Epidermal stem cell diversity and quiescence

Fiona M. Watt[1,2] & Kim B. Jensen[1]*

Mammalian epidermis is maintained by self-renewal of stem cells and terminal differentiation of their progeny. New data reveal a diversity amongst stem cells that was previously unrecognized. Different stem cell populations have different locations and differ in whether they are quiescent or actively cycling. During normal epidermal homeostasis, each stem cell population feeds a restricted number of differentiated lineages. However, in response to injury or genetic manipulation the different pools of stem cells demonstrate multi-lineage differentiation ability. While it is well established that Wnt signalling promotes hair follicle (HF) differentiation, new observations suggest a role for EGF receptor signalling in promoting differentiation of interfollicular epidermis. NFATc1 maintains quiescence in the HF, while Lrig1 exerts the same function in the junctional zone. The stage is now set for exploring the relationship between the different epidermal stem cell populations and between quiescence and lineage selection.

Introduction

The epidermis forms the outer layer of the skin. It comprises a multi-layered epithelium, the interfollicular epidermis (IFE) and associated adnexal structures that include hair follicles (HFs) and sebaceous glands (SGs; Fig 1). The outermost, cornified layers of the IFE are continually shed from the surface of the skin and are replenished through proliferation of cells in the basal layer. Within the SGs, fully differentiated sebocytes burst, releasing their lipid content onto the skin surface, and are replaced by proliferation of cells at the periphery of the gland.

The HF has a more complex organization than the IFE and SG, since it is made up of eight distinct cell layers, representing different differentiated lineages (Fig 1). Nevertheless, the terminally differentiated cells of the HFs, including the hair shaft, are also lost in adult skin and replenished by proliferation of less highly differentiated cells. HFs go through phases of growth (anagen), regression (catagen) and rest (telogen) (Alonso & Fuchs, 2006), allowing new hairs to form to replace those that are lost in the previous cycle.

It has been appreciated for many years that the epidermis contains stem cells that are responsible for replacing the differentiated cells of the IFE, HFs and SGs. However, it has only recently become apparent that there are multiple populations of stem cells. In addition, there is emerging evidence for intersection between the pathways that regulate stem cell proliferation and lineage selection.

Stem cells of the interfollicular epidermis

Some of the earliest studies of turnover of cells within the epidermis focused on the organization of adult mouse IFE (reviewed by Hall & Watt, 1989; Potten & Morris, 1988). It was noted that within the basal layer of the epidermis there is heterogeneity in cell cycle time, and this was incorporated into a concept that stem cells divide infrequently, while their non-stem progeny, named transit amplifying cells, undergo a small number of rounds of division before withdrawing from the cell cycle and undergoing terminal differentiation in the suprabasal cell layers. A single stem cell, surrounded by transit amplifying cells, was hypothesized to lie at the base of a column of differentiated cells, forming an 'epidermal proliferative unit' (Potten & Morris, 1988).

(1) Wellcome Trust Centre for Stem Cell Research, Tennis Court Road, Cambridge, UK.
(2) CRUK Cambridge Research Institute, Li Ka Shing Centre, Robinson Way, Cambridge, UK.
Corresponding author: Tel: +44 1223 404400; Fax: +44 1223 404573; E-mail: fiona.watt@cancer.org.uk

Lineage tracing experiments performed in both human and mouse epidermis confirm that differentiating, suprabasal cells are the progeny of the undifferentiated, proliferative cells directly beneath them (see, *e.g.* Ghazizadeh & Taichman, 2001, 2005; Jensen et al, 2009). Nevertheless, the model that the IFE is organized into self-limiting epidermal proliferative units is not supported by recent lineage tracing and clonal analysis (Clayton et al, 2007; Jones et al, 2007). In adult mouse tail epidermis, IFE

Glossary

Anagen
The phase of the hair growth cycle when the hair is growing.

Basal layer
The layer of epidermal cells that is attached to the basement membrane (extracellular matrix) that separates the epidermis from the underlying dermis.

Bulge
The bulge lies at the insertion point for the HF arrector pili muscle and marks the bottom of the permanent portion of the HF.

Catagen
The phase of the hair growth cycle when the HF stops growing and regresses.

Hair follicle
The multi-layered epithelium that forms the hair.

Hair peg
A stage in the development of the HF during embryogenesis.

Hair shaft
The dead cells that form the hair that protrudes from the skin surface.

Infundibulum
The portion of the HF that lies above the SG and is continuous with the IFE.

Interfollicular epidermis
The multi-layered epithelium that forms the outer covering of the skin.

Isthmus
The region of the HF that extends from the bulge to the SG.

Lineage tracing
A technique that marks all the progeny of a single cell.

Mesenchymal condensate
A cluster of fibroblastic cells in the embryonic dermis that marks the location of the future HFs.

Placode
The epithelial bud in embryonic skin that will give rise to the HF.

Quiescence
State in which cells that are competent to proliferate have not undergone cell division for a prolonged period of time.
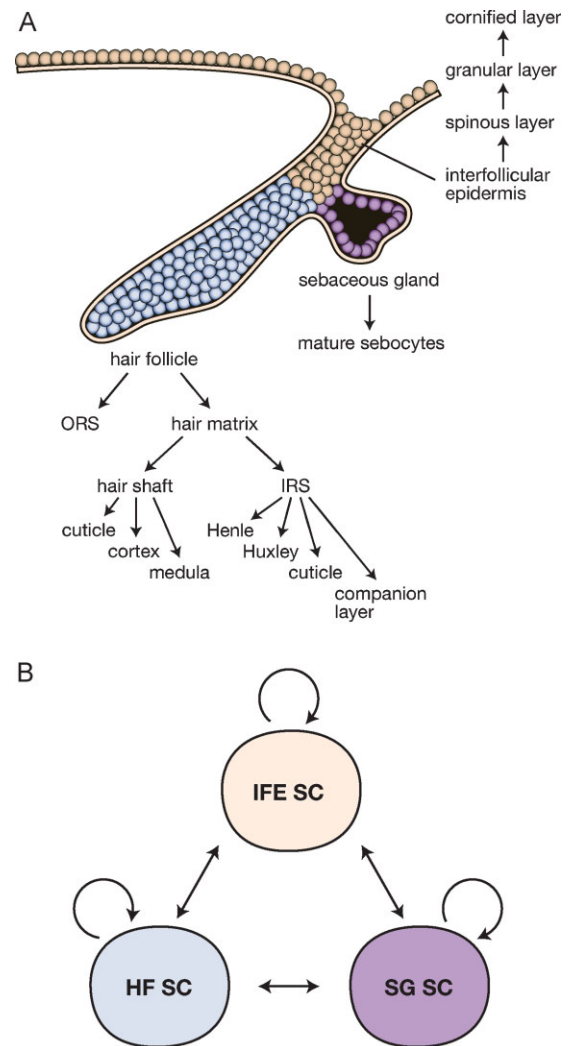
Sebaceous glands
Glands at the junction between the HF and the IFE that produce sebum to lubricate the skin surface.

Stem cells
In adult tissues these are cells with extensive ability to self-renew and the ability to produce cells that undergo further differentiation.

Telogen
The resting phase of the hair growth cycle.

Figure 1. Organization of adult mouse epidermis.
A. The differentiated lineages of the interfollicular epidermis (IFE), sebaceous gland (SG) and hair follicle (HF) are shown. ORS, outer root sheath; IRS, inner root sheath.
B. In response to appropriate stimuli, stem cells (SC) in any region of the epidermis can give rise to any of the epidermal differentiated lineages. SCs are therefore represented as being interconvertible. Based on Owens and Watt (2003).

homeostasis can be explained using a very simple model in which a single population of cells has three stochastic choices upon cell division, resulting in the production of two postmitotic cells destined to undergo terminal differentiation, two cells that remain undifferentiated in the basal layer, or one of each. Balanced symmetrical cell division ensures steady-state homeostasis, and individual clones die out or expand progressively over time (Clayton et al, 2007; Jones et al, 2007). Whether these cells are stem cells is, to some extent, a semantic issue. While we would argue that, on a population level, they are stem cells, since they maintain the IFE and have the dual properties of self-renewal and terminal differentiation (Hall & Watt, 1989), their

ability to repair the epidermis following injury has not been tested.

In the basal layer of human IFE, as in mouse, there is proliferative heterogeneity. Clonal growth assays of cultured human epidermal cells have been used as a surrogate readout of stem and transit amplifying cells (Barrandon & Green, 1987; Jones & Watt, 1993; Jones et al, 1995). This in turn has led to the identification of a number of markers that enrich for highly clonogenic cells, the putative stem cells, including high expression of β1 integrins and the EGF receptor antagonist Lrig1 (Jensen & Watt, 2006; Jones & Watt, 1993; Jones et al, 1995, 2007). These markers are expressed by clusters of basal cells that have a low proliferative index and tend to lie where the dermis comes closest to the skin surface (Jensen & Watt, 2006; Jensen et al, 1999; Jones et al, 1995). Clustering of stem cells has been proposed both to maintain their location within a specific microenvironment and to protect against terminal differentiation stimuli, such as Notch activation (Estrach et al, 2007; Jensen et al, 1999; Lowell et al, 2000; see also Gurdon, 1988).

Hair follicle stem cells

Within the HF, the best characterized stem cell compartment lies in the bulge, the lower permanent part of the HF (Cotsarelis et al, 1989, 1990; Oshima et al, 2001; Rochat et al, 1994) (Fig 2). Mouse bulge cells express a number of markers, including K15 and CD34 (Lyle et al, 1998; Morris et al, 2004; Trempus et al, 2003). Markers expressed in the human bulge have also been documented, including CD200, follistatin and frizzled homologue 1 (Ohyama et al, 2006). The bulge contains quiescent,
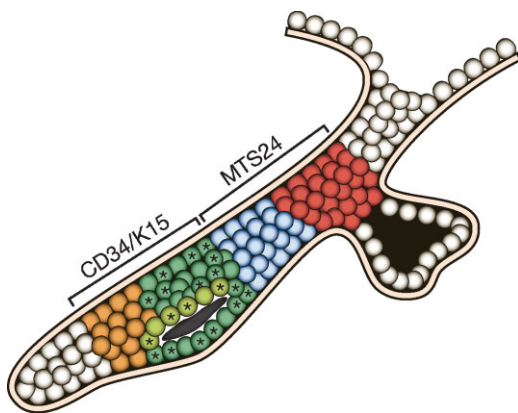
Figure 2. Different epidermal SC populations in adult mouse HF and junctional zone. Red: Lrig1 expressing cells of the junctional zone (Jensen et al, 2009). Blue: CD34 negative, α6 low cells of the upper isthmus (Jensen et al, 2008). MTS24 is expressed by both populations. Orange and green: CD34 positive, K15 positive cells of the bulge (Lyle et al, 1998; Trempus et al, 2003). The suprabasal, α6 low subpopulation is shown in pale green (Blanpain et al, 2004). Orange: Lgr5 expressing cells (Jaks et al, 2008). Label retaining cells are indicated by asterisks. The club hair shaft is shown in black within the bulge region.

DNA label retaining cells (LRCs) (Braun et al, 2003; Cotsarelis et al, 1990; Tumbar et al, 2004), which can be recruited into the cell cycle by a number of stimuli, including exposure to the phorbol ester, TPA (Braun et al, 2003).

It is now clear that there is more than one stem cell population within the mouse HF bulge (Fig 2). Two distinct CD34-positive bulge stem cells can be distinguished on the basis of location and α6 integrin expression (Blanpain et al, 2004). One population has high levels of α6 integrin and is attached to the basement membrane. The other has low α6 integrin levels, a suprabasal location and arises only after the start of the first postnatal hair cycle, coincident with the onset of CD34 expression.

Lower down the bulge, a third population of CD34 positive cells is distinguished by expression of Lgr5 (Fig 2), originally identified as a marker of intestinal stem cells (Jaks et al, 2008). In contrast to other CD34 positive cells, cells that express Lgr5 are actively cycling. Lgr5 positive cells can reconstitute all the epidermal lineages in skin reconstitution assays, in which disaggregated epidermal cells are combined with neonatal dermal cells in chambers implanted onto the back of syngeneic or immunocompromised mice. *In situ* lineage tracing shows that Lgr5 expressing cells maintain the HF lineages under steady-state conditions and migrate down the lower outer root sheath to contribute to anagen hair growth.

Lying above the CD34 positive bulge are cells that express the epitope MTS24, now known to correspond to Plet1 (Depreter et al, 2008) (Fig 2). MTS24-positive cells express α6 integrin but not K15 or CD34 (Jensen et al, 2009; Nijhof et al, 2006); nevertheless, they do express some bulge markers (Nijhof et al, 2006). Some MTS24 positive cells are quiescent LRC; they are clonogenic in culture, but have not been tested in epidermal reconstitution assays of long-term self-renewal and differentiation potential (Nijhof et al, 2006).

One further stem cell population that has been documented in the HF lies between the infundibulum and bulge (Jensen et al, 2008, Fig 2). These cells have low α6 integrin expression and lack CD34 and Sca-1, but express MTS24 (Jensen et al, 2008, 2009). They can reconstitute the IFE, HF and SGs in skin reconstitution assays, but have a gene expression profile that differs from bulge stem cells. Like Lgr5 expressing cells, this stem cell population is actively cycling and does not have characteristics of long-term quiescence.

While it is evident that there are multiple stem cell populations in the HF, it is not clear whether they are independent or organized in a hierarchy. For example, since stem cells are reported to move downwards from the bulge during anagen (Oshima et al, 2001), are CD34 positive LRC the source of proliferative, Lgr5 cells? Lineage tracing to simultaneously track the fate of two or more stem cell populations is now required.

Sebaceous gland stem cells

Sebaceous glands are formed as outgrowths from HFs during embryonic development (see, *e.g.* Collins & Watt, 2008; Jensen et al, 2009) and loss of HFs can often result in failure to maintain the SG (discussed by Lo Celso et al, 2008). Nevertheless, using

long term, retroviral-mediated lineage tracing, Ghazizadeh and Taichman (2001) demonstrated that SGs do not obligatorily share the lineage of cells in the adjacent HF. This is clear evidence for the existence of a distinct SG stem cell compartment.

One proposed marker of SG stem cells is the transcriptional repressor, Blimp1 (Horsley et al, 2006). Although Blimp1 is also expressed in the IFE and HFs (Lo Celso et al, 2008; Magnúsdóttir et al, 2007), loss of Blimp1 primarily affects the SG (Horsley et al, 2006). In the absence of Blimp1, there is increased Myc expression, SG hyperplasia and enhanced proliferation in the bulge stem cell compartment. It is proposed that when normal SG homeostasis is perturbed by loss of Blimp1, bulge stem cells are mobilized to migrate upwards and replenish the SG compartment (Horsley et al, 2006).

Junctional zone stem cells

Lrig1 was identified as a marker of human IFE stem cells by single cell gene expression profiling (Jensen & Watt, 2006). In adult mouse epidermis, the Lrig1 positive population is located in the junctional zone between the IFE, SG and bulge (Fig 2) and expresses Lgr6 but not Lgr5 and CD34. In skin reconstitution assays, the Lrig1 positive population is as effective as CD34 positive bulge cells in giving rise to IFE, SG and HF. However, lineage tracing shows that in homeostatic conditions these junctional zone stem cells are only bipotent, contributing to the SG and IFE (Jensen et al, 2009). On treatment with all-trans retinoic acid, which stimulates IFE proliferation (Collins & Watt, 2008), there is selective enlargement of junctional zone clones, and they extend upwards into the IFE.

The existence of stem cells that contribute to SG and IFE was predicted by earlier studies. Thus, sebocytes can be induced in the IFE by Myc activation (Braun et al, 2003) and clonogenic human SG cells generate progeny that differentiate into sebocytes and IFE, both in culture and in xenografts (Lo Celso et al, 2008).

There is evidence that in some tissues, including epidermis, lung and pancreas, stem cells do not actively participate in homeostasis (Clayton et al, 2007; Dor et al, 2004; Giangreco et al, 2009), but are mobilized for tissue repair following injury (Giangreco et al, 2009; Jensen et al, 2009). In adult mouse epidermis, cells from within the HF are required for an effective wound response (Langton et al, 2008) and lineage analysis has demonstrated that those cells come from the upper isthmus or infundibulum rather than the bulge (Levy et al, 2007). These observations would lend support to the proposal that the junctional zone cells defined by Lrig1 expression are a quiescent reservoir of IFE stem cells that are stimulated to repair the IFE on injury or hyperplasia (Jensen et al, 2009).

Epidermal stem cell plasticity

The recent studies on junctional zone stem cells highlight a general principle: that stem cells with the ability to generate all
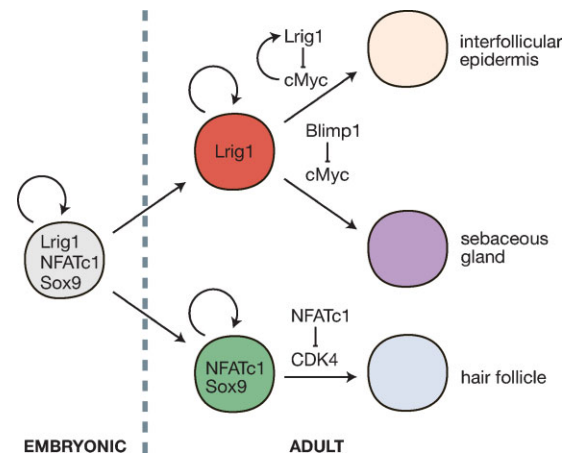
Figure 3. Regulation of epidermal SC quiescence and lineage selection. All SCs may arise from a common population of embryonic SCs that reside in the developing HF (Lrig1, NFATc1 and Sox9 positive). In adult epidermis, Myc promotes proliferation of interfollicular epidermal and SG SCs. Quiescence is maintained by negative regulation of Myc by Lrig1 and Blimp1 in the IFE and SG, respectively. Lrig1 is a Myc target gene and Lrig1 expression reduces Myc activity. NFATc1 maintains quiescence of bulge SCs by negatively regulating CDK4.

the epidermal lineages in skin reconstitution assays or following genetic manipulation may exhibit more restricted lineage selection *in situ* in undamaged skin (Clayton et al, 2007; Ito et al, 2005, 2007; Jensen et al, 2009; Levy et al, 2005, 2007; Oshima et al, 2001; Silva-Vargas et al, 2005; Taylor et al, 2000). This leads to the concept that different epidermal stem cell populations are functionally equivalent and interconvertible, and their differentiation potential is largely, if not solely, determined by their local microenvironment (Owens & Watt, 2003; Fig 3).

These observations suggest a number of interesting possibilities. First, it may be that any epidermal cell that has not lost the ability to divide and embarked on a programme of terminal differentiation can self-renew and exhibit other stem cell properties. If this is so, then the markers of the different stem cell pools may be expressed in response to the local environment rather than being inherent characteristics of those cells. Second, transit amplifying cells may not be a distinct cell population, but rather part of a continuum between cells of high self-renewal probability and cells that have initiated terminal differentiation (Jones et al, 2007; Niemann & Watt, 2002).

Do all the different adult stem cell populations arise from a common embryonic cell population?

While distinct epidermal stem cell populations are present in adult epidermis, there is evidence that they may all arise from a common multi-potent stem cell in embryonic skin (Fig 3). Lineage tracing studies demonstrate that cells within the early developing HF contribute to all lineages of the epidermis (Levy et al, 2005, 2007; Nowak et al, 2008).

In mouse skin, the HF placode develops at approximately embryonic day 14 (E14), induced by an evenly patterned mesenchymal condensate (Sick et al, 2006). The placode subsequently elongates into the underlying dermis, becoming the hair peg. The development of the peg coincides with the appearance of a subpopulation of HF cells that co-express markers of adult HF stem cells (NFATc1, Sox9) (Horsley et al, 2008; Nowak et al, 2008) and Lrig1, a marker of interfollicular and SG stem cells (Jensen et al, 2009) (Fig 3).

As HF development proceeds further, NFATc1 and Sox9 are no longer co-expressed with Lrig1, and the bulge and junctional zone become distinct regions of the HF (Jensen et al, 2009). We speculate that the Sox9 positive cells are the future adult HF stem cell populations (Nowak et al, 2008), while the Lrig1 positive cells will become the stem cells of adult IFE and SG (Fig 3).

Lineage selection: contrasting roles of Wnt and EGF

The Wnt pathway plays a central role in specifying HFs during development and in directing HF differentiation in adult epidermis (Andl et al, 2002; Blanpain & Fuchs, 2009; Gat et al, 1998; Lo Celso et al, 2004; Watt et al, 2006). In adult epidermis, different levels of β-catenin activation have different biological effects, ranging from initiation of anagen, to formation of ectopic follicles and tumour development (Gat et al, 1998; Lowry et al, 2005; Silva-Vargas et al, 2005). β-Catenin activation in adult epidermis can also lead to expansion of the CD34 positive and Lrig1 positive stem cell compartments (Jensen et al, 2009; Silva-Vargas et al, 2005), although this is not always observed (Lowry et al, 2005).

Constitutive activation of β-catenin during development leads to uncontrolled placode formation, but the placodes do not progress to mature HFs or express adult stem cell markers such as Sox9, K15 and Lrig1 (Narhi et al, 2008; Zhang et al, 2008). Excessive placode induction reflects β-catenin induced Shh expression (Suzuki et al, 2009), as also observed upon activation of β-catenin in adult epidermis (Lo Celso et al, 2004; Silva-Vargas et al, 2005). Thus, β-catenin activation in embryonic and adult epidermis induces similar pathways but with strikingly different consequences for HF morphogenesis. One possible explanation is that HF formation cannot proceed until the multi-potent stem cell pool (expressing Sox9, NFATc1 and Lrig1) has been specified.

While the role of β-catenin in specifying the HF lineages is well established, traditionally EGF signalling is thought of more in terms of controlling proliferation. EGF promotes proliferation of human epidermal stem cells in culture (Rheinwald & Green, 1977). In vivo, reduced EGFR signalling within the epidermis (Hansen et al, 1997; Luetteke et al, 1994; Miettinen et al, 1995; Sibilia & Wagner, 1995; Threadgill et al, 1995) leads to decreased proliferation, while constitutive activation of EGFR results in increased proliferation and development of epidermal tumours (papillomas and squamous cell carcinomas) (Ferby et al, 2006; Sibilia et al, 2000; Vassar & Fuchs, 1991). Nevertheless, a role for EGFR signalling, acting in concert with FGF7, in specifying IFE is now emerging. During embryonic development, receptors for EGF and FGF7 (KGF) are downregulated in the HF placodes (Richardson et al, 2009). Increased levels of EGFR ligands and FGF7 inhibit follicle formation by promoting epidermal differentiation at the expense of HF fate (Richardson et al, 2009). In postnatal skin, inhibition of EGFR leads to defects in the hair growth cycle (Ballaro et al, 2005; Hansen et al, 1997; Miettinen et al, 1995; Schneider et al, 2008; Sibilia & Wagner, 1995; Threadgill et al, 1995). Overexpression of FGF7 leads to IFE hyperproliferation and inhibition of HF morphogenesis (Guo et al, 1993).

A potential role for EGFR signalling in lineage selection would be consistent with the ability of EGF ligands to activate Myc (Jensen & Watt, 2006). Myc activation not only stimulates epidermal proliferation but also promotes differentiation of the IFE and SG lineages (reviewed by Watt et al, 2008). In cultured human sebocytes overexpression of Myc stimulates sebocyte differentiation, whereas overexpression of β-catenin stimulates expression of IFE and IRS differentiation markers (Lo Celso et al, 2008). While high levels of Myc promote IFE and SG differentiation they do not prevent anagen entry (Arnold & Watt, 2001) and indeed expression of the dominant interfering Myc bHLHZip dimerization domain mutant Omomyc inhibits the hair growth cycle (Soucek et al, 2008). This suggests that, as in the case of Wnt signalling, different levels of Myc activity may yield different outcomes in the epidermis.

Stem cell quiescence

While quiescence (infrequent cell division) is not an obligatory characteristic of epidermal stem cells (Jaks et al, 2008; Jones & Watt, 1993), the bulge, junctional zone and IFE all contain quiescent, DNA LRCs (Braun et al, 2003; Cotsarelis et al, 1990; Jensen et al, 2009; Nowak et al, 2008; Potten & Morris, 1988; Tumbar et al, 2004), and the putative stem cell clusters in human IFE contain fewer cycling cells than other regions of the basal layer (Jensen et al, 1999; Jensen & Watt, 2006; Jones et al, 1995).

Pending issues

- Are there more stem cell populations to be discovered and, if so, is it possible that all undifferentiated epidermal cells are stem cells?

- Are the different epidermal stem cell pools autonomous or do they exist in a hierarchy?

- Are actively dividing stem cells more likely to undergo lineage commitment than quiescent stem cells?

- Do Myc and Wnt induced differentiation act as fail-safe devices to prevent uncontrolled epidermal stem cell proliferation?

- How does communication between epidermal stem cells and other cells in the skin influence their behaviour?

Some of the pathways that regulate epidermal quiescence have now been elucidated. Quiescence of bulge stem cells is regulated by the transcription factor NFATc1 (nuclear factor of activated T cells c1), which is activated by BMP signalling (Horsley et al, 2008). The telogen phase of the hair growth cycle comprises a refractory phase, during which HF stem cells are exposed to high levels of BMP, and a competent phase, when BMP levels are reduced (Plikus et al, 2008). Reduced BMP levels not only create a permissive environment for β-catenin activation (Kobielak et al, 2007; Zhang et al, 2006), but also reduce NFATc1 expression (Horsley et al, 2008). NFATc1 expression maintains quiescence by repressing CDK4 (Horsley et al, 2008). In addition, NFATc1 signalling stimulates expression of p21(WAF1/Cip1) and p27(KIP1), two cyclin-dependent kinase inhibitors associated with keratinocyte differentiation (Santini et al, 2001).

Loss of *NFATc1* in the epidermis causes constant HF cycling and HF stem cells show no sign of quiescence (Horsley et al, 2008). Cyclosporin A, which acts as an inhibitor of calcineurin, a phosphatase that positively regulates NFATc1 activity, also induces HF cycling (Gafter-Gvili et al, 2003, 2004) and epidermal deletion of the calcineurin B1 gene results in loss of telogen (Mammucari et al, 2005). These observations demonstrate that NFATc1 plays a central role as gate-keeper of quiescence in HF stem cells (Fig 3).

In the IFE and SG two negative regulators of Myc, Lrig1 and Blimp1, are involved in the maintenance of stem cell quiescence (Horsley et al, 2006; Jensen & Watt, 2006; Jensen et al, 2009) (Fig 3). Blimp1 is a transcriptional repressor that binds to elements within the Myc promoter and controls Myc expression (Horsley et al, 2006; Lin et al, 1997). Loss of Blimp1 triggers proliferation of SG stem cells, resulting in SG hyperplasia (Horsley et al, 2006). Lrig1 negatively regulates Myc by decreasing EGFR signalling, and is part of a negative feedback loop, since the Lrig1 gene is transcriptionally regulated by Myc (Jensen & Watt, 2006; Jensen et al, 2009). Loss of Lrig1 stimulates proliferation specifically within the IFE via increased proliferation of bipotent stem cells in the junctional zone (Jensen et al, 2009). It seems likely that the stimulation of IFE and SG proliferation and differentiation resulting from activation of a Myc transgene driven by a keratin 14 promoter (Arnold & Watt, 2001) is a consequence of overcoming the negative effects of Blimp1 and Lrig1 (Jensen et al, 2009).

Conclusions

Recent studies point to the existence of multiple epidermal stem cell pools that are competent to differentiate along all the epidermal lineages, but are normally lineage-restricted as a result of specific environmental cues. Quiescence is not an obligatory characteristic of epidermal stem cells, but some stem cell populations divide infrequently and the mechanisms that govern quiescence are being elucidated. The relationship between proliferation and differentiation is an interesting one, given that the Wnt pathway drives both HF lineage selection and proliferation, while EGFR signalling stimulates

proliferation and, at least in embryonic skin, IFE lineage selection. It has been proposed that Myc induced differentiation acts as a fail-safe devise to prevent uncontrolled epidermal stem cell proliferation (Jensen & Watt, 2006; Watt et al, 2008), and it would be interesting to discover whether this also applies to Wnt-mediated differentiation. Finally, the complex interplay between different signalling pathways, including Wnt, Hedgehog, Notch and BMP, and communication between epidermal stem cells and other cells in the skin are important areas for further exploration.

For more information

Stembook:
http://www.stembook.org/

Author's website:

Fiona Watt:
http://www.bio.cam.ac.uk/∼wattlab/index.html

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