From The DEPARTMENT OF BIOSCIENCES AND NUTRITION
Karolinska Institutet, Stockholm, Sweden

EPIDERMAL STEM CELLS IN PHYSIOLOGICAL TISSUE REGENERATION, WOUND HEALING AND CANCER

Anja Füllgrabe

Karolinska Institutet

Stockholm 2016

Published by Karolinska Institutet.
Printed by AJ E-print AB

ISBN 978-91-7676-177-9

Epidermal stem cells in physiological tissue regeneration, wound healing and cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Anja Füllgrabe

Principal Supervisor:
Dr. Maria Kasper
Karolinska Institutet
Department of Biosciences and Nutrition

Co-supervisor(s):
Dr. Viljar Jaks
Tartu University
Department of Molecular and Cell Biology

Prof. Rune Toftgård
Karolinska Institutet
Department of Biosciences and Nutrition

Opponent:
Prof. Vladimir Botchkarev
University of Bradford
Bradford School of Medical Sciences
Centre for Skin Sciences

Examination Board:
Prof. Kristian Pietras
Lunds Universitet
Department of Translational Cancer Research

Prof. Johan Ericson
Karolinska Institutet
Department of Cell and Molecular Biology

Dr. Maria Eriksson
Karolinska Institutet
Department of Biosciences and Nutrition

Though this be madness, yet there is method in ‘t.
- William Shakespeare, Hamlet

ABSTRACT

The mammalian skin is a versatile organ that protects from external harm, regulates the body temperature, and provides the touch sensation. Its epithelium, the epidermis, forms several highly regenerative structures as the hair follicle (HF), the sebaceous gland (SG), and the interfollicular epidermis (IFE). Lineage tracing experiments in mice have demonstrated that several basal cell populations in the IFE and HF have the capacity to renew the epidermis during homeostasis, and also contribute to wound healing and cancer formation. However, clear insights distinguishing the different stem cell populations and defining their exact spatio-temporal patterns of contribution were lacking.

Expression of *Lgr5* marks stem cells located in the HF bulge and hair germ. We used lineage tracing of *Lgr5*-expressing (*Lgr5*$^+$) cells in mice to investigate how wound healing affects the capacity of epidermal stem cells to initiate skin cancer. Induction of basal cell carcinoma (BCC) through activation of Hedgehog signalling in the entire basal layer confirmed that wounding strongly increases the incidence and severity of BCC-like lesions. Targeting the oncogenic mutation to *Lgr5*$^+$ cells revealed that transformed HF cells are able to leave their natural environment and establish tumours in the IFE in response to wounding. Thus, wounding activates HF stem cells to expand and migrate to unaffected tissue areas, thereby augmenting BCC development.

Since it was discovered that *Lgr6* is another epidermal stem cell marker, we set out to explore the role of *Lgr6*$^+$ stem cells during epidermal homeostasis. Detailed analysis of the lineage-tracing pattern of the *Lgr6*$^+$ cells populations in the isthmus, SG, and IFE disclosed that these resident *Lgr6*$^+$ populations independently maintain their respective compartment. The mode of tissue renewal displayed by all three *Lgr6*$^+$ cell populations was in accordance with a stochastic division of one type of progenitor cell. These results highlight that stochastic stem cell renewal is relevant in many types of rapidly proliferating epithelia.

We further investigated the susceptibility of the different epidermal compartments harbouring *Lgr6*$^+$ stem cells towards BCC initiation and evaluated the influence of the microenvironment on tumour formation. Knockout of *Ptch1* in *Lgr6*$^+$ cells resulted in highly accelerated BCC development within the HF isthmus and the touch dome niches in the IFE. The touch dome and the isthmus are both associated with cutaneous nerve fibres, and show several morphological and molecular features that are highly similar to BCC. This demonstrates that these two niches promote the response of the epithelial cells to the oncogenic stimulus.

In summary, tracking the fate of *Lgr5*- and *Lgr6*-expressing epidermal stem cells during homeostasis, wound healing and early cancer formation shed light on the similarities and differences between distinct stem cell populations in the skin. The results illustrate how stem cell renewal is achieved, elucidate the early steps of skin cancer development, and underline the influence of the microenvironment on the behaviour of tissue stem cells.

LIST OF SCIENTIFIC PAPERS

I. Maria Kasper, Viljar Jaks, Alexandra Are, Åsa Bergström, **Anja Schwäger**, Jessica Svärd, Stefan Teglund, Nick Barker and Rune Toftgård

Wounding enhances epidermal tumorigenesis by recruiting hair follicle keratinocytes

Proceedings of the National Academy of Sciences of the United States of America, 2011, 108(10):4099-4104.

II. **Anja Füllgrabe**, Simon Joost, Alexandra Are, Tina Jacob, Unnikrishnan Sivan, Andrea Haegebarth, Sten Linnarsson, Benjamin D. Simons, Hans Clevers, Rune Toftgård and Maria Kasper

Dynamics of *Lgr6*$^+$ progenitor cells in the hair follicle, sebaceous gland, and interfollicular epidermis

Stem Cell Reports, 2015, 5(5):843-855.

III. **Anja Füllgrabe**, Alexandra Are, Rune Toftgård and Maria Kasper

Healthy skin harbors pre-existing micro-niches that promote tumor formation

Manuscript

CONTENTS

1 INTRODUCTION 1
1.1 The skin in homeostasis 1
1.1.1 The epidermis 1
1.1.2 The hair follicle – morphogenesis and cycling 3
1.1.3 Stem cell niches in the hair follicle 4
1.1.4 The sebaceous gland 7
1.1.5 Stem cell dynamics in the interfollicular epidermis 7
1.1.6 *Lgr5* and *Lgr6* as stem cell markers 9
1.2 Wound healing 12
1.3 Skin cancer 14
1.3.1 Cancer cells-of-origin – the role of stem cells in tumourigenesis 15
1.3.2 Hedgehog signalling 16
1.3.3 Basal cell carcinoma 18
1.4 Genetic tools 19
2 AIMS OF THE THESIS 23
3 RESULTS AND CONCLUSIONS 25
3.1 Paper I 25
3.2 Paper II 26
3.3 Paper III 28
4 GENERAL DISCUSSION AND PERSPECTIVES 31
4.1 Significance and discussion of the work 31
4.2 Implications for future research 34
4.2.1 Interactions between *Lgr5*$^{+}$ and *Lgr6*$^{+}$ cell populations 34
4.2.2 Regulation of *Lgr6* expression 35
4.2.3 Function of the LGR6 receptor in the skin 35
4.2.4 Regulation of stem cell renewal 36
4.2.5 Wound healing and tumourigenesis 38
5 ACKNOWLEDGEMENTS 39
6 REFERENCES 41

LIST OF ABBREVIATIONS

BCC	Basal cell carcinoma
Bmp	Bone morphogenic protein
DMBA	7,12-dimethylbenz[a]anthracene
DNA	Deoxyribonucleic acid
E	Embryonic day
EGFP	Enhanced green fluorescent protein
HF	Hair follicle
Hh	Hedgehog
IFE	Interfollicular epidermis
IRES	Internal ribosomal entry site
IRS	Inner root sheath
K	Keratin
Lgr4/5/6	Leucine-rich repeat-containing G protein-coupled receptor 4/5/6
NCPC	Neural crest precursor cell
NMSC	Non-melanoma skin cancer
ORS	Outer root sheath
P	Postnatal day
Ptch1/2	Patched 1/2
R26	Rosa26 *(gene locus)*
RNA	Ribonucleic acid
SG	Sebaceous gland
Shh	Sonic hedgehog
Smo	Smoothened
TA	Transit-amplifying
tet	Tetracycline
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRE	Tetracycline response element
tTA	Tetracycline transactivator

1 INTRODUCTION

1.1 THE SKIN IN HOMEOSTASIS

The skin is the outer shell of the mammalian body. It protects other organs from external harm, such as pathogens and UV radiation, regulates the body temperature, and provides the touch sensation. The epithelium of the skin contains several appendages that are highly regenerative throughout the life of the organism, such as the hair follicle (HF), sebaceous gland (SG), sweat gland and nails [1]. The skin is thus an excellent model tissue to study the capabilities of adult mammalian stem cells during homeostatic tissue maintenance.

Structurally, the skin is divided into three main layers: the epidermis, the dermis and the skin-associated adipose tissue, called subcutis (**Figure 1**). The subcutis lies beneath the dermis and is composed of adipocytes and fibroblasts, and harbours larger blood vessels [2,3]. The dermis is a connective tissue supporting the epithelial structures and constitutes the thickest layer of the skin. The main cellular components of the dermis are fibroblasts, myofibroblasts, macrophages and other immune cells [2]. The fibroblasts form an extracellular matrix composed of collagen and other elastic fibres [4]. The main focus of this thesis will be on the topmost layer of the skin, the epidermis and its appendages.

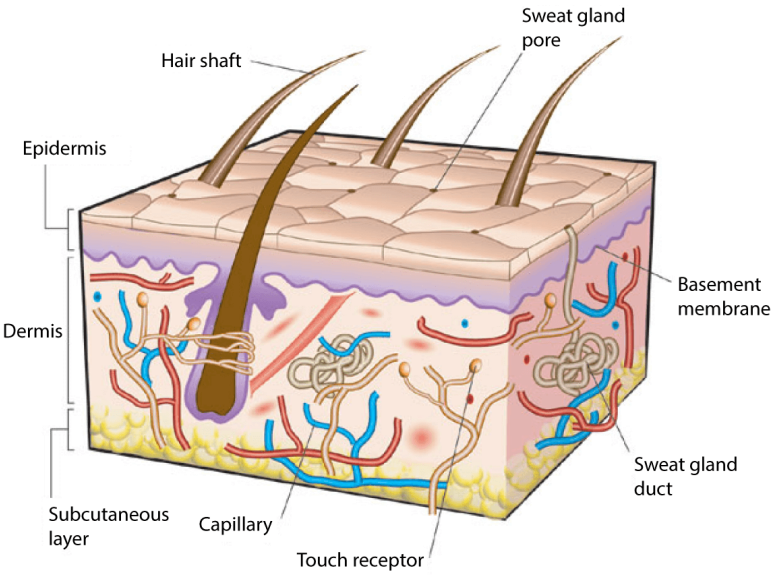

Figure 1. Overview of the major components of human and murine skin.
The skin is composed of the epidermis, the underlying dermis and the subcuteanous layer. The epidermis comprises hair follicles, sebaceous and sweat glands. Nerves, blood vessels and muscles are found within the dermis. The subcutaneous layer consists mainly of fat cells. Note that sweat glands are lacking in murine hairy skin (Reprinted with permission from [5])

1.1.1 The epidermis

The epidermis is the outer epithelial lining of the skin. The vast majority of cells within the epidermis are keratinocytes, with minor populations of Langerhans cells, melanocytes and Merkel cells [2]. Keratinocytes are named after their characteristic expression of keratin

intermediate filaments. Keratins always occur in heterodimers and are crucial for the rigidity of the epithelial cells forming the outer surface of the body [6]. Remarkably, keratin expression differs strongly among the epidermal keratinocyte populations, with typical keratin pairs being associated with cells in distinct differentiation stages or different locations in the epidermal structures [7].

The stratified epidermis consists of four layers (**Figure 2**) and its main function is to provide a barrier to water loss. The bottom layer is called basal layer and the cells residing here are termed basal cells. In mice, they form a single layer of cells that are tightly anchored to the underlying basement membrane. This anchoring, which is mainly achieved by expression of integrin receptors, prevents them from differentiation and keeps them in an undifferentiated state [8,9]. Basal cells are characteristically marked by expression of keratin 5 (K5) and K14, and are primarily responsible for the mitotic cell divisions in the epidermis [10]. When the cells start to differentiate, they move upwards into the spinous and granular layer, where they change their biochemical composition and form tight junctions. They express the keratins K1, K10 and involucrin. Eventually, the suprabasal cells will lose their nucleus and become a flattened keratinized layer (stratum corneum) that is continuously shed from the body. Typical markers of the terminally differentiated cells are loricrin and filaggrin [11].

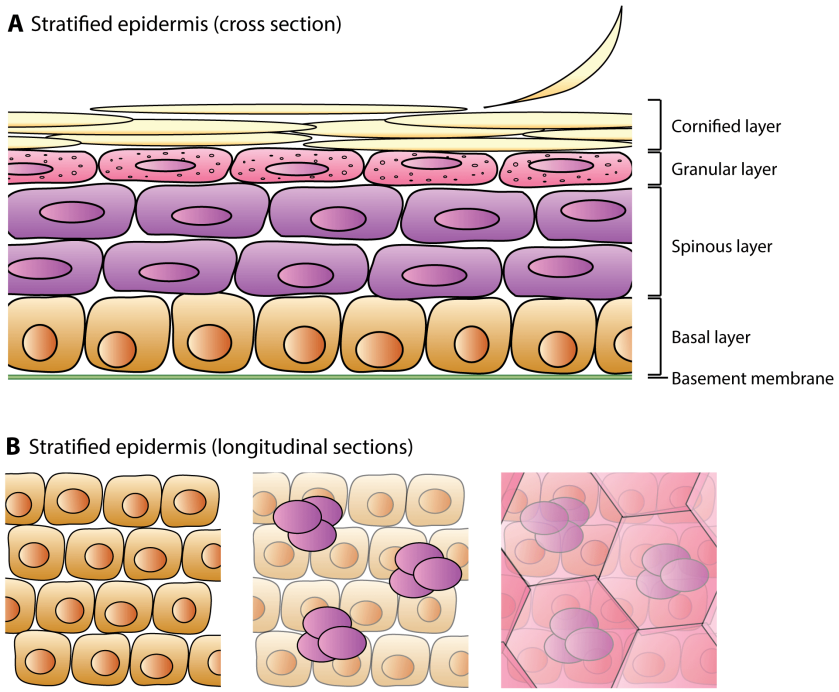

Figure 2. Architecture of stratified epidermis in murine dorsal skin.
(**A**) The epidermis consists of four distinct layers of keratinocytes. The basal layer contains undifferentiated cells attached to the basement membrane. Upon differentiation cells move upwards to the spinous layer. In the granular layer cells prepare for terminal differentiation, where they lose their nuclei and form a layer of keratinized cells, which is eventually shed from the body. (**B**) Longitudinal sections through the epidermis at the level of basal cells (left), suprabasal cells (middle) and keratinized cells (right). The small basal cells form a densely packed layer. The nuclei of the flattened suprabasal cells usually cluster together. The keratinized cells form regular hexagonal shapes.

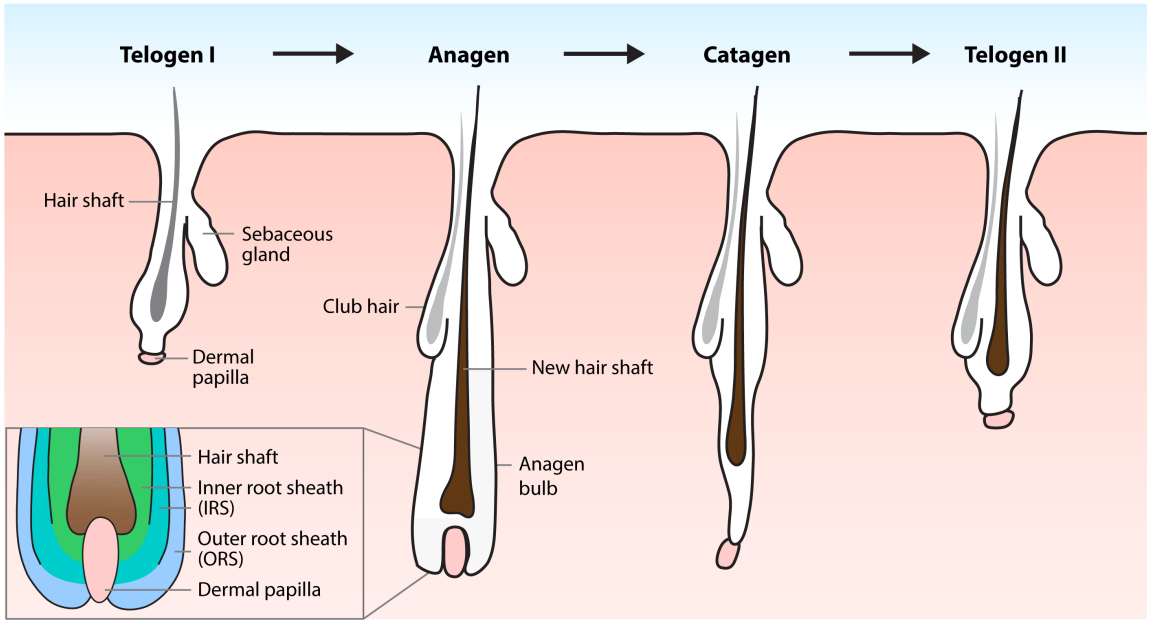

Figure 3. The hair cycle and simplified structure of the anagen bulb.
Throughout the lifetime of an organism, the hair follicle undergoes cycles of growth (anagen), regression (catagen) and rest (telogen), changing the morphology of the lower part of the hair follicle. Hair production takes place during the anagen phase. In mice, the old hair shaft is not shed like in humans but retained as a club hair. The inset illustrates the arrangement of the keratinocyte layers in the lower anagen bulb. Closest to the dermal papilla, which is fully engulfed by keratinocytes, occurs most cell proliferation. The outer root sheath consists of a single layer of basal cells, while the inner root sheath comprises seven layers that expand upwards, accompanying the growing hair shaft.

1.1.2 The hair follicle – morphogenesis and cycling

Hair is a distinctive feature of the mammalian body. The visible hair shaft is produced by the HF, which is one of the appendages that are formed by the epidermal layer, invaginating into the underlying dermis. The HF is the most intriguing epidermal structure to study activation of tissue stem cells because it constantly undergoes morphological changes in order to produce hair. Moreover, HF cells follow several distinct differentiation paths, allowing investigation of stem cell lineage specification.

In mice, the first HFs on the dorsal skin start to develop at embryonic day 14.5 (E14.5). This process begins with the induction of an epithelial hair placode, a localised compaction of epithelial cells, in response to signals from the dermis [12]. These first inductive signals are believed to be activation of the Wnt/β-catenin pathway [13]. Reverse signals from the placode stimulate the condensation of the mesenchymal cells into the so-called dermal papilla. The dermal papilla acts as the major signalling centre for hair growth and stays closely associated with every HF [14,15]. Following signals from the dermal papilla, the epidermal placode then develops into the hair germ, which elongates and forms the mature HF. The lower part of the HF now engulfs the dermal papilla, where several concentric layers of keratinocytes emanate (**Figure 3**, inset). The outer root sheath (ORS) is the outermost layer, which is continuous with the basal layer of the interfollicular epidermis (IFE). Seven

distinct layers form the inner root sheath (IRS), which surround the developing hair shaft [16]. The HF in this growth phase is called anagen HF. In the lower anagen HF segment, also called bulb, most proliferation is done by the cells that are in closest contact with the dermal papilla. These cells are termed matrix cells and give rise to the different lineages of the IRS and hair shaft [17].

When the hair shaft has reached its final length, at around postnatal day 16 (P16), the HF starts to regress. During this phase called catagen most of the cells of the bulb undergo apoptosis. Between P19 and P21 the HF exists in its resting conformation, termed telogen HF [18]. Soon after that the lower part of the HF starts to re-grow into its anagen conformation to produce the next hair shaft. This sequence of regression and growth continues throughout life and is referred to as the hair cycle (**Figure 3**) [19]. An important difference between the hair cycle in humans and mice is that the previous hair shaft is retained in mouse dorsal skin while the next one is growing. The hair remains anchored in its own dormant HF, which is referred to as club hair. In contrast, the human hair cycle contains a fourth phase following catagen, which is called exogen and initiates the loss of the hair shaft [20]. Moreover, in mice, dorsal HFs are synchronized in their hair cycle stage until 2-3 months of age, which is advantageous for research to start experiments at a distinct hair cycle stage.

1.1.3 Stem cell niches in the hair follicle

The continuous supply of new cells for epidermal homeostasis and hair cycling is secured by epidermal stem cells. Stem cells are defined by their capacity to extensively self-renew, generate cells that undergo terminal differentiation, and repair the tissue upon injury. To date, numerous stem cell markers have been identified within almost all epidermal HF compartments (**Figure 4**).

The bulge of the telogen HF was the first stem cell compartment discovered in the epidermis. A labelling that is incorporated into the cell's DNA and is diluted out in dividing cells can be used to identify slowly dividing cells, which is a putative property of stem cells [21]. The cells located in the bulge area retain DNA labelling over long periods, thus marking them as the most quiescent epidermal cells [22,23]. Subsequent lineage tracing experiments revealed that bulge cells are able to generate all lineages of the anagen HF [23,24]. Identification of marker genes expressed by bulge cells, such as *CD34* [25], *Krt15* (K15) [26], *Lgr5* [27] and *Krt19* (K19) [28], enabled further analysis of their characteristics and stem cell potential. Isolation and transplantation of *CD34*-positive (*CD34*$^{+}$) bulge stem cells demonstrated that these cells are capable of regenerating the entire epidermis including full HFs [29]. However, during homeostasis bulge and hair germ cells are only activated upon anagen entry and their contribution to the other compartments above the bulge is limited [30,31]. This implies that stem cells outside the bulge niche must be responsible for maintenance of the upper HF compartments. In the narrow segment above the bulge, called isthmus, cells expressing *Plet1* [32], *Lgr6* [33] and *Gli1* [34] were found to possess stem cell properties by *in vivo* lineage

tracings. The junctional zone around the opening of the SG was shown to be populated by *Lrig1*-expressing cells that give rise to cells in the infundibulum and the SG [35,36]. Interestingly, all HF stem cell populations that were tested in transplantation assays had the capacity to regenerate epidermis and HFs even though their contributions during homeostasis are restricted to specific compartments, which demonstrates an enormous plasticity among epithelial stem cells [37].

The questions remain how this heterogeneity of stem cell markers is established and what regulates the specific fate choices of stem cells in particular areas. By investigating signalling pathways and microenvironmental components, we are only beginning to understand the factors that orchestrate epidermal homeostasis.

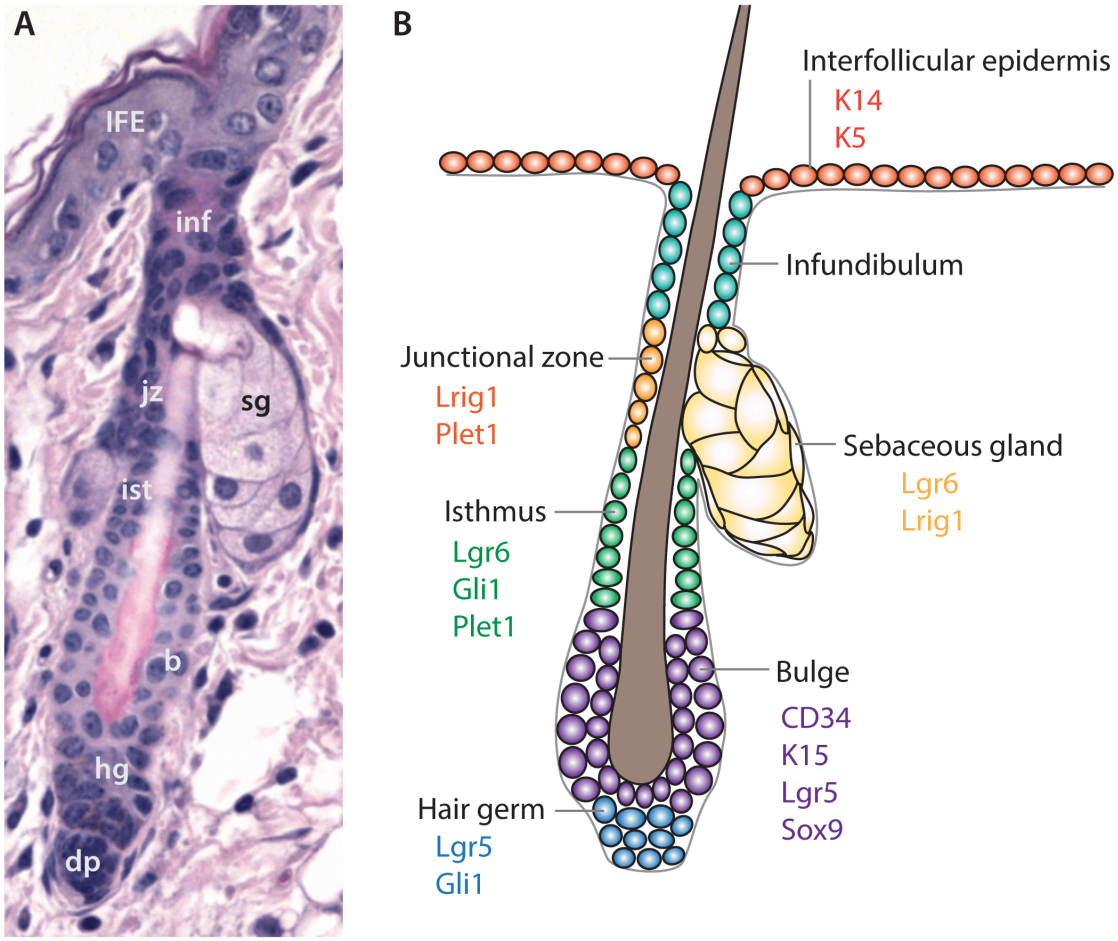

Figure 4. The telogen hair follicle - compartments and stem cell markers.
(**A**) Hematoxylin and eosin staining of paraffin-embedded dorsal skin shows the typical morphology of the telogen hair follicle. IFE: interfollicular epidermis; inf: infundibulum; jz: junctional zone; sg: sebaceous gland; i: isthmus; b: bulge; hg: hair germ; dp: dermal papilla. (**B**) The hair follicle structure is subdivided into distinct compartments. In each compartment exist basal cell populations that possess stem cell properties and are defined by distinct molecular markers.

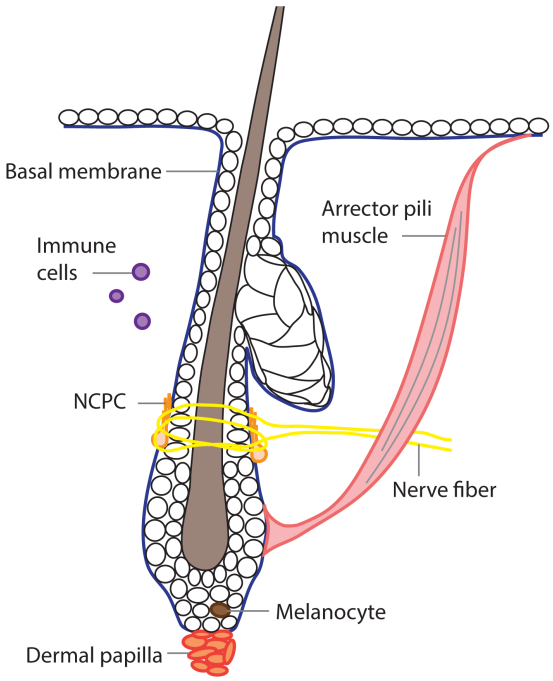

Figure 5. Cellular and non-cellular interactions affecting hair follicle stem cells.
In the bulge and hair germ, cells are influenced by dermal papilla fibroblasts, melanocyte precursor cells and the arrector pili muscle anchorage site. Above the bulge epidermal cells interact with sensory nerve fibres and nerve-associated neural crest-derived precursor cells (NCPCs). All basal cells are encompassed by the basement membrane, which provides specific binding sites and structural support. Also immune cells in the dermis and epidermis, and mechanical forces can alter the fate of hair follicle stem cells.

The Wnt and the bone morphogenic protein (Bmp) pathways are important mechanisms to control activation and quiescence of hair germ and bulge cells. The dermal papilla situated just underneath the hair germ, is a condensate of specialized mesenchymal fibroblasts and is essential for initiating anagen entry and hair growth [14,15]. Dermal papilla cells have been shown to secrete Wnt molecules and Bmp inhibitors to activate proliferation in the nearby hair germ cells, as the first step of hair regeneration [30].

Moreover, a variety of structures and cell types in the dermis have been shown to influence epidermal stem cells (**Figure 5**). The arrector pili muscle, which is responsible for erection of the hair, is anchored to the basal lamina of bulge cells, which in turn provide signals that attract the muscle to this site [38]. At the upper bulge and isthmus, epidermal cells closely interact with neural crest-derived precursor cells (NCPCs) that are associated with the endings of sensory nerve fibres, wrapping around each HF [39,40]. The nerve fibres have been shown to also influence epidermal stem cells by secretion of Sonic hedgehog (Shh) ligand, which activates Hedgehog (Hh) signalling in the upper bulge [34]. Melanocyte stem cells are another neural crest-derived cell type within the bulge niche. The survival and activation of melanocytes closely relies on signals provided by epidermal stem cells [41,42]. Evidence is accumulating that immune cells can modulate HF stem cells, for example by secretion of growth factors during wound healing [43]. Lastly, the composition and rigidity of the extracellular matrix have profound effects on the properties of epidermal stem cells. The basement membrane is mainly composed of laminins and type IV collagen and the basal cells attach to it via integrin receptors. This interaction is crucial for maintaining stem cell potential and tissue homeostasis as cells that lose this connection start to differentiate [9]. Furthermore, it was discovered that the mechanical properties of the surroundings, such as matrix stiffness

and density, can alter the stem cell fate [44]. Constraining the physical shape of keratinocytes to small cell sizes induced differentiation while stretched out cells kept dividing, which implies that epidermal differentiation can be directed by mechanical signals [45].

1.1.4 The sebaceous gland

SGs are holocrine glands, producing sebum from fatty acids that lubricates the hair shaft and supports the barrier function of the IFE. One or two SGs are typically connected to the upper part of dorsal HFs and are regarded as a HF compartment in this thesis. Basal cells in the SG are frequently proliferating, giving rise to differentiating sebocytes within the gland. Mature sebocytes travel towards the SG duct, where they burst and release their oily contents into the hair canal [46].

During embryonic development, the SG is generated from *Lrig1*$^+$ cells in the upper part of the HF shortly after the induction of the hair germ [47]. The signalling pathways that positively regulate SG differentiation include c-Myc [48] and Hh signals [49]. Conversely, activation of the Wnt/β-catenin pathway blocks SG development [50], and inhibition of the Wnt-associated transcription factor LEF1 results in *de novo* SG formation [51], implicating canonical Wnt signalling as a key regulator of the fate choice between SG and HF lineage. Moreover, sebocyte differentiation is significantly affected by sex hormones; especially androgens stimulate SG growth [52].

The mode of SG renewal and the identity of SG stem cells are still a matter of debate. *Blimp1* was first presented as a marker specific to SG progenitor cells, locating SG progenitors at the opening of the gland [53]. However, inducible lineage tracing from the *Blimp1* promoter revealed that *Blimp1* is in fact expressed in differentiated cells within the SG and the IFE, and thus *Blimp1*$^+$ cells unlikely represent SG precursor cells in adult mice [54]. Nevertheless, several other studies presented evidence for the existence of a local, self-maintaining SG progenitor cell population. HF-independent maintenance of the SG was observed by randomly labelling epidermal cells for lineage analysis [55]. Furthermore, *Lrig1* and *Lgr6*, which are both expressed in the SG basal layer, were suggested as putative markers of SG progenitor cells [35,54]. In contrast, contributions of bulge (*Krt15*$^+$), isthmus (MTS24$^+$ and *Lgr6*$^+$) and junctional zone (*Lrig1*$^+$) cells to SG maintenance were proposed by lineage tracing experiments using different HF stem cell markers [32,33,36,56]. Thus, it remains to be resolved if and how different HF stem cells contribute to the SG during homeostasis.

1.1.5 Stem cell dynamics in the interfollicular epidermis

Since the HF bulge is the only epidermal compartment containing slow-cycling, quiescent stem cells, it was questioned whether the IFE contains an autonomous stem cell population or if it is also maintained by HF stem cells. Several early reports concluded that HF cells

generally take part in the homeostatic turnover of the IFE [24,57]. However, more recent studies, using genetic labelling techniques, presented compelling evidence for the existence of independent IFE stem cells. By permanently labelling all HF cells, using either the *Shh* or *Sox9* promoter during development, two studies demonstrated that the IFE is not traced during normal tissue maintenance, indicating that HFs and IFE contain separate stem cell pools [58,59].

Until today, no specific stem cell markers for the IFE have been identified. The basal markers K5, K14 and p63 are found in all epidermal basal cells and generally mark the undifferentiated status throughout the epidermis [60]. In human IFE, a few markers have been suggested, such as high levels of β1 integrin [61]. So the question remained how epidermal stem cells are defined. The first theories originated from the observations of keratinocytes in culture and murine epidermis after irradiation. Seeding keratinocytes at low density, distinct types of clonal colonies are detected, indicating a proliferative heterogeneity amongst basal epidermal cells. From the number of clonogenic cells and the diverse differentiation capacities, a hierarchical model was proposed of slowly dividing stem cells that give rise to transit-amplifying (TA) cells with limited proliferation potential, which eventually give rise to differentiated, suprabasal cells [62,63] (**Figure 6A**). This hypothesis was supported by the architecture of stratified epidermis, forming regular stacks of keratinized cells with an underlying group of basal cells (**Figure 2**). In each of these epidermal proliferative units a central slow-cycling stem cell is surrounded by a number of TA cells, which generate the differentiated cells on top.

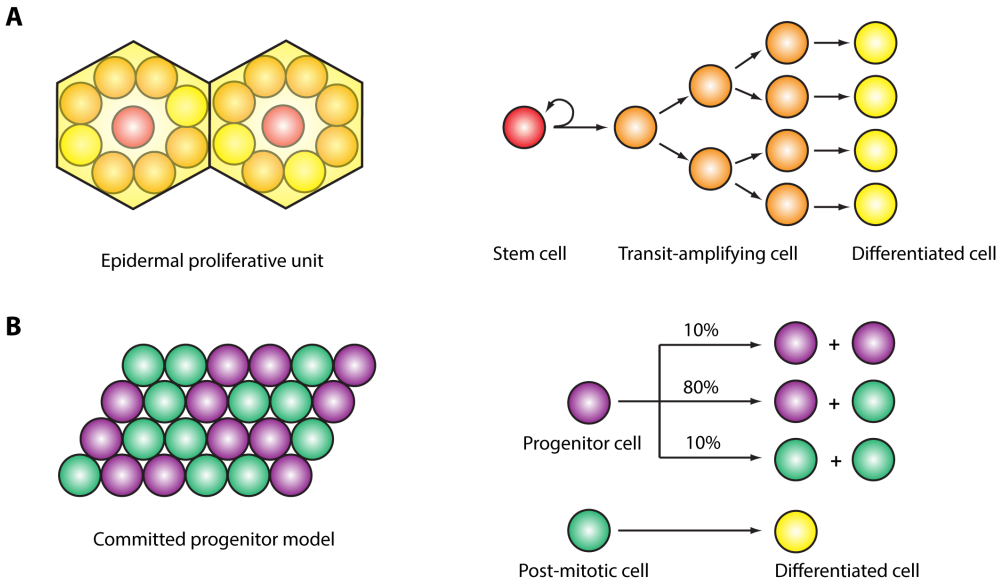

Figure 6. Models of stem cell renewal in the interfollicular epidermis.
(**A**) This model describes a cluster of a stem cell (red) with surrounding transit-amplifying (orange) and differentiated cells (yellow) as an epidermal proliferative unit, each one underlying a stack of hexagonally shaped keratinocytes in the strateum corneum. The different cell types arise in a strict hierarchy and have distinct characteristics. (**B**) The committed progenitor model describes the basal layer as composed of cells with progenitor potential (purple) and post-mitotic cells (green). Progenitor cells stochastically follow one of the three depicted fates to generate progenitor and post-mitotic cells, which then give rise to differentiated cells in the upper layers of the IFE.

In 2007, Clayton and colleagues conducted a pioneering study, uncovering that progenitor cells in the IFE basal layer chose their cell fate stochastically, instead of following a strict hierarchy [64]. Based on the clone size distributions of randomly labelled basal cells in tail epidermis, they reconstructed the underlying mode of stem cell renewal using mathematical probability models. The "committed progenitor" model states that all IFE progenitor cells have the same proliferation potential and follow one of three fate options after division: two progenitor cells, one progenitor cell and one post-mitotic basal cell, or two post-mitotic basal cells (**Figure 6B**). Post-mitotic cells will eventually differentiate and exit the basal layer. Hence, by symmetric divisions the progenitor cell pool is either enlarged (two progenitors) or diminished (two post-mitotic cells). To ensure tissue homeostasis, both processes must occur at the same rate. This mode of epithelial regeneration was later also confirmed in the epidermis of the ear [65] and paw [66], and in many other rapidly renewing tissues, such as the intestine [67], oesophagus [68], and stomach [69]. It should be noted that the committed progenitor model is a convincing model, which describes the experimental observations accurately. However there are no means (yet) to distinguish progenitor cells from the post-mitotic basal cells experimentally. Hence, the factors that determine the progenitor cells remain to be discovered.

1.1.6 *Lgr5* and *Lgr6* as stem cell markers

Lgr5, a leucine-rich repeat containing G protein-coupled receptor, became known as the first marker gene for intestinal stem cells, the so-called crypt base columnar cells [70]. The group of Hans Clevers originally identified *Lgr5* as a Wnt target gene specifically expressed in the intestinal crypt [71,72]. Since no functional antibodies were available against LGR5, they subsequently developed knock-in mouse strains to detect *Lgr5*-expressing cells and test their stem cell properties by lineage tracing experiments. The $Lgr5^+$ stem cells that gave rise to all lineages of the intestine in long-term lineage tracing were rapidly proliferating and did not sustain DNA-label incorporation [70]. Thus, the discovery of $Lgr5^+$ stem cells challenged the paradigm of the slow-cycling, label-retaining tissue stem cell that gives rise to transit-amplifying cells, which had been the prevailing view of how adult tissues are maintained by mostly quiescent stem cells [73].

Shortly after this breakthrough, $Lgr5^+$ cells were discovered within the murine HF, using the same genetic tools [27]. $Lgr5^+$ cells are located in the lower bulge and hair germ of the telogen HF (**Figure 7A**), and are not DNA label-retaining, in contrast to most $CD34^+$ bulge cells. In the anagen HF, *Lgr5* is expressed in the ORS of the lower HF bulb. Upon isolation and transplantation onto the back of immune-compromised nude mice, $Lgr5^+$ cells demonstrated the potential to give rise to all lineages of the epidermis, including HFs, SGs, and IFE, indicating that $Lgr5^+$ cells are multipotent stem cells [27]. At the molecular level, $Lgr5^+$ cells are characterized by active Hh signalling and transcription of Wnt pathway components. During homeostasis, telogen $Lgr5^+$ cells in the hair germ are the first cells to respond to the anagen-inducing signals by the dermal papilla and start proliferating to

generate the anagen HF. Lineage tracing revealed that *Lgr5*$^{+}$ cells of the lower bulge and hair germ contribute to all layers of the anagen bulb and sometimes to the isthmus [27]. Interestingly, *Lgr5*$^{+}$ cells in the lower ORS, labelled in anagen, survive the catagen phase and repopulate the hair germ in the subsequent telogen, showing that ORS cells also have a certain stem cell potential and are not entirely lost by apoptosis during catagen. These results emphasize the notion that long-term surviving stem cells are not necessarily slow-cycling and label-retaining [74]. The quiescent cells in the bulge may serve as a back-up population that replenish the actively cycling stem cell pool and participate in wound repair, rather than contributing to routine tissue turnover [73]. *Lgr5* has since been defined as stem cell marker in various other organs such as stomach [75], mammary gland [76], taste buds [77] and kidney [78], underlining the importance of *Lgr5* as a general marker of adult epithelial stem cells.

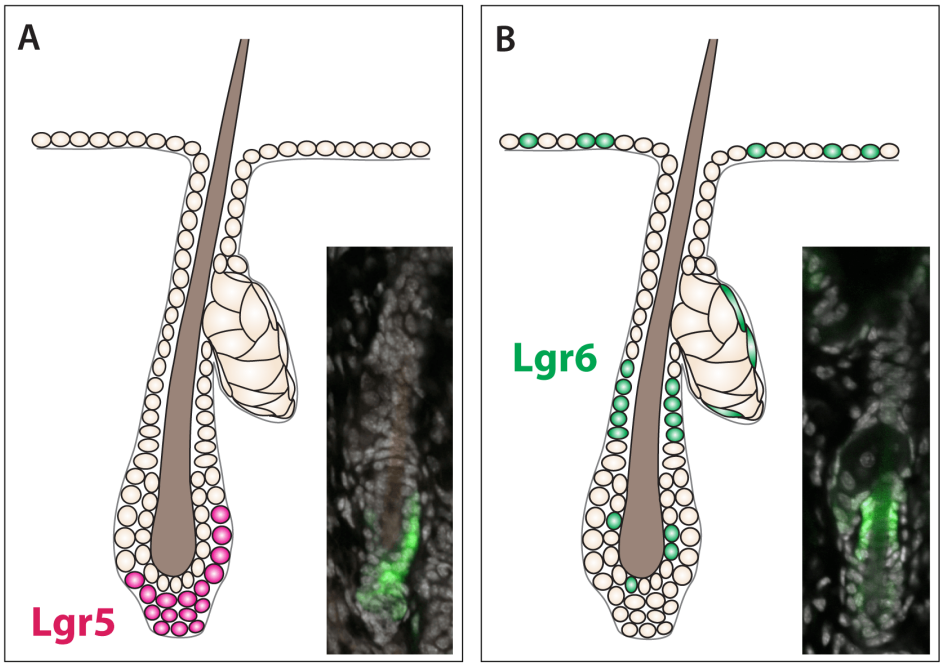

Figure 7. Expression of Lgr5 and Lgr6 in the telogen hair follicle.
(**A**) Lgr5 is exclusively expressed in cells of the hair germ and lower bulge. (**B**) Lgr6 expression is strongest in the isthmus region, but is also detectable in the basal layer of the sebaceous gland and interfollicular epidermis, as well as in some cells in the inner layer of the bulge. Insets show EGFP expression in *Lgr5/6-EGFP-IRES-CreERT2* dorsal skin tissue.

Shortly after *Lgr5* was established as a HF stem cell marker, the expression of the homologous genes *Lgr4* and *Lgr6* was analysed in the skin using *LacZ* reporter mice [33]. The highest levels of *Lgr6* were found in the central HF isthmus, while *Lgr4* expression was detected in the entire HF below the infundibulum. During HF morphogenesis, *Lgr6* starts to be expressed already in the earliest hair placodes at E14.5. Like *Lgr5*$^{+}$ HF cells, *Lgr6*$^{+}$ cells are rapidly dividing and do not retain DNA labelling. Lineage tracing in adult mice, using an

Lgr6 knock-in mouse line, revealed that *Lgr6*$^+$ cells mainly replenish cell pools in the isthmus, SG, and IFE during homeostasis, demonstrating a long-term stem cell capacity. When isolated and transplanted, *Lgr6*$^+$ keratinocytes were able to reconstitute entire HFs and epidermis, implying an intrinsic multipotent stem cell potential [33].

In contrast to *Lgr5*, *Lgr6* gene expression does not seem to be regulated by Wnt signals [33]. Recently, it was implicated that *Lgr6* expression in the epidermis is more widespread than previously recognized, being additionally detected in the basal layer of the IFE and the SG (**Figure 7B**) [35,79]. Moreover, a connection between *Lgr6* reporter activity and sensory nerve fibres in the skin was observed [79], providing a new indication of the regulation and function of *Lgr6*.

Molecularly, LGR4, LGR5 and LGR6, belong to the class of 7-transmembrane, G protein-coupled receptors. On the extracellular domain they contain 13-17 characteristic leucine-rich repeat elements (17 in LGR4 and LGR5, 13 in LGR6), forming a ternary horseshoe shape [80]. When the *Lgr5* gene is deleted in mice, the pups die shortly after birth due to malformation of the palate, indicating an involvement of *Lgr5* in murine development [81]. *Lgr6*-null mice, however, are viable and fertile [33].

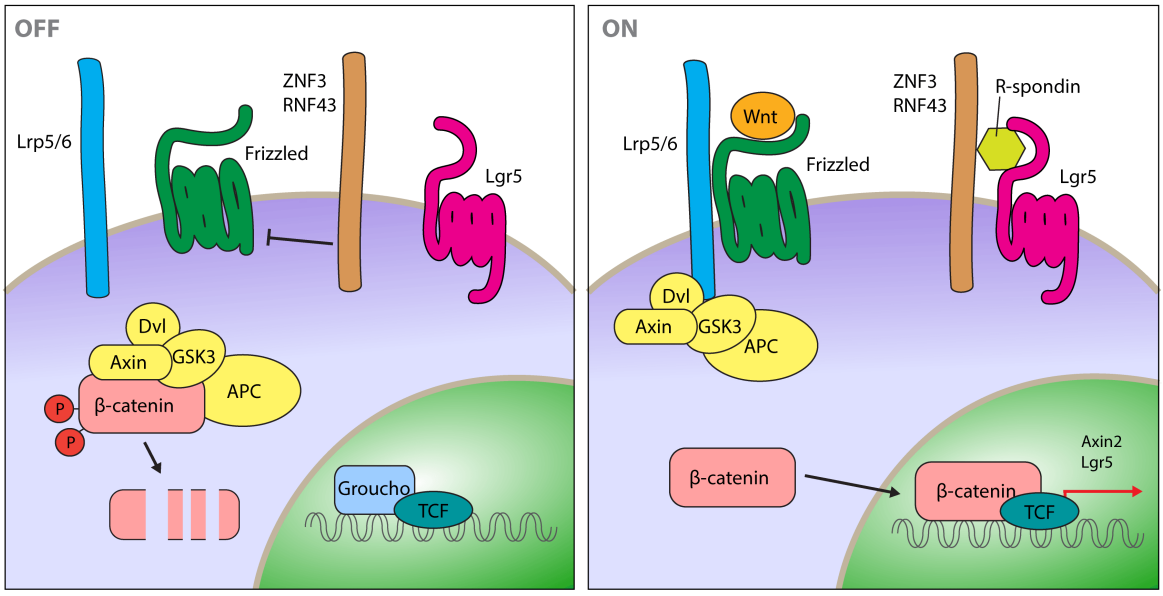

Figure 8. The canonical Wnt signalling pathway and the putative role of Lgr5.
In absence of Wnt ligands, β-catenin is tagged for degradation in the cytoplasm by a destruction complex, composed of Axin, adenomatosis polyposis coli (APC), and glycogen synthase kinase 3 (GSK3) (left panel). When Wnt binds to the Frizzled receptor, they form a complex with the co-receptors Lrp5/6 at the cell surface (right panel). This leads to the recruitment of Axin via the scaffolding protein Dishevelled (Dvl), resulting in inactivation of the destruction complex and accumulation of β-catenin, which translocates to the nucleus, where it binds to TCF transcription factors to activate Wnt target genes. The proposed role of Lgr5 is to enhance Wnt signalling by binding R-spondin molecules, which inactivates the E3 ubiquitin ligases ZNF3 and RNF43 that normally down-regulate Frizzled.

Recently, it was uncovered that R-spondins are the ligands for LGR4 and LGR5 [82-86]. R-spondins were already known to be secreted activators of Wnt/β-catenin signalling, acting as mitogens during development [87]. Being a Wnt-responsive gene, the LGR5 receptor now classifies as a part of the Wnt signalling complex at the cell surface (**Figure 8**). However, the exact molecular mechanism how the LGR5/R-spondin interaction leads to enhanced Wnt signalling is not clear. It is speculated that the LGR5/R-spondin complex increases the Wnt receptor levels on the cell surface by sequestering the E3 ligases RNF43/ZNF3 that tag the receptors for degradation [88].

1.2 WOUND HEALING

Wound healing is a vital mechanism to rebuild tissue following an injury, which often results in the formation of a scar. The sequence of this complex process is very similar in various tissues and involves the orchestration of many different cell types [89]. The cutaneous wound healing process can be divided into four distinct phases: (1) immediate response, (2) inflammation, (3) new tissue formation, and (4) remodelling [89,90]. The first stage of wound healing in the skin is the formation of a platelet clot that coagulates the blood vessels to stop the bleeding. The blood clot also serves as a temporary scaffold for recruited cells and releases various growth factors and other signalling molecules [90]. In the second phase, circulating and skin-resident immune cells appear at the wound site. First are the neutrophils and later monocytes arrive, which differentiate into macrophages to defend the body against pathogens and clear cell debris [91]. In the third phase, the cells regenerating the new tissue invade the wound. Keratinocytes migrate and proliferate as they move from the wound edge to the centre of the wound to establish the new epidermis, a process called re-epithelialisation [89]. Within the dermis, fibroblasts synthesise collagen, the main substrate of the scar tissue, and differentiate into contractile myofibroblasts, which promotes wound closure by contraction [92,93]. Moreover, angiogenesis and lymphangiogenesis are initiated. The last phase describes the restoration of the skin structure and function by remodelling and refinement of the structures over longer periods of time. All of these processes need to be tightly regulated to ensure the arrest of proliferation and prevent overproduction of tissue, resulting in fibrosis or excessive scarring [89].

As impressive as the regeneration of skin is, the newly formed scar tissue does not completely regain all of its functionality and components as before wounding. First and foremost, all appendages are lacking, which usually contribute to the skin's physiology [94]. In addition, scar tissue shows altered biomechanical properties, such as reduced elasticity [95]. Interestingly, it was recognised that human foetal skin has the ability to heal without scarring [96,97]. The main difference between foetal and adult skin is the lower number of immune cells in foetal skin, which led to the establishment of the theory that the

inflammatory reaction constrains the regenerative potential of adult skin and promotes scarring [94,98].

The role of epidermal stem cells in the wound healing process has been intensely investigated. The keratinocytes required for wound re-epithelialisation derive from the surrounding IFE and the appendages. A study using mice which display a lack of tail HFs (ectodysplasin-A receptor-associated death domain knockout mice), proved that HFs are not essential for wound healing but they accelerate wound closure [99]. Intriguingly, the rate of wound healing is correlated with the number of HFs in anagen and is consequently fastest in areas such as the scalp, where the density of anagen HFs is the highest [100]. Lineage tracing of HF cells revealed that HF stem cells participate substantially in the re-epithelialisation of cutaneous wounds [101]. Even dormant stem cells located in the HF bulge, marked by K15 expression, are activated and recruited to aid in wound closure [102]. Also stem cells located in the lower isthmus, junctional zone and infundibulum were shown to contribute to epidermal regeneration [34,35,99]. These studies demonstrate the plasticity of epidermal stem cells, which are able to convert their fate from an appendage stem cell to an IFE stem cell in the newly generated epithelium covering the scar [103].

It will be interesting to explore the molecular mechanism governing this conversion of cell fates. Several experiments started elucidating the lineage conversion between IFE and HF cells. It was shown that canonical Wnt signalling is able to induce the formation of ectopic HFs from existing HFs or IFE when constitutively activated, e.g. in the form of β-catenin with a nuclear translocation domain [50,104,105]. Conversely, repression of Wnt signalling by expression of a dominant negative ΔN-LEF1 leads to a switch from HF differentiation to stratified squamous epithelium [51].

In a striking experiment Ito and co-workers showed that *de novo* formation of fully functioning HFs is possible in wild type mice, by introducing sufficiently large full-thickness wounds on dorsal skin [106]. The growth of the HFs in the middle of the scar epidermis closely resembled the naturally occurring HF morphogenesis, with Wnt/β-catenin as the initial inductive signal. Further studies revealed that an increased number of fibroblasts in the upper dermis, which is permissive for HF formation, and the secretion of fibroblast growth factor 9 from γδ T cells within the dermis are important factors in this process [43,107]. The cellular source for *de novo* HFs were the IFE and the infundibulum, and not bulge stem cells [106]. This again is very similar to the developmental HF morphogenesis and highlights the flexibility of keratinocyte lineages.

The activation of stem cells to repair damaged tissue is crucial, however, the cell proliferation and recruitment during wound healing must be tightly controlled to prevent cancer development. It has long been observed that tumour formation is promoted at sites of chronic wounds and fibrotic tissues, and there are many intriguing similarities between wounds and tumours [108]. Both, healing wounds and tumours exhibit leaky vessels, deposition of fibrin/fibronectin, and ongoing angiogenesis [109]. There is an established connection between tumour development and the inflammatory response, which is characterised by the

infiltration of symptomatic immune cells, such as M2 macrophages, and the production of reactive-oxygen species [109-111]. Further similarities between wounds and skin tumours are the migration and proliferation of keratinocytes, which are often stimulated by the same set of mitogens, the differentiation of fibroblasts into myofibroblasts, and the conversion of epithelial cells to mesenchymal-like cells [108,109,112]. During normal wound healing those processes are naturally controlled and terminated to re-establish tissue homeostasis. But when cells are transformed by oncogenic mutations, the wound environment can start to advance tumour growth.

1.3 SKIN CANCER

As the outermost layer of the body, the epidermis is constantly exposed to UV radiation and potentially harmful chemical substances. Thus, skin cancer is the most commonly diagnosed form of cancer [113]. The different types of keratinocyte-derived tumours are referred to as non-melanoma skin cancer (NMSC) and comprise the two most common forms, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), and other rare cancers, such as trichofolliculoma and sebaceous adenoma.

The epidermis and other epithelial tissues, such as the intestinal epithelium, proliferate intensely during homeostasis. On the one hand this ensures a constant replacement of damaged and dead cells. But on the other hand high cellular turnover rates increase the chances that a cell carrying a DNA mutation propagates. The transformation of a normal cell into a cancer cell usually requires multiple genetic mutations and each round of cell division raises the probability of such events to manifest [114]. The most common genetic lesion detected in NMSC is the mutation of the p53 tumour-suppressor gene. More than 90% of SCCs and 50% of BCCs show UV-induced p53 mutations [115]. The same p53 mutations are even found in normal skin and the precancerous stages of skin neoplasms, like actinic keratosis [116,117].

Modelling the process of skin carcinogenesis in mice facilitates the comprehension of the early steps of tumour development. Tumour induction is classically achieved by treatment with carcinogenic chemicals, mimicking the natural evolution of a tumour with successive mutational events. This model is referred to as multistage carcinogenesis and involves the topical application of a carcinogenic compound e.g. 7,12-dimethylbenz[a]anthracene (DMBA) followed by the repetitive treatment with a tumour promoting substance such as the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [118]. This procedure typically induces mutations of H-Ras as the driver oncogene, leading to the emergence of benign papillomas, which then progress to SCC [118,119]. Furthermore, UV irradiation and genetic mouse models with specific activation of certain oncogenes or knockout of tumour-

suppressor genes are used to study the initiation and progression of skin tumours. Even though there are certain differences between humans and mouse models that need to be taken into consideration, the general mechanism of NMSC development is comparable in both species [120].

1.3.1 Cancer cells-of-origin – the role of stem cells in tumourigenesis

The variety of NMSC types, or generally the fact that different types of cancer arise within the same tissue, raises several questions: Do different tumour types arise from different cell types? Can one cell type give rise to multiple tumour phenotypes, depending on the acquired mutations? Or can the same type of tumour even arise from different cell types? Especially for therapeutical intervention it is important to elucidate whether some cells are more prone to generate cancer than others and which cell types are the main targets for tumourigenic transformation.

Defining the "cancer cell-of-origin" and answering the above questions is therefore a major goal in basic cancer research. The term cancer cell-of-origin describes the cell which has experienced the first mutagenic event that led to tumour development, and should not be confused with "cancer stem cell", which often denotes certain cells belonging to an already established tumour that sustain tumour growth [121].

As many tumour tissues resemble one particular cell type, in regards to histology and marker expression, it seems intuitive that this cell type is also the source of tumour cells. Hence, the cellular origin of the tumour was, and still is, indirectly inferred based on these correlations. However, several examples have shown that this is not always the case, and only experiments *in vivo*, for example targeted transformation and lineage tracing, can prove that a given cell type or stem cell population can give rise to a certain cancer [121]. It is possible that the altered signalling cascades in a transformed cancer cell lead to transdifferentiation into another cell type or change the lineage fate of a cancerous stem cell. Likewise, cells further down the differentiation pathway may dedifferentiate to a stem cell-like state upon malignant transformation, which has been experimentally demonstrated in the mammary gland and the intestine [122,123].

There are several examples where the apparent cell type of the tumour cells did not match that of the experimentally determined cell-of-origin. Breast cancer arising in *BRCA1* mutant individuals with a stem cell-like (basal-like) appearance were shown to have developed from expanding luminal cell populations and not from the basal mammary gland stem cells [124]. Similarly, BCC cells generally resemble undifferentiated HF cells and were therefore believed to arise from ORS cells [114]. However, lineage-tracing experiments in mice have demonstrated that BCCs do not only arise from stem cells of the HF but can also evolve from IFE basal cells [28,125,126].

Tissue stem cells have been in the focus of the search for the cancer cell-of-origin because stem cells already possess unlimited life-span, active growth signalling pathways, and a multipotent status [114]. Experiments using multistage carcinogenesis have shown that mutations induced by DMBA treatment can persist for a long time before the final promotion phase triggers cancer development, implying that long-lived stem cells are targeted by the mutagenesis [127]. Directly introducing activated H-Ras into epidermal keratinocytes showed that more aggressive tumours (such as SCC) occur when basal cells are targeted instead of suprabasal cells, which only generate benign pre-cancerous papillomas [128,129]. Moreover, the signalling pathways that are deregulated in NMSCs are associated with stem cell regulation in normal tissue, such as Hh, Wnt/β-catenin and Bmp signalling [113].

1.3.2 Hedgehog signalling

The Hh pathway has been recognised as the main driver of BCC and medullablastoma, which is exceptional because a single event, i.e. the mutation of one pathway component is sufficient to initiate tumour formation [130]. In normal physiology, the three mammalian Hh molecules Sonic hedgehog, Desert hedgehog and Indian hedgehog act primarily as morphogens during embryonic development [131]. By forming long- or short-ranged concentration gradients, the Hh ligands are involved in the specification of a plethora of different cell types and the development of different organs, including limbs, bones, muscles, and neurons [131]. In the adult organism, Hh signalling plays a role in stem cell maintenance in the brain, skin, teeth, and several other organs [132].

The Hh signalling pathway exhibits a unique signalling cascade to transduce signals from the cell surface to the nucleus (**Figure 9**). The secreted ligands are bound by the 12-transmembrane receptor Patched 1 (PTCH1) and its homologue PTCH2. The receptor forms a complex with several co-receptors to increase Hh ligand binding affinity [133]. When no ligands are present, Ptch receptors are present at the cell surface, in a specialised protuberance called primary cilium, and prevent the appearance of the G protein-coupled receptor Smoothened (Smo) at the cell surface. Signalling along the primary cilium, involving casein kinase 1α (CK1), protein kinase A (PKA), and glycogen synthase kinase 3β (GSK3β), leads to proteolytic processing of the Gli transcription factors into their repressor form [132]. There are three Hh-mediating transcription factors in mammals, GLI1, GLI2 and GLI3. The C-terminus of both GLI2 and GLI3 can be proteolytically cleaved off. In this truncated form they act as a transcriptional repressor, while the full-length conformation activates transcription of Hh target genes in the nucleus. In contrast, GLI1 only exists in its full-length form as a transcriptional activator. Interestingly, *Gli1* and *Gli2* are themselves target genes activated by Hh signalling, generating a positive feedback loop [134]. Upon binding of the Hh ligands to Ptch, it moves out of the primary cilium, releasing the blockage of Smo, which is consequently recruited to the cilium. This leads to the accumulation of the full-length activator forms of the Gli transcription factors, which then translocate to the nucleus to induce expression of Hh target genes [132]. Among the primary Hh-induced genes is *Ptch1*,

resulting in a negative feedback response. However, the molecular mechanisms underlying the suppression of Smo activity by Ptch and how the downstream signalling from Smo to Gli is mediated are still largely unsolved [133].

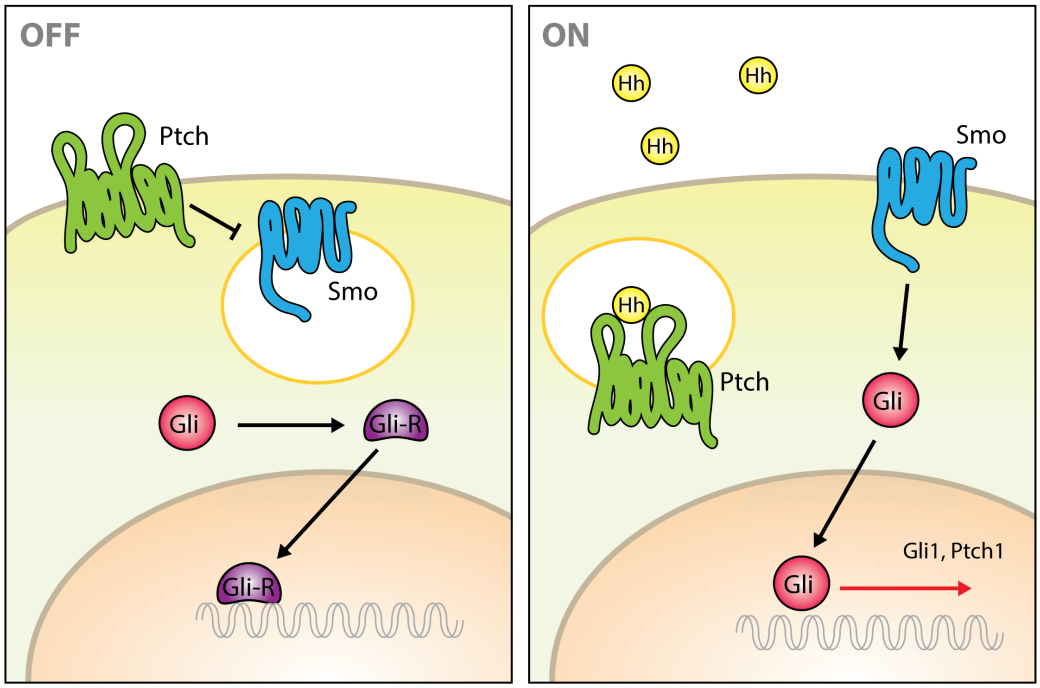

Figure 9. The Hedgehog signalling pathway (simplified).
In absence of ligand, Patched (Ptch) receptors are present at the cell surface and inhibit signalling of Smoothened (Smo). This causes cleavage of Gli transcription factors into a repressor form (Gli-R). Upon binding of the Hedgehog ligand (Hh) to Ptch, the inhibition of Smo is released. Through a largely unknown mechanism, Smo now permits the accumulation of the Gli activator form, which acts as a transcriptional inducer. Important target genes of the Hh pathway are Gli1 and Ptch1, creating regulatory feedback loops.

In the skin, Shh signalling was demonstrated to have a vital role in the early HF morphogenesis as development beyond the placode stage is abrogated in *Shh* knockout mice [135,136]. During the normal cycling of mature HFs, Shh is responsible for anagen induction, along with Wnt/β-catenin and Noggin (Bmp inhibition) [16,137]. *Shh* is also expressed in the matrix of the anagen HF, where it promotes proliferation of epithelial cells [138]. In line with the importance of the Hh pathway in HF specification and proliferation, ectopic activation of Hh signalling in epidermal cells leads to BCC initiation. The tumourigenic process closely mimics the early phases of HF development, which is reflected in a similar palisading cell morphology and overlapping gene expression profiles [139,140].

Less is known about the function of active Hh signalling in the resting telogen HF. The telogen dermal papilla and hair germ were shown to express *Gli1*, *Gli2*, and *Gli3*, with *Gli2* expression additionally being present in the basal layer of the bulge [34]. As a reliable readout for Hh activity, *Gli1* expression was also detected at the bulge-isthmus border of the

HF, where the sensory nerve fibres come into contact with the epithelial HF cells [34]. Potential sources of the Hh ligands are the nerve fibres close to the isthmus [34], and the dermal papilla, providing Hh ligands to the hair germ [141]. Low levels of *Shh* mRNA were also detected in the *Lgr5*$^{+}$ hair germ and bulge cells [27].

1.3.3 Basal cell carcinoma

BCC belongs to the group of NMSC and is the most common form of cancer diagnosed in individuals of European descent [142]. In contrast to SCC, which is often invasive and can cause mortality, BCCs are typically benign tumours and very rarely metastasise [142-144]. However, the fact that the aetiology of BCC is very well defined, compared to other types of cancer, renders it an ideal tumour type to study cancer initiation and development in pre-clinical models [145].

UV light is a major mutagenic driver of BCC carcinogenesis, as there is a high correlation between cumulative sun exposure and BCC incidence, and BCC more often develops in sun-exposed body areas, such as the face and limbs [146]. BCCs frequently appear as pale lesions that may be ulcerated or pigmented and grow into the dermis as nodules or strings [147]. The primary cell mass of the tumours consists of keratinocytes that resemble undifferentiated basal cells, and express K5, K14, K17, Sox9 and CDP, which are also markers of the early HF buds during embryonic development [139].

The development of BCC is closely linked to the specific deregulation of the Hh signalling pathway. Genetic linkage analyses of sporadic BCCs and patients with basal-cell nevus syndrome (or Gorlin syndrome) revealed mutation or loss of the *PTCH1* gene locus as the primary cause of BCC [148-150]. Up to 90% of sporadic BCCs carry mutations of the PTCH1 receptor [151]. The role of the Ptch receptor is to release suppression of Hh signalling upon ligand binding. Thus, inactivating mutations or loss of *PTCH1* activates the pathway even in absence of ligands. On the other hand, 10% of BCC patients show activating mutations of Smo, which acts downstream of Ptch [142]. Patients with Gorlin syndrome predominantly carry a heterozygous germ line mutation of *PTCH1*, rendering them highly susceptible to development of BCC and other neoplasms [152].

The primary target for clinical therapy of advanced BCC is inhibition of Smo activity. The most common drug in clinical use, Vismodegib, acts in a similar manner as the naturally occurring Hh inhibitor cyclopamine [153]. Yet, around 20% of cases develop Smo mutations, causing resistance to Vismodegib and recurrence of the disease [154,155].

A remarkable fact about BCC is that in contrast to other cancer types, it is not possible to maintain BCC cells in culture conditions for extended periods nor to transplant these cells as xenografts [142]. Therefore, animal models of BCC are a major resource for gaining insights into the molecular mechanisms of BCC development, interactions with the tumour stroma,

and drug responses. Importantly, cutaneous BCC development can be easily triggered by introduction of mutant components of the Hh pathway [145,156].

1.4 GENETIC TOOLS

Genetic mouse models are ideal to decipher the expression and function of a specific gene, and study the effects of activating a certain pathway *in vivo*. In the studies of this thesis several different systems were combined to detect, label and manipulate epidermal stem cells in mice.

To detect the expression of *Lgr5* and *Lgr6*, and initiate lineage tracing or other genetic manipulations in $Lgr5^+$ or $Lgr6^+$ cells, *Lgr5/6-EGFP-IRES-CreERT2* knock-in mice were used. In these mice that will be referred to as *Lgr5Cre* and *Lgr6Cre* mice, the enhanced green fluorescent protein (EGFP) gene sequence was inserted behind the endogenous promoter, replacing the *Lgr5*/*6* transcript. Thus, all cells that naturally express *Lgr5*/*6* can be identified by EGFP fluorescence, which is of particular advantage because no antibodies specific to LGR5 or LGR6 were available.

The second part of the inserted gene sequence contains an internal ribosomal entry site (IRES) and a fusion protein of the Cre recombinase with a modified oestrogen receptor (CreERT2). The IRES sequence allows a second protein (CreERT2) to be generated from the same mRNA, containing both the EGFP and CreERT2 coding sequences. The Cre recombinase is a bacteriophage P1 enzyme which is able to cut and re-ligate DNA sequences that are flanked by a specific Cre recognition sequence, called LoxP site [157]. With the addition of the oestrogen receptor-binding domain, the activity of the Cre enzyme can be controlled by exploiting the nuclear shuttling mechanism of the oestrogen-bound receptor [158]. When the oestrogen analogue tamoxifen is administered to the cells expressing CreERT2, the Cre relocates to the nucleus where it can exert DNA recombination (**Figure 10A**). This mechanism provides a tight temporal control of the genetic manipulation.

By placing the expression of Cre under a specific promoter, the Cre/LoxP system can be used for inducible lineage tracing of cells expressing the gene-of-interest. To this end, an additional allele carrying a conditional reporter gene has to be present in the mouse genome. These reporter alleles contain a LoxP-flanked stop sequence in front of the protein coding sequence that is removed upon Cre recombination, allowing subsequent stable expression of the reporter (**Figure 10B**). The reporter sequences are typically inserted into the genomic locus of a gene called *Rosa26* (*R26*), which is ubiquitously expressed [159]. Genes commonly used as reporter include fluorescent proteins (EGFP and derivatives) or *LacZ*, coding for β-galactosidase. Hence, after successful Cre recombination, the targeted cell (or

cell population) is permanently labelled by the reporter expression. Since the genetic modification is not reversible, all progeny of this cell is also labelled, which makes it possible to distinguish the cells that are derived from the originally targeted cell. In lineage tracing experiments, the entity of labelled cells that are derived from one cell are referred to as a "clone".

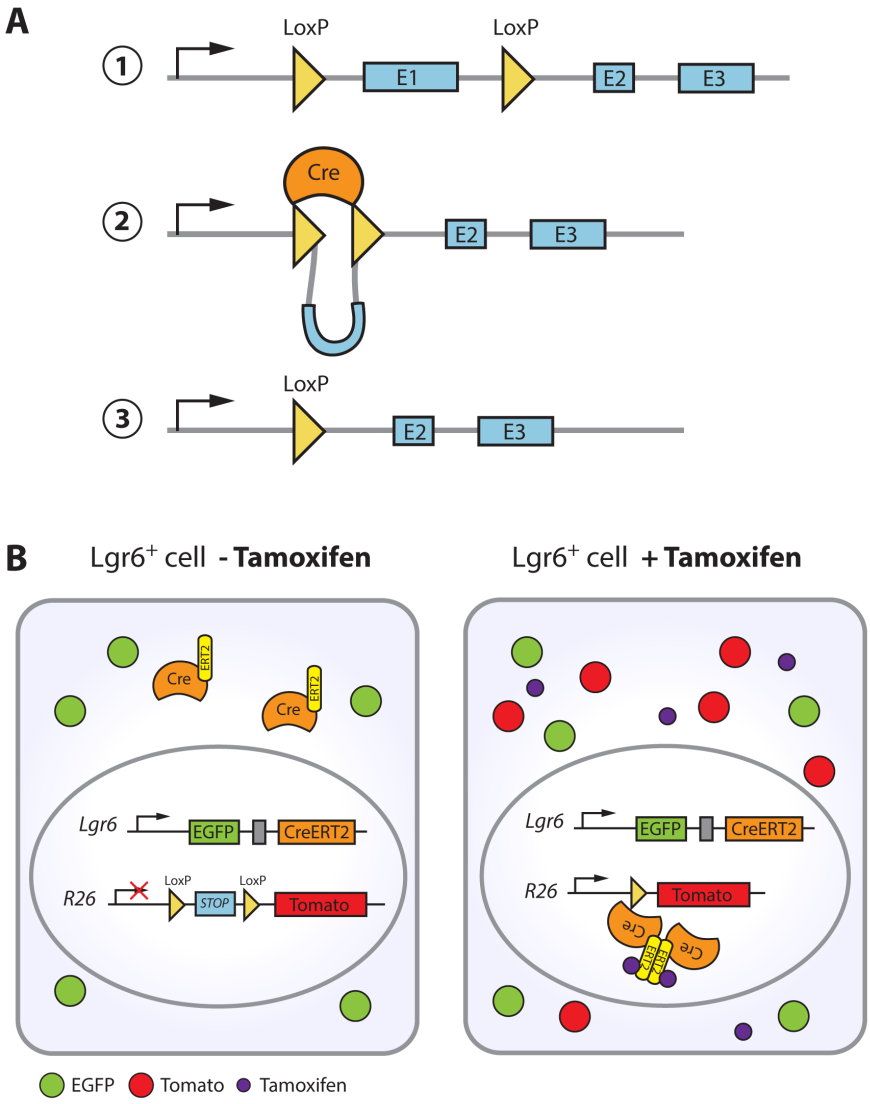

Figure 10. Mechanism of Cre/LoxP recombination and genetic lineage tracing.
(**A**) 1. The first exon (E1) of a gene is flanked by LoxP sequences (yellow triangles). The LoxP sites serve as recognition sites for the Cre recombinase. 2. The DNA sequence located between two forward-facing LoxP sites is removed by a Cre-mediated enzymatic reaction. 3. After re-ligation of the DNA, one LoxP sequence is retained. (**B**) A cell of an *Lgr6Cre/Tomato* mouse with active Lgr6 promoter expresses EGFP and CreERT2 recombinase. Tamoxifen binds to the ERT2 domain of the Cre fusion protein, leading to nuclear translocation of the Cre complex. Within the nucleus, Cre mediates the recombination of genomic DNA flanked by two LoxP sites. Thereby the stop codon in front of the reporter allele is removed. Subsequently, the Tomato fluorescent dye is permanently expressed in this cell and all of its progeny.

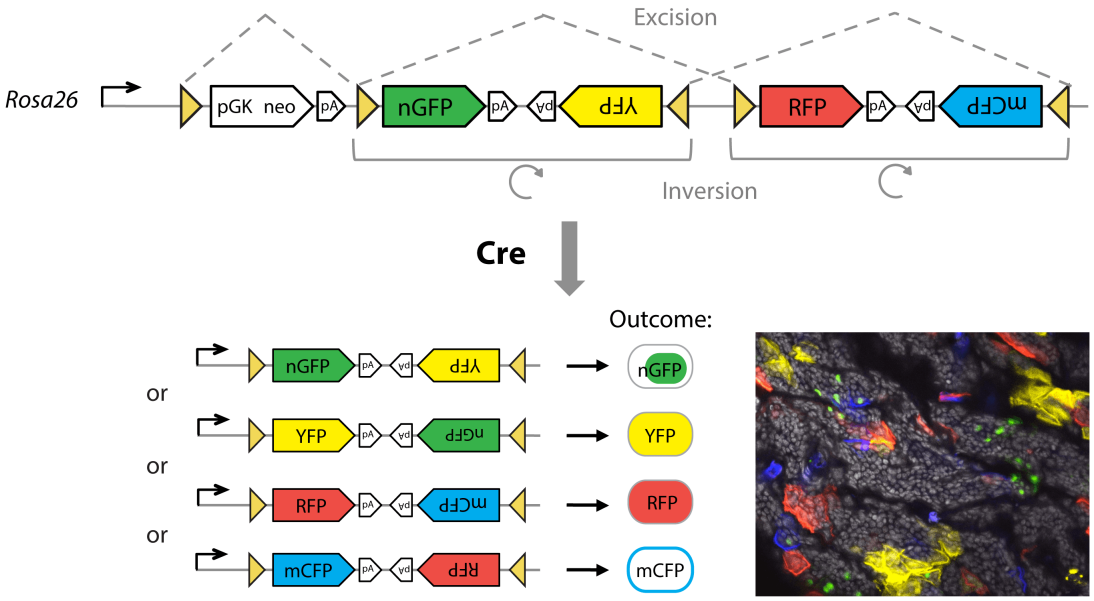

Figure 11. The Confetti multicolour reporter construct.
The Confetti cassette, inserted in the Rosa26 locus, contains a stop codon (pGK neo) and the genes encoding nuclear GFP (nGFP), cytosolic YFP and RFP, and membranous CFP (mCFP). The LoxP sites (yellow triangles) are arranged around the elements so that they permit either excision of the sequence between two LoxP sites oriented in the same direction or inversion of the sequence between two opposing LoxP sites. Thus, Cre recombination generates a random outcome of reporter expression. The inset depicts *Lgr6Cre/Confetti* traced epidermis with clones in all four colours, scanned by confocal microscopy. pA: polyadenylation signal.

To track the fate of individual stem cells or tumour-initiating cells, a high clone frequency and merging of clones need to be avoided. For this purpose multicolour reporters, in which the clone colour is randomly chosen for each labelled cell, offer considerable advantages over monochromatic reporters. Making use of the wide range of engineered fluorescent proteins, the first fluorescent multicolour reporter constructs, called Brainbow, were developed by Jeff Lichtman's group in Harvard and successfully used in mice to individually label neurons during development [160]. The Confetti construct used in the studies at hand is a modified version of the Brainbow-2.1, which has been inserted into the *R26* locus and includes a stop codon that renders the reporter expression inducible [67]. Recombination of this construct leads to random excision and flipping of the LoxP-flanked parts, which is governed by the specific arrangement of LoxP sites around the genes encoding the four fluorescent proteins (**Figure 11**). The final state of the recombined allele will stably express one of the four fluorescent markers, thereby permanently distinguishing the offspring of the individual, targeted cell.

Besides lineage tracing, the Cre/LoxP system was used to activate the Hh signalling pathway by conditional knockout of *Ptch1*. Exon 2 of the *Ptch1* gene was encompassed by LoxP sites, to create a knockout upon Cre recombination [161]. This strategy closely mimics the aetiology of sporadic BCC in humans, as up to 90% of patients show inactivating mutations

of *PTCH1* [151]. Another mechanism that was used to induce BCC development in a mouse model is the tet-off system. This strategy is based on the bacterial tetracycline (tet) operon, and allows control of gene expression by administration of tetracycline or derivatives, such as doxycycline [162]. To specifically express a gene-of-interest, the promoter must be replaced by a tet-response element (TRE) and the cells have to express the tet-transcriptional activator (tTA). In absence of tet, tTA binds to the TRE sequence and initiates transcription of the target gene but when tet is present and bound to the tTA, target-gene transcription is blocked. In our studies we employed the tet-off system to express the human GLI1 protein in basal cells. To this end, we used mouse lines with transgenic insertion of tTA under the control of the *Krt5* promoter and the *GLI1* gene sequence under the control of a TRE, and induced gene expression by doxycycline withdrawal.

The biggest advantage of using mouse models to address biological research questions is that the cells are kept within their natural environment with all influencing factors present. In contrast, isolated cells or cell lines which have been sustained in culture over longer periods of time are subjected to highly artificial *ex vivo* conditions that change the nature of the cells, limiting the relevance of the conclusions. Hence, uncovering the natural behaviour of stem cells in homeostasis is only possible within a living organism. Moreover, organ development and contributions of distinct stem cell populations are difficult to recapitulate in cell culture.

However, there are also certain drawbacks in the current *in vivo* models used in stem cell research. The inducing agents (e.g. tamoxifen and doxycycline) may cause side effects that perturb homeostasis and influence the outcome of the experiment [163,164]. Also the expression of the fluorescent proteins may be toxic or place the labelled cell population at a disadvantage [165]. Another challenge is real-time imaging, which is much harder to achieve in living animals compared to cell cultures. Thus, in our studies we had to rely on static “snap shots” of the traced clones to infer the dynamics of the stem cell expansion. A more general problem is the activation of a pathway beyond the physiological levels (e.g. by overexpression of a transcription factor), which limits the conclusions and the possible translation of the results to the human body. Moreover, ethical considerations come into play when using animals for research. It is critical to keep the number of mice to a minimum and ensure that the mice experience the lowest amount of inconvenience and suffering during the experiments.

2 AIMS OF THE THESIS

The general aim of this study was to investigate the role of *Lgr5*- and *Lgr6*-expressing keratinocytes during epidermal homeostasis, wound healing and tumour formation, and consequently compare the properties of stem cells in different compartments of the skin.

Specific aims:

Paper I Investigate the effect of wounding on BCC formation and the role of $Lgr5^+$ HF stem cells in this process

Paper II Identify the stem cell characteristics and molecular identity of $Lgr6^+$ stem cells in different epidermal compartments (isthmus, SG, and IFE) during homeostasis

Paper III Study the potential of $Lgr6^+$ stem cells in different compartments to give rise to BCC and analyse the niche factors that influence their susceptibility

3 RESULTS AND CONCLUSIONS

3.1 PAPER I

Wounding enhances epidermal tumourigenesis by recruiting hair follicle keratinocytes

BCC is one of the most common forms of cancer in humans and its incidence has been repeatedly associated with cutaneous wounds [109,166]. Although a prolonged inflammatory response has been shown to be one of the contributing factors [167], the cellular and molecular mechanisms how exactly wound healing affects skin cancer development were unknown. In this study we investigated the mechanism how wounding influences the tumourigenic capacity of epidermal and HF stem cells.

To induce BCC in mice, we used several different mouse models, which activate Hh signalling in the skin. The first model enables ectopic expression of human GLI1 in all basal keratinocytes, by placing the *GLI1* gene under the control of a tetracycline-responsive element (*TREGLI1*) and combining it with a K5-dependent tetracycline activator (*K5tTA*). In the second model, the *Ptch1* gene is deleted in basal cells, by inducing a Cre-mediated knockout of *Ptch1* in K5-expressing cells (*K5Cre/Ptch*$^{fl/fl}$). Following activation of GLI1 expression or *Ptch1* knockout, both models showed extensive development of BCC-like lesions, arising in the HFs and IFE.

When full-thickness wounds were introduced in *K5tTA/TREGLI1* and *K5Cre/Ptch*$^{fl/fl}$ mice, we found that at the wound sites the BCC-like lesions were significantly larger and more progressed than in unwounded skin. In *K5Cre/Ptch*$^{fl/fl}$ mice additionally the number of lesions was increased at the sites of wounding compared to normal IFE. These results show that wounding has a profound impact on tumour growth, and in the *Ptch1* deletion model also on the tumour initiation frequency.

Since it is known that HF cells are mobilised to the wound during re-epithelialisation, we tested if the recruited HF cells are responsible for the enhancement of BCC development in wound areas. Using *Lgr5Cre/LacZ/K5tTA/TREGLI1* mice to label and trace *Lgr5*$^{+}$ cells during tumour initiation revealed that progeny of *Lgr5*$^{+}$ HF cells indeed migrate to the IFE of scar epidermis and contribute to the formation of BCC-like lesions, while no contribution to IFE-associated lesions was detected in unwounded skin.

To further support the notion that HF progeny can establish tumours in the wound epidermis, we generated *Lgr5Cre/Ptch*$^{fl/fl}$ mice to limit oncogenic transformation to cells of the lower bulge and hair germ. Activation of Hh signalling in *Lgr5*$^{+}$ cells caused development of BCC-like lesions that were restricted to the HF compartment. However after wounding, we detected multiple lesions in the wound epidermis, which shows that HF cells contribute to tumour formation in the IFE as a response to wound healing. Interestingly, only full-thickness

incision and excision of skin, or TPA treatment were sufficient to trigger emigration of *Lgr5*$^+$ progeny to the IFE. Superficial wounding did not affect the tumour incidence.

Taken together, our results indicate that the wound healing response causes HF cells carrying tumourigenic mutations to migrate and establish BCC growth in other epidermal compartments, thereby enhancing tumour development in wounded skin.

3.2 PAPER II

Dynamics of *Lgr6*$^+$ progenitor cells in the hair follicle, sebaceous gland, and interfollicular epidermis

Lgr6 was initially described to be a marker of multipotent stem cells in the HF, giving rise to cells in the isthmus, SG, and IFE [33]. However, the dynamics of stem cell renewal and the relationship between the lineages in the different HF compartments above the bulge were not known. The aim of this study was to fully characterize *Lgr6* expression in adult dorsal skin and then study the fate of *Lgr6*$^+$ cells in the different compartments, in order to elucidate the mechanism of epidermal regeneration by *Lgr6*$^+$ stem cells.

We employed heterozygous *Lgr6Cre* knock-in mice to visualise the expression of *Lgr6* in the telogen HF and epidermis by EGFP fluorescence in tissue whole-mounts. The highest level of EGFP expression was detected in the lower and mid-isthmus of the HF, followed by medium EGFP expression levels in scattered cells in the basal layer of the IFE and SG, and in the inner bulge layer. When enhancing the signal EGFP by immuno-staining, we found rare cells expressing low levels of *Lgr6* in the outer bulge and the dermal papilla. Cell sorting of *Lgr6Cre* keratinocytes revealed that around 20% of IFE basal cells are *Lgr6*-positive. However, there was no difference in the *Lgr6*-positive and -negative IFE population regarding the fraction of dividing cells, indicating that *Lgr6* expression is not a general marker of proliferative epithelial cells, even though it is preferentially expressed in highly proliferative epidermal regions.

To show that *Lgr6*$^+$ IFE cells possess stem cell properties, we analysed lineage tracing using a multicolour fluorescent reporter strain (Confetti). Clones – labelled cells originating from a single labelled cell – developed from all compartments expressing high and medium level of EGFP (*Lgr6*): isthmus, SG, IFE, and inner bulge. Long-term analysis revealed that clones survived and expanded in all of these compartments except in the inner bulge, where cells do not divide. On the basis of the Confetti clone colours, we were able to determine that IFE clones evolve independently of HF clones. The colours of IFE clones did not match the clone colours in nearby HFs to a greater extent than expected for a random colour correlation of independent clones. These results confirm existing data that the IFE contains a separate stem cell pool [55,59,102] and establish that *Lgr6*$^+$ cells function as IFE stem cells.

Since SGs also displayed *Lgr6* expression as well as long-term surviving clones, we investigated the relationship between the *Lgr6*$^+$ populations in the isthmus and the SG. It was not entirely clear if independent resident stem cells exist within the SG or if HF stem cells also replenish the SG, as no exclusive SG stem cell marker has been identified. We found that *Lgr6*$^+$ derived SG clones were maintained over long-term tracing periods without any connection to the HF, indicating that *Lgr6* also marks resident stem cells in the SG. Moreover, colour correlation analysis revealed that the percentage of SG clones with colour-matched clone in the isthmus was not significantly greater than expected for a random correlation. Thus, the two compartments are presumably independent, although a minimal exchange of cells between the isthmus and the SG cannot be excluded.

The next goal was to define the mode of stem cell renewal of the *Lgr6*$^+$ populations in the three epidermal compartments. Recent work strongly supports that murine IFE is maintained by a single basal progenitor cell population that divides in a stochastic manner [64,65]. Thus, tissue regeneration is governed by the balanced stochastic fate outcome of the progenitor population as a whole (population asymmetry) as opposed to a selected stem cell population that strictly divides asymmetrically (stem cell asymmetry). Using lineage-tracing experiments the underlying population asymmetry can be detected by a stochastic clonal expansion, leading to increasing clone size and decreasing clone frequency over time. We tested these measures in the *Lgr6*$^+$-derived isthmus, SG, and IFE Confetti clones. Remarkably, in all compartments the clonal behaviour accorded with the predicted outcome for population asymmetry. This suggests that even in other epidermal compartments with a more complex architecture than the IFE, population asymmetry is the underlying mode of tissue renewal, and the different populations likely have similar stem cell potency.

Lastly, we investigated the differences between the individual *Lgr6*$^+$ populations at the transcriptional level, to investigate whether the *Lgr6*$^+$ populations are more similar to each other or rather represent the signature of their respective compartment. We isolated *Lgr6*$^+$ cells from the IFE (SCA-1$^+$) and the HF/SG (SCA-1$^-$) by flow cytometry and performed RNA-sequencing. The gene expression profile of *Lgr6*$^+$ IFE or HF/SG cells closely resembled the signature of the *Lgr6*-negative cells in the respective compartment and did not disclose a general gene set associated with *Lgr6* expression. However, *Lgr6*$^+$ HF/SG cells revealed several isthmus-specific genes such as defensin beta 6 (*Defb6*), cystatin E/M (*Cst6*) and parathyroid hormone-like peptide (*Pthlh*).

In summary, this study closely examined the location, interaction and transcriptional networks of *Lgr6*-expressing stem cells in the skin. We found that *Lgr6* expression in basal cells is not specific to any HF compartment, hair cycle phase or differentiation course, and is not strictly associated with the activity of a specific pathway. Moreover, the mode of stem cell renewal was identical in all independent *Lgr6*$^+$ populations, which denotes population asymmetry as a robust mechanism of tissue regeneration in all permanent compartments of the epidermis.

3.3 PAPER III

Healthy skin harbours pre-existing micro-niches that promote tumour formation

It is increasingly recognized that the tumour microenvironment has a profound influence on the growth of a tumour [110]. Cancer-associated fibroblasts, inflammatory cells, endothelial cells, and many other cell types jointly create a milieu for solid cancer cells to thrive. Hence, we asked if such external factors also govern tumour development at the early tumour-initiation stage. Having characterized the stem cell dynamics of *Lgr6*$^+$ cells during homeostasis, we employed this model to target epidermal progenitor cells in various epithelial compartments (isthmus, SG, IFE, bulge) that are subjected to distinct environmental cues. Activation of the Hh pathway enabled us to study whether any of these compartments is more susceptible to BCC development than the others and what are the underlying differences that cause this diversity.

To induce BCC formation in mice, we generated *Lgr6Cre/Tomato/Ptch1*$^{fl/fl}$ mice, in which the expression of *Ptch1* is abrogated upon Cre recombination. Initiating simultaneous lineage tracing and *Ptch1* knockout in *Lgr6*$^+$ cells of adult mice, we observed rapid development of lesions, resembling early BCCs. These lesions, however, prevailed at the lower isthmus region. The response of IFE cells to Hh pathway activation was much less pronounced and SG cells did not react during the observation period. Strikingly, we found large IFE lesions that appeared in a regular pattern, progressing at a similar rate as the isthmus tumours. To validate *Lgr6*$^+$ IFE cells as a possible source for these rare lesions, we induced BCCs by ectopic expression of GLI1, which activates Hh signalling more strongly than *Ptch1* deletion, and simultaneously traced *Lgr6*$^+$ cells. Continuous expression of GLI1 in all basal cells indeed led to the rapid appearance of BCC-like lesions in the IFE, which originated from *Lgr6*-positive and -negative cells. This shows that *Lgr6*$^+$ IFE cells have the capacity to initiate IFE tumours.

But why are only a few of them susceptible to mild activation of Hh signalling? Examining the niche composition and factors influencing gene expression in the isthmus, we hypothesised that the cutaneous nerve fibres, which provide Hh ligand to the lower isthmus, may be a dominant external factor promoting BCC emergence in the HF. When inspecting *Gli1* expression, which indicates active Hh signalling, in the IFE, we found that *Gli1* is indeed expressed by a rare subset of IFE epithelial cells and adjacent mesenchymal cells, alike the isthmus niche. These *Gli1*$^+$ IFE cells localize to specialized touch perception structures, called touch domes, which harbour nerve fibre endings connected to neuroendocrine Merkel cells. Moreover, *Lgr6*$^+$/*Gli1*$^+$ cells were detected within touch domes, accounting for another similarity to the isthmus niche, where *Lgr6*$^+$/*Gli1*$^+$ cells are also present.

To provide further evidence that *Lgr6*$^+$/*Gli1*$^+$ touch dome IFE cells are able to generate BCC-like lesions in the IFE we generated *Gli1Cre/Tomato/Ptch*$^{fl/fl}$ mice. Upon tamoxifen

administration, these mice developed large Merkel cell-associated tumours within the IFE, confirming that touch dome cells are founders of BCC. To substantiate that also in the *Lgr6* model IFE tumours predominantly arise from $Lgr6^{+}$ touch dome cells, we analysed the presence of Merkel cells around the IFE lesions in $Lgr6Cre/Tomato/Ptch^{fl/fl}$ mice. BCC-like lesions that were associated with Merkel cells were significantly larger than tumours in the remaining IFE. This collectively indicates that the isthmus and the touch dome niche promote BCC initiation and progression. Epithelial cells within both niches are influenced by extrinsic nerve signals, which facilitate a constitutive activation of the Hh pathway, thereby lowering the threshold for BCC growth.

Remarkably, the isthmus and touch dome niche do not only resemble each other by the presence of nerve fibres and active Hh signalling. We found several additional genes that are expressed in both niches, e.g. extracellular matrix protein *versican* and transcription factor *Sox9*. Intriguingly, many of those genes have been associated with BCC in mouse models and human BCC. This suggests that there are pre-existing micro-niches within the skin that predispose to BCC development by inducing the expression of BCC-related genes.

4 GENERAL DISCUSSION AND PERSPECTIVES

4.1 SIGNIFICANCE AND DISCUSSION OF THE WORK

The main goal of this thesis was to study the behaviour of epidermal stem cells under different conditions, and to get insights into the capabilities and flexibility of stem cells within different niches of the epidermis. The marker genes *Lgr5* and *Lgr6* allowed targeting cells within different locations of the skin so that the individual potential of those subsets could be determined. The work presented in this thesis reinforces the enormous plasticity of epidermal cells, demonstrated by their involvement in wound healing, their distinct compartmental contributions during homeostasis as well as their tumour initiation potential.

Although many experiments had been conducted in previous research to identify and characterise numerous different stem cell populations in mouse skin [168,169], several important questions regarding the homeostasis of the epidermis remained. How are the separate parts of the epidermis precisely maintained and how are the different stem cell populations coordinated? To better understand the interactions between cell populations in the HF and the IFE, we performed lineage tracing of *Lgr6*$^+$ keratinocytes during tissue homeostasis in **Paper II**. An important prerequisite for these experiments was to accurately determine the expression pattern of the marker gene and test the floxed reporter expression with *Lgr6Cre* as a driver (which turned out to be highly distinct for the different reporter alleles, see **Paper III**). The results of the Confetti lineage tracing signified that the targeted *Lgr6*$^+$ basal cells exhibit self-renewal potential irrespective of their location within the different epidermal compartments. Moreover, the three main *Lgr6*$^+$ populations in the isthmus, SG, and IFE were largely independent of each other, maintaining predominantly their respective compartment. These findings match other predictions that the progeny of stem cell populations in the permanent part of the HF do not cross compartment boundaries [35,170]. The concept of tissue compartmentalisation maintains that compartments are delimited by tightly regulated boundaries that prevent stem cell progeny from leaving their home territory [171]. Stem cell compartmentalisation is also found in organs with a less distinct morphological demarcation of the compartments as in the HF, such as the mammary gland [172], the prostate [173] and the *Drosophila* midgut [174,175]. However, it is still not fully clear how these compartment borders are established.

Moreover, our results imply that there is no inherent hierarchy between the progenitors within different locations, even though they follow distinct differentiation pathways, e.g. stratified epidermis in the IFE, sebocyte maturation in the SG, and differentiation in the isthmus without formation of a granular layer [176]. The fact that the dynamics of the clone expansion over time was very similar in the three compartments, overall matching the predictions of the committed progenitor model, suggests that the stem cell populations are

equipotent rather than following a hierarchy or being subject to non-neutral drift. However, this assumption must be substantiated with more precise data, which allow the determination of the modelling parameters, such as stem cell division rate and stem cell fraction [64]. Only if the parameters of the stochastic model are concordant for the three populations, equal potency can be inferred. Several examples have shown that a proliferation bias between two populations undergoing population asymmetry, e.g. by introduction of tumourigenic mutations leading to dominant expansion of clones, is reflected in altered stem cell division rate and stem cell fraction [177-179]. Another recent observation supports equal intrinsic potency of all basal HF cells. Cells from the upper HF compartments were able to replace bulge cells after laser ablation and adopted a bulge cell fate in the new niche [31]. This also highlights that the location seems to be the major determining factor for the different differentiation paths in the epidermal compartments.

One drawback of cell population-based lineage-tracing experiments is the fact that the fate of an individual stem cell cannot be tracked directly. Hence, correlations between the exact position in the tissue and the associated fate outcome are hard to draw, if not impossible. However, these questions are highly interesting, e.g. if there is a pre-defined hierarchy in the telogen bulge, determining which cells will generate each layer of the IRS and ORS in the next anagen cycle, respectively. Using intra-vital fluorescent imaging, the group of Valentina Greco has now started to illuminate single-cell contributions during anagen induction and catagen, and correlated positional cues to a particular fate outcome [31,180,181].

In **Paper I**, we have shown that wounding and tumour induction can profoundly change the behaviour of cells compared to homeostasis, and how wounding and tumour development act together to alter the cell fate. The discovery that bulge and hair germ cells can preserve tumour-initiating mutations and transfer them to another epidermal compartment offers an intriguing explanation of how a wound environment accelerates BCC development.

Expression of full-length GLI1 in K5-positive basal keratinocytes demonstrated that BCC can also develop from IFE cells, and not only from HF cells, even though BCC cells have a similar phenotype to HF cells and BCC initiation resembles HF morphogenesis [139,140]. This finding was confirmed by several publications, using other genetic models, e.g. expression of constitutively active Smo under the K5 promoter and expression of a GLI2 activator form (GLI2ΔN) in various epidermal compartments [28,126]. Similar to Hh activation the wound response also seems to re-program the cells into a developmental-like state [89]. The regeneration process relies on stimulating the same pathways and fate-switch mechanisms to restore tissue function that are also active during HF morphogenesis, which is reflected in its extreme case by *de novo* HF induction in large wounds [106,108,109]. Hence, when ectopic Hh coincides with a chronic wound environment the factors promoting BCC are potentiated and might thus result in accelerated cancer formation.

In **Paper III**, we have deepened the understanding of which epidermal populations are most susceptible to tumour formation and which factors play a role. As was shown in **Paper I**, almost all basal keratinocytes are able to initiate BCC formation in response to augmented Hh

signalling, albeit with varying sensitivity [182]. This became most apparent when *Ptch1* knockout was driven by the broadly expressed promoters of *Krt5* and *Lgr6*. Thus, it is interesting to compare the dynamics of BCC development in *Lgr5Cre/Ptch*$^{fl/fl}$ and *Lgr6Cre/Ptch*$^{fl/fl}$ mice to identify the differences between individual epidermal stem cell populations. It is difficult to compare results obtained in different studies using diverse mouse models and types of Cre activation, but in this case the same genetic modifications were introduced and the same protocols were followed. Overall, tumours were formed significantly faster by *Lgr6*$^{+}$ isthmus cells, which responded within days, compared to *Lgr5*$^{+}$ bulge/hair germs cells, which took months to grow into advanced lesions. This discrepancy evidently highlights that the niche factors have a profound effect in adjusting the sensitivity of cells to becoming tumour-initiating cells.

The results presented in **Paper III** support the concept of pre-existing micro-niches in healthy tissue that predispose for tumour development by providing a tumour-like microenvironment for early transformed (cancer-initiating) cells. It is more and more recognised that the microenvironment of a cancer plays a decisive role in promoting tumour growth and progression [183,184]. Examples of non-transformed cells that support tumour growth are fibroblasts in contact with the epithelial cancer cells that produce growth factors, or endothelial and immune cells that are recruited by signals from the tumour and promote blood vessel sprouting. Our study expands this concept to the pre-tumour stage by attributing cancer-promoting properties to the microenvironment of healthy epithelium.

The key finding that the nerve-proximal niches, isthmus and touch dome, promote BCC formation was confirmed in a simultaneous study using various other Cre drivers [185]. Furthermore, this study presented evidence that de-nervation reduces *Ptch1* knockout-driven tumour development in the touch dome, indicating that nerve-derived factors contribute to the cells' enhanced sensitivity to transformation.

The observations in **Paper III** that *Gli1*-expressing keratinocytes are most susceptible to ectopic Hh and BCC formation, may appear contradictory to the fact that *Gli1* is also expressed in the hair germ of telogen HFs [34] and should overlap with *Lgr5* expression. Yet *Lgr5Cre/Ptch*$^{fl/fl}$ cells were much less responsive than the *Gli1Cre/Ptch*$^{fl/fl}$ populations in other parts of the epidermis (see **Paper I**). Cells in the isthmus and touch dome are normally not primed to generate cells of the hair lineage but follow differentiation into stratified epidermis. Generalising these observations, basal cells of the epidermal lineage seem to be more prone to develop BCC than cells participating in hair regeneration. It could thus be speculated that *Gli1*$^{+}$ hair germ cells may already have a machinery in place that is adapted to control fluctuating Hh signals in the context of the hair cycle. Therefore, these cells might be less sensitive to enhanced Hh and do not quickly transform into cancer cells.

The general concept supported by all three studies tracing *Lgr5*- and *Lgr6*-expressing epidermal stem cells is that basal cells in the different epidermal compartments have their specific function during homeostasis without conforming to a hierarchy. Moreover, they demonstrate a high plasticity in switching fates in response to perturbations. In summary, the

results highlight the influence of environmental factors on tissue stem cell behaviour, which can determine cellular properties, such as susceptibility to tumourigenic transformation, and contribution to wound healing.

4.2 IMPLICATIONS FOR FUTURE RESEARCH

4.2.1 Interactions between *Lgr5*$^+$ and *Lgr6*$^+$ cell populations

Lineage tracing performed by different groups are difficult to compare due the variations in the methodology. Especially, different transgenic mouse models to induce Cre recombination or direct reporter expression, different tracing protocols, and non-standardised analysis methods, result in significant variation between individual studies. Therefore, the interactions between the multitude of HF stem cell lineages marked by distinct genes have remained unclear. The results of the studies presented here support that during homeostasis the progeny of distinct stem cell populations do not mix significantly. This implicates a compartmentalisation of the different parts of the HF, with borders being maintained between the compartments.

Despite the same genetic strategy and lineage tracing protocols, comparing the lineage tracing patters between *Lgr5Cre* and *Lgr6Cre* mice is still not trivial because the progeny of the two populations cannot be labelled and discriminated simultaneously. Nevertheless, it seems that during the telogen phase there is only minimal cellular interchange between the bulge and the isthmus, insinuating a border between the two compartments. *Lgr5*$^+$ cells do not frequently trace into the isthmus and likewise contribution of *Lgr6*$^+$ progeny to the bulge was only detected towards very long tracing periods (~1 year). In a similar fashion, it was observed that HF infundibulum cells do not trace into the IFE during homeostasis, maintaining a similar "invisible border" [58,59].

Future studies, potentially involving alternative recombination strategies (e.g. combining Cre/LoxP with Flp/FRT recombination to label two individual populations simultaneously), will be necessary to resolve the exchange between compartments and discover the factors that define the borders in the epidermis. In the case of the bulge-isthmus border, extrinsic signals may play a role in specifying the cellular border, e.g. the interactions with nerve fibres and NCPCs, the arrector pili muscle, or the presence of the inner bulge cells may modify the cell behaviour [186]. Moreover, the properties of the cells in the isthmus and bulge are intrinsically distinct; bulge cells are quiescent and have large nuclei whereas isthmus basal cells are mitotically active and have a small cell size. These inherent differences may also act in preventing a mixing of the cell lineages. Additionally, cell-cell interactions are guided by surface receptor expression such as ephrin receptors, which are associated with boundary

formation [187]. Ephrins and their receptors are present in the epidermis, and thus are a potential subject for further research [188].

4.2.2 Regulation of *Lgr6* expression

The gene expression analysis of *Lgr6*-expressing keratinocytes presented in **Paper II** aimed at expanding the knowledge about the regulation of *Lgr6* expression in skin and what discriminates *Lgr6*-expressing cells from *Lgr6*-negative cells. On the global level, combining cells from all the different sampled compartments, there was no significantly enriched gene signature or pathway, which was specifically correlated with *Lgr6*. This implies that *Lgr6* gene regulation is context dependent; in each niche different signals might be able to activate the gene expression. In support of this hypothesis, different studies reported *Lgr6* up-regulation in response to various conditions: dominant negative LEF1 expression in K15-positive bulge cells [56], Wnt3a stimulation of bulge cells [189] and overexpression of *Stat3c* in basal cells [190]. Conversely, β-catenin knockout [189] and splicing factor *SRSF6* induced *Lgr6* down-regulation [191].

A study by Liao and Ngyen in 2014 proposed that *Lgr6* expression, similarly to *Gli1* expression, is established by signals arising from proximal nerve fibres, based on the observation that de-nervated skin did not exhibit *Lgr6* reporter expression [79]. Led by the assumption that *Lgr6* expression in the HF and IFE may be governed by extrinsic signals from the microenvironment such as cutaneous nerve endings, we conducted a preliminary study to analyse the dynamics of *Lgr6* expression in the IFE. Strikingly, short-term lineage tracing, after labelling almost all *Lgr6*$^+$ cells with permanent Tomato expression, revealed that *Lgr6* expression seems to be an invariable state of certain IFE cells and is not fluctuating between different cells. The small, unlabelled fraction of *Lgr6*$^+$ cells did not increase over time, which would have been expected if *Lgr6* expression was turned on in unlabelled *Lgr6*-negative cells during the tracing period (Füllgrabe and Kasper, unpublished). Thus, *Lgr6* expression is probably not a stochastic event but more likely a permanent state that is maintained in certain cells. To confirm that peripheral nerves induce *Lgr6* expression, these results must be substantiated by further research, additionally assessing the co-localisation of *Lgr6*$^+$ cells with nerve endings protruding into the IFE.

4.2.3 Function of the LGR6 receptor in the skin

A yet unanswered question of tremendous interest is the function of the LGR6 receptor and its role in keratinocytes. It has been claimed that LGR6 acts similarly to LGR4 and LGR5 as an R-spondin receptor, enhancing canonical Wnt signalling [82,84-86]. However, convincing experimental data is lacking. Only one study presented results that attest human LGR6 a certain R-spondin binding affinity [192]. Nevertheless, the downstream functional effect of this interaction remains elusive [193]. It may be possible that LGR6 instead mediates

suppression of Wnt signals by acting as an R-spondin decoy receptor. The partial overlap of the *Lgr6* and *Lgr4/5* expression patterns in murine epidermis [33] could be an indication that LGR6 may sequester R-spondins and prevent their binding to LGR4 and LGR5 as part of a negative feedback loop. LGR6 would thereby aid in establishing an activity gradient of the Wnt signals between the bulge (active Wnt), and the upper part of the HF and the SG (suppressed Wnt).

In order to gain further insights into the role of the LGR6 receptor in the murine skin, we assessed the phenotype of *Lgr6* knock-in mice in more detail. In a preliminary study, we analysed gene expression of keratinocytes from homozygous *Lgr6Cre* mice, in which the knock-in cassette should abrogate transcription of the *Lgr6* gene, starting at the first exon. Comparing homozygous *Lgr6Cre* cells to heterozygous or wild type cells by microarray analysis revealed a general two-fold down-regulation of *Lgr6* expression, indicating that *Lgr6* transcription is not completely suppressed in these mice (Füllgrabe and Kasper, unpublished). This partial knockout may be due to alternative transcriptional start sites further downstream in the gene locus. This was substantiated by real-time PCR analysis, showing that transcripts of *Lgr6* from exon 4 are present in homozygous mice, which were thus not influenced by the presence of the knock-in cassette. However, it is unclear whether these transcripts are translated into proteins and if the potential N-terminally truncated version of the receptor exerts a different function as the full-length version. On the global level, homozygous *Lgr6Cre* keratinocytes showed increased transcription of a significantly enriched cluster of olfactory receptors. Down-regulated genes were associated with lipid synthesis, pointing towards a co-regulation of *Lgr6* expression with SG-specific genes and thus a role of *Lgr6* within sebocytes. The correlation of *Lgr6* reduction with the increased expression of olfactory receptors seems odd and further experiments are needed to validate these results. Remarkably, *Lgr6* is highly expressed in the olfactory brain of adult C57BL/6 mice (Expression Atlas/ArrayExpress accession E-MTAB-3579) [194,195]. Moreover, *Lgr6* expression was found in taste bud stem cells and in cells of the developing mouse cochlea in two very recent studies [196,197]. This insinuates a general association of *Lgr6* with sensory perception.

4.2.4 Regulation of stem cell renewal

The recent discoveries deciphering the stochastic nature of stem cell divisions have challenged old paradigms of stem cell biology and opened up new theories. The results presented in **Paper II** deepen the understanding of epithelial tissue renewal in adult organisms. The actively proliferating epidermal regions such as the upper HF compartments, the SG, and the IFE, all show the same pattern of stem cell renewal. This behaviour is also inherent to many other rapidly renewing epithelia throughout the whole body [198]. The high conservation of this process demonstrates its significance. However, the mathematical modelling only describes the behaviour of the cells we can measure, it does not expose the

underlying cause of the cell division outcome. So how is stochastic stem cell division regulated molecularly?

The view that there is a dedicated set of stem cells within a certain tissue, marked by a specific gene expression signature, does not seem to hold true [199]. The current models of stochastic stem cell renewal seem to be in line with the conception that all basal cells have the same intrinsic potential to be long-lived and thus all basal cells could be considered as stem cells. But at the same time this means that all basal cells also have the same likelihood to terminally differentiate.

The question remains if there are marker genes that correlate with a pre-determined fate outcome already before cell division is taking place. To find out if we really can distinguish between the three kinds of cell divisions, we would need to make use of sophisticated live-fate tracking of single-cells [31,181,200]. Combining live imaging *in vivo* or *in vitro* with fluorescent gene-expression reporters would enable testing if certain genes are specifically expressed in cells that choose a certain fate after division, e.g. a gene that is only expressed in those 10% of basal cells that are about to undergo symmetrical division, yielding two stem cells. This method would also yield some clues whether the position of a certain cell and its microenvironment play decisive roles in this decision.

Without marker genes or any other indication that there is indeed a difference between basal cells with a specific fate outcome or differentiation status, it is not sensible to distinguish stem cells based on fate outcome because the fate cannot be determined at the stage of the cell division. At this stage it is difficult to define what is a stem cell at all. Cells that would normally undergo differentiation can even turn back and change fate to regain stem cell capacity in response to injury or stress, and thus the cells may retain their stem cell *potential* all the time [199].

Another concept of how stem cell division and self-renewal might be regulated in the epidermis is an entirely self-organising system. Nothing is pre-determined and stem cells divide as long as there is space. If cells lose their contact with the basement membrane because they are pushed upwards after division, the differentiation programme is induced. When basal cell density becomes higher, the cells pause replicating. Research on the effect of mechanical forces on cells and their gene expression has already effectively shown that the physical environment alone can have a major impact on the activated pathways [45,201]. Moreover, certain pathways have been identified that are regulated by physical signals and can transmit these stimuli to the nucleus, such as the Hippo pathway and Yap/Taz transcription regulators as key mechanotransducers [202-204]. Again interactions between cells and the extracellular matrix as well as cell-cell interactions are central for signal transduction in such a system. This is a compelling theory because of its simplicity, offering much potential for future research.

4.2.5 Wound healing and tumourigenesis

Some questions also remain unanswered regarding the response of epidermal stem cells to wound healing. HF stem cells migrate to the IFE during wound repair and become integrated as IFE stem cells. But it is unknown what signals activate stem cell migration from the HF to the IFE, how these signals are mediated and how the response changes the transcription of the keratinocytes. Different keratinocyte populations might react differently to these stimuli, as they also show differing properties such as survival time within the newly formed scar epidermis [27,34,35,102].

Also the molecular response of early transformed keratinocytes is not fully understood yet. Deciphering the transcriptional signatures of transformed epidermal cells within different niches might help to understand the dynamics of cells in different locations in the HF. Why do some populations remain dormant whereas others quickly form lesions when challenged with the same mutation?

Finally, comparing the transcriptional changes during early tumourigenic transformation to those occurring in normal stem cells that are activated by wound healing may uncover a set of genes that is associated with the re-epithelialisation response but is not involved in cancer development. Those genes can potentially be harnessed to safely stimulate epidermal stem cells in order to improve healing of chronic wounds. A better understanding of the signalling mechanism and the transcriptional changes that occur during the "natural reprogramming" of epithelial stem cells will also advance *ex vivo* skin reconstruction, and gene-based therapy of chronic wounds and other skin diseases [89].

5 ACKNOWLEDGEMENTS

This work would not have been possible without the help and guidance by numerous people. First and foremost, I would like to thank my PhD supervisors.

Maria, thank you for accepting me as your first PhD student and taking this "journey" together with me. On the day we met, you convinced me in one hour that the hair follicle is the coolest thing one can study in biology. And your excitement about science in general and all those little details has not faded since. You inspired me to try out new methods and develop my own ideas. And I most enjoyed our hour-long theory-crafting discussions. You gave me the opportunity to manage my own project and learn so much. Thank you for always leaving your door open, being approachable, and immediately jumping up to find a solution to even the strangest, little problem.

Thank you **Viljar**, for being my mentor during the first year. Your affectionate and outspoken way made me feel comfortable and helped me to integrate in the lab, when I started. Thank you for teaching me that taking the bad, the worse and the boring with a little humour makes life much easier.

Rune, thank you for welcoming me as a PhD student at KI and the BioNut department. You always had some wise advice and guidance, when I was stuck. Thank you for imparting your knowledge and experience.

I would like to thank all my half-time review examiners, **Maria Eriksson**, **Inderpreet Sur**, and **Chritian Göritz** for the excellent suggestions helping my research during the last years.

I am grateful to all my collaborators that contributed to the work presented in this thesis. **Benjamin D. Simons** for the enlightening discussions about stem cell and tissue maintenance models, and of course the help with our manuscript. **Sten Linnarsson's group** for performing the RNA-sequencing analysis. And **Hans Clevers** for providing the mouse strains.

I would like to thank all my colleagues in the MKA group, who contributed in many ways to this work. **Sasha**, thank you for managing the lab and always being supportive. You taught me so many tips and tricks in the lab, and always helped me out when I tried new protocols or didn't manage to make a buffer. **Tina**, you are wonderful, and probably the most cooperative person in the world. Thanks especially for the correction of this thesis and the taming of the bachelor students in the last month. Thank you **Simon** for your awesome computer and statistics skills, and simulating all my experiments. **Unni** and **Xiaoyan**, thank you for being great company and super nice colleagues. I could always rely on you when I needed your help. Good luck with your postdocs! I also want to thank the Master students that were visiting the lab, **Gioele**, **Eduardo** and **Karl**. You were all fun to work with and I hope I could teach you at least something useful.

I am very grateful to the members of the RTO group that I have been a part of during the first two years. **Maria H.** my PhD and HEALING companion. Without you the struggle through HEALING would have been much harder. Thank you for being a friend (and of course lots of pancakes). **Leander**, **Marco** and **Fabian** thank you for the weirdest, funniest and of course most helpful discussions. It has been a pleasure. Thank you, **Agneta** for always being open and helpful in any situation, **Elin** for your very nice company and great help with the mouse work, **Åsa B.** and **Maryam** for managing the lab and all the help with the mouse work. **Peter**, thank you for always being interested in my work and asking helpful questions. **Stephan**, **Csaba**, **Uta**, **Katharina**, **Ferdous**, **Yumei**, **Ani** and **Raoul**, thank you for the nice company and journal club discussions. **Gunnel**, **Britt-Marie** and **Robert**, thank you for the nice fika company. My former colleagues (in the old office), **Åsa K.**, **Vicky**, **Karin** and **Ulrika**, thank you for all your advice.

Thank you **Sylvie LeGuyader** for the microscopy help and cookies. Thank you **Åsa-Lena Dackland** and **Iyadh Douagi** for the support with FACS sorting and analysis. The staff of the **BEA** core facility, especially **Marika Rönnholm** and **Patrick Müller**, thank you for being super cooperative and helpful with my transcriptomics work. Thank you to the **BioNut administration** and **service team** for all the support during the years.

Jens, my husband, soul-mate and best friend. Thank you for always motivating me and making life more exciting, but also holding me back in the right moments. Thank you for always being there for me. I will love you forever. Ich mag dich! **Sofia**, the most wonderful detour on this journey. When you hold my hand, I know what I am living for. My parents, **Manuela** and **Richard**. Thank you for all the love and support. And all the train rides to Stockholm! My parents-in-law, **Gabi** and **Burkhard**, you are the best. And my grandparents, **Helga**, **Rita** and **Theo**, thank you for being so interested and proud.

Thank you also to my new manager and colleagues at the EBI **ArrayExpress/Atlas team** for the lovely welcome and all the support during the writing of this thesis.

I would also like to thank the **ImageJ** and **R/Bioconductor** open source communities, who develop great software that make life as a bioscience researcher much easier.

Last but not least, I would like to thank my sponsors. The EU FP7 Marie Curie initial training network **HEALING**, and its organisers for the wonderful scientific meetings and the exchange with other young researchers. **Radiumhemmet** and **Cancerfonden** that allowed me to visit the fantastic ISSCR stem cell conference in 2014.

6 REFERENCES

1 Blanpain, C. and Fuchs, E. Epidermal homeostasis: a balancing act of stem cells in the skin. *Nature reviews. Molecular cell biology* **10**, 207-217, (2009).

2 Velazquez, E. F. and Murphy, G. F. in *LEVER'S Histopathology of the Skin, 10th Edition* (eds David E Elder, Rosalie Elenitsas, Bernett L Johnson, George F Murphy *et al.*) 8-66 (Lippincott Williams & Wilkins, 2004).

3 Higgins, C. A. and Christiano, A. M. Regenerative medicine and hair loss: how hair follicle culture has advanced our understanding of treatment options for androgenetic alopecia. *Regenerative medicine* **9**, 101-111, (2014).

4 Venus, M., Waterman, J. and McNab, I. Basic physiology of the skin. *Surgery (Oxford)* **29**, 471-474, (2011).

5 MacNeil, S. Progress and opportunities for tissue-engineered skin. *Nature* **445**, 874-880, (2007).

6 Fuchs, E. Keratins and the skin. *Annual review of cell and developmental biology* **11**, 123-153, (1995).

7 Moll, R., Divo, M. and Langbein, L. The human keratins: biology and pathology. *Histochemistry and cell biology* **129**, 705-733, (2008).

8 Watt, F. M., Kubler, M. D., Hotchin, N. A., Nicholson, L. J. and Adams, J. C. Regulation of keratinocyte terminal differentiation by integrin-extracellular matrix interactions. *Journal of cell science* **106 (Pt 1)**, 175-182, (1993).

9 Watt, F. M. Role of integrins in regulating epidermal adhesion, growth and differentiation. *The EMBO journal* **21**, 3919-3926, (2002).

10 Penneys, N. S., Fulton, J. E., Jr., Weinstein, G. D. and Frost, P. Location of proliferating cells in human epidermis. *Archives of dermatology* **101**, 323-327, (1970).

11 Candi, E., Schmidt, R. and Melino, G. The cornified envelope: a model of cell death in the skin. *Nature reviews. Molecular cell biology* **6**, 328-340, (2005).

12 Duverger, O. and Morasso, M. I. Epidermal patterning and induction of different hair types during mouse embryonic development. *Birth defects research. Part C, Embryo today : reviews* **87**, 263-272, (2009).

13 Millar, S. E. Molecular mechanisms regulating hair follicle development. *The Journal of investigative dermatology* **118**, 216-225, (2002).

14 Chi, W., Wu, E. and Morgan, B. A. Dermal papilla cell number specifies hair size, shape and cycling and its reduction causes follicular decline. *Development* **140**, 1676-1683, (2013).

15 Jahoda, C. A., Horne, K. A. and Oliver, R. F. Induction of hair growth by implantation of cultured dermal papilla cells. *Nature* **311**, 560-562, (1984).

16 Schneider, M. R., Schmidt-Ullrich, R. and Paus, R. The hair follicle as a dynamic miniorgan. *Current biology : CB* **19**, R132-142, (2009).

17 Legue, E. and Nicolas, J. F. Hair follicle renewal: organization of stem cells in the matrix and the role of stereotyped lineages and behaviors. *Development* **132**, 4143-4154, (2005).

18 Müller-Röver, S., Handjiski, B., van der Veen, C., Eichmüller, S., Foitzik, K. *et al.* A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. *The Journal of investigative dermatology* **117**, 3-15, (2001).

19 Alonso, L. and Fuchs, E. The hair cycle. *Journal of cell science* **119**, 391-393, (2006).

20 Stenn, K. S. and Paus, R. Controls of hair follicle cycling. *Physiological reviews* **81**, 449-494, (2001).

21 Braun, K. M. and Watt, F. M. Epidermal label-retaining cells: background and recent applications. *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research* **9**, 196-201, (2004).

22 Cotsarelis, G., Sun, T. T. and Lavker, R. M. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* **61**, 1329-1337, (1990).

23 Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W. E. *et al.* Defining the epithelial stem cell niche in skin. *Science* **303**, 359-363, (2004).

24 Morris, R. J., Liu, Y., Marles, L., Yang, Z., Trempus, C. *et al.* Capturing and profiling adult

hair follicle stem cells. *Nat Biotechnol* **22**, 411-417, (2004).

25 Trempus, C. S., Morris, R. J., Bortner, C. D., Cotsarelis, G., Faircloth, R. S. *et al.* Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. *The Journal of investigative dermatology* **120**, 501-511, (2003).

26 Liu, Y., Lyle, S., Yang, Z. and Cotsarelis, G. Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. *The Journal of investigative dermatology* **121**, 963-968, (2003).

27 Jaks, V., Barker, N., Kasper, M., van Es, J. H., Snippert, H. J. *et al.* Lgr5 marks cycling, yet long-lived, hair follicle stem cells. *Nature genetics* **40**, 1291-1299, (2008).

28 Youssef, K. K., Van Keymeulen, A., Lapouge, G., Beck, B., Michaux, C. *et al.* Identification of the cell lineage at the origin of basal cell carcinoma. *Nature cell biology* **12**, 299-305, (2010).

29 Blanpain, C., Lowry, W. E., Geoghegan, A., Polak, L. and Fuchs, E. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* **118**, 635-648, (2004).

30 Greco, V., Chen, T., Rendl, M., Schober, M., Pasolli, H. A. *et al.* A two-step mechanism for stem cell activation during hair regeneration. *Cell stem cell* **4**, 155-169, (2009).

31 Rompolas, P., Mesa, K. R. and Greco, V. Spatial organization within a niche as a determinant of stem-cell fate. *Nature* **502**, 513-518, (2013).

32 Nijhof, J. G., Braun, K. M., Giangreco, A., van Pelt, C., Kawamoto, H. *et al.* The cell-surface marker MTS24 identifies a novel population of follicular keratinocytes with characteristics of progenitor cells. *Development* **133**, 3027-3037, (2006).

33 Snippert, H. J., Haegebarth, A., Kasper, M., Jaks, V., van Es, J. H. *et al.* Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. *Science* **327**, 1385-1389, (2010).

34 Brownell, I., Guevara, E., Bai, C. B., Loomis, C. A. and Joyner, A. L. Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. *Cell stem cell* **8**, 552-565, (2011).

35 Page, M. E., Lombard, P., Ng, F., Gottgens, B. and Jensen, K. B. The epidermis comprises autonomous compartments maintained by distinct stem cell populations. *Cell stem cell* **13**, 471-482, (2013).

36 Jensen, K. B., Collins, C. A., Nascimento, E., Tan, D. W., Frye, M. *et al.* Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis. *Cell stem cell* **4**, 427-439, (2009).

37 Blanpain, C. and Fuchs, E. Stem cell plasticity. Plasticity of epithelial stem cells in tissue regeneration. *Science* **344**, 1242281, (2014).

38 Fujiwara, H., Ferreira, M., Donati, G., Marciano, D. K., Linton, J. M. *et al.* The basement membrane of hair follicle stem cells is a muscle cell niche. *Cell* **144**, 577-589, (2011).

39 Amoh, Y., Li, L., Katsuoka, K., Penman, S. and Hoffman, R. M. Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 5530-5534, (2005).

40 Johnston, A. P., Naska, S., Jones, K., Jinno, H., Kaplan, D. R. *et al.* Sox2-mediated regulation of adult neural crest precursors and skin repair. *Stem cell reports* **1**, 38-45, (2013).

41 Tanimura, S., Tadokoro, Y., Inomata, K., Binh, N. T., Nishie, W. *et al.* Hair follicle stem cells provide a functional niche for melanocyte stem cells. *Cell stem cell* **8**, 177-187, (2011).

42 Rabbani, P., Takeo, M., Chou, W., Myung, P., Bosenberg, M. *et al.* Coordinated activation of Wnt in epithelial and melanocyte stem cells initiates pigmented hair regeneration. *Cell* **145**, 941-955, (2011).

43 Gay, D., Kwon, O., Zhang, Z., Spata, M., Plikus, M. V. *et al.* Fgf9 from dermal gammadelta T cells induces hair follicle neogenesis after wounding. *Nature medicine* **19**, 916-923, (2013).

44 Watt, F. M. and Huck, W. T. Role of the extracellular matrix in regulating stem cell fate. *Nature reviews. Molecular cell biology* **14**, 467-473, (2013).

45 Connelly, J. T., Gautrot, J. E., Trappmann, B., Tan, D. W., Donati, G. *et al.* Actin and serum response factor transduce physical cues from the microenvironment to regulate epidermal stem cell fate decisions. *Nature cell biology* **12**, 711-718, (2010).

46 Niemann, C. Differentiation of the sebaceous gland. *Dermato-endocrinology* **1**, 64-67, (2009).

47 Frances, D. and Niemann, C. Stem cell dynamics in sebaceous gland morphogenesis in

mouse skin. *Developmental biology* **363**, 138-146, (2012).

48 Arnold, I. and Watt, F. M. c-Myc activation in transgenic mouse epidermis results in mobilization of stem cells and differentiation of their progeny. *Current biology : CB* **11**, 558-568, (2001).

49 Allen, M., Grachtchouk, M., Sheng, H., Grachtchouk, V., Wang, A. *et al.* Hedgehog signaling regulates sebaceous gland development. *The American journal of pathology* **163**, 2173-2178, (2003).

50 Baker, C. M., Verstuyf, A., Jensen, K. B. and Watt, F. M. Differential sensitivity of epidermal cell subpopulations to beta-catenin-induced ectopic hair follicle formation. *Developmental biology* **343**, 40-50, (2010).

51 Niemann, C., Owens, D. M., Hulsken, J., Birchmeier, W. and Watt, F. M. Expression of DeltaNLef1 in mouse epidermis results in differentiation of hair follicles into squamous epidermal cysts and formation of skin tumours. *Development* **129**, 95-109, (2002).

52 Schneider, M. R. and Paus, R. Sebocytes, multifaceted epithelial cells: lipid production and holocrine secretion. *The international journal of biochemistry & cell biology* **42**, 181-185, (2010).

53 Horsley, V., O'Carroll, D., Tooze, R., Ohinata, Y., Saitou, M. *et al.* Blimp1 defines a progenitor population that governs cellular input to the sebaceous gland. *Cell* **126**, 597-609, (2006).

54 Kretzschmar, K., Cottle, D. L., Donati, G., Chiang, M. F., Quist, S. R. *et al.* BLIMP1 is required for postnatal epidermal homeostasis but does not define a sebaceous gland progenitor under steady-state conditions. *Stem cell reports* **3**, 620-633, (2014).

55 Ghazizadeh, S. and Taichman, L. B. Multiple classes of stem cells in cutaneous epithelium: a lineage analysis of adult mouse skin. *The EMBO journal* **20**, 1215-1222, (2001).

56 Petersson, M., Brylka, H., Kraus, A., John, S., Rappl, G. *et al.* TCF/Lef1 activity controls establishment of diverse stem and progenitor cell compartments in mouse epidermis. *The EMBO journal* **30**, 3004-3018, (2011).

57 Taylor, G., Lehrer, M. S., Jensen, P. J., Sun, T. T. and Lavker, R. M. Involvement of follicular stem cells in forming not only the follicle but also the epidermis. *Cell* **102**, 451-461, (2000).

58 Levy, V., Lindon, C., Harfe, B. D. and Morgan, B. A. Distinct stem cell populations regenerate the follicle and interfollicular epidermis. *Developmental cell* **9**, 855-861, (2005).

59 Nowak, J. A., Polak, L., Pasolli, H. A. and Fuchs, E. Hair follicle stem cells are specified and function in early skin morphogenesis. *Cell stem cell* **3**, 33-43, (2008).

60 Beck, B. and Blanpain, C. Mechanisms regulating epidermal stem cells. *The EMBO journal* **31**, 2067-2075, (2012).

61 Jones, P. H., Harper, S. and Watt, F. M. Stem cell patterning and fate in human epidermis. *Cell* **80**, 83-93, (1995).

62 Potten, C. S. The epidermal proliferative unit: the possible role of the central basal cell. *Cell and tissue kinetics* **7**, 77-88, (1974).

63 Ghadially, R. 25 years of epidermal stem cell research. *The Journal of investigative dermatology* **132**, 797-810, (2012).

64 Clayton, E., Doupe, D. P., Klein, A. M., Winton, D. J., Simons, B. D. *et al.* A single type of progenitor cell maintains normal epidermis. *Nature* **446**, 185-189, (2007).

65 Doupe, D. P., Klein, A. M., Simons, B. D. and Jones, P. H. The ordered architecture of murine ear epidermis is maintained by progenitor cells with random fate. *Developmental cell* **18**, 317-323, (2010).

66 Lim, X., Tan, S. H., Koh, W. L., Chau, R. M., Yan, K. S. *et al.* Interfollicular epidermal stem cells self-renew via autocrine Wnt signaling. *Science* **342**, 1226-1230, (2013).

67 Snippert, H. J., van der Flier, L. G., Sato, T., van Es, J. H., van den Born, M. *et al.* Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* **143**, 134-144, (2010).

68 Doupe, D. P., Alcolea, M. P., Roshan, A., Zhang, G., Klein, A. M. *et al.* A single progenitor population switches behavior to maintain and repair esophageal epithelium. *Science* **337**, 1091-1093, (2012).

69 Leushacke, M., Ng, A., Galle, J., Loeffler, M. and Barker, N. Lgr5(+) gastric stem cells divide symmetrically to effect epithelial homeostasis in the pylorus. *Cell reports* **5**, 349-356, (2013).

70 Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M. *et al.* Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**, 1003-1007, (2007).

71 van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I. *et al.* The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111**, 241-250, (2002).

72 Van der Flier, L. G., Sabates-Bellver, J., Oving, I., Haegebarth, A., De Palo, M. *et al.* The Intestinal Wnt/TCF Signature. *Gastroenterology* **132**, 628-632, (2007).

73 Li, L. and Clevers, H. Coexistence of quiescent and active adult stem cells in mammals. *Science* **327**, 542-545, (2010).

74 Morgan, B. A. A glorious revolution in stem cell biology. *Nature genetics* **40**, 1269-1270, (2008).

75 Barker, N., Huch, M., Kujala, P., van de Wetering, M., Snippert, H. J. *et al.* Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell stem cell* **6**, 25-36, (2010).

76 de Visser, K. E., Ciampricotti, M., Michalak, E. M., Tan, D. W., Speksnijder, E. N. *et al.* Developmental stage-specific contribution of LGR5(+) cells to basal and luminal epithelial lineages in the postnatal mammary gland. *The Journal of pathology* **228**, 300-309, (2012).

77 Yee, K. K., Li, Y., Redding, K. M., Iwatsuki, K., Margolskee, R. F. *et al.* Lgr5-EGFP marks taste bud stem/progenitor cells in posterior tongue. *Stem cells* **31**, 992-1000, (2013).

78 Barker, N., Rookmaaker, M. B., Kujala, P., Ng, A., Leushacke, M. *et al.* Lgr5(+ve) stem/progenitor cells contribute to nephron formation during kidney development. *Cell reports* **2**, 540-552, (2012).

79 Liao, X. H. and Nguyen, H. Epidermal expression of Lgr6 is dependent on nerve endings and Schwann cells. *Experimental dermatology* **23**, 195-198, (2014).

80 Kumar, K. K., Burgess, A. W. and Gulbis, J. M. Structure and function of LGR5: an enigmatic G-protein coupled receptor marking stem cells. *Protein science : a publication of the Protein Society* **23**, 551-565, (2014).

81 Morita, H., Mazerbourg, S., Bouley, D. M., Luo, C. W., Kawamura, K. *et al.* Neonatal lethality of LGR5 null mice is associated with ankyloglossia and gastrointestinal distension. *Molecular and cellular biology* **24**, 9736-9743, (2004).

82 Carmon, K. S., Gong, X., Lin, Q., Thomas, A. and Liu, Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 11452-11457, (2011).

83 Carmon, K. S., Lin, Q., Gong, X., Thomas, A. and Liu, Q. LGR5 interacts and cointernalizes with Wnt receptors to modulate Wnt/beta-catenin signaling. *Molecular and cellular biology* **32**, 2054-2064, (2012).

84 de Lau, W., Barker, N., Low, T. Y., Koo, B. K., Li, V. S. *et al.* Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature* **476**, 293-297, (2011).

85 Glinka, A., Dolde, C., Kirsch, N., Huang, Y. L., Kazanskaya, O. *et al.* LGR4 and LGR5 are R-spondin receptors mediating Wnt/beta-catenin and Wnt/PCP signalling. *EMBO reports* **12**, 1055-1061, (2011).

86 Ruffner, H., Sprunger, J., Charlat, O., Leighton-Davies, J., Grosshans, B. *et al.* R-Spondin potentiates Wnt/beta-catenin signaling through orphan receptors LGR4 and LGR5. *PloS one* **7**, e40976, (2012).

87 Kim, K. A., Zhao, J., Andarmani, S., Kakitani, M., Oshima, T. *et al.* R-Spondin proteins: a novel link to beta-catenin activation. *Cell cycle* **5**, 23-26, (2006).

88 Hao, H. X., Xie, Y., Zhang, Y., Charlat, O., Oster, E. *et al.* ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Nature* **485**, 195-200, (2012).

89 Gurtner, G. C., Werner, S., Barrandon, Y. and Longaker, M. T. Wound repair and regeneration. *Nature* **453**, 314-321, (2008).

90 Shaw, T. J. and Martin, P. Wound repair at a glance. *Journal of cell science* **122**, 3209-3213, (2009).

91 Baum, C. L. and Arpey, C. J. Normal cutaneous wound healing: clinical correlation with cellular and molecular events. *Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al.]* **31**, 674-686; discussion 686, (2005).

92 Werner, S., Krieg, T. and Smola, H. Keratinocyte-fibroblast interactions in wound healing. *The Journal of investigative dermatology* **127**, 998-1008, (2007).

93 Hinz, B. Formation and function of the myofibroblast during tissue repair. *The Journal of investigative dermatology* **127**, 526-537, (2007).

94 Takeo, M., Lee, W. and Ito, M. Wound healing and skin regeneration. *Cold Spring Harbor perspectives in medicine* **5**, a023267, (2015).

95 Corr, D. T., Gallant-Behm, C. L., Shrive, N. G. and Hart, D. A. Biomechanical behavior of scar tissue and uninjured skin in a porcine model. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* **17**, 250-259, (2009).

96 Rowlatt, U. Intrauterine wound healing in a 20 week human fetus. *Virchows Archiv. A, Pathological anatomy and histology* **381**, 353-361, (1979).

97 Adzick, N. S. and Longaker, M. T. Scarless fetal healing. Therapeutic implications. *Annals of surgery* **215**, 3-7, (1992).

98 Walmsley, G. G., Maan, Z. N., Wong, V. W., Duscher, D., Hu, M. S. *et al.* Scarless wound healing: chasing the holy grail. *Plastic and reconstructive surgery* **135**, 907-917, (2015).

99 Langton, A. K., Herrick, S. E. and Headon, D. J. An extended epidermal response heals cutaneous wounds in the absence of a hair follicle stem cell contribution. *The Journal of investigative dermatology* **128**, 1311-1318, (2008).

100 Ansell, D. M., Kloepper, J. E., Thomason, H. A., Paus, R. and Hardman, M. J. Exploring the "hair growth-wound healing connection": anagen phase promotes wound re-epithelialization. *The Journal of investigative dermatology* **131**, 518-528, (2011).

101 Levy, V., Lindon, C., Zheng, Y., Harfe, B. D. and Morgan, B. A. Epidermal stem cells arise from the hair follicle after wounding. *Faseb J* **21**, 1358-1366, (2007).

102 Ito, M., Liu, Y., Yang, Z., Nguyen, J., Liang, F. *et al.* Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nature medicine* **11**, 1351-1354, (2005).

103 Plikus, M. V., Gay, D. L., Treffeisen, E., Wang, A., Supapannachart, R. J. *et al.* Epithelial stem cells and implications for wound repair. *Seminars in cell & developmental biology* **23**, 946-953, (2012).

104 Gat, U., DasGupta, R., Degenstein, L. and Fuchs, E. De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. *Cell* **95**, 605-614, (1998).

105 Silva-Vargas, V., Lo Celso, C., Giangreco, A., Ofstad, T., Prowse, D. M. *et al.* Beta-catenin and Hedgehog signal strength can specify number and location of hair follicles in adult epidermis without recruitment of bulge stem cells. *Developmental cell* **9**, 121-131, (2005).

106 Ito, M., Yang, Z., Andl, T., Cui, C., Kim, N. *et al.* Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. *Nature* **447**, 316-320, (2007).

107 Driskell, R. R., Lichtenberger, B. M., Hoste, E., Kretzschmar, K., Simons, B. D. *et al.* Distinct fibroblast lineages determine dermal architecture in skin development and repair. *Nature* **504**, 277-281, (2013).

108 Arwert, E. N., Hoste, E. and Watt, F. M. Epithelial stem cells, wound healing and cancer. *Nature reviews. Cancer* **12**, 170-180, (2012).

109 Schafer, M. and Werner, S. Cancer as an overhealing wound: an old hypothesis revisited. *Nature reviews. Molecular cell biology* **9**, 628-638, (2008).

110 Hanahan, D. and Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, (2011).

111 Lewis, C. E. and Pollard, J. W. Distinct role of macrophages in different tumor microenvironments. *Cancer research* **66**, 605-612, (2006).

112 Kalluri, R. and Weinberg, R. A. The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation* **119**, 1420-1428, (2009).

113 Lorz, C., Segrelles, C. and Paramio, J. M. On the origin of epidermal cancers. *Current molecular medicine* **9**, 353-364, (2009).

114 Owens, D. M. and Watt, F. M. Contribution of stem cells and differentiated cells to epidermal tumours. *Nature reviews. Cancer* **3**, 444-451, (2003).

115 Brash, D. E., Ziegler, A., Jonason, A. S., Simon, J. A., Kunala, S. *et al.* Sunlight and sunburn in human skin cancer: p53, apoptosis, and tumor promotion. *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research* **1**, 136-142, (1996).

116 Ziegler, A., Jonason, A. S., Leffell, D. J., Simon, J. A., Sharma, H. W. *et al.* Sunburn and p53 in the onset of skin cancer. *Nature* **372**, 773-776, (1994).

117 Martincorena, I., Roshan, A., Gerstung, M., Ellis, P., Van Loo, P. *et al.* Tumor evolution. High burden and pervasive positive selection of somatic mutations in normal human skin. *Science* **348**, 880-886, (2015).

118 DiGiovanni, J. Multistage carcinogenesis in mouse skin. *Pharmacology & therapeutics* **54**, 63-128, (1992).

119 Balmain, A., Ramsden, M., Bowden, G. T. and Smith, J. Activation of the mouse cellular Harvey-ras gene in chemically induced benign skin papillomas. *Nature* **307**, 658-660, (1984).

120 Schwarz, M., Munzel, P. A. and Braeuning, A. Non-melanoma skin cancer in mouse and man. *Archives of toxicology* **87**, 783-798, (2013).

121 Visvader, J. E. and Lindeman, G. J. Cancer stem cells: current status and evolving complexities. *Cell stem cell* **10**, 717-728, (2012).

122 Schwitalla, S., Fingerle, A. A., Cammareri, P., Nebelsiek, T., Goktuna, S. I. *et al.* Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell* **152**, 25-38, (2013).

123 Chaffer, C. L., Brueckmann, I., Scheel, C., Kaestli, A. J., Wiggins, P. A. *et al.* Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 7950-7955, (2011).

124 Lim, E., Vaillant, F., Wu, D., Forrest, N. C., Pal, B. *et al.* Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nature medicine* **15**, 907-913, (2009).

125 Kasper, M., Jaks, V., Are, A., Bergstrom, A., Schwager, A. *et al.* Wounding enhances epidermal tumorigenesis by recruiting hair follicle keratinocytes. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 4099-4104, (2011).

126 Grachtchouk, M., Pero, J., Yang, S. H., Ermilov, A. N., Michael, L. E. *et al.* Basal cell carcinomas in mice arise from hair follicle stem cells and multiple epithelial progenitor populations. *The Journal of clinical investigation* **121**, 1768-1781, (2011).

127 Van Duuren, B. L., Sivak, A., Katz, C., Seidman, I. and Melchionne, S. The effect of aging and interval between primary and secondary treatment in two-stage carcinogenesis on mouse skin. *Cancer research* **35**, 502-505, (1975).

128 Brown, K., Strathdee, D., Bryson, S., Lambie, W. and Balmain, A. The malignant capacity of skin tumours induced by expression of a mutant H-ras transgene depends on the cell type targeted. *Current biology : CB* **8**, 516-524, (1998).

129 Bailleul, B., Surani, M. A., White, S., Barton, S. C., Brown, K. *et al.* Skin hyperkeratosis and papilloma formation in transgenic mice expressing a ras oncogene from a suprabasal keratin promoter. *Cell* **62**, 697-708, (1990).

130 Wicking, C., Smyth, I. and Bale, A. The hedgehog signalling pathway in tumorigenesis and development. *Oncogene* **18**, 7844-7851, (1999).

131 Ingham, P. W. and McMahon, A. P. Hedgehog signaling in animal development: paradigms and principles. *Genes & development* **15**, 3059-3087, (2001).

132 Petrova, R. and Joyner, A. L. Roles for Hedgehog signaling in adult organ homeostasis and repair. *Development* **141**, 3445-3457, (2014).

133 Briscoe, J. and Therond, P. P. The mechanisms of Hedgehog signalling and its roles in development and disease. *Nature reviews. Molecular cell biology* **14**, 416-429, (2013).

134 Regl, G., Neill, G. W., Eichberger, T., Kasper, M., Ikram, M. S. *et al.* Human GLI2 and GLI1 are part of a positive feedback mechanism in Basal Cell Carcinoma. *Oncogene* **21**, 5529-5539, (2002).

135 St-Jacques, B., Dassule, H. R., Karavanova, I., Botchkarev, V. A., Li, J. *et al.* Sonic hedgehog signaling is essential for hair development. *Current biology : CB* **8**, 1058-1068, (1998).

136 Chiang, C., Swan, R. Z., Grachtchouk, M., Bolinger, M., Litingtung, Y. *et al.* Essential role for Sonic hedgehog during hair follicle morphogenesis. *Developmental biology* **205**, 1-9, (1999).

137 Sato, N., Leopold, P. L. and Crystal, R. G. Induction of the hair growth phase in postnatal mice by localized transient expression of Sonic hedgehog. *The Journal of clinical investigation* **104**, 855-864, (1999).

138 Botchkarev, V. A. and Kishimoto, J. Molecular control of epithelial-mesenchymal interactions during hair follicle cycling. *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research* **8**, 46-55, (2003).

139 Yang, S. H., Andl, T., Grachtchouk, V., Wang, A., Liu, J. *et al.* Pathological responses to oncogenic Hedgehog signaling in skin are dependent on canonical Wnt/beta3-catenin signaling. *Nature genetics* **40**, 1130-1135, (2008).

140 Youssef, K. K., Lapouge, G., Bouvree, K., Rorive, S., Brohee, S. *et al.* Adult interfollicular tumour-initiating cells are reprogrammed into an embryonic hair follicle progenitor-like fate during basal cell carcinoma initiation. *Nature cell biology* **14**, 1282-1294, (2012).

141 Driskell, R. R., Giangreco, A., Jensen, K. B., Mulder, K. W. and Watt, F. M. Sox2-positive dermal papilla cells specify hair follicle type in mammalian epidermis. *Development* **136**, 2815-2823, (2009).

142 Epstein, E. H. Basal cell carcinomas: attack of the hedgehog. *Nature reviews. Cancer* **8**, 743-754, (2008).

143 Sahl, W. J. Basal cell carcinoma: influence of tumor size on mortality and morbidity. *International journal of dermatology* **34**, 319-321, (1995).

144 Lo, J. S., Snow, S. N., Reizner, G. T., Mohs, F. E., Larson, P. O. *et al.* Metastatic basal cell carcinoma: report of twelve cases with a review of the literature. *Journal of the American Academy of Dermatology* **24**, 715-719, (1991).

145 Kasper, M., Jaks, V., Hohl, D. and Toftgard, R. Basal cell carcinoma - molecular biology and potential new therapies. *The Journal of clinical investigation* **122**, 455-463, (2012).

146 Rass, K. and Reichrath, J. UV damage and DNA repair in malignant melanoma and nonmelanoma skin cancer. *Advances in experimental medicine and biology* **624**, 162-178, (2008).

147 Crowson, A. N. Basal cell carcinoma: biology, morphology and clinical implications. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **19 Suppl 2**, S127-147, (2006).

148 Gailani, M. R., Bale, S. J., Leffell, D. J., DiGiovanna, J. J., Peck, G. L. *et al.* Developmental defects in Gorlin syndrome related to a putative tumor suppressor gene on chromosome 9. *Cell* **69**, 111-117, (1992).

149 Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W. *et al.* Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science* **272**, 1668-1671, (1996).

150 Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S. *et al.* Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. *Cell* **85**, 841-851, (1996).

151 Gailani, M. R., Stahle-Backdahl, M., Leffell, D. J., Glynn, M., Zaphiropoulos, P. G. *et al.* The role of the human homologue of Drosophila patched in sporadic basal cell carcinomas. *Nature genetics* **14**, 78-81, (1996).

152 Gorlin, R. J. Nevoid basal cell carcinoma (Gorlin) syndrome: unanswered issues. *The Journal of laboratory and clinical medicine* **134**, 551-552, (1999).

153 Rudin, C. M. Vismodegib. *Clinical cancer research : an official journal of the American Association for Cancer Research* **18**, 3218-3222, (2012).

154 Sharpe, H. J., Pau, G., Dijkgraaf, G. J., Basset-Seguin, N., Modrusan, Z. *et al.* Genomic analysis of smoothened inhibitor resistance in basal cell carcinoma. *Cancer cell* **27**, 327-341, (2015).

155 Atwood, S. X., Sarin, K. Y., Whitson, R. J., Li, J. R., Kim, G. *et al.* Smoothened variants explain the majority of drug resistance in basal cell carcinoma. *Cancer cell* **27**, 342-353, (2015).

156 Nitzki, F., Becker, M., Frommhold, A., Schulz-Schaeffer, W. and Hahn, H. Patched knockout mouse models of Basal cell carcinoma. *Journal of skin cancer* **2012**, 907543, (2012).

157 Sauer, B. Inducible gene targeting in mice using the Cre/lox system. *Methods* **14**, 381-392, (1998).

158 Feil, R., Wagner, J., Metzger, D. and Chambon, P. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochemical and biophysical research communications* **237**, 752-757, (1997).

159 Soriano, P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nature genetics* **21**, 70-71, (1999).

160 Livet, J., Weissman, T. A., Kang, H., Draft, R. W., Lu, J. *et al.* Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature* **450**, 56-62, (2007).

161 Svärd, J. *Repressing the Hedgehog Signalling Pathway* Ph.D. thesis, Karolinska Institutet, (2007).

162 Sprengel, R. and Hasan, M. T. Tetracycline-controlled genetic switches. *Handbook of experimental pharmacology*, 49-72, (2007).

163 Bhatia, A., Singh, B., Amarji, B. and Katare, O. P. Tamoxifen-loaded liposomal topical formulation arrests hair growth in mice. *The British journal of dermatology* **163**, 412-415, (2010).

164 Moullan, N., Mouchiroud, L., Wang, X., Ryu, D., Williams, E. G. *et al.* Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for Caution in Biomedical Research. *Cell reports*, (2015).

165 Liu, H. S., Jan, M. S., Chou, C. K., Chen, P. H. and Ke, N. J. Is green fluorescent protein toxic to the living cells? *Biochemical and biophysical research communications* **260**, 712-717, (1999).

166 Ozyazgan, I. and Kontas, O. Previous injuries or scars as risk factors for the development of basal cell carcinoma. *Scandinavian journal of plastic and reconstructive surgery and hand*

surgery / Nordisk plastikkirurgisk forening [and] Nordisk klubb for handkirurgi **38**, 11-15, (2004).

167 Eming, S. A., Krieg, T. and Davidson, J. M. Inflammation in wound repair: molecular and cellular mechanisms. *The Journal of investigative dermatology* **127**, 514-525, (2007).

168 Jaks, V., Kasper, M. and Toftgard, R. The hair follicle-a stem cell zoo. *Exp Cell Res* **316**, 1422-1428, (2010).

169 Kretzschmar, K. and Watt, F. M. Markers of epidermal stem cell subpopulations in adult mammalian skin. *Cold Spring Harbor perspectives in medicine* **4**, (2014).

170 Schepeler, T., Page, M. E. and Jensen, K. B. Heterogeneity and plasticity of epidermal stem cells. *Development* **141**, 2559-2567, (2014).

171 O'Brien, L. E. Regional specificity in the Drosophila midgut: setting boundaries with stem cells. *Cell stem cell* **13**, 375-376, (2013).

172 Van Keymeulen, A., Rocha, A. S., Ousset, M., Beck, B., Bouvencourt, G. *et al.* Distinct stem cells contribute to mammary gland development and maintenance. *Nature* **479**, 189-193, (2011).

173 Ousset, M., Van Keymeulen, A., Bouvencourt, G., Sharma, N., Achouri, Y. *et al.* Multipotent and unipotent progenitors contribute to prostate postnatal development. *Nature cell biology* **14**, 1131-1138, (2012).

174 Marianes, A. and Spradling, A. C. Physiological and stem cell compartmentalization within the Drosophila midgut. *eLife* **2**, e00886, (2013).

175 Buchon, N., Osman, D., David, F. P., Fang, H. Y., Boquete, J. P. *et al.* Morphological and molecular characterization of adult midgut compartmentalization in Drosophila. *Cell reports* **3**, 1725-1738, (2013).

176 Vogt, A., McElwee, K. J. and Blume-Peytavi, U. in *Hair growth and disorders* (eds U. Blume-Peytavi, D. A. Whiting, and R.M. Trüeb) Ch. 1, 6 (Springer, 2008).

177 Mascre, G., Dekoninck, S., Drogat, B., Youssef, K. K., Brohee, S. *et al.* Distinct contribution of stem and progenitor cells to epidermal maintenance. *Nature* **489**, 257-262, (2012).

178 Snippert, H. J., Schepers, A. G., van Es, J. H., Simons, B. D. and Clevers, H. Biased competition between Lgr5 intestinal stem cells driven by oncogenic mutation induces clonal expansion. *EMBO reports* **15**, 62-69, (2014).

179 Alcolea, M. P., Greulich, P., Wabik, A., Frede, J., Simons, B. D. *et al.* Differentiation imbalance in single oesophageal progenitor cells causes clonal immortalization and field change. *Nature cell biology* **16**, 615-622, (2014).

180 Mesa, K. R., Rompolas, P., Zito, G., Myung, P., Sun, T. Y. *et al.* Niche-induced cell death and epithelial phagocytosis regulate hair follicle stem cell pool. *Nature* **522**, 94-97, (2015).

181 Rompolas, P., Deschene, E. R., Zito, G., Gonzalez, D. G., Saotome, I. *et al.* Live imaging of stem cell and progeny behaviour in physiological hair-follicle regeneration. *Nature* **487**, 496-499, (2012).

182 Blanpain, C. Tracing the cellular origin of cancer. *Nature cell biology* **15**, 126-134, (2013).

183 Balkwill, F. R., Capasso, M. and Hagemann, T. The tumor microenvironment at a glance. *Journal of cell science* **125**, 5591-5596, (2012).

184 Hanahan, D. and Coussens, L. M. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer cell* **21**, 309-322, (2012).

185 Peterson, S. C., Eberl, M., Vagnozzi, A. N., Belkadi, A., Veniaminova, N. A. *et al.* Basal cell carcinoma preferentially arises from stem cells within hair follicle and mechanosensory niches. *Cell stem cell* **16**, 400-412, (2015).

186 Hsu, Y. C., Li, L. and Fuchs, E. Emerging interactions between skin stem cells and their niches. *Nature medicine* **20**, 847-856, (2014).

187 Mellitzer, G., Xu, Q. and Wilkinson, D. G. Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81, (1999).

188 Genander, M., Holmberg, J. and Frisen, J. Ephrins negatively regulate cell proliferation in the epidermis and hair follicle. *Stem cells* **28**, 1196-1205, (2010).

189 Lien, W. H., Polak, L., Lin, M., Lay, K., Zheng, D. *et al.* In vivo transcriptional governance of hair follicle stem cells by canonical Wnt regulators. *Nature cell biology* **16**, 179-190, (2014).

190 Rao, D., Macias, E., Carbajal, S., Kiguchi, K. and DiGiovanni, J. Constitutive Stat3 activation alters behavior of hair follicle stem and progenitor cell populations. *Molecular carcinogenesis* **54**, 121-133, (2015).

191 Jensen, M. A., Wilkinson, J. E. and Krainer, A. R. Splicing factor SRSF6 promotes hyperplasia of sensitized skin. *Nature structural & molecular biology* **21**, 189-197, (2014).

192 Gong, X., Carmon, K. S., Lin, Q., Thomas, A., Yi, J. *et al.* LGR6 is a high affinity receptor of R-spondins and potentially functions as a tumor suppressor. *PloS one* **7**, e37137, (2012).

193 Peng, W. C., de Lau, W., Forneris, F., Granneman, J. C., Huch, M. *et al.* Structure of stem cell growth factor R-spondin 1 in complex with the ectodomain of its receptor LGR5. *Cell reports* **3**, 1885-1892, (2013).

194 FANTOM Consortium, RIKEN PMI and CLST (DGT). A promoter-level mammalian expression atlas. *Nature* **507**, 462-470, (2014).

195 Petryszak, R., Burdett, T., Fiorelli, B., Fonseca, N. A., Gonzalez-Porta, M. *et al.* Expression Atlas update--a database of gene and transcript expression from microarray- and sequencing-based functional genomics experiments. *Nucleic acids research* **42**, D926-932, (2014).

196 Zhang, Y., Chen, Y., Ni, W., Guo, L., Lu, X. *et al.* Dynamic expression of Lgr6 in the developing and mature mouse cochlea. *Frontiers in cellular neuroscience* **9**, 165, (2015).

197 Ren, W., Lewandowski, B. C., Watson, J., Aihara, E., Iwatsuki, K. *et al.* Single Lgr5- or Lgr6-expressing taste stem/progenitor cells generate taste bud cells ex vivo. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 16401-16406, (2014).

198 Simons, B. D. and Clevers, H. Strategies for homeostatic stem cell self-renewal in adult tissues. *Cell* **145**, 851-862, (2011).

199 Krieger, T. and Simons, B. D. Dynamic stem cell heterogeneity. *Development* **142**, 1396-1406, (2015).

200 Ritsma, L., Ellenbroek, S. I., Zomer, A., Snippert, H. J., de Sauvage, F. J. *et al.* Intestinal crypt homeostasis revealed at single-stem-cell level by in vivo live imaging. *Nature* **507**, 362-365, (2014).

201 Mammoto, T., Mammoto, A., Torisawa, Y. S., Tat, T., Gibbs, A. *et al.* Mechanochemical control of mesenchymal condensation and embryonic tooth organ formation. *Developmental cell* **21**, 758-769, (2011).

202 Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S. *et al.* Role of YAP/TAZ in mechanotransduction. *Nature* **474**, 179-183, (2011).

203 Zhang, H., Pasolli, H. A. and Fuchs, E. Yes-associated protein (YAP) transcriptional coactivator functions in balancing growth and differentiation in skin. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 2270-2275, (2011).

204 Zhao, B., Tumaneng, K. and Guan, K. L. The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. *Nature cell biology* **13**, 877-883, (2011).