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Involvement of histone deacetylation in MORC2-mediated down-regulation of carbonic anhydrase IX

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ABSTRACT

Carbonic anhydrase IX (CAIX) plays an important role in the growth and survival of tumor cells. MORC2 is a member of the MORC protein family. The MORC proteins contain a CW-type zinc finger domain and are predicted to have the function of regulating transcription, but no MORC2 target genes have been identified. Here we performed a DNA microarray hybridization and found *CAIX* mRNA to be down-regulated 8-fold when MORC2 was overexpressed. This result was further confirmed by northern and western blot analysis. Our results also showed that the protected region 4 (PR4) was important for the repression function of MORC2. Moreover, MORC2 decreased the acetylation level of histone H3 at the *CAIX* promoter. Meanwhile, trichostatin A (TSA) had an increasing effect on *CAIX* promoter activity. Among the six HDACs tested, histone deacetylase 4 (HDAC4) had a much more prominent effect on *CAIX* repression. ChIP and ChIP Re-IP assays showed that MORC2 and HDAC4 were assembled on the same region of the *CAIX* promoter. Importantly, we further confirmed that both proteins are simultaneously present in the PR4-binding complex. These results may contribute to understanding the molecular mechanisms of *CAIX* regulation.

INTRODUCTION

Carbonic anhydrase IX (CAIX), also called MN or G250, is a member of the carbonic anhydrase family that catalyzes the reversible hydration of carbon dioxide ($H_2O + CO_2 \Leftrightarrow H^+ + HCO_3^-$) and as such are vital to many biological and physical functions. CAIX is a transmembrane isozyme and has been implicated in the control of tumor cells growth and survival (1). Because CAIX expression correlates with lowered O_2 tension in tumors, CAIX is proposed as an intrinsic marker of hypoxia (2). CAIX may be involved in early gastric carcinogenesis as CAIX deficient mice show increased cellular proliferation and develop gastric hyperplasia (3). And it has been reported that the expression of CAIX correlates with an extremely poor prognosis in gastric adenocarcinomas (4). In human malignancy, overexpression of CAIX is consistently seen in a strikingly high proportion of carcinomas of the cervix (5) and clear cell carcinoma of the kidney (6), and, to a lesser degree, in other types of human tumors, such as carcinomas of the breast (7,8), head and neck (9,10), lung (11) and tumors of the brain (12,13). However, expression of CAIX is low or even lost in most gastric cancers (14,15). In addition, a subgroup of gastric cancers retain CAIX expression in cancer cells at the invasion front, implying that increased CAIX expression may contribute to invasion and thus advanced disease and tumor progression in a subset of gastric cancers (16). Therefore, in order to understand the roles of *CAIX* gene expression in the carcinogenesis and progress of gastric cancer, it is essential to gain a more intensive insight into the expression control of this gene.

The promoter of *CAIX* gene has been characterized and localized in the region between −173 and +31 bp in respect to the transcription start site (17). Hypoxia activates *CAIX* gene transcription through hypoxia inducible factor-1 (HIF-1), which binds to the hypoxia-response element (HRE) in the *CAIX* promoter immediately

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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upstream of the transcription start site (18). There are five protected regions (PRs) within the *CAIX* promoter, of which PR1 and PR2 are the most critical for transcriptional activity (17). Transcription factors Sp1/Sp3 are described to up-regulate *CAIX* by binding to the PR1 and PR5 position of *CAIX* promoter (19,20). HIF-1α and Sp1, in combination with CBP/p300, are identified as the crucial elements for CAIX expression in clear cell renal cell carcinoma (21). Although a repressor complex, which directly contacts with PR4, is found to negatively regulate *CAIX* transcription (17), the composition of the repressor complex and the repression mechanism remain unknown.

MORC2 (microrchidia 2) is a member of the MORC protein family characterized by conserved structures consisting of an ATPase domain, a zinc finger type CW domain, a nuclear localization signal (NLS) and coiled-coil domains. There are four predicted MORC family proteins (MORC1, MORC2 [KIAA0852, ZCWCC1], MORC3 [KIAA0136, ZCWCC3, NXP-2] and MORC4 [ZCWCC2]) in human. The MORC family proteins belong to CW-domain-containing subfamilies I (MORC1 and MORC2) or IX (MORC3 and MORC4) (22). MORC1 is expressed specifically in male germ cells (23), whereas MORC2 and MORC3 are ubiquitously expressed. A recent study has showed that MORC3 regulates p53 activity by localization to a nuclear subdomain (24). And it is reported that MORC4 is a potential biomarker as it is highly expressed in a subset of diffuse large B-cell lymphomas (25).

MORC family proteins are conserved in higher eukaryotes, predicting an important function for them in the biology of multicellular organisms, however, few studies have been made to identify the possible molecular function of MORC family proteins. MORC2 contains a CW zinc finger motif and the CW domain is predicted to play a part in DNA binding and/or promoting protein–protein interactions, therefore MORC2 may have a role in gene transcriptional regulation. To search for hitherto unidentified MORC2 target genes, we performed a DNA microarray hybridization (affymetrix). We found *CAIX* mRNA to be down-regulated 8-fold when MORC2 was overexpressed. We also confirmed the result by northern and western blot analysis, as well as the luciferase reporter assays. Moreover, we showed that the PR4 in the *CAIX* promoter was important for the repression function of MORC2 on *CAIX* transcription. In addition, MORC2 decreased the acetylation level of histone H3 at the *CAIX* promoter. Meanwhile, trichostatin A (TSA) had an increasing effect on *CAIX* promoter activity. Among the six histone deacetylases (HDACs) tested, histone deacetylase 4 (HDAC4) had a much more prominent effect on *CAIX* repression. Finally, we found that MORC2 and HDAC4 were assembled on the same region of *CAIX* promoter. Importantly, we further confirmed that both proteins are simultaneously present in the PR4-binding complex. These data provide a basis for the further investigation into the mechanisms of *CAIX* gene regulation.

MATERIALS AND METHODS

Plasmids construction

For the construction of *CAIX* promoter-driven luciferase reporter plasmid pGL3-E-CP, the *CAIX* promoter fragment comprising −173 to +31 bp was amplified by PCR from human genomic DNA with sense primer 5′-GTATTC GGGGTACCACCTGCCCCTCACTCC-3′ and antisense primer 5′-ATCGAGGAAGATCTGGGTGTGTCCCAG CAC-3′. The amplified fragment was digested with *Kpn*I/*Bgl*II and inserted into the firefly luciferase reporter vector pGL3-Enhancer (Promega). A series of 5′-deleted constructs were derived from pGL3-E-CP by PCR. The sense primers were 5′-GTATTCGGGGTACCCTTTGG TATGGGGGAGA-3′ (pGL3-E-C5, −132 to +31 bp), 5′-GTATTCGGGGTACCGGAGAGGGCACAGGGC CA-3′ (pGL3-E-C4, −107 to +31 bp), 5′-GTATTCGGG GTACCCTGTGAGACTTTGGCTCC-3′ (pGL3-E-C3, −71 to +31 bp), 5′-GTATTCGGGGTACCGGCTTGCT CCTCCCCCAC-3′ (pGL3-E-C2, −45 to +31 bp) and 5′-GTATTCGGGGTACCCACGTACAGCCCGTACA C-3′ (pGL3-E-C1, −8 to +31 bp). The antisense primer was the same as that used for pGL3-E-CP construction. For the construction of pcDNA3.1/MORC2, pcDNA3.1/MORC1 and pcDNA3.1/MORC3, human MORC2, MORC1 and MORC3 cDNA were obtained from NIH-MGC (http://mgc.nci.nih.gov) (Invitrogen) by PCR. The primers were as follows: MORC2 sense primer 5′-ATA GATATTTAGGTACCAAGACGAGAG-3′, MORC2 antisense primer 5′-CGACCACCAACTCGAGAATGA AG-3′, MORC1 sense primer 5′-GTCTACGGGATCCA TGGACGACAGGTACCCT-3′, MORC1 antisense primer 5′-CGGGTCGACTCGAGTTAATTTTCCGAA GTCTT-3′, MORC3 sense primer 5′-GTCTACGGGAT CCATGGCGGCGCAGCCACC-3′ and MORC3 antisense primer 5′-CGGGTCGACTCGAGTTAAGTA CTACTGATTTC-3′. The amplified MORC2 fragment was digested with *Kpn*I/*Xho*I and inserted into pcDNA3.1/HisA plasmid (Invitrogen). The amplified MORC1 and MORC3 fragments were digested with *Bam*HI/*Xho*I and inserted into pcDNA3.1/HisC plasmid (Invitrogen), respectively. All plasmids were confirmed by restriction mapping and DNA sequencing analysis. Plasmids expressing human HDAC1-6 (fused to the Flag-epitope) were kind gifts from Dr E. Seto (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA).

Cell culture, transfections and luciferase assays

Human embryonic kidney (HEK)-293 cells, human gastric cancer cell lines, BGC-823, MGC-803, SGC-7901, human colorectal cancer cell lines LS174T and Clone A were grown in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (fetal bovine serum), 100 units/ml penicillin and 100 μg/ml streptomycin. Transient and stable transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For stable transfections, SGC-7901 cells were transfected with the recombinant vector pcDNA3.1/MORC2 or the empty (control) vector pcDNA3.1/HisA.

Geneticin (800 µg/ml) (Invitrogen) was used to select for stable clones. For transient transfections, cells were cotransfected with the listed constructs. After 24 h of transfections, cells were harvested, washed and lysed in 50–200 µl of lysis buffer. Luciferase activities were analyzed using a Promega dual-luciferase reporter assay system. Firefly luciferase activity was normalized to the activity of *Renilla* luciferase control. Relative luciferase activity was analyzed using the luminometer Lumat LB 9507 (Berthold Technologies, Germany). All the results represent the means ± SD based on at least three independent experiments.

Total RNA isolation and northern blot analysis

Total cellular RNA was extracted using a total RNA isolation system (Invitrogen) according to the manufacturer's instructions. Twenty microgram of total RNA was fractionated in a 1.2% agarose–formaldehyde gel and then transferred to a nylon membrane Hybond-N (Amersham Biosciences). The probes of *CAIX* and *MORC2* cDNA labeled with [α-^{32}P] dCTP were used in hybridization. rRNAs (28S and 18S) were used to assess the integrity of the RNA. The blots were routinely reprobed with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA for RNA loading and transfer control.

Protein isolation and western blot analysis

Cells were lysed using ice-cold RIPA lysis buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mM EDTA and protease inhibitor cocktail] for 15 min at 4°C. Total cell extracts were obtained by sonication and centrifugation. Equal amounts of denatured proteins were separated by SDS–PAGE (10% gels) and transferred to a PVDF membrane (Millipore). The samples were incubated with anti-MORC2 (Bethyl Laboratories Inc.), anti-His (GenScript), anti-CAIX (Santa Cruz) or anti-GAPDH (Kang Chen, as a loading control) antibodies. The samples were detected using the chemiluminescent detection system (Pierce Technology).

Gene silencing

Silencing of *MORC2* was achieved with an RNA interference (RNAi) approach using small interfering RNA (siRNA) for transient knockdown. The target sequences for *MORC2* were 5′-GAAAGCAGAGCACGTAGCAA GGATT-3′ and 5′-GAAGAAGACAGAGTCACCCAT CAAA-3′. BGC-823 cells were transfected with 50 nM of either *MORC2* siRNAs or non-silencing siRNA (as a negative control) (Shanghai GeneChem Co. Ltd) using lipofectamine 2000 (Invitrogen) as recommended by the manufacturer.

Reverse transcription and quantitative real-time PCR

Total RNA (1 µg) was reverse transcribed to cDNA in a total volume of 20 µl using a RT (reverse transcriptase) reaction kit (Promega). Real-time PCR was performed using an ABI 7500® real-time PCR system (Applied Biosystems) according to the manufacturer's instruction and SYBR® Premix Ex Taq (TaKaRa) as a DNA-specific fluorescent dye. PCR was carried out for 40 cycles of 95°C for 10 s and 60°C for 40 s. Primer sequences for detection of *CAIX* mRNA expression were synthesized as 5′-AGATGAGAAGGCAGCACAGAA-3′ (sense) and 5′-GAAGTGGCATAATGAGCAGGA-3′ (antisense). Primer pairs for detection of *MORC2* mRNA expression were synthesized as 5′-TCGGAAGCGGAGTGTC-3′ (sense) and 5′- CGTGCAGCCCTTTATCT-3′ (antisense). The primers used for analysis of *β-actin* mRNA were: 5′-TCGTGCGTGACATTAAGGAG-3′ (sense) and 5′-ATG CCAGGGTACATGGTGGT-3′ (antisense). All the reactions were repeated at least three times. Gene expression levels were calculated relative to the housekeeping gene *β-actin* by using ABI 7500® System SDS software.

ChIP (chromatin immunoprecipitation) and ChIP Re-IP

Transfected SGC-7901 cells were cross-linked with 1% formaldehyde (final concentration) after washing. Cells were lysed with lysis buffer [50 mM Tris/HCl (pH 8.1), 10 mM EDTA, 1% SDS and protease inhibitor cocktail] and sonicated on ice, then precleared with protein A-agarose. Following immunoprecipitation with anti-acetyl-H3 (Millipore), anti-MORC2 (Bethyl Laboratories, Inc.) or anti-Flag (Sigma) antibodies, protein complexes were immunoprecipated and washed in turn with low salt, high salt, lithium chloride buffer and TE buffer [10 mM Tris/HCl (pH 8.0) and 1 mM EDTA]. After elution and reverse cross-linking, the purified DNA was resuspended in TE buffer. DNA samples (2 µl) were then amplified by PCR. Primer pairs for the *CAIX* promoter (−173/ + 31), up- and down-stream regions of the *CAIX* gene were 5′-A CCTGCCCCTCACTCC-3′ (*CAIX* promoter sense), 5′-G GGTGTGTCCCAGCAC-3′ (*CAIX* promoter antisense), 5′-CATATCGTGGCAGGCAGTG-3′ (upstream sense), 5′-CCAGGAAGCAGGTAAAGAGGT-3′ (upstream antisense), 5′-TCTGCGTTTGTGACATCGT-3′ (downstream sense) and 5′-GGGACCAGTGTTCAGGGAC-3′ (downstream antisense).

For ChIP Re-IP, complexes were eluted from the primary immunoprecipitation by incubation with 10 mM DTT at 37°C for 30 min and diluted 1:50 in buffer [1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris/HCl (pH 8.1)] followed by reimmunoprecipitation with the second antibodies. ChIP Re-IPs of supernatants were done essentially as were the primary IPs. The PCR primers for ChIP Re-IPs were the same as those for ChIP assays.

GST-pull down assays

pcDNA3.1/MORC2 was transcripted and translated *in vitro* using the TNT-coupled transcription and translation system (Promega). GST-HDAC4 fusion proteins prebound to glutathione Sepharose 4B (GE Healthcare) were incubated with MORC2 proteins and rotated at 4°C for 3 h followed by extensive washing with binding buffer. The bound proteins were then eluted in 30 µl 2× SDS loading sample buffer and boiled for 5 min prior to centrifugation. Attached proteins were separated by SDS–PAGE (10% gel), blotted onto PVDF membrane

and immunostained using standard procedures. ECL chemiluminescence reagents (Amersham) were used for detection. Ponceau stain indicated the loading amounts of the GST-fusion proteins.

Immunoprecipitation

HEK-293 cells were transiently transfected with His-MORC2 and GFP-HDAC4 or GFP empty vectors. The immunoprecipitation (IP) procedure was performed using the Protein A Sepharose CL-4B (GE Healthcare). After 36 h transfection, the cells were harvested and resuspended in IP lysis buffer. The cells were lysed for 30 min followed by centrifugation at $12\,000 \times g$ for 30 min. The supernatants were incubated with mouse monoclonal anti-GFP antibody overnight at 4°C, followed by addition of 50 µl Protein A agarose beads. The incubation proceeded for 3 h at 4°C. The complexes were collected by centrifugation followed by extensive washing by IP lysis buffer. Following centrifugation, the complexes were dissolved in 30 µl 2× SDS loading sample buffer. Attached proteins were separated by SDS–PAGE (8% gel), blotted onto PVDF membrane and immunostained using standard procedures.

Electrophoretic mobility shift assays (EMSA)

Nuclear protein extracts from BGC-823 cells were prepared with NE-PER® nuclear and cytoplasmic Extract reagents (Pierce) and *in vitro* translated MORC2 were made by *in vitro* TNT-coupled transcription and translation system (Promega). The PR4 oligonucleotides were labeled with the Biotin 5′ end DNA Labeling Kit (Pierce) and hybridized to form duplexes. Gel shift assays were performed using the Pierce LightShift Chemiluminescent EMSA Kit (Pierce) with 20 fmol duplex per binding reaction. Competition reactions containing a 50-, 100- or 500-fold molar excess of unlabeled duplex PR4 were performed. Antibody supershift reactions were performed using anti-MORC2 (Bethyl Laboratories Inc.) or anti-HDAC4 (Santa Cruz) antibody.

RESULTS

MORC2 down-regulates the mRNA and protein levels of *CAIX*

The MORC proteins are predicted to have the function of regulating transcription (23), but no MORC2 target genes have been identified. To search for hitherto unidentified MORC2 target genes, we carried out stable transfection experiment. SGC-7901 cells, which had low endogenous MORC2 expression (Figure 1A), were stably transfected with pcDNA3.1 or pcDNA3.1/MORC2 plasmid. Then we performed a DNA microarray hybridization experiment using RNA from the two stable-transfected cell lines and found a lot of MORC2 target genes, most of them were down-regulated. The genes were involved in a variety of biological functions including ion transport, lipid metabolism, inflammatory response, response to hypoxia, etc. *CAIX* is one of the genes which are down-regulated markedly. In order to examine whether MORC2 expression is relative to the expression of CAIX in different colorectal and gastric cancer cell lines, we carried out northern and western blot analysis. Total mRNA was isolated and equal amounts of mRNA were subjected to northern blot analysis. The endogenous mRNA level of *MORC2* was high in CloneA, LS174T and BGC-823 cells, in which *CAIX* mRNA level was obviously low. Whereas in SGC-7901 cells, which did not express detectable level of *MORC2* mRNA, the endogenous *CAIX* mRNA level was distinctly high (Figure 1A). We performed western

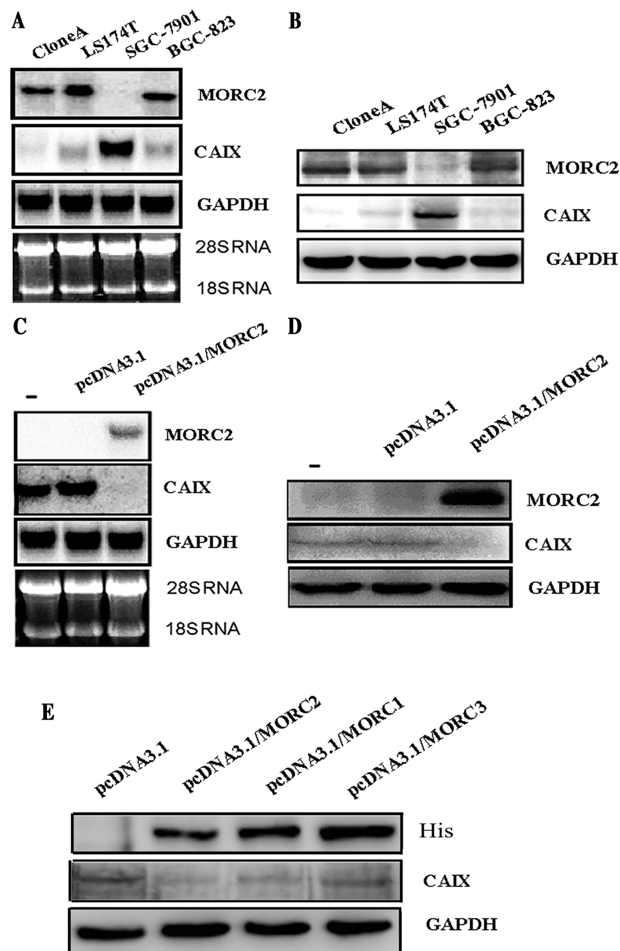

Figure 1. MORC2 down-regulates the mRNA and protein levels of *CAIX*. (**A**) Northern blot analysis of *MORC2* and *CAIX* mRNA levels in different colorectal and gastric cancer cell lines. Total RNA was isolated and 20 µg total RNA was analyzed by northern blot analysis as described in 'Materials and methods' section. GAPDH mRNA was used to assess the integrity of the RNA and to control for the RNA loading. (**B**) Western blot analysis of MORC2 and CAIX protein levels in different colorectal and gastric cancer cell lines. Cells were lysed as indicated in 'Materials and methods' section. Equal amounts of protein (60 µg) were separated by SDS–PAGE and blotted with anti-MORC2 or anti-CAIX antibodies. The expression of endogenous MORC2 and CAIX were detected using the ECL staining method. The expression of MORC2 and CAIX in SGC-7901 cells stable-transfected with pcDNA3.1 or pcDNA3.1/MORC2 were analyzed by northern blot (**C**) and western blot analysis (**D**). −, untransfected SGC-7901 cells. (**E**) Western blot analysis of MORC family members and CAIX protein levels in SGC-7901 cells transfected with pcDNA3.1, pcDNA3.1/MORC2, pcDNA3.1/MORC1 or pcDNA3.1/MORC3.

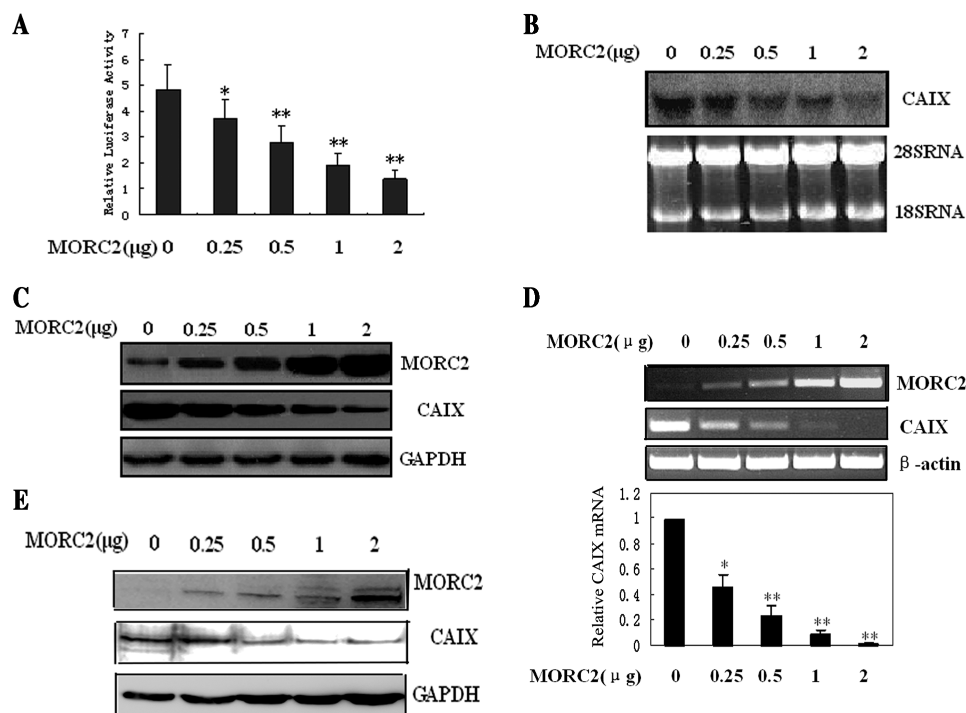

Figure 2. MORC2 down-regulates *CAIX* promoter activity, mRNA and protein levels in a dose-dependent manner. (**A**) MGC-803 cells were transfected with pGL3-E-CP reporter construct (firefly luciferase expression vector), pRL-TK plasmid (renilla luciferase expression vector) and MORC2 expression vector as indicated. Total DNA of the plasmid was adjusted to the same amount by transfecting pcDNA3.1 empty vector. After 24 h of transfection, the cells were harvested and firefly and renilla luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega), with the results expressed as the ratio of firefly to renilla luciferase activity (Fluc/Rluc). The renilla luciferase activity was used as a control for normalizing the assay. All the results represented means ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$. (**B**) MGC-803 cells were transfected with the MORC2 expression vector as indicated. After 30 h of transfection, the mRNA levels of *CAIX* were measured by northern blot analysis. (**C**) The same transfection experiment as (B), after 30 h of transfection, cells were lysed and equal amounts of protein (100 μg) were separated by SDS–PAGE, the protein levels of MORC2 and CAIX were measured by western blot analysis. (**D**) MORC2 expression vectors were transiently transfected into SGC-7901 cells as indicated, and the mRNA level was estimated by RT–PCR and real-time PCR analysis. Values are means ± SD ($n = 3$). $**P < 0.01$. (**E**) SGC-7901 cells were transfected with MORC2 expression vector as indicated, after 30 h of transfection, the protein levels of MORC2 and CAIX were measured by western blot analysis.

blot analysis using the same cell lines as used in the northern blot analysis. The result showed that the protein levels of MORC2 and CAIX in these cells were similar to their mRNA expression levels (Figure 1B). Then we confirmed the MORC2-dependent decrease of the *CAIX* mRNA and protein levels by northern and western blot analysis, respectively (Figure 1C and D). These data suggested that MORC2 down-regulated the expression of CAIX. In order to know whether other MORC family members have the same repressive effect on CAIX expression, we carried out western blot analysis and found that ectopic expressed MORC1 also down-regulated CAIX expression. But MORC3 did not down-regulate the expression of CAIX obviously.

MORC2 down-regulates *CAIX* promoter activity, mRNA and protein levels in a dose-dependent manner

In order to elucidate whether the decrease in *CAIX* mRNA is dependent on MORC2 as a regulator of transcription, we examined the regulation of the *CAIX* promoter. As a reporter, we used the −173 to +31 fragment of the *CAIX* promoter fused to the luciferase reporter gene. This region has been shown to be sufficient for the transcriptional induction of *CAIX* gene in MaTu and HeLa cells (17). MGC-803 cells were transfected with the pGL3-E-CP reporter construct and increasing amounts of MORC2 expression vector. The results showed that MORC2 down-regulated *CAIX* promoter activity in a dose-dependent manner (Figure 2A). To test the expression of MORC2 after the transfection, we carried out western blot analysis, at the same time, *CAIX* mRNA and protein levels were also examined. As can be seen in Figure 2B and C, with increasing amounts of MORC2 expression plasmid transfected, the mRNA and protein levels of *CAIX* were decreased. We also performed RT-PCR, real-time PCR and western blot analysis in SGC-7901 cells. The mRNA and protein levels of *CAIX* were reduced with the ectopic MORC2 expression level enhancing (Figure 2D and E). In sum, these results show that MORC2 is able to down-regulate the expression of *CAIX* in a dose-dependent manner.

Specific knockdown of *MORC2* increases *CAIX* expression

To further address the down-regulation of *CAIX* by MORC2, we tested whether specific knockdown

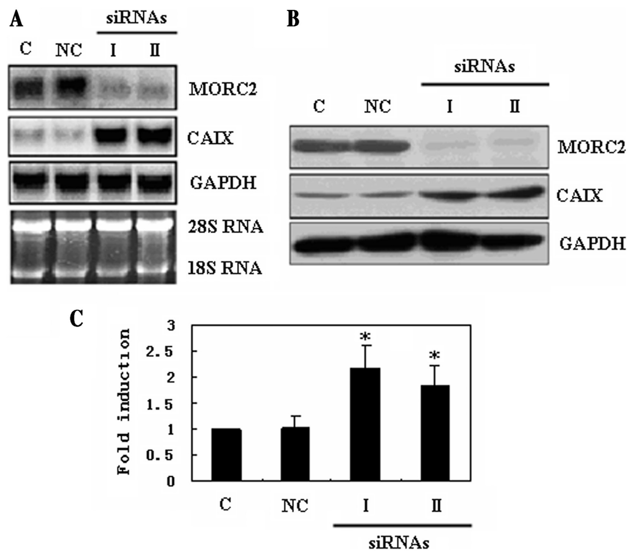

Figure 3. Specific knockdown of *MORC2* leading to the up-regulation of *CAIX*. BGC-823 cells were transfected with siRNAs targeting MORC2 or non-silencing control. C, untransfected BGC-823 cells, NC, BGC-823 cells transfected with non-silencing control. (**A**) After 30 h of transfection, the mRNA levels of *MORC2* and *CAIX* were measured by northern blot analysis. GAPDH mRNA was used to assess the integrity of the RNA and to control for the RNA loading. (**B**) After 30 h transfection, the protein levels of MORC2 and CAIX were measured by western blot analysis. (**C**) After 24 h of transfection, the cells were transfected with the reporter constructs pGL3-E-CP (firefly luciferase expression vector) and pRL-TK (renilla luciferase expression vector), 24 h later, the cells were lysed, firefly and renilla luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega). First we got the ratio of firefly to renilla luciferase activity (Fluc/Rluc, Rluc was used as a control for normalizing the assay) and then the results were shown as fold induction relative to that of cells transfected without siRNA. The results were the means ± SD of three individual experiments. $^{*}P < 0.05$.

of *MORC2* increased *CAIX* expression. BGC-823 cells were transfected with siRNAs targeting *MORC2* or non-silencing control. After 24 h of transfection, northern and western blot analysis were carried out to detect the expression of *MORC2* and *CAIX* using these cells. The effective knockdown of *MORC2* enhanced the mRNA level of *CAIX* (Figure 3A), western blot analysis showed the specific knockdown of *MORC2* increased the protein level of *CAIX* (Figure 3B). We also performed luciferase assays to detect the promoter activity of *CAIX* after the expression of MORC2 was knockdown. The result showed that after the specific knockdown of MORC2, the promoter activity of *CAIX* was elevated (Figure 3C). All these observations indicate that specific knockdown of *MORC2* increases *CAIX* expression.

The PR4 in the *CAIX* promoter is important for the suppression function of MORC2 on *CAIX* transcription

Five PRs within the *CAIX* promoter have been identified (17), to determine which regions are required for the suppression function of MORC2 on *CAIX* transcription, a series of luciferase reporter constructs containing 5′-flanking deletions of the *CAIX* promoter were generated (Figure 4A) and transiently transfected into BGC-823 cells. Luciferase activities generated from these constructs upon transfection were shown in Figure 4B. Compared to deletion to −132, the deletion to −107 had an increased luciferase activity, implying that PR4 was a negative regulatory element (Figure 4B), which is consistent with the obvious observation of another group (17). Our attention was focused on the association of the negative element PR4 with the MORC2-mediated decreasing of *CAIX* promoter activity. Therefore, we carried out luciferase assays to test the effect of PR4 on the *CAIX* transcriptional inhibition by MORC2. We used the same luciferase reporters as shown in Figure 4A. Significant decrease of *CAIX* promoter activity was observed in pGL3-E-CP and pGL3-E-C5 constructs, but not in pGL3-E-C4, pGL3-E-C3, pGL3-E-C2 and pGL3-E-C1, which had not the PR4 (Figure 4C). We therefore conclude that the PR4 region plays an important role in the suppression function of MORC2 on *CAIX* transcription.

Histone deacetylation is involved in the *CAIX* transcriptional repression

Histone deacetylation is one of the best-characterized covalent modifications associated with a repressed chromatin state (26,27). In order to detect the influence of MORC2 on the acetylation level of histone H3 at the *CAIX* promoter, ChIP assay was carried out. The chromatin fragments from SGC-7901 cells transfected with MORC2 expression plasmid or pcDNA3.1 empty vector were immunoprecipitated with antibody against acetylated histone H3. Following the isolation of precipitated DNA, the −173 to +31 region of the *CAIX* promoter was amplified. The result showed that the acetylation level of histone H3 was markedly decreased by the transfection of *MORC2* (Figure 5A). Furthermore, inhibition of HDAC activity by TSA, a known HDAC inhibitor, resulted in the elevation of *CAIX* promoter activity (Figure 5B, compare column 3 with 1). Thus, the activity of *CAIX* promoter was associated with HDACs (Figure 5B). We performed the same experiment in two cell lines, HEK-293 cells and BGC-823 cells, and obtained similar result as above (Figure 5B). These data strongly suggest that HDACs play important roles in the *CAIX* transcriptional repression. We further tested the effects of the six HDACs (HDAC1-6) on *CAIX* gene regulation. We transfected the pGL3-E-CP plasmid together with the empty vector or the HDAC constructs expressing HDAC1-6, respectively. After 24 h of transfection, luciferase activities were measured and normalized to *Renilla* activity. The result was shown in Figure 5C, which indicated that the six HDACs tested exerted distinct repressive effects on the *CAIX* promoter activity, among which HDAC4 had a much more prominent effect on *CAIX* repression, bringing about a nearly 3-fold repression of the promoter activity (Figure 5C). To further investigate how the endogenous mRNA level of *CAIX* was affected by MORC2 and HDAC4, we performed RT-PCR and real-time PCR experiments. The results showed that HDAC4 was able to inhibit

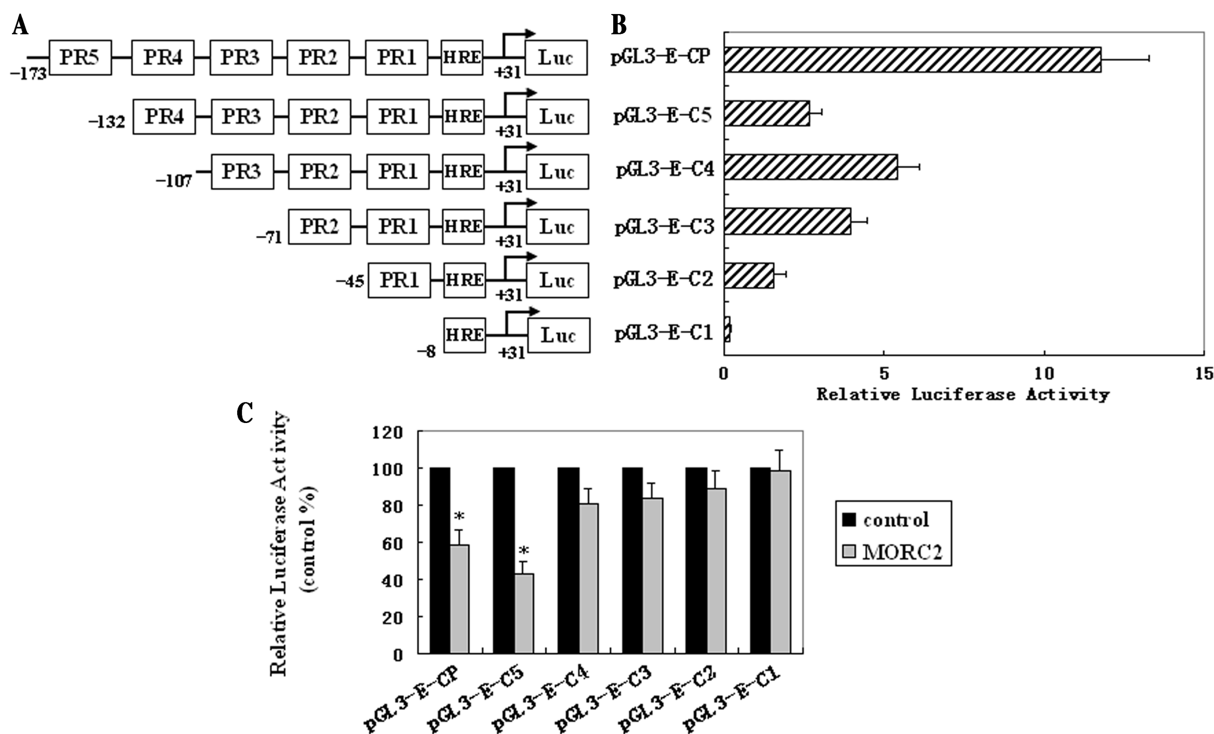

Figure 4. Identification of the *cis*-elements involved in the suppression function of MORC2 on *CAIX* transcription. (**A**) Schematic representation of a series of 5′-deleted *CAIX* promoter/luciferase constructs. The transcription initiation site is indicated by a bent arrow. Numbers indicate base pairs from the transcription start site. (**B**) The basal activities of the corresponding constructs in BGC-823 cells. Luciferase activities were normalized to *Renilla* activities. The bar is mean ± SD from three independent experiments, in duplicate for each construct. (**C**) The comparison of the effects of MORC2 on the activities of 5′-deleted *CAIX* promoter/luciferase constructs in BGC-823 cells. BGC-823 cells were transfected with the indicated constructs and with (the grey bar) or without (the black bar) MORC2. Results are expressed as a percentage of the MORC2-untransfected control that is taken as 100%. $^*P < 0.05$ compared with control.

CAIX mRNA level independently and cumulatively with MORC2 (Figure 5D). We performed additional luciferase assays with HDAC4 expression plasmid and *CAIX* promoter deletion constructs used in Figure 4A. As can be seen in Figure 5E, obvious decrease of *CAIX* promoter activity was exhibited only in pGL3-E-CP and pGL3-E-C5 constructs, which had the PR4 region. The result indicates that HDAC4 probably also works through the PR4 element similar to MORC2. All these data demonstrate that histone deacetylation is involved in the *CAIX* transcriptional inhibition and PR4 plays an important role in the *CAIX* transcriptional repression by HDAC4.

HDAC4 and MORC2 act in a combinatorial fashion on the *CAIX* promoter

In an attempt to test the binding of MORC2 or HDAC4 to the *CAIX* promoter, we performed two ChIP assays. SGC-7901 cells were transfected with pcDNA3.1 empty vector or pcDNA3.1/MORC2 expression plasmid, ChIP was carried out using antibody against MORC2, followed by PCR with primers specific for the *CAIX* promoter region. As shown in Figure 6A, binding of MORC2 was detected at the −173 to +31 region in the *CAIX* promoter, but not the more up- or down-stream region of the *CAIX* gene. For another ChIP experiment, SGC-7901 cells were transfected with pcDNA3.1 empty vector or Flag-HDAC4 expression plasmid, the chromatin fragments from these cells were immunoprecipitated with antibody against Flag. Following the isolation of precipitated DNA, the *CAIX* promoter were amplified. The result showed that HDAC4 was associated with the *CAIX* promoter, but not the more up- or down-stream region of the *CAIX* gene (Figure 6B). To further examine whether MORC2 and HDAC4 were assembled on the same promoter, ChIP Re-IP assays were carried out. We divided the soluble chromatin derived from MORC2 and Flag-HDAC4 cotransfected or pcDNA3.1 transfected cells into two aliquots. One was immunoprecipitated with anti-Flag antibody followed by release of the immune complexes and reimmunoprecipitated (Re-IP) with anti-MORC2 antibody. The other was first immunoprecipitated with anti-MORC2 antibody followed by release and Re-IP with anti-Flag antibody. The same Re-IP was also performed on the unbound supernatant fractions from the primary immunoprecipitation. While both Flag and MORC2 antibodies were able to immunoprecipitate the *CAIX* promoter (−173/+31) after cells were cotransfected with MORC2 and HDAC4 (Figure 6C), subsequent supernatant Re-IPs with either MORC2 antibody or Flag antibody were unable to do so. On the other hand, subsequent Re-IPs of the eluted primary immunoprecipitates were able to bind the *CAIX* promoter (−173/+31) ('bound' in Figure 6C). Figure 6C

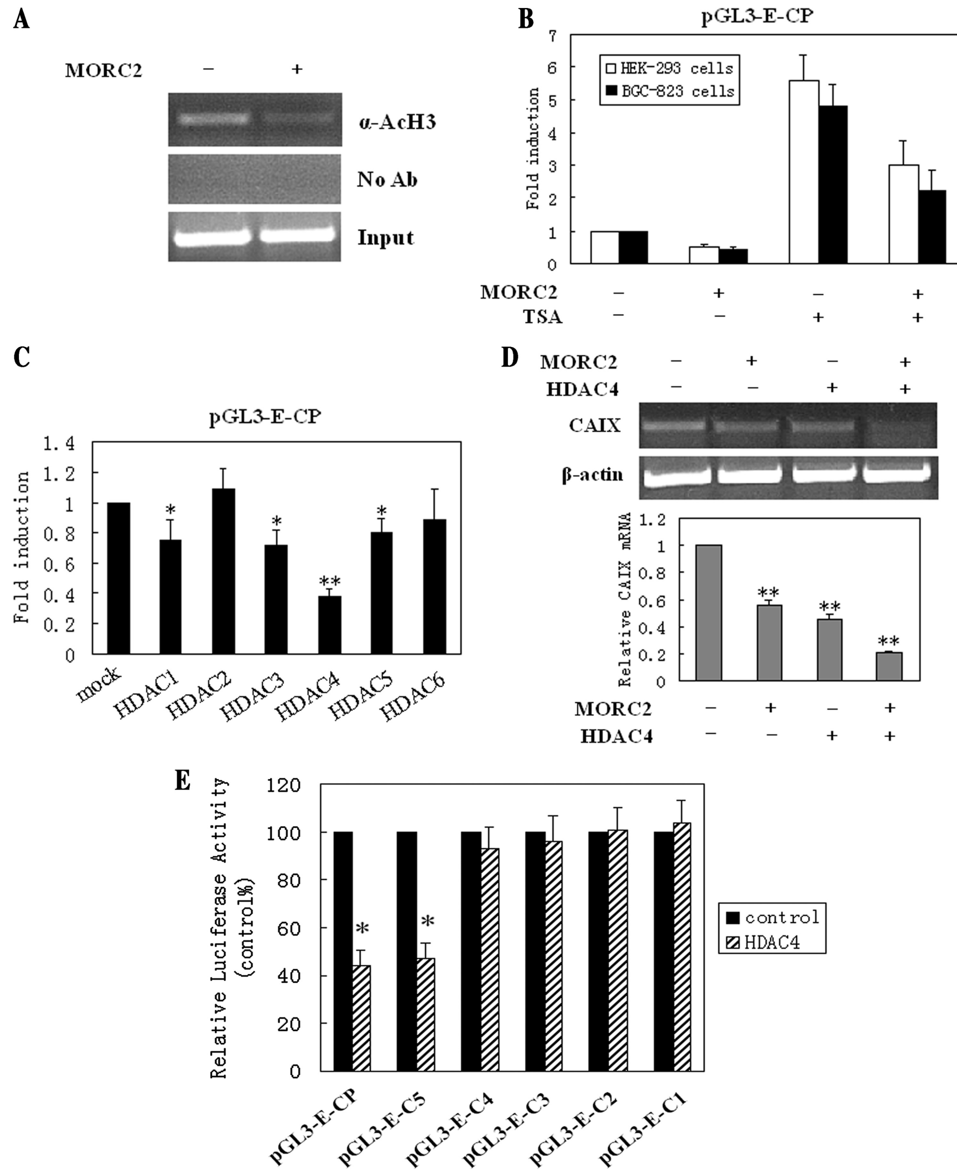

Figure 5. Histone deacetylation is involved in the *CAIX* transcriptional repression. (**A**) The acetylation level of histone H3 at *CAIX* promoter (−173/ + 31) was decreased after the overexpression of MORC2. SGC-7901 cells were transfected with 8 µg MORC2 expression vector or pcDNA3.1 empty vector as the control. ChIP was carried out using antibody against acetylated histone H3 (α-AcH3), followed by PCR with primers amplifying the *CAIX* promoter region (−173/ + 31). (**B**) HEK-293 cells and BGC-823 cells were transfected with the pGL3-E-CP reporter construct, pRL-TK and MORC2 or pcDNA3.1 empty plasmid for 6 h followed by treatment with or without TSA (200 nM) for 24 h. Results are shown as fold induction relative to that of the cells transfected with pGL3-E-CP and pRL-TK, and without treatment of TSA. Values are means ± SD (n = 3). (**C**) BGC-823 cells were transfected with pGL3-E-CP plasmid together with HDAC constructs expressing HDAC1-6, respectively. Luciferase activities were determined and normalized to *Renilla* activity 24 h after transfection. Results are shown as fold induction relative to that of the cells transfected without HDAC plasmid and are the means ± SD from at least three individual experiments. $*P < 0.05$, $**P < 0.01$. (**D**) MORC2 and HDAC4 were transiently transfected into BGC-823 cells as indicated, and the mRNA level was estimated by RT-PCR and real-time PCR analysis. Values are means ± SD (n = 3), $**P < 0.01$. (**E**) BGC-823 cells were transfected with *CAIX* promoter deletion constructs used in Figure 4A and with or without HDAC4 expression plasmid as indicated. Luciferase activities were determined and normalized to *Renilla* activity 24 h after transfection. Results are expressed as a percentage of the MORC2-untransfected control that is taken as 100%. $*P < 0.05$ compared with control.

showed that both HDAC4 and MORC2 were bound to the *CAIX* promoter, apparently in the same complex. In order to show the specificity of such a co-interaction, we performed ChIP Re-IP assay with HDAC2 and MORC2. The result showed that HDAC2 neither bound the *CAIX* promoter nor interacted with MORC2 (Figure 6D). These experiments support a model in which MORC2 and HDAC4 act in a combinatorial fashion on the *CAIX* promoter.

MORC2 binds to HDAC4 and both of them bind to the PR4 region of the *CAIX* promoter

To test whether MORC2 could physically interact with HDAC4, *in vitro* binding study was done using *in vitro*

Figure 6. ChIP and ChIP Re-IP assays for MORC2 and HDAC4 in association with the *CAIX* promoter. (**A**) ChIP assay to examine the binding of MORC2 to the *CAIX* promoter. SGC-7901 cells were transfected with 4 µg MORC2 expression vector or pcDNA3.1 empty vector as the control. ChIP was carried out using antibody against MORC2, followed by PCR with primers amplifying the *CAIX* promoter region (−173/+31), up- and down-stream regions of *CAIX* gene. (**B**) ChIP assay for detection of the recruitment of HDAC4 to the *CAIX* promoter. SGC-7901 cells were transfected with 4 µg pcDNA3.1 empty vector or Flag-HDAC4 expression plasmid. ChIP was carried out using antibody against Flag, followed by PCR with primers amplifying the *CAIX* promoter region. (**C**) ChIP Re-IP to examine whether MORC2 and HDAC4 were assembled on the same promoter. Soluble chromatin was prepared from SGC-7901 cells transfected with MORC2 and HDAC4 or pcDNA3.1 empty vector and divided into two aliquots. One aliquot was first immunoprecipitated with antibody against Flag (1° IP). The supernatant was collected and reimmunoprecipitated with antibody against MORC2 (Supernatant Re-IP). The other aliquot was first immunoprecipitated with antibody against MORC2 (1° IP), followed by reimmunoprecipitation with antibody against Flag. Similar reciprocal Re-IPs were also performed on complexes eluted from the 1° IPs (Bound Re-IP). (**D**) The same ChIP Re-IP experiment as (C) to examine whether MORC2 and HDAC2 were assembled on the same promoter. SGC-7901 cells were transfected with MORC2 and Flag-HDAC2 or pcDNA3.1 empty vector. The antibodies used were against MORC2 and Flag.

transcripted and translated full-length MORC2 and GST-tagged full-length HDAC4. The results indicated that MORC2 bound to the full-length HDAC4 (Figure 7A). To confirm the binding of MORC2 and HDAC4 *in vivo*, we carried out immunoprecipitation followed by western blot analysis. We transfected HEK-293 cells with His-tagged MORC2 and GFP-tagged HDAC4 or GFP empty vector. The results indicated that MORC2 and HDAC4 were in the same complex *in vivo* (Figure 7B). To identify whether both MORC2 and HDAC4 bound to the PR4 region, PR4 oligonucleotides were synthesized and labeled with biotin as the probe. Nuclear extracts from BGC-823 cells were used. The results showed that PR4 probe formed a complex with nuclear extracts. The complex was competed by 50-, 100- or 500-fold molar excess of unlabeled PR4 (Figure 7C). Further studies showed that the complex formed was super-shifted with anti-MORC2 or anti-HDAC4 antibody (Figure 7C). PR4 probe also formed a complex with *in vitro* transcripted and translated MORC2 protein. The complex was also competed by 50- or 100-fold molar excess of unlabeled PR4 (Figure 7D). The complex was also super-shifted by antibody specific to MORC2 (Figure 7D).

DISCUSSION

MORC2 belongs to the MORC protein family. Although the biological functions of MORC proteins have remained poorly understood, studies of the prokaryotic MORCs and their relatives suggest that their eukaryotic counterparts are likely to carry out chromatin remodeling function (28). MORC2 contains a CW zinc finger motif and the CW domain is predicted to play a part in DNA binding and/or promoting protein–protein interactions (22), therefore MORC2 may have a role in gene transcriptional regulation. The main contribution of this study is the demonstration of the involvement of MORC2 and histone deacetylase 4 in transcriptional control of *CAIX* gene in gastric cancer cells.

Previous studies have concentrated on *CAIX* gene regulation in non-gastric cancer cells, such as renal cell

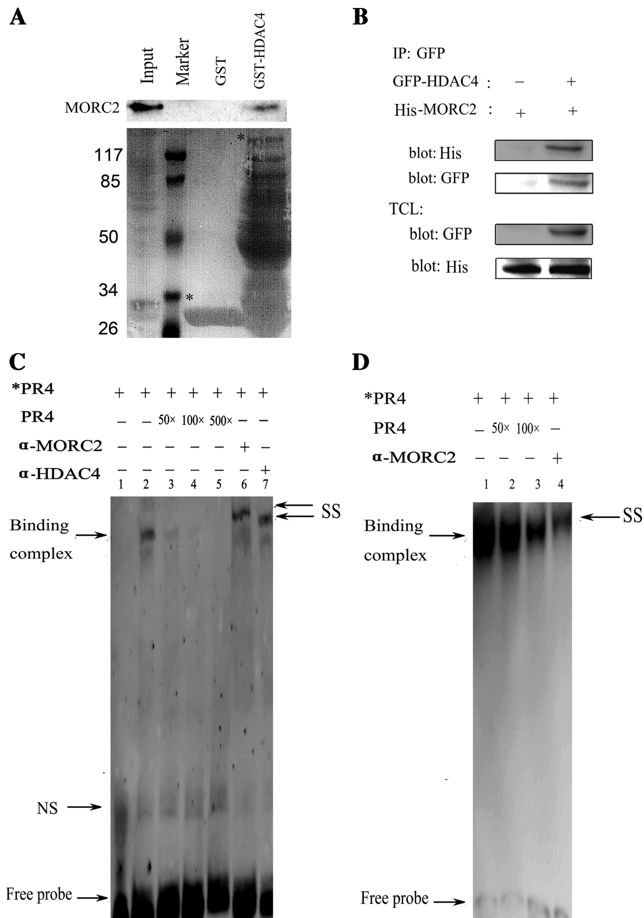

Figure 7. MORC2 binds to HDAC4 and both of them bind to the PR4 region of the *CAIX* promoter. (**A**) For GST pull-down assay, GST or GST fusion proteins were incubated with MORC2 protein *in vitro* translated. Bound proteins were subjected to SDS–PAGE and western blot with anti-MORC2 antibody. (**B**) For the immunoprecipitation assay, cell lysates were immunoprecipitated by anti-GFP antibody, and precipitates were immunoblotted with anti-His antibody. The expression were checked using 20 µg of total cell lysate (TCL) blotted with the indicated antibodies (bottom panels). (**C**) EMSA was performed with nuclear extracts from BGC-823 cells and the PR4 oligonucleotide (*PR4) labeled with biotin. Lane 1 is *PR4 probe without nuclear extracts. The binding complexes were competed by 50-, 100- or 500-fold molar excess of unlabeled PR4, respectively, and supershifted by anti-MORC2 or anti-HDAC4 antibody. NS, non-specific; SS, super-shift; *PR4, PR4 probe labeled with biotin; PR4, unlabeled PR4. (**D**) EMSA was performed with *in vitro* translated MORC2 protein using a TNT quick coupled transcription-translation system (Promega) and PR4 oligonucleotide (*PR4) labeled with biotin. The binding complexes were competed by 50- or 100-fold molar excess of unlabeled PR4, respectively, and super-shifted by anti-MORC2 antibody.

carcinoma (RCC) cells, breast carcinoma MCF-7 and osteosarcoma Saos-2 cells, etc. (21,29), and focused on the identification of *cis*-acting elements and their cognate transcription factors (cofactors) that activated *CAIX* gene. Six *cis*-acting elements were functionally characterized in the *CAIX* promoter, they are HRE and five PRs, among which five have a positive and one has a negative influence on promoter activity (17). HRE is a positive regulatory element, which activated by the hypoxia-inducible factor (HIF). Two of the positive *CAIX cis*-acting elements, PR1 and PR5, bind Sp1/Sp3 factors (19–21). The PR2 and PR3 are also positive regulatory elements, which bind AP1 and proteins from nuclear extracts respectively (17,19). Although the PR4 has a negative effect on the *CAIX* promoter activity, the presumed repressor binding PR4 has not been identified. In the present study, the transcriptional regulation of *CAIX* gene in gastric cancer cells was examined. We found that MORC2 repressed the mRNA and protein levels, as well as the promoter activity of *CAIX* in a dose-dependent manner in gastric cancer cells (Figure 2). And this result was also proved by the specific knockdown of MORC2 (Figure 3). The luciferase assays using the deletion mutants showed that the PR4 region was important for the suppression function of MORC2 on *CAIX* transcription (Figure 4).

Epigenetic modifications, such as methylation of CpG sites in the proximity of promoters and/or post-translational modifications of histones, play a significant role in the control of gene expression, presumably by limiting access of transcription factors to *cis*-acting elements. Histone deacetylation is one of the best-characterized covalent modifications associated with a repressed chromatin state (26). The methylation status of the −74 and −6 CpG sites in the *CAIX* promoter has been reported to negatively correlate with *CAIX* expression in renal cells (30–33), but no one has studied the histone acetylation status at the *CAIX* promoter. The study presented here showed that the acetylation level of histone H3 was markedly decreased by the overexpression of MORC2 (Figure 5A). Thus, we presumed that histone deacetylase participated in the regulation of *CAIX* gene transcription. And this presumption was further supported by the result that inhibition of HDAC activity by TSA resulted in the elevation of *CAIX* promoter activity (Figure 5B, compare column 3 with 1).

Among the six human HDACs tested in the present study, HDAC4 was found to be much more effective in suppressing the transcriptional activity of the *CAIX* promoter (Figure 5C). HDAC1-6 represents the enzymes belonging to two different classes, which have distinct features in structure and exert different functions. Compared with Class I members, Class II HDACs, including HDAC4, 5, 6 and 7, display more specific responses to various signaling molecules. Such specificities allow specific target genes to be regulated by individual Class II HDAC members in a type-specific pattern. We have demonstrated that over-expression of HDAC4 resulted in markedly reduction of *CAIX* promoter activity and mRNA level (Figure 5C and D), suggesting that members of Class II HDACs might be more effective in inhibiting transcription of the *CAIX* gene.

As co-repressors, HDACs require specific transcription factors for recruiting them to target DNA elements for regulatory functions. In this study, we showed that both of MORC2 and HDAC4 were associated with the *CAIX* promoter by ChIP assays (Figure 6A and B). We also described experimental results to show that MORC2 and HDAC4 were assembled on the same promoter (Figure 6C). We performed additional ChIP Re-IP experiment to prove the specificity of such a co-interaction

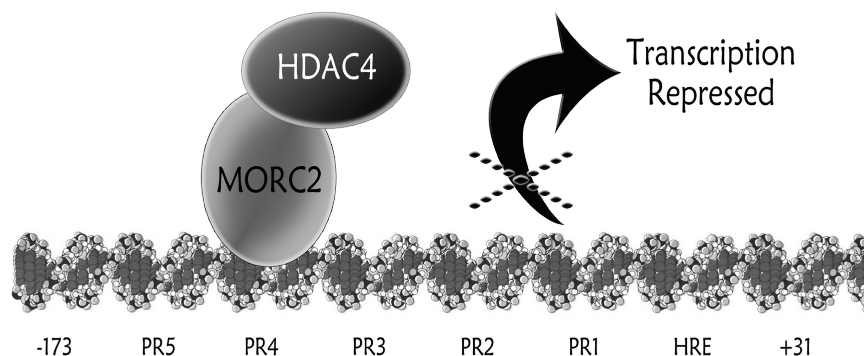

Figure 8. Proposed model showing roles of MORC2 and HDAC4 in regulation of the *CAIX* gene. MORC2 binds the protected region 4 in the *CAIX* promoter, and recruits HDAC4 that decreases the acetylation level of histone H3 at the *CAIX* promoter, leading to a closed chromatin structure and thus the transcriptional repression of *CAIX* gene.

(Figure 6D). In addition, the *in vitro* and *in vivo* interaction of MORC2 and HDAC4 was confirmed by GST-pull down and IP experiments (Figure 7A and B). Furthermore, we performed EMSA with PR4 and proved by super-shift that both proteins were simultaneously present in the PR4-binding complex (Figure 7C and D). HDAC4 is often found to form multisubunit complex with other corepressors, such as N-CoR etc, to regulate the transcription of target genes (34). Our findings indicated that MORC2 and HDAC4 were in the inhibitory complex to suppress the expression of the *CAIX* gene. Further studies are needed to identify other corepressors which might form a complex with HDAC4 to inhibit the transcription of the *CAIX* gene. Based on the previous knowledge, as well as findings from this study, we propose a hypothesized model in which MORC2 binds the PR4 in the *CAIX* promoter, and recruits HDAC4 that decreases the acetylation level of histone H3 at the *CAIX* promoter, leading to a closed chromatin structure and thus the transcriptional repression of *CAIX* gene (Figure 8).

The exact molecular mechanisms of gastric tumorigenesis are still unclear. The studies in *CAIX* knockout mice have indicated that CAIX is an important factor in gastric morphogenesis and homeostasis of the gastric epithelium possibly acting through the control of cell differentiation and proliferation (3). Moreover, it has been reported that the expression of CAIX correlates with an extremely poor prognosis in gastric adenocarcinomas (4). CAIX was highly expressed in the normal gastric mucosa and the expression declined in carcinomas with less differentiation (14). Till now, few studies have been given to clarification the mechanisms of *CAIX* expression regulation in gastric cancer cells. Our findings may help to achieve a better understanding of the *CAIX* expression control in gastric cancer cells.

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