Molecular and Cytoskeletal Regulations in Epidermal Development

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Abstract

At the surface of the body, the epidermis covers great depth in its developmental regulation. While many genes have been shown to be important for skin development through their associations with disease phenotypes in mice and human, it is in the past decade that the intricate interplay between various molecules become gradually revealed through sophisticated genetic models and imaging analyses. In particular, there is increasing evidence suggesting that cytoskeleton-associated proteins, including adhesion proteins and the crosslinker proteins may play critical roles in regulating epidermis development. We here provide a broad overview of the various molecules involved in epidermal development with special emphasis on the cytoskeletal components.

Keywords

skin development; molecular mechanism; cytoskeleton; cell junction; spectraplakin

I. Introduction

The skin lies at the surface of the human body, providing a physical barrier against microbial infection and environmental hazards, and help maintain homeostasis by preventing water loss [1]. The skin can be divided into the inner dermis and outer epidermis. The former consists primarily of fibroblasts and extracellular collagen, while the latter consists mostly of keratinocytes but also includes other cell types that form the skin appendages including hair follicles, sweat glands and sebaceous glands. Development of these structures requires cooperative roles from the dermis and epidermis [2]. The epidermis and the dermis are separated by the basement membrane, a thin layers of extracellular matrix material composed of collagen and lamin secreted by epidermal keratinocytes and dermal fibroblasts [3]. This review will focus on epidermal development and the molecular and cytoskeletal regulators involved in this process.

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II. Overview of epidermis development

Skin epidermis can be divided into the basal, spinous, granular, and cornified layers, the cells in each layers have distinct morphology and specific protein expression (figure 1). Basal cells are undifferentiated stem cells population and are positioned and attached on the basement membrane. They are the only proliferative cells in the epidermis, whereas differentiated keratinocytes in the other layers are non-proliferative [4].

How basal cells contribute to the suprabasal layer is still under intense investigation. Earlier models suggest that epidermal development is organized into clusters containing a basal stem cell and multiple transit amplifying cells, which give rise to differentiated keratinocytes [5, 6]. Another model is that a single type of progenitor cell is able to repopulate the skin through symmetrical and asymmetrical divisions [7]. Later experiments suggest that both slow-cycling stem cells and the committed progenitors may co-exists and both contribute to skin homeostasis [8]. Finally, a recently proposed model is that basal stem cells are uncommitted at birth, and receive spatial and temporal coordination from sibling stem cells to determine whether it dives or differentiated. The committed cells are integrated into metastable vertical columns similar to the EPU, and termed epidermal differentiation units (EDUs) [9].

A unique process during skin cell development is the formation of an enclosure called the cornified envelope located just inside of the cell membrane of each granular and cornified cell [10]. This formation begins when cells enter the granular layer. The cornified envelope consists of lipids, cholesterol, desmosome proteins and other proteins. While at the granular layer, most of the components that will later constitute the cornified envelope are within vesicles called keratohyalin granules, hence the name granular layer. Upon transition to the cornfied layer, these vesicles fuse to the membrane and deposit the cornified proteins. Concurrent with this process, other cellular events such as enucleation or degradation of DNA and the cellular dehydration occurs. Upon completion of differentiation, keratinocytes become flattened dead cells, utilizing their cell body, membrane and cornified envelope to provide structural rigidity and mechanical strength that is the hallmark of skin.

Interestingly, cornified cell squamation is synchronized with differentiation of basal cells, suggesting some unknown mechanism of skin homeostasis. Disruption of epidermal skin development is associated with many human genetic skin disorders [4, 11]. Furthermore, despite our knowledge of the morphogenetic changes that occur during skin development, much remains to be learned about the molecular signaling pathways involved in this process.

III. Molecular mechanisms regulating skin development

Epidermal specification occurs at embryonic day development 8.5 (E8.5), and the subsequent keratinocyte differentiation process is well characterized by expression of various keratin filaments. During development, undifferentiated ectodermal cells express keratin 8 (K8) and K18 [12]. Undifferentiated skin basal cell markers, K5 and K14, can be detected as early as embryonic day of development 9.5 (E9.5), but full expression of K5 and K14 can take up to E14.5 [13, 14]. Committed skin cells undergoing differentiation will

express suprabasal cell markers K1 and K10. K1 mRNA is expressed even earlier at E10, while K10 mRNA is expressed at E12, steadily increasing until day 16 of development [15]. K1 and K10 are thought to be markers of keratinocytes committed to differentiation, which include cells of the spinous, granular, and cornified layers. More differentiated cells display cellular markers such as loricrin and profilaggrin in granular layer cells, and filaggrin in the cornified layer (figure 1). Once keratinocytes differentiate to spinous cells, the subsequent differentiation process into granular and cornified layers is cell-autonomous.

While the morphologic and immunophenotypic changes that occur during skin development have been well characterized, the molecular mechanisms regulating this process is less clear. Summarized below are the important regulatory pathways identified in skin development, from epidermal specification to terminal keratinocyte differentiation.

1. Epidermal specification and formation of the basal layer

The major transcription factors and signaling pathways involved in epidermal specification includes p63, AP-2γ, and Wnt pathway. The transcription factor p63 is thought to be the master regulator of stratified epithelial cell fate, as it has been found to be the first factor for specifying cells to the epidermal lineage [16]. Loss of p63 leads to loss of all stratified epithelium [17, 18]. In addition to the full length form of p63, the protein also contains an isoform which lacks the N-terminal transactivation domain, Np63 [19], and the two isoforms are regulated under their specific promoters [20]. Full length p63 is found to be expressed first during epidermal morphogenesis. As with initial specification of the epidermal cell fate, p63 is considered to be a key player for specification of basal cell fate. Full length p63, but not Np63, is thought to indirectly induce the initial expression of the basal cell marker K14 through activation of transcription factor AP-2γ, a key factor for epidermal differentiation. It has previously been shown that loss of AP-2γ leads to loss of Keratin 14 expression [21, 22]. However, full length p63 is not required for maintaining K14 expression in basal cells, as loss of p63 does not alter K14 expression in mature epidermis [23]. On the other hand, Np63 is thought to function in basal keratinocytes by maintaining cell proliferation, presumably by inhibiting genes that direct differentiation, such as transcription factor p21 and cellular signaling protein stratifin (14-3-3σ) [24–26]. In the case for p21, Np63 directly represses the p21 promoter and prevents notch signaling, which normally feeds back to upregulates p21.

Increased β-catenin signaling has been found to be sufficient to induce transdifferentiation to epidermal fate [27, 28]. However, there are conflicting reports regarding Wnt/β-catenin signaling in interfollicular epidermal development as loss of Wnt activation leads to hair follicle defects, while the interfollicular epidermis appears normal [29].

2. Initiating keratinocyte differentiation

It is currently unclear how basal keratinocytes are initially triggered to undergo differentiation, though recent evidence suggests that the cells may receive spatiotemporal cues from sibling stem cells [9]. Basal cells undergoing differentiation will lose expression of the basal markers K5 and K14, and express high levels of K1 and K10 [30]. While this switch in keratin filaments is well characterized, its significance is unknown. Mice with

homozygous deletion of both K1 and K10 can still develop all four layers of the epidermis, suggesting that the two keratin filaments are not required for skin keratinocyte differentiation [31]. As in basal cell development, the initial stages of keratinocyte differentiation is also regulated by several key factors, such as ΔNp63, Notch, and IRF6.

The p63 isoform ΔNp63 may play a complex role in basal cell regulation. As mentioned previously, ΔNp63 is necessary for basal cell development and maintenance. However there is also evidence that the protein is required for terminal differentiation and spinous specification [19]. This contrasting regulatory roles may result from differential activation of a set of downstream factors. Specifically, ΔNp63 have been shown to activate p57Kip2, a cyclin-dependent kinase inhibitor that facilitates keratinocyte terminal differentiation [32]. ΔNp63α may also repress proliferation and induce differentiation by downregulating cyclin B2 and cdc2 cell cycle proteins by directly repressing their promoters [33]. Finally, ΔNp63 can also control epidermal differentiation through the NF-κB pathway by regulating IKKα. Indeed, IKKα knockout mice have been shown to have expanded region of K1 expressing cells that are still mitotically active in the epidermis, suggesting defects in transition into mitotically inactive spinous cells.

Notch signaling has been shown to play an important role in switching proliferating undifferentiated keratinocytes to non-proliferating differentiated keratinocytes [26, 34]. Notch1 conditional knockout in the skin leads to basal cell hyperproliferation, and the keratinocytes do not express the differentiation marker K1 and involucrin. In contrast, overexpression of the Notch intracellular domain (NICD) will induce spinous cell fate [35]. Notch is also a target of ΔNp63, and it has been shown that ΔNp63 upregulates Notch, which in turn regulates Hes1 to induce K1 expression during terminal differentiation.

Other proteins, including IRF6, stratifin, Ovol1, RIPK4 and KDF-1 have also been implicated in the spinous cell differentiation process. Mice deficient for transcription factor IRF6 show skin differentiation and proliferation defects and failure to develop spinous cells [36, 37]. Similarly, repeated epilation (*Er*) mice which have truncated stratifin show epidermis with differentiation defects [38], including failure to undergo terminal differentiation, separation of keratinocytes, and variation of spinous and granular layers of the epidermis [39]. Based on the Er model, it has been shown that stratifin regulates proliferation and differentiation by changing transcription factor YAP1 localization by binding to YAP1 and facilitating nuclear transcriptional function [40]. Intriguingly, a recently generated mouse model with Cre-mediated stratifin depletion did not show significant epidermis defects, suggesting that the Er model may not entirely recapitulate the effects of stratifin ablation [41]. Further experiments will be needed to resolve the contradiction between the two models and clarify the role of stratifin in skin development.

OVOL1 is a transcription factor expressed in epithelial tissue of the skin, kidneys and germinal epithelium [42]. OVOL1-dificeient mice shows epidermal defects and is caused by impaired inhibition of factor c-Myc [43]. OVOL1 is thought to directly regulate c-Myc, as *OVOL1* knockout mice have mitotic suprabasal keratinocytes with sustained upregulation of c-Myc. The OVOL1-c-Myc has also recently been implicated in squamous cell carcinoma progression [44].

RIPK4 belongs to the receptor-interacting protein (RIP) family kinase. RIPK4 knockout mice are perinatally lethal and display expanded spinous layer and granular layer defects. In addition, keratinocytes at the superficial layers expressed basal cell markers and retain the nucleus, indicating impaired differentiation [45]. In humans, germline mutations in RIPK4 have been linked to popliteal pterygium syndrome which is characterized by developmental defects in skin, craniofacial and genital systems [46, 47]. RIPK4 regulates epidermal differentiation through phosphorylation of the desmosome component, plakophilin-1 (PKP1) [48].

KDF-1 is a novel protein recently identified through forward genetic screening and found to be involved in skin differentiation [49]. Interestingly though, KDF-1 is a short protein without any recognizable domains, and its molecular interaction is unclear. *KDF-1* is also found to be mutated in a case of heritable ectodermal dysplasia, though the significance of this finding will need further evaluation [50].

3. Transition into the granular and cornified layers

The transition of spinous cells to granular cells is highly dependent on calcium signaling. Increased levels of Ca^{2+} leads to basal keratinocytes differentiation *in vitro* [51, 52]. This is reflected *in vivo* where Ca^{2+} gradient (lowest in basal layer and highest in cornified layer) is established in utero during development and maintained for homeostasis.

Molecular studies of skin development suggest that Ca^{2+} levels increases phosphatidylinositol metabolism and diacylglycerol levels, components of the PKC pathway [53]. PKC proteins are implicated to function between the transitions of spinous cells to granular cells. PKC proteins of serine/threonine kinases become fully functional upon phosphorylation [54]. PKC isoforms alpha and nu are expressed in keratinocytes, while only alpha is Ca^{2+}-sensitive [55]. PKC function is associated with decreased K1 and K10 expression and increased expression of granular cell markers: loricrin, filaggrin, transglutaminase [56]. However, it should be noted that none of the individual PKC isoform knockout mice display any *in vivo* skin developmental defects.

The extracellular calcium-sensing receptor (CaR) is specifically expressed in granular keratinocytes and regulates granular differentiation [57]. Loss of CaR leads to decreased loricrin and filaggrin expression. In contrast, CaR overexpression in basal cells leads to expanded spinous and granular cell layers with increased filaggrin expression. The homeobox transcription factor DLX3 is another protein implicated in granular differentiation. DLX3 is a downstream target of both p63 and PKC, and DLX3 expression induces keratinocyte proliferation and terminal differentiation [58, 59].

Granular cells subsequently undergo cornification and form the cornified envelope, and the terminally differentiated cells form a barrier of dead flattened cells at the skin surface. The transcription factor KLF4 is implicated in the cornification process. KLF4 is expressed in the spinous and granular layer and *KLF4* knockout mice display mutant granular and cornified layers while having unaffected basal and spinous layers [60]. Aberrant expression of KLF4 in the basal layer results in thinner, more differentiated epidermis. Microarray analysis of KLF4-dependent genes identified connexin-26 (GJB2) and SPRR proteins as

targets of KLF4. KLF4 directly inhibits connexin-26 [61]. GRHL3/GET1 is another transcription factor essential for epidermal development. While GRHL3 seems dispensable for adult epidermal differentiation, it regulates cell adhesion and lipid metabolism, and is required for epidermal barrier repair [62, 63].

IV. Adhesion proteins in skin development

To maintain proper function, skin development is not only regulated at the individual keratinocyte level, but requires coordination across multiple cells. This is achieved through a host of adhesion proteins which allows for communication between epithelial cells as well as cells and mesenchyme. The adhesion proteins include tight junctions, adherens junctions, and desmosomes, which play different regulatory roles in skin development (figure 2).

1. Tight junctions

Tight junctions are present in the granular layer epidermis and are essential for the barrier function of the skin. It is currently unknown whether these proteins play a role in epidermal development. Tight junction proteins primarily consist of the transmembrane proteins claudins and occludins, as well as the intracytoplasmic MGUK family proteins ZO-1, ZO-2 and ZO-3 (encoded by *TJP1*, *TJP2* and *TJP3*, respectively). Claudins and occludins are responsible for creating barriers through their intercellular interactions. On the other hand, the ZO proteins link tight junctions to adherens junctions and actin filaments. It is unclear whether these linking proteins act solely as cytoskeleton anchors or may have some regulatory role. Analysis has been limited because *TJP1* and *TJP2* knockout mice are embryonically lethal, whereas *TJP3* knock out mice appears to be phenotypically normal [64, 65].

2. Adherens junctions

Adherens junctions are formed by transmembrane cadherin family proteins which interact with other cadherins intercellularly and with catenin proteins intracellularly. In the epidermis, E-cadherins make up the majority of adherens junctions and play an important role in regulating skin development. Indeed, conditional ablation of E-cadherin in skin leads to hyperproliferation and defective differentiation of basal cells [66].

The regulatory effect of E-cadherin is mediated through the catenin proteins, p120, α-, and β-catenin. The main function of p120 is to stabilize the cadherin complex, although loss of p120 results in decreased level of adherens junctions, it is not required for epidermal development per se (Perez-Moreno et al., 2006). As mentioned in the previous section, β-catenin is a downstream effector of the Wnt singling pathway and can induce epidermal differentiation. Although the Wnt/β-catenin pathway is implicated primarily in hair follicle regeneration, recent evidence suggests that Wnt/β-catenin pathway can also contribute to epidermal homeostasis [67].

The primary role of α-catenin is to associate with actin filaments and maintain cell polarity, which is essential for epidermal development. Loss of α-catenin causes epidermal cells to lose polarity, become dissociated and hyperproliferative, and can subsequently undergo malignant transformation [29, 68]. It has been proposed that α-catenin may help maintain

cell polarity by localizing atypical PKC (aPKC) and PAR proteins [69], though the precise mechanisms is unclear. Additionally, α-catenin can also regulate YAP1 and control proliferation [70, 71].

3. Desmosomes

Desmosomes are adhesive intercellular junctions that associate with intermediate filaments to generate a coordinated three-dimensional intercellular scaffold and provide mechanical strength in epithelial cells [72–74]. The desmosome complex consists of three major components. The transmembrane desmosomal cadherins, intracellular armadillo domain-containing proteins, and plakins. While originally identified for its structural function, desmosome proteins are now recognized to also facilitate signaling pathways of various cellular processes, including epidermis development [74, 75].

The desmosomal cadherin desmoglein1 (dsg1) is highly expressed in the suprabasal layer and has been shown to regulate keratinocyte differentiation through suppressing EGFR signaling [76, 77]. Plakoglobin, the armadillo family protein within the desmosome complex, shares structural and functional similarities with β-catenin. Plakoglobin is also found in adherens junctions, and like β-catenin, it is expressed both at cell junction and nucleus and is a substrate of the APC/proteasome degradation machinery [78]. In plakoglobin-null epidermis, β-catenin can associate with desmosomes in place of plakoglobin, though it cannot completely restore the function of plakoglobin [79, 80]. Unlike β-catenin, the downstream signaling pathway of plakoglobin, as well as its possible role in epidermis development, is less well characterized. Plakoglobin has been shown to upregulate p53-target proteins including stratifin in squamous cell carcinoma, however whether this interaction is relevant in the context of skin development remains to be determined [81].

V. Spectraplakins in skin development

Spectraplakins are large cytoskeletal proteins that bind to and coordinate other cytoskeletal proteins. Structurally, spectraplakins contain an actin-binding domain in the N-terminal domain, a plakin domain that link intermediate filaments and adhesive proteins, EF-hand and GAR domains at the C-terminal which interacts with microtubules, and spectrin repeats that constitute spacer regions in between. Although the mammalian spectraplakins are encoded by only two genes, *MACF1* (MT and actin crosslinking factor 1, also known as *ACF7*) and *BPAG1* (Bullous Pemphigoid Antigen 1)/*DST* (Dystonin), they generate a large variety of isoforms through differential promoter regulation and alternative splicing, allowing them to coordinate a variety of biological processes in different tissues[82] (figure 2).

1. BPAG1

BPAG1 consists of several isoforms, including BPAG1e, BPAG1a (also known as BPAG1n) and BPAG1b. BPAG1e is the major epithelial variant and expressed in basal keratinocytes, whereas BPAG1a and BPAG1b are expressed in nerves and muscles, respectively. BPAG1e is initially identified as an autoantigen in patients with the skin blistering disease bullous

pemphigoid, and hence the designation bullous pemphigoid antigen1 [83]. BPAG1 deletion in mice epidermis causes the base of the basal cells to become fragile, the cells will dissociate upon pressure, resulting in easy blistering and poor wound healing [84]. The skin defects caused by BPAG1 loss likely reflect the role of BPAG1e in the hemidesmosome, a junctional protein that links basal keratinocytes with the basement membrane. Indeed, BPAG1e, together with plectin, are intracellular components of the hemidesmosome and crosslink intermediate filaments K5 and K14 to the transmembrane hemidesomosome components, BPAG2 and $\alpha_6\beta_4$ integrin [4, 85]. While BPAG1e provide mechanical strength by linking the cytoskeletons, its interaction between BPAG2 and $\alpha_6\beta_4$ integrins is also important for regulating keratinocyte polarity and migration, which is mediated through Rac1 signaling [86].

Recently, two patients presented clinically with trauma-induced skin fragility have been found to have truncating mutations in BPAG1e [87]. Interestingly, the mutant keratinocytes isolated from the patients showed adhesion defects but enhanced migration [88]. The contrasting roles of BPAG1e on keratinocyte migration may be due to the different assays used, i.e. *in vivo* random migration versus *in vivo* wound healing, which implied that the interaction between keratinocytes and the basement membrane may also provide important cues during wound healing. Another possibility is that the truncated BPAG1e is expressed in the patient's keratinocytes and harbor aberrant functions that differentiate them from BPAG1e knockdown skin [88]. Further studies will be needed to delineate the role of BPAG1e in regulating keratinocyte migration and skin development.

2. MACF1

Like BPAG1, MACF1 also consists of several isoforms expressed in a wide range of cell types, including neurons, intestinal epithelial cells, cardiomyocytes, and basal keratinocytes. However, instead of linking intermediate filaments, MACF1 coordinates microtubule and actin dynamics. Several major functions of MACF1 have been identified so far. First, MACF1 acts as a microtubule plus-end trafficking protein and mediates cortical interaction by linking the microtubule with actin and the cell membrane [89]. Second, MACF1 helps stabilize microtubules, and cells lacking MACF1 show unstable, long microtubules with skewed trajectories [90]. Finally, MACF1 interacts with the trans-Golgi network protein, p230, facilitating vesicle transport along the microtubule and actin cytoskeleton [91].

In the epidermis, the MACF1 is essential in maintaining microtubule and focal adhesion dynamics during keratinocyte migration. Indeed, conditional knockout of *MACF1* in mice epidermis results in impaired wound healing response. At the cellular level, *MACF1* deletion in keratinocytes prevents the microtubules from converging at the peripheral focal adhesion, and cell migration capability is significantly impaired [92]. It has recently been shown that the interaction between MACF1 and microtubules is regulated by two phosphorylation events. First, the microtubule-binding domain of MACF1 can be phosphorylated by GSK3, and the modification results in uncoupling of MACF1 to microtubules. During skin wound healing, Wnt signaling is activated and inhibits GSK3, allowing MACF1 to associate with microtubules and promote stem cell migration [93]. In addition to the microtubule domain, a tyrosine residue within the CH domain can also be phosphorylated by the Src/FAK complex

at focal adhesions [94]. Phosphorylation at the CH domain changes MACF1 structurally from a closed conformation to an extended conformation, which is required for its interaction with f-actin [95].

Interestingly, MACF1 has also been implicated in Wnt signaling pathway during mouse embryonic development. Indeed, embryos that lack MACF1 show defects in the primitive streak and mesoderm, which is similar to the defects observed when Wnt3 or Wnt co-receptors LPR5 and LPR6 are lost [96, 97]. MACF1 may positively regulate Wnt signaling by interacting with axin, traslocating it to the Wnt receptor, and allowing β-catenin to stabilize [98]. Since Wnt signaling also plays a major role in epidermis differentiation, the natural question is whether MACF1, in addition to regulating keratinocyte migration during wound healing, also plays a role in skin development. However, the role could be indirect or subtle, as mice with conditional MACF1 knockout in K14 keratinocytes appears to have normal skin when uninjured [92].

VI. Conclusion

The past decade has seen many exciting new insights into skin development. New models have been proposed along with better understanding of the underlying molecular mechanisms. In particular, advanced knowledge in cytoskeletal proteins and spectraplakins have opened up new perspectives on the subject. Future efforts on strengthening the link between cell structure and signaling, for example through conditional knockout models or lineage tracing experiments, will allow for a more comprehensive view over the complex spatial and temporal regulations involved in this vital process.

Acknowledgments

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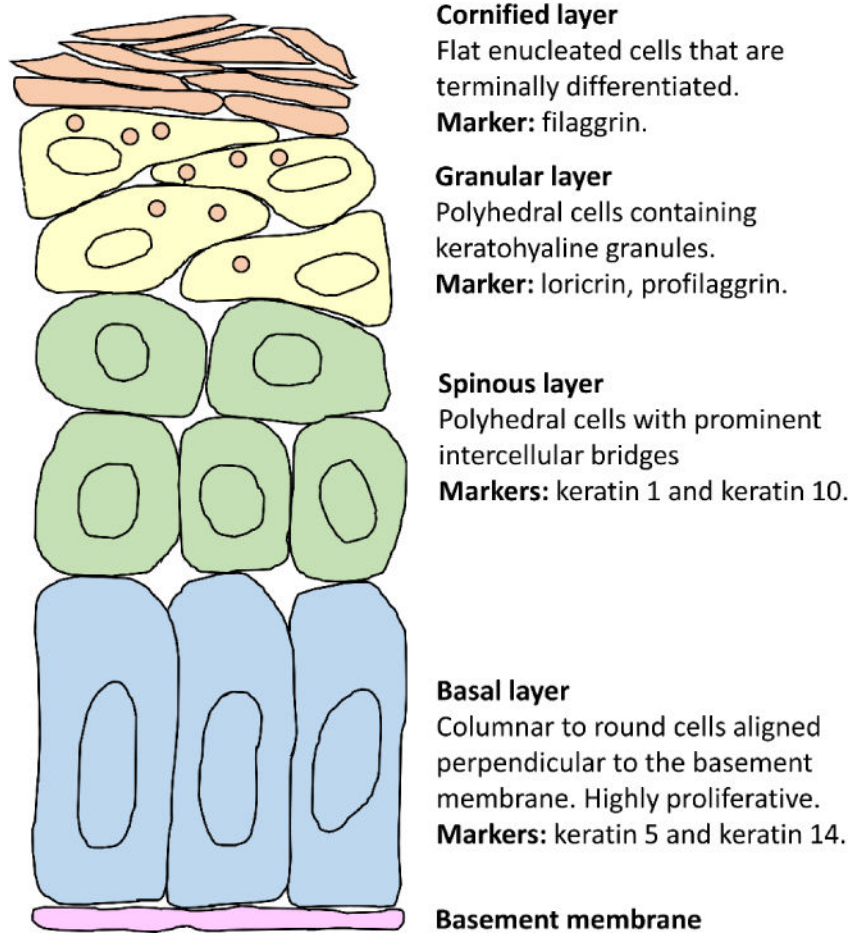
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Cornified layer
Flat enucleated cells that are terminally differentiated.
Marker: filaggrin.

Granular layer
Polyhedral cells containing keratohyaline granules.
Marker: loricrin, profilaggrin.

Spinous layer
Polyhedral cells with prominent intercellular bridges
Markers: keratin 1 and keratin 10.

Basal layer
Columnar to round cells aligned perpendicular to the basement membrane. Highly proliferative.
Markers: keratin 5 and keratin 14.

Basement membrane

Figure 1. Epidermis layers and skin differentiation
The cross section of the epidermis and the basement membrane underneath is depicted. Basal keratinocytes are attached to the basement membrane and are the only proliferative cells in normal skin epidermis. When basal cells are committed to differentiation, they move away from the basement membrane and generate three layers of differentiated cells: the spinous layer, granular layer and cornified layer. Key morphological features and protein markers of each layer are shown in the right.
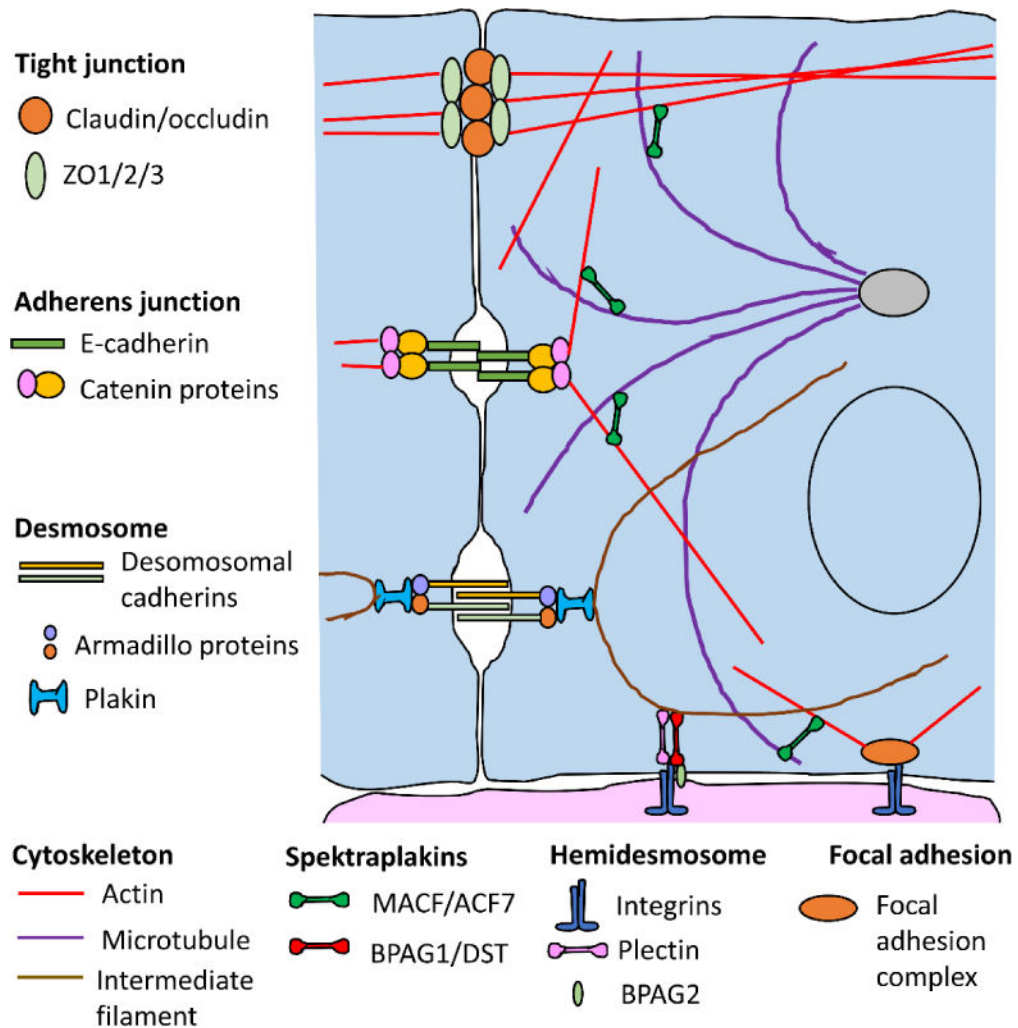
Figure 2. Cytoskeletal proteins in keratinocytes
Schematic view of a basal keratinocyte attached to the basement membrane, with key cytoskeletal proteins depicted. Cytoskeletal proteins not only provide mechanical strength, but may also play important regulatory roles. Tight junctions seal the intercellular space between neighboring cells and also interact with actin filaments in the cell. Adherens junction interact with actin through α- and β-catenins and regulates differentiation through Wnt/β-catenin. Desmosomes interacts with keratin filaments and also regulate keratinocyte differentiation. The two spetraplakins, BPAG1 and ACF7 are both important for cell motility and establishing polarity. BPAG1 is part of the hemidesmosome and crosslinks it to keratin filaments. ACF7 crosslinks microtubules and actin, and is essential for focal adhesion dynamics during cell migration.