Transcription Factor p63 Regulates Key Genes and Wound Repair in Human Airway Epithelial Basal Cells

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The airway epithelium in asthma displays altered repair and incomplete barrier formation. Basal cells are the progenitor cells of the airway epithelium, and can repopulate other cell types after injury. We previously reported increased numbers of basal cells expressing the transcription factor p63 in the airway epithelium of patients with asthma. Here we sought to determine the molecular consequences of p63 expression in basal human airway epithelial cells during wound repair. Because at least six isoforms of p63 exist (N-terminally truncated [Δ N] versus transcriptional activation promoter variants and α , β , or γ 3' splice variants), the expression of all isoforms was investigated in primary human airway epithelial cells (pHAECs). We modulated p63 expression, using small interfering RNA (siRNA) and adenoviral constructs to determine the effects of p63 on 21 candidate target genes by RT-PCR, and on repair using a scratch wound assay. We found that basal pHAECs from asthmatic and nonasthmatic donors predominantly expressed the N-terminally truncated p63 α variant (Δ Np63 α) isoform, with no disease-specific differences in expression. The knockdown of Δ Np63, using specific siRNA, decreased the expression of 11 out of 21 genes associated with epithelial repair and differentiation, including β -catenin, epidermal growth factor receptor, and Jagged1. The loss of Δ Np63 significantly inhibited wound closure (which was associated with the decreased expression of β -catenin and Jagged1), reduced epithelial proliferation as measured by Ki-67 staining, and increased E-cadherin expression, potentially preventing cytokinesis. In conclusion, Δ Np63 α is the major isoform expressed in basal pHAECs, and is essential for epithelial wound repair. The role of Δ Np63 α in epithelial barrier integrity requires further study to understand its role in health and disease.

Keywords: p63; airway epithelium; wound repair; asthma

The airway epithelium is the first structural barrier to the inhaled environment. The epithelium of patients with asthma displays several structural and functional abnormalities, including goblet cell metaplasia, a loss of ciliated epithelial cells, increased susceptibility to oxidant-induced stress, abnormal cytokine and extracellular matrix release, and mitotic dyssynchrony (1–5). Our group (6, 7) has also described a marked expansion of the population of basal cells expressing the transcription factor p63 in the epithelium of patients with asthma. Importantly, this phenotype is

conserved when these cells are grown *in vitro* under air–liquid interface (ALI) culture conditions (7), indicating that this expansion is an intrinsic response and not solely a product of an *in vivo* inflammatory milieu.

The transcription factor p63 is a homologue of the p53 tumor suppressor, and is absolutely required for the appropriate development of stratified epithelial tissues. The genetic deletion of p63 in mice results in the absence of stratified epithelia, most notably in the epidermis (8, 9). The subsequent deficiency of barrier function results in perinatal lethality because of dehydration. Similarly, the airway epithelium is the first structural barrier to the inhaled environment, and its ability to repair and regenerate after injury is crucial to maintaining normal tissue function. The basal cells of the airway epithelium exhibit progenitor capacity, and are able to repopulate other cell types, including ciliated and mucus-producing cells, during cellular differentiation (6, 10) and in response to injury (11). In contrast to the normal pseudostratified airway epithelium consisting of basal, ciliated, and mucous cells, the tracheobronchial epithelium of newborn p63^{−/−} mice displays only a single layer of columnar ciliated cells and a complete absence of basal and mucous cells (12). Thus, the lack of stratified epithelium in p63^{−/−} mice has led to two hypotheses, namely, (1) p63 may play a role in maintaining stem-cell populations (8, 13), or (2) p63 may regulate the commitment to specific epithelial lineages (9).

Studies of p63 have demonstrated that the gene can be expressed in at least six different mRNA/protein isoforms (14). The initiation of transcription at the canonical promoter gives rise to isoforms containing the full-length transcriptional activation (TA) domain, whereas initiation from an alternative promoter within intron three produces the N-terminally truncated (Δ N) isoforms (Figure 1A). All isoforms share a common DNA-binding domain (DBD) and an oligomerization domain, which are required for appropriate function (15). Alternative splicing at the 3' end produces transcripts of varying length, termed α , β , and γ . The α and β splice variants are highly similar. The α variant contains only one additional exon, which encodes a sterile alpha motif and a transcription inhibition domain. The γ variant is much shorter and contains a novel 3' end. Two additional 3' variants, δ and ϵ , have been reported, which function similarly to the N-terminally truncated p63 α variant (Δ Np63 α) (16), although most current work remains focused on the α , β , and γ variants. Δ Np63 α is by far the most predominant endogenous isoform in basal cells of stratified epithelial tissues such as the skin (14), cornea (17), and prostate (18), and thus has been the most frequent subject of functional studies. To date, the expression and role of specific p63 isoforms in the maintenance and repair of the human airway epithelium have not been explored.

In this study, we hypothesized that an overabundance of p63 contributes to the observed abnormalities in cellular function and repair observed in the asthmatic epithelium. We sought to determine which specific isoforms of p63 are expressed in the human airway epithelial basal cell, and how these isoforms

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Figure 1. The N-terminally truncated p63 α variant (Δ Np63 α) is the most abundant isoform in human airway epithelial cells *in vitro*. (A) Schematic of p63 isoforms arising from the TP63 gene locus, indicating the two possible promoter variants (transcriptional activation [TA] versus N-terminally truncated [Δ N]), a shared DNA-binding (DBD) and oligomerization (OD) domain, and at least three 3' splice variants (α , β , and γ). The α isoforms contain a sterile α motif (SAM). Approximate binding sites of small interfering (siRNA) to the Δ N, DBD, and α/β domains are indicated. (B) Quantitative RT-PCR for Δ N versus TAp63 mRNA in cultured primary human airway epithelial cells (pHAECs) indicates robust expression of the Δ N but not the TA isoforms ($n = 7$). (C) Semiquantitative PCR for full-length p63 isoforms in cultured pHAECs from $n = 3$ donors indicates that Δ Np63 α/β are the predominant mRNA isoforms. Adenoviral (AdV) overexpression provides a positive control for TAp63 α and TAp63 γ , which were not endogenously detected. Distilled water (dH₂O) was used as a no-template control. (D) Immunoblot for total p63 in monolayer pHAEC cultures (from $n = 5$ each) nonasthmatic (N) and asthmatic (A) donors (left). Adenoviral overexpression of Δ Np63 α , TAp63 α , and TAp63 γ in human

embryonic kidney (HEK) cells (right), included as positive controls. β -tubulin (β -Tub) is included as a loading control. (E) Densitometry analysis of Δ Np63 α protein, followed by a two-tailed, unpaired *t* test, indicates that Δ Np63 α expression is not significantly different between disease groups ($P = 0.394$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

contribute to the regulation of key genes involved in processes such as proliferation and repair.

MATERIALS AND METHODS

Human Airway Epithelial Cell Culture

This study was approved by the Research Ethics Board of the University of British Columbia. Primary human airway epithelial cells (pHAECs) were isolated from nonasthmatic and asthmatic donor lungs deemed unsuitable for transplantation, as described previously (6). In addition, pHAECs were obtained by bronchial brushings of nonasthmatic and asthmatic subjects (19) or from commercial sources (normal human bronchial epithelial cells; Lonza, Basel, Switzerland). pHAECs were maintained in bronchial epithelial growth media (Lonza) and supplemented with antibiotics/antimycotics (Gibco, Burlington, ON, Canada). Experiments were performed on pHAECs at Passage 2 or 3. A549 cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% FBS and antibiotics (all from Gibco). Minimally immortalized bronchial epithelial cells (HBEC6-KTs) were generously provided by Dr. John Minna (20) and maintained in keratinocyte serum-free media (Invitrogen, Burlington, ON, Canada) with growth supplements and antibiotics. Experiments were performed on HBEC6-KTs at Passages 12 and 13. For full culture conditions, see the online supplement.

Small Interfering RNAs and Adenoviruses

Small interfering RNAs (siRNAs) were designed to target specific domains of p63. Sequences can be found in the online supplement. pHAECs were transfected with 50 nM siRNA using HiPerfect (5% vol/vol; Qiagen, Valencia, CA), according to the manufacturer's instructions.

Adenoviruses encoding Δ Np63 α (21), TAp63 α , TAp63 γ , and β -galactosidase (LacZ) (22) were kindly provided by Dr. Wendy Weinberg. pHAECs were infected at 70% confluence at a multiplicity of infection (MOI) of 0.15 plaque-forming units (pfu) per cell. A549 cells were infected at 30% confluence with an MOI of 1.0 or 3.0 pfu/cell. Samples were collected after 48 hours (RNA) or 72 hours (protein).

Gene Expression Analysis

RNA was extracted using an RNeasy Mini Kit (Qiagen) and reverse-transcribed. Gene expression was determined by TaqMan quantitative RT-PCR, according to the manufacturer's instructions (Applied Biosystems, Burlington, ON, Canada) or by semiquantitative PCR. Refer to the online supplement for primers and conditions.

Epithelial Gene Expression Array

Five hundred nanograms of total RNA were used to synthesize cDNA for use on custom-designed, preloaded RT² Profiler Arrays (SABiosciences, QIAGEN, Valencia, CA) containing quantitative RT-PCR primers for

TABLE 1. DONOR DEMOGRAPHICS OF PHAEC CULTURES USED FOR IMMUNOBLOTTING OF p63 ISOFORMS

Donor	Sex	Age (yr)*	Asthma Status†	Sample Type	Cause of Death
N1	F	26	None; PC ₂₀ > 8.0	Bronchial brushing	
N2	M	20	None	Donor lung	Head trauma
N3	F	23	None; PC ₂₀ > 8.0	Bronchial brushing	
N4	F	47	None	Donor lung	Head trauma
N5	M	29	None; PC ₂₀ > 8.0	Bronchial brushing	
A1	F	23	PC ₂₀ = 0.757	Bronchial brushing	
A2	F	23	PC ₂₀ = 0.25	Bronchial brushing	
A3	M	11	Diagnosed; age 2 years	Donor Lung	Anoxia; asthma
A4	F	29	PC ₂₀ = 0.42	Bronchial brushing	
A5	M	25	Diagnosed; age 11 years	Donor lung	Anoxia; asthma

Definition of abbreviations: A, asthmatic; F, female; M, male; N, nonasthmatic; PC₂₀, provocative concentration of methacholine causing a 20% fall in FEV₁; PHAEC, primary human airway epithelial cell.

* Unpaired, two-tailed *t* test confirmed no significant difference in mean age between disease groups (*P* = 0.267).

† For cells obtained by bronchial brushing, methacholine challenge data either confirmed (PC₂₀ < 1.0 mg/ml) or excluded (PC₂₀ > 8.0 mg/ml) a diagnosis of asthma.

21 potential p63-regulated genes. Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase, and subsequently to untreated control values. Data are expressed as means ± SEMs between all experiments.

Wound Repair

HBEC6-KT cells were seeded in four-well chamber slides and transfected at 70% confluence with 25 nM siRNA and 3% vol/vol HiPerfect. After 24 hours, cells were scratch-wounded in a cross pattern, rinsed, and retransfected. Wounds were photographed at 0, 4, 8, and 24 hours. Repair was quantified using ImagePro Plus software (Media Cybernetics, Rockville, MD). Samples were then either lysed for quantitative RT-PCR, the immunoblotting of proteins of interest, or fixed for 20 minutes in 4% paraformaldehyde to determine proliferation. A full description is presented in the online supplement.

Statistical Analysis

One-way ANOVA followed by the Dunnett *post hoc* test was used to assess statistical significance of treatment conditions versus control conditions (Prism version 5.0; GraphPad Software, La Jolla, CA) for multiple conditions. A two-tailed, unpaired *t* test was used for two-category comparisons. *P* < 0.05 was considered statistically significant.

RESULTS

ΔNp63α Is the Most Abundant p63 Isoform in Human Airway Epithelial Basal Cells

When submerged in culture, airway epithelial cells form a relatively homogenous cuboidal monolayer that is representative of basal cells within the airway epithelium. We have shown that p63 is only expressed in basal cells identified by positive staining for cytokeratin-5, CD151, and tissue factor (6). Given the multiple possible isoforms of p63, we first needed to determine the relative contributions of the ΔN and TA isoforms in pHAECs. Using quantitative PCR, we show abundant expression of ΔNp63 in pHAECs, whereas TAp63 expression is virtually undetectable (Figure 1B). We then used semiquantitative PCR to detect the presence of full-length mRNA isoforms of p63 in pHAECs (Figure 1C). As shown in Figure 1A, to design primers that are specific for the p63β isoform is not possible, so we used primers that were specific for both the p63α/β isoforms and γ isoforms. Using this strategy, we determined that the most predominant isoforms expressed were ΔNp63α/β, followed by ΔNp63γ. Although TAp63α/β was detected, it was at a very low level even after 40 cycles of amplification, which is in agreement with our quantitative PCR data for the TA isoform. However, endogenous TAp63γ mRNA was not detected. As TAp63

isoforms were expressed at such low levels, we used the adenovirus-mediated overexpression of TAp63α or TAp63γ in pHAECs as positive controls, and were able to detect robust expression, indicating that endogenous levels of TAp63 isoforms are indeed extremely low in pHAECs.

We then used immunoblotting to confirm our mRNA findings and demonstrate that the ΔNp63α/β isoform is the most abundantly expressed protein isoform in basal pHAECs (Figure 1D, left). The adenovirus-mediated overexpression of ΔNp63α, TAp63α, and TAp63γ in human embryonic kidney cells are included as positive controls and for molecular weight comparisons (Figure 1D, right), indicating that although our system was also able to detect TAp63, the endogenous levels were too low to detect in pHAECs.

We have previously shown that the number of basal epithelial cells expressing p63 is elevated in asthmatic compared with nonasthmatic donors, both in the airway and in ALI cultures (7). To determine whether the expression of p63 was altered in a homogenous basal culture system, we compared the expression of ΔNp63α protein in monolayer cultures of pHAECs derived from nonasthmatic (N) and asthmatic (A) donors (Figure 1D; see Table 1 for patient data). In contrast to our findings in differentiated airway epithelium, no significant difference in the expression of ΔNp63α protein was evident between basal pHAECs derived from asthmatic and nonasthmatic donors, as measured by densitometry (Figure 1E; *P* = 0.394).

Knockdown of ΔNp63α Decreases the Expression of Genes Involved in Epithelial Differentiation and Repair

To understand the effects of loss of function of ΔNp63α in pHAECs, we designed siRNAs to target different regions of

TABLE 2. DONOR DEMOGRAPHICS OF NONASTHMATIC PHAEC CULTURES USED FOR EPITHELIAL GENE EXPRESSION ARRAY

Donor	Sex	Age (yr)	Sample Type*	Cause of Death
N1	M	20	Donor lung	Head trauma
N2	F	20	Donor lung	Head trauma
N3	M	21	NHBEs (Lonza, Basel, Switzerland)	
N4	F	23	Bronchial brushing	
N5	M	47	Donor lung	Head trauma

Definition of abbreviations: F, female; M, male; N, nonasthmatic; NHBEs, normal human bronchial epithelial cells; PHAEC, primary human airway epithelial cell.

* Airway epithelial cells were obtained from the upper airways of human lungs deemed unsuitable for transplantation, from bronchial brushing or commercially available NHBEs. All donors lacked doctor-diagnosed asthma.

TABLE 3. EPITHELIAL GENE EXPRESSION ARRAY OVERVIEW: FOLD CHANGE IN GENE EXPRESSION AFTER 48-HOUR TREATMENT WITH siRNA TO Δ N OR α/β p63 ISOFORMS

Gene	Δ N siRNA		α/β siRNA	
	Fold Change $\pm$ SEM*	P Value†	Fold Change $\pm$ SEM	P Value
CTNNB1	0.71 $\pm$ 0.04	< 0.001	0.64 $\pm$ 0.06	< 0.001
LAMC2	0.46 $\pm$ 0.07	< 0.001	0.68 $\pm$ 0.05	< 0.01
TJP1	0.62 $\pm$ 0.02	< 0.001	0.62 $\pm$ 0.07	< 0.001
CD44	0.68 $\pm$ 0.05	< 0.01	0.64 $\pm$ 0.08	< 0.001
EGFR	0.40 $\pm$ 0.15	< 0.01	0.50 $\pm$ 0.06	< 0.05
EP300	0.66 $\pm$ 0.06	< 0.01	0.54 $\pm$ 0.07	< 0.001
F11R/JAM1	0.57 $\pm$ 0.07	< 0.01	0.56 $\pm$ 0.07	< 0.001
GSK3B	0.74 $\pm$ 0.05	< 0.01	0.81 $\pm$ 0.06	< 0.01
PLEC1	0.50 $\pm$ 0.08	< 0.05	0.50 $\pm$ 0.10	< 0.05
CCND1	0.84 $\pm$ 0.06	< 0.05	0.77 $\pm$ 0.11	< 0.01
JAG1	0.48 $\pm$ 0.10	NS	0.39 $\pm$ 0.09	< 0.05
CDKN1A	0.86 $\pm$ 0.07	NS	0.91 $\pm$ 0.08	NS
CEBP	1.14 $\pm$ 0.18	NS	0.92 $\pm$ 0.09	NS
KRT18	0.68 $\pm$ 0.07	NS	0.69 $\pm$ 0.10	NS
KRT5	0.57 $\pm$ 0.07	NS	0.52 $\pm$ 0.13	NS
CLDN1‡	0.95 $\pm$ 0.05	< 0.001	0.66 $\pm$ 0.08	P < 0.001
NOTCH2‡	0.83 $\pm$ 0.04	< 0.001	0.75 $\pm$ 0.06	P < 0.001
BMP7	Low expression	Low expression		
EGF	Low expression	Low expression		
FGFR2	Low expression	Low expression		
WNT1	Low expression	Low expression		

Definition of abbreviations: Δ N, N-terminally truncated; NS, no significance; siRNA, small interfering RNA.

*Values expressed represent fold change $\pm$ SEM, relative to untreated control samples ($n = 5$ donors).

† One-way ANOVA, followed by the Dunnnett *post hoc* test, was used to assess statistical significance versus scrambled siRNA construct.

‡ Indicates genes that were significantly altered by scrambled siRNA treatment versus untreated control samples.

this mRNA, including the 5' (Δ N), DNA-binding (DBD), and 3' α/β domains of this isoform. The knockdowns using Δ N, DBD, or α/β siRNA each significantly decreased total Δ Np63 mRNA expression in pHAECs ($P < 0.001$; Figure 2A), but did not significantly affect TAp63 mRNA expression (Figure 2B). We next determined the impact of Δ Np63 mRNA knockdown on Δ Np63 α protein expression by immunoblotting (Figure 2C). The densitometry analysis of three independent experiments showed a significant reduction in Δ Np63 α protein expression after transfection with each of the siRNAs ($P < 0.01$; Figure 2D). Given that Δ N and α/β are most specific for the Δ Np63 α isoform, we used these siRNA constructs to understand which candidate target genes are regulated by Δ Np63 α in pHAECs from nonasthmatic donors ($n = 5$; see Table 2 for patient data).

To determine the impact of Δ Np63 knockdown on genes involved in key epithelial differentiation and repair functions, we designed an array of PCR primers for 21 genes (Table 3) that have been shown to be regulated by p63 in other epithelial tissues and/or that contain a p63 response element in their promoter region (according to the p53 FamTaG database) (23). We found that 11 of 21 candidate target genes were significantly down-regulated by the knockdown of Δ Np63 α with either the Δ N or α/β siRNA constructs (Table 3), compared with the scrambled control samples. Interestingly, we found no genes that showed increased mRNA expression after the knockdown of Δ Np63 α . As demonstrated by three of the 11 p63-regulated genes, CTNNB1 (β -catenin; Figure 2E), epidermal growth factor receptor (EGFR; Figure 2F), and JAG1 (Jagged1; Figure 2G), both the Δ N and α/β siRNA constructs induced similar mRNA down-regulation, indicating that Δ Np63 α/β is the predominant isoform of Δ Np63 inducing this phenotype. These three genes in particular were selected according to their potential contributions to epithelial differentiation and repair in asthma. β -catenin is a subunit of the adherens junction that anchors the actin cytoskeleton to regulate cell growth and adhesion between cells, and can also function as a transcriptional

coactivator (24, 25). EGFR regulates responses to the epithelial mitogen epidermal growth factor, and is crucially involved in the process of repair in the epithelium (26, 27). JAG1 encodes the developmental gene Jagged1, a ligand for the Notch family of proteins that is involved in cell-fate determination in tissue-resident progenitor cells (28).

Overexpression of Δ Np63 α Alters the Expression of Genes Involved in Epithelial Function

Having shown that the knockdown of Δ Np63 α/β reduces the mRNA expression of β -catenin, EGFR, and Jagged1, we investigated the effects of overexpressing Δ Np63 α using adenoviral vectors (21). Infection with adenovirus encoding Δ Np63 α but not LacZ (MOI = 0.15 pfu/cell) led to a statistically significant increase in Δ Np63 mRNA after 48 hours (Figure 3A; $P < 0.01$) and an up-regulation of Δ Np63 α protein, as shown by immunoblot after 72 hours (Figure 3B). When quantified by densitometry, Δ Np63 α adenovirus infection resulted in a statistically significant eightfold increase in Δ Np63 α protein expression (Figure 3C; $P < 0.001$). Surprisingly, the analysis of mRNA expression for any of the three candidate genes (β -catenin, EGFR, or Jagged1) showed no significant changes with Δ Np63 α overexpression (Figures 3D–3F). Because endogenous Δ Np63 α is highly expressed within basal pHAECs, we hypothesized that the lack of effect on target gene expression was attributable to the saturation of Δ Np63 α signaling, despite further exogenous Δ Np63 α expression. To test this hypothesis, we infected an alveolar Type II cell line (A549) that lacks endogenous Δ Np63 α with Δ Np63 α -encoding adenovirus. Infection with Δ Np63 α -encoding adenovirus at an MOI of 3.0 pfu/cell significantly induced Δ Np63 mRNA expression in A549 cells (Figure 4A; $P < 0.001$), to a level comparable to that observed after infection with an MOI of 0.15 pfu/cell in pHAECs (as seen in Figure 3A). Δ Np63 α protein expression (Figure 4B) was also induced by Δ Np63 α adenovirus in A549 cells. Importantly, the adenovirus-mediated overexpression of Δ Np63 α (MOI 3.0 pfu/cell) in A549

Figure 2. Loss of p63 significantly down-regulates genes involved in epithelial function. (A) Quantitative RT-PCR of Δ Np63 mRNA in pHAECs, 48 hours after transfection with siRNA to N-terminally truncated (Δ N), DNA binding (DBD), or α/β domains of p63, indicates significantly decreased expression of Δ Np63 with each of the p63-specific siRNAs, but no change with HiPerfect (Hip) transfection reagent or scrambled siRNA (Scr) construct. Con, control. (B) Quantitative RT-PCR of TAp63 mRNA in pHAECs indicates no change at 48 hours after transfection with any siRNA constructs. (C) Representative immunoblot of Δ Np63 α protein, 72 hours after transfection with 50 nM siRNA to Δ N, DBD, or α/β domains of p63. (D) Densitometry analysis of Δ Np63 α expression in $n = 3$ pHAEC experiments. Values were normalized to β -tubulin as a loading control, and are shown as fold change over untreated control values (denoted with dashed line). (E–G) Quantitative RT-PCR of candidate p63 target genes by epithelial gene expression array, 48 hours after the knockdown of Δ N or α/β isoforms of p63 in $n = 5$ pHAECs. (E) β -catenin. (F) Epidermal growth factor receptor (EGFR). (G) Jagged1. Gene ex-

pression (relative to glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) is shown as fold change over untreated control condition (dashed line). One-way ANOVA with the Dunnett *post hoc* test was used to assess statistical significance between treatments and scrambled siRNA control. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

cells significantly increased the expression of β -catenin (Figure 4C; $P < 0.01$), EGFR (Figure 4D; $P < 0.001$), and Jagged1 (Figure 4E; $P < 0.001$) mRNA, compared with LacZ-infected conditions, supporting our hypothesis that Δ Np63 α is indeed a positive regulator of these three target genes in human pulmonary epithelial cells.

Loss of Δ Np63 Significantly Impairs Epithelial Cell Wound Repair

Having shown that Δ Np63 α regulates several genes known to affect epithelial function, we wanted to determine whether the knockdown of Δ Np63 α/β isoforms affects epithelial wound repair. For these experiments, we used minimally immortalized HBEC6-KT cells, which, like pHAECs, express predominantly Δ Np63 α , which is responsive to the knockdown of protein expression using either the Δ N or α/β siRNA constructs (Figure 5A). We used a scratch wound repair model and, as shown by representative light microscopy images (Figure 5B) of HBEC6-KT cultures at time 0 and 24 hours after wounding, Δ Np63 siRNA constructs noticeably slowed wound repair. Further, by quantifying repair over time, we demonstrate that although control or scrambled siRNA-treated cultures were equally able to reconstitute more than 90% of the wound area within 24 hours,

the knockdown of total Δ Np63 inhibited wound closure to only $71\% \pm 5\%$ (Figure 5C; $P < 0.01$). In contrast, specific knockdown with the α/β siRNA construct did not significantly alter wound repair. To confirm that the knockdown of Δ Np63 α in wounded HBEC6-KT cells still down-regulated the candidate genes identified in Figure 2, we performed quantitative RT-PCR. We demonstrated that the knockdown of Δ Np63 α , using either Δ Np63 or α/β siRNA constructs, maintained the down-regulation of Δ Np63 (Figure 4D), β -catenin (Figure 4E), and Jagged1 (Figure 4G) mRNA 24 hours after wounding. EGFR mRNA was reproducibly down-regulated by Δ N or α/β siRNA constructs (Figure 4F), but this did not reach statistical significance.

To determine whether the decreased repair induced by decreased Δ Np63 expression was attributable to effects on proliferation, we examined the expression of the proliferation marker Ki-67 (Figure 6A), 24 hours after wounding. The knockdown of Δ Np63 significantly reduced the number of Ki-67–positive cells (Figure 6B; $P < 0.05$), but the use of the α/β siRNA construct did not. Interestingly, although Ki-67 expression is generally thought to be restricted to the nucleus (Figure 6B, *gray arrow*), we observed abundant cytoplasmic staining (Figure 6B, *red arrow*). As such, we separately quantified the expression of

genes, 48 hours after adenoviral transduction in $n = 4$ pHAECs. (D) β -catenin. (E) Epidermal growth factor receptor. (F) Jagged1. Statistical significance was assessed using an unpaired two-tailed t test between LacZ and Δ Np63 α adenovirus treatment. Dashed line represents values in untreated control samples. ** $P < 0.01$. *** $P < 0.001$.

nuclear (Figure 6B, gray bars) and cytoplasmic (Figure 6B, red bars) Ki-67 expression, and demonstrated that knockdown with the Δ Np63 siRNA construct primarily reduced cytoplasmic staining compared with control samples ($P < 0.05$), and this accounted for the significant loss of Ki-67.

Because both proliferation and migration occur during wound repair, and have been shown to be linked to cell adhesion,

we quantified the expression of the adherens junction protein E-cadherin according to immunoblotting (Figure 6C), and found that Δ Np63 siRNA significantly increased E-cadherin protein expression compared with scrambled control samples ($P < 0.05$), suggesting that Δ Np63 is a negative regulator of E-cadherin. After scratch wounding, E-cadherin protein expression was up-regulated, but this was not statistically significant. We propose

Figure 4. Exogenous Δ Np63 α significantly induces expression of candidate gene expression in p63-null A549 cells. (A) Quantitative RT-PCR analysis of Δ Np63 expression, 48 hours after transduction with adenovirus expressing LacZ or Δ Np63 α at MOI = 1.0 or 3.0 pfu/cell in p63-null A549 cells ($n = 6$), demonstrates negligible endogenous Δ Np63 mRNA, but a significant up-regulation of Δ Np63 mRNA after treatment with Δ Np63 α adenovirus, as assessed by one-way ANOVA with the Dunnett *post hoc* test. (B) Representative immunoblot for total p63 protein, 72 hours after adenoviral infection, indicates expression of Δ Np63 α protein only in the presence of Δ Np63 α adenovirus. β -tubulin is included as loading control. (C–E) Quantitative RT-PCR for target gene mRNA, 48 hours

after the adenoviral (AdV) transduction of A549 cells with LacZ or Δ Np63 α (MOI = 3.0 pfu/cell), indicates a significant up-regulation of all target genes by Δ Np63 α . (C) β -catenin. (D) EGFR. (E) Jagged1. Expression data were normalized to GAPDH for $n = 6$ experiments. Dashed line represents values in untreated control samples. Statistical significance was assessed using an unpaired, two-tailed t test between LacZ and Δ Np63 α adenovirus treatment. ** $P < 0.01$. *** $P < 0.001$.

Figure 5. Loss of ΔNp63 significantly impairs repair in scratch-wounded monolayer cultures of human bronchial epithelial cells. (A) Representative immunoblot of monolayer culture of minimally immortalized bronchial epithelial cells (HBEC6-KT) cells, 72 hours after transfection with 25 nM p63-specific siRNA, indicates decreased ΔNp63α protein expression. (B) Phase-contrast images of confluent monolayer cultures of HBEC6-KT cells at time of scratch wounding (0 h) and after 24 hours (24h) indicate repair progress in the presence of scrambled, N-terminally truncated (ΔN), or α/β siRNA constructs. White lines denote edges of wound areas. Images are representative of $n = 6$ independent experiments. (C) Analysis of wound repair in $n = 6$ independent experiments at 0, 4, 8, and 24 hours after scratch wounding. Wound repair was calculated as percent area reconstituted compared with the initial wound area (time 0), where the horizontal dashed line indicates 100% closure. Statistical significance was assessed at each time point using one-way ANOVA with the Dunnett *post hoc* test versus Scr siRNA (Scr si) conditions. ΔN si, ΔN siRNA. (D–G) Quantitative RT-PCR of target gene mRNA, 24 hours after media change or scratch wounding in the presence of scrambled, ΔN, or α/β siRNA, indicates sustained down-regulation of (D) ΔNp63, (E) β-catenin, (F) EGFR, and (G) Jagged1 mRNA expression. Statistical significance was assessed at each time point, using one-way ANOVA with the Dunnett *post hoc* test versus Scr siRNA conditions. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

that, because E-cadherin contains a p63 response element and its loss and redistribution are essential for cytokinesis during epithelial cell proliferation and for the dissolution of adherens junctions during migration, a loss of ΔNp63 leads to the increased expression of E-cadherin, and as a consequence, a less migratory and proliferative cell phenotype.

DISCUSSION

We and others have previously shown that the expression of the transcription factor p63 is confined to the basal cells of the airway epithelium (6, 12). Here we demonstrate that

ΔNp63 is the exclusive promoter isoform expressed, and more specifically that the ΔNp63α splice variant is the most abundantly expressed isoform in human airway epithelial basal cells. We show that the loss of function of ΔNp63α, using targeted siRNA, induces a significant decrease in the expression of a number of genes involved in epithelial differentiation and repair. Further, we demonstrate a strong role for ΔNp63 in epithelial repair through the regulation of epithelial proliferation and E-cadherin expression during wound closure *in vitro*. These data support our hypothesis that ΔNp63α is an important regulatory protein in the human airway epithelial cell.

Figure 6. Loss of Δ Np63 significantly impairs epithelial proliferation in scratch-wounded monolayers of human bronchial epithelial cells. (A) Immunocytochemistry for proliferation marker Ki-67 (brown) indicates both nuclear (gray arrow) and cytoplasmic (red arrow) staining. Nuclei are counterstained with Harris hematoxylin (blue staining). (B) Quantification by manual point-counting of Ki-67 nuclear (gray bars) and cytoplasmic (red bars) staining was performed in five images from each of $n = 3$ independent experiments, 24 hours after wounding, and indicates significantly decreased Ki-67 expression with Δ Np63 siRNA treatment. Dashed lines represent untreated control conditions. (C) Densitometry quantification of $n = 3$ immunoblots for E-cadherin protein, 24 hours

after media change or scratch-wounding of confluent HBEC6-KT monolayers pretreated with N-terminally truncated (Δ N), DBD, or α/β siRNA constructs, indicates a significant up-regulation of E-cadherin protein with the Δ Np63 siRNA construct. Statistical significance was assessed using one-way ANOVA with the Dunnett *post hoc* test versus Scr si conditions. $*P < 0.05$.

This finding of the predominance of Δ Np63 α in airway epithelial basal cells, which we and others have shown to be the progenitor cells of the airway epithelium (6, 10), is supported by studies demonstrating the expression of Δ Np63 α in the basal cells of several nonpulmonary epithelial tissues (specifically skin, prostate, cornea, and breast) (8, 17, 18, 29). The role of Δ Np63 in maintaining the basal-cell population within epithelial tissues has been supported by several homozygous null p63 murine studies that demonstrated a striking lack of epithelial tissues and, in particular, the epidermis (8, 9). Further, Candi and colleagues found that complementation of the p63 null background with Δ Np63 was able to rescue the basal-cell layer of the epidermis (30). Finally, the recent study from Romano and colleagues, in which the specific knockout of Δ Np63 was generated, found that these mice phenocopied the epidermal abnormalities of the total p63 knockout, indicating the crucial role of Δ Np63 in the development and maintenance of epithelial tissues (31). Interestingly, we have shown that the number of basal cells expressing Δ Np63 is significantly greater in the epithelium of patients with asthma *in vivo* (7), which may indicate a defect in the normal mechanism of epithelial repair and/or differentiation. We therefore wanted to determine the expression levels of each p63 isoform in pHAECs from asthmatic and nonasthmatic donors. When cultured as a submerged monolayer culture, pHAECs form a cuboidal epithelium consisting of relatively undifferentiated basal cells (32). When we measured the expression of Δ Np63 isoforms, we found that Δ Np63 α is the predominant isoform in basal pHAECs, with no differences in expression between asthmatic and nonasthmatic subjects. These data indicate that although the number of basal cells expressing Δ Np63 α is elevated in patients asthma compared with nonasthmatic subjects, the total protein levels of Δ Np63 α do not differ in basal cells from diseased donors. In addition, our finding that TAp63 mRNA expression is exceptionally low ($\sim 2,000$ -fold less than the Δ N promoter variant, whereas protein expression was not detectable) is consistent with previous studies demonstrating that TAp63 is not expressed in noncancerous adult epithelial basal

cells (33, 34). Thus, Δ Np63 α is the most abundant isoform expressed in human airway epithelial basal cells.

We then further investigated the role of Δ Np63 α in epithelial repair by modulating its expression, using pHAECs from non-asthmatic donors. Epithelial repair is a highly integrated series of processes that includes migration, proliferation, and differentiation. As such, we selected 21 candidate target genes involved in these processes, based on the knowledge that they have been shown to be regulated by p63 in other epithelial tissues (21, 33, 35–38) and/or to contain a p63-response element in their promoter region (23). We found that 11 of 21 candidate genes were significantly down-regulated by the knockdown of either Δ N or α/β p63 isoforms (Table 3; CTNNB1, LAMC2, TJP1, CD44, EGFR, EP300, F11R/JAM1, GSK3B, PLEC1, CCND1, and JAG1), in pHAECs. Further, four genes showed no significant differences after siRNA treatment (CDKN1A, CEBPA, KRT18, and KRT5). Interestingly, of these, CDKN1A (p21^{WAF1}) is known to be directly repressed by Δ Np63 α in keratinocytes (39), and thus we would have expected increased expression with the loss of Δ Np63 α . This mechanism of regulation may not be predominant in airway epithelial cells, or may be a consequence of pro-proliferative growth conditions. Our model also involved adult-derived pHAECs. This may partly explain why we found four genes involved in development (BMP7, EGF, FGFR2, and WNT1) to be expressed at very low levels.

Because the asthmatic epithelium is widely accepted to display abnormal differentiation and repair, we subsequently focused our efforts on three candidate genes involved in these processes: CTNNB1 (β -catenin), EGFR, and JAG1 (Jagged1). Although β -catenin has been shown to be dispensable in the repair of mouse airway epithelium after naphthalene-induced injury (40), the inhibition of β -catenin in cytokeratin 14-expressing basal cells significantly delayed normal epithelial repair processes after chemical injury (41), and the activation of β -catenin in these cells increased proliferation. The expression of EGFR has been shown to be increased in the airway epithelium of patients with asthma (26), suggesting an important role for this receptor in epithelial repair.

In addition, Δ Np63 α has been shown to be a direct transcriptional activator of EGFR expression in pancreatic cancer cell lines (43) and keratinocytes (43). Reciprocally, p63 may also be regulated by EGFR (44), providing a further rationale for its inclusion in this analysis. Jagged ligands are involved in epithelial differentiation and repair through signaling through Notch family ligands. Jagged1 has also been shown to be directly transcriptionally regulated by p63 in keratinocytes (43). Jagged1 is also a β -catenin-dependent gene. Therefore, its upstream regulation by p63 may have implications for dysregulated epithelial wound repair responses in asthma (3).

The adenoviral-mediated expression of Δ Np63 α in pHAECs exerted no effect on target gene expression, despite a significant increase in the expression of Δ Np63 α itself. This surprising finding led us to postulate that endogenous levels of Δ Np63 α were constitutively high or not modifiable. Indeed, this turned out to be the case, because infection of the alveolar Type II cell line (A549) that lacks p63 expression induced a significant increase in all target genes, verifying that p63 indeed regulates these targets. In support of this, King and colleagues demonstrated that the overexpression of Δ Np63 α was able to maintain primary murine keratinocytes in a basal phenotype (38), and the Δ N domain appeared to be sufficient to maintain cells in a proliferative state. These findings highlight the idea that endogenous levels of Δ Np63 α in pHAECs in monolayer culture may in fact be sufficient for target gene induction, but further increases in expression may exert no additive effect. Interestingly, a loss of p63 expression in pHAECs did not completely abrogate the expression of β -catenin, EGFR, and Jagged1, indicating the existence of other transcriptional regulators of these genes. β -catenin is known to be regulated by the Wnt ligands at the protein level and at the transcriptional level by the TCF4 transcription factor and by β -catenin protein itself, as has been shown during the migration of cancer cells (45). EGFR is known to be regulated by the mitogen EGF, and also by multiple other growth factors. Jagged1 expression is regulated by Notch signaling, and interestingly is also significantly regulated by Wnt/ β -catenin/TCF signaling (46). Chromatin immunoprecipitation experiments in human keratinocytes previously demonstrated that p63 can directly bind to the EGFR and Jagged1 promoters (43). In contrast, the mechanism of action by p63 on β -catenin expression is somewhat less clear, because it may function either by forming protein complexes with β -catenin, suggesting that Δ Np63 acts as a regulator of β -catenin signaling through nonpromoter binding properties (47), or by complexing with the TCF/LEF family of Wnt-related transcription factors, to recruit transcriptional modifiers to Wnt-responsive genes, such as β -catenin, as shown in urinary bladder carcinoma cells (24). Many factors can contribute to the ability of a transcription factor to bind a given promoter, including epigenetic mechanisms such as promoter methylation, histone modifications, microRNAs, and additional transcription factors. Although the elucidation of specific regulatory mechanisms of Δ Np63 α in pHAECs would be of interest, these experiments are beyond the scope of the present study.

To examine the functional involvement of Δ Np63 α in epithelial repair, we scratch-wounded minimally immortalized human bronchial epithelial cell (HBEC6-KT) monolayers after the knockdown of Δ Np63, and found that this significantly compromised the capacity of airway epithelial cells to undergo repair. In contrast, in a scratch-wound model using immortalized keratinocytes, the loss of Δ Np63 α by siRNA treatment increased the migration of cells into the wounded area, independent of proliferation (48), a finding that has also been reported *in vivo* (49). However, because p63 has been shown to exhibit distinct cell-type specificity, we believe that Δ Np63 α is in fact

necessary for repair in airway epithelial basal cells, primarily by regulating cellular proliferation, although specific targeting of the α/β isoforms did not significantly affect wound repair. Two explanations may account for this: first, TAp63 α may be induced upon wounding, and may exert an inhibitory effect on repair, as has been shown in a murine deep-skin wound model (50). However, we did not observe any induction of TAp63 mRNA after wounding (data not shown). The second possibility states that Δ Np63 γ may be involved in wound repair although its role appears to be cell-type specific. Indeed, in mammary epithelial cells, the loss of Δ Np63 α/β allowed Δ Np63 γ to confer a more migratory phenotype, leading to epithelial-mesenchymal transition (EMT) (51). However, in human epidermal keratinocytes, the overexpression of Δ Np63 α itself resulted in enhanced transforming growth factor (TGF)- β 1-induced EMT (52). We have previously shown that Δ Np63-expressing airway epithelial basal cells are highly susceptible to TGF- β 1-induced EMT (7). In this scenario, the expression of Δ Np63 γ may confer a degree of cell plasticity, enabling repair via epithelial migration or EMT-like effects. However, because we did not observe enhanced repair beyond or even to the same degree as that under control conditions, Δ Np63 γ alone is unlikely able to compensate for the Δ Np63 α -dependent loss of proliferation.

We tested the effects of Δ Np63 knockdown on wound repair by examining the expression of Ki-67, which has been shown to be a specific marker of proliferation. Interestingly, we saw the vast majority of Ki-67 staining within the cytoplasm, with lesser amounts in the nuclei of cells. The cytoplasmic expression of Ki-67 has been documented previously, although the functional effect of this expression remains unclear. For example, cytoplasmic Ki-67 in breast cancer biopsies has been found to correlate with worse prognoses (53), but it has also been described as a disease-independent phenomenon in rat atrial cells undergoing postnatal remodeling (54). Whether our finding is physiological or pathological in nature remains unclear at this time, but it is noteworthy that we observed dramatically decreased cytoplasmic Ki-67 expression in response to a loss of Δ Np63, indicating that wound closure was inhibited because of decreased proliferation.

The process of wound repair requires epithelial cells to both proliferate and migrate. E-cadherin is known to be involved in the maintenance of adherens junctions that tether the actin cytoskeleton between adjacent cells. Thus the loss of E-cadherin expression is required for cytokinesis during proliferation (55) and to permit cell migration (56). We found that Δ Np63 siRNA significantly up-regulated E-cadherin protein expression, but α/β siRNA did not. These data support our finding that the knockdown of Δ Np63 was able to prevent wound repair significantly, which we propose could be attributable to enhanced E-cadherin expression. Also in agreement with this finding, we have previously shown both increased Δ Np63 (57) and decreased E-cadherin (58) in the asthmatic airway epithelium *in vivo* and in ALI culture *in vitro*, indicating the potential for a negative regulation of E-cadherin by Δ Np63 α .

In this study, we have attempted to elucidate the roles of Δ Np63 α in pHAECs. We have focused here on the use of epithelial cells in a monolayer culture, which results in a relatively homogenous model consisting of cells with a basal-like phenotype, rather than on the use of differentiated cell culture models. Although this may seem a limitation with regard to *in vivo* comparisons, we believe that a monolayer culture offers the most appropriate model, because Δ Np63 α is known to be expressed primarily in basal cells *in vivo*. In addition, studies of isoform-specific functions are somewhat limited by the high degree of shared domains between various isoforms. As such, targeting a specific region of the p63 molecule with siRNA will

affect a subset of isoforms rather than one specific isoform. Here we targeted either the Δ Np63 domain, knocking down all Δ Np63 isoforms, or a region shared by both the α and β isoforms, which leaves only the γ isoform expressed. We chose this as a two-pronged approach to target Δ Np63 α , based on the abundance of this isoform under our study conditions. To minimize the ambiguity of this approach, we have described reciprocal regulatory effects using the adenovirus expressing Δ Np63 α , further showing that Δ Np63 α is the primary isoform responsible for target-gene regulation.

In conclusion, we have demonstrated that of the six potential p63 isoforms, Δ Np63 α is the predominant isoform expressed in pHAECs. This isoform appears to be active in regulating several epithelial genes, and plays a key role in epithelial repair in basal cells in monolayer culture. Further elucidation of the role of Δ Np63 α , as well as its upstream regulators, will have important implications in our understanding of epithelial differentiation and regeneration, and in our understanding of how the role of Δ Np63 α pertains to the pathophysiology of epithelial injury in asthma.

Author disclosures are available with the text of this article at www.atsjournals.org.

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