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DECODING THE HETEROGENEITY OF SKIN IN HOMEOSTASIS AND REGENERATION AT SINGLE-CELL RESOLUTION

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Cover image: Microscopy images of different skin cell types projected onto a t-SNE representation of skin single-cell RNA-sequencing data. Shown are hair follicle matrix cells expressing *Foxq1* (green, magenta), traced Lgr5 stem cells contributing to wound healing (red), dermal fibroblasts expressing *Sparc* (cyan), adipocytes stained with PLIN (orange) and γδ T cells stained with TCR Vγ5 (yellow).

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Decoding the heterogeneity of skin in homeostasis and regeneration at single-cell resolution

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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ABSTRACT

The skin plays a critical role in securing homeostasis in the mammalian body. Its epidermis forms a tight barrier, which separates the internal from the external environment, thereby shielding the body from physical and chemical insult. Due to the exposed position of skin as the outermost organ of the body, skin cells need to be replaced continuously. Cellular maintenance and regeneration of the skin and its associated hair follicles is orchestrated by a variety of stem cell populations. Because of its regenerative properties, the mouse skin is one of the most important model organs in stem cell research and regenerative medicine.

The skin is a complex multicellular system composed of a large variety of molecularly and functionally distinct cell populations. The physiology of the skin is a result of the intricate interplay of these diverse cell types. Accordingly, knowledge about the cellular composition of the skin is an essential step in understanding its biology. For a long time, cell populations in the skin were defined based on the expression of individual molecular markers, thus making a comprehensive analysis of cellular heterogeneity impossible. In this thesis, I describe how we used single-cell transcriptomics to create systematic cell type maps of the skin in order to analyze complex molecular processes at single-cell resolution.

In the first part of this thesis, I provide an overview of the morphology, function and cellular heterogeneity of the skin. I put particular emphasis on the skin as a self-maintaining tissue and model organ for stem cell research, describing regenerative process such as skin barrier maintenance, cyclical regeneration of hair follicles and cutaneous wound healing in great detail. Then, I introduce single-cell RNA-sequencing as a technique, which has revolutionized the way we analyze and conceptualize cellular heterogeneity in complex tissues.

Next, I portray how we championed the application of single-cell transcriptomics in skin biology with three key papers. In **Paper I**, we used single-cell RNA-sequencing to analyze the mouse epidermis including hair follicles during its resting stage (telogen). We discovered previously unknown cellular heterogeneity in the epidermis and demonstrated that the complexity of this tissue is the result of just two vectors of variation: differentiation stage and spatial position. In **Paper II**, we analyzed the complete mouse skin, including both epidermal and stromal cells, during hair growth (anagen) and rest (telogen). In addition to describing novel cell types in the stromal part of the skin, we model cellular differentiation and lineage specification in the growing hair follicle at unprecedented resolution. In **Paper III**, we use single-cell transcriptomics to track molecular changes in different stem cell populations during wound healing and answer several key questions related to stem cell identity and plasticity during regenerative processes.

In the last section of this thesis, I demonstrate that our studies have not just allowed us to analyze the cellular heterogeneity of the mouse skin at unprecedented detail, but have also enabled us to address a variety of critical questions such as how stem cell identity is shaped and how regenerative processes are orchestrated in the skin. I thus outline how our endeavors mark the first step towards a systems biology of the skin.

LIST OF SCIENTIFIC PAPERS

I. **Simon Joost**, Amit Zeisel, Tina Jacob, Xiaoyan Sun, Gioele La Manno, Peter Lönnerberg, Sten Linnarsson, and Maria Kasper. *Single-cell transcriptomics reveals that differentiation and spatial signatures shape epidermal and hair follicle heterogeneity.* Cell Systems 3, 3 (2016): 221-237.

II. **Simon Joost**, Xiaoyan Sun, Tina Jacob, Karl Annusver, Inês Sequeira, Rickard Sandberg, and Maria Kasper. *A single-cell atlas of mouse skin during hair growth and rest.* Manuscript.

III. **Simon Joost**, Tina Jacob, Xiaoyan Sun, Karl Annusver, Gioele La Manno, Inderpreet Sur, and Maria Kasper. *Single-cell transcriptomics of traced epidermal and hair follicle stem cells reveals rapid adaptations during wound healing.* Cell Reports 25, 3 (2018): 585 – 597.

Papers not included in this thesis

IV. Saiful Islam, Amit Zeisel, **Simon Joost**, Gioele La Manno, Pawel Zajac, Maria Kasper, Peter Lönnerberg, and Sten Linnarsson. *Quantitative single-cell RNA-seq with unique molecular identifiers.* Nature Methods 11, 2 (2014): 163.

V. Anja Füllgrabe, **Simon Joost**, Alexandra Are, Tina Jacob, Unnikrishnan Sivan, Andrea Haegebarth, Sten Linnarsson, Benjamin D. Simons, Hans Clevers, Rune Toftgård, et al. *Dynamics of Lgr6+ progenitor cells in the hair follicle, sebaceous gland, and interfollicular epidermis.* Stem Cell Reports 5, 5 (2015): 843-855.

VI. Marco Gerling, Nikè VJA Büller, Leonard M. Kirn, **Simon Joost**, Oliver Frings, Benjamin Englert, Åsa Bergström, Raoul V. Kuiper, Leander Blaas, Mattheus C.B. Wielenga, et al. *Stromal Hedgehog signalling is downregulated in colon cancer and its restoration restrains tumour growth.* Nature Communications 7 (2016): 12321.

VII. Juan R. Acosta*, **Simon Joost***, Kasper Karlsson, Anna Ehrlund, Xidan Li, Myriam Aouadi, Maria Kasper, Peter Arner, Mikael Rydén, and Jurga Laurencikiene. *Single cell transcriptomics suggest that human adipocyte progenitor cells constitute a homogeneous cell population.* Stem Cell Research & Therapy 8, 1 (2017): 250.

VIII. Xiaoyan Sun, Alexandra Are, Unnikrishnan Sivan*, **Simon Joost***, Karl Annusver*, Tina Jacob, Anja Füllgrabe, Tim Dalessandri, Marco Gerling and Maria Kasper. *Coordinated hedgehog signaling induces de novo hair follicles in primed epithelial structures.* SSRN (2018): 3288530.

* Equal contribution

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LIST OF ABBREVIATIONS

APM	Arrector pili muscle
BMP	Bone morphogenetic protein
CL	Companion layer
CU	Cuticle
CX	Cortex
DC	Dendritic cell
DP	Dermal papilla
DPT	Diffusion pseudotime
DS	Dermal sheath
ECM	Extracellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FISH	Fluorescence *in situ* hybridization
GL	Germinative layer
HE	Henle layer
HF	Hair follicle
HG	Hair germ
HGF	Hepatocyte growth factor
HS	Hair shaft
HU	Huxley layer
ICU	IRS cuticle
IFE	Interfollicular epidermis
ILC	Innate lymphoid cell
ICA	Independent component analysis

IRS	Inner root sheath
IVT	In vitro transcription
JZ	Junctional zone
KGF	Keratinocyte growth factor
kNN	k-nearest neighbor
LPC	Lower proximal cup
LRC	Label retaining cell
ME	Medulla
MMLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinase
MST	Minimum spanning tree
NB	Negative binomial
NMF	Non-negative matrix factorization
ORS	Outer root sheath
PCA	Principal component analysis
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator-activated growth factor
PSU	Pilosebaceous unit
scRNA-seq	Single-cell RNA-sequencing
SG	Sebaceous gland
TD	Touch dome
TGF	Transforming growth factor
t-SNE	t-distributed stochastic neighbor embedding
TSO	Template switching oligonucleotide
UMAP	Uniform Manifold Approximation and Projection
UMI	Unique molecular identifier
VEGF	Vascular endothelial growth factor

1 THE MOUSE SKIN AS A MODEL ORGAN IN STEM CELL RESEARCH

The skin is by far the largest organ of the mammalian body and plays a crucial role in maintaining the body's physical and physiological integrity. The cells of the epidermis – the epithelial part of the skin – form a strong, watertight barrier, which secures homeostasis by separating the internal from the external environment. A variety of epidermal appendages play additional roles in mammalian physiology. Hair follicles (HFs) form the pelage, which contributes to thermoregulation, camouflage and physical defense in most mammals, while sweat glands help to uphold fluid and thermal homeostasis. Additionally, a variety of touch and pain receptors located in the skin create a neurosensory interface with the outside world (Fuchs, 2007; Hsu, Li, & Fuchs, 2014a).

As the skin is continuously exposed to harmful outside influences such as radiation, infectious agents and physical insult, the cells of the epidermis need to be replaced constantly. Diverse stem cell pools in the epidermis ensure homeostatic maintenance of the skin barrier and contribute to wound healing upon large-scale damage of the skin. Likewise, the integrity and function of epidermal appendages is secured by various specialized stem cell populations (Blanpain & Fuchs, 2009). Due to its distinct microanatomy, experimental accessibility, cellular heterogeneity and the cyclical nature of hair follicle regeneration, the skin of the mouse (*Mus musculus*) has become one of the key model systems for developmental biology, stem cell research and regenerative medicine. The study of stem cells in the murine skin has not only yielded important insights into medical conditions such as baldness, skin tumors and chronic wounds, but has also established many paradigms of stem cell identity and function (Rompolas & Greco, 2014; Schepeler, Page, & Jensen, 2014; Watt, 2014).

The skin is a complex multicellular organ consisting of many different cell types with distinct molecular identities and functional roles. The physiology, or pathophysiology, of the skin is the result of the intricate interplay of these diverse cell populations (Fuchs, 2007). Accordingly, a large portion of skin research has always been focused on elucidating the molecular identities, cellular dynamics or functional properties of distinct skin cell populations. This thesis describes how we used single-cell transcriptomics to systematically analyze the transcriptomic identities and molecular dynamics of skin (stem) cell populations during homeostasis and regeneration.

1.1 MICROANATOMY OF THE SKIN

Histologically, the mouse skin can be divided along the distal-proximal axis into the epidermis and its appendages, the dermis, and the hypodermis **(Figure 1A)**. As sweat glands are only found at specific sites such as the paws, the epidermis covering most of the murine body is composed of interfollicular epidermis (IFE) interspersed with hair follicles (Rognoni & Watt, 2018). The IFE is a multilayered sheet of highly proliferative keratinocytes that

differentiate upwards to form the keratinized skin barrier (Fuchs, 1990). Intermingled with the IFE are immune cells, most importantly γδ T cells and Langerhans cells, which play a crucial role in modulating the immunological barrier function of the skin. Hair follicles are morphologically and histologically complex mini-organs **(Figure 1B)**, which pass through regular cycles of rest (telogen), growth (anagen) and regression (catagen) to maintain the mammalian coat. During telogen, the hair follicle can be anatomically divided into the infundibulum, the junctional zone, the isthmus, the bulge and the hair germ. All these structures are lined by multiple layers of keratinocytes (Kretzschmar & Watt, 2014; Schepeler et al., 2014). Connected to each hair follicle at the height of the upper bulge and junctional zone respectively are the arrector pili muscle responsible for piloerection (Fujiwara et al., 2011) and the sebaceous gland, which releases sebum into the hair canal to lubricate the skin (Niemann & Horsley, 2012). The hair germ region is furthermore linked to the dermal papilla, a condensate of fibroblast-like cells that play a key signaling role during hair follicle regeneration (Driskell et al., 2011). Together, hair follicle, sebaceous gland, arrector pili and dermal papilla form the pilosebaceous unit. The mouse coat is formed by four different types of hair – guard, awl, auchene and zigzag hair – distinguished by developmental induction time point, microanatomy and structure of the hair shaft. Each hair follicle type is innervated by a unique combination of mechanosensory neurons (Li et al., 2011). Additionally, the IFE around the guard hair openings is lined with touch domes, mechanoreceptors composed of epidermal palisade and Merkel cells (Doucet et al., 2013).

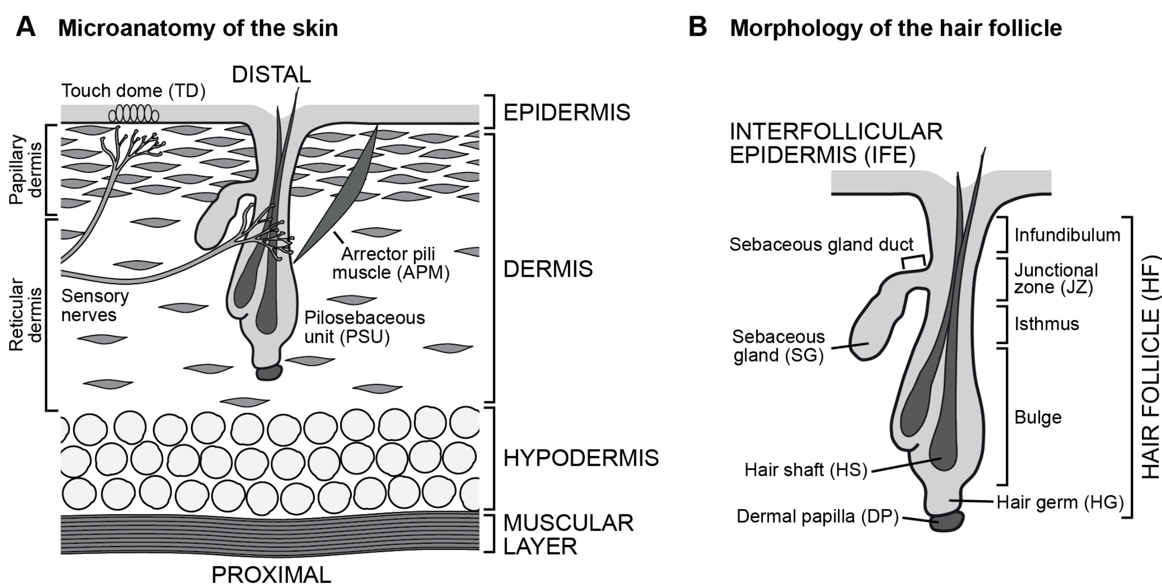


Figure 1: Microanatomy of the mouse skin **(A)** and the telogen hair follicle **(B).**

The dermis is a layer of fibrous, irregular connective tissue dominated by collagen-producing fibroblasts. It can be further subdivided into the denser, more distal *papillary dermis* and the more proximally located *reticular dermis*. In addition to dermal fibroblasts, the dermis contains immune cells, blood and lymph vessels, as well as mechanosensory and nerve fibers. In

contrast, the hypodermis/subcutis is dominated by subdermal adipose tissue and skeletal muscle. Dermis and hypodermis ensure the elasticity of skin, serve as layers of protection against physical damage and create a scaffold for the attachment of epidermal cells (Hsu, Li, & Fuchs, 2014a; Rognoni & Watt, 2018). Furthermore, many cell types of the dermis and hypodermis form signaling relationships with keratinocytes of the epidermis and are crucial in modulating epidermal stem cell identity and function (Chacón-Martínez, Koester, & Wickström, 2018).

In **Paper I**, we used single-cell RNA-sequencing to disentangle the cellular heterogeneity of the epidermis during rest (telogen). Using an unsupervised approach, we identified a variety of cell types connected to the IFE and different spatial compartments of the hair follicle. A supplementing dataset of touch dome palisade cells was analyzed in Paper VIII, which is not included in this thesis. In **Paper II**, we broadened our scope and analyzed epidermis, dermis and hypodermis during both rest (telogen) and hair follicle growth (anagen) at single-cell resolution, creating among other things the first unbiased census of cellular heterogeneity in the stroma of the skin.

1.2 DEVELOPMENT OF MOUSE SKIN

While less is known about the specific developmental pathways of dermal and subcutical cell types, the development of the murine epidermis is a well-defined process (**Figure 2**) (Fuchs, 2007). Early in embryogenesis, WNT signaling enforces epidermal fate in the neuroectoderm by suppressing FGF and inducing BMP signaling (Stern, 2005). The result is the single-layered embryonic epidermis, which begins to stratify at E14.5 and interacts with the under-lying mesoderm to induce the development of epidermal appendages. A reaction-diffusion system of WNT and its inhibitor Dickkopf leads to the formation of even spaced placodes – small epidermal downgrowths that constitute the basis of hair follicle development (Andl, et al., 2002; Huelsken et al., 2001; Sick et al., 2006). In the mouse skin, placode formation occurs in three major waves, with the development of primary hairs (guard: E14.5) preceding the formation of secondary hairs (awl, auchene: E16.5; zigzag: E18.5) by several days (Schmidt-Ullrich & Paus, 2005). Placode cells express SHH, FGFs and other morphogens to attract the formation of dermal condensates – which later mature into the dermal papilla – below the hair follicle placode (germ phase: E15.5) (St-Jacques et al., 1998). Molecular crosstalk between epidermis and dermis subsequently leads to the downgrowth and matura-tion of hair follicles. Markers such as *Sox9*, *Lgr6* and *Lrig1* are initially co-expressed in the placode, but become increasingly compartmentalized as the follicle matures – thus setting the foundation for stem cell heterogeneity in the adult hair follicle (Jensen et al., 2009; Nowak, Polak, Pasolli, & Fuchs, 2008; Snippert et al., 2010). About two days after induction, hair follicle cells begin to engulf the dermal papilla and a highly proliferative matrix forms in the hair follicle center. Cells from this matrix terminally differentiate into the inner lineages of the anagen follicle to form the hair shaft (described in detail in Section 1.5). At the same time, sebaceous gland precursors begin to appear in the distal parts of the developing hair follicle (Niemann & Horsley, 2012).

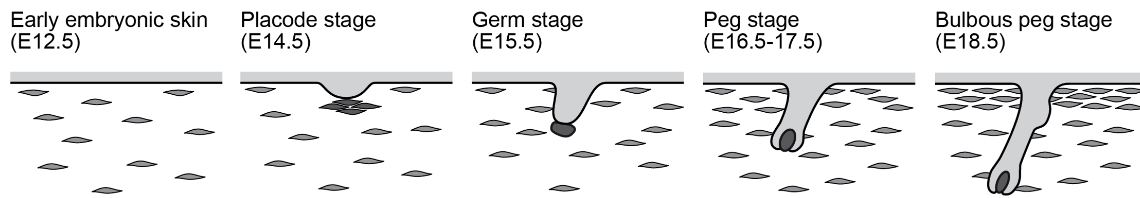


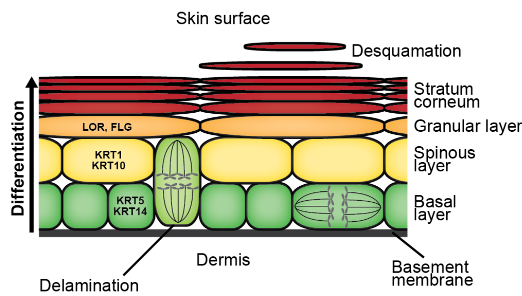
Figure 2: Development of the mouse epidermis and dermis. Time points correspond to guard hair development.

The dermis of the dorsal skin is derived from the paraxial mesoderm (Driskell & Watt, 2015). While the dermis is homogenous at E12.5, fibroblast progenitors soon after split into two distinct lineages with one lineage creating the differentiated fibroblasts of the papillary dermis, the dermal papilla and the arrector pili, while the other lineage forms the fibrous tissue of the reticular dermis and the adipose tissue of the hypodermis. Papillary and reticular dermis are clearly discernable at E18.5 (Driskell et al., 2013). Skin innervation in the mouse is highly coordinated with hair follicle development (Peters et al., 2002). Melanocytes are neural crest derived and enter the epidermis from E14.5 (Lin & Fisher, 2007).

1.3 INTERFOLLICULAR CELL HETEROGENEITY AND CREATION OF THE SKIN BARRIER

The IFE is a squamous, stratified, multilayered epithelium, which creates and maintains the keratinized skin barrier by continuous proliferation and upwards differentiation of keratinocytes. Based on histological and molecular features, the IFE can be divided into four distinct layers (**Figure 3A**): the basal layer, which is attached to the basement membrane and marked by KRT14 and KRT5, the spinous layer marked by KRT1 and KRT10 and distinguished by elevated lipid metabolism, the granular layer mass-producing cornified envelope proteins such as LOR and FLG, and the stratum corneum – the keratinized end product of the differentiation process (Fuchs, 1990; Fuchs, 2007). The combination of cross-linked cornified envelope proteins, keratins and barrier lipids, such as cholesterol and ceramides, in the stratum corneum ensures the function of the IFE as both a physical and chemical barrier. In addition, a variety of antimicrobial peptides are produced in the IFE (Elias, 2008).

Two loosely linked cellular processes orchestrate epidermal stratification and differentiation: asymmetric cell division perpendicular to the basement membrane, which pushes one daughter cell into the suprabasal layer, and loss of basement membrane contact (delamination) (Fuchs, 2007; Hsu, Li, & Fuchs, 2014a). Notch signaling is known to be important in epidermal stratification (Watt, Estrach, & Ambler, 2008). Other transcription factors, which play a role in IFE differentiation are *Hoxa7*, *Grhl1* and *Prdm1* (Kretzschmar et al., 2014; La Celle & Polakowska, 2001; Mlacki et al., 2014). A variety of proteolytic enzymes, most importantly kallikreins, have been linked to desquamation, the consistent shedding of stratum corneum layers (Brattsand et al., 2005; Elias, 2008).



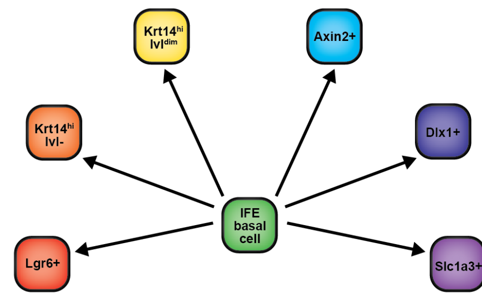


Figure 3: (A) Cellular structure of the stratifying interfollicular epidermis (IFE). **(B)** Described cellular heterogeneity in the basal layer of the mouse IFE. Note that it is unknown if and how subpopulations overlap.

Since the cells of the stratum corneum are shed regularly, a constant supply of new keratinocytes has to be provided. As suprabasal cells are post-mitotic, proliferative activity is restricted to stem and/or progenitor cells and their progeny in the basal layer of the IFE (Blanpain & Fuchs, 2009). A variety of molecular mechanism have been linked to the maintenance of IFE basal cell identity, most importantly the expression of integrins (particularly types $\alpha_3\beta_1$ and $\alpha_6\beta_4$) and the deposition of laminin-5 in the basement membrane (Watt & Fujiwara, 2011). For decades, the identity and function of interfollicular stem cells has been one of the focal points of skin research. In addition to untangling the cellular mechanisms of stem cell homeostasis in the IFE, many studies attempted to determine whether one or more privileged populations of stem cells – distinct from other cells of the basal layer – exist in the IFE. Two models of stem cell dynamics have been proposed for the IFE (Klein & Simons, 2011; Simons & Clevers, 2011). According to the *hierarchical model*, IFE stem cells are long-lived, slowly cycling cells that divide asymmetrically into one stem cell and one transit-amplifying cell, whose progeny eventually leaves the basal layer. As the IFE shows a distinct cellular organization with several basal and suprabasal cells located below a sheet of hexagonal stratum corneum cells, it was assumed that these structures represent epidermal proliferative units maintained by a single basal stem cell (Potten, 1974). In contrast, the *stochastic model* assumes that IFE stem cells can divide both symmetrically into either two stem cells or two cells entering differentiation, or asymmetrically. According to this model, IFE homeostasis is achieved stochastically by neutral competition of stem cells. In recent years, several studies have come to support the stochastic model of stem cell homeostasis in the IFE (Clayton et al., 2007; Doupé et al., 2010; Füllgrabe et al., 2015; Mascré et al., 2012). Rompolas et al., 2016 reconciled the stochastic model with the distinct cellular organization of the IFE by showing that although basal cells divide according to neutral competition, their progeny integrates into distinct vertical columns (epidermal differentiation units).

Over the years, a variety of cellular subpopulations have been identified in the IFE basal layer and implicated as discrete stem cell pools (**Figure 3B**). Expression of the WNT target *Axin2*

has been described as a marker for epidermal stem cells (Lim et al., 2013). In contrast, Mascré et al. (2012) suggest that *Krt14*-cre and *Ivl*-cre cells represent a hierarchy of slowly-cycling stem cells and committed progenitor cells respectively. In a recent study, it has furthermore been suggested that label-retaining cells (LRC) marked by *Dlx1* and non-LRCs marked by *Slc1a3* constitute two different IFE stem cell pools (Sada et al., 2016). We observed that a subset of IFE basal cells expresses *Lgr6* and that these cells constitute a self-renewing population following neutral competition. However, we were unable to find any robust transcriptomic differences between *Lgr6*+ and *Lgr6*- IFE basal cells (Paper V, not included in this thesis).

In **Paper I**, we used our single-cell RNA-sequencing dataset to model IFE differentiation along a pseudotemporal axis, detecting a variety of known and novel genes including transcription factors likely involved in the execution and regulation of epidermal stratification and skin barrier formation. Furthermore, we detected a subset of IFE basal cells marked by high levels of *Thbs1*. However, our data suggest that overall heterogeneity in the IFE basal layer is mostly shaped by gradual changes in cellular differentiation stage ($Krt14^{hi}/Krt10^{lo}$ vs. $Krt14^{dim}/Krt10^{dim}$) and not by discrete populations of stem and/or progenitor cells.

1.4 CELL HETEROGENEITY IN THE RESTING HAIR FOLLICLE

The telogen hair follicle harbors a surprising diversity of stem cell populations necessary to maintain the hair follicle at rest and to orchestrate its cyclical growth and regression during anagen and catagen. Under the assumption that tissue stem cells are proliferatively quiescent, the first populations of follicular stem cells in the bulge region were identified in the 1990s based on their label-retaining characteristics after pulse chase with radioactive tracers, BrdU or H2BGFP (Braun et al., 2003; Cotsarelis, Sun, & Lavker, 1990; Tumbar et al., 2004). Since then, the combination of transgenic reporter mice and lineage tracing approaches has facilitated the identification of a large number of different stem cell populations in all compartments of the resting hair follicle (**Figure 4**).

It has been shown that *Cd34* and *Krt15* mark cells in the bulge that are slowly cycling, maintain the lower hair follicle during homeostasis, and are able to form all epidermal lineages in skin reconstitution assays (Blanpain et al., 2004; Morris et al., 2004; Trempus et al., 2003). In addition to expression in the hair follicle placode, the transcription factor *Sox9* is also expressed in the adult bulge and knockdown of *Sox9* during skin developments leads to loss of *Cd34*+/*Krt15*+ stem cells (Nowak et al., 2008; Vidal et al., 2005). A similar role in shaping bulge stem cell identity has been reported for the transcription factors *Lhx2*, *Nfatc1*, *Tbx1* and *Tcf3* (Chen et al., 2012; Horsley et al., 2008; Nguyen, Rendl, & Fuchs, 2006; Rhee, Polak, & Fuchs, 2006). While *Cd34* expression is restricted to the bulge area, *Lgr5* marks cells of both the lower bulge and hair germ (Jaks et al., 2008). Hair germ cells are sensitive to WNT signals and proliferate rapidly upon telogen-to-anagen transition. One model of stem cell hierarchy in the hair follicle thus postulates that *Cd34*+ cells in the bulge represent a more quiescent population of stem cells that replenishes the hair germ stem cell pool after hair regeneration (Greco et al., 2009; Jaks et al., 2008). However, not all *Lgr5*+ cells

are lost after catagen (Hsu, Pasolli, & Fuchs, 2011). In addition to *Lgr5*, cells in the hair germ have been shown to express effectors of the Hedgehog signaling pathway such as *Gli*1, as well as the Id-proteins *Id1* and *Id3*, linked to modulation of WNT and BMP signaling (Brownell et al., 2011; Genander et al., 2014). An additional population of *Gli1*+ cells was identified in the upper bulge region in direct proximity to the sensory nerve enwrapping the hair follicle (Brownell et al., 2011). In contrast to the basal cells of the hair follicle bulge (outer bulge), the *Krt6*+ cells of the inner bulge do not possess stem / progenitor cell potential themselves but are important for the maintenance of adjacent follicular stem cells (Hsu, Pasolli, & Fuchs, 2011).

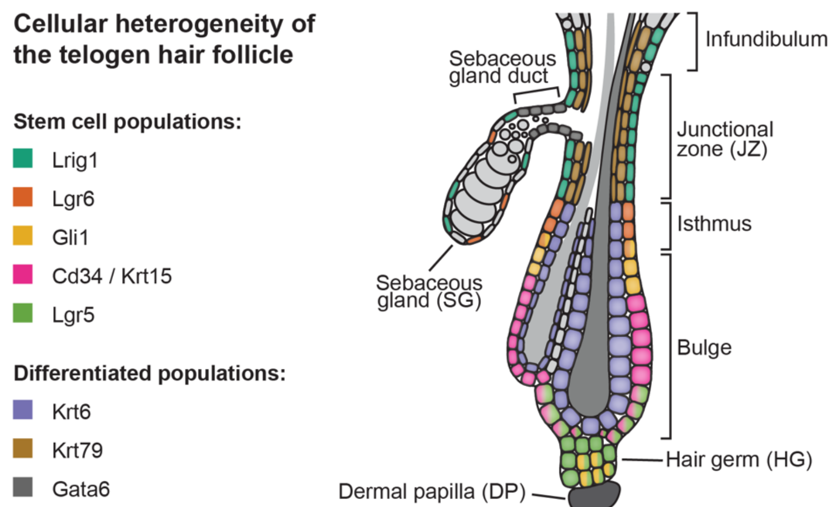


Figure 4: Previously described cell populations in the telogen hair follicle.

First evidence that the upper hair follicle, which includes isthmus, junctional zone, sebaceous gland and infundibulum, can be maintained without the contribution of bulge stem cells was acquired from lineage tracing experiments of randomly labeled cells (Ghazizadeh & Taichman, 2001). Shortly after, *Plet1*/MTS24 was identified as a first potential marker of follicular stem cells outside the bulge (Nijhof et al., 2006). In addition to a subset of IFE basal cells, *Lgr6* marks cells of the isthmus region directly distal to the bulge as well as individual cells in the basal layer of the sebaceous gland. *Lgr6*+ cells contribute to the maintenance of the isthmus and sebaceous gland and can reconstitute complete hair follicles when grafted into nude mice (Füllgrabe et al., 2015; Snippert et al., 2010). In contrast, *Lrig1*+ cells are situated in the basal layer of the junctional zone and maintain junctional zone and infundibulum. Like *Lgr6*+ cells, individual *Lrig1*+ cells found in the basal layer of the sebaceous gland also contribute to sebaceous gland maintenance (Jensen et al., 2009; Page et al., 2013). A variety of markers for non-basal cell populations in the upper hair follicle have also been described in recent years. KRT79 marks cells of the suprabasal inner layers of the upper hair follicle that line the hair shaft (Veniaminova et al., 2013). In contrast, the transcription factor *Gata6* specifies cellular identity in the sebaceous duct (Donati et al., 2017). Despite the high

number of stem and progenitor cells identified in the hair follicle, it is still disputed which cellular or molecular factors specify stemness in either IFE or hair follicle.

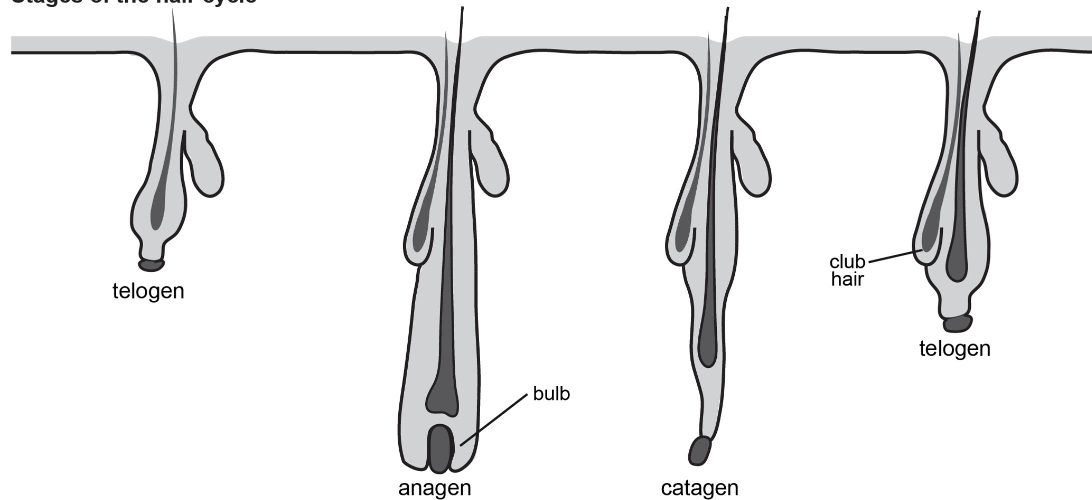
In **Paper I**, we used our single-cell sequencing data to demarcate populations of the telogen hair follicle in an unbiased, systematic fashion. We robustly identified five outer bulge, seven upper hair follicle, and three inner bulge populations. We furthermore showed that cellular heterogeneity in the hair follicle is shaped by an interplay of gradually changing spatial gene expression signatures along the proximal-distal axis and the differentiation status of cells. In order to screen for gene expression signatures linked to stem cell identity, we systematically compared the transcriptomes of *Krt14*^{hi}, *Lgr6*+, *Lrig1*+, *Cd34*+, *Gli1*+ and *Lgr5*+ cells in our dataset. Interestingly, we did not find any distinct stem cell signature setting these cells apart from other basal cells. Instead, epidermal stem cells shared a particularly high expression of markers linked to overall basal layer identity.

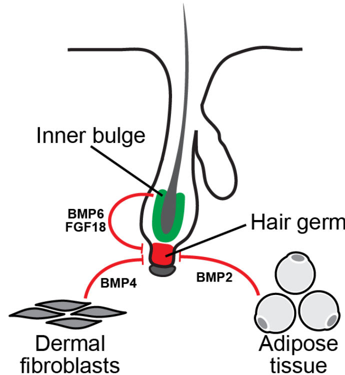
1.5 CELLULAR DYNAMICS DURING HAIR FOLLICLE REGENERATION

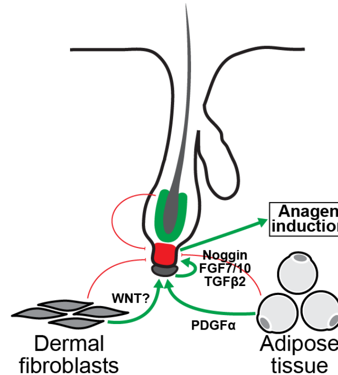
In contrast to the continuous activity of stem cells in the IFE, follicular stem cells act in a highly cyclical fashion to orchestrate the growth (anagen) and regression (catagen) of the hair follicle (**Figure 5A**) (Schneider, Schmidt-Ullrich, & Paus, 2009). Signals driving and modulating hair cycling are derived from both neighboring epidermal cells and the stromal microenvironment, which undergoes significant changes in cellular and molecular composition between anagen and telogen. As the first two hair cycles are synchronized and follow a highly predictable timing in mice, the hair cycle is one of the best-studied regenerative processes in the mammalian body (Hsu, Li, & Fuchs, 2014a).

The resting stage (telogen) is the energy-efficient default state of the hair follicle during which the fully matured club hair is maintained (Geyfman et al., 2014). During refractory telogen, hair follicle stem cells are quiescent and unresponsive to activation due to inhibitory signals such as BMP2 from the subcutaneous fat, BMP4 from dermal fibroblasts as well as BMP6 and FGF18 from the inner layer of the bulge (Hsu, Pasolli, & Fuchs, 2011; Plikus & Chuong, 2014; Plikus et al., 2008). In contrast, competent telogen is marked by a decrease of inhibitory stimuli, thus making follicular stem cells more receptive towards anagen induction (Plikus et al., 2008). The dermal papilla located proximal to the hair germ is essential for anagen induction and follicles lacking dermal papillas are unable to enter the hair cycle (Rompolas et al., 2012). Signals from the dermal papilla that activate hair follicle stem cells include the BMP inhibitor Noggin, as well as FGF7, FGF10 and TGFβ2 (Greco et al., 2009; Oshimori & Fuchs, 2012). Activation of the dermal papilla is in part modulated by PDGFα expressed in adipocyte precursor cells (Festa et al., 2011). Active WNT signaling is likewise crucial for the activation of both dermal papilla and hair germ (Greco et al., 2009), even though the specific WNT effectors and their cellular sources remain to be identified. Anagen entry is achieved when activatory signals override the inhibitory telogen microenvironment and induce proliferative activity in the hair germ (**Figure 5B**) (Hsu, Li, & Fuchs, 2014a). In contrast to human hair, which cycles in a mosaic-like fashion, the coat of most rodents is maintained in synchronized, wave-like patterns (Plikus et al., 2011). In fact, it has been

shown that WNT activation in single pilosebaceous units does not translate into stem cell activation and hair cycle entry. This highlights the requirement for signal-coupling between individual pilosebaceous units to reach the threshold for anagen induction and emphasizes the role of spatially extended changes in the microenvironment – e.g. in the dermis and hypodermis – during hair cycling (Geyfman et al., 2014; Plikus & Chuong, 2014).







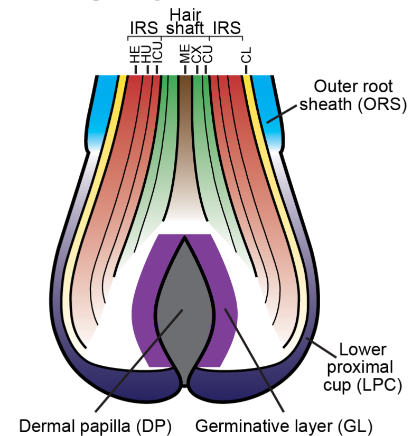


Figure 5: (A) Hair follicle morphology during the telogen, anagen and catagen stages of the hair cycle. **(B)** Activatory and inhibitory signals during refractory and competent telogen. **(C)** Cellular heterogeneity in the mature anagen hair follicle bulb. CL: companion layer; HE: Henle layer; HU: Huxley layer; ICU: IRS cuticle; CU: cuticle; CX: cortex; ME: medulla.

Upon anagen induction, stem cells of the hair germ and bulge start to proliferate and hair follicles with their associated dermal papillas begin to extend downwards into the dermis and hypodermis. During this process, the transit-amplifying cells of growing hair follicles begin to engulf the dermal papilla, forming the characteristic bulbar structure of the anagen follicle (Alonso & Fuchs, 2006; Schneider et al., 2009). The cells of the hair germ, which start to proliferate first (Greco et al., 2009), form the bulbar matrix, whose cells will terminally differentiate into the inner layers and hair shaft of the anagen follicle. In contrast, the cells of the bulge will form the basal layer on the outside of the anagen follicle – called outer root

sheath (ORS) (Hsu, Pasolli, & Fuchs, 2011; Rompolas, Mesa, & Greco, 2013). While hair germ stem cells are activated by the aforementioned signals, recent evidence shows that SHH secreted by hair germ-derived transit-amplifying cells is required to activate bulge stem cells, thus explaining their delayed proliferative activity (Hsu, Li, & Fuchs, 2014b). The mature anagen hair follicle possesses a distinct cellular anatomy (**Figure 5C**). In addition to the ORS marked by high levels of KRT5 and KRT14, and the lower proximal cup (LPC) expressing high levels of *Lgr5* (Sequeira & Nicolas, 2012), the anagen hair follicle is made up of seven concentric inner cell layers: the companion layer, the three layers of the inner root sheath (IRS: Henle, Huxley and IRS cuticle), as well as the cuticle, cortex and medulla layers forming the hair shaft (Fuchs, 2007). Companion layer, IRS and hair shaft are marked by KRT75, KRT71 and KRT35 respectively (Langbein et al., 2010).

How matrix differentiation is orchestrated on the cellular and molecular level is disputed. It has been shown that precursor cell fate becomes more constrained as anagen proceeds, and that the spatial organization of matrix progenitors in the hair follicle bulb determines lineage identity with more central cells entering hair shaft lineage while more peripheral cells acquire companion layer or IRS identity (Legué & Nicolas, 2005; Legué, Sequeira, & Nicolas, 2010; Sequeira & Nicolas, 2012). A recent study postulates that micro-niches generated by heterogeneous dermal papilla cells specify lineage fate in matrix precursor cells adjacent to the dermal papilla (Yang et al., 2017). Likewise, it has been reported that early matrix cells preferentially enter companion layer fate, while later matrix cells primarily contribute to hair shaft and IRS lineages (Mesler et al., 2017). Studies also show that balancing of WNT and BMP signaling via Id proteins specifies follicle lineage, with IRS marked by high BMP and hair shaft by strong WNT signaling (Genander et al., 2014). In addition, MAF, MAFB, CUX1 and GATA3 have been identified as transcription factors specifying IRS lineage (Ellis et al., 2001; Kaufman et al., 2003; Miyai et al., 2010), while HOXC13 and FOXN1 have been linked to hair shaft identity (Bazzi et al., 2009; Mecklenburg et al., 2001).

During catagen, widespread apoptosis and phagocytosis removes all matrix and most lower ORS cells leading to an involution of follicle and dermal papilla (Hsu, Pasolli, & Fuchs, 2011; Mesa et al., 2015). Meanwhile, the cells of the middle and upper ORS form a new hair germ and bulge respectively, harboring the regenerative potential for the next hair cycle. It has been suggested that a small number of lower ORS cells is not subjected to apoptosis and differentiates to form the KRT6+ inner layer of the new bulge (Hsu, Pasolli, & Fuchs, 2011). FGF5, BMP2/4 and the TNFα-KRT17 axis have been identified as molecular regulators of catagen (Hébert et al., 1994; Schneider et al., 2009; Tong & Coulombe, 2006). Importantly, crosstalk between hair follicle and dermal papilla via TGFβ has been described as an important modulator of catagen, with dermal papilla ablation inhibiting follicular regression (Mesa et al., 2015).

Melanocytes are neural crest-derived cells, which protect the skin against DNA damage by producing the UV-absorbing pigment melanin. During telogen, melanocyte stem cells reside in the bulge area (Nishimura et al., 2002). Upon anagen entry, signals including endothelin

from the follicular matrix and KITL from the dermal papilla induce proliferation and differentiation of melanocytes, which locate to the bulb and transfer melanin to nearby differentiating matrix cells. Differentiated melanocytes degenerate during catagen. (Lin & Fisher, 2007).

In **Paper II**, we used single-cell RNA-sequencing data from anagen skin to disentangle cellular heterogeneity in the mature anagen follicle. We were able to describe more than 20 subpopulations of cells in the ORS, matrix, companion layer, IRS and hair shaft of the anagen hair follicle. We furthermore combined analysis of RNA velocity and pseudotemporal ordering of cells to model the differentiation trajectory of matrix cells forming the inner layers of the hair follicle, identifying new potential regulators of hair shaft and IRS differentiation.

1.6 CELLULAR HETEROGENEITY IN DERMIS AND HYPODERMIS

In addition to serving as layers of protection against physical damage, dermis and hypodermis have essential roles in creating both the overall molecular conditions and the specialized signaling microenvironments necessary for the maintenance and function of the epidermal sheath. A large variety of distinct dermal and subcutical cell populations create the extracellular matrix (ECM) scaffold required for epidermal attachment, provide signals orchestrating the hair cycle, ensure the function of the skin as an immune barrier, maintain the vasculature and innervate the skin (Hsu, Li, & Fuchs, 2014a; Rognoni & Watt, 2018). While the cell composition of the epidermis and the hair follicle is relatively well-described, the true extent of dermal and hypodermal cell heterogeneity is still largely unknown.

Dermal fibroblasts are the most abundant cell type in the dermis and produce the fibrous connective tissue, which makes up most of the dermal ECM (**Figure 6A**). In line with the morphological distinction between papillary and reticular dermis, it has been shown that fibroblast precursor cells split into two lineages that remain morphologically and molecularly distinguishable in postnatal skin (Driskell et al., 2013). Likewise, it has been suggested that embryonic expression of *En1* can be used to define a population of fibroblasts that is located in the lower dermis of adult mice, transcriptionally distinct from other fibroblasts, marked by DPP4 and responsible for most ECM deposition in healthy skin (Rinkevich et al., 2015). A single-cell RNA-sequencing study recently described additional heterogeneity among dermal fibroblasts in human skin, demarcating at least four molecularly distinct populations (Philippeos et al., 2018). In addition, several specialized cell populations are derived from fibroblasts of either the papillary or the reticular dermis lineage **(Figure 6A**). During development, the papillary dermis lineage creates follicle-associated structures including the arrector pili, the dermal sheath and the dermal papilla, while the reticular dermis lineage forms the adipose tissue of the subcutis (Driskell et al., 2013). The dermal sheath consists of myofibroblast-like cells encapsulating the anagen follicle. It has been shown that in adult murine skin, both the dermal papilla and the dermal sheath are maintained by a population of hair follicle-associated progenitor cells (Rahmani et al., 2014). In contrast, the adipocytes of the hypodermis are maintained by a population of adipocyte precursor cells, which are derived from

the reticular dermis lineage and whose activity is highly hair cycle stage dependent (Festa et al., 2011).

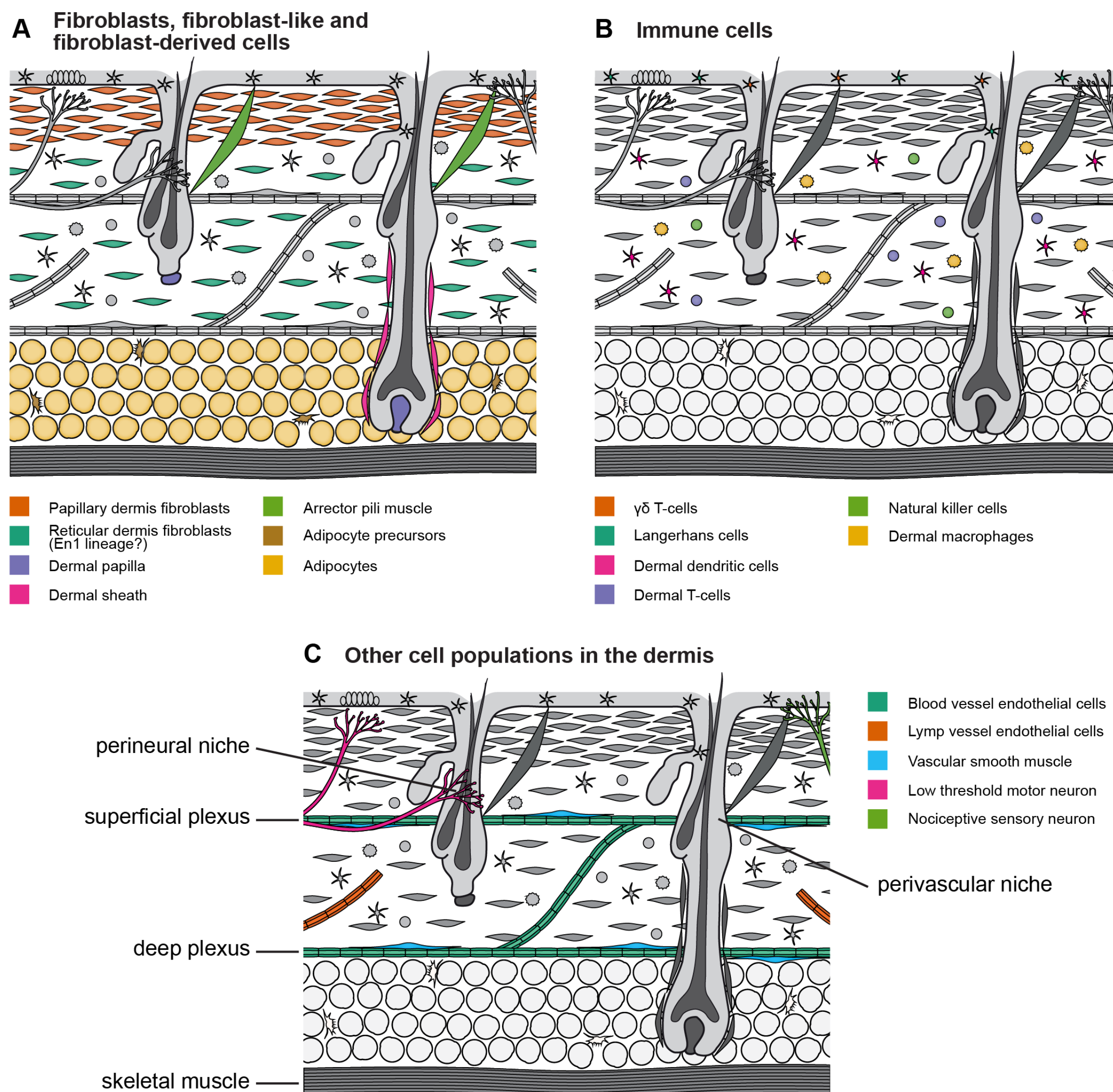


Figure 6: (A-C) Diversity of fibroblasts and fibroblast-derived cells **(A)**, immune cells **(B)** and other cell types **(C)** in the dermis and hypodermis.

In addition to its function as a barrier against physicochemical insult, the skin plays an important role as the first line of immune defense against microbes. In consequence, a large variety of specialized immune cell types can be found in both epidermis and dermis (**Figure 6B**). The epidermis contains a network of Langerhans cells and T cells interspersed with keratinocytes. Langerhans cells are dendritic cells, which are located above the basal layer of the epidermis and whose projections can reach into the stratum corneum. They have been implicated in sensing antigens on the surface of the skin and priming the immune system towards potentially dangerous agents, including induction of a T_H17 immune response upon the detection of certain pathogens. In contrast, epidermal γδ T cells, which likewise exhibit a

dendritic morphology, can sense keratinocyte stress and barrier dysfunction in the epidermis and play a key role during wound healing and cancer. The dermis harbors a more heterogeneous mix of immune cells, including various subsets of T cells, macrophages, mast cells, dendritic cells and innate lymphoid cells (ILC). At least three subtypes of dermal dendritic cells (DCs) – plasmacytoid DCs, $Cd103^+$ DCs and $Cd11b^+$ DCs – have been described in healthy mouse skin. Like epidermal Langerhans cells, these dermal DC populations have roles in antigen presentation and induction of T_H1 and T_H2 cells. In contrast to DCs, most dermal macrophages have a poor antigen presenting capacity and do not migrate to the lymph nodes, but instead contribute to tissue homeostasis by scavenging cellular breakdown products and invading microorganisms. In addition, ILC1 natural killer cells, which play a crucial role in the skin's antiviral response, as well as $Cd4^+$ regulatory (T_{REG}), effector memory (T_{EM}) and resident memory (T_{RM}) T cells, which orchestrate the adaptive immune response, have been described in the healthy dermis (Heath & Carbone, 2013; Malissen, Tamoutounour, & Henri, 2014).

The skin vasculature including the lymphatic system has an important role in ensuring nutrient supply, thermoregulation, hormonal communication and immune function. The blood supply of the skin is histologically well-defined (**Figure 6C**), with interconnected blood vessel networks running perpendicular to the skin surface at the level of the hair follicle bulge (superficial plexus) and the reticular dermis (deep plexus). In addition, a mesh of capillaries envelops the hair follicle and has been shown to form a perivascular niche for follicular stem cells (Xiao et al., 2013). The skin vasculature, which is formed by a variety of cell types including endothelial cells, vascular smooth muscle cells, and pericytes, is subject to significant remodeling during the hair cycle and inhibition of angiogenesis impairs anagen entry (Mecklenburg et al., 2000). Likewise, chemotherapy-induced hair loss is at least partially linked to the disruption of the skin vasculature. (Amoh et al., 2007).

A variety of afferent and efferent nerve fibers traverse the skin (**Figure 6C**). These include specialized nociceptive neurons that terminate in the epidermis (Zylka, Rice, & Anderson, 2005), at least four different types of mechanosensory neurons that innervate the hair follicle and touch dome (Li et al., 2011), as well as motor neurons regulating arrector pili contraction (Furlan et al., 2016). In addition, a variety of nerve-associated cells can be found in the dermis and epidermis, including Schwann cells (Gresset et al., 2015) and mechanoreceptive Merkel cells forming part of the touch domes (Doucet et al., 2013).

In **Paper II**, we used single-cell RNA-sequencing to create a systematic and unbiased census of cellular heterogeneity in the anagen and telogen dermis and hypodermis. We defined seven populations of fibroblast-like cells, six populations of immune cells, four populations of vascular cells and three populations of neural crest-derived cells. We furthermore quantitatively assessed differences in cellular composition of dermis and hypodermis between anagen and telogen skin.

1.7 CUTANEOUS WOUND HEALING

As physical injury is the most serious threat to the barrier function of the skin, a complex cellular program involving nearly all cell types in the skin is activated immediately upon wounding to regenerate the skin barrier (Gurtner et al., 2008). While the main hallmarks of the wound healing process are evolutionarily conserved, distinct phyla and species show clear differences in their regenerative capacity. Cutaneous wound healing in humans is dominated by fibrosis, scar formation and frequent loss of epidermal appendages, whereas mice can regenerate hair follicles under certain circumstances (Gurtner, Callaghan, & Longaker, 2007; Takeo, Lee, & Ito, 2015). Furthermore, wound size reduction due to muscle contraction plays a much larger role in mice than in humans (Zomer & Trentin, 2018). Overall, the wound healing process can be divided into three main stages: the inflammatory stage, which is defined by blood clotting and immune cell infiltration, the tissue formation stage during which new dermal and epidermal structures are formed and the skin barrier is re-established, and the remodeling stage leading to partial regeneration with scar formation or complete regeneration of the skin (**Figure 7**) (Gurtner et al., 2008). Likewise, the cutaneous wound healing program can broadly be conceptualized as a stromal response – involving immune cells, fibroblasts and endothelial cells – and a delayed epidermal response culminating in the re-epithelialization of the wound site.

Shortly after wounding, hemostasis is achieved by infiltration of platelets and activation of the coagulation cascade. The fibrin matrix of the resulting blood clot serves as a scaffold for invading cells and thus forms the fundament of the wound's granulation tissue. Immune cell infiltration is initially dominated by primarily bactericidal neutrophils attracted to invading microorganisms and signals from the blood clot. In later stages, macrophages, which are recruited from the wound-adjacent tissue or differentiate from circulating monocytes, and which play an important role in clearing cellular debris and bacterial particles, become the most abundant immune cell type in the wound. Platelets, neutrophils and macrophages release various growth factors including PDGF, VEGF and TGFβ to promote the activation of fibroblasts, endothelial cells and keratinocytes (Eming, Martin, & Tomic-Canic, 2014; Gurtner et al., 2008; Martin & Leibovich, 2005). TGFβ has been described as a main factor in regulating fibroblast activation during wound healing. Upon activation, fibroblasts in the wound-adjacent dermis acquire a myofibroblast-like phenotype marked by expression of alpha smooth muscle actin and collagen III, migrate into the wound matrix and proliferate heavily. After migration into the granulation tissue, wound fibroblasts mass-produce a variety of ECM proteins including collagens and fibronectin and thus prime the wound matrix for the migration of epidermal cells (Werner, Krieg, & Smola, 2007). While fibroblasts from the reticular dermis make up most wound fibroblasts, it has been shown that papillary dermis fibroblasts show a delayed migratory response contemporaneous with re-epithelization, exclusively re-locate to the upper wound dermis and are crucial for *de novo* hair follicle regeneration in wounds (Driskell et al., 2013). It has furthermore been suggested that DPP4$^+$ fibroblasts from the lower dermis are responsible for the fibrotic response in mouse wounds and that inhibition of these cells reduces scarring (Rinkevich et al., 2015). Adipocyte pre-

cursor cells have been shown to migrate into the wound and adipogenesis is important for fibroblast recruitment during wound healing (Schmidt & Horsley, 2013). Meanwhile, dermal papilla cells have been found to not contribute to the wound response (Kaushal et al., 2015). In order to satisfy the need for nutrients of the metabolically active granulation tissue, large scale angiogenesis takes place simultaneously to cell migration into the wound matrix (Gurtner et al., 2008). VEGF has been implicated in regulating wound angiogenesis, and suppression of VEGF production in macrophages significantly reduces the blood supply of the wound tissue (Shaw & Martin, 2016). During the wound remodeling stage, many immune cells, endothelial cells and fibroblasts leave the wound or undergo apoptosis. At the same time, wound fibroblasts lose their myofibroblast identity and collagen III in the ECM is replaced by collagen I (Xue & Jackson, 2015). In many organisms including humans and mice, wound remodeling however does not lead to a complete regeneration of the dermis and fibrotic scar tissue is formed instead (Gurtner et al., 2008). A variety of cellular and molecular processes have been implicated in scar formation. While immune cells and the inflammatory environment, as well as specialized fibroblast subpopulations, have been suggested to contribute to scar formation (Martin & Leibovich, 2005; Rinkevich et al., 2015), several signaling and ECM molecules such as matrix metalloproteinases (MMPs) and TGFβ3 have been linked to scarless wound healing (Xue & Jackson, 2015).

The formation of new epidermal tissue on top of the granulation tissue is a central step in restoring the skin barrier (Gurtner et al., 2008). Re-epithelialization of the wound requires the induction of migratory and proliferatory activity in epidermal stem cells. Two recent studies combined lineage tracing, live cell microscopy and gene expression profiling to describe the cellular dynamics of epidermal cells during wound healing (Aragona et al., 2017; Park et al., 2017). These studies showed that keratinocytes directly adjacent to the wound front possess a highly migratory phenotype. These cells express a distinct gene signature – including α5β1 integrin – which allows them to attach to the granulation tissue and to slowly migrate towards the wound front, thus forming the leading edge of the wound epidermis. In contrast, the cells located behind the leading edge are less migratory but highly proliferative. The balance of migration and proliferation leads to gradual closure of the wound and thickening of the wound epithelium. While suprabasal cells have been shown to contribute to wound closure, most keratinocytes that are recruited into the wound are basal cells of the IFE (Park et al., 2017). Moreover, less differentiated cells (*Krt14*$^{+}$) contribute stronger to wound healing than more differentiated cells (*Ivl*$^{+}$) (Mascré et al., 2012). A large variety of signaling molecules involved in the activation of keratinocytes upon wounding have been described. Among these factors, most of which are secreted by fibroblasts and immune cells of the granulation tissue, are EGFs, FGFs, HGFs, KGFs, PPARs, TGFβ, acetylcholine and catecholamines. Interestingly, suppressing one of these factors has at most a moderate detrimental effect on wound healing, which points to a large degree of redundancy in the signaling environment of the wound (Werner et al., 2007).

Intriguingly, it has been shown that follicular stem cells can bridge compartmental boundaries, migrate into the wound and contribute to wound closure. In fact, contribution to wound

healing and retention of long-term clones at the wound site has been described for virtually all telogen hair follicle stem and basal cell populations including label-retaining cells, *Krt15*$^+$, *Lgr5*$^+$, *Lgr6*$^+$, *Gli1*$^+$ and *Lrig1*$^+$ cells (Brownell et al., 2011; Füllgrabe et al., 2015; Ito et al., 2005; Jaks et al., 2008; Kasper et al., 2011; Page et al., 2013; Snippert et al., 2010; Tumbar et al., 2004). According to location along the follicular axis, different stem cell populations show distinct temporal recruitment dynamics, with upper hair follicle cells reaching the wound front already after 24 hours while the wound response of bulge stem cells is more delayed. Although differences in long-term contribution to the wound epithelium have been described, with certain stem cell populations being more likely to create long-term clones than other populations, it is disputed whether this phenomenon represent diverging stem cell potential or is merely a result of different numbers of cells migrating into the wound (Page et al., 2013). Studies conducted on mutant mice lacking hair follicles show that, although hair follicle cells slightly accelerate wound closure, they are not essential for wound healing (Langton, Herrick, & Headon, 2008).

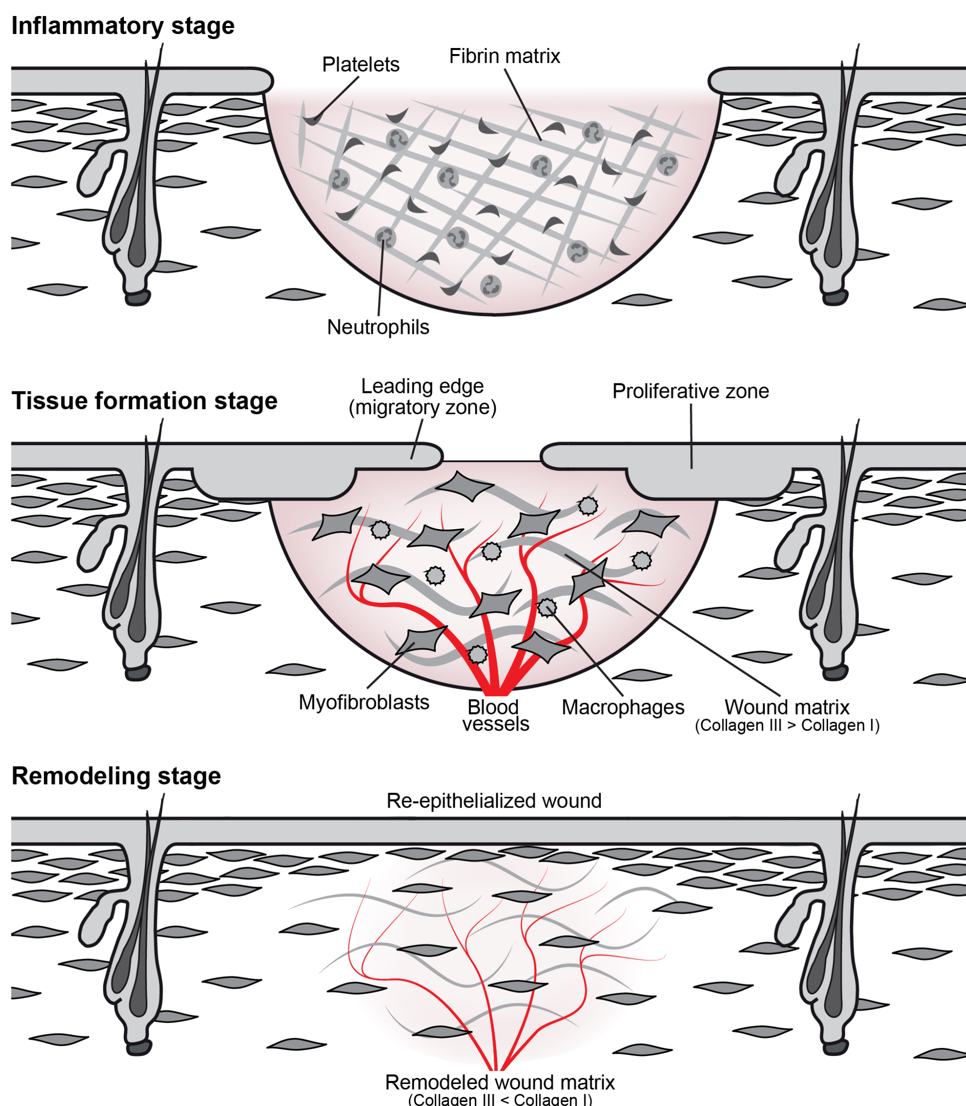


Figure 7: Stages of cutaneous wound healing.

Although most wounds in mice heal without the regeneration of epidermal appendages, *de novo* hair follicle formation has been described in wildtype mice (Takeo et al., 2015). Hair neogenesis can occur in large full thickness wounds (diameter > 1 cm) and recapitulates many hallmarks of embryonic hair follicle induction (Ito et al., 2007). A variety of cell populations and signaling factors have been implicated in *de novo* hair follicle regeneration. As of today, papillary dermis fibroblasts, γδ T cells, as well as FGF and WNT signaling have been described as positive regulators of hair neogenesis in wounds (Driskell et al., 2013; Gay et al., 2013; Ito et al., 2007).

Many aspects of the epidermal stem cell response to wounding are still unknown or disputed. It is not known how epidermal stem cells, recruited from the hair follicle to the wound epidermis, break the compartment barriers which limit their IFE contribution during homeostasis. Likewise, it is not clear whether different stem cell populations pass through the same wound healing program upon recruitment or if stem cells contributing to wound healing enter a specialized wound stem cell state (Donati & Watt, 2015). Interestingly, it has recently been described that keratinocytes in the vicinity of the wound enter a state of lineage infidelity, which is characterized by the co-expression of markers from both the IFE and hair follicle lineage (Ge et al., 2017). Furthermore, it has been suggested that differentiated *Gata6*$^{+}$ cells from the sebaceous duct can contribute to wound healing by dedifferentiating into a more stem cell like state (Donati et al., 2017).

In **Paper III**, we employed single-cell transcriptomics to study the molecular responses of Lgr5 and Lgr6 stem cell progeny as they contribute to wound healing. We show that Lgr5 and Lgr6 wound cells pass through distinct molecular stages, which persist over several time points and can occur simultaneously in the wound. We furthermore show that both stem cell populations rapidly upregulate a wound signature, which allows them to attach to and communicate with the wound matrix, and that Lgr5 and Lgr6 cells transcriptionally converge during the wound healing process.

2 THE ERA OF SINGLE-CELL TRANSCRIPTOMICS

For a long time, the methodological repertoire of skin research has been limited to techniques studying single markers or individual parameters. Cell populations were distinguished from each other based on the expression of individual genes or proteins, which were qualitatively or quantitatively analyzed using antibody-based immunostainings or qPCR. Likewise, most stem cell populations in the skin were identified and characterized based on knock-in reporter mouse models that mark cells according to the activity of individual promoters.

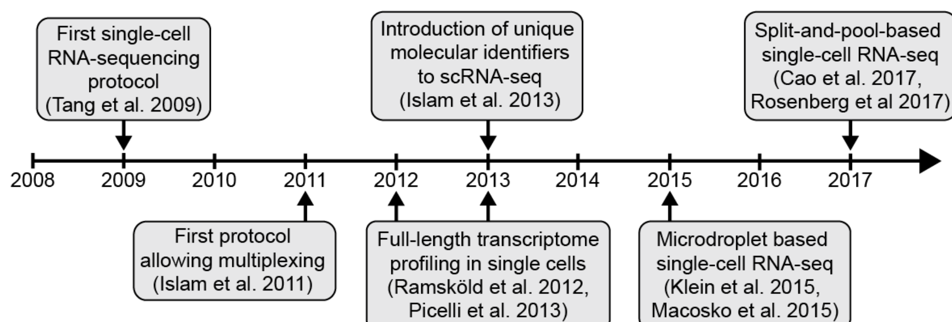
During the last two decades, genomic, transcriptomic and epigenomic techniques focused on measuring thousands of markers or parameters in parallel have become increasingly available in skin research. Whole transcriptome gene expression analysis has been used to establish the molecular identity of a variety of epidermal (stem) cell populations (Brownell et al., 2011; Füllgrabe et al., 2015; Jaks et al., 2008; Morris et al., 2004; Page et al., 2013; Sada et al., 2016; Snippert et al., 2010; Tumbar et al., 2004) and to analyze gene expression changes during hair cycling (Lin et al., 2004) as well as wound healing (Aragona et al., 2017). Likewise, a variety of epigenomic techniques including the study of global DNA methylation, histone modification, and chromatin accessibility patterns has been used to disentangle stem cell identity (Adam et al., 2015; Bock et al., 2012), hair follicle lineage specification (Adam et al., 2018; Lien et al., 2011) and wound healing (Ge et al., 2017). Whole genome RNAi screens have meanwhile been used to identify new genes regulating stem cell identity or modulating skin tumorigenesis (Beronja et al., 2013; Chen et al., 2012). However, all these approaches require the input of thousands or even millions of cells, which are usually isolated using pre-defined markers. Therefore, the resulting datasets represent averaged signals from potentially heterogeneous pools of cells. This and other factors such as the low number of samples have until very recently prevented the systematic and unbiased analysis of cellular heterogeneity in the skin.

In recent years, many genomic, transcriptomic and epigenomic techniques have been adapted to perform at single-cell resolution. Whole-genome- and exome sequencing methods for individual cells have been described (Gawad, Koh, & Quake, 2016), as have been the single-cell analysis of DNA methylation, histone modification and chromatin status (Kelsey, Stegle, & Reik, 2017). Of all these methods, single-cell RNA-sequencing (scRNA-seq), which allows the analysis of global gene expression in thousands of individual cells, has found the most widespread use. Application of scRNA-seq has greatly advanced the study of cellular heterogeneity and the system biology of tissues and organs (Tanay & Regev, 2017).

2.1 MEASURING GENE EXPRESSION IN SINGLE CELLS

While single-cell microarrays found some limited application (Kurimoto et al., 2006), high throughput single-cell transcriptomics only became viable after the introduction of next generation sequencing technology. Since the first single-cell RNA-sequencing protocol was intro-

duced in 2009 (Tang et al., 2009), a large number of techniques for the generation of transcriptome libraries from single cells have been described (reviewed in Chen, Teichmann, & Meyer, 2018). While many of these methods draw on chemistry introduced for bulk-cell cDNA library preparation, they were specifically adapted to the challenges of working with single cells, especially to the minuscule amount of RNA, which can be extracted from each cell. Measures to prevent sample loss included one-tube-reactions, reduced washing steps, shrinking of reaction spaces and pooling tagged cDNA from many different cells (Chen, Teichmann, & Meyer, 2018). An additional goal in the evolution of scRNA-seq protocols was increasing throughput while reducing sequencing costs per cell (**Figure 8A**) (Angerer et al., 2017). Nearly all single-cell cDNA library preparation protocols share the same general workflow (**Figure 8B**): a single cell is captured and lysed in a separated reaction environment, the released mRNA is reverse transcribed and marked with a cell-specific identifier sequence, and the tagged cDNA from many individual cells is pooled, amplified and sequenced. However, protocols differ substantially in specific details related to the single-cell capturing approach and reaction environment, the reverse transcription and amplification chemistry, as well as in their transcriptome and gene coverage (Chen, Teichmann, & Meyer, 2018).



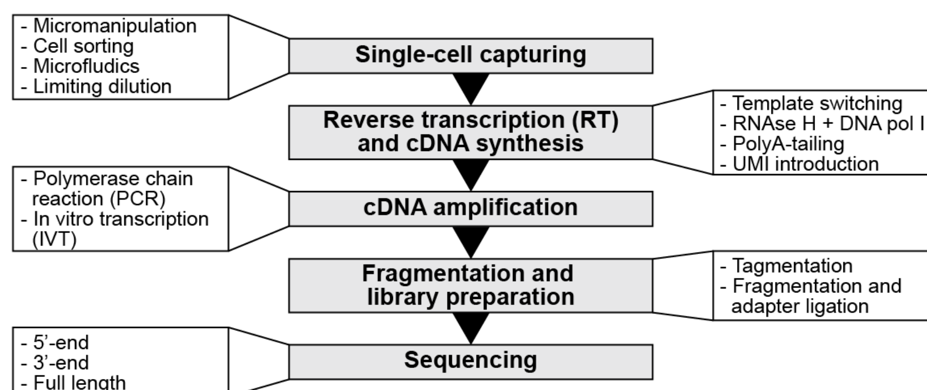


Figure 8: (A) Milestones in the evolution of single-cell RNA-sequencing technology. **(B)** The general single-cell RNA-seq library preparation workflow.

The first described scRNA-seq techniques relied on manual cell picking for single-cell capturing, leading to long preparation times and low throughput (Islam et al., 2011; Tang et al., 2009). A first increase in capturing efficiency was linked to the introduction of microfluidic single-cell capturing devices – most notably the Fluidigm C1 – and the adaption of single-cell library preparation protocols to cells sorted into 96 and 384 microwell plates (Hashimshony et al., 2012; Islam et al., 2014; Jaitin et al., 2014; Ramsköld et al., 2012). Throughput of single-cell experiments was dramatically increased with the introduction of droplet-based microfluidic systems, which capture single cells in lipid droplets that serve as the subsequent reaction space (Klein et al., 2015; Macosko et al., 2015). With these techniques, which have been commercialized in the *10X Chromium* system (Zheng et al., 2017), it became possible to analyze thousands of single-cell transcriptomes in a single experiment. Nanowell based methods allow a similarly high throughput (Gierahn et al., 2017; Hochgerner et al., 2017). In recent years, several approaches that circumvent the need to capture single cells in individual reaction spaces altogether by combinatorial indexing of randomly distributed pools of cells have been described (Cao et al., 2017; Rosenberg et al., 2018). These split-pool approaches promise additional improvements regarding complexity, throughput and costs associated with scRNA-seq library preparation.

As most scRNA-seq protocols selectively target mRNA, reverse transcription is usually primed with an oligo-dT primer while second strand synthesis is more varied between protocols. Template switching has been used for second strand synthesis by a variety of protocols (Islam et al., 2011; Islam et al., 2014; Macosko et al., 2015; Picelli et al., 2013; Ramsköld et al., 2012; Rosenberg et al., 2018; Zheng et al., 2017). Template switching requires the terminal transferase activity of Moloney murine leukemia virus (MMLV) reverse transcriptase, which adds several cytosines to the 3'-end of the first strand. These cytosines serve as binding partners for template switching oligonucleotides (TSOs) – short primers which contain several guanines at their 3'-end –, allowing them to prime the synthesis of the second strand of cDNA. The use of template switching allows the introduction of additional sequences such as primer binding sites and sequencing adapters into the second strand via the TSO. In contrast, a number of other protocols rely on the use of RNase H and DNA polymerase I, which have been used in cDNA synthesis since the 1970s, for second strand synthesis (Cao et al., 2017; Hashimshony et al., 2012; Hashimshony et al., 2016; Jaitin et al., 2014; Klein et al., 2015). As this approach does not allow introduction of additional sequences into the second strand, most of the protocols using this method are based on *in vitro* transcription (IVT) for cDNA amplification. More rarely, terminal transferase is used to add a poly-A tail to the 3'-end of the first strand, allowing second strand priming with poly-T primers (Sasagawa et al., 2013; Sasagawa et al., 2018; Tang et al., 2009). While most scRNA-seq studies focus exclusively on poly-A mRNA expression, protocols for the analysis of total or non-coding RNA are available (Faridani et al., 2016; Hayashi et al., 2018).

Due to the minuscule amount of cDNA retrieved from each single-cell reaction, library creation requires heavy amplification of single-cell cDNA. Most protocols rely on PCR for library amplification with second strand primers either introduced during template switching

or ligated to the cDNA (Cao et al., 2017; Islam et al., 2011; Islam et al., 2014; Macosko et al., 2015; Picelli et al., 2013; Ramsköld et al., 2012; Rosenberg et al., 2018; Sasagawa et al., 2013; 2018; Tang et al., 2009; Zheng et al., 2017). Other protocols use IVT from cDNA for library amplification (Hashimshony et al., 2012; Hashimshony et al. 2016; Jaitin et al., 2014; Klein et al., 2015). As IVT amplifies cDNA in a linear – as opposed to an exponential – fashion, it is less prone to amplification bias than PCR. Because cDNA libraries from hundreds or thousands of cells are commonly pooled before sequencing, cDNA retrieved from each single cell must be barcoded with a unique identifier sequence. Pooling usually occurs after cDNA synthesis or amplification, and cells are commonly barcoded during first strand synthesis, second strand synthesis, during or after amplification. A large number of protocols now allow the introduction of unique molecular identifiers (UMI), small randomized oligonucleotide sequences introduced into each mRNA molecule during reverse transcription or second strand synthesis (Islam et al., 2014; Kivioja et al., 2011). UMIs allow deduction whether two reads are derived from the same original mRNA molecule and can thus help to reduce amplification and sequencing bias.

Next generation sequencing technologies require the fragmentation of libraries and the addition of sequencing adapters. Either enzymatic or physical fragmentation in conjuction with adapter ligation, or tagmentation are commonly used in this step (Chen, Teichmann, & Meyer, 2018). Although many scRNA-seq library preparation protocols generate cDNA libraries covering the full length of mRNAs, most of these methods only sequence the 5'-end (Hochgerner et al., 2017; Islam et al., 2011; Islam et al., 2014) or 3'-end of the transcript (Cao et al., 2017; Hashimshony et al., 2012; Hashimshony et al., 2016; Jaitin et al., 2014; Klein et al., 2015; Macosko et al., 2015; Rosenberg et al., 2018; Sasagawa et al., 2013; 2018, Zheng et al., 2017). While allowing a general census of all mRNA molecules expressed in a particular cell, these approaches are incompatible with a more in-depth analysis of transcriptomic organization, such as the detection of splice isoforms. In addition, single-cell sequencing data derived from protocols with full length gene coverage are usually less sparse since the detection rate of each mRNA molecule is increased. However, full-length protocols are not yet available with UMIs and generally have a lower throughput (Picelli et al., 2013; Ramsköld et al., 2012).

During the evolution of scRNA-seq technology, most methodological advances increased throughput and decreased sequencing costs per cell, whereas the sensitivity and sampling depth remained mostly constant (Angerer et al., 2017; Ziegenhain et al., 2017). While early protocols allowed the parallel processing of only hundreds of cells, the introduction of droplet microfluidics and split-pool protocols made it possible to analyze ten-thousands of cells in a single experiment. Accordingly, datasets of several hundred-thousand cells are not uncommon anymore. The optimal ratio between sampling depth and cell number in the design of single-cell experiments is still disputed. However, it has been suggested that the analysis of a large number of cells at limited depth can be effectively used to infer idealized cell populations (Tanay & Regev, 2017).

In **Paper I** and **Paper III**, we used the microfluidics-based STRT-C1 protocol to analyze several thousand cells from epidermis during rest (telogen) and during wound healing. In **Paper II**, we use the commercialized, droplet-based *10X Chromium* protocol to analyze around 6000 cells from telogen and anagen skin (**Figure 9**).

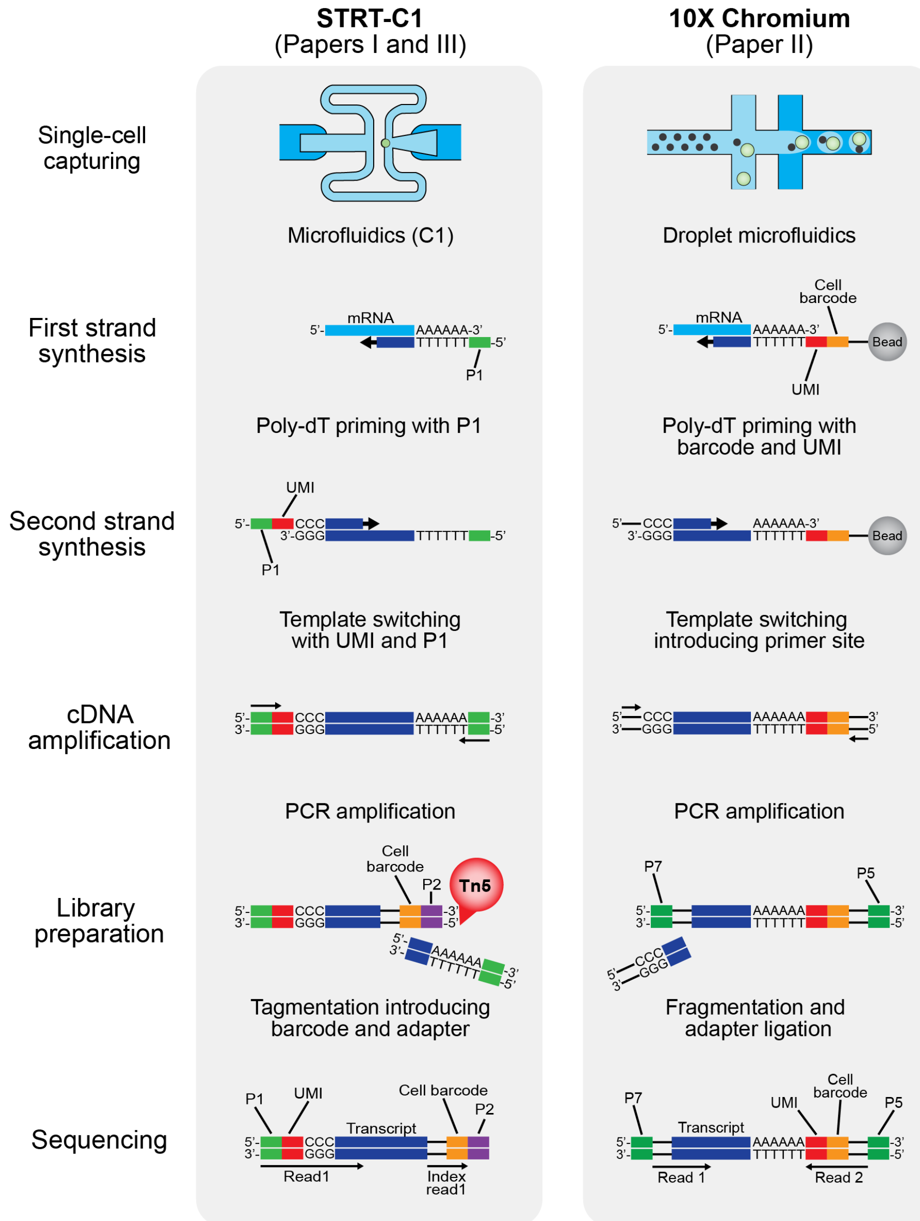


Figure 9: Overview of the sequencing protocols used in Papers I - III.

2.2 DISENTANGLING HETEROGENEITY IN SINGLE-CELL DATASETS

Data derived from scRNA-seq experiments are in many respects different from classical gene expression data, i.e. bulk-cell microarray and bulk-cell RNA-sequencing data. Due to the low amount of mRNA recovered from each cell, only between 1000 and 20000 unique reads per

cell (based on UMIs) distributed over 500 to 5000 genes are commonly detected in single cell experiments. Furthermore, many mRNA molecules or mRNA species expressed in a cell are not recovered at all. scRNA-seq data is therefore much sparser and zero-inflated compared to typical bulk-cell RNA-sequencing data. Accordingly, it is more susceptible to experimental biases, technical biases and batch effects (Wagner, Regev, & Yosef, 2016). These peculiarities limit the application of many tools developed for the analysis of bulk-cell data. Additionally, the analysis goals of bulk- and single-cell experiments oftentimes differ. While the main analytical focus of most bulk-cell experiments is the identification of differentially expressed gene signatures, most single-cell studies are aimed at defining and characterizing heterogeneous cell populations within the dataset. Parallel to the evolution of scRNA-seq technology, a large variety of tools and methods specifically tailored to the analysis of scRNA-seq data has been developed. These methods cover the preprocessing and normalization of data, the clustering and visualization of cell populations, and the identification of differentially expressed genes and cellular markers in single-cell datasets (Hwang, Lee, & Bang, 2018).

Single-cell data is influenced by different sources of variation including technical variation (amplification bias, cell quality, library quality), gene-specific variation (transcription bursts, RNA processing) and cell-specific variation (cell identity, cell state, cell cycle phase) (Wagner, Regev, & Yosef, 2016). Accordingly, the aim of data preprocessing is to separate undesired variation (e.g. technical variation, confounding biological variation such as cell cycle phase) from the focus of analysis (e.g. cell type identity). Normalization aims to bring individual transcriptomes to a common scale of comparison, thus reducing for instance differences in sequencing depth and mRNA recovery. Many commonly used normalization approaches were introduced for bulk-cell RNA-sequencing experiments. However, these methods perform poorly with zero-inflated single-cell data and are outperformed by approaches adapted to the peculiarities of single-cell datasets (Vallejos et al., 2017). For instance, a recently introduced normalization approach circumvents the problems associated with zero-inflation by randomly pooling cells, calculating pool-based size factors and deriving cell-specific size factors by deconvolution (Lun, Bach, & Marioni, 2016). Batch effects, i.e. systematic variations between technical or biological replicates, can have a strong influence on single-cell datasets. Most approaches attempt to regress out batch effects by fitting linear regression models with batches as predictors (Johnson, Li, & Rabinovic, 2007). Similar regression models have been used to control for other sources of variation such as cell cycle stage (Barron & Li, 2016). Recently, several novel batch correction approaches, which are robust towards differences in cellular composition between batches and which can merge datasets generated with different library preparation protocols, have been presented (Butler et al., 2018; Haghverdi et al., 2018). Likewise, a variety of methods to deal with dropout events / false negatives have been introduced. These include zero-inflated models and false negative curves, as well as approaches imputing missing values from co-expression patterns (Wagner, Regev, & Yosef, 2016). With the advent of large scale datasets, data smoothing and imputation based on k-nearest-neighbor (kNN) relationships have become increasingly common (Li & Li, 2018; van Dijk et al., 2018; Wagner, Yan, & Yanai, 2018). Feature selection – i.e. the

selection of genes, which likely show biological variation in their expression – is a critical step in many preprocessing and downstream analysis pipelines. As scRNA-sequencing data can be modeled using a negative binomial (NB) distribution (Grün, Kester, & van Oudenaarden, 2014), many feature selection approaches use NB or non-parametric models to predict dispersion in gene expression as a function of mean expression. In these approaches, genes with the highest residuals are considered to be most likely differentially regulated between cell populations and thus selected as features for clustering and dimensionality reduction. While we did not use any normalization in **Paper I**, data in **Paper III** was normalized using the size factor method introduced by Anders & Huber, 2010. In **Paper II,** the single-cell adaptation of the size factor method (Lun et al., 2016) was used. Since our data was derived from inbred mouse strains, we observed a surprisingly high amount of consistency between biological replicates. Therefore, batch correction was not necessary.

The clustering of single-cell transcriptomes is commonly the first step in the identification of distinct cell populations or cell states in single-cell datasets. Many clustering approaches used in the analysis of single-cell datasets are classical general-application machine learning algorithms, with techniques such as hierarchical clustering and k-means clustering being among the most widely used (Andrews & Hemberg, 2018; Hwang et al., 2018). Graph-based methods such as the Louvain algorithm for community detection in networks have also become increasingly popular as they scale well with large datasets (Blondel et al., 2008; Levine et al., 2015). Initial dimensionality reduction with principal component analysis (PCA) or non-negative matrix factorization (NMF) is commonly performed before clustering (Andrews & Hemberg, 2018; Lee & Seung, 1999). This step does not only serve to reduce calculation time, it also aggregates co-expressed genes into modular "metagene" structures and thus minimizes problems linked to zero-inflation and transcriptional variation. Cell clustering relies on a measure of distance or similarity between cells, most commonly Euclidean or correlation distance. As cell-cell distance is usually calculated using all selected features – either scaled or unscaled – with equal weight, these measures do not take into consideration organizational principles of gene expression such as regulatory networks and transcriptional hierarchies. Accordingly, cell identity as defined by clustering of single-cell transcriptomes is mostly based on purely mathematical measures of data point (dis-)similarity. Therefore, it is usually necessary to validate clustering results by spatial remapping – e.g. antibody staining or fluorescence *in situ* hybridization (FISH) of marker genes – or functional studies (Andrews & Hemberg, 2018). As many clustering algorithms have limits regarding resolution and minimal cluster size, they often fail to detect rare cell populations even if those populations are very distinct. Accordingly, specific protocols for the detection of rare cells in single-cell datasets have been developed, most importantly RaceID based on a NB model (Grün et al., 2015). In **Papers I** and **II**, we used affinity propagation, a relatively novel machine learning approach (Frey & Dueck, 2007), to cluster single-cell data based on correlation distance in high dimensional space (**Paper I**) or Euclidean distance in PCA-reduced space (**Paper II**). Clustering in **Paper III** was performed using k-means clustering in PCA- or NMF-reduced space. To define wound cells in the same paper, we used a naïve

Bayes classifier, which is similar in its main assumptions to the RaceID rare cell detection approach.

Manifold learning methods are widely used to visualize complex single-cell datasets in low-dimensional space. T-distributed stochastic neighbor embedding (t-SNE) in particular has become the method of choice in nearly every single-cell study (Maaten & Hinton, 2008). However, t-SNE is limited by its stochasticity, slow runtime and misleading representation of inter-cluster distances. Recently, Uniform Manifold Approximation and Projection (UMAP) has been presented as an alternative to t-SNE (McInnes & Healy, 2018). UMAP has been shown to scale well with larger datasets and to preserve cellular continua and global population structures better than t-SNE (Becht et al., 2018). Additionally, diffusion maps are oftentimes used instead of t-SNE when modeling cellular trajectories, as they more faithfully represent gradual identity changes (Haghverdi, Buettner, & Theis, 2015). In **Paper I and III**, t-SNE was used for dimensionality reduction, data visualization and as input for trajectory reconstruction. In **Paper II**, both t-SNE and UMAP were used to visualize data, while lineage reconstruction was based on diffusion maps.

Subsequent characterization of cell populations or cell states requires the identification of differentially regulated genes. A large variety of methods have been employed to screen for differentially expressed genes in single-cell transcriptome clusters. These include classical parametric or nonparametric statistical method such as the Wilcoxon rank-sum test, as well as methods developed for bulk-cell gene expression data including DESeq2 (Love, Huber, & Anders, 2014) and limma (Ritchie et al., 2015). In addition, many methods specifically adapted to peculiarities of scRNA-seq data, such as drop-out events, have been described (Delmans & Hemberg, 2016; Finak et al., 2015; Kharchenko, Silberstein, & Scadden, 2014). Interestingly, it has been shown in several benchmark studies that these specialized methods do not consistently outperform general use or bulk-cell methods for calling differential expression (Jaakkola et al., 2016; Soneson & Robinson, 2018). While differential expression analysis is oftentimes used to find population-specific marker genes for validation or downstream functional studies, it may also be employed to identify gene modules, regulatory networks and transcription factors shaping cell type identity. Although potential regulatory modules and their associated transcription factors are often defined according to co-expression patterns alone (e.g. based on correlation or mutual information), more complex tools for gene regulatory network inference have been released. For instance, the widely used single-cell regulatory network inference and clustering (SCENIC) toolkit screens co-expression modules for enrichment of cis-regulatory motifs to remove false positive associations (Aibar et al., 2017). Differentially expressed genes were defined in **Papers I** and **III** based on a NB Bayesian regression approach, which models gene expression using cluster membership as predictors (Zeisel et al., 2015). In contrast, the Wilcoxon rank-sum test was used to identify differentially expressed genes in **Paper II**. Modules of co-expressed genes and transcription factors associated with gene modules were identified based on correlation in all three papers.

2.3 MODELING CELLULAR TRAJECTORIES FROM SINGLE-CELL DATA

In most tissues, cellular identity is not immutable. Instead, many cells continuously undergo dynamic identity changes in line with processes such as lineage specification during development, differentiation during tissue maintenance and regeneration upon tissue damage. Single-cell transcriptomics made it possible to resolve these changes in high resolution at the transcriptome level. In actively self-maintaining adult tissues, where cells pass continuously trough all stages of differentiation, sampling from a single time point is oftentimes sufficient to resolve complex cellular dynamics, as even such singular transcriptomic "snapshots" capture all the differentiation substates present in a tissue. In other cases, such as embryogenesis, time series protocols need to be employed to dissect developmental processes occurring over large time frames. Various methods to infer and model cellular trajectories from scRNA-seq data have been developed. While these methods differ in their specific computational approach, they all aim to reconstruct cellular trajectories by ordering single-cell transcriptomes gradually along a unidirectional or branching "pseudotemporal" axis. Subsequent downstream analysis oftentimes involves modelling gene expression along the pseudotime axis in order to identify factors or regulatory networks critical for the differentiation process. Likewise, some methods allow inference about cell directionality and identification of stem and terminally differentiated cell populations (Kester & van Oudenaarden, 2018; Saelens et al., 2018).

Despite many differences in their specific implementation, most lineage reconstruction algorithms can be divided into two classes. Interestingly, this division already became apparent in the two earliest described methods, Monocle (Trapnell et al., 2014) and Wanderlust (Bendall et al., 2014). Monocle is based on dimensionality reduction via independent component analysis (ICA) and the subsequent construction of a minimum spanning tree (MST) in low-dimensional space. The longest path of the MST is used as the backbone of the pseudotime axis and branching cells are condensed onto the backbone path using the PQ-tree algorithm. The pseudotime position of a cell is based on its geodesic distance from one end of the condensed graph **(Figure 10)**. Many other methods such as TSCAN (Ji & Ji, 2016), Waterfall (Shin et al., 2015), SCUBA (Marco et al., 2014) and Slingshot (Street et al., 2018) have since used a similar approach. However, these methods differ in their dimensionality reduction approach with PCA or t-SNE having been used in place of ICA. Likewise, principal curves have been employed by some approaches such as SCUBA and Slingshot instead of MSTs to find the pseudotime backbone. Orthogonal projection instead of PQ-trees is most commonly used to project cells onto the main path.

In contrast to Monocle, Wanderlust represents high dimensional data as kNN networks. The pseudotime distance of a cell is then determined based on a series of random walks through the network from a pre-defined starting point. Similarly, the diffusion pseudotime (DPT) approach (Haghverdi et al., 2016) uses cells projected into diffusion space to create a weighted kNN graph and determines pseudotime from a starting cell based on random walks **(Figure 10)**. The identification of bifurcations and multiple lineages is one of the key challenges for lineage reconstruction algorithms. Many methods including TSCAN, Waterfall and

Slingshot use condensed data, for instance cluster centroids, for MST construction. Such an approach does not only simplify calculations, it also reduces the probability of misidentifying spurious bifurcations in the MST. Network-based approaches such as Wishbone (Setty et al., 2016), the successor of Wanderlust, and DPT aim to identify branching points based on disagreements between random walks in- and excluding certain points. In contrast to both these techniques, StemID (Grün et al., 2016) uses a guided topology approach to define branch differentiation trajectories. In this approach, cluster medoids are calculated and connected in high dimensional space. Single cells are subsequently projected onto the edges between clusters and edges with none or few projected cells are pruned, leaving the final topology.

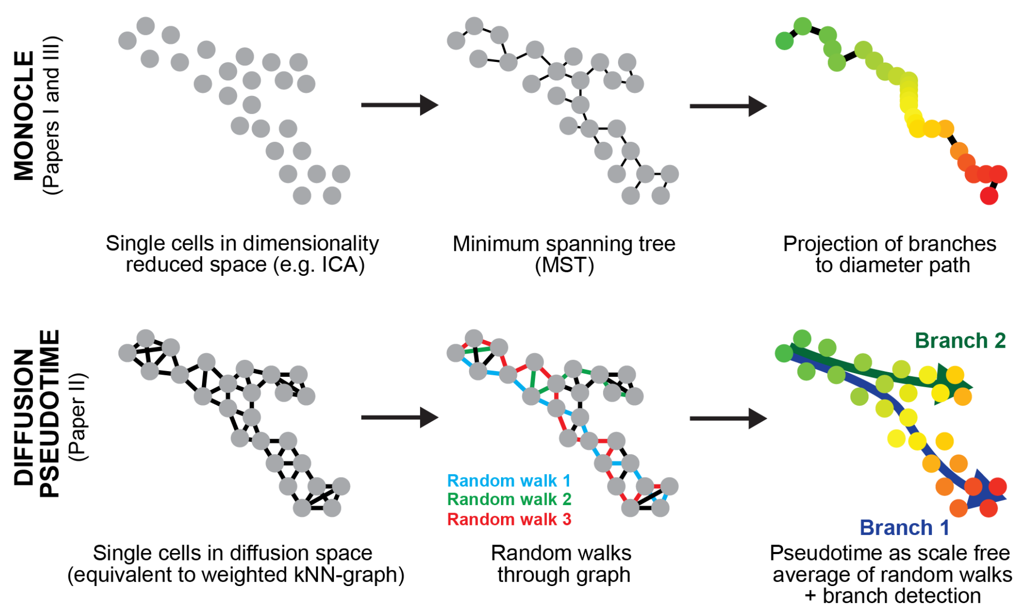


Figure 10: Overview of trajectory reconstruction approaches used in Papers I - III.

Although lineage reconstruction methods have been steadily improving, most of these methods still rely on prior assumptions about the topology of the system and/or *a priori* biological knowledge. Most importantly, the directionality of reconstructed lineages is oftentimes not directly apparent. While methods such as Monocle can construct pseudotemporal models without knowledge about the direction of lineage progression, other methods such as Wishbone and DPT require the explicit specification of a root cell (Saelens et al., 2018). Given that lineage reconstruction approaches are most often used to infer the lineage trajectory in developing or differentiating cellular systems, the specified root cells should represent the stem or progenitor cells of the analyzed system. A variety of approaches have been used to infer stem cell identity or pseudotime directionality before or after lineage reconstruction. It is assumed that differentiating cells acquire a more specialized, narrow and invariant transcriptomic identity in line with increasingly restrictive fate specification. In contrast, stem cells are thought to exhibit more uncommitted and less tightly regulated gene expression programs. Accordingly, entropy, a statistical measure of uncertainty or disorder in a system, has been used to infer differentiation status. In the lineage reconstruction method SLICE, the

transcriptome entropy of single cells is used as a factor in pseudotemporal ordering with entropy expected to decrease during differentiation (Guo et al., 2016). Likewise, high entropy is used in StemID as a factor informing stem cell identity (Grün et al., 2016). Differentiation is associated with decreased multipotency and increased fate bias. FateID, an extension of StemID, infers lineage relationship by assessing fate bias using an iterative classification approach proceeding from pre-specified terminally differentiated cell populations. (Herman, Sagar, & Grün, 2018). A fundamentally different approach to model lineage relationships from single-cell data has been recently presented by La Manno et al., 2018: velocyto uses expression data from spliced and unspliced transcripts to infer gene expression dynamics (RNA velocity) such as active up- or downregulation in single cells. In consequence, velocyto is able to directly predict cell fate from single-cell expression data.

In **Papers I and II**, we use a modified version of Monocle to model IFE differentiation and gradual identity changes during wound healing, respectively. In **Paper III**, we use a combination of velocyto and DPT to model the differentiation trajectories of hair follicle matrix cells **(Figure 10)**.

3 SINGLE-CELL TRANSCRIPTOME ANALYSIS OF SKIN

As of today, single-cell transcriptomics have been employed to study cellular heterogeneity in nearly all tissues of the mammalian body. scRNA-seq was, for instance, used to dissect the cellular composition of various parts of the brain (reviewed in Ofengeim et al., 2017), heart (DeLaughter et al., 2016; Skelly et al., 2018), lungs (Treutlein et al., 2014), intestinal tract (Grün et al., 2015; Haber et al., 2017), hematopoietic and immune system (reviewed in Stubbington et al., 2017), kidney (Park et al., 2018) and pancreas (Baron et al., 2016; Muraro et al., 2016; Segerstolpe et al., 2016). With increasing throughput and higher cost-effectiveness, atlas projects mapping the cellular heterogeneity of complex organs, such as the mouse nervous system (Zeisel et al., 2018), or even complete organisms (Cao et al., 2017; Plass et al., 2018) became feasible in recent years. These efforts are currently culminating into large scale, consortium-driven projects such as the Tabula muris (Schaum et al., 2018) or Human Cell Atlas (Regev et al., 2017).

Although other widely used systems for stem cell research such as the immune system and the small intestine are well-characterized at the single-cell transcriptome level, only very few single-cell studies focusing on skin are available. Since our first publication pioneering single-cell RNA-seq in skin (**Paper I**), a small number of other studies followed. scRNA-seq of approximately 1100 cells has been used to characterize heterogeneity in the dermal papilla and matrix of the anagen follicle, revealing the presence of distinct micro-niches relevant for hair follicle lineage specification (Yang et al., 2017). In addition, scRNA-seq has been used to study the WNT responsiveness of cultured keratinocytes (n = 254) (Ghahramani et al., 2018) and the heterogeneity of human dermal fibroblasts (n = 184) (Philippeos et al., 2018). Furthermore, a recent study uses single-cell transcriptomics (n = 1371) to show that dermal condensate identity is specified before niche formation (Mok et al., 2018). Two years after we published the first single-cell map of mouse epidermis, a similar study validated many of our findings in human epidermis (n = 92889) (Cheng et al., 2018).

Taken together, the three papers covered in this thesis represent the most detailed and comprehensive characterization of adult mouse skin at the single-cell transcriptome level available to date. In **Paper I**, we used single-cell transcriptome analysis to decode the heterogeneity of the murine epidermis at resting stage (telogen) and showed that said heterogeneity can be explained by the interplay of a pan-epidermal differentiation process and spatial niches. In **Paper II**, we expanded our analysis by creating a cell type atlas of full thickness skin at telogen and anagen. We identified more than 50 different subpopulations of cells from both the stromal and epidermal compartment of the skin and we were able to model cellular heterogeneity and differentiation dynamics of the anagen follicle at unprecedented resolution. In **Paper III**, we analyzed the molecular behaviour of Lgr5 and Lgr6 stem cells as they contribute to wound healing using single-cell transcriptome analysis. We show that both Lgr5 and Lgr6 stem cells pass through a shared wound healing program marked by distinct wound

cell states. This program is initiated rapidly upon wounding while the cells are still in their stem cell niches and eventually results in the molecular convergence of Lgr5 and Lgr6 stem cells in the healed wound.

3.1 PAPER I: SINGLE-CELL TRANSCRIPTOMICS REVEALS THAT DIFFERENTIATION AND SPATIAL SIGNATURES SHAPE EPIDERMAL AND HAIR FOLLICLE HETEROGENEITY

In this study, which was the first large-scale analysis of skin cells using scRNA-seq, we aimed to create a cell map of the epidermis and hair follicle during rest (telogen). For this purpose, we sequenced 1422 randomly sampled single cells from the back skin of 19 female C57BL/6 mice at second telogen (8 weeks of age). Single-cell libraries were created using the STRT-seq protocol with cells being captured on the Fluidigm C1 platform (Islam et al., 2014).

We were able to map the cellular heterogeneity of the skin at unprecedented resolution. In a first round of unsupervised clustering (1st level clustering), we identified and validated nine major cell populations in the epidermis: basal, suprabasal (two clusters), and terminally differentiated cells (two clusters) of the IFE, cells of the upper hair follicle (three clusters), cells of the sebaceous gland, cells of the outer bulge, cells of the inner bulge, and resident immune cells including Langerhans and γδ T cells. We were able to subcluster (2nd level clustering) basal cells of the IFE, identifying two distinct basal cell populations as well as cells of the infundibulum. Subclustering of upper hair follicle cells yielded seven distinct subpopulations including two previously unknown populations linked to the sebaceous gland opening. Likewise, outer bulge and inner bulge cells could be subclustered into five and three robust subpopulations respectively.

We next modeled cellular differentiation of the keratinized skin barrier in the IFE as a pseudotemporal process. We identified more than 1600 genes, which were significantly differentially regulated along pseudotime, thus providing the largest compendium of genes linked to epidermal stratification currently available. Likewise, we identified a variety of novel and known transcription factors that might play a role in IFE differentiation. Intriguingly, we observed that nearly all keratinocytes in our dataset – even the ones from the hair follicle – exhibited a gene expression signature, which places them at a particular point on the differentiation pseudotime. Cells of the upper hair follicle pass through all stages of differentiation, while cells of the bulge – though not actively differentiating – show a clear basal signature. Accordingly, we classified differentiation stage as a pan-epidermal source of gene expression heterogeneity.

Considering all keratinocytes that show a basal differentiation signature, we show that these cells self-organize according to spatial gene expression identity along the hair follicle axis from the IFE to the bulge. Spatial gene expression signatures are not discretely compartmentalized but instead gradually change into each other along a "pseudospatial" axis. We identified nearly 600 genes and key transcription factors differentially expressed along this axis,

and classified spatial axis position as a second vector of gene expression heterogeneity among keratinocytes.

Wondering how much of the cellular heterogeneity in the epidermis is explained by just differentiation stage and spatial niche of keratinocytes, we created a NB regression model using binned pseudotime and pseudospace as predictors. Comparing the modeled data to our observed gene expression data, we show that more than 90% of gene expression is explained by baseline expression, differentiation axis and spatial axis alone. Sebaceous gland and immune cell identity constitute additional sources of heterogeneity.

Next, we compared cells expressing stem cell markers such as *Cd34*, *Gli1*, *Lrig1, Lgr5*, *Lgr6*, and high *Krt14* levels to basal cells, which do not express any of those markers. Interestingly, we were unable to find any specific stem cell signature shared by these stem cells. Instead, we observed that the cells expressing stem cell markers show a less differentiated and accordingly more basal identity, as evident from higher levels of genes such as *Krt14*, *Krt5*, *Apoe* and *Col17a1*.

Overall, we created the most comprehensive transcriptome analysis of the epidermis available at the time of publication. We showed that the epidermis is a highly heterogeneous tissue composed of at least 25 robust subpopulations of cells. We furthermore established that most cellular heterogeneity in the epidermis can be explained by the interplay of differentiation stage and spatial position, thus illustrating that complex tissue identities can be shaped by only few vectors of heterogeneity.

3.2 PAPER II: A SINGLE-CELL ATLAS OF MOUSE SKIN DURING HAIR GROWTH AND REST

The physiology of the mouse skin is shaped by the intricate interplay of epidermal and stromal cell types and by the cyclical nature of hair follicle regeneration. Similar to **Paper I**, we sequenced randomly captured cells from the dorsal skin of female C57BL/6 mice. However, in this study, cells were captured from full skin cell suspensions containing cells from the epidermis, dermis and hypodermis isolated during second anagen (5 weeks of age) or second telogen (9 weeks of age). Single-cell libraries were created using the 10X Chromium system (Zheng et al., 2017).

Overall, we analyzed nearly 6000 single cells from anagen and telogen skin. We identified cells from all main tissues of the skin: IFE cells, cells from the permanent part of the hair follicle, cells from the cycling part of the hair follicle, skin fibroblasts and fibroblast-derived cells, vascular cells, immune cells, neural crest-derived cells such as melanocytes and Schwann cells, as well as skeletal muscle cells. Overall, we were able to subcluster these main populations of cells into more than 50 robust subpopulations. While most populations were present during both anagen and telogen, some cell types showed clear statistical enrichment in either anagen or telogen skin.

In this dataset, we were able to re-identify nearly all epidermal cell populations that we defined in **Paper I**. Overall, we show that telogen-to-anagen transition has only minor effects on gene expression and cellular identity in the IFE and the permanent part of the hair follicle. Proliferative cells are enriched in the IFE basal layer and upper hair follicle compartment during anagen. Likewise, anagen cells from the IFE and the hair follicle show a low-level increase of gene expression signatures linked to metabolic and physiological activity.

The cycling part of the anagen follicle is a highly heterogeneous structure containing more than 20 subpopulations of cells. We show that the ORS of the anagen follicle is composed of two intermixed and transcriptionally highly distinct clusters of cells, each containing three subpopulations. One population marks ORS cells enriched in the upper half of the hair follicle and, interestingly, suprabasal cells of the companion layer and lateral disc. The other cluster meanwhile contains cells enriched in the lower ORS and lower proximal cup.

The remaining follicular cells form a large branching cluster representing the matrix and differentiating inner layers of the anagen hair follicle. We were able to define a central cluster of germinative layer cells (containing four subpopulations) and three branches representing differentiating IRS (six subpopulations), cortex and cuticle (five subpopulations) and medulla cells (three subpopulations). Using RNA velocity analysis, we show that germinative layer cells are uncommitted matrix progenitors and that clear lineage commitment begins in cell populations with mixed germinative layer / branch-specific signatures at the root of each branch. We next modeled IRS, cortex/cuticle and medulla differentiation from matrix progenitors and discovered novel transcription factors associated with lineage commitment in each branch. Interestingly, cells of all lineages pass through an intermediate state marked by genes neither found in the progenitors, nor in the terminally differentiated cells.

We show that in addition to dermal papilla and dermal sheath cells, at least four distinct subclusters of fibroblasts can be distinguished in the mouse skin. Interestingly, these four skin fibroblast populations show a gradual topology and clear overlaps in their gene expression profiles. Using markers for each subcluster, we show that this graduality reflects the spatial organization of different skin fibroblast populations along a proximal-distal axis from the papillary dermis to the adventitia. In addition, we show that the gene expression of dermal fibroblasts changes substantially between anagen and telogen.

In addition to fibroblasts, fibroblast-like cells and keratinocytes of IFE and hair follicle, we were able to distinguish six populations of immune cells, four populations of cells from the vasculature including blood and lymph vessel cells, three populations of neural crest-derived cells including Schwann cells and melanocytes, as well as skeletal muscle cells. For each of the defined populations, we were able to infer statistically whether they are enriched in anagen or telogen skin.

All in all, this study is the first analysis of complete murine skin using scRNA-seq. It enabled us to dissect cellular heterogeneity in the dermal and hypodermal compartments of the skin in an unbiased and systematic fashion and to quantitatively compare the cellular composition of

skin during telogen and anagen. In addition, we provide the most detailed analysis of cellular identity in the anagen follicle available to date and model gene expression changes during inner layer differentiation at unprecedented resolution.

3.3 PAPER III: SINGLE-CELL TRANSCRIPTOMICS OF TRACED EPIDERMAL AND HAIR FOLLICLE STEM CELLS REVEALS RAPID ADAPTATIONS DURING WOUND HEALING

In this study, we used scRNA-seq to study the molecular response of Lgr5 and Lgr6 stem cells during wound healing. For this, we crossed *Lgr5-EGFP-Ires-CreERT2* and *Lgr6-EGFP-Ires-CreERT2* mice with *R26-tdTomato* reporter mice and started tracing Lgr5 (Lgr5-Tomato) and Lgr6 progeny (Lgr6-Tomato) at 7 weeks of age. We introduced two 4 mm full thickness wounds into the back skin of Lgr5 and Lgr6 mice at 8 weeks of age and sequenced Tomato-traced cells from the wound area and an unwounded control area 1 day, 4 days, 7 days, 10 days and more than one month after wounding. In addition, we sequenced cells from unwounded mice at 8 weeks of age (0-day control). Overall, we included 1873 cells in the study.

In order to distinguish Lgr5 and Lgr6 stem cell progeny contributing to wound healing from homeostatic stem cells, we devised a naïve Bayes classification approach. Wound area cells with expression profiles very unlikely to be observed in homeostatic cells were defined as wound(-influenced) cells. Overall, we identified 106 Lgr5 and 106 Lgr6 wound cells.

We show that Lgr5 and Lgr6 wound cells are not only transcriptionally distinct from Lgr5 and Lgr6 stem cells during homeostasis but also exhibit internal heterogeneity. We identified several wound cell states in Lgr5 and Lgr6 wound cells, which peak at different time points in the wound healing process, persist over several sampling time points and can co-occur in the same wounds. Interestingly, Lgr5 and Lgr6 wound cells pass through homologous wound cell states marked by similar gene expression patterns.

While most traced Lgr6 cells are located in the IFE directly adjacent to the wound front, Lgr5 stem cell progeny needs to migrate out of the bulge niche into the IFE in order to contribute to re-epithelialization. Accordingly, we were wondering whether Lgr5 cells contributing to wound healing retain their bulge identity in the wound. Analyzing the expression of bulge and IFE marker genes, we show that Lgr5 progeny becomes gradually more IFE-like while downregulating typical hair follicle bulge niche genes. In consequence, Lgr6 and Lgr5 cells transcriptionally converge during wound healing.

Interestingly, we observed the partial upregulation of IFE markers already in 1 day old Lgr5 wound cells. Given that Lgr5 wound cells only started migrating out of their niche between 3 and 4 days after wounding, this suggests that Lgr5 cells upregulate some IFE-related signatures already in their original bulge niche. Wondering whether this phenomenon could be related to signal responsiveness, we screened for ligands and receptors expressed in Lgr5 and Lgr6 control and early wound cells. We defined the repertoire of ligands and receptors expressed in the general wound environment including the wound stroma using bulk-cell RNA-

sequencing data and quantified the interaction potential of Lgr5 and Lgr6 wound cells with the wound environment using a receptor-ligand database. We show that Lgr6 cells already express many receptors, which would allow them to interact with the wound environment, during homeostasis. In contrast, homeostatic Lgr5 stem cells are non-receptive to wound signals and the required receptors need to be rapidly upregulated in early Lgr5 wound cells. We define *Itgb1* and *Cd44* as key receptors for wound signals and show that these receptors are upregulated in both the wound-adjacent bulge and IFE in early wounds.

Using gene set enrichment analysis, we show that both Lgr5 and Lgr6 wound cells pass through a functionally similar wound healing program. Early wound cells are enriched in signatures linked to migration, cytoskeletal reorganization and cell proliferation. In contrast, late wound cell states are characterized by increased energy metabolism and protein synthesis required for the re-establishment of the skin barrier.

This study was first in using single-cell transcriptomics to characterize distinct stem cell populations during wound healing, and answered several key questions linked to stem cell plasticity during regeneration. For the first time, we clearly show that stem cells from different niches lose their original identity, go through a similar regenerative program and transcriptionally converge during wound healing. Interestingly, we demonstrate that this adaption occurs much more rapidly than previously expected.

4 DECODING THE SKIN

Mouse skin is one of the most important model systems for stem cell research. Many paradigms of stem cell biology, tissue maintenance and regenerative medicine have been established or validated in murine skin. Accordingly, the skin is extraordinarily well-characterized on both the cellular and molecular level. However, nearly all attempts to study the cellular heterogeneity of skin on a molecular or functional level were based on inherently reductionist approaches involving *a priori* defined markers or *ex vivo* tissue systems. In consequence, a systematic and unbiased comparative analysis of cellular heterogeneity in the skin has been unfeasible until recently. This thesis describes three studies, which championed the early adaption of scRNA-seq to skin biology, and which provided important new insights into the transcriptomic organization of the skin.

4.1 PREVIOUSLY UNKNOWN CELLULAR HETEROGENEITY OF SKIN

In addition to addressing important questions linked to epidermal stem cell biology and function, **Paper I** allowed us to construct an unbiased cell map of the IFE and hair follicle for the first time. We observed a previously unknown degree of transcriptomic heterogeneity among follicular and interfollicular cells. Over both the 1st and 2nd level clustering, we observed 25 distinct populations of epidermal cells including both well-established and novel populations (**Figure 11**). For example, we identified several populations of cells located in the sebaceous gland duct (later also described by Donati et al., 2017), which express high levels of anti-microbial peptides such as Defensin β6. The specific functions of these cells remain to be elucidated. One possibility is that these cells, similar to Paneth cells in the intestinal crypt, modulate the microbiome in the hair follicle opening. Likewise, we observed a population of cells located in the outer bulge of the club hair, which express a mixture of outer and inner bulge signatures. Since cells of the inner bulge are highly quiescent and express genes that suppress stem cell activation (*Fgf18*, *Bmp6*) (Hsu, Pasolli, & Fuchs, 2011), the co-expression of an inner bulge signature in club hair basal cells might explain why the club hair does not contribute to hair regeneration.

In **Paper II**, we expanded our single-cell transcriptome map to include full thickness mouse skin during anagen and telogen. This did not only allow us to molecularly characterize many less well-defined cellular components of the skin such as dermal fibroblasts or vascular cells, it also enabled us to quantitatively analyze the changes in cellular composition and transcriptomic identity that occur during the hair cycle. Our data presents the skin as a highly heterogeneous tissue, which is composed of more than 50 robust subpopulations of cells and is subjected to significant cyclical remodeling during hair regeneration. In addition to the cycling part of the hair follicle, we observed substantial differences in gene expression or cell abundance in some stromal populations during anagen, most importantly among dermal fibroblasts.

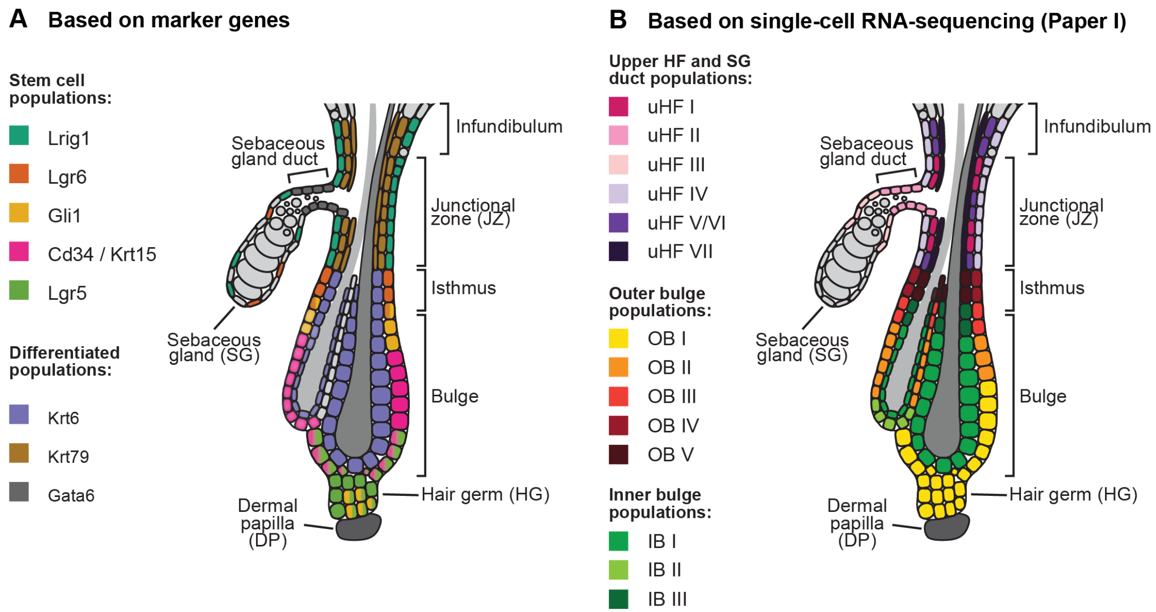


Figure 11: Cellular heterogeneity of the telogen hair follicle based **(A)** on previous studies using individual marker genes or **(B)** on single-cell RNA-sequencing analysis performed in Paper I.

A variety of studies have analyzed fibroblast heterogeneity in the mouse skin. One of these studies identified a functional and molecular compartmentalization of fibroblasts in the papillary and reticular dermis during embryogenesis, but did not show whether the molecular diversity of papillary and reticular fibroblasts is preserved through adulthood (Driskell et al., 2013). A second study claims that there are two distinct populations of fibroblasts in the adult murine skin (Rinkevich et al., 2015). However, these populations were traced based on marker expression during skin development and it is not clear whether these two populations are internally heterogeneous. In **Paper II**, we define four subpopulations of skin fibroblasts. Interestingly, these subpopulations are not distinguished by fully discrete gene expression signatures. Instead, fibroblasts in the skin self-organize along an unknown axis and the expression of many genes gradually changes along this axis. Using a remapping strategy, we show that this transcriptomic graduality mirrors the spatial organization of fibroblasts along a proximal-distal axis from papillary dermis to adventitia. Skin fibroblasts thus change their transcriptomic identity based on their location in the skin with intermediate states found at the boundaries of all fibroblast compartments. The source for this identity change is not clear. A likely scenario would be that fibroblast identity is shaped by microenvironmental factors, which are differentially distributed throughout the skin.

4.2 NO EVIDENCE FOR DISTINCT INTERFOLLICULAR STEM CELLS

The establishment and maintenance of the skin barrier is the primary physiological function of the skin, and most skin pathologies are directly and indirectly linked to barrier disruption. Accordingly, most early skin research was focused on identifying and characterizing the cellular and molecular components that constitute the skin barrier. In consequence, the physiolo-

gical and biochemical processes occurring in cells of the spinous layer, granular layer or stratum corneum have been well-known for decades. Likewise, the core enzymes and proteins, which orchestrate skin keratinization are well-characterized on both the functional and structural level. In contrast, the gene-regulatory networks, which govern epidermal differentiation are less well established. This is mainly due to the limited applicability of genomic techniques when it comes to studying epidermal stratification *in vivo*. While a variety of studies used bulk-cell microarrays or RNA-sequencing to characterize epidermal differentiation on the level of the transcriptome (Gazel et al., 2003; Radoja et al., 2006), nearly all of these studies were based on *in vitro* differentiation of isolated keratinocytes – an inherently artificial system. scRNA-seq of randomly sampled cells from the IFE enabled us to transcriptionally characterize cells belonging to different layers of the epidermis without having to resort to marker-based sorting or *in vitro* differentiation strategies. At the same time, it allowed us to define intermediate differentiation states, which would have been lost in bulk-cell approaches. Overall, our scRNA-seq data allowed us to reconstruct the epidermal differentiation program at unprecedented resolution in **Paper I**. Accordingly, we were able to define a variety of previously unknown gene modules and regulatory factors in our analysis.

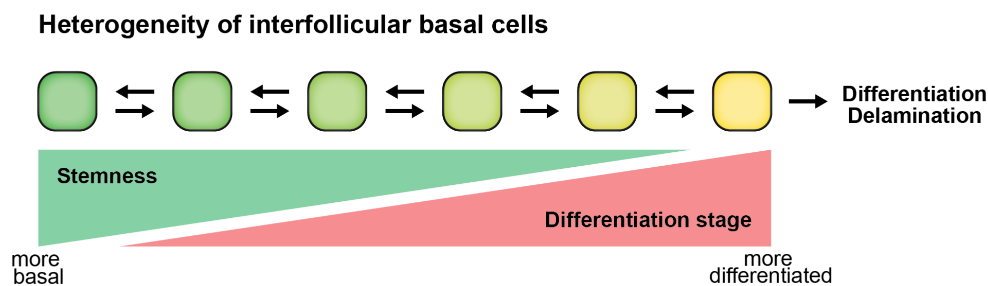


Figure 12: Model for the heterogeneity of interfollicular basal cells. In brief, IFE basal cells show a variety of differentiation states (purely basal to co-expression of basal and differentiation genes). Purely basal IFE cells likely have higher stem cell potential.

In recent decades, the focus of epidermal biology slowly shifted from characterizing the cellular and molecular components of the skin barrier, to analyzing how stem cells can maintain epidermal integrity. Initially, it was claimed that molecularly and functionally distinct stem cells located in the basal layer of the IFE maintain clearly defined proliferative units. In line with a general paradigm shift in stem cell biology, this restrictively asymmetric stem cell model was challenged by the neutral competition model, which allows both symmetric and asymmetric stem cell division. Nevertheless, it is still disputed whether distinct stem cell populations indeed exist in the epidermis. A variety of studies claim to have identified populations in the basal layer of the epidermis that harbor distinct degrees of stem cell potential (Lim et al., 2013; Sada et al., 2016). Most of these studies are based on reporter mouse models and if genomic analysis is used at all, it is usually statistically underpowered and based on comparisons to heterogeneous control populations (e.g. all basal cell not expressing the potential stem cell marker). While we were able to distinguish between proliferating (S or

M phase) and non-proliferating cells (G1 phase) in the basal layer, we were unable to detect any transcriptionally highly distinct subpopulation of epidermal basal cells in either **Paper I** or **Paper II**. At most, we saw that interfollicular basal cells showed small differences when it comes to the deposition of ECM proteins such as *Thbs1*. However, it is unlikely that these minute differences in gene expression discriminate between stem and non-stem cells. In line with our results, a recent single-cell study of the human epidermis was likewise unable to define discrete basal layer stem cell subpopulations (Cheng et al., 2018). Given the limited sensitivity of scRNA-seq, it is possible that we were unable to resolve distinct stem cell states in our basal cell data, as many of the reported potential stem cell marker genes were very lowly expressed in our dataset. However, given the assumption that a distinct stem cell state would be maintained by a specific regulatory module containing tens or even hundreds of genes, we should still be able to resolve the overall differences between stem and non-stem basal cells. Overall, our data does not support the existence of discrete stem cell populations among basal cells of the IFE. Instead, our data shows that basal cells are highly heterogeneous in regards to the expression of differentiation related genes. All basal layer cells can be placed on a continuum between cells expressing purely basal gene expression signatures (including *Krt14*) and cells expressing a mixture of basal and differentiation related genes (including *Ivl*). In line with insights from Mascré et al., 2012, who show that *Krt14*+ basal cells have a higher potential to form long term clones than *Ivl*+ basal cells, this suggests a model in which basal cells with a higher level of differentiation markers are more likely to leave the basal layer and differentiate compared to cells with exclusively basal signatures (**Figure 12**).

4.3 UNCOVERING THE MODULAR ORGANIZATION OF HAIR FOLLICLE (STEM) CELL IDENTITY

A large variety of stem cell populations have been defined in the hair follicle during the last decade. During homeostasis, these stem cells maintain clearly delineated compartments of the hair follicle, such as the bulge, isthmus or junctional zone. What sets stem cells in the hair follicle apart from other cells is still not known. Using our single-cell approach in **Paper I**, we show that nearly all basal cells in the hair follicle express one or more stem cell markers. And similar to our observations in the basal layer of the IFE, we did not observe a distinct transcriptional state, which sets apart basal cells expressing stem cells markers from basal cells, which do not. Instead, our data suggest that follicular stem cells are basal cells expressing additional spatially restricted niche markers. Overall, the interplay between cellular differentiation stage and spatial signatures explains most of the cellular heterogeneity observed in the telogen follicle (**Figure 13**). We therefore show for the first time how even few distinct vectors of gene expression can create complex tissue phenotypes. Interestingly, spatial or niche signatures are not clearly separated but instead gradually bleed into each other along the proximal-distal hair follicle axis. It still remains to be discovered how the compartmentalization of stem cell progeny observed in lineage tracing experiments is maintained at the cellular and molecular level. Interestingly, we observed that many genes expressed along the spatial axis encode ECM proteins and corresponding receptors. It is therefore possible, that

spatially distinct stem cell populations seed their own niche and that these specific microenvironments restrict cell migration during homeostasis.

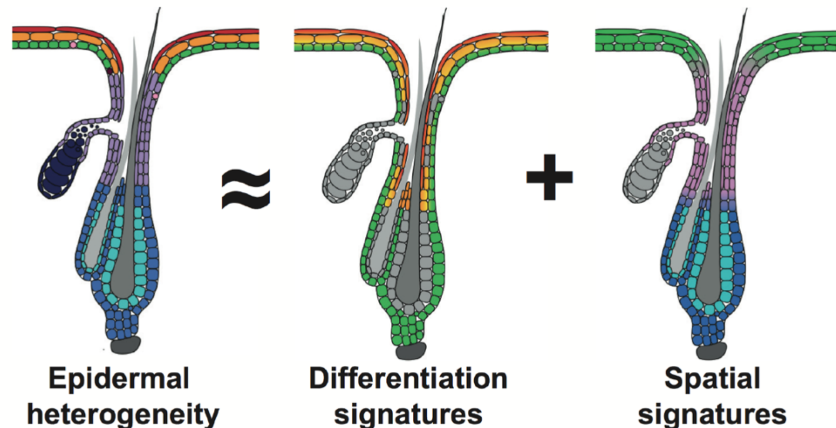


Figure 13: Organization of heterogeneity in IFE and hair follicle. Overall, most cellular heterogeneity in the epidermis can be explained by the interplay of a pan-epidermal differentiation axis and specific spatial niches along the hair follicle.

4.4 NEW INSIGHTS INTO STEM CELL DYNAMICS DURING HAIR REGENERATION

Despite important insights in the last years, many aspects of stem cell dynamics during hair follicle regeneration are still unknown or disputed. The ORS of the anagen follicle has never been fully characterized on the gene expression level. Likewise, inner layer differentiation has mostly been studied using only a few functional markers. Most importantly, it is still highly controversial how inner lineage identity in the follicular matrix is specified.

In most models of the anagen hair follicle, the ORS – the basal layer of the cycling part of the hair follicle, which spans the area between bulge and bulb – has been represented as one continuous cellular compartment. However, a variety of markers have been described to be differentially expressed in the ORS along the hair follicle axis. In **Paper II**, we defined six robust subpopulations of ORS-associated cells, which group into two transcriptionally highly distinct clusters of cells (called ORS1 and ORS2, each having three subpopulations). While ORS1 and ORS2 are slightly enriched in the upper and lower ORS respectively, both populations seem to intermix along most of the hair follicle. However, RNA-FISH analysis revealed that the lower proximal cup located in the bulb region is composed entirely of cells from ORS2. Most interestingly, cells belonging to the ORS1 cluster form the companion layer and the lateral disc, a suprabasal structure with unknown function located in the bulge (Panteleyev, Jahoda, Christiano, 2001). That companion layer cells have an ORS-like identity

is highly surprising since it is commonly assumed that these cells originate from the matrix. The functional role of these distinct ORS populations and their genesis during anagen induction remains to be determined.

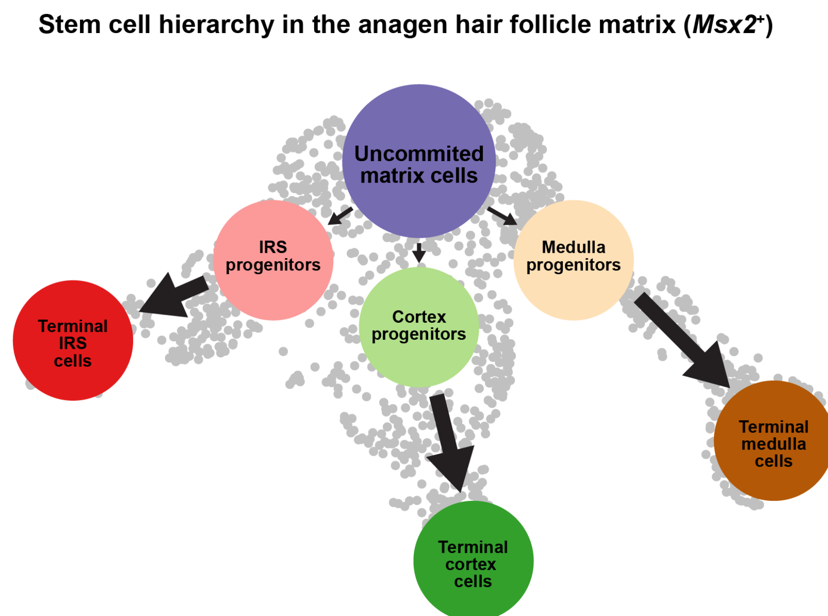


Figure 14: Potential stem cell hierarchy of cells in the anagen hair follicle matrix. Projected onto the UMAP representation of *Msx2+* cells introduced in Paper II.

How progenitor cells in the matrix of the bulb give rise to the inner lineages of the hair follicle remains controversial. In a recent study using scRNA-seq of marker-sorted cells, Yang et al., 2017 claim that matrix progenitors are located in distinct micro-niches, which prime these cells on the transcriptome level towards a certain lineage identity. In contrast, we observed that differentiating cells of the IRS, cortex/cuticle and medulla lineage branch off from one central cluster of germinative layer cells. While these germinative layer cells show some internal heterogeneity along a spatial axis, most importantly in the expression of transcription factors such as *Lef1* and *Id3*, they do not yet show any gene expression signature which would point deterministically to a specific cell fate. Accordingly, RNA velocity analysis suggests that these cells are uncommitted progenitors. In addition, we observed cells with matrix progenitor identity, which also express low level inner lineage signatures, at the root of each differentiation branch. These mixed-identity cells are highly committed towards a certain lineage and could be equivalent to the cells sequenced by Yang et al., 2017 However, these cells likely represent a more committed state compared to the matrix progenitors which we identified (**Figure 14**). Overall, our data indicate the presence of a population of mostly uncommitted matrix progenitor cells, which can in principle give rise to all inner lineages in the anagen follicle.

While the inner lineages of the anagen follicle can be easily identified morphologically, their molecular characteristics are still poorly defined. We computationally reconstructed differen-

tiation trajectories in the inner hair follicle and modeled gene expression changes during IRS, cortex/cuticle and medulla differentiation. We identified thousands of novel genes and a variety of novel transcription factors involved in inner lineage specification. Interestingly, all inner lineage trajectories encompassed an intermediate cell state expressing genes neither found in progenitor, nor terminally differentiating cells. These intermediate states mapped mostly to the bulb area below the hair shaft and thus most likely play a role in early lineage specification.

4.5 HIGH DEGREE OF STEM CELL PLASTICITY DURING WOUND HEALING

The ability to repair damage is one of the central functions of any self-maintaining adult tissue. Studying cutaneous wound healing is therefore not only important from a clinical perspective, but could also provide important insights into how skin stem cells operate. After wounding, stem cells from all compartments of the IFE and the hair follicle are recruited into the wound front to contribute to re-epithelialization. During this process, most stem cell populations leave their original niche. Studying different stem cell populations on the transcriptome level during wound healing could answer several critical questions related to stem cell identity and plasticity: Is stem cell identity purely dependent on niche factors or is there an inherent regulatory program, which maintains a specific stem cell identity even outside the niche? And are stem cells from different compartments of the skin able to execute different regenerative programs? (**Figure 15**) (Donati & Watt, 2015). Such questions are difficult to answer using bulk-cell transcriptome profiling.

In **Paper III**, we used scRNA-seq to track the molecular response of two stem cell populations – Lgr5 and Lgr6 stem cells – during wound healing. In homeostatic skin, these cells are found in two distinct niches, the follicular bulge and the IFE. During wound healing, Lgr6 stem cells do not leave their interfollicular niche. In contrast, Lgr5 stem cell progeny needs to migrate through the upper hair follicle into the IFE in order to reach the wound front. We show that during this migration process, Lgr5 stem cells gradually lose their bulge identity and instead upregulate an interfollicular identity similar to Lgr6 cells. This change in transcriptomic identity was so pronounced that a classifier, which was trained to distinguish Lgr5 and Lgr6 cells, had problems in correctly classifying Lgr5 stem cell progeny in the wound. These results clearly highlight a high degree of plasticity among skin stem cells and underline the importance of niche factors in shaping stem cell identity. As such, they are in agreement with previous studies showing that stem cells can easily change their identity in order to repopulate different compartments of the hair follicle (Rompolas et al., 2013). Overall, these observations suggest that different stem cell identities in the IFE and hair follicle are not restricted by rigid regulatory networks. Instead, stem cells can quickly adapt – molecularly and functionally – to changes in the microenvironment. This is in line with observations from **Paper I**, suggesting that stem cell identity is equivalent with basal identity and that niche factors only contribute additional, compartment specific input.

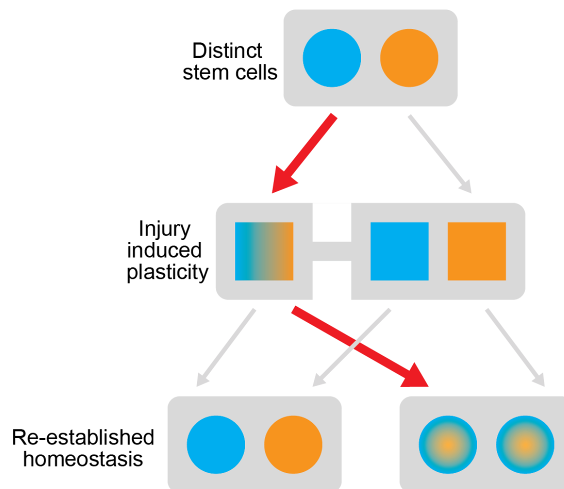


Figure 15: Cellular plasticity in cutaneous wound repair. Shown are different models of stem cell plasticity during tissue injury repair (inspired by Donati and Watt, 2015) including the acquisition of mixed or stem cell specific wound states, and the re-establishment of distinct or mixed stem cell identity in the healed tissue. The red arrows highlight the mode of stem cell plasticity observed for Lgr5 and Lgr6 stem cells in Paper III.

Interestingly, we found that partial acquisition of interfollicular gene expression signatures can already occur while Lgr5 stem cells are still in their original niche. This suggests that the observed identity change in Lgr5 wound cells is not a purely passive process occurring during transition between niches. Instead, it seems that Lgr5 cells are able to actively and rapidly adapt their transcriptome. In particular, we found that Lgr5 cells primed for wound healing quickly upregulate receptors more commonly found in interfollicular cells – including Lgr6 cells – in order to become more responsive to signals from the wound. Since re-epithelialization and reestablishment of the skin barrier is the main purpose of wound healing, and since follicular stem cells are dispensable for wound regeneration (Langton et al., 2008), it seems reasonable that the wound healing program is primarily adapted to activating interfollicular cells. In consequence, acquisition of interfollicular characteristics may be necessary for a hair follicle cell in order to contribute to wound healing.

In addition to the aforementioned change in niche identity, we observed that both Lgr5 and Lgr6 stem cell progeny execute a similar wound healing program during which they pass through distinct wound cell states. While these states peak at distinct time points in the wound healing process, wounds can contain cells in different states. Intriguingly, in addition to increased migration and proliferation, wound cell identity was mainly linked to an upregulation of genes involved in basic cell physiology and metabolism. Our results indicate that wound cells are characterized by increased energy production, synthesis of intermediary metabolites and production of cellular machinery linked to transcription and translation. Therefore, wound cells seem to be metabolically primed for the large-scale mRNA and protein synthesis, which is necessary for cell proliferation and skin barrier reestablishment.

4.6 TOWARDS A SYSTEMS BIOLOGY OF SKIN

Using single-cell transcriptomics, we were able to create a detailed map of cellular heterogeneity in the telogen epidermis including the hair follicle (**Paper I**) and in the full thickness skin during anagen and telogen (**Paper II**). In addition, we were able to transcriptomically analyze stem cells at the single-cell level during homeostasis (**Paper I**), hair regeneration (**Paper II**), and wound healing (**Paper III**), thus providing new insights into the molecular identity, regulation and plasticity of epidermal stem cells. This thesis covers three of the earliest examples of the application of scRNA-seq to skin. While studies using single-cell transcriptomics to analyze aspects of skin biology are still comparatively rare, we expect that this technique will be more commonly used over the next years. We furthermore predict that most future studies will follow the experimental approaches, which were outlined in this thesis. Similar to **Papers I – II**, a large number of studies will analyze randomly sampled cells from the skin in order to create unbiased cell maps. With the higher throughput of droplet microfluidic and split-pool approaches, we soon expect large scale datasets of mouse and human skin, allowing an even better resolution of cellular heterogeneity. Likewise, many future studies will aim to resolve temporal changes of the skin even further. This includes a detailed temporal analysis of the hair cycle, as well as studies focusing on skin development and aging. In addition, single-cell RNA-sequencing will be increasingly used to analyze aspects of skin pathology such as cancer development and diseases such as psoriasis or chronic wounds. On top of these "atlas projects", many future studies will furthermore employ tracing methodologies similar to those introduced in **Paper III**. Given the large variety of stem cell models and lineage tracing approaches used in skin biology, many labs will aim at tracing specific cellular progeny at single-cell resolution. This will include a detailed analysis of the contribution of distinct epidermal stem and progenitor cells to hair regeneration, as well as studies focusing on the molecular role of specific stromal cell populations during development, hair cycling and wound regeneration. Overall, with the increased accessibility of single-cell technologies, we expect a huge increase in transcriptomic studies focusing on all aspects of skin biology in the near future. These studies will provide a more systematic and unbiased view of the physiology and pathophysiology of the skin than is currently available. Therefore, our studies mark the first step towards a systems biology of the skin.

5 ACKNOWLEDGEMENTS

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Sten Linnarsson, I met you shortly after I met Maria at a time when single-cell RNA-sequencing was still in its infancy and many people were saying that the aims of this project were completely unrealistic. Your vision in the revolutionizing potential of single-cell transcriptomics convinced us to push this project forward, and without your technical expertise we would have never completed it. I remember that in the first years of my PhD, we often came together to discuss the technique, its application and future potential. And even though we met less frequently in later years, as I became more independent in my work, you were always available for discussion, advice and inspiration.

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