Regulation of the cyto-architecture in epithelial morphogenesis and homeostasis

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Abstract
Intercellular adhesion and polarity are crucial determinants of tissue morphogenesis and tissue architecture. They couple intercellular communication to cell shape, fate, migration, and orientation of cell division (Niessen et al., 2011; Nelson, 2003; Macara, 2004; Niessen and Gottardi, 2008). The goal of the Niessen laboratory is to understand how regulation of the cyto-architecture controls the formation and maintenance of stratifying epithelia such as the skin epidermis and how interference with this regulation contributes to disease. Our laboratory asks how key regulators of cell architecture, the aPKC/Par polarity complex, or upstream niche signals of this complex, e.g. insulin/IGF-1 or classical cadherins (Tunngal et al., 2005; Seifert et al., 2009; Farese and Sajan, 2010) balance epidermal barrier homeostasis, cell fate and oriented cell division and thereby control growth, differentiation, and stem cell behavior. In this focus we will concentrate on the role of the aPKC/Par complex in the regulation of mammalian epidermal barrier function, cell fate and homeostasis.

Polarity: a short introduction
Polarity is a fundamental property of cells and tissues and defined as the unequal distribution of molecules (RNAs, lipids, proteins) within a cell to produce asymmetry in structure and function at the cellular, tissue and organismal level. Establishment and maintenance of polarity is a critical determinant of cell and tissue architecture crucial for the regulation of cell behavior in tissue morphogenesis, differentiation and homeostasis (Iden and Collard, 2008; Macara and Mili, 2008; Simons and Mlodzik, 2008; St Johnston and Ahringer, 2010).

Polarity is established on two levels: 1) on the cellular level resulting in asymmetry in individual cells, e.g. apico-basolateral polarity, asymmetric cell division and the leading and trailing edge of migrating cells. 2) on the tissue level, in which subcellular structures and/or cells are aligned in the plane of the tissue. This is also known as planar cell polarity (PCP). Examples of tissue polarity are convergent extension movements, in which cells rearrange their cytoskeleton in the plane of tissue to drive directional intercalation of cells or the orientation of cilia, signal sensing cell organelles within a tissue seen e.g. in the kidney or in the skin.

A core set of polarity proteins that are highly conserved throughout Metazoa establish and maintain cell polarity or tissue polarity. These basic signal pathways that cover either cell or PCP pathways integrate to coordinate individual cell shape with tissue architecture (Simons and Mlodzik, 2008). In addition, polarity signals also regulate and interact with other key determinants of cell shape and tissue architecture, such as cytoskeletal components, membrane trafficking, and adhesive junctions (Li and Bowerman, 2010).

Initially mostly studied in lower organisms or in epithelial cell culture systems, it is now clear that polarization is also a fundamental requirement for the proper functioning of mammalian tissues (McCaffrey and Macara, 2009) and alterations in the establishment and/or maintenance of asymmetry results in a variety of human disease, such as kidney disease, hearing dysfunction, inflammatory diseases and cancer (Huang and Muthuswamy, 2010).

Polarity in stratifying epithelium: the epidermis as an example
The epidermis of the skin is a stratifying multi-layered epithelium that forms a barrier against external challenges and water loss (Fig.1B). It consists of the interfollicular epidermis (IFE) and epidermal appendages: hair follicles, sebaceous and sweat glands. In the proliferating basal layer, epidermal keratinocytes balance life long self-renewal with a spatiotemporally strictly regulated terminal differentiation program necessary to form the stratum corneum, a dead, cornified and water impermeable cell layer (Candi et al., 2005; Koster, 2009). Different populations of stem and progenitor cells located in the basal layer of the IFE and in specific areas of hair follicles guarantee constant self-renewal under steady state conditions. In case of injury, these progenitors also provide sufficient plasticity for the fast replacement of lost tissue e.g upon wounding (Blanpain and Fuchs, 2009; Watt and Jensen, 2009).

Many features within the epidermis are polarized and, more importantly, this polarization is crucial for the formation and function of the IFE and its appendages. During stratification basal keratinocytes differentiate, move suprabasally while undergoing controlled polarized cell shape changes until they reach

the stratum corneum. This process requires intercellular rearrangements to allow cells to migrate through the layers. Another example of polarity is oriented cell division of basal cells in the IFE and in hair follicles. By orienting the mitotic spindle either parallel (symmetric cell division, SCD) or perpendicular (asymmetric cell division, ACD) with respect to the underlying basement membrane, stem and progenitor cells can control cell fate while guaranteeing renewal (Fig.2B). Wound closure is a highly polarized process that requires the coordinated secretion and deposition of extracellular matrix to allow for directional migration of keratinocytes. Cilia are positioned in a polarized manner on keratinocytes and this is likely important for their role in signal transduction. Not only individual cells or subcellular structures are highly polarized but the orientation of multicellular structures such as sebaceous glands and hair follicles are organized in the plane of the tissue. All of these processes depend on cell and tissue polarity and work by several groups including ours have started to unravel how polarity genes contribute to these processes in stratifying epithelia.

The atypical protein kinase C: a key regulator of polarity

Atypical protein kinase C (aPKC) is a serine/threonine kinase that has emerged as an evolutionary conserved central regulator of all forms of polarity and thus of cell and tissue architecture in almost all cell types (Suzuki and Ohno, 2006; Goldstein and Macara, 2007). aPKC binds the scaffolding proteins Par6 and Par3 to form the Par3/Par6/aPKC polarity complex. Par6 regulates aPKC kinase activity whereas Par3 can function both as an upstream regulator and downstream effector of aPKC and may have independent functions outside of the complex (Goldstein and Macara, 2007). By coupling to different downstream interaction partners or substrates this complex drives the asymmetric distribution of proteins and thus functional activity. For example, by coordinating the establishment of apical membrane identity and the formation and positioning of barrier forming tight junctions, which separate apical from basolateral membranes, aPKC regulates the proper function of simple epithelial ion and size barriers that separate tissues (Suzuki and Ohno, 2006). The aPKC/Par complex has also been implicated in the regulation of insulin/IGF and NFκB signaling (Moscat et al., 2009) and may thus couple control of cyto-architecture to the regulation of metabolism and inflammation (Martin-Belmonte and Perez-Moreno, 2012).

Atypical PKCs belong to the protein kinase C family of cytosolic serine and threonine kinases. Unlike the other members of this family, atypical PKCs are not activated by either phorbol esters or Ca^{2+} due to the lack of binding motifs. In mammals two isoforms of aPKC exist, aPKCzeta (aPKCζ) and aPKCiota/lambda (aPKCι/λ), which are highly related but encoded by separate genes (reviewed in Rosse et al., 2010). In vitro studies have implicated both aPKCs in the regulation of polarity. However, complete inactivation of aPKCζ resulted in viable mice with reduced B-cell survival and altered NFkB signaling (Leitges et al., 2001) whereas aPKCλ knockout mice die early during embryogenesis due to gastrulation defects (Soloff et al., 2004; Seidl et al., 2013). This suggests separate functions for the two isoforms

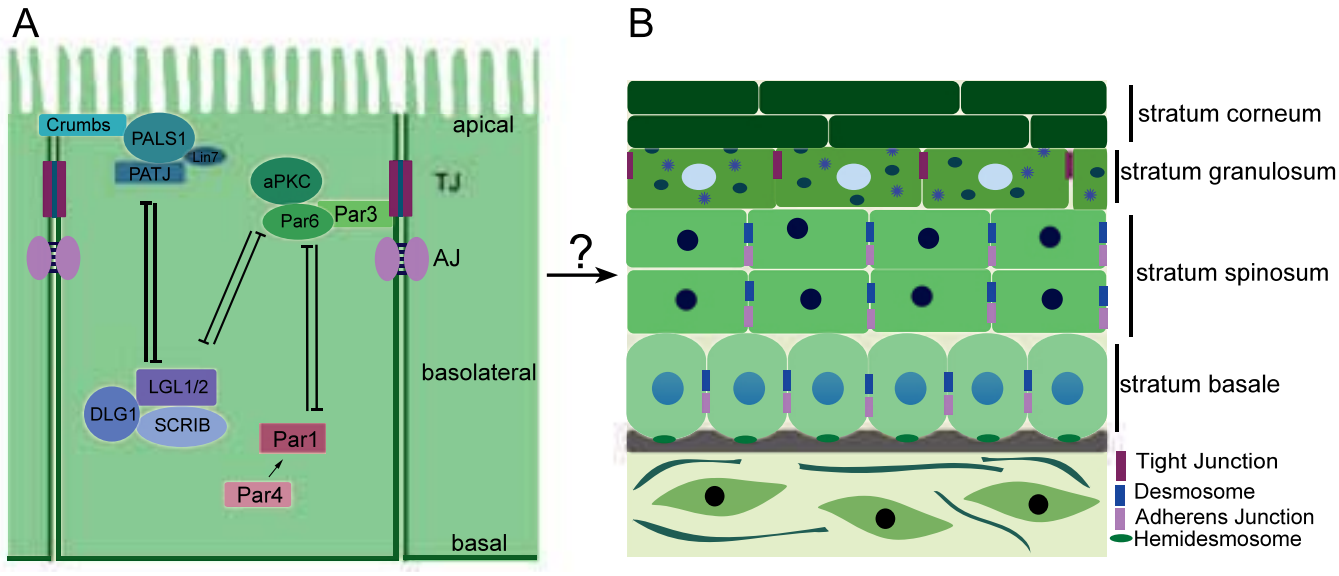

Figure 1. Functional similarities and differences of polarity in simple epithelia and stratifying epithelia.
At present it is unclear if the same molecular mechanisms controlling apico-basal polarity in simple epithelia are involved in the establishment of epidermal polarity (question mark). (A) Apico-basal polarity in simple epithelia. The apical junctional complex, consisting of tight junctions (TJ) and adherens junctions (AJ) forms a border to establish apico-basal polarity. Mutual interactions between polarity proteins and complexes regulate apico-basal polarity and barrier formation in simple epithelia. (B) Polarity in the murine epidermis. In contrast to simple epithelia, the epidermis has no distinct apical and basolateral membrane domains, but displays apico-basal polarity from the stratum basale as the most basal layer and the stratum spinosum to the stratum granulosum forming the viable apical border and the stratum corneum, the outermost dead water impermeable layer. Polarity in this multilayered tissue is also reflected in cell shapes and adhesive junctions. Moreover, lamellar bodies (blue circles) and keratohyalin granules (purple asterisks) are targeted (indicated by arrows) towards the upper layers to form the cornified envelope.

in the regulation of e.g. innate immunity versus polarity. On the other hand, both isoforms are necessary to polarize T-cells in vivo and couple this to an effective Th2-response (Martin et al., 2005; Yang et al., 2009). Thus, at present it is unclear if both aPKCs are functionally redundant or have separate functions in the regulation of polarity, metabolism and immunity. Although both aPKC isoforms are expressed in the skin, real time PCR analysis revealed that aPKCλ is expressed around 10-fold more strongly in mouse epidermis. In addition, whereas aPKCζ is confined to the basal layer of the epidermis, aPKCλ is strongly enriched at sites of intercellular junctions in the suprabasal layer of the epidermis, suggesting that this isoform may regulate epidermal polarity and barrier function.

aPKC and the regulation of barrier function in stratifying epithelia

Perhaps the best characterized example of cell polarity is apico-basolateral polarity, also known as epithelial polarity, in which simple epithelia such as the intestine establish two different membrane domains, the apical and basolateral domains that are separated by the apical intercellular junctional complex consisting of tight junctions, adherens junctions and desmosomes (Roignot et al., 2013). Apico-basolateral polarity is important for barrier function, vectorial transport and sensory and signal perception. The stratifying epidermis is not a classically polarized epithelium like the intestine, in which tight junctions separate basolateral and apical membrane proteins and lipids (Fig.1A). Instead, the epidermis establishes polarity along the basal to apical axis of the tissue, with the stratum granulosum forming the viable apical boundary (Fig.1B). The formation of the stratum corneum depends on the fusion of lamellar bodies, specialized secretory granules containing enzymes and lipids necessary to build up the stratum corneum with plasma membranes at the transition between stratum granulosum and corneum layers. As in simple epithelia, tight junctions in the stratum granulosum may thus have a fence function that may be necessary for "apical" targeting of these lipid vesicles directly towards the stratum corneum.

From C. elegans to humans, the formation and maintenance of intercellular junctions and apical membrane domain identity in simple epithelia is tightly linked to the Par3/Par6/aPKC complex (Nelson, 2003; Goldstein and Macara, 2007). It is thus well possible that the positioning of functional tight junctions and the formation/maintenance of the apical domain in stratifying epithelia would also require the activity of the apical Par3/Par6/aPKC complex. Par3/Par6/aPKC coordinates simple epithelial polarity through mutual inhibitory and activating interactions with other polarity complexes, such as the LGL/Scribble and the Crumbs complex, within the same cell (Fig. 1A). The mechanisms that regulate the formation of stratifying apico-basolateral tissue polarity are largely unknown. If similar mechanisms are in place as in simple epithelia then the mutual antagonistic actions of polarity complexes have to be established over several cell layers. A relatively simple system could consist of counter-gradients of mutually inhibiting complexes over the basal-apical axis of the epidermis (Fig.1B). Interestingly, as in simple epithelia, both Rac and Par3 are necessary for tight junctional barrier function in stratifying keratinocytes (Mertens et al., 2005; Iden et al., 2012), suggesting a similar mechanism as in simple epithelia at least for formation of functional tight junctions.
To examine whether aPKC regulates epidermal barrier function we exogenously expressed aPKC in primary keratinocytes and observed that whereas wt aPKC would enhance epidermal

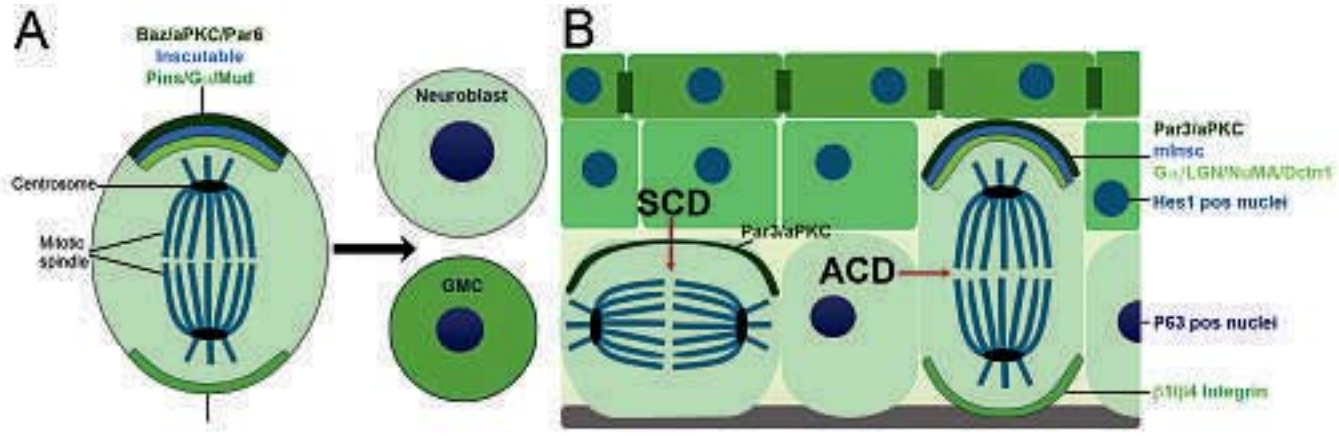

Figure 2. Mechanisms of asymmetric cell division.
Schematic overview of asymmetric localization of polarity proteins and spindle orientation regulators during asymmetric cell division in Drosophila (panel A) and in the interfollicular epidermis (panel B), illustrating that similar molecular mediators are involved in the establishment of asymmetric cell divisions of neuroblasts and keratinocytes. (A) Asymmetric cell division (ACD) in Drosophila neuroblasts. The apical aPKC-Baz-Par6 complex is connected to the Pins-Ga1-MUD complex via Inscuteable. This complex directs the asymmetric basal localization of the cell fate determinants Numb, Brat and Prospero. GMC, Ganglion mother cell. (B) ACD in the developing IFE. ACD contribute to stratification by producing one basal, proliferating cell (light green) and one suprabasal cell (dark green), whereas symmetric cell divisions (SCD) result in two daughter cells residing in the basal layer. aPKC-Par3, mInsc and Ga1-LGN-NuMA-Dcnt1 localize to one side of the dividing cell and are important for the establishment of epidermal ACD, as reported for their Drosophila homologues in neuroblast ACD. Suprabasal activity of the Notch signaling pathway (indicated by nuclei positive for Hes1, a well known Notch target) are crucial for the regulation of this process.

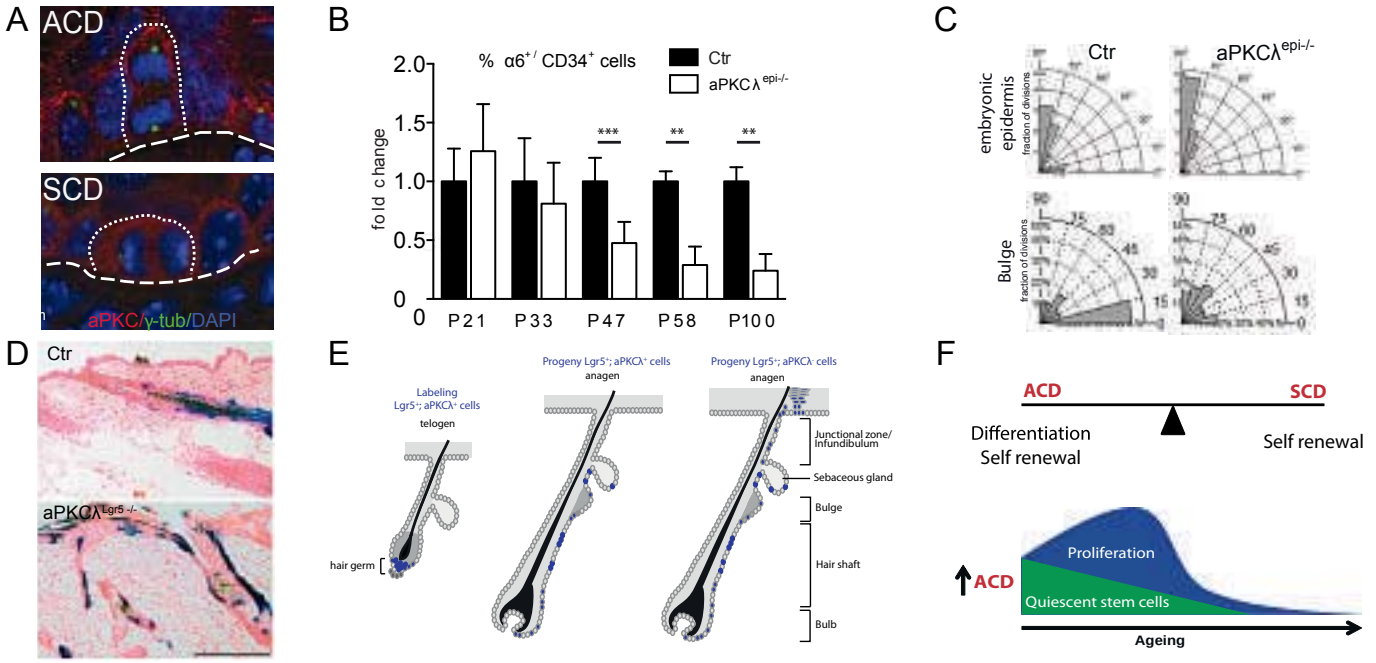

Figure 3. aPKCλ controls oriented cell division and cell fate in the mammalian epidermis.
(A) Apical localization of aPKC in asymmetric and symmetric divisions in the interfollicular epidermis. (B) Gradual loss of hair follicle bulge stem cells in epidermal specific aPKCλ knockout mice. Quantification of FACS analysis of integrin α6+/CD34+ bulge hair follicle stem cells from epidermis at indicated time points. (C) Spindle orientation plots reveal that epidermal loss of aPKCλ induces a shift towards more asymmetric divisions in the developing interfollicular epidermis (embryonic epidermis) and in the bulge hair follicle stem cell compartmet in adults (bulge). (D and E) Loss of aPKCλ alters the fate of lower bulge stem cells. Genetic lineage trace analysis reveal that aPKCλ-negative lower bulge stem cells do not only contribute to lower hair follicle regeneration, as controls, but now fuel the upper hair follicle (junctional zone/infundibulum), sebaceous glands and interfollicular epidermis. (F) Model proposing that a shift towards more asymmetric cell division promotes loss of quiescent hair follicle bulge stem cells that become more committed progenitors that initially expand but as these also undergo increased asymmetric division, these cells also are depleted leading to increased differentiation and premature skin aging.

barrier function overexpression of dominant negative aPKC mutants interfered with TJ function (Helfrich et al., 2007). In collaboration with Michael Leitges (Biotechnology Centre of Oslo, University of Oslo) we deleted aPKCλ in mouse epidermis using the Cre-LoxP system. Isolated keratinocytes from these mice showed a reduced TER that in vivo was associated with cytoskeletal changes, altered differentiation and proliferation accompanied by inflammation, similar to what is observed in very common skin barrier associated diseases such as ichtyosis or psoriasis. Together, these results suggest a specific function of aPKCλ in skin barrier regulation. However, initial characterization of mice in which both aPKCs are absent showed a much more severe morphogenetic and barrier dysfunction phenotype, indicating specific and overlapping functions of the two aPKCs in the epidermis. Thus, aPKCs integrate cell polarity, nutrient signaling and regulation of innate immunity to coordinate tissue architecture and barrier function.

The role of aPKC in mammalian cell division orientation and cell fate

In lower organisms aPKC controls cell fate and asymmetric cell division (ACD) (Lee et al., 2006; Knoblich, 2010), resulting in two daughter cells with differential fate. In Drosophila neuroblasts, the initial polarization cue comes from the apical enrichment of the aPKC/Par complex (Fig.2A). This apical distribution is essential for asymmetric localization of cell fate determinants, which is coupled to spindle orientation by binding to the adaptor protein Inscuteable (Insc). Insc then recruits a protein complex consisting of the heterotrimeric G protein α1-subunit (Gα1), PINS and MUD, which provides attachment sites for astral microtubules (Knoblich, 2010).

Whether oriented division regulates adult tissue homeostasis or if aPKCs determine division orientation and cell fate in mammals is not known. Whereas in vitro and ex vivo studies indicate an important role for aPKCλ and/or aPKCζ, in spindle orientation and cell fate (Dard et al., 2009; Hao et al., 2010; Durgan et al., 2011), in vivo inactivation in the hematopoetic or neuronal systems indicate no essential role for aPKCs in these processes (Imai et al., 2006; Sengupta et al., 2011).

The epidermis contains different progenitor cell populations and at least in the IFE it was shown that ACD at least in part drives differentiation (Lechler and Fuchs, 2005; Niessen et al., 2012). This tissue thus provides an excellent model system to address the role of balancing SCD and ACD and its regulators in tissue homeostasis, differentiation and cell fate determination. As in Drosophila neuroblasts, Par3 and aPKC show an apical distribution in murine epidermis that is independent of cell division (Lechler and Fuchs, 2005). This apical polarity might have been

inherited from the polarized single layer epithelium before the onset of stratification. Par3 binds mInsc in the epidermis and this likely drives the apical recruitment of the mammalian homologues of Pins and Mud, LGN and NuMA (Fig.2B). However, differential localization of aPKC/Par localization alone is not sufficient to drive ACD as aPKC/Par also show an apical localization in cells that undergo symmetric divisions in the epidermis.

To examine whether aPKCλ regulates oriented cell division, epidermal cell fate, stem cell behavior and tissue homeostasis in the epidermal lineage we analyzed epidermal specific aPKCλ knockout mice. Loss of aPKCλ strongly disturbs epidermal homeostasis, stem cell maintenance, hair follicle cycling and lineage differentiation causing progressive morphological changes in different epidermal compartments, e.g. hair follicle and sebaceous glands. This is accompanied by a gradual loss of quiescent bulge stem cells (Fig.3B) and a temporary increase in different proliferating progenitors. This ultimately leads to loss of proliferative potential, stem cell exhaustion, increased differentiation, complete alopecia and premature skin aging. Unexpectedly, inactivation of aPKCλ increased asymmetric divisions, known to promote differentiation, not only in the developing IFE but also in other stem/progenitor compartments of the epidermis, including the bulge stem cell compartment (Fig.3C).

To examine whether loss of aPKCλ would alter the cell fate of bulge hair follicle stem cells towards more committed progenitors, thus explaining the increase in more committed progenitors, we crossed aPKCλfl/fl mice with Lgr5CreERT2eGFP mice (Barker et al., 2007) and with Rosa26RLacZ Cre-reporter mice (Soriano, 1999). At P21, when HFs are in telogen, Lgr5 is exclusively expressed in the lower bulge and hair germ HFSCs and its progeny only contribute to the lower part of the hair follicle but not to the JZ, infundibulum and interfollicular epidermis (Jaks et al., 2008). Upon tamoxifen-induced activation of Cre at P21, control Lgr5-progeny were labeled by β-galactosidase and contributed exclusively to the lower non-permanent part of control hair follicles (Fig. 3D,E). In contrast, aPKCλ-/-/β-galactosidase positive Lgr5-progeny were not only found in the lower HF, but also in JZ, infundibulum, and IFE (Fig.3D). Thus, loss of aPKCλ is crucial for homeostasis of self-renewing stratifying epithelia, regulation of cell fate and differentiation and maintenance of epidermal bulge stem cells and epidermal progenitor cells likely through its role in balancing symmetric and asymmetric division (Fig.3F).

The mechanisms by which aPKCλ regulates the balance between symmetric and asymmetric divisions are less clear. Whereas loss of aPKCs resulted in random spindle orientation in C. elegans or in vitro (Dard et al., 2009; St Johnston and Ahringer, 2010; Durgan et al., 2011), in vivo epidermal loss of aPKCλ caused a shift towards more ACDs. In contrast, in vivo knockdown of known regulators of spindle orientation, such as NuMA or LGN, promote SCDs in the developing interfollicular epidermis (Williams et al., 2011). This suggests that aPKCλ does not directly interfere with the machinery crucial for spindle orientation. In agreement, the localization of NuMA was also not obviously altered in asymmetrically dividing aPKCλ-/- keratinocytes at E16.5. As keratinocytes still express aPKCζ, albeit in low amounts, this might be sufficient to drive spindle orientation in the absence of aPKCλ. Together, our data identify aPKCλ as essential for balancing ACD/SCD, which likely controls cell fate in the epidermis and suggest that aPKC may either actively inhibit ACDs or promote SCDs in the epidermis.

Concluding remarks

These data from stratifying epithelia reveal that the mammalian aPKC/Par complex is not only a crucial regulator of simple epithelial polarity but also controls stratifying epithelial barrier function. In addition, there is a specific role for aPKCλ in regulating mammalian epidermal cell fate choices, likely by controlling the balance between ACD and SCD. A central remaining question is the identification of the physiological relevant substrates by which mammalian aPKCs regulate epidermal barrier function, cell fate, oriented cell division, stem cell dynamics and thus skin morphogenesis and homeostasis.

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