p63 Gene is Regulated by Grainyhead-Like 2 (GRHL2) Through Reciprocal Feedback and Determines Epithelial Phenotype in Human Keratinocytes

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Running title: p63 and GRHL2 determine epithelial plasticity in human keratinocytes

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Background: p63 isoforms ΔNp63α, ΔNp63β, ΔNp63γ and Grainyhead-like 2 (GRHL2) play distinct roles in regulating epithelial phenotype.

Results: p63 modulation leads to epithelial-mesenchymal transition in human keratinocytes, and GRHL2 binds directly to p63 promoter.

Conclusion: GRHL2/p63 reciprocal regulation maintains epithelial phenotype and plasticity.

Significance: GHRL2/p63 model is crucial to understanding epithelial plasticity and metastasis, epithelial wound healing, and tissue regeneration.

ABSTRACT

In the current study, we investigated the effects of p63 modulation in epithelial plasticity in human keratinocytes. p63 isoforms ΔNp63α, ΔNp63β, and ΔNp63γ were ectopically expressed in normal human epidermal keratinocytes (NHEK). Epithelial or mesenchymal state was determined by morphological changes and altered expression of various markers, e.g., fibronectin (FN), E-Cadherin (E-Cad), and keratin 14 (K14). Overexpression of ΔNp63α and ΔNp63β, but not ΔNp63γ isoforms, led to morphological changes consistent with epithelial-mesenchymal transition (EMT). However, only ΔNp63α overexpression was able to maintain the morphological changes and molecular phenotype consistent with EMT.

Interestingly, knockdown of all p63 isoforms by transfection of p63 siRNA also led to EMT phenotype, further confirming the role of p63 in regulating epithelial phenotype in NHEK. EMT in NHK accompanied loss of Grainyhead-Like 2 (GRHL2) and miR-200 family gene expression, both of which play crucial roles in determining epithelial phenotype. Modulation of GRHL2 in NHK also led to congruent changes in p63 expression. Chromatin immunoprecipitation (ChIP) revealed direct GRHL2 binding to p63 promoter; GRHL2 knockdown in NHK led to impaired binding of GRHL2 and changes in the histone marks consistent with p63 gene silencing. These data indicate the presence of a reciprocal feedback regulation between p63 and GRHL2 in NHEK to regulate epithelial plasticity.

p63 is a transcription factor that regulates epithelial phenotype and keratinocyte proliferation and is an important marker of epidermal and stratified keratinocyte stem cells (KSCs) (4,5). p63 is expressed as six isoforms. Three of these isoforms contain the N-terminal transactivation domain (TAp63α, TAp63β, TAp63γ), whereas
the remainder (ΔNp63α, ΔNp63β, ΔNp63γ) do not (6). Our recent study demonstrated that transduction of the ΔNp63α isoform and intact TGF-β signaling is necessary to induce epithelial-mesenchyme transition (EMT) in normal human epidermal keratinocytes (NHEK) (4).

EMT is a process by which epithelial cells exhibit morphologic and molecular changes consistent with mesenchymal phenotype and is involved in tissue and organ development and repair (7). Cells with EMT phenotype exhibit loss of epithelial morphology and development of elongated, spindled, mesenchymal morphology (8). EMT is facilitated by the loss of cell-cell adhesion mediated by E-Cadherin (E-Cad), and loss of E-Cad expression serves as a marker for EMT (9). Also, loss of keratin 14 (K14) and enhanced expression of fibronectin (FN) and vimentin serve as mesenchymal markers (9-11).

GRHL2 is a novel transcription factor involved with epithelial morphogenesis, cell proliferation, and differentiation (12). Our recent studies showed that GRHL2 transcriptionally regulates broad spectrum of target genes including the human telomerase (hTERT) gene, proliferating cell nuclear antigen (PCNA), and epidermal differentiation complex (EDC) genes (12,13). Phenotypically, GRHL2 promotes epithelial cell proliferation and inhibits keratinocyte differentiation in NHEK (12). Other studies suggest its inhibitory role in EMT and apoptosis in breast cancer cell lines, demonstrating its involvement in cancer progression (14). Mechanistically, GRHL2 downregulates zinc finger E-box binding protein 1 (ZEB1), which triggers EMT through suppressing E-Cad expression (15). Thus, GRHL2 is a determinant of epithelial phenotype through transcriptional network of its target genes.

Our previous study demonstrated that ectopic ΔNp63α expression in NHEK yielded EMT phenotype in a TGF-β dependent manner (4). In this report, we show that induced EMT phenotype is specific to ectopic overexpression of ΔNp63α among the p63 isoforms. Alternatively, knockdown of all isoforms of p63 also led to EMT phenotype through loss of GRHL2 and miR-200 family genes. Lastly, we found that GRHL2 directly binds to the p63 promoter, and that p63 and GRHL2 regulate their mutual expression. This reciprocal feedback loop between p63 and GRHL2 maybe critical in determining epithelial plasticity.

EXPERIMENTAL PROCEDURES

Cells and cell culture - Primary cultured single cell-suspension of NHEK were isolated from discarded foreskin epidermal tissues and grown in EpiLife medium supplemented with growth factors (Invitrogen, Carlsbad, CA), as described elsewhere (16). Normal human oral keratinocytes (NHOK) isolated from separated keratinizing oral epithelial tissues were prepared similar to NHEK. NHEK were serially subcultured and passed at 70% confluence until they reached replicative senescence. Replication kinetics were recorded as described previously (16). Normal human oral fibroblasts (NHOF) were obtained from discarded gingival connective tissue and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Bone marrow mesenchymal stromal cells (BM-MSCs) and dental pulp stem cells (DPSCs) were cultured in α-MEM medium (Invitrogen) supplemented with 10% FBS (Invitrogen), and 5 mg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA). SCC4 and SCC9 cancer cell lines were cultured in DMEM/F12 (Invitrogen) and supplemented with 10% FBS and 0.4 pg/ml hydrocortisone, as described elsewhere (17). Immortalized keratinocyte cell line HaCaT cells were grown in EpiLife medium supplemented with growth factors (Invitrogen). Cells were maintained at 37°C at 5% CO₂ in a humidified chamber.

Retroviral vector construction and transduction of cells – We constructed retroviral vectors expressing human wild-type ΔNp63α, ΔNp63β, and ΔNp63γ. Full-length cDNAs of ΔNp63 were cloned from the cDNA library obtained from primary human keratinocytes by PCR amplification. ΔNp63α, ΔNp63β, and ΔNp63γ cDNAs were subcloned into pLXSN retroviral expression vector (Clontech, Mountainview, CA) at XhoI/BamHI restriction sites, and isoforms were confirmed by sequencing. Retroviral construction and infection were performed as described elsewhere (18), and the infected cells were selected with 200 µg/ml G418 (Sigma, St. Louis, MO). G418-resistant cells were continually
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maintained by serial subculture as outlined above.

**Transwell Migration Assay** – Transwell chambers with polycarbonate membranes were used to measure cell migration (Corning Inc., Corning, NY), according to previously described methods (19). NHEK/LXSN, NHEK/ΔNp63α, NHEK/ΔNp63β, and NHEK/ΔNp63γ cells were cultured in EpiLife (Invitrogen) and seeded in the upper chamber of the transwell. Cells were allowed to migrate for 24 hours, and the transwell was washed with 1x phosphate-buffered saline (PBS) and fixed in 10% formalin for 10 min. Cells were then stained with 1% crystal violet dissolved in 10% formalin for a duration of 1 hour. Non-migratory cells were removed from the chamber and transwells were photographed.

**Immunofluorescence Staining** – Cells were fixed for 15 minutes in 3.7% formaldehyde prior to permeabilization with 0.25% Triton X-100 for 10 minutes. Cells were then blocked in 10% normal goat serum for 1 hour. Antibodies against p63 (A4A), E-Cad, β-catenin, ZEB1 (Santa Cruz Biotech, Santa Cruz, CA), GRHL2 (Abnova, Taipei City, Taiwan), and anti-FN (Sigma) were used as primary antibodies, and Alex Fluor 488 IgG (Invitrogen) was used as secondary antibody. Cells were counterstained with DAPI, and Olympus BH2-RFCA fluorescence microscope was used to obtain images.

**siRNA Transient Transfection** – NHEK were transfected with non-specific, scrambled siRNA (Si-SCR) or siRNA targeting human p63 (Si-p63) at a concentration of 10 nM using Lipofectamine reagents (Invitrogen) according to the manufacture’s instructions. NHEK were transfected with Si-p63 once every 3 days over the course of 9 days. NHEK growth medium was replaced with mesenchymal growth medium (α-MEM medium/10% FBS, 5 mg/mL gentamicin sulfate) upon morphological transition to mesenchymal phenotype.

**Western Blotting** - Whole cell extracts were isolated from the cultured cells, fractionated by sodium dodecylsulfate polyacrylamide gel electrophoresis, and transferred to Immobilon membrane (Millipore, Billerica, MA). Antibodies against p63 (A4A), p63 (H129), E-Cad, K14, ZEB1, N-Cad, GAPDH (Santa Cruz Biotech), GRHL2 (Abnova), FN and Snail (Sigma) were used. Chemiluminescence signal was detected by using the HyGLO Chemiluminescent HRP antibody detection reagent (Denville Scientific, South Plainfield, NJ).

**Real-time qRT-PCR** - Total RNA was extracted from cultured cells by using RNeasy Plus Mini kit (Qiagen, Valencia, CA). Reverse transcription (RT) was performed with 5 μg RNA by using the method described elsewhere (20). qRT-PCR was performed for the relative mRNA expression of TAp63, ΔNp63, and GRHL2, relative microRNA expression of miR-200a, miR-200b, miR-200c, miR-141, miR-429 and enrichment of GRHL2, H3K4Me3 and H3K27Me3 in triplicates for each sample with LC480 SYBR Green I master (Roche, Basel, Switzerland) using LightCycler 480 (Roche). Second derivative Cq value was calculated after running a total of 50 cycles in order to compare relative change in mRNA and microRNA expression. The primer sequences and the PCR conditions will be available on request.

**F-Actin Staining / Stress Fiber Formation** – Cells were fixed for 10 minutes in 3.7% formaldehyde prior to permeabilization with 0.1% Triton X-100 for 5 minutes. Cells were stained with Alexa Fluor 594 Phalloidin (Invitrogen) for 20 minutes at room temperature. Cells were counterstained with DAPI, and Olympus BH2-RFCA fluorescence microscope was used to obtain images.

**Lentiviral vector construction and knockdown of endogenous GRHL2** – Lentiviral vector expressing short hairpin RNA (shRNA) against GRHL2 (Sh-GRHL2) and control vector expressing enhanced green fluorescence protein (EGFP) were constructed as described earlier (21). Endogenous GRHL2 was knocked down with Sh-GRHL2. All lentiviral vectors used allow identification of infected cells by GFP fluorescent signal under epifluorescence microscope.

**Dual-Luciferase Reporter Assay** – Promoter regions of GRHL2 and p63 were cloned into pGL3B-Luc reporter plasmid (Promega, Madison, WI) expressing firefly luciferase. The promoter-luciferase constructs were transfected into SCC4 or SCC9 using Lipofectin Reagent (Invitrogen), along with pRL-SV40 containing Renilla luciferase cDNA under the control of SV40 enhancer/promoter. After 48 hours post-transfection, cells were collected and the lysates were prepared using Dual Luciferase Reporter Assay System (Promega). Firefly and Renilla
Overexpression of ΔNp63α in NHEK yields EMT morphology and phenotype. NHEK were stably infected with retroviruses containing empty vector LXSN or those expressing ΔNp63α, ΔNp63β, or ΔNp63γ isoforms. Among the transduced cells, cells expressing ΔNp63α and ΔNp63β isoforms showed morphological changes indicative of EMT, e.g., elongated, flattened, and spindle-like morphology (Figure 1A, left panel). However, we observed that in NHEK expressing ΔNp63β isoforms, few mesenchymal-like cells were formed and were not able to be maintained in culture. Transwell migration assay also showed enhanced cellular motility, a hallmark of EMT (22) in NHEK/ΔNp63α, while NHEK/ΔNp63β, NHEK/ΔNp63γ, and NHEK/LXSN showed limited transwell migration (Figure 1A, right panel).

To further determine the molecular phenotype of EMT in NHEK transduced with various ΔNp63 isoforms, we performed Western blotting for the markers of epithelial or mesenchyme state. Cells transduced with ΔNp63α, ΔNp63β, and ΔNp63γ were harvested at two different population doubling (PD) levels at 24 and PD28; EMT phenotype occurred between these two PD levels (Figure 1C). We used both pan-p63 antibody (p63 A4A) and ΔNp63α-specific antibody (p63 H129) to assess protein expression of ΔNp63 isoforms. We observed specific bands corresponding to the α, β, and γ isoforms of ΔNp63 that were distinctly visible at 60, 50, and 40 kDa, respectively (Figure 1B, C). We also noted that high molecular weight p63 band denoted with asterisk (*) disappeared with occurrence of EMT only in the culture infected with LXSN-ΔNp63α at PD28, concomitant with appearance of the EMT phenotype. We also found that only prolonged subculture of NHEK/ΔNp63α led to reduced protein expression of epithelial markers E-Cad and K14 and increased expression of mesenchymal marker FN (Figure 1B, C). Prolonged subculture of NHEK/ΔNp63α also led to downregulation of GRHL2, a transcriptional regulator and EMT-inhibitor that regulates epithelial proliferation and differentiation (12). Loss of E-Cad and enhanced FN expression in NHEK/ΔNp63α cells were also confirmed in situ (Figure 2). In this particular culture, both cell types exhibiting the mesenchymal or epithelial phenotype were visible; those cells with the mesenchymal morphology also demonstrated enhanced FN and reduced E-Cad signal. Collectively, these data indicate that...
ectopic expression of ΔNp63α, but not ΔNp63β and ΔNp63γ, in NHEK triggers the EMT phenotype.

**Knockdown of all p63 isoforms in NHEK by transient transfection of siRNA results in EMT phenotype.** Since EMT occurrence by ΔNp63α overexpression was accompanied by loss of the major p63 band (denoted with an asterisk in Figures 1B, C), we asked whether the loss of all p63 isoforms would lead to EMT. NHEK were transiently transfected with the control, scrambled siRNA (Si-SCR) or siRNA targeting p63 (Si-p63). In order to assess phenotypic and morphological changes upon p63 knockdown, we examined the changes in protein expression of p63, E-Cad, and FN after 3, 6, and 10 days post-transfection with Si-SCR or Si-p63. After 10 days post-transfection, NHEK/Si-p63 cells exhibited complete loss of p63 and E-Cad protein expression, whereas FN expression was notably enhanced (Figure 3A). These cells also exhibited mesenchymal cell morphology after 17 days when compared to NHEK/Si-SCR (Figure 3B).

We confirmed successful knockdown of p63 by qRT-PCR (Figure 3C). In the control cells (Si-SCR), the levels of ΔNp63 and GRHL2 were substantially higher than those in NHOF; upon Si-p63 transfection, p63 was almost completely knocked down to the level similar to those of NHOF. TAp63 expression level was negligible in all tested samples. Concomitantly, we found that GRHL2 level was also drastically reduced by p63 knockdown, indicating a link between p63 and GRHL2 in epithelial phenotype.

We also determined mesenchymal cell surface markers (e.g., CD44, CD73, CD90, and CD105) in NHEK/Si-p63 cells with EMT phenotype. For comparison, we included BM-MSCs, DPSCs, and parental NHEK. The NHEK/Si-p63 cells with EMT phenotype showed elevated CD44, CD90, and CD105 expression compared with the parental NHEK, while the level of CD73 was reduced, consistent with other MSC cell types (Figure 4), further supporting EMT in cells after loss of p63 expression.

EMT is a process by which epithelial cells lose the cell-to-cell contact adhesion and associated protein expression and exhibit mesenchymal phenotype, including stress fiber formation and migration (22). As additional evidence of EMT, the NHEK/Si-p63 cells demonstrated stress fiber formation with organized F-actin (Figure 3D). We also performed fluorescence microscopy to determine changes in the EMT marker expression upon p63 knockdown in NHEK. NHEK/Si-p63 cells showed loss of p63 and GRHL2 expression, reduced expression of E-Cad and β-catenin, and enhanced expression of ZEB1 and FN (Figure 3E). We used NHEK treated with 5 ng/ml TGF-β for 10 days as a positive control for EMT, which similarly showed reduced GRHL2, p63, K14 and β-catenin expression and enhanced FN expression by immunofluorescence staining (Figure 5). Collectively, these data indicate that knockdown of all p63 isoforms in NHEK led to EMT.

**Knockdown of p63 in NHEK results in loss of GRHL2 and miR-200 family gene expression.** In the next experiments, we investigated the mechanism underlying the p63-associated EMT. In both NHEK/ΔNp63α and NHEK/Si-p63 cells, EMT occurred with enhanced expression of ZEB1 and N-Cad, which are linked with mesenchymal and stem characteristics (23) (Figure 6A). We also found that both NHEK/ΔNp63α and NHEK/Si-p63 exhibiting EMT phenotype lost GRHL2 and E-Cad expression. Since GRHL2 is a direct upstream regulator of E-Cad (24), our data suggest that p63 modulation could lead to EMT through loss of GRHL2.

Recent studies showed that GRHL2 suppressed EMT by upregulating miR-200 family genes (25), which then downregulate ZEB1 (26). In the current study, acquisition of EMT phenotype in NHEK/ΔNp63α and NHEK/Si-p63 resulted in strong downregulation of miR-200a, miR-200b, miR-200c, miR-141, and miR-429 microRNA expression (Figure 6B). To test whether p63 directly binds to and regulates the gene expression of miR-200 family genes, we performed ChIP assay; NHEK/ΔNp63α and NHEK/Si-p63 with EMT phenotype showed loss of p63 enrichment on the promoter regions of the miR-200a, miR-200b, miR-200c, and miR-429 loci, compared to those of parental NHEK (Figure 6C). No change in p63 enrichment on miR-141 was observed between NHOF, parental NHEK, and NHEK/ΔNp63α and NHEK/Si-p63. These data suggest that p63 may regulate the epithelial phenotype in
keratinocytes by direct regulation of GRHL2 and miR-200 family genes, although miR-141 may be bound and regulated by other factors downstream of p63.

Modulation of GRHL2 alters p63 expression and EMT induction. In another experiment, we induced EMT in SCC4 and HaCaT epithelial cell lines by exposure to TGF-β. In time-dependent manner after TGF-β treatment, protein expression of p63 and GRHL2 were reduced and the expression of FN, N-Cad, ZEB-1, and Snail were enhanced in SCC4 compared to the untreated cells (Figure 7A). Similarly, HaCaT cells showed reduced expression of GRHL2, p63, and E-Cad and enhanced expression of FN and ZEB-1 upon TGF-β treatment in time-dependent manner (Figure 7B). These data further confirm the regulatory relationship between p63 and GRHL2 in EMT.

Since GRHL2 is a transcription regulator for diverse gene targets, including those in the epidermal differentiation complex (EDC) (12), we tested whether p63 itself would be under the regulation of GRHL2. GRHL2 was ectopically expressed in NHOF and NHOK by using retroviral vectors. Overexpression of GRHL2 resulted in a weak but notable upregulation of p63 and E-Cad in NHOF and very strong upregulation in NHOK (Figure 7C). Also, GRHL2 overexpression in SCC9, which lack endogenous GRHL2 gene expression, led to visible p63 band and enhanced mRNA expression. Knockdown of GRHL2 in SCC4, which express high endogenous GRHL2, almost completely abolished p63 expression (Figure 7C, E). Furthermore, in situ staining for GRHL2, p63, and β-catenin revealed protein expression only in SCC9 cells transduced with GRHL2, and not in the control cells (Figure 7D). Notably, there was heterogeneity in the level of p63 staining in the SCC9 cells expressing exogenous GRHL2, presumably due to the varied level of GRHL2 expression in cells.

Since our prior data indicated that p63 was necessary for GRHL2 expression (Figure 3C), the above data indicate reciprocity in regulation between p63 and GRHL2 in determining the epithelial phenotype. We explored this possibility by performing a dual-luciferase reporter assay. Knockdown of GRHL2 in SCC4 resulted in diminished p63 promoter activity, whereas GRHL2 overexpression in SCC9 resulted in enhanced p63 promoter activity (Figure 7F). Interestingly, knockdown of p63 in SCC4 also led to a notable reduction of GRHL2 promoter activity. Hence, GRHL2 and p63 mutually regulate their promoter activity through a reciprocal feedback loop.

To examine whether GRHL2 directly binds to the promoter region of p63, ChiP assay was performed with SCC4 and NHEK cells. In both cell types, GRHL2 pull-down co-immunoprecipitated the p63 promoter fragment near the TSS. Also, GRHL2 enrichment on the p63 promoter was altered according to the level of GRHL2 protein, i.e., GRHL2 knockdown in SCC4 or overexpression in NHEK (Figure 8A, B). As positive control, we included GRHL2 binding to the promoter region of the hTERT gene, which is a known transcriptional target of GRHL2 (21). ChiP revealed concordant GRHL2 binding signal to the hTERT promoter according to the GRHL2 protein level (Figure 8C). ChiP-qPCR quantitatively showed the altered GRHL2 binding to the p63 promoter in concordance to the GRHL2 protein levels in SCC4 and NHEK (Figure 8D,E). Furthermore, ChiP-qPCR analysis revealed reduced enrichment of trimethylated histone 3 at lysine 4 (H3K4Me3) indicative of active chromatin on the p63 promoter when GRHL2 was knocked down in SCC4 (Figure 8F), in keeping with the loss of p63 expression in SCC4 with GRHL2 silencing. On the contrary, the repressive mark (H3K27Me3) was slightly elevated with GRHL2 knockdown. Since p63 modulation showed regulatory effects on GRHL2 expression, these data indicate a reciprocal regulation between GRHL2 and p63 to establish the epithelial phenotype.

DISCUSSION

Our laboratory previously showed that overexpression of the ΔNp63α isoform conferred EMT phenotype in NHEK in TGF-β dependent manner (4). The current study further showed that ΔNp63β or ΔNp63γ do not fully transform NHEK to mesenchymal state, although there was morphological transition by ΔNp63β expression in NHEK. One of the speculations from our prior study was that the ΔNp63α isoforms abolished the expression of the high molecular weight (~75 kDa;
band marked with an asterisk in Figures 1B, 1C) p63 protein with unknown identity, which invariably occurred during EMT (4). As an alternative approach, in the current study we knocked down all p63 isoforms using pan-p63 siRNA. This transient knockdown of p63 isoforms led to EMT phenotype after 10 days post-transfection. Acquisition of the EMT phenotype in the NHEK/ΔNp63α and NHEK/Si-p63 cells was assessed by altered morphology and expression of the EMT markers, e.g., loss of E-Cad, K14, β-catenin expression and enhanced FN, and ZEB1 expression. Whereas NHEK/ΔNp63α required serial subculture and several passages to induce molecular and phenotypic EMT-associated changes, NHEK/Si-p63 acquired these altered phenotype quite readily within 10 days after the gene knockdown (Figures 1C and 3A).

In our previous paper, we showed that the NHEK/ΔNp63α cells with EMT phenotype acquired stemness characteristics and expressed high levels of the reprogramming factors, e.g., Lin28 and Nanog (4). Likewise, the NHEK/Si-p63 showed stem characteristics, e.g., transdifferentiation ability into osteogenic and adipocytic lineages (data not shown). We also found that NHEK/Si-p63 expressed cell surface markers CD44, CD73, CD90, and CD105, which are characteristically expressed in mesenchymal stem cell (MSC) population (Figure 4) (27,28). Therefore, our studies demonstrate that EMT in NHEK is associated with acquisition of stem characteristics, whether it is triggered by overexpression of ΔNp63α or knockdown of all p63 isoforms. We coined the term “induced mesenchymal stem cells” (iMSCs) to describe these epithelial cells that have acquired multipotency through EMT to distinguish them from endogenous MSCs or induced pluripotent stem cells (iPSCs), which acquire embryonic stem cell characteristics after stable transduction of the reprogramming factors (29). These iMSCs may be derived from cultured primary epithelial cells from skin via transient transfection of p63 siRNA, and may have tissue-engineering potential for cases requiring autologous cell transplantation approach.

The current study reveals GRHL2 as the regulatory partner of p63, which is critical for epithelial cell identity and embryonic morphogenesis (30,31). GRHL2 is a transcription factor responsible for regulating epithelial proliferation (12), and is known to play an important role in inducing many epithelial cancers and has been found to regulate EMT (14,21). The current study showed that EMT in NHEK/ΔNp63α and NHEK/Si-p63 accompanied downregulation of GRHL2 and E-Cad, and upregulation of ZEB1 and N-Cad expression (Figure 6A). GRHL2 has also been shown to suppress EMT in breast cancer cells by suppressing ZEB1 signaling, and a reciprocal feedback loop has been found between GRHL2 and ZEB1 in regulating EMT (25,32). Furthermore, GRHL2 has been found to inhibit ZEB1 expression and EMT by activating miR-200b and miR-200c (32). We found that NHEK/ΔNp63α and NHEK/Si-p63 resulted in downregulation of miR-200 family gene expression, including miR-200a, miR-200b, miR-200c, miR-141, and miR-429 (Figure 6B), and that p63 binds directly to the promoter of miR-200 family genes (Figure 6C). It is important to note that p63 did not bind to the promoter of miR-141, and that miR-141 may be bound and regulated by a downstream target of p63. Thus, p63 modulation may alter miR-200 family gene activation and induce EMT by facilitating upregulation of ZEB1 expression. These findings indicate a novel role for p63 in GRHL2-mediated regulation of EMT. In addition, GRHL2 was found to be required and necessary for the expression of p63. In NHOF, NHOK and SCC9 cells, GRHL2 overexpression led to notable increase in the p63 expression level. Also, GRHL2 knockdown in SCC4 abolished the expression of p63, suggesting p63 regulation by GRHL2. This was confirmed by ChiP analyses showing direct GRHL2 binding on the p63 gene promoter and concordant alteration of the histone mark at the gene promoter. We also demonstrated through dual luciferase reporter assay that GRHL2 is required for p63 promoter activity, and vice versa. Hence, we propose a model in which p63 and GRHL2 regulate each other’s expression, through direct promoter binding, and this reciprocal regulatory loop between GRHL2 and p63 is critical for maintaining the epithelial phenotype through transcriptional regulation of their target genes (Figure 9). This model may provide a framework to study the molecular
mechanism that regulates epithelial plasticity, a key player in physiologic and disease processes, including cancer metastasis, epithelial wound healing, and tissue damage response to environmental insults.

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CONFLICT OF INTEREST
The authors declare no conflict of interest with the content of this article.

AUTHOR CONTRIBUTIONS
SM, WC and MKK planned the study. RHK, KS, NHP, MKK participated in the design of experiments. SM wrote the paper. SM and WC performed and analyzed the experiments shown in Figures 1, 3, 5, 6, and 8. JEO and ZXL performed and analyzed the experiments in Figure 2 and 4. SM, WC, KLK and JKY performed, and analyzed the experiments shown in Figure 7. All authors analyzed the results, revised the manuscript, and approved the final version of the manuscript.

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FOOTNOTES
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2The abbreviations used are: EMT, Epithelial-Mesenchymal Transition; NHEK, Normal Human Epithelial Keratinocyte; FN, Fibronectin; E-Cad, E-Cadherin; K14, Keratin 14; MSC, Mesenchymal Stem Cell; iMSC, induced Mesenchymal Stem Cell; DPSC, Dental Pulp Stem Cell; GRHL2, Grainyhead-like 2; BM-MSC, Bone-Marrow Mesenchymal Stem Cell; SCC, Squamous Cell Carcinoma; NHOK, Normal Human Oral Keratinocytes

FIGURE LEGENDS

FIGURE 1. Overexpression of ΔNp63α in NHEK yields EMT phenotype. (A) NHEK were stably transduced using retroviruses containing an empty vector (LXSN), or ΔNp63 isoforms α, β, or γ. Morphological changes were observed in cells after stable transduction of ΔNp63 isoforms by phase contrast. Change in cell motility was assessed by transwell migration assay. (B) NHEK stably transduced using retroviruses containing an empty vector (LXSN), or ΔNp63 isoforms α, β, or γ were serially subcultured and assessed for change in protein expression of ΔNp63α, ΔNp63β, and ΔNp63γ isoforms, GRHL2, E-Cad and FN at two different population doubling levels (PD 24 and PD28) by Western blotting. Asterisk (*) indicates high molecular weight band of p63. (C) NHEK stably transduced using retroviruses containing an empty vector (LXSN), or ΔNp63α isoforms at PD 22, PD24 and PD28 were assessed for change in protein expression of ΔNp63α (using ΔNp63α specific antibody H129), GRHL2, K14, E-Cad and FN. NHOF was used as a negative control. GAPDH was using as a loading control. Asterisk (*) indicates high molecular weight band of p63.

FIGURE 2. Overexpression of ΔNp63α in NHEK results in EMT molecular profile. Changes in the expression of E-Cad and FN were assessed in NHEK transduced with ΔNp63α, ΔNp63β, or ΔNp63γ by means of fluorescence microscopy. Arrow indicates the NHEK/ΔNp63α cells exhibiting EMT phenotype.

FIGURE 3. Knockdown of p63 in NHEK by transient transfection with p63 si-RNA (Si-p63) results in EMT phenotype. (A) Western blotting was performed using whole cell lysates of NHEK transiently transfected using scramble siRNA (Si-SCR) and NHEK transiently transfected using siRNA targeting p63 (Si-p63) to confirm loss of p63 and E-Cad protein expression, and enhanced FN expression (arrow) consistent with EMT. NHOF was used as a positive control for mesenchymal phenotype. GAPDH was used as a loading control. (B) Morphological changes were observed in Si-SCR and Si-p63 transfected NHEK to determine onset of EMT phenotype. (C) Relative mRNA expression of TAp63, ΔNp63, and GRHL2 was assessed in NHOF, and NHEK transiently transfected using Si-SCR or Si-p63 by qRT-PCR. (D) Stress fiber formation in NHEK/Si-SCR and NHEK/Si-p63 was determined by F-actin staining using Alexa Fluor 594 Phalloidin. (E) Changes in p63, GRHL2, ZEB1, FN, E-Cad and β-catenin expression were determined in NHEK/Si-SCR and NHEK/Si-p63 by immunofluorescent staining.

FIGURE 4. Modulation of p63 results in acquisition of stem-like molecular profile in NHEK. Flow cytometry was performed to assess change in expression of stem cell-associated cell surface markers CD44, CD73, CD90, and CD105 in Parental NHEK, early and late passage Si-p63 (PD 42 and PD 59, respectively). BM-MSC and DPSC were used as positive controls.

FIGURE 5. TGF-β induces EMT phenotype in NHEK. NHEK were cultured in 5 ng/mL TGF-β for 10 days and expression of GRHL2, p63, FN, K14, and β-catenin were assessed by immunofluorescent staining. Cells were counterstained using DAPI.
**FIGURE 6.** Modulation of p63 results in the loss of GRHL2 and miR-200 family expression. (A) NHEK ectopically expressing ΔNp63α and transiently transfected with p63 siRNA were harvested before and after the onset of EMT. Non-transduced/transfected NHEK were used as an epithelial cell control, and NHOF were used as a mesenchyme control. Whole cell lysates were collected, and Western blot was performed for p63, ZEB1, GRHL2, E-Cad, and N-Cad. GAPDH was used as a loading control. (B) Change in expression of miR-200a, miR-200b, miR-200c, miR-141, and miR-429 was assessed by qRT-PCR using QuantiMir cDNA. (C) ChIP assay and ChIP-qPCR was performed to examine binding of p63 to the miR-200a, miR-200b, miR-200c, miR-141, and miR-429 promoter regions in NHOF, parental NHEK, NHEK/ΔNp63α and NHEK/Si-p63 cells showing EMT phenotype.

**FIGURE 7.** Modulation of GRHL2 alters p63 expression and EMT phenotype in human keratinocytes and epithelial cancer cell lines. (A) SCC4 cells were treated with 10 ng/ml TGF-β for 0, 3, 5, and 10 days and harvested. Whole cell lysates were collected, and Western blotting was performed for p63, GRHL2, FN, N-Cad, ZEB1, and Snail. GAPDH was used as a loading control. (B) HaCaT cells were treated with 10 ng/ml TGF-β for 0, 1, 3, 7, and 10 days and harvested. Whole cell lysates were collected, and Western blotting was performed for GRHL2, p63, FN, ZEB-1, and E-Cad. GAPDH was used as a loading control. (C) GRHL2 was overexpressed in NHOF, NHOK, and SCC9, and knocked down in SCC4 cells using shRNA. Whole cell lysates were collected, and Western blot was performed for GRHL2, p63, and E-Cad. GAPDH was used as a loading control. (D) Change in p63, GRHL2, and β-catenin expression was determined in SCC9 overexpressing GRHL2 by immunofluorescence staining. (E) Change in mRNA expression of p63 was assessed in SCC9 overexpressing GRHL2 and SCC4 after GRHL2 knockdown by qRT-PCR. (F) GRHL2 and p63 were independently knocked down in SCC4 and GRHL2 was overexpressed in SCC9, and dual luciferase reporter assay was performed to assess the promoter activity.

**FIGURE 8.** GRHL2 directly binds to and activates p63 promoter in human keratinocytes and epithelial cancer cell lines. (A) ChIP assay was performed to examine binding of GRHL2 to p63 promoter in SCC4 infected with Sh-GRHL2 and (B) in NHEK overexpressing GRHL2. (C) ChIP assay was performed to examine binding of GRHL2 to the hTERT promoter in NHEK with or without GRHL2 overexpression. Pull down using α-IgG was used as a negative control. (D) ChIP-qPCR was performed to quantify enrichment of GRHL2 on the p63 promoter in SCC4 with GRHL2 knockdown and (E) in NHEK overexpressing GRHL2. (F) Enrichment of H3K4Me3 and (G) H3K27Me3 on the p63 promoter in SCC4 with GRHL2 knockdown was also assessed by ChIP-qPCR.

**FIGURE 9.** Role of p63 and GRHL2 in epithelial plasticity. Current data establish a reciprocal regulation between ΔNp63 and GRHL2 through direct promoter binding. Both ΔNp63 and GRHL2 are required for the expression of miR-200 family genes, which then suppress EMT through negative regulation of ZEB1. In this model, GRHL2 appears to be intricately involved with many different factors that determine epithelial phenotype.
p63 and GRHL2 determine epithelial plasticity in human keratinocytes

Figure 1

A

Phase Contrast

Transwell migration

B

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- p63 (H129)
- GRHL2
- K14
- E-Cad
- FN
- GAPDH

* α
Figure 2

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Figure 3

A

B

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Figure 4

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Figure 5

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p63 and GRHL2 determine epithelial plasticity in human keratinocytes
Figure 6

A

B

C

NHEK

p63
GRHL2
E-Cad
N-Cad
GAPDH

miR-200a

miR-200b

miR-200c

miR-141

miR-429

Relative microRNA expression

Relative microRNA expression on p63

Relative microRNA expression on miR-200 genes

Relative microRNA expression on p63

Downloaded from http://www.jbc.org/ by guest on September 1, 2017
p63 and GRHL2 determine epithelial plasticity in human keratinocytes

Figure 7

A

B

C

D

E

F
Figure 8

A

SCC4

p63 promoter

EGFP

Sh-GRHL2

B

NHK

p63 promoter

LXSN

GRHL2

C

NHK

p63 promoter

LXSN

GRHL2

HTERT promoter

D

E

F

G

p63 and GRHL2 determine epithelial plasticity in human keratinocytes
Figure 9
p63 Gene is Regulated by Grainyhead-Like 2 (GRHL2) Through Reciprocal Feedback and Determines Epithelial Phenotype in Human Keratinocytes

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