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Transcription factor Sox4 is required for PUMA-mediated apoptosis induced by histone deacetylase inhibitor, TSA

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ABSTRACT

PUMA is a crucial regulator of apoptotic cell death mediated by p53-dependent and p53-independent mechanisms. In many cancer cells, PUMA expression is induced in response to DNA-damaging reagent in a p53-dependent manner. However, few studies have investigated transcription factors that lead to the induction of PUMA expression via p53-independent apoptotic signaling. In this study, we found that the transcription factor Sox4 increased PUMA expression in response to trichostatin A (TSA), a histone deacetylase inhibitor in the p53-null human lung cancer cell line H1299. Ectopic expression of Sox4 led to the induction of PUMA expression at the mRNA and protein levels, and TSA-mediated up-regulation of *PUMA* transcription was repressed by the knockdown of Sox4. Using luciferase assays and chromatin immunoprecipitation, we also determined that Sox4 recruits p300 on the *PUMA* promoter region and increases *PUMA* gene expression in response to TSA treatment. Taken together, these results suggest that Sox4 is required for p53-independent apoptotic cell death mediated by PUMA induction via TSA treatment.

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1. Introduction

PUMA is a member of the BH3-only Bcl-2 family, and mediates apoptotic cell death through mitochondria dysfunction-mediated activation of caspases [1–3]. In many human cancer cell types, expression of PUMA is barely detected. However, it is rapidly induced in response to a variety of stress leading to apoptotic cell death [1,2]. Especially, DNA-damaging agents can induce PUMA expression via the function of tumor suppressor p53. Genotoxic stress leads to p53 recruitment to the consensus sequences at the *PUMA* promoter [4,5]. p53 and the p53 binding elements in the *PUMA* promoter are necessary for the induction of PUMA by DNA damaging stimuli [5]. In addition to genotoxic stress, histone deacetylase (HDAC) inhibitor induces apoptotic cell death in gastric cancer cells by enhancing p53 binding to the *PUMA* promoter, whereas HDAC3 is dissociated from the *PUMA* promoter [6]. These reports supported the contention that PUMA induction by various cellular stimuli occurs in a p53-dependent fashion. Recent studies have revealed that PUMA expression is also induced by p53-independent mechanisms in response to several stimuli. Transcription factor p73, which is a p53 homologue, can regulate PUMA expression in p53-deficient cancer cells by binding to p53 consensus sites in the *PUMA* promoter in a serum starved condition [7,8]. The transcriptional level of *PUMA* is controlled by the forkhead family member FOXO3a in $p53^{-/-}$ mouse embryonic fibroblast cell lines in response to cytokine withdrawal [9]. In this regard, the finding of a stimulus-mediated novel molecular mechanism that can induce PUMA expression might be important in the understanding of p53-independent apoptotic cell death. However, these correlations between PUMA-inducible stimuli and transcriptional modulators remain to be elucidated.

Sox4, a transcription factor of the sex-determining gene on the Y chromosome (SRY), is characterized by a highly conserved sequence in the high-mobility group (HMG) DNA-binding domain (DBD) [10]. Sox4 plays important roles in many developmental processes, including embryonic cardiac, thymocyte and nervous system development [11–13]. In addition, Sox4 is also involved in tumorigenesis. Especially, Sox4 has dual-functions – oncogenic or tumor-suppressive – in a number of tumors. For example, the expression of Sox4 is highly increased in carcinomas of the lung, breast and colon [14–16]. In contrast, Sox4 functions as a tumor suppressor in the bladder and hepatocarcinoma [17,18]. Moreover, Sox4 displays pro-apoptotic effects when Sox4 is overexpressed in HeLa and HEK293 cells [18–20]. Furthermore, many genes related to the inhibition of cancer cell viability have been identified as putative Sox4 target genes by microarray analysis [21]. In addition,

Abbreviations: TSA, trichostatin A; DBD, DNA binding domain; TAD, transactivation domain.

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a recent report described that increased Sox4 expression by DNA-damage acts as a co-activator of p53 in colon and lung cancer, implicating Sox4 as a tumor suppressor [22]. However, the precise roles of Sox4-mediated inhibition of cancer progression remain unclear.

In this report, we demonstrate that trichostatin A (TSA), a HDAC inhibitor, induces PUMA expression in the p53-deficient human lung cell line, H1299, by recruitment of Sox4 and Sox4-dependent p300 co-activator in the *PUMA* promoter. These findings suggest that Sox4 is required for TSA-mediated apoptotic cell death in p53-deficient cancer cells.

2. Materials and methods

2.1. Cell culture and transfection

H1299 cells were obtained from the ATCC (American type culture collection; Manassas, VA). H1299 cells were maintained RPMI 1640 medium. This medium was supplemented with 10% fetal bovine serum (Invitrogen) and penicillin–streptomycin (50 units/ml). Transient transfection was performed by Lipofectamine 2000 (Invitrogen) with different plasmid DNA according to the manufacturer's instructions.

2.2. Plasmid constructs

Details for plasmid constructions are described in the Supplemental material.

2.3. Western blotting

Western blot analysis was performed as described previously [24]. The monoclonal antibody against GFP (11814460001) was purchased from Roche Diagnostics (Indianapolis, IN, USA) and PUMA (1652-S) was purchased from Epitomics Inc. (Burlingame, CA). PARP1 (51-6639GR) was purchased from BD Biosciences (Franklin. 162. Lakes, NJ). Polyclonal antibodies against β-tubulin (sc-9104) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Western blotting was visualized by chemiluminescence using an ECL system (Santa Cruz Biotechnology Inc.).

2.4. RNA preparation and quantitative real-time PCR

Total RNA extraction and quantitative real-time PCR was performed as described previously [25]. The expression levels of human *PUMA* and *18SrRNA* in H1299 cells were measured by qRT-PCR with the following specific primers: *PUMA*, forward, 5′-GAC GAC CTC AAC GCA CAG TA-3′; reverse, 5′-CAC CTA ATT GGG CTC CAT CT-3′; *18SrRNA*, forward, 5′-GAT TAA GCC ATG CAT GTC TA-3′; reverse, 5′-GTC GGG CGC CGG CGG CTT TG-3′.

2.5. TUNEL assay

H1299 cells were incubated for 24 h with or without 1 μM TSA and the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) assay was performed with the use of the DeadEnd™ Fluorometric TUNEL System (Promega), according to the manufacturer's instructions.

2.6. Cell viability assays

Cell viability was measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously [24]. For investigation of apoptosis by nuclear staining, transfected H1299 cells were washed with phosphate-buffered saline (PBS) and fixed for 10 min in 4% paraformaldehyde. The cells were permeabilized with 0.3% Triton X-100 for 15 min. After incubation in blocking solution for 30 min, cells were incubated in 300 nM DAPI in PBS for 2 min at room temperature. Chromatin condensation in cells was visualized using a Carl Zeiss LSM-510 META laser scanning microscope (Oberkochen, Germany). To evaluate cell death rate, TSA-treated H1299 cells were stained by trypan blue solution (0.4%) and the number of apoptotic cells was counted and presented as a percentage versus the total population of control cells.

2.7. Luciferase assay

H1299 cells were cultured in 60 mm dishes and were transfected using Lipofectamine 2000, with the luciferase reporter constructs (0.1 μg), pCMV-β-galactosidase and GFP-Sox4 or siRNA-Sox4. The cells were lysed in reporter lysis buffer 48 h after transfection (Promega). Cell lysates were then analyzed with the luciferase reporter assay system, using a glomax luminometer (Promega). Luciferase activities were normalized on the basis of the β-galactosidase activity of the cotransfected vector. All transfection experiments were repeated independently at least three times.

2.8. Polyclonal antibody production of Sox4

Details of Sox4 antibody production are described in the Supplemental material.

2.9. Chromatin immunoprecipitation (ChIP)

A ChIP assay was conducted following the protocol provide by Upstate Biotechnology (Lake Placid, NY, USA), and as described previously [25]. The chromatin fragments from H1299 cells were immunoprecipitated with antibodies against Sox4, p300, acetylated histone H3 and H4, and HDAC1. DNA of the immunoprecipitates and control input DNA was analyzed by quantitative real-time PCR with the *PUMA* promoter-specific primers: forward, 5′-TCT CCA AAC CCC GCG AGG GAC-3′; reverse, 5′-ACC CCT GGG GTC GAC CCT CTT-3′.

2.10. Statistical analysis

Statistical analysis of variances between two different experimental groups was conducted with Tukey's post hoc comparison test using SPSS, version 12 (SPPS Inc., Chicago, IL, USA). All experiments were repeated at least three times. The levels were considered significant at $p < 0.05$ (*) and very significant at $p < 0.01$ (**), obviously significant at $p < 0.001$ (***), or not significant (*n.s.*).

3. Results

3.1. TSA increases H1299 lung carcinoma cell death through the enhancement of PUMA mRNA and protein expression

Although the HDAC inhibitor TSA modulates the transcriptional activity of p53, leading to cell cycle arrest or apoptotic cell death, the p53-independent molecular mechanisms by HDAC inhibitors are currently unclear. To test the TSA-mediated cell death through the p53-independent pathway, H1299 cells were treated for three days either in 1 μM TSA or in the same volume of ethanol, as a control, and cell viability and death were determined by MTT assay and trypan blue staining. As shown in Fig. 1A, the viability of H1299 cells was decreased (upper panel) and cell death was significantly increased (lower panel) twenty-four hours after TSA treatment. Furthermore, TUNEL positive cells, apoptotic cell death markers, were detected in TSA-treated H1299 cells (Fig. 1B). It

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