Depletion of *TFAP2E* attenuates adriamycin-mediated apoptosis in human neuroblastoma cells

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Abstract. Neuroblastoma is a childhood malignancy originating from the sympathetic nervous system and accounts for approximately 15% of all pediatric cancer-related deaths. To newly identify gene(s) implicated in the progression of neuroblastoma, we investigated aberrantly methylated genomic regions in mouse skin tumors. Previously, we reported that *TFAP2E*, a member of activator protein-2 transcription factor family, is highly methylated within its intron and its expression is strongly suppressed in mouse skin tumors compared with the normal skin. In the present study, we analyzed public data of neuroblastoma patients and found that lower expression levels of *TFAP2E* are significantly associated with a shorter survival. The data indicate that *TFAP2E* acts as a tumor suppressor of neuroblastoma. Consistent with this notion, *TFAP2E*-depleted neuroblastoma NB1 and NB9 cells displayed a substantial resistance to DNA damage arising from adriamycin (ADR), cisplatin (CDDP) and ionizing radiation (IR). Silencing of *TFAP2E* caused a reduced ADR-induced proteolytic cleavage of caspase-3 and PARP. Of note, compared with the untransfected control cells, ADR-mediated stimulation of CDK inhibitor $p21^{WAF1}$ was markedly upregulated in *TFAP2E*-knocked down cells. Therefore, our present findings strongly suggest that *TFAP2E* has a pivotal role in the regulation of DNA damage response in NB cells through the induction of $p21^{WAF1}$.

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Introduction

Neuroblastoma (NB) is an embryonal tumor originating from the sympathetic nervous system including the adrenal medulla and paravertebral nerve trunk. NB is the most common extracranial solid tumor in children and accounts for approximately 15% of all pediatric cancer deaths (1). NB displays a wide variety of biological and clinical features with a heterogeneous prognosis, ranging from spontaneous regression to rapid tumor progression and death. For example, NB diagnosed at 12 months of age or younger typically regresses and/or spontaneously differentiates, whereas NB in patients older than 12 months typically become aggressive and are associated with an unfavorable prognosis. *MYCN* gene amplification is often observed in advanced NB. *MYCN* encodes a sequence-specific transcription factor and transactivates its target genes implicated in crucial cellular processes such as cell cycle progression, proliferation, apoptosis, differentiation and metabolism (2). In addition to *MYCN* gene amplification, a growing body of evidence indicates that gain of chromosome 17q (3) and deletion of the distal part of chromosome 1p are tightly associated with poor prognosis in patients with NB (4). Unfortunately, despite multimodal therapy such as chemotherapy, surgical tumor removal, radiation therapy and hematopoietic stem cell transplantation, the 5-year survival rate of patients with high-risk NB remains less than 40% (1). Consequently, there is an urgent clinical need to clarify the precise molecular mechanisms underlying advanced NB and develop novel treatment strategies.

To identify novel cancer-related genes, we have screened genome areas based on aberrant methylation status in mouse skin tumors compared with the normal skin. During the

analysis of mouse skin cancers induced by a 2-stage carcinogenesis protocol using 7.12-dimethylbenz(a)anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), several skin tumor-specific differentially-methylated regions (ST-DMRs) and genes differentially expressed in tumor tissues compared with the normal tissues were identified. Subsequent studies revealed that some of the ST-DMRs such as zygote arrest 1 (*ZAR1*), are aberrantly methylated and genes within these loci are expressed in numerous types of human tumor tissues including NB (5-8).

TFAP2E, located within one of the ST-DMRs, has been shown to be highly methylated in SCC tissues. In our recent study (9), we demonstrated that the expression levels of *TFAP2E* are significantly lower in SCC tissues than in the normal skin. *TFAP2E* encodes a nuclear transcription factor activator protein-2 (AP-2) epsilon and is largely expressed in normal skin tissues (10). Intriguingly, aberrant methylation of *TFAP2E* genomic locus and/or its expression is associated with prognostic outcome or drug resistance in certain human tumors. Indeed, hypermethylation and lower expression levels of *TFAP2E* have been shown to correlate with resistance to fluorouracil in patients with colon cancer (11). In gastric cancer, hypermethylation and lower expression levels of *TFAP2E* were much more frequently observed in tumors with lower differentiation grades (12). In addition, hypermethylation of *TFAP2E* has been frequently detected in genomic DNA prepared from urine samples of prostate cancer patients relative to that of urine samples of normal males (13).

In the present study, we focused on *TFAP2E* and extended the findings of our previous study of NB. We demonstrated that *TFAP2E* plays a vital role in the regulation of DNA damage responses in NB.

Materials and methods

Cell lines and culture conditions. Human neuroblastoma-derived NB1 and NB9 cells were obtained from RIKEN Cell Bank (Ibaraki, Japan). Cells were maintained in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% (NB1) or 15% (NB9) heat-inactivated fetal bovine serum (FBS; Nichirei Bioscience, Tokyo, Japan), 100 IU/ml of penicillin (Life Technologies, Carlsbad, CA, USA) and 100 μl/ml of streptomycin (Life Technologies).

Cell viability. NB1 cells were seeded in 24-well plates at a density of $2x10^4$ cells/well and allowed to attach. Cells were then transfected with control siRNA or with *TFAP2E* siRNA using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were treated with adriamycin (ADR), cisplatin (CDDP), hydrogen peroxide (H_2O_2) or irradiated with ionizing radiation (IR) (X-ray linear accelerator MBR-1520R-3; Hitachi Medical, Tokyo, Japan). Twenty-four hours post-treatment, number of viable cells was determined using a Millipore Sceptor.

FACS analysis. For the analysis of cell cycle distribution, floating and attached cells were collected 24 h after ADR exposure. Cells were washed in phosphate-buffered saline (PBS) and then fixed in 75% ice-cold ethanol for 2 h. After washing in PBS, cells were incubated with 0.1% FBS, 25 μg/ml of propidium iodide and 200 μg/ml of RNase A in PBS for 15 min at room temperature in the dark and subsequently subjected to the flow cytometric analysis (FACSCallibur). The analysis was performed 3 times and the flow cytometry graphs were created by calculating the total data.

The percentage of apoptotic cells was determined 24 h after the ADR exposure by using Annexin V-FITC apoptosis detection kit (BioVision, Inc., Milpitas, CA, USA) according to the instructions of the manufacturer. Fluorescence was detected by flow cytometry. The analysis was conducted 3 times and the average percentages of apoptotic cells were calculated.

Immunoblotting. Cells were lysed in RIPA buffer containing a protease inhibitor cocktail (Nacalai Tesque). The protein concentration of lysates was measured using Bio-Rad DC kits (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates (20 μg of protein) were separated by 4-12% SDS-polyacrylamide gel electrophoresis and then electro-transferred onto Immobilon-P membrane (Millipore). Membranes were blocked with Blocking One (Nacalai Tesque) overnight at 4˚C and incubated with polyclonal anti-TFAP2E (ProSci, Inc., Poway, CA, USA), polyclonal anti-caspase 3 (Cell Signaling Technology, Beverly, MA, USA), polyclonal anti-PARP (Cell Signaling Technology), monoclonal anti-p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-phospho-p53 at Ser-15 (Cell Signaling Technology), polyclonal anti-$p21^{WAF1}$ (H-164; Santa Cruz Biotechnologies), monoclonal anti-γ-H2AX (2F3; BioLegend, San Diego, CA, USA) or with polyclonal anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA) at 4˚C. Twenty-four hours after incubation, membranes were washed in PBS containing 0.1% Tween-20 (PBS-T) followed by incubation with horseradish peroxidase-conjugated secondary antibody (GE Healthcare Life Sciences, Buckinghamshire, UK) for 1 h at room temperature. The membrane was washed extensively in PBS-T and then treated with Chemi-Lumi One Super (Nacalai Tesque) to visualize immunoreactive signals using ImageQant LAS 4000 (Fujifilm Corp., Tokyo, Japan).

Quantitative real-time PCR (qPCR). Total RNA was isolated from cells using RNeasy mini kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For cDNA synthesis, 500 ng of total RNA were reverse-transcribed using iScript cDNA synthesis system (Bio-Rad Laboratories). qPCR was performed using a SYBR Premix Ex Taq™ system (Takara, Shiga, Japan) according to the manufacturer's recommendations. Relative gene expression was expressed as relative fold-change in mRNA levels compared with reference cDNA. Primer sets used for qPCR-based amplification were as follows: *TFAP2E*, 5'-cggttacgtctgtgagacgga-3' (sense) and 5'-tgcaaactccttgcagatctgc-3' (antisense); *CDKN1A* (encoding $p21^{WAF1}$), 5'-gcagaccagcatgacagattt-3' (sense) and 5'-ggattagg gcttcctcttgga-3' (antisense); and 18S rRNA, 5'-ggccctgtaattgga atgagtc-3' (sense) and 5'-ccaagatccaactacgagctt-3' (antisense). 18S rRNA was used as a reference gene.

Statistical analysis. Statistical analyses were performed using Student's t-test. Data were presented as means ± SD from at least three independent experiments. $P<0.05$ was considered statistically significant.

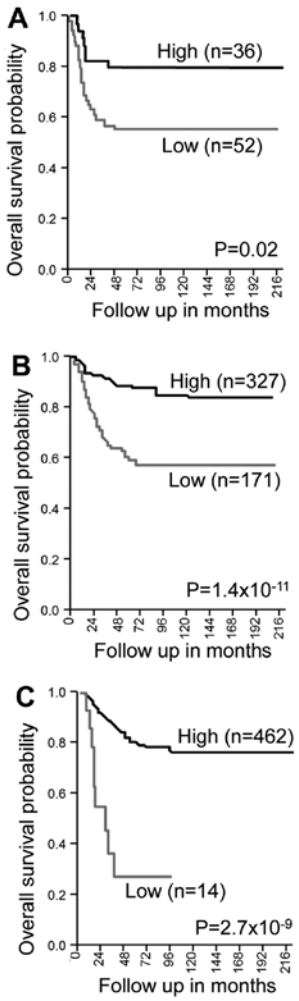

Figure 1. Lower expression level of TFAP2E is associated with poor prognosis of the patients with neuroblastoma (NB). (A-C) Kaplan-Meier survival analysis based on 3 independent public microarray data sets.

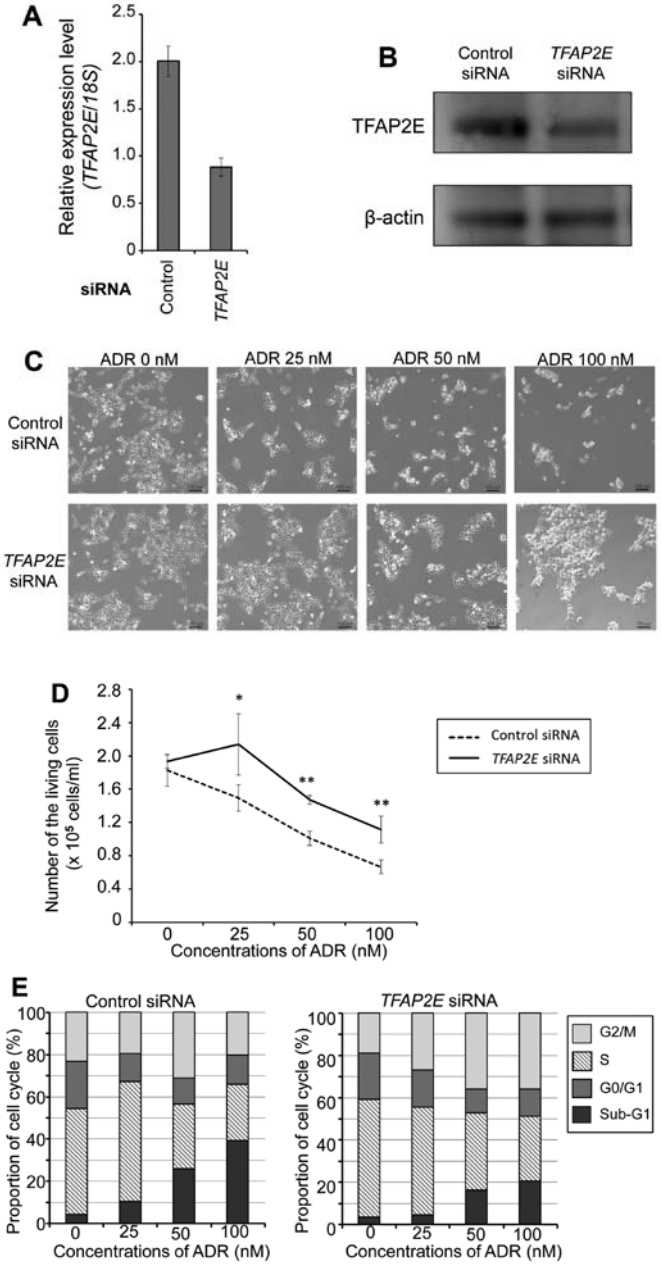

Figure 2. Knockdown of *TFAP2E* suppresses ADR-mediated cell death of NB-derived NB1 cells. (A and B) siRNA-mediated silencing of *TFAPT2E*. NB1 cells were transfected with *TFAPT2E* siRNA or with control siRNA. Forty-eight hours after transfection, total RNA and cell lysates were prepared and analyzed by real-time PCR (A) and immunoblotting (B), respectively. (C) Phase-contrast micrographs. Cells were transfected as in (A) and cultured for 24 h, and then treated with the increasing amounts of ADR. Twenty-four hours after treatment, representative pictures were taken. (D and E) Effects of *TFAPT2E* depletion on NB1 cells in response to ADR. Cells were transfected as in (A), and exposed to the indicated concentrations of ADR. Twenty-four hours after treatment, number of viable cells was scored by Millipore Sceptor (D) or floating and adherent cells were harvested, stained with propidium iodide, and their cell cycle distributions were analyzed by FACS (E).

Results

A lower expression level of TFAP2E is closely related to a poor prognosis of the patients with neuroblastoma (NB). To examine the clinical significance of TFAP2E in the genesis and/or progression of NB, we performed Kaplan-Meier survival analysis based on three independent public microarray data sets. As shown in Fig. 1, a lower expression level of *TFAP2E* was closely associated with an unfavorable clinical outcome of patients with NB. These results indicate that *TFAP2E* might have a role in the suppression of malignant progression of NB such as the acquisition of anticancer drug resistance.

siRNA-mediated knockdown of TFAP2E attenuates ADR-dependent cell death of NB-derived NB1 cells. To determine whether *TFAP2E* could affect anticancer drug sensitivity of NB cells, we performed siRNA-mediated knockdown of *TFAP2E* in NB-derived NB1 cells. Forty-eight hours after transfection, total RNA and cell lysates were prepared and analyzed to determine the expression level of *TFAP2E* by real-time PCR and immunoblotting, respectively. As expected, *TFAP2E* expression was significantly reduced in *TFAP2E*-depleted cells at both mRNA and protein levels (Fig. 2A and B). Under the same experimental conditions, non-depleted and *TFAP2E*-depleted cells were treated with the indicated concentrations of the anticancer drug ADR. Twenty-four hours after treatment, representative pictures were taken. As shown in Fig. 2C, *TFAP2E*-knocked down cells became much more resistant to ADR compared with non-depleted cells exposed to ADR. Consistent with these

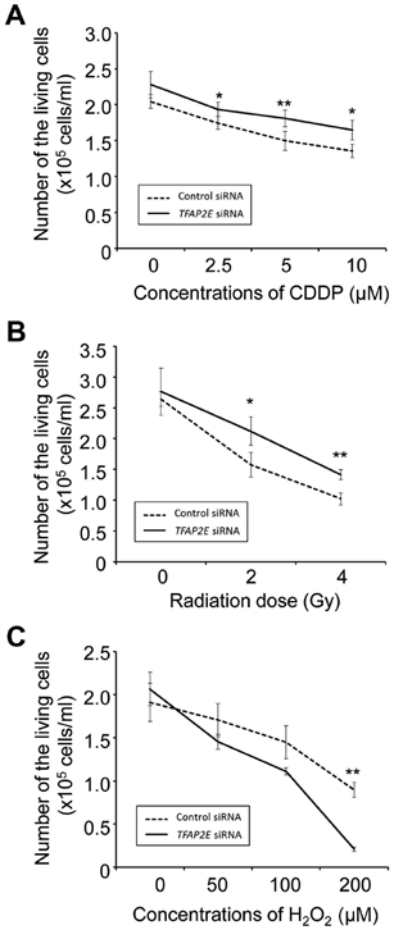

Figure 3. Knockdown of *TFAP2E* causes a development of resistance to DNA damage arising from CDDP and ionizing radiation but not from H_2O_2. NB1 cells were transfected as in Fig. 1, and exposed to the indicated doses of CDDP (A), X-ray (B) or H_2O_2 (C). Twenty-four hours after treatment, number of viable cells was measured using Millipore Sceptor. Data are shown as mean ± SD. *P<0.05, **P<0.01.

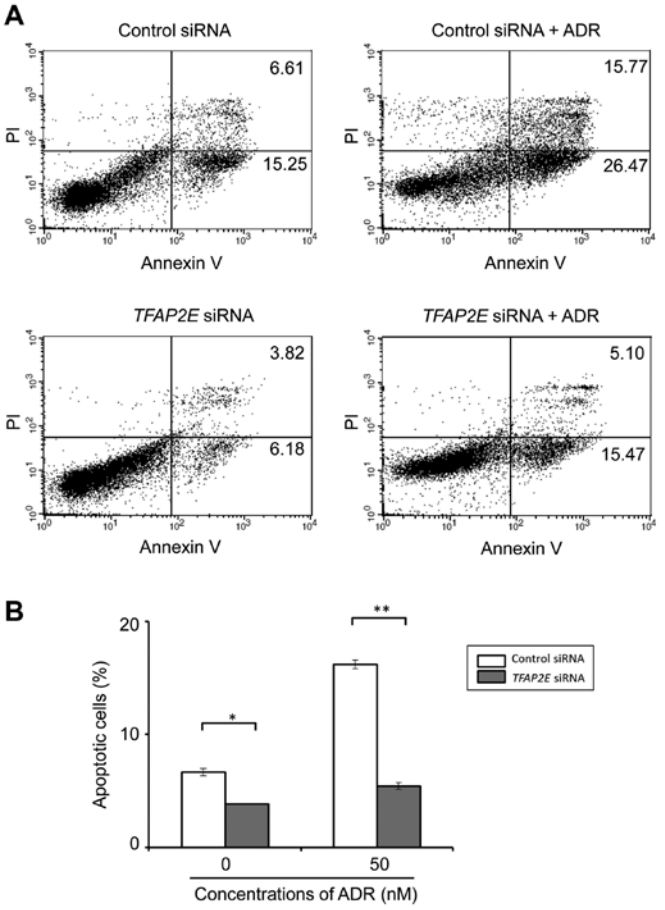

Figure 4. Knockdown of *TFAP2E* suppresses ADR-mediated apoptosis of NB1 cells. Cells were transfected with siRNA for 24 h, followed by the treatment with 50 nM of ADR. Twenty-four hours after ADR addition, both dead cells and living cells were harvested and analyzed by FACS to detect the cells stained with Annexin V and propidium iodide. (A) Representative quadrants, and (B) percentages of late apoptotic cells (upper right quadrant) are shown (mean ± SD). *P<1x10^{-3}, **P<1x10^{-5}.

observations, silencing of *TFAP2E* significantly increased number of viable cells as compared to the control transfection (Fig. 2D). *TFAP2E* knockdown cells also became resistant to CDDP and IR but not to H_2O_2 (Fig. 3).

FACS analysis demonstrated that the relative number of cells with sub-G1 DNA content markedly declined by *TFAP2E* depletion in the presence of ADR (Fig. 2E). On the other hand, the cell population in G2/M phase was increased by *TFAP2E* depletion depending on the dose of ADR. Moreover, FACS analysis after Annexin V/PI staining revealed that number of apoptotic cells in response to 50 nM of ADR was markedly suppressed in *TFAP2E*-knocked down cells compared with the control cells (Fig. 4). Late apoptotic cell population (Annexin V, PI-double positive cells) of control and knocked down cells in the absence of ADR were 6.65±0.31 and 3.82±0.03%, respectively. Upon ADR treatment, number of control cells with late apoptotic property was markedly increased (16.19±0.38%), whereas *TFAP2E* depletion had a negligible effect on ADR-mediated apoptosis (5.42±0.302%) (Fig. 4). Similar results were also obtained in NB-derived NB9 cells (data not shown). Together, these findings indicate that *TFAP2E* plays a vital role in the regulation of DNA damage response of NB cells.

ADR-dependent induction of p21^{WAF1} is further augmented in TFAP2E-knocked down NB1 cells. To gain insight into understanding the molecular mechanisms behind *TFAP2E* depletion-mediated ADR resistance, we sought to examine the tumor suppressor p53-dependent cell death pathway under our experimental conditions. According to the IARC TP53 database (http://p53.iarc.fr/CellLines.aspx), NB1 cells carry wild-type *p53*. NB1 cells were transfected with control siRNA or with siRNA against *TFAP2E* and then incubated in the presence of ADR. At the indicated time-points after ADR exposure, the cell lysates were prepared and subjected to immunoblotting. As shown in Fig. 5, ADR-induced accumulation and phosphorylation of p53 at Ser-15 were basically unchanged regardless of *TFAP2E* depletion. In accordance with the results shown in Fig. 3, ADR-mediated proteolytic cleavage of caspase-3 and its substrate, PARP, was substantially downregulated in *TFAP2E*-knocked down cells compared with that in the non-depleted cells. Of note, an obvious reduction of γ-H2AX, which has been considered to be a reliable DNA damage marker, was detectable in *TFAP2E*-depleted cells exposed to ADR. Furthermore, silencing of *TFAP2E* stimulated ADR-dependent induction of cell cycle-related p21^{WAF1}. Real-time PCR analysis

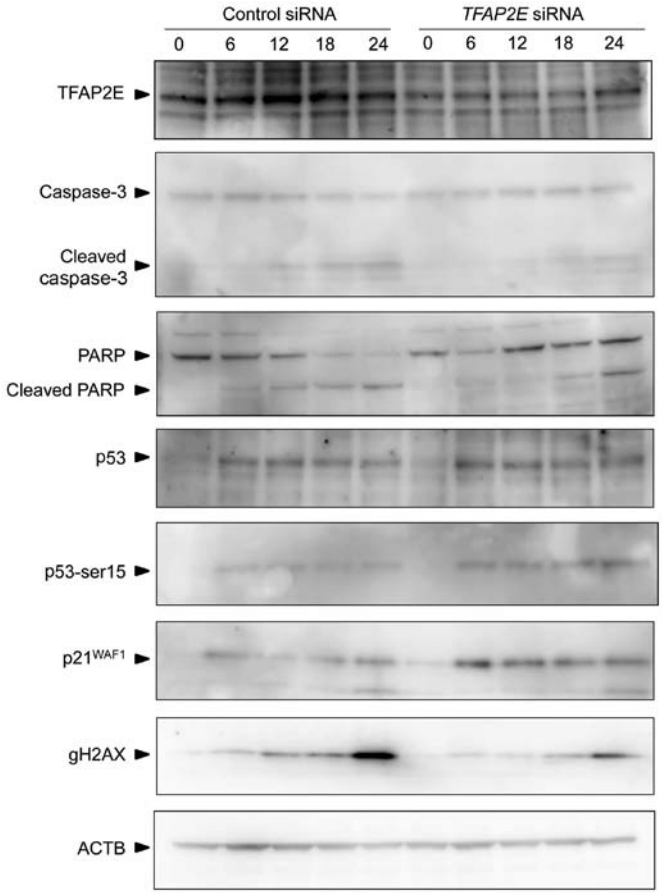

Figure 5. Depletion of *TFAP2E* attenuates ADR-mediated activation of caspase-3 in association with upregulation of cell cycle-related $p21^{WAF1}$. NB1 cells were transfected as in Fig. 1, and incubated with 50 nM of ADR. At the indicated time periods after treatment, cell lysates were prepared and subjected to immunoblotting. β-actin was used as a loading control.

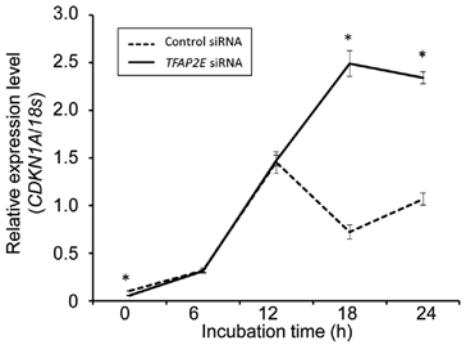

Figure 6. Silencing of *TFAP2E* augments ADR-mediated induction of $p21^{WAF1}$ at mRNA level. NB1 cells were transfected as in Fig. 1 and treated with 50 nM of ADR. At the indicated time-points after treatment, total RNA was extracted and analyzed for the expression of $p21^{WAF1}$ by real-time PCR.

revealed that the expression of $p21^{WAF1}$ is regulated at mRNA level (Fig. 6).

Discussion

In the present study, we demonstrated that depletion of *TFAP2E* attenuates ADR-dependent apoptosis but promotes mitotic arrest in NB cells. In addition to ADR, *TFAP2E* gene silencing prohibited apoptosis induced by the other DNA damaging agents such as CDDP and IR, suggesting that TFAP2E might act as a tumor suppressor of NB.

TFAP2E belongs to the AP-2 transcription factor family, which consists of five members including TFAP2A, TFAP2B, TFAP2C, TFAP2D and TFAP2E. All of AP-2 family proteins share a highly conserved structure such as a helix-span-helix motif at the carboxyl terminus and act as transcription factors. According to the previous studies (14), AP-2 proteins affect the transcription of numerous number of genes involved in the crucial biological processes including cell proliferation and differentiation. The possible functional roles of AP-2 family members in carcinogenesis vary among individual proteins. For example, reduced expression levels of TFAP2A are closely associated with unfavorable phenotypes of many cancers such as gastric adenocarcinoma, prostate cancer and melanoma (15-17). Overexpression of TFAP2B has been shown to contribute to poor prognosis of lung adenocarcinoma (18). In contrast, low expression level of TFAP2B was related to unfavorable prognostic markers in neuroblastoma (19). Additionally, elevated expression of TFAP2C has been found in testicular carcinoma, advanced-stage of ovarian carcinoma and advanced grade of breast cancer (20-22). Collectively, it is likely that TFAP2A is a potent tumor suppressor, whereas TFAP2C has a tumor-promoting function.

Recently, it has been reported that hypermethylation of *TFAP2E* genome locus and lower expression of its transcript are associated with unfavorable outcome and non-responsiveness to chemotherapy in colorectal cancer and gastric cancer (11,12). Analysis of the public database revealed that a lower expression of *TFAP2E* is also related to a shorter survival of neuroblastoma patients. Although these findings indicate that *TFAP2E* is a potent tumor suppressor, it remains elusive how TFAP2E could regulate the expression of cancer-related genes. It has been described that TFAP2E exerts its tumor suppressive function through the downregulation of Dickkopf WNT signaling pathway inhibitor 4 (DKK4) in CRC (11). However, we were unable to detect *DKK4* expression in NB1 cells under our experimental conditions (data not shown).

According to our results, ADR-dependent stimulation of cell cycle-related $p21^{WAF1}$ was further augmented by *TFAP2E* depletion. Although it is well known that $p21^{WAF1}$ inhibits CDK activity of cyclin A-CDK2 and cyclin E-CDK2 complexes and thereby functions as a tumor suppressor, $p21^{WAF1}$ also has an anti-apoptotic potential (23). Thus, it is possible that *TFAP2E* depletion-mediated upregulation of $p21^{WAF1}$ prohibits ADR-dependent apoptosis and induces mitotic arrest of NB cells. Since $p21^{WAF1}$ is one of p53-target gene products, it is suggestive that TFAP2E might participate in p53-dependent DNA damage response of NB cells. However, it was not the case. Firstly, *TFAP2E* gene silencing had an undetectable effects on ADR-mediated induction of p53 and accumulation of phosphorylated p53 at Ser-15. Secondary, the expression level of p53-target 14-3-3σ implicated in mitotic arrest (24) was unaffected by *TFAP2E* depletion (data not shown). Thirdly, the complex formation between *TFAP2E* and p53 was not detectable in the presence or absence of ADR as examined by co-immunoprecipitation experiments (data not shown). Therefore, it is indicative that *TFAP2E* depletion-dependent mitotic arrest is regulated in a p53-independent manner.

In conclusion, our present findings suggest that TFAP2E acts as a tumor suppressor and potentiates proper DNA damage response in NB.

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