

TAp73 and Δ Np73 Have Opposing Roles in 5-aza-2'-Deoxycytidine-Induced Apoptosis in Breast Cancer Cells

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The *p73* gene contains an extrinsic P1 promoter and an intrinsic P2 promoter, controlling the transcription of the pro-apoptotic TAp73 isoform and the anti-apoptotic Δ Np73 isoform, respectively. The DNA methylation status of both promoters act equally in the epigenetic transcriptional regulation of their relevant isoforms. The aim of this study was to analyze the different effects of these *p73* isoforms in 5-aza-2'-deoxycytidine (5-aza-dC)-induced apoptosis in breast cancer cells. We investigated the effects of the DNA demethylation agent, 5-aza-dC, on the T-47D breast cancer cell line, and evaluated the methylation status of the *p73* promoters and expression of TAp73 and Δ Np73. Furthermore, we assessed the expression of p53 and *p73* isoforms in 5-aza-dC-treated T-47D cells and *p53* knockout cells. 5-aza-dC induced significant anti-tumor effects in T-47D cells, including inhibition of cell viability, G1 phase arrest and apoptosis. This was associated with *p73* promoter demethylation and a concomitant increase in TAp73 mRNA and protein expression. In contrast, the methylation status of promoter P2 was not associated with Δ Np73 mRNA or protein levels. Furthermore, demethylation of P2 failed to inhibit the expression of Δ Np73 with 5-aza-dC in the *p53* knockdown cell model. Our study suggests that demethylation of the P1 and P2 promoters has opposite effects on the expression of *p73* isoforms, namely up-regulation of TAp73 and down-regulation of Δ Np73. We also demonstrate that p53 likely contributes to 5-aza-dC-induced Δ Np73 transcriptional inactivation in breast cancer cells.

INTRODUCTION

The *p73* gene was the first identified homolog of the *p53* tumor suppressor gene, and encodes a protein with extensive structural and functional similarities to *p53*, such as the potential to activate *p53* responsive genes and the ability to induce apoptosis (Ozaki and Nakagawara, 2005). However, unlike *p53*, *p73* is rarely mutated in human cancers (Ikawa et al., 1999). In marked contrast to *p53*, the *p73* gene contains sophisticated splicing loci, leading to the generation of different isoforms with antagonistic functions, including TAp73 and Δ Np73, which are encoded by two different promoters. The P1 promoter generates the TAp73 isoform containing a transactivation domain (TA), which shares significant homology to *p53*. This isoform is capable of activating the transcription of numerous *p53* target genes and inhibiting cell growth in a *p53*-like manner, via the induction of cell cycle arrest and apoptosis. The Δ Np73 isoform encoded by the P2 promoter lacks the TA domain, and is capable of inhibiting the oncogenic activity of *p53* and TAp73 by competing with DNA-binding sites or oligomerizing with the full-length proteins (Ishimoto et al., 2002; Rossi et al., 2004; Stiewe et al., 2002). In addition, another group of N-terminal truncated *p73* isoforms (*p73* Δ ex2, *p73* Δ ex2/3 and Δ N'-*p73*), collectively referred to as Δ TAp73, also function via alternative splicing of transcripts generated from the P1 promoter (Melino et al., 2002; Moll and Slade, 2004; Murray-Zmijewski et al., 2006). As described in previous studies, Δ TAp73 may be up-regulated by TAp73 (Grob et al., 2001; Nakagawa et al., 2002). Interestingly, the P2 promoter in intron 3 contains a *p53*/TAp73 responsive element, thus enforcing a negative feedback loop between the *p53*, TAp73 and Δ Np73 proteins, allowing tight control of mechanisms triggering cell death (Grob et al., 2001; Kartasheva et al., 2002; Nakagawa et al., 2002). Functional reports show that mice with selective deficiency of TAp73 develop spontaneous tumors, particularly lung adenocarcinomas, and are more sensitive to chemical carcinogens (Tomasini et al., 2008). Furthermore, neuronal apoptosis caused by infection of sympathetic neurons with a *p53* adenovirus was reversed following co-infection with the Δ Np73 β (Pozniak et al., 2000). Taken together, these studies reinforce a model whereby the two *p73* protein isoforms exhibit opposite effects: TAp73 exhibits pro-apoptotic effects similar to a tumor suppressor, while Δ Np73 has an anti-apoptotic function, akin to an oncogene. Indeed, the relative expression of these two pro-

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teins is associated with prognosis of several cancers. Therefore, the balance between TAp73 and ΔNp73 finely regulates cellular sensitivity to death (Ramadan et al., 2005).

DNA methylation is an epigenetic regulatory mechanism, establishing long-term gene silencing during development and cell commitment. DNA methylation occurs extensively at CpG-rich regions that, in many instances, are located at promoter regions (Han et al., 2013; Holliday and Pugh, 1975; Jones and Baylin, 2002; Robertson, 2005). Abnormal DNA methylation is observed at gene promoters in the majority of cancers and can lead to aberrant silencing of tumor suppressor genes. The DNA methyltransferase (DNMT) inhibitor, 5-aza-dC, which has been approved by the FDA, has been widely used in demethylation studies and clinical practice to reverse DNA methylation and induce the re-expression of silenced genes (Gore, 2005). Recent studies have shown that methylation of *p73* is observed in hematological malignancies such as acute lymphoblastic leukemia (ALL) (Sahu and Das, 2005), adult T-cell leukemia/lymphoma (Sato et al., 2010) and acute myeloid leukemia (Griffiths et al., 2010). Treatment of cells with 5-aza-dC led to re-expression of *p73*, as a consequence of promoter demethylation (Schmelz et al., 2005). The methylation status of the two *p73* gene promoters was also tested in certain solid tumors, including lung cancer (Daskalos et al., 2011), gastric carcinoma (Ushiku et al., 2007) and cervical cancer (Jha et al., 2012). However, to date, the methylation status of *p73* promoters and ability of 5-aza-dC to reactivate these promoters in breast cancer remain unclear.

In the present study, we used sensitive, quantitative assays to determine the methylation levels of P1 and P2 promoters in the T-47D breast cancer cell line and investigate their correlation with mRNA and protein levels. In addition, we also investigated the potential mechanism(s) regulating the expression of TAp73 and ΔNp73. We also analyzed the anti-neoplastic effects of 5-aza-dC in breast cancer cells, including effects on cell proliferation, cell cycle and apoptosis. Our studies suggest that 5-aza-dC-induced anti-neoplastic activity in breast cancer cells is closely associated with TAp73 and ΔNp73 expression and function.

MATERIALS AND METHODS

Cell culture and 5-aza-dC treatment

The T-47D breast cancer cell line was purchased from the American Type Culture Collection (USA), and was cultured in RPMI 1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C, 5% CO₂. The Hct116p53^{+/+} and Hct116p53^{-/-} cell lines were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University). Cells were plated in 96-well dishes or six-well dishes, allowed to attach for x h, and subsequently treated with 5-aza-dC (Sigma) at various concentrations for 48 h. 5-aza-dC was dissolved in dimethylsulfoxide (DMSO, Sigma). Culture medium was replaced every other day for 2 days prior to harvesting cells for analysis.

Cell survival (MTT) assay

Cells were plated in 96-well plates at approximately 8 × 10³ cells/well, treated with various concentrations of 5-aza-dC and incubated at 37°C for 48 h or treated with 40 μmol/L for distinct times. MTT solution (Sigma, 10 μl of a 5 mg/ml stock) was added to each well and cells were incubated at 37°C for 4 h. The medium was then replaced with DMSO (200 μl) and absorbance was measured at 490 nm using a microplate reader (BIO-RAD, USA). Each assay was performed in triplicate. IC₅₀ values were

determined by plotting a linear regression curve. The percentage of cell viability was calculated as follows: The inhibitory rate of cell viability (%) = [1-(OD of treatment-OD of blank) / (OD of control - OD of blank)] × 100%.

Cell cycle analysis

T-47D cells were treated with 5-aza-dC (5-40 μmol/L) for 48 h. Cells were then harvested by trypsinization (not with EDTA), washed once with PBS, and cell cycle was analyzed by flow cytometry using the KeyGEN Biotech cell cycle test Detection Kit (KeyGEN Biotech, USA) in accordance with the manufacturer's instructions. Briefly, cell pellets were fixed with 500 μl ice-cold 70% ethanol and incubated at 4°C for at least 4 h. Cells were then washed twice with PBS, dissolved in 100 μl RNase and incubated for 30 min at 37°C. Cells were incubated with propidium iodide (400 μl) and analyzed by flow cytometry (BD FACS Calibur, USA) within 1 h. Each sample was tested in triplicate and untreated cells were used as controls.

Apoptosis analysis

Apoptosis assays were performed using the KeyGEN Biotech FITC Annexin V Apoptosis Detection Kit (KeyGEN Biotech), according to the manufacturer's instructions. Briefly, T-47D cells from each treatment group were incubated in six-well dishes for 48 h. All cells were subsequently harvested (including cells in the supernatant), centrifuged and washed twice with PBS. Cell pellets were resuspended in 500 μl binding buffer and incubated with annexin V-fluorescein isothiocyanate (FITC) (5 μl) and propidium iodide (PI) (5 μl) in the dark, at room temperature for 15 min. Apoptotic cells (FITC⁺/PI⁻) were analyzed by flow cytometry (BD FACS Calibur).

DNA extraction and methylation analysis

T-47D cells were incubated with 5-aza-dC (20 μmol/L) for 48 h and harvested. Genomic DNA was extracted from cell pellets using a DNeasy kit (Biotech). DNA quality and quantity was assessed by spectrophotometry at 260/280 nm. Primers were designed by methyprimer (<http://www.urogene.org/methyprimer/index1.html>) and are as follows: M primer of P1 (5'-GCGCGTAGTAGGTTGTAATAGTC-3' and 5'-CTAATCGAAA-AAAACGTAAACGTT-3'), product size: 166 bp; U primer of P1 (5'-TGGGTGTGTAGTAGGTTGTAATAGTTG-3' and 5'-CCTA-ATCAAAAAAACATAAACATT-3'), product size: 170 bp; M primer of P2 (5'-CGAGTTTTTTTTTAGGTAGGTAGTATTTC-3' and 5'-CTTACCCGATAATATATTAACCGC-3'), product size: 130 bp; U primer of P2 (5'-TGAGTTTTTTTTTAGGTAGGTAGTATTTC-3' and 5'-TTACCCAATAATATATTAACCGC-3'), product size: 129 bp. Genomic DNA (1 μg) was treated with sodium bisulfate using the EZ DNA methylation Kit (ZymoResearch, USA). Bisulfite-treated genomic DNA (2 μg) was used in a 25-μl PCR reaction. Thermal cycling conditions were: 95°C for 2 min [94°C for 30 s, 62°C for 45 s and 72°C for 45 s], 40 cycles and 72°C for 10 min. PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. Pyrosequencing was performed as previously described (Daskalos et al., 2009). The methylation index (Mtl) for each promoter was calculated as the average % methylation of the examined CpGs.

Measurement of DNMT activity

Nuclear proteins were isolated using the EpiQuik™ Nuclear Extraction Kit I (Epigentek, USA) from cells exposed to designated concentrations of 5-aza-dC. Following protein quantification using a BCA kit (Thermo Scientific), total DNMT activity was

assessed in 5 μg of nuclear protein using the EpiQuik™ DNA Methyltransferase Activity/Inhibition Assay (Epigentek) in accordance with the manufacturer's instructions. Absorbance was measured at 450 nm/499 nm with a microplate reader (BIO-RAD). DNMT activity was calculated using the formula: DNMT activity (OD/h/mg) = 1000 × (Sample OD – Blank OD) / Protein amount (μg) / h.

RNA extraction, cDNA synthesis and mRNA expression analysis by RT-PCR

RNA extraction was performed with TRIzol (Invitrogen, USA) and the quality and quantity of the RNA were assessed by capillary electrophoresis on an Agilent 2100 Bioanalyser (Agilent Technologies, USA). For cDNA synthesis, 1 μg total RNA was reverse transcribed using a PrimeScript 1st Strand cDNA synthesis kit (Takara, CA, USA). PCR analysis was performed in a final volume of 25 μl using PCR Master Mix (Takara). Amplification conditions were 95°C for 2 min [94°C for 30 s, 56°C for 45 s and 72°C for 45 s], 35 cycles and 72°C for 10 min. Primers for TAp73, ΔNp73 and GAPDH were synthesized by Invitrogen. The sequences are as follows: TAp73 (5'-CCAGGCTCTTTCAGCTTCA-3' and 5'-GACGGAATTCACCACCATCCT-3'), product size: 169 bp; ΔNp73 (5'-GCCACGGCCAGTTCAAT-3' and 5'-GAAGGTGGAGCTGGGTTGTG-3'), product size: 138 bp and GAPDH (5'-CCATGGAGAAGGCTGGGG-3' and 5'-CAAAGTTGTCATGGATGACC-3'), product size: 195 bp. PCR products were separated on 1.5% agarose gels stained with ethidium bromide. GAPDH was used as a loading control. All RT-PCR experiments were performed in triplicate from a single RNA preparation from each tumor specimen.

Western blotting

Whole cell lysates were prepared from 5-aza-dC-treated cells and untreated controls as previously described. Total protein was extracted using RIPA buffer supplemented with protease and phosphatase inhibitors, and quantified using a BCA kit. Protein lysates (20 μg/per lane) were separated on a sodium dodecylsulfate-polyacrylamide (SDS-PAGE) gel and blotted onto nitrocellulose membranes. Blots were blocked with 5% dry milk in Tris-buffered saline/0.1% Tween-20 and incubated with mouse anti-p73 (ab17230), mouse anti-p73 Delta N (ab13649) or rabbit anti-p53 (RS1913) primary antibodies overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) for 2 h. Proteins of interest were normalized to GAPDH expression. Results were analyzed from at least two independent experiments.

Immunofluorescence analysis

Immunofluorescent staining was used to verify the expression and examine the subcellular localization of TAp73 and ΔNp73 proteins. Cells were plated onto glass coverslips in six-well plates and treated with 5-aza-dC (20 μmol/L) for 48 h. Cells were then washed twice with PBS, fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% TritonX-100 for 10 min. Immunofluorescent staining was then performed by incubating cells with the following antibodies: mouse anti-p73 antibody (1:200) or mouse anti-p73 Delta N antibody (1:100), for 1 h at 37°C. Cells were washed twice with PBS and incubated with anti-mouse IgG-FITC secondary antibody (Invitrogen; 1:200) for 30 min at 37°C. Subsequently, nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for 10 min. Samples were photographed using a fluorescent microscope (Axiovert 200; Carl Zeiss).

Transient transfection

The pcDNA3-HA-p53 plasmid and pcDNA3 negative control empty vector were generously provided by Dr. Marin (Gonzalez-Cano et al., 2013). The pGPU6/GFP/Neo plasmid used for constructing the p53 short hairpin RNA (shRNA) vector was purchased from GenePharma (China). The p53 shRNA target sequence was TACCACCATCCACTACAATA and the new plasmid was named Si-p53. A random DNA sequence (Si-control) was used as a negative control. Cells were seeded in six-well plates at 1×10^6 /well and incubated overnight. pcDNA3-HA-p53, pcDNA3, Si-p53 and Si-control plasmids were transfected using TurboFect Transfection Reagent (Thermo Scientific) according to the manufacturer's protocol. After incubation for 48 h at 37°C, cells were harvested and the expression of TAp73 and ΔNp73 was assessed by western blot analysis.

Statistical analysis

Statistical analyses were performed using SPSS Statistics 16.0 (SPSS Inc.) software. All experiments were repeated in biological triplicate. Data are presented as means ± standard deviation (SD). Comparisons were made using one-way ANOVA or Student's *t*-test. The statistical significance was defined by **P* < 0.05 or ***P* < 0.001.

RESULTS

Effects of 5-aza-dC treatment on cell proliferation, cell cycle and apoptosis in T-47D cells

To explore the effect of 5-aza-dC on cell proliferation, T-47D cells were treated with increasing concentrations of 5-aza-dC (0–160 μmol/L) for 48 h (Fig. 1A) and cell viability was assessed by MTT assay at 40 μmol/L after 12, 24, 48, 72 and 96 h (Fig. 1B). Treatment of T-47D cells with 5-aza-dC led to inhibition of cell proliferation in a dose- and time-dependent manner. Cell viability was decreased ~2-fold following treatment with 5-aza-dC (40 μmol/L) for 48 h (*P* < 0.05). Based on this, further studies were performed using these conditions (0–40 μmol/L 5-aza-dC, 48 h).

Previous studies demonstrated that 5-aza-dC inhibited cell growth by inducing cell cycle arrest at the G1 and G2-M phases of the cell cycle (Hassler et al., 2012; Tosi et al., 2005). We therefore used flow cytometry to determine whether the growth inhibition of T-47D cells following 5-aza-dC treatment was associated with specific cell cycle arrest (Fig. 1C). Treatment of cells with 5-aza-dC led to a significant increase in the percentage of cells in the G1 phase compared with untreated controls in a dose-dependent manner, and a concomitant decrease in S phase cells (*P* < 0.05).

To further characterize mechanisms underlying the decrease in cell viability, we assessed apoptosis in T-47D cells following 5-aza-dC treatment, using an Annexin V flow cytometric assay. Treatment of cells with 5-aza-dC (40 μmol/L) led to an increase in the proportion of Annexin V positive cells compared with untreated controls (37.47 ± 4.5% vs 6.36% ± 1.6%, respectively, *P* < 0.05), and this trend was dose dependent (Fig. 1D).

5-aza-dC induces demethylation of p73 promoters in T-47D cells

Analysis of CpG-rich regions in the promoters of the *p73* gene using the CpG island prediction software, methyprimer (<http://www.urogene.org/methyprimer/index1.html>), identified abundant CpG islands in both *p73* gene promoters. We next performed pyrosequencing to quantitatively determine P1 and P2 promoter methylation levels in T-47D cells. We also detected the forecast sites in the P1 and P2 promoters, while only three sites in P1 and one site in P2 successfully detected methylation.

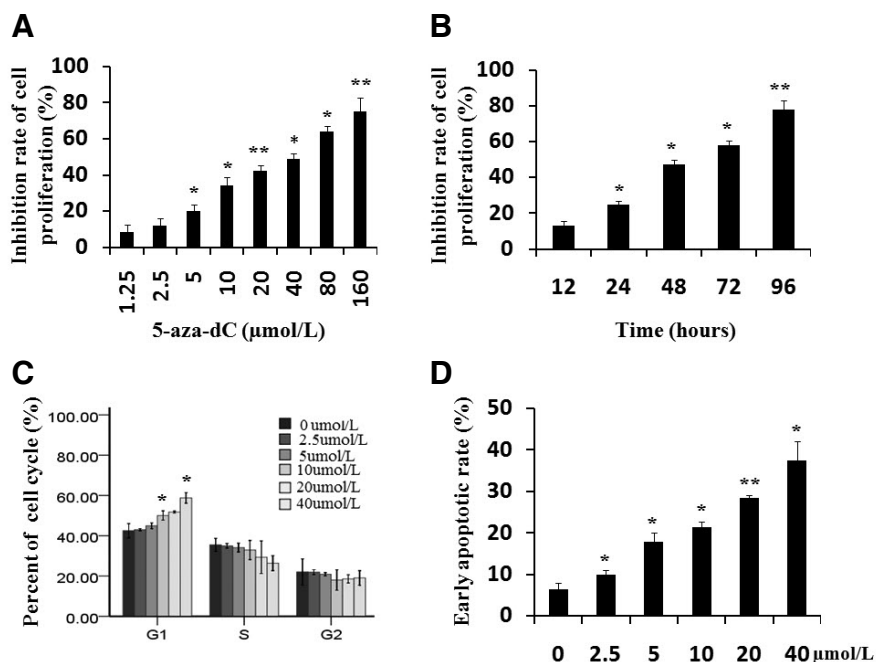


Fig. 1. Effect of 5-aza-dC on cell proliferation, cell cycle and apoptosis in T-47D cells. (A) Cell viability of T-47D cells was measured by MTT assay following treatment with 5-aza-dC at various concentrations for 48 h. (B) Viability of T-47D cells was measured by MTT assay following treatment with 5-aza-dC (40 μmol/L) for different time intervals. (C) Cell cycle distribution was analyzed by flow cytometry following exposure of cells to various concentrations of 5-aza-dC for 48 h. (D) Cell apoptosis was analyzed by flow cytometry following treatment of cells as in (C). Results are presented as the mean ± SD of triplicate observations. * $P < 0.05$, ** $P < 0.001$.

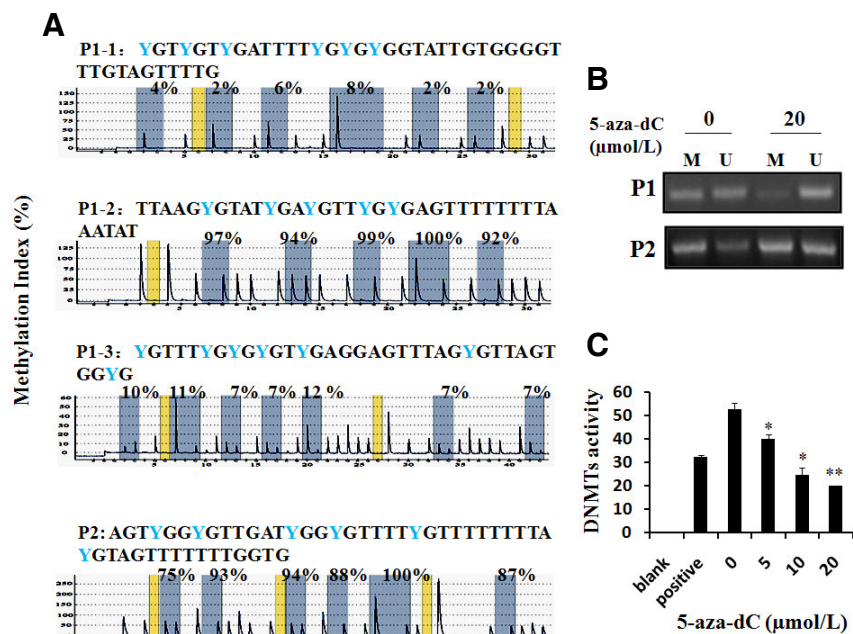


Fig. 2. Methylation status of P1 and P2 p73 promoters in T-47D cells. (A) Representative pyrograms from DNA methylation analysis of the P1 and P2 promoters in T-47D cells. Methylation was detected in three sites in P1 and one site in P2. Gray columns depict regions of CpG sites; the sequence including CpG sites of methylation and the percentage methylation at each CpG site are shown on the top. The percentage of methylation is calculated as the C/(C + T) peak ratio per CpG. (B) Demethylation of P1 and P2 promoter regions with 5-aza-dC determined by methylation specific PCR (MSP). "M" indicates the methylated gene, "U" indicates the unmethylated gene. (C) Total DNMT activity was evaluated in T-47D cells treated with 5-aza-dC (0-20 μmol/L) for 48 h. Results are expressed as the mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.001$.

Hypermethylation of both P1 and P2 promoters was observed, as shown in representative pyrograms (Fig. 2A). We next investigated whether the methylation status of these promoters was reversed by 5-aza-dC treatment, using MSP (Fig. 2B). Treatment of T-47D cells with 5-aza-dC (20 μmol/L) led to a decrease in the methylation status of both P1 and P2 promoters compared with control cells, with a concomitant increase in the demethylation states. We also observed a significant decrease in the total DNMT activity in 5-aza-dC-treated cells (Fig. 2C).

5-aza-dC induces the expression of TAp73 and inhibits the expression of Δ Np73 via both transcriptional and translational mechanisms

To further understand the role of DNA demethylation in the expression of p73 in T-47D cells, we next investigated gene and protein expression by RT-PCR and western blotting, respectively. TAp73 and Δ Np73 mRNA transcript levels from each promoter of p73 are shown in Fig. 3A. Levels of TAp73 mRNA were increased in 5-aza-dC-treated T-47D cells compared with control

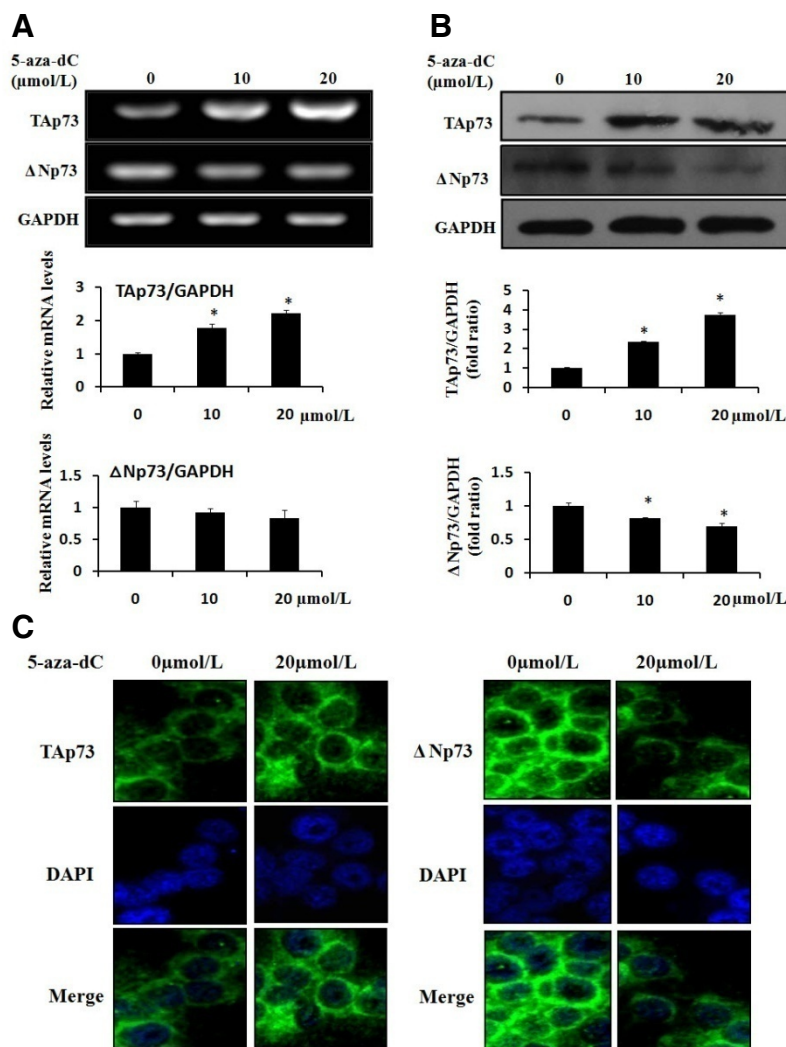


Fig. 3. Analysis of TAp73 and ΔNp73 mRNA and protein levels and correlation with the methylation status of their corresponding promoters. (A) T-47D cells were exposed to 5-aza-dC (0-20 μmol/L) for 48 h, and TAp73 and ΔNp73 mRNA levels were determined by RT-PCR and normalized to GAPDH expression. The relative levels of TAp73 and ΔNp73 mRNA are represented by column graphs. (B) T-47D cells were treated by the same process as (A) and the levels of TAp73 and ΔNp73 proteins were determined by western blot normalized to GAPDH expression. The relative levels of TAp73 and ΔNp73 proteins are represented by column graphs. (C) T-47D cells were exposed to 5-aza-dC (20 μmol/L) for 48 h and the localization and expression of TAp73 and ΔNp73 proteins were determined by fluorescent microscopy and compared with untreated cells. Results are the mean ± SD of three independent experiments. **P* < 0.05, ***P* < 0.001.

cells, and this was associated with a decrease in DNA methylation at the *P1* promoter. In contrast, this trend between ΔNp73 mRNA levels and the *P2* promoter was not observed.

We next examined the levels of TAp73 and ΔNp73 proteins by Western blot, using GAPDH as a control. As shown in Fig. 3B, treatment of T-47D cells with 5-aza-dC led to up-regulation of TAp73 protein and down-regulation of ΔNp73. It should be noted that this decrease in ΔNp73 protein levels was not completely reflective of ΔNp73 mRNA levels, which were largely unchanged by 5-aza-dC treatment. In contrast, TAp73 protein levels were in accordance with observed TAp73 mRNA levels.

To verify the expression of TAp73 and ΔNp73 proteins and examine the subcellular localization of these proteins, we next performed immunofluorescent staining. As shown in Fig. 3C, we detected TAp73 and ΔNp73 expression in the cytoplasm of T-47D cells. Consistent with the Western blot analyses, TAp73 expression was significantly increased in T-47D cells treated with 5-aza-dC (20 μmol/L) compared with untreated cells, while ΔNp73 expression was decreased.

The role of p53 in regulating TAp73 and ΔNp73 expression induced by 5-aza-dC

As a p53 family member and a critical molecule involved in apoptosis, cell cycle regulation and differentiation, p73 is capable of inducing apoptosis/growth arrest in cells with mutant p53 (Willis et al., 2003). However, the impact of p53 gene expression on TAp73 and ΔNp73 transcription differs. Inactivation of p53 leads to up-regulation of TAp73 in cancer cells, while up-regulation of p53 induces ΔNp73 transcription and expression, suggesting a feedback network between p53 and p73 (Kartasheva et al., 2002; Nakagawa et al., 2003; Tophkhane et al., 2012; Yu et al., 2007).

To investigate the role of p53 in regulating TAp73 and ΔNp73 expression, we transfected T-47D cells with pcDNA3-HA-p53 overexpression or Si-p53 shRNA constructs, and assessed levels of p53, TAp73 and ΔNp73 proteins after 48 h by Western blot (Fig. 4A). Transfection of T-47D cells with pcDNA3-HA-p53 enhanced p53 protein levels compared with empty vector control. Overexpression of p53 was associated with an increase in the expression of TAp73 and ΔNp73, while silencing of p53 following transfection of cells with Si-p53 was associated with decreased expression of TAp73 and no change in ΔNp73 levels. We also assessed the

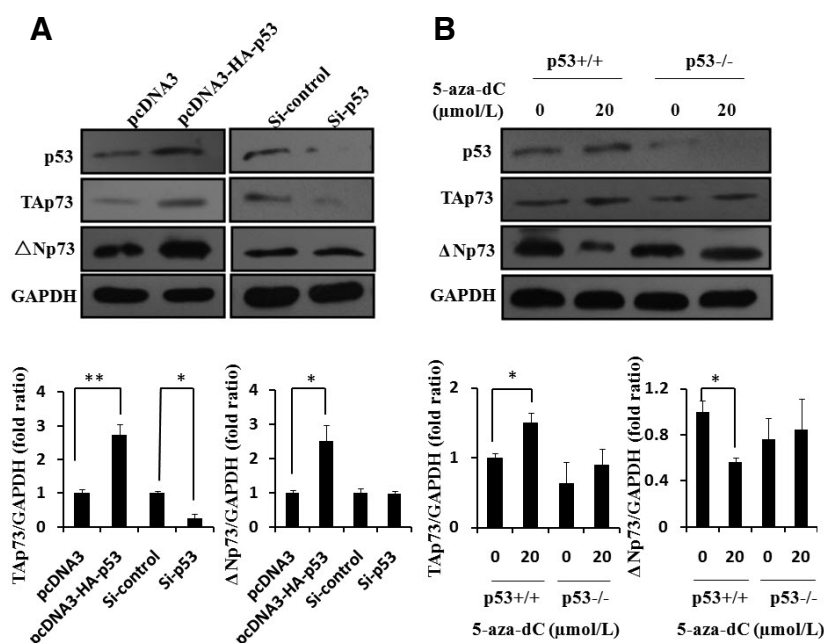


Fig. 4. The roles of p53 in 5-aza-dC-induced TAp73 and Δ Np73 expression. (A) Levels of TAp73 and Δ Np73 proteins were detected by Western blotting following transfection of T-47D cells with pcDNA3-HA-p53 or Si-p53 for 48 h. The relative protein levels are represented by column graphs following normalization to GAPDH expression. (B) 5-aza-dC-induced changes in TAp73 and Δ Np73 protein levels in Hct116p53+/+ (WT) and Hct116p53-/- (knockout) cell lines. p53+/+ and p53-/- cells were treated with 5-aza-dC (20 μ mol/L) for 48 h, and TAp73 and Δ Np73 protein levels were determined by Western blotting following normalization to GAPDH expression. The relative levels of TAp73 and Δ Np73 proteins are represented by column graphs. Results represent the average of three independent experiments. * $P < 0.05$, ** $P < 0.001$.

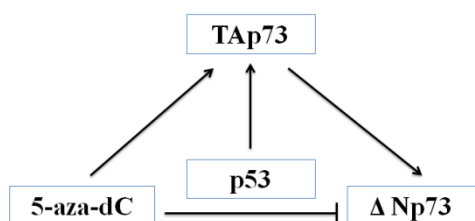


Fig. 5. Schematic model of the effect of 5-aza-dC in the p53 loop 73 and Δ Np73 expression. Demethylation of the P1 and P2 promoters led to opposing effects on the expression of p73 isoforms, up-regulating TAp73 and down-regulating Δ Np73. p53 may play a role in regulating 5-aza-dC-induced Δ Np73 transcriptional inactivation.

levels of p53, TAp73 and Δ Np73 proteins in Hct116 p53+/+ and Hct16 p53-/- cell lines, following treatment with 5-aza-dC (20 μ mol/L) for 48 h (Fig. 4B). Similar to changes in TAp73 and Δ Np73 protein levels observed in Fig. 3B, we observed an increase in the expression of TAp73 and a decrease in the expression of Δ Np73 in p53+/+ cells. This trend of increased TAp73 expression was lower in p53-/- cells compared with p53+/+ cells, however. Interestingly, treatment of p53-/- cells with 5-aza-dC (20 μ mol/L) was not associated with decreased expression of Δ Np73.

DISCUSSION

DNA methylation is a dynamic, reversible mode of epigenetic regulation, which can modify the functionality of numerous genes. 5-aza-dC, which was developed as an inhibitor of DNMTs, can reactivate aberrantly hypermethylated genes by preventing maintenance of the methylation state. 5-aza-dC may therefore exert anti-neoplastic actions by inducing apoptosis, cell cycle arrest and differentiation (Gonzalez-Gomez et al., 2004;

Kong et al., 2005; Tosi et al., 2005). In this study, we demonstrate that the DNMT inhibitor, 5-aza-dC, significantly inhibits the proliferation of breast cancer cells in a dose- and time-dependent manner (Figs. 1A and 1B). Treatment of the T-47D breast cancer cell line with a suitable concentration of 5-aza-dC led to an increased percentage of cells in the G1 phase and a concomitant decrease in S phase cells, and progressive induction of apoptosis (Figs. 1C and 1D). Taken together, these data demonstrate that 5-aza-dC inhibits cell proliferation by inducing cell cycle arrest and apoptosis.

Previously, it was reported that methylation of gene promoter regions could suppress gene expression (Holliday and Pugh, 1975; Jones and Baylin, 2002; Robertson, 2005). The anti-neoplastic role of p73, via the regulation of cell cycle and apoptosis, is well documented (Ishimoto et al., 2002; Stiewe et al., 2002). In this study, we assessed the methylation levels of p73 P1 and P2 promoters in the T-47D breast cancer cell line by pyrosequencing, and investigated their association with mRNA and protein levels. We observed differences in the expression of two p73 isoforms and in the extent of DNA methylation of P1 and P2 promoters (Fig. 2). Our results show that treatment of cells with 5-aza-dC leads to decreased methylation of the P1 promoter and an increase in the unmethylated status of both P1 and P2 p73 promoters. Meanwhile, we also observed a significant decrease in total DNMT activity. Taken together, our results indicate that the methylation status of the two p73 promoters may be reversed by treatment with the DNMT inhibitor, 5-aza-dC. Indeed, previous reports revealed that methylation-dependent silencing of p73 transcription could be reversed by 5-aza-dC (Hatzimichael et al., 2009). In non-small cell lung cancer, particularly squamous cell carcinomas, overexpression of Δ Np73 mRNA is associated with P2 hypomethylation (Daskalos et al., 2011).

DNA methylation generally displays modifications of transcription, especially the initial transcripts (Schubeler et al., 2000), demonstrated by both the TAp73 mRNA and Δ Np73 mRNA levels with histological significance, which have an opposite biological function (Yu et al., 2007). In our report, we observed

changes in the levels of TAp73 and Δ Np73 mRNA; however, only TAp73 mRNA levels were dependent on promoter methylation. Indeed, higher levels of P1 methylation were associated with lower expression of TAp73 mRNA. Treatment of cells with specific concentrations of 5-aza-dC led to re-expression of TAp73 and similar trends were observed at the protein level. Immunofluorescence analysis revealed expression of TAp73 protein in the cytoplasm, and these levels were increased following demethylation of the P1 promoter. However, while P2 hypermethylation was observed, the transcriptional activity of P2 following 5-aza-dC treatment was not consistent with promoter methylation status. According to this model, Δ Np73 mRNA levels should be higher after P2 demethylation; however, we observed the opposite result. Complex mechanisms, such as dynamic changes in DNA methylation modification, might explain these results.

Previous studies have shown that the P1 and P2 promoters of the p73 gene may be regulated by p53. In keeping with these reports, we observed that expression of TAp73 and Δ Np73 isoforms were altered following overexpression or inhibition of p53. Indeed, induction of TAp73 by 5-aza-dC was reduced in p53 knockout cells. The effect of 5-aza-dC on Δ Np73 expression was also abrogated in p53 knockout cells (Fig. 4B). The results indicate that p53 likely plays a role in mediating 5-aza-dC-induced Δ Np73 transcriptional inactivation in breast cancer cells (Fig. 5).

In conclusion, our results demonstrate that 5-aza-dC inhibits the growth of breast cancer cells via activation of cell apoptosis and cell cycle arrest. These anti-neoplastic activities are likely associated with the relative levels of TAp73 and Δ Np73. Furthermore, we demonstrate that the P1 and P2 promoters controlling p73 gene expression are regulated by DNA methylation, and this may be reversed by treatment with 5-aza-dC. The increased expression of TAp73 is likely induced by demethylation of P1, whereas expression of Δ Np73 is not explained by P2 demethylation. Further studies investigating the relationship between P2 promoter methylation and Δ Np73 expression are necessary.

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