Loss of ΔNp63α Promotes Invasion of Urothelial Carcinomas via N-Cadherin/Src Homology and Collagen/Extracellular Signal-Regulated Kinase Pathway

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Abstract

p63 plays a critical role in normal development and maintenance of stratified epithelia, including the urothelium. In the normal urothelium, urothelial cells in the basal layers abundantly express the predominant p63 isoform ΔNp63α. We previously showed that (*a*) ΔNp63α expression at the similar level to the normal urothelium is retained in most low-grade papillary noninvasive (LPN) tumors, whereas frequently lost in high-grade invasive carcinomas, and that (*b*) loss of ΔNp63α is associated with poor prognosis of invasive bladder urothelial carcinoma patients. However, a functional role of ΔNp63α in progression of urothelial carcinomas remains to be elucidated. Here, we show that loss of ΔNp63α expression promotes invasion of urothelial carcinoma cells. In 5637 cells substantially expressing only ΔNp63α isoform at the protein level, knockdown of endogenous p63 upregulated N-cadherin, which recruited more Src homology and collagen to N-cadherin and activated extracellular signal-regulated kinase (ERK) signaling, and consequently potentiated cell motility, excretion of matrix metalloproteinase-9, and invasion. In T24 cells originally lacking endogenous ΔNp63α expression, exogenous expression of ΔNp63α attenuated invasion by downregulating N-cadherin expression and ERK activity, confirming an invasion-suppressive role of ΔNp63α in urothelial carcinoma cells. We further documented loss of ΔNp63 expression accompanied by N-cadherin upregulation during muscle-invasive recurrence in patients whose bladder cancer had progressed from LPN tumors to muscle-invasive disease. These results suggest that loss of ΔNp63α and subsequent upregulation of N-cadherin is one of the mechanisms underlying progression of bladder cancer. [Cancer Res 2009;69(24):9263–70]

Introduction

A homologue of the *p53* tumor suppressor gene, *p63*, also known as *p51, p40, p73L*, or *KET* (1–5), encodes for two major classes of isoforms, termed TAp63 and ΔNp63. The former has an acidic NH_2 terminus analogous to the transactivating domain of p53, whereas the latter has a truncated NH_2 terminus that lacks this region. In addition, alternative splicing at the COOH terminus yields three p63 isoforms (α, β, and γ; refs. 1, 5). Unlike p53, which is induced in response to a myriad of stress, p63 is constitutively expressed in the basal cell compartment of stratified epithelial tissues, including the skin, prostate, breast, and urothelium, in which ΔNp63α is the predominant isoform expressed (6). The p63-null mice show severe structural defects in their stratified epithelia, strongly suggesting the key role of p63 in normal development and in maintaining normal structure of these epithelial tissues (7, 8).

Whereas the *p53* tumor suppressor is mutated in the half of human cancers of various origins, *p63* is rarely mutated in human cancers (1, 9, 10). Instead, *p63* is shown to play a role of a putative oncogene in human squamous cell carcinomas that overexpress ΔNp63α (11, 12), which functions as oncoprotein displaying a dominant-negative function against the p53 (5), activating β-catenin signaling (13), and upregulating the heat shock protein 70 expression (14). At the same time, loss of ΔNp63α expression is associated with more advanced disease and poorer prognosis in urothelial carcinomas (15–18). In addition, recent works suggest that p63 functions as a tumor suppressor (19) and a potent metastasis suppressor (19, 20). Thus, p63 plays a complex role in tumor formation and progression.

Bladder cancer is the fifth most common cancer for both sexes in the United States and 70,980 newly diagnosed cases are estimated in 2009 (21). Histologically, >90% of bladder cancer consists of urothelial carcinoma. From the viewpoint of biological aggressiveness and clinical management, bladder urothelial carcinomas are divided into two major phenotypic variants: low-grade papillary noninvasive (LPN) tumors and high-grade invasive carcinomas (22). In contrast to relatively benign features of LPN tumors (22–24), high-grade invasive carcinomas frequently progress to incurable distant metastases (25). Previous studies including ours showed that high-grade invasive urothelial carcinomas frequently diminish ΔNp63α expression, whereas LPN tumors highly preserve the normal p63 expression pattern characterized by the abundant and well-organized ΔNp63α expression (16, 17). Furthermore, the loss of ΔNp63 expression associates with poor prognosis of patients with high-grade invasive urothelial carcinomas of the bladder (15). Although these data suggest that loss of ΔNp63α expression results in tumor progression, a functional role of ΔNp63α in biological aggressiveness of urothelial carcinomas remains to be elucidated.

Patients with LPN bladder tumors commonly encounter intravesical tumor recurrences despite curative resection of the tumors (22–24, 26). Although the majority of recurrent tumors remain the LPN phenotype, a small subset (2%) of patients develops life-threatening, muscle-invasive recurrent cancer (26). Our previous cross-sectional study (16) showed impaired ΔNp63 expression in

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~20% of LPN tumors, raising a question whether ΔNp63 expression diminishes at or before muscle-invasive recurrences. To address this question, we longitudinally analyzed ΔNp63 expression status in tumor samples obtained from patients who had developed muscle-invasive recurrences following resection of LPN tumors.

Here, we show that the loss of ΔNp63α increased an invasive potential of human bladder carcinoma cells with epithelial phenotype. Silencing of the ΔNp63α expression resulted in upregulation of N-cadherin, which recruits an adaptor protein, Src homology and collagen (Shc), to activate extracellular signal-regulated kinase (ERK), consequently promoting *in vitro* invasion. In addition, exogenous ΔNp63α expression inhibits *in vitro* invasion via downregulation of N-cadherin and subsequent ERK inactivation in ΔNp63α-null, highly invasive human bladder carcinoma cells. Longitudinal expression analysis in clinical tumor samples documented loss of ΔNp63 expression accompanied by N-cadherin upregulation at muscle-invasive bladder cancer recurrences. These results indicate that loss of ΔNp63α and subsequent upregulation of N-cadherin contributes to progression of bladder cancer.

Materials and Methods

Antibodies and reagents. Anti-Shc antibody (BD Transduction Laboratories) was used for immunoprecipitation and immunoblotting. Anti-ΔNp63 rabbit polyclonal antibody Ab-1 (Oncogene Research Products) was used for immunoblotting and immunohistochemistry. For immunoblotting, antibodies for N-cadherin, E-cadherin (BD Transduction Laboratories), p44/42 ERK/mitogen-activated protein kinase (MAPK), phospho-Thr^{202}/Tyr^{204} p44/42 ERK/MAPK, Akt, phospho-Ser^{473} Akt, β-actin (Cell Signaling Technology), and anti-p63 mouse monoclonal antibody 4A4 (Santa Cruz Biotechnology) were used. Anti–N-cadherin mouse monoclonal antibody 3B9 (Invitrogen) was used for immunofluorescence. A MAPK/ERK kinase (MEK) inhibitor, PD98059, was purchased from Cell Signaling Technology.

Cell culture. Four human bladder urothelial carcinoma cell lines were used: JTC-30 (low-grade papillary, epithelial morphology), 5637 (low-grade, epithelial morphology), T24 (high-grade, mesenchymal morphology), and JTC-32 cells (high-grade, epithelial morphology). JTC-30 and JTC-32 cells were established in our laboratory (27). 5637 and T24 cells were obtained from the American Type Culture Collection. JTC-30 cells have a wild-type *p53* gene, whereas 5637, T24, and JTC-32 cells missense mutations of R280T, Y126X, and R280T, respectively (28). JTC-30, T24, and JTC-32 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO_2. For JTC-32 cells, 1% sodium pyruvate and 25 mmol/L HEPES were also added to the medium. 5637 cells were maintained in RPMI 1640 supplemented with 10% FBS, 1% sodium pyruvate, 25 mmol/L HEPES, and 1% penicillin/streptomycin at 37°C and 5% CO_2.

Small interfering RNA, transfection, and infection. 5637 and JTC-30 cells were transfected with small interfering RNAs (siRNA) specific for p63 or Shc, or control nonspecific siRNA (Santa Cruz Biotechnology) using HiPerFect transfection reagent (Qiagen K.K.) according to the manufacturer's protocol. Replication-deficient adenovirus carrying *ΔNp63α* was described elsewhere (29). T24 cells were infected with adenoviral vectors at 300 multiplicities of infection in serum-free medium. JTC-32 cells were transiently transfected with pcDNA3.1/Hygro-*ΔNp63α* or its empty vector using FuGene HD (Roche Applied Science) according to the manufacturer's protocol. All experiments were done 48 to 96 h after transfection or infection because, within this time frame, effects of p63 siRNA and vectors carrying *ΔNp63α* on p63 protein expression level were equally stable.

Immunoblotting and immunoprecipitation. Cells were lysed by scraping in TNESV lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 1 mmol/L EDTA, 100 mmol/L NaCl, 2 mmol/L Na_3VO_4] supplemented with Complete proteinase inhibitors (Roche Applied Science). Following clarification by centrifugation, protein concentration was measured by the bicinchoninic acid assay (Pierce). For immunoprecipitation, 1.5 mg cell lysate was incubated with 6 μg of anti-Shc antibody for 2 h at 4°C and then incubated with protein G plus agarose bead suspension (Calbiochem) overnight at 4°C. Cell lysates or immunoprecipitates were resolved by 10% to 20% or 8% to 16% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies. Protein expression was visualized with the enhanced chemiluminescence protein detection system (Pierce) and densitometrically measured using the NIH Image.

Reverse transcription-PCR. Total RNA was extracted from cell lysates using NucleoSpin RNA II (Macherey-Nagel GmbH) and reverse transcription-PCR (RT-PCR) was performed using ThermoScript RT-PCR system (Invitrogen) according to the manufacturer's protocol. Design of oligonucleotide primers for ΔNp63, TAp63, and hypoxanthine-guanine phosphoribosyltransferase 1 and optimal PCR conditions were described in detail elsewhere (16). ΔNp63 and TAp63 primer pairs were designed to consist of a shared common downstream primer and distinct upstream primers unique for each first exon but with similar melting temperature and to yield RT-PCR products with similar size, allowing for comparison of *ΔNp63* and *TAp63* transcript levels with reasonable accuracy (16).

***In vitro* invasion assay.** Following serum starvation for 24 h, 2.5×10^5 or 5×10^4 cells were suspended in serum-free medium and seeded onto 24-well Matrigel-coated invasion chambers (8-μm pores; BD Biosciences). The lower chamber was filled with complete medium containing 10% FBS. After incubation for 24 or 48 h, cells remaining in the upper chamber were removed with cotton swabs. The cells that invaded through Matrigel and attached to the lower surface of the inserts were fixed, stained with the Diff-Quik kit (Dade Behring, Inc.), and counted in five 400× fields per an insert under a light microscope. All experiments were conducted in four replicates for each condition and mean number of invading cells per 400× field was determined.

Wound-healing assay. After serum starvation for 24 h, confluent cell monolayer was scratched with a sterile pipette tip. After additional incubation for 48 h, random cell migration was assessed under an inverted microscope.

Gelatin zymography. Equal numbers of cells were plated on 6-cm dishes and serum starved for 24 h. Cells were incubated for additional 48 h in 4 mL fresh serum-free medium, which was then collected and concentrated using Amicon Ultra-4 Centrifugal Filter Devices (Millipore). Proteins in concentrated medium were eluted in sample buffer without DTT and separated onto a 10% zymogram gelatin gel (Invitrogen). Gels were washed in Zymogram Renaturing Buffer (Invitrogen), incubated for 24 h in Zymogram Developing Buffer (Invitrogen) at 37°C, and stained with SimplyBlue SafeStain (Invitrogen).

Cell proliferation assay. Cell proliferative potential was measured using CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega K.K.). Cells (3×10^3 or 6×10^3) suspended in 100 μL of serum-free or complete medium were plated in 96-well plate. After incubation, 20 μL of MTS solution were added to each well. After 1 h of incubation, the absorbance at 490 nm was recorded using a microplate reader. Six wells were assayed for each condition and the mean absorbance was determined.

Patients, immunohistochemistry, and immunofluorescence. Expression of ΔNp63 and N-cadherin protein was evaluated in surgically resected tumor specimens just before and at muscle-invasive recurrence obtained from seven patients whose bladder cancer had progressed from LPN tumors to muscle-invasive recurrent disease. The seven patients consist of three female and four male. Their median age (range) at initial transurethral resection of LPN tumor was 67 y (64–79 y). Immunostaining was performed in paraffin-embedded tissue specimens using anti-ΔNp63 Ab-1 antibody as described elsewhere (16). To detect N-cadherin expression, immunofluorescence was done as described elsewhere (30) using anti–N-cadherin antibody and Alexa Fluor 488–labeled anti-mouse secondary antibody (Invitrogen) at 1:200 for each. Protein expression of each tumor was evaluated for intensity (none, weak, intense) and extent (none, <10%; heterogeneous, 10–50%; homogeneous, ≥50%). Normal urothelium and proximal renal tubules were used as positive controls for intense expression of ΔNp63 and N-cadherin, respectively. In negative controls, the primary antibody was substituted for PBS.

Results

Associations of p63 expression with morphology, *in vitro* invasion, and expression profile of cadherins in urothelial carcinoma cell lines. JTC-30 and 5637 cells, both of which are derived from moderately differentiated bladder cancer, show epithelial morphology characterized by formation of cobblestone-like clusters, whereas T24 cells, derived from high-grade invasive bladder cancer, have spindle-shaped mesenchymal features without cluster formation. On *in vitro* invasion assay, T24 cells were most invasive among the three bladder cancer cell lines examined (Fig. 1*A*).

Expression profile of p63 protein was determined by immunoblotting. Using the 4A4 antibody, which recognizes all p63 isoforms, only a single band at ~70 kDa was detected in JTC-30 and 5637 cells (Fig. 1*B*). In terms of molecular weight, candidates of p63 isoforms responsible for the band are ΔNp63α, TAp63α, and TAp63β (5, 11). The band also reacted with the Ab-1 antibody, which exclusively recognizes ΔNp63 isforms, and *ΔNp63* isforms were predominantly expressed over *TAp63* by RT-PCR (Fig. 1*C*), confirming that ΔNp63α is the predominant p63 isoform expressed in JTC-30 and 5637 cells. In contrast to JTC-30 and 5637 cells, T24 cells did not express any form of p63 protein. An inverse association between ΔNp63α expression status and invasive potential of these urothelial carcinoma cell lines is consistent with our clinical observation that loss of ΔNp63α expression is relevant to progression of bladder cancer (15, 16).

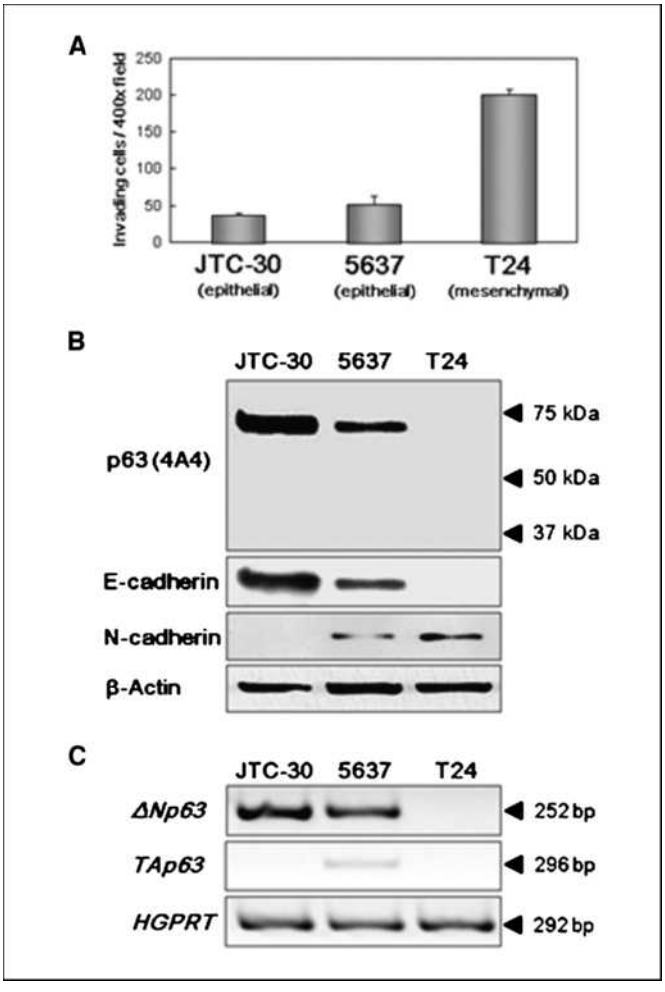

Figure 1. Associations of p63 expression with invasive potential and expression profile of cadherins in bladder urothelial carcinoma cell lines JTC-30, 5637, and T24. *A,* T24 cells are the most invasive among the three cell lines. Invasive potential was measured by Matrigel invasion assay. An equal number of JTC-30, 5637, and T24 cells were plated onto 24-well Matrigel-coated invasion chambers, and after 48 h, cells that had invaded through the membranes were counted. Each experiment was conducted in four replicates. *Bars,* SE. *B,* expression profile of p63 and cadherins in JTC-30, 5637, and T24 cells. Equal amount of protein extracted from the three cell lines was loaded and protein levels of p63, E-cadherin, and N-cadherin were measured by immunoblot. The 4A4 anti-p63 antibody, which recognizes all p63 isoforms, reacts with a band at ~70 kDa. Representative of three independent experiments. *C,* agarose gel electrophoresis of RT-PCR products at 30 cycles of PCR amplification using p63 isoform-specific primer sets. *HGPRT,* hypoxanthine-guanine phosphoribosyltransferase.

Loss of endogenous ΔNp63α promotes invasion of 5637 cells via activation of the ERK signaling pathway. To investigate whether loss of endogenous ΔNp63α expression facilitates invasion of urothelial carcinoma cells, we silenced the p63 expression in 5637 and JTC-30 cells and evaluated their invasive potentials. p63 siRNA, which targets all p63 isoforms, successfully disrupted expression of >90% of endogenous ΔNp63α in both cell lines 48 to 96 hours after transfection (Fig. 2*A*) without apparent morphologic changes (data not shown).

If loss of ΔNp63α enhances invasive potential of urothelial carcinoma cells, critical signaling cascades involved in invasion may be activated, including Akt and ERK pathways (34, 35). We thus analyzed phosphorylation status of Akt and ERK in the urothelial carcinoma cells transfected with p63 siRNA and control siRNA. In 5637 cells, loss of ΔNp63α induced consistent activation of ERK but not of Akt (Fig. 2*A*). Neither pathway was activated after p63 knockdown in JTC-30 cells (Fig. 2*A*).

Next, invasive potential was compared in 5637 and JTC-30 cells transfected with p63 siRNA versus control siRNA. In parallel with the ERK activation, loss of ΔNp63α potentiated *in vitro* invasion of 5637 cells by 2.5-fold (Fig. 2*B*) but not of JTC-30 cells (Supplementary Fig. S1*A*). Incubation with a MEK inhibitor, PD98059, which completely inactivated ERK in 5637 cells with p63 knockdown (Fig. 2*C*), hampered the increase in invasion induced by loss of ΔNp63α (Fig. 2*B*). Simultaneously, cell proliferation was compared between 5637 cells with p63 knockdown and those with intact ΔNp63α. Loss of ΔNp63α increased proliferation of 5637 cells by only 1.2-fold in the same condition as the invasion assay (data not shown), confirming that loss of ΔNp63α substantially potentiates *in vitro* invasion.

Cell motility and degradation of extracellular matrices are critical cellular processes involved in cancer invasion. We thus assessed effects of loss of ΔNp63α on migration and secretion of matrix metalloproteinases (MMP), major enzymes to degrade extracellular matrices. To determine whether loss of ΔNp63α augments production of MMPs via ERK activation, secretion of

MMPs was compared by gelatin zymography between 5637 cells with p63 knockdown and those with intact ΔNp63α in the presence or absence of PD98059. Loss of ΔNp63α increased MMP-9 secretion (Fig. 2*C*) in parallel to ERK activation (Fig. 2*C*). Secretion of MMP-2, another major subtype of MMPs, was not different between cells with p63 knockdown and those with intact ΔNp63α (Supplementary Fig. S2). Incubation with PD98059 inhibited increase in MMP-9 secreted from 5637 cells with p63 knockdown (Fig. 2*C*), indicating that the MMP-9 secretion depends on the ERK activity. Similarly, loss of ΔNp63α facilitated migration of 5637 cells in an ERK-dependent manner (Fig. 2*D*). Thus, loss of ΔNp63α activates the ERK signaling and consequently potentiates MMP-9 secretion, cell migration, and cell invasion in 5637 cells.

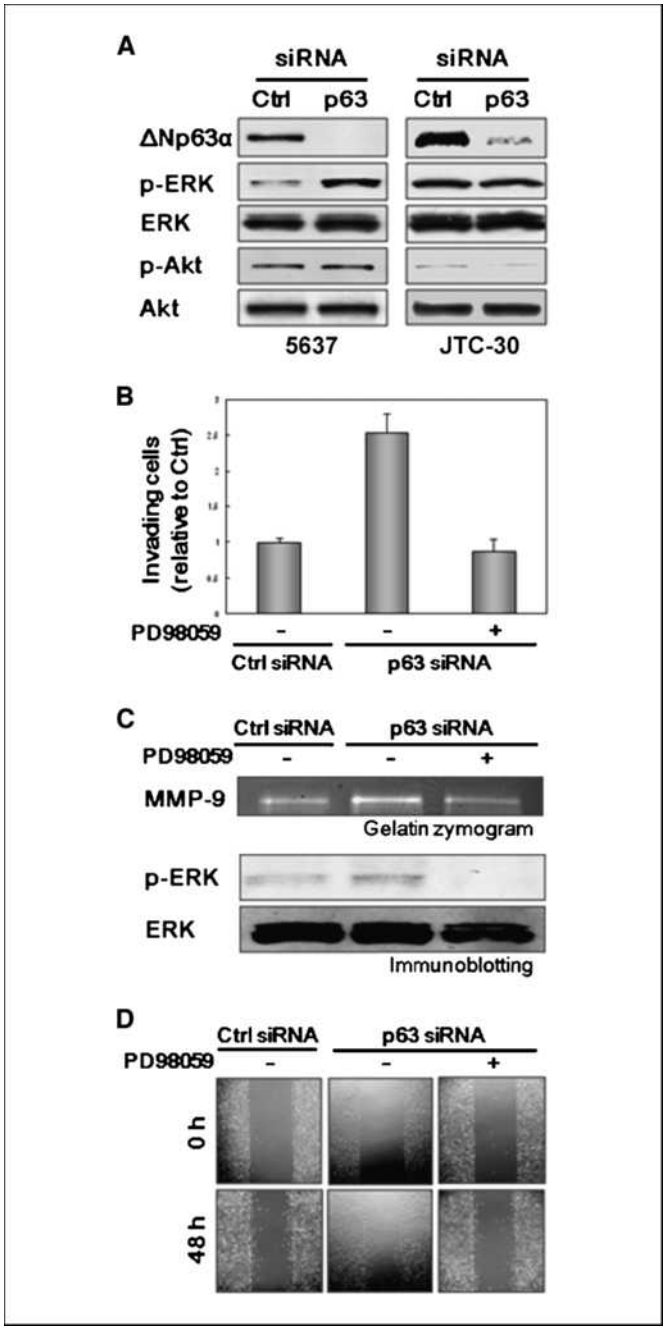

Figure 2. Loss of ΔNp63α expression potentiates invasion, production of MMP-9, and cell motility via activation of ERK signaling in 5637 cells. *A,* loss of ΔNp63α activates ERK in 5637 cells but not in JTC-30 cells. 5637 and JTC-30 cells were transfected with control or p63 siRNA. After 48 h, protein levels of ΔNp63α, phospho-ERK (*p-ERK*), ERK, phospho-Akt (*p-Akt*), and Akt were measured by immunoblot. Representative of at least three independent experiments. *B,* loss of ΔNp63α increases invasive potential of 5637 cells in an ERK-dependent manner. 5637 cells were transfected with control or p63 siRNA. After 48 h, an equal number of cells were plated onto 24-well Matrigel-coated invasion chambers in the presence or absence of 40 μmol/L PD98059. After 48 h, cells that had invaded through the membranes were counted. Each experiment was conducted in four replicates. *Bars,* SE. Control was set to 1.0. *C,* loss of ΔNp63α increases MMP-9 production in 5637 cells in an ERK-dependent manner. An equal number of 5637 cells transfected with control or p63 siRNA were incubated in 6-cm dishes for 24 h and serum starved for 24 h. Cells were incubated for additional 48 h in 4 mL of fresh serum-free medium with or without 40 μmol/L PD98059. The medium was processed to measure MMPs by gelatin zymography. The cell lysate was processed to measure expression of phospho-ERK and ERK by immunoblot. Representative of three independent experiments. *D,* loss of ΔNp63α increases cell migration of 5637 cells in an ERK-dependent manner. 5637 cells transfected with control or p63 siRNA were incubated for 24 h and serum starved for 24 h. Confluent cell monolayers were scratched, and random cell migration was assessed after incubation for additional 48 h in serum-free condition in the presence or absence of 40 μmol/L PD98059. Representative of three independent experiments. Reduced from ×40.

Loss of ΔNp63α upregulates N-cadherin and subsequently increases N-cadherin/Shc interaction to activate ERK in 5637 cells. To elucidate molecular mechanisms by which loss of ΔNp63α activates the proinvasive ERK signaling in 5637 cells, we compared expression profile of proteins associated with EMT and cell invasion, including E-cadherin, N-cadherin, β-catenin, vimentin, and cytokeratins (36), between 5637 cells with p63 knockdown and those with intact ΔNp63α. Among these proteins, only N-cadherin was upregulated by 2-fold after disruption of ΔNp63α expression (Fig. 3*A*).

Shc is an adapter protein, which has phosphotyrosine-binding and Src homology 2 (SH2) domains and mediates ERK signaling in response to extracellular stimuli (37). Via the two domains, Shc binds to phosphotyrosine residues of various molecules. When tyrosine residues of Shc are phosphorylated, growth factor receptor binding protein 2 (Grb2) and Sos are recruited to Shc, consequently activating the Ras/MEK/ERK pathway (38). N-cadherin also has tyrosine residues, which are phosphorylated by Src kinase, and Shc can bind to the phosphotyrosine sites of N-cadherin via the SH2 domain (39). We hypothesized that the loss of ΔNp63α upregulates N-cadherin, which allows more amount of Shc to bind to N-cadherin, leading to the ERK activation.

To verify this hypothesis, we first investigated whether loss of ΔNp63α increases N-cadherin/Shc interaction in 5637 cells. Strikingly, more amount of N-cadherin bound to Shc in cells with p63 knockdown compared with those with intact ΔNp63α (Fig. 3*B*). Second, we investigated a role of Shc in the p63 knockdown-induced ERK activation. Transfection of Shc siRNA substantially disrupted endogenous Shc expression and attenuated the basal ERK activity in 5637 cells (Fig. 3*C, right lane*). Dual knockdown of Shc and p63 failed to activate ERK despite upregulation of N-cadherin to the similar level to p63 knockdown alone (Fig. 3*C, middle two lanes*). Thus, Shc plays an essential role in maintaining the basal ERK activity and in mediating the loss of ΔNp63α-induced ERK activation in 5637 cells. Taken together, loss of ΔNp63α induces upregulation of N-cadherin, which recruits more amount of Shc to N-cadherin and activates ERK signaling, consequently promoting invasion in 5637 cells. In this context, the reason why loss of ΔNp63α brought neither increase in ERK activity nor invasive potential of JTC-30 cells would be lack of induction of

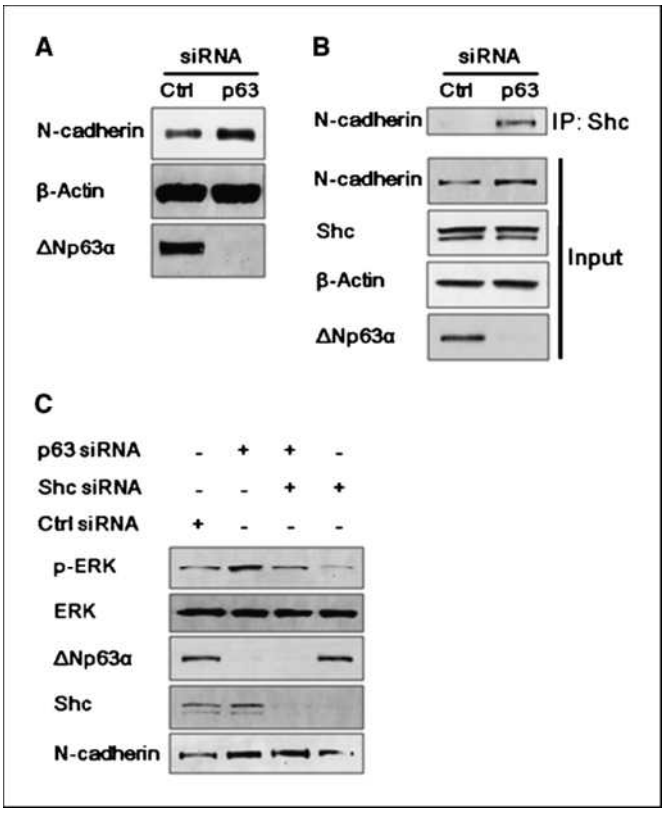

Figure 3. Loss of ΔNp63α upregulates N-cadherin and subsequently increases N-cadherin/Shc interaction to activate ERK in 5637 cells. *A,* loss of ΔNp63α upregulates N-cadherin in 5637 cells. N-cadherin and ΔNp63α protein levels were analyzed in 5637 cells transfected with control and p63 siRNA by immunoblot. *B,* N-cadherin/Shc interaction is increased by loss of ΔNp63α in 5637 cells. Shc protein was immunoprecipitated from cells transfected with control and p63 siRNA and the blot was probed with anti–N-cadherin antibody. Representative of three independent experiments. *C,* Shc is necessary for the loss of ΔNp63α-induced ERK activation in 5637 cells. Protein levels of phospho-ERK, ERK, ΔNp63α, Shc, and N-cadherin were measured in 5637 cells transfected with control, p63, and/or Shc siRNA by immunoblot. Representative of at least three independent experiments.

endogenous N-cadherin expression in the cell line (Supplementary Fig. S1*B*). It may be also possible that complete loss of ΔNp63α is required for induction of N-cadherin because p63 siRNA, even when applied at higher concentrations, did not completely eliminate ΔNp63α expression in JTC-30 cells (Fig. 2*A*; Supplementary Fig. S1*B*).

Exogenous expression of ΔNp63α downregulates N-cadherin and attenuates ERK signaling and invasion in T24 cells. N-cadherin expression and ERK activity were measured in ΔNp63α-null T24 cells infected with an adenoviral vector carrying *ΔNp63α* and a control vector. T24 cells infected with an adenoviral vector carrying *ΔNp63α* successfully expressed ΔNp63α protein (Fig. 4*A*) without apparent morphologic difference from control and parental T24 cells (data not shown). Exogenous expression of ΔNp63α repressed N-cadherin expression and attenuated ERK activity (Fig. 4*A*). Introduction of ΔNp63α expression diminished invasive potential of T24 cells by 60% compared with that of control cells (Fig. 4*B*) but did not influence cell proliferation in the same condition as the invasion assay (data not shown). These findings are consistent with data obtained from the loss of function approach for ΔNp63α in 5637 cells, further confirming that ΔNp63α functions as an invasion suppressor via the N-cadherin/ERK pathway in urothelial carcinoma cells.

JTC-32 cells, derived from high-grade bladder cancer and having epithelial morphologic features, lack expression of both ΔNp63α and N-cadherin (Fig. 4*C*). By RT-PCR in the same condition as Fig. 1*C*, neither *ΔNp63* nor *TAp63* mRNA was detectable (data not shown). To confirm a role of N-cadherin in the ΔNp63α-mediated modulation of ERK activity, ERK activity was measured in JTC-32 cells transfected with pcDNA3.1-*ΔNp63α* or its empty vector. Exogenous expression of ΔNp63α did not influence either ERK activity or N-cadherin expression in this N-cadherin–null urothelial carcinoma cell line (Fig. 4*C*). Given that loss of ΔNp63α did not influence ERK activity in N-cadherin–null JTC-30 cells, N-cadherin expression is likely to be essential for the ΔNp63α-mediated modulation of ERK activity.

Loss of ΔNp63 and upregulation of N-cadherin in clinical tumor tissues in the course of muscle-invasive bladder cancer recurrence. To investigate (*a*) whether ΔNp63α expression diminishes at muscle-invasive recurrences or is already impaired in LPN tumors before the recurrence and (*b*) whether loss of

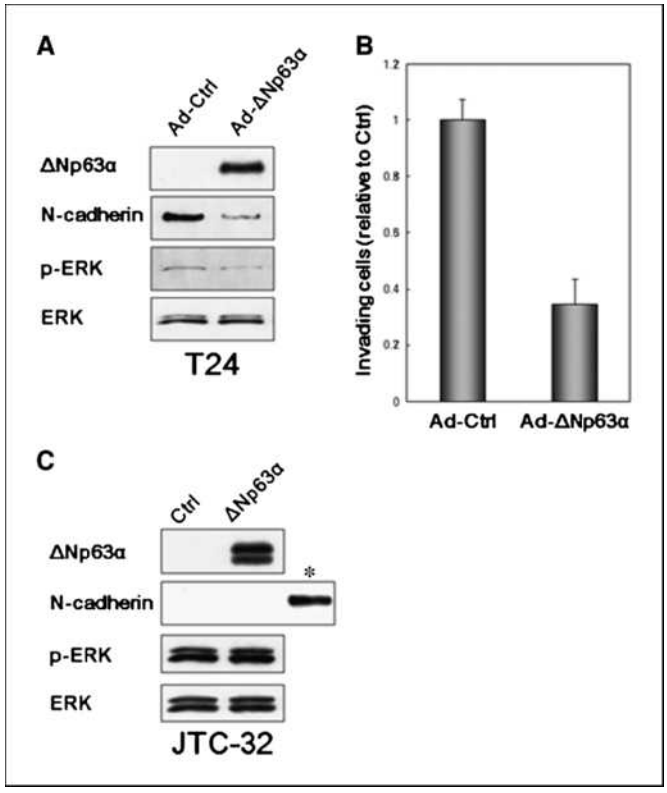

Figure 4. Exogenous expression of ΔNp63α downregulates N-cadherin and attenuates ERK signaling and invasion in T24 cells. *A,* exogenous expression of ΔNp63α downregulates N-cadherin and attenuates ERK signaling in T24 cells. Protein levels of ΔNp63α, N-cadherin, phospho-ERK, and ERK were measured in T24 cells infected with control and ΔNp63α adenoviral vectors at 300 multiplicities of infection by immunoblot. Representative of at least three independent experiments. *B,* exogenous expression of ΔNp63α attenuates invasive potential of T24 cells. T24 cells were infected with control and ΔNp63α adenoviral vectors at 300 multiplicities of infection. After 48 h, an equal number of cells were plated onto 24-well Matrigel-coated invasion chambers. After 24 h, cells that had invaded through the membranes were counted. Each experiment was conducted in four replicates. *Bars,* SE. Control was set to 1.0. *C,* exogenous expression of ΔNp63α did not influence ERK signaling in N-cadherin–null JTC-32 cells. Protein levels of ΔNp63α, N-cadherin, phospho-ERK, and ERK were measured in JTC-32 cells transfected with pcDNA3.1-*ΔNp63α* and its empty vector by immunoblot. Representative of at least three independent experiments. *, lysate from T24 cells as positive control for N-cadherin expression.

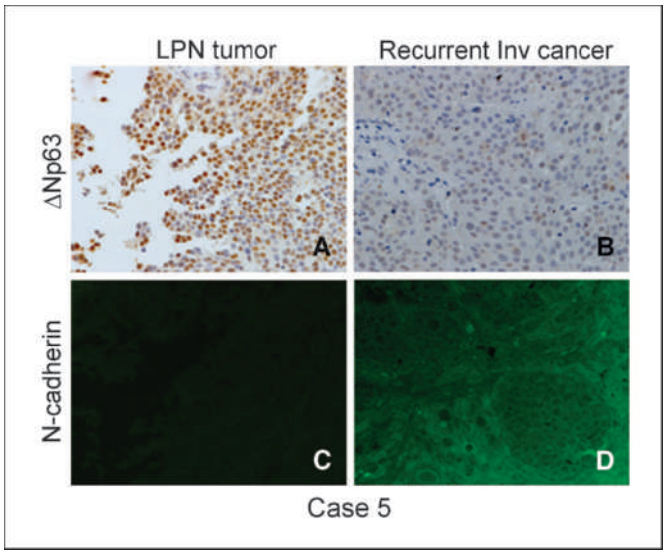

Figure 5. Loss of ΔNp63 expression and increased N-cadherin expression in the course of muscle-invasive (*Inv*) bladder cancer recurrence in a representative case (case 5 in Table 1). *A,* intense and homogeneous ΔNp63 expression in a LPN tumor at the latest resection before invasive recurrence. *B,* weak and focal ΔNp63 expression in recurrent invasive cancer. *C,* weak and heterogeneous N-cadherin expression in the LPN tumor shown in *A*. *D,* intense and homogeneous N-cadherin expression in the recurrent invasive cancer shown in *B*. Immunoreactivity was localized using 3,3′-diaminobenzidine and Alexa Fluor 488 for ΔNp63 and N-cadherin, respectively. Reduced from ×200.

ΔNp63α expression is accompanied by N-cadherin upregulation, expression status of ΔNp63 and N-cadherin was examined in surgically resected, metachronous tumor tissues obtained from seven patients just before and at diagnosis of muscle-invasive recurrences. Figure 5 shows representative photomicrographs of expression of ΔNp63 and N-cadherin in tumor tissues. As shown in Table 1, six of the seven initial LPN tumors (86%) expressed ΔNp63 intensely and homogeneously, which is similar to the finding obtained from the previous cross-sectional studies (16, 17). All of the seven initial LPN tumors expressed N-cadherin weakly (heterogeneous in four and homogeneous in three). In recurrent muscle-invasive cancer tissues, ΔNp63 expression was reduced in five (71%) when compared with their LPN counterparts. Of the five cases, three (60%) showed upregulation of N-cadherin in the course of invasive recurrence, whereas N-cadherin expression remained unchanged in the two rest cases whose invasive cancer retained ΔNp63 expression at comparable level to their LPN counterparts. These findings in clinical material suggest that loss of ΔNp63α and subsequent upregulation of N-cadherin could be one of actual molecular mechanisms underlying muscle-invasive recurrence of bladder carcinomas.

Discussion

Previous studies including ours have shown an association between loss of ΔNp63α expression and more aggressive nature of urothelial carcinomas in clinical settings (15–17). However, a functional role of ΔNp63α in progression of urothelial carcinomas remains to be elucidated. The current study has clearly shown that loss of ΔNp63α promotes invasion of urothelial carcinoma cells and that ΔNp63α functions as an invasion suppressor. Loss of endogenous ΔNp63α upregulated N-cadherin, which recruited more amount of Shc to N-cadherin and activated ERK signaling, and consequently promoted cell motility, excretion of MMP-9, and invasion in human bladder carcinoma cells with epithelial phenotype. In addition, exogenous expression of ΔNp63α attenuated invasion by downregulating N-cadherin expression and ERK activity in ΔNp63α-null human bladder carcinoma cells with mesenchymal phenotype, confirming a suppressive role of ΔNp63α in invasion of urothelial carcinoma cells. Such a role of ΔNp63α was observed in N-cadherin–expressing urothelial carcinoma cells but not in those lacking N-cadherin, suggesting that N-cadherin expression is required for the control of invasive potential of urothelial carcinoma by ΔNp63α. We further documented loss of ΔNp63 concomitantly with upregulation of N-cadherin in clinical tumor samples in the process of muscle-invasive bladder cancer recurrences. These data suggest that loss of ΔNp63α and subsequent upregulation of N-cadherin is one of the mechanisms underlying progression of bladder cancer.

EMT is characterized by functional changes, including the increased motility of cells and the loss of cell-cell adhesion as well

Table 1. ΔNp63 and N-cadherin expression in LPN tumors at the latest resection just before muscle-invasive recurrence and in the recurrent muscle-invasive carcinomas in seven bladder cancer patients

Case no.	Age/sex	TNM stage at progr.	Time to progr.*	ΔNp63 expr.		N-cadherin expr.		Clinical course	Follow-up period*
				LPN	Rec Inv	LPN	Rec Inv		
1	69/F	T3N2M0	16 mo	++	+	±	+	DOD	32 mo
2	64/M	T4N2M0	97 mo	++	±	±	+	DOD	112 mo
3	67/M	T2N0M0	158 mo	++	+	±	±	DOD	170 mo
4	65/M	T4N0M0	15 mo	++	±	+	±	DOD	31 mo
5	69/F	T2N0M0	9 mo	++	±	±	++	NED	94 mo
6	65/M	T4N0M0	27 mo	+	+	+	+	DOD	38 mo
7	79/F	T2N0M0	43 mo	++	++	+	+	NED	58 mo

Abbreviations: TNM, tumor-node-metastasis; Inv, invasive carcinoma; progr., progression; expr., expression; Rec Inv, recurrent invasive cancer; DOD, dead of disease; NED, no evidence of disease. ++, intense and homogeneous expression; +, weak and homogeneous expression; ±, weak and heterogeneous expression.
*Period (months) from initial diagnosis of LPN tumors.

as morphologic changes to mesenchymal traits (36). EMT is a crucial process in tumor progression, providing tumor cells with the ability to escape from the primary tumor and to invade the surrounding tissue. EMT involves changes in various molecules, such as N-cadherin, E-cadherin, vimentin, cytokeratin, and desmoplakin (36). In 5637 bladder carcinoma cells, loss of endogenous ΔNp63α potentiated cell motility and invasion by upregulating N-cadherin but not induced changes in morphology or expression of E-cadherin, vimentin, and cytokeratin. Given that N-cadherin induces functional EMT lacking morphologic changes in mammary epithelial cells (40), loss of ΔNp63α would induce functional EMT by upregulating N-cadherin in 5637 cells. In the squamous cell lineage, loss of ΔNp63α induces both functional and morphologic EMT *in vitro* by modulating expression of genes, including N-cadherin (41). We also showed that exogenous expression of ΔNp63α functionally recovers EMT by downregulating N-cadherin in ΔNp63α-null T24 cells. Considering that *p63* physiologically plays an essential role in maintaining the stratified epithelial features (7, 8), it is conceivable that ΔNp63α functions as an invasion suppressor in urothelial carcinoma cells.

Distant metastasis is the most common causes of death from malignancies including urothelial cancer. Loss of ΔNp63α is relevant to the induction of EMT, which favors the early processes of metastasis, including invasion of carcinoma cells into surrounding tissues. A very recent work showed that ΔNp63α functions as a metastasis suppressor by regulating *Sharp-1* and *Cyclin G2* expression in breast cancer (20). Thus, loss of ΔNp63α might promote cancer metastasis besides potentiating invasiveness of urothelial carcinomas. Indeed, previous clinical studies showed that loss of ΔNp63 expression is significantly associated with shorter survival of patients with urothelial carcinomas of the bladder (15) and upper urinary tracts (18).

Although N-cadherin has been extensively studied as a key molecule to mediate cancer invasion, mechanisms by which N-cadherin promotes cell invasion are not yet fully understood. The current study provided the N-cadherin/Shc/ERK pathway, which links loss of ΔNp63α to enhanced invasive potential of 5637 cells. Loss of ΔNp63α upregulates N-cadherin, increasing N-cadherin/Shc complex formation and subsequently activating the MEK/ERK signaling, which is integral to cell motility, degradation of the extracellular matrix, and cell invasion in 5637 cells. Rieger-Christ and colleagues (34) reported that pharmacologic inhibition of Akt and Src but not ERK potentially inhibits invasion enhanced by exogenous N-cadherin expression in two bladder cancer cell lines, CUBIII and HT1376, which express E-cadherin but lack N-cadherin. Given that Shc could mediate phosphoinositide 3-kinase (PI3K)/Akt signaling by forming Shc/Grb2/Grb2-associated binding protein 2 complex (42) and that Src phosphorylates tyrosine residues in the cytoplasmic domain of N-cadherin to which Shc is recruited (39), Shc binding to N-cadherin might universally trigger activation of the MEK/ERK and/or PI3K/Akt pathway, depending on the cellular context, to promote cell invasion.

We showed for the first time the loss of ΔNp63 expression at clinical muscle-invasive recurrences following resection of LPN tumors. Of seven LPN tumors at the latest resection before the diagnosis of muscle-invasive recurrences, six retained intense and homogeneous ΔNp63 expression. ΔNp63 expression diminished not before but at the diagnosis of muscle-invasive recurrences in five of the six patients, indicating that ΔNp63 expression status in LPN tumors is unlikely to predict subsequent development of muscle-invasive bladder carcinomas.

Some studies showed that loss of *TAp63*, a minor component of *p63* isoforms expressed in normal and neoplastic stratified epithelial cells, possibly promotes progression of bladder urothelial carcinomas (10, 19). Further evaluation on p63 isoform-specific functions is needed to clarify the role of *TAp63* in bladder cancer formation and progression.

Although the role of p63 in tumor formation and progression has been emerging, mechanisms underlying the loss of ΔNp63α expression in urothelial carcinomas still remain to be elucidated. Previous studies on urothelial carcinomas revealed that ΔNp63α expression is lost at the mRNA level (16, 17). Because of rare mutations or allelic deletions of *p63* gene in human carcinomas, including urothelial carcinomas (1, 9, 10), the loss of *ΔNp63α* mRNA is attributed to epigenetic alterations. At least, hypermethylation of the promoter regions seems to be one of the causes for the loss of *ΔNp63* mRNA in urothelial carcinoma cell lines (10). Recently, Snail, a zinc finger transcription factor, is reported to downregulate *ΔNp63α* mRNA in squamous cell carcinoma cells *in vitro* (43). Systematic evaluation on clinical samples for such possible alterations may achieve further understanding of mechanisms underlying progression of urothelial carcinomas, allowing development of a novel, rational therapeutic strategy against this lethal disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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