

Dynamic Change in p63 Protein Expression during Implantation of Urothelial Cancer Clusters^{1,2}

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Abstract

Although the dissemination of urothelial cancer cells is supposed to be a major cause of the multicentricity of urothelial tumors, the mechanism of implantation has not been well investigated. Here, we found that cancer cell clusters from the urine of patients with urothelial cancer retain the ability to survive, grow, and adhere. By using cell lines and primary cells collected from multiple patients, we demonstrate that Δ Np63 α protein in cancer cell clusters was rapidly decreased through proteasomal degradation when clusters were attached to the matrix, leading to downregulation of E-cadherin and upregulation of N-cadherin. Decreased Δ Np63 α protein level in urothelial cancer cell clusters was involved in the clearance of the urothelium. Our data provide the first evidence that clusters of urothelial cancer cells exhibit dynamic changes in Δ Np63 α expression during attachment to the matrix, and decreased Δ Np63 α protein plays a critical role in the interaction between cancer cell clusters and the urothelium. Thus, because Δ Np63 α might be involved in the process of intraluminal dissemination of urothelial cancer cells, blocking the degradation of Δ Np63 α could be a target of therapy to prevent the dissemination of urothelial cancer.

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Introduction

Urothelial cell carcinomas are often multifocal and synchronous at primary diagnosis, and 17% of patients with upper urinary tract urothelial carcinomas reportedly present with concomitant bladder cancer [1]. In addition, approximately one half of the patients develop intravesical recurrences after transurethral resection (TUR) of non-muscle-invasive bladder cancer [2]. Two theories have been proposed to explain the multifocality of urothelial cell carcinoma: the field cancerization hypothesis and the monoclonality hypothesis. The field cancerization hypothesis proposes that tumors arise from different clones, probably because of severe carcinogenic insults to the urinary tract. On the other hand, the monoclonality hypothesis proposes that the multifocal tumors originate from a single transformed cell and that the tumor cells spread by intraluminal implantation or intraepithelial migration [3,4].

Dissemination of the cancer cells through urine is supported by clinical observations and the molecular profiling of the cancers. The risk of bladder cancer recurrence is 30% to 51% in upper urinary tract

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cancer (UUTC) after nephroureterectomy [5,6] despite a much lower risk (1.8%-7.5%) of UUTC recurrence after TUR for bladder cancers [7,8]. The risk increases to 6% to 20% (15- to 22-fold) in patients with vesicoureteral reflux [9,10]. In addition, coincidental genetic alterations in UUTC and bladder cancer are reported in the same patients [11–13]. Thus, there is evidence that cancer cells disseminate through the urinary stream, although the biological interaction between the urothelium and urothelial cancer cells is poorly understood.

The TP63 gene is a member of the TP53 gene family. The TP63 gene contains two transcriptional start sites that are used to generate transcripts encoding two isoforms. One contains an N-terminal transactivation (TA) domain (TAp63), but the others do not (Δ Np63). Both genes can be alternatively spliced to generate proteins with three different C-termini: α , β , and γ [14,15]. p63 is expressed in embryonic ectoderm and in the nuclei of basal cells of many epithelial tissues in the adult, including the urothelium, and plays an important role in the development and homeostasis of the epithelium [16,17]. In normal adult urothelium as well as urothelial cancer cells, Δ Np63 is predominantly abundant compared with TAp63 [18–21]. The functional role of p63 in cancer is controversial. Studies using bladder cancer cell lines revealed that suppression of Δ Np63 α induces N-cadherin expression and promotes motility and invasiveness [21,22]. The expression of Δ Np63 α is lower in muscle-invasive cancer than non-muscle-invasive cancer [17,20]. These reports indicate that Δ Np63 α is an oncosuppressor. On the other hand, because Δ Np63 expression in muscle-invasive cancer correlates with a worse prognosis [17,20,23], Δ Np63 α can be considered an oncogene.

The concept that the collective behavior of a group of cancer cells defines malignant function is rapidly developing. Within the group, cells remain cohesive by expressing cell–cell junction molecules. These groups are represented by collective cancer cell invasion [24] and the clusters of circulating tumor cells [25]. In breast cancer, circulating tumor cell clusters derived from multicellular groupings of primary tumor cells retain cell–cell contact through plakoglobin-dependent intercellular adhesion, which greatly contributes to the metastatic spread of cancer [26]. We demonstrate here that cancer cell clusters in urine are viable, able to proliferate, and can attach to the cell matrix. By using bladder cancer cell lines and primary cultured cancer cells, we further demonstrate that Δ Np63 α expression levels change dramatically during attachment and play a critical role in implantation.

Materials and Methods

Ethics Statement

The institutional ethics committees at the Osaka Medical Center for Cancer and Cardiovascular Diseases (OMCCCD) approved this study. Human urothelial cancer specimens and human urothelial tissues were collected from patients treated at the Department of Urology, OMCCCD. Animal studies were approved by the OMCCCD Institutional Animal Care and Use Committee and were performed in compliance with institutional guidelines.

Cells and Cell Culture

Bladder cancer cell lines RT4, 5637, and J82 and an immortalized urothelial cell line, SV-HUC-1, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All cell lines were authenticated by short tandem repeat DNA profiling using GenePrint 10 System (Promega, Madison, WI). RT4 cells were cultured at 37°C

under 5% CO₂ in McCoy's 5A; 5637 and J82 cells were cultured in RPMI 1640; and SV-HUC-1 cells were cultured in F12-K medium supplemented with 10% fetal bovine serum. Clusters from each cell line were prepared by seeding 4×10^5 cells in 2 ml of medium in poly-2-hydroxyethyl methacrylate-coated dishes prepared by dissolving 5 mg/ml poly-2-hydroxyethyl methacrylate (Sigma-Aldrich, Saint Louis, MO) in 95% ethanol and then adding 500 μ l of the solution to six-well culture dishes. Clusters were subjected to further experiments 24 hours after seeding. Culture conditions are referred to as "2D" for conventional adhesive culture and as "3D" for multicellular spheroid culture in suspension.

Primary cultures of urothelial cancer cells were prepared according to the cancer-tissue-originated spheroid (CTOS) method [27,28]. Briefly, surgical samples or xenograft tumors were mechanically dissociated, partially digested with liberase DH (Roche Applied Science, Mannheim, Germany), and filtered through cell strainers. Fragments on 100- μ m or 40- μ m cell strainers (BD Falcon, Franklin Lakes, NJ) were collected. CTOSs were cultured in StemPro hESC human embryonic stem cell culture medium (Invitrogen, Carlsbad, CA). All bladder and upper urinary tract tumor samples were confirmed as urothelial carcinoma by institutional pathologists.

Patient-derived xenografts of BC23 and UT35 were described in our previous report [28]. In experiments on attachment, CTOSs were cultured on type I collagen (Cellmatrix, Nitta Gelatin, Osaka, Japan)-coated dishes for 20 to 24 hours. Culture conditions are referred to as "floating" for spheroid cultures in suspension and "attached" for culture after attachment of floating spheroids.

Urine Samples

Urine samples were collected from patients diagnosed with urothelial cancer by cytology. Voided urine or urine from catheters was collected and centrifuged, and sediments were recovered and cultured in StemPro hESC human embryonic stem cell culture medium (Invitrogen). Subsequently, cell clusters in the culture medium were picked up using a pipette and further cultured in Matrigel growth factor reduced (BD Biosciences, Bedford, MA) or type I collagen-coated dishes for growth or adhesion assays, respectively.

Western Blotting

Cells were lysed with cell lysis buffer (10 mM Tris [pH 7.4], 0.15 M NaCl, 1% NP40, 0.25% sodium deoxycholate, 0.05 M NaF, 2 mM EDTA, 0.1% SDS, 2 mM NaVO₄, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM PMSF). Western blotting was performed as previously described [28]. Primary antibodies raised against Δ Np63 (anti-p40) were obtained from Calbiochem (Merck Millipore, Darmstadt, Germany); E-cadherin and N-cadherin, from Abcam (Cambridge, MA); and β -actin, from Sigma-Aldrich.

Immunohistochemistry and Analysis

Immunohistochemistry was performed as previously described [28]. Primary antibodies against total p63 (4A4), Δ Np63 (anti-p40), and E-cadherin were obtained from Santa Cruz Biotechnology (Dallas, TX), Calbiochem, and Abcam, respectively. For assessment of the percentage of p63-positive cells in spheroids, the number of nuclei staining for p63 expression and the total number of nuclei were counted in each spheroid in each group. Intense Δ Np63 nuclear staining was scored as 2; moderate, as 1; and negative, as 0. The Δ Np63 score was then calculated by multiplying the staining intensity by the rate of positivity. For evaluation of the

immunoreactivity of E-cadherin, tissue sections were assessed according to preceding reports [29,30]; *positive staining for E-cadherin* was defined as the proportion of tumor cells with membranous staining >90%. For whole-mount immunostaining, cell lines and cell clusters were fixed and permeabilized with 4% paraformaldehyde/PBS containing 1% Triton X-100 at 4°C for 1 hour. After blocking with 5% goat serum/PBS-T for 1 hour, cells were incubated with anti-p63 antibody (4A4) (Santa Cruz Biotechnology) and Ki67 (Leica Biosystems, Newcastle, UK) overnight, then with Alexa-488 and Alexa-555 conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) overnight. After counterstaining with Hoechst33342 (Molecular Probes), cells were mounted with FluorSave Reagent (Calbiochem, Merck millipore). Images of fluorescent cells were obtained using confocal microscopy (TCS SPE, Leica Microsystems, Wetzlar, Germany).

Flow Cytometry

Spheroids derived from 5637 cells or CTOSs were dissociated into single cells using 0.25% trypsin/EDTA acid (Life Technologies, Carlsbad, CA, USA). For cell death assays, single cells were incubated with 1 µg/ml of propidium iodide (PI) (Molecular Probes) at 37°C for 15 minutes without fixation. Subsequently, flow cytometry was conducted using an Attune Acoustic Focusing Cytometer (Applied Biosystems, Foster City, CA), and the results were analyzed using FlowJo software (Tree Star, Ashland, OR).

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cell lines or CTOSs cultured under floating or attached conditions using TRIzol Reagent (Life Technologies). For semiquantitative RT-PCR, 1 µg of total RNA was reverse transcribed to obtain cDNA using Superscript III (Invitrogen) according to the manufacturer's protocol. PCRs were performed using the Gene Amp VR PCR System (Life Technologies). The primer sequences and PCR cycle numbers are shown in Supplementary Tables S1 and S2, respectively.

Proteasome Inhibition Experiments

Approximately 80 spheroids derived from 5637 cells or CTOSs were plated in 24-well tissue culture dishes (Corning Inc., Corning, NY) or type I collagen (Cellmatrix)-coated dishes, and cultured with 0.1% dimethyl sulfoxide or the indicated dose of MG132 (Sigma-Aldrich) or SB216763 (Sigma-Aldrich). The spheroids were collected after 7 hours for cell lines and after 20 hours for CTOSs, and then the proteins from lysed cells were subjected to Western blotting. For the spheroids cultured in collagen-coated dishes, collagen was removed by incubation at 37°C for 10 minutes with collagenase type IV (Worthington Biochemical Corporation, Lakewood, NJ) before collection.

Urothelium Clearance Assay

GFP-expressing SV-HUC-1 cells or primary human urothelial cells were seeded on 96-well plates with or without coating with type I collagen (Cellmatrix). Cells were maintained in culture until confluent (24-36 hours after plating). In co-culture experiments, a spheroid was placed onto a confluent urothelial monolayer. Images under bright-field and fluorescent microscopy were captured using an OLIMPUS IX70 microscope (OLUMPUS, Tokyo, Japan). To quantify the clearance of urothelial cells by a spheroid, the area devoid of the urothelial monolayer was measured and divided by the initial area of the spheroid.

Plasmid Construction and Gene Transfer

To construct the ΔNp63α expression vector, the BamHI-NotI fragment of pCDNA3.1hygro/deltaNp63alpha1xflag (Addgene, Cambridge, MA) was transferred to pPBiresPuro to make pPB/deltaNp63alpha1xflag. For short hairpin RNA vectors targeting p63, two targeting sequences, ACATGTGAGTGACGATGAT (#1) or AACCATGAGCTGAGCCGTGAATT (#2), were cloned into pPB shRNA [31] to make pPB/shp63 #1 and pPB/shp63 #2. EGFP was introduced by subcloning into pPB-Ubc.eGFP-neo [32]. The vectors were co-transfected with pCMV-hyPBase [32] by lipofection, using X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science) according to manufacturer's protocol. After transfection, cells permanently expressing the transfected genes were selected with G-418 (Roche Applied Science) or puromycin.

Statistical Analysis

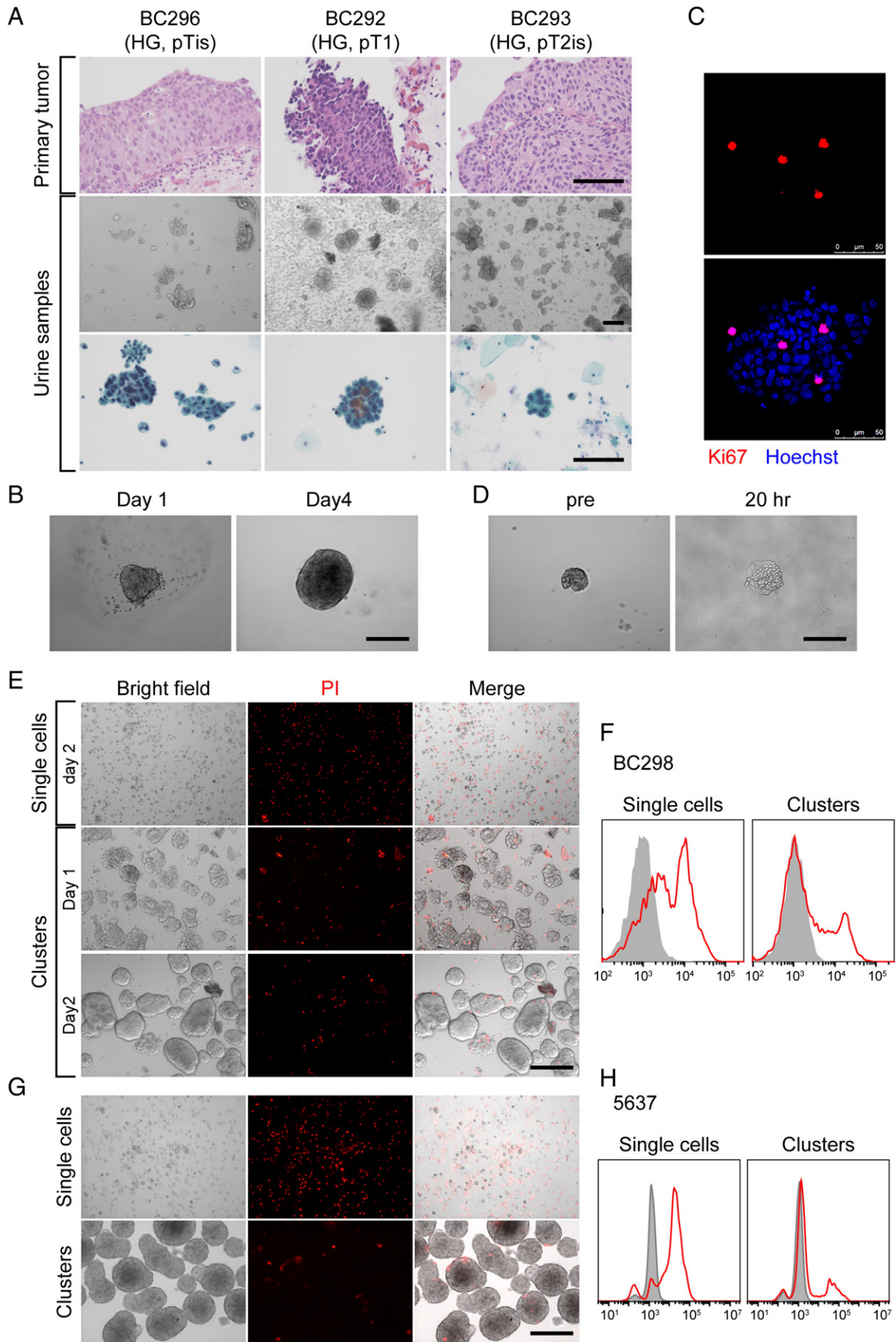
Statistical analysis was conducted using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Data are expressed as means with standard deviation. Statistical significance was tested using unpaired *t* test for single comparisons or one-way analysis of variance followed by Tukey test for multiple comparisons. A value of *P* < .05 was considered statistically significant.

Results

Cancer Cell Clusters in the Urine of Patients with Urothelial Cancer Retain the Ability to Grow and Adhere

We first examined the urine samples collected from patients with urothelial cancer to assess the status of cancer cells in the urine. We collected cancer cell clusters, known from clinical cytology to exist in patients' urine, from patients' urine (Figure 1A, Supplementary Figure 1). Some of the clusters (8 of 28 clusters from 5 patients, 28.6%) grew as spheroids *in vitro* with Ki67-positive proliferating cells (Figure 1, B and C). In addition, 5 of 12 clusters from 3 patients (41.7%) were able to attach to type I collagen-coated dishes (Figure 1D). Thus, the cancer cells also existed as clusters in the urine of patients with urothelial cancer and were able to grow and attach to the matrix. When cancer cells detach from the extracellular matrix,

Figure 1. Cancer cell clusters in the urine of patients with bladder cancer retain the ability to grow and adhere. (A) Hematoxylin and eosin staining of primary bladder urothelial tumors (upper row), phase contrast images (middle row), and Papanicolaou staining of sediments in urine derived from the same patients (lower row). HG, high grade. Scale bar, 100 µm. (B) Phase contrast images of cell clusters collected from the urine of a patient (BC289) with urothelial cancer and cultured in Matrigel growth factor reduced. The culture periods are indicated. Scale bar, 200 µm. (C) Immunostaining for Ki67 in cell clusters collected from the urine of a patient (BC287) with urothelial cancer. (D) Phase contrast images of cell clusters collected from the urine of a patient (BC297) with urothelial cancer and cultured in adhesive conditions. The culture periods are indicated. Scale bar, 200 µm. (E, F) Cell death assay by PI incorporation with single cells or cell clusters prepared from surgically resected urothelial tumors (BC298). Bright-field and fluorescent images of PI (E) and histograms of flow cytometry at day 2 (F). Red lines are for PI staining, and gray shades are for nonstained cells. (G, H) Bright-field and fluorescent images of PI (G) and histograms of flow cytometry at day 2 (H) with a bladder cancer cell line (5637). Scale bar, 200 µm.



they usually die because of anoikis [33,34]. We previously reported that cancer cells from urothelial cancers, as well as other cancers from patient tumors, can be stably prepared and cultured by maintaining cell–cell contacts throughout the preparation and culture procedure using a CTOS method [27,28,31,35]. When the clusters of cancer cells prepared from surgically resected urothelial tumors were dissociated into single cells, the dead cells with PI-positive staining drastically increased after 24 hours in culture under floating conditions compared with intact clusters (Figure 1, E and F). The clusters formed spheroids with smooth surface after 24 hours (Figure 1E). The same phenomenon was observed in the bladder cancer cell line 5637 (Figure 1, G and H). These results suggest that urothelial cancer cells in the urine have a better chance of surviving as clusters than as single cells under floating conditions.

Δ Np63 α Is Highly Expressed in the Nucleus of Relatively Differentiated Bladder Cancer Cell Lines in Spheroids Compared with 2D-Cultured Cells

We focused on Δ Np63 α because it was reported to play a role in motility and invasion as well as N-cadherin expression in urothelial cancer cells [21,22]. In addition, decreased staining levels of Δ Np63 are reported to correlate with recurrence of non–muscle-invasive bladder cancer after TUR [17,36]. We confirmed this in patients from our institution by using a different antibody than that previously used, showing that the staining score was higher in patients without recurrence (Figure 2, A and B; Supplementary Table 3). All the tumors analyzed were primary, solitary, pathological T1, and less than 3 cm in diameter without concomitant carcinoma *in situ*. None of the patients received intravesical therapy after TUR.

To analyze the Δ Np63 α status in the clusters, we first analyzed multiple bladder cancer cell lines with various differentiation levels: RT4 (well), 5637 (moderately), and J82 (poorly). The shape of the clusters differed among cell lines. RT4 and 5637 formed spheroids with a smooth surface, whereas J82 formed irregular clusters (Figure 2C). The protein levels of Δ Np63 in 3D clusters and in 2D cells were assessed by Western blotting. All isoforms of Δ Np63 (α , β , and γ) were detected in the spheroids of RT4 and 5637 cells, with the α isoform having the highest expression level (Figure 2D). The levels of α isoforms were higher in cells in 3D than 2D cultures. In contrast, only low levels of the γ isoform were detected in J82, and the levels were the same in 3D clusters and 2D cells. When spheroids of the 5637 cells were attached to the dish, they quickly grew out as monolayer cells (Figure 2E). The levels of Δ Np63 α decreased over time (Figure 2F). Immunocytochemistry revealed that p63 was localized in the nucleus of all the cells in the 5637 spheroids, whereas nuclear p63 was lost in many of the 2D cells, with some cells retaining the signal in the cytoplasm (Figure 2G). Thus, Δ Np63 α was highly expressed in the nucleus of relatively differentiated bladder cancer cells in 3D spheroids compared with cells in 2D cultures.

Δ Np63 α Expression Was High in Primary Cultured Spheroids and Decreased after Attachment to the Cell Matrix

We further examined whether the phenomena observed in the 5637 cell line are common in primary cultured cancer cells. The spheroids prepared using the CTOS method from patient urothelial cancers or patient-derived xenografts (PDXs) retain original characteristics [28,37]. CTOSs were directly prepared from operatively resected bladder or upper urinary tract tumors or PDX tumors. When CTOSs were placed in collagen-coated dishes, almost all of them attached to the surface and then started to form a monolayer (Figure 3A). Floating CTOSs showed higher levels of p63 than matrix-attached CTOSs in all cases examined (Figure 3, B and C). It should be noted that even the cancer cells in CTOSs that did not directly attach to the collagen matrix showed decreased p63 expression (Figure 3B). Western blotting showed decreased expression of Δ Np63 α after matrix attachment in all primary CTOSs examined, whereas the levels of Δ Np63 α varied among samples (Figure 3D). When the attached CTOSs were forcibly detached from the collagen matrix, they again formed spheroids, and Δ Np63 α expression was recovered (Figure 3E). Thus, in clinical samples, the levels of Δ Np63 α were high in floating clusters and then decreased when attached to the matrix, and the expression levels were highly reversible.

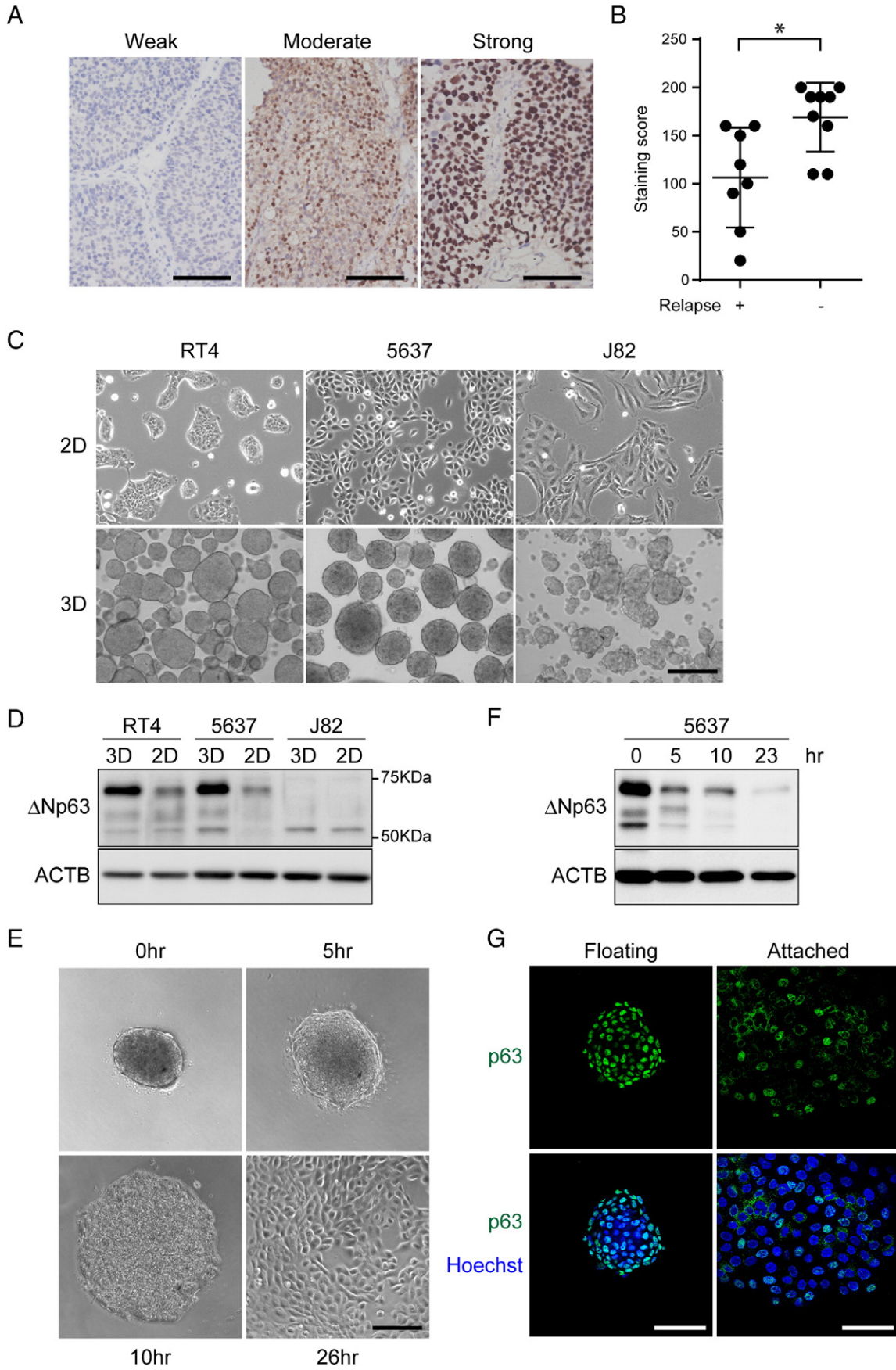
It is reported that Δ Np63 α decreases after induction of differentiation [38]. Using RT-PCR for various cytokeratins (CKs), we examined whether the decrease in Δ Np63 α was due to differentiation after attachment. CK5 and CK14 are basal cell markers, and CK18 and CK20 are differentiation markers [39]. Because changes in the expression of these CKs after attachment vary among the cell lines and CTOSs (Supplementary Figure 2A), differentiation alone cannot explain the decrease in Δ Np63 α expression after attachment.

Decrease in Δ Np63 α Expression after Attachment to the Matrix Was Due to Degradation by the Proteasome

We investigated the mechanisms of the attachment-induced decrease in Δ Np63 α protein levels in urothelial cancer cell clusters. In contrast to the protein levels, Δ Np63 mRNA levels were about the same in floating clusters and 2D-attached RT4 and 5637 cells, and were quite low under both conditions in J82 cells (Figure 4A). In all the CTOSs examined, mRNA levels were about the same in floating and attached CTOSs (Figure 4B).

Δ Np63 α is reportedly degraded by the ubiquitin-proteasome system after DNA damage [40] or induction of differentiation [38]. After phosphorylation by glycogen synthase kinase 3 (GSK3), Δ Np63 α is recognized by ubiquitin ligases such as Fbw7 [41]. We investigated the involvement of the proteasome in the attachment-induced decrease in Δ Np63 α protein levels in urothelial cancer cell clusters. The decrease in Δ Np63 α protein levels after attachment was suppressed by the proteasome inhibitor MG132 (Figure 4C). An

Figure 2. Δ Np63 α expression in a patient with bladder cancer and the dynamic change in the expression *in vitro* under 2D or 3D culture conditions. (A) Representative images of the indicated intensity of Np63 immunostaining in pT1 bladder cancers. Scale bar, 100 μ m. (B) Comparison of Np63 staining scores between patients with or without intravesical recurrence after transurethral resection. * $P < .05$. (C) Phase contrast images of the indicated cell lines under 2D or 3D conditions. Scale bar, 200 μ m. (D) Western blot analysis of Δ Np63 in the indicated cell lines cultured under 3D or 2D conditions. ACTB, β -actin. (E) Time course images showing attachment of a 5637-derived spheroid placed on a culture dish. Scale bar, 200 μ m. (F) Western blot analysis of Δ Np63 in 5637 cells in E. (G) Whole mount p63 immunostaining of a 5637-derived spheroid and of a spheroid 24 hours after attachment. Scale bar, 75 μ m.



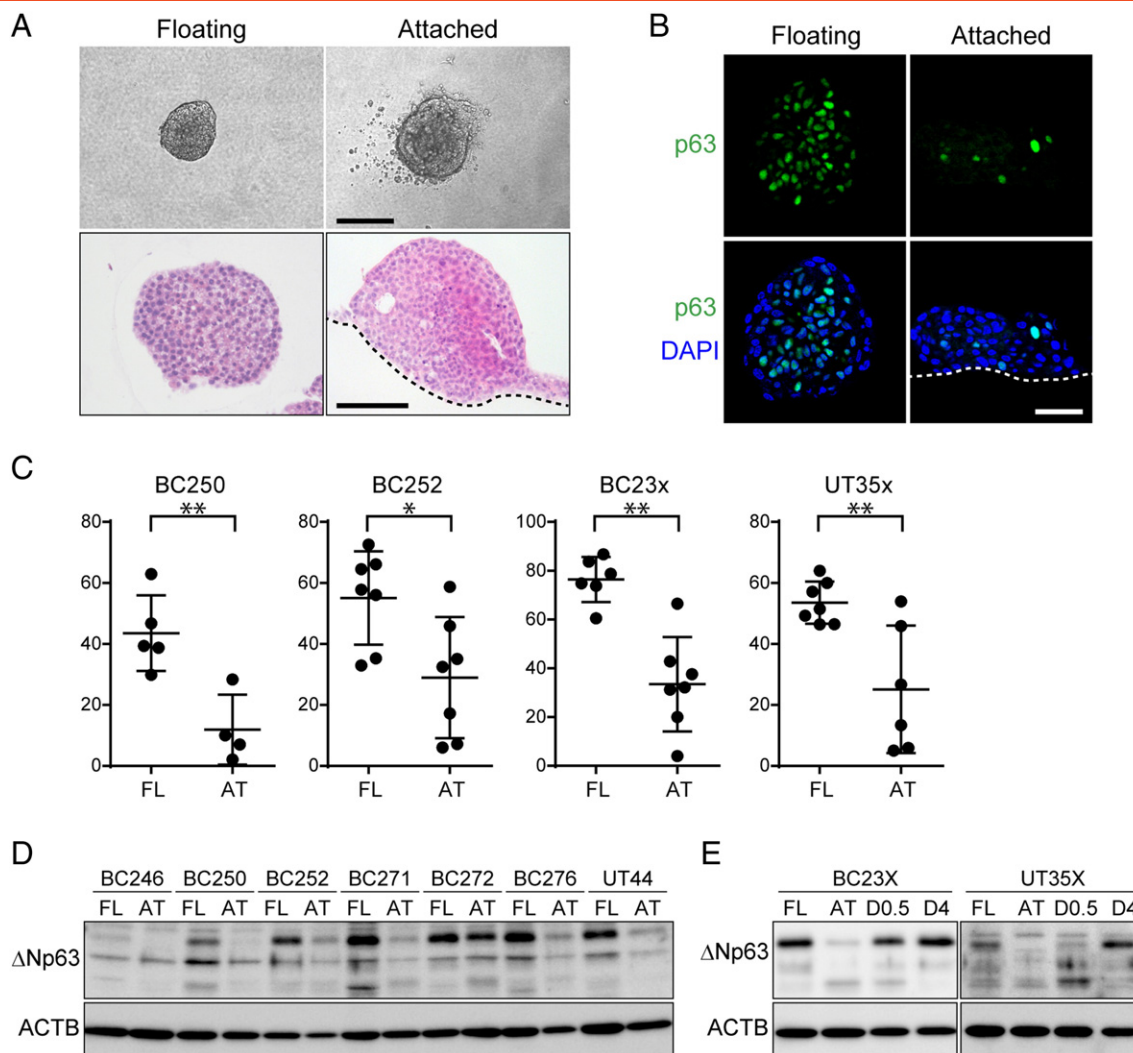


Figure 3. Dynamic change in Δ Np63 α expression in primary cultured spheroids after attachment to the cell matrix. (A) Images of phase contrast (upper row, scale bar 200 μ m) or hematoxylin and eosin (lower row, scale bar 100 μ m) of CTOSs prepared from urothelial cancer (BC246) cultured in floating conditions or attached on a type I collagen gel. (B) Images showing p63 expression by immunohistochemistry of a CTOS under floating or attached conditions (BC250). Scale bar, 50 μ m. A sagittal section is shown for the attached CTOSs. Dotted lines indicate the surface of the gel (A, B). (C) Percentage of p63-positive cells in various CTOSs. Each dot indicates one CTOS. * $P < .05$, ** $P < .01$. (D) Western blot of Np63 in CTOSs cultured under floating (FL) or attached (AT) conditions. CTOSs were prepared from surgically resected tumors. (E) Western blot of Np63 in CTOSs from two PDX tumors (BC23x and UT35x). CTOSs were attached to type I collagen gels and then detached from the gels and cultured for 0.5 (D0.5) and 4 hours (D4).

inhibitor of GSK3, SB216763, suppressed the decrease in Δ Np63 α protein levels after attachment (Figure 4D). The same effect was observed in CTOSs from MG132 and SB216763 (Figure 4, C and D). Thus, the decrease in Δ Np63 protein levels after attachment to the matrix is generally due to degradation through the proteasome.

Decreased Δ Np63 α Protein Levels in Urothelial Cancer Cell Clusters Parallels Cadherin Switching

We examined the effect of decreased Δ Np63 α on the molecular events related to the attachment to the matrix. We focused on cadherins as being downstream of Δ Np63 α because forced expression of Δ Np63 α is reported to negatively regulate N-cadherin expression and suppress motility and invasion in 2D cell lines [21,22]. We first assessed the relationship between the expression levels of Δ Np63 α and E-cadherin in clinical samples. Immunohistochemistry of non-muscle-invasive tumors revealed that cases negative for

E-cadherin expression had lower Δ Np63 expression levels (Figure 5, A and B). Thus, the levels of E-cadherin parallel those of Δ Np63 α in clinical samples. Next, we examined whether the levels of cadherins change under different culture conditions. 5637 cells in 3D spheroids expressed higher levels of E-cadherin and lower levels of N-cadherin than 2D cells with regards to both mRNA and protein levels (Figure 5, C and D). Meanwhile, the gene expression of other epithelial-to-mesenchymal transition (EMT)-related genes was not enriched in 2D cells (Supplementary Figure 2B). When CTOSs were attached to the matrix, E-cadherin signals decreased markedly in cells within CTOSs (left panels) in parallel with the decline in p63 (right panels) (Figure 5E). Here again, even the cancer cells in CTOSs that did not directly attach to the collagen matrix showed decreased E-cadherin expression. Taken together, changes in the Δ Np63 α levels during attachment of the cancer cell clusters were related to the cadherin levels.

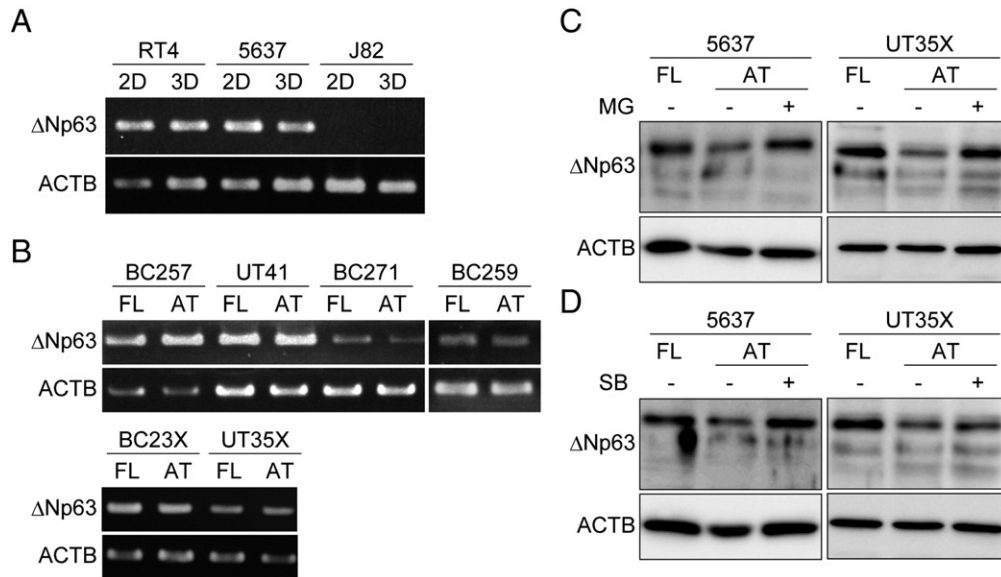


Figure 4. Decrease in Δ Np63 α protein levels after attachment to the matrix was due to degradation by the proteasome. (A, B) Semiquantitative RT-PCR for Np63 mRNA in bladder cancer cell lines (A) and CTOSs from patient tumors or patient xenograft tumors (B). FL, floating and AT, attached conditions. (C, D) Western blot of Δ Np63 in the 5637 cell line and UT35x CTOSs under floating or attached conditions, with or without the proteasome inhibitor MG132 (C) or the GSK3 inhibitor SB216763 (SB) (D). MG132 1 μ M for 5637 and 0.2 μ M for UT35x; SB216763 10 μ M for 5637 and 5 μ M for UT35x.

Degradation of Δ Np63 α in Urothelial Cancer Cell Clusters Is Involved in the Clearance of Urothelium

According to the dissemination theory, floating cancer cells first implant in the sheet of urothelial cells covering the wall of the urinary tract. Implantation of ovarian cancer cell clusters on mesothelial cells was previously demonstrated to be accompanied by exclusion of the mesothelium by the cancer cells [42]. Using a similar assay, we investigated the interaction between the clusters of urothelial cancer cells and the urothelium. When clusters of the 5637 bladder cancer cell line were placed onto a sheet of GFP-labeled SV-HUC-1 (normal immortalized human urothelial cell), the clusters or spheroids attached to the dish by clearing the urothelial cells (Figure 6A). The clearance of the urothelium and attachment to the dish by the clusters proceeded over time (Figure 6B). Spheroids prepared from primary bladder cancer cells also attached to the dish by clearing the primary cultured urothelial cells (Supplementary Figure 3).

Next, we generated 5637 cell lines in which Δ Np63 α was forcedly overexpressed or suppressed (Figure 6C). Both cell lines formed the same spheroids as control vector-transfected cells, but the Δ Np63 α -overexpressing cells were more roundly shaped under 2D conditions (Supplementary Figure 4, A and B). E-cadherin levels were slightly higher in Δ Np63 α -overexpressing cell spheroids but were clearly lower in the p63 knockdown cell spheroids (Figure 6C). Reciprocally, the N-cadherin levels in spheroids were lower in the Δ Np63 α -overexpressing cells and higher in the p63 knockdown cells (Figure 6C). The differences in Δ Np63 α expression levels between 3D and 2D conditions were observed also in the Δ Np63 α -overexpressing and the p63 knockdown cells (Supplementary Figure 4, C and D). We examined whether the decrease in Δ Np63 α has a function in the clearance of urothelium and attachment to the matrix underneath the urothelium. Urothelium clearance by the Δ Np63 α -overexpressing 5637 spheroid cells was suppressed compared with the control vector-transfected cells

(Figure 6, D and E). On the contrary, clearance by the p63 knockdown spheroid cells was enhanced (Figure 6, F and G). Thus, the decrease in Δ Np63 α in urothelial cancer cell clusters is involved in the clearance of the urothelium and attachment of the underlying matrix.

Discussion

We demonstrate that Δ Np63 α expression levels in urothelial cancer cell clusters dramatically decrease during the attachment to the cell matrix. The decrease in Δ Np63 α plays a critical role in cadherin switching, one of the aspects of the EMT in which cells shift expression from E-cadherin to N-cadherin [43]. Cadherin switching has a profound effect on tumor phenotype. In clinical reports, lower E-cadherin levels and higher N-cadherin levels correlate with intravesical recurrence of bladder cancer after TUR [29,30]. Taken together with the clinical reports, our results suggest a model of intraluminal dissemination; clusters of cancer cells detach from the primary site of urothelial cancer, drift in the urine, and attach to distant urothelium, resulting in decreased expression of Δ Np63 α followed by a cadherin switch and becoming the origin of disseminated foci.

The ability of epithelial cells to reversibly change their phenotype is referred to as epithelial plasticity [44]. Tumor metastasis is an example of epithelial plasticity in which tumor cells undergo EMT and acquire a migratory and invasive phenotype and disseminate, whereas the reversion of EMT, mesenchymal-to-epithelial transition, is essential to settle the metastasis in the target organ [45]. Mutations or deletions in TP63 are rarely reported in malignant tumors, including urothelial cancers [46,47], whereas gene manipulation of Δ Np63 α revealed that suppression of Δ Np63 α promotes EMT in human bladder cancer cells [21,22]. As we demonstrate in our study, the expression levels of Δ Np63 α were flexibly regulated in the dissemination model, and Δ Np63 α might be a key molecule for epithelial plasticity in urothelial cancer.

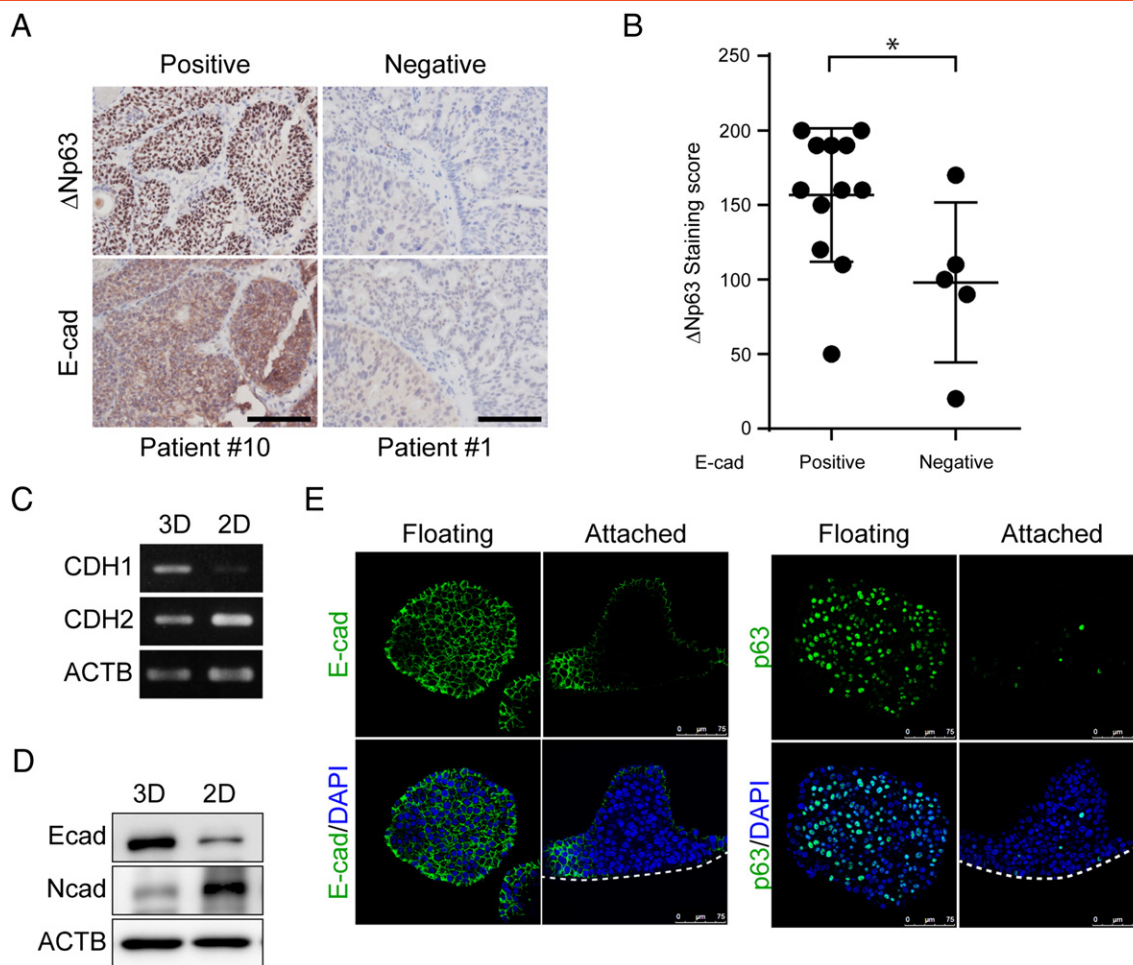


Figure 5. Decreased Δ Np63 α protein levels in urothelial cancer cell clusters parallels cadherin switching. (A) Representative images of positive or negative patterns of Δ Np63 and E-cadherin immunostaining in pT1 bladder cancers. Scale bar, 100 μ m. (B) Comparison of Δ Np63 staining scores between patient samples with positive or negative E-cadherin expression (same cohort of patients as in Figure 2B). * $P < .05$ (C, D) Semiquantitative RT-PCR (C) and Western blot (D) of E-cadherin and N-cadherin in the 5637 cell line cultured under the indicated conditions. (E) Immunohistochemical staining for E-cadherin (left panels) and p63 (right panels) in a CTOS under floating or attached conditions (BC246). A sagittal section is shown for the attached CTOSs. Dotted lines indicate the surface of the gel.

To study the interaction between clusters of urothelial cancers and the urothelium, we applied a model developed for peritoneal dissemination of ovarian cancer: an ovarian cancer-mesothelium clearance assay [42,48]. Using this assay, Davidowitz et al. reported that the ability to clear mesothelium in clusters of ovarian cancer cells is increased when the mesenchymal character is artificially rendered to the cancer cells [48]. In this study, we observed cadherin switching during the attachment of cancer cell clusters to the cell matrix, which was regulated by changes in endogenous Δ Np63 α levels. Among the EMT-like events, cadherin switching might play a central role in promoting clearance of urothelium by clusters of urothelial cancer cells.

The inner lumen of the urinary tract is covered by urothelium, which is a transitional epithelium consisting of distinct types of cell layers including basal, intermediate, and superficial [39]. In this study, we applied a monolayer culture of immortalized urothelial cells (SV-HUC-1) as a model of the epithelium so that the interaction between cancer cell clusters and superficial-layer cells was not assessed. Indeed, experiments using reconstituted urothelium from porcine urinary bladders revealed that the cancer cell line would not implant on umbrella cells but would implant on the cells in the basal

and intermediate layers after removing the umbrella cells [49]. Conditions of the urothelium, such as injury by operative procedures, might affect implantation.

The p63 protein levels are regulated by ubiquitin-dependent degradation through the proteasome [47,50]. Several E3 ligases for p63 and kinases that modulate the binding of E3 ligases to p63 proteins have been identified. In this study, a decrease in Δ Np63 α protein levels was suppressed by inhibition of the proteasome. Because translocation of Δ Np63 α from the nucleus to the cytoplasm was observed after attachment of the clusters in the 5637 cell line and the decrease in Δ Np63 α was suppressed by a GSK3 inhibitor, the degradation might be through the pathway reported by Galli et al.; MDM2 binds Δ Np63 α in the nucleus, promoting its translocation to the cytoplasm where p63 is targeted for degradation by the Fbw7 E3-ubiquitin ligase [41]. Degradation of Δ Np63 α is reportedly promoted by DNA damage [40,41] or induction of differentiation [38,41]. Because we did not observe clear evidence of differentiation after attachment (Supplementary Figure 2A), it is unlikely that the degradation of Δ Np63 α is due to differentiation induced by attachment.

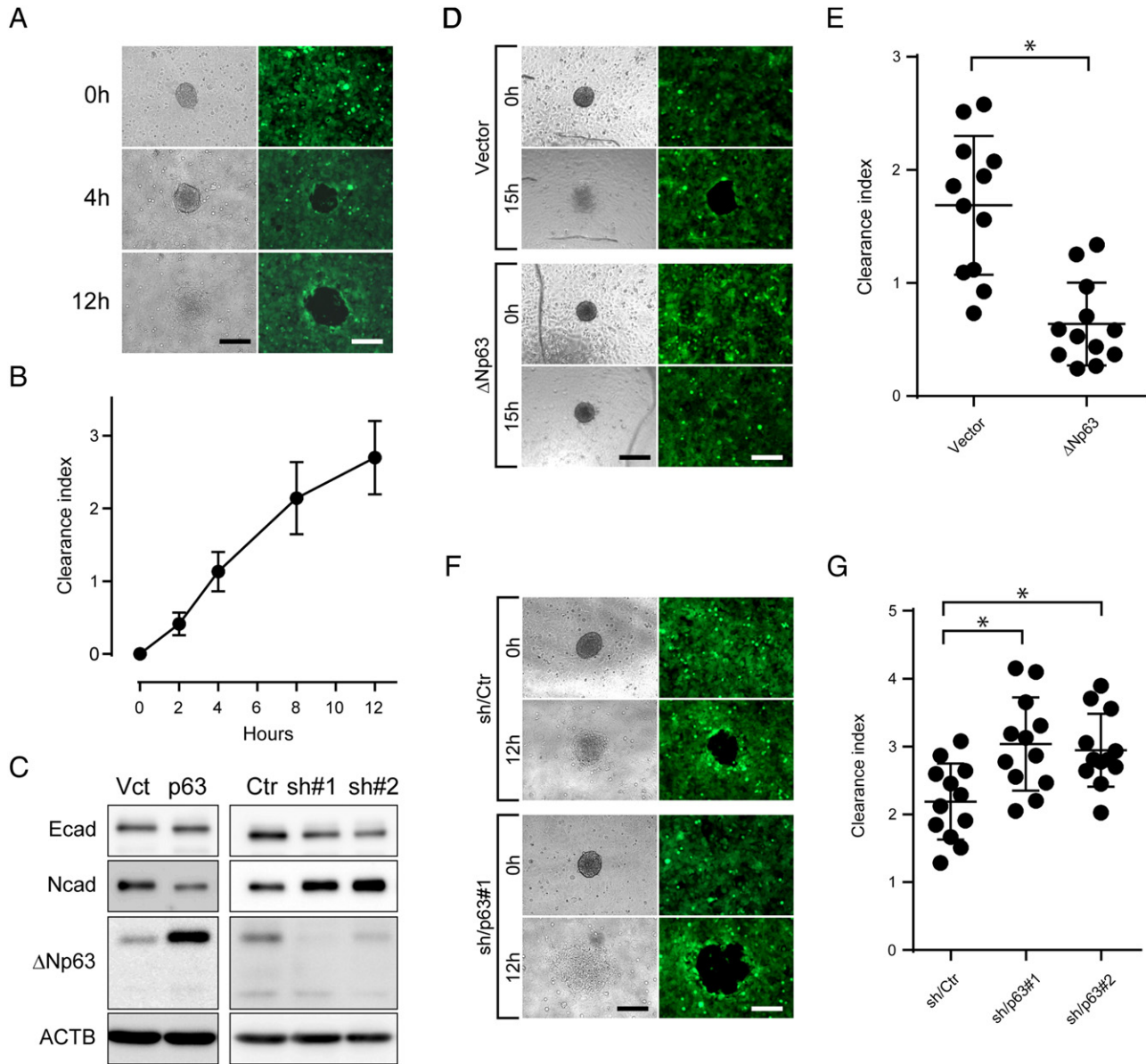


Figure 6. Degradation of $\Delta\text{Np63}\alpha$ in bladder cancer cell clusters after attachment was involved in the clearance of urothelium. (A) Time course images of urothelium clearance by bladder cancer cell clusters from the 5637 cell line. Bright-field images (left column) and fluorescent images (right column) of GFP-labeled SV40-immortalized normal human urothelial cells (SV-HUC-1). Scale bar, 200 μm . (B) Quantification of urothelial clearance by bladder cancer cell clusters in A. (C) Western blot analysis of E-cadherin (Ecad), N-cadherin (Ncad), and ΔNp63 in the spheroids of 5637 cells transfected with expression vectors $\Delta\text{Np63}\alpha$ (p63) and p63 shRNA (sh/p63#1 and sh/p63#2). Vct, empty vector; Ctr, scrambled shRNA. (D) Bright-field images (left column) and fluorescent images (right column) showing clearance of urothelium at the indicated time points by control vector or $\Delta\text{Np63}\alpha$ -overexpressing 5637-derived spheroids. Scale bar, 200 μm . (E) Quantification of urothelium clearance in D. * $P < .01$. (F) Bright-field images (left column) and fluorescent images (right column) showing clearance of urothelium at the indicated time points by control vector (shCtr) or p63-silenced (sh/p63#1) 5637-derived spheroids. Scale bar, 200 μm . (G) Quantification of urothelium clearance in F. * $P < .01$.

The upstream signaling pathway transmitting the attachment cue to the ubiquitin-proteasome system for $\Delta\text{Np63}\alpha$ degradation was not identified. Direct integrin signaling or secondary events, such as cytoskeletal remodeling, might be candidates for the upstream signaling pathway. It is intriguing that the decrease in ΔNp63 occurred not only in the cells directly attached to the matrix but also in cells within the clusters apart from the attachment interface with the matrix. These results suggest the existence of an intercellular communication pathway within the cluster. The downstream

signaling pathway, which transfers the decrease in $\Delta\text{Np63}\alpha$ levels to cadherin switching or later attachment and migration, is also unknown. Transcription factors involved in EMT were not increased in this study (Supplementary Figure 2B), suggesting that mechanisms other than typical EMT are involved. Although it requires further study, blocking the degradation of ΔNp63 could be a target of therapy for preventing dissemination of urothelial cancer.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2015.07.004>.

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