Role of TAp73α in induction of apoptosis by transforming growth factor-β in gastric cancer cells

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Abstract **Transforming growth factor-β (TGF-β) is implicated as a tumor suppressor because it eliminates cancer cells from normal tissues by inhibiting cell growth and inducing apoptosis. Although p53 tumor suppressor is required for TGF-β-induced p21[WAF1] expression and cell growth inhibition, its role in TGF-β-induced apoptosis remains unclear. Here, we report that TAp73α, which is a member of the p53 family, binds to p53-binding sites in the promoters of proapoptotic *Bax* and *Puma* to activate their transcription, and mediates TGF-β-induced apoptosis in gastric cancer cells. Our findings reveal a novel role of TAp73α in the induction of apoptosis by TGF-β in cancer cells.**
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Keywords: p73; TGF-β; Apoptosis; Gastric cancer

1. Introduction

Transforming growth factor-β (TGF-β) is a multifunctional cytokine regulating various biological processes, such as tissue homeostasis and development. In adult tissues, TGF-β negatively controls cell numbers by inducing growth inhibition and apoptosis. Recent studies have elucidated the mechanism of TGF-β signal transduction [1]. Following the binding of TGF-β, the TGF-β receptor complex activates Smad transcription factors (Smads). Activated Smads translocate into the nucleus, where they bind to promoter regions of their target genes to regulate transcription through direct interactions with their cofactors. The TGF-β signaling pathway is considered to be a tumor suppressor pathway because most carcinomas lose cellular responsiveness to TGF-β, which eliminates primary tumor cells from normal tissues by inducing apoptosis [2]. This proapoptotic effect of TGF-β has been demonstrated in various cancer cells such as hepatoma, B cell lymphoma, and prostate cancer cells. Although previous studies have shown that several proapoptotic genes are activated by TGF-β, the activa-

tion mechanism of proapoptotic target genes by TGF-β remains unclarified.

p53 is one of the Smad cofactors that associate with Smads to induce transcription of TGF-β target genes. A recent report has shown that the N-terminal phosphorylation of p53 by the Ras/mitogen-activated protein kinase signaling pathway enables the interaction of p53 with Smads to induce TGF-β-induced expression of the cyclin-dependent kinase inhibitors p21[WAF1] and p15[Ink4b] [3]. Although these results suggest that the p53 and TGF-β signaling pathways cooperate to activate the transcription of TGF-β target genes for cell growth inhibition, the role of this cooperation is unclear in relation to TGF-β-induced apoptosis. *p73* belongs to the *p53* family consisting of *p53*, *p63*, and *p73*, and shares sequence homology and functional similarity with *p53* and *p63* [4]. TAp73α is one of the alternatively spliced C-terminal isoforms of full-length p73 and mediates apoptosis induced by DNA damage and chemotherapeutic agents. DNA damage-dependent accumulation and acetylation of TAp73α increase its ability to activate proapoptotic *p53AIP1* and induce apoptosis in colon carcinoma cells [5]. TAp73α is also induced by various chemotherapeutic agents [6]. Inhibition of TAp73α induction by *p73* siRNA leads to the suppression of chemotherapy-induced apoptosis in human colon carcinoma cells. Moreover, functional inactivation of TAp73α through overexpression of the inhibitory isoform of p73 lacking the transactivation domain (ΔNp73) is reported in neuroblastoma [7], and ovarian, cervical, and breast carcinomas [8]. These observations suggest that TAp73α has tumor suppressive functions by inducing apoptosis in cancer cells. We report here that TAp73α is responsible for TGF-β-induced apoptosis via the activation of proapoptotic *Bax* and *Puma* in gastric cancer cells.

2. Materials and methods

2.1. Cell culture, transfection, and reagents
SNU-16 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. Transfection of cDNA and siRNA was performed using FuGENE 6 (Roche Applied Science, Indianapolis, IN) and Lipofectamine 2000 (Invitrogen), respectively. Human recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

2.2. Plasmid construction
Mutagenesis of the *p73PF* promoter (−2713/+77) [9] was performed using a QuikChange multi site-directed mutagenesis kit (Stratagene, La Jolla, CA), and the mutations were confirmed by DNA sequencing.

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The primers used for mutagenesis were 5′-GAGAGAACGAATTTG-CATCTGACTGGGCAGAGCG-3′, 5′-GGCAAGCCCTGCTTGG-ATGAAAGCAGCCGTTCCCC-3′, 5′-GAGCGACGCGATCCAA-AAGGCGGATGGAAG GAGGC-3′, and 5′-GCTGCCTTCCATC-GCGCCGGGCTAAAAAGATGCTAACGCCC-3′.

2.3. Apoptosis detection

Apoptosis was quantified using the cell death detection enzyme-linked immunosorbent assay (ELISA) PLUS assay kit (Roche Applied Science) according to the manufacturer's instructions. Apoptosis was also quantified using an annexin V-fluorescein isothiocyanate apoptosis detection kit II (BD Biosciences, San Jose, CA). After staining with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (PI), cells were analyzed by flow cytometry using a FACS vantage (BD Biosciences). Data were analyzed using Cell Quest software (BD Biosciences).

2.4. Real-time quantitative RT-PCR analysis

Total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany) and treated with DNase I using an RNase-free DNase set (Qiagen). Equal amounts of total RNA were reverse-transcribed using an Omniscript reverse transcriptase kit (Qiagen). The first-strand cDNA was used as a template. The primers for human $p53$, $p63$, $p73$, Bax, $Puma$, $p21^{WAF1}$, and $GAPDH$ were purchased from Applied Biosystems (Foster City, CA).

2.5. Luciferase assay

Cells were transiently transfected with luciferase reporter constructs and an internal control pRL-TK vector (Promega, Madison, WI). Luciferase activity was measured after 20 h using the dual-luciferase reporter assay system (Promega) in an LB 960 Microplate Luminometer Centro (Berthold Technologies, Bad Wildbad, Germany).

2.6. Western blot analysis and immunoprecipitation

Whole-cell lysates were resolved by SDS–PAGE and transferred to Immobilon-P membranes (Millipore, Temecula, CA). The membranes were sequentially probed with an appropriate primary antibody and a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, UK), and immunocomplexes were detected using ECL Western blotting detection reagents (Amersham Biosciences). The mitochondria-enriched heavy membrane fractions were prepared as described previously [10] and subjected to SDS–PAGE. For immunoprecipitation, whole-cell lysates were incubated overnight with an anti-p73 (H-79) or agarose-conjugated anti-p53 (DO-1) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and the immune complexes were analyzed by Western blot analysis with an anti-p73α (C-17) or horseradish peroxidase-conjugated anti-p53 (DO-1) antibody (Santa Cruz Biotechnology). Anti-p63 (4A4) and anti-ΔN p63 (N-16) antibodies were purchased from Santa Cruz Biotechnology; anti-p73β (GC15) antibody was purchased from Millipore; anti-δNp73 (38C674.2) antibody was purchased from Imgenex (San Diego, CA); an anti-β-actin (AC-15) antibody was purchased from Sigma–Aldrich (St. Louis, MO); anti-Bax (#2772), anti-Puma (#4976), and anti-COX (IV) (#4844) antibodies were purchased from Cell Signaling (Danvers, MA); anti-Bax (6A7) antibody was purchased from BD Biosciences.

2.7. Chromatin-immunoprecipitation (ChIP) assay

ChIP assay was performed using the chromatin-immunoprecipitation assay kit (Millipore) according to manufacturer's instructions. Anti-p73 (H-79) antibody (Santa Cruz Biotechnology) and normal rabbit IgG were used for immunoprecipitation. The promoter-specific primer sequences were as follows: Bax (forward) 5′-TAATCC-CAGCGCTTTGGAAG-3′, Bax (reverse) 5′-TGCAGAGACCTGG-ATCTAGC-3′, $Puma$ (forward) 5′-TTGCGAGACTGTGGCCTTG-
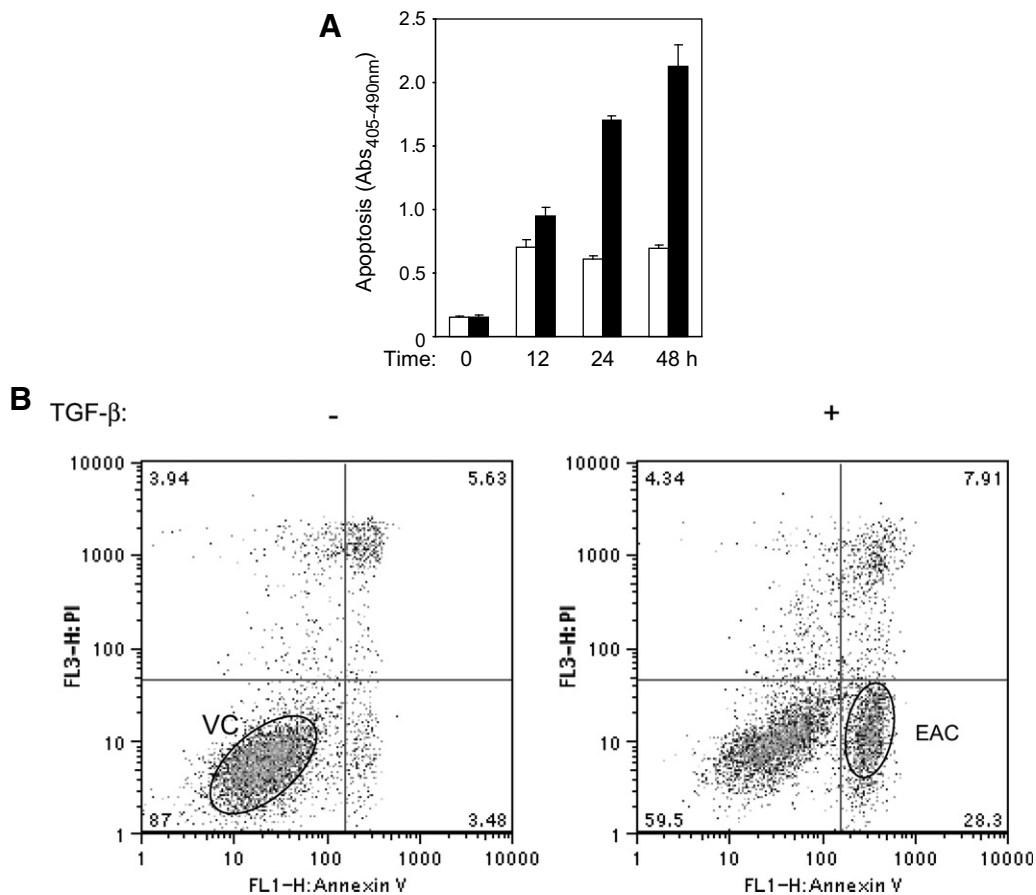

Fig. 1. TGF-β-induced apoptosis in SNU-16 cells. (A) Cells were cultured with (filled bars) or without (open bars) TGF-β1 for the indicated times. Apoptosis induction was assessed by cell death detection ELISA assays. (B) Cells were cultured with or without TGF-β1 for 24 h. The cells were thereafter stained with annexin V and PI, and then analyzed by flow cytometry. VC, viable cell population; EAC, early apoptotic cell population.

TGTC-3′, *Puma* (reverse) 5′-GTCGGACACACACACTGACTGG-GA-3′, *GAPDH* (forward) 5′-TACTAGCGGTTTTACGGGCG-3′, *GAPDH* (reverse) 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′.

3. Results

3.1. TGF-β-induced apoptosis in SNU-16 cells

Although most human gastric cancer cell lines lose responsiveness to TGF-β to undergo apoptosis, the SNU-16 cell line is highly responsive [11]. Cell death detection ELISA assays showed that apoptosis was induced after 24 h and increased during the 48 h after TGF-β stimulation (Fig. 1A). To quantify the apoptotic cell fraction, we stained the cells with annexin V and PI and analyzed them by flow cytometry. The percentage of early apoptotic cells (EAC; annexin V$^+$, PI$^-$) increased dramatically from 3.48% to 28.3% following TGF-β stimulation (Fig. 1B).

3.2. TAp73α plays an essential role in TGF-β-induced apoptosis

To assess the role of p53 family members in TGF-β-induced apoptosis, we sorted the unstimulated viable cell (VC; annexin V$^-$, PI$^-$) and TGF-β-stimulated EAC populations using a flow cytometer (Fig. 1B) and analyzed the mRNA levels of *p53*, *p63*, and *p73* by real-time quantitative RT-PCR. The *p73* mRNA level in the EAC population increased by 3.8-fold, whereas those of *p53* and *p63* hardly increased (Fig. 2A).

We then examined whether the transcriptional activity of the *p73* promoter is upregulated by TGF-β stimulation. SNU-16 cells were transfected with a luciferase reporter construct under the control of the *p73* promoter or *p21^{WAF1}* promoter, which is one of the target genes of TGF-β. Luciferase assays were performed in the presence or absence of TGF-β. The transcriptional activity of the *p73* promoter was greatly increased by TGF-β, similar to that of the *p21^{WAF1}* promoter (Fig. 2B).

The protein levels of p73 isoforms were examined in SNU-16 cells following TGF-β stimulation. The expression of TAp73α, but not of ΔNp73, was increased by TGF-β stimulation (Fig. 2C); TAp73β was hardly detected. The expression levels of p53, TAp63, and ΔNp63 were low and not increased by TGF-β stimulation. Next, SNU-16 cells were transfected with control siRNA or *p73* siRNA, and were then either left unstimulated or stimulated with TGF-β. Transfection with *p73* siRNA inhibited the induction of TAp73α but not that of basal p53 expression (Fig. 2D), and led to a significant decrease in TGF-β-induced apoptosis (Fig. 2E), indicating that TGF-β-induced TAp73α mediates apoptosis.

3.3. TAp73α-induced Bax and Puma mediate apoptosis induced by TGF-β

We analyzed the induction of proapoptotic *Bax* and *Puma* whose promoters have p53-binding sites in the VC and EAC populations by real-time quantitative RT-PCR. The mRNA levels of *Bax* and *Puma* in the EAC population significantly increased, as well as that of *p21^{WAF1}* (by 3.0-, 2.6-, and 4.0-fold, respectively) (Fig. 3A). Next, we examined whether TAp73α activates the *Bax* and *Puma* promoters. SNU-16 cells were transfected with a luciferase reporter construct under the control of the *Bax* or *Puma* promoter, with or without the TAp73α expression plasmid. TAp73α markedly increased the transcriptional activities of the *Bax* and *Puma* promoters by 4.5- and 4.1-fold, respectively. In contrast, the activities of the mutant *Bax* and *Puma* promoters, both of which lack the p53-binding sites, were not stimulated by TAp73α (Fig. 3B). To confirm direct binding of TAp73α to the *Bax* and *Puma* promoters, we performed ChIP assay. As shown in Fig. 3C, TGF-β stimulation enhanced TAp73α binding to both the *Bax* and *Puma* promoters in SNU-16 cells.

The protein levels of both Bax and Puma were significantly increased in the EAC population (Fig. 3D). We performed Western blot analysis on the mitochondria-enriched heavy membrane fraction using an anti-Bax 6A7 antibody that detects active Bax. Bax was found when the cells were stimulated with TGF-β (Fig. 3E). Following apoptotic stimuli, Puma promotes conformational change and mitochondrial localization of Bax. We investigated the effect of siRNA-mediated *Puma*
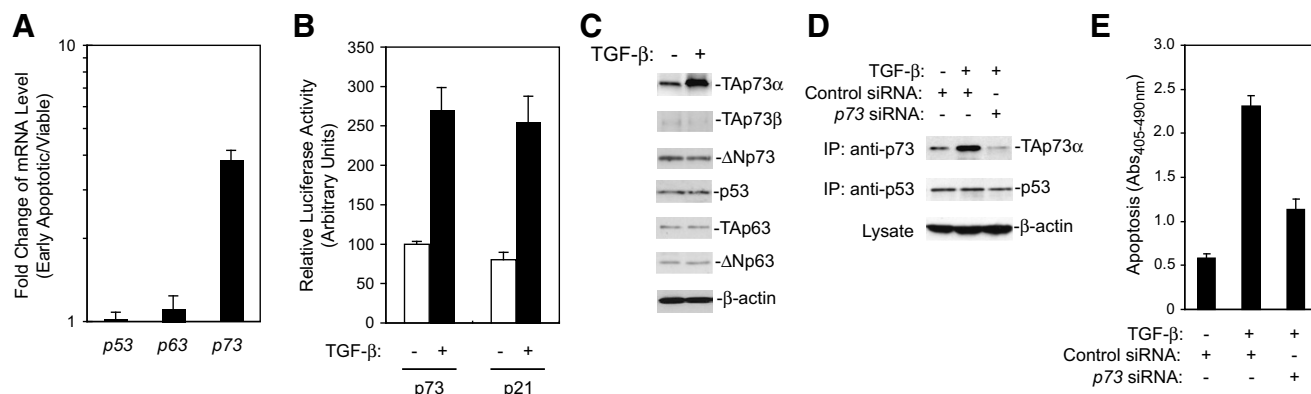

Fig. 2. *p73* is induced in the EAC population. (A) Cells were either left unstimulated or stimulated with TGF-β1 for 24 h. The VC and EAC populations were sorted by flow cytometry, and the mRNA levels of *p53*, *p63*, and *p73* were determined by real-time quantitative RT-PCR. (B) Cells were transfected with the *p73* (p73) or *p21^{WAF1}* (p21) promoter reporter construct and left unstimulated (open bars) or stimulated with TGF-β1 (filled bars). Relative luciferase activity was measured in cell lysates after 20 h. (C) Cells were either left unstimulated or stimulated with TGF-β1. Whole-cell lysates were resolved by SDS–PAGE and analyzed by Western blot analysis with an anti-p73α (C-17), anti-p73β (GC15), anti-δNp73 (38C674.2), anti-p53 (DO-1), anti-p63 (4A4), anti-ΔN p63 (N-16), or anti-β-actin (AC-15) antibody. (D and E) Cells were transfected with control siRNA or *p73* siRNA (Santa Cruz Biotechnology) and were then left unstimulated or stimulated with TGF-β1. (D) p73 and p53 were immunoprecipitated from whole-cell lysates. Immune complexes were resolved by SDS–PAGE and analyzed by Western blot analysis. (E) Apoptosis induction was assessed by cell death detection ELISA assays.
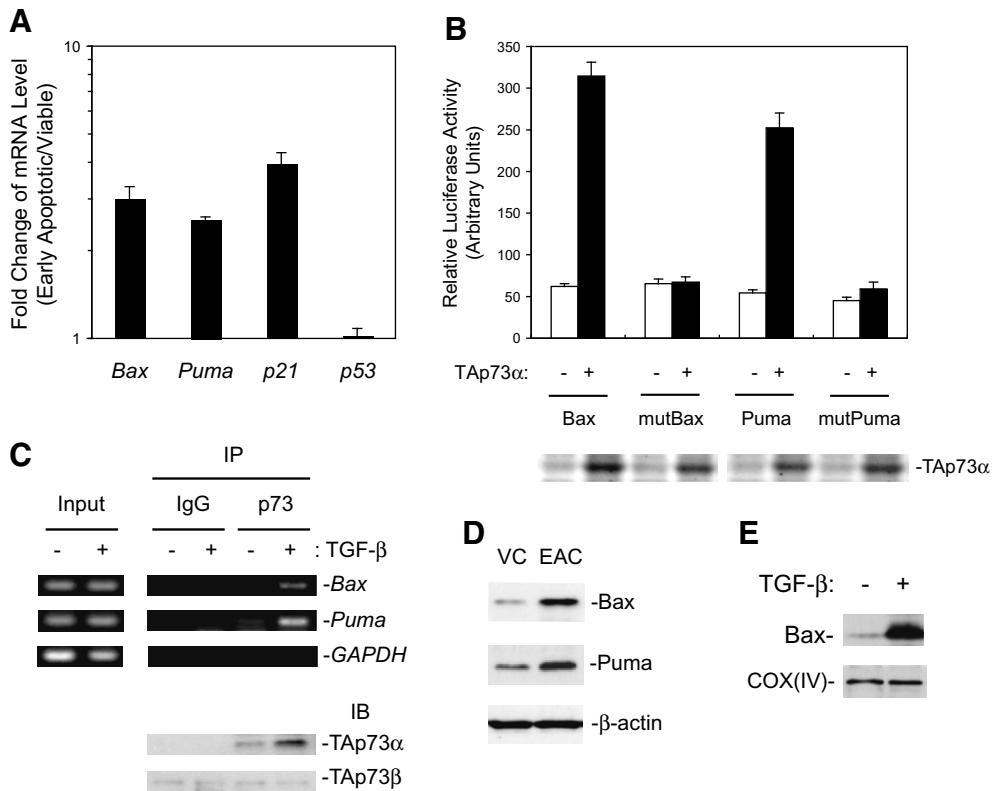
Fig. 3. Activation of *Bax* and *Puma* in the EAC population. (A) The VC and EAC populations were sorted by flow cytometry. mRNA levels of *Bax*, *Puma*, *p21*[WAF1], and *p53* were determined by real-time quantitative RT-PCR. (B) SNU-16 cells were transfected with (filled bars) or without (open bars) the expression plasmid for TAp73α together with the *Bax*, *Puma*, or their mutated version promoter reporter construct. Relative luciferase activities were measured, and TAp73α protein levels were determined by Western blot analysis. (C) SNU-16 cells were either left unstimulated or stimulated with TGF-β1, and then the binding of TAp73 to the *Bax*, *Puma, or GAPDH* promoters was analyzed by ChIP assay. The presence of TAp73α or TAp73β in the immunoprecipitated complex was examined by Western blot analysis. (D) Protein levels of Bax and Puma were investigated in the VC and EAC populations by Western blot analysis with an anti-Bax, anti-Puma, or anti-β-actin antibody. (E) SNU-16 cells were cultured with or without TGF-β1 for 24 h. The mitochondria-enriched heavy membrane fractions were then prepared and subjected to SDS–PAGE and Western blot analysis with an anti-Bax 6A7 or anti-COX (IV) antibody as a mitochondrial loading control.
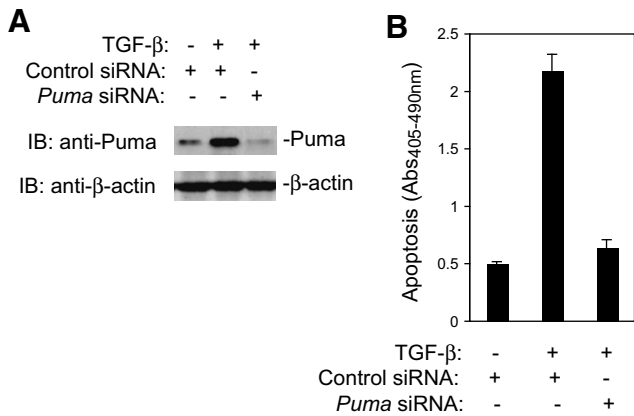

Fig. 4. Role of Puma in TGF-β-induced apoptosis. Cells were transfected with control siRNA or *Puma* siRNA (Dharmacon, Lafayette, CO) and were then left unstimulated or stimulated with TGF-β1. (A) Whole-cell lysates were resolved by SDS–PAGE and analyzed by Western blot analysis with an anti-Puma or anti-β-actin antibody. (B) Apoptosis induction was assessed by cell death detection ELISA assays.
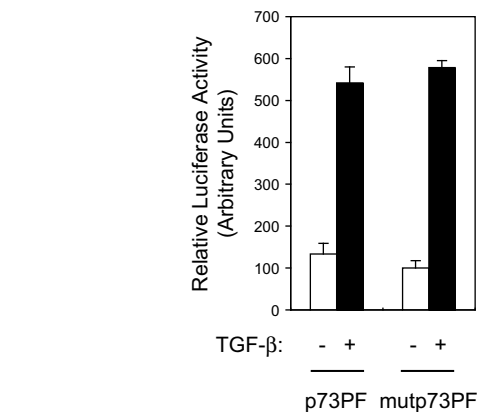
Fig. 5. E2F1 is not required for *p73* activation by TGF-β. Cells were transfected with the *p73PF* luciferase reporter construct (p73PF) or mutant *p73* construct (mutp73PF) and were then left unstimulated (open bars) or stimulated with TGF-β1 (filled bars). Relative luciferase activity was measured in cell lysates after 20 h.

silencing on TGF-β-induced apoptosis. *Puma* siRNA, but not control siRNA, suppressed TGF-β-induced Puma expression

(Fig. 4A) and apoptosis (Fig. 4B). These results demonstrate that TGF-β-induced apoptosis in SNU-16 cells occurs through the mitochondrial pathway.

3.4. E2F1 is not responsible for activation of p73 following TGF-β stimulation

The E2F1 transcription factor regulates the expression of various genes involved in DNA synthesis, cell proliferation, and apoptosis. The *p73PF* promoter (−2713/+77) has six consensus E2F-binding sites [9]. Inactivation of the six E2F-binding sites by mutations (the mutant *p73* promoter) dramatically decreased the transcriptional activation of the promoter by E2F1 in the SAOS2 osteosarcoma cell line (data not shown). SNU-16 cells were transfected with a luciferase reporter construct under the control of the *p73PF* promoter or the mutant *p73PF* promoter. Luciferase assays were performed in the presence or absence of TGF-β. The transcriptional activities of both promoters were similarly increased in the presence of TGF-β (Fig. 5), suggesting that E2F1 does not mediate *p73* induction by TGF-β in SNU-16 cells.

4. Discussion

The apoptotic and cytostatic functions of TGF-β suggest its role in tumor suppression. Loss of responsiveness to TGF-β leads to hyperproliferative disorders and cancer progression in vivo. Here, we show that TAp73α is an essential mediator of TGF-β-induced apoptosis in gastric cancer cells and that both *Bax* and *Puma* are targeted by TGF-β-induced TAp73α.

It has been reported that wild-type p73 is frequently overexpressed in gastric adenocarcinoma [12]. However, it was demonstrated that not only TAp73 but also ΔNp73 is overexpressed in more than 60% of primary adenocarcinomas of the stomach and esophagus, and that ΔNp73 strongly inhibits the transcriptional and apoptotic activities of TAp73α and TAp73β [13]. Recent studies have shown that the frequency of the CpG island methylation of the *p73* promoter, which causes loss of p73 expression, is specifically high in Epstein–Barr virus-associated gastric carcinoma [14,15]. Moreover, TAp73α is an important mediator of apoptosis in response to anticancer chemotherapeutic agents [6]. TAp73α expression is induced by various chemotherapeutic agents, such as camptothecin, etoposide, and cisplatinum, which induce apoptosis in cancer cells. These observations suggest that TAp73α acts as a tumor suppressor in vivo. Here, we demonstrated that TAp73α was responsible for the tumor suppressive proapoptotic function of TGF-β in gastric cancer cells. Since *p73* is rarely mutated in cancer unlike *p53*, therapeutic modulation of TAp73α expression might be used to target various cancers with *p53* mutations.

Several proapoptotic genes, including the TGF-β-inducible early-response gene (*TIEG*), *death-associated protein kinase* (*DAPK*), *Src homology 2 domain-containing 5′ inositol phosphatase* (*SHIP*), and *GADD45b* have been shown to be activated by TGF-β in the induction of apoptosis [1]. Although the promoters of *DAPK*, *SHIP* and *GADD45b* are activated by Smads, the consensus Smad-binding site is only found in the *DAPK* promoter. We could not find any consensus Smad-binding sites in the *p73*, *Bax*, and *Puma* promoters. Although overexpressed E2F-1 directly binds to the *p73* promoter and increases its transcriptional activity to promote apoptosis in an osteosarcoma cell line [16,17], our present results showed that E2F1 was not involved in the activation of the *p73* promoter by TGF-β in gastric cancer cells (Fig. 5). Further studies will be required to ascertain the activation mechanism of proapoptotic target genes by TGF-β.

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