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Mini-Review

Keratinocyte Stem Cells: friends and foes

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Abstract

Skin and its appendages provide a protective barrier against the assaults of the environment. To perform its role, epidermis undergoes an ongoing renewal through a balance of proliferation and differentiation/apoptosis called homeostasis. Keratinocyte stem cells reside in a special microenvironment called niche in basal epidermis, adult hair follicle and sebaceous glands. While a definite marker has yet to be detected, data raised part in humans and part in the mouse system, point to a critical role of stem and its progeny transit amplifying cells in epidermal homeostasis. Stem cells are protected from apoptosis and are long-resident in adult epidermis. This renders them more prone to be the origin of skin cancer. In this review, we will outline the main features of adult stem cells in mouse and humans and discuss their fate in relation to differentiation, apoptosis and cancer.

Epidermis serves as a barrier that protects against environmental stresses, such as harmful microbes, water loss, physical, thermal and mechanical injuries. To support this role, the epidermis generates a number of appendages, including nails, hair follicles, sebaceous and sweat glands. Epidermis must renew constantly throughout life to maintain normal homeostasis and to repair damage after wounding. The regenerative capacities of epithelial populations have been studied for many years with the development of cultured keratinocytes derived from the epidermis, used to produce autologous grafts that regenerate an epidermis over a full-thickness wound (Coolen et al., 2007). Epidermal homeostasis depends on a balance between proliferation and differentiation/apoptosis of keratinocytes. The epidermis contains a basal layer of proliferative keratinocytes that adhere to the underlying basement membrane (BM). Periodically, these cells detach from the basement membrane, withdraw from the cell cycle and initiate a program of terminal differentiation, moving upward to the skin surface. Upon commitment to terminal differentiation, keratinocytes progress through different stages and form three distinct layers: the spinous, the granular and the stratum corneum. Eventually, keratinocytes lose the nuclei and are continuously shed from the skin surface. Although the morphogenetic changes associated with epidermal differentiation are well established, the molecular mechanisms underlying this process are complex, as they involve many genes and they are still poorly understood (Blanpain and Fuchs, 2009).

Keratinocyte stem cells (KSC) are responsible for maintaining epidermal homeostasis and for repairing the tissue following injuries. KSC self-renew and generate the differentiated lineages that form the mature tissue. There are three pools of KSC in different locations. The interfollicular (IF) stem cells of the basal layer, the hair follicle (HF) stem cells of the bulge and the sebaceous gland (SG) stem cells that are located

above the bulge and below the hair shaft orifice (Fig. 1). During physiological tissue renewal, the three skin cell populations are each believed to be maintained by their own stem cells. When tissue homeostasis is disrupted, however, any of the three stem cell populations is capable of producing all three structures (Fuchs and Hosley, 2008; Levy et al., 2007). Human epidermis is thick with frequent cellular turnover and a tendency to produce long term epidermal cultures in vitro. On the other hand, while the mouse can be genetically manipulated, it displays a thin, less active epidermis, whose cells fail to yield long-term cultures. In addition, some of the markers used to identify stem cells are different in mouse versus human skin. Despite these differences, common signaling pathways appear to control epithelial stem cell maintenance, activation, lineage determination, and differentiation. Data and discussion reported in this review will be based on studies performed in both mouse and human epidermis.

Interfollicular stem cells

Regeneration of epidermis depends on the proliferation of a subpopulation of basal keratinocytes, known as IF stem cells. The current dogma of the epidermal proliferative unit (EPU) defines a model where a single stem cell divides infrequently to produce a stem cell daughter and a non-stem committed progenitor cell, named transit amplifying (TA) cell. TA cells divide a small number of times before withdrawing from the cell cycle and undergoing terminal differentiation in the suprabasal layer. The EPU model has been questioned recently by lineage tracing experiments demonstrating that normal adult epidermis is maintained by a single population of committed progenitor cells with different stochastic choices (Clayton et al., 2007). In any event, the EPU model implies that in the basal layer of epidermis there is proliferative heterogeneity. Clonogenicity assays allow to distinguish three types of clones: holoclones that arise from stem cells and found large colonies with the greatest regenerative capacity in long-term culture; paraclones which give rise to abortive colonies and undergo differentiation, and meroclones that originate from TA and have an intermediate proliferative capacity (Barrandon and Green, 1987). The different clonogenic potential well correlates with the expression of β_1 integrin (Jones and Watt, 1993). Indeed, keratinocytes expressing highest levels of β_1 integrin give rise to holoclones, whereas cells with low levels of β_1 integrin produce smaller colonies. This has allowed to select a population of stem keratinocytes based on the rapid adherence to type IV collagen. A number of markers to enrich for stem cells has been reported, including high expression of α_6 integrin (α_6^{bri}) and low expression of CD 71 (CD71^{dim}). In particular, $\alpha_6^{\text{bri}}\text{CD71}^{\text{dim}}$ basal keratinocytes exhibit many features of stem cells, such as quiescence and the greatest long-term regenerative capacity (Li et al., 1998). Furthermore, melanoma chondroitin sulphate proteoglycan (MCSP) is confined to non-cycling keratinocytes expressing highest levels of β_1 integrin, and can thus be considered a marker for epidermal stem cells (Legg et al., 2003). Also the EGF receptor antagonist Lrig1 has been proposed as a marker of human IF stem keratinocytes, since it maintains these cells in a

quiescent state (Jensen et al., 2006). While these markers have greatly helped in isolating and characterizing keratinocyte subpopulations enriched in stem cells, a definite identification of epidermal stem cells and their niche is still vague. Clusters of putative stem cells expressing β_1 integrin, MCSP and Lrig1 are mostly detected in the upper part of the epidermal rete ridge, whereas $\alpha_6^{\text{bri}}\text{CD71}^{\text{dim}}$ keratinocytes are found at the tip of the rete ridges. On the other hand, xenograft studies using lentiviral vectors have shown that stem cells are dispersed throughout the basal layer (Ghazizadeh and Taichman, 2001; Ghazizadeh and Taichman, 2005). Irrespective of the markers and the location, stem cells can be identified based on the fact that they divide infrequently, thus maintaining the incorporated radioactive thymidine for long periods of time. This provides evidence that at least mouse epidermis contains slow cycling stem keratinocytes. On the other hand, radioactive labeling cannot be applied to humans in vivo. Yet, using a scaffold-based organotypic skin culture, it is now possible to mark keratinocytes with iododeoxyuridine and follow them for 8 to 10 weeks, when only less than 1% of basal cells have retained the label. Using whole mount preparations, Muffler and co-workers were able to demonstrate that LRC are individually dispersed throughout the basal layer in a random distribution with no evidence of a pattern suggestive of EPU. As a similar pattern was also observed for the IFE of mouse skin (Braun et al., 2003), this is likely to be the kind of distribution of stem cells in the basal layer. Consistent with this finding, we have shown recently that survivin identifies a population of IF stem cells expressing highest levels of β_1 integrin, and these cells are dispersed randomly in the basal layer (Marconi et al., 2007). Actually, one could comment that such a random distribution throughout the entire basal layer better fulfills the tasks of IF stem cells that would thus be ready to reconstitute epidermis wherever it is needed. While stem cells should reside in protected locations, factors, adhesion molecules and cells participating in the niche are evenly expressed throughout the epidermis and not just at the tip or in the upper part of the rete ridges (Muffler et al., 2008).

Hair follicle stem cells

In contrast to continuous regeneration of the IF epidermis, the HF undergoes cycles of growth (anagen), apoptosis-mediated regression (catagen) and quiescence (telogen) (Alonso and Fuchs, 2006). The new hair shaft is regenerated from hair specific SCs resident in the so-called bulge region of the HF epithelium. The bulge provides a unique differentiation-restricted environment for different types of adult SCs. In fact, the bulge area also contains melanocytes SCs and SCs with neural crest characteristics (Yu et al., 2010). Major differences exist between rodent and human HFs (Cotsarelis, 2006). For example, as opposed to the mouse HFSC niche, the human bulge is less morphologically distinct. Another difference is that HFs grow for years in humans and for weeks in rodents. Moreover, human and murine follicular epithelial SCs have a different biomarker expression.

Human bulge cells express CD200, K15, K19, as the most reliable markers, while CD34, K15 are preferentially expressed by mouse bulge cells. *In vivo*, the mouse CD34⁺ keratinocytes are label-retaining cells, while *in vitro* they show enhanced colony-forming ability (Trempeus et al., 2003). Bulge cells targeted by the K15 promoter have revealed a gene expression profile substantially overlapping that of CD34⁺ cells, consistent with stem cell properties (Morris et al., 2004; Trempeus et al., 2007). Using different biomarkers, Inoue and co-workers have recently described two stem cell subpopulations in human HF: CD200⁺CD34⁻ K15^{+bright} cells are located in the basal layer, and form few but large-sized colonies, while CD200⁺CD34⁻ K15^{+low} cells are mostly found in suprabasal location and form smaller colonies (Inoue et al., 2009). Two distinct CD34⁺ bulge stem cell populations with stem cell properties have also been isolated in the mouse HF based on α_6 integrin expression and on the attachment to the basal lamina (Blanpain et al., 2004). Recently, Lgr5⁺ [leucine-rich repeat containing G protein-coupled receptor 5] stem cells were identified in the mouse telogen bulge and in the lower outer root sheath of anagen HF (Jaks et al., 2008). Lgr5⁺ population was strongly enriched for bulge markers such as CD34 (Snippert et al., 2010). Although Lgr5⁺ cells rarely include LRCs and actively proliferate, these cells act as fully functional HFSCs *in vitro* and *in vivo*, but do not contribute to the SG or IF epidermis (Jaks et al., 2008). Stem cells can also be found in areas of the mouse HF located above the bulge: the isthmus, the infundibulum and the SG. In the upper isthmus, resides a subpopulation of multipotent and highly clonogenic keratinocytes, expressing Lrig1 and Plet-1 markers, recognized by MTS24 antibody (Nijhof et al., 2006). MTS24⁺ cells are distinguished by low α_6 integrin levels and are negative for CD34, K15 and Sca-1. On the other hand, Sca-1, that is a marker of mouse IFE, also identifies a population of cells in the HF infundibulum that can regenerate epidermis but not HF (Jensen et al., 2008). More recently, Snippert et al have identified a Lgr6⁺ cell population located in central isthmus of the HF, directly above CD34⁺K15⁺ bulge cells. Lgr6⁺ cells do not retain DNA label and overlap only marginally with MTS24⁺Lrig1⁺ cells but give rise to all lineages of the skin. Moreover, Lrg6⁺ stem cell pool can renew sebaceous cells and seed the epidermis throughout life (Snippert et al., 2010). A discrete population of progenitor cells has also been identified in the SG that arises from the hair follicle. SG stem cells are marked by Blimp-1, and conditional ablation of Blimp-1 induces SG hyperplasia. Lineage tracing experiments confirm that SG stem cells can give rise to the entire gland, thus ensuring SG homeostasis (Horsley et al., 2006). Finally, another reservoir of mouse stem cells has been identified in the junctional zone between the IFE, SG and the bulge. Junctional zone cells are a pool of quiescent stem cells identified by Lrig1 (Jensen et al., 2009).

Stem cells and apoptosis

KSC reside in a special microenvironment, the niche, that allows them to maintain their unique features and “stemness”. Namely, IF KSCs are located within the basal layer of epidermis and rest upon the BM that is rich in extracellular matrix (ECM) proteins and growth factors. As basal keratinocytes exit the niche, they move into suprabasal layers, and different microenvironmental stimuli influence their destiny. Under normal conditions, basal keratinocytes leave the basal niche to undergo terminal differentiation. This critical process is regulated by an increasing number of signals. Notch ligands expressed in basal keratinocytes bind to Notch receptors in suprabasal cells, thus promoting the classical change between keratins 5/14 and K1/10 that characterizes the commitment of basal keratinocytes to differentiation (Watt et al., 2008; Wang et al., 2008). While these and other molecular markers finely regulate the program of epidermal differentiation some of these signals are also involved in apoptosis (Lippens et al., 2005), suggesting that, under different regulatory inputs, basal cells exit the niche to undergo cell death. Conceivably, the niche concept in itself implies that KSC, in order to maintain longevity and to ensure continuous tissue renewal, need to be protected from apoptosis. Indeed, transition from KSC to TA cells is associated with increase rate of apoptosis both in the murine (Tudor et al., 2007) and in the human system (Tiberio et al., 2002). Moreover, K15+ stem cells are more resistant than TA cells to apoptosis in a murine model of graft versus-host disease (Zhan et al., 2007), while epithelial cells with stem-like properties are less prone to cell death than the remainder population (Harper et al., 2010). Δ Np63, a member of the p53 family, is critical for epidermal commitment of embryonic stem cells (Medawar et al., 2008), and identifies epidermal stem cells (Senoo et al., 2007). Interestingly, KSC lacking Δ Np63 undergo apoptosis, while down-regulation of Δ Np63 is required for UVB-induced apoptosis (Senoo et al., 2007; Liefer et al., 2000). Moreover, up-regulation of microRNA-203 that is associated with keratinocyte differentiation, not only inhibits the Δ Np63-dependent clonogenicity of epidermal stem cells (Yi et al., 2008), but it also induces KSC apoptosis (Lena et al., 2008). It is likely that, during epidermal homeostasis, Δ Np63 represses a number of genes, including p53, and protects KSC from cell death, which in turn contributes to maintain “stemness”. Additional insights into the relationship between KSC and apoptosis come from the study of the integrin pathway. Indeed, both $\alpha_6\beta_4$ and β_1 integrin are highly expressed in KSC and, upon binding to ligands of the BM component, promote proliferation and survival (Piwko-Czuchra et al., 2009; Manohar et al., 2004) Blocking β_1 integrin induces KSC anoikis, an apoptotic signaling cascade caused by cell detachment from the ECM and characterized by down-regulation of Bcl-2 (Tiberio et al., 2002) as well as by the early activation of caspase-8 (Marconi et al., 2004). This indicates that KSC are protected from cell death because of the high levels of β_1 integrin. Thus, it would appear that low levels of β_1 integrin are responsible for KSC to exit the niche and undergo either differentiation or apoptosis. While in epidermis these two events are clearly different at the molecular

level (Lippens et al., 2005), it is interesting to note that blocking β_1 integrin induces keratinocyte anoikis without affecting the markers of differentiation and vice versa (Tiberio et al., 2002; Mitra et al., 1997). The role of β_1 integrin within the KSC niche is even more complex, since the β_1B isoform cooperates with unligated β_1A to induce anoikis (Lotti et al., 2010). The best example of the close correlation between KSC and apoptosis derives from survivin, an antiapoptotic molecule that also regulates cell cycle (Mita et al., 2008). Survivin identifies KSC, whereas it is almost undetectable in TA cells. When keratinocytes undergo anoikis induced by blockade of β_1 integrin, survivin protein disappears (Marconi et al., 2007), and survivin overexpression protects keratinocytes from UVB-induced apoptosis (Dallaglio et al., 2009). Although the molecular switch determining differentiation or apoptosis of KSC remains to be defined, it should be taken into account that epidermis is a unique tissue under the constant physiological stress of UV irradiation that is one of the major causes of apoptosis. Interestingly, UVB irradiation induces keratinocyte apoptosis mainly in the proliferative basal layer (Qin et al., 2002). The balance between KSC resistance to UVB-induced apoptosis (Dallaglio et al., 2009) versus the higher susceptibility of the TA populations is likely to play a role in several skin disorders, including skin cancer where KSC carrying UVB-induced p53 mutation resist to apoptosis and expand because of the cell death of adjacent normal keratinocytes outside their boundary (Chao et al., 2008). Little is known about the correlation between stem cells and apoptosis in HF. During catagen, the lower two-thirds of the hair follicle rapidly regress, mainly by apoptosis of inner and outer root sheath keratinocytes in association with low levels of Bcl-2 and highest levels of the p75 neurotrophin receptor (Lindner et al., 1997), while bulge hair follicle stem cells escape apoptosis (Schneider et al., 2009). Recently, Tiede and co-workers have been able to select vital K15⁺ progenitor keratinocytes from the adult hair follicle by a number of criteria, including resistance to apoptosis (Tiede et al., 2009), and high levels of Bcl-2 mediate resistance of hair follicle bulge stem cells to DNA-damage-induced cell death (Sotiropoulou et al., 2010). On the other hand, alopecia areata is caused by induction of apoptosis in bulge stem cells, and premature ageing in mice is characterized by increased bulge stem cell apoptosis (Espada et al., 2008).

Stem cell, apoptosis and skin cancer

KSC are long-lived residents in the epidermis, do not protect their genome by asymmetric chromosome segregation (Sotiropoulou et al., 2008) and are highly resistant to apoptosis. It is thus likely that they accumulate a number of oncogenic mutations and induce skin cancer formation. It has been shown recently that the same cell subset characterized by high clonogenic potential and by a great resistance to apoptosis is common to both normal and malignant epithelial cells (Harper et al., 2010). In human skin, survivin and its antiapoptotic isoforms are mostly expressed in KSC, whereas the proapoptotic variants are

only expressed in more differentiated cells (Marconi et al., 2007). Survivin is abundantly expressed in squamous cell carcinoma (SCC) (Lo Muzio et al., 2001) that is caused primarily by chronic exposure to UV irradiation and originates from KSC (Zhang et al., 2005). Upon sustained UVB irradiation, KSC, highly expressing survivin and carrying p53 mutations, colonize the adjacent epidermal compartments (Zhang et al., 2001). Moreover, skin specific expression of survivin promotes new clonal formation and malignant progression in a UVB-irradiated transgenic mouse model (Zhang et al., 2005). This suggests that survivin, highly expressed in KSC, contributes to skin carcinogenesis, by protecting cells from UVB-induced apoptosis (Dallaglio et al., 2009). Consistently, Bcl-xl plays a key role in UVB-induced skin carcinogenesis also through the up-regulation of survivin in cells of the bulge region (Kim et al., 2009a). Moreover, Stat3, a transcription factor that regulates the expression of several antiapoptotic genes, including survivin and Bcl-xl, is involved in a number of human tumors. Specific disruption of Stat3 in bulge region KSC inhibits skin tumor initiation by increasing carcinogen-induced apoptosis (Kim et al., 2009b). This indicates that Stat3 is required for the survival of bulge region KSC during the initiation of skin tumors. SCCs are tumors that arise in multilayered epithelia such as the epidermis, esophagus, cervix and the oral cavity. Independent of the tissue of origin, SCC are characterized by changes in integrin expression (Janes and Watts, 2006), and β_1 integrin mutation increases conversion of benign to malignant skin cancers (Ferreira et al., 2009). In the epidermis, SCC originates from KSC (Klein et al., 2009) that are protected from anoikis because of the highest levels of β_1 integrin (Tiberio et al., 2002). While anoikis is important for tissue homeostasis, anchorage-independent growth is a crucial step during tumorigenesis and in particular during the metastatic spreading of cancer cells (Chiarugi and Giannoni, 2008). Conceivably, upon carcinogenic stimuli, integrin expression is perturbed and the basement membrane is extensively damaged (Ferreira et al., 2009; Savoia et al., 1994). Under these conditions, KSC detach from the basement membrane, proliferate and may reattach to an inadequate location to eventually metastasize. We have recently shown that the β_1 B integrin isoform can cause apoptosis, possibly working as a “sensor” of the detachment from the proper ligand, thus acting as a “fail-safe” device by stimulating cell death to prevent spread of SCC (Lotti et al., 2010).

Conclusions

KSC are distributed in different pools within adult epidermis, where they play a critical role because of their self-renewal, high proliferative and multipotent differentiation capacity. These features have been already exploited for regenerative medicine and tissue repair (Pellegrini et al., 1999) as well as for gene therapy in genetic skin diseases (Titeux et al., 2010). Moreover, alterations of stem cells and their niche are of great relevance to the ageing process (Giangreco et al., 2008). On the other hand, despite a great deal of research, a number of questions still remain unanswered. First, no definite marker has been identified yet

that allows the isolation of a pure population of KSC. Secondly, the role of KSC progeny, namely of TA cells should be better defined. Clayton and co-workers have recently challenge the existence of TA cells, by proposing a model whereby a single cell population has three stochastic choices upon cell division (Clayton et al., 2007; Jones et al., 2007). On the other hand, TA cells have always been overlooked and there are no clear markers to identify them. According to the early methods of enrichment based on the levels of β_1 integrin, cells not adhering to the ECM within 20 minutes are considered TA cells (Jones and Watt, 1993). Our group has modified this technique and has defined TA cells as cells adhering in a longer period of time, while cells not adhering were considered as postmitotic (Tiberio et al., 2002). Because of the lack of markers for TA cells, it is also difficult to assess their precise location within the basal or suprabasal layer. It is interesting to note that data are now emerging on the relevance of TA in physiological and pathological processes. It would appear that aging epidermis is characterized by changes in TA, but not in stem cell kinetics (Charruyer et al., 2009). In psoriasis, a skin disease characterized by altered epidermal homeostasis, TA and not KSC seem to carry the intrinsic defect leading to the development of the lesion (Grabe and Neuber, 2007; Franssen et al., 2005). While the microenvironment of HF KS seems to be better characterized, the IF KSC niche needs to be fully understood mostly because KSC are individually dispersed throughout the basal layer that in turn could be considered itself as a niche (Fuchs, 2009). This would imply that adjacent keratinocytes, other skin cells, adhesion molecules and growth factors in any area of the basal cell compartment could contribute to the niche. In this respect, we have shown that a complex neurotrophin network exists in the skin and perform a number of autocrine and paracrine functions (Botcharev et al., 2006) In particular, nerve growth factor is highly expressed only in KSC (Marconi et al., 2003). and stimulates KSC, but not TA cell proliferation (personal observation), suggesting an autocrine loop that could participate in the KSC niche. Finally, although many evidence point to KSC as the origin of skin cancer, more studies are needed to fully define both the stem pool involved in each type of tumor and the pathways through which a stem cell drives carcinogenesis, also in relation to UVB. Interestingly, a recent work points to the IF KSC as the origin of basal cell carcinoma (BCC) (Youssef et al., 2010), as opposed to the dogma that the cell of origin of BCC resided in the HF bulge. This is actually conceivable, since BCC is a UV-induced tumor and IF KSC are closer than HF KSC to the skin surface.

In conclusion, a better knowledge of the different keratinocyte stem cell pools in relation to their niche and to apoptosis will allow to design new therapeutic strategies either to protect “stemness” and allow tissue repair and regenerative processes or to target KSC when they acquire mutations and give raise to skin carcinogenesis.

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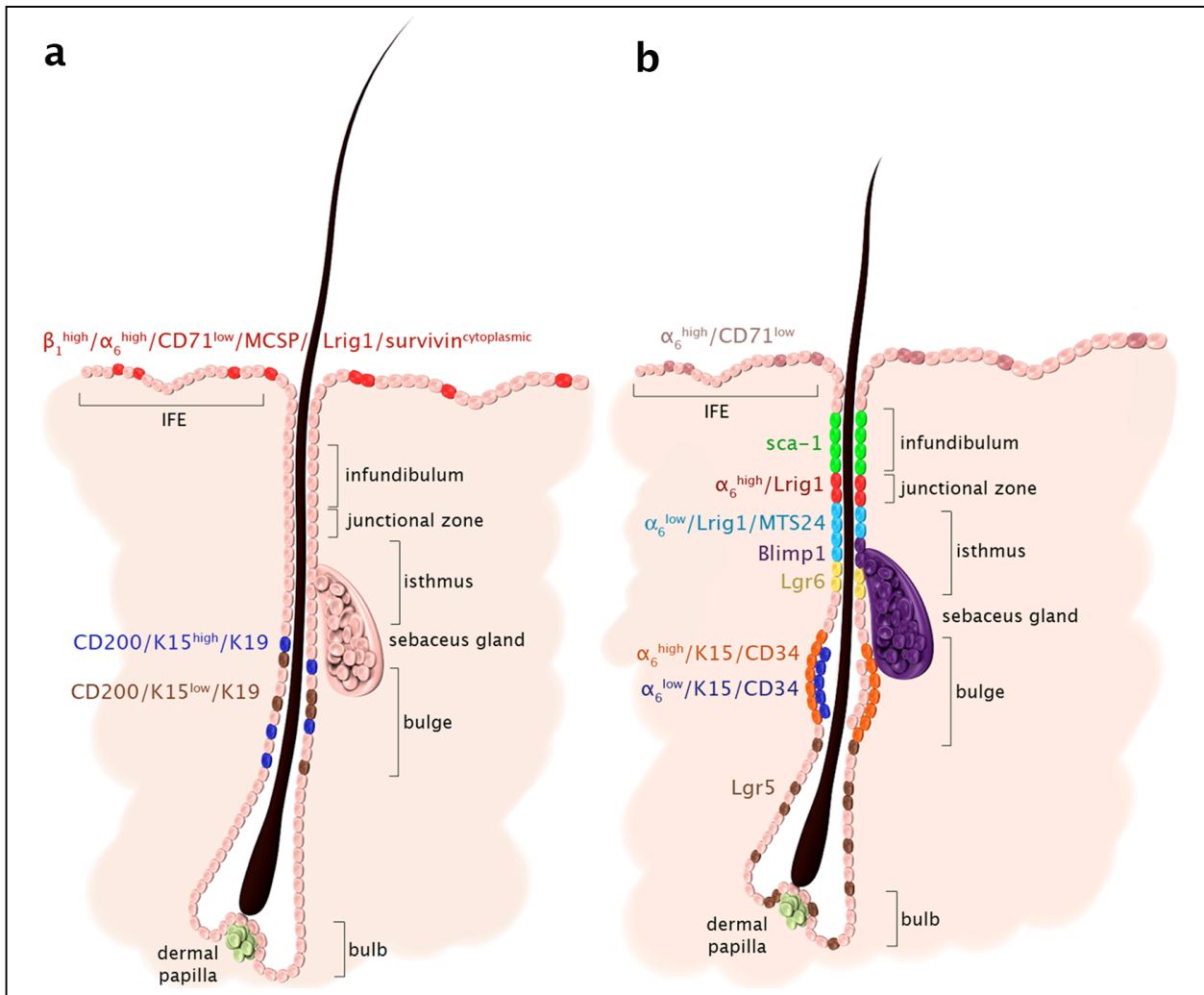


Fig. 1 A schematic representation of the location of the KSC subpopulation in human and mouse epidermis. Multiple epidermal stem cell pools are competent to differentiate along all the epidermal lineages, and during tissue homeostasis, each pool contribute to maintenance of its specific population. Upon injury, each stem cell compartment is capable of regenerating all epidermal structures. In humans, IFE KSC are better characterized than HF KSC (a), whereas in mouse most stem cell markers were detected in the HF (b).

Table. 1 Apoptotic molecules in HF and IFE keratinocyte subpopulations

Anti-apoptotic markers are almost exclusively expressed in KSC, whereas pro-apoptotic molecules are mainly detected in more differentiated cells.

Anti-apoptotic molecules		References
Δ np63	p53 family	Seeno et al. 2007
β_1 integrin	adhesion molecule	Tiberio et al.2002
survivin	IAP family	Marconi et al. 2007
bcl-2	Bcl-2 family	Tiberio et al. 2002, Sotiropoulou et al. 2010
bcl-xL	Bcl-2 family	Kim et al. 2009a
mcl-1	Bcl-2 family	Tiberio et al. 2002, Kim et al. 2009a
Pro-apoptotic molecules		
miRNA-203	small non-coding RNA class	Lena et al. 2008
Bax	Bcl-2 family	Lindner et al. 1997, Tiberio et al. 2002
Bad	Bcl-2 family	Tiberio et al. 2002
p75NTR	TNF-R super family	Lindner et al. 1997