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A dominant negative form of p63 inhibits apoptosis in a p53-independent manner

Hae-ock Lee [a], Jung-Hwa Lee [a], Eunhee Choi [a], Ja Young Seol [b], Yungdae Yun [b], Hyunsook Lee [a,*]

[a] *Department of Biological Sciences, Research Center for Functional Cellulomics, College of Natural Sciences, Seoul National University, San 56-1 Shillim-dong, Gwanak-ku, Seoul 151-742, Republic of Korea*
[b] *Division of Molecular Life Sciences, Ewha University, 11-1 Daehyun-dong, Seodaemoon-ku, Seoul 120-750, Republic of Korea*

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Abstract

Stem cells are a source of differentiated cells in multiple tissues. If genetic alterations occur in stem cells, the problem persists and malignant cancers may arise. ΔNp63α—a homologue of the tumor suppressor p53—is exclusively expressed in proliferating undifferentiated epithelial cells and cancer cells of epidermal origin. Here, we show that ΔNp63α antagonizes DNA damage-induced apoptosis in a p53-independent manner. We found that upon cellular injury, ΔNp63α must be downregulated before apoptotic program can be activated. The 5637 cell line has abundant levels of ΔNp63α and mutant p53, and it is resistant to DNA damage-induced apoptosis. The knockdown of ΔNp63α by RNA interference sensitized these cells to apoptosis upon genotoxic insult. This suggests that ΔNp63α plays an anti-apoptotic role regardless of the p53 status. Considering the frequent mutations of p53 in tumor cells, our results provide important implications for the treatment of cancers in which p63 is amplified.

Keywords: ΔNp63α; p53; Apoptosis; Tumorigenesis; DNA damage

p63 is the most recently identified member of the emerging p53 tumor suppressor family. The *p63* (*AIS/KET/CUSP/p40/p51/p73L*) gene encodes two different transcripts that have apparently opposite functions in transcriptional control. One transcript encodes TAp63 that is structurally and possibly functionally similar to p53. ΔNp63, which lacks the transcription-activating (TA) domain of TAp63, is encoded from a second promoter [1]. ΔNp63 is interesting in that it acts as a dominant negative for p53 and TAp63 in vitro and in vivo [1,2]. The biology of p63 is complicated by alternative splicing at the C-terminal that generates three splice variants. Thus, the *p63* allele encodes at least six different isotypes.

Using antibodies generated against the DNA-binding domain that is shared by TAp63 and ΔNp63, the high and constitutive expression of p63 has been detected in the immature basal cell layers of epithelial tissues [1]. This expression is lost in the differentiated layers. Consistent with this expression pattern is that mice whose *p63* gene has been knocked out lack all stratified epithelia, including lacrymal epithelia, salivary epithelia, mammary glands, and hair follicles [3,4]. Therefore, p63 is proposed to be essential for the maintenance and regenerative proliferation of epidermal stem cells. However, since *p63*-null mice are devoid of all p63 isoforms, studies using these mice were unable to determine the potentially disparate functions of TAp63 and ΔNp63.

Studies using zebrafish embryos as a model system demonstrated that ΔNp63, but not TAp63, is absolutely required for epidermal cell proliferation during embryogenesis [2]. Moreover, the timed overexpression of p53 in the epidermis has the same effect as the inhibition of ΔNp63 expression; this suggests that there is interplay

* Corresponding author. Fax: +82 2 886 4335.
E-mail address: HL212@snu.ac.kr (H. Lee).

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between p53 and ΔNp63 that is essential for the maintenance of epidermal cell proliferation during embryonic development [2]. P63 also participates in epidermal lineage commitment [3,5], cellular senescence [6], and neuronal apoptosis [7]. Although these studies warrant isoform-specific knockouts to address the precise roles of TAp63 vs. ΔNp63α, p63 undoubtedly plays a major role in diverse cell fate decisions. It is thought that the interplay between the members of the p53 family determines the cellular fate.

The initial identification of *p63* attracted considerable attention because it was a potential novel tumor suppressor gene. However, evidence supporting the hypothesis that p63 is a tumor suppressor is lacking because while the functional inactivation of the murine *p63* gene results in severe defects in development, enhanced tumor development is not observed [3,8]. Indeed, there are reports suggesting that Δ*Np63* may in fact be an oncogene since it is amplified in cervical squamous carcinomas, head and neck squamous cell carcinomas (HNSCC), and a subset of tumor cell lines [9–15]. However, the molecular mechanism by which Δ*Np63* could act as an oncogene remains unknown.

In order to understand how malignant tumors arise in the epithelia, we focused on the following two key characteristics of p63: (1) p63 is a marker for epidermal stem cells [2,15–20] and (2) ΔNp63 is a dominant negative regulator of p53 in vivo during embryonic development [2]. Stem cells are a source of differentiated cells in multiple tissues. When genetic alterations occur in stem cells, the problem persists, and it may lead to malignant cancers. Considering that more than 50% of human cancers carry p53 mutations [21] and the rest are thought to harbor problems in the p53 pathway, misregulated interplay between ΔNp63 and p53 in stem cells might contribute to the transformation process of epithelial cells, which comprise the majority of human cancers.

Once mutant p53 takes over cancer cells, is the presence of ΔNp63 a mere remnant of misregulated interplay or is it involved in facilitating the growth of cancer cells? Here, we show that the inhibition of ΔNp63α sensitizes cancer cells with p53 mutations to DNA damage-induced apoptosis. Therefore, we propose that ΔNp63 contributes to tumorigenesis at two different levels. First, ΔNp63 would downregulate p53 activity in response to DNA damage (p53 dependent) and next, ΔNp63 would facilitate the growth of cancer cells in a p53-independent manner.

Materials and methods

Cell lines and cell culture. 5637 (bladder cancer) and A549 (lung cancer) cells were grown in RPMI-1640 supplemented with 10% v/v fetal bovine serum, penicillin, and streptomycin. Human foreskin keratinocytes (HFK) were isolated and cultured as described previously [11]. Human telomerase reverse transcriptase (hTERT)-introduced human mammary epithelial (HME) cells were kindly provided by Dr. William Hahn (Dana Farber Cancer Center, Boston).

Antibodies, plasmids, and chemical reagents. Antibodies specific for p53 (DO-1; Oncogene Science, Cambridge, MA), phospho-p53 (Ser20; Cell Signaling Technology, Danvers, MA), and phospho-p53 (Ser15; Cell Signaling Technology) were obtained commercially. Monoclonal antibodies to lamin A/C were gifted by Dr. Frank McKeon (Harvard Medical School, Boston). Mitomycin C, nocodazole, doxorubicin, and cisplatin were purchased from Calbiochem (San Diego, CA). The p53 and p63 expression plasmids and antibody (4A4) were kind gifts from Dr. Frank McKeon. The Bax and Mdm2 promoter reporter constructs were kind gifts from Dr. Jaewoon Lee (Baylor College of Medicine, Houston).

UV and γ irradiation. The cells were plated at the required density 24 h before irradiation. Prior to UV irradiation, the media were removed, and the culture dishes were irradiated with UV rays at 120 mJ/cm^2 using CL-100 UV crosslinker (UVP, Upland, CA). For γ irradiation, culture dishes containing the media were exposed at 15 Gy to a ^{137}C source (GC 3000 Elan; MDS Nordion, Ontario, Canada). The cells were reincubated with fresh media for a defined time period.

Treatment with chemical reagents. The cells (5×10^5) were plated in a 60-mm dish on the night before the treatment. Cisplatin (5 μg/ml), doxorubicin (2 μg/ml), mitomycin C (2 μg/ml), and nocodazole (2 μg/ml) were added to the culture medium for defined time periods.

RT-PCR. Total RNA was extracted from HME, A549, and 5637 cell lines using the TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was performed using Superscript one-step RT-PCR (Invitrogen) using 1 μg of total RNA. The following primers were used to amplify p63, TAp63, SAM domain, and β-actin. p63: 5′ ATGTTGTACCTGGAAAACAATGCCCAGACT and 5′ GGTGGG GTCATCACCTTG. TAp63: 5′ ATGTCCCAGAGCACACAG and 5′ TG GTCCATGCTGTTCAG. SAM: 5′ CCCACAGATTGCAGCATTGTC AGTTTCTT and 5′ TCACTCCCCCTCCTCTTTGATGCGCTGTT. β-actin: 5′ GTGGGGCGCCCCAGGCACCA and 5′ CCCTTAATG TCACGCACGATTTC.

siRNA preparation and transfection. Small interfering RNAs (siRNAs) were obtained from Dharmacon Research (Chicago, IL). The sequences that were selected for synthesizing siRNAs are listed as follows: p63: AACCATGAGCTGAGCCGTGAATT and TAp63: GAGCACCCAGA CAAGCGAGTT. Synthetic oligonucleotides were deprotected, desalted, and annealed. Twenty-four hours before transfection, 1×10^5 cells were plated on 60-mm dishes. The transfection of siRNAs was carried out for targeting endogenous genes by using siPORT Lipid (Ambion, Austin, TX). The cells were incubated for 18 h in the presence of doxorubicin (0.5 μg/ml), 72 h after the transfection.

DNA transfection and reporter assays. Site-directed mutagenesis was performed on the p53 construct to substitute arginine at 280 with threonine (R280T). The wild-type p53 or the mutant p53 containing R280T was transfected to BHK cells by using FuGENE 6 transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. In order to test ΔNp63α-mediated repression on p53-dependent transcriptional activation, Bax-luc or Mdm2-luc reporter constructs were employed. The DNA quantities for all transfections were equalized with the pcDNA3-Myc vector. Luciferase activity was measured 48 h after the transfection by the Luciferase Assay System (Promega, Madison, WI) and MicroLumat Plus LB 96 V luminometer (Berthold Technologies, Oak Ridge, TN).

TUNEL staining. Cells were seeded onto coverslips one day prior to UV treatment and subjected to the TUNEL assay using the In Situ Cell Death Detection kit (Roche).

Apoptosis assay and cell cycle analysis using a flow cytometer. The cells were stained with fluorescein-conjugated annexin-V and propidium iodide (PI; BD Biosciences, San Diego, CA) according to the manufacturer's instructions and analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) by using the CellQuest software. The percentage of apoptotic cells was calculated by scoring the cells that were positive only for annexin-V (early apoptotic stage) or positive for both annexin-V and PI (late apoptotic and necrotic stages). Cell cycle analysis was performed by measuring the DNA content by PI staining and using a FACSCalibur flow cytometer.

Results

ΔNp63α is downregulated following various cellular damages that induce apoptosis

In order to test our hypothesis that ΔNp63α may antagonize p53 in tumorigenesis as observed previously during embryogenesis [2], we checked the p63 status of 15 cancer cell lines of epithelial origin by using Western blot analysis and RT-PCR. This screening revealed that A549—a non-small cell lung carcinoma line—was positive for ΔNp63α expression while the 5637 bladder carcinoma cell line was a ΔNp63α-overexpressing cell line. As compared to human foreskin keratinocytes HFK, the level of ΔNp63α in A549 was low but it was significantly higher in 5637 cells (Fig. 1A). On comparing the sizes of the various isoforms of p63 expressed in the transfected 293T cells, ΔNp63α was the p63 isotype that was detected in both cell lines (Fig. 1A). RT-PCR analysis using specific primers that distinguish between the p63 DNA-binding domain, N-terminal TA domain of p63, and C-terminal SAM domain showed that TA domain-specific primers did not amplify a product. However, primers specific for the SAM domain—which only exists in the α-isotypes [1]—were able to amplify a product (Fig. 1B). Therefore, the form of p63 that is expressed in the A549 and 5637 cells is ΔNp63α. It is noteworthy that the A549 cell line expresses wild-type *p53* whereas the 5637 bladder cancer cell line, which has abundant levels of ΔNp63α, expresses mutant *p53* [22]. We determined the function of ΔNp63α on cellular stress using these cell lines.

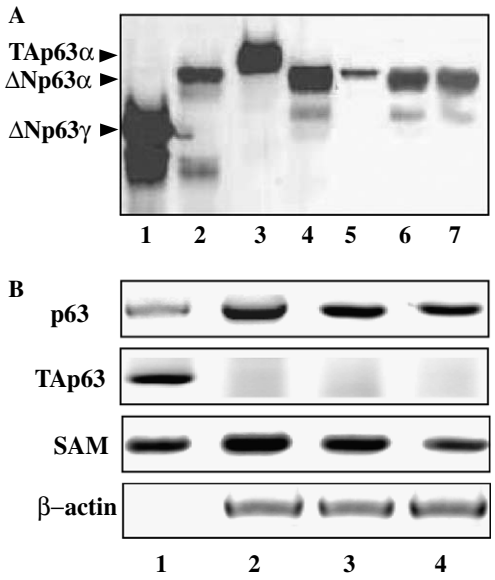

Fig. 1. ΔNp63α is expressed in proliferating primary epithelial cells and a subset of cancer cell lines. (A) Lysates of the 5637 bladder carcinoma (lane 4) and A549 non-small cell lung carcinoma (lane 5) cell lines, human mammary epithelial cells immortalized with hTERT (HME, lane 6), and human foreskin keratinocytes (HFK, lane 7) were analyzed by Western blotting with anti-p63 (4A4) antibodies. All the lanes were loaded with 30 μg of the total cell lysates. The 293T cells transfected with Δ*Np63γ* (lane 1), Δ*Np63α* (lane 2), and *TAp63α* (lane 3) expression plasmids were used as controls to identify the p63 isoform that is expressed in the cells. (B) RT-PCR analysis to confirm that ΔNp63α is the predominant isoform in the HME (lane 2), A549 (lane 3), and 5637 cells (lane 4). PCR primers specific for the common domain (upper panel, p63), TA domain (middle, TAp63), and p63 SAM domain (lower panel, SAM) of *p63* were employed to distinguish between the different p63 isotypes. The PCR products of ΔNp63α and TAp63α expression plasmids were loaded side by side (lane 1) as controls. RT-PCR for β-actin shows that the same amount of RNAs was employed for each reaction.

In order to determine whether ΔNp63α antagonizes p53 function on cellular stress, A549 cells were treated either with various drugs that cause double-stranded DNA breaks or microtubule disruption or with UVB irradiation that generates single-stranded DNA breaks. All these act as p53-activating signals. In A549 cells, treatment with doxorubicin, cisplatin, and mitomycin C (all of which introduce double-stranded DNA breaks), and nocodazole (which inhibits microtubule polymerization) led to the elevated levels of p53, thereby suggesting p53 activation [23,24] (Fig. 2). On genotoxic stress, tumor suppressor p53 undergoes multiple posttranslational modifications. The N-terminal of p53 is phosphorylated by kinases such as ataxia telangiectasia mutated (ATM) kinase and checkpoint kinase (Chk2), while its C-terminal is heavily acetylated [25,26]. This stabilizes the protein and allows it to function. Therefore, the same blot was reprobed with phospho-p53-specific antibodies to demonstrate the activation of p53 on genotoxic insult. In agreement with the upregulation of p53 protein levels, the phosphorylation of p53 was increased by the treatment of A549 cells with doxorubicin, cisplatin, or mitomycin C (Fig. 2). This confirms that in A549 cells, p53 is indeed activated by DNA breakage. The phosphorylation of Ser15 or Ser20 residues of p53 was not increased by treatment with the microtubule polymerization inhibitor nocodazole since these phosphoryla-

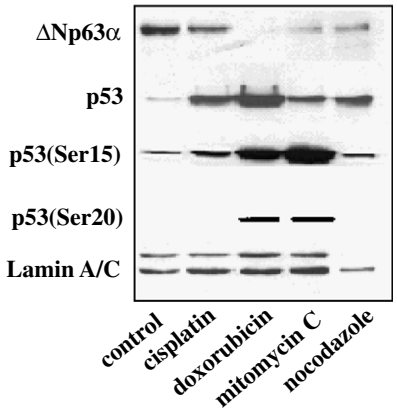

Fig. 2. ΔNp63α downregulation is associated with various apoptotic signals that activate p53. The A549 cells were treated with 5 μg/ml cisplatin, 2 μg/ml doxorubicin, 2 μg/ml mitomycin C, or 2 μg/ml nocodazole for 18 h and then subjected to Western blot analysis using antibodies specific for p63 (4A4), p53 (DO-1), and phosphoserine 15 and phosphoserine 20 in p53. All the lanes were loaded with 60 μg cell lysates. The same blot was reprobed with antibodies to lamin A/C as the loading control.

tions occur only upon DNA damage. However, the p53 levels were upregulated by nocodazole treatment; this suggested that p53 was activated by microtubule disruption, as has been reported previously [27] (Fig. 2). In sharp contrast, the ΔNp63α levels were downregulated when challenged with the various cellular insults that activate p53 in A549 cells (Fig. 2). Similar results were obtained when we used untransformed mammary epithelial cells (data not shown). These observations suggest that the downregulation of ΔNp63α accompanies p53 activation in response to various types of cellular damage.

Liefer et al. [28] had reported earlier that ΔNp63α downregulation is required for UV-induced apoptosis. Our observations on UVB-irradiated A549 cells that showed the downregulation of ΔNp63α levels (Fig. 3A) are in agreement with this finding. Therefore, it appears that the downregulation of ΔNp63α is involved in the response to single-stranded and double-stranded DNA breaks and microtubule disruption.

Interestingly, while UVB treatment downregulates ΔNp63α levels in A549 cells (Fig. 3A, upper panel), ΔNp63α levels in the 5637 bladder cancer cells remained unaffected (Fig. 3A, lower panel). In order to determine whether there is a mutation in ΔNp63α in this cell line, RT-PCR, subcloning, and sequencing of at least four independent clones were performed. This confirmed that the ΔNp63α protein in the 5637 bladder cancer cell line is the wild-type protein (data not shown). Thus, on DNA damage, 5637 cells with p53 mutation overexpress wild-type ΔNp63α that is resistant to degradation. Further, we observed that the apoptotic response of 5637 bladder cancer cells to UVB irradiation was low compared to that of the A549 cells (Fig. 3B).

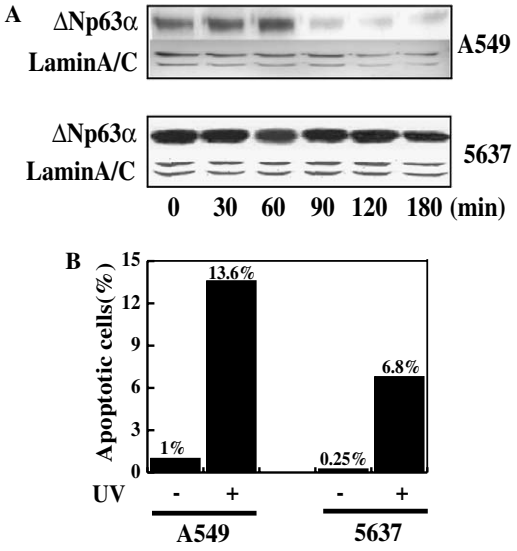

Fig. 3. UVB-induced ΔNp63α downregulation in the 5637 bladder cancer cell line is impaired as compared to the A549 cell line. (A) The A549 and 5637 cells were irradiated with UVB for a defined time period, and their cell lysates were then subjected to Western blot analysis using p63-specific antibodies (upper lanes). The same blot was reprobed with antibodies to lamin A/C (Lamin A/C) as the loading control (lower lanes). For the A549 cells, 60 μg of the lysates was employed for Western blot analysis whereas 20 μg of 5637 cell lysates was employed due to the high levels of ΔNp63α in these cells. (B) The A549 and 5637 cells were irradiated with same dose of UVB irradiation and subjected to the TUNEL assay to detect cell death in response to DNA damage. The percentage of TUNEL-positive cells increased to 13.6% in A549 cells, whereas the percentage of TUNEL-positive cells in 5637 cells was half of the level observed in A549 cells (6.8%).

Abrogation of ΔNp63α expression sensitizes the cells to apoptosis on DNA damage

We determined whether resistance to DNA damage-induced apoptosis in 5637 cells was due to the elevated levels of ΔNp63α. For this purpose, we employed the RNA interference technology in mammalian cells [29]. The sequence and region that were selected for synthesizing siRNAs used in this study have been described in the

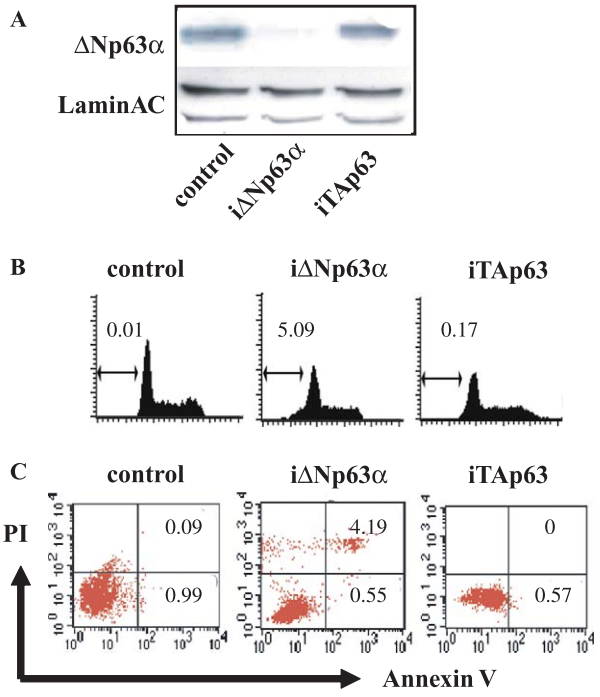

Fig. 4. The knockdown of ΔNp63α expression in the 5637 cancer cell line affects apoptosis. (A) Western blot analysis showed that Δ*Np63α* expression is specifically inhibited by employing p63 siRNA (iΔNp63α), whereas the siRNAs for TAp63 (iTAp63) did not affect ΔNp63α expression. The blot was reprobed with the lamin A/C antibody as the loading control. (B) The cells were subjected to cell cycle analysis 3 days after introducing various siRNAs. The overall cell cycle progression, as assessed by measuring the DNA content by PI staining, was not significantly affected by the introduction of the siRNAs. (C) 5637 bladder cancer cells were subjected to apoptosis analysis 3 days after transfection with siRNAs as indicated. The reduction of ΔNp63α levels by transfecting the cells with *p*63 siRNA (iΔNp63α) induces apoptosis, as determined by flow cytometry analysis of annexin-V- and PI-stained cells. The viable cells are negative for both annexin-V and PI (lower left quadrant), while the cells in the early (annexin-V positive/PI negative) or late (annexin-V positive/PI positive) stage of apoptosis appear in the lower right and upper right quadrants, respectively. The numbers indicate the percentage of cells (%) in each quadrant.

Materials and methods. The region selected for *p*63 siRNA is actually located in the common DNA-binding domain and can inhibit the gene expressions of both *TAp63* and Δ*Np63*. Since extensive Western blot analysis and RT-PCR (Fig. 1) revealed that ΔNp63α, but not TAp63, is expressed in A549 and 5637 cells, we predicted that the use of this siRNA will specifically interfere with Δ*Np63* expression. However, the siRNA that recognizes the TA domain—present only in *TAp63*—was also employed to confirm that *p*63 siRNA specifically blocks ΔNp63α gene expression.

The 5637 cells transfected with various siRNAs were subjected to Western blot analysis 3 days after the transfection. A marked reduction was detected in the ΔNp63α levels of the cell lysates when the cells were transfected with *p*63siRNA (iΔNp63α, Fig. 4A). However, *TAp63* siRNA did not block the expression of Δ*Np63*α in the 5637 cells (iTAp63, Fig. 4A). Thus, transfection with *p*63 siRNA specifically blocks Δ*Np63*α gene expression (Fig. 4A).

We then analyzed the cell growth and survival of the 5637 cells after the specific knockdown of Δ*Np63*α expression. Cell cycle progression, which was assayed by measuring the DNA content with PI staining, showed that cell cycle progression was not significantly affected by transfection with any of the siRNAs. Instead, the sub-G1 population—indicative of apoptosis—increased when ΔNp63α expression was abrogated (Fig. 4B).

Apoptosis in these cells was further analyzed by staining the cells with annexin-V and PI. The cells in which Δ*Np63*α expression was inhibited by Δ*Np63* siRNA transfection showed a 4.5-fold increase in apoptosis in the absence of external stimuli (iΔNp63α, Fig. 4C); this is consistent with the data shown in Fig. 4B. These results suggest that the reduced expression of Δ*Np63*α in 5637 cells interferes with cell survival.

Next, we determined whether the response of 5637 cells to DNA damage will be altered when ΔNp63α gene expression is abrogated. We challenged 5637 cells with 0.5 μg/ml doxorubicin—a radiomimetic drug—for 18 h 3 days after transfection with various siRNAs. The 5637 cells in which ΔNp63α expression was inhibited were very sensitive to doxorubicin-induced apoptosis since 79.6% of these cells were positive for the apoptosis marker annexin-V. However, less than 10% of the control cells and iTAp63-challenged cells showed apoptosis (Figs. 5B and C). Cell cycle analysis showed that DNA damage due to doxorubi-

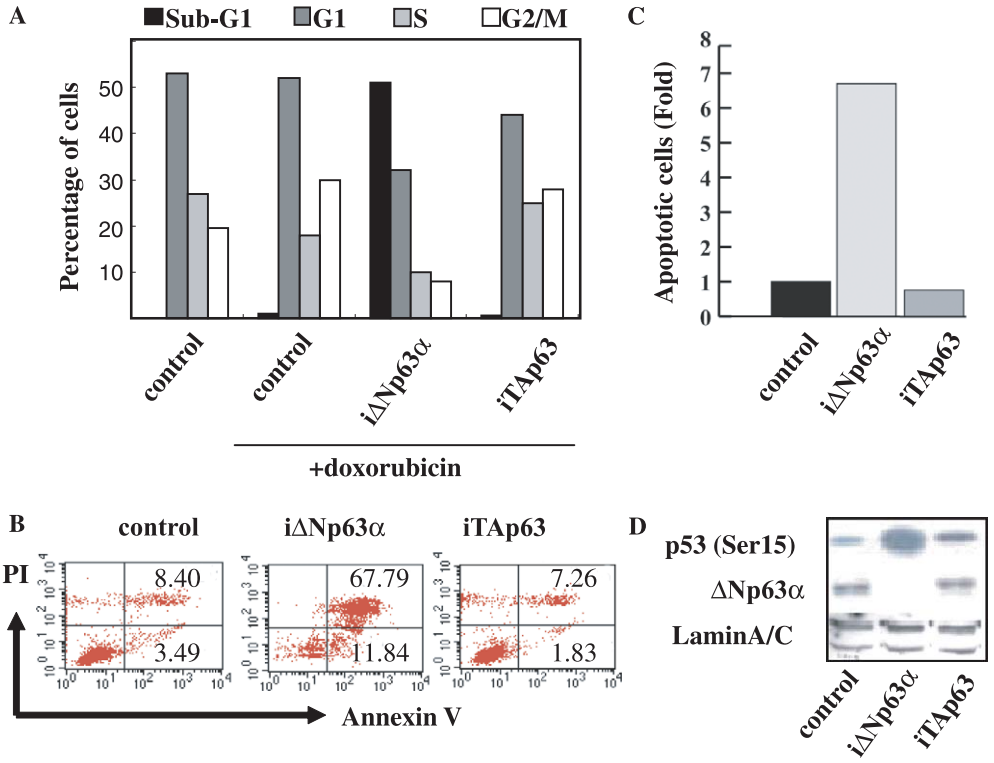

Fig. 5. Elimination of ΔNp63α from 5637 bladder cancer cells makes them sensitive to DNA damage-induced apoptosis. (A) Cell cycle analysis shows that the knockdown of Δ*Np63*α increases the susceptibility of the cells to 0.5 μg/ml doxorubicin since the sub-G1 population increased to 51% (iΔNp63α + doxorubicin). The control cells showed an increase in the G2 population level after treatment with doxorubicin (control + doxorubicin), thus indicating that the indicated dose of doxorubicin leads to G2 arrest in 5637 cells. Cells transfected with siRNAs for TAp63 also showed an increase in the levels of the G2 population, but not in the sub-G1 population, after DNA damage. (B) Cells challenged with various siRNAs were treated with 0.5 μg/ml doxorubicin for 16 h and subjected to apoptosis analysis. Knockdown of Δ*Np63*α expression by employing *p*63 siRNA (iΔNp63α) leads to a marked increase in apoptotic cell number (79.6%) as compared to the control untransfected cells (11.1%). (C) The apoptotic cell levels in (B) are presented as graphic bars. The elimination of ΔNp63α and treatment of cells with doxorubicin increased apoptosis by 7-fold as compared to that in the control cells. (D) Western blot analysis with antibodies to phosphor-p53 (Ser15) and p63 shows that knockdown of ΔNp63α expression and phosphorylation of p53 in response to DNA damage.

cin treatment led to G2 arrest in the control and iTAp63-challenged cells, whereas the ΔNp63α siRNA-transfected cells undergo massive apoptosis as measured by sub-G1 population (Fig. 5A). Surprisingly, Western blot analysis shows that in the presence of DNA damage, p53 Ser15 phosphorylation dramatically increases in the cells in which Δ*Np63*α expression is inhibited, although the 5637 cells harbor the mutant p53 (R280T) that has been reported to be non-functional [30] (Fig. 5D). Therefore, we investigated the behavior of this mutant p53. As shown in Fig. 6A, p53-R280T is extremely stable as compared to its wild-type, recapitulating the high expression levels of the mutant p53. DNA damage induced the phosphorylation of p53-R280T at Ser15; this is also consistent with Fig. 5D. However, the mutant barely activated Bax or Mdm2 promoters on transient transfection into BHK cells (Fig. 6C); this demonstrates the compromised function of R280T-p53, although p53 phosphorylation in response to DNA damage is intact. These data indicate that the abrogation of ΔNp63α sensitized the 5637 cells to DNA damage-induced apoptosis independent of p53-mediated transcriptional activation. Taken together, we propose that ΔNp63α operates downstream of the DNA damage response pathway and may function as an antiapoptotic factor. The antiapoptotic function of ΔNp63α bypasses p53-dependent transcriptional activation.

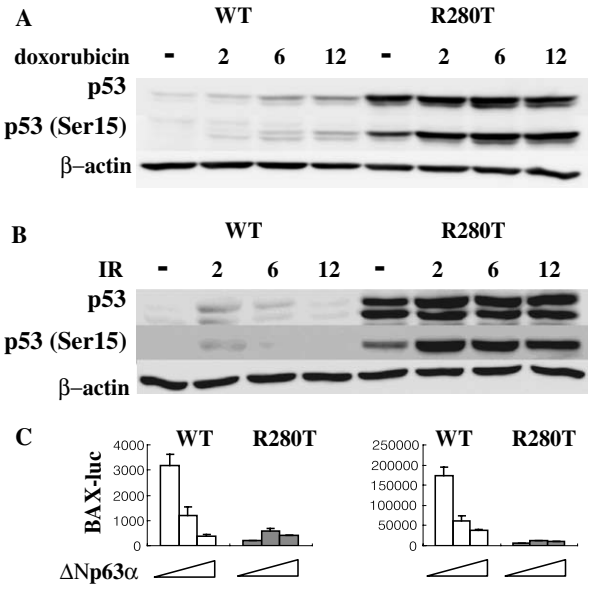

Fig. 6. The R280T p53 mutant is non-functional. (A,B) Wild-type and R280T mutant p53 were transfected to BHK cells. The cells were split after 24 h and were treated either with 2 μg/ml doxorubicin (A) or γ irradiation (B) for a defined time period. The levels of p53 and phosphorylation at Ser15 were determined by Western blot analysis. (C) Wild-type and mutant p53 (1 μg) were cotransfected with the BAX or Mdm2 reporter constructs (100 ng) and pRL-TK (100 ng) for normalization. ΔNp63α was also cotransfected (0.2 or 1 μg) to test transcriptional repression. The DNA quantities for transfection were equalized with the pcDNA3-Myc vector. Reporter activity was plotted as the peak luminescence per second.

Discussion

Here, we have presented several lines of evidence suggesting that ΔNp63α plays an antiapoptotic role in the cellular response to DNA damage. Our results demonstrate that the overexpression of ΔNp63α, which has been observed in a number of epithelial cancer cell lines, makes the cells resistant to apoptotic signals on DNA damage. Defective apoptotic responses make the cells highly susceptible to acquiring new mutations that can then lead to malignancy.

The most well-known function of ΔNp63α is the inhibition of transcriptional activation by p53, TAp63, and possibly p73 [1]. Reciprocally, ΔNp63α is subjected to regulation by p53 [10]. In this context, p53 mutation could lead to the deregulation of ΔNp63α proteolysis and growth advantage in consequence. In agreement with this, a study on squamous cell carcinoma samples showed that the overexpression of the ΔNp63α protein was always accompanied by a *p*53 mutation [15]. Surprisingly, in our study, silencing ΔNp63α elicited DNA damage-induced apoptosis in cells with p53 mutation. As compared to the wild-type, the mutant p53 possessed >10-fold lower activity for the transcriptional activation of p53 target promoters. However, this mutant (R280T) can be phosphorylated on DNA damage. Taken together, we consider that the phosphorylation of p53 in response to DNA damage is intact in the 5637 cells; however, this does not necessarily indicate the contribution of p53 in apoptosis since this mutant lacks the ability to activate downstream target gene expression. As we were unable to detect the TAp63 message or protein in the 5637 cells (Fig. 1), we speculate that the antiapoptotic function of ΔNp63α in 5637 cells is independent of the transcriptional activity of p53 or TAp63. Recently, Rocco et al. [31] reported that ΔNp63α promotes cell survival in squamous epithelial cancers by repressing the p73-dependent proapoptotic transcriptional program. A similar situation might have occurred when 5637 cells encounter genotoxic insult in this study. On the other hand, recent progress has identified more isoforms of p53 [32]. On the basis of these reports, it is reasonable to think that the p53 network is more complex than it is currently recognized. Therefore, ΔNp63α may have antagonized a yet-unidentified member of the p53 family network in 5637 cells. This high level of complexity is probably required for proper damage responses under diverse stress conditions.

We found that the downregulation of ΔNp63α is not restricted to certain drug treatments because this phenomenon occurs with cellular damaging signals that introduce single-stranded or double-stranded DNA breaks and microtubule disruption. Further, by employing RNA interference technique, we clearly showed that ΔNp63α downregulation is a prerequisite for the induction of apoptosis in ΔNp63α-overexpressing cells. Our results show that the downregulation of Δ*Np63*α expression markedly increases the sensitivity to apoptosis of cells that were otherwise insensitive to apoptosis. These observations suggest that

a combined therapy to regulate the ΔNp63α level and activation of apoptosis by DNA damage may be a viable approach for the treatment of tumors in which p63 is amplified, namely the cancers of epidermal origin.

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