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ΔNp63α mediates proliferation and apoptosis in human gastric cancer cells by the regulation of GATA-6

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The oncogenic isoform of the p63 protein, ΔNp63α, has been found to be overexpressed in numerous human squamous cell carcinomas. However, the role of ΔNp63α in human gastric cancer remains unknown. To evaluate this role, we screened a panel of gastric cancer cell lines for ΔNp63α expression and found that they are correlated with the differentiation status of the cell lines. Using the MKN28 gastric cancer cell line for loss-of-function or gain-of-function of ΔNp63α in our experiments, we observed that forced expression of ΔNp63α promoted cell proliferation as assessed by the MTT and colony formation assays, and increased the GATA-6 expression. In contrast, down-regulation of ΔNp63α *via* small interfering RNA suppressed cell proliferation, induced cell apoptosis, and reduced the expression of GATA-6. In conclusion, our data suggest that ΔNp63α plays an important role in cell growth and proliferation of gastric cancer cells, which may be associated with the regulation of GATA-6 expression. This is the first study exploring the biological functions and the underlying mechanism of ΔNp63α during gastric cancer development. It also identifies potential targets for anti-tumor treatment.

Key words: ΔNp63α, gastric cancer, proliferation, apoptosis, GATA-6

According to the latest global estimates of cancer incidences, gastric cancer remains the fourth most common malignancy and the second most frequent cause of cancer-related deaths worldwide. High incidence of gastric cancer mainly aggregates in East Asia, with 41% new cases occurring in China and 11% in Japan [1]. Despite significant advances in multidisciplinary treatment approaches, such as surgery, chemotherapy and radiotherapy, 5-year survival in patients amenable to definitive treatment remains only 40% in China, largely as a consequence of late detection and distant metastasis [2]. Therefore, the identification of novel targets responsible for gastric cancer progression is critical for both early detection and the development of novel therapeutic approaches for the successful treatment of gastric cancer.

The transcription factor p63 belongs to the p53 and p73 family of proteins that share a highly conserved DNA-binding domain and can bind to very similar DNA sequences in promoters and enhancers of eukaryotic genes [3, 4]. Unlike p53 with a single promoter, the human p63 gene has two separate transcriptional initiation sites that result in two classes of transcripts: those encoding proteins with the N-terminal transactivation domain (TAp63) and those encoding proteins lacking this domain (ΔNp63). Additionally, alternative splicing within the 3' end of mRNA yields α, β and γ isoforms, leading to a total of six p63 isoforms [5]. p63, in particular ΔNp63α, is constitutively expressed in basal layers of stratified epithelial tissues, such as the skin, prostate, breast, and urothelium [6]. Consistent with its distribution, a major physiological role for p63 in epithelial morphogenesis during embryonic development was revealed using p63 knockout mice, which display severe structural defects in their stratified epithelia [7, 8]. As the predominant isform of p63, ΔNp63α has been shown to be involved in a diverse array of cellular processes by regulating the transcription of many target genes [9-12]. Recently, ΔNp63α has been proposed to be a putative oncogene in human squamous cell carcinomas by displaying a dominant-negative function against the p53 [5], activating β-catenin signaling [13], upregulating the heat shock protein 70 expression [14], inducing a stem cell phenotype [15, 16], and conferring drug resistant properties [17]. However, loss of ΔNp63α expression is associated with more advanced disease and poorer prognosis in urothelial carcinomas [18-21].

In addition, downregulation of ΔNp63α expression leads to an increase in cell motility and invasiveness of squamous cell carcinoma cells [22, 23]. Taken together, these findings suggest a complex role of ΔNp63α in tumor formation and progression. To our knowledge, however, the functional role of ΔNp63α in gastric cancer remains to be elucidated.

In the present study, we investigated the functional characterization of ΔNp63α as a candidate oncogene and its engagement in multiple processes which influence proliferation and apoptosis in human gastric cancer cells.

Materials and methods

Cell culture and clinical tissue samples. Human gastric cancer cell lines MGC803, AGS, MKN45, SGC7901, and MKN28 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 100 U/ml penicillin/streptomycin at 37°C in a humidified 5 % CO_2 atmosphere. The cells were routinely passaged, and cells at logarithmic growth phase were used for the experiments.

Specimens of gastric mucosa were obtained from five patients who had undergone subtotal gastrectomy for benign gastric ulcers. A research protocol to use tissue specimens was approved by the Institutional Review Board for the Use of Human Subjects at China Medical University (Shenyang, China). Each patient signed a written informed consent form before surgery. A portion of tissue specimens from each case was immediately frozen in liquid nitrogen after surgical resection.

ΔNp63α overexpression vector construction. The pEGFP-N1 vector (BD Bioscience, Clontech, Palo Alto, CA, USA) was used as the start plasmid to construct the ΔNp63α overexpression vector. The open-reading frame (ORF) of human ΔNp63α gene was amplified by PCR using the primers as follows: forward: 5'-AAACTCGAGATGTTGTACCTGGAAAACAATGCC-3', and reverse: 5'-AAAGGATCCTTCTCCCCCTCCTCTTTGATGCGCTGTTG-3'. The cycling conditions were: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 40 s of annealing at 60°C, 30 s of extension at 72°C. The resulting PCR fragment was purified and ligated into BamHI/XhoI (TAKARA, Dalian, China) digested pEGFP-N1 vector to yield the pEGFP-N1-ΔNp63α construct. The identity of the recombinant constructs was confirmed by sequencing.

ΔNp63α small interfering RNA (siRNA) synthesis. The siRNA targeting ΔNp63α mRNA was designed and synthesized by Genechem Co., Ltd (Shanghai, China). The siRNA sequence for ΔNp63α targeting was 5'-ACAAUGCCCAGACUCAAUU-3'. Negative control siRNA consisted of a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA. Both siRNAs mentioned above underwent PAGE purification in an environment free of RNase contamination. In accordance with the manufacturer's instructions, the siRNA was dissolved at the appropriate concentration.

Transfection studies. For ΔNp63α overexpression, MKN28 cells were seed at a density of 2×10^5 cells per well in a six-well plate. At 70-80% confluence, the cells were transiently transfected with 4 μg of the ΔNp63α-expressing plasmid pEGFP-N1-ΔNp63α or the control plasmid pEGFP-N1 by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfection efficiency of >80% was achieved at 48 h post-transfection, as determined by green fluorescent protein under a fluorescence microscope. For knockdown assay, a specific siRNA targeting ΔNp63α transcripts and a non-specific control siRNA were designed and synthesized. At 70-80% confluence, the cells were transfected with the siRNAs using Lipofectamine 2000. If not stated otherwise, at 48 h post-transfection, the transfected cells were used for the different experiments described below.

Western blot analysis. Total proteins were extracted from tissue specimens and transfected cells using radioimmunoprecipitation (RIPA) assay buffer (50mM Tris pH 7.5, 150mM NaCl, 10mM EDTA, 1% NP-40, 0.1% SDS, 1mM PMSF, and 0.5% sodium deoxycholate), and protein concentrations were determined by the Bradford method. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% skimmed milk at room temperature for 2h and then incubated overnight (4°C) with anti-ΔNp63, anti-GATA-4, anti-GATA-5, anti-GATA-6 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1: 5,000, Sigma-Aldrich, St. Louis, MO, USA), followed by horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized with ECL plus chemiluminescence kit (Millipore, Bedford, MA, USA).

MTT assay. MKN28 cells were seeded into 96-well plates at a density of 5×10^3 cells/well for 72h. Twenty microliters of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Amresco, Solon, OH, USA, 5 mg/ml) was then added to each well, and the cells were incubated continuously at 37°C for 4 h. After removal of medium, the crystals were dissolved in dimethyl sulfoxide (DMSO) and absorbance was assessed at 490 nm with a microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Colony formation assay. MKN28 cells were seeded at 1×10^3 cells/well in six-well plates and maintained in sterile 37°C, 5% CO_2 incubator. After 2 weeks, visible cell colonies were fixed with methanol for 15 min and washed in phosphate buffered saline (PBS) solution. The colonies were then stained with 0.1% crystal violet for 1 h, rinsed with water, and finally counted manually.

Direct cell counting. Cells (1×10^4) were seeded into six-well plates in triplicate. Total cell numbers were counted every 12h with a hematocytometer and an inverted microscope. The cell viability was assessed by using trypan blue. The number of cells per well was reported as the mean ± standard deviation (SD) at the indicated number of hours after plating.

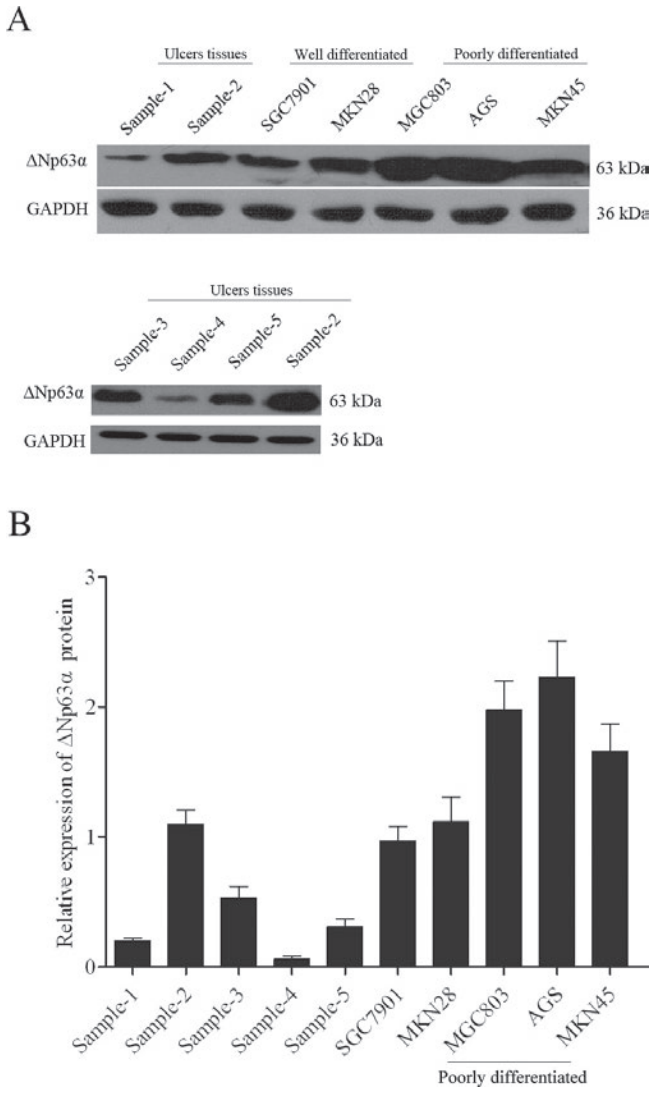

Figure 1. Western blot analysis of ΔNp63α in benign gastric ulcers tissues and various gastric cancer cells. (A) Representative blots are shown, and protein size is expressed in kDa. GAPDH was used as a loading control. (B) Densitometric values were normalized by GAPDH levels, and data are expressed as the mean ± SD.

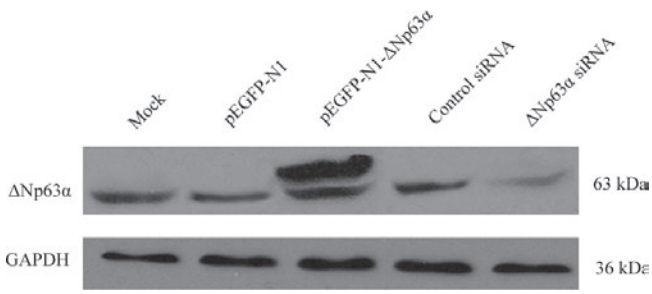

Figure 2 Western blot analysis of ΔNp63α in MKN28 cells transfected with the pEGFP-N1-ΔNp63α, the control pEGFP-N1, ΔNp63α siRNA, or control siRNA. The experiments were repeated at least three times.

Flow cytometry analysis. The effects of ΔNp63α on the apoptosis of MKN28 cells were determined by flow cytometry using the Annexin V: propidium iodide (PI) Apoptosis Detection Kit (Jingmei Biotech, Shenzhen, China) according to the recommended protocol. Briefly, cells were harvested at 72h post-transfection, washed twice and resuspended in 200 μl of binding buffer. FITC-conjugated Annexin V (10 μl) was then added to give a final concentration of 0.5 μg/ml. The staining sample was incubated at room temperature for 20 min. Subsequently, 5 μl of PI was added to the samples (final concentration of 1 μg/ml) and 10,000 cells were immediately analyzed using a MoFlo FACS flow cytometer (Dako Cytomation, Fort Collins, CO, USA).

Statistical analysis. All experiments were performed in triplicate and all data were expressed as means ±SD. Raw data were analyzed by one-way ANOVA followed by the Bonferroni post-hoc test using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered to be statistically significant.

Results

ΔNp63α expression in benign gastric ulcers tissues and gastric cancer cell lines. The expression of ΔNp63α in gastric cancer cells is still unknown. Here, we examined its expression in five benign gastric tissues from patients with gastric ulcers and in a panel of gastric cancer cell lines by Western blot analysis. As shown in Figure 1, ΔNp63α expression was detectable in all the examined tissues and cancer cells. However, the levels of ΔNp63α in gastric cancer cells were significantly higher than those in benign gastric ulcers tissues, suggesting a potential role of ΔNp63α in the development and progression of gastric cancer. Additionally, three poorly differentiated cancer cells (MGC803, AGS and MKN45) expressed a relatively high level of ΔNp63α than two well differentiated cancer cells (SGC7901 and MKN28), implying that ΔNp63α overexpression may be correlated with differentiation of gastric cancer. The MKN28 cell line was used for the following experiments because of its moderate expression of ΔNp63α.

ΔNp63α is involved in proliferation of MKN28 cells. To investigate whether ΔNp63α plays a crucial role in gastric cancer progression, we performed gain-of-function and loss-of-function studies in MKN28 cells. Specific knockdown or overexpression of ΔNp63α in these cells was achieved by transient transduction of a specific siRNA targeting ΔNp63α transcripts or the wild-type human ΔNp63α expression plasmid, respectively. Transfection of a non-specific control siRNA or the empty vector pEGFP-N1 plasmid was served as control for the knockdown or overexpression assay, respectively. Western blot analysis confirmed that the ΔNp63α specific siRNA was efficient in blocking the ΔNp63α expression, and transfection of the pEGFP-N1-ΔNp63α plasmid led to a significant overexpression of ΔNp63α in MKN28 cells. In contrast, the controls had little effects on the ΔNp63α expression (Figure 2). We next examined the effect of ΔNp63α on cell viability. In MTT assay, knockdown of ΔNp63α expression caused a sig-

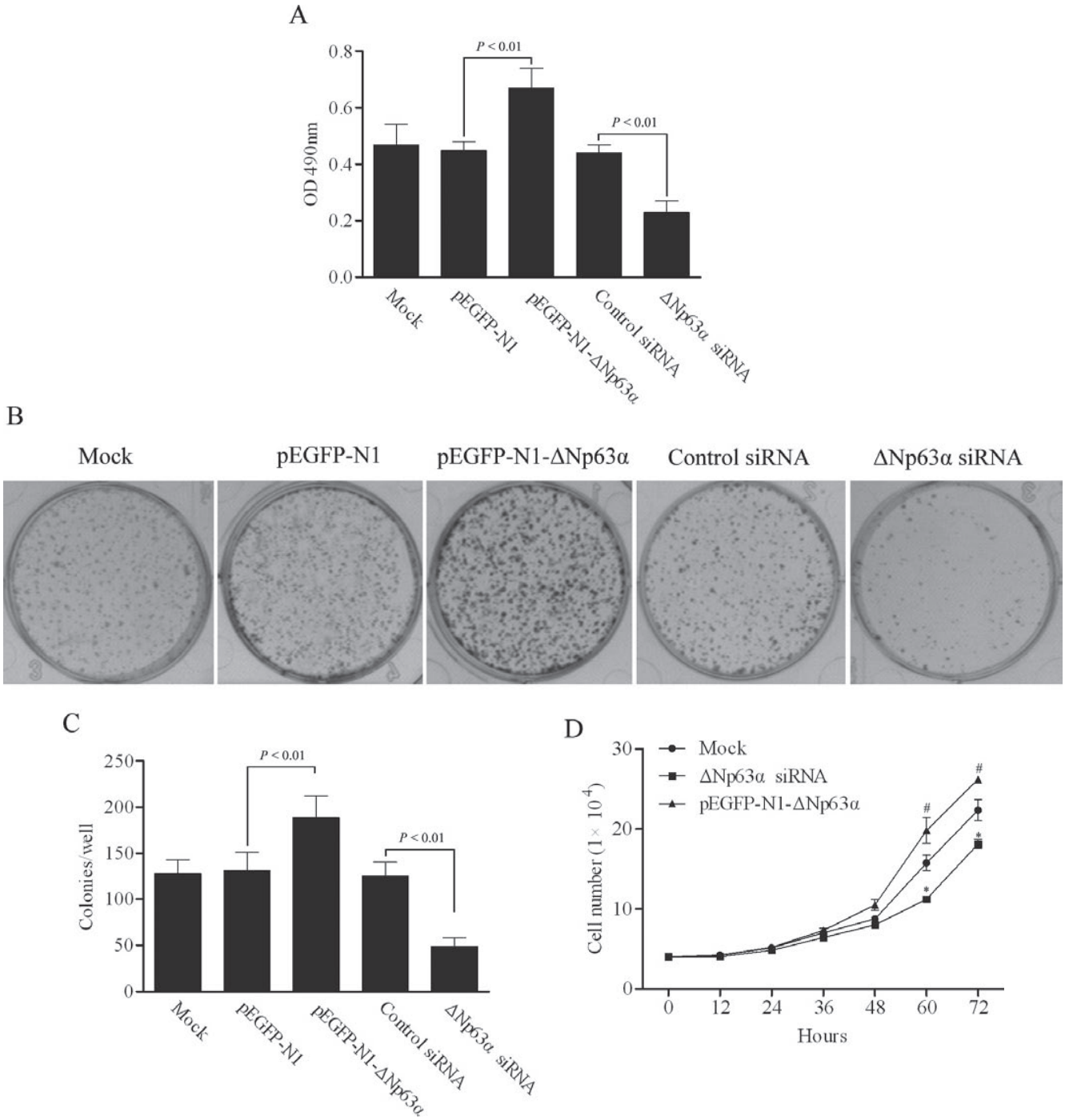

Figure 3. Effects of ΔNp63α on the growth of MKN28 cells. (A) Triplicate of 5×10^3 MKN28 cells were seeded into each well of a 96-well plate, and cell viability was determined by MTT assay at 72h after seeding. Data are presented as the mean ± SD of values from three independent experiments. (B) A typical microphotograph of colony derived from MKN28 cells with different treatments. (C) Quantification of the colonies formed in colony formation assay. Data are presented as the mean ± SD of values from three independent experiments. (D) Direct cell counting of MKN28 cells. #, $P < 0.05$; *, $P < 0.05$ *vs* mock cells.

nificant decrease in the growth rate of MKN28 cells, whereas the growth rate was higher for ΔNp63α-overexpressing cells than for control cells (Figure 3A). To further determine the effects of ΔNp63α on the colony-forming ability of MKN28 cells, we performed an *in vitro* soft agar assay. As shown in Figure 3B and C, down-regulation of ΔNp63α decreased anchorage-independent cell growth of MKN28 cells, whereas up-regulation of ΔNp63α increased it. In addition, direct cell

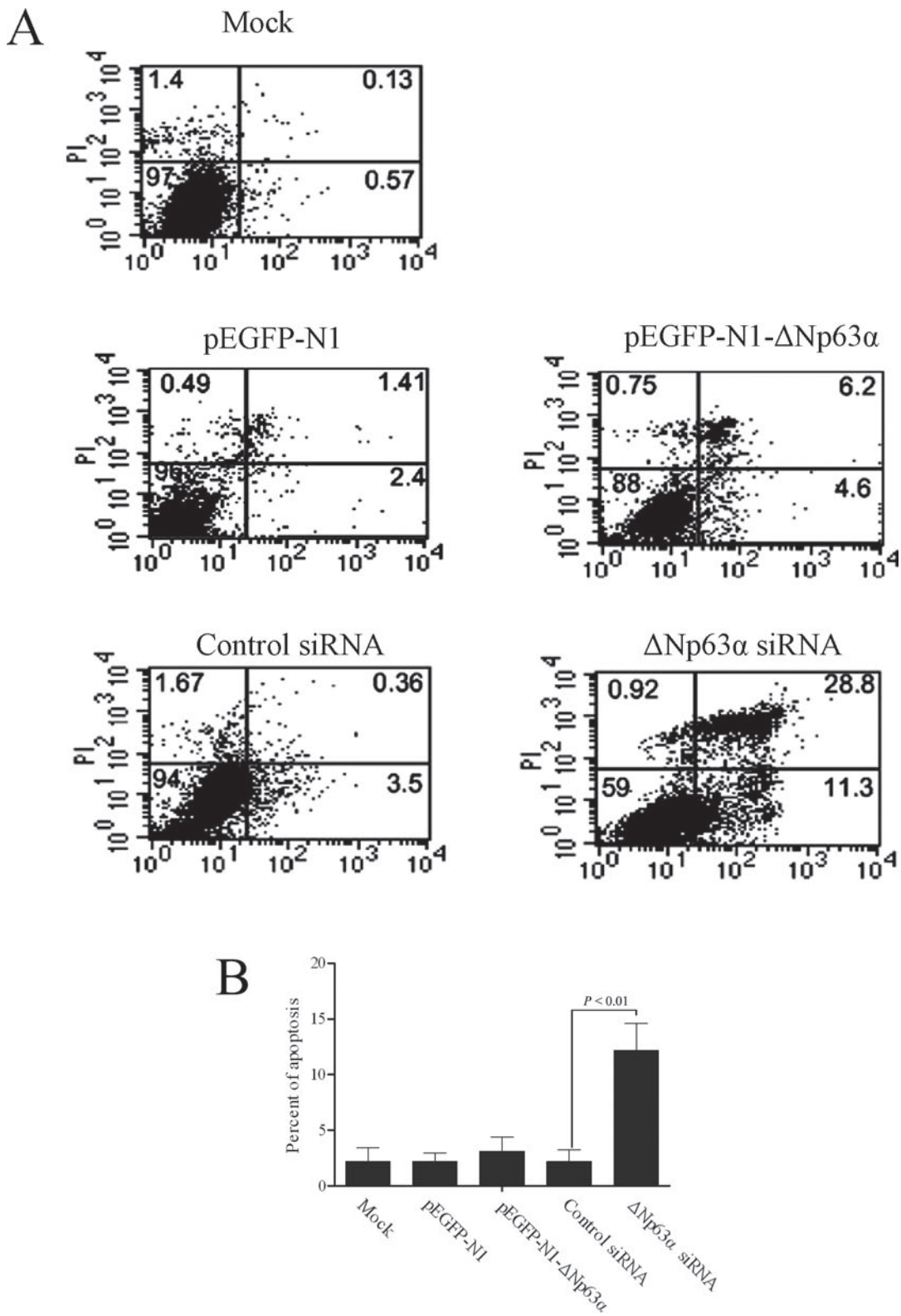

Figure 4. Effects of ΔNp63α on the apoptosis of MKN28 cells. (A) Cell apoptosis was determined by flow cytometry using the Annexin V: propidium iodide (PI) Apoptosis Detection Kit. The numbers of PI/Annexin V single positive and Annexin V/PI double positive cells were calculated as the measurement of apoptotic cells. (B) Results of flow cytometry showing the percent apoptosis of MKN28 cells with different treatments. Data are presented as the mean ± SD of values from three independent experiments.

counting showed that pEGFP-N1-ΔNp63α plasmid promoted and ΔNp63α specific siRNA inhibited cell growth of MKN28 cells in a time-dependent manner (Figure 3D). These results provided evidence that ΔNp63α may function as a key growth stimulator during gastric cancer progression.

ΔNp63α knockdown induces apoptosis in gastric cancer MKN28 cells in vitro. Cell viability is the net result of cell proliferation and apoptosis. To further characterize the putative properties of ΔNp63α on MKN28 cells, we examined the effects of ΔNp63α on MKN28 cells by using flow cytometry analysis. As shown in Figure 4, percentage of apoptotic cells was significantly increased in MKN28 cells transfected with ΔNp63α specific siRNA, as compared to those with control siRNA and mock transfection. However, percentage of apoptotic cells was not altered in MKN28 cells transfected with pEGFP-N1-ΔNp63α plasmid, compared to cells transfected with pEGFP-N1. The data suggest that ΔNp63α knockdown might trigger cells apoptosis in MKN28 cells.

ΔNp63α regulates the expression of GATA-6, but not GATA-4 and GATA-5 in gastric cancer MKN28 cells. GATA transcription factors are a family of transcription factors that regulates the development of diverse tissues. Several lines of evidence suggest GATA-3 as a target gene of p63 [24, 25]. To investigate the possible mechanism underlying the above observations, we evaluated the protein levels of GATA-4, GATA-5 and GATA-6 in MKN28 cells with different treatments. As shown in Figure 5, transient transfection of a ΔNp63α expression plasmid into MKN28 cells led to a significant up-regulation of GATA-6, whereas knockdown of ΔNp63α by siRNA caused a marked reduction in GATA-6 protein expression. However, no significant changes in expression of GATA-4 and GATA-5 were observed. These results suggest that overexpression of ΔNp63α promotes gastric cancer cell proliferation by the up-regulation of GATA-6.

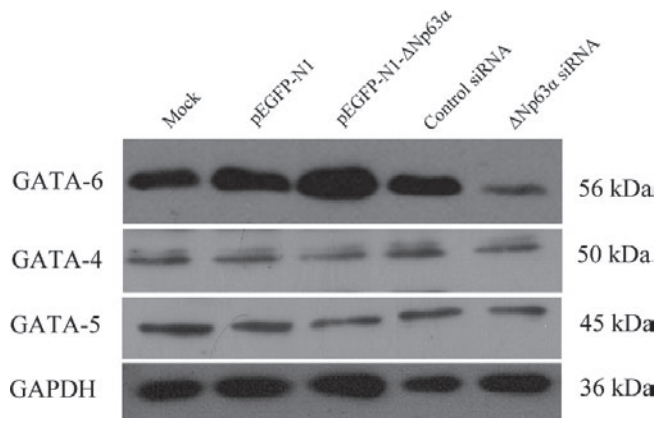

Figure 5. Effects of ΔNp63α on the expression of GATA-4, GATA-5 and GATA-6. Representative blots are shown, and protein size is expressed in kDa.

Discussion

In the present study, we demonstrated that ΔNp63α is differentially expressed in gastric cancer cell lines. High expression of ΔNp63α was detected in three poorly differentiated gastric cancer cell lines (MGC803, AGS and MKN45). However, the well differentiated cell lines (SGC7901 and MKN28) had a low level of ΔNp63α protein, suggesting an association between ΔNp63α expression levels and differentiation status of gastric cancer cells. To dissect the role of ΔNp63α in gastric cancer progression, we used gain-of-function and loss-of-function approaches to investigate the effects of manipulating this gene on cellular behaviors of gastric cancer cells. Exogenous expression of ΔNp63α in MKN28 cells promoted cell proliferation, but had no effects on cell apoptosis. In contrast, down-regulation of endogenous ΔNp63α expression in MKN28 cells resulted in a decreased proliferation and increased apoptosis, coupled with down-regulation of GATA-6. These data highlight an important role for ΔNp63α in the regulation of gastric cancer development.

Several previous reports have documented that amplification of ΔNp63 gene and overexpression of ΔNp63 protein are frequent in human squamous carcinomas, including that of the head and neck [26], bladder [27], esophagus [28], lung [29], and nasopharynx [30]. These observations suggest that ΔNp63 may have important implications in carcinogenesis. However, the expression of ΔNp63 in gastric cancer cells has not been examined. In this study, we collected five gastric cancer cell lines and five benign gastric tissues and compared their ΔNp63α expression levels. We found that the protein levels of ΔNp63α in gastric cancer cells were significantly higher than those in benign gastric tissues, which is consistent with previous findings in other types of human cancers [26-30]. Further studies are needed to confirm the alterations of ΔNp63α in gastric cancer tissues and to explore the correlation between ΔNp63α expression and clinicopathological factors of patients with gastric cancer.

Previous studies have shown that knockdown of ΔNp63α expression by siRNA inhibits the cell proliferation and induces apoptosis in human bladder cancer cells and nasopharyngeal carcinoma cells in vitro and in vivo [31, 32]. In agreement with the previous findings, our data revealed a similar function of ΔNp63α in the regulation of gastric cancer cell growth [31, 32]. We found that ΔNp63α expression levels were positively correlated with cell proliferation as assessed by the MTT and colony formation assays. In addition, we also found that ΔNp63α silencing promoted apoptosis of gastric cancer cells, which is consistent with previous observations in other types of cancer cells. Unexpectedly, overexpression of ΔNp63α has no effects on the apoptosis of gastric cancer cells. However, keeping in mind all these observations, it seems reasonable to conclude that ΔNp63α may play an oncogenic role in gastric cancer progression through promoting cell survival and proliferation.

GATA factors are a group of transcription regulatory proteins that recognize a target site conforming to the consensus

motif GATA through two conserved zinc finger DNA-binding domains [33]. On the basis of their tissue distribution, the GATA family is divided into two subfamilies: GATA-1, -2, and -3 and GATA-4, -5, and -6. GATA-1, -2, and -3 are essential regulators for the development and differentiation of the hematopoietic cell lineage, whereas GATA-4, -5, and -6 are predominantly associated with the development and differentiation of endoderm-derived organs [34, 35]. Recently, a number of microarray-based investigations have demonstrated that several GATA family members are up-regulated by p63 [24], and GATA-3 has been identified as a target gene of p63 [36, 37]. Furthermore, GATA-4, -5, and -6 have been implicated in cancer development [38]. These previous findings led us to explore whether ΔNp63α could regulate the expression of GATA-4, -5, and -6 in gastric cancer cells. In the present study, we clearly showed that ΔNp63α overexpression increased, whereas knockdown of ΔNp63α reduced the expression of GATA-6. However, overexpression or knockdown of ΔNp63α did not affect the expression of GATA-4 and GATA-5. Taken together, our data not only suggest that ΔNp63α exerts its biological functions at least in part through the regulation of GATA-6, but also support the notion that ΔNp63α might have oncogenic effects since it is predominantly expressed in proliferating progenitor cells [6]. Despite the observations, we do not have direct evidence to show that the influence of ΔNp63α on cellular behaviors is due solely to its regulation of GATA-6. Further explorations are therefore needed before the inter-relationship between ΔNp63α and GATA factors can be clearly defined. Additionally, the major limitation of transient transfection assays is that, within a transfected cell, the plasmid DNA exists in a highly artificial configuration with variable copy number that can profoundly influence results, leading, for example, to inactivity or dysregulation of regulatory elements. Thus, further studies using stable transfection assays are required to confirm our findings.

In summary, the present study provides the first evidence that ΔNp63α is expressed in gastric cancer cells and plays crucial roles in cell growth and apoptosis in addition to the regulation of GATA-6. These results suggest that elevated expression of ΔNp63α in gastric cancer may lead to gastric cancer activation and disease progression.

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