Multipotency, Differentiation and Malfunction of Epidermal Stem Cells

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Summary

During prenatal life, cell division supplies the developing organism with new cell types and contributes to the growing cell number. On the other hand, in the adult, cell proliferation is mainly responsible for maintaining a more or less stable number of cells in each tissue, for example, after cell death or injury. However, in adult tissues most cells are short-lived and terminally differentiated thus reflecting an insufficient ability to proliferate. A specific population of adult stem cells is therefore essential to ensure cellular turnover throughout the postnatal life. Such population is constituted by undifferentiated, multipotent (ability to differentiate into different cell types) cells that self-renew, and that are usually found in specific niches. These cells retain their stem cell characteristics throughout life while giving rise to progenitor cells that then undergo terminal differentiation.

The clinical success achieved with stem cell therapy highlights the crucial importance of this field. Due to its accessibility and well-studied developmental stages, the skin stem cells are an excellent model for the study of adult stem cells. The study of skin stem cells will potentially yield new clinical approaches to skin injury, skin diseases, hair loss and skin cancer.

The present work aims to highlight the importance of skin stem cells as a scientific model. We start of by describing the main structures and developmental stages of the skin as well as some of the important molecular pathways so far linked to skin development and homeostasis. We describe the different skin stem cell niches identified to date and some of their defining characteristics. Finally, we explore some of the consequences of skin stem cell malfunction and molecular cues that have been studied in association with ageing and skin cancer.

Abstract

During embryonic development, cell division supplies the organism with new cell types and contributes to the growing cell number. In the adult, cell proliferation is mainly responsible for maintaining a more or less stable number of cells in each tissue, for example, after cell death or injury. However, in adult tissues most cells are short-lived and terminally differentiated thus lacking the ability to proliferate. A specific population of adult stem cells is therefore essential to ensure cellular turnover throughout the postnatal life. Such population is constituted by multipotent, undifferentiated cells that self-renew, and that are usually found in specific niches. These cells retain their stem cell characteristics throughout life while giving rise to progenitor cells that then undergo terminal differentiation.

The clinical success achieved with stem cell therapy highlights the crucial importance of this field. Due to its accessibility and well-studied developmental stages, the skin and, specifically, the epidermal stem cells are an excellent model for the study of adult stem cells. The study of epidermal stem cells will potentially yield new clinical approaches to skin injury, skin diseases, hair loss and skin cancer.

The present work aims to highlight the importance of epidermal stem cells as a scientific model. We start of by describing the main developmental stages of the epidermis and the hair follicle as well as some of the important signalling pathways so far linked to skin development and homeostasis. These include Wnt/β-catenin, BMP, p63 and Shh signalling. We describe the epidermal stem cell niches identified to date and some of their defining characteristics. Finally, we explore some of the consequences of epidermal stem cell malfunction and molecular cues that have been studied in association with ageing and skin cancer.

Index

Abbreviations List

BCC - Basal cell carcinoma
Blimp1 - B lymphocyte-induced maturation protein 1
BMP - Bone morphogenic protein
CPs - Committed progenitor cells
DNMAML1 - Dominant negative Mastermind Like 1, notch inhibitor
DP - Dermal papilla
E - Embryonic day
ECM - Extracellular matrix
Egf - Epidermal growth factor receptor
Fgf - Fibroblast growth factor
Get-1 - Grainyhead-like epithelial transactivator
GFP - Green fluorescence protein
HF - Hair follicles
HG - Hair germ
H3K27me3 - Histone mark trimethylated Lys27 of histone H3
IFE - Interfollicular epidermis
IRF6 - Interferon regulatory factor 6
IRS - Inner root sheath
Klf4 - Kruppel-like factor 4
KO - Knockout
K - Keratin
MCSP - Melanoma chondroitin sulphate proteoglycan
miRNA - MicroRNA
mRNA - Messenger RNA
Mx - Matrix compartment
NF-κB - Nuclear factor-κB
ORS - Outer root sheath
P - Postnatal day
SCC - Squamous cell carcinoma
Shh - Sonic hedgehog
TA - Transiently amplifying cells
TAp63 - p63 isoforms that contain a p53-like N-terminal transactivation domain
TGF-β - Transforming growth factor beta family of growth factors
TPA - 12-O-tetradecanoylphorbol-13-acetate
UI - Upper isthmus
WT - Wild-type
ΔNp63 - p63 isoforms that lack a p53-like N-terminal transactivation domain

Introduction

In the embryo, cell division contributes to new cell types or to the growing cell number of the developing organism. By contrast, in the adult, cell proliferation is mainly responsible for maintaining the number of cells more or less constant in each tissue, for instance, after cell death or injury. However, most cells in adult tissues are short-lived and terminally differentiated hence lacking the ability to proliferate. To circumvent this problem, tissues host a particular population of adult stem cells that ensures cellular turnover throughout the postnatal life. These are undifferentiated cells that self-renew, tend to be multipotent, and are usually found in specific niches. They retain these characteristics throughout life and give rise to progenitor cells that then undergo terminal differentiation (Watt, 1998).

Epidermal stem cells were first described in the early 70s as slow cycling and long-lived cells with the capability of differentiating and renewing all the cell lineages and layers of the epidermis (Cotsarelis et al., 1990). Due to its accessibility and well-studied developmental stages, the skin and, specifically, the epidermal stem cells are an excellent model for the study of adult stem cells. Additionally, the clinical success achieved with stem cell therapy highlights the crucial importance of this field. Indeed, the study of epidermal stem cells will potentially yield new clinical approaches to skin injury, skin diseases, hair loss and skin cancer.

The degree of complexity and organisation of this model has become more obvious over the years. Studies indicate the existence of more than one stem cell compartment within the epidermis. The different stem cell niches are believed to separately maintain the interfollicular epidermis, the hair follicles and the glands. Nevertheless, the cross-talk between these niches and the molecular pathways that govern each one still need extensive investigation. Furthermore, it is of high interest to study how epidermal stem cells are maintained, exactly in how many compartments they reside and what epidermal cell lineages they are able to originate.

In the present work we aim to address the epidermal stem cells as an important scientific model. To do so, we first describe the main stages and molecular signals so far implicated in skin development and homeostasis. Next, some of the most important findings in the epidermal stem cell research field are discussed. Finally, we address the role of epidermal stem cells regarding wound healing, ageing and cancer.

The skin

Vertebrates are covered by skin, which protects them from pathogens, radiation and water loss, and helps regulate the body temperature. The skin also plays an essential role in social and reproductive interactions, providing exchange of important information about the species. Animals become aware of the surrounding environment also through the skin's tactile function and numerous species resort to skin camouflage to escape from predators (Blanpain and Fuchs, 2009).

The skin is the largest mammalian organ and in the adult it is composed of a superficial layer – epidermis- and a subjacent layer – dermis **(Fig. 1)**. The epidermis is the first barrier against external

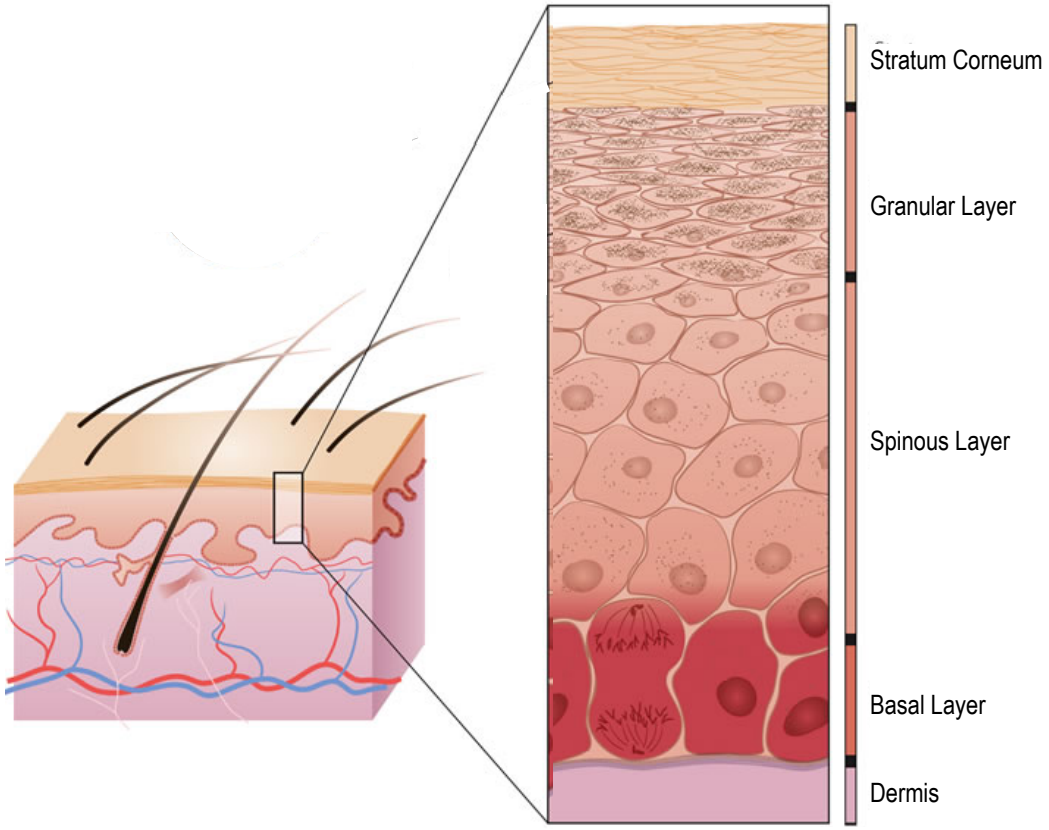

Figure 1 – The adult mammalian skin is constituted by the epidermis and the dermis. The epidermis is further divided in four strata: the Stratum Corneum, the Granular Layer, the Spinous Layer and the Basal Layer (adapted from www.skinipedia.org).

threats and is mainly constituted of keratinocytes. It is a non-vascular multilayered epithelium, formed of four different strata: the stratum corneum, the granular layer, the spinous layer and the basal layer. This stratified epithelium is also designated as interfollicular epidermis (IFE). The dermis is where blood and lymph vessels are located, supporting and nourishing the epidermis. Skin structures such as the hair follicles (HF), sebaceous and sweat glands, which lubricate and protect the hair and skin surface, project from the epidermis into the dermis (Gilbert, 2000).

Embryonic development and stratification of the skin

The epidermis is formed early after gastrulation during the embryonic development, phase during which the embryonic blastula (single layered) develops into the three layered gastrula. The formation of these three germ layers, defined as ectoderm, mesoderm and endoderm, is followed by organogenesis when each layer forms specific tissues and organs. The ectodermal cells located at the surface of the embryo commit to an epidermal fate. In mice, this happens at embryonic day (E) 8.5 and complete epithelial stratification takes approximately 10 days (Byrne et al., 1994; Moll et al., 1982) **(Fig. 2 A)**.

Still during embryonic development, asymmetrical division of proliferating basal keratinocytes originates the periderm and then the intermediate cell layer in cooperation with mesenchymal cues. While the periderm is lost before birth, the cells of the intermediate layer become less proliferative and differentiate first into spinous cells and later into granular and cornified cells. In the embryo as well as in the adult, the basal keratinocytes continue to divide and their daughter cells move up towards the skin surface maturating into the different epidermal layers. This process is accompanied by alterations in expression of differentiation markers such as keratins, intermediate filament proteins that provide structural support. Expression of keratins 8 and 18 (K8 and K18) is characteristic of uncommitted surface ectodermal cells. Commitment to the epidermal fate leads to replacement of K8 and K18 by K5 and K14 expression, which is maintained by the basal layer. Stratification of the epidermis, however, results in expression of K1 and K10 at the level of the spinous layer (Byrne et al., 1994; Moll et al., 1982).

Keratins are particularly important in the formation of the outer layers of the epidermis. They are required for the generation of more robust networks that support cell-cell junctions and protect the body surface against physical stresses. In the granular layer, for instance, cells soon loose their cytoplasmic organelles and retain keratins as the main cytoplasmic protein, together with enzymatically cross-linked proteins. Additionally, in the extracellular space between dead corneum cells, lipid bilayers are deposited and covalently linked, waterproofing the epidermis. As corneum cells are shed from the surface of the skin, basal cells continue to proliferate in order to restore and maintain the different epidermal strata. The differentiation and migration of a basal cell until the skin surface can take between 2 to 3 weeks (Blanpain and Fuchs, 2009; Fuchs and Green, 1980; Koster and Roop, 2007).

The dermis, on the other hand, is formed by mesoderm-derived fibroblasts that secrete collagen, arrecator pili muscles that support the HFs, vasculature, subcutaneous fat and immune cells. Neural crest cells originate melanocytes, melanin-producing cells located in the basal layer of the epidermis, the dermis of the head and sensory nerves of the skin (Blanpain and Fuchs, 2006).

Molecular signalling during epithelial differentiation and stratification

The development of the skin and stratification of the epidermis are complex processes. Therefore they ought to be tightly controlled during embryogenesis but also throughout adult life due to the continued renewal of the epidermis. Within this context, particular molecular cues are essential for appropriate differentiation of basal cells into the layers that constitute the epidermis **(Fig. 2 B)**.

In vertebrates, expression of p63 (p53 related) is strongly associated with epidermal commitment. It was found to be the first transcription factor expressed specifically by the epidermal lineage (Green et al., 2003; Lee and Kimelman, 2002; Yasue et al., 2001). Due to the existence of two promoters, p63 isoforms can be expressed either containing a p53-like N-terminal transactivation (TA) domain or lacking the domain s

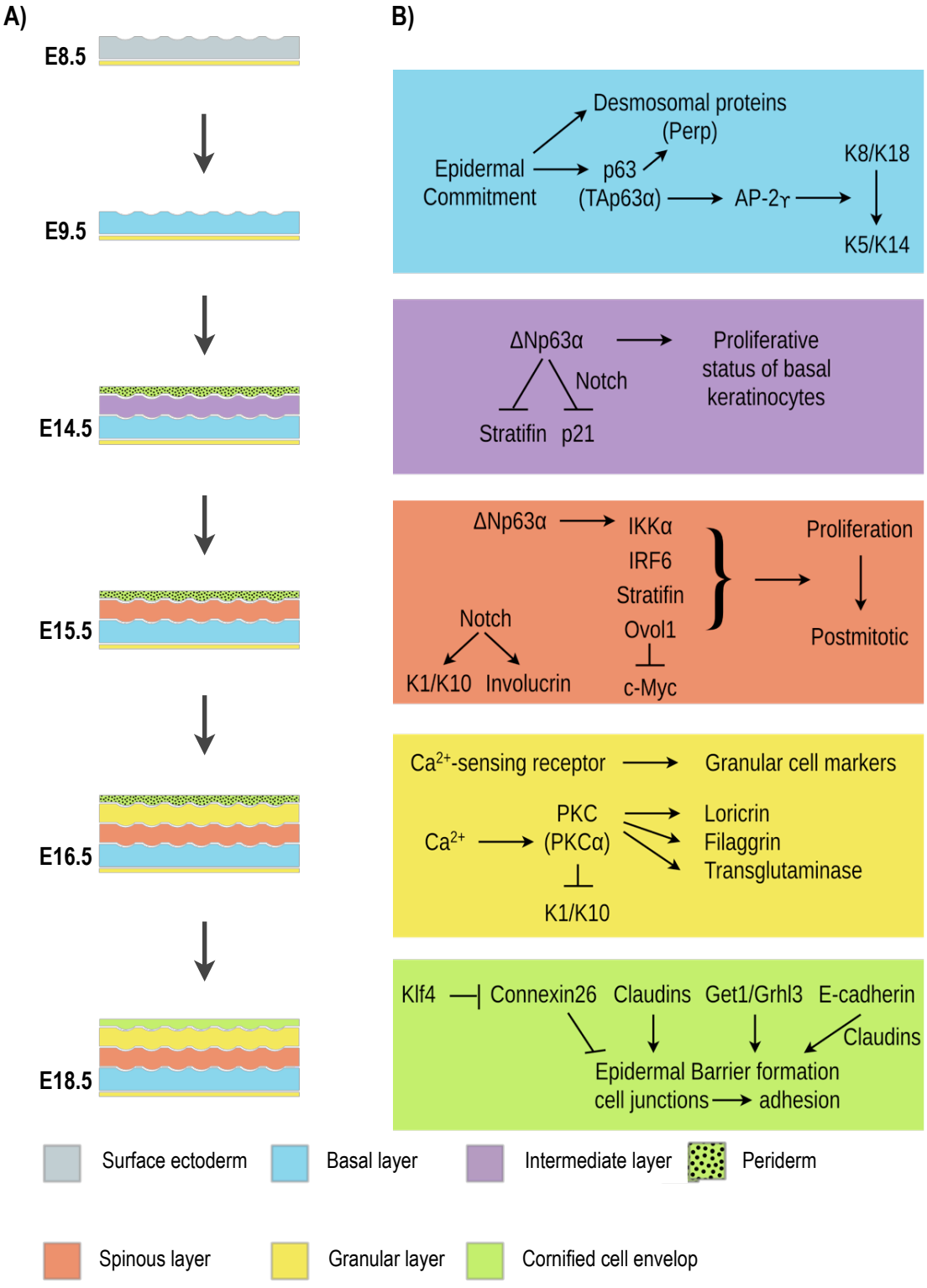

Figure 2 – Epidermal stratification and molecular mechanisms involved. **A)** In mice, epidermal stratification takes approximately 10 days, starting with epidermal commitment of the surface ectoderm at E8.5. In cooperation with mesenchymal cues, asymmetrical division of embryonic basal keratinocytes originates the periderm and then the intermediate layer. While the periderm is lost before birth, the cells of the intermediate layer become less proliferative and differentiate into spinous cells. Postmitotic spinous keratinocytes mature into granular and cornified cells. In the granular layer, cells soon loose their cytoplasmic organelles. The barrier formation requires cell adhesion, and lipid synthesis for deposition within the extracellular space between dead corneum cells, waterproofing the epidermis. **B)** The main molecular mechanisms required for the development of each layer are summarised in boxes with the colour of the respective layer (left) (based on (Koster and Roop, 2007)).

(ΔN) (Yang et al., 1998). TAp63 isoforms are the first to be expressed during embryonic development of epidermis, even before the expression of K14 (Koster et al., 2004). In particular, the isoform TAp63α was shown to regulate expression of K14. This is partially dependent on the neural crest- and epithelial-specific transcription factor AP-2Ɣ, a known inducer of K14 expression (Koster et al., 2006; Romano et al., 2007).

Commitment to the epidermal fate is also linked to the expression of desmosomal proteins, which further ensure cell adhesion. Interestingly, p63 regulates the expression of at least one desmosomal protein, Perp, essential for proper epidermal development (Cheng and Koch, 2004; Ihrie et al., 2005). Together, these findings highly support p63 as a key player in the epidermal commitment process.

The TAp63 isoforms are essential in the early establishment of the epidermal fate. On the other hand, the ΔNp63α isoform is only induced after epidermal commitment but strongly expressed in basal keratinocytes. These facts suggest that p63 might also contribute to the maintenance of the basal layer characteristics (Candi et al., 2006; Senoo et al., 2007). Indeed, the ΔNp63α isoform appears to contribute to the proliferative status of basal keratinocytes through repression of the *p21* and *14-3-3σ* (also named stratifin) genes, both expressed by differentiating epidermal cells (Truong et al., 2006; Westfall et al., 2003). Repression of *p21,* in particular, was observed directly, by promoter-binding, but also through ΔNp63α-meditated inhibition of Notch signalling. The Notch pathway acts upstream of p21 and has been implicated in the differentiation and self-renewal processes of the epidermis (Nguyen et al., 2006; Rangarajan et al., 2001; Westfall et al., 2003).

The differentiation process of the intermediate layer into the spinous layer is mainly characterised by the switch of the mitotic state of cells to a postmitotic one. A number of studies have implicated different proteins as regulators of this process. Among these is *IKKα*, positively regulated by ΔNp63α and a player in epidermal morphogenesis. *IKKα* deficient mice die perinatally and exhibit very proliferative epidermal cells as well as altered differentiation (Koster et al., 2007; Takeda et al., 1999). Other mice models that resemble this phenotype include mice lacking the interferon regulatory factor 6 (IRF6) function (Ingraham et al., 2006; Richardson et al., 2006) and mice with mutant *stratifin* (Herron et al., 2005; Li et al., 2005). *Ovol1*-deficient mice also provided evidence that Ovol1 directly represses *c*-Myc in suprabasal cells and arrests proliferation (Nair et al., 2006).

Moreover, differentiation of basal cells into spinous cells is again linked with the canonical Notch signalling. In fact, expression of activated Notch1 by adenoviral infection induced the expression of spinous cells markers (such as K1, K10 and involucrin) (Rangarajan et al., 2001). Additionally, RBPJ conditional null epidermis, a Notch required canonical signalling partner (Blanpain et al., 2006), and loss of *Hes1*, an epidermal Notch target gene, both result in altered differentiation of the spinous layer. Notch signalling is also essential for granular layer differentiation (Moriyama et al., 2008). The AP-2 transcription factor family was found to cooperate with the Notch pathway as well and to regulate suprabasal differentiation (Wang et al., 2008).

Differentiation of epidermal spinous cells into granular cells is characterised by the expression of loricrin, filaggrin and transglutaminase (enzyme involved in cross-linking structural proteins at the superficial epidermis). The PKC protein induces the expression of these granular markers and downregulates both K1 and K10. Therefore, it seems to be important for the spinous to granular transition process. It (Dlugosz and Yuspa, 1993, 1994).

In addition, a link between PKC activity and the extracellular Ca^{2+} gradient formed during embryogenesis has been proposed. The extracellular Ca^{2+} concentration is lower in the basal and spinous layers, increasing until the granular layer and decreasing in the stratum corneum. Studies suggest a specific role for the calcium gradient in the terminal differentiation of the spinous and the granular layers (Elias et al., 1998; Menon et al., 1985; Yuspa et al., 1989). The extracellular Ca^{2+}-sensing receptor, for instance, is found expressed in granular cells and specifically required for terminal differentiation of keratinocytes and expression of the granular cell markers (Komuves et al., 2002; Turksen and Troy, 2003). In primary keratinocytes, Ca^{2+} promotes phosphatidylinositol metabolism and increases diacylglicerol (involved in PKC activation) (Lee and Yuspa, 1991). On the other hand, although a number of PKC isozymes are regulated during keratinocyte differentiation, PKCα appears to be the only one inducing expression of granular markers in a Ca^{2+}-dependent manner (Denning et al., 1995; Koizumi et al., 1993; Ohba et al., 1998; Yang et al., 2003).

Finally, the formation of the epidermal barrier and differentiation of cornified cells is associated with the transcription factor Kruppel-like factor 4 (Klf4). *Klf4*-deficient mice lack the skin barrier function, mostly due to impaired formation of the cornified envelope, and die soon after birth (Segre et al., 1999). Conversely, ectopic expression of Klf4 accelerates the formation of the epidermal barrier and epidermal differentiation in mice (Jaubert et al., 2003). One molecular mechanism through which Klf4 is thought to act is by directly repressing the expression of the gap junctional protein Connexin26. Connexin26 expression promotes the epidermal proliferative state and delays epidermal barrier formation (Djalilian et al., 2006; Patel et al., 2006). Cellular junctions proteins like Connexin26 further ensure the function of the epidermal barrier. As another example, *claudin-1*-deficient mice, a tight junctional protein, display extensive water loss and die perinatally (Furuse et al., 2002). E-cadherin-deficient (core adherens junctions protein) mice die due to permeable cell junctions and consequent water loss. The authors found that E-cadherin regulates the association of claudins with tight junctions, hence, controlling barrier formation. Interestingly, also in these mice, PKC was found mislocalised which correlates with its known role in tight junction formation (Suzuki et al., 2002; Tunggal et al., 2005). The *Grainyhead-like epithelial transactivator (Get-1)/Grhl3*-deficient mice exhibit strong defects of barrier formation and differentiation of the corneum layer as well. This transcription factor controls the expression of numerous epidermal differentiation proteins, including cell adhesion and structural proteins, and enzymes involved in lipid metabolism (Ting et al., 2005; Yu et al., 2006).

Interestingly, recombination experiments have provided strong evidence that epithelial stratification is stimulated by signals produced by mesenchymal cells in the skin. Grafting of rabbit corneal epithelium together with mouse embryonic dermis showed how the dermis is able to influence epithelial commitment. Corneal cells differentiated into the epithelial basal layer, formed epithelial appendages like sweat glands, and started expressing keratins characteristic only of the epidermis (Ferraris et al., 2000). Epidermal transdifferentiation of other epithelial tissues, such as the prostate and the mammary epithelium, has also been reported. Importantly, the process was further associated with β-catenin signalling, a central player of the active Wnt pathway (Bierie et al., 2003; Miyoshi et al., 2002; Pearton et al., 2005). Corroborating these data is the observation that *Dkk2* knockout mice, a Wnt antagonist, results again in epidermal transdifferentiation of the corneal epithelium (Mukhopadhyay et al., 2006). It seems that Wnt signalling might also cooperate with bone morphogenic protein (Bmp) signalling (Wilson et al., 2001). Bmps belong to the transforming growth factor beta (TGF-β) family of growth factors and have been established as epidermal-fate inducers (Hawley et al., 1995; Nikaido et al., 1999; Suzuki et al., 1997). A recent study showed in chick embryo that the transcription factor AP-2Ɣ acts also downstream of Bmp4 to promote epidermal differentiation (Qiao et al., 2012).

Other pathways have also been identified as players during terminal differentiation and stratification of the skin. For instance, the nuclear factor-κB (NF-κB) that regulates epidermal proliferation. Nevertheless, the network that connects and balances the signalling of all these pathways, ultimately guiding skin terminal differentiation, still needs further investigation (further reviewed by (Dai and Segre, 2004; Koster and Roop, 2007)).

The study of the epigenetic regulators' role in skin development is starting to gain more importance over the years. It has become clear that histone modifications do influence the overall chromatin state and regulate expression of key genes throughout epidermal differentiation (Frye and Benitah, 2012). The histone mark trimethylated Lys27 of histone H3 (H3K27me3), for example, occupies and represses the promoter of many genes important for epidermal differentiation. Upon loss of this mark, differentiation is induced (Ezhkova et al., 2009; Mejetta et al., 2011). Silencing of the histone demethylase JMJD3 (binds differentiation promoters marked by H3K27me3) blocked the expression of JMJD3-bound differentiation genes. Accordingly, ectopic JMJD3 expression induced premature differentiation (Sen et al., 2008). Additionally, it has been described that Myc, known to control epidermal proliferation, can induce histone modifications that significantly influence the exit from the stem cell compartment (Frye et al., 2007).

MicroRNAs (miRNAs) are known to play a role in numerous developmental mechanisms by regulating gene expression (Yi et al., 2006). The miR-203 is a key example of the implication of miRNAs in the development of the skin. Already during epithelial development, the expression of this miRNA leads to skin differentiation and stratification through the repression of the suprabasal layers proliferative potential. MiR-203 targets and

represses *p63*, amongst other genes, decreasing the proliferative potential of the cells where it is expressed (Jackson et al., 2013; Yi et al., 2008). It is still possible that miR-203 or other miRNAs regulate the expression of more crucial genes that control the level of differentiation and 'stemness' of epidermal cells.

Morphogenesis of the hair follicle

The hair follicle and the respective sebaceous gland constitute a pilosebaceous unit. In the adult, the HF is continuously cycling and, thus, renewed. This reflects the need for a population of stem cells that proliferates and maintains the homeostasis of this structure.

The HF is formed by concentric layers of cells that develop from the projection of the epidermis into the dermis **(Fig. 3 A)**. After the embryonic epidermal stratification starts, undifferentiated basal cells receive the first signal from the dermis to initiate the HF development. Epithelial-mesenchymal interactions are established at sites of dermal condensation, inducing the formation of the hair placode, an elongation of undifferentiated basal epithelium. The epidermal hair placode sends the second signal and instructs the formation of the imminent dermal papilla (DP), a specialised mesenchymal structure. The DP then communicates with the elongating basal epithelium and sends the third signal inducing differentiation of the different HF cell lineages.

Located in the base of the HF are the matrix cells (Mx), TA cells that stimulated by the DP continue to proliferate until the HF starts to maturate. At that moment, these cells start to differentiate and give rise to the companion layer, the hair shaft, to the inner root sheath (IRS), and the hair channel. The IRS is characterised by the expression of trichohyalin, an intermediate-filament-associated protein (O'Guin et al., 1992), and GATA3, a central player in T-cell lineage commitment and required for IRS formation (Kaufman et al., 2003). The outer root sheath (ORS) is formed by the basal layer connecting with the epidermis and externally covered by a basement membrane. ORS cells express K5 and K14, in line with the expression pattern of the basal layer of the IFE. In the meantime, the other HF structures including the sebaceous glands, are also formed. In mice, the HF downgrowth is only concluded eight days after birth. In the following seven days, the Mx continues to proliferate and differentiate to form seven concentric layers of follicle **(Fig. 3 B)** (reviewed by (Blanpain and Fuchs, 2006).

The hair follicle cycle

Throughout postnatal life, HF undergoes cyclical bouts of growth, regression and quiescence. Interestingly, the formation of a new mature follicle is highly similar to the embryonic development of the HF.

It is 16 days after birth that the proliferation of the Mx is suspended and that the lower two-thirds of the HF quickly degenerate **(Fig. 4)**. This apoptosis-driven phase of the cycle is designated as the catagen stage. The remaining IRS moves upwards the connected DP to rest next to the non-cycling segment of the follicle. This permanent portion of

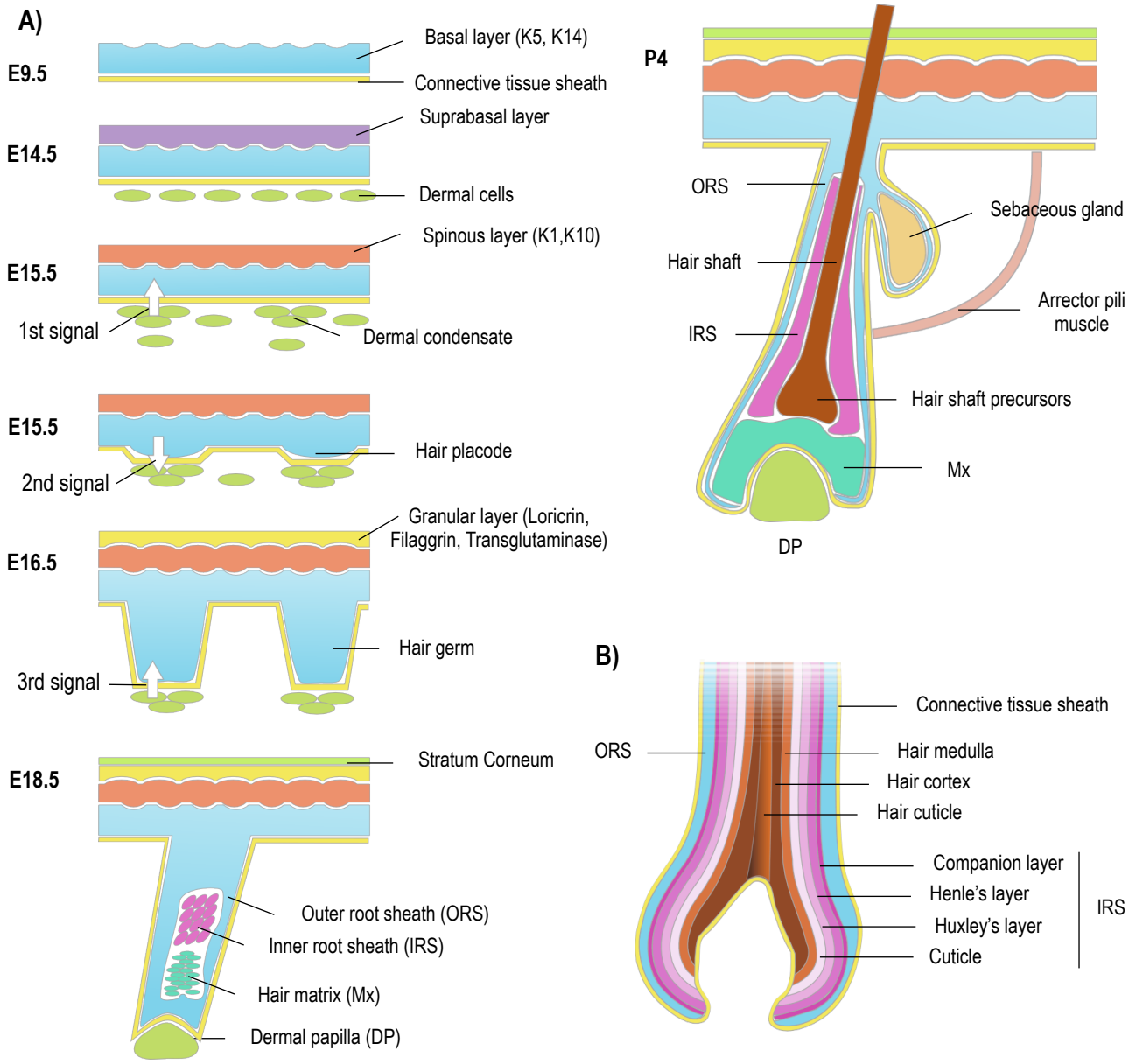

Figure 3 – Hair follicle morphogenesis and anatomy. **A)** During embryonic development, the epidermis gradually stratifies and forms layers of terminally differentiating cells. The underlying dermis sends the first signal and instructs some of the undifferentiated basal cells to form a hair follicle. Subsequently, the epidermis responds with a second signal that commands the dermis to make the imminent dermal papilla (DP). The maturing DP then sends a third signal to the developing hair germ, promoting its growth and differentiation into the hair follicle lineages, hair and sebaceous glands. Encased by connective tissue, the outer root sheath (ORS) is contiguous with the epidermal basal layer and surrounds the inner root sheath (IRS). Located in the base of the mature follicle are proliferating cells that constitute the matrix (Mx). **B)** Mx cells differentiate and give rise to concentric rings of cells forming the three hair shaft layers and the four different IRS layers (based on (Blanpain and Fuchs, 2006; Cotsarelis, 2006)).

the follicle, right below the sebaceous gland, is where the bulge area is located. There, stem cells that specifically fuel the HF cycle form a niche. The resting period is named telogen and is characterised by the quiescent state of the bulge stem cells (Fuchs, 2009; Morris et al., 2004; Tumbar et al., 2004). In mice, the first telogen phase that takes place after birth is as short as one day. However, in the following HF cycles, the telogen becomes longer which also results in less synchronous cycles. The start of a new cycle - anagen stage - is marked by the reactivation of bulge stem cells and formation of

a proliferating hair germ (HG), a small cluster of cells below the bulge. This results in new hair growth by replenishing of the previously lost cells (Legue and Nicolas, 2005; Muller-Rover et al., 2001) .

The HF cycle is sensitive to many hormones, cytokines, growth factors, neuropeptides and pharmaceutical compounds (Paus and Cotsarelis, 1999). However, the molecular networks that control the development and homeostasis of the HF have yet to be further explored.

Signalling pathways in the cycling hair follicle

A few signalling pathways have been identified as central regulators of HF morphogenesis and homeostasis. The understanding of how they cooperate during adult HF cycling will be crucial for the design of therapeutic strategies against skin pathologies.

Transgenic mice have been used to study the role of pathways like Wnt/β-catenin in the skin **(Fig. 5 A&B)**. Lef-1 can participate in Wnt signalling, partnering with β-catenin to form a functional transcriptional complex (Behrens et al., 1996). Some studies have initially implicated the Lef-1/Tcf family of DNA-binding proteins in the follicular epithelial-mesenchymal signalling (Kratochwil et al., 1996; van Genderen et al., 1994; Zhou et al., 1995). Mice expressing a transgenic stabilised form of β-catenin under the control of the epidermal *K14* promoter, display *de novo* hair morphogenesis, including DP and sebaceous gland formation. The growth of existent follicles was also stimulated. Ultimately, these mice develop skin tumours addicted to β-catenin signalling (Gat et al., 1998; Lo Celso et al., 2004; Van Mater et al., 2003).

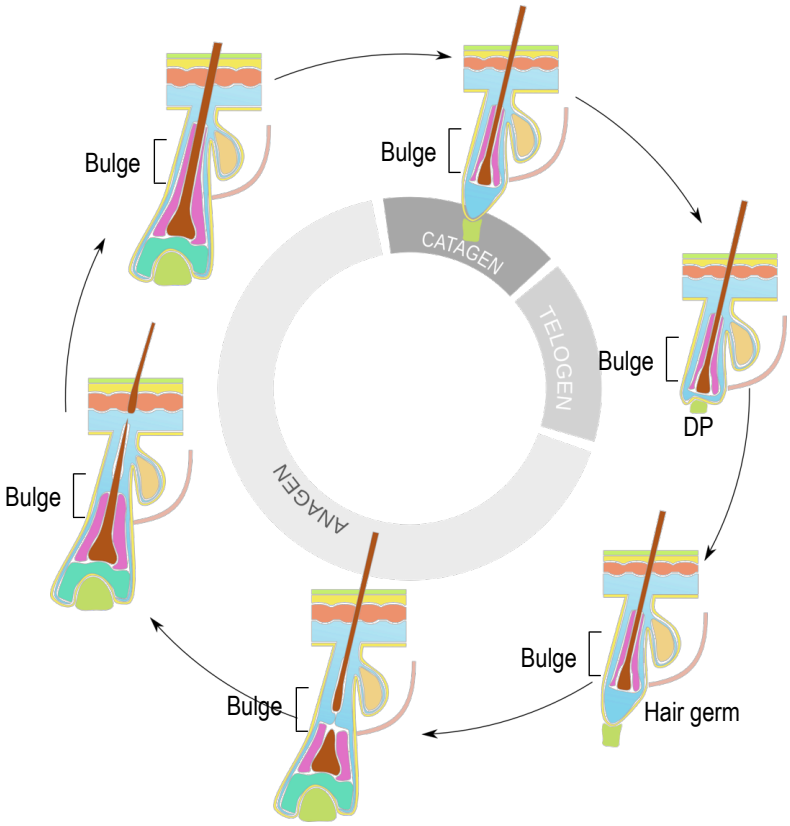

Figure 4 – The hair follicle cycle. Sixteen days after birth, matrix cells proliferation stops and the lower two-thirds of the hair follicle (HF) quickly degenerate. This is the catagen stage, an apoptosis-driven process. The dermal papilla (DP) moves upwards connected to the remaining inner root sheath, to rest next to the non-cycling segment of the follicle. This permanent portion, right below the sebaceous gland, is where the bulge area and stem cells that specifically fuel the HF cycle are located. The resting period during which bulge stem cells remain quiescent is named telogen. The anagen stage is the start of a new cycle, marked by the reactivation of bulge stem cells and formation of a new hair germ, a small cluster of cells below the bulge. This results in growth of a new hair and replacement of the previously lost cells (based on (Blanpain and Fuchs, 2006)).

A number of studies have also linked Notch signalling to the postnatal homeostasis (Lin et al., 2000; Pan et al., 2004; Uyttendaele et al., 2004). Inactivation of *Notch1* results in almost complete hair loss after birth and in the adult mice (Vauclair et al., 2005). In contrast with other progenitor cell populations, *in vitro* Notch signalling in keratinocytes was described as a promoter rather than a repressor of differentiation (Lowell et al., 2000; Rangarajan et al., 2001). Deletion of *jagged-1*, a Notch ligand, leads to postnatal conversion of HFs into IFE differentiating phenotype (Estrach et al., 2006). Remarkably, Notch signalling has been proposed to act downstream of β-catenin, which promotes *jagged-1* expression. Experiments showed active Notch signalling in a region of the HF with high Wnt activity, where commitment to hair lineages takes place (pre-cortex, the region above the Mx). More importantly, β-catenin-driven formation of new HFs in the adult is blocked in the absence of Notch signalling. Conversely, activation both pathways results in faster growth and differentiation of the ectopic follicles (Estrach et al., 2006).

The Sonic hedgehog (shh) pathway is also affected by β-catenin, which presumably acts upstream of this pathway both during hair morphogenesis and HF cycling. In *β-catenin*-negative skin, *Shh* is not expressed (Huelsken et al., 2001). Inhibition of Shh signalling diminishes the β-catenin-induced effect of new HF formation (Silva-Vargas et al., 2005). Smoothened deletion, a Shh pathway member, results in *de novo* hair morphogenesis but no alteraton in β-catenin signalling activity (Gritli-Linde et al., 2007). Collectively, these findings indicate that β-catenin pathway acts upstream and promotes the Shh signalling. More experiments further support the role that Shh plays in the control of the ingrowth and morphogenesis of the HF (Chiang et al., 1999; St-

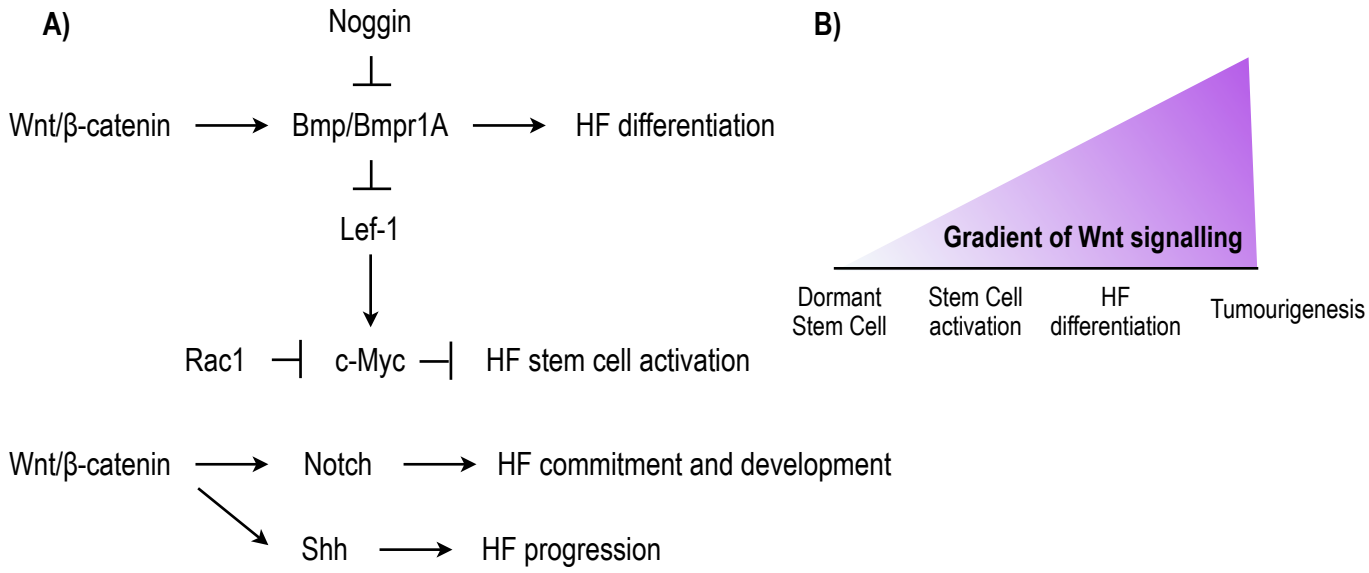

Figure 5 – Signalling pathways involved in hair follicle morphogenesis. **A)** Interactions between the major signalling pathways implicated in hair follicle formation and cycling. **B)** A gradient of Wnt signalling could explain the distinct roles and contributions of the pathway to hair follicle morphogenesis. Such gradient concerns the Tcf/Lef/β-catenin status of transcriptional activity within the cell, and does not necessarily involves the Wnt proteins (based on (Blanpain and Fuchs, 2006)).

Jacques et al., 1998). In particular, *Shh*-negative mouse embryos display obstructed formation of the DP (Karlsson et al., 1999).

During the skin development, Bmp2 is expressed by the hair placode, Mx and precortex epithelium (Lyons et al., 1990). Bmp4, on the other hand, is found in the underlying mesenchymal cells. This suggests the involvement of Bmps in epithelial-mesenchymal interactions. In the adult, Bmp4, 6 and 7 are expressed in the DP. Bmp7 and Bmp8 are expressed also by the IRS, whereas Bmp2 and 4 are expressed by hair shaft precursor cells. This suggests distinct non-redundant roles for these proteins (Blanpain and Fuchs, 2006; Huelsken et al., 2001). The Bmps are secreted and recognised by a cell membrane receptor complex formed by Bmpr1 and Bmpr2 receptors. In zebrafish, Bmp directly induces epidermal differentiation of uncommitted ectodermal cells (Nikaido et al., 1999). This observation highlights how evolutionary conserved the role of the Bmp family is in skin development.

Expression of Bmps appears to be depend on β-catenin since Bmp2, 4 and 7 are not expressed in *β-catenin*-negative skin during hair morphogenesis and hair cycling (Huelsken et al., 2001). Conditional ablation of *Bmpr1* leads to impaired differentiation of the hair shaft and IRS. Mutant HFs fail to undergo catagen, and instead continue to proliferate, forming follicular cysts and matricomas. Furthermore, although *Lef-1* expression is retained, nuclear β-catenin is lost from the epithelium of severely mutated follicles (Andl et al., 2004). However, in another study, mice lacking the Bmp inhibitor Noggin do not express Lef-1. The authors proposed a model in which inhibition of Bmpr1a allows *Lef-1* expression, required for stem cell activation. Then, subsequent activation of Bmpr1a is necessary for differentiation of *Lef-1*- and *β-catenin*-expressing hair progenitor cells into the low-Wnt activity hair lineages (Kobielak et al., 2003). These data underscore the existence of complex regulatory loops between the Wnt and Bmp signalling pathways in postnatal follicles.

Genetic studies in mice have significantly contributed to the current scientific knowledge. Consequently, other pathways have been implicated in HF morphogenesis and cycling. For instance, the fibroblast growth factor (Fgf), the epidermal growth factor receptor (Egf) and the NF-κB signalling pathways are important players (Blanpain and Fuchs, 2006). Finally, epidermal deletion of Rac1 promotes proliferation and terminal differentiation of hair follicle stem cells, disturbing the homeostasis of the IFE, HFs, and sebaceous glands. Rac1 is a Rho guanosine triphosphatase known to repress *c-Myc* in the epidermis (Benitah et al., 2005).

Epidermal stem cells and skin homeostasis

The skin epidermis and its ornaments are continuously being renewed. Niches of multipotent adult stem cells strategically localised in the skin are responsible for skin postnatal homeostasis. In the IFE, cells located in the basal layer compensate for the high turnover of the epidermis. However, structures like the HFs and sweat glands are mostly dependent on the homeostasis of their respective stem cell niches.

Located near the base of the 'permanent' region of the HF is the stem cell niche that fuels each anagen stage of the hair cycle. Such stem cells give rise to TA Mx cells that then proliferate in the hair bulb and undergo terminal differentiation, resulting in the formation of a new IRS and hair shaft. Recent findings describe the existence of other stem cells niches located above the HF bulge are. These will be also addressed further below.

Epidermal stem cells in the interfollicular epidermis

Throughout postnatal life, the epidermis has to be continuously renewed as dead cornified cells are lost at the surface of the skin and replaced by the subjacent differentiating cells.

A hypothesis that explains the basal layer contribution and fuelling of the differentiated suprabasal cells is the asymmetric cell division of the IFE stem cell compartment (Smart, 1970). The human skin is composed of thicker epidermis with epidermal cavities that anchor the epidermis to the dermis. Each one of these structures is proposed to work as an epidermal proliferative unit (EPU), where a potential stem cell is located and surrounded by the transiently-amplifying (TA) progeny (Allen and Potten, 1974; Potten, 1975). Indeed, IFE slow-cycling cells are located at the bottom of these undulations, assumedly the most protective region of the IFE (Lavker and Sun, 1982). It has been further suggested that after a certain number of cell divisions these TA cells then differentiate into postmitotic keratinocytes, balancing the epidermal turnover (Potten, 1975). Asymmetric cell division of the stem cell compartment into proliferating TA basal cells can occur by orienting the mitotic spindle parallel to the basal layer. This allows the positioning of one of the daughter cells away from the IFE stem cell compartment. Such mechanism leads to unequal distribution of cell fate and growth factors between the two daughter cells. These factors may include specific RNAs and polarity proteins. On the other hand, if the asymmetric division occurs parallel to the basal membrane, both daughters remain temporarily inside the basal layer. One daughter cell receives the signals necessary to remain a stem cell, while the other soon starts the basal differentiation program and moves towards the surface of the epidermis. (Lechler and Fuchs, 2005).

However the stem-TA-cell model has been broadly accepted (Brash et al., 2005; Fuchs, 2007; Kaur, 2006), it has recently been challenged by the work of Clayton and her colleagues. The authors have proposed a different model in which committed progenitor (CPs) cells represent a single proliferating basal cell compartment. Upon division, these cells can stochastically adopt three possible

fates: two daughter progenitor cells, two daughter differentiated cells, or asymmetric division. The model included no role for IFE stem cells in the steady state, but a function in growth or wound healing was not excluded (Clayton et al., 2007). It was only not long ago that a study demonstrated the existence of two discrete epidermal progenitors populations: a slow-cycling stem cell population and a more proliferating CP one. Both progenitor types share a similar pattern of asymmetric self-renewal (Mascre et al., 2012). The balance between proliferation and differentiation reflects stochastic fate choice, as described for CPs by (Clayton et al., 2007). However, these two populations were found to differentially contribute to the homoeostasis and repair of the epidermis. While rapidly cycling CPs are the main players in routine maintenance, dormant stem cells become active and very efficiently contribute to epidermal wound repair (Mascre et al., 2012).

In vitro studies identified β1-integrin as a potential marker for IFE stem cells. In cultured human keratinocytes, a higher proliferative status was associated with higher levels of this cell surface receptor. Furthermore, keratinocytes with the highest levels of β1-integrin also express melanoma chondroitin sulphate proteoglycan (MCSP). Resorting to an antagonist of MCSP, the authors showed how MCSP is required for proper cadherin-mediated cell-cell adhesion and maintenance of cortical actin cytoskeleton of high β1-integrin-expressing cells. This marker for IFE stem cells thus potentially contributes to their clustering in the EPU (Legg et al., 2003). Conversely, proliferating keratinocytes that adhere more slowly to extracellular matrix (ECM) proteins display properties of TA cells and differentiate after one to five rounds of division (Jones and Watt, 1993). *In vivo*, β1-bright cells localise to the basal layer where they form clusters. However, these cells did not localise at the base of every epidermal ridge (Jones et al., 1995).

TGF-β is a well established proliferation inhibitor. Epithelial deletion of TβRII, an essential receptor for TGF-β signalling, results in hyperproliferation which, however balanced with increased apoptosis, highlights the role of this pathway in IFE proliferation (Guasch et al., 2007).

The above described studies have unveiled some characteristics of the IFE stem cells. Nevertheless, purification and analyses of this population of cells will likely require the identification of more markers. The exact location of this stem cell niche awaits further investigation as well. Finally, it will be very interesting to understand if IFE stem cells also contribute to the homeostasis of the other epidermal structures.

Epidermal stem cells of the hair bulge

HF stem cells were first thought to reside in the Mx area of the hair bulb due to the rather undifferentiated state of these cells. However, upon removal of the Mx-containing hair bulb, a new and complete HF is still formed (Cotsarelis et al., 1990). Since then, it has been proposed instead that stem cells of the bulge migrate downwards during anagen. Once they reach the Mx area, terminal differentiation into the different HF lineages occurs (Oshima et al., 2001; Tumbar et al., 2004).

It was early in the 1990s that nucleotide experiments allowed the labelling and identification of the HF stem cells and their niche. Experiments were carried based on one of the main stem cell characteristics: their slow-cycling nature that potentially protects them from replication-caused DNA errors. According to this, stem cells require repeated administration of tritiated thymidine and for longer periods. On the other hand, once the isotope is incorporated, in contrast to other cells, stem cells should retain it for a prolonged period of time. They were thus designated as label-retaining cells (LRCs). Studies demonstrated that these LRCs are, indeed, not located in the Mx region but rather in the bulge area of the HF. Remarkably, the bulge constitutes a strategic location for stem cells, since, in contrast with the hair bulb, it does not suffer cyclic degeneration; it is protected from damage caused by hair plucking; its location coincides with the downgrowth region and allows interactions with the DP during the specific hair cycle stages; and the tissue itself is rather vascularised, ensuring the nourishment of the stem cells (Cotsarelis et al., 1990; Morris and Potten, 1999).

It is noteworthy that already during embryonic development a population of slow-cycling cells is formed in the non-cycling portion of the HF, right below the imminent sebaceous gland. This population and the respective progeny, assumedly stem cells, were found to contribute to the epidermal cell lineages and to be essential for HF and sebaceous gland formation. Tracking these cells until the adult bulge confirmed them to be early bulge LRCs. Importantly, four transcription factors were identified in the early bulge LRCs: Sox9, NFATc1, Tcf3, and Lhx2 (Nowak et al., 2008). On the other hand, in the adult hair bulge, stem cells express, along with the above mentioned factors, green fluorescence protein (GFP) under the control of *K15* promoter, and CD34. K15 and CD34 are now very often used as markers (Morris et al., 2004; Trempus et al., 2003). Moreover, albeit the origin of bulge stem cells has been traced to the HF embryogenesis, with the start of the first HF cycle after birth, the bulge acquires a second layer of cells. Both embryonically- and postnatally-formed bulge populations were shown to be slow-cycling in the niche but able to self-renew *in vitro*, and to originate epidermis and hair *in vivo* (Blanpain et al., 2004).

Although generally quiescent, these stem cells can also be stimulated to proliferate, for instance, upon 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment (strong tumour promoter) (Cotsarelis et al., 1990). Moreover, when dissected from rat, cultured bulge cells give rise to more colonies than any other region of the HF (Oshima et al., 2001). Transgenic mice expressing H2B-GFP under the control of *K5* promoter have further confirmed the bulge as the residing niche for the slow-cycling HF stem cells. Additionally, purification of this population of cells allowed the profiling and identification of a vast number of messenger RNAs (mRNAs) specially expressed by the LRCs. Some of these are skin-specific, while expression of others is shared with other stem cell populations (Tumbar et al., 2004). A different study investigated whether Lgr5, a leucine-rich repeat-containing G protein expressed by cycling stem cells of the intestine (Barker et al., 2007), is also markedly expressed by HF stem cells. Indeed, *Lgr5* marks actively cycling cells in the HG and bulge during telogen and in the

lower ORS during anagen. Over long periods of time, *Lgr5*-expressing cells are capable of forming new HFs and to give rise to all HF cell lineages. The activity of this population of cells was associated with Shh signalling but its significance remains to be determined (Jaks et al., 2008). *Sox9* has also been identified, downstream of the Shh pathway, as a player during ORS differentiation and in formation of the hair stem cell niche. Moreover, the progeny of $Sox9^+$ cells give rise to all epidermal lineages. Conditional ablation of *Sox9* results in absence of the hair stem cell compartment and, likely as a consequence, severe HF and sebaceous gland defects (Nowak et al., 2008; Vidal et al., 2005).

During late catagen, bulge stem cells were also shown to repopulate and fuel the future HG formation (Ito et al., 2004). Regarding patterns of expression, albeit distinct, the HG is more similar to bulge cells than to the proliferating Mx cells. Additionally, both HG and bulge cells act rather quiescent during most telogen (Greco et al., 2009). Bmp signalling, also involved in HF development, appears to control and maintain such quiescent state of bulge cells. Conditional ablation of *Bmpr1a* and overexpression of *Noggin* leads to the activation and proliferation of otherwise quiescent stem cells (Plikus et al., 2008). This causes accumulation of undifferentiated masses and loss of slow-cycling cells (Kobielak et al., 2007). During telogen bulge cells, including some slow-diving cells, leave the niche and migrate to the HG. By the end of this stage, HG cells located at the base of the bulge start to differentiate (Greco et al., 2009; Zhang et al., 2009).

As described above, the start of anagen is associated with downregulation of the Bmp pathway and concomitant activation of Wnt and β-catenin signalling in bulge stem cells (Greco et al., 2009; Huelsken et al., 2001; Kobielak et al., 2007). β-catenin is, in fact, central during the transition of bulge stem cells from dormancy to TA proliferating progeny. This mechanism is directly linked to temporary Lef1/Tcf activation, which then activates expression of target genes that promote proliferation and TA cell conversion (Lowry et al., 2005). During the grow phase (anagen), clonogenic potential was also observed in cells residing outside the bulge (Oshima et al., 2001). Accordingly, $Lgr5^+$ stem cells extend down to the lower ORS but retain its stem cell characteristics (Jaks et al., 2008). Lastly, it has been hypothesised that a gradient of Wnt signalling affects many, if not all, of the HF developmental stages (Moore and Lemischka, 2006). However, exactly how, when and to which extent different levels of signalling affect the HF stem cells is still undetermined.

Other epidermal stem cell niches

Studies have suggested that every stem cell compartment in the skin is capable of originating all the epidermal cell lineages, for instance, in the case of wounding (Nijhof et al., 2006; Snippert et al., 2010; Taylor et al., 2000). In spite of this, there is evidence for other stem cell compartments besides the IFE and the bulge. Experiments show that most likely, and although each niche is capable of compensating for another, they are directly responsible for the homeostasis of the nearest

structures. Accordingly, while the IFE stem cells are mainly responsible for the homeostasis of the epidermis, bulge stem cells maintain the HFs. In the same line of reasoning, recent reports support the existence of yet other stem cell compartments mainly responsible for the renewing of the sebaceous gland.

The Lrig1$^+$ niche, for example, is located at the HF junctional region near the sebaceous gland, and is directly implicated in its the maintenance. Nonetheless, loss of *Lrig1* leads to hyperproliferation of the IFE, underscoring the contribution of this niche to the homeostasis of other skin compartments besides the sebaceous glands (Jensen et al., 2009).

The population located between the bulge and the sebaceous gland of the HF, a region designated as the upper isthmus (UI), expresses the cell surface marker MTS24 labels HF cells. MTS24$^+$ cells are found in the early HF developmental stages and in the adult. They are positive for α6-integrin and K14, resembling basal keratinocytes. MTS24$^+$ cells do not however express the bulge stem cell marker CD34 or k15. Despite this, gene expression profiling revealed some similarities between MTS24$^+$ and CD34$^+$ cells. MTS24$^+$ keratinocytes have higher clonogenic capacity than MTS24$^-$ cells (Nijhof et al., 2006). Furthermore, transplanted UI stem cells originate all three - IFE, HF and sebaceous - epidermal lineages arguing in favour of their multipotency (Jensen et al., 2008b).

More recently, a Lgr5-closely related protein - Lgr6 - was also studied in the context of HF stem cells. Whereas in the embryo, Lgr6 was found in the earliest hair placodes, in the adult it is expressed in a restricted cluster at the central isthmus. Curiously, those cells do not express any of the so far studied bulge stem cell makers. Embryonic Lgr6$^+$ cells originate the IFE, the sebaceous gland and the HF, whereas after birth their capacity to contribute to the HF lineages decreased with age. Finally, Lgr6 was not found to be regulated by Wnt signalling (Snippert et al., 2010).

Another transcription factor has been directly implicated in the maintenance of the sebaceous glands: the B lymphocyte-induced maturation protein 1 (Blimp1). Downregulation of Blimp1 leads to hyperproliferation of the sebaceous gland (Horsley et al., 2006). Interestingly, Blimp1 is a transcriptional repressor of Myc which is also known to affect the sebaceous glands (Arnold and Watt, 2001; Horsley et al., 2006; Waikel et al., 2001).

Future research should address the exact origin of these emerging HF stem cell niches. It has been proposed that bulge Lgr5$^+$ stem cells might originate from the Lgr6$^+$ pool during the early stages of skin embryogenesis (Snippert et al., 2010). Lineage-tracing experiments will be necessary to understand whether these novel stem cell populations are part of the activated progeny of another stem cell niche like the bulge. Agreeing with this is the activated state of MTS24$^+$ keratinocytes (Jensen et al., 2008b). They might nevertheless constitute independent niches that mainly maintain the homeostasis of the sebaceous gland compartment.

The contribution of stem cells to wound healing

The process of regeneration of the skin and its underlying mechanisms have great clinical significance. Upon wounding of the skin, epidermal cells proliferate and migrate towards the lesion in order to repopulate it. Due to the longevity of stem cells, it was proposed that they contribute to epidermal renewal and repair. Currently, there is significant evidence for the role that stem cells play in this process **(Fig. 6 A)**.

As previously mentioned, IFE stem cells are now believed to mainly contribute to long-term repair of the epidermis, rather than to routine epidermal homeostasis (Mascre et al., 2012). HF stem cells were also shown not to be essential for IFE homeostasis in the absence of injury (Ito et al., 2005; Levy et al., 2005). Upon genetic ablation of K15-expressing bulge stem cells, and although HFs are lost, skin homeostasis is not disturbed. Nevertheless, bulge stem cells are actively recruited after wounding. Curiously, bulge stem cells that reach the wound and their progeny are short-lived and in time replaced by $K15^-$ epidermal cells (Ito et al., 2005). On the other hand, wound healing is considerably delayed in the absence of HFs (Langton et al., 2008). These observations suggest the existence of two wound healing strategies. One in which $K15^+$ bulge stem cells quickly migrate but are eventually replaced by other epidermal cells. Another where isthmus stem cells and other epidermal cells are slowly but permanently recruited to the lesion **(Fig. 6 B)**.

Regarding the first strategy in which bulge stem cells play the main role, Taylor and her colleagues performed groundbreaking experiments using a double pulse-chase labelling technique. They showed how in case of injury bulge stem cells migrate and contribute to the newly-formed IFE (Taylor et al., 2000). The progeny of bulge stem cells migrates from the HFs towards the wound approximately five days after trauma. The K15-expressing cells start to disappear 20 days after wounding. By the 50th day, barely any $K15^+$ cells are found at the wound edge (Ito et al., 2005).

As a second strategy, other follicular stem cells can permanently contribute to repopulation after wounding. Two studies first established the long-lasting presence of follicular cells around the wound (Levy et al., 2007; Nowak et al., 2008). More recently, these follicular stem cells were identified as belonging to the isthmus and junctional zone areas of the HF. Both $Lgr6^+$ and $Lrig1^+$ stem cells migrate and permanently reside at the wounded zone or after treatment with promoters of IFE-proliferation (Jensen et al., 2009; Snippert et al., 2010).

Remarkably, wounds surrounded by telogen HFs heal significantly slower than the ones ringed by anagen HFs (Ansell et al., 2011). Since follicular stem cells become activated during anagen and considering the previous observations, the direct effect of the HF cycle on the wound healing process is extremely plausible.

The bulge stem cell marker Lhx2 has been implicated as a promoter of the re-epithelialisation process (Mardaryev et al., 2011). Nonetheless, deeper understanding of the epidermal wound healing process should unveil other pathways and molecular mechanisms involved. Ultimately, research on the field should contribute to the

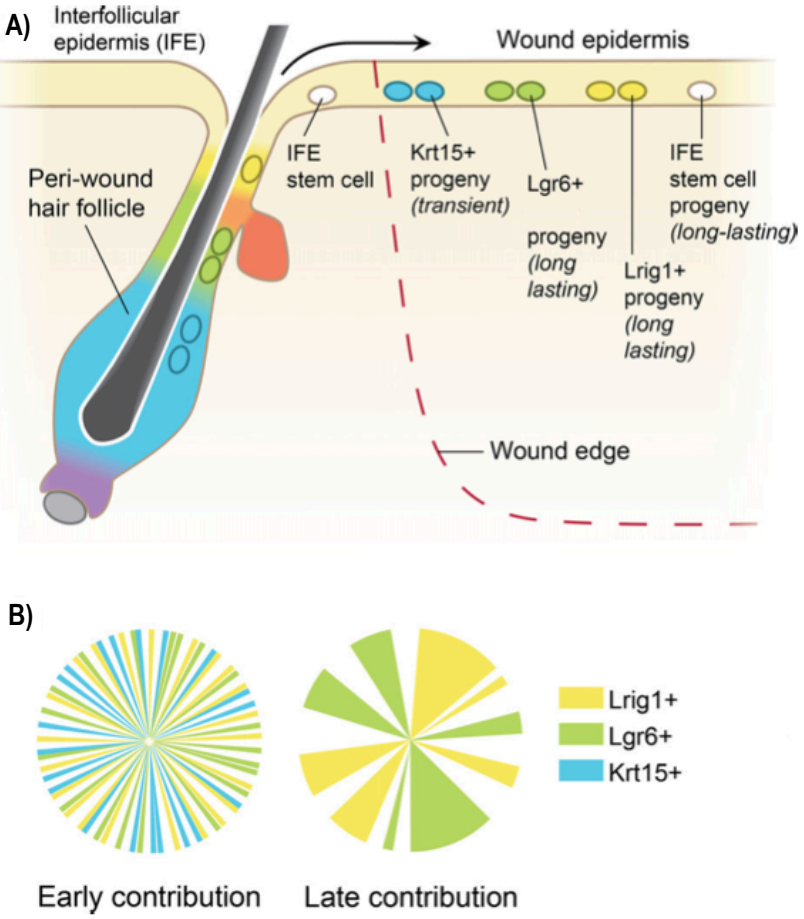

Figure 6 – The contribution of different epidermal stem cells to wound healing. **A)** Upon wounding, Krt15$^+$ bulge stem cells (blue) become activated and give rise to progeny that migrates out of the follicles and contributes to rapid wound re-epithelialization. Interfollicular (IFE) (white), Lrig1$^+$ (yellow) and Lgr6$^+$ (green) stem cells also participate in this process. **B)** With time, the progeny of Krt15$^+$ bulge stem cells disappears from the wound edge, while the Lrig1$^+$ and Lgr6$^+$-derived populations permanently contribute to the repopulation of the wound (adapted from (Plikus et al., 2012)).

development of therapies against wound healing pathologies and severe burn injuries.

Epidermal stem cell heterogeneity

There is growing evidence for the heterogeneity of stem cell response to activating signals. It appears that different subsets of stem cells react to such stimuli in different time frames. In fact, a small subset of bulge cells are dormant LRCs (Morris and Potten, 1999). This thus results in heterogenous populations composed of both activated and dormant stem cells. Consequently, they may differentiate at different times, in response to different signals, and give rise to progenitor cells with discrete fates (Greco et al., 2009; Zhang et al., 2009).

The circadian-clock has recently been proposed to explain the mixed responsive status of the HF stem cells. Strikingly, Bmal1 and Clock, core clock transcription factors, were found to bind the promoter of several key epidermal stem cell genes such as modulators of Wnt, Bmp and Notch signalling. The transcription levels of several of these genes were affected by conditional deletion of

Bmal1. Perhaps more importantly, in stem cells purified from wild-type (WT) mice the expression of some of these genes differed within a 12h period. The same was not observed for cells from *Bmal*-deletion mice (Janich et al., 2011). This study has further substantiated the "stem cell-heterogeneity" hypothesis by linking the clock machinery to the predisposition of epidermal stem cells to react to quiescence or activation cues.

The regulatory mechanisms that explain such heterogeneity are still poorly understood. Nevertheless, the regulation of the epithelial stem cell status is of clear importance, since disturbance of this balance leads to anticipated ageing and higher risk of carcinogenesis (Flores et al., 2005; Morris, 2004; Owens and Watt, 2003).

Malfunction of epidermal stem cells

Considering the fundamental role that stem cells play both during the embryonic development and postnatal tissue homeostasis, the potential consequences of stem cell malfunction can be severe. It will be extremely important to understand which molecular contexts reflect stem cell malfunction and what diseases may or may not result from that.

Ageing

The current knowledge on the causes underlying ageing is still very limited. It has been hypothesised that a decrease over time on stem cells function may significantly contribute to the changes perceived as ageing. The duration of the telogen phase of the hair cycle increases with age, perhaps reflecting molecular changes in the stem cell compartment (Fuchs, 2009). However, previous studies had shown no detectable differences between young and old epidermal stem cells (Giangreco et al., 2008; Stern and Bickenbach, 2007).

It was only more recently that alterations in the bulge subset of epidermal stem cells were observed as a consequence of skin ageing. An increasing number of bulge stem cells accompanied by decreased function and lower tolerance to stress conditions was detected in association to skin ageing. Interestingly, in these cells Jak-Stat signalling, implicated in molecular reprogramming (Yang et al., 2010), was also disturbed with ageing (Doles et al., 2012). Stat3 skin-specific KO mice exhibit compromised hair cycles and wound healing activity. In addition, with age mice suffered from ulcers and hair formation became seldom (Sano et al., 1999). Curiously, epidermal *Dll1*-deficiency, a Notch ligand, leads to tumour formation only in mice aged over 1.5 years (Estrach et al., 2008).

Recently the accumulation of DNA damage in HF stem cells was explored in the context of ageing. Over time, HF stem cells start to loose their DNA repair capacity. Consequently, aged-HF stem cells accumulate significant amount of DNA double-strand breaks that result in chromatin rearrangements. In spite of this, the physiological ageing process did not cause HF stem cells to undergo cellular senesce nor apoptosis (Schuler and Rube, 2013).

One possible explanation for the divergence between these reports might rely on spacial and temporal stem cell heterogeneity. In light of Janich et al. (2011) work regarding the circadian rhythms' influence on stem cell activity, and the existence of at least three epidermal stem cell niches, heterogeneity may have masked the effects of ageing. Nevertheless, future research should help elucidate the putative connection between the process of ageing and epidermal stem cells.

Stem cells in skin cancer

It has been hypothesised that stem cells may be the ones accumulating sufficient mutations to develop cancer. Several skin tumours such as squamous cell carcinomas (SCC), sebaceous adenomas, basal cell carcinomas (BCC) and different types of hair tumours, exhibit capacity for

multilineage differentiation, a common feature of multipotent stem cells (Ambler and Maatta, 2009; Locke et al., 2005). Indeed, stem cells appear to be attractive targets since they reside and proliferate in their niches throughout the adult life. Moreover, epidermal stem cells do not protect their genome by means of asymmetrical chromosome segregation (Sotiropoulou et al., 2008).

Genetic ablation of CD34 followed by TPA treatment clearly showed how CD34 is required for HF stem cell activation and TPA-induced tumour formation. Even when subjected to high TPA concentrations, CD34 knockout (KO) mice developed lower number of papillomas then the control mice (Trempus et al., 2007). In SCC the subset of cells with tumour initiating potential has been reported to express bulge stem cell markers such as CD34 and Sox9 (Malanchi et al., 2008; Prince et al., 2007). Notoriously, these SCCs and their tumourigenic potential were strictly dependent on β-catenin signalling (Malanchi et al., 2008). In spite of that, β-catenin stabilisation does not lead to SCC formation but rather Mx-derived hair tumours (Chan et al., 1999; Gat et al., 1998).

On the other hand, development of BCC, HF-derived tumours, is strongly associated with Shh signalling upregulation. BCC formation resembles the HF cycle, in which the Shh pathway is also very much involved (Hutchin et al., 2005). Approximately 50-60% of all sporadic BCC have *Patched* mutations, a Shh pathway antagonist (Athar et al., 2006; Daya-Grosjean and Couve-Privat, 2005). Curiously, such tumours were dependent of Wnt/β-catenin signalling as well (Yang et al., 2008). Other studies have further corroborated the impact of Wnt/β-catenin signalling in skin carcinogenesis (Bhatia and Spiegelman, 2005; Braun, 2008). Sox9 upregulation has also been observed in human BCC (Vidal et al., 2005).

Perturbation of TGF-β signalling, an epidermal growth-suppressor, promotes SCC formation in the IFE (Glick, 2012; Guasch et al., 2007). The subunit β4 of α6-integrin, a marker for epidermal stem cells, has been observed to act upstream of TGF-β signalling. By preventing TGF-β from inhibiting clonal growth of keratinocytes, α6-β4-integrin promotes carcinogenesis (Owens et al., 2003). Indeed, upregulation of integrins, namely the α6-β4-integrin has been associated with poorer prognosis (Van Waes et al., 1995). In addition, expression of α6-β4-integrin at the suprabasal layer can be used as an early predictive marker for aggressive SCC (Tennenbaum et al., 1993). However, additional deletion of α6-β4-integrin in $p53^{-/-}$,$Smad4^{-/-}$ cells from mice skin, led to enhanced tumour growth upon subcutaneous injection into nude mice. By contrast, the presence of α6-β4-integrin induced tumour growth once these cells were further transformed with oncogenic Ras (Raymond et al., 2007). This work showed that α6-β4-integrin tumour promoter activity might be dependent on the oncogenic mutations of the tumour cells.

Such as the TGF-β, the Notch signalling activity is another epidermal tumour suppressor pathway. One described mechanism by which Notch1 inhibits growth is through the activation of the cell-cycle inhibitor $p21^{Cip1}$ expression (Rangarajan et al., 2001). Furthermore, Notch is a target gene of tumour suppressor protein p53 (Lefort et al., 2007). Mice with epidermal *Notch1*-deficiency develop BCC-like tumours (Nicolas et al., 2003).

Mice aged over 1.5 years with epidermal *Dll1*-deficiency developed spontaneous tumours (Estrach et al., 2008). Presenilin 1 KO mice, a player in the Notch pathway, also develop spontaneous skin tumours (Xia et al., 2001). Finally, epidermal expression of a Notch inhibitor, the dominant negative Mastermind Like 1 (DNMAML1), in mice results to sporadic SCC formation (Proweller et al., 2006).

p63, another protein intimately involved in skin stratification, is important for tumourigenesis. In contrast with its tumour suppressor family member p53, $p63^{+/-}$ mice are not tumour prone. When studied in a heterozygous p53 background, absence of one *p63* copy sill led to fewer tumours than $p53^{+/-}$,$p63^{wt/wt}$ mice. Furthermore, p63 was found expressed in human carcinomas highlighting its potential role also in human cancer formation (Keyes et al., 2006). Similarly to p63, *c-Myc* also behaves as an epidermal oncogene. Mice overexpressing *c-Myc* under the control of *K5* promoter develop spontaneous tumours (Rounbehler et al., 2001). Deletion of *c-Myc* in basal cells leads to resistance against Ras-mediated, TPA induced tumourigenesis. Activation of the Ras-ERK pathway is known to induce $p21^{Cip1}$ expression. Conversely, c-Myc represses $p21^{Cip1}$ expression causing cells to bypass this cell-cycle checkpoint. Therefore, trough the repression of $p21^{Cip1}$, c-Myc plays a key role in Ras-driven epidermal tumour formation (Oskarsson et al., 2006).

A recent study identified another pathway essential for epidermal homeostasis: the Hippo signalling pathway. Activation of Yap1, a transcriptional activator of the pathway, induces proliferation of progenitor and stem cells and can lead to SCC-like tumour formation. These findings highlight Yap1 as a tumour suppressor in the epidermis (Schlegelmilch et al., 2011).

Finally, normal epidermal stem cell markers have been studied and found expressed by poorly differentiated human SCCs and immortalised SCC cell lines. Moreover, the authors proposed that SCC cells not only display stem cells characteristics but also downregulate the pathways involved in stem cell quiescence (Jensen et al., 2008a). Discovery of other epidermal stem cell markers and profiling of skin tumour cell may unveil more properties shared between the stem cell and the cancer setting. However, it still remains elusive whether skin tumours directly derive from epidermal stem cells or if they arise from progenitor or differentiated cells that acquired stem cell properties. Transgenic mice like the above described ones, coupled with cell tracing *in vivo* experiments might help answer this question.

Final remarks

The present work succinctly covered the developmental stages of the epidermis and the HF as well as some of the important pathways that contribute to such processes. These include Wnt/β-catenin, BMP, p63 and Shh signalling. Epidermal stem cell differentiation, multipotency and the consequences of their malfunction have been also addressed. We therefore explored the epidermal stem cells and their niches as a model system for the study of adult stem cells.

Numerous studies have contributed over the past few decades to the current knowledge on epidermal stem cells and their vital function within the human skin. Their important throughout both embryonic development and postnatal life is unquestionable. Nonetheless, numerous questions have arisen and the network of pathways that govern the development and homeostasis of the skin is quickly growing. Clearly, there is an increasing necessity to bridge and integrate the numerous discoveries on the field.

As more transcription factors are being discovered to regulate stem cell activity, it becomes more important to unveil the respective target genes and find the potentially overlapping networks. Genome-wide chromatin immunoprecipitation and transcriptional analyses will be essential to answer those questions. This should help understand if such transcription factors cooperate to control epidermal homeostasis and HF regeneration.

Regarding stem cell spacial heterogeneity, it will be interesting to study the interplay between the different epidermal stem cell compartments. Moreover, identification of the mechanisms that allow the migration of bulge and isthmus stem cells in the HF and into the IFE during wound healing will be crucial. There is still the possibility for other unidentified progenitor or stem cell niches within the epidermis. The identification of specific stem cell markers remains a prime goal. On the other hand, it is still unclear whether all the so far identified niches originate from a single stem cell compartment. Epidermal stem cells are known to have the capacity to originate any cell lineage of any structure of the epidermis. This argues in favour of a common ancestor that during early embryogenesis establishes the different niches.

Another key question concerns the influence of the microenvironment on stem cell behaviour. Dedifferentiation of terminally differentiated cells has been shown possible *in vitro*. This raises the question whether differentiated cells *in vivo* also dedifferentiate in response to specific molecular cues. Indeed, experiments have demonstrated how progenitor cells from the HG are capable of re-establishing the bulge stem cell niche in case of injury (Ito et al., 2002). Moreover, as shown by recombination techniques, dermal signals can induce adult cornea epithelium reprogramming and expressing epidermal markers. Committed basal cells of the adult corneal epithelium were converted into forming functional HFs, thus, probably including the HF stem cell niches (Ferraris et al., 2000; Pearton et al., 2005). Therefore, the microenvironment obviously plays an important role in lineage commitment and terminal differentiation. It may also be responsible for triggering cells in a particular niche into maintaining a certain level of stemness (Blanpain et al., 2004). Taking into account the abundance and accessibility of HFs and

epithelial stem cells, this epidermal plasticity certainly promises great clinical applications. However, the plasticity of the adult epidermis and the role of the microenvironment both in normal skin homeostasis and in case of injury is yet poorly studied.

The type of tumour formed and its aggressiveness depends on the acquired oncogenic mutations and the cell that has acquired them. Some of the proteins that are important in epidermal tumour formation have been already identified and extensively studied. Those include p53, RAS, SHH and β-catenin. Oncogenic mutations in these or other proteins can occur in any epidermal cell. Nevertheless, stem cells are highly clonogenic and reside in the organism throughout most postnatal life. These properties make them prime candidates for accumulation of DNA damage and, hence, tumour formation. Nevertheless, the surrounding differentiated cells and the microenvironment play a preponderant role on whether a mutant stem cell will develop a tumour or not. More work is needed to define the role and interplay between normal differentiated cells and tumour stem cells.

Finally, significant effort is being directed at revealing the molecular mechanisms that trigger stem cells either to enter quiescence or to become active, to self-renew or to differentiate. Understanding or identifying the signals responsible for these decisions may help in the expansion and differentiation of specific cell lineages or even tissues. This will be extremely useful in the clinic for transplantation and grafting strategies but also in the field of cancer research.

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