ELSEVIER

Transcriptional control of epidermal specification and differentiation

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Recent experiments reveal the role of transcription factors in integrating upstream signals to execute specification and differentiation of epidermal cells. Based on the skin phenotype observed with misregulation of transcription factors such as p63, c-Myc, RelA, pRb, Klf4 and others, their function in controlling proliferation and differentiation is dissected. Understanding the pathways regulated by these factors and their coordinate interactions remains a challenge for the future.

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Abbreviations
IFE interfollicular epidermis
K keratin
SC stem cell
TA transit-amplifying
TF transcription factor

Introduction

Recent experiments have provided extensive new insight into the regulation of proliferation, differentiation, cell-fate determination, and pattern formation of mammalian epidermis. The epidermis is established *in utero* and replenished from a pool of adult stem cells (SCs), which give rise to interfollicular epidermis (IFE), hair follicles, and sebaceous and sweat glands. Perturbations to the normal balance of differentiation and proliferation can result in skin disorders including cancer. Therefore, understanding the molecular and genetic control of this process is of immense medical and pharmaceutical relevance.

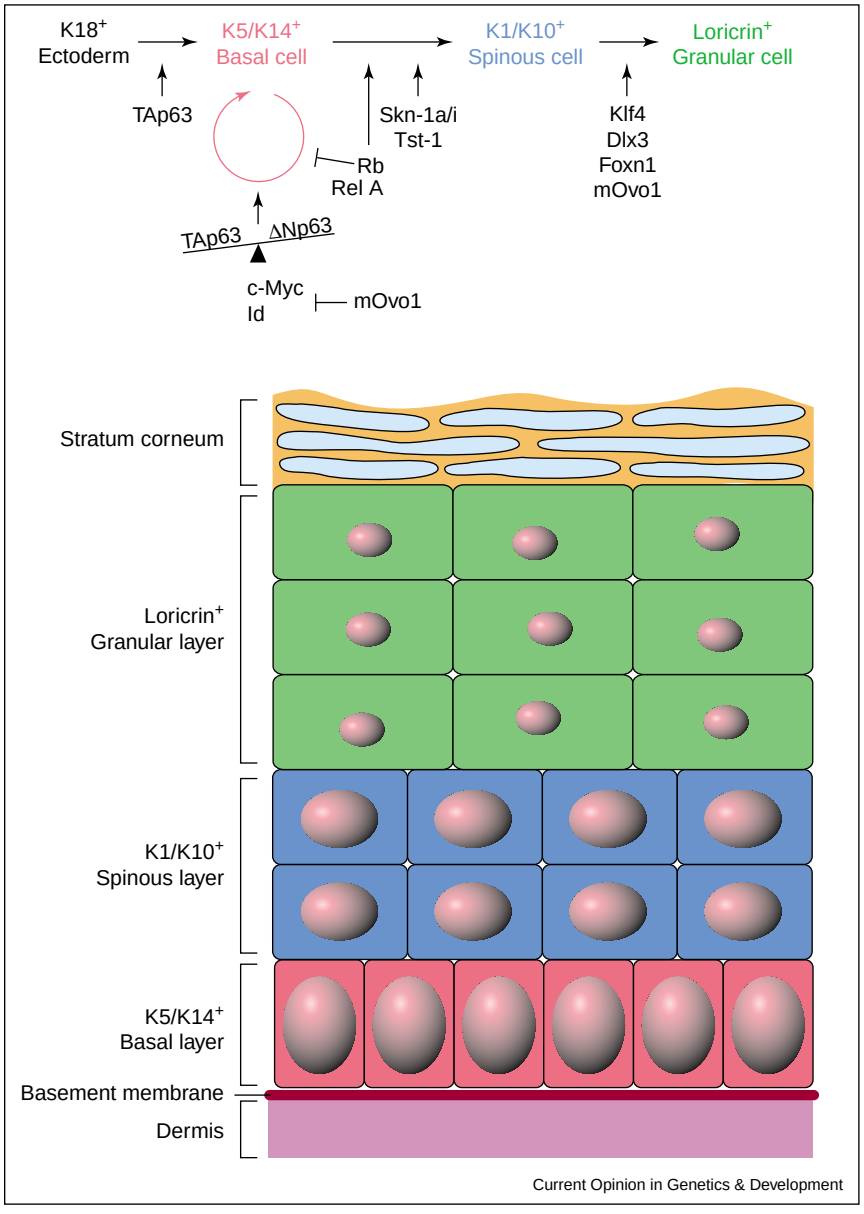
Regulation of gene expression is at the heart of all development and differentiation processes. Transcription factors (TFs) integrate and interpret signals from upstream developmental/growth factor signaling pathways in a coordinate and complex fashion to execute downstream differentiation/morphogenetic events. Here, we review recent progress made on understanding the role of TFs in regulating epidermal SC maintenance and the orderly progression of interfollicular terminal differentiation.

Stem cell maintenance and proliferation

An emerging topic of intense focus has been defining and characterizing the epidermal SCs (for reviews, see [1,2]). Recent transcriptional profiling studies provide fodder for years of future experimentation and insights into how gene expression might be regulated during SC renewal and differentiation [3••,4••]. Key regulators of SC maintenance and epidermal proliferation include p63, c-Myc, Gli and Id TFs (Figure 1).

The essential role of p63, a homolog of the p53 tumor suppressor, in skin development was revealed by two independent studies of p63-deficient mice. These mice lack stratified epidermis, producing a disorganized single-layered surface epithelium that is negative for epidermal markers such as keratin (K) 5 and 14 [5,6]. Although the mutant phenotypes in these two studies are similar, the interpretation of p63's mechanism remains controversial. McKeon's group [5] proposed that p63 is involved in SC maintenance but not epidermal maturation, as sporadic spots of cells positive for late-epidermal differentiation markers were observed. Roop's group [6,7••] suggested that p63 is a determining factor of stratification, as they did not detect the expression of any early or late epidermal differentiation markers in the p63 mutants. Although these two mechanisms are not mutually exclusive, that p63 is an epidermal master regulator maintains strong experimental support. Complicating the dissection of the specific role of p63 are the splicing variants that exist and antagonize each other's functions (reviewed in [8]). Overexpressing ΔNp63 in cells already committed to terminal differentiation does not affect the overall proliferation of the transgenic epidermis [9], suggesting that ΔNp63 maintains the proliferative state rather than promoting it. Complementary experiments show that overexpressing ΔNp63 in cultured keratinocytes blocks Ca^{2+}-induced growth arrest and terminal differentiation [10]. Conversely, ectopic expression of TAp63, but not ΔNp63, converts a normal K18-positive simple epithelium into a K5/K14-expressing stratified epithelium, suggesting that TAp63 is sufficient to drive epidermal specification [7••]. Based on the embryonic expression pattern and

Figure 1



TFs regulating specification, maintenance and differentiation of interfollicular epidermis. Shown at top are the TFs that regulate this process with the stages of differentiation color coded to match the schematic diagram of epidermis, shown below.

these functional data, Roop hypothesizes that TAp63 specifies an epidermal epithelia with the subsequent balance between the levels of ΔNp63 and TAp63 determining the proliferative state of the keratinocyte.

The protooncogene c-Myc has traditionally been viewed as inducing proliferation by controlling the G_1–S cell cycle transition. Consistent with this, c-Myc epidermal expression is confined to the proliferating basal cells [11]. However, increasing expression of c-Myc in cultured keratinocytes promotes terminal differentiation and causes a progressive reduction in growth [12]. This seemingly counter-intuitive finding was reconciled with the model of a selective action of c-Myc to drive SCs into transit-amplifying (TA) cells, thereby initiating the differentiation pathway. K14 promoter driving c-Myc transgenics, with elevated expression in the SCs, have severe skin defects including hyperproliferation, hair loss, and defective wound healing, indicative of an excessive cellular expansion at the cost of an SC reservoir [13,14]. Misexpression of c-Myc in the postmitotic suprabasal cells leads to ectopic proliferation, further underscoring

c-Myc as a key positive regulator of epidermal proliferation [15,16]. Possible mechanisms to explain the physiological role of c-Myc in the conversion of SC to TA cell include a direct involvement in cell–substratum adhesiveness because c-Myc activation decreases integrin levels [13,17••]. Ectopic c-Myc expression stimulates IFE and sebaceous gland fate at the expense of hair fate, raising the possibility that c-Myc regulates lineage specification of the epidermal SCs. Watt's group [17••] presents an intriguing idea that epidermal lineage specification is subject to niche regulation and that hair fate is compromised in the K14–c-Myc transgenic skin because precursor cells fail to migrate to their designated location and receive the proper stimuli to differentiate further.

The Shh signaling pathway also plays a positive role in epidermal proliferation by opposing growth arrest [18]. Gli2, a member of the Gli family of zinc finger TFs, is the main mediator of Shh signaling in skin, while Gli1 appears to play only a potentiating role. Overexpression of Gli2 in the proliferating cells of the epidermis leads to basal cell carcinomas in transgenic mice [19]. Conversely, $Gli2^{-/-}$ mice phenocopy the $Shh^{-/-}$ mice in their reduced proliferation in the hair follicles [20]. No apparent proliferation defect was noted in the IFE of $Gli2^{-/-}$ mice, although further *in vitro* experiments may still reveal a role.

The Id family of proteins are positive regulators of proliferation and negative regulators of differentiation in multiple tissue/cell lineages. Three (Id1–3) of the four Id genes are expressed in proliferating epidermal cells [21]. Overexpression of Id1 in keratinocytes leads to increased proliferative potential and compromised terminal differentiation [22,23]. A quarter of the Id2 knockout mice die neonatally, a key feature of mice with a dysfunctional epidermal permeability barrier, but these mice were not characterized for possible epidermal phenotypes [24]. Id proteins function in a dominant negative fashion to interfere with lineage-specific basic helix-loop-helix (bHLH) TFs (e.g. MyoD in muscle), but curiously no epidermal-restricted bHLH protein has yet been identified. Given the recent identification of Id as epidermal SC-enriched markers [3••,4••], the function of Id genes and their associated bHLH factors in epidermal differentiation should be revisited.

Initiation and progression of terminal differentiation

Terminal differentiation of the IFE can be divided arbitrarily into three steps: growth arrest, initiation of differentiation, and terminal differentiation. Not all studies offer this resolution because strong homeotic control of skin often leads to concomitant defects in growth and differentiation. Where a step-specific function is suggested, clues have often come from other systems or analysis of putative downstream targets. Here we discuss TFs that are involved in driving the differentiation process (Figure 1).

The well-demonstrated role of the pRb family of transcriptional regulators, pRb, p107 and p130, in growth suppression inspired an investigation of these proteins in skin. To circumvent the gastrulation defect of pRb null embryos, pRb was specifically ablated in the epidermis [25••,26••]. These mice exhibit increased proliferation in both basal and suprabasal layers along with a loss in the label-retaining SC population. The differentiation program is altered with aberrant co-expression of K5 and K10. *In vitro*, pRb-deficient keratinocytes re-enter the cell cycle after undergoing differentiation. The two studies separately show that the phenotypes are augmented either with the loss of p107 or the expression of HPV E7 oncogene. Reporter assays indicate that pRb may be acting through E2F whose role in promoting epidermal proliferation and differentiation in the epidermis has been studied independently. Although a single deletion of either p107 or 130 had no apparent effect on skin development, p107/p130 double knockout mice displayed histological and biochemical skin alterations only in the late stage of terminal differentiation [27]. Increased proliferation was observed in grafted skin but not analyzed in embryonic skin, precluding any conclusion about whether this is either a direct or secondary effect.

Initial molecular studies demonstrated that inhibiting NF-κB signaling either by pharmacologic agents or IκBα overexpression led to epidermal hyperplasia [28]. Blocking NF-κB signaling while activating Ras leads to a bypass of the cell-cycle arrest, generating malignant epidermal tissue resembling squamous cell carcinoma [29]. With all of this tantalizing functional evidence, the mechanism by which NF-κB regulates the balance of epidermal differentiation, proliferation, and apoptosis appears more complex with every experiment. All five members of the NF-κB family (c-rel, RelA, RelB, p50 and p52) are expressed in epidermis, and since they function as hetero- and homodimers, this leaves multiple possibilities for functional redundancy and regulation. Mice deficient in four of the five NF-κB subunits develop immune deficiencies but lack epidermal abnormalities. RelA/p65 null mice die *in utero* too early to examine the epidermal homeostatic defects. Finally Khavari's group has done the careful analysis of the intrinsic epidermal defect by grafting E15.5 $RelA^{-/-}$skin. They were rewarded for their efforts by the discovery that $RelA^{-/-}$ epidermis displays hyperproliferation with normal differentiation, independent of immune response [30•]. Analyses of the epidermal phenotype of mice deficient in IκB kinase α, a key regulator of NF-κB activation, has brought investigators into a new realm. These mice die shortly after birth because of a severe cell-autonomous defect in epidermal terminal differentiation, which is independent of IκB kinase-α's protein kinase activity and NF-κB

signaling [31–33]. Grafting and culturing with conditioned media led Karin's group to speculate that IκB kinase-α is necessary for keratinocytes to produce a differentiation-inducing protein, that has yet to be purified biochemically or identified genetically [34]. Intriguingly, IκB kinase-α contains a nuclear localization domain that is independent of kinase activity and essential to induce differentiation of keratinocytes [35].

The zinc finger protein mOvo1 is expressed in differentiating epidermal cells and its expression is regulated by the β-catenin/LEF1 transcriptional complexes [36,37]. Recent studies uncovered a strain-specific requirement for mOvo1 in epidermal development and differentiation (A Teng, M Nair, X Dai, in preparation). Developing mutant epidermis displays increased proliferation during late embyrogenesis, accompanied by up-regulation of c-Myc and Id2, as well as morphological and biochemical changes in the late-terminal differentiation. In reporter assays, mOvo1 represses the activity of c-Myc, Id2, and loricrin promoters, suggesting a cell-autonomous coordinate regulation of growth arrest and terminal differentiation.

Three genes encoding the Oct class of POU domain TFs are expressed in the epidermis: Oct-1, Tst-1 (Oct-6/SCIP), and Skn-1a/i (Epoc/Oct-11). Overexpression of the epidermis-restricted Skn-1a/i in keratinocytes facilitates differentiation, and ablation of both Skn-1a/i and Tst-1 in mice leads to ectopic expression of K14 in suprabasal cells [38]. Possible mechanisms of action include cell-autonomous repression of basal-specific keratins, and activation of early and late stage differentiation genes.

Functional AP-2 and AP-1 binding sites have been defined in the promoters of many epidermal structural proteins. Multiple members of these TF families are expressed in the skin with unique and overlapping patterns of expression. Bioinformatic analysis has identified a fifth (and probably final) member of the AP-2 family with skin-specific expression [39]. Targeted ablations of individual AP-2 and AP-1 family members have failed to elucidate any specific role for a factor in skin development and differentiation, either because of an earlier embryonic lethality or potential functional redundancy. Mice with epidermal specific targeted ablation of AP-1 family member c-Jun show defects in EGF signaling [40,41]. The c-Jun null epidermis was unremarkable but insights came from culturing primary keratinocytes, which showed reduced proliferation and altered differentiation caused by the loss of paracrine factors provided by adjacent dermal cells of skin [40]. Analysis of epidermal-specific ablation and dominant negative transgenic models may help to define the role of additional AP-1 and AP-2 family members in epidermal homeostasis.

Three TFs — Klf4, Dlx3 and Foxn1/nude — are expressed predominantly in the suprabasal layers of the epidermis and direct distinct aspects of terminal differentiation. Klf4 is both necessary and sufficient (given a field of competence) to establish a functional permeability barrier because targeted ablation results in a loss of barrier and ectopic expression earlier in development accelerates normal barrier acquisition in a dose-dependent fashion [42••,43]. Structural protein components of the cornified envelope are misexpressed in the $Klf4^{-/-}$mutants [43]. A more extensive search for genes differentially expressed in both the Klf4 transgenic and deficient mice may yield more direct targets. Ectopic expression of Dlx3 in the basal proliferating cells results in improper transcription of terminal differentiation markers in this layer and a greatly reduced level of proliferation. Analysis of the necessary function of Dlx3 in the epidermis has been hampered by an earlier essential role in placental development and needs to be revisited with an epidermal-specific deletion [44]. Although the most prominent feature of a deletion of Foxn1/nude is the lack of hair (and thymus), deficient keratinocytes have an increased propensity to differentiate even under proliferative conditions [45]. Over-expression of Foxn1 in differentiated keratinocytes stimulates the transcription of early differentiation markers and suppresses the expression of later-stage markers [46]. Although Klf4, Dlx3 and Foxn1 are all involved in the terminal differentiation process, modulating their expression gives rise to distinct alterations in gene expression.

All-*trans* retinoic acid, acting through retinoic acid receptors and retinoid X receptors, has a dramatic effect on the regulation of epidermal proliferation and differentiation. Again, dissection of the function of these nuclear receptor TFs in skin was challenged by embryonic lethalities and functional redundancies between family members. Compound epidermal specific knockouts have revealed defects in hair cycling and wound healing, but only subtle defects in IFE proliferation and expression of differentiation markers [47–49]. Deficiencies of either glucocorticoid or peroxisome proliferator-activated receptor α signaling result in delays of the late stages of epidermal maturation but compensatory mechanisms are evoked [50,51].

Conclusions and perspectives

A comprehensive picture of the genetic pathways governing epidermal differentiation remains in infant form. Two major advantages of this system are the ability to move between *in vitro* and *in vivo* and to modulate gene expression with promoters specific to the different layers of the epidermis. Looking into the future, sophisticated genetic approaches such as crossing mutants of different components of a particular pathway, analysis of tissue-specific, isoform-specific, and double even triple knockouts, will be necessary to illustrate the function of various

TFs. One wide-open issue is whether as yet unidentified lineage-specific factors act together with common TFs to govern gene expression or whether simple modular combinatorial expression of common TFs imbues specificity.

Ultimately, we must recognize that we have not identified and characterized all (or perhaps even many) of the TFs that regulate fate specification and terminal differentiation of keratinocytes. However, we are in a period of tremendous gene discovery that has very recently yielded many new targets of study.

Two complementary strategies are emerging to identify and prioritize the study of new keratinocyte transcriptional regulators: transcriptional profiling and genome-wide approaches to identify putative regulatory elements. Independently, Cotsarelis's and Fuchs's groups published *tour de force* purification of hair follicle and epidermal SCs and queried their transcriptional profile [3••,4••]. As microarray technology improves, both with specificity and number of targets, these types of experiments will define candidate regulators of epidermal specification and terminal differentiation. Relative expression levels or knowledge of the TF's function in other systems can be used to prioritize the genes for future experimental study. Alternatively, gene selection can be based on the prevalence of the TF's cognate binding sites upstream of keratinocyte specific structural or regulatory proteins. Computational methods to identify conserved TF binding sites between evolutionarily diverged vertebrates may aid in predicting functional sites [52]. One would predict that TF binding sites would lie within DNaseI hypersensitive sites. Although identifying hypersensitive sites is a laborious method when applied to each individual gene, novel approaches have been reported recently on how to clone hypersensitive sites from specific cell types, greatly increasing efficiency [53,54]. Conserved non-coding sequences that contain epidermal-specific DNaseI hypersensitive sites may predict key regulatory elements. Major advances have also been made to predict direct targets of a TF by immunoprecipitating and querying the chromatin pulled down with antibodies directed against the specific TF. This technique has the potential to identify individual targets and to construct transcriptional regulatory networks by examining the genome-wide profile of sequences immunoprecipitated by multiple TFs [55].

An intriguing unexplored feature of keratinocyte biology is that many of the structural proteins are arranged in clusters — for example, the acidic keratins, basic keratins and epidermal differentiation complex on human chromosome 17,12,1 respectively. Not only is it important to understand how individual genes within these clusters are regulated, but it is fascinating to speculate whether the genes are tandemly arrayed to enable coordinate regulation.

In summary, experiments have demonstrated that TFs can regulate epidermal specification and differentiation. Beyond discovering additional TFs involved in these processes, the future lies in elucidating the pathways regulated by key factors.

Update

Gerondakis's group [56•] circumvents the embryonic lethality of RelA and c-*rel* deficient mice by placing them on a tumor necrosis factor alpha deficient background. The compound c-$rel^{-/-}$ $RelA^{-/-}$ epidermis is thinner with more proliferative basal cells, which fail to form colonies *in vitro*. The c-$rel^{-/-}$ $RelA^{-/-}$ neonates do not survive, and the grafted skin is relatively normal although immune responsive hyperproliferation is observed.

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Fuchs's group used the bipartite tetracycline transgenic system to pulse label the slow cycling SCs. The histones of K5 positive cells were GFP labelled transiently and then chased for 1 to 4 months after which time only the SCs retained high GFP signal. This group isolated GFP^{high}, GFP^{low} and $\beta4integrin^{+}$ GFP^{lower} cells and obtained transcriptional profiles for the three populations. Approximately one-third of the same genes in the list of 'upregulated in stem cells' were shared between this study and [4••] and many genes were shared between epidermal and other lineage SCs.

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Cotsarelis's group characterized cells expressing the K15 promoter as present in epidermal SCs and capable of generating all epidermal cell types. Driving EGFP from this promoter, Cotsarelis's group isolated GFP-bright SCs and GFP-negative basal keratinocytes. Transciptional profiling identified genes, including TFs, differentially expressed in SCs. Approximately one-third of the same genes in the list of 'upregulated in stem cells' were shared between this study and [3••].

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Vooijs's laboratory continued their elegant analysis of the role of pRb family members by making an epidermal-specific targeted ablation of pRb. pRb-deficient epidermis has increased basal and suprabasal proliferation, fewer label retaining cells (i.e. depletion of the SC population), and an altered program of differentiation. After induction of terminal differentiation, pRb-deficient keratinocytes can be stimulated to re-enter the cell cycle. In a dose-dependent fashion, loss of p107 exacerbates the phenotypes observed with pRb deficiency.

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