Loss of *p63* Expression Is Associated with Tumor Progression in Bladder Cancer

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***p63*, a member of the *p53* gene family, encodes multiple proteins that may either transactivate *p53* responsive genes (TAp63) or act as a dominant-negative factor toward *p53* and *p73* (Δ Np63). *p63* is expressed in many epithelial compartments and *p63*^{-/-} mice fail to develop skin, prostate, and mammary glands among other defects. It has been previously shown that *p63* is expressed in normal urothelium. This study reports that *p63* is regulated in bladder carcinogenesis and that *p63* expression is lost in most invasive cancers whereas papillary superficial tumors maintain *p63* expression. Examination of bladder carcinoma cell lines reveals that certain lines derived from invasive carcinomas maintain expression of Δ Np63, as demonstrated by both immunoblotting and confirmed by isoform-specific quantitative reverse transcriptase-polymerase chain reaction. Another novel finding reported in this study is the fact that *p63*^{-/-} mice develop a bladder mucosa epithelial layer yet fail to complete uroepithelial differentiation, producing a nontransitional default cuboidal epithelium. These data indicate that in contrast to the skin and prostate, *p63* is not required for formation of a bladder epithelium but is indispensable for the specific differentiation of a transitional urothelium. (Am J Pathol 2002, 161:1199–1206)**

The discovery of *p63* and *p73*, two paralogues of *p53*, has revealed an additional level of complexity in the analysis of *p53* function.¹ Both *p63* and *p73* encode multiple proteins with transactivation, DNA-binding, and tetramerization domains. Through alternate promoter usage, certain isoforms of *p63* (TAp63) and *p73* (TAp73) are capable of transactivating *p53* target genes and in-

ducing apoptosis, whereas other isoforms (Δ Np63 and Δ Np73) act in a dominant-negative manner to counteract the transactivation-competent isoforms of not only *p63* and *p73*, but *p53* as well. Additionally, TA and Δ N forms are alternatively spliced at the carboxy-terminus, designated α , β , γ , and so forth. These C-termini may regulate *p63* transcriptional activity.²

In normal tissues, *p53* levels are undetectable and only reach significant levels after genotoxic stress or mutational inactivation causing *p53* protein stabilization. In contrast, *p63* exhibits a strikingly varied expression pattern in normal tissues. Work from our group and others has shown that *p63* is expressed at high levels in squamous epithelium and urothelium, as well as the basal cell compartment of glandular epithelium in prostate, breast, and bronchi.^{2–4} The normal expression pattern anticipates in part the defects observed in *p63*-null mice, which have made it apparent that *p63* plays a key role in regulating epithelial differentiation and maturation programs. The absence of *p63* leads to nonregenerative epidermal differentiation, as well as agenesis of mammary glands, lacrimal glands, and the prostate.^{5–7}

A number of studies have investigated the role of *p63* in neoplastic transformation and tumor progression. Squamous cell carcinomas (SCCs) from different organs express high levels of Δ Np63.^{8–10} The *p63* gene may be the target of 3q27-29 gains common in SCC.^{10,11} However, 3q changes have not been consistently implicated in cytogenetic studies of transitional cell carcinomas (TCC) of the bladder.^{12,13} So far, only a single study has examined *p63* in bladder carcinomas.¹⁴ The observation that the urothelium expressed high levels of *p63* prompted us to investigate the role of *p63* in TCCs of the bladder.³

Supported by the Leukemia and Lymphoma Society (special fellowship no. 3956-01 to C. J. D.) and the National Institutes of Health (grants CA-87497, CA-47179, and DK-47650 to C. C.-C.).

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Accepted for publication June 20, 2002.

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Materials and Methods

Tissue and Patient Characteristics

Tumors from 160 patients with TCC of the bladder were analyzed. Tumor specimens included 54 papillary superficial tumors (T_a), of which 18 were low grade and 36 were intermediate or high grade. The analysis also included 106 invasive tumors (T_2 to T_4).

Cell Lines

Seven cell lines derived from invasive transitional carcinomas (J82, T24, HT-1197, 5637, UM-UC-3, TCC-SUP, HT-1376), a cell line derived from SCC of the bladder (SCaBER), and a single line derived from a superficial TCC (RT4) were obtained from and maintained as recommended by the American Type Culture Collection (Manassas, VA).

Plasmid, Cell Culture, and Transfection

Murine myc-tagged TAp63 α , TAp63 γ , Δ Np63 α , and Δ Np63 γ constructs (in pcDNA3) were kindly provided by Dr. Xinbin Chen (University of Alabama at Birmingham). H1299 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 5% CO₂ at 37°C. Cells were transfected as per the manufacturer's specifications using Lipofectamine 2000 reagent and OptiMem I Media (Invitrogen, Carlsbad, CA) with 1.0 μ g of DNA and harvested at 24 hours after transfection. For Western blotting, total cell extracts of cultured cells were prepared as described previously.¹⁵

Tissue Microarray Construction

Tumor and normal tissues were embedded in paraffin and 5- μ m sections stained with hematoxylin and eosin (H&E) were obtained to identify viable, representative areas of the specimen. Core biopsies were taken from the defined areas with a precision instrument (Beecher Instruments, Silver Spring, MD) as previously described.¹⁶ Tissue cores with a diameter of 0.6 mm from each specimen were punched and arrayed in triplicate on a recipient paraffin block.¹⁷ Five- μ m sections of these tissue array blocks were cut and placed on charged polylysine-coated slides. These sections were used for immunohistochemical analysis. Tissues and cell lines known to express p63 were used as positive controls.

Immunohistochemistry

Sections from tissue microarrays were deparaffinized, rehydrated in graded alcohols, and processed using the avidin-biotin immunoperoxidase method. Briefly, sections were submitted to antigen retrieval by microwave oven treatment for 15 minutes in 10 mmol/L of citrate buffer at pH 6.0. Slides were subsequently incubated in 10% normal horse serum for 30 minutes followed by appropriately

diluted primary antibody incubation overnight at 4°C. The mouse anti-human p63 monoclonal antibody 4A4 (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1/200 dilution for a final concentration of 1.0 μ g/ml. Samples were then incubated with biotinylated anti-mouse immunoglobulins at 1/500 dilution for 30 minutes (Vector Laboratories, Inc., Burlingame, CA) followed by avidin-biotin peroxidase complexes (1/25, Vector Laboratories, Inc.) for 30 minutes. Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain. Immunoreactivities were classified as continuum data (undetectable levels or 0% to homogeneous staining or 100%) for the p63 nuclear identification. Slides were reviewed by several investigators (CC-C, MU, and CJD) and results were scored by estimating the percentage of tumor cells showing characteristic nuclear staining as well as the intensity of nuclear staining (–, undetectable; +, moderate; ++, strong).

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from tissue samples using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. Total RNA (1.0 μ g) was then amplified using p63 isoform-specific primers using the Superscript One-Step RT-PCR Kit with Platinum Taq (Life Technologies, Inc.) using the manufacturer's protocol (50- μ l reaction volume). All reverse transcriptase (RT) reactions were performed for 30 minutes at 50°C, then 3 minutes at 94°C, followed by isoform-specific PCR conditions for each primer set: set A: p63 α -C-terminal variant (nucleotides 1380 to 1568 of Δ Np63 α), two cycles at 94°C (30 seconds), 57°C (40 seconds), 72°C (30 seconds), then 38 cycles at 94°C (30 seconds), 55°C (40 seconds), and 72°C (30 seconds) using SKO28 (sense, 5'-GAGGTTGGGCTGTTTCATCAT-3') and SKO29 (anti-sense, 5'-AGGAGATGAGAAGGGGAGGA-3'); set B: p63 β -C-terminal variant (nucleotides 1345 to 1550 of TAp63 β), two cycles at 94°C (30 seconds), 57°C (40 seconds), 72°C (30 seconds), then 38 cycles at 94°C (30 seconds), 55°C (40 seconds), and 72°C (30 seconds) using SKO30 (sense, 5'-AACGCCCTCACTCCTACAAC-3') and SKO31 (anti-sense, 5'-CAGACTTGCCAGATCCTGA-3'); set C: p63 γ -C-terminal variant (nucleotides 1057 to 1270 of TAp63 γ), two cycles at 94°C (30 seconds), 57°C (40 seconds), 72°C (30 seconds), then two cycles at 94°C (30 seconds), 55°C (40 seconds), 72°C (30 seconds), then 36 cycles of 94°C (30 seconds), 53°C (40 seconds), and 72°C (30 seconds) using SKO22 (sense, 5'-ACGAAGATCCCCA-GATGATG-3') and SKO23 (anti-sense, 5'-GCTCCA-CAAGCTCATTCCTG-3'). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was chosen as an endogenous expression RT-PCR standard using SKO36 (sense, 5'-GAAGGTGAAGGTCTGGAGT-3') and SKO37 (anti-sense, 5'-GAAGATGGTGTATGGGATTTC-3'). Isoform-specific RT-PCR (including *GAPDH* and a 1 primer-only control) was performed in triplicate. Twenty-five μ l of RT-PCR products were resolved in 1.8% agarose gels.

Quantitative RT-PCR (Q-PCR)

The Δ Np63-specific primer and probe set has been described previously.⁷ Glyceraldehyde-3-phosphate (GAPDH) was used as an endogenous control to standardize for the amount of RNA in each reaction (TaqMan GAPDH control reagents; PE Applied Biosystems, Foster City, CA). Primers were synthesized by DNAgency (Malvern, PA) and the probe was synthesized by σ -Genosys (The Woodlands, TX). RNA was amplified using the TaqMan One-Step RT-PCR Kit (PE Applied Biosystems) according to the manufacturer's instructions on an ABI Prism 7700 Sequence Detector (PE Applied Biosystems). Each reaction was performed in a 50- μ l volume containing 100 ng of total RNA template and 1.25 U of 40 $\times$ Multiscribe Enzyme Mix. The primers were added to a final concentration of 50 nmol/L and the final probe concentration was 100 nmol/L. The cycling conditions were 48°C for 30 minutes, 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. All samples were amplified in triplicate. Seven serial 100-fold dilutions of HaCaT total RNA as template were performed with the GAPDH and Δ Np63 for each amplicon to generate standard curves that relate cycle threshold to the log input amount of template. The relative amount of p63 message in each cell line was determined by using the standard curve method as described in the ABI Prism Sequence Detection System User Bulletin No. 2 (PE Applied Biosystems).

Western Blotting

For Western blotting, total cell extracts of cultured cells were prepared as described previously.¹⁵ The mouse anti-human p63 monoclonal antibody (4A4, sc-8431) was purchased from Santa Cruz Biotechnology and used at a dilution of 1/250. The goat anti-human Ran polyclonal antibody (C-20, sc-1156) was purchased from Santa Cruz Biotechnology and used at a dilution of 1/1000. The anti-myc monoclonal antibody (9E10) was obtained from Santa Cruz Biotechnology and used at a dilution of 1/500. Horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham, Arlington Heights, IL) was used as the secondary antibody at a 1/3000 dilution. Proteins were visualized with an enhanced chemiluminescence-plus detection system (Amersham).

Analysis of p63^{-/-} and Wild-Type Mice

Targeted disruption of the murine p63 gene was performed as previously described,⁵ and ES cell lines heterozygous for the mutation were microinjected into the blastocysts of B57BL/6 and BALB/c mice. Mice heterozygous for the mutation were interbred and the genotype of the progeny determined by Southern blotting.⁵ Harvard Medical School is an A.A.A.L.A.C. accredited institution and the mice were cared for in accordance with institutional guidelines. Wild-type and null p63 newborn mice were fixed in 10% neutral buffered formalin, embedded in paraffin in the sagittal orientation, and serially sectioned. Selected sections were stained with H&E) and sections

containing the urinary tract were culled for comparative histological and immunohistochemical analysis.

Statistical Analysis

In this study, the association between p63 expression levels and clinicopathological parameters, including stage (superficial versus invasive disease) and tumor grade (low versus intermediate/high) was evaluated. These variables were assessed at time of cystectomy (or immediately before cystectomy). p63 expression was treated as a continuous variable because no predetermined cutoff exists. The Wilcoxon-Mann-Whitney test was used to test the hypothesis of no differences between the above groups (defined for each variable).

Results

Expression of p63 in Human Bladder Tumor Tissues

Several studies have reported that p63 protein is strongly expressed in normal human uroepithelial cells whereas stromal cells have undetectable p63 levels (Figure 1A).^{2,3,14} To determine the frequency and potential clinicopathological implications of altered patterns of p63 expression, we examined 160 bladder tumors compiled onto three tissue microarrays.^{17,18} Table 1 summarizes the association between the percentage of p63-positive tumor cells in each tissue array core and pathological grade. Within superficial stage tumors, we found a statistically significant inverse association between the percentage of tumor cells positive for p63 and increasing pathological grade. Low-grade papillary superficial bladder tumors expressed p63 in 93% of tumor cells (Table 1, Figure 1B). However in the intermediate- to high-grade superficial tumors, we observed a significant reduction in p63 positivity to 68% (Table 1). For papillary superficial tumors, the difference between low grade and intermediate/high grade was statistically significant ($P = 0.0002$). Invasive tumors expressed low levels of p63 with only an average of 16% of cells positive, and the difference between superficial and invasive TCC was significant ($P = <0.0001$) (Table 1, and Figure 1, C and D). A subset of invasive TCC did retain significant p63 expression (Figure 1C). In the small subset for which the original pathological grade was known, maintenance of p63 was unrelated to the histology of the tumor biopsied for the tissue core (data not shown). In many tumors, we failed to detect any p63 immunoreactivity (Figure 1D). We conclude that diminished p63 protein expression is associated with grade in papillary superficial TCC's and with progression from superficial to invasive tumors.

Expression of p63 in Human TCC Cell Lines

Because the antibody used for immunostaining recognizes all of the various isoforms of p63, we studied a group of nine bladder tumor cell lines at the RNA and

Figure 1. Representative photomicrographs of immunophenotypes of p63 in normal human urothelium and TCC using the anti-p63 4A4 monoclonal antibody. Normal urothelium (**A**); low-grade superficial TCC (**B**); invasive TCC (**C**, **D**). Arrayed tissues are formalin-fixed and paraffin-embedded. DAB was used as the chromogen and hematoxylin as the nuclear counterstain. Original magnifications: $\times 400$ (**A**); $\times 200$ (**B–D**).

protein level to allow for a more detailed study of the p63 isoforms expressed in TCC. Immunoblotting with the mouse anti-human p63 monoclonal 4A4 antibody used for immunohistochemistry revealed a pattern similar to that of the patient samples (Figure 2A). As predicted from the immunohistochemistry result, RT-4, a cell line derived from a superficial TCC, appeared to express multiple p63 isoforms and differed significantly in this respect from the other cell lines derived from invasive TCC and SCC. Furthermore, the invasive derivatives either failed to express any p63 protein (TCC-SUP, T24, J82, UM-UC-3, HT-1197) or expressed a single prominent species mi-

grating at ~ 75 kd (SCaBER, 5637, H-1376). Previously published data suggests that this isoform is likely to be $\Delta Np63\alpha$.^{2,10} SCaBER, the only cell line established from a SCC of the bladder, contained a second isoform migrating near 59 kd, which was also seen in RT-4. Given the amino- and carboxy-terminal variation of p63, it is difficult to assign exact isoform designation to the proteins seen by immunoblot analysis. Because of these reasons, we raised anti-sera against the TA and ΔN isoforms of p63. However, although these reagents performed well by immunoblotting using extracts from cells overexpressing p63, they could not identify endogenous

Table 1. Loss of p63 Expression Is Associated with Histopathological Parameters

Tumor type	Mean % (+/- SE) of tumor cells p63-positive	Range	Number	P value
Invasive*	16 $\pm$ 2	0–75	106	<0.0001 [†]
Superficial [†]	76 $\pm$ 4	10–100	54	
LG [§]	93 $\pm$ 4	25–100	17	
HG [¶]	68 $\pm$ 4	10–100	36	0.0002

*All invasive tumors were pT₂–pT₄ high-grade lesions.

[†]One patient listed as superficial, but grade unknown.

[‡]Wilcoxon-Mann-Whitney test for superficial versus invasive.

[§]LG, low grade; WHO grade 1.

[¶]HG, intermediate and high grade; WHO grades 2 and 3.

^{||}Wilcoxon-Mann-Whitney test for superficial LG versus superficial HG.

Figure 2. Analysis of p63 RNA and protein expression in human TCC cell lines. **A:** Western blot analysis in which 100 μ g of total cell extract was loaded onto 10% SDS-PAGE, followed by Western blotting using the mouse anti-human p63 monoclonal antibody 4A4 and the mouse anti-human Ran monoclonal antibody as a loading control. **B:** Western blot analysis in which 30 μ g of H1299 total cell extract was loaded onto 12% SDS-PAGE, followed by Western blotting using the mouse anti-myc monoclonal antibody 9E10 (to detect the transfected myc-tagged p63 isoforms) and the mouse anti-human actin monoclonal antibody AC-40 as a loading control. For both **A** and **B**, horseradish peroxidase-conjugated rabbit anti-mouse IgG was used as the secondary antibody, followed by enhanced chemiluminescence.

p63 in extracts from the bladder cancer cell lines. Thus, to further characterize the identity of the isoforms seen by the 4A4 antibody, we transfected plasmids encoding myc-tagged TAp63 α , TAp63 γ , Δ Np63 α , and Δ Np63 γ into the p53-null cell line H1299. Western blotting of the resultant total cell extracts with the anti-myc antibody 9E10 revealed that myc- Δ Np63 α migrates at approximately the same molecular weight as the isoform seen in the invasive cell lines 5637 and H-1376 (Figure 2B).

Furthermore, we could not find any relationship between the p63 expression pattern and p53 status in these cell lines. RT-4 is wild type for p53 whereas the other eight lines carry p53 mutations (data not shown). Examination of TCC cell lines recapitulated the p63 expression pattern *in vivo*, in which superficial-derived cell lines and those from invasive exhibited differing p63 expression patterns. The cell line derived from superficial TCCs expressed a greater variety of p63 isoforms than those lines established from invasive tumors that lacked any p63 or expressed a single dominant isoform.

RT-PCR Analysis of p63 in Human TCC Cell Lines

The maintenance of Δ Np63 in invasive TCC lines was significant in that this same species accounted for the majority of p63 in a bladder SCC cell line (SCaBER). Previous work has demonstrated that SCCs tend to up-regulate the dominant-negative N-terminal variant, Δ Np63, which is postulated to be a feature of squamous oncogenesis.¹⁹ Our Western blot data suggested that up-regulation and/or maintenance of Δ Np63, as the predominant isoform, might be important in the development of a subset of invasive TCC as well. The N-terminal vari-

ants of p63 are transcribed from a distinct promoter(s) suggesting that the major regulation of Δ Np63 *versus* TAp63 might be at the level of transcription. Accordingly, we hypothesized that transcript levels of Δ Np63 would parallel the protein levels as derived from band intensity by immunoblot. Quantitative RT-PCR of Δ Np63 was performed using a primer and probe set binding in the unique Δ N region described previously.⁷ We observed a marked correlation between the levels of Δ Np63 RNA and the band intensity observed by Western blotting (compare Figures 2A and 3). Δ Np63 was expressed at the highest levels in RT-4 followed by SCaBER, 5637, HT-1376, and HT-1197. This order was preserved at the protein level with the exception of HT-1197, which lacked detectable p63 by Western blot. This data strengthened our belief that certain invasive derived TCC cell lines maintain high level Δ Np63, but not TAp63.

Figure 3. RT-PCR for p63 isoforms in TCC cell lines. Quantitative RT-PCR analyses of Δ Np63 in which 100 ng of total RNA template, 1.25 U of 40 $\times$ Multiscribe enzyme mix, probe (final concentration, 100 nmol/L), and Δ Np63 primers (final concentration, 50 nmol/L) were added in a 50 μ l reaction volume. The y axis represents equivalent nanograms of HaCat total RNA normalized to the endogenous control glyceraldehyde-3-phosphate (GAPDH) in each sample. All samples were amplified in triplicate.

Table 2. RT-PCR of C-Terminal Splice Variants in Human TCC Cell Lines

Cell lines	p63 α	p63 β	p63 γ
Superficial: RT-4	+	+	+
Invasive: TCC-SUP 5637	+	+	+
HT-1376	+	+	+
HT-1197	+	+	+
J82	—	+	—
T24	+	+	—
UM-UC-3	+	+	+
Squamous: SCaBER	+	+	+

RT-PCR for p63 α , - β , and - γ C-terminal splice variants was performed on total RNA isolated from nine cell lines using p63 isoform-specific primers.

In an attempt to further clarify the identity of the p63 variants seen by Western blot, we performed RT-PCR for p63 α , p63 β , and p63 γ C-terminal splice variants on total RNA isolated from the nine cell lines. Although T-24 lacked the p63 γ -tail-containing transcripts and J82 did

not express p63 β or p63 γ , we were able to amplify all three C-terminal variants in the remaining seven cell lines (Table 2). This result is in agreement with the recent observation that there is no direct correlation between mRNA transcript expression and protein translation for many p63 isoforms.²⁰

The Role of p63 in Urinary Tract Development

p63 is required for the development of many epithelia.^{5–7} Given the expression of p63 in normal uroepithelial cells, we investigated its role in bladder development. Comparison of p63^{+/+} and p63^{-/-} newborns revealed that p63 is required for urothelial differentiation (Figure 4). In the absence of p63, a cuboidal epithelium forms that lacks morphological characteristics of a transitional epithelium (Figure 4D, inset). In particular, p63 null embryos do not form the umbrella cells that comprise the apical cell layer in normal bladder epithelium (Figure 4, compare A and B and C and D). Examination of p63^{-/-} embryos indicates that p63 is not required for formation but rather for differentiation of uroepithelium.

Figure 4. Analysis of bladder development in p63^{-/-} mice. Cystic (A) and ureteric (C) transitional epithelium of p63^{+/+} mice. **Arrows** indicate terminally differentiated umbrella cells. Cystic (B) and ureteric (D) transitional epithelium of p63^{-/-} mice. Note lack of umbrella cells. Formalin-fixed and paraffin-embedded mouse embryos were sectioned and stained with H&E.

Discussion

p63 is expressed at high levels in normal human urothelium and neoplastic change is associated with disruption of the normal expression pattern. Altered p63 expression in TCC may occur with progressive loss of urothelial differentiation as tumors advance in both stage and grade. In low-grade papillary superficial tumors an average of 93% of tumor cells expressed p63, whereas in intermediate- and high-grade superficial tumors 68% of neoplastic cells were p63-positive. More striking was the decrease observed when tumors progressed in stage from superficial to invasive in which p63 was expressed in only 16% of cells (Table 1). This suggests that, in the normal urothelium, p63 may play a growth-suppressive and differentiation-associated role. However, certain invasive tumors maintained widespread expression of p63 (Figure 1C). Similar data were reported by Park and colleagues,²¹ in that they found reduced expression of TAp63, as assessed by RT-PCR, to be associated with tumor stage and grade in a cohort of 47 bladder cancer patients. Moreover, these authors also noted a trend toward overexpression of Δ Np63 in certain bladder cancer cases.²¹ Taken together, data indicates that loss of p63 in TCC generally occurs with a progressive loss of urothelial differentiation associated with stage and grade.

An explanation for our observation of the two p63 expression patterns in TCC could be differential regulation of the p63 isoforms in oncogenesis. In many SCCs, Δ Np63 has been hypothesized to facilitate carcinogenesis by inhibiting maturation of tumor cells.^{8–11} One explanation for these two patterns of p63 expression could be differential regulation of the p63 isoforms in oncogenesis. The maintenance of the Δ Np63 isoforms in squamous cancers may contribute to a more immature cellular phenotype, thereby promoting tumor growth. Whereas, the diminished p63 expression in TCCs may represent the loss of differentiation-associated, and therefore, growth-inhibitory p63 isoforms. Interestingly, squamous metaplasia of the bladder epithelium was associated with elevated p63 expression even in the context of invasive SCCs of the bladder (data not shown).

Pursuant to this possibility, we investigated the isoform distribution in a group of TCC cell lines. Immunoblotting for p63 protein in these lines revealed a pattern similar to that seen *in vivo*. RT-4, established from a superficial TCC, strongly expressed a wide variety of p63 isoforms whereas the invasive cell lines either lacked p63 expression or expressed one prominent isoform. Interestingly, SCaBER, a bladder tumor cell line of squamous origin, expressed the same prominent species migrating near 75 kd (Figure 2A). Given that SCC of the bladder is likely to share its p63 phenotype with SCC at other sites, previous work suggests that the dominant isoform is Δ Np63 α .^{2,10,22} As this is the major product in all invasive TCC lines expressing p63, up-regulation of Δ N-containing isoforms may be tumor growth facilitating as hypothesized for a majority of SCCs. To confirm the identity of this isoform, we transfected several isoforms of epitope-tagged murine p63 into H1299 cell and found that Δ Np63 α migrates near 75 kd (Figure 2B).

Δ Np63 and TAp63 are transcribed from distinct promoters, suggesting that their differential regulation is likely to be transcriptional. Therefore, we performed quantitative RT-PCR for Δ Np63 to demonstrate that the p63-positive TCC cell lines also have correspondingly high Δ Np63 transcript. We found a nearly exact correlation between transcript and protein levels for Δ Np63 (Figures 2A and 3), indicating further that Δ Np63 predominates in the p63-positive subset of invasive TCC. To investigate patterns of C-terminal variation, we performed RT-PCR to specifically amplify the p63 α , - β , and - γ splice variants in the TCC cell lines (Table 2). We found no obvious pattern of expression among the cell lines or any correlation between protein expression and mRNA expression, indicating that future work must demonstrate that a given p63 isoform is indeed translated.

To determine the role of p63 in bladder development and differentiation, we performed a histological examination of embryos from p63^{+/+} and p63^{-/-} mice (Figure 4). In contrast to other tissues such as skin and prostate, p63 is not required for the formation of a bladder epithelium. However, this epithelium is defective in its ability to differentiate into a transitional epithelium from its default precursor, indicating that certain p63 isoforms are required for urothelial differentiation.

In summary, certain invasive tumors maintain high levels of p63 and examination of bladder carcinoma cell lines suggests that these tumors express Δ Np63 (Figures 1C and 2A). There is convincing experimental evidence to hypothesize that Δ Np63 may function as an oncogene. Δ Np63 is anti-apoptotic in response to UV treatment and is down-regulated in squamous differentiation.^{8,23,24} Δ Np63 δ (p40^{ΔIS}) enhanced colony formation in transformation assays, as well as tumor size in nude mice.¹⁰ However, Δ Np63 was also present in the RT-4 cell line, derived from a superficial TCC. Thus, what may be important is not the presence or absence of a given isoform, but rather the total p63 aggregate. As the p63 isoform population changes throughout tumor formation and progression, distinct functions of p63 may appear and recede. Future work will focus on the various TA and Δ N isoforms functioning as oncogenes in tumor promotion and as growth-suppressive differentiation factors throughout urothelial carcinogenesis. Designation of p63 as an oncogene or a tumor suppressor could be problematic as the many isoforms may perform opposing functions.

Acknowledgments

We thank Maria Dudas for her technical assistance with immunohistochemistry; Michael Overholtzer for assistance with quantitative RT-PCR; Carol Prives for assistance and guidance in transfection experiments; and Drs. Pallavi Sachdev, Pere Puig, and Eric Green for critical comments on the manuscript.

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