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Original Article

Regulation of CRADD-caspase 2 cascade by histone deacetylase 1 in gastric cancer

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Abstract: CRADD, also referred as RAIDD, is an adaptor protein that could interact with both caspase 2 and RIP that can promote apoptosis once activated. HDAC inhibitors are promising anti-cancer agents by inducing apoptosis of various cancer cells. In this study, we found that CRADD was induced by TSA (trichostatin A) to activate caspase 2-dependent apoptosis. CRADD was downregulated in gastric cancer and the restoration of its expression suppressed the viability of gastric cancer cells. HDAC1 was responsible for its downregulation in gastric cancer since HDAC1 siRNA upregulated CRADD expression and HDAC1 directly bound to the promoter of CRADD. Therefore, the high expression of HDAC1 can downregulate CRADD to confer gastric cancer cells the resistance to caspase 2-dependent apoptosis. HDAC inhibitors, potential anti-cancer drugs under investigation, can promote caspase 2-dependent apoptosis by inducing the expression of CRADD.

Keywords: CRADD, HDAC1, TSA, caspase 2, HDACi, gastric cancer

Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of cancer death worldwide [1]. Despite the improvement in the diagnosis and treatment, it remains a highly lethal cancer with poor prognosis. Although the incidence of gastric cancer in the developed countries such as US has fallen dramatically, it remains a major public health problem in many areas especially the East Asia.

As previously reported, trichostatin A (TSA), one of various HDAC inhibitors (HDACi), has a significant inhibitory effect on gastric cancer cells in vitro [2]. TSA treatment hurts gastric cancer cells by inhibiting histone deacetylase 3 (HDAC3) which inhibits expression of PUMA, whereas it forces lung cancer cells into cell death by inhibiting HDAC6 which leads to lower expression of ADAMTS1 [3]. In addition to classical caspase-8 and caspase-9 that are critical to the apoptosis induced by exogenous and endogenous signals respectively [3-5], caspase 2 has been reported to participate in apoptosis activated by various stimuli [6-9]. However, whether and how TSA induces caspase 2-dependent apoptosis in gastric cancer cells remains unknown.

CRADD (CASP2 and receptor-interacting protein kinase 1 domain containing adaptor with death domain), also referred as RAIDD (receptor-interacting protein-associated ICH-1/CED-3 homologous protein with a death domain), is an adaptor protein important for the activation of caspase 2. It has bipartite architectures containing a carboxy-terminal death domain (DD) that interacts with RIP (receptor-interacting protein) and an N-terminal caspase homology domain that interacts with caspase 2 [10]. The death domain and caspase recruitment domain (CRAD) are two related protein-protein interaction domains in proteins involved in apoptosis [11-13]. The interaction between CRADD and RIP is mediated through their DDs, while the

interaction between CRADD and caspase 2 is mediated through their CARDs [10, 12, 14].

Apoptosis caused by PIDD depends on CRADD activated caspase 2 and later activated caspase 3 and caspase 7 [15].

In the current study, we found that HDACi could promote caspase 2-dependent apoptosis by activating the expression of CRADD, which was found to be the direct target of HDAC1 and downregulated in gastric cancer. The epigenetic downregulation of CRADD confers gastric cancer cells the resistance to caspase 2-dependent apoptosis. HDAC inhibitors such as TSA can promote caspase 2-dependent apoptosis by inducing the expression of CRADD.

Materials and methods

Cell lines and tissue samples

GES-1 cells and GC cell lines (MKN-45, MKN-28, AGS, BGC-823, SGC-7901, N-87) were obtained from American Type of cell collection (ATCC, Manassas, VA, USA) or RIKEN BioResource center (Ibaraki, Japan). GES-1, MKN-28, AGS, BGC-823, SGC-7901 and N-87 cells were cultured in RPMI 1640 medium (Invitrogen, Carsbad, CA, USA) supplemented with 10% fetal bovine serum and incubated at 5% CO_2, 37°C and 95% humidity. MKN-45 were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum. For plasmid transfection, FuGENE HD (Roche Applied Science, Mannheim, Germany) was used after the protocol provided. Total RNA was extracted after 48h transfection using Trizol RT reagent (Invitrogen) following manufacturer's instruction. The concentrations were quantified by NanoDrop 2000 (Nanodrop, Wilmington, DE, USA).

Plasmids and antibodies

CRADD ORF (1-600 bp) was amplified by PCR using the SuperScript® III Reverse Transcriptase (Invitrogen). The primers sequence are 5'-GAGATCTGAGGCCAGAGACAAAC and 5'-GGTCGACTCACTCCAACATGTGCA. The PCR product was cloned into the pCR4-TOPO vector (Invitrogen). After sequence verification, the insert was sub-cloned using BamH I and Sal I restriction sites into the hygromycin resistant mammalian expression vector pCMV-3Tag-7 (Agilent, La Jolla, CA, USA). Anti-CRADD antibody (#1957-1) and anti-Caspase2 antibody (#1552-1) was purchased from Epitomics Inc (Epitomics, Burlingame, CA, USA). GAPDH (#3683) and PARP (#9542) antibodies were from Cell Signaling Technology (Cell Signaling, Boston, MA, USA). HDAC1 antibody (#05-100) was purchased from Millipore (Temecula, CA, USA).

SiRNA transfection

CRADD, Caspase2 and HDAC1 depletion were achieved by transfection with siRNA (Gene Pharma, Shanghai, China). Cells were seeded 24 h in 6-well plates ($2×10^5$/well) and transfected with siRNA duplxes (20 nM) using Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested for RNA and protein extraction after 48 hours. SiRNA against Nonesense (SI04380467, Qiagen) was used as the control. SiRNA duplxes used for CRADD knockdown are 5'-GGGCCCUAAAGCAUUUGAUTT and 5'-AUCAAAUGCUUUAGGGCCCTT. SiRNA duplxes used for caspase 2 knockdown are 5'-UGGAAGUAUUUGAGAGAGATT and 5'-UCUCUCUCAAAUACUUCCATT. HDACs were knocked down as previously described [2].

Quantitative real-time RT-PCR

Reverse transcription reaction was performed using 1 μg of total RNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Genes' expression was determined by quantitative real-time PCR using SYBR Green Master Mix Kit and ABI 7500 Real-Time PCR System (Applied Biosystems). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control of RNA integrity. Primers used for CRADD are 5'-AAGATACGTGGTTGCAGACG (Forward) and 5'-TAGGATATCCAGCAGGAGCA (Reverse).

Cell growth assay

Cell growth assay was performed with The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (MTS assay) (Promega). Briefly, 48 hours after transfection, the transfected cells were transferred into a 96-well plate for one day more. Then the cell growth was measured following the manufacture's instruction. Samples were prepared in triplicates, and the cell viability was determined as the mean±SD.

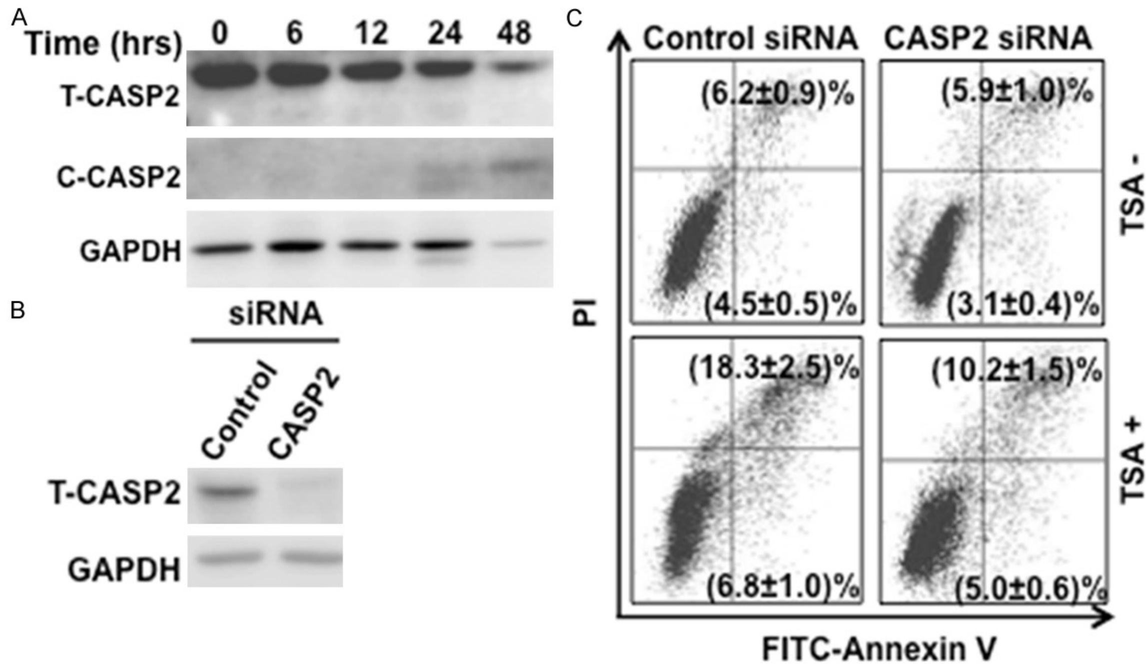

Figure 1. Activation of caspase 2 in TSA-induced apoptosis. A. Cells were cultured with 150 nM TSA for 6, 12, 24, 48 hours respectively. Caspase 2 activation induced by TSA in SGC-7901 was determined by western blot analysis. GAPDH served as loading control. T-/C-CASP-2 represented for total caspase 2 or cleaved caspase 2, respectively; B. SGC-7901 cells were transiently transfected with negative control siRNA or Caspase 2 targeting siRNA, and the expression of caspase 2 was determined by western blotting; C. SGC-7901 cells were transiently transfected with negative control siRNA or caspase 2 targeting siRNA, with or without 150 nM TSA for 48 hours. The apoptosis effect was determined by flow cytometry (). All experiments were repeated three times.

Colony formation assay

Anchorage-dependent growth of tumor cells was investigated by monolayer colony formation assay. MKN-28 and SGC-7901 Cells were cultured overnight in a 6-well plate (2.0×10^5 per well) and transfected with CRADD and control vector, using FuGENE HD (Roche Applied Science). Forty-eight hours later, the transfectants were replated in triplicate and cultured for 16-20 days with complete medium RMPI1640 containing G418 (200 μg/ml for MKN-28, 150 ug/ml for SGC-7901). Surviving colonies were stained with Gentian Violet after methanol fixation, and visible colonies (≥ 50 cells) were counted.

Flowcytometry (FCM) analysis

Apoptosis analysis was determined by flow cytometry analysis. Briefly, cells transfected with caspase 2 siRNA, CRADD siRNA, HDAC1 siRNA or control siRNA were harvested and were washed twice with cold 1×PBS and then resuspended cells in 1×Binding Buffer. Then the cells were stained with FITC Annexin V apoptosis detection kit (BD Bioscience, Bedford, MA, USA) for 30 min at room temperature in dark. The apoptosis detection was determined using a FACS can flow cytometry (Becton Dickinson, Mountain View, CA, USA) within 1 hour.

Western blotting

Same amount of protein from each lysate was harvested and boiled in sample buffer. The boiled lysates were resolved by SDS-PAGE, transferred to PVDF membranes. And then the protein on PVDF membranes was probed with the indicated primary antibodies, washed with TBS-T (TBS with 0.1% of Tween-20), incubated with suitable HRP-conjugated second antibodies and visualized with enhanced chemiluminescence (Millipore).

Chromatin Immunoprecipitation

A ChIP assay was performed using a ChIP assay kit (Cell Signaling) according to the manufacturer's protocol. Briefly, cells were cross-linked with 1% formaldehyde (Sigma) in a culture

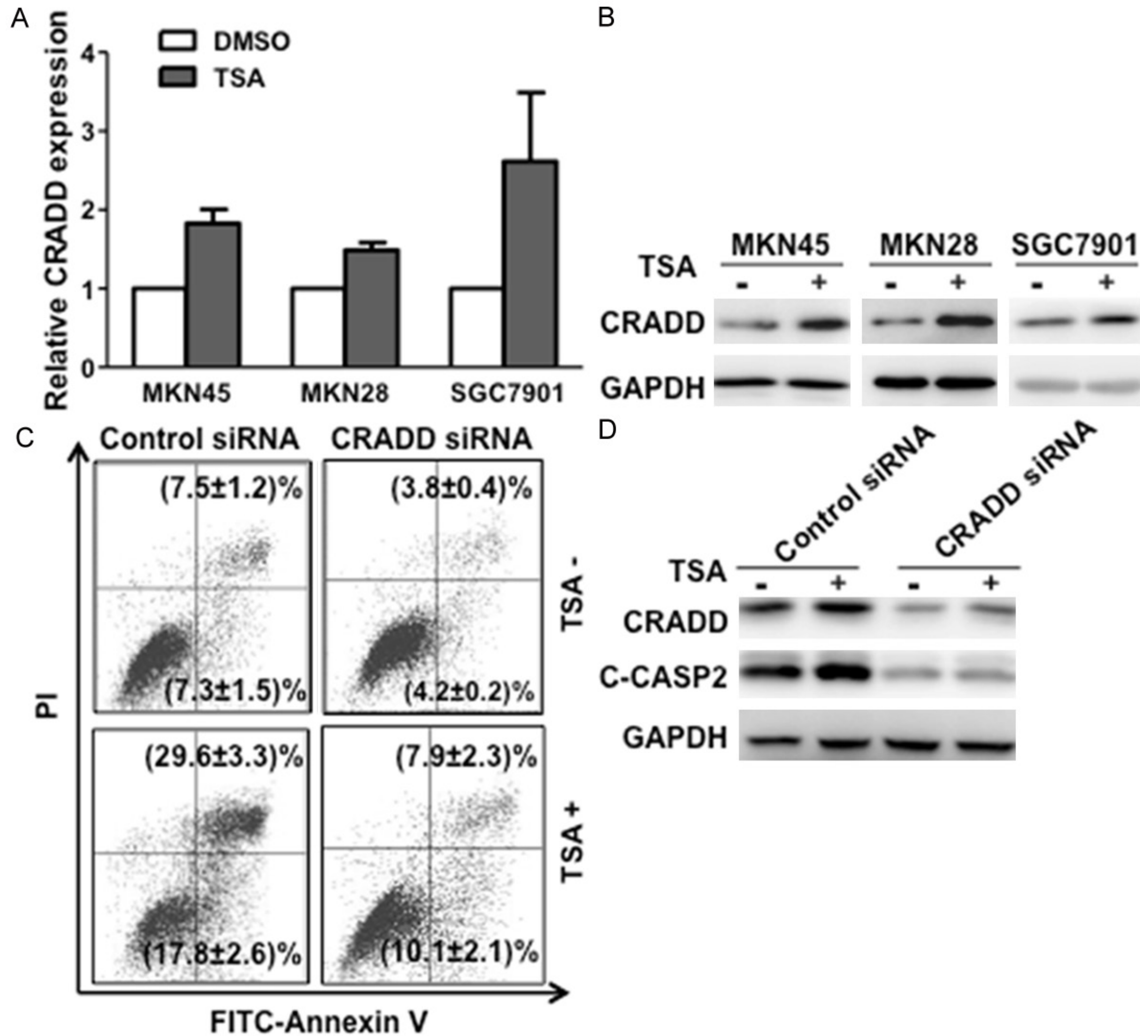

Figure 2. Upregulation of CRADD after TSA treatment induces apoptosis. Cells were cultured with TSA for 48 hours. The concentrations of TSA were 400 nM, 200 nM and 150 nM for MKN45, MKN28 and SGC-7901 respectively. RT-real time PCR was performed to detect mRNA upregulation of CRADD (A), and western blot analysis was performed to detect upregulation of CRADD protein (B). GAPDH served as the loading control. SGC-7901 cells were transiently transfected with negative control siRNA or CRADD targeting siRNA, with or without 150 nM TSA for 48 hours. The apoptosis was determined by flow cytometry (C, $p < 0.05$, Student's t test) and western blotting (D). All experiments were repeated three times.

medium at room temperature for 10 min. Cells were then suspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH8.1 and 1×protease inhibitor) and sonicated on ice. Next, the chromatin solution was precleared, incubated with HDAC1 antibody (Santa Cruz), and immune complexes were eluted. Then, quantitative real-time PCR was performed to determine relative occupancy of HDAC1 around the promoter region of CRADD. The primers used for ChIP assay are 5'-AGTAACTAGCTG-AGTGCTGC and 5'-AGCCTTCTCTGACCTATCTG.

Immunohistochemistry staining

For immune-detection of CRADD and HDAC1 expression in normal and tumor tissue sections, tissues were fixed in formalin and subsequently embedded in paraffin, then were dried on glass slides overnight at 37°C. The sections were pre-treated to achieve antigen retrieval. Then pre-incubation was performed for 30 min followed by an overnight incubation at 4°C with 1:500 CRADD antibody (Epitomics), 1:500 HDAC1 antibody (Millipore). An undiluted HRP

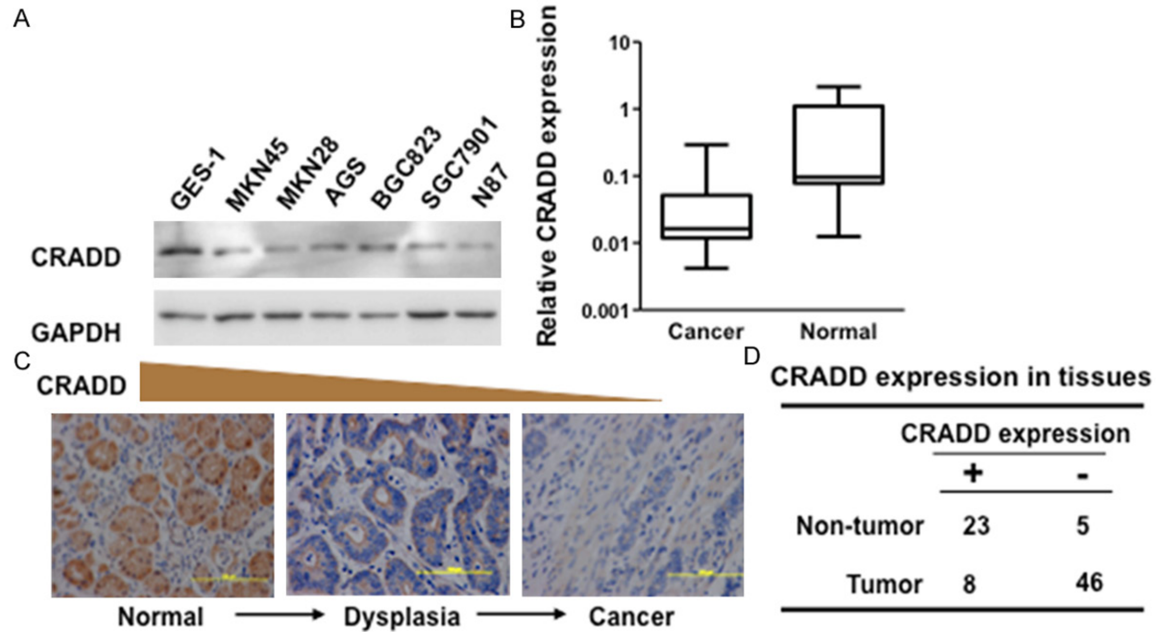

Figure 3. Downregulation of CRADD expression in gastric cancer. A. CRADD protein expression in gastric cancer cell lines and non-tumor gastric epithelial GES-1 cells were determined by Western blot analysis; B. Real time RT-PCR was performed to compare CRADD mRNA expression in primary gastric carcinoma tissues and in adjacent non-tumor stomach tissues ($p < 0.05$, n=17, Wilcoxon matched pairs t-test); C and D. CRADD expression in primary tissues were analyzed by immunohistochemistry staining ($p < 0.05$, Fisher's exact test).

conjugated compact polymer system (PowerVision poly-HRP-Anti-Rabbit IgG, ImmunoLogic) was used as secondary antibody. Finally sections were viewed under a fluorescence microscope.

Results

TSA activates caspase 2 to promote apoptosis

Upon the treatment of TSA, total caspase 2 was decreased with the accumulation of cleaved caspase 2 in a time-dependent manner in gastric cancer SGC-7901 cells (**Figure 1A**). It indicated that caspase 2 could be relevant to TSA-induced apoptosis. Indeed, TSA-induced apoptosis was impaired once caspase 2 expression was reduced by siRNA (**Figure 1B** and **1C**).

CRADD was induced by TSA to activate apoptosis

In an effort to screen TSGs suppressed by histone deacetylation, we profiled gene expression in gastric cancer cells before and after TSA treatment. Among 23,152 active signals, 122 genes were found to be upregulated at least 2 folds after TSA treatment [2]. CRADD, which has been reported to be essential for caspase 2 activation [10, 16], was one of these genes upregulated significantly. Therefore, we wondered whether CRADD is relevant to TSA-induced apoptosis in gastric cancer cells. CRADD expression in gastric cancer cells was indeed elevated both in the mRNA and protein level after TSA treatment (**Figure 2A** and **2B**). Interestingly, TSA-induced apoptosis was impaired when CRADD expression was reduced by siRNA (**Figure 2C**). Moreover, CRADD knockdown disturbed TSA-induced activation of caspase 2 (**Figure 2D**), confirming the relevance of CRADD to TSA-induced apoptosis.

Downregulation of CRADD expression in gastric cancer

To further explore the relevance of CRADD to gastric carcinogenesis, we examined the expression of CRADD in gastric cancer cells as well as primary gastric tissues. It was downregulated in a panel of gastric cancer cell lines compare to GES-1, a non-tumor gastric epithelial cell line (**Figure 3A**). Importantly, CRADD expression levels in primary gastric carcinoma tissues were significantly lower than its expression in adjacent non-tumor stomach tissues by

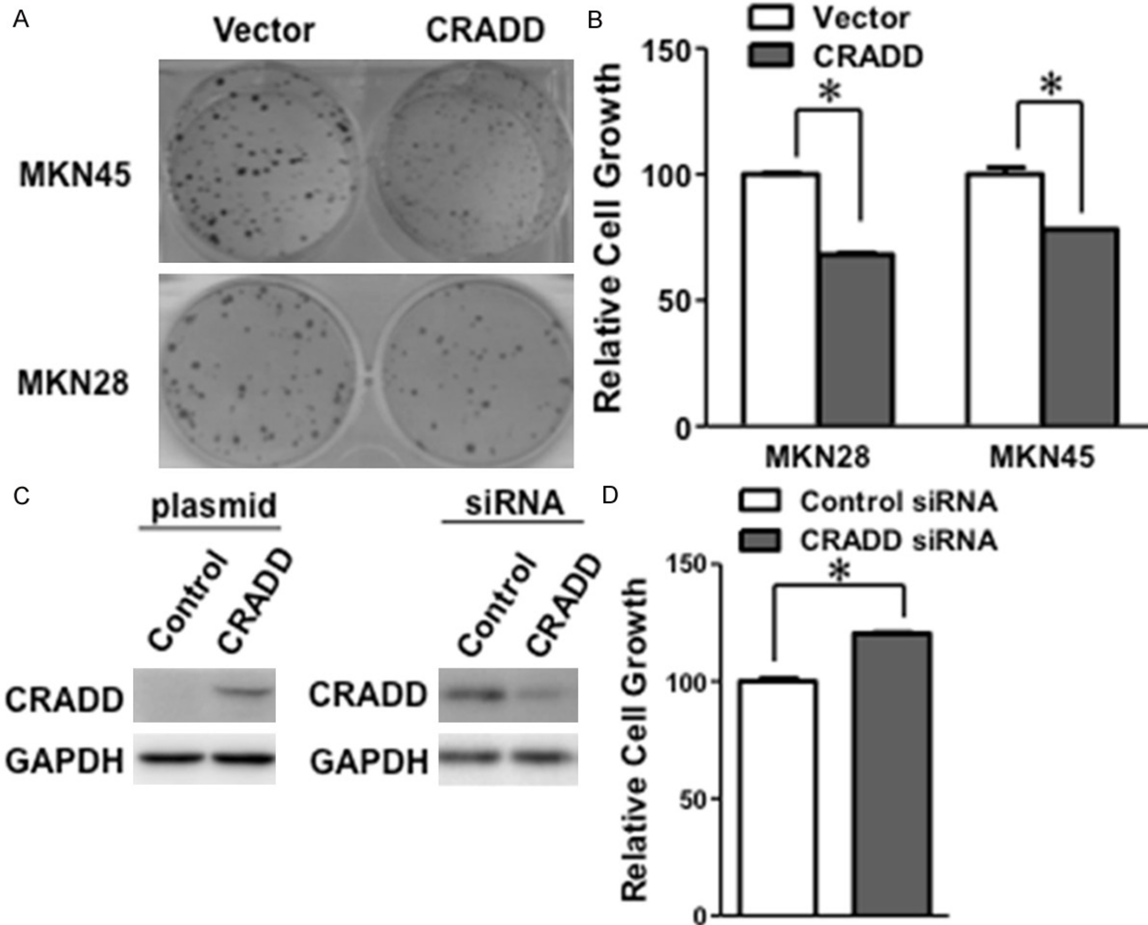

Figure 4. CRADD suppressed proliferation of gastric cancer cells. A. The colony formation assay was performed to compare the growth of gastric cancer cells before and after the restoration of CRADD; B. The growth of gastric cancer cells before and after the restoration of CRADD expression was detected by MTS assay. The asterisks indicate statistical significance ($p < 0.05$, Student's t test); C. Western blot analysis was used to confirm CRADD expression after the transfection of CRADD expressing plasmid or knock down of CRADD; D. The growth of gastric cancer cells before and after transiently knock down CRADD was detected by MTS assay. All experiments were repeated three times. The asterisks indicate statistical significance ($p < 0.05$, Student's t test).

real-time RT-PCR (n=17, p=0.0098, Wilcoxon matched pairs t-test) (**Figure 3B**). Furthermore, immunohistochemistry staining was performed to evaluate CRADD expression in primary gastric cancer tissues. CRADD expression was gradually decreased with the development of gastric cancer (**Figure 3C**). Its expression in gastric carcinoma tissues was significantly lower than its expression in adjacent non-tumor tissues (**Figure 3D**, $p < 0.01$, Fisher's exact test).

CRADD suppressed in vitro growth of gastric cancer cells

All of these results_indicated that CRADD may function as a tumor suppressor in gastric cancer. Therefore, the in vitro growth of gastric cancer cells before and after the restoration of CRADD was compared by the colony formation assay and MTS assay. As expected, ectopic CRADD expression in two independent gastric cancer cell lines significantly suppressed cellular growth (**Figure 4A-C**). In contrast, suppression of CRADD expression promoted the growth of GES-1 cells (**Figure 4C** and **4D**), indicating that CRADD functions as a tumor suppressor in gastric cancer cells.

Relevance of HDAC1 to the regulation of CRADD expression

Next we would like to explore which HDAC was responsible for TSA-activated CRADD expression in gastric cancer cells as TSA can inhibit various HDACs. Presently, it is widely recog-

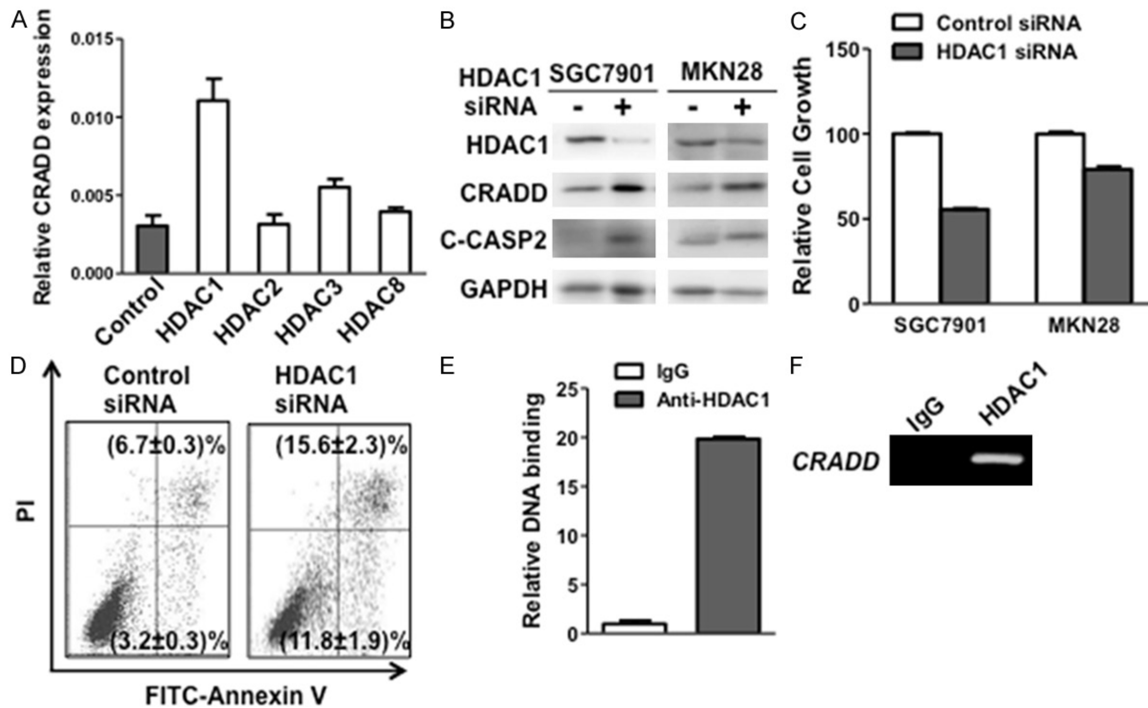

Figure 5. Relevance of HDAC1 to the regulation of CRADD expression. A. Real time RT-PCR was performed to compare CRADD mRNA expression in gastric cancer cells before and after the knockdown of HDACs as indicated; B. Western blot analysis of HDAC1, CRADD and caspase 2 in gastric cancer cells after transiently knock down of HDAC1; C. The growth of gastric cancer cells before and after transiently knock down HDAC1 was detected by MTS assay. All experiments were repeated three times. The asterisks indicate statistical significance ($p < 0.05$, Student's t test); D. The apoptosis of SGC7901 cells with or without HDAC1 knockdown were determined by flow cytometry ($p < 0.05$, Student's t test). E and F. ChIP assay was performed to explore the binding of HDAC1 to CRADD promoter ($p < 0.05$, Student's t test).

nized that class I family of HDACs has the closest relationship with cancer [17]. To further determine which HDAC in the class I family of HDACs is important to regulate CRADD expression, we compared CRADD expression in gastric cancer cells before and after the knockdown of four class I HDACs individually. The data showed that CRADD expression was significantly upregulated after the depletion of HDAC1 but no other HDACs (**Figure 5A**), indicating that CRADD might be a target regulated by HDAC1. Moreover, along with the knockdown of HDAC1 and the upregulation of CRADD, we found significant inhibition of cell proliferation and cleavage of Caspase2 in SGC-7901 and MKN-28 cells, comparing to the control group (**Figure 5B** and **5C**). Consistently, more apoptotic cells were detected after the knockdown of HDAC1 (**Figure 5D**). Furthermore, ChIP assay showed that the binding of HDAC1 to CRADD promoter was significantly decreased after the TSA treatment, confirming that CRADD is directly regulated by HDAC1 (**Figure 5E** and **5F**).

Discussion

By affecting the expression of many genes critical to cancer development, reversible histone acetylation plays a crucial role in the pathogenesis of various human cancers including gastric cancer [18, 19]. Generally, histone acetylation catalyzed by histone acetyltransferases (HATs) relaxes the chromatin to activate gene expression while histone deacetylation by HDACs is often associated with transcriptional silencing [20, 21]. During cancer development, many important tumor suppressor genes (TSGs) could be transcriptionally silenced due to aberrant histone deacetylation. Therefore, HDAC inhibitors (HDACis) were proposed to be a new approach for the treatment and prevention of human cancers [22-24].

Based on domain organization and sequence similarity, HDACs can be classified in four groups [25, 26]. As the most dominate class, the first two groups of HDACs could be inhibited

by TSA and many other molecules currently under investigation as new anti-cancer agents. Being the selective inhibitor for the class I and II HDACs, TSA was widely used to reactive the expression of TSGs in cancer cells [27-29]. In an attempt to identify tumor suppressor genes silenced through histone deacetylation in gastric cancer cells, we have compared gene expression profiling in gastric cancer cell lines with or without TSA treatment. PUMA (p53 Up-regulated Modulator of Apoptosis) or BBC3 (bcl-2 binding component 3), was found as one of the genes up-regulated after TSA treatment [2]. As a BH3 only proapoptotic member of the Bcl-2 family, PUMA plays an important role in p53-dependent and -independent apoptosis in several human cancer tissues and cell types with DNA damage or stress [30-33].

In addition to the activation of apoptosis by PUMA, we found herein that inhibition of HDACs by TSA can activate caspase 2-dependnet apoptosis through stimulating the expression of CRADD. The induction of CRADD to activate caspase 2-dependent apoptosis by TSA seemed not to be specific to gastric cancer cells. It has been reported that TSA can also stimulate CRADD expression to initiate caspase 2 activation in prostate cancer cells [34]. Together with PIDD and pro-Caspase 2, it can form a multi-protein complex called PIDDosome to activate caspase in response to many apoptosis-inducing stimuli such as genotoxic stresses. As such cellular responses are critical for tumor suppression, deregulated expression of individual PIDDosome components has been noted in many human cancers [35-37]. In this study, we indeed found that CRADD was downregulated along the development of gastric cancer. However, CRADD was found to be a target of HDAC1 while PUMA was directly regulated by HDAC3. Therefore, TSA can inhibit HDAC1 to activate CRADD and caspase 2-dependent apoptosis in addition to inhibit HDAC3 to activate PUMA-dependent apoptosis. More specific agents to target HDAC1 or HDAC3 might be developed for the target therapy of gastric cancer after analyzing the dependence of cancer cells on HDAC1-CRADD or HDAC3-PUMA.

In conclusion, we demonstrated that TSA induced caspase 2-dependent apoptosis of gastric cancer cells through stimulating CRADD expression. CRADD was directly downregulated by HDAC1 and functioned as a TSG in gastric cancer cells. Selectively suppressing HDAC1 may induce CRADD to activate caspase 2-dependent apoptosis, thus representing a novel approach for the treatment of gastric cancer.

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Disclosure of conflict of interest

We declare no conflict of interest.

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