell population, giving rise to more differentiated cells that retain a finite ability to divide and eventually form the heterogeneous bulk of the tumor. Stem cells are also thought to be associated with resistance to chemotherapy due to their quiescence and the presence of ATP-binding cassette (ABC) transporters that allow them to efflux drugs and toxins from their cytoplasm. Given these characteristics, tumor stem cells could be responsible for relapse, the formation of a new tumor, and even metastasis.
3.3 The neural crest

One frequently used model to study stem cell biology is the neural crest.

3.3.1 Formation and induction of neural crest stem cells (NCSCs)

Vertebrate neurogenesis commences with the formation of the neural plate, which is initially a flat, single-cell-layer thick, neuroepithelial sheet that extends the length of the body axis. The bilateral halves of the neural plate then curl up and fuse to form a neural tube that ultimately gives rise to the entire vertebrate CNS. Neural crest cells (NCCs) are born at the interface between the non-neural ectoderm (surface ectoderm and presumptive epidermis) and the dorsal region of the neural plate at the neural plate border (Crane and Trainor, 2006) (Fig. 2).

Studies of the development of the NC have been carried out in *Xenopus*, avians, zebrafish, and mice. Depending on the animal model, there are slight differences in the process. Induction of NCCs requires contact-mediated tissue interactions between neural plate, surface ectoderm and lateral mesoderm. Lineage-tracing experiments have shown that both neuroepithelium and surface ectoderm give rise to NCCs in avians (Selleck and Bronner-Fraser, 1995). A dorso-ventral BMP gradient (Liem et al., 1995; Marchant et al., 1998; Mayor et al., 1995; Selleck et al., 1998), the signalling pathways Notch (Wakamatsu et al., 2000), FGF (Monsoro-Burq et al., 2003; Monsoro-Burq et al., 2005) and Wnt (Garcia-Castro et al., 2002) have also been implicated in the induction and patterning of NCCs.

During the folding of the neural tube, NC precursors are contained within the neural folds and subsequently within the dorsal portion of the neural tube when closure is complete (Barembaum and Bronner-Fraser, 2005) (Fig. 2).
3. Introduction

Figure 2. Neurulation and formation of the neural crest
Interactions between the non-neural ectoderm (blue) and the mesendoderm (green) with the prospective neural plate (purple) induce the neural plate border (light blue). As neurulation proceeds, the neural plate rolls up and the neural plate border forms the neural folds. Near the time of neural tube closure, the NCCs undergo an epithelial to mesenchymal transition and delaminate from the neural folds or dorsal neural tube and migrate along defined pathways (from Basch et al., 2004).

3.3.2 NCC delamination and migration
In mice NC formation and migration commence well before fusion of the bilateral halves of the neural plate is complete. NCCs undergo an epithelial to mesenchymal transformation that will confer on them the ability to migrate (Trainor, 2005). This implies deep changes in cell morphology and in the expression of cell surface adhesion and recognition molecules (Morales et al., 2005).

The emergence of NCCs from the neural tube begins at the region of the caudal midbrain and rostral hindbrain, and proceeds simultaneously as a wave rostrally towards the
forebrain and caudally towards the tail. NCCs can be subdivided into at least four broad distinct axial populations: cranial, cardiac, vagal and trunk. Each migrates along unique pathways, contributing to a unique set of specific cell and tissue types that are distinct for each axial level (Crane and Trainor, 2006) (Fig. 3).

Figure 3. Fate map of the neural crest-derived phenotypes along the neural axis
The various cell phenotypes yielded by NCCs at different levels of the neural fold (light blue) are shown in chick embryos of 7 (left) and 28 (right) somites. Left, tissues that arise from the cephalic NC. Right, tissues that arise from the trunk NC in cervical, thoracic and lumbosacral regions of the spinal cord. The region that gives rise to mesectoderm (green) extends from the level of mid-diencephalon down to rhombomere 8 (corresponding to S4). Melanocytes (grey) are produced along the entire length of the neural axis. The parasympathetic ciliary ganglion (yellow) derives from the mesencephalic NC. Enteric ganglia (orange) arise from both vagal (S1-S7) and lumbosacral (posterior to S28) NC. Caudal to S4, the trunk NC yields PNS sympathetic ganglia (red), whereas the sensory ganglia (dark blue) are generated by the mesencephalic NC and by the NC from posterior rhombencephalic to lumbosacral levels. Endocrine cells (violet) originate from the NC of S2-S4 and S18-S24 levels (from Le Douarin et al., 2004).

Cranial NC migrate throughout the head and contribute to most of the bones and cartilage of the head, connective tissue of the face, as well as neurons and all the glia of the cranial ganglia. The cardiac NC give rise to some tissues of the heart like the articopulmonary septum and aortic arch smooth muscle cells, and to parasympathetic cardiac ganglia (Kirby et al., 1983; Kirby and Stewart, 1983). The vagal NCCs contribute to nearly the entire gut and the majority of neurons and glia that constitute the enteric nervous system, and finally
trunk NCCs give rise to sympathetic and parasympathetic ganglia (peripheral nervous system (PNS)), the enteric nervous system (Burns and Douarin, 1998) and secretory cells from the endocrine system. NCCs from all levels give rise to pigment cells and sensory neurons (Crane and Trainor, 2006; Trainor, 2005).

3.3.3 Growth factor responsiveness and multipotency of NCSCs

Several growth factor/receptor signaling pathways are implicated in the development of specific NC derivatives. Gliogenesis in the PNS is regulated by epidermal growth factor-like neuregulins that signal through the ErbB receptor family. Neuregulin (NRG) instructively promotes gliogenesis in cultured NCSCs. Soluble NRG1 triggers differentiation into satellite glia (Hagedorn et al., 1999; Morrison et al., 1999; Paratore et al., 2001; Shah et al., 1994) whereas membrane-bound NRG1 induces a Schwann cell phenotype (Leimeroth et al., 2002).

Sympathetic neuron development is driven by bone morphogenetic proteins (BMPs) and transforming growth factor (TGF) β (Hagedorn et al., 2000; Hagedorn et al., 1999; Mehler et al., 1997; Morrison et al., 1999; Shah et al., 1996). Growing evidence has also been provided that BMPs control NCC differentiation into parasympathetic and enteric lineages (White et al., 2001).

In addition to their effects on autonomic neuronal sublineages, BMPs and TGFβ also induce smooth muscle-like cells from NCSCs in vitro (Hagedorn et al., 2000; Hagedorn et al., 1999; Morrison et al., 1999; Shah et al., 1996). Smooth muscle cells, however, do not differentiate from trunk NCCs in vivo, suggesting that additional mechanisms suppress inappropriate non-neural fates in the PNS. These mechanisms might involve cell-cell interactions in developing ganglia, as communities of NCSCs behave differently than single NCSCs, and undergo apoptosis or neurogenesis instead of producing smooth muscle cells in response to TGFβ (Hagedorn et al., 2000; Hagedorn et al., 1999).

Melanocyte lineage differentiation in the skin is stimulated by stem cell factor (SCF) and endothelin (ET)-3, which activate the tyrosine receptor kinase c-Kit and ETRB/B2, respectively. In cultures, SCF supports early NCC and melanocyte precursor survival in conjunction with other cues (Lahav et al., 1998; Lahav et al., 1994). Melanocytes also depend on Wnt proteins for both precursor expansion and lineage specification (Hari et al., 2002; Jin et al., 2001; Le Douarin and Dupin, 2003).
Sustained β-catenin activity in neural crest cells promotes the formation of sensory neurons in vivo at the expense of virtually all other neural crest derivatives, while in culture Wnt1 instructs NCSCs to adopt a sensory neuronal fate in a β-catenin dependent manner (Hari et al., 2002; Lee et al., 2004).

Enteric gangliogenesis by NCCs depends upon ligands of the glial-derived neurotrophic factor (GDNF)-family and on the ET-3/ETRB pathway (Le Douarin and Dupin, 2003). The first data demonstrating multipotency of NCCs came from in vitro studies in quail embryos. The developmental potential of single pre-migratory trunk neural crest cells was investigated, and three types of clones were found: unpigmented clones consisting of adrenergic neurons, pigmented clones consisting of melanocytes, and mixed clones (Sieber-Blum and Cohen, 1980). Reintroducing cultured quail NC colonies into host chick embryos resulted in contributions of the transplanted cells to various NC derivatives in the host (Bronner-Fraser et al., 1980). Subsequently it was shown that both pre-migratory and migratory trunk NCCs in avian embryos are pluripotent (Bronner-Fraser and Fraser, 1989). Similar to the trunk NC, quail cranial NCCs yield neuronal and glial lineages of the PNS as well as melanocytes. Additionally they yield cartilage, bone, and connective tissue (Baroffio et al., 1991), but the majority of clones give rise to only one or two lineages. Therefore, these cells are rather progenitors than stem cells, because they become progressively committed or restricted to different developmental fates (Baroffio et al., 1991).

Also the cardiac NC consists of a heterogeneous population of pluripotent, multipotent and unipotent progenitor cells in avians (Ito and Sieber-Blum, 1991) as well as in mammals (Youn et al., 2003). In vitro cultures of individual trunk NCCs demonstrated the presence of low numbers of pluripotent cells that could generate glial, neuronal, myofibroblast and melanocyte cells in avians (Trentin et al., 2004). However, NCSCs from rat explants, DRGs or embryonic sciatic nerves were also cultured in the presence of instructive growth factors. 70-90% of the individual crest-derived cells challenged with changing environmental conditions were competent to respond to BMP2, TGFβ, NRG1 or Notch signalling and adopted a particular fate at the expense of alternative fates. This high percentages show that most of the undifferentiated cells isolated from neural crest explants or from early postmigratory neural crest derivatives must be uncommitted, multipotent progenitor cells (Sommer, 2001).
3.3.4 Persistence of NCSCs postnatally and into adulthood

The first experiments to test the persistence of NCSCs in vivo used E14.5 rat sciatic nerve tissue. Flow cytometry was used to identify a cell population positive for low affinity neurotrophin receptor (p75) and negative for Myelin protein 0 (P0). This cell population self-renewed and was capable of generating neurons, Schwann cells, and smooth muscle-like cells in clonal cultures (Morrison et al., 1999). Similar multipotent progenitors, on the contrary, could not be isolated from postnatal sciatic nerves. Dorsal root ganglia (DRGs) from E14 and E16 rat embryos also contain multipotent progenitors (Hagedorn et al., 1999).

Surprisingly, multipotent NC progenitors could also be isolated from the adult gut. They self-renewed extensively and formed neurons, glia, and smooth muscle cells in clonal culture, although they showed altered factor responsiveness (Kruger et al., 2002).

Cardiac NCSCs could be isolated from the adult in the form of cardiospheres via fluorescence activated cell sorting (FACS), using a NC-specific promoter. They expressed markers like nestin and musashi-1, and differentiated into NC lineages including neurons, Schwann cells and smooth muscle. After transplantation into chicken embryos they migrated along with endogenous neural crest and colonized the PNS within the DRGs and peripheral nerves (Tomita et al., 2005).

Adult vibrissal follicles, the hair follicles in the face of some mammals, were also shown to contain multipotent progenitors, as well. Clones derived from cells from the upper part of vibrissal follicles contained neurons, smooth muscle, rare Schwann cells, chondrocytes and melanocytes (Sieber-Blum and Grim, 2004; Sieber-Blum et al., 2004). NC-derived cells from the vibrissal dermal papilla (DP) of young mice also differentiated into smooth muscle cells and neurons, similar to NC-derived cells from the trunk skin, although their single-cell potential and, in the case of back skin, their in vivo localization was not established clearly (Fernandes et al., 2004; Toma et al., 2001).

3.3.5 Identification of NCSCs

To study the potential of a specific cell type it must first be clearly identified. In our study we used mainly two methods: marker expression and lineage tracing.

3.3.5.1 P75 and Sox10 as markers for NCSCs

The low affinity neurotrophin receptor p75 is a transmembrane glycoprotein that is
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expressed in many different tissues both during embryogenesis and in the adult. In the PNS, p75 is expressed by NCSCs, Schwann cells (Bernd, 1986; Smith-Thomas and Fawcett, 1989) and some types of neurons. It has been widely used to enrich multipotent NCSCs using FACS (Kruger et al., 2002, Bixby, 2002 #4809; Morrison et al., 1999; Stemple and Anderson, 1992) or to perform prospective identification of NCSCs on the culture dish (Hagedorn et al., 1999; Kleber et al., 2005; Lee et al., 2004; Paratore et al., 2001). Nevertheless, p75 doesn’t seem to mark precursors for all neural crest derivatives (Crane and Trainor, 2006).

Soxl0 is a member of the Sry HMG-box family, a large group of transcription factors that bind DNA by means of a high mobility group (HMG) domain. Soxl0 expression is activated as a consequence of NC induction at the neural plate border; in mouse, Soxl0 is not expressed in the migrating cranial NC and appears to be confined at all times to the trunk (Hong and Saint-Jeannet, 2005) Soxl0 sustains survival of multipotent postmigratory NCCs before lineage segregation (Kim et al., 2003; Paratore et al., 2001). Soxl0 is also required for the acquisition of the glial fate (Paratore et al., 2001), its expression persisting in differentiated glia (Britsch et al., 2001; Kuhlbrodt et al., 1998). It is also detectable during melanocyte development in mammals (Potterf et al., 2001; Southard-Smith et al., 1998).

3.3.5.2 Lineage tracing of NC-derived cells

Lineage tracing or fate-mapping is a frequently used method to identify NC-derived cells. It consists of following back the derivation of a differentiated cell to a tissue of the embryo, to find out the ancestry of the adult cell. Fate mapping in avians is possible due to accumulations of heterochromatin in quail nucleoli that are not found in chicken cells. This permits transplantations between the two species and identification of cells from the host and the donor. This technique facilitated the fate mapping of the avian NC and the identification of cellular derivatives of corresponding domains of the dorsal neural tube from chick and quail embryos (Le Lièvre and Le Douarin, 1975).

For studies in mice, the most widely used model for lineage tracing is a two-component system based on Cre/lox recombination to mark the cell lineage of interest by gene expression. One component is a transgene expressing Cre recombinase under the control of a lineage specific promoter. The second component is a conditional reporter gene termed
R26R, which expresses β-galactosidase from the ROSA26 locus upon Cre-mediated excision of a loxP-flanked transcriptional “stop” sequence (Soriano, 1999). The ROSA26 locus has been shown to be ubiquitously and uniformly active at all developmental and postnatal times, and its expression has no apparent sensitivity to genetic or environmental manipulation (Soriano, 1999; Zambrowicz et al., 1997). Without recombination, the transcript originating from the ROSA26 promoter encodes an irrelevant sequence, whereas after Cre-mediated recombination the transcript produces a functional β-galactosidase protein. Importantly, the progeny of cells that have undergone recombination will continue to be β-galactosidase positive, even though the Cre transgene is no longer active (Jiang et al., 2000). Recently, a mouse reporter line has been constructed in which cells express enhanced yellow fluorescent protein (EYFP) from the ROSA26 locus instead of β-galactosidase (Srinivas et al., 2001). This is a big advantage, because EYFP expression can be monitored in living tissues, in contrast to lacZ expression (Fig. 4).

Figure 4. Cre-mediated recombination in the ROSA26 locus
Cre recombinase (under the control of a lineage-specific promoter) excises a STOP sequence from the R26R gene, allowing the expression of either β-galactosidase or EYFP. The cells of interest that have undergone recombination become labeled and their progeny continues to express the reporter.

In the case of lineage tracing of NC-derived cells, the most widely used promoter to control Cre is Wnt1 (Danielian et al., 1998). The Wnt1 gene is expressed specifically in the neural plate, in the dorsal neural tube, and in the early migratory NC population at all axial levels excluding the forebrain. Expression of Wnt1 is extinguished as the crest cells migrate away from the neural tube, and appears not to be expressed at any other time or in any other place during development or in postnatal life (Echelard et al., 1994; Jiang et al.,
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Wnt1-Cre R26R mice have been used in many NC lineage tracing studies (Chai et al., 2000; Fernandes et al., 2004; Jiang et al., 2000; Hari et al., 2002; Lee et al., 2004; Sieber-Blum and Grim, 2004; Sieber-Blum et al., 2004; Szeder et al., 2003).

Another promoter used to control the expression of Cre recombinase specifically in NCCs is the human tissue plasminogen activator (Ht-PA) promoter. It labels all the known NC derivatives. However, in contrast to Wnt1-Cre, the Ht-PA-Cre does not target cells in the dorsal neural tube. NCCs are labelled only as they begin to migrate, but not before they undergo their epithelial-to-mesenchymal transition (Pietri et al., 2003).

To track NC-derivatives such as Schwann cells, a transgenic mouse has been used where Cre recombinase is under the control of the Desert hedgehog (Dhh) promoter. The Dhh gene is prominently expressed in Schwann cell precursors of the developing nerves and in Sertoli cells of the testis (Bitgood and McMahon, 1995). Dhh-Cre activity was observed in peripheral glia from E12 onwards (Jaegle et al., 2003).

In the case of the melanocyte lineage, several promoters have been used to identify either melanoblasts or melanocytes, all from genes involved in melanogenesis. In the case of Dopachrome tautomerase (Dct) also known as Trp2, the gene is expressed at E12.5 in the presumptive retinal pigment epithelium, migrating melanoblasts, and in the telencephalon (Guyonneau et al., 2002).

3.4 The skin

The skin is the largest organ in the body. It provides protection against environmental insults and dehydration, and is an important gateway for sensory input. In addition to forming an environmental barrier, the skin has evolved to produce an amazing variety of appendages including scales, feathers, hair follicles, sweat glands and mammary glands (Millar, 2005). Mammalian skin consists of mainly two layers; epidermis and dermis, and it harbors many different cell types: epithelial cells that form the stratified epidermis and contain specialized intermediate filament proteins called keratins, mesenchymal cells that form the underlying dermis, melanocytes that provide skin pigmentation, and glial cells that myelinate some of the axons that innervate the skin. In addition, skin also contains antigen-presenting cells like dendritic Langerhans cells, Merkel mechanoreceptor cells, which form complexes with sensory axons, and mast cells that produce histamine (Millar, 2005). Both melanocytes and glial cells are derivatives of the neural crest. In the case of
Merkel cells, a neural crest origin has also been suggested, but is still debated (Moll and Moll, 1992; Szeder et al., 2003).

To maintain its high level of regeneration and homeostasis, the skin also harbors different types of stem cells: epidermal stem cells in the basal layer of the epidermis and hair follicle and melanocyte stem cells in the upper permanent part of the hair follicle. Multipotent, neural crest-derived cells have also been identified, but their exact localization and in vivo role in skin has not been clearly investigated yet.

### 3.4.1 The epidermis

The epidermis is a thin but tough layer of stratified squamous epithelium made of keratinocytes. It rests on top of a basement membrane of extracellular matrix (ECM), which separates it from the underlying dermis (Fuchs and Raghavan, 2002). The epidermis originates from the outer layer of the embryo, the surface ectoderm, which proliferates and migrates from the dorsal midline to cover the embryo and persists as a single-layered epithelium until approximately E9.5 of mouse embryogenesis. At this stage, epidermal stratification begins as basal cells withdraw from the cell cycle, lose their ability to adhere to the basal membrane and start to migrate. BMPs activate the epidermal differentiation program and induce the expression of keratin proteins (Fuchs and Raghavan, 2002).

By birth, the epidermis consists of a basal layer that differentiates to form the suprabasal (spinous and granular) layers, and an outer, “cornified”, enucleated shell, the stratum corneum, which constitutes the epidermal barrier and is continuously shed and replenished through life (Fig. 5).

### 3.4.2 The dermis

The mammalian dermis consists of loosely packed fibroblasts. Fate mapping experiments have shown that the lateral plate mesoderm gives rise to ventral trunk dermis, while the dermis of the head arises, at least in part, from the neural crest. Analysis of chick-quail chimeras revealed that in avian embryos the dorsal dermis arises from the dorsal region of somites (Millar, 2005).
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Figure 5. Diagram of the histology of the epidermis

The basal layer of the epidermis is composed of mitotically active cells that are attached to the basement membrane. When they start to differentiate, basal cells stop dividing, detach from the basement membrane and migrate towards the skin surface. As they enter the spinous layer, they strengthen their cytoskeletal and intercellular connections becoming more resistant to mechanical stress. Once this task is completed, the cells enter the granular layer, where they synthesize cornified envelope precursor proteins and lamellar granules filled with lipid bilayers. When the cells enter the final phases of terminal differentiation, the cornified envelope proteins are cross-linked and the lipids extruded. Cell death follows leaving dead, flattened squames at the skin surface, which are periodically sloughed and replenished by cells from the inner layers moving outward (from Alonso and Fuchs, 2003).

3.4.3 The hair follicles

The hair follicle is a skin appendage exclusive to mammals and it is in charge of producing pigmented epithelial fibers, the hair. The hair follicle is the only organ in the mammalian body that undergoes cyclic transformations during its entire lifetime (Stenn and Paus, 2001). There are different kinds of hair follicles depending on their location on the body surface.

3.4.3.1 Pelage hair follicles

Hair follicle formation occurs only once in the lifetime of an individual and therefore, mammals are born with a fixed number of follicles (Stenn and Paus, 2001). Murine fur consists of four different hair types, which all start to develop between E14 and E17. The initial signal in specifying any skin appendage is a dermal cue (Hardy, 1992), which probably involves Wnt signaling (Andl et al., 2002). Transplantation experiments suggest that the epithelium possesses the information as to what kind of appendage to make, while the mesenchyme presents the stimulus (Garber et al., 1968).

In response to the dermal cue, the epithelium thickens into distinct placodes of
keratinocytes, which grow downward to form a hair follicle (Fig. 6). At this stage, the underlying mesenchymal cells organize into a dermal condensate of specialized fibroblasts, the DP, which will become the permanent mesenchymal portion of the hair follicle (Fuchs et al., 2001). The developing hair germ cells that lose contact with the DP become the outer root sheath (ORS), which is contiguous with the developing, now stratifying epidermis. Those cells that maintain contact with the DP, the matrix cells, then proliferate and form the three cylinders of the inner root sheath (IRS) and the three layers of the hair shaft (Fuchs et al., 2001), which contains bundles of keratins that confer high tensile strength and flexibility (Alonso and Fuchs, 2006). Shortly thereafter, a sebaceous gland emerges as an appendage to the upper segment of each hair follicle, formed from keratinocytes from the hair follicle epithelium. The sebaceous gland secretes lipids (sebum) into the hair canal at the skin surface. At this stage melanin begins to be synthesized in the newly formed hair follicle pigmentary unit and mast cells, Langerhans cells, and macrophages populate the hair follicles and the perifollicular mesenchyme.

Figure 6. Hair follicle morphogenesis (following page)
Hair follicle morphogenesis consists of eight developmental stages. The process starts with a signal from the dermis, which instructs the epidermis to thicken at distinct places to form placodes made of keratinocytes. A placode then grows downward to form a cup of uniform cells, the hair germ that opens to the ectoderm. Probably it is the hair germ that cues the underlying mesenchymal cells to condensate into the DP. The bulb of the hair germ epithelium forms a cloak around the DP, which in turn stimulates the epithelium to proliferate and grow downward. The developing hair germ cells that lose contact with the DP become the ORS. The cells that maintain contact with the DP, the matrix cells, withdraw from the hair cycle and move upwards in concentric cylinders of cells, each of which adopts a morphologically distinct differentiation program. The three cylinders that compose the IRS form first until finally the hair appears at the center of the developing follicle within this cylinder. As hair shaft cells terminally differentiate, they extrude their organelles and become tightly packed with bundles of keratins. Shortly thereafter, the sebaceous gland is formed from keratinocytes of the hair follicle epithelium. The development of hair follicles is accompanied by incoming nerve fibers, which are present before hair morphogenesis starts and will eventually innervate the newly formed follicles at FNA and FNB (see skin innervation). Dp, dermal papilla; IRS, inner root sheath; ORS, outer root sheath; sg, sebaceous gland (from Peters et al., 2002a).
3.4.3.1.1 The hair cycle
All mature follicles undergo a cycle consisting of phases of growth (anagen), regression (catagen), rest (telogen), and shedding (exogen) (Fig. 7). The reason for this cycling is not obvious, but cleansing of the body surface, adapting to a changing environment, and protection from malignant degeneration are some speculations (Stenn and Paus, 2001). In
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humans, hair follicles grow synchronously only before and around birth. After that, each follicle has its own inherent rhythm and thus the cycles are asynchronous (Chase, 1954). In most rodents, however, large collections of follicles cycle together in waves. The completion of the first two hair cycles is largely synchronous. However, as the mouse ages, the waves become less frequent and synchronous hair growth occurs only in small patches (Stenn and Paus, 2001).

Figure. 7 The stages of the hair cycle
The hair cycle can be divided into phases of growth (anagen), regression (catagen), rest (telogen) and shedding of the hair shaft (exogen) (from Alonso and Fuchs, 2006).

3.4.3.1.1.1 Anagen

In mice, the first anagen phase after morphogenesis is completed in back skin by about P8 and lasts until about P16. Subsequent anagen phases follow most of the steps of morphogenesis. At the point when the epithelial finger reaches its deepest level, the cylinders of the follicle form. The outermost cylinder forms the ORS and maintains itself
via basal cell growth while the cells in the central cylinders reverse their growth direction and progress upward forming the IRS and hair shaft, fed by cells from the matrix. Morphologically, there are at least eight cell lineages in the anagen follicle: ORS, companion layer, Henle’s layer, Huxley’s layer, cuticle of the IRS, cuticle of the shaft, shaft cortex and shaft medulla (see inset in Fig. 7) (Stenn and Paus, 2001). However, matrix cells have a finite proliferative capacity, setting a biological limit to the number of hair and IRS cells they can generate. The timer appears to be genetically determined, but is influenced by the size and stimulatory output of the DP as well as other factors in the surrounding environment (Fuchs et al., 2001).

3.4.3.1.1.2 Catagen

When the proliferative capacity of the matrix cells is exhausted, a destructive phase follows. In mice, the onset of the first catagen ranges from P14 at the upper back to P18 in the lower back near the tail and lasts three to four days (Alonso and Fuchs, 2006). The first catagen begins in a wave spreading from the top of the head caudally towards the tail and laterally down the sides of the animal (in contrast to anagen which starts caudally and spreads rostrally to the head). In humans, the first entry into catagen occurs already in utero (Paus and Foitzik, 2004).

In mice, the progression of the hair cycle is evident from the color and thickness of the skin, which changes from the dark gray or black of anagen to the pale pink of catagen, becoming thinner. The lower two-thirds of the hair follicle undergo apoptosis and as the follicle shrinks, the DP cells maintain contact with the basement membrane that separates the epithelium and mesenchyme. This draws the DP up to the base of the permanent epithelial portion of the hair follicle, a region known as the bulge. The purpose of catagen is to delete the old hair shaft factory (Stenn and Paus, 2001). After extensive apoptosis, the follicle enters a resting phase known as telogen (Fig. 7).

3.4.3.1.1.3 Telogen

During telogen, the proliferative and biochemical activity of the hair follicle reach the lowest point during the hair cycle (Paus and Foitzik, 2004). The old hair shaft, called club hair, left without anchorage, becomes fragile and can be liberated from its site when physically stressed. In mice, the first telogen is short, lasting only one or two days, from approximately P19 to P21 in the mid back. The second telogen, however, lasts more than
two weeks beginning around P42 (Alonso and Fuchs, 2006). Telogen can last for months in humans or barely at all in the case of whisker follicles. Following the resting period, a new hair cycle is spontaneously initiated, as a fresh hair germ-like structure streams down from the bulge (Alonso and Fuchs, 2006) (Fig. 7).

3.4.3.2 Vibrissal hair follicles

The vibrissae or whiskers are found in the face of some mammals (excluding humans) and are produced by extremely large and densely innervated follicles. They serve an important sensory function (Fig. 8).

Vibrissae are the first follicles to develop in mice and their formation starts around E12. They consist, as do all hair follicles, of an epithelial core surrounded by a mesenchymal sheath, which is in continuity with the DP in the hair bulb. However, vibrissal follicles differ from pelage hair follicles by the presence of vascular sinuses encompassed by a rigid capsula, surrounded by bundles of striated muscle fibers. In contrast to the pelage follicle, cycling of the vibrissal follicle is different in that the lower follicle does not regress upward. Nevertheless, there is a cyclical change in the diameter of the follicle (Stenn and Paus, 2001) (Fig. 9).

Figure 8. The whisker pad of rodents

Vibrissae are localized in the whisker pads of some mammals and have an important sensory function. The hair cycle stage of a vibrissa at a given time depends on its localization in the pad. (Adapted from Nishimura et al., 2005)

Figure 9. Schematic representation of the various structures of a vibrissal follicle (following page)

Vibrissal follicles are the biggest hair follicles and share many structures of pelage follicles like the sebaceous gland (SG), outer root sheath (ORS), dermal papilla (DP) and hair shaft. Not shown are the dermal sheath that covers the ORS and is not of epidermal origin, and the nerve. The bulge is the epidermal stem cell compartment of hair follicles and belongs to the ORS (Adapted from Oshima et al., 2001).
3.4.4 Skin innervation

The skin is abundantly innervated by sensory, and motor neurons (sympathetic and parasympathetic axons) residing in the DRGs and sympathetic ganglia. In mice, nerve fibers begin to be seen at E14, when hair follicles are still absent (Peters et al., 2002a). Nerve fibers that invade the subepidermal mesenchyme form an increasingly dense network that eventually becomes organized into three horizontally oriented nerve plexuses: the subepidermal, the deep cutaneous and the subcutaneous nervous plexus. The subepidermal nervous plexus is located close to the epidermis and consists of thin, mostly unmyelinated nerve fibers that end freely in the subepidermal layer of the dermis or penetrate the epidermal basal membrane and innervate the epidermis. Single branches of the deep cutaneous plexus, which is located on the dermis-subcutis border, innervate hair follicles and arrector pili muscles. Some single fibers arising from this plexus are very thick and are probably myelinated, as they are positive for S-100 and myelin basic protein (MBP) when stained with antibodies against these proteins. Some fibers end freely in the dermis. Nerve fibers from the subcutaneous nervous plexus innervate subcutaneous blood vessels (see references in Botchkarev et al., 1997).

3.4.4.1 Hair follicle innervation

The bulge region has the highest density of nerve fibers compared to other innervated skin regions. All telogen pelage follicles in murine back skin contain two distinct follicular neural networks, termed follicular neural network (FN) A and B. Numerous nerve fibers arising from the subepidermal nervous plexus form the FNA, which extends from the
perifollicular epidermis to the level of the sebaceous gland enwrapping the infundibulum of the hair follicle. The FNB consists of longitudinal and circular fibers arranged in a highly organized manner around the bulge of the follicle just below the sebaceous gland and above the insertion of the arrector pili muscle. These two types of fibers are associated with distinct types of nerve endings. The longitudinal fibers arise from the deep cutaneous nervous plexus and are formed from myelinated nerve fibers that penetrate the DS of the follicle, where they lose their myelin sheath. However, these nerve fibers still have a coat of Schwann cell processes. They are thought to function as rapidly adapting type I mechanoreceptors and have close contacts to the ORS basal lamina. The circular fibers arise from both the deep cutaneous and the subepidermal nervous plexus. They are located distally to the longitudinal nerve fibers, in the DS and have an unmyelinated Schwann cell sheath ending freely. They could possibly function as slowly adapting type II mechanoreceptors. Another type of nerve endings, which has no Schwann cells, contacts the basal lamina of the ORS (see references in (Botchkarev et al., 1997) (Fig. 6).

3.4.4.1 Hair follicle innervation during the hair cycle

There are substantial and highly selective changes in the innervation of both the hair follicle and various skin compartments during the hair cycle. In general, a significant transient increase in fiber number is associated with early anagen in both FNA and FNB, although an increase only in the number of circular but not longitudinal fibers can be detected in FNB. The number of circular fibers declines again during later stages of the hair cycle (Fig. 10).

How are these changes in innervation achieved? A collapse-and-extension concept could not accurately explain the changes of innervation during the hair cycle, at least not in murine skin, because the most prominent innervation changes occur in the permanent portion of the follicle, not in the cycling part. The increase in fiber number most probably has to be attributed to sprouting of existing nerve fiber processes. It is accompanied by NCAM and growth associated protein (GAP) 43 expression, both of which indicate growth of nerve fibers. As differentiated neurons do not have the ability to proliferate, it is highly unlikely that the detected fibers derive from new neurons, which would have to grow within days to the skin periphery (Botchkarev et al., 1997).
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Figure 10 Hair cycle-dependent plasticity of murine skin and hair follicle innervation
The schematic drawing summarizes the observed changes in protein gene product (PGP) 9.5 immunoreactive nerve fibers during the murine hair cycle. An increase in fiber density of the FNA and of circular, but not longitudinal fibers of the FNB is observed during anagen II. The innervation of epidermis, dermal compartments and subcutis is denser during anagen II than during telogen and anagen VI. APM, arrector pili muscle; DP, dermal papilla; FNA+B, follicular neural network A+B; HM, hair matrix; HS, hair shaft; I+B, bulge; Mel, melanocytes; ORS, outer root sheath; SG, sebaceous gland (from Botchkarev et al., 1997).

3.4.5 Skin pigmentation
Melanocytes, the pigment producing cells of skin, hair follicle, inner ear, and uveal tract of the eye (iris, ciliary body, and choroid) are derived from the neural crest. On the other hand, the outer layer of the neuroectodermal optic cup, which gives rise to the retinal
pigment epithelium is derived from the developing forebrain of the embryo (Guyonneau et al., 2004).

The pigments produced by melanocytes, the melanins, are polymorphous and multifunctional biopolymers that protect the skin against ultraviolet (UV)-induced damage. Their biosynthesis starts with L-Tyrosine, which is transformed through complex oxidation and reduction reactions into the different types of melanin. Under physiological conditions, melanin synthesis in melanocytes is restricted to melanosomes, specialized vesicles that contain the relevant enzymes for melanogenesis (Slominski et al., 2004).

In humans, melanocytes are localized in the basal layer of the epidermis in close contact with keratinocytes at various stages of progression to the upper cornified layer and in hair follicles. In contrast, many furred mammals, including the mouse, lack melanogenically active melanocytes in their adult truncal epidermis. Instead, melanin is only produced in the hair follicle bulb and only during anagen, to pigment the hair shaft cortex (Slominski et al., 2004). Independent of their location, the task of melanocytes is to transfer melanin granules to keratinocytes, but how this is achieved is still debated. Nevertheless, melanocyte growth, dendricity, spreading, cell-cell contacts, and melanization can all be regulated by keratinocyte-secreted factors (Tenchini et al., 1995).

3.4.5.1 Pigmentation during the hair cycle

*Dct-lacZ* transgenic mice express lacZ under the control of the dopachrome tautomerase (*Dct*) promoter (see section 3.5.2.1). Dct is an early melanocyte marker. In the *Dct-lacZ* mice at least one lacZ+ cell is maintained in the lowest permanent portion throughout the hair cycle. At the transition from telogen to anagen, the lacZ expression level and cell size of melanocyte lineage cells increases and eventually they divide. Other lacZ+ cells, surrounded by the growing hair germ, extended their processes towards the DP and became localized to the hair matrix, dividing and differentiating into pigmented melanocytes (Nishimura et al., 2002) (Fig. 11).

The earliest signs of imminent hair follicle regression (catagen) are retraction of melanocyte dendrites and attenuation of melanogenesis. The melanogenically active melanocytes of the anagen phase are no longer detectable at this stage (Slominski et al., 1994). Keratinocyte proliferation, however, continues for some time so that the most proximal telogen hair shaft remains unpigmented.
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Figure 11. Melanoblast and melanocyte distribution during hair cycling
Stem cells (blue) are maintained in the lowest permanent portion of the hair follicle throughout the hair cycle. They are reactivated at early anagen to supply amplifying progeny (red) to the hair matrix, where most of them mature into differentiated melanocytes (green). AC, amplifying cells; C, cycling portion; P, permanent portion; SC, stem cells; SG, sebaceous gland; Mel, mature melanocytes (Adapted from Nishimura et al., 2002).

3.5 Stem cell types in skin

3.5.1 Epidermal stem cells
Several techniques have been used to identify and isolate stem cells from the skin. One takes advantage of the characteristic expression of cell surface markers, a standard procedure used for the isolation of haematopoietic stem cells (HSCs). In skin, specific markers for stem cells could not be identified to date. It has been shown, however, that epidermal stem cells express high levels of β1 (Jones and Watt, 1993) and α6 integrins and low levels of the transferrin receptor, in contrast to transient amplifying cells that express high levels of both α6 integrin and the transferrin receptor (Tani et al., 2000). Nevertheless the integrins could not be considered to be exclusive stem cell markers.

Another way to isolate stem cells is by sorting the so-called side population. This idea is based on the presence of ABC drug transporters in stem cells. Cells with these transporters show efflux of the Hoechst 33342 dye (Wagers and Weissman, 2004). This technique in combination with selection by size, yielded a virtually homogeneous population of epidermal stem cells (Dunnwald et al., 2001; Liang and Bickenbach, 2002). However, their specific location in vivo cannot be shown in this way.

Another method used to identify stem cells in a tissue takes advantage of the fact that they are slow cycling, relatively quiescent. After a pulse with bromodeoxyuridine (BrdU) or [3H] thymidine, all dividing cells incorporate the nucleotide into newly synthesized DNA. When the label is chased over a long period, only those cells that divide rarely and still reside in the tissue over time will retain their label (Alonso and Fuchs, 2003). Recent
experiments showed that the majority of label-retaining cells (LRCs) in the skin reside in the bulge region of the hair follicle with only a fraction of LRCs in the basal layer of the interfollicular epidermis (Cotsarelis et al., 1990). The bulge resides within the ORS in a small niche just below the sebaceous gland, at or near the site of insertion of the arrector pili muscle. Bulge cells retain the label for longer than stem cells of the epidermis, and they can give rise to all the layers of the hair follicle (IRS, hair shaft), the epidermis (Taylor et al., 2000) and sebaceous glands (Oshima et al., 2001). In rat whisker follicles, 95% of colony-forming cells were found in the bulge, and 5% were found in the matrix region (Kobayashi et al., 1993).

Epidermal stem cells in the hair follicle are important for the hair cycle. It seems likely that the process of activating these stem cells to initiate anagen depends on factors that are both intrinsic and extrinsic to the bulge cells themselves. In vibrissal follicles it has been shown, that bulge stem cells leave the niche and migrate down into the matrix toward the DP, where they receive their cue to divide and differentiate (Oshima et al., 2001). Clonogenic keratinocytes located in the lower region or the bulb are still multipotent and participate in long-term hair follicle renewal when transplanted (Claudinot et al., 2005).

3.5.2 Neural crest-derived stem cells

3.5.2.1 Melanocyte stem cells

Cutaneous melanocytes originate in the NC, where the emergence of a committed melanocyte lineage appears to be determined mainly by MITF, FGF2 and ET-3 amongst others factors (Dupin and Le Douarin, 2003). Melanocyte precursors, melanoblasts, migrate from the dorsal portion of the closing neural tube and move dorsolaterally to populate the basal layer of the epidermis and the hair follicle. Human melanocytes enter the dermis and are already present in the epidermis two weeks before hair morphogenesis (Holbrook et al., 1989).

In mice, melanoblasts migrate through the epidermis until all are localized in the hair follicles. Once localized there, they are separated into two populations: differentiated melanocytes, which are localized in the hair matrix region and are responsible for hair pigmentation, and melanocyte stem cells that are localized at the lower permanent portion of the hair follicle and are responsible for the repopulation of the melanocyte system in subsequent hair cycles (Osawa et al., 2005).
Melanocyte stem cells were identified using a transgenic mouse carrying the *lacZ* reporter under the control of the *Dct* promoter (Mackenzie et al., 1997). *Dct* encodes the enzyme dopachrome tautomerase (also known as Trp-2), which is involved in melanogenesis together with Trp-1 and Tyrosinase. *Dct* is specifically expressed in melanoblasts and melanocytes, with an onset of expression in skin at about E11 (Guyonneau et al., 2004). *Dct-lacZ*+ cells were localized in the ORS of the lower permanent portion of the hair follicle, the bulge and in the sub-bulge area. They were also abundant in the hair matrix (Nishimura et al., 2002).

The tyrosine kinase receptor c-Kit is expressed in melanoblasts during migration (Botchkareva et al., 2001; Peters et al., 2002b; Wilson et al., 2004) and also involved in differentiation (Luo et al., 1995) and survival/apoptosis of melanocytes (Ito et al., 1999). However, the melanocyte stem cells of the bulge seem to lose c-Kit expression and are independent of its signalling pathway. To show this, neonatal mice were treated with an anti c-Kit antibody (ACK2), which selectively depleted amplifying populations of melanoblasts. Only cells in the bulge were labelled with lacZ in *Dct-lacZ* transgenic mice. Additionally, the first hairs to develop were almost all unpigmented, but pigmentation was restored in the next hair cycle. This indicates the existence of a population of melanoblasts that survive independently of stem cell factor (SCF)/c-Kit signalling (Nishimura et al., 2002).

The melanocyte cells in the bulge region were shown to be slow-cycling, activated only during anagen. When pulsed during anagen, but not at other times, they retained a BrdU label at least 70 days (Nishimura et al., 2002). Single-cell-based gene expression profiling and immunohistochemistry revealed that melanocyte stem cells express Dct, Pax3, and Sox10. The latter was downregulated in 60% of the melanocyte stem cells of the bulge (Osawa et al., 2005). In contrast, differentiated melanocytes from the matrix express proteins involved in melanogenesis, like Tyrosinase, Dct and Trp-1, in addition to c-Kit, Mitf, Pax3 and Sox10, which play an important role in melanocyte development (Mollaaghababa and Pavan, 2003).

Hair graying is associated with a loss of melanocyte stem cells that clearly precedes the loss of pigmented melanocytes in the hair matrix. It is caused by an incomplete maintenance of melanocyte stem cells and associated with ectopic pigmentation and differentiation within the melanocyte stem cell niche (Nishimura et al., 2005).
3.5.2.2 Skin-derived precursors (SKPs)

SKPs were isolated from embryonic, neonatal, juvenile and adult skin from mice, and cultured in the presence of EGF and FGF as spheres (Fernandes et al., 2004; Toma et al., 2001). The earliest time point where sphere formation could be observed was E14, with the highest number of spheres formed from E15 to E19 skin. Sphere-forming cells comprised 0.5-1% of the whole plated population. In neonatal skin there was already a tenfold decrease in stem cells (Fernandes et al., 2004).

Many cells in the undifferentiated spheres were shown to express nestin (a marker for neural precursors), fibronectin, and Sca-1 (a stem cell marker). Some cells also expressed the transcription factors snail, slug and twist (important in neural crest induction), Pax3, and Sox9. Undifferentiated cells did not express p75 and Sox10 (markers for neural crest stem cells and glial cells), and no melanocyte or glial markers. They could only be isolated from the dermis. After differentiation in high density, some cells from spheres expressed different neuronal markers from various stages of differentiation, and several glial markers. Non-neural cell types like smooth muscle cells and adipocytes were also contained in the cultures. Embryonic and neonatal spheres produced p75 only after differentiation (Fernandes et al., 2004; Toma et al., 2001).

After injection of neonatal skin-derived primary spheres into the chick NC migratory stream in ovo, SKPs migrated into peripheral NC targets like the spinal nerve, DRG, peripheral nerves and the dermis of skin. A subpopulation of these cells expressed S100 (Fernandes et al., 2004).

SKPs were also isolated from whisker pads of Wnt1-Cre/R26R mice. Spheres expressed β-galactosidase and some cells expressed βIII tubulin upon differentiation. In addition, cells cultured from microdissected DPs also formed spheres and SKPs were shown to express some DP markers, therefore it was concluded that the DP is one niche for SKPs. Evidence for the exact localization of SKPs in back skin was not conclusive, although the DP was mentioned (Fernandes et al., 2004).

To assess the neuronal potential of SKPs in vivo, undifferentiated SKP spheres were transplanted into neonatal rat hippocampal slice cultures. Cells showed no migration or neuronal differentiation, but when the cells were predifferentiated, they migrated and expressed p75, βIII tubulin and tyrosine hydroxylase (TH) four to six weeks after transplantation. Patch-clamp recordings of differentiated cells from primary E18 spheres in
culture showed that SKP-derived neurons did not possess voltage-gated inward sodium currents (Fernandes et al., 2006). Neonatal SKPs could also be pushed to a glial phenotype with forskolin or NRG1 treatment. When differentiated SKPs were transplanted into the distal part of crushed sciatic nerves of shiverer mice they associated with axons and expressed MBP. They also integrated into white matter tracts of newborn shiverer organotypic cerebellar slice cultures and expressed MBP (McKenzie et al., 2006).

SKPs were also isolated from adult human scalp (Toma et al., 2001) and neonatal foreskin tissue. As foreskin contains no hair follicles at all, the localization of SKPs in foreskin is unknown (Toma et al., 2005).

3.5.2.3 Epidermal neural crest stem cells (EPI-NCSCs)

EPI-NCSCs were isolated from whisker follicles of adult Wnt1-Cre/R26R mice by microdissecting the “bulge” region and culturing it in a medium with chicken embryo extract (CEE) and fetal calf serum (FCS). Whole explants were put on the plate and cells migrated out after 48h of culture. The cells were β-galactosidase positive and some expressed Sox10. Treatment with NRG1 induced differentiation into Schwann cells and treatment with BMP2 induced differentiation into chondrocytes. Self-renewal was shown by serial cloning, and clonal analysis showed clones containing βIII tubulin, smooth muscle actin, melanocytes, chondrocytes, and rarely some Schwann cells (Sieber-Blum et al., 2004). However, it was not clear if all of these cell types arose in the same clone. These cells were proposed to be localized in the epidermal part of the vibrissal follicle, although the DS (neural crest-derived) was not removed before culture.

Gene profiling by LongSAGE was performed with differentiated NCSCs (after 7 days of differentiation), embryonic mouse NCSCs (after 2 days of culture) and mouse EPI-NCSCs (after 2 days of culture). Nineteen genes were proposed as a common signature of EPI-NCSCs and undifferentiated NCSCs from explants (not expressed in differentiated NCSCs). This list of genes included genes involved in lineage choice, Wnt signalling, cell migration, cell invasiveness, cell proliferation and prevention of apoptosis. These results were compared to the gene profile from LRCs of the bulge (Tumbar et al., 2004) of transgenic mice that express histone H2B-green fluorescent protein (GFP) restricted to skin epithelium (K5 promoter and tetracycline regulation). Of the bulge exclusive population
Only one of 91 genes was in common. When the profiles were compared to the $GFP_{\text{high}}$ population that may also include basal layer and other ORS cells, 51 from 91 genes were in common, but differentially expressed (Hu et al., 2006). None of the NC signature genes occurred in DP signatures published before from either back skin (Rendl et al., 2005) or vibrissae (O'Shaughnessy et al., 2004).

After acute implantation of EPI-NCSCs in the contusion lesioned murine spinal cord, the cells survived and intermingled with host neurons. They did not migrate, proliferate, or form tumors. Some cells of the grafts expressed $\beta_{III}$ tubulin (46%), glutamic acid decarboxylase (GAD67) (a GABAergic marker) (19.4%), regulation of phenobarbitol-inducible P450 (RIP) (an oligodendrocyte marker), or MBP, while GFAP was not expressed (Sieber-Blum et al., 2006).

3.5.3 Other cells with stem cell properties isolated from skin

Cells with stem cell-like potential have also been isolated from the dermis of humans. These cultures contained cells that express nestin and musashi but no p75, and were induced to differentiate with conditioned medium of hippocampal astrocytes. The cells differentiated into about 10% of neurons, and clonal spheres produced smooth muscle cells (Joannides et al., 2004).

Similar stem-like cells were isolated from pig fetal skin. Upon differentiation by growth factor withdrawal they generated $\beta_{III}$ tubulin-positive neurons, GFAP-positive glia, and adipocytes from clonal spheres (Dyce et al., 2004). Media from both studies contained EGF and FGF.

In a similar medium, but containing 10% FBS, cells from adult human scalp were isolated and cultured in adherent conditions. Their surface antigen profile was similar to that of bone marrow mesenchymal stem cells, and they had an osteogenic, chondrogenic, and adipogenic potential also similar to that of the mesenchymal cells. The cells of interest comprised 0.5-2% of the isolated tissue cells. The differentiation into neurons was tested with RT-PCR, and microtubule-associated protein (Map) 2 and neurofilament (NF) were found to be upregulated after differentiation. GFAP-positive cells could be found under all different culture conditions (Shih et al., 2005).

Cells with stem cell properties were also identified in skin via nestin expression (transgenic mice carrying EGFP under the control of the nestin second-intron enhancer). They were
3. Introduction

described to be located in the upper ORS of the back skin hair follicles including the bulge, colocalizing with keratin 5/8 and 15 expressing cells (Li et al., 2003). Cells expressing EGFP were isolated from vibrissal bulge and pelage hair follicles in telogen phase from nestin EGFP mice under the microscope. The cells were cultured as spheres in medium containing FGF. In an undifferentiated state they were keratin 15-negative, but positive for CD34. The EGFP-expressing cells were induced to differentiate in RPMI 1640 medium containing 10% FBS, and produced neurons, glia, keratinocytes, smooth muscle cells, and melanocytes with an amazing variety of marker expression and an amazing neuronal potential of 48% in vibrissae and 68% in pelage. Single cells from the vibrissal bulge were cultured, formed a colony, and such colonies were induced to differentiate. These colonies were described to contain neurons, astrocytes, oligodendrocytes, keratinocytes and smooth muscle cells although it is not completely clear if all the lineages arose from the same clone. If a cell colony or sphere was transplanted into the subcutis of nude mice, the cells differentiated into neurons (Amoh et al., 2005).

Few attempts have been made to isolate cells and try to establish their potential in vivo. Cells from skin of 14-week old human fetuses and individuals between 12-65 years of age were enriched for cells expressing the HSC marker AC133 by magnetic cell sorting (MACS). AC133 is a marker found on HSCs (Yin et al., 1997) and in cells from cord blood (de Wynter et al., 1998). Cultured in the presence of EGF and FGF, these cells express nestin but not p75 in an undifferentiated state. They are able to differentiate in vitro into neurons, astrocytes, and rare oligodendrocytes. Spheres were also generated from single cells. Short-term predifferentiated cells were transplanted into the lateral ventricles of adult mice, where they engrafted into the ventricular zone and migrated from there mainly into host cortex, but also into striatum and hippocampus. 69% of the cells that engrafted expressed GFAP, while there were only few neurons (Belicchi et al., 2004).

There are many different reports describing the isolation of cells with stem cell properties from the skin. Most of these publications focus on the in vitro potential of the isolated cells with the aim of future therapeutic use. However, it is just as important to describe the origin and localization of a cell type in the tissue, because the origin might influence its behaviour in a new environment. It will be crucial now, to distinguish clearly between different cell types isolated from skin to choose the one best suited for a certain application.
4 MULTIPLE ORIGINS OF MULTIPOTENT NEURAL CREST-DERIVED CELLS IN THE ADULT SKIN

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Figures 1, 2 and 8 and most of figures 3, 5, 6 and 7 of Chapter 4.3 was work done exclusively by myself. Figures 3 and 4 were done in collaboration with Christian Paratore. The table in figure 5 was done in collaboration with Prof. Yann Barrandon, EPFL, Lausanne. Immunofluorescent stainings in Figures 6 and 7 were done in collaboration with M.T Dours-Zimmermann.
4.1 Abstract

Given their accessibility, multipotent skin-derived cells might be useful for future cell replacement therapies. Here, we describe the isolation of sphere-forming multipotent cells from the adult trunk skin of mice and humans. Unlike previously characterized skin-derived precursor cells, these cells express the neural crest stem cell (NCSC) markers p75 and Sox10, pointing to different origins of multipotent cells in the skin. To determine the nature of multipotent skin-derived cells, we genetically mapped the fate of neural crest cells in face and trunk skin of mouse. In whisker follicles of the face, many mesenchymal structures are neural crest derived and, intriguingly, contain cells with sphere-forming potential. In the trunk skin, however, sphere-forming neural crest cells are restricted to the glial and melanocyte lineages. Thus, multipotent cells in the adult skin can be obtained from many neural crest derivatives, and these are of distinct nature in face and trunk skin. These findings are relevant for the design of therapeutic strategies, because the potential of a multipotent cell in vivo likely depends on its nature and origin.
4.2 Introduction

Embryonic, fetal, and adult tissues are used as sources to investigate the developmental and therapeutic potential of stem cells. Because of their accessibility and the possibility that the patient could act as stem cell donor, adult stem cells from the skin have received particular attention (Slack, 2001). Apart from multipotent epithelial stem cells that form hair follicles, sebaceous glands, and epidermis (Blanpain et al., 2004; Claudinot et al., 2005; Oshima et al., 2001; Taylor et al., 2000) and so-called melanocyte stem cells that generate pigmented cells (Nishimura et al., 2002), a multipotent cell dubbed skin-derived precursor cell (SKP) has been isolated from both the murine and human dermis (Toma et al., 2001). SKPs have the potential to produce in vitro cell types normally not found in the skin, such as neuronal cells. Subsequently, several laboratories provided evidence for the existence of multipotent cells present in the skin of mice, pigs, and humans (Amoh et al., 2005; Belicchi et al., 2004; Dyce et al., 2004; Joannides et al., 2004; Shih et al., 2005; Sieber-Blum et al., 2004; Toma et al., 2005). These cells have been expanded either in floating sphere cultures or in adhesive clonogenic cell cultures, indicating their self-renewing capacity. Moreover, skin-derived cells have been reported to undergo differentiation in vitro to varying degrees into cells expressing neuronal, glial, osteoblast, chondrocyte, smooth muscle, melanocyte, and adipocyte lineage markers.

The formation of cells normally not present in skin might be due to transdifferentiation, which describes the conversion of a cell type of a specific tissue lineage into a cell type of another lineage (Wagers and Weissman, 2004). Alternatively, cells from a given lineage might dedifferentiate into a more naive state that allows the cell to redifferentiate along new lineages. Finally, multipotent cells with stem cell features might persist until adulthood, able to generate a broad variety of cells depending on their environment. To distinguish among these possibilities, the origin and nature of the cell in question has to be determined, and its developmental potential has to be analyzed at the single cell level (Wagers and Weissman, 2004).

The developmental origin and exact localization of skin cells giving rise to neural and non-neural progeny is in many of the reported cases unclear. Multipotent skin-derived cells have been enriched by means of markers found on hematopoietic stem cells (Belicchi et al., 2004) or have been isolated from transgenic animals expressing green fluorescent marks.
protein (GFP) from promoter elements of Nestin (Amoh et al., 2005), a gene also expressed in neural progenitor cells. One source unambiguously associated with multipotent SKPs is the dermal papilla from whisker follicles (Fernandes et al., 2004). Whisker follicles are large hair follicles of the face that serve as sensory organs for a wide range of mammals. Genetic in vivo cell fate mapping revealed that the dermal papilla of these follicles is of neural crest origin (Fernandes et al., 2004). Similarly, culturing explants of bulge and dermal sheath of whisker follicles allowed the identification of stem cells in the upper part of the whisker follicle (Sieber-Blum et al., 2004). These were also shown to derive from the neural crest, but were characterized by markers not expressed in SKPs.

A neural crest origin might explain the multipotency of at least some stem and progenitor cells in the skin. Indeed, the neural crest—a transient structure in vertebrate development induced at the dorsal part of the closing neural tube—contributes to a variety of tissues, including the peripheral nervous system, craniofacial structures, smooth muscle cells in the heart outflow tract, and melanocytes in the skin (Le Douarin and Dupin, 2003). Clonal analysis revealed that multipotent, self-renewing neural crest stem cells (NCSCs) are not only present during migration in early development, but can also be isolated from different tissues at later stages and even from the adult organism (Bixby et al., 2002; Kruger et al., 2002). Thus, it is conceivable that apart from the whisker follicle, other neural crest-derived compartments in the skin, such as the niche for melanocyte stem cells (Nishimura et al., 2002), might contain multipotent neural crest-derived cells, although this remains to be elucidated.
4.3 Results

p75/Sox10-positive neural crest cells with stem cell properties can be isolated from the adult murine and human skin

Floating sphere cultures have previously been used to identify self-renewing multipotent cells in both murine and human skin (Belicchi et al., 2004; Fernandes et al., 2004; Joannides et al., 2004; Toma et al., 2001; Toma et al., 2005). To further characterize sphere-forming cells derived from the trunk skin of adult mice, dorsal and ventral skin biopsies comprising both dermis and epidermis were dissociated and cultured in uncoated tissue culture flasks in the presence of EGF and FGF2. Similar to previous reports, formation of spheres was observed within four to seven days of culture. These spheres could be passaged for several months without overt morphological changes (Fig. 1A), pointing to the self-renewing capacity of cells present in the spheres. Intriguingly, unlike SKPs enriched by marker selection (Belicchi et al., 2004) or cultured in slightly different conditions than used here (Fernandes et al., 2004), 100% of all primary, secondary, and later passage spheres generated from mouse trunk skin (n > 50 spheres) contained cells expressing the low affinity neurotrophin receptor p75 and also the transcription factor Sox10, both markers for NCSCs (Paratore et al., 2001; Stemple and Anderson, 1992). In spheres passaged more than 20 times, 67.0±10.5% of all cells expressed p75, 76.6±4.5% of all cells expressed Sox10, and 58.6±10.5% of all cells were double positive for p75 and Sox10. 15.0±6.2% of all cells were negative for these markers, pointing to a cellular heterogeneity within skin-derived spheres as also observed in sphere cultures from other tissues (Reynolds and Rietze, 2005). The data indicate that skin-derived cells expressing NCSC markers can be propagated in culture for prolonged time periods.

To address whether spheres with p75/Sox10-positive cells can also be generated from adult human skin, surgical samples of human thigh and face skin were dissociated and treated as described above for mouse skin. Spheres readily formed (Fig. 1B) and could be expanded by passaging, such that after three months more than $10^9$ cells had been generated from a 16 cm$^2$ skin sample used as starting material. Similar to mouse cultures, all spheres contained p75/Sox10-positive cells, which accounted for more than 60% of all cells (Fig. 1D, F). However, other markers for premigratory or migratory NCSCs, such as Sox9 and HNK-1, were not expressed.
Figure 1. Skin-derived spheres contain cells that express the NCSC markers p75 and Sox10
Spheres were generated from murine adult trunk skin (A) and human adult thigh skin (B). Spheres passaged for more than 22 times were allowed to spread on FN-coated plates (C-F) and fixed after 8 h. Immunocytochemical analysis revealed that both murine and human skin-derived spheres contain numerous cells expressing p75 (visualized by Alexa488 fluorescence, green) and Sox10 (revealed by Cy3 fluorescence, red) (E, F). Arrow: double positive cell, arrowhead: negative cell. (C, D) corresponding DAPI staining. Scale bar 20 μm.

As p75 and Sox10 are markers for NCSCs (Paratore et al., 2001; Stemple and Anderson, 1992), we next examined whether the mouse trunk skin-derived spheres originate from the neural crest. To this end, we mapped the fate of neural crest cells in vivo by mating ROSA26 Cre reporter (R26R) mice, which express β-Galactosidase upon Cre-mediated recombination, with mice expressing Cre recombinase under the control of the Wnt1 promoter (Jiang et al., 2000; Lee et al., 2004). In Wnt1-Cre/R26R double transgenic mice, virtually all NCSCs express β-Galactosidase (Brault et al., 2001; Lee et al., 2004). Importantly, despite the transient expression of Cre recombinase, the progeny of neural crest cells continue to express β-Galactosidase because of the genomic recombination event, therefore allowing monitoring the fate of the neural crest cells in vivo. This method has successfully been used before to demonstrate the neural crest origin of SKPs from the dermal papilla of whiskers, of multipotent cells present in explant cultures of the upper part of whisker follicles, and of neuronal cells and smooth muscle generated in back skin.
4.3 Results

Explant cultures (Fernandes et al., 2004; Sieber-Blum and Grim, 2004; Sieber-Blum et al., 2004). Anti-ß-Galactosidase antibody staining revealed that all primary and late passage spheres generated from the back skin of adult Wnt1-Cre/R26R double transgenic mice were comprised of neural crest-derived cells (Fig. 2; and data not shown). In particular, 100% of all p75-positive cells co-expressed ß-Galactosidase, as revealed by a typical punctuated staining pattern (Lutolf et al., 2002). Since 87.3 ± 6.0% of all p75-positive cells also expressed Sox10 (3 independent experiments with spheres obtained after 20-35 passages), the data demonstrate that sphere-forming, p75/Sox10-expressing cells from the adult mouse skin are indeed neural crest derivatives.

Figure 2. Skin-derived spheres are of neural crest origin
Neural crest-derived cells were identified by lineage-tracing in spheres generated from Wnt1-Cre/R26R adult mouse skin samples. Primary spheres (not shown) and spheres at passage 20 were analyzed immunocytochemically for p75 (green) (A) and ß-Galactosidase (red) (B) expression. Note the predominantly perinuclear expression of ß-Galactosidase (Lutolf et al., 2002). Overlay of (A) and (B) reveals that spheres contain cells co-expressing p75 and ß-Galactosidase (D). (C), corresponding DAPI staining. Scale bar 20μm.

Migratory and postmigratory p75/Sox10-positive NCSCs are able to give rise to various neural and non-neural cell lineages (Bixby et al., 2002; Hagedorn et al., 1999; Stemple and Anderson, 1992). To test the developmental potential of cells in spheres derived from murine and human skin, spheres containing p75/Sox10-positive neural crest cells were allowed to settle on culture dishes and to differentiate at high cellular density. The formation of glia expressing glial fibrillary acidic protein (GFAP), βIIIITubulin (TuJ1)-positive neuronal cells, and smooth muscle actin (SMA)-expressing non-neural cells was readily detectable both in mouse and human cell cultures (Fig. 3A-C, G-I), although the
number of neuronal cells generated was highly variable and low in comparison to that of glia and smooth muscle cells. Moreover, upon addition of ascorbic acid and BMP2, the generation of chondrocytes stained by Alcian blue was observed (Fig. 3D, J), while treatment with stem cell factor (SCF) in combination with endothelin (ET)-3 resulted in formation of a few melanocytes, as revealed by DOPA reaction (Fig. 3E, K). Finally, occasional adipocytes were detected (Fig. 3F, L). However, we never observed the generation of keratinocytes as assessed by staining with a pan-keratin antibody (data not shown), demonstrating that neural crest-derived sphere-forming cells are distinct from epithelial stem cells of the skin.

Figure 3. Cells from skin-derived spheres differentiate into neural crest cell lineages and adipocytes
Late passage spheres obtained from murine and human skin were allowed to spread at high densities on different substrates and were incubated in media permissive for cell differentiation. Marker expression indicated the generation of GFAP-positive glia (A, G), TuJ1-positive neuronal cells (B, H), and SMA-positive smooth muscle cells (C, I). Some plates (A, B, G, H) were counterstained with DAPI. Cells with features of chondrocytes, melanocytes, and adipocytes were identified by Alcian blue staining (D, J), DOPA reaction (E, K), or with Oil Red O (F, L), respectively. Scale bar 20μm.

The above data are consistent with the idea that skin-derived spheres contain multipotent cells capable of generating neuronal cells, glia, smooth muscle, and other non-neural cell types. In analogy to NCSCs isolated from other stages and locations, it is likely that this broad potential is inherent to the p75/Sox10-expressing neural crest-derived cells found in the spheres. To address this hypothesis, we plated neural crest cells from mouse trunk skin-
derived spheres at clonal density, and prospectively identified and mapped single undifferentiated, unpigmented p75-positive clone founder cells, as described before for migratory and postmigratory multipotent neural crest cells (Hagedorn et al., 1999; Kleber et al., 2005; Lee et al., 2004; Stemple and Anderson, 1992). The clone founder cells were then incubated in culture conditions permissive for neurogenesis, gliogenesis, and non-neural cell formation (Stemple and Anderson, 1992). As illustrated in Fig. 4A, 57.9% of all p75-positive founder cells were at least tripotent, giving rise to clones consisting of neural and non-neural cell types. Virtually no p75-positive cell was restricted to a single cell lineage. Thus, p75/Sox10-positive neural crest-derived cells prepared from the adult trunk skin are multipotent and can be expanded in culture. Upon isolation, these cells therefore exhibit properties of NCSCs.

Several instructive growth factors—including Wnt, bone morphogenic protein (BMP), neuregulin (NRG), and transforming growth factor (TGF)β—have been shown to promote specific fate decisions in NCSCs at the expense of other possible fates. While Wnt responsiveness is lost at later developmental stages (Kleber et al., 2005), postmigratory NCSCs isolated from various structures maintain their responsiveness to BMP2, NRG1, and TGFβ, although the biological activity of these factors changes with time and location (Bixby et al., 2002; Kruger et al., 2002). Similarly, prospectively identified p75-positive neural crest cells isolated from the adult back skin and plated at clonal density were sensitive to BMP2, NRG1, and TGFβ, respectively (Fig. 4B-D). In particular, all three instructive growth factors suppressed multipotency without affecting survival of founder cells. However, the effect of these factors on adult skin-derived neural crest cells was different from their reported activities on other types of NCSCs: all three factors promoted the generation of clones containing non-neural cells that were mostly SMA positive, and we were unable to identify growth factors inducing exclusively neuro- or gliogenesis in skin-derived, multipotent neural crest cells. In contrast, NCSCs isolated from other sources but cultured in the same conditions give rise to neurons and glia, respectively, in response to BMP2 and NRG1 (Le Douarin and Dupin, 2003; Sommer, 2001). Hence, adult skin-derived neural crest cells, although displaying NCSC features, are intrinsically different from other types of NCSCs and show altered factor responsiveness.
Figure 4. Clonal analysis of skin-derived multipotent neural crest-derived cells
Spheres derived from mouse skin were dissociated after passage 17 and single cells were plated at clonal
density in standard medium (SM) with or without the addition of instructive growth factors. Cells were
prospectively identified with p75 staining and mapped on the plate. A clone was counted positive for a given
cell type when it contained at least one cell expressing the appropriate marker (TuJ1, neuronal cells; NG2,
glia; SMA, smooth muscle). N (neuronal cells), G (glia), S (smooth muscle), O (other), death (lost clones).
(A) Culture in SM without addition of instructive growth factors was permissive for the generation of
heterogeneous clones. Multipotent cells generated 3 or 4 different fates: G/N/S/O, G/S/O, or N/S/O. Bipotent
cells generated mostly S/O clones. Clones containing exclusively S, N, or O were not identified. (B) Cultures
containing BMP2 generated S-only clones, S/O, and N/S/O clones. Clone types containing glial cells were
significantly decreased or not generated at all. (C) Cultures containing NRG1 also generated S/O and S-only
clones. Multipotent cells generated mostly G/S/O clones. Clone types containing neuronal cells were
decreased or not generated. (D) TGFβ addition increased the number of S/O clones and of S-only clones.
Each bar represents the mean ± SD of three independent experiments, counting at least 50 clones per
experiment.
Multiple sources of sphere-forming neural crest cells in the whisker follicle

Apart from back skin-derived p75/Sox10-positive multipotent cells (Figs. 1, 2), the neural crest origin of sphere-forming cells in the adult skin has been demonstrated for SKPs from the dermal papilla of whisker follicles, which however are negative for the NCSC markers p75 and Sox10 (Fernandes et al., 2004). This could either reflect differential regulation of NCSC markers in the same cell type due to varying culture conditions, or indicate sphere-forming capacity of skin cells from different neural crest derivatives. To address this issue, we first performed a detailed mapping of neural crest derivatives in the adult skin and investigated which of these neural crest derivatives express the NCSC marker Sox10 in vivo. We initially focused on the whisker follicle because this structure has been identified before as a source of multipotent neural crest-derived cells (Fernandes et al., 2004; Sieber-Blum et al., 2004). In the head, the neural crest contributes to craniofacial bones and cartilage, teeth, eyes, blood vessels, and other mesenchymal structures (Santagati and Rijli, 2003). This contribution can be monitored in Wnt1-Cre/R26R double transgenic mice, in which virtually all neural crest cells as well as their progeny express β-Galactosidase (Jiang et al., 2000; Lee et al., 2004; Wurdak et al., 2005). Many mesenchymal structures in whisker follicles isolated from Wnt1-Cre/R26R double transgenic mice expressed β-Galactosidase (Fig. 5A). In particular, the capsula, the ringwulst, the dermal sheath, and – as previously published (Fernandes et al., 2004; Sieber-Blum et al., 2004) – the dermal papilla turned out to be neural crest derived. The neural crest origin of all these structures was confirmed by fate mapping experiments performed in Ht-PA-Cre/R26R mice, in which Cre recombinase is expressed in neural crest cells independently from Wnt1 promoter activity (Pietri et al., 2003) (Fig. 5B). Of all neural crest-derived structures within the whisker follicle, only the dermal sheath appeared to contain Sox10-expressing cells (Fig. 5C), as revealed by X-Gal staining of whisker follicles isolated from Sox10^osoz mice that express β-Galactosidase from the Sox10 locus (Britsch et al., 2001). Capsula, ringwulst, and dermal papilla did not express Sox10 in vivo, while glial cells in nerve endings and melanocytes were Sox10-positive (Fig. 5C), in agreement with earlier reports (Kuhlbrodt et al., 1998; Potterf et al., 2001). Thus, the whisker follicle comprises various Sox10-positive and Sox10-negative tissues of neural crest origin.
4.3 Results

Figure 5. Many mesenchymal structures of whisker hair follicles derive from the neural crest and harbor cells capable of generating spheres.

(A) Whisker follicles from Wnt1-Cre/R26R mice were dissected out and incubated in X-Gal solution to reveal neural crest-derived cells in the dermal sheath (DS), capsula (C), dermal papilla (DP), ringwulst (RW), nerve (N), and regions above the sebaceous gland (SG). The SG itself was negative. Melanocytes (M) were not or only very weakly X-Gal positive, as reported (Fernandes et al., 2004). (B) Whiskers dissected from Ht-PA-Cre/R26R mice and treated with X-Gal solution. The same structures as in the Wnt1-Cre/R26R follicles were shown to be derived from the neural crest. (C) Whiskers dissected from Sox10lacZ mice and incubated in X-Gal solution. Cells in which the Sox10 promoter was active were identified in DS, N, and M. C, DP, RW and SG were negative. (D) Different neural crest-derived structures were microdissected from Wnt1-Cre/R26R whisker follicles (A) and cultured in medium permissive for sphere formation. All structures have the potential to form X-Gal positive spheres. The RW was isolated from rat whisker follicles and therefore not stained with X-Gal (n.d. = not done). Six independent experiments were performed for each structure. Scale bar 50 μm.

In order to investigate which of these neural crest derivatives contain cells with sphere-forming potential, dermal papilla, capsula, the upper part of the dermal sheath (without the bulge), and the lower part of the dermal sheath were isolated from whiskers of adult Wnt1-Cre/R26R double transgenic mice by microdissection, dissociated, and cultured in the same conditions as used before for trunk skin-derived multipotent neural crest cells. In addition,
rat whiskers were used to dissect the ringwulst, which in mice was too small to be isolated without contamination from other tissues. Strikingly, all these whisker follicle structures harbor cells with the capacity to generate spheres (Fig. 5D). X-Gal staining of Wnt1-Cre/R26R mouse cell cultures confirmed that the spheres were neural crest-derived. Therefore, neural crest cells with sphere-forming potential are not confined to a particular niche in the whisker follicle. Likewise, multipotency is not restricted to a neural crest cell type from a particular location, given that multipotent neural crest cells have been isolated from both the dermal papilla and the upper part of the whisker follicle (Fernandes et al., 2004; Sieber-Blum et al., 2004).

**Glial cells in nerve endings as well as the melanocyte lineage are associated with sphere-forming p75/Sox10-positive neural crest cells in the adult back skin**

Unlike in the head, the mesenchyme in the trunk is not derived from the neural crest (Santagati and Rijli, 2003). Accordingly, β-Galactosidase expression in back skin of Wnt1-Cre/R26R mice was not as broad as in whisker follicles, and was restricted to a few locations (Fig. 6A, G). The same structures were also labelled in the back skin of Ht-PA-Cre/R26R mice (Fig. 6B, H). In particular, both in the anagen and telogen stage, X-Gal staining was found in the permanent part of the pelage follicle including the bulge region below the sebaceous gland (Fig. 6A, B, K). This area comprises the location of melanocyte stem cells (Nishimura et al., 2002) and glial cells in nerve endings (Botchkarev et al., 1997). In addition, pigmented melanocytes in the bulb region (the lower part of the hair follicles) (Fig. 6G, H) and nerves expressed β-Galactosidase. In contrast, other hair follicle structures such as the dermal papilla, dermal sheath, and the outer and inner root sheaths were X-Gal negative and in the trunk skin do not originate from the neural crest (Fig. 6A, B, G, H).

To determine the potential origin of Sox10-positive sphere-forming neural crest cells isolated from the adult trunk skin (Fig. 1), we assessed Sox10 expression by virtue of β-Galactosidase activity in the back skin of Sox10lacZ mice *in vivo*. Interestingly, Sox10-expressing cells were confined to exactly the same areas as were X-Gal-positive cells in Wnt1-Cre/R26R and Ht-PA-Cre/R26R mice, including nerves, melanocytes, and a domain consistently found below the sebaceous gland in anagen and telogen stage that encompasses the hair follicle bulge with the niche for melanocyte stem cells and nerve
endings (Fig. 6I, J, L). Importantly, both in \textit{Wnt1-Cre} /\textit{R26R} and \textit{Ht-PA-Cre} /\textit{R26R} mice, X-Gal-positive cells in the region below the sebaceous gland co-expressed Sox10 and p75 protein (Fig. 6C, D, E, F). Thus, p75/Sox10-positive multipotent neural crest-derived cells from the trunk skin (Figs. 1-4) are connected to the glial or the melanocyte lineage, or to both of these lineages.

\textbf{Figure 6. Localization of neural crest-derived and Sox10-positive cells in murine back skin}

Back skin from \textit{Wnt1-Cre} /\textit{R26R} (A, C, E, G), \textit{Ht-PA-Cre} /\textit{R26R} (B, D, F, H) and \textit{Sox10^{lacZ}} (I, J) mice with pelage follicles in anagen phase was stained with X-Gal solution and embedded in resin. In all three transgenic mouse models, positive cells identified on sections were localized inside hair follicles (in the bulge region below the SG (arrows) and in the bulb (G, H, J)) and in glial cells outside hair follicles. (A, B, I) overview. Enlarged areas show X-Gal-positive cells in the bulge area co-expressing Sox10 (C, D) and p75 (E, F) visualized by immunofluorescence. The panels to the right represent overlays of X-Gal and marker stainings. Melanocytes (M) in the bulb stained with X-Gal. The dermal papilla (DP) was X-Gal negative in all three mouse lines. Back skin from \textit{Wnt1-Cre} /\textit{R26R} (K) and \textit{Sox10^{lacZ}} (L) mice was collected in telogen phase, stained with X-Gal solution and embedded in resin. Positive cells identified on sections are clearly visible below the SG in both transgenic mouse models. Scale bars in (A, G) 20\textmu m, scale bar in (C) 10\textmu m.
To elucidate whether p75/Sox10 expression and the capacity to form spheres are associated with glial cells from skin, we made use of Dhh-Cre mice that express Cre recombinase in the peripheral glial lineage from early stages onwards, but not in migrating neural crest cells or in neural crest-derived cells of other than glial lineages (Jaegle et al., 2003). β-
Galactosidase activity was detectable in nerves and nerve endings in the back skin of adult \textit{Dhh-Cre/R26R} mice (Fig. 7A). As predicted from the proposed location of glial cells associated with nerve endings in the hair follicle (Botchkarev et al., 1997), X-Gal staining in pelage follicles of \textit{Dhh-Cre/R26R} mice was confined to a region around the bulge (Fig. 7A), corresponding to the area that also contains β-Galactosidase-expressing cells in \textit{Wnt1-Cre/R26R}, \textit{Ht-PA-Cre/R26R}, and \textit{Sox10\textsuperscript{loxP/loxP}} mice (Fig. 6A, B, I). In \textit{Dhh-Cre/R26R} mice, X-Gal-labelled cells of the bulge region were also labelled with anti-Sox10 antibody (Fig. 7C) and anti-p75 antibody (Fig. 7E) Pigmented melanocytes in the hair follicle bulb were X-Gal negative, however, indicating that cells labelled in \textit{Dhh-Cre/R26R} mice do not give rise to melanocytes and thus are not related to the melanocyte lineage (Fig. 7G).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure_7.png}
\caption{Lineage-tracing of cells from the glial or the melanocyte lineage in murine back skin}
Back skin from \textit{Dhh-Cre/R26R} (A, G) and \textit{Dct-Cre/R26R} (B, H) mice was collected, stained with X-Gal solution, and embedded in paraffin to identify progenitors and differentiated cells from the glial and melanocyte lineage, respectively, by lineage-tracing. (A) X-Gal expressing cells on sections from \textit{Dhh-Cre/R26R} mice were localized inside hair follicles in the bulge region below the sebaceous gland (arrow) and in glial cells outside hair follicles. (G) Melanocytes (M) in the hair follicle bulb were not stained. (B) X-Gal-expressing cells on \textit{Dct-Cre/R26R} sections were localized inside hair follicles in the bulge region (arrow). (H) Melanocytes also stained for X-Gal in \textit{Dct-Cre/R26R} skin. Enlarged areas show X-Gal positive cells in the bulge area co-stained for Sox10 (C, D) and p75 (E, F). Scale bar in (A, G) 20μm, scale bar in (C) 10μm.
\end{figure}
To directly demonstrate that cells from the glial lineage tracked by *Dhh-Cre* promoter activity possess sphere-forming potential, these cells have to be prospectively identified and freshly isolated. One possibility to achieve this would be by using specific surface antigen markers. However, such markers for the early glial lineage are currently unavailable. In particular, p75 is not suitable for this purpose, because it is expressed in several distinct structures of the skin, many of which are not neural crest derived (Botchkareva et al., 1999; Rendl et al., 2005). Furthermore, nerve endings present in the skin cannot be isolated by microdissection. Therefore, we used a genetic strategy to prospectively identify and directly isolate cells associated with the glial lineage. To this end, *Dhh-Cre* mice were mated with *R26R-EYFP* mice that express EYFP upon Cre-mediated recombination (Srinivas et al., 2001). Cells expressing EYFP in the trunk skin of *Dhh-Cre/R26R-EYFP* double transgenic mice were isolated by FACS and transferred into medium permissive for sphere formation (Fig. 8A, B). While from unselected skin samples more than $10^6$ cells were used to generate about 50 spheres (Fig. 1; Materials and methods), less than 10'000 cells from both the EYFP-positive and the EYFP-negative cell fraction were seeded in these experiments, in order to assess a possible enrichment in the spherogenic potential of FACS-selected cells. In two independent experiments, the EYFP-positive cell population (Fig. 8A, green frame) gave rise to 15 and 12 spheres out of 8000 and 6000 cells, respectively (Fig. 8D). In contrast, the same number of EYFP-negative cells (Fig. 8A, blue frame) did not form any spheres. Moreover, acutely fixed primary spheres of EYFP-positive cells were composed of cells expressing both p75 and Sox10 (Fig. 8C). Thus, p75/Sox10-positive cells related to the glial lineage can be isolated from the skin and form spheres.
We next analyzed whether sphere-forming potential is a common feature of peripheral glia or whether this potential is confined to cells of the glial lineage in the skin. Therefore, we investigated whether sphere cultures can also be established from adult peripheral nerves. Sciatic and trigeminal nerves from adult mice were dissociated and the cells were cultured exactly as described above for skin-derived cells. However, in agreement with others (Toma et al., 2001), we were unable to obtain spheres from peripheral nerve cultures (data...
not shown). Thus, nerve endings in skin comprising specialized glial cells, but not peripheral nerves in general, contain cells with sphere-forming potential.

However, in cell preparations from the trunk skin of Dhh-Cre/R26R mice, only a fraction of all p75/Sox10-positive cells also expressed β-Galactosidase (data not shown). This could point to inefficient Cre-mediated recombination in Dhh-Cre/R26R mice. Alternatively, sources in the skin other than the glial lineage might yield sphere-forming neural crest-related cells. To address whether spherogenic neural crest-derived cells might be connected to the melanocyte lineage, we traced the fate of trunk skin cells in Dct-Cre/R26R mice (Guyonneau et al., 2002). Dct codes for the enzyme dopachrome tautomerase (also called Trp-2), which is required for melanin synthesis and already expressed in melanocyte stem cells (Nishimura et al., 2002). As reported (Guyonneau et al., 2002), β-Galactosidase activity in the back skin of Dct-Cre/R26R mice was detectable in melanocytes (Fig. 7H) and in the hair follicle bulge region corresponding to the location of melanocyte stem cells (Fig. 7B) (Nishimura et al., 2002). Moreover, the X-Gal-positive cells in the bulge region also expressed Sox10 (Fig. 7D) and p75 (Fig. 7F).

To investigate whether—in addition to cells of the glial lineage—the early melanocyte lineage also comprises undifferentiated neural crest-derived cells with the capacity to generate spheres, we isolated EYFP-expressing cells prospectively identified in the skin of Dct-Cre/R26R-EYFP mice. Intriguingly, in two independent experiments, FACS isolation and culturing of less than 10^6 cells revealed that only EYFP-expressing (Fig. 8E, green frame), but not EYFP-negative cells (Fig. 8E, blue frame), were able to form spheres (Fig. 8H). Analysis of acutely fixed primary spheres revealed many cells co-expressing p75 and Sox10, while pigmented differentiated melanocytes were absent (Fig. 8G). These data indicate that the early melanocyte lineage comprises p75/Sox10-positive cells that can be propagated as spheres. Thus, as in whisker follicles of the face, the trunk skin contains more than one source of sphere-forming neural crest-derived cells, namely glial cells in nerve endings and cells of the melanocyte lineage.
4.4 Discussion

In the present study, we show that cells with NCSC features can be isolated from the adult trunk skin of both mouse and human. Like NCSCs from other embryonic and postnatal sources, these neural crest-derived cells in the skin express p75 and Sox10, and are multipotent, able to generate several neural and non-neural lineages. Moreover, multipotent neural crest cells from the adult skin display self-renewing capacity, in that mouse and human skin-derived cells can be grown and expanded for months in floating sphere cultures. However, both with respect to marker expression and to tissue origin, the multipotent, spherogenic cells described here are distinct from other sphere-forming neural crest-derived skin cells such as SKPs found in the dermal papilla of whisker follicles (Fernandes et al., 2004). In fact, several structures of neural crest origin in the whisker follicle have the capacity to generate spheres. In the trunk skin, genetic cell fate mapping, Sox10 expression analysis in vivo and, importantly, prospective identification and direct isolation demonstrate that cells displaying NCSC properties do not reside in mesenchymal structures of hair follicles such as the dermal papilla and dermal sheath, but rather are associated with the melanocyte lineage and nerve endings. Thus, multipotent sphere-forming neural crest-derived cells in the skin are not confined to a particular niche but can be attributed to distinct locations in face and trunk skin.

Sphere-forming multipotent neural crest cells reside in distinct structures of the adult skin

Several reports have described the isolation of multipotent cells from murine and human skin (Amoh et al., 2005; Belicchi et al., 2004; Dyce et al., 2004; Joannides et al., 2004; Shih et al., 2005; Sieber-Blum et al., 2004; Toma et al., 2005), raising the question about the origin of the endogenous cells able to self-renew and to generate multiple cell lineages, including cell types normally not found in the skin. Multipotent cells expressing GFP under the control of a Nestin regulatory element have been isolated from the hair follicle bulge area of transgenic mice and reported to undergo neurogenesis in vivo upon transplantation into the murine subcutis (Amoh et al., 2005). In these mice, Nestin-GFP-expressing cells are associated with the outer root sheath (Li et al., 2003), which however does not originate from the neural crest (Figs. 5 and 6). The nature of Nestin-expressing
cells in the skin remains to be determined, though, as Nestin-GFP in another transgenic mouse line (Kawaguchi et al., 2001) marks the inner but not outer root sheath (Wong C., Okano H., and Barrandon Y., unpublished). In human skin Nestin may be widely expressed in the epidermis, the bulge region, the inner root sheath, in some cells of the basal layer, and more weakly in the sebaceous gland (Wang et al., 2006).

As mentioned before, the dermal papilla from whisker follicles has been shown to be of neural crest origin and to harbor multipotent sphere-forming SKPs that are p75/Sox10-negative (Fernandes et al., 2004). However, many structures of the whisker follicle turned out to be neural crest derived, including the dermal sheath, the ringwulst, and the capsula, apart from the dermal papilla (Fig. 5). Intriguingly, upon microdissection, all these tissues were shown to contain cells with sphere-forming potential. In agreement with these findings, multipotent neural crest-derived cells were isolated from explant cultures of the upper part of whisker follicles comprising dermal sheath and the bulge region (Sieber-Blum et al., 2004). Thus, multipotency and the capacity to self-renew appear to be a widespread feature of neural crest-derived cells in the adult skin of the face.

It has been proposed that similar to SKPs from face skin, p75-negative cells isolated from the mouse back skin or the human foreskin are also neural crest-derived, although this has not been addressed yet (Fernandes et al., 2004; Toma et al., 2005). Most likely, however, these latter sphere-forming cells are not associated with the dermal papilla of hair follicles, because the human foreskin is devoid of hair follicles. Moreover, using in vivo cell fate mapping we demonstrate that, unlike in whisker follicles of the face, only few structures of pelage follicles in the trunk skin are actually neural crest derived. Dermal papilla, dermal sheath, and other supportive structures do not appear to be neural crest derivatives (Figs. 5 and 6). This is consistent with studies in chicken and mouse, which showed that in the face, neural crest cells form multiple non-neural tissues, while in the trunk mesenchymal tissues are not neural crest-derived (Santagati and Rijli, 2003). These differences in facial vs. trunk neural crest contribution might also be relevant for the study of hair follicle development, given that whisker follicles are a widely used model system to investigate mechanisms regulating follicular cell fates (Alonso and Fuchs, 2003; Gambardella and Barrandon, 2003).

Given that hair follicles in the trunk skin receive less neural crest contribution than whisker follicles, endogenous multipotent neural crest-derived cells in the trunk skin must be
confined to fewer tissues. Skin structures harboring such cells are presumably marked by Sox10 expression *in vivo*, given that cells displaying NCSC properties after isolation from the adult trunk skin express Sox10. Indeed, in *Wnt1-Cre/R26R, Ht-PA-Cre/R26R, Dhh-Cre/R26R*, and *Dct-Cre/R26R* mice, β-Galactosidase-expressing cells positive for p75 and Sox10 were found in the bulge region encompassing melanocyte stem cells and glial cells in nerve endings (Botchkarev et al., 1997; Nishimura et al., 2002) (Figs. 6, 7). However, the only way to unambiguously demonstrate that these lineages contain resident multipotent cells with the potential to self-renew is by prospectively identifying such cells in the adult skin and testing their potential upon acute isolation. Due to the lack of specific surface markers, we were not able to use antibodies to directly isolate multipotent cells from back skin. In particular, p75 expression does not distinguish between glial and melanocyte lineages (Fig. 7) and, in addition, is found in regions of the skin which are not neural crest-derived, such as the outer root sheath of hair follicles, and –at early stages of hair follicle morphogenesis– the dermal papilla (Botchkareva et al., 1999; Rendl et al., 2005). Similarly, the melanoblast marker c-Kit is not suitable for isolation of prospective multipotent cells from the melanocyte lineage, because it is not expressed in the bulge of anagen hair follicles and because *in vivo* it is also found in epithelial skin cells not originating from the neural crest (Peters et al., 2003; Peters et al., 2002). Moreover, in preliminary experiments we failed to obtain spheres from c-Kit-positive skin cells isolated by FACS (C.W. and L.S., unpublished observation). Nonetheless, we were able to identify spherogenic neural crest-derived cells in the skin by using a genetic approach. Thereby, the lineage-specific activity of *Dhh-Cre* and of *Dct-Cre*, respectively, combined with a Cre reporter allele (Srinivas et al., 2001), led to EYFP expression in cells from either the glial or the melanocyte lineage. Consistent with a dual origin of multipotent neural crest-derived cells in the trunk skin, EYFP-positive cells isolated from both *Dhh-Cre/R26R-EYFP* and *Dct-Cre/R26R-EYFP* mice formed spheres of p75/Sox10-positive cells (Fig. 8). Thus, we conclude that in the trunk skin of mice, spherogenic neural crest-derived cells are associated with the glial as well as the melanocyte lineage. Based on their high similarities to mouse cells in terms of marker expression and potential, we assume that the p75/Sox10-positive multipotent cells obtained from human skin (Figs. 1, 3) are also related to glial and melanocyte rather than mesenchymal lineages.

An alternative explanation of our data would be that there is only one source of
endogenous multipotent neural crest cells in the trunk skin, simultaneously marked in *Dhh-Cre/R26R* as well as in *Dct-Cre/R26R* mice. However, the *in vivo* cell fate mapping experiments (Fig. 7) reveal that cells labelled in *Dhh-Cre/R26R* mice do not give rise to the same cell types as cells labelled in *Dct-Cre/R26R* mice and *vice versa*. That cells of the bulge region marked in *Dct-Cre/R26R* mice generate melanocytes is in agreement with earlier findings (Nishimura et al., 2002), which identified *Dct<sup>lacZ</sup>*-positive cells of the bulge region as so-called melanocyte stem cells generating differentiated melanocytes in the lower part of the hair follicle. Our analysis of neural crest-derived cells isolated from *Dct-Cre/R26R* mice supports the hypothesis that melanocyte progenitors in the bulge region are not only self-renewing (Nishimura et al., 2002) but indeed represent multipotent cells (Sommer, 2005).

The combined data indicate that the adult skin is host to sphere-forming cells with different identities. In addition to multipotent, undifferentiated neural crest-related cells (Fernandes et al., 2004; this study), the potential to self-renew has also been attributed to pigment cells and possibly other developmentally restricted neural crest-derived cell types (Dupin et al., 2000; Trentin et al., 2004). Similar to NCSCs isolated at different time points and from different PNS regions (Kleber and Sommer, 2004), the multipotent neural crest-derived skin cells described in this study display altered responsiveness to instructive growth factors as compared to migratory NCSCs (Fig. 4). Thus, multipotent, self-renewing neural crest-derived cells change intrinsic properties with time and location. This presumably reflects their specific functional requirements, although the physiological role of multipotent neural crest-derived cells in the skin remains to be determined. In particular, it is unclear whether these cells display properties of NCSCs only upon transfer into culture, or whether they are multifated and self-renewing *in vivo* as well. Moreover, it will be interesting to elucidate whether and how these cells functionally interact with other, unrelated stem cell types of the skin, such as epithelial stem cells also found in the bulge region of hair follicles (Blanpain et al., 2004; Claudinot et al., 2005; Oshima et al., 2001; Taylor et al., 2000). Finally, it should be addressed whether spherogenic neural crest-derived cells persisting in the adult skin might represent a target for mutational transformation leading to cancers such as melanoma (Pardal et al., 2003).
Limited developmental and therapeutical potential of multipotent neural crest cells from the adult skin

Adult stem cells as an alternative to embryonic stem cells are a prime target of applied research that seeks to treat degenerative diseases by cell replacement therapies (Wagers and Weissman, 2004). The skin might represent an ideal source for adult stem cells in tissue repair, because it is easily accessible. Because skin-derived multipotent cells described here are of neural crest origin, their capacity to give rise to neural and non-neural cell types at clonal density (Fig. 4) does apparently not reflect transdifferentiation, but rather the broad potential inherent to NCSCs. The fact that these cells can be easily expanded in culture, even when isolated from the skin of adult humans, might make them a valuable source for cell replacement therapies, because sufficient cell material could be obtained for such purposes. However, in our hands spherogenic neural crest-derived cells from the adult mouse skin displayed a rather restricted potential in vivo (data not shown). In particular, skin-derived spheres of neural crest origin, when dissociated and injected into the lateral ventricles of rat and chicken embryos or transplanted onto hippocampal brain slices, remained largely undifferentiated in cell aggregates close to the injection site, and failed to integrate into the host CNS tissue (Paratore C., Gossrau G., Perez-Bouza A., Stoeckli E., Brüstle O., and Sommer L., unpublished). This is in contrast to neural progenitors obtained from embryonic stem cells or neural stem cells from the CNS assessed in the same experimental paradigms (Benninger et al., 2003; Brustle et al., 1997; Wernig et al., 2004). Moreover, we observed neither neural differentiation, tissue integration, nor behavioral improvement upon transplantation of skin-derived multipotent neural crest cells into the striatum of a 6-hydroxydopamine-lesioned mouse model for Parkinson’s disease (Paratore C., Ferger B., and Sommer L., unpublished) (Bjorklund et al., 2002), or upon intravenous injection into mice with experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis previously used to test the therapeutic potential of adult neural stem cells from the CNS (Paratore C., Bargsten P., Becher B., and Sommer L., unpublished) (Pluchino et al., 2003). These trials suggest that multipotent cells of neural crest origin that are present in the adult skin cannot transdifferentiate into neural cell types of the CNS. Likewise, SKPs failed to generate CNS neurons upon transplantation into hippocampal slices (Fernandes et al., 2006). This does not exclude that adult neural crest-related cells with stem cell properties might be of high
value for the generation of Schwann cells, cartilage, or other neural crest-derived tissues potentially useful in clinical applications. Hence, our study underlines the importance of choosing the appropriate stem cell type for a given task.
4.5 Acknowledgements

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5 OUTLOOK

I have isolated multipotent progenitor cells from adult murine and human skin. Co-expression of p75 and Sox10 at late passages suggested that my cells are neural crest-derived progenitors, a hypothesis confirmed by lineage tracing experiments using the Wnt1-Cre/R26R mouse model. Skin sphere-derived cells could differentiate into many neural crest lineages like neurons, glia, smooth muscle cells, melanocytes and chondrocytes in vitro, forming higher numbers of non-neural cells. Fate mapping and immunohistochemistry on skin sections showed that a distinct cell population localized below the sebaceous gland of hair follicles stains for X-Gal, p75 and Sox10, and likely possesses the sphere-forming potential. This region, called the bulge, contains different kinds of stem cells including the neural crest-derived melanocyte stem cells. In addition, it receives innervation of myelinated axons, therefore also containing neural crest-derived glial cells. Cells isolated from both the melanocyte and glial lineages by FACS using Dhh-Cre/R26R-EYFP (glial lineage) and Dct-Cre/R26R-EYFP (melanocyte lineage) transgenic mice had the ability to form spheres containing p75/Sox10 positive cells, indicating that there are several probably multipotent neural crest progenitors in skin. In addition, vibrissal follicles contained many different structures derived from the neural crest with sphere-forming ability.

5.1 Potential use for cell transplantation therapies

As mentioned before, adult stem cells have many advantages over embryonic stem cells for use in transplantations, as long as they can be induced to produce the desired cell type. Adult stem cells taken from an accessible tissue of the patient would be a big asset as not only ethical issues could be avoided, but also lifelong immunosuppression. The interest of most researchers lies in the neuronal potential of isolated adult stem cells, because of the attractive prospect of healing or at least improving the life quality of patients affected by neurodegenerative diseases such as Parkinson’s disease.

We also tested the potential of our skin-derived cells to integrate into the striatum of a 6-hydroxydopamine-lesioned mouse model for Parkinson’s disease. Cells survived in the brain of the mice for long periods of time, but did not show neuronal differentiation, or
tissue integration and the mice showed no behavioral improvement (Paratore C., Ferger B., and Sommer L., unpublished). After injection of our cells into the tail vein of mice with EAE, cells were found in the brain near blood vessels, but showed no glial phenotype (Paratore C., Bargsten P., Becher B., and Sommer L., unpublished). These results suggest that the neuronal and glial potential of our cells is too limited for use in transplantations at least into the CNS. This is not altogether surprising given the fact that our cells are derived from the neural crest, which contributes to the PNS, but not the CNS.

Testing the neural potential of our cells in a disease model known to be induced by neural crest deficiencies, such as Hirschsprung disease might yield more promising results. This disease is characterized by the absence of enteric ganglia from varying lengths of the terminal colon so that the peristaltic movements of the distal gut are impaired and the gut dilated. Often, Hirschsprung disease is accompanied by melanocyte deficiencies leading to white patches of skin and hair (Shah-Waardenburg syndrome). Mouse models for Hirschsprung disease include the Dom mouse (Lane and Liu, 1984) and mice carrying a targeted Sox10 null allele (Britsch et al., 2001; Paratore et al., 2002). Skin-derived neural crest progenitor cells would have to be tested \textit{in vitro} for responsiveness to GDNF, and then transplanted into aganglionic gut organ cultures or mice with Hirschsprung disease.

Neural crest-derived precursor cells from skin could also be used in models of disease where non-neural cells are needed. Pigmentary disorders like vitiligo, where melanocytes are destroyed via an autoimmune attack, are sometimes treated with autologous, noncultured melanocyte-keratinoocyte cell transplantations with good success rates (Rusfianti and Wirohadidjodjo, 2006). This would require that our cells home to the melanocyte niche and produce pigment. As there is no mouse model for vitiligo, it would be very difficult to address this issue. Treatment of diseases caused by problems in melanocyte development, such as piebaldism (Guerra et al., 2004) are also a possible target for NC-derived precursors from the skin. Nevertheless, it would have to be evaluated if the use of these cells would have any advantage in comparison to the techniques already used.

Another possibility would be transplantations for replacement of chondrocytes, although preliminary experiments to differentiate our cells into compact chondrocyte pellets were difficult to evaluate due to problems with the control cells.
5.2 Comparison to other stem cells isolated from skin

There are many reports of multipotent stem cells isolated from skin, but differences in isolation protocols, culture conditions, in vitro differentiation assays, marker expression and models used for testing the in vivo potential makes it very difficult to compare these cells and sort out whether they all represent different or similar kinds of stem or progenitor cells.

Only two reports confirmed the isolation of NCSCs from skin. One population was found in the upper part of the whisker follicle. These cells were termed EPI-NCSCs, because it is suspected that they are localized in the epidermal part of the follicles, the stem cell-containing bulge region (Sieber-Blum et al., 2004). However, in vibrissal follicles, there is a neural crest-derived mesenchymal sheath covering the ORS and the bulge that was not removed before the experiment. Therefore it is not certain that the cells migrating onto the culture dishes are really NCCs coming from the epidermal part and not from the mesenchymal sheath. Given the fact that these cells are from vibrissal follicles makes them uninteresting for transplantations, as humans don’t have whisker follicles. We did not find evidence for neural crest-derived cells in the epidermis of trunk skin, and also could not show that any mesenchymal part of trunk follicles was derived from the neural crest, making our cells distinctly different from EPI-NCSCs.

The group of Freda Miller also isolated neural crest-derived cells from the dermis of mostly embryonic and neonatal mice, and from adult humans, with very similar isolation and culture conditions (Fernandes et al., 2004; Toma et al., 2001). However, their cells never expressed p75 or Sox10 in an undifferentiated state, an important characteristic of our cells. In addition, many SKPs if not most of them, expressed nestin, whereas only 10% of our cells expressed this marker, even at late passages. SKPs also seem to have a broader neuronal potential than our cells. In our differentiation assays, neurons were present, but always in very limited numbers. When transplanted onto hippocampal brain slices or into the lateral ventricles of rat embryos, they remained largely undifferentiated in cell aggregates close to the injection site and failed to integrate into the host CNS tissue (Paratore C., Gossrau G., Perez-Bouza A., Brüstle O., and Sommer L., unpublished). In contrast, SKPs seem to integrate into hippocampal brain slices when transplanted after a pre-differentiation period, although patch-clamp recordings indicated that SKP-derived
neurons from primary spheres were not fully functional (Fernandes et al., 2006).

In the case of glial differentiation, our cells did not help in the recovery of mice with EAE when injected into the tail vein, suggesting that glial differentiation potential in vivo (at least in the CNS) is also limited. All these differences strongly suggest that our cells are distinctly different from SKPs, but different media could also play a role. The DP of whisker follicles is one niche that contains SKPs. Accordingly, the DP of back skin hair follicles was also suggested to represent a niche for SKPs, but our data clearly show that the DP in trunk skin is not NC-derived. Thus, it cannot be a niche for our neural crest-derived cells isolated from the back skin.

Most other studies describing the isolation of multipotent stem cells from skin have not addressed the localization or embryonic origin of the cells. Some papers clearly state that their undifferentiated cells do not express p75 (Belicchi et al., 2004; Joannides et al., 2004) or did not test it (Amoh et al., 2005; Dyce et al., 2004; Shih et al., 2005). Furthermore, Sox10 was never tested in the studies (Amoh et al., 2005; Belicchi et al., 2004; Dyce et al., 2004; Joannides et al., 2004; Shih et al., 2005). Most of the cell populations seem to be positive for nestin (Amoh et al., 2005; Belicchi et al., 2004; Joannides et al., 2004) also setting them apart from our cells.

Cells isolated from transgenic mice expressing EGFP under the control of the nestin promoter seem to have an in vitro potential similar to that of my cells, with many lineages being clearly NC-derived such as melanocytes and smooth muscle (Amoh et al., 2005). That all these cell types could arise in the same differentiation medium sequentially at different time points is quite amazing but in my cultures I never observed epidermal (keratinocytes) and NC-derived cell types arising from the same progenitor. In my opinion, such a conversion is quite unlikely. As nestin-EGFP cells were isolated under the microscope and not using FACS, a contamination with epidermal bulge cells cannot be ruled out, as they are supposed to be localized in the same region. Results from clonal analysis were not clear in this study. In addition, in our hands, nestin expression in transgenic mice was found in the IRS, a layer of the hair follicle that consists of differentiated cells (Wong C., Okano H., and Barrandon Y., unpublished). Therefore, I conclude, that my cells seem to be different from the cells isolated by other groups, and that a lot of work still needs to be done in this field to carefully test all these cell types and evaluate their real use for transplantation therapies.
5.3 Potential involvement in cancer

As some of our sphere-forming cells are probably early dct-positive precursors of the melanocyte lineage or maybe even melanocyte stem cells, an interesting possibility would be their involvement in the formation of melanoma. Melanoma is the most common skin cancer. It is thought to be caused by a malignant transformation of melanocytes with disruptions in proliferation, differentiation and apoptosis. Melanoma is a very metastatic disease and resistant to most cancer therapies including chemotherapy (Chudnovsky et al., 2005). The fact that melanoma is very difficult to treat with chemotherapy and that it metastasizes very frequently, suggests that stem cells could be involved in the formation of the tumor. One publication states that 20% of metastatic melanoma tumors tested contained a subpopulation of cells that propagated as spheres. Cells from these spheres could differentiate into melanocytic, adipocytic, osteocytic and chondrocytic lineages. When injected into mice, the animals developed tumors. Multipotent cells were enriched in a CD20 positive fraction from melanoma-derived spheres in one case (Fang et al., 2005). In preliminary experiments we tested if melanoma cell lines contained cells that were positive for the neural crest stem cell markers p75 and Sox10 and found small populations that co-expressed these proteins.

5.4 Open questions

One of the most important questions that has not been addressed in this study is the role of our cells in vivo in skin. A possible role involves replenishment of a particular cell type either for regeneration in connection with the hair cycle or with disease. In the case of the melanocyte lineage, it is not known if our cells are melanocyte stem cells that are activated during anagen and have a role in the pigmentation of the hair shaft during the hair cycle (Nishimura et al., 2002), or if they are different progenitor cells that also express dct. About 60% of the melanocyte stem cells from the bulge were shown to downregulate Sox10 (Nishimura et al., 2002), but their differentiation and spherogenic potential was not tested yet. Some of the dct positive cells identified on sections of our Dct-Cre/R26R mice clearly still expressed Sox10 and p75.

In the case of the glial cells with the spherogenic potential, it would be conceivable that new glial cells are needed during the hair cycle, because it seems that hair follicle
innervation is more plastic than previously thought. Immunohistochemistry showed that PGP9.5 positive circular fibers of the FNB are increased at early anagen. These nerve fibers are associated with Schwann cells, but probably not myelinated. However, a clear role for multipotent neural crest-derived cells is not known.

It would be particularly interesting to address the interaction of our cells with other cell types possibly localized in their vicinity. Given that some of our cells belong to the melanocyte lineage, their culture with keratinocytes could give interesting information concerning survival, melanization or dendricity. These processes have been shown to be controlled by keratinocytes (Tenchini et al., 1995).

Another interesting experiment would be to assess label retention with BrdU, to see if the cells that express EYFP or β-Galactosidase in our transgenic mouse models retain the label and are quiescent. Label-retention would be expected, because stem cells in general and particularly also epidermal stem cells (Cotsarelis et al., 1990) and melanocyte stem cells from the bulge were proven to be label-retaining.
6 MATERIALS AND METHODS

6.1 Skin sphere cultures

Human thigh skin from an adult man (approx 45 yrs. of age) and face skin from an adult woman (approx. 57 yrs. of age) (provided by Dr. G. Beer, Reconstructive Surgery, University Hospital Zurich) were obtained in the frame of cosmetical surgery according to the guidelines of the University Hospital Zurich. Murine skin was taken from adult C57/BL6 mice (Elevage Janvier) of at least 8 weeks of age. The hair was shaved away, about 4cm$^2$ of back skin (composed of both dermis and epidermis) was dissected, and cut into small pieces in a sterile culture cell dish using scissors. The pieces were transferred into a 50ml falcon tube containing 20ml 0.1% Trypsin-EDTA (Invitrogen) in Hank’s balanced salts solution (HBSS) without Ca$^{2+}$ and Mg$^{2+}$ (Amimed) and digested for 50 min at 37°C in a shaker rotating at 75 rpm. Trypsin activity was stopped with D-MEM (Invitrogen) containing 10% FCS. Skin pieces were centrifuged at 1400 rpm for 3 min and washed 3 times with D-MEM-F12 (Invitrogen) containing 100 Units/ml Penicillin, 100µg/ml Streptomycin (P/S) and 1µg/ml Amphotericin B (Fungizone, Invitrogen). After centrifugation, the partially-digested skin pieces were dissociated mechanically in 1ml D-MEM (see above) using a 1000µl pipette with a cut filter tip. The skin pieces were filtered through a 40µm cell strainer (BD Falcon). The cell suspension was centrifuged, washed with medium and the cell pellet resuspended in 1ml Growth medium (GM) consisting of D-MEM-F12 (1:1) containing 1x B-27 Supplement (Invitrogen), 20ng/ml FGF2 (PeproTech), 10ng/ml EGF (PeproTech) (final concentration), P/S and Fungizone as described for the washing medium above. In addition, GM for human cells contained 10ng/ml Leukemia Inhibitory Factor (LIF) (Sigma). Cells were counted using a Neubauer chamber and 2.5 to 4 million cells were plated in GM into an uncoated T-25 cell culture flask (BD Falcon). After 4 to 7 days in culture sphere formation was observed.

For FACS skin was taken from Dhh-Cre/R26R-EYFP or Dct-Cre/R26R-EYFP mice between 10 and 16 days of age (hair follicles in anagen phase). The hair was shaved away, about 4cm$^2$ of back skin (composed of both dermis and epidermis) was dissected and incubated in 2.5ml of 0.5mg/ml Dispase neutral protease (Roche) in HBSS (containing P/S and Fungizone) in a 35mm dish for 30min at 4°C. The adipose tissue was removed with...
forceps under a dissection microscope until the follicles were clearly visible and the sample was cut into small pieces as described above. The pieces were transferred into a 50ml falcon tube containing 20ml of 1mg/ml Collagenase type I (Worthington) in HBSS and incubated for 45 min at 37°C shaking at 110rpm. Samples were centrifuged, decanted and a 0.1% Trypsin-EDTA solution in HBSS was added. Skin pieces were further digested for 5 min at 37°C in a shaker rotating at 110 rpm. All solutions contained 100 Units/ml Penicillin, 100μg/ml Streptomycin (P/S) and 1μg/ml Amphotericin B (Fungizone, Invitrogen). Samples were further treated as described above, resuspended in GM, counted, and the GM switched to PBS containing 5% FCS, P/S and Fungizone and transported on ice. FACS was performed with a FACS Aria (Becton Dickinson) into GM.

6.2 Skin sphere passaging

Once a week the sphere suspension was transferred into a 15ml Falcon tube. Cells adhering to the flask bottom were discarded. Spheres were centrifuged 3 min at 1300rpm and one third of the supernatant was transferred as conditioned medium into a new T-25 flask. The rest of the supernatant was discarded. Spheres were incubated with 300μl Trypsin-EDTA solution (0.25%) for 3 to 5min at room temperature (RT) (depending on the size of the spheres), mixing well. 400μl of Ovomucoid solution (25mg Trypsin Inhibitor (Sigma), 10mg DNase (Roche) in 25ml medium (D-MEM or L-15)) were added and spheres were dissociated mechanically with a 1000μl pipette, centrifuged, resuspended in fresh GM and plated into a new flask containing one third conditioned medium. After some passages spheres were cultured in flasks coated with Poly(2-hydroxyethylmethacrylate) (Poly-Hema) (Sigma). Coating was performed at RT with a solution of 16mg/ml Poly-Hema in 95% ethanol and left to dry in a sterile environment (stock solution 120mg/ml diluted 1:7.5).

6.3 Microdissection of vibrissae and vibrissal sphere cultures

The whisker pad was carefully cut away from the face of mice and rats. Vibrissal follicles were dissected out of the pad using forceps and transferred into a dish containing medium with 10% FCS. Some were used without further microdissection for resin sections as described for mouse back skin (see below). Eight vibrissal follicles in anagen phase were
microdissected for vibrissal sphere cultures as follows: the capsula was removed as a first step. The rest of the follicle was divided into three parts: the upper part containing the bulge and still containing the dermal sheath (DS), the lower part still containing the DS and the bulb still containing the DP. As a last step the DP was removed from the bulb. The different structures were incubated separately but pooled from the eight vibrissae in 35mm dishes. The upper and lower parts were incubated for 30 min with 1mg/ml collagenase/dispase in PBS at 37°C. After this step the DS were teased away from the upper parts and transferred into another plate. The DS from the lower part was not teased away because it was too small. All structures were then incubated with a 0.05% Trypsin/EDTA solution in D-MEM for 1.5 to 2 hours at 37°C. Trypsin activity was stopped with D-MEM containing 10% FCS. The dishes were rinsed and the contents were centrifuged in a 50ml falcon tube at 1290rpm for 5min. After two washing steps with GM cells were plated on an uncoated 24 well for each structure and sphere formation was observed within 1 to 2 weeks in culture. Six independent experiments were done. Passaging was performed as described for skin spheres using 0.05% Trypsin-EDTA instead of 0.25% for 2 min at RT.

6.4 Coating of dishes and plating of spheres

Dishes were coated either with fibronectin (FN) only or poly-D-lysine (pDL) and FN.

*FN*: A solution of 1mg/ml FN (Sigma) was diluted 1:5 in PBS Dulbecco (Biochrom KG). This solution was added to the plates and withdrawn immediately. Medium was added without letting the plates dry.

*pDL/FN*: pDL (Sigma) was dissolved in ddH2O at a concentration of 0.5mg/ml. Dishes were rinsed with this solution and air dried. They were washed two times with tissue culture water and air-dried again. FN was added as described above.

Spheres were plated at low density and let settle. After some hours spheres spread and cells migrated out.
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6.5 Cell differentiation

All differentiation assays were performed using spheres plated on FN as described above. GFAP-positive glia, TuJ1-positive neurons and SMA-positive smooth muscle cells were observed when spread spheres were cultured 3 to 7 days in GM (see above). Alcian blue-positive chondrocytes were observed in cultures of spread spheres on uncoated dishes left for 9 days in D-MEM containing 10% FCS, 50μg/ml ascorbic acid 2-phosphate (Sigma), 10ng/ml FGF2 and P/S. Then the medium was switched to D-MEM containing 10% FCS, 50μg/ml Ascorbic acid 2-phosphate, 10ng/ml BMP2 (PeproTech) and P/S for another 3 days. Adipocytes were occasionally observed when spheres were cultured in the same BMP2-containing medium as chondrocytes. DOPA-positive melanocytes were observed when cultured in MEM (Invitrogen) containing 10% FCS, 50ng/ml SCF (murine, PeproTech), 100nM ET-3 (Sigma) and P/S for at least 10 days.

6.6 Clonal analysis

Murine skin-derived spheres from late passages were dissociated with Trysin-EDTA as described above for passaging and plated at clonal density on pDL/FN coated 35mm dishes (Corning) (see above) in Standard medium (SM). Cells were left at 37°C for 2 hours until they adhered to the plate. The medium was replaced with fresh medium containing αp75 antibody (see below) at a dilution of 1:150. Cells were incubated for 30 min at RT with the primary antibody and then washed 3 times with PBS. The PBS was replaced with SM containing the secondary antibody (Cy3-conjugated Goat anti Rabbit, see below) at a dilution of 1:200. After an incubation period of 30 min at RT, cells were washed again 3 times with PBS. Fresh SM alone or supplemented with 100ng/ml BMP2 (PeproTech), or 1nM NRG1 (R&D Systems) or 0.1ng/ml TGFβ (R&D Systems) was added. After a few hours, p75 positive live-labeled single cells were identified under the microscope (Axiovert, see below), and mapped on the plate with adapted forceps. After 10 days the cells were fixed and analyzed immunocytochemically as described below. Neurons and glia were identified using the markers TuJ1 and NG2 respectively and smooth muscle cells were stained with anti SMA antibodies as described above. Clones containing the different
cell types were counted from 3 independent experiments.

6.7 **Immunocytochemistry**

Cells were fixed with a solution of 4% Formaldehyde (FA) in PBS for 10 min at RT and washed 3 times with PBS. Blocking was performed for 10 min at RT with a solution of 10% Goat serum (Invitrogen), and 0.1% BSA (Fluka) in PBS. Where permeabilization was needed the solution included 0.3% Triton X-100 (Fluka). Primary antibodies were used in blocking buffer in the following dilution with an incubation time of 2 hours at RT: \( \alpha \text{Sox10} 1:3 \) (kind gift from Dr. Michael Wegner), \( \alpha \text{p75} 1:200 \) (Chemicon), \( \alpha \text{GFAP} 1:200 \) (Dako), \( \alpha \text{TuJ1} 1:200 \) (Sigma), \( \alpha \text{SMA} 1:200 \) (Sigma). The following antibodies were used with an incubation period overnight at 4°C: \( \alpha \text{ß-Galactosidase} 1:100 \) (Roche), \( \alpha \text{NG2} 1:200 \) (Chemicon). Cells were washed 3 times with PBS and then incubated with the following secondary antibodies for 1 hour at RT: Cy3-conjugated Goat anti mouse 1:200 (Jackson), Cy3-conjugated Goat anti Rabbit 1:200 (Jackson), Alexa 488-conjugated Goat anti Mouse 1:100 (Molecular Probes), Alexa 488-conjugated Goat anti Rabbit 1:100 (Molecular Probes). After washing 3 times with PBS, nuclei were stained with DAPI at a dilution of 1:1000. Cells were kept at 4°C in PBS. Immunofluorescence was analyzed using an Axiovert 100 microscope and Axiovision 4.1 software (Carl Zeiss MicroImaging, Inc.).

6.8 **Immunohistochemistry**

Antigen retrieval was done in 10mM Tris, 1.7mM EDTA, 1mM sodium citrate, total pH 7.8 in a controlled antigen retrieval device FSG 120-T/T (Milestone). The sections were washed 2 times in PBS for 5 min and subsequently blocked first in 0.2% gelatine and 0.5% BSA in PBS for 30 min and then in blocking buffer from an M.O.M kit (Vector Laboratories). Slides were incubated with anti Sox10 monoclonal antibody (described above) overnight at 4°C. After washing in PBS the sections were incubated with Alexa594 labeled Goat anti mouse IgG secondary antibody at a dilution of 1:200. Sections were washed in PBS and mounted in fluorescence mounting medium (DAKO). Heat unmasking for the p75 staining was done in 10mM tri-sodium citrate pH 6 using the same antigen retrieval device. The antibody was used at a dilution of 1:5000. Alexa594-conjugated goat anti mouse and Alexa488-conjugated goat anti rabbit (1:200; Molecular Probes) were used.
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as secondary antibodies.

6.9 Other stainings

*X-Gal*: Skin was fixed in 4% PFA in PBS for 10 min at RT on a shaker and washed 3 times with PBS. The pieces were then transferred into X-Gal solution (4mM K$_3$Fe(CN)$_6$, 4mM K$_4$Fe(CN)$_6$, 2mM MgCl$_2$, 1mg/ml X-Gal (Axonlab) in PBS) and incubated overnight at 37°C. Samples were washed 3 times with PBS.

Cells were fixed with 4% FA in PBS for 10 min at RT, washed 3 times with PBS and incubated overnight at 37°C in X-Gal solution. Next morning they were washed 3 times with PBS and fixed again for 10 min with 4% FA.

*Alcian Blue*: Cells were fixed with 4% FA as described above. After washing with PBS cells were incubated with a 3% solution of glacial acetic acid in distilled water for 3 min at RT. This solution was replaced with a 1% Alcian blue solution (Chroma Gesellschaft) in 3% acetic acid for 5 min at RT. Plates were rinsed with tap water.

*Oil Red O*: Cells were fixed with 4% FA as described above. After washing with PBS, 60% isopropanol in water was added and cells were incubated for 15 min at RT. Cells were incubated another 15 min in an Oil Red O mixture (0.35g Oil Red O (Sigma) in 50ml Isopropanol and water in a 3:2 dilution). Washing was performed with tap water.

*DOPA reaction*: Cells were fixed with 2% PFA for 10 min at RT and washed 3 times with PBS. They were incubated in a 0.1% solution of 3-(3,4-Dihydroxyphenyl)-L-alanine (L-DOPA) in PBS for 5 hours at 37°C and then washed with PBS.

6.10 Resin sections

Skin from P12 mice (anagen) or 8 week old mice (telogen) and single vibrissae from Wnt1-Cre/R26R, Ht-PA-Cre/R26R, and Sox10lacZ transgenic mice were collected. The skin was cut into small rectangular pieces with scissors. The pieces were fixed and incubated in X-Gal solution as described above. Samples were washed 3 times with PBS and incubated for 24 hours at RT in Bouin’s fixative. The skin was washed 2 times 15 min in ddH$_2$O and transferred into 80% Ethanol. One further dehydration step in 95% and 3 subsequent dehydration steps in 100% ethanol followed. Samples were transferred into a 1:1 mixture of 100% ethanol and glycomethacrylate, left for infiltration at 4°C for 1 to 2 weeks and
embedded in glycomethacrylate resin. 4-5μm sections were cut on a microtome using a glass knife. Counterstains were done with nuclear Fast red.

### 6.11 Paraffin sections

Skin from P12 mice from *Dhh-Cre/R26R* and *Dct-Cre/R26R* was collected and treated with X-Gal solution as described above. Skin pieces were run through a paraffin machine overnight and embedded in paraffin on a membrane for western blots. Sections were cut to a thickness of 7μm and dried at 37°C overnight. For viewing under the microscope they were deparaffinized and mounted with moviol.
7 REFERENCES


7. References


7. References


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9 CURRICULUM VITAE

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Languages

German: excellent
Spanish: excellent
English: excellent
French: good

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