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EPIDERMAL STEM CELLS AT THE POINT OF NO RETURN

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Cover illustration: Cover art depicts the duality of epidermal cell behaviour, highlighting the cells that are differentiating and the cells that have retained plasticity. Highlighting is overlaid on the UMAP representations of the differentiation timeline presented in Paper I.

Epidermal Stem Cells At The Point Of No Return
Thesis for Doctoral Degree (Ph.D.)

By

Karl Annusver

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"The universe is full of magical things, patiently waiting for our wits to grow sharper."

Eden Phillpotts

Popular science summary of the thesis

Our bodies are made up of trillions of cells, forming our every tissue and performing crucial functions. Our biggest organ, the skin, mediates many of our senses such as touch, temperature, pressure, and pain, regulates our body temperature, and it forms the first line of defence against outside harm (e.g., pathogens, UV-radiation). To understand how skin performs all these actions, it is important to know the characteristics and behaviour of skin cells. Importantly, knowing what the cells are doing when we are healthy will help us understand what has gone wrong when we develop skin diseases or how to improve recovery from injuries.

This thesis is mainly focusing on understanding the functions of cells in the outermost layer of the skin – keratinocytes in the epidermis (**Figure 1**.). To maintain the barrier function that our skin has, keratinocyte stem cells (cells that are responsible for maintaining the tissue) need to renew themselves and the tissue by dividing. Moreover, they need to take on more and more specific characteristics, a process that is called differentiation, to convert from being a stem cell to a terminally differentiated cell in the most upper layer of skin.

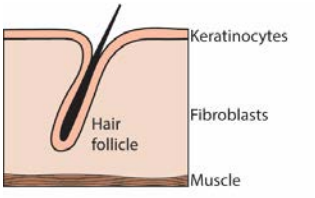


Figure 1. Overview schematic of skin and its main cell types

To study this process, we need to first understand what happens during this differentiation. While keratinocytes share the same genetic material in the form of DNA with every other cell in our body, they use skin-specific parts of it (likewise immune cells use immune-specific parts etc) – these parts are called genes. Genes are short segments of DNA that the cells "read" and "translate" to a form called RNA to make all the proteins, the building blocks of the cells, that they need. Over the last decade, research methodologies have improved to a point where it is possible to read the RNA molecules in individual cells, thus knowing exactly which genes and how much of the gene the cell is using. This method is called single-cell RNA sequencing (scRNA-seq).

In **Paper I**, we studied skin stem cells in order to understand when they commit to becoming differentiated, specialized cells. We performed scRNA-seq on these cells and unified the results with previously well-characterized datasets. First, we realized that many of the cells previously thought to be in the upper skin layers were unexpectedly still in the bottom basal layer, interspersed with the stem cells. Moreover, we found that about 40% of cells in the basal layer had already committed to becoming differentiating cells, a lot more than was expected. Finally, we saw that many of these committed cells showed signs that they were undergoing cell division which was also unexpected. To

confirm all these findings, we turned to a powerful microscopy method, called intra vital microscopy, which makes it possible to look at individual cells in a living mouse without causing harm to the mouse or to the cells. Furthermore, it is possible to return to the same spot multiple times after hours and days, thus being able to track the behaviour of the cells over long time periods. Using a specific laboratory mouse model that highlights the differentiating cells with fluorescence, we did indeed find confirmation for the high number of differentiating basal cells and their ability to divide. Finally, when we blocked the cell division capability of these differentiating cells, we found that it did not impact the skin's health. However, recovery from injury took longer than in mice where these cell divisions were not blocked, indicating that these cells could be important for a rapid response to injury, while in healthy skin they serve a rather supportive role.

In **Paper II**, we looked at all the different cell types in the skin of adult mice using scRNA-seq to understand what genes these different cells express. We were able to characterize all main cell types, including keratinocytes, fibroblasts that produce the structural components in the deeper skin layers, several types of immune cells, cells of the blood and lymph vessels, muscle cells, pigment producing melanocytes, and Schwann cells that wrap around nerve fibres to insulate them. Notably, keratinocytes are not only involved in maintaining the outer layer of skin, but they also make up the hair follicles and produce hair. Because hair growth cycles between active growth and periods of rest, we wanted to understand how these cycles influence skin cells. Moreover, as hair follicles cells have a strict organization into concentric layers (like onion layers), we aimed to characterize the differences between the keratinocytes located in different parts of the hair follicle. Interestingly, we found that a cell layer, that has previously been considered to be part of the inner layers, was more similar to the outer layer cells. Moreover, we found that the hair follicle stem cells were likely more flexible in producing the different concentric layers than previously thought, as demanded by their location within the hair follicle. Finally, we described how skin fibroblasts change their identity while the hair is actively growing.

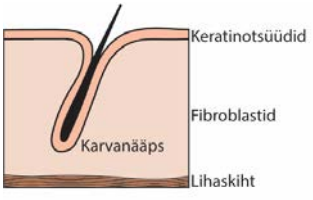
Paper III performed a similar analysis as **Paper II**, but this time studying mouse skin during embryonic development at timepoints when the crucial skin components are first established. We used a combination of scRNA-seq and microscopy to establish anatomical landmarks in the developing skin and to characterize all major cell types, which resemble cell types in adult skin. Curiously, we found that the embryonic fibroblast showed a separation into many more specific types than what was expected for this stage in development. A group of these fibroblasts were involved in creating the skin-associated muscle layer called panniculus carnosus muscle, while another group of specific fibroblasts participates in the formation of the first hair follicles. Finally, we looked in-depth at the embryonic keratinocytes. To our surprise, they contained a big group of cells that form the outside layer of embryonic skin, called periderm, which protects the different parts of the embryo from sticking to each other. Finally, we described the identities of keratinocytes that will form the outer layers of skin, as well as the keratinocytes that will produce the first hair follicles.

Taken together, the three papers presented in this thesis help us to better understand what happens when different skin cells commit to their ultimate fate, whether in adult skin or during development. Our findings challenge some of the currently held beliefs, while specifying and supporting other ideas. The papers also give a broad overview of all the skin cell types and we have made the results easily searchable in online tools to facilitate future research in the field of skin biology.

Populaarteaduslik lõputöö kokkuvõte

Meie kehad koosnevad triljonitest rakkudest, mis moodustavad kõik meie koed ja täidavad olulisi funktsioone, et hoida meid tervena. Kõige suurem organ, nahk, vahendab paljusid meie meeli (puudutus, temperatuur, rõhk, valu), aitab reguleerida meie kehatemperatuuri ja moodustab peamise kaitsekihi väljapoolt tulevate kahjustuste (patogeenid, UV-kiirgus) vastu. Et aru saada, kuidas kõik need funktsioonid töötavad, on oluline mõista naha rakkude omadusi ja käitumist. Lisaks, teades mida rakud teevad siis kui me oleme terved, saame ka paremini aru, mis on valesti läinud nahahaiguste korral või kuidas parandada vigastustest taastumist.

See lõputöö keskendub peamiselt naha välimises kihis asuvate rakkude - epidermise keratinotsüütide - funktsioonide mõistmisele (**Joonis 2**. Naha peamiste struktuuride skeem). Naha kaitsefunktsiooni säilitamiseks peavad keratinotsüüdid jagunema, et ennast ja kude uuendada, neid rakke kutsutakse ka tüvirakkudeks. Lisaks peavad need rakud järk-järgult spetsialiseeruma, tuntud kui diferentseerumine, et muutuda jagunevast tüvirakust lõplikult diferentseerunud naha väliskihi rakuks.



Joonis 2. Naha peamiste struktuuride skeem

Selle protsessi uurimiseks peame esmalt mõistma, mis toimub diferentseerumise käigus. Kuigi keratinotsüüdid jagavad täpselt sama geneetilist materjali (DNA kujul) nagu kõik teised raku meie kehas, kasutavad nad ainult nahaspetsiifilisi osi sellest materjalist (sama kehtib immuunrakkude ja immuunspetsiifiliste osade kohta jne) - neid osi nimetatakse geenideks. Geenid on lühikesed DNA järjestused, mida rakud "loevad" ja "tõlgivad" RNA vormi, millest nad seejärel toodavad kõiki valke (rakkude ehituskivid) mida neil vaja on. Viimase kümnendi jooksul on uurimismeetodid arenenud sellisele tasemele, et on võimalik üles lugeda kõik RNA molekulid igas üksikus uuritavas rakus, saades täpselt teada, milliseid geene ja kui palju need rakud kasutavad. Seda meetodit nimetatakse üksikraku RNA sekveneerimiseks (*single-cell RNA sequencing* - scRNA-seq).

Artiklis I uurisime naha tüvirakke, et mõista, millal nad diferentseeruvad spetsialiseerunud rakkudeks. Me teostasime üksikraku RNA-sekveneerimise analüüsi nendel rakkudel ning kombineerisime tulemused varasemalt hästi iseloomustatud andmehulkadega, et koostada ühildatud arusaam saadud andmetest. Esiteks märkasime, et eelnevalt naha ülemiste kihtide hulka arvatud keratinotsüüdid kuulusid tegelikult alumistesse kihtidesse, koos naha tüvirakkudega. Lisaks sellele leidsime, et umbes 40% aluskihis olevatest rakkudest oli juba diferentseerumas, palju rohkem kui me eelnevalt ootasime. Lõpuks nägime, et paljud diferentseeruvad rakud olid jagunemas, sisaldades

jagunemise jaoks vajalikke geene. Kõikide nende leidude kinnitamiseks pöördusime võimsa mikroskoopia meetodi - eluskoe (*intra vital*) mikroskoopia - poole, mis võimaldab vaadata üksikuid rakke elava hiire nahas seda kahjustamata. Lisaks on võimalik mitmeid kordi samade rakkude juurde tagasi tulla tundide ja päevade pärast, et jälgida rakkude käitumist pikema aja jooksul. Kasutades laborihiire mudelit, mis märgib diferentseeruvaid rakke fluorestseeruva tähisega, leidsime kinnituse, et naha aluskihis oli tõe poolest suur hulk diferentseeruvaid rakke, mis suutsid endiselt jaguneda. Lõpuks, kui blokeerisime diferentseeruvate rakkude jagunemisvõime, siis see ei mõjutanud tervet nahka, ainult vigastustest paranemine võttis kauem aega. See näitas, et need rakud võivad olla tähtsad kiireks reageerimiseks vigastustele, aga tavaolukorras on nad peamiselt kude toetavas rollis.

Artiklis II uurisime täiskasvanud hiire naha erinevaid rakutüüpe, kasutades taaskord scRNA-seq meetodit. Me iseloomustasime kõik peamised naha rakutüübid, sealhulgas keratinotsüüdid, sügavamate naha kihtide struktuurikomponente tootvad fibroblastid, mitmed erinevad immuunrakud, veresoonte ja lümfisoonte rakud, lihasrakud, pigmendirakud melanotsüüdid, närvikiude isoleerivad Schwanni rakud ja isegi punalibled. Tähelepanuväärne on see, et keratinotsüüdid ei ole mitte ainult seotud naha välimise kihi säilitamisega, vaid nad on ka karvanääpsude moodustajad, et karvu pikemaks kasvatada. Karva kasv on tsükliline, kus aktiivne kasv vaheldub puhkeperioodiga, seetõttu tahtsime teada, kas karvakasvu tsükkel mõjutab rakkude omadusi. Kuna karvanääpsu rakud moodustavad väga korrapäraseid struktuure (nagu erinevad sibula kihid), siis püüdsime kirjeldada erinevusi keratinotsüütide vahel, mis asuvad erinevates karvanääpsu osades. Huvitaval kombel leidsime, et rakukiht, mida seni peeti sisekihi osaks, oli sarnasem karvanääpsu välimise kihi rakkudega. Lisaks leidsime, et karvanääpsu tüvirakud olid palju paindlikumad kui seni arvatud, käitudes vastavalt nende täpsele asukohale. Lõpuks näitasime ka, kuidas naha fibroblastid muudavad oma omadusi aktiivse karva kasvu ajal.

Artikkel III viis läbi sarnase analüüsi nagu **Artikkel II**, kuid uuris seekord hiire embrüonaalset nahka ajahetkedel, mil olulised naha komponendid on esmalt välja kujunemas. Kasutasime taaskord scRNA-seq ja mikroskoopia meetodeid, et täpselt kirjeldada arenevas nahas anatoomiat ning iseloomustada kõiki peamisi rakutüüpe nagu täiskasvanud nahas. Huvitaval kombel leidsime, et embrüonaalsed fibroblastid jagunesid rohkemateks spetsiifilisteks fibroblasti tüüpideks, kui enne selles arenguetapis arvati. Leidsime, et üks nendest fibroblastide rühmadest on oluline välimise lihaskihi, *panniculus carnosus* kihi, moodustamisel. Samuti iseloomustasime spetsiifilisi fibroblaste, mis osalevad esimeste narvanääpsude moodustamisel. Lõpuks uurisime ka embrüonaalseid keratinotsüüte, mis meie üllatuseks sisaldasid suurt rühma rakke, mis moodustavad embrüonaalse naha pealiskihi (*periderm*), mis kaitseb erinevaid embrüo osi teineteise külge kleepumast. See rakupopulatsioon ei ole seotud teiste naha keratinotsüütidega, kuid järgib sarnast diferentseerumise mustrit, ehkki omab oma eripärasid. Lõpuks kirjeldasime ka nende keratinotsüütide omadusi, millest arenevad täiskasvanud naha väliskihid ja esimesed karvanääpsud.

Kokkuvõttes aitavad selles lõputöös esitatud kolm artiklit meil paremini mõista, mis juhtub erinevate naharakkudega, kui nad pühenduvad oma lõplikule saatusele nii täiskasvanu nahas kui ka naha arengu käigus. Meie tulemused seavad kahtluse alla mõned hetkel valitsevad arvamused, samas täpsustades ja toetades teisi ideid. Artiklid annavad ka laia ülevaate kõigist naharakutüüpidest ning oleme tulemused teinud kergesti otsitavaks veebitööriistades, et hõlbustada tulevasi uurimistöid nahabioloogia valdkonnas.

Abstract

The skin is our main barrier against outside harm, and it helps to maintain internal homeostasis. These functions are mostly fulfilled by the epidermis, which is the outermost layer of the skin, formed from keratinocytes that are constantly being shed and replaced through proliferation, differentiation, and subsequent delamination of tissue resident stem cells. Keratinocytes also give rise to hair follicles which go through regular cycles of growth and rest. These processes are supported by, for example, fibroblasts, immune cells, nerves and vessels, helping to maintain the protective function of the skin. The wealth of different cell types and stem cell behaviours makes skin an attractive model system for research.

The overarching aim of this thesis was to study the epidermal stem cells at their onset of differentiation – to determine their point of no return beyond which they cannot revert back to a less differentiated state. To address this question, a combination of single-cell RNA-sequencing, *in situ* mRNA stainings and *intra vital* imaging methods were used, allowing for the assessment of cells' transcriptional states, their location in the tissue and their behaviour over time.

In Paper I, we present a thorough characterization of differentiation-committed basal cells in the interfollicular epidermis. We study their behaviour in living tissue and describe their transcriptional states upon commitment and delamination. Finally, we show how mitosis of committed cells is not integral to their differentiation journey.

In Paper II, we analysed full thickness adult skin in the growth and resting stages of the hair cycle to create an unbiased transcriptional atlas of all major cell types. We uncover unexpected heterogeneity in the anagen hair follicle lineages and highlight how fibroblast populations can undergo transcriptional state changes during hair cycling.

In Paper III, we expanded the transcriptional atlas to embryonic development, describing the transcriptional and anatomic landmarks at the time when crucial skin structures are established. We show early heterogeneity within the fibroblast population, as well as the first signs of hair follicle development and the formation of the panniculus carnosus muscle.

Taken together, this thesis investigates skin stem cells molecularly, spatially, and behaviourally when they commit to specific lineages whether in the adult or developing epidermis, in the growing hair follicle or in the embryonic fibroblast compartment. Finally, we speculate on what could be the underlying cause that pushes the stem cells over the point of no return.

List of scientific papers

I. Katie Cockburn*, **Karl Annusver***, David G. Gonzalez, Smirthy Ganesan, Dennis P. May, Kailin R. Mesa, Kyogo Kawaguchi, Maria Kasper#, Valentina# Greco. *Gradual differentiation uncoupled from cell cycle exit generates heterogeneity in the epidermal stem cell layer*. Nat Cell Biol. 2022 Dec;24(12):1692-1700.

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III. Tina Jacob, **Karl Annusver**, Paulo Czarnewski, Tim Dalessandri, Christina Kalk, Chiara Levra Levron, Nil Campamà Sanz, Maria Eleni Kastriti, Marja L Mikkola, Michael Rendl, Beate M Lichtenberger, Giacomo Donati, Åsa Björklund, and Maria Kasper. *Molecular and spatial landmarks of early skin development*. Manuscript.

* These authors have contributed equally.

Co-corresponding authors

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° These authors have contributed equally.
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Contents

List of abbreviations

bp	Base pair
cDNA	Complementary DNA
DETCs	Dendritic epidermal T cells
DNA	Deoxyribonucleic acid
DP	Dermal papilla
E (e.g., E8.5)	Embryonic day
ECM	Extracellular matrix
EPU	Epidermal proliferative unit
FIB	Fibroblast
GL	Germinative layer
HF	Hair follicle
IFE	Interfollicular epidermis
IRS	Inner root sheath
LPC	Lower proximal cup
mRNA	Messenger RNA
ORS	Outer root sheath
PCM	Panniculus carnosus muscle
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
scRNA-seq	Single-cell RNA-sequencing
SNP	Single nucleotide polymorphism
TAC	Transit amplifying cell
t-SNE	t-distributed stochastic neighbor embedding
UMAP	Uniform manifold approximation and projection
UMI	Unique molecular identifier
UV	Ultraviolet

1 Introduction

1.1 Overview of adult mouse skin

Skin is the largest mammalian organ covering the outside of the body, protecting it from harm through providing an impermeable barrier. The outer layers of skin are constantly replenished by tissue resident stem cells, which proliferate and differentiation in response to natural skin-cell shedding, and damage, such as mechanical abrasions, UV radiation and pathogens (Blanpain & Fuchs, 2009). Apart from its protective function, skin is also essential in sensing the outside world via touch, pressure and temperature responsive nerves, and it facilitates thermoregulation (Fuchs, 2007; Hsu et al., 2014). The skin can be broadly divided into two main compartments: the epidermis, including the hair follicles (HFs) and their associated sebaceous glands, and the underlying dermis, including the hypodermis (**Figure 3**).

1.1.1 Epidermis

Epidermis is the outermost skin layer, formed by specialized epithelial cells called keratinocytes, and functioning as the main barrier for the body. The epidermis can be further divided into two main parts: the HF and the interfollicular epidermis (IFE) **(Figure 3)**. As the name implies, IFE is the epithelium in-between the HFs, which is comprised of keratinocytes responsible for maintaining the epidermis as well as a variety of other cell types such as immune cells and touch-sensitive Merkel cells. The HFs are essentially self-maintaining mini organs with their own resident stem cells and micro-niches. They include hair-producing keratinocytes, mesenchymal cells controlling hair growth signalling, melanocytes, sebocytes in the sebaceous gland and resident immune cells monitoring the hair follicle opening. Moreover, HFs go through regular and coordinated cycles of growth and rest, bringing about changes also in the surrounding cell populations (reviewed in: Fuchs, 2007; Hsu et al., 2014).

1.1.2 Dermis

Dermis is a mesenchymal cell compartment underneath the epidermis, housing all the supportive structures needed for skin homeostasis **(Figure 3)**. The outermost part of the dermis is made up of a thin, but fibroblast-dense area called the papillary dermis, which is in direct contact with the IFE, while the underlying reticular dermis spans the length of the HF and is composed mainly of extracellular matrix (ECM) proteins. The reticular dermis also houses the majority of non-fibroblast dermal cell types such as immune cells, peripheral nerves and Schwann cells, blood vessels and pericytes, as well as the arrector pili muscle. below the reticular dermis lies the hypodermis, which mainly consists of

adipocytes and some scattered fibroblasts (reviewed in: Driskell & Watt, 2015; Hsu et al., 2014).

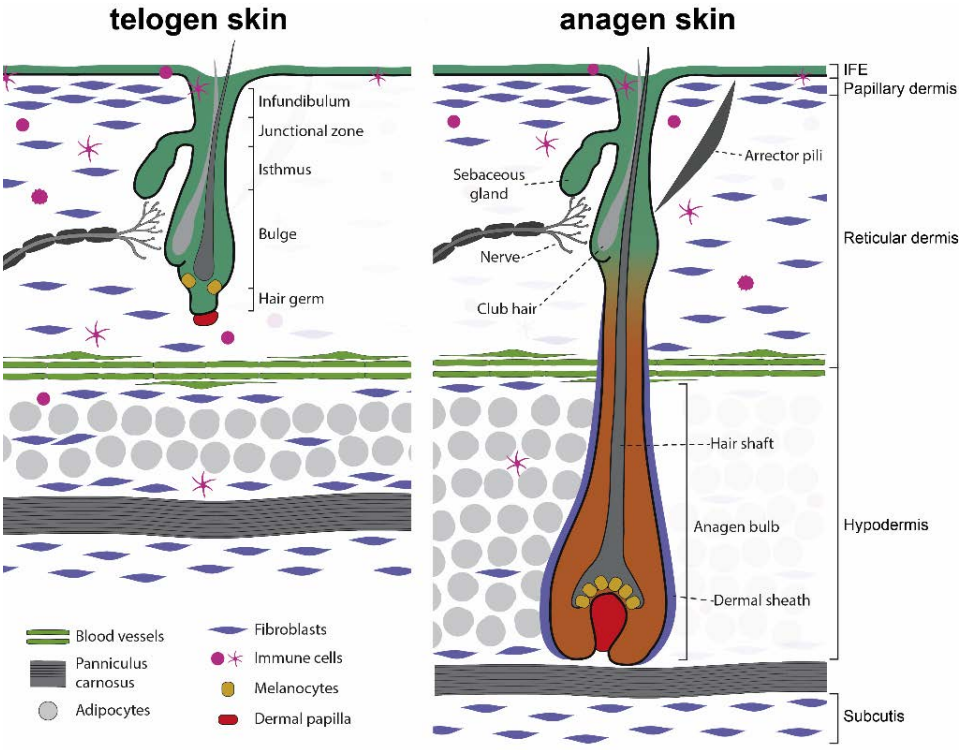

Figure 3. Anatomy of skin and hair follicles during different hair cycle stages. Shown are the different skin compartments and how they change between telogen and anagen. Dark green and orange layers represent keratinocytes (Figure published in: Lichtenberger & Kasper, 2021)

1.2 Skin homeostasis

1.2.1 Interfollicular epidermis

The IFE is a stratified epithelium consisting of multiple distinct layers of differentiating keratinocytes, separated from the underlying dermis by the basement membrane. Based on their functions and microanatomy, four layers can be distinguished: the basal layer harbouring proliferative keratinocytes with high KRT14 and KRT5 expression, the spinous layer with differentiating KRT10 and KRT1 expressing keratinocytes, the granular layer where keratinocytes have a characteristic flat hexagonal shape and produce LOR and FLG, and the stratum corneum composed of terminally differentiated and fully keratinized cells (Fuchs, 1990; Hsu et al., 2014).

1.2.2 Models of epidermal differentiation

Although epidermal differentiation has been extensively studied, it remains an open question how cells decide if they should proliferate and remain a stem cell or if they should differentiate. There are two models explaining stem cell maintenance in skin: the hierarchical model, in which stem cell divisions are asymmetric, giving rise to one differentiated cell and one stem cell, and the stochastic model that balances proliferation and differentiation on the population level (Simons & Clevers, 2011).

Early studies provided evidence for the hierarchical model where an epidermal stem cell would give rise to rapidly proliferating transit-amplifying cells (TACs) which then differentiate upwards (Mackenzie, 1970). Further findings described a group of about ten basal cells, with a slowly cycling central cell, that give rise to about eight suprabasal cells forming the epidermal proliferative unit (EPU) (Potten, 1974). More recently, it was described that the basal layer is maintained by slow-cycling *Krt14*-expressing cells and faster cycling *Ivl*-expressing committed progenitor cells (Mascré et al., 2012), which may reflect the patterned scale and interscale areas of tail skin (Gomez et al., 2013). Indeed, a later study in the tail epidermis described two independent basal epidermis stem cell pools, one being a slow-cycling population in the interscale region and the other one being a fast-cycling population in the scale region (Sada et al., 2016). Furthermore, recent findings indicate that dorsal skin also harbours slow-cycling stem cells that are marked by Thy1 expression and which undergo preferentially asymmetric divisions (Koren et al., 2022).

In contrast, the stochastic model proposes that basal cells follow neutral competition and have an equal chance of proliferating or differentiating. In that model, cell divisions can give rise to either two progenitor cells, two post-mitotic cells, or a progenitor and a post-mitotic cell, which, over time, leads to an overall increase in the average clone size and a decrease in the number of different clones. This model has been shown to hold true in many skin regions (Clayton et al., 2007; Doupé et al., 2010; Füllgrabe et al., 2015; Lim et al., 2013; Piedrafita et al., 2020) as well as in other epithelia (Doupé et al., 2012; Snippert, van der Flier, et al., 2010). Interestingly, a study tracking cell fates in live mice was able to link the two models and solve a few of the discrepancies by showing that,

while basal stem cells divide according to the neutral competition model, differentiating cells follow a pattern similar to what was described for EPUs. The majority of differentiating cells remain constrained within a column of cells directly on top of each other, which the authors termed as the epidermal differentiation unit (Rompolas et al., 2016).

1.2.3 Basal cell differentiation

A crucial step in the epidermal differentiation process is delamination (Hsu et al., 2014; Watt & Green, 1982), during which a basal cell severs contact with the basement membrane and commits to differentiation (**Figure 4**). It was long thought that delamination and differentiation would be initiated by asymmetric division of basal keratinocytes, perpendicular to the basement membrane, with one daughter cell moving directly up into the spinous layer (Lechler & Fuchs, 2005). This has been more recently shown to happen during embryonic and neonatal development, where cells begin to differentiate and delaminate in response to crowding in the basal-layer (Miroshnikova et al. 2018). However, intra-vital imaging in ear skin has unveiled that in adult epidermis, differentiation and delamination precede cell division, i.e. only after a cell has left the basal layer will the neighbouring cells start to proliferate and fill in the empty space (Mesa et al., 2018). These discrepant findings could be due to the different modes of cell division when comparing embryonic development, where there is a rapid expansion of cells, and adult tissue that is in homeostasis and needs to balance cell loss with proliferation. Still, it remains unknown how the basal cells in adult skin sense the empty space left behind by the differentiating cell. An intriguing factor could be that the changes in mechanical forces signal the neighbouring cells through Hippo and Wnt pathways as well as through the mechanosensory Piezo1 ion channel, causing them to proliferate (Benham-Pyle et al., 2015, 2016; Gudipaty et al., 2017; Panciera et al., 2017).

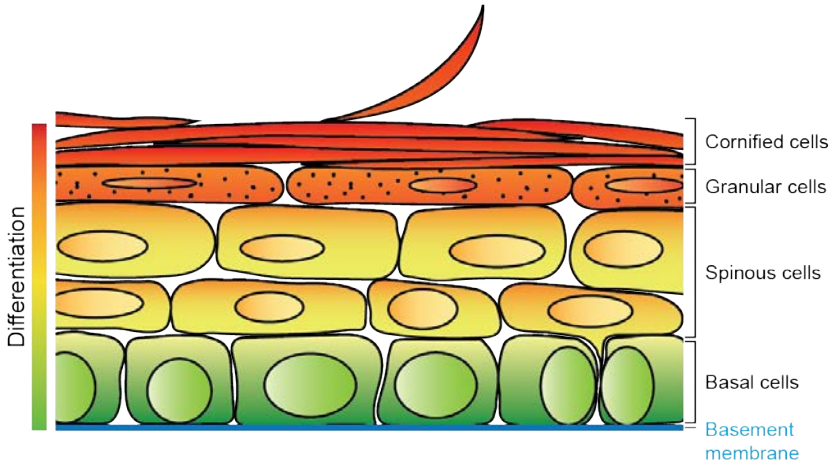

Figure 4. Schematic representation of epidermal keratinocytes and their differentiation from basal layers to the terminal outer layers.

1.2.4 Characteristics of basal cells

As delamination is closely linked to differentiation, for keratinocytes to keep their basal cell identity it is of utmost importance to maintain contact to the basement membrane. For example, secretion of the basement membrane protein laminin-5 as well as cell surface integrins (mainly $\alpha3\beta1$ and $\alpha6\beta4$) are linked to maintaining an undifferentiated basal identity (Watt & Fujiwara, 2011). Moreover, single-cell RNA-sequencing and epigenomic studies have identified the expression of a transcription factors like *Bhlhe40*, *Cebpb, Etv4, Hes1*, *Id1, Ovol1*, *Trp63*, *Zfp36l2* and *Zbed2* together with genes associated with intracellular signalling (e.g., *Wnt7b* and *Fgfr3*) and cell adhesion (e.g., *Cdh3*, *Fat1*, and *Dsg3*) as important for basal-state maintenance (Blanpain et al., 2006; Finnegan et al., 2019; Genander et al., 2014; Joost et al., 2016; Lee et al., 2014; Truong et al., 2006). Differentiating keratinocytes, on the other hand, have been found to express a variety of transcription factors, such as *Gata3*, *Prdm1*, *Grhl1*, *Klf3*, and *Mllt4* among others (Joost et al., 2016; Kaufman et al., 2003; Kretzschmar & Watt, 2014; Mlacki et al., 2014), and to upregulate gene modules involved in terminal differentiation, including genes like *Lor* and *Csta* (Finnegan et al., 2019). This study also found that the chromatin remodelling complex Swr1 components could be important in the switch from basal to differentiated state, however, as the study was performed on neonatal foreskin samples, it is unclear if this finding will also hold true in adult homeostatic conditions. Thus, it is still an open question what causes a basal keratinocyte to enter the differentiation process.

1.3 Cell compartments

1.3.1 Immune cells

Apart from keratinocytes, the epidermis also houses abundant immune cell types throughout the different layers (**Figure 3)**, which continuously monitor for tissue intergrity, pathogenic intrusion or stress (Dalessandri & Strid, 2014; Heath & Carbone, 2013). At steady state, these immune cells are the dendritic Langerhans cells and $\gamma\delta$ T cells known as dendritic epidermal T cells (DETCs). Langerhans cells reside above the basal layer and project their dendrites outwards to the cornified epithelium, where they monitor for pathogenic material and provide a classical antigen processing and presentation role as dendritic cells. DETCs reside mostly in the basal layer and project their dendrites outwards for continuous contact with keratinocytes in the granular layer. In doing so, they scan for keratinocyte stress and have an important role in regulating tissue homeostasis (Dalessandri et al., 2016), wound healing, inflammation, tumour surveillance and response to pathogens (reviewed in: Heath & Carbone, 2013; Malissen et al., 2014).

The dermis, on the other hand, has a big variety of immune cells including multiple dendritic cell subtypes, natural killer cells, helper, regulatory and memory T cells and macrophages. Together they function to monitor for pathogenic intrusion as well as scavenge cellular debris and contribute to tissue homeostasis (Heath & Carbone, 2013; Malissen et al., 2014).

1.3.2 Mesenchymal cells

Dermis contains a diverse group of mesenchymal cells that provide a supportive and signalling environment for other cells (**Figure 3**). The main mesenchymal cell type in the dermis is fibroblasts, producing and depositing extracellular matrix proteins, such as collagens, laminins and proteoglycans to maintain the connective tissue (Driskell & Watt, 2015; Krieg & Aumailley, 2011). These fibroblasts are located in the papillary and reticular dermis, arising from the same mesenchymal population during embryonic development, with a characteristic expression of PDGFRa and LRIG1 (Driskell et al., 2013). Fibroblasts also play an important role in different signalling pathways during homeostasis, wound healing and tumorigenesis (Driskell & Watt, 2015; Plikus et al., 2021). Another population of mesenchymal cells form the dermal papilla (DP), which is a condensed signalling hub just underneath the HFs. The DP plays a crucial role in hair follicle morphogenesis and the coordination of hair cycling (Driskell et al., 2011). Dermal sheath cells surround the HF where they provide a supportive role for HF keratinocytes, but they are also able to repopulate the DP (Rahmani et al., 2014; Cotsarelis, 2010). Finally, the mesenchymal cells also give rise to the arrector pili muscle (responsible for piloerection or so called 'goose bumps'), perivascular cells attached to dermal blood vessels (helping with constriction and homeostasis), and intradermal adipocytes that store energy, participate in long-distance signalling and provide insulation (Driskell & Watt, 2015).

1.3.3 Melanocytes and Merkel cells

Furthermore, the epidermis includes mechano-receptive Merkel cells and pigment-producing melanocytes (**Figure 3**). Both cell types share a common developmental origin from the neural crest, but they perform very different functions in adult skin. Merkel cells are neuroendocrine cells localized in specialized structures within the IFE, called touch domes. These structures form their own specific niche with specialized keratinocytes, fibroblasts and Merkel cells that are responsible for the touch sensation. The perceived signal is conveyed via Piezo2 channels in the Merkel cells that then transmit the signal to peripheral nerve fibres trough their cell-neurite connections (Woo et al., 2015; Xiao et al., 2014). Although in human skin, melanocyte stem cells also reside among the keratinocytes in the basal layer of IFE, in the mouse skin, they are found only in the hair follicle bulge region. They have a dendritic morphology that is used for depositing melanin into their neighbouring keratinocytes (J. Y. Lin & Fisher, 2007). In the human IFE, melanocytes are long-lived and continuously produce melanin that serves a photoprotective function. Hair follicle melanocytes go through cyclical differentiation and apoptosis along with the hair cycle and give colour to newly formed hair shafts by depositing their melanin into the hair-producing keratinocytes (reviewed in: Cichorek et al., 2013; Hsu et al., 2014). Over time, these melanocyte stem cells can accumulate DNA damage, which causes their depletion by differentiation and eventually leads to age-related hair greying (Inomata et al., 2009). Intriguingly, a recent study has identified that stress-related hair greying follows a similar mechanism with melanocyte stem cell differentiation and depletion, brought on by noradrenaline secreted from sympathetic nerves (Zhang et al., 2020).

1.3.4 Panniculus carnosus muscle

The panniculus carnosus muscle (PCM) is the main anatomical structure in mice, but absent in humans, that separates dermis from underlying subcutaneous tissue. It is broadly known that it emerges from *Pax7*$^{+}$ dermomyotome, which is established at E9.5 (Amini-Nik et al., 2011; Atit et al., 2006; Lepper et al., 2011). Moreover However, to the best of our knowledge, its actual histological emergence.

1.4 Hair follicles and hair cycling

Hair follicles are mini-organs that serve both a thermoregulatory and a mechanosensory function in mammals. They undergo regular cycles of hair growth (anagen), regression (catagen), and rest (telogen) (**Figure 3**), with each of these stages having specific cellular and transcriptional dynamics. Anatomically, the HF can be divided into the permanent HF, including infundibulum, junctional zone, isthmus, bulge and hair germ area, and the cycling area formed during anagen (Kretzschmar & Watt, 2014). Furthermore, HFs are associated with several supportive structures and cell types. Sebaceous glands secrete an oily substance called sebum into the junctional zone to lubricate the hair canal and the IFE (Niemann & Horsley, 2012). Arrector pili muscles span from the upper bulge area to the papillary dermis and are responsible for piloerection, also known as goose bumps (Fujiwara et al., 2011). The isthmus area is surrounded by blood vessels, forming a venule annulus (Xiao et al., 2013), as well as nerve fibres that provide SHH to the HF (Brownell et al., 2011). Finally, hair follicles are associated with a mesenchymal cell population called the dermal papilla (DP), which is a signaling hub and regulates hair cycling (Driskell et al., 2011).

1.4.1 Telogen

The telogen hair follicle is characterized by the quiescent state of the constituent keratinocytes and it contains a surprising diversity of stem cells (Geyfman et al., 2015; Jaks et al., 2010). The upper HF, including the infundibulum, junctional zone, and sebaceous gland, is characterized by *Lrig1* and *Krt79* expression (Jensen et al., 2009; Page et al., 2013; Veniaminova et al., 2013). The isthmus area, as well as some sebaceous gland cells, show *Lgr6* expression (Füllgrabe et al., 2015; Snippert, Haegebarth, et al., 2010) with the lower isthmus forming part of a SHH-responsive *Gli1*-expressing cell niche (Brownell et al., 2011). The bulge area contains slow cycling stem cells and is characterized by CD34 and KRT15 expression (Blanpain et al., 2004; Morris et al., 2004; Trempus et al., 2003), with the lower bulge and hair germ showing expression of *Lgr5* (Jaks et al., 2008). Hair germ is further characterized by gene expression such as active Hedgehog signalling (characterized by e.g., *Gli1* expression) and members of the Wnt and Bmp signalling pathway (e.g., genes *Id1* and *Id3*) (Brownell et al., 2011; Genander et al., 2014). As a consequence, these hair germ cells can get activated in response to WNT signals, start to proliferate and can initiate transition into anagen (Greco et al., 2009). Suprabasal *Krt6*-expressing cells in the inner bulge are more differentiated, but they participate in maintaining the stem cells of the basal outer bulge (Hsu et al., 2011), by, for example, producing BMP6 and FGF18, which together with BMP4 and BMP2 secreted by

fibroblasts and subcutaneous adipocytes maintain the quiescent state of the telogen HF (Hsu et al., 2014).

1.4.2 Anagen

Initiation and progression of the hair growth phase – anagen – is a highly orchestrated process where the first two hair cycles are synchronized throughout the murine body. Anagen is further divided into six main stages covering the initiation (ANA I to III) and the maturation (ANA IV-VI) of the follicle. The full-grown anagen follicle is surrounded by the outer root sheath (ORS) and the lower proximal cup (LPC), which encompass seven concentric inner cell layers termed the companion layer, three inner root sheath layers (IRS: Henle, Huxley and IRS cuticle layers), and the three hair shaft-forming layers (cuticle, cortex and medulla) (Fuchs, 2007).

1.4.2.1 Anagen I-III

Anagen initiation begins with a gradual downregulation of BMP4 and BMP2 in late telogen and a concomitant activation of the BMP inhibitor Noggin, as well as FGF7, FGF10 and TGFβ2 signals transmitted from the DP to the hair germ (Hsu et al., 2014). Wnt signalling is also critical for anagen initiation and causes the accumulation of β-catenin in the hair germ (Greco et al., 2009). Once the activatory signals overcome the inhibitory signals, the first anagen stage (ANA I) is induced. ANA I progresses with the proliferation of hair germ cells and the formation of a matrix of rapidly proliferating transit-amplifying cells (TACs), which eventually differentiate into the seven concentric inner layers (Hsu et al., 2014). ORS and the LPC, on the other hand, are formed from outer bulge cells that get activated by SHH secreted from TACs (Hsu et al., 2014). The rapid proliferation of TACs continues in ANA II and leads to an elongation of the growing HF deeper into the dermis. Additionally, the DP starts to be gradually enveloped by the hair germ cells, which form the characteristic anagen bulb (**Figure 3**) (Müller-Röver et al., 2001). First signs of inner layer differentiation become apparent in the beginning of ANA IIIa, when TACs surrounding the DP start to differentiate into IRS cells (Müller-Röver et al., 2001). Signs of the developing hair shaft become visible in ANA IIIb and the HF reaches its full length in ANA IIIc, although the growing hair shaft has not yet reached the hair canal. At this point, the HF bulb resides close to the PCM in the subcutis, the elongated DP is fully enveloped within the bulb and the matrix gives rise to all the differentiated inner cell layers (Müller-Röver et al., 2001).

1.4.2.2 Anagen IV-VI

The fully developed anagen stages ANA IV-VI are characterized by rapid proliferation and differentiation of cells in the cortex, cuticle and medulla, leading to elongation of the hair shaft which finally enters the hair canal and emerges out of the skin (Müller-Röver et al., 2001). Differentiation of the matrix cells into their specific lineages relies on an interplay of different signalling pathways and transcription factors. Main signalling pathways characterizing this process are the Wnt pathway, which is active in the hair shaft, and the Bmp pathway together with ID proteins, which are active in the IRS

(Genander et al., 2014). A more detailed analysis of the transcriptomic changes during lineage specification identified gradual marker and transcription factor gene expression changes in three inner lineages – the IRS, cortex/cuticle, and the medulla (Joost et al., 2020). IRS is characterized by the expression of keratins *Krt71*, *Krt72*, *Krt73* as well as *Ctsc* and transcription factors *Pou3f1*, *Gata3*, *Maf*, *Mafb* and *Cux1* (Ellis, 2001; Joost et al., 2020; Kaufman et al., 2003; Langbein et al., 2010; Miyai et al., 2010). Cortex and cuticle layers have a characteristic *Krt35*, *Krt31* and *Krt36* keratin expression, as well as expression of *Selenbp1* and transcription factors *Cited4* and *Hoxc13* (Bazzi et al., 2009; Joost et al., 2020; Langbein et al., 2010). Finally, medulla cells have an increased expression of *Krt75*, *Csta1*, *Aldh1a3* and the transcription factors *Foxp1* and *Foxq1* (Joost et al., 2020; Kurimoto, 2006; Potter et al., 2006).

1.4.3 Catagen

The third stage of the hair cycle, catagen, is characterized by the regression of the anagen HF back to the telogen stage. Catagen is thought to be initialized and modulated, at least partly, through TGFβ from the DP, as ablating the DP inhibits catagen entry (Mesa et al., 2015). Elimination of cells takes place by two simultaneous mechanisms – basal cells undergo apoptosis and phagocytosis by neighbouring keratinocytes, while the inner layer cells undergo terminal differentiation (Mesa et al., 2015). Interestingly, some ORS cells which underwent limited cell divisions and are still near to the bulge region have been found to not undergo apoptosis, but to rather differentiate into *Krt6*-expressing inner bulge cells (Hsu et al., 2011). Gradual loss of cells causes the anagen bulb to become thinner until finally the hair follicle is back to its original size. The new secondary germ houses the HF stem cells that can initiate the next hair cycle and the hair shaft is retained as a club hair (Hsu et al., 2014; Müller-Röver et al., 2001).

1.5 Skin development

1.5.1 Interfollicular epidermis

Epidermal development starts at around E8.5 when Wnt signals induce surface ectoderm to adopt an epidermal fate. These early keratinocyte progenitors start expressing p63, which in turn induces expression of stemness markers Krt5 and Krt14. During the subsequent days, the epidermis forms a second layer, called periderm, which stays in contact with the amniotic fluid and prevents pathological adhesions between immature surface epithelia during development (Richardson et al., 2014). Next, at E14.5 p63 and Notch signalling promote the formation of an intermediate cell layer that starts to express the differentiation marker Krt1 and matures into spinous cells. At E16.5, continued differentiation due to increase in extracellular Ca^{2+} concentration results in the formation of the granular layer and the epidermal barrier becomes complete at E18.5 with the formation of the cornified layers and disappearance of the periderm (Reviewed in: Koster & Roop, 2007).

1.5.2 Hair follicles

Hair follicle development occurs in waves concomitant to epidermal development. The first wave that gives rise to guard hairs starts at E14.5, followed by a second wave at E16.5 that results in awl and auchene hairs, and a third wave at E18.5 that leads to the establishment of zig-zag hairs, which make up for the vast majority of the adult mouse fur. Hair development starts with an interplay between the hair follicle placode (an epidermal thickening) and the dermal condensate (a condensation of dermal papilla progenitors on the stromal side), and involves several signalling pathways such as Wnt, Eda, Fgf, Hedgehog, Tgfb, and Bmp. Subsequently, the newly formed hair follicles enter the morphogenesis phase, which resembles the anagen stage of mature hair follicles and gives rise to all the different anagen hair follicle layers. First hair shafts emerge around postnatal day P5 and the first hair growth phase (anagen) ends around P13-15. (Reviewed in: Biggs & Mikkola, 2014; Sennett & Rendl, 2012)

1.6 Comparison of mouse body sites

Studies on mouse skin biology are usually performed in either dorsal, ear, paw or tail skin, depending on the research question and utilized methods. The main differences between these body sites lie in the epidermal thickness and composition, the existence of adnexal structures (e.g., hair follicles), differences in developmental fibroblast origin, and the ease of access for different methodologies.

Dorsal skin is one of the most widely used body sites, covering a large area of the murine body, and including all the main cell types and hair follicle types (guard, awl/auchene, and zig-zag) (Sundberg et al., 2005). It is best used for studies requiring a lot of material (tissue or cells) (Joost et al., 2020) and perturbations affecting a large area (e.g., tissue expansion or big wounds) (Aragona et al., 2020; Ito et al., 2007).

Ear skin has gained popularity with *intra vital* imaging due to its ease of access for imaging, an area can be revisited over several days or even weeks without harming the mouse. Furthermore, ears are not affected by breathing motions, the tissue is relatively thin and hair follicles grow at a steep angle, facilitating high-quality imaging (Pineda et al., 2015).

Paw skin in general lacks hair follicles and the epidermis is thicker and more keratinized than in other body sites. It can be subdivided into two regions, the plantar paw and foot pads, of which the latter is the only body site in mice with eccrine sweat glands. Similarly to ear skin, studies in paws have gained popularity with *intra vital* imaging, as hind paws are also readily accessible and the lack of hair follicles benefits studies on dermal cell types and structures (Hung & Williams, 2014).

Tail skin shows a characteristic patterning with two distinct epidermal regions. Hair follicles are located in groups of three in the so-called interscale regions. These regions show similar IFE characteristics as other body sites (i.e., orthokeratotic differentiation). Scale regions, making up the rest of the tail epidermis, follow a parakeratotic

differentiation program instead, where the granular layer is lacking and cell nuclei are retained in the cornified layer (Gomez et al., 2013).

Paper I utilized analysis of both dorsal and ear skin, with scRNA-seq performed with dorsal skin, intra-vital imaging performed in ear skin, and smFISH validation done on both ear and dorsal skin. **Paper II** used dorsal skin acquired at different hair growth cycles in adult mice, and **Paper III** used embryonic dorsal skin.

1.7 Comparison of mouse and human skin

Mice are widely used in biomedical research as model organisms because of their ease of use (fast reproduction, small size) and availability of countless disease models and reporter/tracing mouse lines. While the broad skin anatomy of mice and humans is comparable, there are several differences that should be acknowledged.

Mouse epidermis is thinner than human epidermis and consists generally of 2-3 layers (<25 µm), while tail and paw skin can show more layers (Gudjonsson et al., 2007). Human epidermis is generally around 10 layers thick (~70 µm). The thickness varies between body sites and it decreases with age (Lintzeri et al., 2022). Moreover, human skin also exhibits undulations, called Rete ridges, which enhance mechanical strength of human skin and they include suprabasal proliferating cells (Petrovic et al., 2018; Shen et al., 2023), as well as substantial suprabasal population of cells expressing both stem and differentiation markers (Cohen et al., 2022; Waseem et al., 1999).

Human hair follicles cycle similarly to murine hair follicles; i.e. they both go through cycles of anagen, catagen, and telogen. However, human HFs shed the hair shaft after telogen, in a stage called exogen, while mice retain the grown hairs throughout subsequent cycles. The typical length of the murine hair cycle is measured in weeks, while human hairs can stay in anagen for several years. Also, human hair can be separated into unpigmented vellus hair that covers most of the body although at a rather lower density, and pigmented terminal hair. (Wong et al., 2011; Zomer & Trentin, 2018)

Apart from the differences in the aforementioned structures, human skin is rich in sweat glands, and it generally lacks the PCM that covers the entire mouse trunk (Wong et al., 2011). Furthermore, there are notable differences in melanocyte and immune cell populations. Melanocytes are present in the basal layer of both the IFE and the HF of human skin and thus provide pigment for the skin and hair, while mouse melanocytes are mostly associated with hair follicles, where they produce pigment for the growing hair (J. Y. Lin & Fisher, 2007). Finally, human epidermis lacks the resident γδ T cell population (DETCs)), which in mouse skin is critical for epidermal homeostasis (Rahmani et al., 2020).

2 Research aims

The fate of epidermal cells during tissue homeostasis and development has been an area of intense research for a long time, but many open questions remain. The overarching aim of this thesis is to study epidermal stem cells at the point of no return i.e., finding the transcriptional and behavioural points when epidermal stem cells commit to specific terminal fates.

Paper I analyzes adult epidermal stem cells during homeostasis and injury, and asks five key questions:

- When do epidermal stem cells commit to differentiation?
- What happens to committed epidermal stem cells immediately after crossing the point of no return?
- How is basal cell delamination facilitated at the behavioural and molecular level?
- How does the identified committed K10+ basal cell population affect tissue homeostasis?
- Are these K10+ committed cells necessary for regeneration?

Paper II focuses on understanding what happens in the skin during active hair growth. Key questions addressed are:

- What is the single-cell transcriptional landscape of full-thickness skin?
- Which cell populations are involved in active hair growth and how can they be transcriptionally characterized?
- How are the hair follicle progenitor cells differentiating into the different hair follicle lineages?
- How does the hair cycle affect the stromal remodelling and the transcriptional identities of its cell populations?

Paper III deconstructs the developing skin when the first skin-specific structures are being established, asking the following questions:

- When is fibroblast heterogeneity established in embryonic skin?
- When and how are embryonic fibroblasts differentiating into functionally distinct lineages?
- What is the transcriptional landscape of hair follicle formation?
- What happens when a single-layered epidermis turns into a multilayered epidermis?

3 Materials and methods

3.1 Single-cell transcriptomics

To understand how a tissue maintains and restores itself, it is necessary to characterize all its cells and how they work in concert. While all cells in an organism share a common DNA sequence, they obtain specific phenotypes by regulating, for example, their gene and protein expression, as well as undergo post-transcriptional modifications. Single-cell RNA-sequencing (scRNA-seq) was the first method that enabled comprehensive tissue analysis at the single-cell level. Analysing the mRNA levels in individual cells made it possible to group similar cells together into clusters, which enabled unbiased cell-type characterisation and with it, new cell-type discovery. Furthermore, transcriptomics data can then be analysed for cell-type specific marker gene expression, developmental trajectories or interactions with other cells. Since its first application in 2009 (Tang et al., 2009), single cell RNA-sequencing has become an essential tool for characterizing tissues in homeostasis (Cao et al., 2017; Chen et al., 2018; Han et al., 2018; Joost et al., 2016, 2020), during wound repair (Guerrero-Juarez et al., 2019; Joost et al., 2018), as well as for different diseases, including cancers (e.g., Cheng et al., 2018; Suvà & Tirosh, 2019).

Broadly, scRNA-seq starts with cell lysis followed by reverse transcription, second strand synthesis, cDNA amplification, library preparation and finally sequencing. However, the major challenge for single-cell approaches is the low amount (10-30pg) of RNA present in each cell. Thus, it is of crucial importance to retain as much starting material as possible and to ensure a very sensitive and reproducible library preparation. After tissue has been dissociated, individual cells are isolated from the cell suspension and captured into a reaction space. The main options for this are droplet-based methods (Klein et al., 2015; Macosko et al., 2015; Zheng et al., 2017), split-pool methods with combinatorial barcoding (Cao et al., 2017; Rosenberg et al., 2018) and plate based methods with cell sorting (Gierahn et al., 2017; Hagemann-Jensen et al., 2022; Han et al., 2018). Next, cDNA library preparations start with capturing mRNA with oligo-dT primers binding their poly-A tails and performing reverse transcription. This step also includes addition of PCR primer sites, sequencing adapters and unique molecular identifiers (UMIs) to the cDNA, utilizing either template-switching oligos or *in vitro* transcription, dependent on the exact protocol used. Notably, addition of UMIs enables to uniquely tag each starting cDNA to counteract any bias introduced during PCR amplification. Although library preparation is commonly done with full length cDNA, sequencing is most often performed for either the 5´or 3´end to further reduce costs while retaining information about transcript abundance. However, full-length sequencing can achieve better transcriptome coverage for identification of lowly expressed genes, isoforms or SNPs (Hagemann-Jensen et al., 2022). Finally, the cDNA libraries are sequenced using a range of deep sequencing platforms (Reviewed in: Jovic et al., 2022; Tanay & Regev, 2017).

Analysis of scRNA-seq data has undergone a tremendous leap over the last decade, where the first datasets were analysed using the same methods that were established

for bulk RNA-sequencing to currently having more than a thousand specialized computational methods and packages available (Zappia & Theis, 2021). Briefly, once the generated reads are mapped to the genome and annotated, the data need to be normalized and corrected to account for technical variability, before continuing with dimensionality reduction, clustering, differential gene expression analysis, visualization, and more specific downstream analysis. However, there are a few important aspects to keep in mind. For example, it is important to include several biological replicates, whenever possible, especially in the case of analyzing rare cell types. Presenting data from a single sample can hide biological variation behind technical noise, which can happen easily and is very difficult to remove without proper controls. While these replicates can result in technical noise, it can be corrected for with powerful batch correction and integration methods, but care has to be taken so as not to remove true biological variation in the dataset (Tran et al., 2020). Before the data is further analyzed, one should also make sure to remove any doublets, including those formed by attachment of only cellular components (e.g., dendritic cell dendrites) to the sequenced cells, distorting their transcriptomes (Reviewed in: Jovic et al., 2022; Luecken & Theis, 2019).

In all three papers presented in this thesis scRNA-seq has been used. **Paper I** included isolation and sorting of basal epidermal cells which were sequenced with the Fluidigm C1 technology, and then integrated with published datasets. Sequencing in **Paper II** and **Paper III** was performed using the 10X Chromium technology on unsorted cells that were isolated from adult or embryonic full-thickness skin, respectively.

3.2 Intra-vital imaging

Sequencing methods provide only a snapshot of cellular identities, without the ability to study the same cells at later timepoints. Moreover, to understand the connections between transcriptional states and cell behaviour, it is important to study the tissue without major perturbations. *Intra vital* imaging is a powerful method that fulfils these criteria. It allows for the visualization of individual cells over several hours or days without adverse effects on the cells or the tissue. This is achieved by focusing two (or even three) near infra-red (>680nm) photons, which can penetrate deep into the tissue without photodamage, onto a single focal point, where their combined energy can excite a fluorophore usually requiring shorter wavelength lasers (e.g, 960nm two-photon laser can excite GFP that has an excitation peak at around 480nm). Imaging deep within the tissue still requires highly sensitive detectors, as the emitted light from the excited fluorophores is of lower wavelength and has reduced tissue penetrance. Two-photon systems provide an additional benefit called second harmonic generation, where two photons combine into a single higher-energy photon to interact with highly ordered multimers, producing light of half the original wavelength. This effect enables label-free visualization of, for example, collagen in the dermis (Reviewed in: Larson, 2011; Pineda et al., 2015).

Multiphoton imaging techniques can be used to image tissues at or close to the body surface (e.g., skin or cornea) (Farrelly et al., 2021; Pineda et al., 2015) and with the help of surgical techniques, it can also be used for imaging of internal organs and structures (e.g., intestines or developing embryos) (Q. Huang et al., 2020; Jang et al., 2018). Furthermore, these imaging approaches can also be readily combined with different genetic mouse lines, such as reporter and lineage-tracing lines with inducible fluorophore expression (Farrelly et al., 2019). Additionally, photoactivatable fluorophores can be employed to mark and track single cells or cell groups that are otherwise difficult to target (Xin et al., 2018). Finally, due to the phototoxicity of high-energy lasers, it is possible to use two-photon systems to focus on and ablate specific structures to study tissue regeneration (Rompolas et al., 2012).

Paper I utilized two-photon microscopy to visualize and track cells expressing *Krt10* over several days in a reporter mouse line where *Krt10* expression is coupled with H2BGFP expression.

4 Results

4.1 Paper I: Gradual differentiation uncoupled from cell cycle exit generates heterogeneity in the epidermal stem cell layer

This paper focuses on understanding the transcriptional and behavioural aspects of epidermal stem cells when they commit to differentiation. We addressed these questions by leveraging two orthogonal methods: scRNA-seq of epidermal keratinocytes and *intra vital* imaging of keratinocyte behaviour over several days.

In the transcriptional analysis, we first sorted and sequenced basal IFE keratinocytes (Live/ITGA6^{+}/SCA1^{+}/CD34^{-}) and subsequently integrated their transcriptomes with our previously described IFE differentiation timeline (Joost et al., 2016, 2020). This allowed us to assign a transcriptional basal-suprabasal border according to the sorted cells, revealing that the cells which were previously assigned an intermediate (*Krt10*^{dim}/*Ptgs1*^{dim}/*Mt4*$^{+}$) or even mature differentiation identity (*Krt10*^{high}/*Ptgs1*^{high}) are still part of the basal layer. Notably, about 40% of all basal cells showed expression of *Krt10* while retaining the expression of basal markers (e.g., *Krt14*). This surprisingly high number of *Krt10*-expressing basal cells was confirmed with smFISH stainings, where *Krt10* mRNA did indeed show expression in approximately 40% of the basal dorsal and ear IFE, respectively. Finally, investigating how gene expression changes during the differentiation, we found that *Krt10* was the first gene to show upregulation during the initiation of differentiation, followed closely by other differentiation-associated genes, such as *Krt1* and *Krtdap*.

Using *intra vital* imaging of *Krt10*-H2BGFP reporter mice (*K10rtTA/pTRE-H2BGFP*; GFP expression in *Krt10*-expressing cells) allowed us to study cells' behaviour when they activate *Krt10* expression and to track the fate of these cells. First, we found that the majority of *Krt10*-expressing cells delaminated from the basal layer within 5 days of tracking and none of the *Krt10*-expressing cells lost its *Krt10* expression to return to a more basal state. Thus, *Krt10*-expression is a marker for a basal cell that has committed to differentiation and will eventually delaminate. However, during the early stages of commitment, these differentiating cells are morphologically indistinguishable from their neighbouring uncommitted cells, showing a similarly high basement membrane footprint that starts to shrink only 1-2 days before completing delamination from the basal layer. Moreover, we saw that about 15-25% of these committed cells still divided at least once before delamination, with some cells even going through a second round of cell division prior to final delamination. These divisions were invariably symmetric, with all daughter cells retaining *Krt10* expression and delaminating within short. Moreover, we confirmed that these division events occurred as a response to a neighbouring cell delaminating, rather than being a cell-intrinsic effect.

Using a genetic model to inhibit proliferation in *Krt10*-expressing cells (*K10rtTA; pTRE-Cdkn1b; pTRE-H2BGFP*), we found that the division events of *Krt10*$^{+}$ cells were not necessary for maintaining tissue homeostasis. Epidermal thickness, number of

delamination events, basal layer cell density and total number of cell divisions were not significantly changed due to neighbouring basal cells compensating for the lack of *Krt10*$^{+}$ divisions. However, upon injury with tape stripping, tissue with inhibited *Krt10*$^{+}$ cell divisions was slower to recover, indicating that these cell divisions are not obligatory for tissue homeostasis, however they provide a pool of cells that can be mobilized upon injury for quicker recovery.

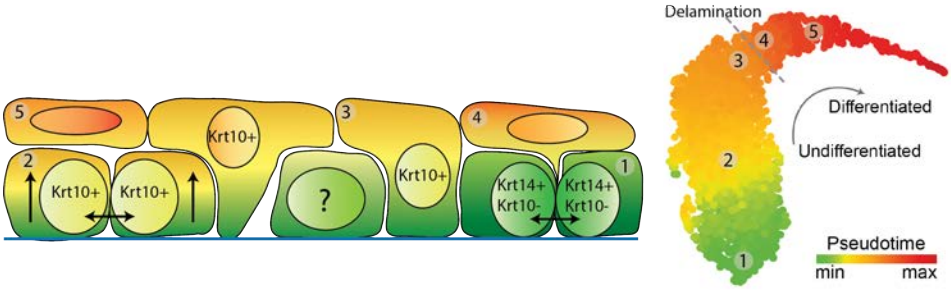

Figure 5. Schematic representation of results acquired in Paper I. Shown are epidermal cells with different behaviours and their corresponding cell populations on the differentiation pseudotime.

Taken together, the results of **Paper I** uncover for the first time the transcriptional state of cells at the point of no return (i.e. when they commit to differentiation) and when they delaminate (**Figure 5**). Moreover, this paper paints a detailed picture of how differentiation and exit from cell cycle are disconnected events in mouse skin, with differentiation-committed cells retaining the non-obligatory ability to divide dependent on local tissue needs.

4.2 Paper II: The molecular anatomy of mouse skin during hair growth and rest

This study generated the first full-thickness scRNA-seq atlas of mouse skin, covering both the telogen (9 weeks old) and anagen (5 weeks old) stage of the hair cycle. Thanks to unbiased cell capturing, we were able to robustly identify 56 cell populations covering almost all major cell types in murine skin: keratinocytes (IFE, permanent HF and cycling HF), fibroblasts and fibroblast-like cells, immune cells (lymphocytes, myeloid cells), vascular cells (endothelial and lymphatic), skeletal muscle cells, and red blood cells.

As keratinocytes from the anagen stage are rapidly proliferating and differentiating into a multitude of specific lineages, we first focused on understanding the identities and dynamics of these anagen hair follicle cells. To our surprise, outer layer cells divided transcriptionally into two separate groups, basal and suprabasal outer layer cells, with the companion layer cells clustering together with the suprabasal ORS cells, rather than forming a part of the inner lineages. Basal outer layer cells include two basal ORS populations and lower proximal cup cells, and show more receptor-ligand interactions with stromal cells compared to suprabasal outer layer cells.

The inner lineages encompass the proliferative germinative layer (GL) cells, also called matrix cells, the IRS, cortex/cuticle, and medulla cells (**Figure 6**). RNA-velocity analysis

suggested that the GL cells are not yet transcriptionally committed but that they still have the potential to differentiate into all the terminal lineages. However, their differentiation potential gets more restricted the closer these GL cells get transcriptionally to the medulla lineage. Complete commitment to a specific lineage occurs through a transit-amplifying cell (TAC) state, where GL cells upregulate lineage-specific gene expression and detach from the basement membrane, while still undergoing cell division before finally assuming the terminal differentiated fate.

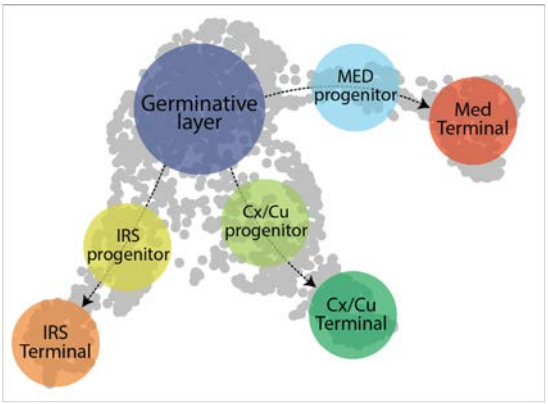

Figure 6. Schematic of anagen inner layer lineage differentiation from GL cells towards each of the terminal endpoints. Lineage identities are projected into UMAP presented in Paper II (Fig. 4a) (adapted from Joost 2019)

In parallel to the epidermal compartment, the stroma also undergoes major changes between telogen and anagen. Thus, we next focused on understanding the changes happening within the fibroblast populations during the hair cycle. We identified four main fibroblast clusters as well as four hair follicle-associated fibroblast-like cell populations (dermal sheath and dermal papilla cells from anagen or telogen stage). Three of the fibroblast clusters showed spatial separation and could be mapped to the dermis, hypodermis, and adventitia/fascia, respectively. The fourth fibroblast cluster was specific to the anagen stage and reflects how dermal fibroblasts change their transcriptional state in response to the hair cycle. Anagen fibroblasts showed upregulation of several genes involved in ECM remodelling and deposition (*Sparc*, *Col1a1*) and energy metabolism (*Ndufs4*), while gene expression of telogen fibroblasts was dominated by proteoglycan genes (*Dcn*, *Lum*, *Mfap4*).

Overall, **Paper II** resulted in a comprehensive atlas of full-thickness adult mouse skin, together with an accompanying online tool to make the gene expression profiles available for everyone.

4.3 Paper III: Molecular and spatial landmarks of early skin development

The aim of this study was to characterize the early embryonic development of mouse skin. We performed scRNA-seq at three consecutive embryonic timepoints (E12.5, E13.4,

and E14.5), allowing us to resolve the earliest steps in the development of several important epidermal and dermal compartments and structures. Histological samples taken from the same timepoints made it possible to define anatomical landmarks and to validate our findings *in situ*.

Overall, we captured >30,000 high quality transcriptomes covering all major cell types including fibroblasts, epidermal cells, immune cells, muscle cells, vessel-associated cells, and neural crest-derived cells. Fibroblasts constituted the majority of cells reflective of the fact that they are the most abundant cell type at the selected embryonic periods.

We wanted to understand how heterogeneous the developing fibroblasts are and if they contain progenitors for other mesenchymal cell types such as e.g., adipocytes. Early fibroblasts from E12.5 skin already separated into two main groups that we termed *FIB Origin* and *FIB Deep*, with *FIB Origin* cells residing close to the epidermis. In-depth analysis of *FIB Origin* cells revealed how they contribute and differentiate into several lineages found at later timepoints, including muscle-associated fibroblasts (*FIB Muscle*), fibroblasts located between the muscle layers (*FIB Inter*), lower dermal fibroblasts (*FIB Lower*) and the upper dermal fibroblasts (*FIB Upper*). (**Figure 7**)

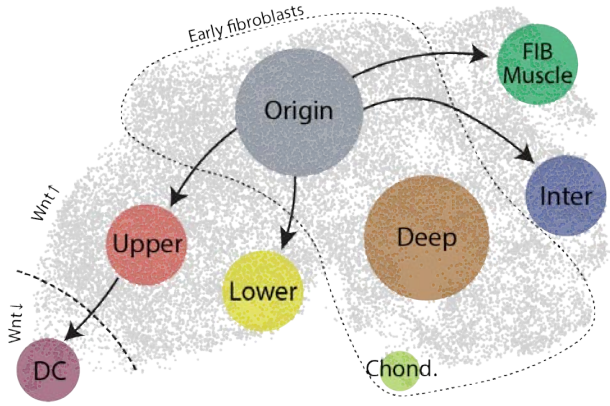

Figure 7. Main fibroblast subpopulations and the likely differentiation trajectories of FIB Origin cells. Early fibroblasts correspond to cells sequenced from E12.5. Wnt↑ and Wnt↓ denote areas with active or inhibited Wnt signaling, respectively. Lineages are projected onto UMAP presented in Paper III (Fig 2). Chond.: chondrocytes. (Adapted from: Jacob 2021).

Based on our smFISH mapping, *FIB Upper* cells locate underneath the epidermis, where they feed into the forming dermal condensates (DC) and support the formation of hair placodes and hair morphogenesis. The switch to a DC cell identity comes with a marked down-regulation of Wnt pathway genes (*Dkk2*, *Dkk1*) and a concomitant increase in Wnt-inhibitors (*Notum*, *Cav1*). Notably, as fibroblasts commit to the DC fate, they also exit the cell cycle (upregulate *Cdkn1a* and *Btg1*). Another interesting fibroblast differentiation trajectory is the establishment of muscle-associated fibroblasts (*FIB Muscle*). First signs of the *FIB Muscle* cell populations in transcriptomic data as well as *in situ* can be seen at E13.5. In the tissue, *Nppc*-expressing *FIB MUSCLE* cells can be found

interspersed with *Pax7*-expressing muscle progenitor cells. smFISH for these early muscle cells also provided novel evidence for the emergence of the PCM layer.

Epidermal cells show heterogeneity already at E12.5, with two distinct subpopulations *EPI Basal1* and *EPI BasalTagln*. Transcriptional analysis indicates that *EPI BasalTagln* cells might be involved in extensive communication with other cell types, however, their exact function remains unknown. Intriguingly, *EPI BasalTagln* cells also express genes commonly associated with smooth muscle cells (*Tagln*, *Myl9)*. *EPI Basal1* cells seem to be the progenitors for the later basal IFE populations that show a more homogeneous transcriptional profile. Basal IFE cells are highly proliferative and go on to differentiate and delaminate resulting in epidermal stratification at around E13.5. This differentiation process is driven by *Notch1, Cdh1* and *Ghrl3*, markers known for their involvement in epidermal differentiation. Finally, epidermal cells also included a population showing signs of early hair placode formation already at E13.5 (*EPI EarlyPlacode*). These early placode cells express markers such as *Fgf20* and *Dkk4*, while the more mature placodes that become morphologically visible at E14.5 *(EPI LatePlacode)* also start expressing more mature placode markers such as *Shh*. Interestingly, placode cells do not seem to downregulate any specific genes, but rather upregulate several markers in addition to the baseline transcriptional program. Finally, epidermal cells also included a substantial *EPI Periderm* cell population that persisted throughout the experimental timepoints. This protective layer is signalling-rich, matures independently but shares several markers with the differentiating IFE (*Krtdap*, *Dkkl1*). Surprisingly, it also expresses multiple genes better known from HF formation (*Sox9*, *Krt8*) and differentiation (*Foxq1*, *Krt4, Tchh*).

In conclusion, **Paper III** gives a comprehensive overview of early skin development, uncovering previously unknown cellular heterogeneity and describing the emergence and fate of important skin structures.

5 Discussion

5.1 Crossing the point of no return

In **Paper I** we are describing how *Krt10* expression is a marker for epidermal cells that have committed to differentiation. To our best knowledge, keratins have not been shown to directly influence gene expression, so it is highly likely that KRT10 is just a marker and not the cause that pushes cells across the point of no return. While we know that e.g., Notch, Wnt and Yap-Taz pathways are involved in regulation and IFE keratinocyte differentiation (Aragona et al., 2020; Hu et al., 2010; Lim & Nusse, 2013), the ultimate cause for the cell's "decision" to commit remains elusive. Broadly, we could classify the possible causes for commitment into three categories: i) stochastic changes in gene expression, ii) cell-extrinsic effects, or iii) cell-intrinsic effects.

Stochastic commitment to differentiation would mean that every basal cell has a random chance to initiate gene expression that starts a cascade leading to differentiation commitment (*Krt10* expression). Basal cells could exist in a dynamic equilibrium, with constant fluctuations in transcription factors, non-coding RNAs, the chromatin landscape and other elements combining into gene regulatory networks (Semrau & Van Oudenaarden, 2015). Thus, a temporary differentiation signal, for example a long-range pulse of Ca^{2+} signaling in the basal layer (Moore et al., 2023; Yuspa et al., 1989), could be sufficient to push an otherwise transiently differentiation-prone cell over the point of no return, without the explicit need from the surrounding tissue for immediate differentiation. This could explain why we see such a high number (≈40%) of epidermal cells with *Krt10*-expression, even though many of those cells remain morphologically indistinguishable from neighboring *Krt10*$^{-}$ basal cells and still contribute to epidermal homeostasis by retaining the ability to undergo mitosis. In this case, they could be considered as sentinels waiting for their chance to continue their differentiation journey by delaminating in order to maintain the suprabasal layers. However, as there is constant demand on tissue stem cells to supply new progeny even under homeostatic conditions, it is conceivable that one mechanism alone would not be enough. Thus, it is highly likely that skin integrity is maintained by the interaction of several mechanisms, including cell-intrinsic and cell-extrinsic factors.

Cell-extrinsic factors resulting in commitment to differentiation could include cues coming from the niche environment, such as neighboring cells, extracellular matrix composition or mechanical forces. For example, the influence of mechanical forces on keratinocyte behavior is supported by various works. Studies on embryonic and early postnatal epidermis have shown how differentiation and subsequent delamination is greatly influenced by the crowding effect, where decreased cortical tension and increased cell-cell adhesion initiate differentiation and subsequent delamination (Miroshnikova et al., 2018). Similarly, injecting saline under skin is an established method in reconstructive surgery to induce cell proliferation and produce more skin for crafting (Zöllner et al., 2013). Investigations in a comparable mouse model have shown how this expansion leads directly to increased proliferation and differentiation in order to

maintain tissue integrity (Aragona et al., 2020). However, in adult homeostasis, cell proliferation occurs as a response to a delamination event (Mesa et al., 2018), suggesting that when a delaminating cell is "pulling" its neighbors along while leaving the basal layer, it increases the forces acting on basal cells. This would in turn induce mitosis of a neighboring cell so that the daughter cells can fill the gap and relieve the local tension.

Another mechanism to mediate the differentiation could be via signaling crosstalk with other cell types, such as immune cells that monitor the suprabasal layers and sense if it needs to be replenished from the basal layers (Heath & Carbone, 2013; Malissen et al., 2014). For example, the presence of dendritic epidermal T-cells (DETCs), which are located in the basal layer of epidermis, have a clear influence on epidermal integrity (Dalessandri et al., 2016). Whether DETCs directly signal to stem cells and instruct their behavior is still unresolved, yet it is conceivable that DETCs are involved in sensing and mediating changes e.g. in trans-epidermal stiffness (Chakraborty et al., 2021) due to loss of suprabasal cells, prompting a committed basal cell to delaminate. While these observations are highly interesting, they do not fully explain why so many basal epidermal cells have already committed to differentiation but rather implicate the role of DETCs in influencing the delamination decision (Dalessandri et al. manuscript in preparation).

Lastly, differentiation could also be induced by cell-intrinsic signals, i.e., cells might commit to differentiation as a way of removing damaged or old cells from the basal stem cell pool and thus maintaining a healthy epidermis. Skin is our main protection against UV-radiation, which increases the chance that epidermal keratinocytes accumulate oncogenic mutations, making it plausible that commitment to differentiation is a way for the tissue to protect itself from these mutations. For example, a study looking at the somatic mutation rate in tissues with quiescent stem cells (liver, lung) and in tissues with more proliferative stem cells (skin, intestine) found them to be similar, i.e., despite being exposed to harmful radiation, skin didn't accumulate more mutations (Ren et al., 2022). Additionally, recent findings are describing how organelle localization and segregation can influence or initiate cell division and subsequent fate choice of the daughter cells. Peroxisomes, for example, have been shown to take on specific locations during keratinocyte division and preventing this localization causes delayed mitosis barrier defects (Asare et al., 2017). Moreover, old and young mitochondria have been shown to be asymmetrically segregated during cell division, where the daughter cell receiving old mitochondria inherit more oxidative energy metabolism, leading to differentiation, while new mitochondria promote purine biosynthesis and redox balance, helping to maintaining stemness (Döhla et al., 2022; Katajisto et al., 2015). However, it remains to be seen if similar effects exist in epidermal stem cells and to what extent they influence cells' fate decisions and commitment to differentiation.

5.2 Models of epidermal differentiation

Over the last decades of intense research, two predominant seemingly opposing models of epidermal stem cell behavior have manifested – the hierarchical model and the neutral competition model (see Introduction). In the light of our new results presented in **Paper I**, it is more tempting to think of these stem cell populations as different states of the same uncommitted basal cell type. Uncommitted (*Krt10*-negative) basal cells are in a transcriptionally dynamic equilibrium state, changing their gene expression as a response to a multitude of different factors, such as signals or influences from nerves (S. Huang et al., 2021), immune cells (Dalessandri et al., 2016), neighboring keratinocytes (Mesa et al., 2018; Moore et al., 2023), mechanical force (Aragona et al., 2020; Miroshnikova et al., 2018), stiffness of the underlying ECM (Ichijo et al., 2022), or even proximity to a hair follicle opening (Roy et al., 2016). Thus, analyzing static snapshots of the tissue with lineage tracing or scRNA-seq studies could reveal cells in slightly different transcriptional states along the epidermal differentiation timeline within the uncommitted population. Dependent on their specific state, they may be more or less prone to undergo cell division, respond to injury, or commit to differentiation, yet they would still share the same core transcriptional profile and shouldn't be considered distinct stem cell types (Arendt et al., 2016; Xia & Yanai, 2019).

5.3 Anagen hair follicle lineage commitment

In **Paper II** we described in detail the transcriptional identities of the different cell layers in the anagen hair follicle, including how the germinative layer (GL) cells branch into the inner hair-producing lineages. These cells have been shown to preferentially differentiate into certain lineages, dependent on their position along the dermal papilla (DP) axis. Earlier stages (sitting at the "beginning" of the DP) rather take on a companion layer identity (Mesler et al., 2017) while GL cells sitting at the middle to upper end of the DP axis preferentially differentiate into IRS and hair shaft lineages, respectively (Legué & Nicolas, 2005; Sequeira & Nicolas, 2012). Furthermore, it has been suggested that micro-niches along the DP, influence the fates of the GL cells immediately surrounding the DP (H. Yang et al., 2017). However, regardless of their position, the fates of GL cells do not seem to be fixed yet, because when GL cells change their position along the DP axis they can take on a new identity (Xin et al., 2018). This is also confirmed by the results of **Paper II** which indicate that GL cells are uncommitted and can still differentiate into all the inner matrix lineages. The results also suggest though that their fate can become more restricted as they move upwards along the DP and come closer to medulla cells. This implies that lineage specification of GL cells comes about as an interplay of transcriptional state and the local micro-niche, which allows GL cells to retain plasticity for rapid response to injury and tissue need, as long as they remain in contact with the basement membrane and DP.

5.4 Epidermal stratification and early fibroblast development

During embryonic and early postnatal development, the epidermis develops from a single-layered to a stratified epithelium, including all the layers that also exist in adult

skin. Notably though, early postnatal epidermis is thicker than adult epidermis. An important hallmark of epidermal development is rapid proliferation, as stem cells need to support the expansion and growth of the tissue. Recently, it has been described how this can lead to a mode of keratinocyte differentiation and delamination that is mainly governed by the crowding effect – newly divided cells start to push on their neighboring cells, inducing delamination coupled with differentiation (Miroshnikova et al., 2018). However, in **Paper III**, we notice a subset of basal keratinocytes that already express differentiation markers (*Krt10*, *Krtdap*), indicating that the mode of differentiation seen in adult epidermis might already exist during embryonic and early postnatal development (Z. Lin et al., 2020), but it doesn't become the main driver of differentiation until the tissue reaches an equilibrium during adult homeostasis after the initial growth stage.

Looking at early fibroblast development in **Paper III**, we see that progenitors for the main dermal fibroblast populations can be broadly separated by expression of *Lef1*. Most likely, *FIB Lower* cells give rise to *Lef1*$^{-}$ hypodermal fibroblasts, and *FIB Upper* cells give rise to *Lef1*$^{+}$ dermal fibroblasts. The *FIB Upper* population does show subclustering into four cell states, but the transcriptional signatures of these clusters are not enough to identify commitment to papillary and reticular fibroblasts fates, seen during later stages of development at E16.5 (Driskell et al., 2013), or even if both of these layers develop from the *FIB Upper* population.

5.5 Best practices in single-cell RNA-sequencing analysis

Ever increasing access to single-cell RNA-sequencing over the last decade has led to an explosion of available new datasets. There has also been a concomitant emergence of analysis methods and also packages, such as Scanpy for Python (Wolf et al., 2018) and Seurat for R (Satija et al., 2015), that gather different methods into an easily usable, streamlined analysis workflow. Availability of clear tutorials makes it possible for both wet and dry-lab scientists to more easily perform basic analysis of their sequencing data without the need of in-depth computational knowledge.

Despite the existence of easy to use tutorials and best practice guidelines (Luecken & Theis, 2019), there are several analysis steps that do not, and cannot, have clear guidelines. From initial normalization, nearest neighbor and primary components selection, the method of dimensionality reduction with its corresponding parameters, to more advanced methods of batch correction, data integration and trajectory analyses. All these steps require the researchers to make individual choices with only some guidance available. Thus, to avoid biased representations, it is imperative to have support for the scRNA-seq findings with orthogonal methods, such as *in situ* validation of the identified expression profiles and/or use of cell and animal models that confirm the findings.

Because of the required individual decisions on the exact methods and parameters to use during data analysis, a major issue that remains with scRNA-seq data is that different research groups tend to find slightly different cell clusters, or similar clusters are assigned a different nomenclature for cell types and cell states even within the same

tissue, both of which are a major hinderance comparison of the different findings. Fortunately, recent efforts by the Human Cell Atlas consortium (Regev et al., 2018) are moving the field towards a more unified representation of cell populations and their transcriptional identities, together with accessible online tools. For example, the first version of the integrated human lung cell atlas has integrated 46 datasets with a total of 2.2 million cells from 444 individuals (Sikkema et al., 2022) and the human skin cell atlas follows close behind (Almet et al., in press). This makes it possible to map new datasets onto the existing representation of known cell types with unified nomenclature, facilitating data exploration, and highlighting if there are any cell types or states in the new dataset that do not have a clear correspondence in the integrated atlas (e.g., newly characterized cancer cells or cell states arising under specific perturbations).

Overall, scRNA-seq has risen to being an indispensable method in the biomedical researchers' toolkit, providing insight into changes in cell populations, what processes different cells are involved in, and how they could be possibly manipulated. Thus, it is important to further improve and simplify the use of existing methods to make them accessible to a broader user group, as well as provide integrative analysis methods to gain a more unified understanding of the data with inclusion of multimodal datasets and analyses, such as ATAC-seq or spatial sequencing.

6 Conclusions

With the papers included in this thesis we have systematically characterized the transcriptional identities and behaviour of several stem cell populations in developing and adult skin, including several points where stem cells commit to different fates.

Paper I focuses on understanding the intricacies of committed basal epidermal cell behaviour. We first show how unexpectedly large this committed population is and how these cells are almost indistinguishable from their neighbouring uncommitted cells. Notably, the committed cells still undertake mitosis, but it's not an obligatory function for tissue homeostasis.

In **paper II** and **paper III**, we have systematically characterized all the major cell types in adult skin during telogen and anagen hair follicle stages, and during embryonic development at the time when the main skin structures start to be established. Together, these papers provide anatomical and transcriptional landmarks in tissue development and hair cycling. Moreover, we have made all the transcriptional signatures of the identified cell populations available via easily searchable online tools.

Altogether, these papers address skin cell fate decisions and provide tools for other researchers to use for addressing their research questions. Importantly, the findings open new avenues for future research to explore, in order to better understand epidermal stem cells at the point of no return.

7 Points of perspective

Understanding how stem cells commit to terminal fates has important implications for understanding tissue biology, regeneration, cancer development and human skin disorders. While **Paper I** provides a good overview of how committed IFE cells behave, it is still an open question of what is the underlying cause that leads to differentiation.

To address this question, an option would be to perform further cell tracking experiments focusing more specifically on the uncommitted population and potentially incorporating reporter lines for the described epidermal stem cell populations. However, as these populations most likely represent different states of uncommitted cells, it is questionable which insights can be obtained by tracking them. Rather, it would be interesting to study potential causes of differentiation such as e.g. cell divisions, mechanical forces, or organelle segregation directly.

A first major question that remains is how tightly stem cell commitment is coupled to cell division i.e., would blocking cell division of basal uncommitted cells still result in normal differentiation behaviour, at least in the short term? Secondly, there is still a lot to be learned about the mechanical forces acting on epidermal cells and how these forces influence fate decisions. While we know that forces parallel to the basement membrane influence proliferation and delamination (Aragona et al., 2020; Miroshnikova et al., 2018), very little is known about the forces acting perpendicular to the basement membrane. Modulation of the perpendicular force could more closely mimic cell shedding as it is seen in homeostatic adult tissue. Finally, organelle segregation has been shown to be an important factor in stem cell differentiation (Döhla et al., 2022), and it remains to be seen how it affects epidermal stem cells in living tissue. Coupling differentiation to inherited cell metabolomics would open a new broad and exciting research field in skin stem cell biology.

While the previous section focused on understanding fate decisions of IFE cells, similar questions remain to be answered for the behaviour of anagen hair follicle stem cells. More and more evidence highlights the plasticity of the GL cells, however there is a lack of data helping to couple transcriptional cell populations to lineages seen in the tissue. In-depth mapping of GL cells to their actual location within the anagen hair follicle and analysing their commitment status will help to better understand how hair is formed and what happens during hair follicle regeneration.

As hair cycling involves stroma remodelling and distinct transcriptional state changes of fibroblasts, it would also be interesting to understand how these changes influence fibroblasts' response to injury and wound healing. Would the fibroblasts that are in an ECM remodelling state during anagen also be more responsive to wound healing and how would this influence scar formation?

With all these potential research avenues, it will be important to use a broad combination of available research tools to gain a comprehensive understanding of cell behaviour. Performing transcriptional analysis together with *intra vital* imaging, ideally on

the same tissue, and expanding the methods to incorporate spatial sequencing, epigenetic studies, as well as metabolomics can help to finally reveal the underlying causes of commitment decisions. And finally, one of the major underlying aims for this research is to gain insight into stem cell biology in both mice and humans. Thus, we eventually aim to extrapolate the most important findings from mouse to human skin and validate the existence of the described paradigms of tissue stem cells.

8 Acknowledgements

I believe that true progress can happen only when people work together towards a shared goal. Everything presented in this work is a shared effort with help from countless people, and as the thesis draws closer to the end, it's a good time to reflect and thank everyone who has made this work possible!

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