Characterization of Hair-follicle Precursors

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Skin-derived precursors (SKPs) are multipotent neural crest-like precursors that derive from Sox2-positive cells in the dermal papilla (DP) and dermal sheath (DS) of hair follicles. This thesis presents evidence that SKPs originate from somites as well as from the neural crest, but that, regardless of their developmental origins, SKPs express markers for neural crest precursors and differentiate into neural crest progeny including Schwann cells. These findings support the idea that some tissue-specific stem cells are flexible enough to cross lineage boundaries, but that this potency is limited by their physiological niche microenvironment. What then are the mechanisms that regulate the stemness and potency of these hair follicle-associated precursors? One candidate is the transcription factor Sox2, which is expressed in SKPs in vivo and in culture. Data in this thesis show that Sox2 defines not just the endogenous SKPs, but also marks additional precursor populations in neonatal, mature and regenerating skin. Specifically, immunostaining of Sox2:EGFP mouse skin defines six distinct subpopulations of neonatal Sox2-expressing skin cells: DP and DS cells in growing hair follicles, melanocyte precursors in the outer root sheath of hair follicles, nerve-terminal (NT) Schwann cell precursors at the junction between hair follicles and skin nerves, Schwann cell/neural crest precursors in skin nerves and
Merkel cell precursors in the follicular/interfollicular epidermis. In adulthood, Sox2 expression becomes limited to NT cells and Merkel cell precursors in adults, and to hair follicle DP and DS cells during the follicle growth phase. However, skin injury led to reexpression of Sox2 in DP and DS cells coincident with the onset of new follicle growth, and in cells with characteristics of Schwann cell/neural crest precursors in skin nerves. These data define multiple precursor populations in adult skin and suggest that Sox2 may play a key role in orchestrating the stemness and/or potency of these distinct precursor populations, including the endogenous SKPs, for normal development and wound healing.
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# Table of Contents

## Chapter 1 Introduction to SKPs

1.1 General Introduction ......................................................... 1

1.2 SKPs are Adult Precursors with Neural Potential. .......................... 4

   1.2.1 Adult Stem Cells & their Potency ..................................... 4

   1.2.2 SKPs are Adult Precursors with Neural Potential ................ 20

   1.2.3 Summary ........................................................................ 22

1.3 SKPs are Similar to Neural Crest Stem Cells ................................. 23

   1.3.1 Neural Crest Generates Both Neural and Mesenchymal Tissues .. 24

   1.3.2 SKPs are Similar to NCSCs ............................................. 37

   1.3.3 Summary ........................................................................ 40

1.4 SKPs Function as Precursors for the Dermis .................................. 40

   1.4.1 Development of Dermis .................................................. 40

   1.4.2 Relations among SKPs, Dermal Precursors & Sox2 ................. 48

   1.4.3 Summary ........................................................................ 51

## Chapter 2 Convergent Genesis of an Adult Neural Crest-Like Dermal Stem Cells from Distinct Developmental Origins ................................. 52

2.1 Abstract .............................................................................. 53

2.2 Introduction .......................................................................... 53

2.3 Materials & Methods ................................................................ 55

   2.3.1 Animals .......................................................................... 55

   2.3.2 Tissue Culture ................................................................... 55

   2.3.3 Cell Sorting & Analysis .................................................... 56

   2.3.4 Microarrays & Bioinformatics ........................................... 57
2.3.5 RT-PCR .................................................................................................................. 57
2.3.6 Immunocytochemistry & Histology ...................................................................... 58
2.3.7 Statistics .................................................................................................................. 59

2.4 Results ........................................................................................................................ 59

2.4.1 Hair follicle DP & DS are neural crest-derived in facial, but not dorsal trunk skin. ................................................................................................................................. 59
2.4.2 Facial, but not trunk, SKPs are neural crest-derived ............................................ 62
2.4.3 Dorsal trunk SKPs, & their hair follicle niche, are somite-derived ......................... 65
2.4.4 Neural crest- & somite-derived SKPs are similar and both populations generate functional Schwann cells. ........................................................................................................ 68
2.4.5 SKPs of distinct developmental origins are highly similar at the transcriptional level, and differ from bone marrow MSCs. .............................................................................. 74
2.4.6 Developmentally-distinct Sox2:EGFP-positive dermal precursors and the SKPs they generate maintain a lineage history at the gene expression level. .... 79

2.5 Discussion ................................................................................................................... 82

Chapter 3 Sox2 defines multiple precursor populations in the developing, mature and regenerating skin .......................................................... 86

3.1 Abstract ...................................................................................................................... 86
3.2 Introduction ................................................................................................................. 86
3.3 Materials & Methods ................................................................................................. 89

3.3.1 Animals ................................................................................................................... 89
3.3.2 Immunocytochemistry ............................................................................................ 90

3.4 Results ......................................................................................................................... 91

3.4.1 Sox2 is expressed in multiple hair follicle precursor populations in neonatal skin. ................................................................................................................................. 91
3.4.2 Sox2-positive hair follicle cells have characteristics of adult dermal and neural crest precursors. ............................................................................................................. 96
3.4.3 Sox2 is induced in dermal and neural crest precursors following skin injury. .... 102

3.5 Discussion ................................................................................................................... 102
Chapter 4 General Discussion

4.1 Developmental Origins of SKPs / Dermal Precursors

4.1.1 Summary

4.1.2 Novelty / Importance of the findings

4.2 Functions of Sox2 in Skin Maintenance

4.2.1 Summary

4.2.2 Novelty / Importance of the findings

4.3 Conclusion

References or Bibliography
List of Figures

Chapter 1: General Introduction

Figure 1.1: Genetic marking of targeted lineage using Cre/LoxP system

Chapter 2: Convergent genesis of an adult neural crest-like dermal stem cell from distinct developmental origins

Figure 2.1: In facial but not dorsal trunk skin, follicle DP and DS derive from the neural crest.
Figure 2.2: In facial but not dorsal trunk skin, SKPs derive from the neural crest.
Figure 2.3: In dorsal trunk but not facial or ventral trunk skin, DP, DS and SKPs derive from the somites.
Figure 2.4: Facial and dorsal trunk SKPs display similar properties.
Figure 2.5: Somite-derived SKPs generate Schwann cells.
Figure 2.6: Microarray analysis of adult dorsal trunk, ventral trunk, and facial SKPs versus MSCs.
Figure 2.7: SKPs of all developmental origins express neural crest signature genes but retain a lineage history at the gene expression level.

Table 2.1: List of genes from multiple correlation analysis as shown in Fig. 7C
Table 2.2: List of genes from multiple correlation analysis as shown in Fig. 7D

Chapter 3: Sox2 defines multiple precursor populations in the developing, mature, and regenerating skin.

Figure 3.1: In neonatal hair follicles, Sox2 is expressed in dermal precursors in the DP and DS and in cells within and at the terminals of skin nerves that express neural crest and Schwann cell precursor markers.
Figure 3.2: Figure 2. In neonatal hair follicles, Sox2 is expressed in potential precursors for melanocytes and Merkel cells.
Figure 3.3: Sox2 is expressed in multiple potential hair follicle precursors in adult skin.
Figure 3.4: Sox2 is expressed in multiple potential neural crest and non-neural crest-derived hair follicle precursors.
Figure 3.5: Sox2 is induced in dermal and neural crest precursors following skin injury.
Chapter 1
Introduction to SKPs

1.1 General Introduction

Stem cells are undifferentiated cells that have the ability to self-renew permanently and produce a specific set of differentiated progeny\(^1\). Because of these abilities, stem cells and their clinical applications have attracted many researchers for the past few decades. In the broad sense, stem cells can be classified as follows: embryonic and adult stem cells. Embryonic stem cells are found in the embryo, and are founder cells essential for tissue and organ development. In contrast, adult stem cells are found in mature tissues and organs, and are responsible for the tissue maintenance. Embryonic stem cells generally tend to self-renew better in vitro and differentiate into a broader range of cells than adult stem cells. The therapeutic potential of embryonic stem cells has been experimentally demonstrated by many studies\(^2\). However, the use of embryonic stem cells for therapies are controversial from an ethical point of view\(^2\). Therefore, two alternative stem cell sources are intensively studied for cell-based therapies today. One of the approaches is to isolate adult stem cells from tissues of patients, and to utilize them for cell-based therapies\(^3\). This approach has been intensively investigated for the past few decades, and many researchers have identified adult stem cells that have therapeutic potential\(^2\). The other approach is to genetically modify differentiated adult cells from patients so that they dedifferentiate to become similar to embryonic stem cells (these are induced pluripotent stem cells, or iPS cells\(^4\)). This approach emerged in 2006, and has also seen some success, although there are several challenges regarding the safety of reprogramming methods\(^5\).

Skin-derived precursors (SKPs) are one adult stem cell population that has been studied from a therapeutic perspective\(^6,16\). SKPs are isolated from mammalian adult skin, and have been defined in vitro as non-adherent cells that self-renew and differentiate into neural and mesenchymal cell types\(^6\), including neurons\(^11\), Schwann cells\(^9,10,12\), myofibroblasts\(^13\), adipocytes\(^15\), chondrocytes\(^15\) and osteocytes\(^15\). Since the functionality of SKP-derived Schwann cells was proven by functional recovery in spinal cord injured rats, SKPs have been considered as a promising source of functional, myelinating Schwann cells\(^9,12\). Although many properties of SKPs had been elucidated since 2001, there were still unsolved questions when I started my
graduate study in 2006. How do SKPs arise during development? Where do SKPs reside in the skin? What kinds of roles do SKPs play in the skin?

One key unanswered question concerned the developmental origin of SKPs. Initial data indicated that SKPs originate from an embryonic cell population called the neural crest. The neural crest is a unique group of embryonic cells that contribute most of the cells in the peripheral nervous system (including Schwann cells), pigment cells of the skin, craniofacial mesenchymal tissues (such as facial dermis) and part of the cardiac outflow tract. Most neural crest cells are a transient population, but some of them are believed to persist into the adult, retaining the ability to self-renew and to differentiate into multiple cell types throughout life. These latter cells are called neural crest-derived stem cells (NCSCs) (reviewed by Olga et al., 2008). In general, it is believed that the characteristics of stem cells of a particular tissue resemble those of embryonic rudiment for that tissue. Since Toma et al. (2001) and Fernandes et al. (2004) initially found that SKPs share several properties with embryonic neural crest stem cells (NCSCs), they suggested that SKPs are likely to be NCSCs located in the adult skin. However, Fernandes et al. (2004) also demonstrated that SKPs are enriched in a niche for hair follicle dermal cells, which was thought to be a niche for dermal mesenchymal cells. In this regard, dermal mesenchymal cells and fibroblasts are known to derive from multiple developmental origins: neural crest for the craniofacial dermis; somites for the trunk dorsal dermis; and lateral plates for the ventral dermis. Do SKPs have multiple developmental origins like dermal mesenchymal cells and fibroblasts? Or, do they originate from the neural crest, as the NCSC-like properties of SKPs indicated? Although there are many possible explanations for these findings, one alternative is that SKPs originate from both the neural crest and from non-neural crest sources and that they then converge on to a neural crest-like dermal lineage during embryogenesis regardless of their developmental origins, thereby sharing several properties with NCSCs, including the expression of characteristic transcription factors and their ability to differentiate into functional peripheral neural cell types in response to exogenous environmental signals.

A general aim of this thesis is to clarify the developmental origins of SKPs, and to test the idea described above. Regardless of their embryonic origins, the robust capacity of SKPs to promote functional recovery from neural injury makes SKPs attractive candidates for cellular transplantation therapies for SCI. However, we have the responsibility to define the biological
relevance of the neural potential in SKPs, before moving into clinical trials. In this regard, I believe that it is valuable to clarify the developmental origins of SKPs, and that indeed the answer I elucidated is a step toward the realization of stem cell-based therapies using SKPs.

Along with this study, Biernaskie et al. in 2009 revealed that SKPs can be isolated from Sox2-expressing hair follicle dermal cells, and that they play roles as adult dermal stem cells that are essential for skin development and maintenance. Briefly, the investigators showed that (1) Sox2 is dynamically expressed in the hair follicle dermal papilla (DP) and dermal sheath (DS), (2) the Sox2-expressing skin cells demonstrate typical stemness properties including de novo hair follicle induction, the ability to home back to the DP and DS of hair follicles, formation of self-renewing spheres and differentiation into dermal and neural cell types, (3) the Sox2-expressing cells exhibit a gene expression profile similar to SKPs, (4) SKPs regenerate the dermis and integrate into DP/DS of the hair follicle when transplanted into adult skin, (5) SKPs collaborate with the epidermis, reconstituting hair follicles, and (6) SKPs clonally reconstitute the dermis and induce hair follicle formation, retaining these abilities for the long term. These data clearly indicate that SKPs derive from Sox2-expressing hair follicle dermal cells and that they have properties of dermal stem cells. Moreover, Le et al. (2009) demonstrated that SKPs with deletion of a tumor suppressor gene contribute to the formation of neurofibromas in response to microenvironmental cues. As well, Su et al. (2009) showed that SKPs with the loss of a p53 family member exhibit hyperproliferation, early senescence, and genomic instability, and that the skin of the TAp63 conditional knockout mice displayed senescence of hair follicle dermal cells, perturbed wound healing, decreased hair follicle morphogenesis, and premature aging. These data indicate that the functionality of hair follicle dermal cells (the major source of SKPs) is essential for the maintenance of the skin, and that disruption of the genes / signals that are involved in their stemness properties may lead to perturbed skin maintenance. However, little is known about which cells express Sox2 in the skin except for dermal

The second aim of this thesis is therefore to elucidate the roles of Sox2 in dermal precursors, based upon the hypothesis that Sox2 is essential for the stemness properties of dermal precursors. Since little is known about which cells express Sox2 in the skin except for dermal
precursors, Sox2-expressing cells were first clarified by immunostaining and in vitro assays. Then, we investigated the impact of Sox2 on the functionality of dermal precursors, utilizing Sox2 mutant mice.

1.2 SKPs are Adult Precursors with Neural Potential.

1.2.1 Adult Stem Cells & their Potency

1.2.1.1 Adult Stem Cells

Adult stem cells are undifferentiated cells that (1) self-renew for the long term, (2) have the ability to differentiate into progeny including transit-amplifying cells and terminally-differentiated cells and (3) reside in adult tissues\(^3\). Together, these properties will be referred to as “stemness” properties. These adult stem cells are also called tissue-specific stem cells, because they are specialized to produce some or all of the mature cell types found within the particular tissues or organs in which they reside. Adult stem cells are found not only in highly-regenerative tissues such as skin\(^3\), blood\(^4\) and gut\(^5\), but also in less-regenerative organs such as the brain\(^6\). It is thought that they function in normal cell turnover and to replenish damaged/dead cells in response to injuries/diseases\(^7\).

1.2.1.2 Stem Cell Niche

Behaviors of stem cells such as the self-renewal, multipotency and quiescence (a distinctively slow-dividing state) in their endogenous environments are strictly regulated by their cellular microenvironment. These microenvironments that regulate stem cells are called stem cell niches\(^8\). Perhaps the best-characterized example of the importance of the stem cell niche for stem cell maintenance and normal homeostasis involves hematopoietic stem cells (HSCs) which reside in the bone marrow\(^9,10\). HSCs are self-renewing cells that can differentiate into the cell lineages of the blood and immune systems, including erythrocytes, platelets, basophils, eosinophils, neutrophils, macrophages, T cells and B cells. HSCs are a heterogeneous population that consists of LT-HSCs (long-term repopulating, quiescent or slow-cycling HSCs) and ST-HSCs (short-term repopulating HSCs)\(^11\). The niche for HSCs in the bone marrow is formed by stromal cells and N-cadherin-positive osteoblasts lining the inner surface of the trabecular bone in the marrow cavity\(^12\). HSCs interact with osteoblasts via N-cadherin in their niche. Many studies have supported the idea that osteoblasts in the HSC niche are essential for
regulation of HSCs. For example, Visnjic et al. (2001) generated mice with a DNA construct bearing a 2.3-kilobase (kb) fragment of the rat α1 type I collagen promoter driving a truncated form of the herpes thymidine kinase gene (Col2.3Δtk)43. This kinase becomes toxic in the presence of nucleotide analogs like ganciclovir (GCV). In this mouse strain, the transgene was detected in early differentiating osteoblasts, and the mice displayed decreased numbers of cells in the osteoblast lineage when treated with GCV for 16 days. In their subsequent publication in 2003, the authors demonstrated that hematopoiesis was also severely altered when osteoblasts were conditionally ablated in the Col2.3Δtk transgenic mice44. In a complementary study, Zhang et al. identified a correlation between the number of N-cadherin-positive osteoblasts and the number of LT-HSCs in experiments that increased osteoblasts by conditionally inactivating BMP signaling45 or by constitutively activating PTH/PTHrP signaling46. These data have indicated that the N-cadherin-mediated contact between osteoblasts and HSCs is essential for retaining the slow-cycling characteristics for the HSCs42,45. Finally, Calvi et al. (2003) showed that the number of HSCs increases after activation of Notch signaling on HSCs via presentation of a Notch ligand Jagged1 on osteoblasts46.

Overall, these studies indicate that stem-cell niche is essential to regulate stem cells. Therefore, understanding stem cell niches is as important as defining the stem cells themselves. Some of the relationships between stem cells and their niches are summarized in Table1 of a recent review by Jones & Wagers (2008)38.

1.2.1.3 Defining Stemness

Stemness represents the minimal set of features by which cells can be defined as stem cells47,48. When self-renewal ability and differentiation capacity of a cell are experimentally proven, we call it a stem cell. Are there any generic properties that are shared amongst all stem cells? It is known that ES cells can be phenotypically defined by a certain combination of transcription factors, including OCT4, SOX2 and NANOG, as well as their self-renewal and differentiation capacities47,49. These transcription factors are all essential for the stemness of ES cells. Since SOX2 is also expressed in diverse adult stem cells, then this suggests that there might be transcription factors that are essential for adult stem cells. However, although generic markers for stem cells have been intensively investigated, no common transcription programs that define adult stem cells have been identified42. Sometimes quiescence, which is thought to permit
retention of adult stem cells throughout the animals lifetime by preventing their depletion, is described as a common feature of adult stem cells\textsuperscript{47}. However, adult stem cells that actively divide have been identified in the gut, indicating that quiescence is not a required state for adult stem cells\textsuperscript{47}. Therefore, we can regard a cell as a stem cell only when it satisfies two functional criteria: self-renewal and multipotency. For further readings about the concept of stemness, these reviews\textsuperscript{48,38,50,51} are helpful.

### 1.2.1.4 Distinguishing Stem cells, Progenitors & Precursors

The term, “skin-derived precursors (SKPs)” will be frequently used in the following chapters. But what are “precursors”, and how can they be distinguished from “stem cells” and “progenitors”? The concept “stem cell” has been used for over 100 years, since it was originally proposed by a histologist Alexander Maksimov at a congress of the hematologic society in Berlin in 1908\textsuperscript{52}. However, stem cell biology is still a relatively-new and rapidly-growing field of study, and as a consequence, definitions of certain terms in this field are still open to discussion. Since stem cells were independently identified by developmental biologists, immunologists and many others, researchers with different backgrounds tend to describe stem cells in different ways\textsuperscript{53}. This situation is problematic both for new comers and for stem-cell professionals\textsuperscript{53}. Most importantly, the term “stem cell” itself is sometimes ambiguous. For example, I described stem cells as undifferentiated cells that (1) self-renew for the long term, and (2) have the ability to differentiate into progeny including transit-amplifying cells and terminally-differentiated cells. However, the length of time stem cells need to self-renew to be called stem cells is still controversial, and it is technically impossible to detect the point in time when a stem cell loses its ability to self-renew. One example of this issue involves embryonic “stem” (ES) cells. The blastocyst cells that are cultured to make ES cells are certainly the founder cells that contribute to multiple organs by their ability to self-renew and differentiate during embryogenesis. However, their self-renewal ability is transient, and they do not persist into adulthood. Nevertheless, the term “embryonic stem cells” is widely accepted. Like “stem cells”, other relevant terms such as “precursors” and “progenitors” have similar problems with their definitions, as well-described in the review by Tajbakhsh (2009)\textsuperscript{53}. 


Thus, there are neither perfect definitions for “stem cells”, “progenitors”, and “precursors”, nor alternative terms that can appropriately describe these cells. For clarity, I will define these terms here as I have used them throughout this thesis.

**Stem cells**: Undifferentiated cells that (1) display high self-renew capacity and that persist throughout an animal’s lifetime and (2) display the capacity to differentiate. However, I have also used this term to apply to cells that are already recognized as stem cells (e.g. ES cells) even if their self-renewal capacity has not yet been verified.

**Progenitors**: Any dividing cell with (1) limited self-renewal and (2) the capacity to differentiate. Progenitors are more committed to certain lineages than stem cells, and they are in an intermediate state between stem cells and fully-differentiated cells.

**Precursors**: Precursors is a less specific term, and includes both stem cells and progenitor cells. This term is useful to describe a newly-identified cell population that has at least transient self-renewal and the multipotency, but is not yet known to be a stem cell.

“Skin-derived precursors (SKPs)” have been shown to self-renew and to have the capacity to clonally differentiate into multiple cell types. Since we have not yet shown that SKPs self-renew and persist throughout the animals lifetime, then we prefer not to describe these cells as “skin-derived stem cells”. Instead, we prefer to call them “skin-derived precursors”.

### 1.2.1.5 Four Tools to Assess Stemness

Assessment of self-renewal and differentiation is the gold standard for identifying a given cell as a stem cell. How can we realistically assess these parameters? Snippert & Clevers (2010) described four assays that have been widely used in adult stem cell biology: (1) DNA/chromatin labeling, (2) in vitro culture, (3) transplantation, and (4) lineage tracing. These techniques have been used in previous studies of SKPs, as well as in many other stem cell studies (for example, see Table 1 of the review by Voog & Jones, 2010). Thus, understanding the concepts, advantages and limitations of these assays is essential to discuss the properties of SKPs. Each approach has limitations in which non-stem cells may be inappropriately identified as stem cells and vice versa. Unfortunately, there is not a single assay by which stemness of a cell can be fully verified. Therefore, the best approach for now is to assess stemness by multiple methods.
DNA/chromatin labeling

Quiescence is unlikely an essential property for adult stem cells\(^47\). However, the visualization of quiescent cells has been a very useful approach for detecting certain types of stem cells. Quiescent cells can be labeled and detected in vivo using either a BrdU assay\(^54\) or transgenic mice called TRE-mCMV-H2B-GFP mice\(^55,56\). BrdU is a DNA analogue, and it can be incorporated into cells in S-phase. After long-term exposure to BrdU, slowly-dividing quiescent cells can retain labeled-DNA for a longer period of time than actively-dividing cells, and therefore quiescent cells can be detected by monitoring the label retention. However, cells that divided during the BrdU exposure and then became terminally differentiated can also retain the DNA label. Since BrdU is detected after cells are fixed, it is not possible to distinguish quiescent cells from differentiated cells by assessing their functionality. To overcome this issue, TRE-mCMV-H2B-GFP mice are a valuable tool. Briefly, every cell in a targeted tissue can be tagged by expressing a GFP-tagged histone 2B in transgenic mice. However, the expression of GFP-H2B can be controlled in time and space by using an inducible expression system and targeted promoters. In the case of the aforementioned transgenic mice, the H2B-GFP expression is induced by doxycycline. Then when doxycycline is stopped, quiescent cells will retain the H2B-GFP tag over time, as in the BrdU assay. Since GFP can be monitored in live cells/animals, then these GFP-positive cells can be isolated by FACS sorting to assess their stemness. However, there are still limitations to this strategy. Some of the adult stem cells in the stomach, small intestine and colon are known to be actively dividing. Also, stem cells in muscle, intestine or the hematopoietic system are thought to segregate their chromatids asymmetrically, meaning that a self-renewal division would lead to one labeled stem cell and one unlabeled stem cell. Therefore, these approaches are not effective for identifying stem cells with either of these characteristics. For example, it was reported that only 0.5% of BrdU-label-retaining hematopoietic cells were HSCs\(^57,58,59,60\).

In summary, quiescence is not a prerequisite for stem cells, and therefore techniques to detect quiescence are only effective in identifying particular types of stem cells.
In vitro culture

In vitro cell culture enables us to selectively grow specific stem cell populations, if appropriate culture conditions are adopted. This allows us to observe stem cell behavior or responsiveness to growth factors or other stimuli. A major disadvantage of this technique is that cells are being exposed to an exogenous environment that may be very different from their endogenous, physiological environment or niche. This often raises a question of whether functions, morphologies and phenotypes of stem cells that were revealed in culture actually reflect their endogenous properties. Nevertheless, this technique helps us study stem cell properties that may be masked or restricted by their surrounding microenvironment and also enables us to directly assess stemness (self-renewal and differentiation). Overall, selective culture methods have contributed significantly to adult stem cell biology over the past 20 years, alongside of rapid progress in the development of technologies such as FACS sorting that allow us to isolate stem cell candidates prospectively.

One approach that has widespread use in the stem cell field involves culturing cells as self-renewing spheres in suspension, frequently called the neurosphere approach because it was first used for neural stem cells by Reynolds et al. in 1992\textsuperscript{61}. This approach has been widely applied to the identification of adult stem cells in other tissues, for example, in pancreas\textsuperscript{62}, mammary glands\textsuperscript{63}, prostate\textsuperscript{64}, muscle\textsuperscript{59}, gut\textsuperscript{65}, trachea\textsuperscript{66} and skin\textsuperscript{67}. After targeted tissues are dissociated to the single-cell level, the cells are plated in serum-free defined media with FGF2 and EGF. As a result, some of the stem cells and progenitor cells will proliferate and form floating cell masses called spheres (some examples are neurospheres, pancreatospheres, and mammospheres). These spheres are regarded as proliferating clones of precursors that originate from a single founding cell. Thus, the sphere assay enables us to conduct clonal analysis without labeling cells\textsuperscript{68}. By repeatedly isolating, dissociating and regrowing these spheres, cells that can self-renew for the long term can be identified. By replating spheres into differentiation media, the multipotency of these self-renewing cells can also be assessed. Today sphere-forming assays have been recognized as a powerful tool that enables us to directly assess the stemness of candidate cells and to retrospectively isolate stem cell populations, which can then be used for high-throughput studies and live-imaging studies, for example\textsuperscript{47}. 
There are however several limitations to this culture system. Briefly, Jensen & Parmer (2006) pointed out the limitations as follows: (1) measurement of self-renewal and/or multipotency in this system is highly dependent upon variables such as cell growth factor concentrations, and methods/frequency of passaging, (2) it is difficult to monitor individual cells because cells always exist as components of spheres, and (3) the microenvironment of the spheres promotes stem cell differentiation, meaning that the spheres themselves are heterogeneous mixtures of cells, including self-renewing stem cells, non-self-renewing progenitors and terminally-differentiated cells. While the first limitation can be prevented by strictly following a consensus standard method, the other limitations are systemic, making it difficult to comprehensively understand the properties of stem cells using this approach. For example, it is estimated that neural stem cells only comprise 1 to 5% of the cells in neurosphere cultures. As well, Singec et al. (2006) demonstrated that spheres can be highly motile in culture and can fuse with each other to form chimeric spheres, regardless of tested cell profiles and even at clonal cell density, at which the cells are considered to retain clonality in the culture. For example, 200 - 20,000 cells/mL has been regarded as a consensus clonal density for sphere assays in the past. However, Singec and colleagues observed the formation of chimeric spheres at very low cell densities, suggesting that more rigorous criteria (e.g. culturing at a single cell per well) is crucial to evaluate stemness of tested cells.

Responding to these recent studies questioning the sphere-forming assays, Morshead and van der Kooy investigated the clonality of the sphere-forming assay by co-culturing EYFP-positive neural cells with dsRed-positive cells. These authors demonstrated that clonal neurospheres are formed when primary cells are plated at 10 cells/uL, but that for passaged neurospheres, clonal spheres are only formed at 1 cell/uL. These authors also demonstrated that experimental error is introduced into this assay when the flask is frequently moved, as this leads to fusion of spheres. Finally, the authors reviewed past reports that raised questions about the accuracy of sphere-forming assays, and found that many of these studies were conducted with passaged neurospheres at densities higher than 1 cell/ul and that these cultures were frequently handled and observed. Thus, sphere-forming assays are an effective approach for isolating stem cells, but they must be used under the appropriate controlled conditions.
For further reading, a protocol review by Pastrana et al (2011) fully describes the historical perspective, critical considerations, alternative approaches, and a flowchart outline for sphere-forming assays.\textsuperscript{70}

**Transplantation**

Stemness can be also assessed by transplanting putative stem cells back into their original tissues.\textsuperscript{47} This is a particularly useful approach for assessing the ability of putative stem cells to reconstitute a tissue structure completely or even partially, thereby assessing functional multipotency, but it can also be used to assess their self-renew ability in tissues. Transplantation of putative stem cells has been intensively studied because reconstitution of fully functional organs by isolated stem cells is one of the major goals in regenerative medicine.\textsuperscript{71} Representative cases of transplantation assays include repopulation of the hematopoietic system by transplantation of hematopoietic stem cells (HSCs),\textsuperscript{72} regeneration of muscle by transplantation of satellite cells (muscle stem cells),\textsuperscript{73,74} and hair reconstitution by transplanting both epidermal stem cells\textsuperscript{75,76} and dermal stem cells.\textsuperscript{77,78} A major uncertainty in the transplantation assay system is heterogeneity of the cells that are transplanted. As described in the previous section, it is critical to describe stemness at a single cell level, something that is almost impossible to do in transplantation assays. In this regard, reconstitution of the hematopoietic system, mammary glands, muscle and hair follicles have been successfully achieved by transplanting single HSCs,\textsuperscript{79} mammary stem cells (MaSCs),\textsuperscript{63} satellite cells,\textsuperscript{80} and hair follicle stem cells (via in vitro expansion).\textsuperscript{81,82}

Another concern with transplantation assays derives from reports that some of transplanted stem cells reconstituted a broader range of cell types than expected.\textsuperscript{47} For example, it is known that distinctive subsets of epidermal stem cells are pooled in different regions of hair follicles. An epidermal stem cell subset that expresses LGR5 is located in the lower region of the hair follicle. In this regard, Jak et al. showed that the LGR5\textsuperscript{+} population contributes to the epidermis for hair follicles but not to sebaceous glands and interfollicular epidermis by tracking Lgr5\textsuperscript{−}GFP-positive cells.\textsuperscript{83} Another epidermal stem cell subset that expresses LGR6 is found in the upper region of the hair follicle. Snippert et al. demonstrated that the LGR6\textsuperscript{+} cells differentiate into sebaceous glands and interfollicular epidermis but not into hair-follicle epidermis by chasing LacZ\textsuperscript{+} cells in Lgr6-lacZ mice.\textsuperscript{84} However, these authors observed that
both \textit{LGR5}^+ and \textit{LGR6}^+ cells gave rise to all of the epidermal cell types (hair-follicle, sebaceous-gland and interfollicular epidermis) after isolation and transplantation\textsuperscript{83,84}. As described in section 2.1.2., stem cell niches regulate the behavior of stem cells. One potential explanation for these data is that the endogenous stem cell populations have the capacity to produce all epidermal cell types, but their differentiation to specific cell types may be restricted by their physiological niches. A second potential explanation is that the isolation and manipulation of these stem cell populations altered their properties somewhat so that they became able to reconstitute all epidermal cell types. Thus, while transplantation approaches are invaluable, they need to be used in conjunction with other approaches to definitively assess stemness.

\textbf{Lineage Tracing}

Lineage tracing is perhaps the most powerful tool for identifying adult stem cells. The concept of lineage tracing was originally developed in the field of embryology (1) to construct a fate map, a diagram showing what each region of the embryo becomes in the course of normal development and (2) to perform clonal analysis (in developmental biology it means a form of fate mapping in which ideally a single cell is labeled and the position and identity of its progeny identified at a later stage)\textsuperscript{85}. According to a review by Fox et al, lineage tracing can be done using a variety of different technical approaches, including direct observation, marker addition, retroviral infection and genetic alteration\textsuperscript{86}. The most frequently used approach in stem cell biology today is a genetic one, where genetic differences between cells are used to trace targeted lineages, and where genetically altered cells can enable us to follow all the progeny of the parent cells\textsuperscript{86,87}. In this regard, cell marking by site-specific recombination is now being used extensively in Drosophila and mice to analyze stem cells\textsuperscript{87}. Of the strategies for clonally marking a specific cell population\textsuperscript{87}, genetic lineage tracing mediated by Cre recombinase has been most frequently used for mouse stem cell studies, and several good reviews for this approach have been published in the past\textsuperscript{47,56,87,88,20}.

The easiest way to understand the concept of Cre-mediated lineage analysis for adult stem cell studies is perhaps to visually grasp its mechanism by illustration (\textbf{Fig. 1.1}). This approach is conducted on genetically manipulated mice that contain at least two genetic elements: (1) a transgene where expression of Cre recombinase (an enzyme to excise DNA
Figure 1.1. Genetic lineage tracing mediated by Cre recombinase in mammalian tissues. (a)
Schematic representation of the genetic strategy to track a specific lineage by permanent genetic marking (modified from Fuchs and Horsley (2011))\(^56\). Expression of Cre recombinase is induced in any cell that (transiently) activates the target promoter. Cre recombinase then excise the stop codon flanked by Cre-recombinogenic \(loxP\) sites upstream of a reporter gene, by which a constitutively-active promoter (such as Rosa26 promoter) drives expression of the reporter permanently, even if the cell inactivates the target promoter. (b) Fate mapping analysis of embryonic neural crest stem cells. Cre recombinase under the control of the \(Wnt1\) promoter\(^89\). Virtually all NCSCs express \(Wnt1\) during embryogenesis, and therefore, the full repertoire of the \(Wnt1\)-expressing NCSC progeny can be traced by using this strain.
segments that are flanked by specialized DNA sequences termed LoxP sites) is controlled by a promoter that is active within the targeted stem cells and (2) a reporter gene (e.g. GFP, YFP, RFP, lacZ, etc) harboring a stop codon flanked by the LoxP sites (described as “floxed”) upstream of the reporter gene, under the control of a ubiquitous promoter like the Rosa26 promoter or CAG promoter. The system is sometimes called the Cre/LoxP system in which a cell is genetically labeled via three steps: (1) expression of the Cre recombinase is activated by the stem-cell-specific promoter; (2) this causes Cre recombinase-mediated excision of the stop codon in the reporter gene in cells in which Cre recombinase is active; and (3) the reporter gene, which is driven by a ubiquitous promoter, is continuously active in the cells that expressed Cre recombinase and their progeny. In this lineage tracing system, it does not matter how long Cre recombinase is expressed in the cell. Even if it is only expressed for a short time, once the stop codon sequence is excised, the cells are genetically marked permanently. For example, if we want to trace hair follicle bulge cells as candidate stem cells, and if we know that the bulge cells express Sox9 exclusively in a short period of embryogenesis, then we can consider the use of the Sox9 promoter as a bulge-cell-specific promoter. Then, in mice that contain transgenes for both Sox9-cre and Rosa26-EGFP, we can trace the prospective bulge stem cells as EGFP+ cells and the position and types of progeny can be identified at a later stage. According to Fuchs et al, Sox9 expression is activated in hair follicle bulge regions at embryonic day (E) 18.5. In this system, it takes 2 days before the Cre/LoxP system reflects the active state of the Sox9 promoter by expressing EGFP, followed by contribution of the EGFP+ progeny to multiple follicle compartments after 21 days. If the system successfully captures the targeted stem cells, then we can also perform in vitro culture or transplantation assays to further dissect their characteristics by isolating the genetically marked cells (e.g. EGFP+ cells) by FACS sorting. Moreover, we can use this system to delete a particular gene or activate a gene ectopically only in the targeted cells. Today there are many characterized Cre drivers for various lineages (e.g. See table 2 of the review by Snippert et al), which provides us with many suitable reagents for targeted stem cell candidates.

One of the problems with the above approach is that it captures not just the stem cells, but also their progeny. In addition, it is unlikely that a specific gene is expressed only in the targeted stem cell candidate. For example, SOX9 is a transcription factor involved in the development of many cell types, including hair follicle epidermis, testis, chondrocytes and their embryonic
rudiment (somites), spinal cord, brain and intestinal epithelium. In the Sox9-cre; Rosa26-EGFP mice, all of these cells and their progeny will be tagged by EGFP permanently. Unless the targeted Sox9+ stem cells and their progeny can be distinguished morphologically, phenotypically or spatially from other Sox9+ lineages, this experimental approach will fail. One way to overcome this issue is to regulate the cell type-specific Cre recombinase activity in a temporally-restricted fashion, thereby narrowing down the cell types that are captured as EGFP+ cells. To do so, a derivative of the Cre system called Cre-ER (or inducible Cre) system was developed. This system contains the Cre recombinase fused to the estrogen receptor (ER); with this fusion protein, Cre recombinase only becomes active when the estrogen receptor is bound to its ligand, with Tamoxifen being the most widely-used ligand. Therefore, Cre recombinase activity is only induced when Tamoxifen is added and if the Cre-ER transgene is driven by a stem-cell-specific promoter, then this greatly increases the accuracy of targeted genetic marking. This approach has been used to characterize stem cells in the testis, skin and intestine, for example.

The lineage tracing approach is essential for modern adult stem cell studies. It enables us to assess the activity of candidate stem cells within a physiologically normal environment, which is different from other stemness assays that expose targeted cells to non-physiological conditions. Furthermore, this approach can be extended to the isolation of candidate stem cells. Once isolated, these cells can be used for in vitro culture and transplantation assays. As well, these Cre/loxP animals can be used to generate mice with stem cell-specific conditional gene knockouts and ectopic overexpression.

1.2.1.6 Distinction of “Stemness Potential” & “Actual Stemness”

The above-described functional assays have been used to define the properties of many adult stem cells. However, the outcomes observed by in vitro culture and transplantation assays sometimes deviate from the properties revealed by in vivo lineage tracing, provoking controversy about the biological significance of the observed stemness. Snippert et al. distinguishes the biological properties that are unmasked in non-physiological conditions (describing them as “stemness potential”) from the “actual stemness” that involves the properties of stem cells in their physiological environment. They also suggest the possibility that the “stemness potential” that is restricted during homeostasis plays an important role in certain
situations such as when tissues are damaged. For example, hair follicle bulge stem cells normally only contribute to hair follicle morphogenesis, but following injury, they contribute to all of the epidermal lineages during the wound healing process.\(^{47,76,83,104,105,106,107}\)

The idea that stemness potential is restricted by the undamaged in vivo environment and that this can be unmasked by transplantation assays is reminiscent of conclusions reached by embryologists. Historically the hierarchy of regional specification during embryogenesis was constructed by investigating which structure is autonomously formed when a tissue explant is isolated from the embryo.\(^{85}\) In these experiments, the specification of a region did not necessarily match the fate of the region during normal development.\(^{85}\) For example, the prospective neural plate of a Xenopus blastula that should contribute to neuroepithelium differentiated into epidermis when cultured in isolation.\(^{85}\) In this regard, Jonathan Slack, an embryologist, stated that “embryologists always recognize that the isolated embryonic cells/tissues are more labile than cells/tissues surrounded by their original environments, so that it is perhaps not surprising that grafted stem cells should often populate unexpected tissues.”\(^{20,108}\)

Bonfanti et al (2010) showed that thymic epithelial stem cells, which originate from endoderm, can adopt the fate of hair follicle stem cells that normally develop from ectoderm when transplanted into the skin environment. This suggests that “microenvironmental cues are sufficient to re-direct (reprogram) epithelial fate, allowing crossing of primitive germ layer boundaries and an increase in potency.”\(^{109}\) The authors reached this conclusion based upon experiments where they isolated and expanded clonogenic thymic epithelial cells (TECs) from embryonic, neonatal and adult EGFP-tagged rats. At this point, the cultured TECs retain the combination of transcription factors that defines the TEC identity and the ability to incorporate into a thymic network on a whole-organ reaggregation assay. Surprisingly, when transplanted into mouse skin, these clonogenic TECs contributed to all epidermal and hair follicle layers, where they persisted for several months. When these EGFP\(^+\) TECs were reisolated from long-term skin grafts, they successfully renewed the epidermis in serial-transplantation assays. Moreover, these cells had lost their TEC-specific transcriptional signature, and acquired epidermal marker expression following exposure to the skin microenvironment. The authors suggested “the existence of a generic program of stratification” and that “skin determination is independent of primary germ line origin, as TECs are of endodermal and not of ectodermal origin”. Also, the authors suggested the possibility that the hair follicle stem cells were locally
induced from the TECs by miroenvironmental-induced transdifferentiation. The preview for this research article written by Biousova & Roop (2010) pointed out that, although it is still unclear whether the cultured TECs were reprogrammed to epidermal lineages or they were endogenously less committed stem cells, at least this study emphasized the possibility of reprogramming one cell type to another without needing experimental genetic manipulations.

The conversion of endoderm-derived TECs to an ectodermally-derived lineage observed in this study seems to deviate from the normal process of decision-making in development. The field of adult stem cells has generated many such challenges against the framework of developmental biology. Many past studies have suggested that certain adult stem cells have the potential to adopt new fates depending on their environment, and that this differentiation potential can cross their lineage boundaries. The “plasticity of adult stem cells” has been argued for over ten years and raises a number of key questions. What is the normal process of decision-making in development? How is the potency of a stem cell defined and restricted? And how plastic are adult stem cells in reality?

1.2.1.7 Lineage Commitment

Lineage commitment of a cell is the process of being programmed to follow a particular lineage, the family tree of a group of cells. During development, any cell in any embryonic region experiences several states of commitment, entering a specific lineage and narrowing down its fate. The zygote is located at the top of developmental hierarchy. As development proceeds, the zygotic progeny are specified to three germ layers: ectoderm, mesoderm and endoderm. Ectoderm gives rise to epidermis and neural lineages, mesoderm contribute to blood and mesenchymal lineages such as bone, muscle, cartilage, fat and dermis, and endoderm generates respiratory tissues and the digestive tract. The segregation of embryonic cells into these groups is followed by expression of different combinations of transcription factors that induce further specification. In normal embryogenesis, the developmental pathway of a cell after formation of the three germ layers is believed to be largely determined by the germ layer from which it derives. Thus, the resultant tissue lineages are thought to never cross the lineage boundary of ectoderm, mesoderm and endoderm during normal development.

How does a cell know which developmental pathway to enter? Historically, this question was asked by what are considered classical embryological experiments, in which a piece of
embryonic tissue was grafted from one place to another to ask whether it developed in accordance with its new position or its old position. If the developmental pathway was unaltered by such a graft, then the tissue was defined as being “determined”. Jonathan Slack explained this determination as a loss of competence as follows: (1) lineage commitment can be regarded as being encoded as a particular combination of transcription factors present in the cell, (2) the loss of competence (responsiveness to the signals that turned on the combination of transcription factors for a specific lineage) occurs in each commitment process, and (3) the cell lineage of any region in the embryo passes through several states of commitment, each defined by a different combination of transcription factors. To explain what developmental commitment means in a visually intuitive manner, the concept of “Waddington’s epigenetic landscape” is often referred to (See some examples). Conrad Hal Waddington was an embryologist and geneticist in the 1930s to 1950s who viewed development as a series of branching decisions, taken under the control of genes. The “epigenetic landscape” describes the decision making process of a cell during development as a ball (a cell) on an illustration of a mountain stream (developmental pathways) bifurcated by several dividing ridges (lineage boundaries). In the epigenetic landscape, a stem cell might be described as a ball that permanently retains its position on a dividing ridge, while producing progeny that can run downward.

1.2.1.8 Molecular Mechanisms of Lineage Restriction

Stem cells are often categorized by their potency, which describes the range of possible cell types or structures into which a particular cell population can develop; stem cells are thus said to be totipotent, pluripotent, multipotent, oligopotent, or unipotent stem cells. It is important to know that potency does not necessarily refer to the nature of the stem cell in the physiological context. Instead, the term covers the range of possible cell types that can be provoked in vitro by environments that may not normally be found within the embryo. As development proceeds, the totipotent zygote forms the pluripotent inner cell mass (ICM) and ES cells, and the progeny of these cells gradually lose their differentiation potency as they progress from a multipotent state to a terminally differentiated state by lineage commitment.

Hemberger et al (2009) explained that the progressive reduction of cell potency is achieved by (1) expression of crucial transcription factors and (2) epigenetic modifications that
impose a cellular memory and thereby stable cell fate\textsuperscript{113}. These authors also defined lineage commitment as cell fate decisions and explained the fixation of cell lineage fate by considering ICM/ES cells\textsuperscript{113}. In these cells, the expression of OCT4 in the ICM is an example of the importance of lineage-specific transcription factors that are involved in lineage decisions and stem cell potency. In particular, Niwa et al. showed that conditional overexpression of OCT4 in ES cells induces differentiation into primitive endoderm and mesoderm, while its repression induces the formation of a trophoblast fate accompanied by the loss of pluripotency\textsuperscript{116}. The co-regulation of other transcription factors is also a key process for lineage commitment. Sometimes interaction between different transcription factors involves reciprocal inhibition, such as that seen between Oct4 in pluripotent cells and Cdx2 in trophectoderm\textsuperscript{117,118}. In addition to these transcriptional networks, epigenetic environment is another crucial process for cell fate decisions. For example, Torres-Padilla et al. showed that manipulation of epigenetic information influences cell fate determination, by demonstrating that overexpression of the histone H3-specific arginine methyltransferase CARM1 in blastomeres directed their progeny to the ICM\textsuperscript{119}. Therefore, the progressive restriction of cellular plasticity is achieved by expression of lineage-specific transcription factors and epigenetic modifications, which is followed by fixation of cell fate through the loss of the ability to switch lineages\textsuperscript{113}. For example, DNA methylation is involved in the lineage-fixing process for blastocyst progeny\textsuperscript{120}.

1.2.1.9 Potency of Adult Stem Cells: Restricted or Labile?

Reversion and conversion of cellular restrictions has always been an important topic in the stem cell biology field. One of the hot issues in this field today is the dedifferentiation or transdifferentiation of adult cells via ectopic expression of pluripotency genes\textsuperscript{113} or lineage-inducing factors\textsuperscript{111,112}. For example, Vierbuchen et al. (2009) reported that they identified neuronal-fate-inducing transcription factors that could directly convert skin fibroblasts to functional neurons\textsuperscript{121}. The underlying assumption in these studies is that adult (stem) cells are highly stable, lacking plasticity\textsuperscript{111}.

However, there was a short period in which the potency of adult stem cells was intensively studied. From the late 1990s to the early 2000s, studies were published leading to the concept that adult stem cells may not be restricted in their own fates, and that they may adopt new cell fates across lineage boundaries by being exposed to ectopic microenvironments\textsuperscript{122}. This
idea was experimentally supported by many publications, including evidence that bone marrow-derived progenitors could differentiate into skeletal muscle\textsuperscript{123,124}, hepatocytes\textsuperscript{123,125?130}, endothelial cells\textsuperscript{131?133}, neurons\textsuperscript{134?136} and cardiac muscle\textsuperscript{133,137,138}, and studies showing the conversion of muscle into bone marrow\textsuperscript{139?141}, oligodendrocytes to neurons\textsuperscript{142}, neural progenitors to blood cells\textsuperscript{143}, muscle\textsuperscript{144} and multiple embryonic tissues\textsuperscript{145}, as reviewed by Tosh and Slack\textsuperscript{20}. However, some of the milestone reports that contributed to generating this emerging concept later suffered from the lack of reproducibility of the data and from other possible explanation of such “unexpected plasticity of adult stem cells”, including contamination of distinct stem cell subpopulations and cell fusion\textsuperscript{39}. Therefore, it is still unclear to what degree adult stem cells are plastic.

1.2.2 SKPs are Adult Precursors with Neural Potential.

1.2.2.1 SKPs are Isolated from Adult Skin.

In terms of cellular transplantation after neuronal injuries, neural stem cells (NSCs) and neural progenitor cells (NPCs) have been considered to have therapeutic potential\textsuperscript{2}. However, the use of exogenous CNS tissues (such as fetal tissues) as stem cell sources requires ethical and political considerations and there are potential problems with immune rejection. Is it possible to obtain NSCs or NPCs that have no ethical and immunological restrictions? Toma et al. tried to answer this question by defining the skin as a novel autologous source for NSCs\textsuperscript{67}. Skin is the niche for Merkel cells, one of the neurosensory receptor cells underlying the skin surface that are innervated by skin nerves. Merkel cells are functionally similar to neurons in terms of neuroendocrine activity. Following skin denervation, Merkel cell numbers decrease, but their numbers recover following reinnervation\textsuperscript{146}. These findings suggest that there are stem cells for Merkel cells in adult skin, leading to the ideas that (1) the putative adult stem cells for Merkel cells could be isolated from adult skin, and (2) that they might not be restricted to generating Merkel cells, but might also be able to differentiate into neurons and glia. This was the assumption underlying the isolation of SKPs (skin-derived precursors).

The existence of self-renewing potential stem cells in skin tissue was tested using the neurosphere culture method\textsuperscript{67}. When dissociated skin cells were plated in culture conditions containing EGF and FGF2, many cells became adherent. However, small number of the cells floated, and some of them formed spheres. According to the original report\textsuperscript{67}, 8 cm\textsuperscript{2} of adult skin
could be dissociated to $12 \times 10^6$ single cells, which generated 8,000 spheres of 5 – 30 cells each. These floating spheres could be passaged (meaning the processes of isolating, dissociating, and re-plating cells in fresh medium with growth factors) several times, indicating that the sphere-forming cells could self-renew. Immunocytochemistry demonstrated that these SKP spheres contained cells that expressed Nestin, an intermediate filament protein that is expressed in NSCs$^{147}$. These cells could be passaged and expanded, and after 3 passages, approximately 60% of cells were Nestin-positive$^{67}$. Therefore, potential adult stem cells in the skin could be isolated by neurosphere cultures and at least some of these cells were similar to NSCs in terms of Nestin expression.

1.2.2.2 SKPs Generate both Neural Cells & Mesenchymal Cells.

Did these Nestin$^+$ SKPs exhibit neural potential like NSCs? When plated on a substrate of laminin/poly-D-lysine (PDL) without growth factors for 4 to 21 days, these sphere cells formed (1) neuronal-like cells expressing βIII-tubulin, neurofilament-M (NFM), a neuron-specific Tau1 α-tubulin:nlacZ transgene$^{148,149}$, and GAD$^{67}$ and (2) glial-like cells expressing GFAP, CNPase and A2B5$^{67}$. GAD and A2B5 are makers for neural cells in both the PNS and CNS that are spatially far from the skin, strongly indicating the de novo formation of neural cell types from the isolated skin cells.

However, subsequent characterization surprisingly revealed that SKPs expressed not only Nestin but also Fibronectin, another type of intermediate filament that is expressed in many mesenchymal cell types. Based on this observation, Toma et al. suggested that the potency of SKPs may not be restricted to neural cell types, but that they might also produce mesenchymal cell types, such as adipocytes and smooth muscle cells. In support of this idea, when SKPs were plated in serum, they actually generated adipocyte-like cells containing lipid droplets and SMA$^+$ smooth muscle cells. At this point, these data suggested that the SKP spheres contained cells that could form neural and mesenchymal lineages.

Stemness should be assessed at a single-cell level as described earlier. In this regard, Toma et al. demonstrated that single isolated SKP cells would form clonal spheres over 5 months, and that these would differentiate into both neural and mesenchymal lineages. Therefore, SKPs were regarded as adult precursors that were isolated from the adult skin that had neural potential like NSCs and mesenchymal potential unlike NSCs.
1.2.2.3  SKPs are distinct from other well-known adult stem cells.

To further characterize SKPs, Toma et al. compared SKPs with NSCs isolated from the CNS. The primary differences were that SKPs expressed Fibronectin, but NSCs did not; and SKPs differentiated into adipocytes and smooth muscle cells, but NSCs did not\(^6\). Also, the authors compared SKPs with MSCs (mesenchymal stem cells or marrow stromal cells) from bone marrow, since SKPs shared several properties with MSCs. The bone marrow-derived MSCs are known to express Fibronectin\(^{150\,150}\) and differentiate into adipocytes\(^{151\,152}\) and smooth muscle cells\(^{153\,155}\). However, the authors found that SKPs differ from MSCs, because SKPs grew in the sphere-forming condition, but MSCs did not and because SKPs were Nestin\(^+\)Vimentin\(^-\) Cytokeratin\(^-\), while MSCs were Nestin\(^-\)Vimentin\(^+\)Cytokeratin\(^+\), when cultured on laminin/PDL without growth factors\(^6\). These data indicated that SKPs are a novel adult stem cell population that had not previously been identified.

1.2.2.4  SKPs are enriched in the dermis of the skin.

What kinds of skin cells generate SKPs? Sensory nerve endings in the skin are known to associate with glial cell types\(^{17\,156}\). Considering the neural potential of SKPs, the nerve-associating glial cells might be a potential source of SKPs. However, nerve-associated cells isolated from sciatic nerve did not grow spheres when plated in the sphere-forming condition, indicating that peripheral glial cells in the skin were unlikely to contribute to SKPs\(^6\). To further narrow down the anatomical origin of SKPs, the skin was separated into the epidermal and underlying dermal layers, both of which were then dissociated and cultured in sphere-forming conditions. This experiment revealed that cells in the dermal but not epidermal layer produced floating spheres\(^6\). These data indicated that SKPs are enriched in the dermal layer of the skin but did not derive from nerve-associated cells. Finally, Toma et al. tested whether the Nestin\(^+\) Fibronectin\(^+\) SKP-like cells could be observed in situ. They occasionally detected Nestin\(^+\) cells in the skin sections, but failed to detect Nestin\(^+\)Fibronectin\(^+\) cells\(^6\).

1.2.3  Summary

Adult stem cells are undifferentiated cells that self-renew and differentiate into multiple cell types, located in diverse adult tissues and organs. They self-renew and differentiate into tissue-specific cell types in response to signals from stem cell niches, physiological microenvironments surrounding the stem cells. Self-renewal and the multipotency are the standard criteria used to
define cells as adult stem cells, characteristics which are commonly assessed by in vitro assays, transplantation assays and lineage tracing analysis. However, many of these approaches expose stem cell candidates to exogenous environments that differ from their endogenous stem cell niches. As a result, the observed properties of the stem cells (“stemness potential”) can deviate from the “actual stemness” observed within the physiological context. It is still unclear what the relationships between the stemness potential and actual stemness are and whether the relatively broad potential of adult stem cells unmasked in culture has any role in vivo.

During development, cells are restricted to specific lineages through several states of lineage commitment, each defined and fixed by a particular combination of transcription factors and by a specific epigenetic program. The lineage restriction of cells in ontogeny has traditionally been considered to be irreversible, and therefore stem cells in adult tissues have been considered to be less plastic than are stem cells from embryo. However, the unexpected plasticity of adult stem cells observed over the past 10 years has challenged the prevailing idea that adult stem cells are only capable of generating cell types found within their adult tissue of origin.

The possibility that skin might be one accessible source of stem cells with neural potential led to the isolation of SKPs from adult skin. SKP spheres expressed Nestin, self-renewed for the long term, and differentiated into neurons and glia, like NSCs. They also expressed Fibronectin and differentiated into adipocytes and smooth muscle cells, like MSCs from the bone marrow. These properties were confirmed at a single-cell level, thereby satisfying the two criteria of stemness: self-renewal and multipotency. Some properties of SKPs are distinct from those of NSCs and MSCs, indicating that SKPs are a novel precursor population in the skin. In addition, SKPs are enriched in the dermal layer of the skin, and do not derive from nerve cells. However, following the initial report of SKPs, it was still unclear which specific skin cell type generates SKPs, where the endogenous niche for SKPs was and whether the in vitro properties of SKPs had biological significance in vivo.

1.3 SKPs are Similar to Neural Crest Stem Cells.

One of the surprising features of SKPs described in the initial report was their ability to generate both neural and mesenchymal lineages. In this regard, a stem cell population called neural crest stem cells (NCSCs) was known to have the ability to generate neural and mesenchymal lineages
like SKPs, and subpopulations of NCSCs have recently been defined within adult skin\textsuperscript{157,160}. What are the relationships between SKPs and NCSCs in the skin? Are SKPs identical to NCSCs? To discuss these topics, past and recent literature regarding the neural crest and neural crest stem cells is reviewed in this section.

1.3.1 Neural Crest Generates Both Neural and Mesenchymal Tissues.

1.3.1.1 Introduction

The neural crest (NC) is a group of embryonic cells that arise at the border between neural ectoderm (the neural primordium) and non-neural ectoderm (primitive epidermis), and that then migrate from the dorsal part of the neural tube to spread out over the embryonic body via several distinctive migratory pathways. The NC gives rise to a diverse array of cell types, including most of the cells in the peripheral nervous system (PNS), pigment cells of the skin (melanocytes), craniofacial mesenchymal tissues (such as facial dermis) and part of cardiac outflow tract\textsuperscript{18}. The astonishingly invasive mobility and diverse range of its potency are the unique characteristics of the NC.

Progress in understanding the NC has been tied to technological advances. Since it was first recognized in 1868\textsuperscript{161}, studies of the NC have often been performed in lower vertebrates such as fish and amphibians\textsuperscript{162}. Then, in the 1960s, intensive investigation of the NC in higher vertebrates began with the development of cell tracking techniques using tritiated thymidine that enabled investigators to label dividing cells\textsuperscript{163} and development of the quail-chick chimera system in which the neural tube and NC in host chick were replaced with those of donor quails, thereby enabling tracking of the donor NC by utilizing the differences between quail and chick cells\textsuperscript{164}. It was clear from these studies that the NC is conserved among diverse species. However, the comparison of data from different model organisms was often difficult because of differences in the timing of NC induction and in the related signaling pathways\textsuperscript{165}. Today, the classic findings in lower vertebrates and birds have largely been verified in mammals, by utilizing genetically engineered mice that enable us to genetically mark neural crest progeny for fate mapping and to investigate the functions of genes of interest in NC development. For example, Wnt1-cre\textsuperscript{89,166}, Ht-PA-cre\textsuperscript{167}, Sox10-cre\textsuperscript{168}, P0-cre\textsuperscript{169} and Sox1-cre\textsuperscript{170} mice have been utilized to reveal some of the properties of the NC in rodents. In addition, FACS sorting technology has enabled the isolation of NC cells for further characterization\textsuperscript{171}.
The NC has been of significant interest to developmental, evolutionary and stem cell biologists for over 100 years. For example, from the point of evolution, the NC that emerges from the neural tube was originally considered to be unique to vertebrates\textsuperscript{18}. However, NC-like cells were recently identified in urochordate embryos\textsuperscript{172}, raising the possibility that establishment of the NC is instead a very early evolutionary event. From a developmental perspective, the neural crest has been thought to be specified by molecular interactions at the neural fold ridges between neural and non-neural ectoderm with adjacent mesodermal signals\textsuperscript{173,176}. However, it has been recently reported that specification of the NC occurs during gastrulation, earlier than neural fold ridges are formed, independently of neural and mesodermal signals\textsuperscript{177}. For the stem cell biologist, the NC has raised many interesting questions as well. For instance, the NC as a population gives rise to diverse cell types, but are all of these cell types derived from single multipotent stem cells? Or is the NC a heterogeneous population that contains several types of more multipotent or unipotent stem and progenitor cells, each with restricted potential? If there are multipotent NC cells, then do they persist into adulthood, thereby fulfilling the criteria for adult stem cells? If subpopulations of NC precursors self-renew and exhibit multipotency, then what kinds of signals and gene regulatory networks are essential to control their differentiation into the diverse NC-derived cellular progeny? How does their niche control manifestation of NCSC potency? If NC stem and progenitor cells persist into adult, then can we utilize them for therapeutic purposes?

The purpose of this section is to provide the background information necessary to understand the concept of neural crest stem cells (NCSCs) and some of these questions, and to discuss the potential relationships between SKPs and adult skin NCSCs.

1.3.1.2 Neural Crest Formation

Neural crest (NC) formation can be roughly categorized into the following phases: (1) induction and specification at the border between neural and non-neural ectoderm, (2) maintenance (cell-cycle control and pooling) of multipotent NC precursors, (3) segregation of the NC progenitors from the dorsal neural tube (that contains the former neural plate border) via an epithelial-mesenchymal transition (EMT), (4) migration and distribution of NC progenitors along distinct pathways to diverse regions of the body, and (5) differentiation into tissue-specific cell types\textsuperscript{178}. As described in the section on lineage commitment, neural crest formation is also thought to pass
through several states of commitment, each defined by a different combination of transcription factors. By analyzing past experimental data, largely obtained in Xenopus, a hypothetical gene regulatory network underlying NC induction was proposed by Sauka-Spengler et al (2008) and Meulemans et al (2004). Although it is important to be cautious about differences in species-specific gene expression, the environmental cues and crucial transcription factors during neural crest formation can be roughly categorized into four types. First are the “inducing signals” (BMP, Wnt, Fgf) that induce the expression of “neural crest specifiers” in the NC precursors and “neural plate border specifiers” in the uncommitted ectoderm. Second are “neural plate border specifiers” (Msx1/2, Dlx3/5, Zic, Pax3/7) that direct the primitive ectoderm to the neural ectoderm (neural plate, being defined by Sox2 and Zic), the non-neural ectoderm (epidermis, being defined by Dlx3/5, AP-2, Msx1/2), and the intermediate neural plate border (defined by the “neural plate border specifiers”). Third are “NC specifiers” (Snail, Slug, FoxD3, Twist, AP-2, Id, Sox9, Sox10) that confer the characteristics of the bona fide NC to the progenitors in the neural plate border. Fourth are “NC effector genes” (Mtf, P0, Trp, cKit, Cad7, RhoB, Col2a, for example) that contribute to the differentiation into specific lineages. Later in Section 3 and in my own results, I will provide data that SKPs and their niche express many NC-related genes and signals, suggesting that SKPs are similar to the NC lineage in terms of gene expression as well as their multipotency.

The processes that regulate the induction and specification of the NC are relatively unknown, particularly when compared to our knowledge of NC migration and differentiation. The classical model of NC induction was built upon heterotopic experiments in vivo and conjugation of different tissues in vitro and has been reviewed by Baker and Bronner-Fraser (1997) and Basch et al. (2004). Briefly, induction is thought to initiate at the neural plate border between neural ectoderm and non-neural ectoderm (the future epidermis), and is mediated by BMP and FGF-mediated signals from the underlying mesoderm, and by Wnt signaling from the mesoderm and the non-neural ectoderm. Accordingly, these signals induce the expression of a particular combination of transcription factors called “NC specifiers”, when the neural plate border is coincidentally exposed to intermediate levels of BMPs. This BMP signaling, together with Notch signaling in the neural plate triggers expression of Snail, Slug, AP-2, Sox9, and FoxD3, all thought to be “NC specifiers”. Wnt and Fgf
signals are also essential for expression of the NC specifiers \cite{182,183,189,191}, and Fgf is thought to be sufficient for NC specifier expression in cultured ectodermal explants \cite{186,192}.

The level of BMPs in this system is adjusted by BMP antagonists secreted in the paraxial mesoderm that underlies the ectoderm \cite{185}. This gradient of BMPs also plays a role in segregation of neural from non-neural ectoderm via activation of “neural plate border specifiers” \cite{193,194}. A high level of BMPs induces expression of Dlx3/5, AP-2 and Msx1/2 in non-neural ectoderm \cite{195,199}. Dlx3/5 represses neural inducers including Sox2 \cite{195,197,200}. AP-2 activates epidermal keratin expression by binding keratin promoters \cite{196,201}. Msx1, an immediate downstream target of BMP2/4 also activates keratin expression, while repressing the neural markers, Zic3 and NCAM \cite{202}. Thus high levels of BMPs eventually causes epidermal differentiation of the non-neural ectoderm. On the other hand, the neural ectoderm, where BMP signaling is inhibited, is directed toward a neural lineage by expression of the Sox2 and Zic genes \cite{203,204}. These genes are thought to activate neural differentiation genes such as NCAM and N-tubulin \cite{196}. Sox2 is also known to repress expression of Slug, a NC specifier \cite{205}. Also, other neural plate border specifiers are thought to be upstream of NC specifiers \cite{179}. Experimentally, Zic was shown to be sufficient to activate Snail,Slug,FoxD3 and Twist in Xenopus \cite{190,203,204,206}. In mice, FoxD3 expression requires Pax3, another neural plate border specifier, which is upregulated by Wnt and Fgf signaling \cite{207}. In frogs, expression of Snail, Slug and FoxD3 is activated by Msx1 \cite{208}.

Therefore, the classical induction model posits that the BMPs, Wnts and FGFs are essential induction signals. However, Basch et al (2006) reported that a restricted region of chick epiblast (primitive ectoderm) was specified to generate NC cells when explanted under non-inducing conditions, thus proposing that specification of the neural crest occurs before the neural plate is formed, independently of mesodermal and neural tissues \cite{177}. In this same study, the investigators identified Pax7 as a key transcription factor for NC induction, because they observed that expression of NC markers such as Slug, Sox9, Sox10 and HNK-1 was blocked by knockdown of Pax7 \cite{177}. The more detailed molecular mechanisms regulating NC induction are well-reviewed elsewhere \cite{165,179}.
1.3.1.3 Neural and mesenchymal derivatives of NC cells

The NC can be divided into the four segments along the anterior-posterior axis, cranial (cephalic), cardiac, vagal, and trunk NC. Each segment migrates out into the embryo by unique pathways that are considered to be conserved in vertebrates\textsuperscript{209}. Historically, the fate of each NC subset was determined by embryological experiments. The cranial NC cells form craniofacial mesenchyme and cranial ganglia, the cardiac NC cells generate the outflow tract of the heart (septum), the vagal and cardiac NC cells contribute to pigment cells and enteric ganglia, and the trunk NC cells form pigment cells, dorsal root ganglia, sympathetic ganglia, adrenal medulla and enteric ganglia\textsuperscript{210}.

One of the intriguing aspects of the NC is its unique ability to generate mesenchymal tissues that are also supplied by the mesoderm, a germ layer that is not the developmental origin of the NC. According to a review by Noden and Schneider\textsuperscript{211}, the progeny of the mesoderm can be grouped as follows: skeletal muscle, cardiac muscle, visceral smooth muscle, endothelium, endocardium, serosa, cartilage*, endochondral bone*, intramembranous bone*, perivascular smooth muscle*, glandular stroma, meninges* and dermis*. Of the thirteen categories, the NC also generates six mesenchymal tissue types (marked by *) in spite of its ectodermal origin. As well, the NC generates a unique cell type that is not made by the mesoderm, odontoblasts\textsuperscript{211}. It was traditionally thought that all of these mesenchymal derivatives were generated only by cranial and cardiac NC cells, based on quail-chick experiments\textsuperscript{212}. However, in zebrafish trunk NC cells contribute to caudal fin mesenchyme during development\textsuperscript{213}. Moreover, genetic tagging experiments in mice demonstrated that trunk NC generates fibroblasts within the sciatic nerve\textsuperscript{214} and, even more surprisingly, that trunk NC cells contribute to the first wave of mesenchymal stem cells (MSCs) in the bone marrow during embryogenesis\textsuperscript{169,170}. The ectoderm-derived NC cells therefore seem to generate mesenchymal cell types across the lineage boundaries.

In spite of the finding that the trunk NC can generate some mesenchymal derivatives, it primarily functions to generate melanocytes and peripheral neural cell types, including peripheral neurons and Schwann cells. The trunk NC cells migrate along two distinct pathways, the dorsolateral pathway between the embryonic epidermis and the somites and the ventromedial pathway between the somites and the neural tube. Until recently, trunk NC-derived melanoblasts
were thought to commit to this lineage as they left the neural tube\textsuperscript{215}, and then these melanoblasts were thought to migrate out into the skin via the dorsolateral pathway. In contrast, most of the PNS, including peripheral autonomic neurons, sensory neurons, peripheral glia and Schwann cells, were thought to be generated by NC cells that migrated out via ventromedial pathway\textsuperscript{216}. Interestingly, however, Adameyko et al (2009) recently demonstrated that Schwann cell precursors on the ventromedial NC migratory pathway contribute to melanocytes in the skin\textsuperscript{217}. These data define an alternative route for melanocyte distribution and demonstrate that Schwann cell precursors, which were thought to be glially-biased, are actually at least bipotent precursors.

The unique nature of the NC, which generates both mesenchymal and neural progeny, has led to a number of interesting hypotheses. For example, Hall et al. suggests that the NC should be regarded as “the fourth germ layer”, because of the way the NC is induced, and the diversity of NC progeny\textsuperscript{218}. In the framework of the germ layer theory, each germ layer is defined as a fundamental embryonic layer from which diverse tissues and organs arise\textsuperscript{218}. Ectoderm and endoderm are recognized as primary germ layers\textsuperscript{218}. Mesoderm is sometimes referred to as the secondary germ layer because it is induced by interactions between primitive ectoderm and endoderm, but also contributes to diverse cell and tissue types\textsuperscript{218}. On the other hand, Weston et al. suggested that the “ectomesenchyme” (the mesenchymal tissues that have been recognized as NC derivatives) actually originates from the “metablast” (a non-neural ectodermal region) rather than the NC\textsuperscript{219}. This model hypothesizes that the NC is not bipotent, generating neural and mesenchymal progeny, but that two completely distinct groups of cells generate the neural cells and mesenchymal cells. For the moment, this idea lacks experimental support\textsuperscript{157}, but it has not been definitively ruled-out in vivo. However, the NC is still considered to have both neural and mesenchymal potential by most investigators, contributing to the entire peripheral nervous system, and to mesenchymal tissues in the bone marrow, the nerve, and the craniofacial region. It is clear, though, that there are still many as-yet-unanswered questions. Are there NC-derived mesenchymal tissues that have not yet been identified? Is it possible that NC-derived mesenchymal precursors (such as MSCs) can regain the potential to generate neural cells by exposure to a non-physiological context? And if so, does this explain the unexpected neural potential of some MSCs\textsuperscript{50,154,220,221} and/or perhaps even the neural/mesenchymal potential of SKPs? Alternatively, is it possible that non-NC-derived cells that experience inductive events similar to the NC (e.g. similar transcription factors, similar environmental cues, similar
interactions between an epithelium and underlying mesenchyme) might acquire NC-like potential?

### 1.3.1.4 Stem Cells of the Neural Crest Persist in Adult Skin.

**Neural Crest Stem Cells (NCSCs)**

Stem cells can be identified by clonally showing their self-renewal and multipotency. Multipotency of individual NC cells was first reported by Sieber-Blum and Cohen (1980). The investigators conducted clonal analysis in vitro using embryonic quail NC cells, demonstrating that the clonal NC cells could be specified to neurons, melanocytes or both. The multipotency of clonal NC cells was then confirmed in vivo by Bronner-Fraser et al (1980). They showed that quail NC cells that were clonally expanded in vitro contributed to diverse cell types, including sympathetic ganglia, adrenal gland and aortic plexus when transplanted to the NC migratory pathways in chick embryo. Thereafter, these investigators assessed multipotency of NC cells under more physiologically normal conditions using single-cell labeling techniques (1988). Specifically, the authors microinjected the vital dye lysinated rhodamine dextran (LRD) into dorsal neural tube of avian embryos, at the exit point for migrating NC cells in order to label single NC precursor cells. The labeled cells individually contributed to sensory neurons, presumptive pigment cells, ganglionic support cells, and adrenomedullary cells. Bronner-Fraser and Fraser further dissected out the multipotency of distinct NC subpopulations using the same single-cell labeling technique. They showed multipotency of the trunk NC cells by demonstrating that LRD-tagged trunk NC cells contributed to sensory and sympathetic neurons, Schwann cells and melanocytes. In these classic studies, the NC cells were sometimes described as “pluripotent” cells, by pointing out their ability to generate all derivatives of a given cell or tissue population. However, in current stem cell biology, pluripotent cells are defined as cells with the ability to form all the body’s cell lineages, including germ cells. Nonetheless, the former definition of “pluripotent” is sometimes used in today’s stem cell papers. In my thesis, I consistently adopt the latter definition.

More recent studies have supported these early conclusions that at least some early NC precursors are multipotent. For example, multipotency of the cranial neural crest was demonstrated by Baroffio et al (1991). Clonal culture of quail cranial NC cells revealed their ability to generate neurons, Schwann cells, melanocytes and mesenchymal tissues such as...
cartilage, bone and other connective tissues. By investigating 533 clones obtained from cranial NC cells, the authors demonstrated that the cranial NC precursor cells are, however, a heterogeneous population that contains many unipotent, bipotent or oligopotent cells with restricted potential to differentiate into a few cell types and relatively few multipotent cells with broader range of the potency. Multipotency of the cardiac NC cells was investigated by Kirby (1983)\textsuperscript{228,229}. Because of their importance to heart development\textsuperscript{230}, the cardiac NC cells have been intensively studied, and now they are known to contribute to the aorta and pulmonary artery of the cardiac outflow tract\textsuperscript{228,229,231}, and smooth muscle of the tunica media\textsuperscript{232,233}. Studies by Ito & Sieber-Blum (1991)\textsuperscript{234} and Youn et al (2003)\textsuperscript{235} showed the heterogeneity of cardiac NC cells, a population that contains both multipotent cells and more restricted cells (for example restricted towards melanocytes, smooth muscle, connective tissue, chondrocytes and sensory neurons)\textsuperscript{234}. To summarize, the NC cells in the craniofacial, cardiac and trunk regions do contain multipotent cells although these are heterogeneous populations of precursors, and many NC precursor cells are unipotent, bipotent or oligopotent cells with more restricted potential.

The term “neural crest stem cells (NCSCs)” was first used by Stemple and Anderson (1992)\textsuperscript{236}. In 1990s, selective markers for NC cells had been elucidated, and the p75 neurotrophin receptor (p75NTR) had been recognized as one of the typical NC markers\textsuperscript{237?239}. Stemple and Anderson therefore isolated p75NTR\textsuperscript{+} NC cells by fluorescence cell sorting and showed that individual p75NTR\textsuperscript{+} NC cells isolated from rodent embryos could self-renew and clonally generate more committed clones such as neuroblasts and glioblasts in vitro.

With regard to these putative NC stem cells, Sommer et al indicated that the multipotent cells are relatively frequent amongst this population in his review\textsuperscript{240}. Quantitatively, of this embryonic NC population, what is the percentage of multipotent NC stem and progenitor cells? This is still an open question today. For example, studies by Trentin et al demonstrated that clones that generate glia, neurons, melanocytes and myofibroblasts were 2.8\% of the total cranial NC clones and 1\% of the total trunk NC clones\textsuperscript{241}. These NC cells were isolated from avian neural tube as migratory NC cells. These data indicated that the NC cell population consisted of a minority of multipotent cells and a majority of unipotent/bipotent cells. On the other hand, Calloni et al. (2009) argued that a highly multipotent progenitor (able to yield neurons, glia, melanocytes and mesenchymal cells) comprises 7-13\% of the total cranial NC clones\textsuperscript{242}, although it seems that the isolation and the expansion of the NC cells were conducted through
protocols similar to those of Trentin et al. Moreover, analyses in zebrafish indicated an absence of multipotent precursors in even premigratory trunk NC cells in this model organism\textsuperscript{243,245}, although this does not mean that there are no trunk NCSCs in zebrafish, since the commitment and segregation of NC progeny apparently occurs earlier than in other species\textsuperscript{159}. While it is still not clear what proportion of the total NC population is multipotent, it is at least agreed amongst scientists that many of the NCSCs transit from a stem cell state to a more restricted progenitor state during embryogenesis. However, these findings raise a number of additional questions. How long do NCSCs persist; are they present in postnatal or adult vertebrates? How do microenvironmental cues regulate NCSC development? What is the range of differentiation capacity in these NCSCs, and can that be unmasked when they are exposed to an exogenous environment?

Regarding this final question, Ruffin et al (1998) demonstrated that postmigratory NC cells can differentiate into motor neurons and floor plate cells of the CNS when ectopically transplanted into ventral neural plate, in spite of the prevailing view that NC cells only contribute to the PNS and not the CNS during development\textsuperscript{246}. These data indicate the remarkable plasticity of NC cells (in other words, the competence of the NC cells to respond to ectopic microenvironmental cues). However, such plasticity of NC cells may not be retained for long or might not be relevant for all NC cells, because similar transplantation experiments conducted by Mckeown et al (2003) showed that cranial NC cells that have migrated into the brachial arch and the trigeminal ganglia lose their competence to generate neural and mesenchymal tissues respectively\textsuperscript{247}. These data indicated that NC cells, as a population, likely become restricted in their lineage potential prior to or soon after their migration from the dorsal neural tube\textsuperscript{247}. However, the findings of Ruffin et al. (1998) raise the possibility that there are subpopulations of postmigratory NC cells that retain their self-renewal capacity and multipotency.

In this regard, a milestone case of identification of postmigratory NCSCs is seen in the publication by Morrison et al (1999)\textsuperscript{248}. These investigators prospectively isolated NCSCs as p75NTR\textsuperscript{+} P0\textsuperscript{−} cells from NC-derived fetal peripheral nerves. These cells exhibited properties that were indistinguishable from the well-characterized embryonic NCSCs that were retrospectively isolated from the dorsal neural tube. These data demonstrated the persistence of NCSCs in peripheral nerve over a week after the onset of neural crest migration. However, in spite of their ability to generate neurons, glia and other cells that were negative for neuronal and
glial markers, NCSCs from the fetal sciatic nerve lacked the ability to generate melanocytes. Later studies showed that postmigratory NCSCs could also be isolated from the embryonic enteric nerve by FACS sorting for p75NTR and α4 integrin\textsuperscript{249}. Bixby et al (2002) compared these enteric NCSCs with the sciatic nerve NCSCs, and demonstrated that the NCSCs in different regions of the PNS are distinct in terms of their differentiation and respond differentially to environmental signals\textsuperscript{249}. This study therefore suggested that differentiation of NCSCs is determined cell-intrinsically as well as by their microenvironmental cues.

Identification of postmigratory NCSCs in the enteric and sciatic nerve accelerated investigation of possible NCSCs in other tissues of embryos, neonates and adults. Today, NCSCs have been identified at various time points and from diverse tissues as reviewed by Shakhova & Sommer (2010)\textsuperscript{158} and Nagoshi et al (2009)\textsuperscript{160}. Moreover, as an alternative strategy to isolate and expand medically-important NCSCs, protocols have now been developed for isolating NCSCs from ES and iPS cells\textsuperscript{250?257}.

**Identification of postmigratory to adult NCSCs**

On what basis can we regard a stem cell population as postmigratory NCSCs? Although there is no gold-standard for the identification of NCSCs, the cells that have been identified as NCSCs generally satisfy several of the following criteria: (1) expression of embryonic NC markers such as p75NTR\textsuperscript{169,248,249,258?262}, α4 integrin\textsuperscript{248,249,258}, Sox10\textsuperscript{169,262} or Slug/Snail\textsuperscript{169,263}; (2) derivation from the NC lineage embryonically, as shown by in vivo fate mapping; (3) in vitro and in vivo responsiveness to proliferation/differentiation/migration signals that are known to regulate embryonic NCSC; (4) multipotency with regard to differentiation into NC progeny including peripheral neurons, Schwann cells, melanocytes, smooth muscle cells, chondrocytes, osteocytes and adipocytes. Each of these criteria is considered in more detail here.

The transcriptional characteristics of embryonic NC cells have been elucidated in classic developmental studies\textsuperscript{165}. It is not surprising that postmigratory NCSCs retain expression of these signature genes. Also, as discussed above, past studies have empirically shown that NCSC-like cells can be isolated by FACS sorting for p75NTR and other extracellular markers\textsuperscript{248,249,258}. However, one of the caveats of this type of analysis is that none of these markers is exclusive for NCSCs, and all are utilized broadly during development, necessitating the use of additional criteria.
To verify that putative postmigratory NCSCs derive developmentally from the NC, most studies have utilized in vivo fate mapping in mice. As an example of this approach, NC cells that will be eventually become Schwann cells pass through several states of commitment, each defined by a different combination of transcription factors and other markers: Sox1 and Wnt1 expression as embryonic NC precursors in the dorsal region of neural tube^{89,170}, Sox10 expression as premigratory NC cells and their progeny committed to the PNS and melanocytes^{165,264,17,265}, P0 expression as NC cells largely committed to being Schwann cell precursors^{17,266}, and Krox20 expression as NC-derived Schwann cell precursors^{17,217}. Thus, genetically engineered mice expressing Cre recombinase under the control of promoters for these different NC markers will enable us to track NC progeny. These approaches therefore allow us to argue the possibility, but not to prove, that cells of interest originate from the embryonic NC, using genetically engineered mice, such as Wnt1-cre^{89,169,214,262,267,270}, Sox10-cre^{271}, P0-cre^{266,170,169}, Sox1-cre^{170}, Krox20-cre^{217,272}, Dct-cre^{262}, Dhh-cre^{262}, and Ht-PA-cre^{167,262}. However, no Cre lines achieve absolute NC-specific targeting. For example, although Wnt1-cre is widely used for fate mapping of the NC lineage, it genetically marks the entire neural tube and its derivatives (e.g. CNS neurons and glia) as well as the NC progeny. Therefore, the data from NC lineage analysis should be carefully interpreted, particularly when combined with systemic problems of the Cre/loxP system (such as transgene penetrance rate, which will be described later). Fate mapping using a number of different Cre mouse report lines (e.g. two independent NC lineage reporters and/or NC lineage reporters combined with reporters for other lineages) can reinforce the argument that cells of interest originate from the NC.

The growth factors and instructive cues that regulate NC development (for example, lineage commitment, migration, survival) have been well-studied (reviewed in Shakhova & Sommer et al, 2010 and Sauka-Spengler & Bronner-Fraser, 2008)^{158,165,273}. For example, neuregulin-1 (NRG1), a ligand for Erb receptors, is thought to be essential for migration, survival, proliferation and maturation of NC-derived Schwann cell precursors, although it is not thought to directly regulate the differentiation of NC cells into Schwann cells^{17}. Because of its crucial role in controlling gliogenesis from the NC, NRG1-containing media has been widely used to assess the gliogenic potential of prospective NCSCs^{274}. The potential of cells to respond to a NC environment can also be tested by assessing their behavior when heterotopically transplanted into embryonic NC migratory pathways^{247}. If a given cell displays a competence
equivalent to embryonic NCSCs, then this supports the argument that they are newly-identified NCSCs. In this regard, newly-identified postmigratory NCSCs have generally been shown to generate neurons, glia and smooth muscle cells. However, postmigratory NCSCs that can differentiate into the full repertoire of embryonic NC progeny have never previously been reported.

There are, however, a number of limitations to previous studies on postmigratory NCSCs. First, the functionality of the cells that were differentiated from these putative NCSCs were often not tested. Second, it has been assumed that cells with neural capacity that are isolated from adult peripheral tissues likely originate from the NC since neural differentiation potential is thought to be limited to stem cells from the central and peripheral nervous systems, and the peripheral nervous system is entirely generated from the neural crest. Intriguingly, however, Seaberg et al (2004) reported an adult stem cell population that they isolated from adult pancreas (named pancreas-derived multipotent precursors, PMPs) that had the ability to generate neurons and glia in vitro. These cells did not express an embryonic NCSC transcriptional profile, so the authors suggested that PMPs are a novel adult precursor with neural potential that has a non-NC developmental origin. While the authors have not yet published a detailed lineage analysis, these findings raise the possibility that adult stem cells with neural potential may not necessarily originate from well-defined neural primordia such as the neural plate or the NC. Perhaps some adult stem cells may retain the ability to generate neurons and glia, regardless of their developmental origins.

Although adult NCSCs have been identified from diverse tissues, one remaining question is whether these adult NCSCs actually function in local adult neurogenesis and/or gliogenesis. In this regard, recent studies have demonstrated that adult carotid body and enteric nervous system undergo adult neurogenesis in response to physiological and/or injury environments, suggesting that the adult NCSCs that have been identified in many tissues may actually participate in local neurogenesis and gliogenesis in these organs.

**Adult NCSCs in the Skin**

It is well-established that NC cells contribute to skin during development, generating melanocytes, the peripheral nerve network and craniofacial dermis, as described earlier. Since these structures are highly regenerative, it is perhaps not surprising that stem cells for these NC
derivatives have been identified in postnatal skin. For example, precursors for melanocytes are the best-characterized NC-derived stem cells in skin\textsuperscript{277}. Comprehensive analyses by our laboratory\textsuperscript{263,278} and others\textsuperscript{77,78,279,281} strongly suggest that dermal cells localized in dermal papillae of whisker follicles (or vibrissae) are NC-derived stem cells. On the other hand, it is not yet clear whether the skin contains adult stem cells that generate new nerve cells (e.g. for Schwann cells or endo-/peri-neurium) or whether melanocyte stem cells generate any other NC-derived cell types in skin. In this regard, Wong et al. (2006) demonstrated that cells of both the glial and melanocyte lineages (genetically marked by Dhh-cre and Dct-cre\textsuperscript{282}) were able to grow in sphere-forming conditions. Simultaneously, they identified a Sox10\textsuperscript{+}p75\textsuperscript{+} skin cell population that formed spheres, and showed that a subpopulation of these Sox10\textsuperscript{+}p75\textsuperscript{+} cells could clonally generate neurons, glia and smooth muscle cells. Based on the colocalization of Sox10, p75 and Dhh/Dct-cre-expressing progeny in vitro and in vivo, they suggested that subpopulations of NC-derived cells that are biased to form glial and melanocyte lineages can self-renew and give rise to a broader range of NC progeny in culture, although they did not show direct evidence for the stemness of Dhh/Dct-cre-expressing progeny\textsuperscript{262}. In addition, Sieber-Blum et al (2006) identified a novel population of NCSCs in the skin, that they called “epidermal NCSCs (epi-NCSCs)” based upon the finding that they were localized in or near the hair follicle bulge where many types of hair follicle stem cells are located. These epi-NCSCs could differentiate into neurons, glia, smooth muscle cells, melanocytes, chondrocytes and osteocytes in vitro, and were positive for a Wnt1-cre-driven NC reporter\textsuperscript{283}. It is still unclear what cell type epi-NCSCs correspond to under normal physiological context, although the hair follicle bulge is closely-associated with peripheral nerves. Independent studies conducted by Amoh et al (2009)\textsuperscript{284} also identified a Nestin-expressing stem cell population localized in the hair follicle bulge region, which they later named “hair follicle pluripotent stem cells (hfPS)”. The hfPS appear to share a niche with epi-NCSCs and differentiate into neurons, glia and melanocytes in vitro. Together, these data indicate that adult NCSCs are present in the skin, but our understanding of these cells is complicated by the fact that these different putative NCSC populations have been identified and assessed by different investigators, approaches and criteria. To further understand each subset of NCSCs, and to investigate the potential relationships amongst them, it might be interesting to assess the characteristics of these different subpopulations in a side-by-side comparison.
1.3.2 SKPs are Similar to NCSCs

1.3.2.1 SKPs satisfy many criteria for NCSCs.

As described in the previous section, adult NCSCs have been identified in the skin, and some of them have been shown to differentiate into neural and mesenchymal cell types. In this regard, SKPs were first defined as skin-derived precursors that originated from the dermis, self-renewed as spheres in the presence of EGF and FGF2 and differentiated into neural and mesenchymal tissues. In addition, SKPs possess neural and mesenchymal transcriptional profiles and express both Nestin, a marker for neural precursors and Fibronectin, a marker for mesenchymal precursors. Interestingly, Nestin has been detected in postmigratory NCSCs found in the DRG and the heart. These data therefore suggest that SKPs might originate from the NC. Fernandes et al (2004) assessed this possibility in detail and provided several lines of evidence that SKPs isolated from craniofacial skin are a NC derivative.

The first line of evidence is that SKPs express NC-associated genes, including Snail/Slug, Twist, Pax3, and Sox9. In the results part of my thesis, I will show that SKPs actually express more NC-related genes. Also, in the last part of the introduction, I will introduce data from other investigators, which indicates that the dermal papilla (DP) of the hair follicle, a niche for SKPs, contains cells expressing some of these NC genes. However, since each of these NC genes is expressed in diverse tissues and organs, regardless of their developmental origins, detection of a certain combination of NC genes in SKPs is not by itself strong proof of the NC origin of SKPs. Although the detection of embryonic NC genes is often described as a property of prospective postmigratory NCSCs, there have been no publications in which the potential roles of these genes in the postmigratory NCSCs have been discussed. Therefore, I will later summarize regulatory mechanisms and functions of the NC-associated genes in NC formation and in the DP (a niche for SKPs), in order to elucidate the relationship between these genes and properties of SKPs.

The second line of evidence was that SKPs could generate the progeny of embryonic NCSCs with the exception of melanocytes, including peripheral neuronal cell types, Schwann cells, smooth muscle cells, chondrocytes, osteocytes and adipocytes in response to in vitro and in vivo environmental cues for embryonic NCSC differentiation. Regarding melanogenic differentiation, it was reported that melanocytes in the interfollicular
dermis originate from endogenous dermal stem cells, and therefore SKPs may also be able to take on a melanogenic fate under appropriate conditions. Also, the odontogenic potential of hair follicle dermal cells was recently reported and odontoblasts are known to originate only from the neural crest during normal development. One of the striking characteristics of SKPs compared to other adult stem cells is that Schwann cells deriving from SKPs are almost identical to endogenous Schwann cells morphologically, phenotypically and functionally. In particular, McKenzie et al (2006) and Biernaskie et al (2006, 2007) clearly demonstrated (1) that SKPs can generate bipolar cells expressing the Schwann cell markers S100b, p75NTR, PMP22, MBP, CNPase and P0 in response to the well-defined gliogenic culture conditions that promote genesis of Schwann cells from embryonic NCSCs, (2) that these SKP-derived Schwann cells (SKP-SCs) associate with the axons of peripheral and cortical neurons in vitro, (3) that when transplanted into the regenerating nerve or injured spinal cord, SKP-SCs associate with and myelinate axons, and exhibit typical Schwann cell phenotypes and morphologies (e.g. Ranvier nodes), (4) that SKP-SCs promote functional recovery in rodent models for spinal cord injury as do real Schwann cells, and (5) that even undifferentiated SKPs will differentiate into myelinating Schwann cells when transplanted into the regenerating peripheral nerve, presumably in response to axonal contact. Thus, SKPs generate bona fide Schwann cells, a cell type that is thought to only be generated by neural crest cells.

In addition to these two major sets of experiments, Fernandes et al. (2004) provided two additional lines of evidence suggesting that SKPs were NC-derived. In one set of experiments, they transplanted SKPs into embryonic chick NC migration pathways, and showed that they migrated into peripheral neural tissues as do the endogenous NC cells, apparently responding appropriately to environmental cues. They also performed lineage tracing, using the Wnt1-cre approach, and showed that SKPs isolated from facial skin were genetically marked as Wnt1-cre expressing progeny and are thus NC derivatives. However, they failed to provide clear results that SKPs from other skin regions are Wnt1-cre expressing progeny. Together, these data indicate that SKPs satisfy many of the criteria for postmigratory NCSCs. Moreover, with the unambiguous data for the NC origin of facial SKPs, we can clearly say facial SKPs are postmigratory NCSCs. Therefore, SKPs have been often introduced as a member of the newly-identified postmigratory NCSC family in many articles.
1.3.2.2 Do SKPs Originate from Neural Crest? Yes (& maybe No).

Why were SKPs from facial skin definitively positive by Wnt1-cre lineage tracing, but SKPs from trunk skin were not? Two possible explanations can be considered. First, SKPs from trunk skin might derive from the NC, but lineage analysis by Wnt1-cre;R26R mice may not be reliable in trunk skin. Second, SKPs from trunk skin not originate from the NC, but they may somehow converge on to a NCSC-like phenotype. With regard to the first explanation, this could occur either because trunk SKPs originated from the Wnt1+ NC, but the reporter was not appropriately expressed in the trunk skin cells because of unexpected systemic issues in Cre/loxP approach\textsuperscript{293,294} or because trunk SKPs originated from the Wnt1- NC, so that trunk SKPs were not positive for the reporter. The former explanation was suggested by Fernandes et al, based on their observation that even known NC derivatives such as Schwann cells and melanocytes were not positive in the back skin of Wnt1-cre;R26RlacZ mice\textsuperscript{263}. On the other hand, while the authors favored this explanation, none of their data addressed the second possibility, that SKPs might have the properties of NCSCs, but derive from another embryonic origin.

1.3.2.3 SKPs may be adult NCSCs in Hair Follicles.

The publication by Fernandes et al (2004) was a milestone in the study of SKPs, not only because it strongly suggested the relationship between SKPs and embryonic NCSCs, but also because it indicated a potential niche for SKPs\textsuperscript{263}. As described earlier, SKPs express a number of typical NC-related transcription factors. The investigators performed in situ hybridization to identify potential endogenous SKPs by detecting skin cells expressing these genes. This experiment clearly showed that the dermal papilla (DP) of hair follicles expressed NC-related genes such as Snail, Slug and Twist. Fernandes et al then showed that cultured SKPs expressed typical markers for the DP including Nexin, Versican and Wnt5a. Moreover, they showed that DP cells dissected from adult whisker follicles generated spheres that were similar to SKPs in terms of their in vitro characteristics. These data indicated that SKPs are enriched in the DP of the hair follicle, an intriguing finding since DP cells have long been considered to be a dermal precursor population of some type, based upon their ability to induce de novo hair follicles\textsuperscript{77,295,297}. In addition, the DP is now known to express several NC genes\textsuperscript{286,287}, and has been proposed to be a niche for multipotent dermal precursors\textsuperscript{280,281}. Together, these findings suggested that SKPs are an adult multipotent precursor that functions as a potential stem cell for
dermal cells in vivo, and that has the potential to generate neural and mesenchymal cell types in vitro and potentially even in vivo.

1.3.3 Summary

The neural crest is a unique group of embryonic cells that contribute to most of the cells in the peripheral nervous system (including Schwann cells), pigment cells of the skin, craniofacial mesenchymal tissues (such as facial dermis) and part of the cardiac outflow tract. Most of the neural crest cells are transient, but some of them are believed to persist into adulthood, retaining the ability to self-renew and their multipotency throughout life, and are therefore called neural crest stem cells (NCSCs) (reviewed by Olga et al., 2008). In general, it has been believed that the characteristics of stem cells of a particular tissue resemble those of the embryonic rudiment for that tissue. Since Toma et al. (2001) and Fernandes et al. (2004) initially found that SKPs share several properties with embryonic neural crest stem cells (NCSCs), they suggested that SKPs are likely to be NCSCs located in the adult skin. However, Fernandes et al. (2004) also provided data indicating that SKPs might be dermal precursors and in this regard, the dermis is known for its multiple developmental origins: neural crest for the craniofacial dermis, somites for the trunk dorsal dermis, and lateral plates for the ventral dermis. However, since even SKPs isolated from the dorsal skin could generate functional Schwann cells, a cell type thought to only be generated by the neural crest, then the relationship between the developmental origin of SKPs in different types of skin relative to their stem cell potential became an important open question.

1.4 SKPs Function as Precursors for the Dermis.

1.4.1 Development of Dermis

In 2009, the Miller laboratory published an important paper demonstrating that SKPs derive from Sox2+ hair follicle dermal cells and that they likely function as endogenous adult stem cells for the dermis. The goal of this section is to discuss the findings in this article. However, before doing this, I will introduce the basics of skin biology, hair follicles and hair follicle dermal cells.

1.4.1.1 Dermis Originates from Mesoderm as well as Neural Crest.

All dermal cells originate developmentally from the neural crest or mesoderm (which can be further subdivided into the somites and lateral plates), depending upon the region where the
dermal cells are located\textsuperscript{21}. As described earlier, the dermis in the craniofacial region was shown to originate from the neural crest by classical quail-chick chimera experiments (Couly & Le Douarin, 1988)\textsuperscript{298}. Likewise, a similar experimental approach revealed that dorsal dermis originates from the somites (Mauger, 1972)\textsuperscript{299}, while the lateral and ventral dermis originates from the lateral plates (Christ, 1983)\textsuperscript{300}. The generation of dermis from spatially distinct embryonic sources has been reconfirmed in more recent studies using genetically-engineered mice such as En1-cre\textsuperscript{301}, HoxB6-cre-ER\textsuperscript{302}, Mesp1-cre\textsuperscript{303} and Wnt1-cre\textsuperscript{263}. However, these more recent experiments simply confirmed something that was known by embryologists long before these experiments were performed. In fact, Marray stated in his 1928 Nature article that “dermis has two sources of origin in the embryo: that of the dorsal and dorso-lateral regions is derived from the dermatomes of the somites; that of the limbs, lateral regions, and by implication also of the ventral regions, derived from somatopleur”\textsuperscript{304}.

1.4.1.2 Mesoderm Provides Somites that Form Dorsal Dermis.

In the section on the neural crest, I explained that the neural crest provides the dermis of the craniofacial region during development. However, with the exception of craniofacial dermis, most dermal cells originate from the mesoderm, one of the three germ layers. At an early stage of vertebrate embryogenesis, the mesoderm is specified into four regions: notochord, paraxial mesoderm (that forms the somites), intermediate mesoderm (that gives rise to the gonads, kidneys and adrenals), and lateral plates\textsuperscript{210}. Somitogenesis is induced by expressing FoxC1 and C2 in the paraxial mesoderm via segmentation, epithelialization and differentiation to two distinctive regions: dermomyotome (that eventually forms the dermis of the dorsal midline and skeletal muscle) and sclerotome (that contributes to vertebrae and ribs)\textsuperscript{210}. Like the neural crest, the epithelial somites undergo EMT (epithelial to mesenchymal transformation)\textsuperscript{305}, then give rise to diverse mesenchymal tissues, which is reviewed elsewhere\textsuperscript{306}. In a prior section, I explained that a small proportion of bone-marrow mesenchymal stem cells (MSCs) originates from the neural crest. Although the primary embryonic source for MSCs has remained largely unknown, it is highly likely that embryonic subpopulations of the mesoderm contain the precursors for MSCs, according to the latest report by Vodyanik et al. (2010)\textsuperscript{307}. Perhaps, this is another interesting property in common between the neural crest and mesoderm.
The developmental commitment and the underlying molecular mechanisms that lead to genesis of the dorsal dermis from the somites has been well reviewed by Olivera-Martinez et al (2004), Scaal & Christ (2004), Kalcheim & Ben-Yair (2005) & Yusuf & Brand-Saberi (2006). Like the neural crest, the dermomyotome gives rise to multiple derivatives, and whether these derivatives originate from single multipotent somitic precursors has long been argued in the field of developmental biology. In this regard, clonal analysis in the chick indicates that the central dermomyotome is composed of a homogeneous population of self-renewing and multipotent epithelial progenitors rather than a mixture of cells with different states of commitment. In terms of the commitment to the muscle lineage from the somites, one of the key events is the expression of transcription factors, called MRFs (Myogenic Regulatory Factors): Myf5, MyoD, myogenin and MRF4. These genes are recognized as “master regulatory genes for myogenesis”, and muscle-specific proteins can be ectopically expressed even in differentiated non-muscle cell types (pigment, nerve, fat, liver and fibroblasts) by forced expression of these genes. In normal embryogenesis, Myf5 is expressed in a subset of muscle precursors, although its expression seems to be not only in the restricted muscle progenitors, but also in broader somitic derivatives. Myf5 expression in a broad range of mesodermal cells has been reported by lineage analysis of Myf5-expressing somitic cells using Myf5-cre; Rosa-26R mice. Gensch et al. suggested that this expression was due to a transient activity of the Myf5 promoter in the paraxial mesoderm, which could be captured by Myf5-cre; Rosa-26R mice, but which was not detectable by direct visualization of Myf5 promoter activity (in Myf5-lacZ mice). Although these authors did not completely describe the somitic cell types that were labeled by this lineage analysis, hair follicle dermal cells in the dorsal back skin from Myf5-cre; Rosa-26R mice appear to be positive for lacZ according to the data in Hosokawa et al. This indicates that dorsal dermis developmentally originates from Myf5-expressing subsets of somites.

The structure of somites can be subdivided into the ventral mesenchymal sclerotome and the dorsal epithelial dermomyotome (DM). The DM in turn becomes subdivided into two parts, the dermatome, which forms the dermis of the dorsal trunk region, and the myotome, which contributes to skeletal muscle. Past fate mapping studies suggested that the dermal progenitors are generated from the central region of the DM, while muscle progenitors arise from the DM edge. Recently, Ben-Yair and Kalcheim investigated the fate of the central DM and found that
the central DM contains proliferative myotomal progenitors (PMPs) which supply both muscle progenitors and dermal progenitors\textsuperscript{313}. This finding called into question whether the central DM is composed of two distinct progenitors or whether it contains bipotent progenitors for dermis and muscle. To ask this question, the authors conducted clonal analysis in ovo, and showed that the central DM contains bipotent progenitors with dermogenic and myogenic potentials. These PMPs express Pax3 and Pax7, two DM markers in the central DM, and downregulate these genes when they are specified to the dermal fate. This may well explain why dorsal dermis can be tagged using lineage tracers that are thought to be specific for the muscle lineage, such as Myf5-Cre.

Regardless of developmental origin, the commitment of cells to a dermal fate is thought to occur around E8.5 – 13.5\textsuperscript{207}. The molecular mechanisms regulating dermal specification are likely to be shared amongst spatially distinct dermal progenitors, and involve expression of Dermo1, a bHLH transcription factor, as regulated by Wnt/β-catenin signaling\textsuperscript{301,302,320}.

1.4.1.3 Dermis Specifies the Overlying Epidermis.

If hair follicle dermal cells that were isolated from the scalp of a human male were transplanted into the arm skin of a female, would she get male scalp-type hairs\textsuperscript{321}? In other words, to what degree does the dermis impact on the development of skin appendages, such as hair follicles? The goal of this section is to briefly answer this question.

From the point of structure, the skin in any region is composed of the dermis, the overlying epidermis and other individual components (such as blood vessels, pigment cells and nerves). The development, maturation, homeostasis and regeneration of the skin are mainly achieved through the molecular interactions between epidermis and dermis\textsuperscript{297,322?325}. Although both the dermis and epidermis at early developmental stages appear to be homogeneous populations, many distinctive skin appendages are eventually formed during development. In this regard, experimentally, the dermis has been shown (1) to play a key role in formation of skin appendages\textsuperscript{322}, (2) to be comprised of anatomically heterogeneous cell populations that are defined epigenetically and cell-intrinsically, and which contribute to regional differences in macro-patterning (e.g. hairy skin, whisker pad and non-hairy palm)\textsuperscript{326,327} and (3) to exhibit heterogeneity on a much smaller scale, thereby influencing micro-patterning such as differentiation of the epidermis to distinct hair follicle types\textsuperscript{287}.
Of the diverse skin appendages, the hair follicle has been intensively studied, largely because the hair follicle is the only organ that cyclically repeats (re)generation and degeneration by recapitulating typical epithelial-mesenchymal interactions, a key aspect of embryonic development, throughout life. In addition, in the more recent past, the hair follicle has been the subject of significant interest because it is a hot bed of developmentally-distinct and medically-useful adult stem cells, and because of the high demand for hair regeneration. Reviews of the structure, morphogenesis, molecular principles and related diseases of hair follicles are available elsewhere. Also, many of the genes responsible for hair follicle formation and alopecia have been elucidated via spontaneously mutant or genetically engineered mice, as reviewed by Nakamura et al (2001). In this regard, the primary interest of my thesis is the hair follicle dermal cells that can be spatially and morphologically subdivided into two subsets: the dermal papilla (DP) and the dermal sheath (DS). The very concise review by Driskell et al (2011) is perhaps enough to understand the basics of the DP and DS. As well, the review by Yang & Cotsarelis (2010) may be helpful for further understanding of the stem cells within follicle, and strategies for studying the DP and DS. Briefly, hair follicle development can be subdivided into two phases, morphogenesis or hair folliculogenesis and cyclical hair growth. The duration of morphogenesis is from approximately E12/E13 to 2 weeks after birth in mice. Morphogenesis can be categorized into three phases: (i) induction between E13 - 14, (ii) organogenesis between E15.5 – E17.5 and (iii) maturation from E18.5. All of these events are the net result of molecular interactions between the epidermis and the dermis. Through these interactions, the dermis and the epidermis themselves are specialized to more hair-follicle-specific cell types. After the completion of morphogenesis, hair follicles enter the hair growth cycle that continues throughout life. The cycle is composed of three phases: anagen (growth phase), catagen (regression phase) and telogen (resting phase). Even if hair follicles are forced to deviate from the hair cycle by depilation or injury, the skin can form de novo hair follicles. The hair follicle dermal cells (DP and DS cells) have been experimentally shown to function as key determinants in all phases of morphogenesis, maintenance and regeneration.

So, did the female who appeared in the first paragraph of this section get male-scalp-type hairs on her arm? Yes - Drs. Amanda J. Reynolds, Colin A. B. Jahoda and their colleagues reported the trans-gender induction of hair follicles in Nature in 1999.
showed that the DS cells dissected from the scalp of a male (C.A.B.J.) induced the thick and pigmented hair follicles typical of the donor hairs when implanted into the forearm of a female recipient (A.J.R.). This article can be regarded as a milestone in the study of hair follicle biology and its clinical applications, because it clearly demonstrated the influence of the dermis in hair folliculogenesis, that adult DS and DP cells are both likely precursors that have the ability to induce hair follicle morphogenesis, and that hair follicle dermal cells have an immune-privileged status. To summarize, the hair follicle dermal cells (DP and DS) are the mesenchymal components of the hair follicle structure, which play key roles in hair follicle morphogenesis, maintenance and regeneration. Interestingly, past experiments have suggested that adult DP and DS cells might function as precursors for the dermis itself, raising the possibility that they retain many and/or all of the stem cell characteristics of embryonic dermal progenitors.

1.4.1.4 Hair Follicle Dermal Cells Seem to Be Multipotent.

As described earlier, the unexpected plasticity of adult stem cells has been a topic of intense interest over the past 10 years. In this section, our current understanding of the differentiation potential of dermal cells will be reviewed. The SKP studies conducted by Toma et al (2001) and Fernandes et al (2004) were the first reports showing that adult dermal stem cells could differentiate into cells outside of their tissue of origin. However, a number of subsequent studies independently defined the unique properties of DP and DS cells. For example, Jahoda et al (2003) and Hunt et al (2008) demonstrated that cultured DP cells differentiate into many neural crest cell types, including adipocytes, osteocytes, neurons and glial cells. In accordance with these findings, microarray analyses conducted by Rendl et al (2005) and Driskell et al (2009) indicated the expression of some neural crest associated genes in DP cells.

In addition to a neural crest transcription factor signature, Rendl et al (2005) first reported that DP cells also express Sox2, a transcription factor essential for maintaining the stemness of diverse stem cell types, including neural stem cells (NSCs), a finding that was later confirmed by other groups. While Sox2 function within the NC lineage is not yet fully understood, Cimadamore et al (2011) recently provided evidence that Sox2 plays a key role in sensory neurogenesis as well as EMT in NC development. Sox2 is also expressed in Schwann cell precursors in developing nerves and is thereafter re-expressed in dedifferentiated...
Schwann cells following nerve injury\textsuperscript{339}. Sox2 is thought to regulate proliferation of immature Schwann cells and to maintain the undifferentiated state of Schwann cells by acting as a negative regulator of myelination\textsuperscript{339}. Recent evidence indicates that Sox2 in this lineage is likely regulated as a function of ephrin-B/EphB signaling\textsuperscript{340}. The function of Sox2 in hair follicle dermal cells is still somewhat enigmatic, but it seems to play a role in the process of hair follicle formation. In this regard, Driskell et al (2009) showed that Sox2 is expressed in the dermal condensates that form the DP in embryonic mouse skin at E14.5 – E16.5 when certain hair follicle types (called guard, awl and auchene) of the mouse pelage are induced\textsuperscript{333}. However, the authors found that Sox2 is not expressed in these condensates at E18.5 when the remaining zigzag hair follicles are specified. Consistent with this, in postnatal mouse skin, they found that Sox2 is expressed in the DP of guard, awl and auchene, but not zigzag, hairs. To ask if the Sox2 expression in the DP is associated with specification of hair follicle types, the authors performed hair-follicle reconstitution assays using (1) unsorted dermal cells, (2) Sox2- dermal cells or (3) dermal cells depleted of DP cells. Although all of the dermal subpopulations induced many zigzag hairs, only the unsorted dermal cells induced awl/awchene hairs. This indicates a requirement for Sox2-expressing DP cells for formation of the awl/au chene hair type. Thus, Driskell et al demonstrated that the DP is composed of Sox2\textsuperscript{+} and Sox2\textsuperscript{-} subpopulations, and that Sox2 coordinates the specification of hair follicle type during hair morphogenesis.

Driskell et al (2009)\textsuperscript{287} found that DP cells in a restricted skin area, dorsal back skin, can be subdivided into two subpopulations, Sox2\textsuperscript{+}CD133\textsuperscript{+} DP cells and Sox2\textsuperscript{-}CD133\textsuperscript{+} DP cells. The authors then demonstrated that each subpopulation specified the overlying epidermis to form distinct hair follicle types. Although it is unclear whether these two subpopulations are interchangeable or cell-intrinsically irreversible, the findings indicated that Sox2 in the DP is likely to play some role in hair follicle induction. However, prior to the work reported in Chapter 3 of this thesis, it was unclear whether Sox2 expression was associated with the dermal precursor function of hair follicle dermal cells and/or the normal development or regeneration of the skin.

Interestingly, Sox2 and Nestin expression in human dermatofibroma and dermatofibrosarcoma were recently shown by Sellheyer et al (2010)\textsuperscript{341}, perhaps indicating some relationship between Sox2-positive dermal stem cells and pathology in the dermis. These findings raise the possibility that while the environment strictly regulates the differentiation of
hair follicle dermal cells under normal conditions, that perhaps under pathological conditions, their broader differentiation potential might be unmasked. Indirect support for this idea comes from other systems. For example, Medici et al (2010) demonstrated that fibrodisplasia ossificans progressive (FOP), in which cartilage and bone are ectopically formed, originates from the conversion of vascular endothelium to cartilage and bone, via a multipotent MSC-like state. Klein et al (2010) showed the spontaneous and frequent dedifferentiation of mature germ cells into spermatogonial stem cells. Thorel et al (2010) reported that the loss of pancreatic β-cells could be replenished by conversion of α-cells to β-cells. Zawadzka et al (2010) identified a common precursor that can generate both CNS oligodendrocytes and PNS Schwann cells from adult injured spinal cord. These studies indicate that at least some adult tissues retain cellular plasticity. Is this also true in the dermis? Current evidence indicates that hair follicle DP and DS cells likely contribute dermal fibroblasts and myofibroblasts within skin, and suggests that they have the potential to contribute adipocytes, all cell types that are normal components of the dermis. In contrast, the neural potential of dermal precursors appears to be unnecessary for normal development, although it is formally possible that they could generate Schwann cells for skin nerves during development, during the normal (re)structuring processes of the neural networks underlying the skin surface and/or following injury. Answers to these questions will come from lineage tracing studies where the progeny of DP and DS cells are specifically followed.

While the idea of a multipotent, endogenous dermal precursor might seem surprising, classical studies of tissue regeneration in lower vertebrates provides precedent for the idea. In particular, lizards can escape from predators by sacrificing their highly-regenerative tails. Moreover, urodeles (newts and salamanders) can regenerate limbs, jaws as well as tails. One of the intriguing questions in these systems is the source of cells that contribute to this regeneration, since the loss of a tissue means the loss of tissue-specific stem cells that are responsible for tissue reconstitution. Briefly, one of the classical ideas in urodele regeneration is that tissue-specific cell types are replenished by dedifferentiation / transdifferentiation of terminally-differentiated cells and/or differentiation of undifferentiated and multipotent “reserve cells” around the lost regions. However, a recent lineage analysis conducted by Kragl et al (2009) indicated that regeneration of amputated limbs in salamanders is achieved not via dedifferentiation of mature cells but via contribution of individual lineages (dermis, cartilage,
muscle, glia, epidermis, bone) while maintaining each lineage restriction\textsuperscript{354,355}. Importantly, the only cell lineage thought to contribute more broadly in this system was the dermis. A similar conclusion was reached in a recent study in mammals. In mammals, newborn and adult mice can regrow their digit tips after amputation at a restricted level\textsuperscript{356,357}. Rinkevich et al (2011) showed that cells of ectodermal and mesodermal origin retain their lineage boundaries while contributing to regeneration of the amputated digits\textsuperscript{361}, although dermal cells were not specifically examined in this study.

1.4.2 Relations among SKPs, Dermal Precursors & Sox2

1.4.2.1 SKPs Derive from Sox2(+)ve Hair Follicle Dermal Cells.

As the final part of the introduction, I review the latest article published by Biernaskie et al (2009), who demonstrated that SKPs derive from Sox2-expressing hair follicle dermal cells and that they have properties of dermal stem cells\textsuperscript{14}.

The DS and DP are the mesenchymal components of hair follicles, and there is evidence that there is some exchange between these two groups of cells\textsuperscript{362}. They interact with the overlying hair follicle epidermis during morphogenesis and during the anagen growth phase of the hair cycle to induce hair follicle formation\textsuperscript{324}. They have also been proposed to contribute to dermal wound healing\textsuperscript{296,363,364}. Because of these properties, DP and DS cells were thought by some to be a precursor population for the dermis, but there was no direct evidence for this idea. The Biernaskie et al (2009) paper directly addressed this issue, and provided strong evidence that the DP and DS cells were indeed dermal precursors\textsuperscript{14}.

Initially, Biernaskie et al analyzed the Sox2-EGFP expression patterns in the adult skin of Sox2-EGFP mice, based upon the previous work showing that Sox2 was expressed in DP cells\textsuperscript{14}. This analysis demonstrated that Sox2-EGFP was expressed in the DP of awl, auchene and guard hairs, and that it was also expressed in the DS, as well as in a small number of \textbf{K5}^+\textbf{CD34}^+ epidermal cells of the hair follicle bulge. Intriguingly, these authors also found that Sox2-EGFP expression was regulated within the DP as a function of the hair cycle; Sox2-EGFP was expressed in DP cells during anagen, but not during catagen or telogen. Based upon these results, the authors prospectively isolated the anagen DP and DS cells by FACS sorting for Sox2:EGFP and for PDGFRα, which is expressed on all dermal cells, including DP and DS cells. These
isolated Sox2:EGFP DP and DS cells were highly enriched for the ability to generate SKP spheres, indicating that they were likely the major source of SKPs, as suggested by the Fernandes et al. (2004) paper. The authors then showed that these Sox2:EGFP+ dermal cells could function as dermal precursors when transplanted; they could integrate back into their DP/DS follicle niche, repopulate the dermal tissues, contribute to wound-healing and promote hair follicle morphogenesis. These data clearly strongly supported the idea that SKPs are derived from the DP/DS of anagen hair follicles, and that they have properties of dermal precursors. The authors then directly compared cultured SKPs versus the prospectively-isolated Sox2:EGFP+ cells by transcriptional profiling. This microarray analysis revealed that the SKPs and Sox2:EGFP cells were transcriptionally similar, arguing that the Sox2:EGFP+ DP/DS cells were not altered by culturing in SKPs conditions, and implying that the properties previously ascribed to the cultured SKPs were likely shared by the endogenous DP and DS cells. Finally, Biernaskie et al performed a series of experiments to ask whether SKPs possess the properties of dermal stem cells. Transplantation assays demonstrated that clonally-generated SKPs could home back to the DP/DS, supply the interfollicular dermis with dermal and myofibroblasts, differentiate to adipocytes in the hypodermis, induce de novo hair follicles, and that they retained these characteristics even after serial isolation and transplantation. Moreover, these clonal SKPs could differentiate into adipocytes, myofibroblasts and neural cells under defined culture conditions. Together these studies not only confirmed that the DP and DS were niches for SKPs, but they also showed that SKPs had the potential to function as dermal stem cells.

A key question raised by these studies is whether the endogenous DP and DS cells function as dermal stem cells under normal conditions. As one way to answer this question, Biernaskie et al. performed grafting experiments. They generated genetically-tagged hair follicles ex vivo, using EGFP-positive SKPs or freshly-isolated dermal cells. They then grafted these hair follicles onto the back of nude mice and asked about the fate of the tagged DP and DS cells. These studies showed that the DP and DS contributed cells to the interfollicular dermis in uninjured skin. Moreover, when wounds were made close to the grafted hair follicles, genetically-tagged DP and/or DS cells moved out of the hair follicles into the wounds, where they contributed dermal fibroblasts and myofibroblasts. Overall, these studies indicate that the DP/DS cells are a novel population of multipotent dermal stem cells that contribute to the formation of hair follicles, maintenance of the dermis, and dermal wound-healing.
For my thesis, the key implication of this article is that SKPs anatomically originate from dermal cells within hair follicles, although the article did not establish that this is the only source for SKPs. If SKPs originate from hair follicle dermal cells, then this predicts that SKPs should developmentally originate from mesoderm as well as from neural crest. The purpose of my graduate study was, in part, to reconcile three apparently contradictory findings that have been summarized in this introduction: (1) SKPs are adult stem cells with neural potential that can generate bona fide functional Schwann cells, a neural cell only ever generated from the neural crest; (2) a subpopulation of SKPs that are capable of making Schwann cells is likely to originate from mesoderm; and (3) mesoderm never provides neural tissues during normal development. To reconcile these findings, I introduced the notion that somatic stem cells may have a broader potential for lineage switching than previously thought, and on this basis, I have constructed a hypothesis that SKPs of mesodermal origin exhibit neural crest potential that is masked in their normal physiological context, but that is unmasked when these precursors are exposed to exogenous microenvironment.

1.4.2.2 Deficits in Dermal Precursors May Be Associated with Skin Aging.

The cellular mechanisms that regulate the aging process are largely undefined. However, recent studies have suggested that some aspects of aging arise from an age-associated decline in the self-renewing adult stem cells that are essential for tissue maintenance. This concept may be relevant for skin, as indicated in a recent paper by Su et al. In this paper, the authors focused upon TAp63, a p53 family member, and showed that TAp63 was essential to maintain precursor populations for epidermis and dermis, and that, when these cells were depleted, skin underwent premature aging and aberrant wound-healing. To understand the cellular basis for these phenotypes, they studied SKPs, and showed that TAp63 deletion in these precursors caused defective proliferation, early senescence, and genomic instability, phenotypes that were also observed in the endogenous dermal precursors. These results provide additional support for the idea that dermal precursors in the hair follicle are essential for dermal maintenance and wound healing. In this regard, it would be interesting to ask if Sox2, a generic stemness regulator, is essential for the proliferation or maintenance of follicle dermal precursors, and if disruption of
Sox2 in dermal precursors causes perturbations in skin maintenance or wound healing, perhaps by depletion of the dermal precursor pool.

1.4.3 Summary

The hair follicle is a mini-organ that is generated, maintained and regenerated via epithelial-mesenchymal cross-talk, and which is sustained by many distinct hair follicle stem cells. Of the hair follicle components, the hair follicle dermal cells within the dermal papilla (DP) and the dermal sheath (DS) function as playmakers in hair follicle development, homeostasis and regeneration, and are historically considered as a potential source of precursors for dermal cell types. Moreover, recent studies have indicated that cells within the DP and DS may have the potential to generate cell types that are not related to the dermis. Specifically, neural identity and neural potential of DP/DS cells have been identified by many independent laboratories, although there is as yet no experimental support for the idea that DP/DS cells may contribute cells for skin nerve development and/or regeneration. However, it is still unclear whether SKPs originate from mesoderm as well as neural crest, whether SKPs derive from skin cells other than dermal cells, and whether SKPs exhibit neural potential in the skin in vivo. Moreover, although it was recently shown that SKPs arise from Sox2-positive DP and DS cells in hair follicles, whether or not these endogenous dermal precursors require Sox2 for their maintenance, as was observed for other Sox2-positive adult stem cells, remains an open question. In the following chapters, I address these major questions.
Chapter 2
Convergent Genesis of an Adult Neural Crest-Like Dermal Stem Cells from Distinct Developmental Origins

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2.1 Abstract

Skin-derived precursors (SKPs) are multipotent dermal stem cells that reside within a hair follicle niche and that share properties with embryonic neural crest precursors. Here, we have asked whether SKPs and their endogenous dermal precursors originate from the neural crest or whether, like the dermis itself, they originate from multiple developmental origins. To do this, we used two different mouse Cre lines that allow us to perform lineage tracing: Wnt1-cre, which targets cells deriving from the neural crest, and Myf5-cre, which targets cells of a somite origin. By crossing these Cre lines to reporter mice, we show that the endogenous follicle-associated dermal precursors in the face derive from the neural crest, and those in the dorsal trunk derive from the somites, as do the SKPs they generate. In spite of these different developmental origins, SKPs from these two locations are functionally similar, even with regard to their ability to differentiate into Schwann cells, a cell type only thought to be generated from the neural crest. Analysis of global gene expression using microarrays confirmed that facial and dorsal SKPs exhibit a very high degree of similarity, and that they are also very similar to SKPs derived from ventral dermis, which has a lateral plate origin. However, these developmentally-distinct SKPs also retain differential expression of a small number of genes that reflect their developmental origins. Thus, an adult neural crest-like dermal precursor can be generated from a non-neural crest origin, a finding with broad implications for the many neuroendocrine cells in the body.

2.2 Introduction

Over the past decade, it has become apparent that most adult tissues contain somatic tissue stem cells that serve, at least in part, to maintain those tissues. However, the genesis of these somatic tissue stem cells is still not very well-understood. In this regard, we previously identified a population of dermal stem cells that originate during embryogenesis and persist into adulthood. These skin-derived precursor cells (SKPs) grow in suspension in response to FGF2 and EGF,
and share properties with neural crest precursors, differentiating into mesenchymal derivatives such as adipocytes and skeletogenic cells and peripheral neural crest cell types such as Schwann cells. Recently, we demonstrated that SKPs derive from a Sox2-positive dermal precursor that resides within the dermal papilla (DP) and dermal sheath (DS) of hair and whisker follicles. These endogenous dermal precursors are transcriptionally similar to SKPs, and, like SKPs, they are able to induce hair follicle morphogenesis and generate differentiated dermal cell types. Moreover, genetic perturbations that disrupt the maintenance of these dermal-derived precursors, such as deletion of the p53 family member, TAp63, lead to aberrant wound-healing and premature tissue aging. Thus, like other adult tissues, the dermis contains a somatic stem cell that is generated during embryogenesis and that may well serve to maintain and even repair that tissue.

Although the existence of dermal stem cells would be predicted by the highly regenerative nature of adult skin, the finding that they display similarities to neural crest precursors is somewhat surprising. In this regard, facial dermis derives from the neural crest embryonically, and SKPs generated from facial skin are neural crest-derived, raising the possibility that their hair follicle niche somehow maintains these dermal precursors in a multipotent, embryonic state. However, the dermis of the dorsal trunk does not come from the neural crest, but is instead generated from the somites, specifically from the dermomyotome. In spite of this, SKPs from dorsal trunk generate cell types only thought to be made from the neural crest such as Schwann cells. One explanation for this finding is that neural crest precursors invade the dorsal dermis during embryogenesis, and that it is these precursors that associate with hair follicles and generate SKPs. Support for this idea comes from our previous work showing that a small subpopulation of cells within and/or adjacent to the DP of dorsal hair follicles express a reporter thought to be limited to neural crest-derived cells, and a recent study showing that melanocytes are, at least in part, generated from multipotent neural crest precursors that enter the skin via peripheral nerves. Moreover, neural crest-like precursors are present and persist in a number of other adult tissues, including peripheral ganglia, gut, heart, skin, cornea, carotid body, and palate. An alternative explanation is that neural crest-like precursors are somehow generated from a different developmental origin. Conceptual support for this idea comes from the fact that many tissues contain neuroendocrine cells of nonneural crest origin, suggesting the existence of a second developmental pathway that
converges on to a neural crest-like phenotype. Precedent for such a convergent development model comes from the dermis itself; cells from the neural crest, somites and lateral plate generate the dermis in the face\textsuperscript{298}, dorsal trunk\textsuperscript{23} and ventral trunk\textsuperscript{24}, respectively.

Here we have tested these two possibilities, taking advantage of genetically-defined lineage tracing approaches. Our data demonstrate that SKPs, and the endogenous dermal precursors from which they derive, originate from the neural crest in the face and the somites in the dorsal trunk, but that in spite of these different origins, they both generate precursors with a highly similar phenotype. These findings provide precedent for the idea that somatic tissue stem cells can derive from multiple developmental origins, and suggest that neural crest-like precursors can derive from non-neural crest origins.

2.3 Materials & Methods

2.3.1 Animals

\textit{Wnt1-cre} mice\textsuperscript{89} (Tg(Wnt1-cre)11Rth Tg(Wnt1-GAL4)11Rth/J) and \textit{Z/EG} reporter mice\textsuperscript{374} (B6.129(Cg)-Tg(CAG-Bgeo/GFP)21Lbe/J) were purchased from JaxMice (Jackson Laboratories, Bar Harbor, ME) and were crossed to obtain \textit{Wnt1-cre;Z/EG} double-transgenic mice. \textit{Myf5-cre} mice\textsuperscript{375} (B6;129S4-\textit{Myf5}\textsuperscript{tm3(cre)Sor}/J) were mated with \textit{R26YFP} reporter mice\textsuperscript{376} (B6.129X1-Gt(Rosa)26Sor\textsuperscript{tm1(EYFP)Co}r/J) or \textit{R26R} mice\textsuperscript{377} (B6;129S-Gt(Rosa)26Sor/J) to obtain \textit{Myf5-cre;R26YFP} or \textit{Myf5-cre;R26R} double-transgenic mice, and were maintained as previously reported\textsuperscript{317,378}. \textit{Sox2-EGFP} mice\textsuperscript{379} were the kind gift of Dr. Larysa Pevny and were maintained as previously reported\textsuperscript{14}. Wild-type mice (C57BL/6 and CD1) and rats (Sprague-Dawley) were purchased from Charles River Laboratory (Wilmington, MA). All animal use was approved by the Animal Care Committee for the Hospital for Sick Children in accordance with the Canadian Council of Animal Care policies.

2.3.2 Tissue Culture

SKPs were cultured as described\textsuperscript{7}. Briefly, dorsal back, ventral trunk, and facial whisker pad skin from neonatal (P0-P5) or adult mice (3 weeks and older) were dissected and cut into 2-3 mm\textsuperscript{2} pieces. For the microarray experiments, tissue was dissected from adult Sprague-Dawley rats. Rats were chosen to provide a direct comparison with adult rat mesenchymal stromal cells (MSCs). Tissue was digested with 1 mg/ml collagenase (type XI; Sigma) for 20-45 min at 37°C,
mechanically dissociated and filtered through a 40 μm cell strainer (BD Falcon). Dissociated cells were pelleted and plated in DMEM-F12 (3:1; Invitrogen), containing 1% penicillin/streptomycin (Cambrex), 2% B27 supplement (Invitrogen), 20 ng/ml epidermal growth factor (EGF), and 40 ng/ml fibroblast growth factor-2 (FGF2) (BD Bioscience), hereafter referred to as proliferation medium. Primary spheres generated after 7-21 days were passaged by collagenase digestion and resuspended as single cells at densities ranging from 1,000 to 25,000 cells/ml. Second passage spheres (or greater) were used for all assays, unless otherwise indicated. MSCs were isolated from the bone marrow of adult GFP-expressing (SLC, Japan) or control Sprague-Dawley rats as described380. MSCs were maintained by plating on uncoated culture dishes at a density of 50,000 cells/ml Mesencult human MSC medium containing 10% fetal bovine serum (FBS; both from Stem Cell Technologies). For SKP self-renewal assays, primary SKPs were dissociated and plated at 2,500 cells/ml in proliferation medium containing 1.6% methylcellulose (Sigma), as previously described7. Sphere formation was scored after 8-14 days. Schwann cell differentiations in mass cultures were performed as previously described9,10. For single sphere differentiations10, single rat SKP spheres, at passage 1 were plated onto 8 well slides (Nunc) coated with poly-L-lysine (Sigma) and laminin (BD Biosciences). Spheres were plated in Schwann cell proliferation medium for 3 days and then changed into Schwann cell differentiation medium for the following 14 days. Schwann cell proliferation medium consisted of DMEM:F-12 (3:1) (Invitrogen), 10% FCS (Stem Cell Technologies), 1% B27 (invitrogen), 1% N2 (Invitrogen), 40ng/ml FGF2 (Peprotech) and 20ng/ml EGF (BD Bioscience) and Schwann cell differentiation medium consisted of DMEM:F-12 (3:1), 2% N2 (Invitrogen), 25ng/ml neuregulin-1β (R&D Systems) and 5μM forskolin (Sigma). Medium was changed every 2-3 days.

2.3.3 Cell Sorting & Analysis

Dorsal back or facial whisker pad skin from neonatal (P0-P5) Wnt1-cre;Z/EG, Myf5-cre;R26YFP or Sox2-EGFP mice was enzymatically digested and dissociated to single cell suspensions as described above. Cells were suspended in PBS containing 1% bovine serum albumin and sorted for EGFP or EYFP expression on either MoFlo (Dako) or FACsAria (Becton Dickinson) cell sorters. Viable cells were identified by propidium iodide exclusion. Fractionated cells were subsequently grown in proliferation medium at densities of 5,000 to 20,000 cells/ml. For Schwann cell assays, SKP spheres generated from sorted cells were passaged once, and then
differentiated under Schwann cell conditions for 3 weeks as described above in preparation for coculture with axons of rat sympathetic neurons grown in compartmented cultures as previously described381. Briefly, sympathetic neurons dissected from the SCG of P1 Sprague-Dawley rats were dissociated and plated on a collagen substrate at a density of 0.6 ganglia/dish in 35mm dishes in compartmented cultures. Neurons were established for 5-7 days in UltraCULTURE (Lonza), 2mM L-Glutamine (Lonza), 1% Penicillin/Streptomycin (Lonza) with 20ng/ml nerve growth factor (NGF, Cedarlane), conditions that allow the growth of axons into side compartments. Cultures were then maintained in the presence of 10ng/ml NGF in both centre and side compartments. Myf5-cre;R26YFP SKPs differentiated in Schwann cell conditions were then plated into the side compartments (8,000 cells/side) in Schwann cell differentiation medium with the addition of 10ng/ml NGF. The cells were maintained on the axons for 8 days with a medium change every 2-3 days.

2.3.4 Microarrays & Bioinformatics

RNA was prepared from twice-passaged adult rat dorsal, facial and ventral SKPs and MSCs using Trizol (Invitrogen), as per the manufacturer’s instructions, followed by the RNeasy Mini Kit (Qiagen). The RNA samples were analyzed on Affymetrix GeneChip Rat Gene 1.0 ST Arrays. The data were background corrected and normalized using standard RMA procedure implemented in the Affymetrix Expression Console software. The preprocessed data were analyzed using the LIMMA Bioconductor package to identify genes that show significant evidence of differential expression between SKPs and MSCs. The F-statistic with Benjamini-Hochberg (BH) multiple testing correction implemented in the eBayes function was used to assess significance of differential gene expression. Those genes with BH-corrected p-value <0.01 were considered statistically significant as per Smyth382. Microarray data are deposited in the NIH GEO repository (accession number: GSE23954).

2.3.5 RT-PCR

RNA was prepared from twice-passaged neonatal mouse dorsal and facial SKPs using Trizol (Invitrogen) and from sorted, uncultured mouse skin cells using Cells-to-cDNA II kit (Ambion) as per the manufacturer’s instructions, followed by the RNeasy Mini Kit (Qiagen). For all analyses, controls were performed without reverse transcriptase. PCR reactions were performed as follows: 94°C, 2min; 25-35 cycles of 94°C, 15s; gene-specific annealing temperature for 30s;
and 72°C for 30s. Primers used in this study were as follows: *Ap2a1*, 5'-TCCCTG
TCCAAGTCCACAGCAAT-3’ and 5'-AAATTCCGTTCGCAACGTACCC-3’; *Eya1*, 5'-CTAAACCAGCCCGCATAGCCG-3’ and 5'-TAGTTGGTGAAGGAGGGTAGG-3’; *Foxd3*, 5'-TCTTACATCGCGCTCCTAC-3’ and 5'-TCTTGACGAAGCAGTCGTTG-3’; *Gapdh*, 5'-CGTAGACAAAATGTGAAGGTTCCGG-3’ and 5'-AAGCAGTTGGTGGTGCAGGATG-3’; *Hoxa5*, 5'-TAGTTCCCGTGAGCGAACAATTC-3’ and 5'-GCTGAGATCCATGCCATTGTAG-3’; *Hoxc4*, 5'-AACCCATAGTCTACCTCTTGAACTC-3’ and 5'-CGGTTGTAATGAAACTCTTTCTCTAATTC-3’; *Hoxc6*, 5'-TCTTACATCGCGCTCCTAC-3’ and 5'-TCTTGACGAAGCAGTCGTTG-3’; *Hoxc9*, 5'-TAGTTCCCGTGAGCGAACAATTC-3’ and 5'-GCTGAGATCCATGCCATTGTAG-3’; *Msx1*, 5'-CGGGCGCCTCACTCTACAGT-3’ and 5'-TCCCGCTGCTCTGCTCAAA-3’; *p75NTR*, 5'-GTGTTCTCCTGCCAGGACAA-3’ and 5'-GCAGCTGTTCCACCTCTTGA-3’; *Pax3*, 5'-TGCCCTCAGTGAGTTCTATCAGC-3’ and 5'-GCTAAACCAGACCTGCACTCGGGC-3’; *Rhob*, 5'-AAGACGTGCCTGCTGATCGTG-3’ and 5'-CTTGCAGCAGTTGATGCAGCC-3’; *Slug*, 5'-CGGCGCCCGTGCCTCATCTCCACTTC-3’ and 5'-TCTTCAGGGGACCCAGGCTACAT-3’; *Snail1*, 5'-CGGCCCGTGTGCTCGCTTTCT-3’ and 5'-GCCCTGGCAGTCTCTCTTC-3’; *Sox9*, 5'-CCGCCCATCACCCGCTCGCAATAC-3’ and 5'-GCCCTCCCCGTGCTCTGTAAG-3’; *Sox10*, 5'-CAAGGGGCCCCTGCTGTGCTCT-3’ and 5'-GCCCGTGCCATGCTAACTCTT-3’; *Twist1*, 5'-CTTCCCGGCCACACCACCATCTCCTT-3’ and 5'-GTCCACGGGCGGTGTCTCGCTTTCT-3’; and *Zic1*, 5'-GCCGCCAGACCCACT-3’ and 5'-TGCCAAAAGCAATGGACACGC-3’.

2.3.6 Immunocytochemistry & Histology

Immunocytochemistry was performed as described14, and immunofluorescence was analyzed using a Zeiss upright fluorescence microscope with Northern Eclipse acquisition software, a Zeiss LSM 5 Pascal confocal microscope, or a spinning disk confocal microscope using Velocity acquisition software (Improvision). Cell nuclei and tissue morphology were visualized using Hoechst 33258 (Sigma), TO-PRO-3 (Invitrogen) or propidium iodide (Sigma). X-gal staining was performed as described7, and cells or tissue sections were counterstained with nuclear fast red (Sigma). Primary antibodies used in this study were as follows: mouse anti-β-galactosidase
(1:50; DSHB), rabbit anti-Fibronectin (1:400; Sigma Aldrich), rabbit anti-GFAP (1:1000; Dako), chicken anti-GFP (1:1000; Millipore), rabbit anti-GFP (1:1000; Millipore), rabbit anti-GFP (1:5000; Abcam), mouse anti-Ki67 (1:200; BD Pharmingen), rat anti-NCAM (1:500; Millipore), mouse anti-Nestin (1:400; BD Pharmingen), chicken anti-P0 (1:250; Aves Labs), rat anti-PDGFRα (1:500; eBioscience), rabbit anti-p75NTR (1:500; Promega), mouse anti-S100β (1:1000; Sigma Aldrich), rabbit anti-TRP1 (1:500; Santa Cruz), rabbit anti-Tyrosinase (1:500; Santa Cruz), rabbit anti-Versican (1:500; kind gift from R. LeBaron, U.T.S.A.), and mouse anti-Vimentin (1:200; BD Pharmingen), anti-βIII-tubulin (1:500, Covance). Secondary antibodies used in this study were as follows: Alexa488-conjugated goat anti-mouse, anti-rabbit, or anti-chicken, Alexa555 goat anti-mouse, anti-rabbit or anti-chicken and Alexa647 goat anti-rabbit, anti-mouse or anti-rat (1:1000; all from Invitrogen).

2.3.7 Statistics

Statistics (with exception of the microarray analyses) were performed using two-tailed t-tests or one-way ANOVA where appropriate, and represented as mean +/- SEM. All experiments were performed at least in triplicate.

2.4 Results

2.4.1 Hair follicle DP & DS are neural crest-derived in facial, but not dorsal trunk skin.

We recently showed that SKPs derive from a Sox2-positive precursor associated with follicle DP and DS in both trunk and facial skin. To ask if these endogenous dermal precursors were neural crest-derived, we performed lineage tracing using a Wnt1-cre mouse, which specifically targets Cre recombinase to neural crest derivatives in the periphery. We crossed this mouse to the Z/EG reporter mouse, where active Cre recombinase will knock in an EGFP reporter, thereby allowing us to trace neural crest progeny by monitoring EGFP expression. We then analyzed neonatal facial and dorsal trunk skin of these crossed mice, performing triple-label analysis for EGFP to trace neural crest cells, NCAM, a marker for the follicle DP and DS, and tyrosinase, a marker for melanoblasts/melanocytes that are present in the hair follicle. This analysis showed that, in facial skin, DP and DS cells of almost all hair and whisker follicles were EGFP-positive (Fig. 2.1A, B), as were the majority of cells within the interfollicular dermis (data not shown), indicating that facial dermis derives from Wnt1-expressing neural crest cells, as
FIGURE 2.1

Facial Skin (Whisker pad)  Facial Skin (Non-whisker pad)

Dorsal Skin

Hair follicles

Schwann cells
Figure 2.1. In facial but not dorsal trunk skin, follicle dermal papilla (DP) and DS derive from the neural crest. (A, B): Immunocytochemistry of facial skin sections from neonatal Wnt1-cre;Z/EG mice. (A): Whisker follicle section immunostained for EGFP (green), the DP marker NCAM (red), and the melanoblast/melanocyte marker tyrosinase (blue). The arrow denotes the whisker follicle DP, and the arrowhead the overlying melanocytes. (B): A section through a hair follicle in facial skin outside of the whisker pad immunostained for EGFP (green) and the DP marker NCAM (red). Nuclei were stained with the dye TOPRO3 (blue). The arrow denotes the hair follicle DP, and the arrowhead the dermal sheath. (C, D): Dorsal trunk skin sections from neonatal Wnt1-cre;Z/EG mice, immunostained for EGFP (green), the DP marker NCAM (red), and the melanoblast/melanocyte marker tyrosinase (blue). Arrows denote the DP of dorsal hair follicles and arrowheads the overlying melanocytes. (E): Dorsal skin from neonatal Wnt1-cre;Z/EG mice, immunostained for EGFP (green) and the Schwann cell markers P0 peripheral myelin protein (red) and p75NTR (blue). Arrows denote a nerve containing myelinating Schwann cells positive for all three markers, whereas arrowheads indicate EGFP-positive, p75NTR-positive nonmyelinating Schwann cells. Scale bar = 50 μm (for all panels). Abbreviations: EGFP, enhanced green fluorescent protein; NCAM, neural cell adhesion molecule.
previously reported\textsuperscript{7,370}. In contrast, in dorsal skin, NCAM-positive DS and DP cells of hair follicles were uniformly EGFP-negative (Fig. 2.1C), as were cells of the interfollicular dermis. However, EGFP was expressed in two neural crest cell types in dorsal skin, tyrosinase-positive melanocytes (Fig. 2.1C,D), and P0-positive myelinating and p75NTR-positive nonmyelinating Schwann cells (Fig. 2.1E). Quantification showed that at least 70-80\% of dorsal skin melanocytes and Schwann cells expressed EGFP, demonstrating that the reporter gene is highly penetrant. EGFP was also expressed in cells located near the bulge region, where they were closely associated with p75NTR-positive nerve fibers (data not shown), consistent with previous work documenting \textit{Wnt1-cre;R26R}-positive cells in the bulge region\textsuperscript{283}. Thus, follicle DP and DS cells are neural crest-derived in the face, while those of the dorsal trunk apparently have another developmental origin.

2.4.2 Facial, but not trunk, SKPs are neural crest-derived.

We\textsuperscript{7} and others\textsuperscript{370} previously showed that SKPs from facial skin are neural crest-derived. To ask whether SKPs from dorsal trunk skin had a different developmental origin, as suggested by the in vivo lineage tracing, we cultured SKPs from dorsal versus facial skin of neonatal \textit{Wnt1-cre;Z/EG} mice. To do this, skin cells were dissociated and cultured at low density under nonadherent conditions in the presence of EGF and FGF2; in these conditions, SKPs will grow and proliferate as spheres. As previously documented, virtually all of the SKP spheres isolated from facial skin were EGFP-positive (Fig. 2.2A). Moreover, when dissociated and passaged at low density, cells in these spheres self-renewed and generated new EGFP-positive spheres. In contrast, almost all primary spheres from dorsal skin were EGFP-negative (Fig. 2.2B), and, after passaging, virtually none of the secondary SKP spheres expressed EGFP, indicating that only the EGFP-negative cells could self-renew.

To ask whether \textit{Wnt1-cre}-positive facial SKPs derive from both whisker and hair follicles, we cultured cells from \textit{Wnt1-cre;Z/EG} whisker pad versus non-whisker-pad frontal facial skin under SKPs conditions. Both tissues generated EGFP-positive SKP spheres (Fig. 2.2C,D) that could be passaged, and that expressed vimentin, PDGFR\textalpha{} (Fig. 2.2C,D), nestin and fibronectin (data not shown), all markers typical of SKP spheres. Thus, neural crest-derived facial SKPs derive from hair as well as whisker follicles.
Figure 2.2. In facial but not dorsal trunk skin, skin-derived precursors (SKPs) derive from neural crest. (A, B): Primary SKP spheres generated from neonatal Wnt1-cre;Z/EG facial (A) and dorsal trunk (B) skin. In the bottom panels, single spheres are shown at high magnification. Scalebar = 50 μm. (C, D): Primary facial SKP spheres from whisker pad skin and from facial skin outside of the whisker pad, immunostained for EGFP (green), and the SKP markers vimentin (red) and PDGFRα (blue). Scale bar = 100 μm. (E, F): Flow cytometry plots of neonatal Wnt1-cre;Z/EG facial (E) and dorsal trunk (F) skin, sorted on the basis of EGFP expression. Left panels show the primary data, with the bottom blue boxes indicating the cells that were collected for culture. Numbers represent the percentage of cells in each quadrant of the plot. Right panels show the data plotted as relative numbers of cells expressing no or high relative levels of EGFP. The percentage of cells in each of those two groups is indicated. (G, H): Skin cells sorted as in (E, F) were cultured in SKPs medium for 1–2 weeks, passaged, and photographed 1 week later. Arrows indicate secondary SKP spheres. For facial skin (G), the EGFP-positive cell fraction contained most of the SKP forming activity, although some SKP spheres were generated from the EGFP-negative population. For dorsal trunk skin (H), EGFP-positive cells did not generate SKP spheres when passaged, but remained as single cells or small clumps of cells, and virtually all of the SKP-forming activity was present within the EGFP-negative cell fraction. Scale bar = 100μm. Abbreviations: EGFP, enhanced green fluorescent protein; PDGFRα, platelet derived growth factor receptor α.
To confirm that facial but not dorsal SKPs derive from the neural crest, we used flow cytometry to sort EGFP-positive and negative cells from Wnt1-cre;Z/EG facial and dorsal skin samples that contained both the epidermis and the dermis (Fig. 2.2E,F). Approximately 30% of facial and 2% of dorsal skin cells expressed robust levels of EGFP, consistent with the immunocytochemical results. When these sorted cells were cultured at low density, very different results were obtained for facial versus dorsal cells. In facial skin, almost all of the primary sphere-forming ability was present in the EGFP-positive cells, although the occasional sphere was generated from the EGFP-negative fraction, potentially because the reporter is not 100% penetrant. These EGFP-positive spheres could be passaged and would self-renew to generate EGFP-positive secondary spheres (Fig. 2.2G). For dorsal skin, virtually all of the sphere-forming ability was present within the EGFP-negative population, and these EGFP-negative spheres self-renewed. In contrast, only very few small primary spheres formed from the EGFP-positive population, and these spheres did not self-renew when passaged (Fig. 2.2H). Thus, SKPs derive from Wnt1-expressing neural crest in facial, but not dorsal dermis, consistent with the in vivo lineage tracing.

2.4.3 Dorsal trunk SKPs, & their hair follicle niche, are somite-derived.

The dorsal trunk dermis arises from the somites\textsuperscript{23,308} suggesting that, despite their similarities to neural crest precursors, dorsal follicle dermal precursors and the SKPs they generate might also derive from this embryonic origin. To address this possibility, we utilized a transgenic Myf5-cre mouse, where cre recombinase is expressed in cells deriving from the somites\textsuperscript{317,375}, and crossed it to R26R reporter mice that carry a floxed β-galactosidase allele in the ROSA26 locus\textsuperscript{377}. In these mice, somite-derived cells will express β-galactosidase. X-gal staining of neonatal dorsal skin from these mice demonstrated that, as predicted by their somite origin, most dermal cells were β-galactosidase-positive (data not shown). Moreover, both X-gal staining and immunocytochemistry for β-galactosidase (Fig. 2.3A) demonstrated that hair follicle DP and DS cells expressed β-galactosidase, indicating that they too were somite-derived. In contrast, dermal cells of facial skin, including those associated with hair follicles, were β-galactosidase-negative (Fig. 2.3B), as predicted by their neural crest origin.

To more definitively identify the cell types that were somite-derived within the dorsal dermis, we crossed the Myf5-cre mice to the R26YFP mouse which has a floxed EYFP allele in
Figure 2.3. In dorsal trunk but not facial or ventral trunk skin, hair follicle DP/dermal sheath (DS) and Skin-derived precursors (SKPs) derive from the somites. (A, B): Dorsal trunk (A) and facial (B) skin sections from neonatal Myf5-cre;R26R mice, stained with X-gal ([A], left panel and [B], blue) or immunostained for \( \beta \)-galactosidase ([A], middle and right panels, red) to detect \( \beta \)-galactosidase. Tissue was counterstained with nuclear fast red (red in [A], left panel and [B]) or propidium iodide (blue in [A], right panel) to show morphology. Arrows denote hair and whisker follicle DP. Scale bar = 50 \( \mu \text{m} \) (A), 500 \( \mu \text{m} \) ([B], left) and 100 \( \mu \text{m} \) ([B], right). (C–F): Dorsal trunk (C, D), facial (E), and ventral trunk (F) skin sections from neonatal Myf5-cre;R26YFP mice, immunostained for EYFP (green), fibronectin ([C], red) or NCAM ([D–F], red). Tissues were counterstained with the nuclear dye TOPRO3 (blue) to show morphology. Arrows denote follicle DP, which are EYFP-positive in dorsal trunk skin (C, D), but not in facial (E) or ventral trunk (F) skin. Arrowheads denote EYFP-positive DS cells in dorsal trunk skin (C, D). Note that muscle cells (M) in facial skin (E) are EYFP-positive. Scale bar = 50 \( \mu \text{m} \). (G): Secondary SKP spheres generated from neonatal Myf5-cre;R26R dorsal trunk skin (left panel), facial skin (middle panel), and ventral trunk skin (right panel), stained with X-gal to detect \( \beta \)-galactosidase (blue) and counterstained with nuclear fast red (red). Scale bar = 200 \( \mu \text{m} \). Abbreviations: d, interfollicular dermis; DP, dermal papilla; epi, epidermis; HF, hair follicle; M, muscle; Mc, melanocytes; NCAM, neural cell adhesion molecule; WP, whisker papilla; YFP, yellow fluorescent protein.
the same ROSA26 locus\textsuperscript{376}. These mice express EYFP in cells of the somite lineage. Double-label immunocytochemistry for EYFP and the dermal fibroblast marker fibronectin on neonatal dorsal skin demonstrated that EYFP was expressed in fibronectin-positive cells of interfollicular and follicular dermis (Fig. 2.3C). A similar analysis for EYFP and NCAM, a DP and DS marker that is expressed in the endogenous sox2-positive dermal precursors\textsuperscript{14}, demonstrated that the follicle dermal precursors also express EYFP (Fig. 2.3D). In contrast, EYFP was not expressed in NCAM-positive follicle DP and DS cells in facial skin (Fig. 2.3E) although it was expressed in muscle cells (Fig. 2.3E). Analysis of EYFP in ventral dermis showed that interfollicular dermal cells as well as follicle DP and DS cells were negative for EYFP (Fig. 2.3F), consistent with a lateral plate rather than somite origin for this region of the trunk dermis.

These findings predict that dorsal, but not ventral trunk SKPs are somite-derived. To test this prediction, we cultured cells from dorsal skin of neonatal Myf5-cre;R26R mice under SKP conditions at low density. For comparison, we cultured facial and ventral skin from the same mice. These cells were then passaged once at clonal density\textsuperscript{7}. X-gal staining of the resultant secondary spheres demonstrated that virtually all SKP spheres isolated from dorsal back, but not ventral trunk or facial skin expressed β-galactosidase (Fig. 2.3G). Thus, follicle dermal precursors and the SKPs they generate derive from a number of distinct developmental origins, as does the dermis itself.

**2.4.4 Neural crest- & somite-derived SKPs are similar and both populations generate functional Schwann cells.**

Our previous work showed that both dorsal and facial SKPs self-renew, and that both can differentiate into βIII-tubulin-positive neurons and SMA-positive myofibroblasts\textsuperscript{7,14}. Moreover, we have previously shown that dorsal SKPs generate bona fide Schwann cells\textsuperscript{10,12}, a cell type thought to be only neural crest-derived. These findings suggest that, in spite of their differing developmental origins, these two populations of neural crest-like stem cells are functionally similar. To address this idea more definitively, we performed a series of side-by-side comparisons. Initially, we characterized the two populations with regard to a series of SKP markers, fibronectin, vimentin, nestin, and versican; immunostaining demonstrated that both populations expressed these markers (Fig. 2.4A). We then quantitatively characterized their proliferation and self-renewal. For proliferation, SKPs were grown under standard conditions in
Figure 2.4. Facial and dorsal trunk skin-derived precursors (SKPs) display similar properties. (A): Secondary SKP spheres generated from neonatal mouse dorsal trunk skin and facial skin, immunostained for fibronectin, vimentin, nestin, and versican. (B): Primary SKP spheres isolated from neonatal wild-type dorsal trunk skin and facial skin, immunostained for Ki67 (green), and counterstained with Hoechst to show cell nuclei (blue). (C): Quantification of Ki67-positive cells from experiments similar to that shown in (B). n = 3 independent experiments. (D): Quantification of the percentage of cells that form a new sphere from primary SKPs plated at clonal density (2,500 cells/ml) in methylcellulose cultures. n = 3 independent experiments.
FGF2 and EGF, were passaged once at similar densities, and 8 days later, these secondary spheres were analyzed by immunostaining for Ki67, a proliferation marker. Approximately 4% of the cells in both facial and back skin SKP spheres were actively proliferating at this timepoint (Figs. 2.4B,C). To assess self-renewal, primary SKP spheres from facial and dorsal skin were dissociated to single cells that were then cultured at clonal density in methylcellulose-containing medium, and the number of new spheres were quantified. This analysis demonstrated that both populations generated approximately 1-2% secondary spheres (Fig. 2.4D). Together with our previous data, these findings indicate that SKPs from both facial and dorsal skin are similar, at least with regard to their proliferation, self-renewal and differentiation ability.

One surprising inference of this work is that somite-derived dermal precursors can apparently generate Schwann cells. To more definitively test this idea, we first confirmed that total dorsal SKPs could generate Schwann cells, as we published previously by differentiating them under previously-defined conditions. Immunocytochemistry revealed that dorsal SKPs generated bipolar cells that expressed the Schwann cell markers, S100β and GFAP (Fig. 2.5A), as did facial SKPs (Fig. 2.5A). To ask whether these two SKP populations were equally efficient at generating these Schwann-like cells, we generated neonatal facial and dorsal SKPs at low density, passaged them once, and then differentiated single, isolated SKP spheres under Schwann cell conditions, as we have described previously. Immunostaining 2 weeks later for S100β and GFAP demonstrated that 48% and 44% of facial versus dorsal SKP spheres generated bipolar, Schwann-like cells.

These data indicate that dorsal SKPs are similar to facial SKPs in their ability to generate cells with Schwann cell characteristics. To definitively establish that somite-derived precursors could generate functional Schwann cells, we sorted EYFP-positive and negative cells from the dorsal skin of neonatal Myf5-cre;R26YFP mice (Fig. 2.5B). We then grew the EYFP-positive and negative skin cells under SKP conditions. As predicted, the vast majority of SKP spheres were generated from the EYFP-positive cells, and all of these spheres were EYFP-positive (Fig. 2.5C). We then differentiated these EYFP-positive, somite-derived SKPs under Schwann cell conditions. After three weeks, cultures were largely comprised of two EYFP-positive cell populations; flat cells with large nuclei, and smaller bipolar cells with a Schwann cell morphology (Fig. 2.5D). Immunocytochemistry revealed that these EYFP-positive spindle-
FIGURE 2.5

A  Facial  Dorsal  
S100 GFAP  Hoechst  

B  Myt5-Cre:R26YFP dorsal trunk  
Relative # of cells  
YFP  

C  Sorted YFP(+ve) cells  Sorted YFP(-ve) cells  

D  
Hoechst  YFP  P0  S100  
Hoechst  YFP  S100  p75NTR  

E  
Hoechst  YFP  bIII-tubulin  P0  

**Figure 2.5. Somite-derived skin-derived precursors (SKPs) generate Schwann cells.** (A): Neonatal mouse dorsal trunk and facial skin cells cultured as SKPs, differentiated under gliogenic conditions for 2–3 weeks, and immunostained for the Schwann cell markers S100β (green) and GFAP (red), and counterstained with Hoechst (blue). (B): Flow cytometry plots of *Myf5-cre;R26YFP* dorsal trunk skin cells, sorted for relative levels of EYFP expression. The left panel shows the primary data, and the blue boxes show the cell fractions that were cultured as EYFP-positive versus negative cells. The right panel shows the same data plotted as relative cell numbers versus EYFP expression. The percentage of cells in each fraction is indicated. (C): Cells sorted as in (B) and cultured for 7 days in SKPs conditions. The left two panels show the SKP spheres derived from the EYFP-positive fraction and the right two panels show the EYFP-negative fraction, with the left panels of each pair showing phase illumination and the right the same fields with fluorescence illumination. (D): *Myf5-cre;R26YFP*-positive dorsal trunk SKPs were differentiated under gliogenic conditions for 2–3 weeks, and immunostained for EYFP (green), and the Schwann cell markers P0 (red, top panels) S100β (blue, top panels; red bottom panels) and p75NTR (blue, bottom panels). Arrows in top panels indicate EYFP-positive, P0-positive, S100β-positive spindle-shaped Schwann cells and in the bottom panels indicate EYFP-positive, S100β-positive, p75NTR-positive Schwann cells. Arrowheads indicate EYFP-positive, flat cells that are negative for the Schwann cell markers. Cells were counterstained with Hoechst (turquoise). Scale bar =100 μm. (E): Differentiated *Myf5-cre;R26YFP*-positive dorsal trunk SKPs were cocultured with axons of sympathetic neurons in compartmented cultures for 8 days, and immunostained for EYFP (green), the Schwann cell marker P0 (blue), and the axonal marker βIII-tubulin (red). Arrows indicate a representative EYFP-positive, P0-positive, spindleshaped Schwann cell associated with a βIII-tubulin-positive axon. Arrowheads indicate an EYFP-positive, flat cell that is not associated with an axon and that is negative for Schwann cell markers. Cells were counterstained with Hoechst (turquoise). Scale bar = 100 μm.

Abbreviations: GFAP, glial fibrillary acidic protein; YFP, yellow fluorescent protein.
shaped cells expressed the Schwann cell markers P0, p75NTR and S100β, whereas the flat cells were negative for these markers (Fig. 2.5D).

We then asked whether these putative Schwann cells were functional by coculturing them with axons of sympathetic neurons that were grown in compartmented cultures. In these cultures neuronal cell bodies are plated in central compartments, and then grow their axons into sealed side compartments. EYFP-positive cells were sorted from dorsal skin of neonatal Myf5-cre;R26YFP mice, were grown as primary SKP spheres, and then differentiated under Schwann cell conditions for 3-4 weeks. These differentiated cultures, which contained a subpopulation of cells with an appropriate Schwann cell morphology, were then plated in side compartments that contained axons, but not neuronal cell bodies. Immunostaining 8 days later showed that many EYFP-positive cells had associated with βIII-tubulin-positive axons and that these expressed the peripheral myelin protein P0 (Fig. 2.5E). These results were indistinguishable from those obtained when cultured, peripheral nerve-derived Schwann cells were plated in similar cultures (data not shown). These data, together with our previous findings showing that SKPs generated from dorsal trunk skin associate with and myelinate axons when transplanted into the peripheral nerve3, provide strong evidence that somite-derived SKPs generate functional Schwann cells, a cell previously thought to only be generated by neural crest precursors.

2.4.5 SKPs of distinct developmental origins are highly similar at the transcriptional level, and differ from bone marrow MSCs.

To more comprehensively define how similar these developmentally distinct populations of SKPs are, we performed global gene expression analysis of adult SKPs derived from skin of dorsal trunk, ventral trunk, and the face. For comparison, we analyzed another adult stem cell with mesenchymal potential, bone marrow-derived mesenchymal stromal cells (MSCs). To perform this analysis, we compared three independent isolates each of dorsal trunk SKPs, ventral trunk SKPs and facial SKPs and four isolates of MSCs, all generated from adult rats. RNA samples deriving from these cells were then analyzed on the Affymetrix GeneChip Rat Gene 1.0 ST Array. Spearman rank correlations, computed between each sample pair based on the microarray expression profiles demonstrated that dorsal, ventral and facial SKPs were virtually identical (Fig. 2.6A). In contrast to this high similarity between dorsal, ventral and facial SKPs, all three populations were different from adult MSCs (Fig. 2.6A). Cluster analysis, performed on the samples using a standard hierarchical clustering algorithm (correlation distance, average
Figure 2.6. **Microarray analysis of adult dorsal trunk, ventral trunk, and facial SKPs versus MSCs.** Microarray analysis was performed to compare gene expression patterns among adult rat dorsal trunk, facial, and ventral trunk SKPs. Adult rat bone marrow MSCs were used as a comparator. Three independent isolates of secondary passage SKPs from dorsal trunk skin (dSKPs 1–3), facial skin (fSKPs 1–3), ventral trunk skin (fSKPs 1–3), and four isolates of MSCs (MSCs 1–4) were compared. (A): Spearman rank correlation matrix computed for the microarray experiments based on the 3,182 probesets showing the most variation across the experiments, as visualized by color-coding, with yellow representing the most highly correlated samples, and blue the least correlated. Note that the dorsal trunk, facial, and ventral trunk SKP samples are highly correlated with each other, whereas they show less correlation with the MSCs. (B): Microarray datasets from all four sets of samples were clustered using hierarchical clustering with correlation distance and average linkage. The significance of the hierarchical clustering result was assessed using AU and BP resampling implemented in the R package pvclust. (C): Venn diagrams of pairwise comparisons between facial SKPs, dorsal trunk SKPs, and MSCs to identify genes differentially expressed between each pair of samples using an analysis similar to one-way ANOVA implemented in the LIMMA bioconductor package. The Venn diagrams show significantly differentially expressed genes (p < .05, Benjamini) that are in common among the pairwise comparisons, revealing that facial SKPs and dorsal SKPs are more similar to each other than either of them are to MSCs. (D): Three-way comparison was conducted across the groups to identify genes that show evidence of differential expression (analysis similar to one-way analysis of variance). Expression profiles of 2,603 genes, identified as differentially expressed (p < .05, Benjamini), are plotted as a heatmap. Abbreviations: AU, approximately unbiased; BP, bootstrap probability; MSC, mesenchymal stromal cell; SKP, skin-derived precursor.
**Figure 2.7. SKPs of all developmental origins express neural crest signature genes but retain a lineage history at the gene expression level.** (A): Microarray expression levels of genes expressed in embryonic neural crest precursors in adult rat facial, dorsal trunk, and ventral trunk SKPs, plotted as a heatmap. Red indicates the lowest relative levels of expression and dark blue the highest, as defined by the color key. (B): Reverse transcription polymerase chain reactions (RT-PCRs) for the same genes shown in (A), in total RNA isolated from neonatal murine dorsal trunk and facial secondary SKP spheres. Total RNA from E8.5 murine embryos was used as a positive control. (C, D): Pairwise differential expression analysis was conducted between (C) facial and dorsal trunk SKPs and (D) ventral trunk and dorsal trunk SKPs from adult rats using the LIMMA bioconductor package. The 35 genes showing most significant differential expression between the two populations on the volcano plots are shown and are listed in Supporting Information Tables 1 and 2, after multiple testing correction. The positive log fold changes indicate genes that are expressed at higher levels in dorsal SKPs (C, D), and the negative log fold changes those that are expressed at decreased levels in dorsal SKPs relative to facial SKPs (C) or ventral trunk SKPs (D). (E): Microarray expression levels of transcription factors that were identified as being among the most differentially expressed in the analysis in (C), plotted as a heatmap. (F): RT-PCRs for the genes highlighted in (E) in total RNA from neonatal murine dorsal and facial secondary SKP spheres, highlighting the differential expression. Total RNA from E8.5 murine embryos was used as a positive control. (G): RT-PCRs for the genes highlighted in (F) in total RNA from uncultured, purified EGFP-positive cells from neonatal Sox2-EGFP mouse dorsal trunk and facial skin, highlighting differential expression in uncultured dermal precursors. Abbreviations: EGFP, enhanced green fluorescent protein; SKP, skin-derived precursor.
linkage clustering), confirmed these conclusions, demonstrating that the three SKPs populations grouped together, and that all three were distinct from the MSC samples (Fig. 2.6B).

To delineate the extent of differences between the transcriptomes of the neural crest-derived facial SKPs and somite-derived SKPs versus MSCs, we performed 3-way differential expression analysis, similar to a one-way ANOVA, using the LIMMA bioconductor package. The Venn diagrams show the numbers of significantly differentially expressed genes (p<0.05, Benjamini-Hochberg) that are in common amongst the comparisons (Fig. 2.6C). Taken together, a total of 2603 genes showed evidence of differential expression among any of the three groups; the expression levels of these genes are provided as a heatmap (Fig. 2.6D). Of these genes, only 106 were significantly different between dorsal and facial SKPs, while 2233 and 2525 differed between MSCs versus dorsal SKPs and MSCs versus facial SKPs, respectively.

These data argue that precursor cells of at least two, and potentially three, different developmental origins converge on to a highly similar phenotype. We therefore directly compared the expression of genes associated with neural crest specification, focusing on Slug, Snail, Twist, Sox9, Sox10, Foxd3, and Ap2a1. Heatmaps of the microarray data showed that these genes were expressed at similar levels in all three of the adult rat SKP samples, as were p75NTR and RhoB, which are also associated with neural crest precursors (Fig. 2.7A). RT-PCR analyses of neonatal murine skin confirmed that these mRNAs were also expressed at similar levels in neonatal murine dorsal versus facial SKPs (Fig. 2.7B).

2.4.6 Developmentally-distinct Sox2:EGFP-positive dermal precursors and the SKPs they generate maintain a lineage history at the gene expression level.

While these analyses indicate that mesenchymal precursors of different developmental origins converge to a very similar adult precursor phenotype, a standard pair-wise differential expression comparison of facial versus dorsal trunk SKPs and dorsal trunk versus ventral trunk SKPs using linear models demonstrated that a subset of genes were significantly differentially expressed (P < 0.05, Benjamini-Hochberg) (Fig. 2.7C,D; Supplemental Tables 1 and 2). Of the 35 most differentially expressed genes in the facial versus dorsal comparison, ten were higher in dorsal SKPs, and 25 in facial SKPs. Of the 35 most differentially expressed genes in the dorsal versus
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**Supplemental Table 1.** List of genes from multiple correlation analysis as shown in Fig. 7C. Negative log fold change (LogFC) indicates genes that are down in facial SKPs (fSKPs) versus dorsal SKPs (dSKPs) while positive log fold change indicates genes that are up in facial SKPs versus dorsal SKPs.
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**Supplemental Table 2.** List of genes from multiple correlation analysis as shown in Fig. 7D. Negative log fold change (LogFC) indicates genes that are down in dorsal SKPs (dSKPs) versus ventral SKPs (vSKPs) while positive log fold change indicates genes that are up in dorsal SKPs versus ventral SKPs.
ventral comparison, 4 were higher in dorsal SKPs, and 31 in ventral SKPs (Tables 1 and 2).

Intriguingly, many of these genes are important during embryogenesis. In particular, dorsal trunk SKPs express high levels of the Zic1 transcription factor relative to both facial and ventral trunk SKPs, and the hox transcription factors Hoxa5, Hoxc4, Hoxc6 and Hoxc9 relative to facial SKPs (Fig. 2.7D; Table 1). In contrast, facial SKPs expressed high relative levels of Pax3, and Msx1, both of which are transcription factors associated with cranial neural crest cells\textsuperscript{387,388}, and Mab-21-like 1 and 2, mammalian homologues of the C. elegans mab-21 cell fate gene that are expressed during embryogenesis\textsuperscript{389} (Fig. 2.7D; Table 1). The relative enrichment of these different mRNAs was confirmed by RT-PCR analysis of neonatal murine dorsal versus facial SKPs (Fig. 2.7E).

To ask whether these differences were also seen in the endogenous dermal precursors that give rise to SKPs, we used flow cytometry to prospectively-isolate EGFP-positive cells from facial versus dorsal skin of Sox2-EGFP neonatal mice. In these mice, EGFP is knocked-in to the Sox2 locus\textsuperscript{379}, and this Sox2-EGFP reporter is selectively expressed in follicle DP and DS cells\textsuperscript{14,333}. We then performed RT-PCR analysis on these two cell populations. This analysis demonstrated that Mab21-like 1 and 2, Eya1, and Pax3 mRNAs were all enriched in facial EGFP-positive precursors (Fig. 2.7F) as they were in facial SKPs. In contrast, Zic1, Hoxa5, Hoxc6, Hoxc4, and Hoxc9 mRNAs were all highly enriched in dorsal EGFP-positive precursors, as they were in dorsal SKPs (Fig. 2.7F). Thus, while these different dermal precursor populations are highly similar, they maintain a history of their distinct developmental origins.

2.5 Discussion

The data presented here support three conclusions. First, they indicate that SKPs and the dermal precursors from which they arise are generated by multiple developmental lineages. Specifically, the lineage tracing indicates that follicle DS and DP cells derive from the neural crest in the face and from the somites in the dorsal trunk, as do SKPs themselves. While we have not definitively established the origin of ventral trunk SKPs, we show that they do not arise from the somites, and it is likely that, like the dermis, they derive from the lateral plate. Second, our findings demonstrate that, in spite of their different developmental origins, facial and dorsal SKPs are functionally similar and, perhaps most surprisingly, that somite-derived SKPs can generate a functional neural crest cell type, Schwann cells. Finally, the microarray analysis
emphasizes the similarities between facial, dorsal trunk and ventral trunk SKPs, showing that they are highly similar to each other at the transcriptional level, but different from another adult mesenchymal precursor, bone-marrow derived MSCs. Intriguingly, these gene expression studies also defined a number of embryonic transcription factors that are highly differentially-expressed in facial versus dorsal trunk SKPs, and in the Sox2-positive dermal precursors from which they derive. Thus, in spite of their similarities, these dermal precursors maintain a memory of their developmental origins. These findings demonstrate that dermal stem cells are generated by distinct convergent developmental pathways, and indicate that adult stem cells with almost indistinguishable phenotypes can be generated from multiple developmental lineages.

Previous work showed that SKPs display similarities to neural crest precursors; they express neural crest markers, differentiate into neural and mesodermal lineages, and migrate like embryonic neural crest precursors when transplanted into the embryonic chick neural crest migratory stream7. We also showed previously that SKPs differentiated into functional Schwann cells, a cell type that is only thought to be generated from neural crest10,12. Importantly, data presented here indicate that dorsal SKPs that are definitively marked as being of a somite origin also generate Schwann cells, a surprising finding. Do the endogenous Sox2-positive precursors that generate SKPs ever differentiate into Schwann cells in vivo? Previous work indicates that follicle dermal precursors differentiate into dermal cell types and induce hair follicle morphogenesis, but there are no data to indicate that they generate other neural crest derivatives in vivo. However, when these Sox2-positive precursors are directly differentiated in culture, without going through a SKPs intermediate, they do in fact generate cells with characteristics of βIII-tubulin-positive neurons14, and microarray analysis indicates that they express many markers similar to neural crest precursors14. Similar results have been obtained for DP cells isolated using other approaches7,281,286,333, confirming these findings. We therefore propose that, as seen with embryonic neural crest precursors367, follicle dermal precursors have a broader differentiation potential than they usually display in vivo, and that their dermal environment restricts their differentiation to appropriate cell types. A key question for the future will be whether or not this broader potential is ever unmasked in vivo either under basal conditions and/or following injury.

Our finding that hair follicle DP and DS from dorsal trunk skin derive from a nonneural crest origin does not rule out the possibility that other neural crest-derived precursors reside in
hair follicles. In particular, previous work defined a population of neural crest precursors within
the follicle bulge region that, like SKPs, can generate peripheral neural progeny and some
mesenchymal derivatives and lineage tracing demonstrated Wnt-1Cre-R26R-positive cells
located in close proximity to the bulge region. Here we have confirmed the presence of Wnt1-
cre;Z/EG-positive cells in p75NTR-positive nerves that were very closely associated with the
bulge region. This finding is particularly intriguing in light of a recent report showing that
multipotent neural crest precursors enter the skin via peripheral nerves and contribute
melanocytes during embryogenesis. While our sorting data argue that these Wnt-1-cre;Z/EG-
positive cells do not generate SKPs, it is nonetheless possible that they play a key role in vivo
either by contributing melanoblasts to the follicle and/or Schwann cells to nerve endings.

The experiments presented here have used a number of different Cre reporter lines to
perform lineage tracing, and the data must therefore be interpreted in light of the limitations of
this approach. We previously utilized β-galactosidase expression in a Wnt1-cre;R26R mouse to
establish that facial SKPs and the follicle DP and DS niche from which they originate are neural
crest-derived. In that same study we reported a small number of β-galactosidase-positive cells
within and/or adjacent to dorsal trunk follicle DP, and interpreted those findings as indicative of
a neural crest origin for at least some of these DP cells. Here, our double-label
immunocytochemical analysis of dorsal trunk skin sections from Wnt1-cre;Z/EG mice showed
that tyrosinase-positive melanocytes, but not NCAM-positive DP cells, expressed EGFP. These
findings, combined with our data showing that dorsal skin DP were genetically-tagged in Myf5-
cre;R26R and Myf5-cre;R26YFP mice strongly argue that dorsal trunk DP and DS cells are
somite-derived.

The concept that cells of two developmental origins can converge on to a similar cellular
phenotype is not a new one, particularly with regard to neural crest derivatives. For example, all
of the mesenchymal derivatives of the face, including bone, cartilage, and dermis, derive from
the neural crest, while those of the body derive from the somites or the lateral plate. Thus, neural crest cells can and do generate mesodermal progeny. While there is no direct
precedent for mesodermal precursors generating neural crest-specific progeny such as cells of the
peripheral nervous system, it is intriguing that many nonneural crest-derived tissues contain
neuroendocrine cell types similar to those generated from the neural crest. Our finding that cells
of nonneural crest origin can converge on to a phenotype with similarities to neural crest
precursors suggests that perhaps a neural crest-like precursor may play an important role in these tissues. A good example of this would be the pancreas which for many years was thought to be at least in part neural crest-derived because of the endocrine and exocrine cells that it contains.

Our large scale gene expression analyses indicate that, in spite of their different developmental origins, facial, dorsal trunk and ventral trunk SKPs are highly similar. However, all of these populations are distinct from another adult mesenchymal precursor, bone marrow-derived MSCs. These results are in good agreement with the functional properties of these different populations. Dorsal and facial SKPs are almost indistinguishable with regard to their growth and differentiation in culture, and transplant studies indicate that they are apparently interchangeable with regard to transplantation into facial or dorsal trunk skin environments (JAB, HJ, FDM, unpublished data). In contrast, although both SKPs and MSCs can generate cells such as adipocytes, cartilage and bone cells in culture, when transplanted into the dermis MSCs do not home to a DP niche, as do SKPs, nor do they induce hair follicle morphogenesis. Thus, SKPs and MSCs are two different precursor populations, in spite of a partially-shared differentiation potential. Intriguingly, during embryogenesis, neural crest stem cells give rise to bone marrow MSCs, suggesting a parent-progeny relationship between these different precursors and raising the possibility that neural crest-like precursors, perhaps of nonneural crest origin, continue to give rise to MSCs in adulthood.

Together, these data support the concept that a stem cell of non-neural crest origin can generate peripheral neural cells that are only thought to derive from the neural crest. These findings raise some interesting questions with regard to the gene-directed transdifferentiation of dermal fibroblasts into neurons. While in studies of this type, it is assumed that dermal fibroblasts represent a homogeneous population that are committed to a fibroblast lineage, findings presented here indicate that a subset of dermal cells are endogenous multipotent precursors and that the genesis of neurons from these precursors may reflect the unmasking of the endogenous potential of these cells rather than transdifferentiation. Whether similar multipotent cells reside within other tissues and/or whether they also have the potential to generate peripheral neural or neuroendocrine cell types is a key question for the future.
Chapter 3
Sox2 defines multiple precursor populations in the developing, mature and regenerating skin.

3.1 Abstract

The transcription factor Sox2 regulates stem cell populations in diverse developing and mature tissues\textsuperscript{30,31}. The Miller laboratory (Biernaskie et al) recently demonstrated that Sox2 marks hair-follicle dermal papilla (DP) and dermal sheath (DS) cells, a dermal precursor that is essential for morphogenesis and wound healing of skin tissue\textsuperscript{14}. This finding raises the possibility that Sox2 might play key roles in skin development and maintenance by regulating these precursors. However, little was known about the full expression pattern of Sox2 in skin. Here, we show that Sox2 defines multiple precursor populations in neonatal, mature and regenerating skin. Specifically, immunostaining of Sox2:EGFP mouse skin defines six distinct subpopulations of neonatal Sox2-expressing skin cells: DP and DS cells in growing hair follicles, melanocyte precursors in the outer rooth sheath of hair follicles, nerve-terminal (NT) Schwann cell precursors at the junction between hair follicles and skin nerves, Schwann cell/neural crest precursors in skin nerves and Merkel cell precursors in the follicular/interfollicular epidermis. Although Sox2 was detected in all of these six cell types in neonates, its expression became limited to NT cells and Merkel cell precursors in adults, as well as to hair follicle DP and DS cells during the follicle growth phase. However, skin injury led to reexpression of Sox2 in DP and DS cells coincident with the onset of new follicle growth, and in cells with characteristics of Schwann cell/neural crest precursors in skin nerves. These data define multiple precursor populations in adult skin and suggest that Sox2 may play a key role in orchestrating these distinct precursor populations for normal development and wound healing.

3.2 Introduction

The mechanisms that regulate the maintenance and repair of adult tissues are still an open question. Intriguingly, recent data suggests that most adult mammalian tissues contain resident stem cells that serve as a reservoir of activity for tissue maintenance and repair\textsuperscript{39}. Moreover, evidence is emerging that when these adult tissue stem cells become depleted or impaired, this
causes premature tissue aging\textsuperscript{37,366}. It is therefore essential to define the cellular mechanisms that promote the maintenance of these adult tissue stem cells over an animal’s lifetime.

One potential candidate gene involved in stem-cell-based tissue maintenance is the transcription factor Sox2 which was recently identified as a signature gene for adult dermal precursors\textsuperscript{30,31}. Sox2 is a member of the SRY-related High Mobility Group transcriptional regulator family, and is involved in maintaining self-renewal and pluripotency in embryonic stem cells\textsuperscript{392,393}, in regulating neural stem and progenitor cells\textsuperscript{205,394,394,398} and in governing fate decision of several epithelial precursors\textsuperscript{395,399-403}. In very early stages of embryogenesis, Sox2 functions as a master regulator to orchestrate self-renewal and pluripotency of embryonic stem cells with other transcription factors such as Oct4 and Nanog\textsuperscript{49}. Recently, Sox2 has also been recognized as one of the key reprogramming factors that induce the pluripotent state in adult fully-differentiated cells\textsuperscript{404}. Additional support for the importance of Sox2 during development comes from the finding that homozygous Sox2 mutant mice die during early embryogenesis\textsuperscript{405}.

In later stages of embryogenesis, Sox2 is expressed in diverse neural and epithelial tissues, and marks precursor populations\textsuperscript{31}. In this regard, human Sox2 mutations cause anophthalmia, defective hippocampal development, cognitive defects and seizures\textsuperscript{406,408}, indicating its importance in neural tissues\textsuperscript{31}. In the mammalian CNS, low Sox2 expression levels result in a loss of striatal and thalamic tissue, epilepsy and neurodegeneration, which might result from decreased stem cell numbers and defective neuronal differentiation\textsuperscript{396,409}. Favaro et al (2009) also developed nervous system-specific conditional Sox2 knockout mice, and demonstrated that Sox2 deletion causes hippocampal defects with neural precursor loss, and showed that neural precursors control their status at least in part via Sox2-dependent cell-autonomous mechanisms\textsuperscript{397}. These authors also showed that adult Sox2 deletion causes loss of hippocampal radial glia and a reduction of doublecortin-positive neuroblasts as well as NeuN-positive early neurons\textsuperscript{397}. These data indicate that Sox2 is required for NSC maintenance both during early development and in the adult brain.

A number of studies have also demonstrated that alterations in the levels of Sox2 have an impact on neural precursors. For example, Graham et al (2003) demonstrated that constitutive expression of Sox2 inhibits neuronal differentiation and results in cells that maintain progenitor cell characteristics, while inhibition of Sox2 signaling results in the delamination of neural
progenitor cells from the ventricular zone and exit from the cell cycle associated with a loss of progenitor markers and the onset of early neuronal differentiation markers\textsuperscript{394}. These data indicate that appropriate levels of Sox2 expression are required for NSC maintenance\textsuperscript{395}.

It is largely unknown whether adult somatic tissue stem cells also require Sox2 for their function\textsuperscript{31}. In this regard, Sox2 is expressed in and plays multiple roles in the trachea and lung from the embryo to the adult\textsuperscript{400,401}. Que et al. (2009) showed that, during embryogenesis, the absence of Sox2 results in the abnormal differentiation of tracheal cells and lung epithelial cells\textsuperscript{401}. The authors then conditionally deleted Sox2 in the adult trachea and lung to investigate its role in adult epithelial maintenance, and demonstrated that epithelial cells lacking Sox2 do not proliferate appropriately in vitro and are impaired in their ability to participate in tissue repair after injury in vivo\textsuperscript{401}. These data indicate that Sox2 is essential for adult epithelial precursor maintenance and thus in repair of the adult trachea and lung.

While Sox2 has been studied extensively in the nervous system, until recently, it was unclear whether Sox2 was expressed broadly in adult tissue stem cells and/or whether it played any broad role in adult tissue homeostasis\textsuperscript{31}. To address this issue, Arnold et al generated genetically engineered mice with a tamoxifen-inducible Cre allele or a suicide gene in the Sox2 locus to conduct fate mapping analysis of Sox2-expressing cells as well as to ablate Sox2-expressing lineages by drug administration\textsuperscript{30}. The authors then used these mice to identify a broad range of Sox2 expressing cells in adult mice, including tissue stem cells of stratified and glandular epithelia (glandular stomach, esophagus, tongue, brain, trachea and bronchiolar epithelium) as well as in sensory cells (Merkel cells and taste bud cells) and spermatogonial stem cells\textsuperscript{30}. To investigate the function of Sox2-expressing cells in maintaining tissue homeostasis, the authors then induced apoptosis of Sox2-expressing cells by drug administration on the adult mice expressing a suicide gene from the Sox2 locus\textsuperscript{30}. These experiments showed that, two weeks after treatment, the mice exhibited major defects in tissue homeostasis that lead to the death of the mice\textsuperscript{30}. Moreover, when the drug was used for a shorter period of time, the tissues recovered concomitant with the replenishment of Sox2-positive progenitors in these tissues\textsuperscript{30}. These data demonstrated that Sox2 marks many types of adult stem cells and regulates their functions, suggesting that Sox2 may act as a common stemness gene of adult stem cells, acting to maintain the homeostasis of diverse adult tissues. Thus, reduction or loss of Sox2 expression in adult stem cells would impair the replicative function of tissue-specific stem cells by limiting
their self-renewal capacity or lead to depletion of stem-cell populations by promoting unexpected and excessive differentiation of them.

As indicated by Arnold et al (2011), Sox2 is expressed in diverse adult tissues and Sox2-expressing cells are likely to function in tissue maintenance, by regulating the stemness of tissue-specific precursors. However, little is known about the full expression patterns of Sox2 and its potential functions in adult skin, a tissue that contains contain multiple precursor populations and that exhibits a high regenerative capacity. Our laboratory has recently identified Sox2-expressing precursors in the adult dermis, and shown that these are the source of most cultured skin-derived precursors (SKPs)\(^{14}\), as summarized in the general introduction. These findings raise the possibility that Sox2 might regulate the stemness of DP and DS cells, a dermal stem cell population. In this regard, Driskell et al. (2009) demonstrated that isolated DP cells that lack the Sox2-expressing subset fail to induce a certain type of hair follicle, thereby elucidating one potential function for Sox2 in DP cells\(^{333}\). However, little is unknown about whether Sox2 is essential for stemness of DP/DS cells or even other precursor types that are enriched in adult skin. Here, I hypothesized that Sox2 is essential for adult stem cell maintenance to ensure homeostasis in adult skin. To test this hypothesis, I started by elucidating the full pattern of Sox2 expression in neonatal and adult skin. Moreover, since Sox2 might also be involved in the stem cell response during tissue repair\(^{31}\), we characterized the expression of Sox2 in skin following injury. These studies define multiple potential Sox2-expressing populations in adult skin, and set the stage for functional studies asking about the role Sox2 plays in these different populations.

### 3.3 Materials & Methods

#### 3.3.1 Animals

All animal use was approved by the Animal Care Committee for the Hospital for Sick Children in accordance with the Canadian Council of Animal Care policies. Sox2-EGFP (Sox2\(^{EGFP+/+}\)) mice were the kind gift of Dr. Larysa Pevny and were maintained as previously reported (ref.). Wnt1-cre (Tg[Wnt1-cre]11Rth Tg[Wnt1-GAL4]11Rth/J) mice, and td-Tomato reporter (Gt[ROSA]26 Soro\(^{tm14(CAG-tdTomato)Hze}\)) mice were purchased from JaxMice (Jackson Laboratories, Bar Harbor, ME, http://jaxmice.jax.org). For in vivo fate mapping experiments, Sox2-EGFP; Wnt1-cre; td-Tomato mice were obtained by crossing Sox2-EGFP mice with Wnt1-cre and td-Tomato mice. For wound healing experiments, 2 month-old Sox2-EGFP mice were
anesthetized, and two 6 mm diameter full thickness punch wounds were made on each side of the
dorsum using 6mm Biopsy Punch (Miltex, KAI, Japan.). Mice were housed individually, and
euthanized at day 5 for immunocytochemical analysis.

3.3.2 Immunocytochemistry

**Fixation, cryoprotection and sectioning:** Skin tissues were dissected out from dorsal trunk or
facial whisker pad. Tissues were fixed with 4% paraformaldehyde (PFA) at 4°C overnight,
transferred to 30% sucrose in 0.1M PB for 24 hours (until the tissues sink), transferred to plastic
molds filled with OCT and frozen on dry ice. Frozen blocks can be stored at -80°C several
months if necessary. Then, 18 μm sections were obtained from frozen tissues on the cryostat,
and mounted on gelatin-coated slides. After sectioning, slides were stored at -30°C for a few
months if necessary.

**Immunocytochemistry:** Sections were dried at 37°C for 10-20 min and washed in phosphate
buffer solution (PBS, Hyclone, Logan, UT) three times. For permeabilization and blocking of
non-specific bindings, sections were then incubated with 0.3% TritonX100 (EMD Chemicals
Inc., Gibbstown, NJ) and 10% normal goat serum (NGS) for 1 hour at room temperature (RT).
Sections were then incubated with primary antibodies overnight at 4°C. After washed in PBS
three times, sections were incubated with secondary antibodies and nuclear staining dyes such as
Hoechst 33258 (Sigma) and TO-PRO-3 (Invitrogen) for 1 hour at RT, washed in PBS three times
and put with mounting media and cover slip. These slides can be stored at 4°C for a few days if
necessary.

**Antibodies:** Primary antibodies used in this study were as follows: rat anti-PDGFRα (1:500;
eBioscience), mouse anti-SMA (1:500; Sigma), rabbit anti-p75NTR (1:500; Promega), rabbit
anti-Nestin (1:500; Abcam), rat anti-Nestin (1:200; Abcam), rabbit anti-Pgp9.5/UCHL-1 (Novus
Biologicals; 1:1000), goat anti-Dct/TRP2 (Santa Cruz; 1:200), rat ant-c-Kit/CD117 (BD
Pharmingen; 1:100), rat anti-K8 (DSHB; 1:200), and mouse anti-Ki67 (1:200; BD Pharmingen).
Secondary antibodies used in this study were as follows: Alexa488/555/647-conjugated goat
anti-mouse, anti-rabbit, or anti-rat antibodies (1:1000; all from Invitrogen).

**Microscopy:** Immunocytochemistry was performed as described, and immunofluorescence was
analyzed using a Zeiss upright fluorescence microscope with Northern Eclipse acquisition
software, a Zeiss LSM 5 Pascal confocal microscope, or a spinning disk confocal microscope using Velocity acquisition software (Improvision).

3.4 Results

3.4.1 Sox2 is expressed in multiple hair follicle precursor populations in neonatal skin.

Sox2 has previously been reported to be expressed in hair follicle dermal precursor cells that are localized within the follicle dermal papilla (DP) and dermal sheath (DS), as well as in some uncharacterized cells in and around the hair follicle bulge\textsuperscript{14}. To ask whether these uncharacterized cells might represent a distinct hair follicle precursor population, we first performed immunocytochemical analysis of markers for hair follicle dermal precursors in skin of neonatal mice where EGFP is knocked-in to the sox2 locus (sox2:EGFP mice). Immunostaining for PDGFR\(\alpha\), a marker for hair follicle dermal cells, and for smooth muscle actin (SMA), a marker for DS cells, confirmed that the SMA and PDGFR\(\alpha\)-positive DS and DP cells expressed sox2:EGFP (Fig. 3.1A-C), as we previously published\textsuperscript{14}. In contrast, the sox2:EGFP-positive cells located in and around the bulge area did not express either of these two markers (data not shown), suggesting they were a distinct population.

We therefore asked whether these sox2:EGFP cells might represent a type of neural crest stem cell (NCSC) since a number of groups have identified NCSC-like activity associated with the bulge region\textsuperscript{19}. Immunostaining for p75NTR, a marker for NCSCs, demonstrated that many of the sox2:EGFP-positive cells associated with the bulge region coexpressed this protein (Fig. 3.1C,D). These bulge-associated sox2:EGFP-positive cells also expressed another NCSC marker, nestin, and displayed a highly distinct morphology and location (Fig. 3.1E). Since the bulge area is highly innervated, we asked whether these sox2:EGFP-positive cells were associated with nerves. Immunostaining for the axonal protein PGP9.5 showed that the sox2:EGFP-positive, nestin-positive, p75NTR-positive cells were localized at the terminals of PGP9.5-positive nerves that innervate the bulge (Fig. 3.1F). We have therefore called these cells NT cells (for nerve terminal cells) as a reflection of their unique location. Intriguingly, we have
FIGURE 3.1

A

B

C

D

E

F

G
Figure 3.1. In neonatal hair follicles, Sox2 is expressed in dermal precursors in the DP and DS and in cells within and at the terminals of skin nerves that express neural crest and Schwann cell precursor markers. (A-C): Dorsal skin sections from neonatal (P2-P7) Sox2:EGFP mice immunostained for EGFP (green) and the DP and DS marker PDGFRα (red, A; blue, C), the DS marker SMA (red, B) or the neural crest/Schwann cell marker p75 (red, C). The arrows denote the EGFP-positive PDGFRα-positive DP/DS or EGFP-positive SMA-positive DS cells while the arrowheads in panel C denote the EGFP-positive p75-positive cells associated with neonatal nerves. (D-F): Sox2:EGFP-positive cells at hair follicle nerve terminals in neonatal Sox2:EGFP mouse dorsal skin (NT cells). The sections were immunostained for EGFP (green) and the neural crest precursor markers p75 (red, D), and nestin (red, E), or the axon marker Pgp9.5 (red, F) to identify nerves. The arrows denote the EGFP-positive Nestin-positive p75-positive NT cells localized at the ends of nerves innervating the hair follicle bulge region. (G) Sox2:EGFP-positive skin nerve cells in the neonatal Sox2:EGFP mouse dorsal skin. The sections were immunostained for EGFP (green) and the neural crest/Schwann cell marker p75 (red). The arrows denote the Sox2:EGFP-positive p75-positive cells in the skin nerve. Note that not all of the p75-positive skin nerve cells are positive for Sox2:EGFP. In all panels except C, cells were counterstained with Hoechst 33258 or Topro3 (blue) to show cell nuclei. Scale bars represent 50 µm in (A-C), and 10 µm in (D-L).
Figure 3.2. In neonatal hair follicles, Sox2 is expressed in potential precursors for melanocytes and Merkel cells. (A–C) Sections through hair follicles in neonatal dorsal skin of Sox2:EGFP mice immunostained for EGFP (green) and the melanoblast marker c-Kit (red, A) or the melanoblast/melanocyte marker Dct (red, B,C). In (A), the arrow denotes an EGFP-positive c-Kit-positive melanoblast located in the upper region of a hair follicle. In (B), the arrows denote an EGFP-positive Dct-positive melanoblast in the upper region of a hair follicle. In (C), the arrows denote EGFP-negative Dct-positive melanocytes in the hair bulb. (D): Sox2:EGFP-positive cells in a heavily innervated portion of a whisker follicle in neonatal Sox2:EGFP mouse facial skin. The section was immunostained for EGFP (green), the neural crest/Schwann cell marker p75 (red), and the Merkel cell precursor/Merkel cell marker K8 (blue). Note that there are two populations of Sox2:EGFP-positive cells, the K8-positive Merkel cell precursor/Merkel cells in the body of the whisker (arrowheads) and the p75-positive cells innervating the outside of the follicle. (E) Section similar to that in (D) immunostained for Sox2:EGFP (green) and the neural precursor marker nestin (red). Arrows denote the Sox2:EGFP-positive Nestin-positive whisker NT cells. In all panels except D, cells were counterstained with Hoechst 33258 or To-pro3 (blue) to show nuclei. Scale bars represent 50µm in (A–C), and 10µm in (D–L).
observed similar sox2:EGFP-positive, nestin-positive NT cells at the ends of nerve terminals in a number of different tissues (data not shown). We also observed a subpopulation of sox2:EGFP-positive, p75NTR-positive cells in skin nerves (Fig. 3.1G) that may be either nerve-associated NCSCs or Schwann cell precursors that have been reported in nerves at this developmental stage. Thus, Sox2:EGFP is expressed not only in dermal precursors of the DP and DS, but also in potential neural crest precursors within and at the terminals of developing nerves.

Melanocytes are another neural crest-derived hair follicle cell type that was recently shown to derive, at least in part, from nerve-associated Schwann cell precursors. We therefore asked whether melanocyte stem cells, which are located in the bulge region, were also sox2:EGFP-positive. Immunostaining of neonatal dorsal skin sections for c-kit and dct, two melanocyte stem cell markers, defined a population of bulge-associated sox2:EGFP-positive cells that expressed both of these proteins (Fig. 3.2A,B). In contrast, mature dct-positive melanocytes, which are located near the base of the hair follicle, immediately above and around the DP, did not express sox2:EGFP (Fig. 3.2C), consistent with the fact that they are terminally-differentiated cells.

We also analyzed neonatal whisker follicles of sox2:EGFP-positive mice. As we have previously reported, the DP and DS of neonatal whisker follicles were uniformly sox2:EGFP-positive (data not shown). In addition, as also reported, many of the keratin-8-positive developing Merkel cells that are associated with whisker follicles expressed sox2:EGFP (Fig. 3.2D). Interestingly, sox2:EGFP-positive NT cells that coexpressed p75NTR and nestin were also present in the bulge region of whisker follicles (Fig. 3.2D,E), and their number was increased relative to hair follicles, consistent with the larger size and increased innervation of whisker follicles.

### 3.4.2 Sox2-positive hair follicle cells have characteristics of adult dermal and neural crest precursors.

These data argue that there are two sox2:EGFP-positive precursor populations in neonatal skin, hair follicle dermal precursors, and NCSC-like precursor cells at the terminals of nerves in hair follicles and within the developing nerves. To ask whether these different populations maintained sox2 expression into adulthood, as would be predicted for adult tissue stem cells, we performed similar analyses with adult skin. Immunostaining for EGFP in adult back skin
showed that dermal precursors within the DP and DS maintained sox2:EGFP expression in anagen hair follicles (Fig. 3.3A) although, as we previously reported\textsuperscript{14}, these dermal precursors lost sox2:EGFP expression during the regressive phase of the hair follicle cycle (data not shown). Immunostaining for nestin, p75NTR and S100beta, all markers for NCSCs and Schwann cell precursors, defined a population of sox2:EGFP-positive cells adjacent to the adult hair follicle bulge region (Fig. 3.3B) that coexpressed all of these proteins (Fig. 3.3C,D). These adult NT cells had a very distinctive morphology. Intriguingly, unlike the follicle dermal precursors, these adult NT cells maintained their sox2:EGFP expression throughout the hair follicle cycle. In contrast to the adult NT cells, sox2:EGFP-positive cells were not observed in skin nerves (Fig. 3.3E), arguing that the hair follicle niche somehow specifically maintains high sox2 expression in the NT cells. Similar results were obtained with regard to adult whisker follicles (Fig. 3.3F), where the DP, DS, and NT cells all expressed sox2:EGFP. Moreover, K8-positive Merkel cells in whisker follicles also maintained their expression of sox2:EGFP, as previously reported (Fig. 3.3G).

The finding that the sox2:EGFP-positive NT cells express both nestin and p75NTR, markers for NCSCs and Schwann cell precursors, argues that they might represent a population of neural crest-derived precursors, particularly in light of previous work showing that NCSC-like cells can be isolated from the bulge region\textsuperscript{19}. To definitively demonstrate that these cells were neural crest-derived, we crossed the sox2:EGFP mice with a Wnt1Cre:R26TdTomato neural crest reporter mouse. In these mice, Cre recombinase is expressed in Wnt1-positive neural crest cells during embryogenesis\textsuperscript{89}, and this causes recombination and expression of a floxed TdTomato reporter gene that is targeted to the ubiquitous R26R locus\textsuperscript{411}, with the net result that all progeny of Wnt1-expressing neural crest cells express a red fluorescent marker protein. Analysis of neonatal skin from these mice demonstrated that in back skin, the TdTomato reporter was expressed in known neural crest populations, including skin nerves and hair follicle melanocytes (Fig. 3.4A,B). TdTomato was not expressed, however, in sox2:EGFP-positive DP or DS cells (Fig. 3.4A,B), consistent with our previous work showing that these cells are not neural crest-derived in dorsal back skin. However, TdTomato was coexpressed with sox2:EGFP in NT cells, and in cells within skin nerves, presumably NCSCs or Schwann cell precursors (Fig. 3.4A,C,D). We also examined neonatal whisker follicles, where the dermis itself is neural crest-derived. As predicted, the sox2:EGFP-positive DP and DS cells of both whisker and hair
FIGURE 3.3
Figure 3.3. Sox2 is expressed in multiple potential hair follicle precursors in adult skin. (A-E): Dorsal skin sections from adult Sox2:EGFP mice immunostained for EGFP (green). (A) Section through the DP of an anagen hair follicle showing expression of Sox2:EGFP in cells within the DP (arrow) and DS (arrowhead). (B) Section through the bulge region of a hair follicle showing expression of Sox2:EGFP in the morphologically distinct NT cells (green, arrows). (C,D) Sections through the bulge region of adult hair follicles that were immunostained for Sox2:EGFP (green), nestin (red), and either S100b (C, blue) or p75 (D, blue). Arrows denote Sox2:EGFP-positive NT cells that coexpress all three of these markers. (E) Section through an adult hair follicle and adjacent nerves that was immunostained for Sox2:EGFP (green) and the neural crest/Schwann cell marker p75 (red). Arrows denote a Sox2:EGFP-positive p75-positive NT cell while the open arrows denote an adult skin nerve that is positive for p75 but negative for Sox2:EGFP. (F,G): Whisker follicle sections from adult Sox2:EGFP mice immunostained for EGFP (green). (F) Section through a whisker follicle to show that Sox2:EGFP was expressed in DP (arrow) and DS (arrowhead) cells. (G) Section through an adult whisker follicle immunostained for Sox2:EGFP (green) and the Merkel cell precursor/Merkel cell marker K8 (red). Sox2:EGFP was expressed in the K8-positive Merkel cells (arrow) and the K8-negative NT cells (arrowhead). In all panels except C and D, cells were counterstained with Hoechst 33258 or Topro3 (blue) to stain nuclei. Scale bars represent 50µm in (B), and 10µm in (A, C - G).
**Figure 3.4. Sox2 is expressed in multiple potential neural crest and non-neural crest-derived hair follicle precursors.** (A-G): Dorsal skin sections (A-D) or facial skin sections (E-G) of neonatal mice that were positive for the neural crest lineage tracer Wnt1-cre;tdTomato (red) and heterozygous for Sox2:EGFP (green). (A) Low magnification section through dorsal skin showing Sox2:EGFP-positive DP cells that are not positive for the neural crest lineage marker (open arrows) and Sox2:EGFP-positive NT cells skin nerve cells that express tdTomato, indicating that they originate from the neural crest (arrows). (B) Higher magnification micrographs of a section through an anagen hair follicle showing that DP cells do not express tdTomato (green, open arrows) while the tdTomato-positive melanocytes (red, arrows) do not express Sox2:EGFP. (C) Higher magnification micrographs of a section through the bulge region of dorsal hair follicles. The arrows denote Sox2:EGFP-positive, tdTomato-positive cells in tdTomato-positive skin nerves while the open arrows denote Sox2:EGFP-positive, tdTomato-positive NT cells in the bulge region of the hair follicle. The arrowhead denotes Sox2:EGFP-positive, tdTomato-negative Merkel cells. (D) Section through the bulge region of a hair follicle immunostained for nestin (blue). The arrow denotes a Sox2:EGFP-positive, tdTomato-positive, nestin-positive NT cell. (E) Low magnification micrograph of a facial skin section through both a whisker follicle and an adjacent hair follicle showing the many Sox2:EGFP-positive, tdTomato-positive cells surrounding the whisker follicle (arrow) and those in the DP and DS of the anagen hair follicle (arrowhead). (F) Higher magnification micrograph through a facial hair follicle, showing the sox2:EGFP-positive, tdTomato positive DP and DS cells (arrow) and the Sox2:EGFP-negative, tdTomato-positive melanocytes (open arrow). (G) The left panel shows a section through a whisker follicle colabelled for the Merkel cell precursor/Merkel cell marker K8 (blue). Note the Sox2:EGFP-positive, tdTomato-positive NT cells (arrow) and the Sox2:EGFP-positive, tdTomato-negative, K8-positive Merkel cell precursors/Merkel cells (open arrow). The right panel shows a higher magnification image of the merge in the left panel, highlighting the NT cells (arrows In all panels except D and G, cells were counterstained with Hoechst 33258 or Topro3 (blue) to stain nuclei. Scale bars represent 50µm in (F), and 10µm in (A-E,G).
follicles in facial skin expressed TdTomato (Fig. 3.4E,F). Moreover, the sox2:EGFP-positive NT cells expressed TdTomato, while the sox2:EGFP-positive, K8-positive Merkel cells did not (Fig. 3.4G), consistent with a non-neural-crest origin for the latter cell type. Thus, sox2:EGFP is expressed in two populations of non-epidermal hair follicle precursors, dermal precursors and a neural crest-derived cell with characteristics of NCSCs or Schwann cell precursors.

3.4.3 Sox2 is induced in dermal and neural crest precursors following skin injury.

These data indicate that sox2 is specifically expressed in a number of hair follicle precursor populations within adult skin, data reminiscent of its expression in precursor populations within other adult tissues. However, while it is clear that sox2 marks a variety of adult stem cell populations, it is still unclear whether sox2 itself is important for the function of these adult precursors in tissue repair and maintenance. To start to address this question, we characterized the cell populations that express sox2 following skin injury, taking advantage of the sox2:EGFP mice. Specifically, we performed 6 mm full thickness punch wounds on the backs of 2 month old sox2:EGFP mice, and then characterized EGFP expression 5 days later. At this age, mouse hair follicles were in telogen, based on morphology of the hairs observed on skin sections, and consistent with this, sox2:EGFP was not expressed in the follicle DP or DS, but was limited to NT cells and Merkel cells (Fig. 3.5A and data not shown). Immunostaining five days following skin injury demonstrated that sox2:EGFP was now expressed in two additional cell populations proximal to the site of injury. One of these was associated with nerves that were located close to the site of injury (Fig. 3.5B). These cells expressed p75NTR and S100beta (Fig. 3.5B,C), consistent with their identification as dedifferentiated Schwann cells, something that is also seen following peripheral nerve injury. The second of these were DP cells localized within hair follicles close to the injury site that were presumably reentering the growth cycle in response to the injury (Fig. 3.5D). In contrast, neither of these sox2:EGFP-positive cell populations were observed at sites distal to the injury in the same animals (Fig. 3.5F).

3.5 Discussion

The data presented here support three conclusions. First, they indicate that Sox2 defines multiple precursor populations in neonatal and adult skin. Specifically, the immunostaining of sox2:EGFP mouse skin defines six distinct subpopulations of Sox2-expressing skin cells: DP
FIGURE 3.5.
Figure 3.5. Sox2 is induced in dermal and neural crest precursors following skin injury.
(A): Photomicrograph of a section through a telogen hair follicle in the dorsal skin of a 2 month-old Sox2:EGFP (green) mouse without injury. The arrow and arrowhead respectively denote EGFP-negative DP cells and EGFP-positive NT cells in the telogen hairs. (B-D): Micrographs of dorsal skin sections of Sox2:EGFP mice 5 days post injury close to the site of injury, immunostained for p75 (B,E, red) or S100β (C, red). Sox2:EGFP was detected in p75-positive and S100β-positive NT cells (arrows, B,C; arrowheads, D) as well as within skin nerves (arrowheads, B,C), something that was not seen in uninjured skin or in skin distal to the injury. In addition, Sox2:EGFP was expressed within the DP of hair follicles close to the injury site (arrow, D), presumably as they were preparing to reenter the growth phase. In all panels, cells were counterstained with Hoechst 33258 or Topro3 (blue) to stain nuclei.
cells, DS cells, NT cells, neural crest or Schwann cell precursors within skin nerves, melanocyte stem cells, and Merkel cells. While we have not tested whether these Sox2-positive cells exhibit stemness, we have identified markers, locations and developmental origin of each subpopulation, all data that indicates that some of these are likely to represent precursor populations that have been reported in past studies. Second, our findings reveal that while these sox2-expressing cells have distinct developmental origins, all of them share neural potential, regardless of their origin. Specifically, our lineage analysis demonstrates that the Sox2-expressing cells we have identified here derive from neural crest and non-neural crest origins. For the neural crest-derived cells NT and nerve cells, the markers we have defined are consistent with their identity as neural crest or Schwann cell precursors, both of which have neural potential. For the non-neural crest-derived sox2-positive DP and DS cells, both of these populations are known to exhibit neural crest precursor-like properties. The third population, Merkel cells, is cutaneous neuroendocrine cells that derive from the epidermis and share properties with neurons. Finally, we found that Sox2 expression is activated and/or elevated in DP cells and nerve-associated cells following skin injury, suggesting that sox2 expression is not a static property of precursors, but that it, and potentially the stem cell state, is modified as a function of the adult tissue environment. A summary of these findings can be found in Fig. 3.6.

We and others previously demonstrated that Sox2 is expressed in the DP/DS cells and Merkel cells of hair follicles. Importantly, data presented here indicate that other skin cell types are also defined by Sox2, a well-known stem cell marker. Do these Sox2-expressing subpopulations actually represent precursors? Previous work showed that Sox2-positive DP and DS cells display characteristics of dermal precursors. However, little was known regarding the Sox2 expression in the other skin cell types, although skin is an enriched source for various precursor populations. Interestingly previous microarray analysis indicates that EGFP-positive cells isolated from the skin of adult Sox2:EGFP mice express many markers associated with neural crest precursors, Schwann cell precursors, melanocyte precursors and epidermal precursors, some of which are not found in the expression profile of SKPs, or of cultured and purified DP and DS cells. We therefore hypothesized that Sox2 defines multiple precursor populations in the skin, and our data support this hypothesis, demonstrating that Sox2 is expressed in a broad range of potential precursor populations in developing and mature skin, and that Sox2-positive cells are increased in response to skin injury. A key question for the future
A. Six distinct precursors were identified as Sox2-positive cells in neonatal skin.

B. Several precursors lose Sox2 expression in adult skin.

C. Several precursors regain Sox2 expression during wound healing in adult skin.
Figure 3.6. The levels of the Sox2 expression in hair-follicle precursors change based on the time course of skin development and wound healing. (A): In the developing mouse pelage skin (P2-7), Sox2 is expressed in the six different hair-follicle precursors: DP cells, DS cells, melanocyte precursors, NT cells, Schwann cell precursors, and Merkel cells. Each precursor type can be distinguished from the others by immunocytochemical markers, morphology, location and lineage analysis. (B): In adult skin (2-month old), Sox2 is continuously expressed in NT cells and Merkel cells. However, Sox2 expression is lost in Schwann cell precursors and melanocyte precursors. Also, Sox2 is expressed in DP and DS cells only in anagen (growing) hair follicles. (C): In adult skin following a punch wound, de novo hair follicles form around the healing tissues. Sox2 is expressed in DP and DS cells of these newly-formed hair follicles. Sox2 is also re-expressed in Schwann cell precursors around the healing wound.
will be whether or not Sox2 expression is essential for maintenance of these precursors and/or for skin repair following injury.

Our findings suggest that Sox2, a prototypic marker for pluripotent stem cells and various tissue stem cells, defines multiple precursor populations in the skin, although we have not directly assessed the stemness of the isolated sox2-positive populations. However, the information obtained from our immunocytochemical analysis of Sox2:EGFP mouse skin strongly supports the idea that some of the identified Sox2-positive cells are precursors. Specifically, Sox2 was detected in the following six skin cell types: DP cells, DS cells, NT cells, nerve-associated cells with properties of neural crest or Schwann cell precursors, melanocyte-lineage cells, and Merkel cells, based on their specific markers, locations and/or morphology. These subpopulations of Sox2-positive skin cells could be categorized into three types: (i) cells that express Sox2 constantly (NT cells and Merkel cells); (ii) cells that activate Sox2 expression during tissue morphogenesis, either during development or in the adult (DP cells, DS cells and nerve-associated cells); and (iii) cells that express Sox2 only during development (melanocyte-lineage cells).

We have confirmed that DP and DS cells expressed Sox2 only during the growth phase of hair morphogenesis, but not during hair regression, as previously demonstrated\textsuperscript{14}. Moreover, previous work has shown that Sox2-positive DP and DS cells have characteristics of dermal precursors\textsuperscript{14}. However, it is still unclear whether or not Sox2 is essential for DP and DS cells to act as dermal precursors. In this regard, previous work from our laboratory demonstrated that Sox2-positive DP cells in hairs close to a wound exited their hair follicle niche and streamed into the inter-follicular dermis towards the wound site\textsuperscript{14}. Moreover, the same study showed that isolated sox2-positive skin cells can contribute to wound-healing hair morphogenesis\textsuperscript{14}. Data presented here demonstrated that when a skin wound is made at a time when hairs are in the regressive telogen phase, then this locally induces hairs to reenter the growth phase and causes a reinduction of Sox2 in DP cells. Together, these data suggest that Sox2 expression is relevant during the injury-induced tissue restructuring process, which involves both genesis of dermal cell types for wound-healing, and new hair morphogenesis. Sox2 expression may be associated with the dermal precursor function of DP and DS cells during tissue regeneration. However, DP and DS cells are likely to represent only a subset of dermal precursors, because dermis lost in injuries is repopulated in non/less-hairy skin such as rodent palm or human skin as well as hairy skin.
such as rodent back skin or human scalp. In this regard, Collins et al (2011) recently demonstrated that remodeling of the dermis in response to the epidermal activation of β-catenin originates from a specific population of fibroblasts located adjacent to the hair follicle junctional zone/sebaceous gland region\(^4\). The authors concluded that fibroblasts in this location are normally quiescent and can retain a DNA label for many months. Thus, this specific subset of fibroblasts may be another precursor subpopulation for dermis\(^4\). It will be interesting to determine whether Sox2 defines not-yet identified precursors for dermal fibroblasts as well as already-identified dermal precursors, DP and DS cells, and if so, whether lineage ablation of Sox2\(^+\) dermal cells induces pathological conditions in adult skin.

Work here has defined a novel population of sox2-expressing cells in hair follicles at the terminals of nerves which we have called NT cells. These NT cells express Sox2 under all of the conditions we have studied. NT cells are located in the hair follicle nerve terminals around the hair follicle bulge. Immunostaining and lineage analysis demonstrated that NT cells have typical characteristics of both neural crest precursors and previously-described Schwann cell precursors. In addition to NT cells, we detected Sox2 in cells of the developing skin nerves that also expressed markers consistent with their identification as neural crest or Schwann cell precursors\(^17,19\). Both of these populations are therefore likely to be some type of developing neural crest or Schwann cell precursor. However, the NT cells maintain Sox2 expression throughout their lifetime, in contrast to the nerve-associated cells, which express Sox2 only during development and following injury. This pattern of expression within skin nerves can likely be explained by previous work showing that Sox2 is a marker for immature Schwann cells and their precursors\(^339\) and that it is reinduced during dedifferentiation of mature Schwann cells during nerve regeneration\(^337,340\). To truly define these nerve-associated cells are Schwann cell precursors will require lineage tracing and/or prospective isolation and characterization.

Another difference between Sox2-expressing NT cells and the Sox2-positive nerve-associated cells is that the NT cells express nestin, a marker for neural stem cells and perhaps multi-lineage progenitor cells\(^414\). Of the various Sox2-expressing skin cell populations, nestin expression was only seen in NT cells. This finding supports the idea that NT cells are distinct from the nerve-associated Sox2-positive cells, and suggests that they may well represent neural crest precursors. In this regard, previous work has identified a neural crest precursor-like activity in the region of
the hair follicle bulge\textsuperscript{19}, and we propose that the Sox2-expressing NT cells may well be these bulge-associated neural crest precursors.

What is the biological function of the NT cells? One potential function may involve modulation of the interactions between the skin nerve endings and the hair follicle itself, by analogy to the as the specialized non-myelinating Schwann cells at the neuromuscular junction\textsuperscript{415}. Intriguingly, we have recently observed that these specialized neuromuscular junction cells also express Sox2. Also, if the NT cells are neural crest-derived precursors as we have proposed, then the NT cells might supply hair follicle melanocytes. This idea comes from the recent finding that Schwann cell precursors contribute to hair follicle melanocytes during embryogenesis, although it is still unclear whether Schwann cell precursors can supply melanocytes in postnatal tissue\textsuperscript{215,368}. In this regard, studies by Wong et al.(2006) indicated that Schwann cell precursors are located in the adult hair follicle bulge\textsuperscript{371}. Therefore, Sox2 may be associated with the functions of Schwann cells and their precursors for skin development and maintenance.

One skin population that only expressed Sox2 during development was a subset of hair follicle cells in the melanocyte lineage. Neonatal and adult melanocyte stem cells are located within the hair follicle bulge. Sox2 is only found in the melanocyte-marker-positive cells around the hair follicle bulge, and not in the more mature melanocytes located in other places in the hair follicle. Thus, Sox2 may define melanocyte stem cells or melanoblasts, although we could not detect Sox2-positive melanocyte lineage cells in the adult skin.

Finally, we confirmed that Sox2 is expressed throughout life in K8-positive Merkel mechanoreceptor cells, as previously described\textsuperscript{333,336}. Because of their phenotypic and functional similarity to neuronal cells, Merkel cells were thought to derive from the neural crest\textsuperscript{416}. However, recently, Merkel cells were shown to derive from epidermis\textsuperscript{412}, and their precursors were identified as Itga6\textsuperscript{+} Sca1\textsuperscript{+} CD200\textsuperscript{+} cells located in the touch domes of hairy skin\textsuperscript{417}. Since data presented here demonstrated that Sox2\textsuperscript{+}K8\textsuperscript{+} Merkel cells also form touch domes and since previous microarray data indicated that a subset of Sox2-positive skin cells express CD200, then we propose that Sox2-expressing Merkel cells may represent Merkel cell precursors. Further support for this idea comes from work on taste bud sensory cells in the tongue that are functionally equivalent to Merkel cells in the skin\textsuperscript{403}. In this system, Sox2 functions in a dose-
dependent manner to regulate the differentiation of epithelial progenitor cells of the tongue into taste bud sensory cells versus keratinocytes\textsuperscript{403}. Therefore, Sox2 may be required for the differentiation of epidermal precursors of the skin into Merkel cells.

Together, these data support the concept that Sox2 defines multiple precursors in the skin. Generally, Sox2 is expressed during development of diverse tissues and organs, and the level of Sox2 is critical for normal organogenesis. In certain organs, Sox2 expression persists into adulthood. For example, Sox2 is expressed in postnatal lung epithelial cells, and essential for tracheal cells to regenerate the epithelium upon injury\textsuperscript{401}. More recently, Arnold et al (2011) performed systemic analysis of the full pattern of Sox2 expression in adult tissues, and elucidated the functional importance of Sox2-positive precursors to maintain homeostasis of different adult tissues\textsuperscript{30}. Whether Sox2 is essential for skin development, maintenance and/or repair is a key question for the future.
Chapter 4
General Discussion

This thesis directly addressed (1) the developmental origins of SKPs/dermal precursors and (2) the definition of multiple Sox2-expressing potential precursors in the skin. By addressing these issues, this thesis also addressed more general themes such as the plasticity of adult stem cells, and the nature and number of different adult stem cell populations, in this case in one complex tissue, the dermis.

4.1 Developmental Origins of SKPs / Dermal Precursors

4.1.1 Summary

Chapter 2 of this thesis described my work identifying the developmental origins of SKPs, something that was previously unclear. The data presented here demonstrated that SKPs can originate from both Wnt1-expressing neural crest and from Myf5-expressing mesoderm\textsuperscript{16}. Specifically, I demonstrated that “dorsal SKPs” (defined in vitro as cells from the dorsal dermis that self-renew in non-adherent conditions and differentiate into neural and mesenchymal cells) can be isolated from cells that have activated the Myf5 promoter at some point in their ontogeny by performing lineage analysis with the \textit{Myf5-cre;Rosa-Reporter} mice. This result demonstrates the mesodermal origin of dorsal SKPs, since (1) Myf5 expression is believed to be restricted to the developing somites that originate from the mesoderm, (2) the somites are known as the primary source of the dorsal dermis, and (3) the Myf5-lineage cells were shown here to contribute to the DP and DS in vivo and SKPs in vitro. To further support this idea, I demonstrated that the neural crest derivatives (labeled by Wnt1-cre;Z/EG) that were isolated from the dorsal skin failed to form self-renewing SKP spheres. Therefore, even if Myf5 is unexpectedly expressed outside of the mesoderm, it is unlikely that neural crest derivatives contribute to dorsal SKPs. More recently, I have generated Dermo1-cre; tdTomato mice that express an RFP variant protein in cells that have expressed Dermo1 at some point in their ontogeny (Dermo1 is a key transcription factor for dermogenesis\textsuperscript{418}). I demonstrated that these \textit{Dermo1-cre; tdTomato} skin cells are not neural crest-derived, but that they were able to generate SKP spheres, and to differentiate into Schwann cells, thereby confirming that mesodermally-derived dermal cells from the back generate SKPs, and that these dermal cells have neural...
potential. Thus, I have demonstrated that in dorsal skin, using three different reporter lines, that SKPs are isolated from the Dermo1-cre;tdT+ dermal cells that originate from Myf5-cre;Rosa- 
EYFP+ somites but not from Wnt1-cre;Z/EG+ neural crest cells. To my knowledge, this is the first report revealing the neural potency of non-CNS/non-PNS precursors that has been backed by genetic marking and positive sorting of non-CNS/non-PNS precursors. For example, while there are many reports of bone marrow stromal cells having neural potential20,419,420, the identity of the parent cells is still vague, and the functionality of the neural derivatives is the subject of debate421,422. Therefore, my results strongly suggest that dermal precursors with neural potential can be generated from sources other than the neural crest.

4.1.2 Novelty / Importance of the findings

4.1.2.1 Adult stem cells may be more plastic than currently thought.

The findings presented in my thesis support the notion that adult stem cells may be plastic enough to generate cell types across lineage boundaries, in response to changes in their microenvironment, and argue against the prevailing idea that the potency of adult stem cells is restricted to tissue-specific cell types111. In the introduction to this thesis, I explained that SKPs isolated from adult dorsal skin can generate functional Schwann cells12, but that little was known regarding their developmental origins. Then, in Chapter 2 of this thesis, I demonstrated that the SKPs isolated from the dorsal skin originate from the somite-derived dermis16. Overall, these data indicate that neural cells can be differentiated from cells that were developmentally biased to form the somites and then the dermis.

How does this conclusion impact on the current landscape of stem cell biology and regenerative medicine? Since Takashima et al reported in 2006 that pluripotent stem cells could be induced from adult fibroblasts by genetic modification, the induced pluripotent stem (iPS) cells have been intensively studied, and have grown to be an essential component of the stem cell research field4,5,423. Moreover, many research groups have applied the concept of iPS cells to genetic modifications that directly convert adult fibroblasts into non-fibroblastic, medically-useful cell types. For example, there are more than 10 articles that describe the direct conversion of fibroblasts to neurons by defined factors391,424,434. In general, these articles argue that (1) a greater number of neuron-like cells (meaning cells that are morphologically and phenotypically similar to the neurons) were identified in cultures of adult fibroblasts induced with.
factors, compared to fibroblast cultures without this induction, and (2) that these cells were functional because they were morphologically similar to neurons and because they expressed a number of neuronal markers. However, these results cannot necessarily be interpreted as indicating the direct conversion of fully-differentiated fibroblasts to neurons. The fibroblastic populations used in these experiments were generally isolated from quite heterogeneous tissues such as the skin, liver and lung, all of which should contain cells that innately have neural potential, such as neural crest-derived precursors or dermal precursors. Therefore, it might be possible that these defined factors induced differentiation into neurons simply by promoting the neural potential of neurally-biased precursor cell types (e.g. dermal precursors) rather than by direct conversion of the fate of differentiated fibroblasts. Thus, my findings argue that these types of experiments need to be performed using fibroblast cultures where neural crest-derived cells and dermal precursors are removed in advance, although this approach has never previously been considered.

My findings also support the value of adult stem cells for cell-based therapies. If adult tissue-specific stem cells are plastic enough to overcome their lineage boundaries, we may be able to use them for cell-based therapies by converting them to more medically-useful cells such as neurons. Although this possibility has been investigated in the past\textsuperscript{39}, the data has not been completely convincing, and instead, iPS cells and their derivatives have been intensively studied as a promising source for regenerative medicine. However, as indicated in this thesis, certain adult precursors may have the potency to convert their lineages without genetic modifications. Therefore, for future regenerative medicine, it might be still worthwhile to pay attention to the innate plasticity of adult tissue-specific cells as well as to seek innovative approaches in the induction of pluripotency or the conversion of determined fates by modifying genetic programs.

4.1.2.2 Why are SKPs similar regardless of multiple distinct developmental origins?

The data presented in my thesis show that SKPs isolated from dorsal skin are functionally similar to SKPs isolated from facial skin, in spite of their different origins (dorsal SKPs from the mesoderm and facial SKPs from the ectoderm by way of the neural crest)\textsuperscript{16}. These results may suggest that the properties of tissue-specific precursors are defined as much by their microenvironment as by their developmental origins. Therefore, although the precursors for the dermis originate from both the ectoderm and the mesoderm, their properties are likely
determined by their skin environment once they acquire a dermal bias upon expression of Dermo1\textsuperscript{118}. In concurrence with this result, I demonstrated that the transcriptional profile of mesenchymal stem cells (MSCs) isolated from the bone marrow is different from those of the mesenchymal precursors isolated from dorsal and facial skin (dorsal and facial SKPs), although MSCs share their neural-crest and mesodermal origins with SKPs. Perhaps the differences I elucidated can be attributed to differences in their niches. The potential impact of the microenvironment on stem cells, and in particular how these environments can override their lineages has also been observed in other reports. For example, thymic epithelial stem cells (TECs) that originate from the endoderm were shown to adopt the fate of ectodermally-derived hair follicle stem cells when they were transplanted into the skin environment\textsuperscript{109}. In this regard, can the skin microenvironment drive MSCs toward a SKP-like phenotype? Or can SKPs acquire the properties of other mesenchymal precursors when transplanted into a tissue other than skin? Because Biernaskie et al (2010) demonstrated that transplanted MSCs do not contribute to hair follicle dermal cells while transplanted SKPs do\textsuperscript{14}, this suggests that cues from the skin environment may not be enough to override the endogenous properties of transplanted precursors. However, it might be still interesting to ask how other mesenchymal cell types (for example, isolated from nerve, lung, or liver) respond to the skin environment. On the other hand, our laboratory has demonstrated that SKPs contribute to structures of hypodermis, bones and nerves as well as dermis, when transplanted into these environments\textsuperscript{15}. These data indicate that SKPs are highly flexible in terms of their cell fate decisions, and suggest that they might be relatively unbiased precursors. In this regard, it would be interesting to ask if SKPs could regain the properties of dermal precursors even after adapting to ectopic environments. Also, because little is known about the molecular mechanisms that allow different environments to influence cell fate decision making, then this would be an important area of research, particularly if we are to maximize the therapeutic potential of SKPs for regenerative medicine in the future. Overall, these data indicate that stem cell niches play a key role in determining the properties of adult tissue stem cells, regardless of their developmental origins.

4.1.2.3 Neural Potential in Dermal Precursors

The neural crest-like potential of dermal cells that are associated with hair follicles has been postulated by many researchers over the past ten years\textsuperscript{7,14,280,281,286,333}. For example, in situ hybridization revealed that hair follicle dermal papillae express typical neural crest markers, and
in vitro assays demonstrated that dermal papilla cells that were dissected out mechanically can form neurons and glial cells\textsuperscript{7,281}. However, it has been unclear whether the dermal hair follicle cells that display these neural crest-like properties are actually the dermal cells, since dermal papillae are not necessarily composed of homogeneous dermal fibroblasts. For example, skin nerves are thought to innervate the dermal papilla of hair follicles, and these nerves contain peripheral glial cell types\textsuperscript{156}. Also, melanocytes, another neural crest-derived cell type, are immediately adjacent to the dermal papillae in hair follicles, and thus represent another cell type that might contaminate mechanically dissected dermal papillae. Therefore, past studies have lacked definitive evidence that the dermal precursors that are associated with hair follicles really have neural crest-like potential. In this regard, I clearly demonstrated the neural potential of dermal precursors regardless of their developmental origins, by conducting three distinct lineage analyses. Now, the question is why and how dermal precursors should retain such neural potential, which appears to be unnecessary for normal skin development and maintenance. Our previous studies have indicated that the neural differentiation capacity of dermal precursors is unmasked in certain exogenous environments (e.g. gliogenic culture conditions or injured sciatic nerve), but restricted in their physiological niche (e.g. dermal papilla of hair follicles). It might be intriguing to ask if the endogenous dermal precursors ever exhibit neural phenotypes within the skin, for example when the microenvironmental cues are disturbed by disease, injury or other experimental manipulation. By asking this, we might ultimately challenge the classical idea that all of the peripheral neural tissues in vivo originate from the neural crest.

4.2 Functions of Sox2 in Skin Maintenance

4.2.1 Summary

Chapter 4 of this thesis revealed Sox2 expression in the Schwann cell and melanocyte lineages in the skin, and described a novel potential neural crest-derived precursor that is associated with nerve terminals in hair follicles that we have termed NT cells. In addition, data presented here showed that Sox2 expression is regulated within these populations. In particular, these data showed that following skin injury Sox2 is reinduced in cells within skin nerves, and is rapidly induced in DP cells coincident with their reentry into the hair growth cycle. While this work was ongoing, other researchers in the Miller laboratory demonstrated that Sox2 plays a key role in the dermal response to injury during skin aging. In particular, Dr. Sibel Naska and Ms. Karen Jones
demonstrated that reduced expression levels of Sox2 in the skin led to perturbed wound healing, and that this disruption of wound healing was more severe in older mice. These data suggest that the potential precursor types I have defined here are important for skin wound-healing, and that Sox2 regulates their function so that when Sox2 is decreased, they cannot participate in wound-healing with the same efficacy. I will therefore discuss the findings presented in Chapter 3 in light of these other concurrent findings.

Sox2 regulates the stemness of diverse precursors and plays key roles in embryonic tissue development and adult tissue maintenance\textsuperscript{30,31}. In this regard, Driskel et al (2009) reported that Sox2 is expressed in the dermal papilla of hair follicles and Merkel cells of the skin surface\textsuperscript{333}. Then, Biernaskie et al identified Sox2 expression in dermal sheath (DS) as well as DP, and showed that these DP and DS cells possess the properties of the dermal precursors\textsuperscript{14}. However, little was known about Sox2 expression in other precursors in skin and whether Sox2 regulate stemness in these precursors. Initially, in order to define the types of skin cells that express Sox2, I reviewed the gene expression profile of the Sox2-expressing skin cells that was published in Biernaskie et al.\textsuperscript{14}, comparing this expression profile to the SKPs transcriptome that I performed in Chapter 2. Unexpectedly, various markers for Schwann cell precursors and melanocyte precursors were detected as enriched in the Sox2:EGFP-positive cells. Second, to ask if Sox2 is expressed in the Schwann cell and melanocyte lineages in the skin, neonatal and adult Sox2:EGFP mouse skin was immunostained for Sox2:EGFP and markers for Schwann cell and melanocyte lineages. The immunocytochemistry detected Sox2:EGFP in Nestin\textsuperscript{+}S100\beta\textsuperscript{+}p75\textsuperscript{+} glial and Dct\textsuperscript{+}cKit\textsuperscript{+} cells near the hair follicle bulge region where skin nerve terminals innervate the hair follicle. These newly-identified Sox2\textsuperscript{+} cells are likely to be potential precursors for Schwann cells and melanocytes, because (1) Sox2\textsuperscript{+} cells in various tissues often represent precursor populations in the tissues\textsuperscript{30,31}, (2) adult neural crest-derived precursors have been thought to be enriched in the hair follicle bulge\textsuperscript{371,435,436,277}, and (3) the markers (e.g. Nestin & cKit) detected in the Sox2\textsuperscript{+} cells are markers identified in past reports that described neural crest-derived precursors in the hair follicle\textsuperscript{19,277}. Therefore, my work indicates that Sox2 defines multiple skin cell types, including hair-follicle dermal precursors, Merkel cells and potential precursors for Schwann cells and melanocytes, although the functions of Sox2 in any skin precursors has never previously been described.
4.2.2 Novelty / Importance of the findings

4.2.2.1 Sox2 may define multiple precursor populations in the skin.

Although Sox2 is known to be essential for precursors, particularly in the embryo, and has recently been implicated in adult stem cells and tissue maintenance, little is known about its expression and function in skin, and it is still unclear precisely how it regulates adult stem cells\(^{30,31}\). The findings presented in Chapter 3 of my thesis revealed six distinct Sox2-expressing skin cell types in neonatal skin: (1) mesoderm-derived PDGFR\(\alpha\)^{High} p75^{Low} SMA^{Low} DP cells localized within the hair bulb, (2) mesoderm-derived PDGFR\(\alpha\)^{High} p75^{Low} SMA^{High} DS cells ensheathing the hair follicle, (3) neural crest-derived Nestin^{High} S100\(\beta\)^{High} p75^{High} cells located near the hair follicle nerve endings, (4) neural crest-derived p75^{High} cells in the skin nerves, (5) neural crest-derived cKit^{High} Dct^{High} cells near the hair follicle bulge region, and (6) epidermis-derived K8^{High} Merkel cells. The markers, location, morphology and/or developmental origins of the Sox2-expressing cells identified in my study match the properties of precursors in adult skin that have been reported elsewhere\(^{19}\). Therefore, Sox2 is likely to define precursor populations for various skin cell types. However, only four of these cell types maintain Sox2 expression into adulthood, with the Sox2-positive skin nerve cells and melanoblast precursors disappearing between the first week and the second month. This suggests either that the state of these precursors changes after development and/or that these particular precursors disappear following development. In this regard, the data showing that Sox2 is reinduced in skin nerves following injury may suggest that the state of the nerve precursors simply changes. Definitively distinguishing these two possibilities will require further study.

My findings are important with regard to a better understanding and classification of the precursors that exist within skin, and the roles of those adult precursors in skin maintenance and repair. In particular, my findings indicate that Sox2:EGFP would be an effective marker for neural crest-derived precursors in the skin and perhaps in other tissues, something that has not been described in previous studies. In past studies, several groups have independently identified multipotent neural crest-derived precursors within skin that exhibit characteristics distinct from SKPs\(^{19}\). Because of their multipotency and their accessibility as a stem cell source, these NCSC-like skin cells, including SKPs, have been intensively studied\(^{19}\). However, the lack of specific markers for these precursors has made it difficult to perform studies that depend upon prospective isolation, something that is essential in the stem cell field. In this regard, the data
presented here indicate that Sox2 is expressed in neural crest-derived precursors in skin, as it is in neural crest-derived precursors in the embryo. Accordingly, we are now capable of sorting the NCSC-like skin cells with higher purity by Sox2:EGFP, thereby enabling better purification and characterization. The robust expression of Sox2 in multiple potential precursors in mature skin also raises the possibility that Sox2 is involved in tissue maintenance and repair as a consequence of its important role in maintaining other populations of adult tissue precursors.

4.2.2.2 Sox2 is essential for the maintenance of adult skin.

Previous work from the Miller and Flores laboratories showed that TAp63 prevents premature skin aging by maintaining the epidermal and dermal precursor populations, indicating that disrupted stemness could lead to premature aging phenotypes such as a delay or disruption of the wound healing response. The work presented in Chapter 3 led to the hypothesis that disruption of Sox2 in adult skin would lead to premature aging, as seen in the TAp63 study, because (1) Sox2 often regulates the self-renewal and proliferation of precursors in various tissues, (2) the TAp63 work argues that depletion of skin precursors causes premature aging, and (3) my work showed that Sox2 is expressed in multiple populations of potential precursors in the skin. To test this idea, Dr. Naska and Ms. Jones analyzed the wound healing process in Sox2 heterozygous mutant mice and Sox2 conditionally null mice using a variety of morphological measures of wound closure. The analysis revealed perturbed wound closure in both Sox2 heterozygous mutant and Sox2 conditionally null mice relative to the Sox2 wild type animals, and interestingly, that this effect was more pronounced in aging mice than in young mice. These results supported the initial hypothesis, and suggested that Sox2 is essential for normal maintenance of skin precursors and that, when it is reduced, this causes depletion of those precursors as animals age, and thus, perturbed wound-healing.

These data therefore demonstrate that Sox2 is not just a marker for skin precursors, but that its expression in those precursors is essential for normal skin repair as animal’s age. This is thus one of the first reports describing the functionality of Sox2 in adult skin, and one of only a few reports of the importance of Sox2 in adult tissue precursors. Specifically, the wound repair data with Sox2\textsuperscript{EGFP/+} mice demonstrated that haploinsufficiency for Sox2 caused a decrease in wound-healing in middle-aged mice that was not present in young adult mice. Previous work in the Sox2\textsuperscript{EGFP/+} mouse line has shown that Sox2 expression levels are approximately 50% of wild-
types\textsuperscript{395}. Other laboratories have studied Sox2 heterozygous mice and demonstrated that, in the CNS, heterozygous levels of Sox2 are sufficient for its proper development, and that defects in neural progenitor cells are only observed when Sox2 levels drop to 20-30% of wild-type levels\textsuperscript{437}. Similar to this, there are no obvious problems in hair growth (HJ, data not shown) or wound-healing in young mice, suggesting that skin precursor development is normal when Sox2 is haploinsufficient. However, the studies showing that aging mice do show deficits in wound-healing suggest that this transcription factor plays an important role in tissue maintenance over time. One possible explanation is that older Sox2\textsuperscript{EGFP/+} mice are exposed to the potential impact of Sox2 reduction for a longer time than younger Sox2\textsuperscript{EGFP/+} mice. However, the wound healing studies in the Sox2 conditional mice revealed that the perturbed wound healing was seen in aging mouse back skin when Sox2 had been deleted three weeks previously, making this explanation unlikely.

An alternative explanation for these results is that Sox2 is important for adult tissue precursors to participate in tissue repair. However, since the first part of Chapter 3 describes multiple potential precursors that express Sox2 in the adult skin, it is still unclear which of these Sox2-expressing skin cell types are responsible for the observed wound-healing phenotype. I propose that much of this phenotype is due to a deficit in dermal precursors for a number of reasons. First, the dermis and overlying epidermis are the key components of wound repair responses. Second, our previous studies indicated that Sox2-expressing hair follicle dermal cells are involved in wound healing events\textsuperscript{14}. Finally, my FACS data (HJ, data not shown) indicates that Sox2-expressing dermal precursors are the major population (approximately 50%) of Sox2-expressing skin cells. Therefore, it is likely that the decrease in wound-healing seen in Sox2 mutants is largely caused by the reduced levels or deletion of Sox2 in dermal precursors. If this is the case, then these studies on Sox2 functional in dermal precursors would support the “stem-cell hypothesis for aging”, a notion that some characteristics of aging reflect a decline in the regenerative capacity of resident stem cells across many different tissues\textsuperscript{366}. In this regard, Su et al (2009) previously demonstrated that the loss of TAp63, a p53 family member, led to hyperproliferation, senescence and genomic instability of cultured dermal precursors, accompanied by premature senescence, reduced hair follicle morphogenesis and disrupted wound healing responses in vivo\textsuperscript{28}. This study indicated a potential link between depletion of the dermal precursor pool and premature aging phenotypes. Thus, in the future it would be
interesting to ask if Sox2 is required for maintenance and/or functionality of dermal precursors and if ablation of Sox2 expression specifically in dermal precursors might lead to defects in wound healing responses and premature aging of the skin.

4.3 Conclusion

My first project has helped define the developmental origins of SKPs. The results indicate that adult mesodermally-derived precursors for the dermis are plastic enough to generate neural tissues in response to exogenous cues. This finding might generalize to adult precursors for other tissues, emphasizing the potential importance of adult precursors as a cell source for stem cell-based therapies. My second project has revealed that Sox2 defines multiple potential precursor populations in the skin, and work by others in the Miller laboratory has shown that Sox2 reduction/depletion in the skin leads to a decline in wound-healing that is exacerbated in older skin. These data indicated that Sox2 expression in adult precursors is essential for proper skin maintenance and repair. From a broader perspective, these results support the idea that depletion of and/or deficiencies in adult tissue precursors cause premature aging, and argue that the aberrant wound repair that is normally associated with skin aging could be ameliorated if we could find ways to recruit and/or enhance dermal precursor populations.
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131


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