Research Article

Molecular Cancer Therapeutics

A Novel Plant Homeodomain Finger 10–Mediated Antiapoptotic Mechanism Involving Repression of Caspase-3 in Gastric Cancer Cells

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Abstract

The mechanisms governing tumorigenesis of gastric cancer have been an area of intense investigation. Currently, plant homeodomain (PHD) finger (PHF) proteins have been implicated in both tumor suppression and progression. However, the function of PHF10 has not been well characterized. Here, we show that various levels of PHF10 protein were observed in gastric cancer cell lines. Alteration of PHF10 expression, which is associated with tumor cell growth, may result in apoptosis in gastric cancer cells both *in vitro* and *in vivo*. Knockdown of PHF10 expression in gastric cancer cells led to significant induction of caspase-3 expression at both the RNA and protein levels and thus induced alteration of caspase-3 substrates in a time-dependent manner. Moreover, results from luciferase assays indicated that PHF10 acted as a transcriptional repressor when the two PHD domains contained in PHF10 were intact. Combined with previous findings, our data suggest that PHF10 transcriptionally regulates the expression of *caspase-3*. Finally, by using systematic reporter deletion and chromatin immunoprecipitation assays, we localized a region between nucleotides −270 and −170 in the *caspase-3* promoter that was required for the efficient inhibition of *caspase-3* promoter activity by PHF10. Collectively, our findings show that PHF10 repressed *caspase-3* expression and impaired the programmed cell death pathway in human gastric cancer at the transcriptional level. *Mol Cancer Ther; 9(6); 1764–74. ©2010 AACR.*

Introduction

Although the incidence of gastric cancer has declined over the past 2 decades, the disease has a high death rate (700,000 per year), making it the second most common cause of cancer mortality (1). For advanced carcinomas, therapeutic options are limited to radiation therapy and chemotherapy, and these modalities do not lead to a cure. The identification of biomarkers that distinguish cancer cells from normal cells is important for the identification of therapeutic targets in human malignancies. Several strategies have been developed to identify relevant tumor antigens that can be used for active immunotherapy strategies in tumor patients. Recently, a novel method termed serologic analysis of antigens by recombinant expression cloning (SEREX; ref. 2) has permitted direct molecular determination of new tumor antigens that elicit an IgG antibody response in tumor patients. This method has enabled the discovery of several novel genes with tumor specificity, such as *MLAA-2* (3), *CAGE* (4), and *T21* (5). Using sera from cancer patients, we have previously reported the identification of immunologically recognized proteins, such as plant homeodomain (PHD) finger 10 (PHF10; refs. 6, 7)and FRZB (8–10), which belong to the zinc finger protein family and are likely candidates for cancer-specific immunotherapy.

The PHD is also called the leukemia-associated protein domain, which was first identified as HAT3.1, a protein involved in plant root development (11). Similar PHD domains have been found in >400 eukaryotic proteins, including transcription factors and other proteins implicated in chromatin-mediated transcriptional regulation (12, 13). Proteins from the PHD zinc finger superfamily are well documented to be capable of translocating to the nucleus and regulating transcription. In the PHF family, the PHD class of transcription factors (e.g., RING, PHF1, PHF2, PHF11, and HBXAP) has been shown to be particularly interesting in cancer and autoimmune diseases for their transcriptional function on targeted proteins.

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We apologize to those whose work could not be cited or discussed due to space limitations.

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These factors are expressed in the nucleus and act in cell proliferation and differentiation in a variety of diseases (14, 15). Potential clues about the relationship between aberrant expression of PHF10 and cancer were observed in a series of studies that found prevalent overexpression of PHF10 in many types of cancers, including colon cancer (16). However, the molecular event in the progression of gastric cancer that involves PHF10 remains unclear.

In this study, we explored the role of PHF10 in the malignant phenotype of gastric cancer and mapped the relationship between PHF10 and its target molecules, which may offer new therapeutic avenues for the treatment of this severe disease.

Materials and Methods

Cell lines and antibodies

Two gastric cancer cell lines, MKN45 and MKN28, were obtained from the Japanese Cancer Research Resources Bank, and the other gastric cancer cell lines were obtained from the American Type Culture Collection. GES-1, an immortalized gastric epithelial cell line, was a gift from Prof. Feng Bi (Institute of Digestive Disease, Xi'jing Hospital). Mouse monoclonal antibodies against cytochrome *c*; β-actin; mouse polyclonal antibody against PHF10; rabbit monoclonal antibodies against procaspase-3, Bid, caspase-8, poly(ADP-ribose) polymerase (PARP), NF-κB, survivin, FasL, Bcl-2, and Bax; and rabbit polyclonal antibody to active+procaspase-3 were purchased from Abcam. The rabbit polyclonal antibody again caspase-9 was from BD Biosciences. Monoclonal mouse anti-actin and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Sigma.

RNA extraction and quantitative reverse transcription-PCR amplification

RNA isolation was done using the Trizol reagent according to the manufacturer's instructions. Total RNA was then subjected to quantitative reverse transcription-PCR (q-RT-PCR) analysis. q-RT-PCR was done using an iCycler IQ Real-Time PCR Detection System (Bio-Rad). Reactions contained 5 pmol forward and reverse primers, 1× iQ SYBR Green Supermix (Bio-Rad), and 2 μL template cDNA from investigated cell lines. The raw quantifications were normalized to the GAPDH values for each sample. The primer sequences used in the q-RT-PCR analyses are available in Supplementary File 1.

Western blot analysis

The M-PER reagents and Halt Protease Inhibitor Cocktail kits (Pierce Biotechnology) were used for cell extract preparation according to the manufacturer's instructions. The protein concentration of the cell extracts was quantified using the bicinchoninic acid protein assay (Pierce Biotechnology). Western blot analysis was carried out as previously described (17, 18). Labeled bands were detected by the ECL chemiluminescent kit (Pierce Biotechnology). Images were captured and the intensity of the bands was quantitated with the VersaDoc image system (Bio-Rad).

Immunofluorescence studies

SGC7901 cells were washed twice with PBS, fixed, and permeabilized with 4% formaldehyde and 0.5% Triton X-100 in PBS for 10 minutes. After washing with PBS, cells were blocked with 1% bovine serum albumin and incubated with mouse polyclonal anti-PHF10 (1:50). After an incubation period of 1 hour, cells were washed thrice with PBS and incubated for 1 hour with secondary antibody (FITC-conjugated rabbit anti-mouse; 1:500; Sigma). Following three washes with PBS, cells were incubated with an actin-specific marker, phalloidin (Sigma). Coverslips were washed thrice, mounted using mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes), and viewed using a Leica TCS-SP2 confocal system.

Mammalian PHF10 expression plasmid construction

By amplifying cDNA of SGC7901 using the hPHF10-F1 (5′-TACAAGCTTCTTCAAGAACAAGTCAGTGAA-3′) and hPHF10-R1 (5′-TACGAATTCTTATCCCTCTTTG-CTGT-3′) primers, full-length PHF10 was obtained and inserted into plasmids, pFLAG-CMV4 and pCMV-BD, to generate fusion proteins. Alignment done in the European Molecular Biology Laboratory protein database showed that the MYST-RELATED PROTEINS subdomain (or PTHR10615:SF8), which ranges from +44 to +391, is evolutionarily conserved among various genes for 2PHD-containing proteins and across different species. Consequently, we hypothesized that this region may be required for PHF10 to exert transcriptional regulation. The remainder of the sequence in the MYST-RELATED PROTEINS subdomain upstream of 2PHD domains was designated as the LEAD domain (Fig. 3B). A series of FLAG-tagged motif fusion proteins were generated using pFLAG-PHF10 expression plasmids as the template. All resulting constructs were designated as indicated in Fig. 4B. The GAL4/LexA promoter activity assay was done using a pL8G5-luc plasmid with or without cotransfection of a pLexA-VP16 construct (17, 18).

The putative 700-bp *caspase-3* promoter sequence was amplified by PCR using human genomic DNA as the template as described in previous studies (19). The promoter sequence was cloned into pGL4-Basic with *Xho*I and *Hin*dIII sites added to each end (Promega). In addition, differently sized fragments of the *caspase-3* promoter, which were generated by progressively deleting from the 5′ end, were cloned into the promoterless pGL4-Basic luciferase reporter vector. The sequences of all promoter fragments derived by PCR were confirmed by sequencing. The luciferase plasmids were transformed into JM109-competent cells (Promega) using the standard protocol for mass production. Short hairpin RNA (shRNA) specific to PHF10 mRNA was designed and prepared as described previously (7) using Psilencer 2.0 vector

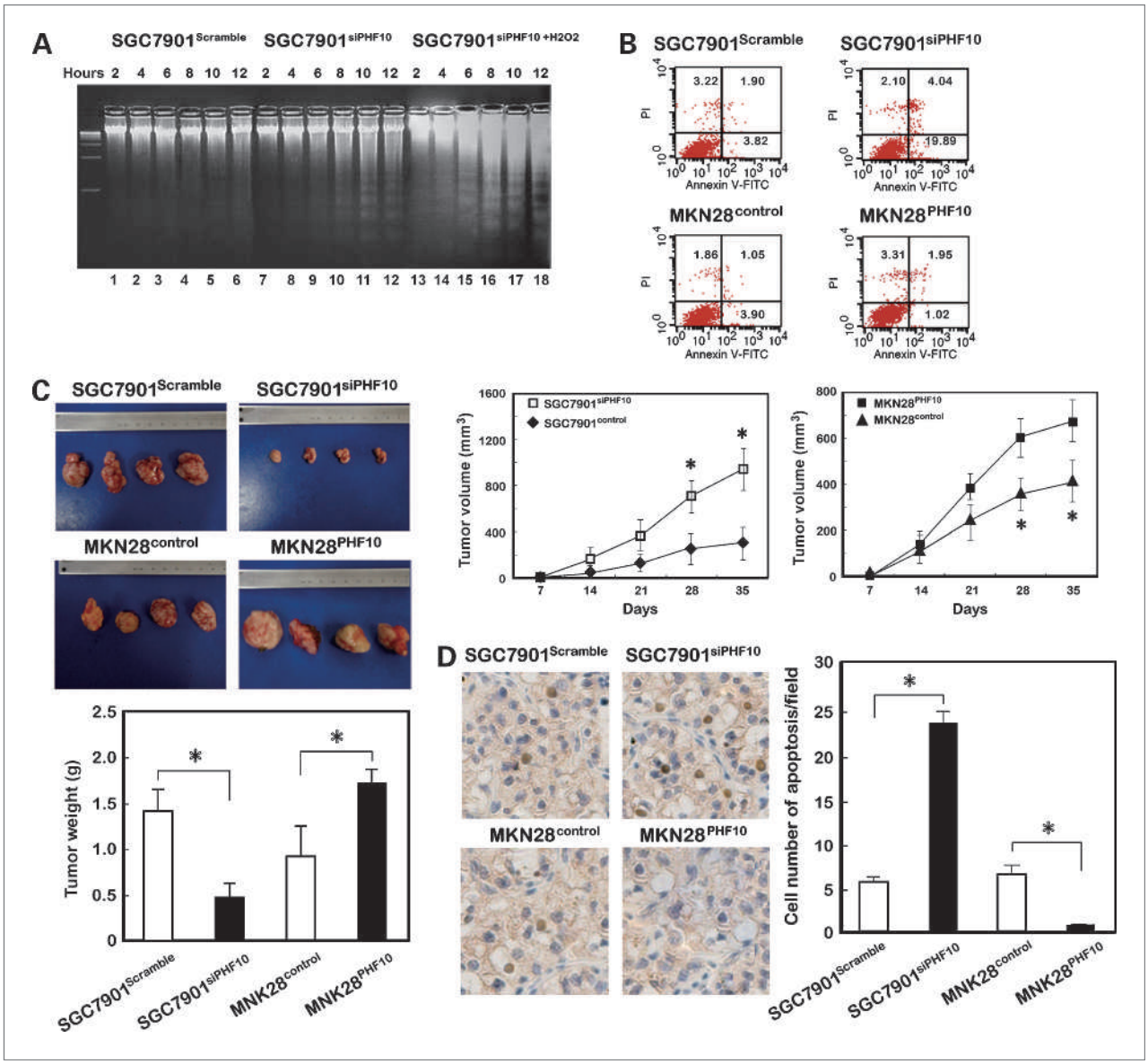

Figure 1. PHF10 knockdown decreased cell viability and increased sensitivity to apoptosis. A, DNA fragmentation in SGC7901 cells transfected with the indicated plasmids and treated with or without H_2O_2 for the indicated times. B, FACS analysis of propidium iodide (PI)–negative and Annexin V–positive cells transfected with plasmids as indicated for 12 h. C, SGC7901^{siPHF10} cells with reduced PHF10 protein expression grew smaller tumors in nude mice than SGC7901^{Scramble} [top, representative tumor sizes (left) and growth kinetics (middle and right); bottom, averages of tumor weight], whereas MKN28^{PHF10} cells grew larger tumors than the control cells. Bars, SD. *, $P < 0.05$ versus control. D, a TUNEL assay was done on tumor specimens from nude mice. Right, representative image; left, average number of apoptotic cells per field.

(Psi-V). The shRNA used in this study is relatively the most effective one after examined via q-RT-PCR. All constructs were verified by sequencing.

Transfection and selection of stable transfectants

Cells were transfected at 60% to 70% confluence with pFLAG-CMV4 empty vector (pFLAG-V), pFLAG-CMV4-PHF10 (pFLAG-PHF10), Psilencer2.0 vectors carrying shRNA for PHF10, and the scrambled sequence using Lipo2000 (Roche) according to the manufacturer's protocol. Cells were cultured in medium supplemented with 1 mg/mL G418 (Promega) for 4 weeks and then maintained in medium containing 350 μg/mL G418. Stably transfected clones were then picked. Psilencer2.0-1333 (Psi-1333) was selected as the most effective vector used for PHF10 knockdown. Clones that had been stably transfected with pFLAG-V and Psilence2.0-scrambled were used as controls.

Induction of s.c. tumors in nude mice

Animal studies were conducted with the approval of the Committee on the Ethics of Animal Experiments at our institution. To examine the growth rate of gastric cancer cells expressing upregulated and downregulated PHF10 in animals, 4×10^6 stably transfected cells in 0.2 mL PBS were injected s.c. into the dorsal flank of 5-week-old male severe combined immunodeficient mice. Mice were monitored weekly, and the tumor volume was measured with a linear caliper. Tumor volume was calculated using the following equation: volume = (width + length)/2 × width × length × 0.5236. Mice were sacrificed at 5 weeks after inoculation of the cells. All tumor grafts were dissected, weighed, harvested, fixed, and embedded. Histologic procedures and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays were done as described previously (7). All experiments were repeated twice.

DNA cleavage analysis

To detect DNA fragmentation, we first did DNA fragmentation assays. Briefly, cells were collected at indicated

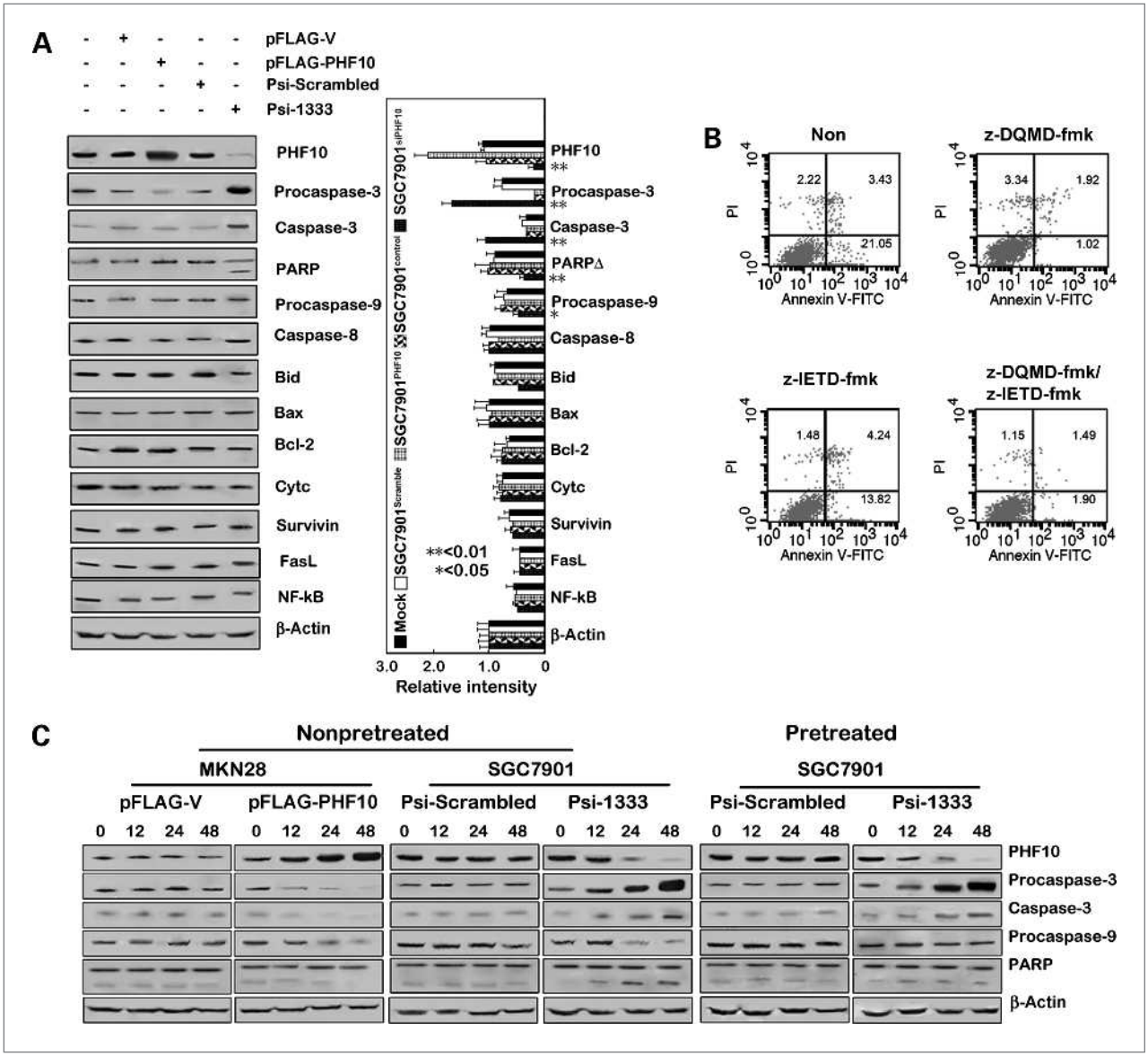

Figure 2. Caspase-3 was a major modulator of apoptosis induced by PHF10 shRNA, which was antagonized by a caspase inhibitor. A, left, effect of PHF10 on the apoptotic protein profile was analyzed using β-actin as a loading control; right, the protein level is quantified. Δ designates intact PARP. *, $P < 0.05$; **, $P < 0.01$. Columns, mean of three experiments; bars, SD. B, cell apoptosis analysis using the indicated caspase inhibitors in SGC7901 cells transfected with Psi-Scrambled/Psi-1333 for 24 h. C, similar to A, protein profile analysis was conducted over a time course. Pretreated and nonpretreated in the figures separately mean pretreated and nonpretreated with the caspase-3 inhibitor z-DQMD-fmk.

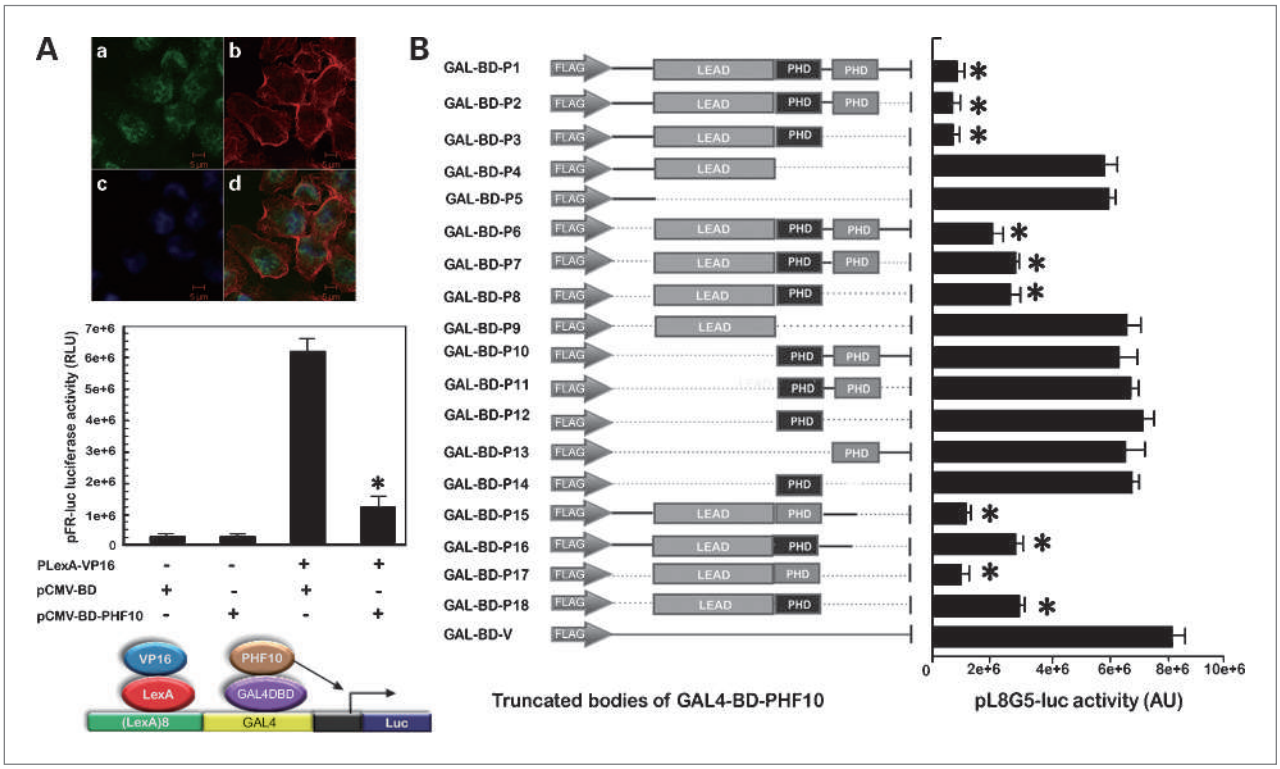

Figure 3. PHF10 acts as a transcriptional repressor. A, top, localization of PHF10 in gastric cancer cells. A coverslip seeded with SGC7901 cells was double stained with PHF10 antibody to permit detection of PHF10 (a) and phalloidin to permit detection of actin (b). A DNA-binding dye, DAPI, was used to counterstain the DNA (c). In SGC7901s cells, PHF10 accumulated in nuclear domains that colocalize with dense regions of DAPI staining (d), indicating association with chromatin. Middle, histogram showing transcriptional activity analysis of PHF10 fusion proteins in 293T cells. Columns, mean of three experiments; bars, SD. *, $P < 0.05$ versus control, cotransfection of pCMV-BD-V and pLexA-VP16. Diagram at the bottom depicts the mechanisms underlying this assay. B, transcriptional activity analysis of full-length and truncated PHF10 fusion proteins in 293T cells. Columns, mean of three experiments; bars, SD. *, $P < 0.05$ versus cotransfection of pCMV-BD-V and pL8G5 luciferase, which was used as control.

times, suspended in lysis buffer [10 mmol/L Tris-HCl (pH 8.0), 10 mmol/L NaCl, 10 mmol/L EDTA, 100 mg/mL proteinase K, 1% SDS], and incubated overnight at 55°C. DNA was extracted with phenol/chloroform (1:1) followed by precipitation with 0.3 mol/L sodium acetate in ethanol. DNA was then washed with 70% aqueous ethanol. The DNA pellet was resuspended in TE buffer [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA] and incubated for 2 hours at 37°C with 0.5 mg/mL DNase-free RNase (Roche). After phenol/chloroform reextraction by the same method, DNA was analyzed on a 1% (w/v) agarose gel supplemented with ethidium bromide. Fluorescence-activated cell sorting (FACS) analysis for each transfectant was done as described previously (20). Additionally, to detect the morphologic changes in apoptosis, we conducted TUNEL assays on cells seeded on coverslips using the Apo-BrdU *In Situ* DNA Fragmentation Assay kit according to the manufacturer's instructions. Similar to confocal analysis, phalloidin and DAPI are used to stain actin and DNA, respectively, and anti-bromodeoxyuridine-FITC was used to detect Br-dUTP ligated to the cleaved DNA.

Luciferase assays

Cells were cotransfected with L8G5 luciferase, pLexA-VP16, and pCMV-BD-PHF10 or other PHF10 truncated bodies according to the protocol provided for the Dual Luciferase kit (Promega) to investigate the effect of PHF10 on transcriptional activity. Determination of luciferase activity was done with a luminometer (Turner Designs TD-20/20). To precisely determine the region involved in the interaction between the *caspase-3* promoter region and PHF10, cells were also cotransfected with fragments of pGL4 *caspase-3* and other truncated pFLAG-PHF10 fusion constructs along with pRL-TK to investigate the effects of individual domains of PHF10 on the transcriptional activity of *caspase-3*.

Chromatin immunoprecipitation assay

For these experiments, SGC7901 and MKN28 cells were subjected to chromatin immunoprecipitation (ChIP) with the ChIP Assay kit (Upstate Cell Signaling Solutions). Briefly, cross-linking of proteins with DNA was done with 4% formaldehyde at 37°C for 15 minutes and quenched with glycine. Cell lysates were sonicated (Branson Sonifier) to shear the DNA to 400- to 1,000-bp

length fragments. Chromatin samples were then precleared with a salmon sperm DNA/protein A agarose 50% slurry for 30 minutes at 4°C and immunoprecipitated overnight in the absence of antibody or with an anti-PHF10 antibody or an anti-FLAG antibody (M5; Sigma). The 3′-untranslated region (3′-UTR) region of the *caspase-3* gene was amplified as a control for the ChIP assay using the following primer set (designated as P1): 5′-TCCCAAGTCCTCGCAGAA-3′ (sense) and 5′-TGGCCTTAAAGAACTCATT-3′ (antisense). The region between −270 and −170 nucleotides of the *caspase-3* promoter was amplified with two pairs of primers (designated as P2 and P3): P2, 5′-TTTCCAAGTCTCCCTCAATT-3′ (sense) and 5′-GGATTTGAAATCTAGG-3′ (antisense); P3, 5′-GGAAGACCTAGATTTC-3′ (sense) and 5′-CGTCTGCACTGCTTCCG-3′ (antisense). The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

Statistical analysis

Each experiment was done independently at least twice with similar results. Findings from one representative experiment are presented. Results are expressed as mean ± SD. Significant differences from *in vitro* and *in vivo* experiments were assessed with the Student's *t* test (two-tailed). Analysis was done using the Statistical Package for the Social Sciences statistical software (version 11.05; SPSS, Inc.). A P value of <0.05 was deemed significant.

Results

Expression of PHF10 is associated with cellular apoptosis

The expression levels of PHF10 in SGC7901 and MKN28 cells were drastically different (data not shown). SGC7901 cells, which were derived from a poorly differentiated gastric cancer, had a relatively higher abundance of PHF10

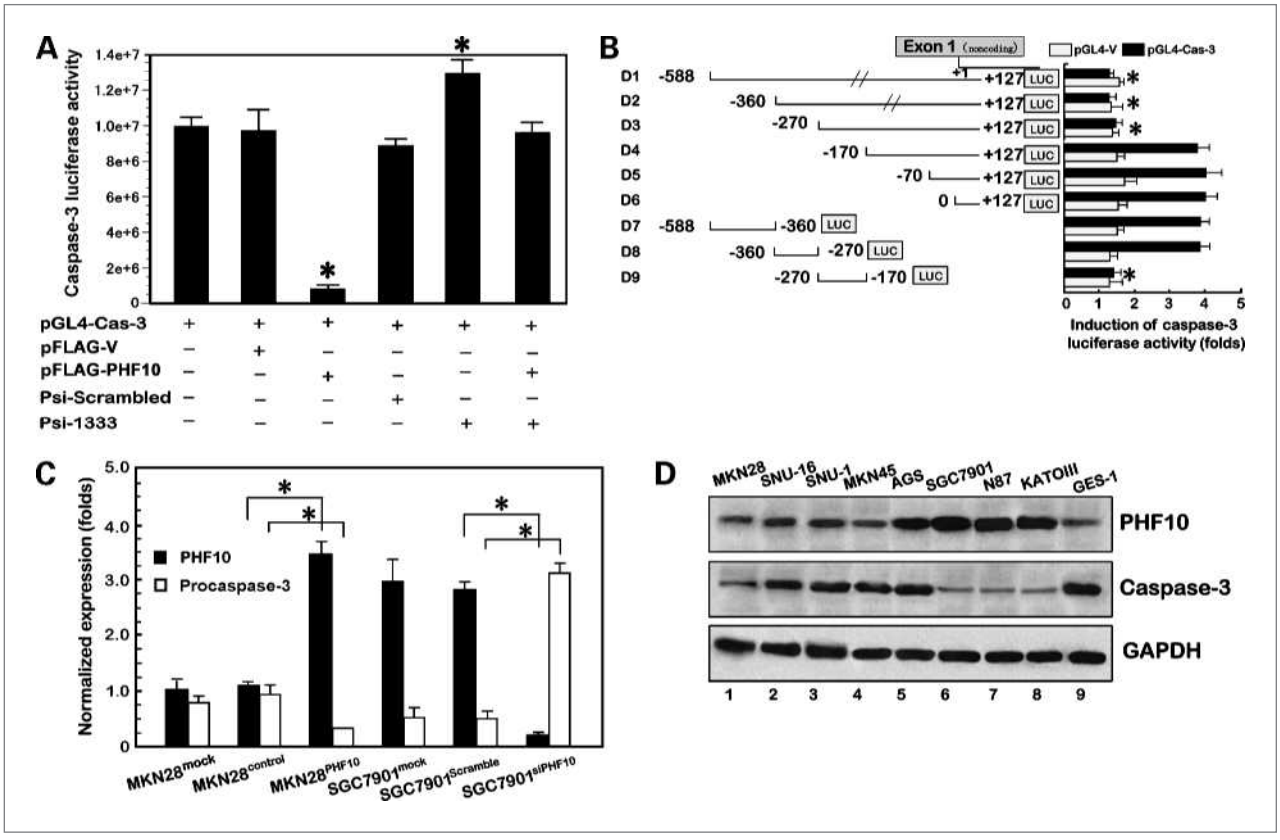

Figure 4. A limited region of PHF10 transcriptionally modulates *caspase-3*. A, PHF10 suppresses transcriptional activation of procaspase-3 in 293T cells. PHF10 suppresses procaspase-3 transcriptional activity, which was reversed or abated by shRNA-mediated knockdown of PHF10. B, deletion analysis of *caspase-3* promoter activity in 293T cells. The 5′ and 3′ end points of each deletion construct are indicated. Each construct containing deletions of *caspase-3* promoter was transiently transfected along with pFLAG-PHF10 plasmid into cultured cells and assayed for luciferase activity. Luciferase activity was normalized to activities of the empty vector of pGL4-luc, expressed as fold difference. Transfections were done in three individual experiments. Bars, SD. $P \leq 0.05$ was considered significant. C, results of q-RT-PCR analyses of PHF10 and procaspase-3 confirm the repression of *caspase-3* by PHF10. Transcriptional activities of *caspase-3* were reversed or abated by siRNA-mediated knockdown of PHF10 in SGC7901 cells. The opposite effect was achieved in $MKN28^{PHF10}$ cells. Expression levels of selected genes (*X* axis) analyzed by q-RT-PCR were quantified. The *Y* axis represents the gene expression level normalized to GAPDH for cells transiently transfected with the indicated plasmids for 24 h. These results represent at least three RNA samples per experimental condition run in triplicate. D, Western blotting analysis of PHF10 and procaspase-3 in nine gastric cancer cell lines and GES-1 cells.

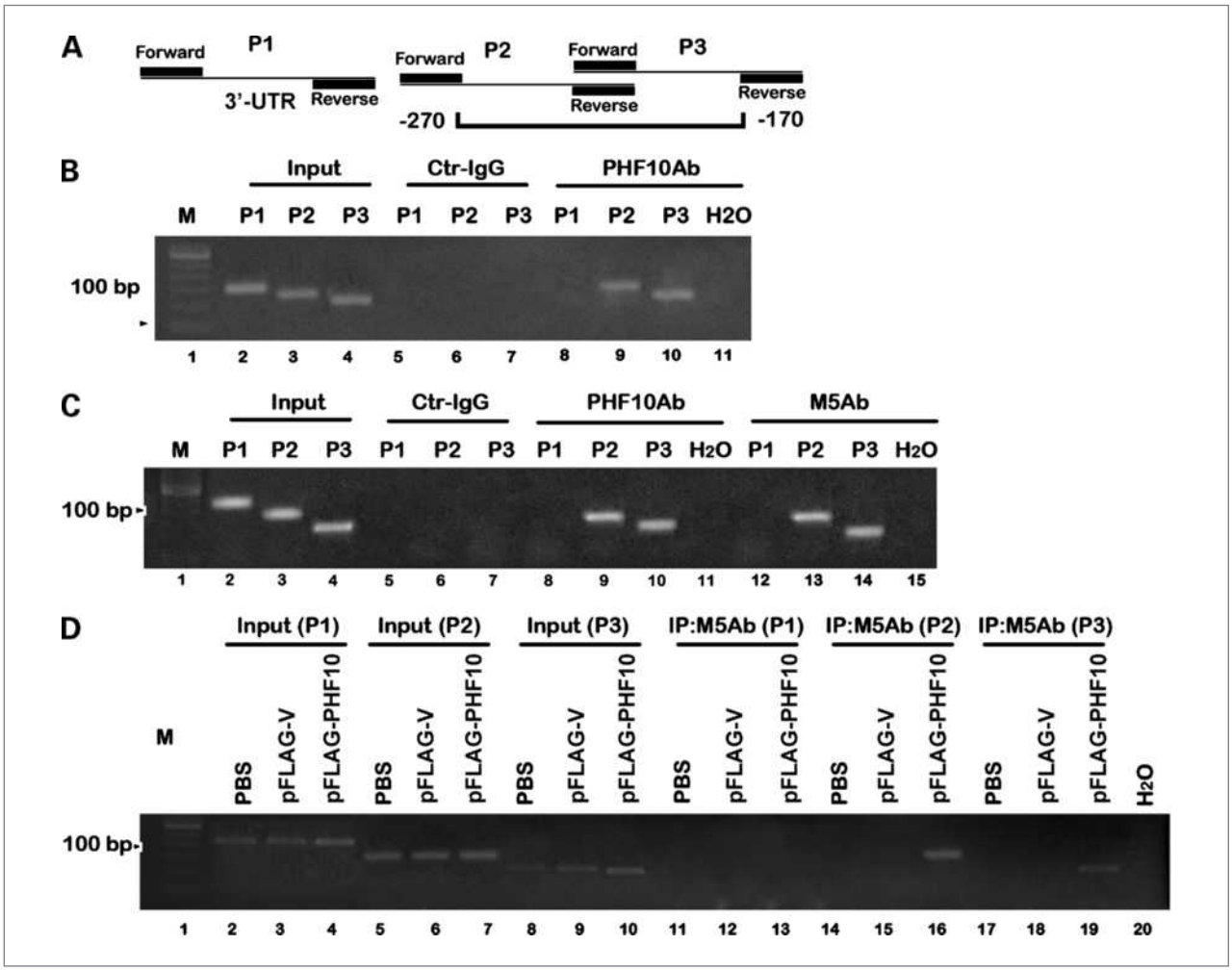

Figure 5. PHF10 binds to the *caspase-3* promoter. A, diagrams illustrating the three pairs of primers used for ChIP analysis. B, binding of endogenous PHF10 to the *caspase-3* promoter. Chromatin fragments were prepared from SGC7901 cells, and the ChIP assay was done using a control IgG or an anti-PHF10 antibody. Chromatin fragments obtained without IgG or specific antibody were used as input controls. PCR was done using each of three sets of primers (P1, for *caspase-3* 3′-UTR as a control; P2 and P3 for two fragments belonging to the region carrying the putative PHF10-binding motif) as described in Materials and Methods. C and D, binding of exogenous PHF10 to the *caspase-3* promoter. C, chromatin fragments for the ChIP assay were prepared from MKN28^{PHF10} cells using input, nonspecific IgG as control. D, the ChIP assay was done on MKN28^{PHF10} cells with MKN28 (PBS), MKN28^{control} as control. PCR was conducted using each of three sets of primers (P1 for lanes 2–4 and 11–13; P2 for lanes 5–7 and 14–16; P3 for lanes 8–10 and 17–19).

protein than MKN28 cells, which were derived from a lesion with moderate malignancy. To explore the role of PHF10 on cell viability, vectors were used to reduce or overexpress PHF10 expression in SGC7901 and MKN28 cells (SGC790^{siPHF10} and MKN28^{PHF10}), respectively. As shown in Fig. 1A, knockdown of PHF10 expression in SGC7901 cells for 10 or 12 hours dramatically increased cell apoptosis as determined by internucleosomal DNA cleavage. Moreover, DNA cleavage was further promoted by H_2O_2 treatment in SGC7901^{siPHF10} cells (Fig. 1A). Similar results were also observed using a FACS assay of apoptosis by propidium iodide and Annexin V staining (Fig. 1B), consistent with TUNEL assays (Supplementary Fig. S1). These results suggest that the reduced expression of PHF10 induced apoptosis in SGC7901 cells. Next, to determine whether expression of PHF10 plays a role in gastric cancer progression *in vivo*, SGC790^{siPHF10}, MKN28^{PHF10}, and respective controls were s.c. implanted into the dorsal flanks of nonobese diabetic/severe combined immunodeficient mice. Tumors generated from SGC7901^{siPHF10} were significantly smaller than tumors derived from the control cells (490 ± 150 mg versus 1,480 ± 260 mg; Fig. 1C). In contrast, tumor growth was dramatically increased in tumors established from MKN28^{PHF10} cells that had overexpressed PHF10 compared with MKN28^{control} (850 ± 370 mg versus 1,750 ± 150 mg; Fig. 1C). To determine whether tumor growth inhibition in SGC7901^{siPHF10} is due to the induction of apoptosis *in vivo*, TUNEL assays of tumor tissues were done. The results revealed that knockdown of PHF10 expression induced apoptosis in SGC7901 cells (6 ± 1 versus 23 ± 2 cells per field). In parallel, overexpression of PHF10

decreased apoptosis of MKN28 cells compared with the control cells (7 ± 2 versus 1 ± 2 cells per field; Fig. 1D).

Caspase-3 is a major modulator of apoptosis induced by PHF10 shRNA

Based on these *in vitro* and *in vivo* studies, we further identified potential downstream targets of PHF10. Analysis of protein profiles by Western blotting assays of SGC7901^{siPHF10} cells showed that, in line with previous findings, procaspase-3 expression was decreased. This decrease in expression was followed by generation of a 17-kDa active form, caspase-3, and cleavage of its substrates, PARP, which appeared with significantly reduced intensity or derivation of additional bands, as expected (Fig. 2A, right and left). However, procaspase-9 displayed less obvious but detectable degradation. As shown in Fig. 2A, there was a slight but detectable accumulation of procaspase-8, which activates Bid and facilitates cytochrome *c* release; however, caspase-8 substrate, Bid, displayed no significant change. In addition, the expression levels of Fas-L, Bcl-2, cytochrome *c*, NF-κB, and survivin were not significantly altered.

Because siPHF10 could induce apoptosis in SGC7901, we sought to determine whether caspase inhibitors antagonized the effect of siPHF10. For this experiment, cells in the exponential growth phase were transiently transfected with shRNA for PHF10 or the control vector and sequentially treated with caspase inhibitors. Treatment of cells with a caspase-3 inhibitor, z-DQMD-fmk (Sigma), or a combination of z-DQMD-fmk and a caspase-8 inhibitor, Z-IETD-fmk (Calbiochem), at indicated doses significantly reduced the percentage of apoptotic SGC7901^{siPHF10} cells as determined by FACS analysis (Fig. 2B). However, Z-IETD-fmk alone did not completely inhibit apoptosis, which suggests that caspase-8 might also contribute to apoptosis in SGC7901^{siPHF10} cells. In addition, inhibitors for caspase-1, caspase-2, caspase-5, caspase-6, and the proteasome were ineffective at rescuing the time-dependent loss of viability induced by shRNA (data not shown).

Time-dependent procaspase-3 accumulation and the resultant generation of caspase-3 and cleavage of caspase-3 substrates were evident in SGC7901 ^{siPHF10} cells without pretreatment with z-DQMD-fmk, whereas the opposite results were observed in MKN28^{PHF10} cells. In the context of z-DQMD-fmk, procaspase-3 was significantly accumulated but with little alteration in its substrates in SGC7901 ^{siPHF10} cells (Fig. 2C; Supplementary Fig. S2A), except for caspase-9. We found that caspase-9 varied time dependently in nonpretreated cells, whereas this trend disappeared when SGC7901 ^{siPHF10} cells are pretreated with z-DQMD-fmk. The variation trend might come from either transcriptional repression or induced cleavage by overexpressed caspase-3, consistent with the fact that caspase-9 acts as one substrate of caspase-3. However, we also noticed that enforced expression of PHF10 in MKN28 cells (Fig. 2C) led to the downregulation of procaspase-9 in a time-dependent manner. Other than transcriptional regulation, one explanation may be that PHD finger proteins could function as an E3 ligase in the ubiquitination complex although this depends on further conclusions (21).

To investigate the effect of PHF10 overexpression on stimuli-induced apoptosis, MKN28 cells were transfected with pFLAG-PHF10 for 12 hours, washed thrice, and then exposed to Fas, H_2O_2, or etoposide. As shown in Supplementary Fig. S2B, pFLAG-PHF10 transfection significantly decreased the ratio of the apoptotic MKN28^{PHF10} cells induced by Fas, H_2O_2, or etoposide, whereas no effect on cellular apoptosis was detected in pretreated MKN28^{control} cells, which further confirms that PHF10 might antagonize the common apoptosis-related molecule caspase-3.

PHF10 is a transcriptional repressor

As shown in Fig. 3A (top), PHF10 protein labeled with anti-mouse FITC was heterogeneously distributed in the nucleus. To examine the potential function of PHF10 in transcriptional regulation, we cotransfected 293T cells with pCMV-BD-PHF10, pL8G5-luc, and pLexA-VP16. As shown in Fig. 3A (middle), coexpression of GAL4-PHF10 with pLexA-VP16 inhibited VP16-activated L8G5 luciferase activity by 75%, suggesting that PHF10 may function as a transcriptional repressor. Together, these observations provide evidence to indicate that PHF10 may function as a transcriptional repressor of *caspase-3*.

As shown in Fig. 3B, expression of GAL4-BD-P2 (without tail) inhibited L8G5 luciferase activity by ~89%, similar to the full-length protein GAL4-BD-P1, suggesting that tail sequence functioned little in transcriptional activity. Repression of GAL-BD-P3 (without the second PHD domain) on pL8G5 was slightly but more significantly presented than both GAL-BD-P4 (without both PHD domains) and GAL-BD-P5 (head without domains). These observations confirmed that both terminal sequences beside the tandem repeat three domains exerted almost no function in transcriptional repression. Similar results were obtained from experiments that used GAL-BD-P3, GAL-BD-P6, GAL-BD-P7, GAL-BD-P8, GAL-BD-P15, GAL-BD-P16, GAL-BD-P17, and GAL-BD-P18, suggesting that the two PHD motifs and the leading chain (LEAD domain) together represent the basal repression domain of PHF10. In addition, the observation that combination of any one single PHD domain and the LEAD domain did not contribute as much as the tandem repeat containing three domains in transcriptional suppression suggests that the second PHD domain exerts a greater effect on luciferase activity repression than the first PHD domain. That no obvious repressive activity was observed from GAL-BD-P9 to GAL-BD-P14 further suggested that deletion of any one PHD domain and/or the LEAD domain would completely abolish the transcriptional suppressive activity of PHF10.

A limited region of PHF10 transcriptionally modulates caspase-3

Procaspase-3 accumulated in SGC7901^{siPHF10} pretreated with z-DQMD-fmk without evident variation of

substrates, which is consistent with the substrates being downstream mediators of procaspase-3. Because PHF10 functions in transcriptional regulation, this finding indicates that procaspase-3 may be transcriptionally modulated by PHF10. To confirm this and distinguish the binding motif for PHF10 on *caspase-3*, we did systematic luciferase assays in 293T cells. PHF10 repression of *caspase-3* promoter activity was confirmed by cotransfecting 293T cells with *caspase-3* luciferase and wild-type PHF10 or the shRNA for PHF10 (Fig. 4A). The promoter activity of *caspase-3* was decreased by 3.5-fold in wild-type PHF10-transfected 293T cells compared with cells transfected with the control vector (pFLAG-V), which is consistent with the presence of a negative regulatory element in the region of *caspase-3*. Opposite results were observed following combined transfection of shRNA and deletions of *caspase-3*. As shown in Fig. 4B, loss of the regions spanning ¡©588 to −270 bp (D1–D3) in the *caspase-3* construct did not affect the suppression of *caspase-3* promoter activity by PHF10; however, further deletion beyond −170 completely abolished the repression of *caspase-3* luciferase activity, suggesting that the D9 region (−270 to −170) contains negative regulatory elements that are required for PHF10 to repress *caspase-3* promoter activity. Many transcription factors have more than one binding site in the promoter region. Therefore, deletions in D7 to D8 (¡©588 to −270) and D4 to D6 (−170 to +127) were also included in our assays. Based on the results of these assays, we conclude that the regions between −270 and −170 contained the repressor site(s).

To rule out a minimal effect due to use of an individual cell line, we did additional luciferase assays by cotransfecting CHO, AGS, SGC-7901, CRL5974, GES-1, and MKN28 cells (Supplementary Fig. S3A). Results from all these cells were similar to those obtained in 293T cells. However, in AGS and SGC-7901 cells, the luciferase activities were significantly different from all other cell lines investigated. High expression of PHF10 in both cell lines might serve as one possible explanation. Furthermore, results in Supplementary Fig. S3B, which were consistent with the L8G5 luciferase assay system results, confirmed that the fragment of the PHF10 protein encompassing three domains in tandem was essential for PHF10 to interact with the target sites. Caspase-3 mRNA levels (tested via q-RT-PCR) were increased after knocking down normal expression of PHF10 by shRNA (Fig. 4C) but were decreased following pFLAG-PHF10 transfection. Furthermore, q-RT-PCR experiments showed alteration of procaspase-3 expression over time (data not shown). The PHF10 protein was expressed at different levels by different cell lines. More specifically, PHF10 was expressed at higher levels in AGS, SGC7901, N87, and KATOIII cell lines than in SNU-1, SNU-16, MKN28, MKN45, and GES-1 cell lines (Fig. 4D). Most gastric cell lines showed an inverse correlation between PHF10 and *caspase-3* expression, which was consistent with the speculation that PHF10 transcriptionally represses *caspase-3* expression. However, a highly similar level of caspase-3 and PHF10 expression was observed in AGS and MKN28 cell lines, which suggests the presence of additional mechanisms contributing to tumorigenesis in these cell lines.

PHF10 binds to the *caspase-3* promoter

We sought to determine whether PHF10 binds to the promoter region of the *caspase-3* gene *in vivo* using ChIP assays. The PCR forward and reverse primers flanking the essential regulatory region are shown in Fig. 5A. Using these primers for the region from −270 to −170, the two fragments derived by PCR overlapped with each other so as not to neglect any possible binding sites. First, to determine whether endogenous PHF10 binds to the *caspase-3* promoter, we conducted ChIP assays on SGC7901 cells, which overexpressed PHF10 (Fig. 5B). Compared with control IgG, we found that PHF10 was clearly bound to caspase-3 promoter sequence using anti-PHF10 antibody (Fig. 5B, lanes 9 and 10). Next, to determine whether exogenous PHF10 (i.e., that produced via gene transfection) binds to the *caspase-3* promoter, we immunoprecipitated chromatin fragments from $MKN28^{PHF10}$ with control IgG and a specific anti-PHF10 or anti-FLAG antibody and found that the DNA fragment of the *caspase-3* promoter was immunoprecipitated by the specific anti-PHF10 or anti-FLAG antibody (Fig. 5C, lanes 9, 10, 13, and 14), whereas the DNA fragment of the *caspase-3* 3′-UTR was not immunoprecipitated by the specific anti-PHF10 or anti-FLAG antibody. In Fig. 5D, exogenous PHF10 clearly bound to the *caspase-3* promoter (lanes 16 and 19) but not to 3′-UTR of *caspase-3* (lane 13) compared with $MKN28^{control}$ and $MKN28^{PBS}$ cells. Thus, both exogenous and endogenous PHF10 bound to the *caspase-3* promoter in gastric cancer cells *in vivo*.

Discussion

Cancers arise from the rare simultaneous acquisition of the two cooperating conditions that permit deregulated cell proliferation and suppressed apoptosis (22, 23). This event, like cancer itself, happens rarely because the two processes are obligatorily interdependent. Deregulated proliferation on its own triggers expeditious cell death, whereas suppression of apoptosis confers no selective advantage in the absence of cell proliferation. Therefore, these two conditions must arise together in the same cell at the same time. This cooperative hypothesis for the emergence of cancer not only is in keeping with the well-characterized synergy exhibited by oncogenes but also has the great benefit of solving a great conundrum of vertebrate biology (22). To date, the extrinsic and intrinsic apoptotic pathways that ultimately lead to activation of effectors (caspase-3, caspase-2, and caspase-7) have been well characterized. The extrinsic pathway is initiated by ligation of death receptors (CD95/Fas, tumor necrosis factor receptor, and tumor necrosis factor–related apoptosis-inducing ligand receptor) to stimulate activator caspases (caspase-8 and caspase-10), which in turn cleave and activate effectors. The intrinsic pathway

requires disruption of the mitochondrial membrane and release of proteins. Disruption of this pathway is extremely common in cancer cells (24). Among the apoptosis participants, the *caspase-3* gene, a key factor in the apoptosis cascade, is widely expressed in tissues and cell lines and has been shown to have negative relationship with cancer malignant behaviors. Its expression is altered during the induction of apoptosis by drugs and during differentiation and development (25). Despite these studies, the mechanism by which the *caspase-3* gene is transcriptionally modulated in cancers remains unknown.

To date, a wide variety of transcription factors have been shown to be involved in the modulation of *caspase-3* expression. Candidate transcription factors involved in caspase-3–independent apoptotic pathways have surfaced from recent studies. Previous studies have indicated that an Ets element for Sp1-binding functions in the regulation of *caspase-3* promoter activity (26). In addition, several other putative binding sites for the Sp1 family identified in the *caspase-3* promoter have been shown to cooperate with p73 to activate the *caspase-3* promoter (19, 26). In rats, a hypoxia-inducible factor-1–binding site has been identified in the *caspase-3* promoter (27). Recently, further evidence has shown that the family of PHF proteins is overexpressed in many types of malignant tumors and might be involved in the progression of cells toward malignancy (28–30). PHD zinc finger proteins probably constitute the largest individual family of such nucleic acid–binding proteins; however, few target genes have been identified for PHD zinc finger proteins. In this study, we isolated and characterized a novel human PHD-containing zinc finger gene, PHF10. shRNA-mediated knockdown of PHF10 expression inhibited tumor growth and induced apoptosis both *in vivo* and *in vitro*, and overexpression of PHF10 did the opposite. These observations are supported by our findings that downregulation of PHF10 induced caspase-3 accumulation and activation of its downstream substrates, whereas PHF10 overexpression decreased the abundance of caspase-3. By assessing the protein profile of the apoptosis pathway, we identified a negative correlation between PHF10 and caspase-3 at the protein level. As we know, function of caspase-3 on its substrates relied on both the form activation and adequate amount. However, previous studies have given evidence that upregulation of the procaspase-3 could lead to the induction of apoptosis factor activation and thus trigger apoptosis, consistent with our results (19, 27, 31, 32).

Based on the results stated above, the next question to be addressed is how PHF10 modulates caspase-3. Indeed, the homeodomain (PHD) has been previously shown to interact with specific DNA sequences as a DNA-binding unit to function in maintaining chromatin stability (29, 30). In this investigation, using systematic transcriptional analysis, we showed that PHF10 might function as a transcriptional repressor, in which the LEAD domain and the PHD domains act in tandem. Knockdown of PHF10 induced apoptosis and gave rise to the accumulation of caspase-3 and activation of its substrates, which raised the possibility that PHF10 might participate in gastric cancer tumorigenesis by its function as a transcriptional repressor of *caspase-3*.

To extend these observations, we used a combination of transcriptional assays and showed that PHF10 seemed to be a transcriptional repressor, which led to the downregulation of caspase-3 protein levels. PHF10 bound to the promoter region of *caspase-3*; the intact PHF10-binding site in the *caspase-3* promoter region is required for the full induction of *caspase-3* promoter activity by PHF10. Furthermore, considering the dynamic nature of the interaction between PHF10 and the *caspase-3* promoter, we conducted assays to determine the transcriptional activities of truncated forms of PHF10. We found that the integrity of the three domains in tandem was essential for PHF10 to achieve its role in transcriptional regulation, which is consistent with observations in the L8G5-luc system (33). The present mechanistic investigations found that downregulation of PHF10 in gastric cancer cells led to significant induction of caspase-3 mRNA and protein and that the opposite trend was achieved in gastric cancer cells with PHF10 overexpression. Evidences are beginning to emerge that some PHD fingers can bind to nucleosomes and chromatin binding is a more widespread property of PHD fingers (34). The role of the PHD fingers seems to be to consolidate, or to strengthen, a separate chromatin-binding activity of either the same or an associated protein, although the interactions between PHD fingers and chromatin need further biochemical characterization (29, 35). PHD fingers also bind to proteins other than histones and rely on the structural integrity of the domain, as originally proposed. Findings that PHD domain participated in transcriptional modulation of caspase-3 in our study suggested that this activity could be a property of this slightly unusual PHD module. This might reveal whether PHD fingers can interact simultaneously with nucleosomes, specific protein ligands, and DNA sequence, thereby tethering these ligands to interaction complex. Anyway, the actual relationship between them needs further studies. Because the regulation of gene expression is complex, involving transcriptional regulators, as well as DNA methylation, histone acetylation, methylation, sumoylation, and other modifications, these results may provide only a limited understanding of PHF10 as a transcriptional regulator of apoptosis in gastric cancer. Moreover, further analysis of the role of PHF10 in human cancers is necessary to assess the involvement of PHF10 in tumorigenesis. In summary, our findings support the notion that PHF10 acts as an oncogene, and the present report provides the basis to develop PHF10 as a potential therapeutic target for the treatment of gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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