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Molecules and Cells

Inhibition of Histone Deacetylase 10 Induces Thioredoxin-Interacting Protein and Causes Accumulation of Reactive Oxygen Species in SNU-620 Human Gastric Cancer Cells

Ju-Hee Lee[1], Eun-Goo Jeong[1], Moon-Chang Choi[1], Sung-Hak Kim[1], Jung-Hyun Park[1], Sang-Hyun Song[1], Jinah Park[1], Yung-Jue Bang[1,2,3], and Tae-You Kim[1,2,3,*]

Histone deacetylase (HDAC)10, a novel class IIb histone deacetylase, is the most similar to HDAC6, since both contain a unique second catalytic domain. Unlike HDAC6, which is located in the cytoplasm, HDAC10 resides in both the nucleus and cytoplasm. The transcriptional targets of HDAC10 that are associated with HDAC10 gene regulation have not been identified. In the present study, we found that knockdown of HDAC10 significantly increased the mRNA expression levels of thioredoxin-interacting protein (TXNIP) in SNU-620 human gastric cancer cells; whereas inhibition of HDAC1, HDAC2, and HDAC6 did not affect TXNIP expression. TXNIP is the endogenous inhibitor of thioredoxin (TRX), which acts as a cellular antioxidant. Real-time PCR and immunoblot analysis confirmed that inhibition of HDAC10 induced TXNIP expression. Compared to class I only HDAC inhibitors, inhibitors targeting both class I and II upregulated TXNIP, indicating that TXNIP is regulated by class II HDACs such as HDAC10. We further verified that inhibition of HDAC10 induced release of cytochrome c and activated apoptotic signaling molecules through accumulation of reactive oxygen species (ROS). Taken together, our results demonstrate that HDAC10 is involved in transcriptional downregulation of TXNIP, leading to altered ROS signaling in human gastric cancer cells. How TXNIP is preferentially regulated by HDAC10 needs further investigation.

INTRODUCTION

There are 18 known human histone deacetylases (HDACs), which can be divided into four classes based on sequence homologies. Class I HDACs (HDACs 1-3, and 8) are most closely related to yeast reduced potassium dependency 3 (RPD3), and are involved in regulation of gene transcription (Gray and Ekstrom, 2001). Class II HDACs are similar to the yeast histone deacetylase 1 (HDA1) proteins and are subclassified into two classes, Class IIa (HDACs 4, 5, 7, and 9) and Class IIb (HDACs 6 and 10) (Fischer et al., 2002; Guardiola and Yao, 2002; Kao et al., 2002; Tong et al., 2002; Zhou et al., 2000; 2001). Class III HDACs (silent information regulators [SIRTs1-7]) are NAD^+-dependent enzymes which are related to yeast SIR2, and HDAC11 is a Class IV HDAC (Gao et al., 2002; Gray and Ekstrom, 2001). HDACs are involved in diverse cell events such as regulation of gene expression, protein expression, protein-protein interactions and cell signaling (Johnstone, 2002; Marks et al., 2001).

Class II HDACs have tissue-specific expression patterns, and are located in both the nucleus and cytoplasm (Lu et al., 2000). Class IIb members, HDACs 6 and 10, have only two deacetylase domains. However in HDAC10, the second deacetylase domain is incomplete and is not functional (Fischer et al., 2002; Guardiola and Yao, 2002; Kao et al., 2002; Tong et al., 2002). HDAC6 functions as a cytoplasmic-protein deacetylase, and its well known substrates are non-histone proteins such as α-tubulin, heat shock protein 90, and ku70 (Boyault et al., 2007; Ding et al., 2008; Rodriguez-Gonzalez et al., 2008). HDAC6 participates in microtubule-associated cell motility, aggresome formation, apoptosis, and autophagy (Boyault et al., 2007; Ding et al., 2008; Rodriguez-Gonzalez et al., 2008). It plays a crucial role in normal biological processes, and its dysfunction leads to many diseases, especially neurodegenerative disorders such as Parkinson's, Alzheimer's, and Huntington's diseases (Ding et al., 2008; Dompierre et al., 2007; Kawaguchi et al., 2003).

In 2002, four different groups identified HDAC10, a novel class IIb HDAC (Fischer et al., 2002; Guardiola and Yao, 2002; Kao et al., 2002; Tong et al., 2002). It has two alternatively spliced transcription variants which encode 669 and 658 residues. HDAC10 is expressed ubiquitously and is found in both the nucleus and cytoplasm. Although HDAC10 is structurally similar to HDAC6, it seems to have different functions. For example, in the nucleus, HDAC10 can indirectly repress gene

[1]Cancer Research Institute, College of Medicine, Seoul National University, Seoul 110-799, Korea, [2]Department of Internal Medicine, College of Medicine, Seoul National University, Seoul 110-799, Korea, [3]Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 151-742, Korea
*Correspondence: kimty@snu.ac.kr

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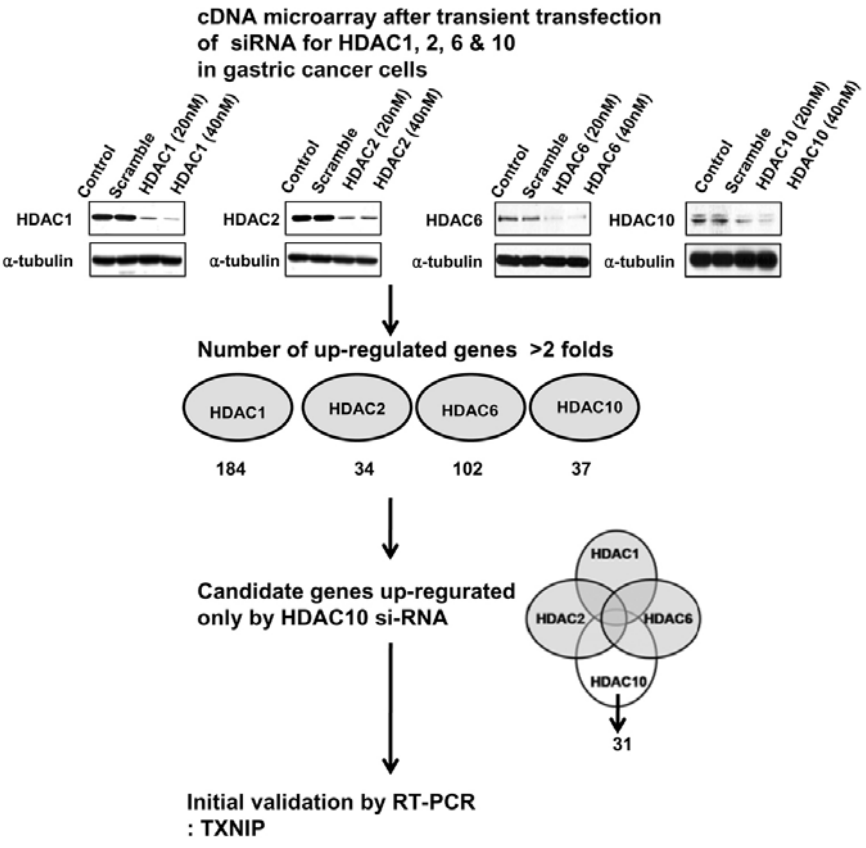

Fig. 1. Experimental design for identifying novel target genes of HDAC10

transcription in a histone deacetylation-independent manner. Moreover, it has a unique leucine-rich domain which distinguishes it from other HDACs (Kobe and Deisenhofer, 1994). A recent study reported that expression of HDAC10 is reduced in lung cancer patients, and that reduced HDAC10 expression may be a good indicator of poor prognosis in cancer patients (Osada et al., 2004). However, its biological function(s) and target gene(s) have not been clarified.

Thioredoxin-interacting protein (TXNIP), which is also known as thioredoxin-binding protein 2 (TBP2) or vitamin D3-upregulated protein 1 (VDUP1), is a negative regulator of thioredoxin (TRX) (Nishiyama et al., 1999). TXNIP can inhibit the reducing function of TRX, through not only binding the active form (reduced form) of TRX but also by reducing TRX expression. By inhibiting cell growth and inducing apoptosis, TXNIP can be regarded as a tumor suppressor. Furthermore, expression of TXNIP is downregulated in various cancers, including solid tumors and leukemia (Butler et al., 2000; de Vos et al., 2003; Ikarashi et al., 2002; Nishinaka et al., 2004; Ohta et al., 2005). It was previously reported that the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) upregulates TXNIP and downregulates TRX in transformed cells, but it is still unknown which HDAC is involved in these activities (Butler et al., 2002).

This study was designed to identify the target genes of HDAC10 in human gastric cancer cells (Fig. 1). Using cDNA microarray analysis after treatment of a gastric cancer cell line with HDAC10-targeting siRNA, we identified *TXNIP* as a target gene of HDAC10. The inhibition of HDAC10 decreases the expression of *TXNIP.* Furthermore, the inhibition of HDAC10 induced accumulation of ROS and activated pro-apoptotic molecules. Our results suggest novel functions of HDAC10 in regulating ROS signaling and apoptosis in human gastric cancer cells.

MATERIALS AND METHODS

Cell culture

The human gastric cancer cell line SNU-620 was purchased from the Korean Cell Line Bank (Seoul) (Ja-Lok and Jae-Gahb, 2005). Cells were maintained in RPMI 1640 supplemented with 10% FBS (WelGENE Inc., Korea) and gentamicin (10 μg/ml). Cells were incubated under standard culture conditions (20% O_2 and 5% CO_2 at 37°C) (Lee et al., 2006).

Reagents

Anti-TXNIP (sc-67134), anti-cytochrome c (sc-7159), and anti-lamin B (sc-6216) antibodies, and rabbit IgG (sc-2027) were purchased from Santa Cruz Biotechnology, Inc. (USA). Anti-TRX1 (#2429), anti-caspase-3 (#9662) and anti-caspase-9 (#9502) antibodies were purchased from Cell Signaling Technology, Inc. (USA). Anti-α-tubulin and anti-BH3 interacting domain death agonist protein (Bid) antibodies were purchased from Sigma, Inc. (USA) and BD Pharmingen, Inc. (Germany), respectively. LAQ824, SAHA, oxamflatin, SK7068, MS275, and sodium butyrate (NaB) were kindly provided by Dr. Dae-Kee Kim of In2Gen, Inc. (Korea).

Transient transfection

SNU-620 cells (1×10^6) were plated on 100 mm dishes and incubated overnight. The next day, after they were washed using a serum-free medium, transfections with siRNAs were performed using Lipofectamine 2000 (Invitrogen, Inc., USA) according to the manufacturer's instructions (Park et al., 2008).

Predesigned siRNAs of HDACs 1, 2, 6, and 10, and control

siRNA were purchased from Qiagen, Inc. (Germany).

cDNA microarray analysis

SNU-620 cells were maintained in culture for 48 h after treatment with siRNAs. Total RNA was extracted using an RNA mini-prep kit (Qiagen, GmbH., Germany) and hybridized to the Affymetrix GeneChip HG-U133 array (Affymetrix, Inc., USA). The results were analyzed by Macrogen, Inc., (Korea) (Kim et al., 2009).

Immunoblot analysis

Cells were collected 48 h after siRNA transfection, washed using ice-cold PBS, and lysed with cell lysis buffer (20 mM Tris-Cl [pH 7.4], 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mM $MgCl_2$) containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM pepstatin A, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 10 mg/ml aprotinin) on ice for 15 min. The lysates were cleared by centrifugation at 13,000 rpm for 20 min at 4°C.

Equal amounts of protein from whole-cell lysate samples were separated on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Proteins were detected using an enhanced chemiluminescence detection kit (Amersham Biosciences, Inc., USA) (Lee et al., 2006).

Immunoprecipitation

Cells were collected after siRNA transfection and washed with PBS. They were then lysed with IP buffer (50 mM Tris-Cl [pH 7.5], 250 mM NaCl, 0.1% NP40, 5 mM EDTA) containing phosphatase and protease inhibitors, and were incubated on ice for 15 min. After the lysates were precleared, 1 μg of anti-TXNIP antibody or rabbit IgG (for the negative control) was added to 500 μg of cell lysate protein in 700 μl of IP buffer, and the mixture was incubated overnight at 4°C.

To capture immune complexes, 30 μl of protein A/G agarose beads was added to the mixture and incubated for 4 h. The immune complexes were obtained by centrifugation at 1,200 rpm for 2 min and washed three times with IP buffer. The protein pellets were eluted with SDS sample loading buffer by boiling them for 5 min in the buffer, and then used for immunoblot analysis (Lee et al., 2006).

Reverse transcription PCR (RT-PCR) and quantitative real-time RT-PCR analysis

Total RNA was isolated using TRI Reagent (Molecular Research Center Inc., USA). After cDNA was synthesized from 1 μg of total RNA using ImProm-II Reverse Transcriptase (Promega Corporation, USA) and random hexamers, PCR was conducted. The primers used in the PCR reaction were as follows: for TXNIP, 5′-CAG AAG CTC CTC CCT GCT ATA TG-3′ and 5′-GAT GCA GGG ATC CAC CTC AG-3′; for GAPDH, 5′-CGG AGT CAA CGG ATT TGG TCG-3′ and 5′-GGG TGG AAT CAT ATT GGA ACA TGT AAA C-3′. Amplifications were performed in 20 μl volumes under the following conditions: 95°C for 1 min; 26 cycles of 95°C for 20 s, 60°C for 20 s and 72°C for 20 s; and a final extension of 72°C for 10 min. GAPDH was used as an internal control. Quantitative real-time PCR was performed using an iCycler iQ detection system (Bio-Rad Laboratories, Inc., USA) with SYBR Green I. The reaction was performed in a final volume of 20 μl containing 4 μl of each cDNA sample, 10 pmol primers, 0.125 mM dNTP, 0.25 mg/ml BSA, 0.05% Tween 20, 1 unit of rTaq DNA polymerase (Takara Bio, Inc., Japan), 1× rTaq reaction buffer (containing 1.5 mM MgCl2) and 1× SYBR Green I (Roche Molecular Biochemicals, Inc., Germany). Reaction conditions were as follows: 95°C for 10 min; and 40 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 30 s. All cDNA samples were synthesized in parallel, and the PCRs were performed in triplicate. The relative mRNA expression levels of TXNIP were normalized to β-actin levels.

Measurement of ROS generation in SNU-620 cells

ROS was detected by CM-H_2DCFDA (5-[and -6]-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester; DCFH-DA) (Molecular Probes, Inc., USA). A fluorescent product (DCF), which is generated after cellular uptake of DCFH-DA and oxidation of its deacetylated form (DCFH), was used to indicate ROS generation. Cells were harvested and resuspended in 2 ml of PBS and incubated for 15 min with 10 μg/μl DCFH-DA at 37°C in a 5% CO_2 atmosphere. The cells were washed with ice-cold PBS, resuspended in ice-cold PBS, and were immediately analyzed by FACSCalibur flow cytometry (Becton, Dickinson and Company, Inc., USA) (Park et al., 2003).

Statistical analysis

Data were expressed as means ± SE. For comparisons, the Student *t*-test or ANOVA were used as appropriate. *P* values of < 0.05 were considered significant.

RESULTS AND DISCUSSION

TXNIP is upregulated in cells trasfected with HDAC10-targeting siRNA

To identify novel target genes of HDAC10, we performed cDNA microarray analysis in SNU-620, a human gastric cancer cell line, after cells were treated with HDAC10-targeting siRNA, which had been previously validated (Park et al., 2008). To identify HDAC10-specific target genes, we also used samples which were treated with HDAC1-, 2-, and 6-specific siRNAs, and then identified HDAC10-specifically-regulated genes, which were not overlapping with targets of HDACs 1, 2, and 6. Several genes upregulated in response to HDAC10 siRNA are shown in Fig. 1. Among the upregulated genes, the mRNA level of thioredoxin-interacting protein (TXNIP) increased 3-fold after HDAC10 knockdown. Since previous researchers have shown that HDAC inhibitors (TSA and SAHA) induced increased mRNA levels of TXNIP in several cancer cell lines including prostate, bladder, myeloma, and breast cancer (Butler et al., 2000; Huang and Pardee, 2000; Richon et al., 1996), we selected TXNIP as a candidate for further study.

In this study, experiments were designed to validate that TXNIP is a target of HDAC10 (Fig. 1). TXNIP expression is reduced in various tumor tissues and cell lines, and overexpression of TXNIP inhibits the proliferation of tumor cells and cell cycle progression.

To verify the results from cDNA microarray analysis, we determined mRNA expression levels of TXNIP after treatment with HDAC1, 2, 6, and 10 siRNAs. Compared to the siRNAs of the Class 1 HDACs, HDAC1, HDAC2, and to HDAC6 siRNA, HDAC10 siRNA significantly increased (4-fold) the expression of *TXNIP* in SNU-620 cells (Figs. 2A and 2B).

It has been reported that *TXNIP* not only has CpG islands in its promoter region, but also *TXNIP* expression levels are increased by 5-aza-2′-deoxycytidine (5-aza) (Ahsan et al., 2006). Therefore, we examined the effect of 5-aza on TXNIP mRNA expression in SNU-620 cells. Combined treatment using TSA plus 5-aza induced a higher increase in the TXNIP mRNA level, compared to treatment using 5-aza alone (data not shown). We next examined TXNIP protein levels after treatment with HDAC

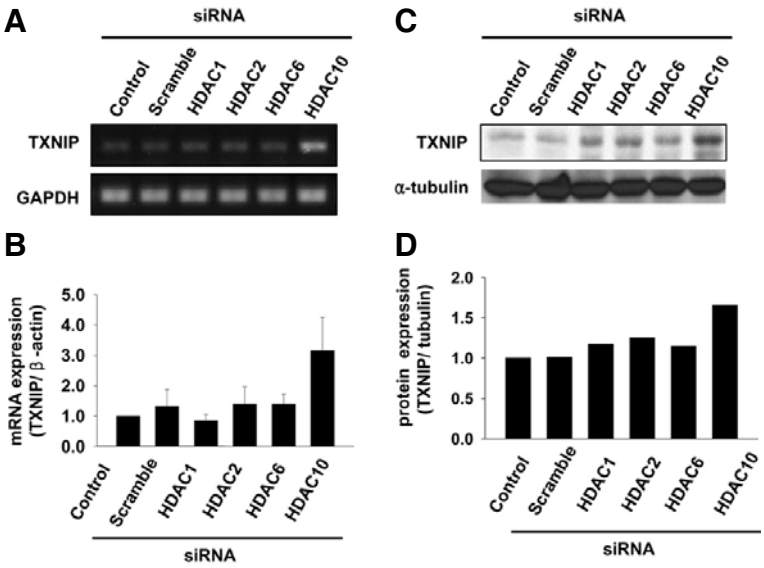

Fig. 2. HDAC10 knockdown increases TXNIP mRNA levels in SNU-620 cells. SNU-620 cells were transfected with 40 nM of HDAC10 siRNA, followed by incubation for 48 h. (A) RT-PCR and (B) quantitative real-time RT-PCR were performed using TXNIP-specific primers to examine TXNIP mRNAs. For quantitative RT-PCR, relative TXNIP mRNA expression levels were normalized to β-actin. Columns and bars represent mean values of data and standard deviations (SD) from three independent PCRs. (C) HDAC10 knockdown increased TXNIP protein levels. SNU-620 cells were transiently transfected with 40 nM of HDAC siRNAs and control siRNA. Proteins from whole cell lysates were used for immunoblot analysis with the indicated antibodies. (D) TXNIP protein levels were quantified using TINA 2.0 software (raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany). The relative protein levels of TXNIP were normalized to a-tubulin levels.

siRNAs. Although HDAC1, 2, and 6 siRNAs induced slight increases in TXNIP protein levels, HDAC10 siRNA induced a significant increase in the TXNIP protein level (Figs. 2C and 2D). These data suggest that TXNIP expression is upregulated by HDAC10 knockdown in SNU 620 cells.

TXNIP protein is upregulated by class II HDAC inhibitors

We next examined changes in TXNIP protein levels in response to several HDAC inhibitors added to SNU620 cells. Pan-HDAC inhibitors (LAQ824, SAHA, and NaB) induced expression of TXNIP protein in a time-dependent manner. However, specific class I HDAC inhibitors (SK7068, MS275, and oxamflatin) did not cause increases in TXNIP protein (Fig. 3A). As shown in Fig. 3B, LAQ824 induced a 2-fold increase in TXNIP protein level; whereas the level of TRX protein was 50% lower compared to the control. SAHA, another pan-HDAC inhibitor, also caused results similar to the results of LAQ824. However, compared to the SAHA pan-HDAC inhibitors, the specific class I HDAC inhibitor SK7068 did not significantly affect expression patterns of either the TXNIP or TRX proteins. Since HDAC10 is resistant to NaB, in addition to trapoxin B (Guardiola and Yao, 2002), NaB treatment did not change TXNIP and TRX expressions. These data suggest that expression of TXNIP is mainly regulated by inhibition of class II HDACs. Therefore, based on targeted and pharmacologic inhibition experiments, *TXNIP* could be a target gene that is primarily regulated by HDAC10. To our knowledge, *TXNIP* is the first identified target gene of HDAC10. Although the mechanism of regulation of *TXNIP* expression by HDAC10 remains to be fully determined, we have determined that *TXNIP* is a target of HDAC10, and it seems clear that inhibition of HDAC10 increases TXNIP mRNA expression.

HDAC10 inhibition induces H_2O_2 accumulation and activates pro-apoptotic molecules

To examine the effect of HDAC10 knockdown on interaction between TRX and TXNIP, we performed immunoprecipitation assays, pulling down TXNIP. We were able to detect the changes in interaction between TRX and TXNIP. As shown in Fig. 4A, although HDAC1 and HDAC2 siRNAs did not induce any change in interaction between TXNIP and TRX, HDAC10 knockdown induced a significant decrease in the binding of TRX to TXNIP. Previous reports have shown that class II HDACs induce deacetylation of non-histone proteins such as Hsp90, Ku70, and mutant p53. However, we could not detect acetylation of TXNIP proteins in SNU-620 cells after HDAC6 and HDAC10 knockdowns (data not shown). Our data clearly showed that HDAC10 inhibition induced expression of TXNIP mRNA as well as TXNIP protein. However, additional studies are needed to determine whether HDAC10 can directly bind to the *TXNIP* promoter and increase *TXNIP* transcription.

Reactive oxygen species (ROS) play important roles in cell signaling and survival. TRX is a key antioxidant regulator of cellular redox balance (Nishinaka et al., 2004; Nishiyama et al., 1999). Overexpression of TXNIP leads to ROS-induced apoptosis; whereas overexpression of TRX increases cell viability by reducing ROS damage (Schulze et al., 2002; Wang et al., 2002). Since we found that inhibition of HDAC10 increased TXNIP levels and decreased TRX, we investigated whether inhibition of HDAC10 affected intracellular ROS levels. To detect ROS, we used a DCFH-DA ROS detection assay (Buxser et al., 1999). The degree of ROS generation tended to correlate well with HDAC10 inhibition (Fig. 4B). Compared to HDAC6, HDAC10 knockdown caused increased ROS in the cytoplasm in a dose-dependent manner. To confirm whether this change is related to the membrane potential of mitochondria, we determined the degree of cytochrome c release into the cytosol of SNU620 cells. Increased levels of cytosol cytochrome c were observed in HDAC10-knockdown cells (Fig. 4C). However, we also observed slightly increased cytosol cytochrome c levels in HDAC1-, 2-, and 6-knockdown cells. To further confirm that HDAC10 inhibition induces release of cytochrome c, we applied various doses of HDAC10 siRNA, as well as a control siRNA. As shown in Fig. 4D, HDAC10 knockdown increased the levels of released cytochrome c in an siRNA-dose-dependent manner. Previous studies in a diabetic mouse model reported that overexpression of TXNIP induced not only increased release of cytochrome c, but also increased levels of cleaved caspase-3 (Chen et al., 2008).

Therefore, we subsequently evaluated the effect of HDAC10 on pro-apoptotic molecules. Inhibition of HDAC10 showed significant cleavages of Bid, caspase-3 and caspase-9 in SNU620 cells (Fig. 4E). Compared to HDAC 1 and 2 knockdowns, HDAC6 and HDAC10 more efficiently activated caspase-3. These results reveal that HDAC10 is involved in regulation of the membrane potential of mitochondria, and apoptosis. Collectively, these data

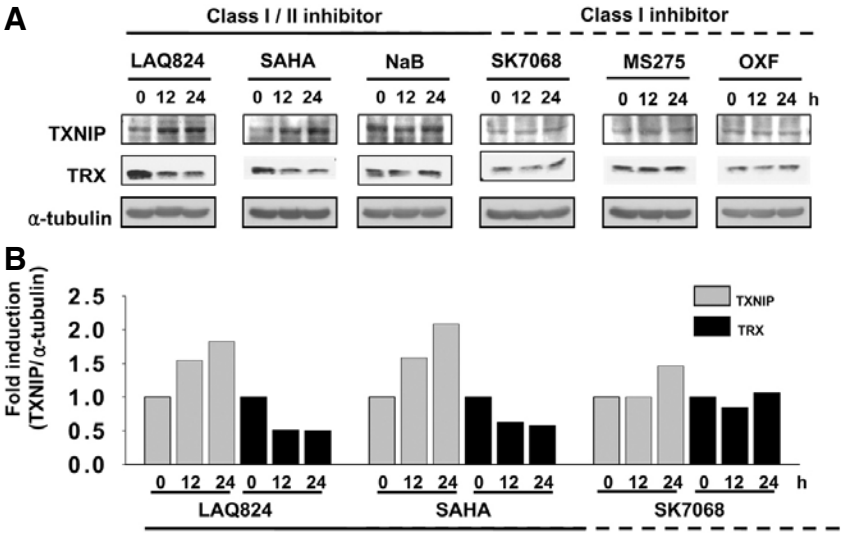

Fig. 3. Class II HDAC-specific inhibitors induce expression of TXNIP. (A) Effects of HDAC inhibitors on levels of TXNIP and TRX in SNU-620 cells. SNU-620 cells were treated with LAQ824 (100 nM), SAHA (1 μM), SK7068 (1 μM), NaB (sodium butyrate, 1 mM), MS275 (1 μM), and OXF (oxamflatin, 1 μM) for indicated times. (B) Protein levels of TXNIP were quantified using TINA 2.0 software. The relative protein levels of TXNIP were normalized to α-tubulin levels. Solid bars and dotted bars indicate pan-HDAC and class I HDAC inhibitors, respectively.

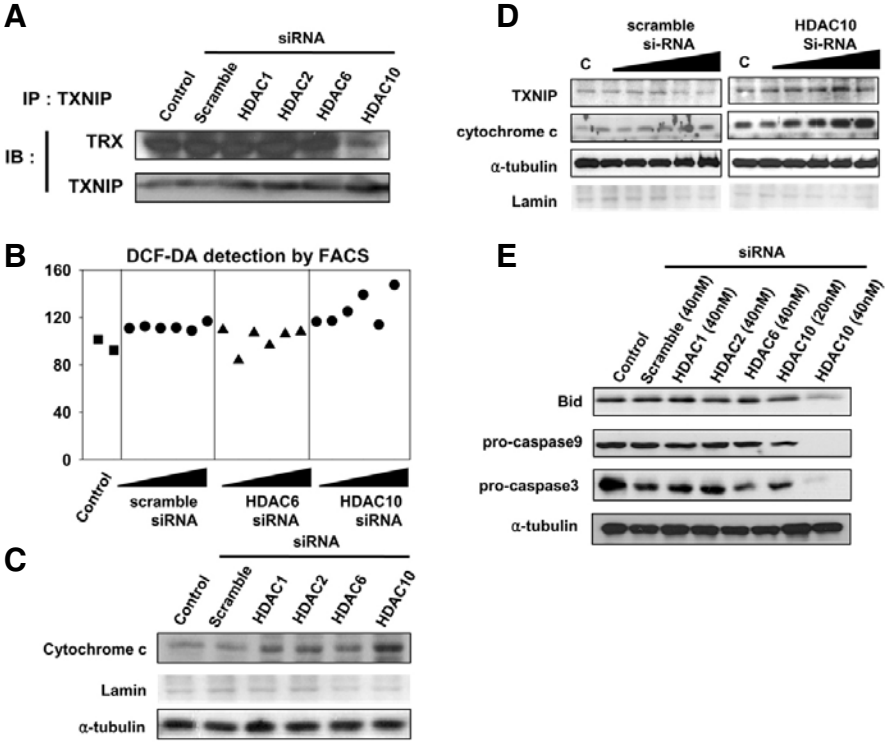

Fig. 4. Inhibition of HDAC10 induces H_2O_2 accumulation. (A) Inhibition of HDAC10 reduced binding of TRX to TXNIP. For immunoprecipitation assays, whole cell lysates were immunoprecipitated with TXNIP antibody, and the captured immune complexes were subjected to SDS-PAGE and immunoblotted using TRX1 antibody. (B) Graph representing ROS levels in SNU-620 cells. After SNU-620 cells were transiently transfected with HDAC6, 10, and control siRNAs, the cells were incubated with CM-H_2DCFDA, an oxidant-sensitive fluorogenic probe. (C), (D) HDAC10 siRNA induces release of cytochrome c from mitochondria to the cytosol in a dose-dependent manner. SNU-620 cells were transfected with 20 nM in (C), and 2.5, 5, 10, 20, 40, and 80 nM in (D) of the indicated siRNAs. Cytoplasmic fractions of SNU-620 cells were detected by immunoblot analysis with anti-cytochrome c antibody. Lamin B, a nuclear protein, was used as a negative control for the cytoplasmic fractions and α-tubulin was used as a loading control. (E) Proteins from whole cell lysates were detected by immunoblotting with pro-Caspase-9, pro-Caspase-3 and Bid.

suggest that HDAC10 is associated with the regulation of cellular redox balance via the regulation of TXNIP, and is also involved in apoptosis through the mitochondrial pathway.

In summary, we first determined that HDAC10 is potentially a regulator of ROS signaling via its regulation of TXNIP and TRX expression. Inhibition of HDAC10 caused an accumulation of ROS, increased release of cytochrome c, and activation of pro-apoptotic molecules such as caspase-3, caspase-9 and Bid. Taken together, the results suggest that in gastric cancer, HDAC10 may be a regulator of ROS through regulation of *TXNIP*, and would be a good candidate molecule for cancer therapy.

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