p63 Regulates Human Keratinocyte Proliferation via MYC-regulated Gene Network and Differentiation Commitment through Cell Adhesion-related Gene Network

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Background: Although the role of p63 in skin development is well known, its action in regulating human adult epidermal homeostasis is not completely elucidated.

Results: p63 regulates keratinocyte proliferation via a MYC-regulated gene network and differentiation through a cell adhesion-related gene network.

Conclusion: The balance between these two networks may control epidermal homeostasis.

Significance: This is important to understand epithelial carcinogenesis.

Although p63 and MYC are important in the control of epidermal homeostasis, the underlying molecular mechanisms governing keratinocyte proliferation or differentiation downstream of these two genes are not completely understood. By analyzing the transcriptional changes and phenotypic consequences of the loss of either p63 or MYC in human developmentally mature keratinocytes, we have characterized the networks acting downstream of these two genes to control epidermal homeostasis. We show that p63 is required to maintain growth and to commit to differentiation by two distinct mechanisms. Knockdown of p63 led to down-regulation of MYC via the Wnt/β-catenin and Notch signaling pathways and in turn reduced keratinocyte proliferation. We demonstrate that a p63-controlled keratinocyte cell fate network is essential to induce the onset of keratinocyte differentiation. This network contains several secreted proteins involved in cell migration/adhesion, including fibronectin 1 (FN1), interleukin-1β (IL1B), cysteine-rich protein 61 (CYR61), and jagged-1 (JAG1), that act downstream of p63 as key effectors to trigger differentiation. Our results characterized for the first time a connection between p63 and MYC and a cell adhesion-related network that controls differentiation. Furthermore, we show that the balance between the MYC-controlled cell cycle progression network and the p63-controlled cell adhesion-related network could dictate skin cell fate.

The outer layer of human skin, the epidermis, is a self-renewing, stratified squamous epithelial tissue. In adult tissue, the maintenance of epidermal homeostasis depends on an exquisitely regulated balance between keratinocyte proliferation and differentiation (1, 2). However, the specific molecular mechanisms governing each of these processes are not completely understood.

p63, a member of the p53 tumor suppressor gene family, is known as a key regulator of epidermis development and keratinocyte differentiation. Striking developmental defects have been discovered during embryonic development in p63-knockout mice (3, 4). In addition, p63 is required for the maintenance of proliferative potential in epithelial stem cells (3, 5, 6), epithelial lineage commitment (4), differentiation of keratinocytes (7), and epithelial cell adhesion and survival (8). Dual roles of p63 in the initiation of epithelial stratification and maintenance of stem cell proliferative potential have been established (6). Because simultaneous p63 and p53 knockdown rescued the cell proliferation defect of p63 knockdown alone but failed to restore differentiation, the roles of p63 in proliferation and differentiation of developmentally mature keratinocytes appear to be distinct (7).

In vivo, as keratinocytes commit to differentiation, they detach from the basal layer and migrate outward into a spinous layer, accompanied by the expression of early differentiation markers keratin 1 (K1) and keratin 10 (K10) (9). The effects of p63 in keratinocyte differentiation have been investigated by gain and loss of function experiments. Ectopic expression of p63 induces the early marker K1 but suppresses the expression of late differentiation markers, such as loricrin and filagrin (10). Loss of p63 inhibited both stratification and differentiation in skin organotypic culture (7). However, little is known about key genes acting downstream of p63 to regulate the dynamic equilibrium of keratinocytes differentiation and proliferation as well as epidermal homeostasis (11).

Similar to p63, the MYC oncogene is predominantly expressed in the basal cell layers of epidermis and is absent in suprabasal layers (12, 13). MYC plays a vital role not only in

1 This article contains supplemental Tables S1–S3 and Figs. S1–S6. All of the microarray data used in this study have been deposited in the NCBI Gene Expression Omnibus under accession number GSE17394.
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keratinocyte proliferation (14–16) but also in accelerating the differentiation of epidermal stem cells (17–20). Specifically, MYC activation leads to epidermal stem cell proliferation, and the sustained elevated expression of MYC in turn stimulates the proliferating stem cells to enter the transit-amplifying compartment, thereby initiating terminal differentiation (21). Although p63 and MYC have been independently demonstrated to be key regulators in the dynamic equilibrium between proliferation and differentiation in epidermal stem cells, no connection between p63 and MYC has been reported so far. The genetic basis underlying the processes regulated by p63 and MYC in mature keratinocytes, as well as the nature of their downstream effectors, remains mostly unknown.

We hypothesized that the regulation of the equilibrium between proliferation and differentiation of keratinocytes could rely on gene networks acting downstream of p63 and MYC rather than on a single gene. To this end, we compared phenotypic outcomes and global gene expression profiles in differentiating human keratinocytes transiently depleted of either p63 or MYC. We demonstrate that p63 regulates the proliferation and commitment to differentiation of human mature keratinocytes by two independent mechanisms. p63 is necessary for the proper expression of MYC and in turn controls keratinocyte proliferation by regulating the expression of MYC via the Wnt/β-catenin and Notch signaling pathways. Moreover, p63 regulates human keratinocyte differentiation by controlling a network of genes implicated in cell migration and adhesion.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Primary human keratinocytes were isolated from human skin biopsy and cultured in KGM2 medium (Clonetics) on flasks coated with collagen type I (Falcon Bio-technologies). HaCaT cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 105 units/liter penicillin, 50 mg/liter streptomycin, and 2 mM GlutaMax (Invitrogen). Human-25K, Réseau National des Génopoles, France) (25). RNA was isolated, amplified, labeled, and hybridized following a published protocol (25). For each condition, three independent biological replicates were obtained, and the knockdown of corresponding genes was verified before RNA extraction (supplemental Fig. S2). Each biological replicate was hybridized to the arrays in four replicates using a dye swap strategy. Slides were scanned with an Agilent G2565AA Microarray Scanner (Agilent Technologies). Data analyses, including intensity-dependent LOWESS normalization of raw data and differential analysis, were performed with GeneSpring 7.0 software (Silicon Genetics). Differentially expressed genes were identified using analysis of variance (p value corrected with Benjamini and Hochberg false discovery rate), and only genes with a -fold change of ≥1.2 and p < 0.01 were used for further analysis.

**Gene pathway and genetic network analyses were performed by Ingenuity Pathway Analysis (IPA) (Ingenuity Systems). Ontologies attached to each gene were used to classify altered genes according to main biological themes. The following criteria enter into generating the networks in the Ingenuity Pathways Analysis application. 1) We have designated the molecules of interest in the analysis parameters before running the analysis. Molecules that (a) meet the cut-off and/or filter criteria and (b) interact with other molecules in Ingenuity’s Knowledge Base are identified as focus molecules (also called “network eli-

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3 The abbreviations used are: NICD, Notch intracellular domain; EdU, 5-ethyl-2′-deoxyuridine; KCF, keratinocyte cell fate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol; qRT-PCR, quantitative RT-PCR; TF, transcription factor; iPS, induced pluripotent stem cell(s).
gible molecules”). Focus molecules serve as the “seeds,” or focal points, for generating networks. 2) Networks are preferentially enriched for focus molecules with the most extensive interactions and for which interactions are specific with the other molecules in the network (rather than molecules that are promiscuous, those that interact with a broad selection of molecules throughout the Ingenuity knowledge base). 3) Additional non-focus molecules from the data set and from the Ingenuity knowledge base are then recruited and added to the growing networks. 4) Networks are scored for the likelihood of finding the focus molecule(s) in that given network. The higher the score, the lower the probability that you would find the focus molecule(s) you see in a given network by random chance. 5) In the current version of the application, there is a cut-off of 35 molecules/network to keep networks to a usable size.

mRNA Expression Analysis—RNA was extracted with an RNeasy mini kit (Qiagen). For real-time quantitative PCR, 2 µg of RNA was reverse-transcribed in a total volume of 20 µl with the SuperScript II RNase H reverse transcriptase system (Invitrogen) and random primers according the manufacturer’s instructions. Reverse transcription reactions were diluted to 500 µl of water, and 5 µl of the diluted cDNA was used for each quantitative PCR. Quantitative PCR was carried out with a Platinum quantitative PCR SuperMix-UDG Kit (Invitrogen) using an ABI 7500 fast real-time PCR system (Applied Biosystems). All experiments were run in triplicate, and the results were normalized to 18 S rRNA expression. Primer sequences are listed in supplemental Table S1.

Immunoblotting—Total cellular protein was extracted using radioimmune precipitation assay buffer supplemented with protease inhibitor mixture (Roche Applied Science) and 1 mM sodium orthovanadate. After quantification with a BCA protein assay kit (Pierce), 20 µg of protein from each sample was run on a NuPAGE BisTris gel (Invitrogen) and then transferred to a PVDF membrane. The membranes were blocked for 1 h at room temperature in 5% nonfat dry milk, TBST and incubated overnight at 4 °C with primary antibody and then for 1 h at room temperature with HRP-conjugated secondary antibody. Detection was performed with an ECL kit (Pierce). Primary antibodies used were as follows: antibodies against TP63 (4A4, sc-8431, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), MYC (N-262, sc-764, Santa Cruz Biotechnology, Inc.), cytokeratin 1 (PRB-149P, Covance), cytokeratin 10 (MMS-159S, Covance), total β-catenin (6B3, 9582, Cell Signaling), cleaved Notch1 (NICD, Val-1744, 2421, Cell Signaling), and (A3854, Sigma). MYC promoter cloned into a luciferase plasmid was described previously (26). A TK-Renilla reporter was used as an internal normalization control. The ratio of firefly luciferase plasmid to Renilla luciferase was 10:1 in each nucleofection (2 µg:200 ng). At 48 h post-transfection, luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega). The luciferase reporter constructions were obtained as follows: pDel-1→pDel-4 reporter (Addgene plasmid 16601, 16602, 16603, 16604) (26), TCF-4/LEF reporter plasmid from SuperArray Bioscience, YY-1 reporter plasmid from Panomics, and JUN and FOS reporter plasmid from H. van Dam (Leiden University, Netherlands).

RESULTS

siRNA-mediated depletion of either p63 or MYC decreased mature keratinocyte proliferation, but only loss of p63 inhibited differentiation.—To investigate the specific roles of MYC and p63 in human keratinocyte differentiation, we knocked down each gene in HaCaT cells using specific siRNA and analyzed the phenotypic consequences. Because of the existence of six different isoforms of p63, we used an siRNA targeting the conserved DNA binding domain in all genes to achieve ablation of all p63 isoforms. We first verified that the expression of either MYC or p63 in siRNA-transfected keratinocytes was specifically knocked down at both the transcript (Fig. 1A) and protein levels (Fig. 1B) 48 h post-transfection. Compared with cells treated with control siRNA, we observed a 70 and 90% down-regulation of MYC and p63 expression, respectively. It is noteworthy that a significant loss of both genes was still observable after 10 days of culture (supplemental Fig. S1). Two days after siRNA transfection, we observed a reduced ATP content (Fig. 1C), whereas both EdU (Fig. 1D) and Ki67 staining (Fig. 1E) significantly decreased, both in p63- and MYC-knockdown HaCaT cells. Together, these data demonstrate a defect in keratinocyte proliferation upon ablation of either p63 or MYC. We next monitored the capacity of these cells to differentiate in vitro during 10 days of culture in the appropriate medium. As we focused on the commitment to differentiation, we chose to monitor the expression of two early differentiation markers, K1 and K10, rather than later ones, such as involucrin or filagrin. Indeed, K1 and K10 are markers of the basal-spinous layer transition in epidermis. Reduction of p63 levels in keratinocytes significantly inhibited the expression of K1 and K10 at the transcript level (Fig. 1F). In contrast, cells lacking MYC still expressed high levels of both markers, suggesting that these cells were still able to differentiate and did so even faster than the control (Fig. 1F). On the protein level, K1 was continuously induced except in cells lacking p63, which exhibited up-regulation of K1 until day 8, followed by a weak decrease in expression (Fig. 1G). Expression of the K10 protein started late in all conditions but became significant in MYC-depleted cells after 8 days of culture (Fig. 1G). In addition, we observed that loss of p63 in cultures of primary human keratinocytes inhibited expression of both K1 and K10 compared with control or MYC-depleted cells (Fig. 1H). These results demonstrate that the ablation of either MYC or p63 significantly reduced human mature keratinocyte proliferation, whereas only the siRNA-mediated loss of p63 inhibited differentiation.
Knockdown of either MYC or p63 Leads to Cell Cycle Arrest—
To determine the potential origin of the proliferation defect in keratinocytes lacking either p63 or MYC, we analyzed cell death and the cell cycle. Knockdown of either p63 or MYC triggered a significant arrest in G0/G1 (Fig. 2A) without affecting cell death (Fig. 2B). To identify the molecular mechanisms controlling this cell cycle arrest, we analyzed the gene expression profiles of keratinocytes depleted of either MYC or p63 after transfection (supplemental Table S2). For these experiments, we have particularly insisted on robustness of the data. We performed three independent siRNA transfections and three independent RNA extractions (supplemental Fig. S2), along with several technical replicates, to generate three independent expression profiles that we then averaged for either MYC- or p63-depleted keratinocytes. This transcriptome analysis showed that a small network of genes strongly associated with cell cycle regulation (Fig. 2C and Fig. 2D) was significantly down-regulated in keratinocytes lacking either MYC or p63 (supplemental Fig. S3A and Fig. 2C). The majority of genes in this network were similarly regulated in both p63-depleted and MYC-depleted cells, except CSK2, GADD45A, and CCND2 (supplemental Fig. S3A). Analysis by qRT-PCR of the major cell cycle inhibitors confirmed that p15 and p21 were both significantly up-regulated in response to the knockdown of either MYC or p63 in HaCaT cells (Fig. 2, D and E). Interestingly, the same trend was observed in primary keratinocytes (Fig. 2, F and G). We also observed that MYC, a major hub (highly connected node) in this cell cycle-controlling network, was down-regulated in both HaCaT and primary keratinocyte cells lacking p63 (Fig. 2, C, D, and F). This result suggests that p63 is necessary for the proper expression of MYC in human keratinocytes.
p63 Is Necessary for Proper Expression of MYC—We first confirmed the down-regulation of MYC expression in cells lacking p63 at the transcript level (Fig. 3A) and at the protein level (Fig. 3B), both in HaCaT cells and in primary human keratinocyte cultures from two different donors. Data mining in the Gene Expression Omnibus (GEO) confirmed that other groups have measured down-regulation of MYC in p63-deficient epithelial cell lines (supplemental Fig. S4) (27). Using specific siRNA targeting either ΔNp63 or TAp63, we were able to demonstrate that only the knockdown of ΔNp63 isoforms triggered down-regulation of MYC expression (Fig. 3C). This was consistent with the fact that ΔNp63 is the isoform predominantly expressed in adult keratinocytes. Conversely, overexpression of either ΔNp63 or TAp63 in HaCaT cells had no effect on MYC expression (supplemental Fig. S5). Together, these results demonstrated that p63 is necessary for the proper expression of MYC.

We next investigated the molecular mechanisms underlying the p63 knockdown-triggered down-regulation of MYC expression. Using luciferase reporter constructs fused to truncated MYC promoter regions (Fig. 3D), we characterized the sequence upstream of MYC and identified a putative p63-controlled region. As demonstrated in Fig. 3E, this region extended from −349 to −607 bp upstream of the transcription start site.
A close-up view of this region revealed the absence of the p63 consensus binding site, in agreement with our previous published results, which showed that MYC was not a direct target of p63 in human keratinocytes (28, 29). These results suggested that the expression of MYC is under the indirect control of p63.

The region upstream of the MYC gene also contains several other binding sites for transcription factors (TFs), including TCF-4 (TF responding to the Wnt/H\(\beta\)-catenin signaling pathway), YY-1 (TF responding to the Notch signaling pathway), c-FOS, and c-JUN (AP1 TFs) (Fig. 3F). Because these TFs are known to play important roles in the control of keratinocyte proliferation and differentiation (30–32), we investigated whether or not they are involved in the p63 knockdown-triggered down-regulation of MYC promoter activity. In keratinocytes lacking p63, we observed a moderate inhibition of both TCF4 and YY1-dependent luciferase activities but no change or a slight activation in JUN- and FOS-driven luciferase expression (Fig. 3G). These results suggest that TCF4 and YY1, the TFs responding to the Wnt/\(\beta\)-catenin and Notch signaling pathways, respectively, were partially responsible for the p63-dependent down-regulation of MYC expression.

p63 Knockdown-triggered Down-regulation of MYC Expression Is Mediated by Notch and Wnt/\(\beta\)-Catenin Signaling Pathways—To determine whether the signaling cascades leading to the activation of YY1 and TCF4 are affected by p63 levels, we scrutinized the expression profiles of keratinocytes lacking either p63 or MYC. Interestingly, we found that the Wnt/\(\beta\)-catenin and Notch signaling cascades were indeed potently inhibited in keratinocytes lacking p63. JAG1 and DLL1, ligands of the Notch signaling pathway, were both down-regulated in p63-knockdown cells (Fig. 4A). In addition, several inhibitors of the Wnt/\(\beta\)-catenin pathway were up-regulated, whereas several activators were down-regulated, probably resulting in the inhibition of this signaling pathway (Fig. 4B). At the protein level, the ablation of p63 resulted in the down-regulation of both cleaved Notch1 (NICD) and total \(\beta\)-catenin (Fig. 4B). Taken together, these results suggest that both pathways were turned down in p63-knockdown cells. Inhibition of the Notch signaling pathway by p63 knockdown therefore likely contributes to the down-regulation of MYC expression.
 signaling pathway in the presence of p63-targeting siRNA was also indicated by the reduced expression of two reporter genes of the Notch-signaling cascade, HES1 and HEY1 (Fig. 4C). To further confirm the potential involvement of the Wnt and Notch pathways in down-regulation of MYC expression, we restored downstream signaling by transfecting HaCaT cells with either WNT3 or NICD expression vectors, together with p63-targeting siRNA or control siRNA. We observed that the overexpression of either WNT3 or NICD induced the up-regulation of MYC expression both at the transcript (Fig. 4D) and protein levels (Fig. 4E). This would partially restore keratinocyte proliferation. However, overexpression of those two genes was not sufficient to restore proper differentiation of keratinocytes lacking p63 (data not shown). These results suggest that the molecular role of p63 in differentiation of human mature keratinocytes is probably different from the ones that dictate their proliferation.

**Cell Migration/Adhesion-related Network Acts Downstream of p63 to Induce Onset of Keratinocyte Differentiation**—Our results demonstrated that MYC is down-regulated in cells lacking p63 (Figs. 2 and 3), thus functionally corresponding, at least partially, to a siRNA-mediated knockdown of MYC. However, these cells exhibited completely opposite differentiation outcomes (Fig. 1E). To investigate the molecular mechanisms enabling keratinocyte differentiation downstream of p63, we compared the expression profiles of p63-depleted and MYC-depleted cells.

As demonstrated in Fig. 5A, 546 genes were common to both expression profiles. It is noteworthy that there were more genes common to both profiles than specific to the p63-depleted cells. This again suggests that part of the transcriptional response to p63 ablation in human keratinocytes was also due to the down-regulation of MYC. The down-regulated genes in keratinocytes lacking either MYC (supplemental Fig. S6A) or p63 (supplemental Fig. S6B) shared similar gene ontology terms (e.g. cell cycle, DNA replication, and DNA repair). Genes up-regulated in either MYC-depleted (supplemental Fig. S6C) or p63-depleted keratinocytes (supplemental Fig. S6D) also shared some gene ontology terms, such as cellular movement or cell death.

Among the 546 genes common to both expression profiles, we found 71 genes that were antagonistically regulated (supplemental Table S3). We hypothesized that these antagonistically regulated genes could mechanistically explain, at least partially, the opposite differentiation outcomes between p63- and MYC-lacking keratinocytes. We used the Ingenuity knowledge base using IPA software to analyze the networks and functions associated with these 71 genes. Strikingly, a network of 41 nodes was extracted and significantly associated with a single function, e.g. cell migration/adhesion (p < 3 x 10^-14). In cells lacking p63, this network was strongly down-regulated (Fig. 5B), whereas in MYC-depleted keratinocytes, this same network was up-regulated (supplemental Fig. S3B). We further validated the expression of some genes in the network by qRT-PCR in HaCaT cells (Fig. 5C) and in primary keratinocytes (Fig. 5D). As expected, the expression of these genes was up-regulated in MYC-depleted cells and down-regulated in keratinocytes lacking p63. These results suggest that this migration/adhesion-related gene network could contain potential effectors acting down-
stream of p63 to induce the onset of terminal differentiation in human keratinocytes.

To validate our hypothesis, we used several functional approaches. First, we searched for known phenotypes associated with this 41-gene network in the Mouse Genome Informatics database. There are 19 knock-out mice with abnormal skin phenotypes reported in that database. Strikingly, 15 genes of these 19 KO mice were present in this network (Table 1). These data suggest that nearly 80% of all known skin dysfunction-related genes belong to the network we have characterized and functionally validate it. We also monitored in vitro differentiation of keratinocytes lacking different genes belonging to this migration/adhesion network: FN1, MMP13, JAG1, IL1B, and CYR61 (Fig. 6A). Except for MMP13, we observed a strong inhibition of differentiation in cells lacking any of these genes, as demonstrated by the delayed expression of K1 (Fig. 6B) and K10 (Fig. 6C) transcripts. It is noteworthy that although the ablation of IL1B was only partial (40%), it significantly inhibited differentiation. Furthermore, ablation of all of these genes together (siCocktail) strongly inhibited keratinocyte differentiation. Finally, if the genes belonging to this network promote commitment to differentiation, we postulated that their expression should be down-regulated in non-differentiated and/or pluripotent cells. We data-mined the NCBI Gene Expression Omnibus database, and interestingly, we found that seven hubs in this network, PLAU, FN1, IL1B, ADM, DUSP10, GADD45A, and RAC2, were significantly down-regulated in induced pluripotent stem cells (iPS) and are even part of the iPS transcriptomic signature (Fig. 6D) (33). Together, these results show that a p63-controlled migration/adhesion-related network plays a key role in the onset of human mature keratinocyte differentiation. As a consequence, we named this network the keratinocyte cell fate (KCF) network.

**DISCUSSION**

Our findings confirm that sustained expression of both p63 and MYC, two major regulators of epidermal homeostasis (5–7, 18, 21, 34, 35), is required to maintain growth and differentiation of human developmentally mature keratinocytes. However, we demonstrate that their respective roles are very differ-
ent, as already suggested by some authors (36). We propose a model to illustrate the distinct mechanisms of action of p63 on human developmentally mature keratinocyte proliferation and differentiation (Fig. 7). p63 is required for the proper expression of MYC through the combined regulation of the Wnt/β-catenin and Notch signaling pathways, leading in turn to cell cycle regulation and cell proliferation. p63 also regulates a KCF network that contains several potential “differentiation effectors,” some located in the extracellular space. The up-regulation of the KCF network would promote the onset of terminal differentiation of keratinocytes (Fig. 7). In this study, we show that the siRNA-mediated loss of MYC triggers down-regulation of the “proliferation network” and up-regulation of the KCF network, promoting human keratinocyte differentiation; in

| Gene ID | GenBank™ accession number | Phenotype                                      |
|---------|--------------------------|------------------------------------------------|
| CCND2   | NM_001759                | Abnormal external granule cell layer morphology |
| COL17A1 | NM_130778                | Abnormal epidermal layer morphology; skin lesions |
| DFNA5   | NM_000403                | Decreased cochlear outer hair cell number       |
| DST     | NM_001723                | Abnormal skin pigmentation                      |
| F2R     | NM_001992                | Abnormal skin condition                         |
| F3      | NM_001993                | Abnormal skin condition                         |
| FN1     | NM_002026                | Skin lesions                                    |
| FST     | NM_006350                | Thickened epidermis                             |
| GADD45A | NM_001924                | Increased sensitivity to skin irradiation       |
| HMGA2   | NM_003483                | Long hair                                       |
| IL1RAP  | NM_002182                | Abnormal skin condition/morphology              |
| ITGA2   | NM_002203                | Abnormal skin condition                         |
| JAG1    | NM_000214                | Abnormal cochlear hair cell morphology          |
| Jnk     | NM_005554                | Decreased hair follicle number                   |
| KRT6A   | NM_002820                | Abnormal coat/hair morphology                   |
| P63     | NM_0003246               | Abnormal skin condition                         |
| PTHLH   | NM_000311                | Abnormal skin condition                         |

FIGURE 6. An extracellular matrix genetic network is involved in keratinocyte differentiation. A, validations of siRNA knockdown targeting FN1, MMP13, JAG1, IL1B, or CYR61. Cells were transfected with 10 nm siRNA for 48 h, and then the gene expression levels were quantified by qRT-PCR. The effect of knockdown of each gene was compared with siCT. B and C, time course analysis of K1 (B) and K10 (C) expression were determined by qRT-PCR 9 days post-transfection of HaCaT cells with siRNA against FN1, MMP13, IL1B, JAG1, CYR61, or siCock (mixture of these five siRNAs). D, expression of oncogene hubs in human iPSC. hFibbr, human fibroblast; e-hiPSC, early human iPSC; l-hiPSC, late human iPSC; hESC, human embryonic stem cells. Each spot represents expression data from one biological sample. Error bars, S.D.
In conclusion, the siRNA-mediated loss of MYC triggers down-regulation of the “proliferation network” and up-regulation of the “KCF migration/adhesion-related network,” promoting human keratinocyte differentiation; in contrast, p63 knockdown down-regulates both cell proliferation and KCF migration/adhesion. These results are consistent with recent reports showing that p63 functions as an inhibitor of cell migration (27) and that a p53 mutant forms a complex with p63 to antagonize its cell migration-inhibitory function, leading to TGFβ-dependent metastasis (49). Similarly, it was shown that p63 regulates a cell adhesion program, including integrins, in epithelial cells (8). Although we cannot exclude the possibility that other genes that are among the 546 common genes but are not found in our network might regulate early differentiation, we have clearly demonstrated that this p63-controlled cell migration/adhesion network contains several effectors acting downstream of p63 to trigger differentiation of mature keratinocytes.

Some of the differentiation effectors we have identified (such as integrins, FN1, PLAU, JAG1, IL1, and CYR61) are also involved in cancer progression in inducible human tissue neoplasia (50). Integrin signaling plays an important role in epidermal adhesion, growth, and differentiation (51, 52). JAG1 is a ligand of the Notch signaling pathway and acts on keratinocyte differentiation (32, 53). JAG1 is also a transcriptional target of p63 (54). Interleukin-1 (IL1) is implicated in human epidermal keratinocyte proliferation (55) and even in the regeneration of epidermal tissue in vitro (56). IL1α is the active form of interleukin-1 in human epidermis. IL1β was considered non-functional in keratinocytes, but our data suggest that IL1β is necessary to induce keratinocyte differentiation.

Although most of these “differentiation effectors” do not seem to be direct transcriptional targets of p63, the control exerted by p63 on this network was dominant. Indeed, we were unable to rescue differentiation of p63-depleted keratinocytes by ectopic expression of NICD or JAG1 with the use of JAG1 or IL1β recombinant proteins or even with an acellular matrix obtained from normal fully differentiated keratinocytes (data not shown).

We report for the first time the role of this p63-regulated cell migration/adhesion network in the commitment of developmentally mature keratinocytes to differentiation. A normal expression of this network seems to be required to trigger differentiation, whereas its down-regulation prevents it. Furthermore, we believe that misregulation of this gene network may play a major role in tumorigenesis. Indeed, Khavari’s group recently reported a core tumor progression signature network in keratinocytes, which contained 282 nodes and was involved in carcinogenesis (50). This core tumor progression signature network contained several oncogene hubs, and eight of the top 10 nodes were extracellular or cell surface proteins. It is noteworthy that four of these eight extracellular oncogene hubs, PLAU, CYR61, FN1, and IL1, also belong to the p63-regulated cell migration/adhesion network we describe in this study. Other oncogene hubs reported in the core tumor progression signature network (50), such as SERPINE1 and ITGA6, were also down-regulated in human keratinocytes depleted of p63 (supplemental Table S2).

In conclusion, the siRNA-mediated loss of MYC triggers down-regulation of the “proliferation network” and up-regulation of the “KCF migration/adhesion-related network,” promoting human keratinocyte differentiation; in contrast, p63 knockdown down-regulates both cell proliferation and KCF differentiation. These results were observed both in the HaCaT cell line (mutated p53) and in normal human primary keratinocytes (wild-type p53), suggesting that the model we propose is independent of the p53 status of skin cells.

Because MYC is a transcriptional repressor of p15 and p21, two cyclin-dependent kinase inhibitors (37–39), and p63 was reported to repress p21 and p16 (40–42), it was not a surprise to observe reduced proliferation in cells lacking either gene. More surprising was the p63 knockdown-triggered down-regulation of MYC expression that we report here for the first time. This result suggests that although MYC is not a direct target of p63 (28, 29), the expression of this transcription factor is necessary for the proper expression of MYC both in HaCaT cell line and primary human keratinocyte culture. Our results establish that MYC expression is mediated, at least in part, by the Wnt/β-catenin and Notch signaling pathways. Interestingly, both pathways are important regulators of proliferation and differentiation in epidermal stem cell maintenance and wound healing (1, 43–46). The interplay between the Wnt/β-catenin and Notch pathways has been reported in epidermal homeostasis and differentiation as well (47, 48). We show that both pathways act in concert downstream of p63 to control the proper expression of MYC and, in turn, regulate the keratinocyte cell cycle.

Although the inhibition of proliferation of cells lacking p63 was partially MYC-dependent, the differentiation defects appeared to be independent of MYC. Indeed, the siRNA-mediated loss of MYC did not impair keratinocyte commitment to terminal differentiation. The differentiation was even slightly accelerated in MYC-depleted cells. By comparing expression profiles from mature keratinocytes lacking either MYC or p63, we found 546 common genes, of which 71 were antagonistically regulated. Strikingly, from that list of 71 genes, we extracted a gene network containing 41 genes that were significantly associated (p < 3 × 10⁻¹⁴) with a single function, cell migration/adhesion. These results are consistent with recent reports showing that p63 functions as an inhibitor of cell migration (27) and that a p53 mutant forms a complex with p63 to antagonize its cell migration-inhibitory function, leading to TGFβ-dependent metastasis (49). Similarly, it was shown that p63 regulates a cell adhesion program, including integrins, in epithelial cells (8). Although we cannot exclude the possibility that other genes that are among the 546 common genes but are not found in our network might regulate early differentiation, we have clearly demonstrated that this p63-controlled cell migration/adhesion network contains several effectors acting downstream of p63 to trigger differentiation of mature keratinocytes.

Some of the differentiation effectors we have identified (such as integrins, FN1, PLAU, JAG1, IL1, and CYR61) are also involved in cancer progression in inducible human tissue neoplasia (50). Integrin signaling plays an important role in epidermal adhesion, growth, and differentiation (51, 52). JAG1 is a ligand of the Notch signaling pathway and acts on keratinocyte differentiation (32, 53). JAG1 is also a transcriptional target of p63 (54). Interleukin-1 (IL1) is implicated in human epidermal keratinocyte proliferation (55) and even in the regeneration of epidermal tissue in vitro (56). IL1α is the active form of interleukin-1 in human epidermis. IL1β was considered non-functional in keratinocytes, but our data suggest that IL1β is necessary to induce keratinocyte differentiation.

Although most of these “differentiation effectors” do not seem to be direct transcriptional targets of p63, the control exerted by p63 on this network was dominant. Indeed, we were unable to rescue differentiation of p63-depleted keratinocytes by ectopic expression of NICD or JAG1 with the use of JAG1 or IL1β recombinant proteins or even with an acellular matrix obtained from normal fully differentiated keratinocytes (data not shown).

We report for the first time the role of this p63-regulated cell migration/adhesion network in the commitment of developmentally mature keratinocytes to differentiation. A normal expression of this network seems to be required to trigger differentiation, whereas its down-regulation prevents it. Furthermore, we believe that misregulation of this gene network may play a major role in tumorigenesis. Indeed, Khavari’s group recently reported a core tumor progression signature network in keratinocytes, which contained 282 nodes and was involved in carcinogenesis (50). This core tumor progression signature network contained several oncogene hubs, and eight of the top 10 nodes were extracellular or cell surface proteins. It is noteworthy that four of these eight extracellular oncogene hubs, PLAU, CYR61, FN1, and IL1, also belong to the p63-regulated cell migration/adhesion network we describe in this study. Other oncogene hubs reported in the core tumor progression signature network (50), such as SERPINE1 and ITGA6, were also down-regulated in human keratinocytes depleted of p63 (supplemental Table S2).

In conclusion, the siRNA-mediated loss of MYC triggers down-regulation of the “proliferation network” and up-regulation of the “KCF migration/adhesion-related network,” promoting human keratinocyte differentiation; in contrast, p63 knockdown down-regulates both cell proliferation and KCF
networks, thus inhibiting differentiation. We believe that the balance between levels of expression of both cell proliferation and KCF networks could dictate keratinocyte cell fate. Furthermore, we think that this network approach would reconcile much of the existing data on the regulation of the balance between proliferation and differentiation in skin.

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