Role of Activating Transcription Factor 3 on TAp73 Stability and Apoptosis in Paclitaxel-Treated Cervical Cancer Cells

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Abstract

Taxol (paclitaxel) is a potent anticancer drug that has been found to be effective against several tumor types, including cervical cancer. However, the exact mechanism underlying the antitumor effects of paclitaxel is poorly understood. Here, paclitaxel induced the apoptosis of cervical cancer HeLa cells and correlated with the enhanced activation of caspase-3 and TAp73, which was strongly inhibited by TAp73β small interfering RNA (siRNA). In wild-type activating transcription factor 3 (ATF3)–overexpressed cells, paclitaxel enhanced apoptosis through increased α and β isoform expression of TAp73; however, these events were attenuated in cells containing inactive COOH-terminal–deleted ATF3 [ATF3(ΔC)] or ATF3 siRNA. In contrast, paclitaxel-induced ATF3 expression did not change in TAp73β-overexpressed or TAp73β siRNA–cotransfected cells. Furthermore, paclitaxel-induced ATF3 translocated into the nucleus where TAp73β is expressed, but not in ATF3(ΔC) or TAp73β siRNA–transfected cells. As confirmed by the GST pull-down assay, ATF3 bound to the DNA-binding domain of p73, resulting in the activation of p21 or Bax transcription, a downstream target of p73.

Overexpression of ATF3 prolonged the half-life of TAp73β by inhibiting its ubiquitination and thereby enhancing its transactivation and proapoptotic activities. Additionally, ATF3 induced by paclitaxel potentiated the stability of TAp73β, not its transcriptional level. Chromatin immunoprecipitation analyses show that TAp73β and ATF3 are recruited directly to the p21 and Bax promoter. Collectively, these results reveal that overexpression of ATF3 potentiates paclitaxel-induced apoptosis of HeLa cells, at least in part, by enhancing TAp73β’s stability and its transcriptional activity. The investigation shows that ATF3 may function as a tumor-inhibiting factor through direct regulatory effects on TAp73β, suggesting a functional link between ATF3 and TAp73β.

Introduction

Paclitaxel is derived from the needles and bark of the Western yew tree (1, 2). It is widely used to treat a variety of solid tumors including ovarian, breast, non–small cell lung carcinomas, and Kaposi’s sarcoma (3). The antitumor effects of paclitaxel are mediated by binding to and stabilizing microtubules, thereby enhancing microtubule polymerization leading to G2-M cell cycle arrest and ultimately to apoptotic cell death (4). Apoptotic tumor cell death is commonly observed in paclitaxel therapy (5); however, the exact mechanisms by which paclitaxel triggers p53-independent apoptosis are not clearly elucidated. Although treatment with paclitaxel can improve survival and quality of life for patients with cancer (6, 7), the majority of patients will eventually experience disease progression even after initially responding to paclitaxel (8).

Unlike p53, the p73 gene is able to encode transcriptionally active TAp73, as well as an NH2-terminally truncated form, ΔNp73 (DNp73), lacking the transactivation domain (9). TAp73 is expressed as several isoforms, designated as p73α, p73β, p73γ, and p73ε, due to extensive splicing at the carboxy terminal domain (10). Moreover, not all of the same signals that activate p53 can induce p73 overexpression. Only a subset of DNA-damaging signals that increase p53 expression, such as irradiation or anticancer drugs including cisplatin, camptothecin, paclitaxel, and doxorubicin have been shown to induce TAp73 protein expression (11, 12). In addition, steady-state levels of TAp73 are not reduced by complex formation with Mdm2 (13), which targets p53 for ubiquitin-mediated proteolysis (14).
Recent studies have shown that despite disruption in p53 and pRB functioning by E6 and E7 oncoproteins associated with cervical cancer caused by the human papillomavirus (HPV), the TAp73 gene was overexpressed in both radiosensitive and radioresistant cervical cancers (15, 16). Moreover, activated p73 in the absence of functional p53 activates the transcription of p53 target genes such as p21 or Bax, and induces apoptosis in p53-null SAOS2 cells (17). In cervical cancers in which p53 is impaired, it is therefore reasonable to postulate that in response to paclitaxel, TAp73 overexpression is a compensatory mechanism necessary to trigger p53-independent apoptosis. Thus, if TAp73 is functionally active in paclitaxel-treated cervical cancer cells, it may be important to understand the molecular mechanisms involved in the regulation of the TAp73 gene and its downstream effects.

**FIGURE 1.** The effects of paclitaxel on apoptosis in cervical cancer cells. HeLa cells were treated with different paclitaxel concentrations for the indicated times. A. MTT assay. B. Apoptosis (M1, sub-G1 peak) was measured by propidium iodide staining (top), TUNEL assay (bottom; bar, 10 μm), DNA fragmentation (right). C. The cleavage of caspase-3 and poly(ADP-ribose) polymerase was analyzed. Expression (D) and quantification (E) of apoptosis-related proteins in HeLa cells. Whole-cell lysates were separated on SDS-PAGE, followed by Western blotting using the specific antibodies. Similar results were obtained for each of the three experiments.
Transcription factors play key roles in controlling cell proliferation, cell cycle progression, and apoptosis (18, 19), and are therefore subject to targeting by therapeutic drugs. In particular, activating transcription factor 3 (ATF3), which is a member of the ATF/cyclic AMP–responsive element binding protein subfamily, is a stress-inducible transcriptional repressor (20) as well as a basic region-leucine zipper transcription factor. It is induced in cells exposed to a variety of stressful stimuli (e.g., toxic chemicals, anticancer drugs, proteasome inhibitors, genotoxic agents, homocysteine, and ischemia reperfusion) and causes cell cycle arrest and apoptotic cell death (21, 22). It was recently reported that ATF3 transcription is regulated by a variety of signaling pathways and transcription factors, including nuclear factor κB, EGR-1, and c-Jun-NH₂-kinase (23). Moreover, topoisomerase inhibitor, etoposide, as well as camptothecin-induced apoptosis and caspase activity are both potentiated by ATF3 overexpression in human epithelial carcinoma HeLa-S3 cells (24). Recently, ATF3 has been found to interact with p53 proteins stimulated by genotoxic stress and prevent p53 ubiquitination and degradation, which consequently augments p53 function and results in enhanced apoptosis (25). In contrast, there is some evidence showing that ATF3 inhibits doxorubicin-induced apoptosis through down-regulation of p53 in cardiac myocytes (26). Although the induction of ATF3 by stress signals is neither tissue-specific nor stimulus-specific, they all induce cellular damage (27). Therefore, we hypothesize that TAp73 contributes to apoptosis induced by paclitaxel in cervical cancer cells and that ATF3 may potentially be a major regulator of p73 expression.

In this investigation, we showed that ATF3 overexpression potentiates paclitaxel-induced apoptosis of cervical cancer HeLa cells, at least in part, through stimulating TAp73 expression and transcriptional activity. Furthermore, ATF3 interacts directly with TAp73 and enhanced the stability of the latter, and therefore, it may serve as a tumor-inhibiting factor via stimulation of the apoptotic functions of TAp73. Here, we show for the first time that TAp73 is physically, as well as functionally, associated with the novel stress-inducible transcription factor ATF3.

Results

**Paclitaxel Induces Apoptosis in HeLa Cells**

To examine the molecular mechanisms involved in the cytotoxic effects of paclitaxel on a human cervical cancer cell...
line, we first screened three different human-derived cervical cancer cell lines, HeLa, SiHa, and Caski, following treatment with paclitaxel at different concentrations for varying time periods. All of the screened cell types revealed high cytotoxicity to paclitaxel in a time-dependent and dose-dependent manner (data not shown). In particular, HeLa cells seemed to be the most sensitive among the panel of cell lines evaluated and was therefore chosen for our experiments. Cell viability measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay revealed that paclitaxel (25 and 50 nmol/L) induced 9% and 24.6% cytotoxicity, respectively, after 24 hours (Fig. 1A). The majority of the cells were arrested in the G2-M phase (M4, G2-M phase; 76.84 ± 0.05%) 12 h after treatment with paclitaxel. Treatment with 50 nmol/L paclitaxel for 24 hours caused marked apoptosis based on DNA content as measured by fluorescence-activated cell sorting analysis (M1, sub-G1 phase, 29.47 ± 0.05% in paclitaxel-treated cells versus 4.92 ± 0.05% in control cells), whereas most of cells died after 48 hours (M1, 72.53 ± 0.05%). Similarly, paclitaxel significantly increased terminal nucleotidyl transferase–mediated nick end labeling (TUNEL)–positive apoptotic cells and genomic DNA fragmentation dose-dependently (Fig. 1B). The activity of caspase-3–like proteases was significantly increased in a dose-dependent manner, correlating with cleavage of poly(ADP-ribose) polymerase (Fig. 1C) and consequently led to the release of cytochrome c from the mitochondria (data not shown).

Next, we examined the effects of paclitaxel on the expression profile of apoptosis-related proteins in HeLa cells. As shown in Fig. 1D and E, TAp73α and TAp73β were strongly induced by paclitaxel, whereas p53 was weakly induced, consistent with previous reports showing that p53 bound to viral proteins such as HPV E6 oncogene are inactivated in HeLa cells (20). Despite minimal p53 expression, p21, a downstream target gene of p53 and p73 (21, 28), was also induced by paclitaxel, suggesting that TAp73α and TAp73β might be principally responsible for the induction of p21 in the absence of p53. On the other hand, our data show that these inductions of TAp73α and TAp73β are specific because the expressions of dominant negative ΔNp73α and ΔNp73β, which were revealed in a variety of tumors (9, 10), were not changed by paclitaxel. Unexpectedly, paclitaxel also increased the antiapoptotic Bcl-2 protein, which was down-regulated after 24 hours, along with the proapoptotic Bax protein; however, another antiapoptotic protein, Bcl-xL, was down-regulated by paclitaxel in a time-dependent manner.

p73 Plays an Important Role in Paclitaxel-Induced Apoptosis

To determine the roles of TAp73 on paclitaxel-induced apoptosis, HeLa cells were transfected with wild-type TAp73β expression vectors and subsequently treated with paclitaxel. As shown in Fig. 2A, after 24 hours, endogenous TAp73β...
expression increased in Neo vector–transfected cells, but was synergistically potentiated in wild-type TAp73β–transfected HeLa cells. Additionally, the expression of Bax and p21 increased in wild-type TAp73β–transfected cells compared with Neo vector–transfected cells. Similarly, TUNEL-positive apoptotic cells induced by paclitaxel increased significantly in wild-type TAp73β–transfected cells, whereas fewer apoptotic cells were detectable in Neo vector–transfected cells (Fig. 2B, top). To further confirm the role of TAp73β on paclitaxel-induced apoptosis, cells were transfected with TAp73β small interfering RNA (siRNA) and then treated with paclitaxel. TAp73β siRNA completely abolished TAp73β induction (Fig. 2C), DNA fragmentation (Fig. 2D), and TUNEL-positive apoptotic cells (Fig. 2E) by paclitaxel. In addition, normally residing within the cytosol, Bax levels were significantly reduced within cytosolic fractions by paclitaxel treatment and increased in the mitochondrial fraction, but significantly attenuated in TAp73β siRNA–transfected cells. Similarly, cytochrome c release from the mitochondria into the cytosol was significantly attenuated by TAp73β siRNA (Fig. 2F).

These results suggest that in p53-inactivated cervical cancer HeLa cells, TAp73β induced by paclitaxel plays an essential role in the induction of apoptosis.

**TAp73β Induces Apoptosis via a p53-Independent Pathway**

To confirm whether TAp73β plays an essential role in paclitaxel-induced apoptosis via a p53-independent pathway, we performed similar experiments using transfection of p53 siRNA into human breast carcinoma MCF-7 cells, which contains both wild-type p53 and TAp73. As shown in Fig. 3A, p53, which increased significantly in response to paclitaxel treatment were almost completely abolished by p53 depletion in MCF-7 cells, whereas Bax and p21 expression as well as TAp73β induced by paclitaxel were not strongly altered by p53 siRNA transfection, indicating that paclitaxel-induced apoptosis may be regulated in a TAp73β-dependent pathway, not in a p53-dependent pathway.

As expected, paclitaxel-induced TAp73β overexpression and apoptosis was observed concomitantly in both control and p53 siRNA–transfected MCF-7 cells (Fig. 3B). In order to further confirm that paclitaxel-induced apoptosis is mediated by TAp73, not by a p53-independent pathway, we took advantage of

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**FIGURE 4.** The effects of paclitaxel on ATF3 expression. **A** and **B,** Expression of ATF3 proteins. HeLa cells were treated with paclitaxel for the indicated time points (**A**) and at the different concentrations (**B**). **C,** Immunocytochemistry for TAp73β and ATF3 and 4,6-diamidino-2-phenylindole (DAPI) staining. Fluorescent microscopic images were taken for TAp73, ATF3, and the final merged images and 4,6-diamidino-2-phenylindole nuclear staining are shown (bar, 10 μm). **D,** Expression of ATF3 and TAp73β in SiHa and Caski cells.
p53-null human osteosarcoma SAOS2 cell lines, which are p53-deficient and do not express TAp73 at the mRNA or protein levels. Moreover, the SAOS2 cell line may be a particularly suitable in vitro model for evaluating the role of TAp73 on paclitaxel-induced apoptosis, as it is well known that paclitaxel induces TAp73 expression in these cells (29). As shown in Fig. 3C, although p53 protein expression was undetectable in paclitaxel-treated SAOS2 cells, the expression levels of TAp73, Bax, and p21 increased following treatment with paclitaxel, as observed in SAOS2 cells, was even lower than the increase observed in MCF-7 cells (Fig. 3D). However, the upstream regulator involved in TAp73 overexpression is still not clear. To elucidate the mechanism by which TAp73 expression is up-regulated, we screened for changes in the expression profile of several transcription factors after treatment with paclitaxel (data not shown). The results showed that paclitaxel significantly increased the expression of the stress-inducible gene, ATF3, in a time-dependent and dose-dependent manner (Fig. 4A and B), which was concomitantly increased with TAp73 expression (Fig. 1D). In addition, most of the ATF3 induced by paclitaxel was colocalized with TAp73 in the nucleus, although this was not significant in control cells (Fig. 4C, left), which was confirmed by data showing that ATF3 and TAp73 levels were concomitantly increased within the nuclei fractions following paclitaxel treatment (right). This finding suggested that paclitaxel-induced ATF3 might be involved in increasing TAp73 up-regulation to compensate for the apoptotic roles of p53 in p53-inactivated HeLa cells. Because HeLa cells are

ATF3 Potentiates TAp73β-Mediated Apoptosis in Paclitaxel-Treated HeLa Cells

Our data show that paclitaxel-induced apoptosis was regulated in a TAp73-dependent and p53-independent pathway;
HPV-18–positive cell lines, we have examined whether paclitaxel induced ATF3 and TAp73β in other HPV-positive cell lines such as SiHa and Caski cells, which were positive for HPV-16 (Fig. 4D). As expected, paclitaxel abundantly induced ATF3 and TAp73β in both cells, indicating that the inductions of ATF3 and TAp73β by paclitaxel were not specific for the strains of HPV.

Next, to define the direct role of ATF3 on paclitaxel-induced TAp73β expression and apoptosis, HeLa cells were transiently transfected with ATF3 siRNA and then treated with paclitaxel. The expression of TAp73α, TAp73β, Bax, and p21 increased by paclitaxel were attenuated significantly by ATF3 depletion. Also, ATF3 siRNA attenuated paclitaxel-mediated down-regulation of Bcl-xL expression (Fig. 5A). Paclitaxel-induced TAp73 and its downstream target p21 and Bax were potentiated by wild-type ATF3 overexpression, which were significantly decreased in cells transfected with ATF3(AC), a deletion in the COOH-terminal (101-181) region was necessary for interaction with other proteins (30).

As a transcriptional factor, nuclear localization of ATF3 is likely a prerequisite for its activity. Therefore, we next examined whether paclitaxel-induced apoptosis is mediated by the direct effects of ATF3 on p73 activation and localization into the nucleus. As shown in Fig. 5D and E, paclitaxel-induced

**FIGURE 6.** The effects of TAp73β or TAp73β siRNA overexpression on paclitaxel-induced apoptosis. **A.** Effects of TAp73β overexpression on ATF3 induced by paclitaxel. The cells were transfected with GFP-TAp73β alone or TAp73β siRNA, and then treated with paclitaxel. The cell lysates were subjected to Western blotting using anti-ATF3, anti-GFP, anti-TAp73β antibodies. **B.** TAp73β depletion inhibits the potentiation of TAp73β on paclitaxel-induced apoptosis. After transfection with GFP-empty vector, GFP-TAp73β, or TAp73β siRNA, cells were incubated with Annexin V and propidium iodide, and analyzed for cell apoptosis by fluorescence-activated cell sorting analysis (top) and quantified (bottom). **C.** Effects of TAp73β depletion using TAp73β siRNA on paclitaxel-induced ATF3 expression. Cells transfected with GFP-TAp73β or TAp73β siRNA were treated with paclitaxel and fixed. Fluorescent microscopic images were taken for GFP (for TAp73β), ATF3, and the final merged images were shown (top; bar, 10 μm). **D.** TAp73β depletion prevents ATF3 translocation into the nuclear fraction induced by paclitaxel.
TAp73β expression was increased significantly by GFP-ATF3 overexpression, whereas GFP-ATF3(ΔC)-transfected cells resulted in minor increases (Fig. 5D and E, left). Interestingly, most of the GFP-ATF3 activated by paclitaxel translocated into the nucleus, but GFP-ATF3(ΔC) was primarily found around the nucleus (Fig. 5D). Furthermore, it seems that the degree of apoptosis induced by paclitaxel was different based on the location of ATF3. Levels of TUNEL-positive apoptotic cells increased in GFP-ATF3–transfected cells, but decreased significantly in ATF3(ΔC)-transfected cells. These results are also supported by using ATF3 siRNA. Apoptosis increased by paclitaxel or ATF3 overexpression was significantly decreased in ATF3 siRNA, such as that in ATF3(ΔC)-transfected cells (Fig. 5F). These results suggest that ATF3, especially the COOH-terminal region (101-181) of ATF3, may play an important role in the functionality of TAp73 and may function as an upstream regulator of TAp73 induced by paclitaxel treatment. We have also observed similar results in other cervical cancer cell lines (Caski and SiHa cells; see Supplementary Data).

Depletion of TAp73β Affects ATF3 Localization, but not ATF3 Expression in Paclitaxel-Treated HeLa Cells

Our data showed that in HeLa cells, ATF3 strongly augmented TAp73β up-regulation induced by paclitaxel, resulting in apoptosis, but we couldn’t exclude the possibility that TAp73β can reversibly affect ATF3 expression or activation. Therefore, after transfection of GFP-TAp73β or TAp73β siRNA, we examined whether TAp73β overexpression affects ATF3 expression and nuclear translocation induced by paclitaxel. As shown in Fig. 6A, paclitaxel treatment increased ATF3 expression in GFP-empty vector–transfected cells, but paclitaxel-induced ATF3 expression was unaffected by GFP-TAp73β overexpression. Additionally, paclitaxel-induced ATF3 expression was not changed by TAp73β depletion in TAp73β siRNA–transfected cells. This finding further supports previous data that ATF3 is an upstream regulator of TAp73β induced by paclitaxel. We have also observed similar results in other cervical cancer cell lines (Caski and SiHa cells; see Supplementary Data). However, it may be possible that TAp73β plays an essential role in apoptosis induced by paclitaxel-activated ATF3 because compared with GFP-transfected cells, paclitaxel strongly enhanced GFP-TAp73β–induced apoptosis, which was significantly attenuated by the depletion of TAp73β (Fig. 6B). GFP-TAp73β alone also induced early-stage apoptosis (bottom right), whereas cotreatment of paclitaxel in GFP-TAp73β–transfected cells increased Annexin V-propidium iodide double staining cells (late-stage apoptosis). As the data showed that decreased ATF3(ΔC) translocation into the nucleus resulted in reduced paclitaxel-mediated apoptosis through lowered TAp73β expression (Fig. 5D and E), we examined whether TAp73β depletion affected paclitaxel-induced apoptosis by altering ATF3 translocation into the nucleus (Fig. 6C). In GFP-TAp73β–overexpressing cells, ATF3 expression was potentiated by paclitaxel and colocalized with GFP-TAp73β in the nucleus. Meanwhile, nuclear translocation of ATF3 significantly decreased in TAp73β siRNA–transfected cells and TAp73β located primarily around the nucleus, correlating with reduced TAp73β expression. These results suggest that functional TAp73β may be needed for ATF3 activation and its translocation into the nucleus, although TAp73β did not affect...
ATF3 expression. To define this possibility, the expression of ATF3 in the isolated cytosolic or nuclear fraction was examined (Fig. 6D). Paclitaxel-enhanced ATF3 expression was observed in both the cytosol and nuclear fractions of GFP- or GFP-TAp73β-transfected cells, whereas ATF3 in TAp73β siRNA-transfected cells was detected mostly in the cytosolic fraction, and weakly in the nuclear fraction, indicating that TAp73β may play an important role in inducing ATF3 activation and nuclear translocation. Tubulin (cytoplasmic protein) and histone I (nuclear protein) were used to confirm whether cytosolic and nuclear fractions were isolated purely from cell extracts.

**TAp73β Plays an Essential Role in ATF3-Mediated Apoptosis**

To examine the effect of ATF3 on paclitaxel-induced apoptosis, HeLa cells were cotransfected with flag-tagged ATF3 or ATF3(DC) in the presence or absence of GFP-TAp73β and then treated with paclitaxel. First, ATF3 overexpression increased the expression of p21 and Bax, TAp73 downstream target proteins related to apoptosis, which was potentiated by paclitaxel (Fig. 7A). In contrast, p21 and Bax were not increased by paclitaxel cotreatment in flag-ATF3(DC)–overexpressed cells, indicating that ATF3 was required for the induction of p21 and Bax by paclitaxel. Interestingly, weak expression of Bax and p21 in ATF3(DC)-transfected cells was strongly recovered in GFP-TAp73β–transfected cells, which was potentiated by paclitaxel treatment. In addition, paclitaxel-induced apoptosis increased strongly in Flag-ATF3–transfected cells, but was observed to be weak in Flag-ATF3(DC)–transfected cells (Fig. 7B). However, inhibition of apoptosis in ATF3(DC)-transfected cells was recovered in part, but not completely, in cells cotransfected with GFP-TAp73β (Fig. 7B, left). Although paclitaxel did not increase apoptosis in ATF3(DC)-transfected cells, overexpression of GFP-TAp73β alone restored apoptosis.

**FIGURE 8.** Interaction of ATF3 with TAp73β in paclitaxel-treated HeLa cells. HeLa cells were cotransfected with ATF3 in the absence or presence of flag-tagged TAp73β or TAp73β. A, After transfection, cell lysates were immunoprecipitated (IP) with anti-IgG or anti-flag (for TAp73β) and then immunoblotted (IB) using anti-ATF3 and anti-flag antibodies. Total lysates were immunoblotted with anti-ATF3 and anti-TAp73β antibodies. B, Cell lysates were immunoprecipitated (IP) with anti-ATF3 and then immunoblotted (IB) using anti-flag and anti-ATF3. C, In vitro binding assay using GST pull-down assay and purified GST, GST-ATF3 (top), or GST-ATF3(DC) was mixed with paclitaxel-treated cell lysates and subjected to SDS-PAGE, and immunoblotted with anti-TAp73β and anti-GST. Total cell lysates were also immunoblotted with anti-TAp73β antibody. D, ATF3 depletion inhibits the endogenous TAp73β expression and their in vitro binding. HeLa cells were transfected with ATF3 siRNA, then treated with paclitaxel. Cell lysates were incubated with GST or GST-TAp73 and then analyzed by GST pull-down assay. In vivo binding assay (E) and ATF3 depletion (F) inhibits in vivo binding. After treatment with paclitaxel in the absence (E) or presence (F) of ATF3 siRNA, the immunoprecipitated lysates with anti-p73 (left) or anti-ATF3 (right) were subjected to immunoblotting with anti-TAp73β and anti-ATF3 antibodies. Total cell lysates were also immunoblotted with anti-ATF3 and anti-TAp73β antibodies.
similar to apoptosis induced by paclitaxel in control cells (Fig. 7B, right), suggesting that TAp73$\beta$ is an important downstream regulator of ATF3-mediated apoptosis and is required for ATF3-mediated apoptosis.

**ATF3 Directly Binds TAp73$\beta$ in Paclitaxel-Treated Cells**

Previously, TAp73 homologous p53 protein levels were elevated by stress via an increased stability and its protein stability could be regulated by associated proteins (25).
our data indicate that paclitaxel-mediated TAp73 up-regulation was dependent on active ATF3 expression, we considered the possibility that through interaction with ATF3, TAp73 stability was enhanced. To answer this question, ATF3 was first cotransfected with flag-TAp73α or flag-TAp73β and commu-

noprecipitation assays were done. ATF3 was detectable in immunocomplexes of TAp73α and TAp73β captured by the anti-flag antibody (Fig. 8A). The immunoprecipitated ATF3 was also found to strongly interact with both TAp73α and TAp73β (Fig. 8B). Immunoprecipitated normal serum IgG was

**FIGURE 10.** ATF3 enhances TAp73β mRNA expression and TAp73β-mediated transcriptional activity. A. TAp73β mRNA expression. Cells were treated with paclitaxel for the indicated times and total mRNA was subjected to reverse transcription-PCR using TAp73β-specific primers. B. ATF3 overexpression delays the stability of TAp73β protein induced by paclitaxel. Top, HeLa cells transfected with the indicated combinations of expression plasmids were pretreated with cycloheximide (CHX) and then treated with paclitaxel. C. ATF3 potentiates TAp73β-mediated transcriptional activities. Cells were cotransfected with p21- or Bax-Luc together with ATF3 or ATF3ΔC and then treated with paclitaxel. After treatment, luciferase activity was measured. Columns, average from three independent experiments; bars, SE (P < 0.05). D. Cells were cotransfected with p21-Luc or Bax-Luc and ATF3, ATF3 siRNA, TAp73β, or TAp73β siRNA, and then luciferase activities were measured. E. EMSAs were done on the TAp73β binding site of p21 promoter cells using nuclear extracts from cells transfected with ATF3 or TAp73β, and then treated with paclitaxel. For oligonucleotide competition experiments, a 100-fold excess of oligonucleotides was used (lanes 1 and 2). F. Depletion of ATF3 or TAp73β inhibits TAp73 protein-DNA complex. The protein-DNA complexes were supershifted by the TAp73β antibody (lane 7), but not by the STAT5a antibody (lane 8). G. ATF3 increases the amounts of TAp73β-associated with the human p21 and Bax promoters. HeLa cells were transiently transfected with GFP or GFP-ATF3 plasmids. Forty-eight hours after transfection, cells were cross-linked with 1% formaldehyde and subjected to chromatin immunoprecipitation assays, followed by PCR analysis as described under Materials and Methods.


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not found to interact with ATF3 and Flag-Tap73α or Tap73β (Fig. 8A, left), indicating that Tap73 interacts specifically with ATF3 in vivo. The total cell lysates were also determined by Western blotting using ATF3 and flag antibodies (bottom). To corroborate whether ATF3 directly binds endogenous Tap73β, cells were treated with paclitaxel to simulate conditions that stimulate Tap73 expression, and subsequently, the GST pull-down assay was conducted using purified GST, GST-fused ATF3, or GST-ATF3(ΔC). From here, we examined the interaction of ATF3 with Tap73β because this interaction is higher than that with Tap73α. As shown in Fig. 8C, association of Tap73β with GST-ATF3 (left) observed in paclitaxel-treated cells (lanes 6 and 7), but not in control cells (lanes 3 and 4). The association of GST-ATF3 with Tap73β might be due to the increase of Tap73β induced endogenously by paclitaxel because the association was not detected in control cells, and did not express Tap73β. However, GST-ATF3(ΔC) (right) or control GST beads did not associate with Tap73β, although Tap73β was induced in paclitaxel-treated cells (lanes 5-7), suggesting that Tap73β induced endogenously by paclitaxel interacts directly with ATF3, specifically, with the COOH-terminal region. Next, we examined whether ATF3 depletion using ATF3 siRNA affects the association of GST-ATF3 with endogenous Tap73β induced by paclitaxel. As shown in Fig. 8D, the interaction between GST-ATF3 and Tap73β was detected in paclitaxel-treated cells (lanes 5 and 6), which was abolished completely in ATF3 siRNA–transfected cells (lanes 7 and 8), and correlated with the reduction in p73β expression through the silencing of endogenous ATF3 (bottom, lanes 7 and 8). In contrast, Tap73β induced by paclitaxel was not associated with control GST (lanes 1 and 2). These results suggest that ATF3 may be involved in stabilizing Tap73β protein induced by paclitaxel treatment and affects the association between them. Next, we have examined whether endogenous ATF3 and Tap73β induced by paclitaxel could also interact with each other in vivo. As expected, after paclitaxel treatment, both endogenous Tap73β and ATF3 were immunoprecipitated together, which was correlated with the proportional increase of ATF3 and Tap73β by paclitaxel treatment (Fig. 8E). However, an increase of endogenous Tap73β captured by immunoprecipitated ATF3 was also completely abrogated by ATF3 siRNA transfection, which correlated with decreased levels of Tap73β via ATF3 depletion (Fig. 8F). We therefore suggest that stabilization of Tap73β by ATF3 may be due to the increased steady-state levels of these proteins or the interaction of these two proteins.

ATF3 Interacts with the DNA-Binding Domain of Tap73β and Stabilizes the Tap73β

Next, to determine the binding region of Tap73β that is necessary for interactions with ATF3, we constructed a series of truncated mutants that contained distinct domains of Tap73β (Fig. 9A). After cotransfection of the p73β-trasactivation domain, p73β DNA–binding domain, and p73β-oligomerization domain mutants with GFP-ATF3 into HeLa cells, overexpressing mutant flag-p73β were immunoprecipitated with anti-flag antibody, and the immunoprecipitates were Western blotted using anti-GFP antibody to detect ATF3 (Fig. 9B). Neither the p73β-trasactivation domain nor the p73β-oligomerization domain regions seemed to interact with ATF3, whereas the p73β DNA–binding domain–containing region (amino acids 54-310) strongly interacted with ATF3, indicating that the DNA-binding domain region of p73β is responsible for the interaction with ATF3. In general, unlike p53, p73 generate two major protein isoforms, transactivation and ΔN domains, through two different promoters and three alternative splicing sites at the 3’ end, denoted by α, β, γ (9). Furthermore, the transactivated isoform of p73 acts more like p53, whereas the ΔN isoform shows a dominant-negative function to Tap73 isoforms (31). Therefore, we have examined whether ATF3 interacts with dominant negative ΔNp73β similar with Tap73β and thus affects paclitaxel-induced apoptosis. As shown in Fig. 9C, immunoprecipitated ATF3 interacts with ΔNp73β similar with Tap73β. However, Tap73β cotransfection, but not ΔNp73 cotransfection, highly induced apoptosis, which was potentiated by paclitaxel (Fig. 9D), suggesting that ΔNp73β could be opposing effects on apoptosis mediated by Tap73β, although ATF3 interacts with the DNA-binding domain of p73β.

Similar to p53, mdm2 binds to Tap73 and inhibits Tap73-mediated transcriptional activation and apoptosis; however, mdm2 failed to ubiquitinate Tap73 (32). Furthermore, it was reported that ATF3 interacts with p53 and prevents the ubiquitination and degradation of the latter, and consequently, augmenting p53 function (18, 29). Therefore, to investigate whether paclitaxel-induced ATF3 also mediates Tap73β expression and function by preventing ubiquitination and degradation, cells were pretreated with MG132, a proteasome inhibitor, in wild-type ATF3 or ATF3(ΔC)-overexpressing cells (Fig. 9E). Pretreatment with MG132 for 2 hours significantly enhanced Tap73β expression induced by paclitaxel (lane 3). Tap73β expression increased in cells overexpressing ATF3 alone, which was potentiated by MG132 pretreatment (lanes 4 and 5), but not ATF3(ΔC)-overexpressing cells (lane 6). The Tap73β protein levels were increased slightly by MG132 in ATF3(ΔC)-overexpressing cells (lane 7) and even by itself MG132 (lane 8). Similar to this, MG132 significantly enhanced paclitaxel-induced apoptosis, which was potentiated by ATF3 overexpression, but not by ATF3(ΔC)-overexpression. TUNEL-positive apoptotic cells induced by ATF3 overexpression were also almost completely decreased by Tap73β siRNA, indicating that ATF3-induced apoptosis is due to the induction of Tap73β (Fig. 9F). Next, to examine the direct effects of ATF3 on the ubiquitination of Tap73β, we did an in vitro ubiquitination assay by using an in vitro–reconstituted ubiquitination reaction system with E1, E2 enzymes, and GST-ATF3 or GST-ATF3(ΔC) recombinant proteins (Fig. 9G). In the presence of all the required ubiquitination reaction components, the addition of recombinant ATF3 protein completely reduced Tap73β ubiquitination. Unlike the full-length protein, the ATF3 protein in which the COOH-terminal domain is deleted, preventing it from binding with Tap73β, was less effective in blocking Tap73β ubiquitination in vitro. We have determined if ATF3 could also inhibit the ubiquitination of Tap73 in vivo, HeLa cells were transiently cotransfected with the expression plasmids for HA-Tap73β and His-ubiquitin with plasmid for ATF3 or ATF3(ΔC). Forty-eight hours after transfection, whole-cell lysates were prepared.
ATF3 Potentiates the Transactivation of TAp73 Target Promoters

Because transcriptional regulation of the TAp73 gene contributes to its activation in response to various stress stimuli, we hypothesized that ATF3-dependent induction of TAp73 is regulated by enhancement of TAp73 transcriptional levels, which might contribute to the COOH-terminal region of ATF3 inhibits the ubiquitination of TAp73β, thereby increasing the stability of TAp73β.

Next, to define the possibility that ATF3 could also affect TAp73β-dependent transcriptional activation, HeLa cells were transiently transfected with the TAp73β cDNA and a luciferase reporter containing the TAp73-responsive element from p21 or Bax promoters, together with ATF3 or ATF3(ΔC) expression plasmids and then treated with paclitaxel. As shown in Fig. 10C, ATF3 potentiated paclitaxel-induced p21- or Bax-luciferase activities, but was attenuated by cotransfection with ATF3(ΔC). In addition, TAp73β-p21 or Bax-luciferase activities increased significantly in ATF3(cotransfected cells, which were not found in ATF3(ΔC)-transfected cells. The silencing of ATF3 by ATF3 siRNA strongly inhibited ATF3-induced p21- or Bax-luciferase activity. TAp73β siRNA also inhibited TAp73β-p21 and Bax transcriptional activity (Fig. 10D). These data suggest that paclitaxel-induced ATF3 mediates TAp73β transactivation, followed by p21 and Bax gene transcription. Thus, to examine whether ATF3 can mediate direct interactions between the TAp73β and p21 promoter, electrophoretic mobility shift assays were done using nuclear extracts from paclitaxel-treated, ATF3-transfected or TAp73β-transfected cells (Fig. 10E). For these experiments, 42-bp oligonucleotides were designed that span the putative TAp73β-binding site containing the sequence of the TAp73 response element in the p21 promoter. As shown in Fig. 10E, paclitaxel increased the TAp73β-p21 binding complex, which was further potentiated by cotransfection of ATF3, but was completely abolished by 100-fold excess of unlabeled oligonucleotides. In addition, the complexes increased by ATF3 and TAp73β transfection were completely abrogated by ATF3 siRNA and TAp73 siRNA, respectively. To ascertain whether the complex consisted of bound TAp73, a supershift assay was conducted with the antibody against TAp73 and ATF3. Results show that both anti-TAp73 and anti-ATF3 shifted the binding complex, although the shifted locations were different. In contrast, no supershifted complex was detected with anti-STAT5 antibody, which was used as a negative control (Fig. 10F). Thus, it is likely that ATF3 potentiates the direct interaction of TAp73 to the TAp73-binding site located in the p21 promoter, increasing TAp73’s transcriptional activity. Next, to clarify the precise molecular mechanism by which ATF3 mediates the transcriptional activity of TAp73 on p21 or Bax promoters, we did a chromatin immunoprecipitation analysis. Cross-linked chromatin prepared from HeLa cells transiently transfected with GFP-empty or GFP-ATF3 plasmids were treated with paclitaxel and immunoprecipitated with the indicated antibodies, followed by amplification with the indicated promoter-specific primers. As shown in Fig. 10G, TAp73β and ATF3 were efficiently recruited to the p21 and Bax promoters in control cells, which were increased strongly in paclitaxel-treated cells. Furthermore, a significant increase in chromatin binding was higher in the presence of ATF3 than in cells transfected with GFP alone. This observation correlates with our previous finding that ATF3 enhanced the transcriptional activity of TAp73β. Interestingly, ATF3 also binds to the TAp73β binding sites on p21 or Bax promoters. This recruitment of ATF3 to TAp73β binding sites was specific because we did not observe any amplification in STAT5 immunoprecipitated chromatin. These data suggest that ATF3 might directly bind to TAp73β binding sites or play as a cofactor mediating p73 functional activity.

Discussion

In this study, we showed that a novel transcription factor, ATF3, interacts directly with TAp73 and enhances the stability of the latter, thereby acting as a tumor-inhibiting factor through stimulating apoptosis mediated by TAp73 after induction by paclitaxel. Paclitaxel is a broad-spectrum anticancer agent which is currently used in treating many types of advanced cancer including cervical, breast, and ovarian cancers, although resistance to paclitaxel chemotherapy occurs in some patients with cancer (8, 34). The primary antitumor mechanism of action by paclitaxel is through its ability to bind to microtubules and prevent their assembly, causing cells to arrest in G2-M, and consequently, resulting in apoptotic cell death (4, 5). Several studies have been reported in which paclitaxel induces apoptosis through nuclear factor-κB down-regulation and Akt phosphorylation followed by mTOR activation, a downstream target of Akt (35). However, the role of paclitaxel in regulating cell death in various cancer cells, including cervical cancer cells, has been extensively studied, but the exact mechanisms through which paclitaxel triggers apoptosis and promotes chemocytotoxicity are still unclear.
Recently, it has been shown that TAp73-inducing agents such as cisplatin, doxorubicin, and paclitaxel could result in TAp73-mediated cell death in a p53-independent manner (16, 36). Particularly, in cervical cancer HeLa cells, two major HPV oncoproteins, E6 and E7, bind and inactivate p53 and pRb tumor suppressors for ubiquitin degradation, which impairs cell cycle control and induces apoptosis in response to DNA damage (15, 16). Despite the functional similarities between TAp73 and p53, the response against HPV E6 or HPV E7 by TAp73 is controversial. In some studies, TAp73 interacts with HPV E6 and becomes functionally inactivated, whereas in other studies, TAp73 does not interact with HPV E6 or HPV E7 and is followed by growth inhibition and apoptosis in cancer cells (37, 38). Therefore, it is important to determine whether paclitaxel could reversibly activate p53 inactivated by HPV E6 and HPV E7 oncogenes expressed in HeLa cells, and if paclitaxel could activate TAp73 to compensate for the function of inactivated p53. Our data show that paclitaxel induced TAp73α or TAp73β and its downstream targets, Bax and p21, but minimally induced p53 in a time-dependent manner in cervical cancer HeLa cells. Moreover, activated TAp73 enhanced p21 promoter activity and cytochrome c release from mitochondria, resulting in apoptosis. These effects were abrogated in cells transfected with inactive TAp73β siRNA, and further supported by findings that TAp73β was also induced by paclitaxel in p53-null SAOS2 cells and MCF-7 cells with depleted p53, correlating with the induction of Bax and p21 proteins and resulting in apoptosis. These results indicate that TAp73β functions to compensate for impaired p53 function in order to trigger p53-independent apoptosis of HeLa cells in response to paclitaxel. However, we should be very cautious in drawing conclusions comparing the effects of MCF-7 and SAOS cells because these cells have very divergent genetic backgrounds. For example, MCF-7 cells lack pRb. On the other hand, paclitaxel increased the levels of antiapoptotic protein Bcl-2 expression during acute time periods (up to 24 hours), but decreased significantly 24 hours later and was sustained for up to 48 hours. Afterwards, Bcl-xL expression decreased in a time-dependent manner. The acute overexpression of Bcl-2 might be involved in the resistance to paclitaxel chemotherapy as previous studies have reported that up-regulation of Bcl-2, nuclear factor κB, Akt, etc., may be potential signaling mediators contributing to paclitaxel resistance.

In response to stress, ATF3 interacts with p53 and enhances the stability of p53 by preventing ubiquitin degradation and augmenting p53-mediated apoptosis (25). However, contradictory evidence suggests that ATF3 inhibits doxorubicin-induced apoptosis through p53 down-regulation in cardiac myocytes (26). Therefore, we examined whether stress-inducible ATF3 activates or inactivates TAp73 function similar to p53 (Figs. 4 and 5). Our findings indicate that paclitaxel-induced TAp73β overexpression was correlated with the induction of ATF3 and up-regulation of p21 and Bax, resulting in apoptosis. These events were augmented by the overexpression of wild-type ATF3, which was almost completely abolished by transfection with inactive ATF3 siRNA or ATF3ΔC, a COOH-terminally deleted ATF3. Interestingly, our data also showed that activated ATF3 translocated into the nucleus, where p73 localizes, but its translocation was inhibited significantly by transfection with ATF3 siRNA or ATF3ΔC. Furthermore, ATF3ΔC over-expression significantly inhibits TAp73α and TAp73β expression induced by paclitaxel, suggesting that ATF3ΔC may play a dominant-negative role for the induction of TAp73, but whether ATF3ΔC induced the mislocalization of TAp73 out of the nucleus was not exact at present although most of the TAp73 expressed moved to the nuclear membrane (Fig. 5D).

Collectively, these results suggest that the COOH-terminal of ATF3 may be required for the translocation of ATF3 into the nucleus and ATF3 induced by paclitaxel might be an upstream regulator of TAp73. To confirm this, we examined whether TAp73β overexpression reversibly affects ATF3 expression and translocation into the nucleus induced by paclitaxel (Fig. 6). Although endogenous TAp73β expression was completely abolished by TAp73β siRNA, paclitaxel-induced ATF3 expression was unchanged by overexpressing TAp73β or inactive TAp73β (Fig. 6B), supporting our findings that ATF3 is an upstream regulator of TAp73β. Our data also showed that paclitaxel-induced apoptosis was strongly inhibited from TAp73β deletion by TAp73β siRNA (Fig. 6C), correlating with reduced ATF3 translocation into the nucleus (Fig. 6D). This suggests that TAp73β might play an important role in apoptosis induced by paclitaxel-activated ATF3, supporting results that show a reduction of Bax and p21 expression and apoptosis from overexpressing ATF3DC and its restoration by GFP-TAp73β overexpression, which was potentiated by cotreatment with paclitaxel (Fig. 7). Functional TAp73β might be required for ATF3-mediated apoptosis and might be an important downstream effector for ATF3-mediated apoptosis. Hypothetically, ATF3 and TAp73 activation may be regulated reciprocally or in a positive feedback loop to induce the apoptosis of tumor cells. On the other hand, although ATF3 does not enter the nuclei in TAp73β-depleted cells (Fig. 6D), the mechanism involved is still not clear. Recently, it was reported that TAp73 mediated the mitochondrial translocation of Bax proteins by transcriptionally inducing p53 up-regulated modulators of apoptosis involved in mitochondrial translocation (39). Thus, we cannot exclude the possibility that TAp73β may also possibly induce a certain gene involved in the nuclear translocation of ATF3. Future experiments will be necessary to specify the underlying mechanisms involved in this phenomenon.

It is well established that the stability of tumor suppressor p53 is mainly controlled by mdm2, which binds to p53 and catalyzes ubiquitination degradation (13, 14, 22). In contrast, although TAp73 also binds to mdm2, their interaction does not lead to the proteosomal degradation of TAp73 (40). Nevertheless, the binding of TAp73 to mdm2 results in a dramatic inhibition of TAp73 transactivating activity (41). Recently, it was reported that ATF3 interacted and prevented p53 ubiquitination and degradation, consequently augmenting p53 function (25). Our data suggest that endogenous ATF3 and TAp73 induced by paclitaxel interacts with each other in vivo includes a dose-dependent association between TAp73β and GST-ATF3, but not with GST-ATF3ΔC or control GST beads. Moreover, these results indicate that stabilization of TAp73β requires direct interaction with the COOH-terminal region of ATF3, and that the binding region of TAp73β that was required for ATF3 interactions was a p73β DNA-binding domain.
binds to the same binding sites with TAp73 on p21 or Bax the transcriptional activity of TAp73 supporting our previous findings showing that ATF3 enhanced significant increase in chromatin binding was higher in the increased strongly in paclitaxel-treated cells. Furthermore, a recruitment of the p21 and Bax promoters in control cells, which analysis data shows that TAp73 protein synthesis. Chromatin immunoprecipitation of ATF3 to increase the steady-state levels of TAp73 complex in cells transfected with ATF3( amino acids 54-310; Fig. 8). However, ΔNp73β has opposing effects on apoptosis mediated by TAp73 because TAp73β cotransfection, but not ΔNp73 cotransfection, highly induced apoptosis, which was potentiated by paclitaxel (Fig. 9D), although ATF3 interacts with the DNA-binding domain of TAp73β (Fig. 9C), suggesting that the active TAp73 isofrom was essential to induce apoptosis through paclitaxel-induced ATF3.

Therefore, we were interested in studying whether paclitaxel-induced ATF3 could also mediate TAp73β’s stability by preventing ubiquitination degradation similar to p53. Our investigation showed that TAp73β induction was enhanced slightly in MG132-pretreated cells and that overexpression of ATF3 alone increased TAp73β expression (Fig. 9C), which was further potentiated by MG132 pretreatment. These effects were not observed in ATF3(ΔC)-overexpressing cells. Furthermore, our results show that ATF3 enhances TAp73β’s stability by interfering with the ubiquitination of the latter (Fig. 9E and F). This event is achieved by direct interaction of the ATF3 with TAp73β proteins, and deletion of the COOH-terminal domain in ATF3, which interacts with p53, failed to stabilize TAp73β. Previously, we showed that the direct binding of p19Ras to TAp73 strongly enhanced tumor cell apoptosis by completely abolishing mdm2-mediated inhibition of TAp73 activity (34). Although the precise molecular mechanism behind the ATF3-dependent stabilization of TAp73 remains unknown, it is likely that ATF3 might also interact functionally with c-Abl, p300/ CBP, or IKK-α, which are well known to interact with TAp73 (42-44). Furthermore, it is possible that there is another ubiquitin ligase, which is different from mdm2, that does not lead to the proteosomal degradation of TAp73 and instead contributes to the efficiency of TAp73 degradation. Among them, other E3 ligases specific for TAp73, such as ITCH, cannot bind TAp73 in the presence of ATF3. Further studies are needed to investigate the exact molecular mechanisms involved.

TAp73 stabilization has generally been shown to reflect an increase in its transcriptional levels (44). However, our results showed that paclitaxel did not lead to an increase in TAp73β mRNA levels (Fig. 10A). We observed that ATF3 enhanced the stability of TAp73β proteins induced by paclitaxel because TAp73β decreased significantly by cycloheximide was attenuated by ATF3 overexpression (Fig. 10B). Following the stabilization of TAp73β, ATF3 also enhanced the transcription of p21 and Bax promoters downstream of TAp73β as well as significantly inhibiting the TAp73-p21 binding complex in cells transfected with ATF3(ΔC) or ATF3 siRNA (Fig. 10C-F). These results suggest that the effects of ATF3 on paclitaxel-induced reporter activities could be due to the ability of ATF3 to increase the steady-state levels of TAp73β via de novo protein synthesis. Chromatin immunoprecipitation analysis data shows that TAp73β and ATF3 were efficiently recruited to the p21 and Bax promoters in control cells, which increased strongly in paclitaxel-treated cells. Furthermore, a significant increase in chromatin binding was higher in the presence of ATF3 than in cells transfected with GFP alone, supporting our previous findings showing that ATF3 enhanced the transcriptional activity of TAp73β. Interestingly, ATF3 also binds to the same binding sites with TAp73 on p21 or Bax promoters, but this recruitment of ATF3 to TAp73 binding sites was specific because we did not observe any amplification in STAT5 immunoprecipitated chromatin. These data suggest that ATF3 might directly bind to TAp73 binding sites or play as a cofactor mediating p73 functional activity because we have used the primers for TAp73-responsive element within the p21 or Bax promoter. However, we cannot exclude the possibility that ATF3 might interact indirectly, via association with TAp73, with the same regions containing TAp73 sites in p21 and Bax promoters because TAp73 and ATF3 interact with each other, whereas TAp73 interacts with the transcriptional coactivator p300 and CBP to enhance its function. Future experiments will be required to clarify the underlying mechanistic details of ATF3 functional interaction.

Another possibility is that ATF3 regulates the modification of TAp73 such as methylation because several studies using tissues from patients with cervical cancer show that TAp73 is often found hypermethylated, which correlated with reduced expression of TAp73 (45). According to our results, depletion of ATF3 impaired tumor suppressor TAp73 activation in response to paclitaxel. These findings could implicate ATF3 deficiency with the development of cancers, as the absence of ATF3 has been associated with activating a Ras signaling cascade and initiating carcinogenesis (21). This possibility was supported by our preliminary findings that TAp73 and ATF3 were expressed minimally in cervical cancer tissues compared with normal patient samples, but was significantly increased in paclitaxel-treated cervical cancer tissues, thereby reducing tumorigenesis (data not shown). Although there are an extensive number of independent regulations of TAp73 and ATF3, and ATF3 has been shown to be involved in TAp73 up-regulation in this study, the exact molecular mechanisms involved in ATF3 regulation of TAp73 activation and apoptosis are still unclear. The functional consequences of the interaction between ATF3 by paclitaxel and TAp73 up-regulation are likely to be complex and will require further study.

Finally, we provide novel insights into the mechanisms involved in paclitaxel-induced apoptosis through TAp73 up-regulation. We are the first to report that ATF3 is a novel activator of TAp73 function in p53-inactivated cells. We have shown that ATF3 plays an important role in regulating the apoptosis of cervical cancer cells via up-regulating TAp73 expression, which compensates for the cytotoxicity of p53 depleted by HPV E6.

Materials and Methods

Cell Line and Reagents
Cervical cancer HeLa cells containing p53 proteins inactivated by HPV E6 or HPV E7 oncogenes, human osteosarcoma SAOS2 cells with p53-null alleles, and human breast cancer MCF-7 cells with wild-type p53 alleles were obtained from American Type Culture Collection and cultured in DMEM containing 10% fetal bovine serum, 2 mmol/L of glutamine, and 100 IU/mL of penicillin, and 100 μg/mL of streptomycin (Life Technologies). Anti-TAp73α, anti-TAp73β, anti-p53, anti-ATF3, anti-Bax, anti–Bel-2, anti–Bel-xL, anti-p21, anti–caspase-3, anti–poly(ADP-ribose) polymerase,
anti–cytochrome c, anti-GFP, and anti-flag were obtained from Cell Signaling Technology and Santa Cruz Biotechnology, Inc. N-acetyl-cysteine, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, penicillin, streptomycin, propidium iodide, and other common chemicals were from Sigma. Paclitaxel (2 mg/kg/mL) and taxol (6 mg/mL, paclitaxel in cremophor EL and dehydrated ethanol; Bristol-Myers-Squibb) were diluted with saline. To maximize the effects of ATF3 overexpression, the cells were incubated with a low concentration of paclitaxel (50 nmol/L).

**Plasmids**

Human wild-type ATF3 and mutated ATF3(ΔC; 1-100) with a COOH-terminal deletion cDNA expression vectors were generous gifts from Dr. T. Hai (Ohio State University, Columbus, OH). Human ATF3 and ATF3(ΔC) cDNA were amplified separately by PCR and cloned into pEGFP-C2 and pGEX-4T-1 vectors (Clontech). The pcDNA3-HA full-length construct was generously provided by Dr. Daniel Cauput (Sanofi Recherche, France). From pcDNA3-HA-TAp73β, GFP-73β was constructed by subcloning into the BamHI/XbaI restriction sites of pEGFP-C1, pGL3-p21WAF1-luc, and pGL3-Bax-luc.

**Transient Transfection**

HeLa and MCF-7 cells were grown in six-well culture plates at ~60% to 70% confluence. Transfections were done/did using Lipofectin reagent (Life Technologies) following the protocol recommended by the manufacturer. Briefly, after a 16-h incubation period, the medium was replaced with normal growth medium and cells were grown for an additional 24 h.

**Immunoblot and Coimmunoprecipitation**

Cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.5), 1% Nonidet P40, 150 mmol/L sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin] at 4°C, then vortexed and centrifuged at 16,000 rpm for 10 min at 4°C. The supernatant was mixed in Laemmli loading buffer, boiled for 4 min, and then subjected to SDS-PAGE. For immunocomplexes, whole-cell lysates (500 μg) were immunoprecipitated with 2 μg of antibody and immunoblotted.

**Reverse Transcription-PCR Analysis**

Total RNA was isolated from the cell lines using TRIzol reagent (Life Technologies) and reverse transcription-PCR analysis was done using a forward 5'-TCTGGAAACCAGACAGCACCCT-3' primer and a reverse 5'-GTGCTGACATGCTGGAAAGT-3' primer. Actin was used for internal normalization.

**GST Pull-Down Assay**

For in vitro binding assays, 500 μg of lysates were incubated with 3.0 μg of GST or fusion GST-GCK proteins coupled to glutathione Sepharose beads in 300 μL of lysis buffer overnight at 4°C with continuous rocking as described previously (46).

**Immunocytochemistry**

The cells were grown on poly-D-lysine–coated coverslips in six-well plates. After treatment, cells were washed with PBS, and then fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. The coverslips were soaked in a blocking solution (PBS containing 5% bovine serum albumin and 0.2% Triton X-100) for 30 min and incubated with anti-ATF and anti-p73 (1:300) overnight at 4°C and then with Alexa-488 antirabbit IgG antibody (1:400) for 30 min in the blocking solution before mounting. Fluorescence was analyzed by confocal microscopy (28).

**Electrophoretic Mobility Shift Assay**

DNA mobility shift assays were done in 20 μL volumes with 20 mmol/L of Tris-HCl (pH 7.9), 1.5% glycerol, 50 μg/mL bovine serum albumin, 1 mmol/L of DTT, 0.5 mmol/L of phenylmethylsulfonyl fluoride, 2 μg of poly(deoxyinosinic-deoxyctydidylic acid), 1 ng 32P-labeled probe (5'-AGCAGTGAAGCGAACACATGTCGACGC-3'), and 10 μg of nuclear extract. Reactions were incubated at 25°C for 20 min and subsequently analyzed by electrophoresis through nondenaturing stock polyacrylamide gels (6% or 10%) in 0.5× Tris-borate EDTA buffer containing 44.5 mmol/L Tris-HCl (pH 8.2), 44.5 mmol/L boric acid, and 1 mmol/L EDTA. After the gel was prerun at 100 V for 2 h, electrophoresis was done at 270 V for 2 h at 4°C. The gels were exposed to X-ray films using two intensifying screens –70°C.

**Luciferase Reporter Gene Assay**

We did transient transfections by using the Lipofectin reagent (Life Technologies) as described previously (28). Briefly, the cells were washed twice with Opti-MEM I reduced-serum medium (Life Technologies) and 0.5 μg of each plasmid DNA [pGL3-p21WAF1-luc, pGL3-Bax-luc, ATF3 cDNA, ATF3(ΔC), TAp73β], and ATF3 siRNA or TAp73 siRNA] per 35 mm plate was cotransfected with 0.5 μg of β-galactosidase vector (Promega) to allow for adjustments of transfection efficiencies. After transfection, the cells were incubated continuously in serum-reduced medium for 8 h, then changed to DMEM containing 2% serum. Cells were harvested after an additional 24-h period, lysed by freeze-thawing, and assayed for luciferase or β-galactosidase activities. We determined protein concentrations by using the Bio-Rad protein assay solution as described by the manufacturer, and bovine serum albumin as standard. We replicated all transfections at least thrice and found similar results.

**RNA Interference and Transfection**

ATF3 (sc-29757), p53 (sc-29435), and TAp73 siRNA (sc-36167, SC-37475, each 0.5 μg) were purchased from Santa Cruz Biotechnology, and HeLa cells were plated at 50% to 70% confluence and transfected with siRNA complexes or only with transfection reagents using LipofectAMINE 2000 (Invitrogen).

**Chromatin Immunoprecipitation**

The chromatin immunoprecipitation assays were done following a protocol provided by Upstate Biotechnology, Inc.
In brief, H1299 cells were transiently transfected with the expression plasmids for GFP or GFP-ATF3 (0.5 μg). Forty-eight hours after transfection, cells were cross-linked with 1% formaldehyde in medium for 15 min at 37°C. Cells were then washed with ice-cold PBS and resuspended in 200 μL of SDS-sample buffer containing protease inhibitor mixture. The suspension was sonicated 10 times for 30 s with a 1-min cooling period on ice between times and precleared with 20 μL of protein A-agarose beads blocked with sonicated salmon sperm DNA for 30 min at 4°C. The beads were removed and the chromatin solution was immunoprecipitated overnight with anti-ATF3, p73, and GFP monoclonal antibodies at 4°C, followed by incubation with protein A-agarose beads for an additional 1 h at 4°C. The immunocomplexes were eluted with 100 μL of elution buffer (1% SDS and 0.1 mol/L NaHCO3) and formaldehyde cross-links were reversed by heating at 65°C for 6 h. Proteinase K was added to the reaction mixtures and incubated at 45°C for 1 h. DNAs of the immunoprecipitates and control input DNAs were purified using a Qiaquick PCR purification kit (Qiagen Inc.) and then analyzed by regular PCR using the human p21WAF1 and Bax promoter-specific primers. The primer sequences were 5'-CACCCTTCACTTTCCCTTA-3' and 5'-GAGGCCCAAGGAAAATAG-3' for p21WAF1 and 5'-AAAGCTCAGAGGCCCATA-3' and 5'-AGGCTGAGACGGG-GTATGCT-3' for Bax.

In vitro and in vivo Ubiquitination Assays

GST-TAp73 and GST-ATF3 proteins were expressed in E. coli BL21, and purified with glutathione-agarose (Sigma). In vitro ubiquitination assays were done as described previously (25) with some modifications. Recombinant purified GST-TAp73 was preincubated with 250 ng of recombinant full-length or the deleted mutant ATF3 protein at 37°C for 1 h in a 30 μL reaction buffer 1 containing 50 mmol/L of Tris-HCl (pH 7.5), 5 mmol/L of MgCl2, 5 mmol/L of DTT, 4 mmol/L of ATP, 100 μmol/L of E1, 10 μmol/L of E2, 5 μg His-ubiquitin (E1, E2, and His-ubiquitin were obtained from Boston Biochem), and His-ubiquitinated proteins were isolated by incubating at 4°C for 1 h with 20 μL of nickel-nitrilotriacetate agarose (Qiagen) in a final volume of 200 μL in reaction buffer 2 containing 50 mmol/L of sodium phosphate (pH 7.9), 300 mmol/L of NaCl, 0.05% Tween 20, and 10 mmol/L of imidazole. After low-speed centrifugation (735 × g), the nickel-agarose beads containing His-ubiquitinated proteins were washed twice with 1 mL of reaction buffer 2. The nickel-bound proteins were eluted and then subjected to Western blotting for ubiquitinated p73 using anti-polyubiquitin antibody. In vivo ubiquitination assay was done as described previously (47) with some modifications. HeLa cells were cotransfected with the constant amount of HA-TAp73 (0.5 μg) and His-tagged ubiquitin (0.5 μg), together with the full-length ATF3 or the deleted mutant ATF3 (0.5 μg). Forty hours after transfection, cells were treated with 20 μmol/L of MG-132 for 6 h before being harvested. His-tagged ubiquitin-containing protein complexes were pulled down with nickel-agarose beads (Qiagen), and subsequently resolved by 10% SDS-PAGE, followed by immunoblotting with anti-HA antibody.

Statistical Analysis

For comparing values obtained in three or more groups, one-factor ANOVA was used, followed by Tukey’s post hoc test, and P < 0.05 was taken to imply statistical significance.

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Role of Activating Transcription Factor 3 on TAp73 Stability and Apoptosis in Paclitaxel-Treated Cervical Cancer Cells

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