[CANCER RESEARCH 63, 2351–2357, May 15, 2003]

Advances in Brief

ΔNp63α and TAp63α Regulate Transcription of Genes with Distinct Biological Functions in Cancer and Development[1]

Guojun Wu,[2] Shuji Nomoto,[2] Mohammad Obaidul Hoque,[2] Tatiana Dracheva, Motonabu Osada, Chyi-Chia Richard Lee, Seung Myung Dong, Zhongmin Guo, Nicole Benoit, Yoram Cohen, Peggy Rechthand, Joseph Califano, Chul-so Moon, Edward Ratovitski, Jin Jen, David Sidransky,[3] and Barry Trink[3]

Departments of Otolaryngology-Head and Neck Surgery, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2196 [G. W., S. N., M. H., M. O., C-C. R. L., S. M. D., Z. G., N. B., Y. C., P. R., J. C., C. M., D. S., B. T.]; Laboratory of Population Genetics, National Cancer Institute, Rockville, Maryland [T. D., J. J.]; and Department of Dermatology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2196 [E. R.]

Abstract

The *p63* gene shows remarkable structural similarity to the *p53* and *p73* genes. Because of two promoters, the p63 gene generates two types of protein isoforms, TAp63 and ΔNp63. Each type yields three isotypes (α, β, γ) because of differential splicing of the p63 COOH terminus. The purpose of this study was to determine whether there is a functional link between the distinct p63 isotypes in their transcriptional regulation of downstream targets and their role in various cellular functions. TAp63α and ΔNp63α adenovirus expression vectors were introduced into Saos2 cells for 4 and 24 h, and then gene profiling was performed using a DNA microarray chip analysis. Seventy-four genes (>2-fold change in expression) were identified that overlapped between two independent studies. Thirty-five genes were selected for direct expression testing of which 27 were confirmed by reverse transcription-PCR or Northern blot analysis. A survey of these genes shows that p63 can regulate a wide range of downstream gene targets with various cellular functions, including cell cycle control, stress, and signal transduction. Our study thus revealed p63 transcriptional regulation of many genes in cancer and development while often demonstrating opposing regulatory functions for TAp63α and ΔNp63α.

Introduction

Twenty years after the discovery of the *p53* tumor suppressor gene, two related genes (*p73* and *p63*) were cloned giving rise to the notion of a *p53* family of genes (1–4). On the basis of the structural similarity of these two genes with p53, it was expected that their function would be similar to p53 in terms of tumor suppression, induction of apoptosis, and cell cycle control. However, it was since shown that the relationship between this family of genes is much more complex. Structurally, p53 has a single promoter with three conserved domains, namely, the TA[4] domain, the specific DNA-binding domain, and the oligomerization domain. In contrast, p63 and p73 each have two promoters, resulting in two different types of protein products: those containing the TA domain (TAp63, TAp73) and those lacking the TA domain (ΔNp63 and ΔNp73; Refs. 2, 5). In addition, both of these genes undergo alternative splicing at the COOH terminus, giving rise to three isotypes (α, β, γ; Refs. 1, 4, 6). The α-isotypes of both p63 and p73 contain an extended COOH terminus with a conserved SAM domain implicated in protein-protein interactions (7). In general, the TAp63 (TAp73) isotypes might behave like p53 because they transactivate various p53 downstream targets, induce apoptosis, and mediate cell cycle control. However, the ΔNp63 (ΔNp73) isotypes have been shown to display opposing functions vis-à-vis the TAp63 (TAp73)-isotypes, including acting as oncoproteins (8–12).

To better understand both the targets of the *p63* gene transcriptional regulation and the functional differences between the TA and the ΔN isoforms, we undertook the task of looking for downstream targets of *p63* using cDNA microarray technology. We found that p63 can regulate genes with diverse roles in cellular function and possesses opposing regulatory effects based on the expression of two main p63 isotypes.

Materials and Methods

Construction of Recombinant Adenoviruses. The full-length cDNAs for TAp63α (p51B) and ΔNp63α (CUSP) were each subcloned into the shuttle vector, pAdTrack-CMV. The resultant plasmids, pCMV-TAp63α or pCMV-ΔNp63α, were cotransformed into electrocompetent *Escherichia coli* (strain BJ5183) cells together with an adenoviral backbone plasmid, pAdEasy-1. Cells were selected for kanamycin resistance, and homologous recombination was confirmed by restriction digest analysis. The linearized plasmids were transfected into HEK-293 using Lipofectamine-2000 (Invitrogen). Final yields of Ad-TAp63α and Ad-ΔNp63α were generally 10^{11}–10^{12} plaque-forming units (11).

Cell Lines and RNA Preparation. Human osteosarcoma cell line Saos2 (p53-null, no p63 expression) was obtained from American Tissue Culture Collection and grown at 37°C in humidified 5% CO_2 in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum. Cells (10^7) were infected with an empty adenovirus Ad5, Ad-TAp63α, or Ad-ΔNp63α (each one with multiplicity of infection = 1). Total RNA was extracted with the Trizol reagent (Life Technologies, Inc.) after different time periods after adenovirus infection. Quality of RNA preparations was evaluated with a denaturing 2% agarose electrophoresis. To evaluate expression of p63 isotypes at 2, 4, 8, 12, 24, and 36 h of adenovirus infection, we tested p63 protein levels by Western blotting using 4A4 antibody (Santa Cruz Biotechnology).

Biotinylated RNA Probe Preparation and Hybridization. Hu95A.V2 DNA chip arrays (Affymetrix) containing 12,500 human genes were used for gene expression profiling. Seven μg of total RNA were used for the preparation of double-stranded cDNA using a Superscript choice system and an oligo$(dT)_{24}$-anchored T7 primer (Invitrogen). The cDNA was then used as a template to synthesize a biotinylated cRNA for 5 h at 37°C with aid of the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Inc.).

Received 11/12/02; accepted 3/28/03.

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[1] This work was supported, in part, by these grants: National Cancer Institute-Lung Cancer SPORE Grant CA-58184-01 (to D. S., B. T.), National Institute of Dental and Craniofacial Research Grant R01-DE 012588-0 (to D. S., E. R., B. T.), National Institute of Allergy and Infectious Diseases Grant R01-AI47224-01 (to E. R.), American Cancer Society Grant RPG-TBE-98317 (to E. R.), Traylor Research Fund and the Lilly Clinical Investigator Award of the Damon Runyon Foundation (to J. C.), and the Clinical Innovator Award from the Flight Attendant Medical Research Institute (to J. C.).

[2] These authors contributed equally to this manuscript.

[3] To whom requests for reprints should be addressed, at Division of Head and Neck Cancer Research, Department of Otolaryngology-Head and Neck Surgery, The Johns Hopkins University School of Medicine, 818 Ross Research Building, 720 Rutland Avenue, Baltimore, MD 21205-2196. Phone: (410) 502-5153; Fax: (410) 614-1411; E-mail: dsidrans@jhmi.edu or btrink@jhmi.edu.

[4] The abbreviations used are: TA, transactivation; RT-PCR, reverse transcription-PCR; Hsp, heat shock protein; IL, interleukin; JAG1, Jagged 1; JAG2, Jagged 2; VEGF, vascular endothelial growth factor; BPAG1, bullous pemphigoid antigen 1; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; RA, retinoic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SAM, sterile alpha motif.

In vitro transcription products were purified using RNeasy spin columns (Qiagen). Biotinylated RNA was then treated for 35 min at 94°C in a buffer composed of 200 mM Tris-acetate (pH 8.1), 500 mM potassium acetate, and 150 mM magnesium acetate. Affymetrix HU95A-V2 array chips were hybridized with biotinylated cRNA (15 μg/chip) for 16 h at 45°C using the hybridization buffer and control provided by manufacturer (Affymetrix). GeneChip Fluidics station 400 (Affymetrix) was used for washing and staining the arrays. A three-step protocol was used to enhance the detection of the hybridized biotinylated cRNA. Firstly, an incubation with a streptavidin-phycoerythrin conjugate followed by labeling with an anti-streptavidin goat-biotinylated antibody (Vector Laboratories) and a final staining again with the streptavidin-phycoerythrin conjugate. The chips were then scanned using a specific scanner (Hewlett Packard). The excitation source was an argon ion laser, and a photomultiplier tube detected the emission through a 570-nm long pass filter. Digitized image data were processed using the GeneChip software (version 3.1) available from Affymetrix.

GeneChip Data Analysis. Average difference values from each individual chip were scaled such that the average intensity of any given chip was 2500. We discarded genes that were scored absent in each sample according to the Affymetrix Array Suite software package. To identify specific genes misexpressed in samples infected with Ad-TAp63α or Ad-ΔNp63α relative to control Ad5-infected samples, we generated a computer algorithm allowing us to select genes exhibiting $\geq$2-fold expression change in all of our experimental samples relative to control (Ad5 only). We used three biological replicas for each of our samples and considered the genes for additional analysis, showing a common pattern of fold changes in each of the three individual experiments.

RT-PCR and Western Blotting. RT-PCR analysis was carried out essentially as described previously (sequences of RT-PCR primers and PCR conditions will be provided on request; Ref. 13). For immunoblotting, protein concentrations in all of the samples were equalized after measurement with the Bio-Rad protein assay kit. We used the following antibodies: a mouse monoclonal antibody against Hsp-70 (NeoMarkers); β-actin (Sigma); c-fos; and a rabbit polyclonal antibody against FGFR2 (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies were sheep antimouse and donkey antirabbit immunoglobulins (Amersham-Pharmacia Biotech). Signals were detected with an enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech) according to the manufacturer's protocol.

Results and Discussion

Analysis of p63 Effects on Cellular Gene Expression by cDNA Microarray. To identify the downstream targets of p63, we generated an experimental model. Saos2 cells were infected with adenoviruses containing ΔNp63α or TAp63α cDNA under expression of the cytomegalovirus promoter. Because Saos2 cells are p53 null and do not express endogenous p63 (11), we anticipated that target genes expressed after such infection would be directly attributable to ectopic p63 expression. On the basis of expression studies of our adenovirus with other genes (data not shown), we also decided to test two time points: an early time point of 4 h and a late time point of 24 h after adenovirus infection. Of the various p63 isotypes available, we used TAp63α, which is the longest isotype and ΔNp63α without a TA domain, and also the most abundant p63 isotype expressed in tumor cells (Fig. 1*A*; Refs. 11, 14–16). As a negative control, we used the same cells infected with an empty adenovirus vector. At different time points after infection, total protein lysates were analyzed for p63 protein levels by denaturing protein gel electrophoresis followed by Western blotting to confirm expression of each p63 isotype used in this study (Fig. 1*B*). As a control for viability of cells after adenovirus infection, we tested and normalized to β-actin protein levels (Fig. 1*B*).

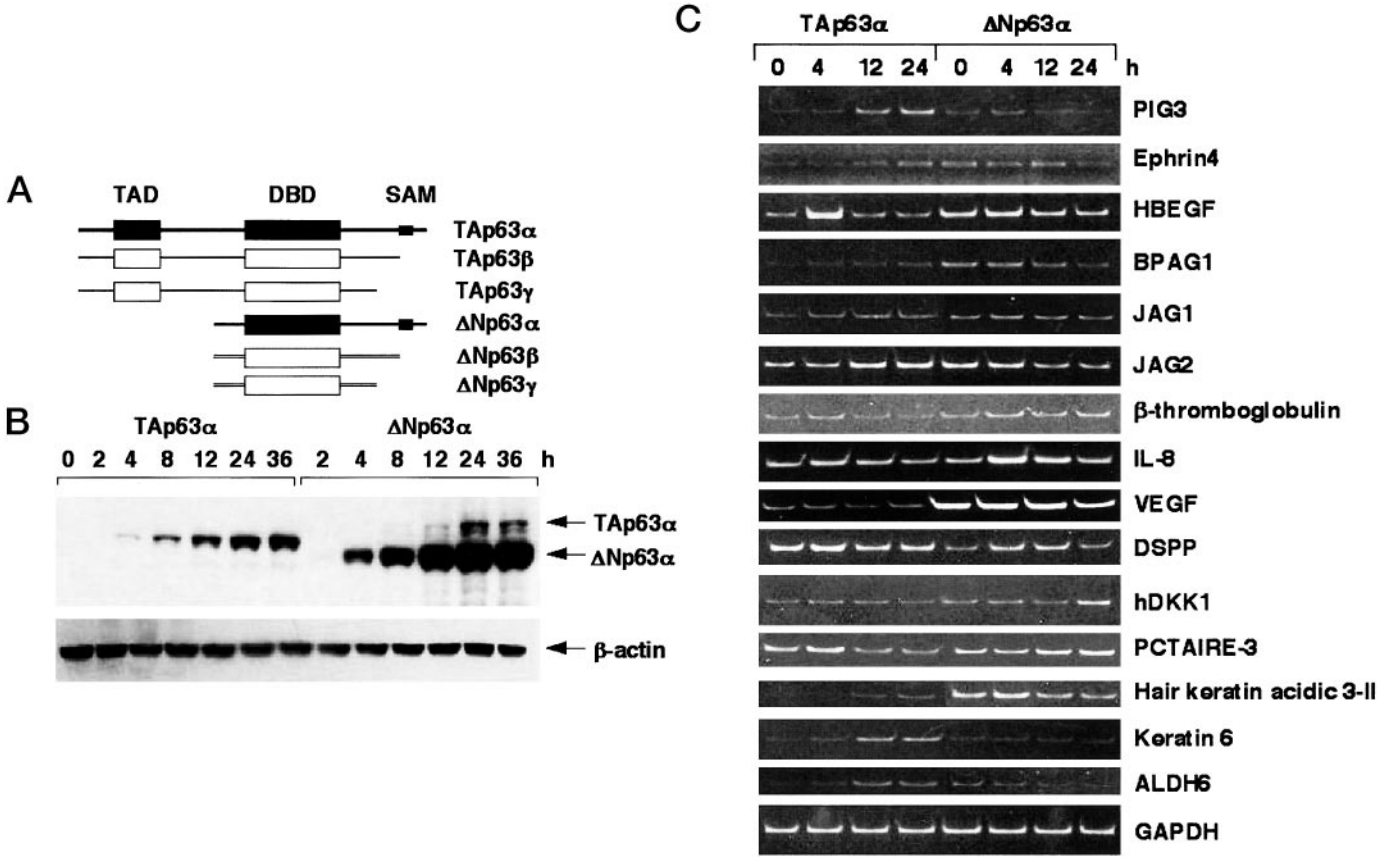

Fig. 1. *A*, the structure of different isoforms of p63. TAp63α and ΔNp63α are the largest members of p63 proteins. *B*, time course of TAp63α and ΔNp63α adenovirus infection. The expression levels were detected by Western blotting using an anti-p63 antibody (4A4; Santa Cruz Biotechnology). *C*, RT-PCR analysis of gene targets mediated by TAp63α and ΔNp63α. Data were taken at different time points after infection as indicated. GAPDH was used as a normalization control for RT-PCR. All of these genes confirmed the initial cDNA microarray data as listed in Table 1.

Moreover, each time point was tested twice, and each experiment was repeated from infection to cDNA microarray analysis under the same conditions. Only those genes that were identified independently in both experiments are listed in Table 1. To additionally confirm these results, we performed Northern blot hybridization analysis or semi-quantitative PCR and/or Western blotting on 35 candidate genes and confirmed >2-fold change in 27 genes. Twenty-two (either cancer or development related) of these 27 genes are shown in Table 2, *A* and *B*. In addition, we tested 10 other potential targets (by RT-PCR) identified on individual microarray results that did not meet all of the above criteria, 6 of which were positive by RT-PCR and also listed in Table 2.

Table 1 consists of 74 genes transcriptionally regulated by p63 and divided into various groups for convenience based on gene function. These groups include cell cycle control, DNA repair, signal transduction, oncogenes and/or tumor suppressor genes, transcription factors, receptors, growth factors or growth inhibitors, stress, development/metabolism, and structural proteins. Ordinary positive numbers represent genes that were up-regulated after p63 infection and minus signs represent down-regulation by a p63 isotype at that particular time point. Approximately 60% of the genes were up-regulated and 40% down-regulated by either ΔNp63α or TAp63α. From Table 1 it can be seen that the expression levels of 53 genes were altered by ΔNp63α compared with 23 genes by TAp63α. The most prominent genes up-regulated by ΔNp63α were various members of the *Hsp-70* family (~15–28 fold; Ref. 17), and ketohexokinase (-52 fold; Ref. 18). The most prominent genes down-regulated by ΔNp63α were the oncogene *c-fos* (~4.6 fold), type VI collagen α-3 (~5.8 fold; Ref. 19), and an adipogenesis inhibitory factor (IL-11 homologue (~5.2 fold; Ref. 20).

Of the genes up-regulated by TAp63α the most prominent were the oncogenes, muscle aponeurotic fibrosarcoma oncogene homolog F (~5.9 fold; Ref. 21), and acute myelogenous leukemia (~5.2 fold; Ref. 22). TAp63α down-regulated *IL-8* (~58-fold; Ref. 23), β-thromboglobulin (~50-fold; Ref. 24), and *LNK* (~7.9 fold), an adaptor protein that can inhibit T-cell activation (25). There was little overlap between the genes differentially expressed by both p63 isotypes (Table 1). In fact, of the genes confirmed by either RT-PCR or Northern blot hybridization, no direct correlation between these two p63 isotypes was seen, consistent with the idea that the TAp63 and ΔNp63 isotypes have different functional roles in the cell. In addition, 12 genes showed an inverse correlation between the two isoforms, suggesting that these isoforms can also have opposing functions (Table 2).

This list may be considered as a partial profile of genes, which are either directly or indirectly regulated by p63. We listed expression profiles of two of six different p63 isotypes in one cell line. A more comprehensive list would also include downstream targets of all of the isoforms of p63 in a variety of cell types. However, this list remains impressive as many key genes from several different functional categories are clearly candidates for transcriptional regulation by p63.

p63 Plays a Role in Cell Cycle Control. *PCTAIRE 2* and *PCTAIRE3*, two downstream targets of p63, are cell cycle control-related genes and both of them were strongly (>3-fold) up-regulated by ΔNp63α and down-regulated by TAp63α (Fig. 1*C*). The PCTAIRE protein kinases are an extended gene family encoding many different cdc2-related serine/threonine-specific protein kinases and are so named for the presence of a cysteine-for-serine substitution in the conserved PSTAIRE amino acid motif found in prototypic cdc2 kinases. Three members of this kinase subfamily, PCTAIRE 1–3, were identified in humans, whereas only two members were identified in mice, PCTAIRE1 and PCTAIRE3 (26, 27). TAp63α and ΔNp63α show opposite effects on these two cell cycle genes. Activation of the cyclin-dependent kinase 2 gene family leads to cell proliferation, and therefore, it is not surprising that ΔNp63α as a potential oncogene would up-regulate, whereas TAp63α would down-regulate this function. The expression pattern of other cell cycle-related genes including cyclin G1, cyclin G2, cyclin T1, and cyclin T2β were also checked by RT-PCR, but no significant changes were confirmed, although the microarray data showed at least a 2-fold change. This can be explained by the limited sensitivity of the RT-PCR in detecting minor changes at the RNA level. It is also possible that p63 is predominantly involved in cell cycle control through regulation of PCTAIRE members.

p63 Regulates Multiple Signaling Pathways. Two notch ligands, *JAG1* and *JAG2*, were identified by the cDNA microarray as up-regulated in TAp63α infected cells. This result was confirmed by RT-PCR, which in addition, showed that ΔNp63α down-regulates *JAG2* but not *JAG1* (Fig. 1*C*). Our results confirm previous studies that *JAG1* and *JAG2* genes are up-regulated by p63 and p73 (28). Furthermore, investigators identified a p63-binding site in the second intron of the *JAG1* gene, which can directly interact with the p63 protein *in vivo*, as assessed by a chromatin immunoprecipitation assay. *JAG1* and *JAG2* were also up-regulated by TAp63γ, suggesting that TAp63γ may have much stronger transcriptional activity than TAp63α (28). Taken together with our data, these studies highlight a potential role of p63 in the Notch pathway.

We also observed a role for p63 in the Wnt pathway. Both DNA chip microarray analysis and RT-PCR demonstrated that ΔNp63α up-regulates *hDKK* expression at the RNA level (Tables 1 and 2, Fig. 1*C*). However, TAp63α had no effect on the hDKK1 transcription (Tables 1 and 2). The *hDKK* gene encodes a secreted glycoprotein that binds to the extracellular domain of LRP5/6 and, in turn, prevents the formation of the active Wnt-Frizzled-LRP5/6 receptor complexes (29). Recently, p53 was reported to transcriptionally activate the *hDKK* gene (30). Although p53 and ΔNp63 often demonstrate opposing functions, in this case, they appear to up-regulate the same target. By additional RT-PCR analysis, we failed to find any significant changes in the RNA levels of other genes associated with the Wnt pathway, including Frizzled, Dishevelled, glycogen synthase kinase-3β, and β-catenin.

Effects of P63 on Proliferation, Apoptosis, Stress, and Angiogenesis Factors. The c-fos proto-oncogene is a member of multigene family encoding a transcriptional factor, which dimerizes with the Jun family proteins c-Jun, JunB, and JunD to form the transcription factor complex activator protein 1 (31, 32). The c-fos protein has been implicated as a key molecule in cell differentiation, proliferation, and transformation. In addition, the c-fos protein has been associated with apoptosis. Moreover, the c-fos gene has been shown to be a target for TA by p53. In mice, this TA of c-fos by p53 is not through the 5′ basic promoter but through the p53-responsive element located in its first intron (32). Our study showed that TAp63α up-regulates the c-fos gene (as early as 4 h), whereas both ΔNp63α and p40 (the smallest ΔNp63 isotype) down-regulate this gene at the RNA and protein levels (Fig. 2, *A* and *B*). We failed to find any *cis*-elements in either the 5′ basic promoter or in the first intron of c-fos using a luciferase reporter assay (data not shown). This may reflect a difference between human and mouse c-fos regulation by p53 and p63.

It is well documented that PIG3 is induced by wild-type p53 but not by mutant p53 proteins, which are unable to induce apoptosis, suggesting the possible involvement of PIG3 in p53-mediated cell death. P53 activates the *PIG3* gene through its interaction with a pentanucleotide microsatellite sequence within the *PIG3* promoter (33). In our study, TAp63α up-regulated the *PIG3* gene ~3-fold (Table 2 and Fig. 1*C*), suggesting that TAp63 and p53 may be involved in the same apoptotic pathway through their interaction with the *PIG3* gene.

Table 1 *Common targets of p63 of two independent Affymetrix*

GenBank No.	Genes	1[a]	2	3	4
	Cell cycle				
x66362	PCTAIRE-3		3.6[b]		
AB025254	PCTAIRE-2		3.3		
AF048732	Cyclin T2b		2		
U61836	Cyclin G1			4.8	
U24152	PAK1				3.2
AF04830	Cyclin T1		2.7		
	DNA repair				
L20046	ERCC5(XPG)			2	
	Signal transduction				
L26318	JNK1		2.4		
AB020315	Hdkk-1		2.3		
M65254	PP2A,65kD	9		−5.2	
X12534	RAP2		−2.3		
U43784	MAPKK3		2.4		
U27193	Tyrosine phosphotase	3.7	3.1		
U08316	Insulin-stimulated pk1			−2.4	
M37190	RAS inhibitor	2.5			
U70987	p62DOK		3.5		
U12592	TRAP3		2.7		
	Oncogene/TSG				
V01512	c-fos	−4.6			
M95712	B-raf		2.7		
U01337	Araf		2.3		
AB02401	p33/ING	2.8			
AF040708	p21	2			
M29039	junB		3.7		
D43968	AML			5.2	
AI021977	MAFF			5.9	
M15024	c-myb		2.5		
M74088	APC	4.3			
X85133	RBQ1	3.2			
u43916	TMP				2.3
X86371	HUGL tumor suppressor protein		3.6		
	Transcription factors				
Y13467	RB18A		3.2		
M87503	IFN-responsive factor		2.4		
X98253	znf183		2.4		
U38904	C2H2 25		2.3		
U38864	C2H2 150		3.3		
U68369	Gfi1		2.6		
	Receptors				
M76446	Adrenergic receptor		3.3		
U88153	PELP1	3.4			
L19872	AH receptor		2.4		
M87770	FGFR2(BEK)		−2.5		
S62539	Insulin receptor		−2.3		
	Growth factors/inhibitors				
M28130	IL8			−58	
M63978	VEGF		−2.6		
M35878	IGBP3		−3		
	Stress				
M11717	HSP70		15.8		
M59830	HSP70B		28		
M17017	β-Thromoboglobulin protein			−50	
	Development/metabolism				
AJ005168	KHK		52		
M14758	PGY1		2.3		
X82634	Hair keratin acidic 3-II				2.4
AF003837	JAG1			3	
AF029778	JAG2			2	
U07919	Aldehyde dehydrogenase 6		−2.2		
X99226	FAA	8.2			
Z29331	Ubiquitin conjugated	6.5			
X95632	ARG binding protein	5.1			
U34044	SEL D	4.2			
L05515	CRE BP1	3.1			
AF032108	Integrin α7		3.7		
X77956	id1		−2.2		
X58377	Adipogenesis inhibitor		−5.2		
M97252	KAL		−2.4		
AB000520	APS		−2.4		
M14083	β migrating plas. activator		−3.3	−2.2	
AF055581	LNK			−7.9	
U89278	HPH2			−3.8	
AB026891	Cystine/glutamine transporter			−2.1	
X52022	trpe VI collagen α 3		−5.8		
AF045800	Gremlin			−3.8	
J03764	Plas. activator inhibitor-1				4.9
U14383	MUC8				2.2
U22029	CYP2A7				3.3
X66403	Acetylcholine receptor				3
L37792	Syntaxin 1A				3.2

[a] 1 = ΔNp63α/4h; 2 = ΔNp63α/24h; 3 = TAp63α/4h; and 4 = TAp63α/24h.
[b] Fold change is the > between two independent experiments.

Table 2 *Gene expression confirmed by RT-PCR/Northern blot analysis*

	A. Oncogenesis related			
GenBank Accession No.[a]	Genes	TA p63α	ΔN p63α	Gene functions
X66362	PCTAIRE-3	Down[b]	Up	CDC2-related serine/threonine-specific protein kinase
AB025254	PCTAIRE-2	Down	Up	CDC2-related serine/threonine-specific protein kinase
M17017	β Thromoboglobulin like	Down	Up	Inflammation, wound repair, and coagulation
AF040708	P21	Up	Down	G_0-G_1 arrest
V01512	c-fos	Up	Down	Proto-oncogene
AB028449	Helicase MOI	Up	Down	RNA interference, repress gene expression
Z29331	UBCH2	Up	Down	Protein degradation
AF010309#	PIG3	Up	x	Antiapoptosis, p53 induced
AB024401	p33	Up	x	Proto-oncogene
M60278#	HB-EGF	Up	x	An epidermal growth factor family growth factor
m63978	VEGF	Down	x	Angiogenesis
M11717	Hsp-70	x	Up	Antiapoptosis, protein folding
M59830	HSP70B	x	Up	Antiapoptosis, protein folding
M76446	Adrenergic receptor	x	Down	Active mitogen-activated protein kinase pathway
U43916	TMP	x	Up	Homologue of mouse tumor-associated membrane protein
m28130	IL-8	x	Up	Chemokine
U15932	DSPP	x	Up	Protein phosphatase
	B. Development			
GenBank Accession No.	Genes	TA p63α	ΔN p63α	Gene functions
AF029778	Jag2	Up	Down	Embryonic development
X82634	Hair keratin acdic3-ii	Up	Down	Hair development
L42611#	Keratin 6 isoform k6e	Up	Down	Hair development
M69225#	BPAG1	Up	Down	Skin disease and sensory neurodegeneration
U07919	Aldehyde dehydrogenase 6	Up	Down	RA synthesis, primary neurogenesis
M87770	FGFR2b	Up	Down	Limb development
AJ006352#	EPHRIN A4	Up	x	Mediating development events in nervous system
AF003837	Jag1	Up	x	Embryonic development
X99226	FAA	Down	x	Acts with other genes to control FA pathway
L38517#	IHH	x	Down	Chondrocyte differentiation, bone development
AB020315	Hdkk-1	x	Up	Inhibit Wnt coreceptor function

[a] These genes were selected from one of the microarray experiments.
[b] Up, up-regulated; down, down-regulated; X, no significant change.

One of the most consistent and strongly up-regulated genes exclusively mediated by ΔNp63α is the *Hsp-70* (17). In general, Hsp's function is to protect cells from adverse stresses, be it environmental, chemical, or physical. Hsps prevent protein aggregation and promote the refolding of denatured proteins. It is now thought that several members of this family, including Hsp-70, can and do play an antiapoptotic role and thereby protect cells from death after DNA damage or stress (17). In normal cells, sublethal damage may activate the antiapoptotic function of Hsp-70 and maintain cell survival (in a nondividing state) until the damage has been repaired and the cell can then resume its normal function. However, an increase in Hsp-70 expression in tumor cells might maintain inappropriate cell survival, promoting damage retention and tumorigenesis. Because ΔNp63α but not TAp63α up-regulates Hsp-70, this observation additionally supports an oncogenic role for ΔNp63α.

The induction of new blood vessels or angiogenesis plays a major role in tumor formation and metastasis. RT-PCR results suggest that both p63 isotypes used in these studies influence two genes known to play a role in angiogenesis, namely, *VEGF* (34) and *IL-8* (23). VEGF binds specific receptors on vascular endothelial cells and thereby promotes both proliferation and neovascularization. Interestingly, TAp63α led to down-regulation of this gene, whereas ΔNp63α had no effect.

However, although others have supported repression of VEGF by TAp63α, they also reported up-regulation by ΔNp63α (35). IL-8's role in cancer is through its mitogenic and angiogenic activities (23, 34, 36). By RT-PCR, we show that ΔNp63α up-regulates IL-8 (Fig. 1*C*). However, we could not confirm the down-regulation of IL-8 by TAp63α as seen in the microarray results (Table 1). These results suggest a possible connection between p63 and angiogenesis and need to be additionally investigated.

The Involvement of p63 in Skin Differentiation and Morphogenesis. Seminal studies, based on generation of p63-knockout mice, clearly established a critical role of p63 gene in differentiation of stratified squamous epithelia leading to severe defects in skin development, skin renewal, and hair follicle morphogenesis (37, 38). For this reason, it is noteworthy that hair keratin acidic3–11 (type I keratin) and keratin-6e are regulated by TAp63α, as shown by cDNA microarray studies and RT-PCR (Table 2 and Fig. 1*C*). Hair keratin acidic3–11 is abundantly expressed in the hair follicle and constitutes the elemental keratin profile of cortex cells (39). In addition, the type II keratin 6 is constitutively expressed in distinct types of epithelia, including the outer root sheath of hair follicles (40). Therefore, our data implicates p63 in the development of the hair follicle.

BPAG1 is a glycoprotein that is one of the components of the basement membrane zone of skin. BPAG1 is made by stratified squamous epithelia, where it localizes to the inner surface of specialized integrin-mediated adherens junctions (hemidesmosomes). In BPAG1-knockout mice hemidesmosomes were found to be normal, but they lacked the inner plate and had no cytoskeleton attached (41). This change compromised the mechanical integrity of epithelia and influenced cell migration without affecting cell growth or adhesion to substrate. Our study indicates that TAp63α up-regulates the *BPAG1* gene expression (Table 2 and Fig. 1*C*), additionally supporting the importance of p63 in normal skin development and function.

In addition, FGFR2 was up-regulated by TAp63α and down-regulated by ΔNp63α (Table 2) with verification by RT-PCR and Western blotting (Fig. 2, *C* and *D*). We also tested FGFR1 and FGFR3 expression by independent RT-PCR analysis, but no significant changes were detected (data not shown). Interestingly, the expression level of several other players of FGF signaling (FGF1, FGF9, FGF18, FGFR1, and FGFR4) were also seen to change, albeit much less

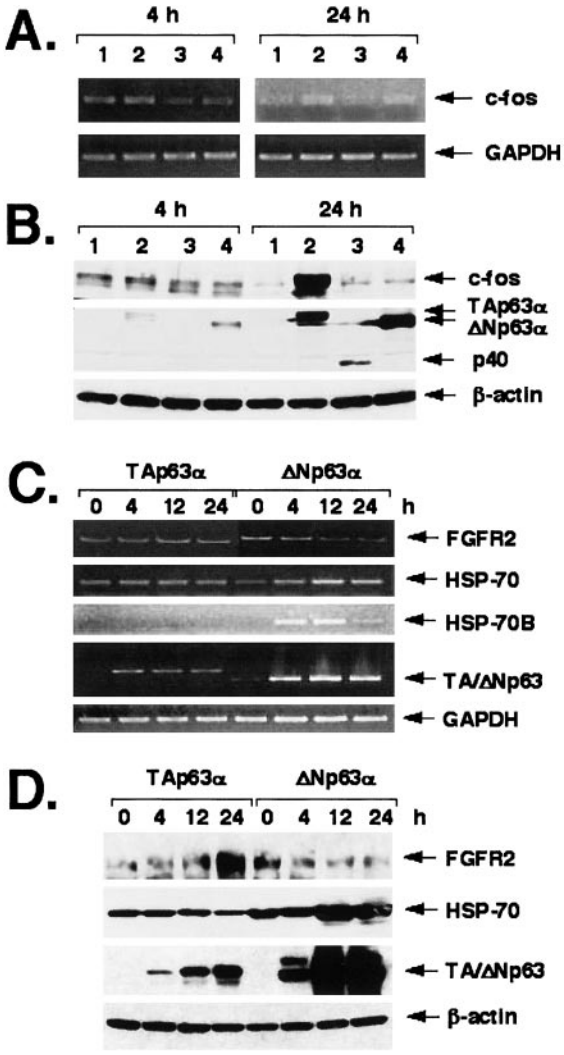

Fig. 2. *A*, RT-PCR analysis of expression in c-fos at different time points as indicated. GAPDH was used as a normalization control. 1, 2, 3, 4 corresponds to vector, TA p63α, p40, and ΔNp63α, respectively. *B*, Western blot analysis of c-fos expression in parallel with TAp63α, p40, and ΔNp63α expression. The expression of c-fos was induced by TAp63α at 4 and 24 h but inhibited by ΔNp63α and p40 at 4 h. β-Actin was used as a protein-loading control. 1, 2, 3, and 4 corresponds to vector, TA p63α, p40, and ΔNp63α, respectively. *C*, RT-PCR of FGFR2, Hsp-70, and Hsp-70B gene expression at different time points. Marked down-regulation of FGFR2 and up-regulation of HSP70 and HSP70B are seen. TAp63α and ΔNp63α expression are shown in parallel. GAPDH was used as a normalization control. *D*, Western blotting analysis of FGFR2 and Hsp-70 expression in parallel with TAp63α and ΔNp63α expression. The expression of FGFR2 was induced by TAp63α but inhibited by ΔNp63α. Expression of Hsp-70 was induced dramatically by ΔNp63α at 12 and 24 h, whereas it was virtually unchanged for TAp63α. β-Actin was used as a protein-loading control.

($\sim$1.2–1.9 fold) in response to p63 overexpression in one or both of our two independent microarray studies. As shown earlier, the p63 gene is essential for limb, craniofacial, and epithelial development (37, 38). FGFRs have been reported to function in cranial suture morphogenesis, calvarial bone development, eyelid, and skin formation (42, 43). Previous studies have shown that FGFR2 is less likely to act upstream of the p63 gene, and therefore, it was suggested that p63 and FGFR2 might control two parallel pathways in skin initiation and development (42). However, our study clearly shows that FGFR2 functions as a downstream target of p63, suggesting that the latter might be involved in limb development and/or skin formation through regulation of the FGF-signaling pathway.

Aldehyde dehydrogenase 6 gene is up-regulated by TAp63α and down-regulated by ΔNp63α (Fig. 1*C*). This gene has also been shown to be important in development (44). It controls one of the steps involved in the generation of retinol (vitamin A) to RA. A defect in RA synthesis might be involved in the transformation process, and RA has been used to reverse such changes (45). TAp63α up-regulates this gene, whereas ΔNp63α leads to its down-regulation. Moreover, p53 was shown to activate this gene (46), supporting the notion of overlap between p53 and TAp63 function.

Similarities and Differences between p53 and p63 Isotypes. As expected, some p53-responsive genes were also identified here as targets of p63. These genes include *PIG3*, *p21*, *JAG1*, *JAG2*, *c-fos*, *ephrin A4*, *IGBP3*, and *ERCC5* (Tables 1 and 2). The existence of common targets for both p53 and p63 indicates that p53 and p63 are not only structurally similar but are also involved in common cell signaling pathways. However, p63 failed to activate most known p53 targets identified by various approaches (Table 2; Refs. 47, 48). This may reflect functional differences between p53 and p63 but also may be caused by the variable conditions used in the different analyses.

In contrast to TAp63 isotypes, ΔNp63 isotypes lack a TA domain, which led to the notion that ΔNp63 proteins do not possess TA properties (4). However, ΔNp63 isotypes were shown to directly activate specific gene targets (48) and, together with our results, support the idea that ΔNp63α may possess direct or indirect transcriptional activity on specific genes in certain cell types. To verify this hypothesis, we tested the ability of TAp63α or ΔNp63α to affect the *Hsp-70* gene promoter activity using a luciferase reporter assay in Saos2 cells. The results show that ΔNp63α has much stronger transcriptional activity ($\sim$10 fold) than TAp63α on the *Hsp-70* promoter (data not shown). This result confirmed our microarray data, RT-PCR analysis, and Western blotting, which showed that the *Hsp-70* and *Hsp-70B* genes were up-regulated by ΔNp63α but not by TAp63α (Fig. 2, *C* and *D*). We have previously demonstrated critical protein-protein interactions between ΔNp63α and p53 (10). Because Saos2 cells lack p53, the p63 activity shown here was not attributable to abrogation of p53 transcriptional activity. We have not shown that this is a result of direct ΔNp63α binding to the promoter nor excluded protein-protein interactions. Regardless, ΔNp63α expression clearly leads to activation of specific downstream targets.

TAp63α and ΔNp63α seem on the whole to influence a different plethora of genes (Table 2 and Fig. 1*C*) and, consequently, may have opposite or different roles to play in the cell. This is in agreement with the idea that the TAp63 isotypes play a more p53-like role, whereas the ΔNp63 isotypes have a more opposing effect or even an inherent oncogenic role in cancer progression (4, 10, 11, 49). Our data suggests that these opposing effects are likely to be involved in many different cellular processes, including cell cycle control, apoptosis, proliferation, and cell migration, and each of these functions deserves additional detailed study in normal development and cancer formation.

Acknowledgments

We thank Bert Vogelstein from Johns Hopkins University School of Medicine for providing pAdTrack-CMV vector, Lela A. Lee (Denver Health Medical Center, Denver, CO) for CUSP (ΔNp63α) cDNA, and Shuntaro Ikawa (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) for TAp63α (p51b).

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