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$_2$O + CO$_2$ ↔ 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 identified as the crucial elements for CAIX expression in HIF-1α, PR1 and PR5 position of CAIX are described to up-regulate which PR1 and PR2 are the most critical for trans-

effect on deacetylase 4 (HDAC4) had a much more prominent effect on CAIX promoter. Meanwhile, trichostatin A (TSA) had decreased the acetylation level of histone H3 at the 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′-GTATCGGGTGTCAGGCAGCCAGCAGCAGGCA−3′. The amplified fragment was digested with KpnI/BglII 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′-GTATTTGGGTACCGCAGGAGGAGA−3′ (pGL3-E-C5, −132 to +31 bp), 5′-GTATCGGGTGTCAGGCAGCAGCAGGCA−3′ (pGL3-E-C4, −107 to +31 bp), 5′-GTATCGGGTGTCAGGCAGCAGCAGGCA−3′ (pGL3-E-C3, −71 to +31 bp), 5′-GTATCGGGTGTCAGGCAGCAGCAGGCA−3′ (pGL3-E-C2, −45 to +31 bp) and 5′-GTATCGGGTGTCAGGCAGCAGCAGGCA−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.nic.nih.gov) (Invitrogen) by PCR. The primers were as follows: MORC2 sense primer 5′-ATA CATATCGGTACCGCAGGA−3′, MORC2 antisense primer 5′-CGGCCATGCCAGGAGA−3′, MORC2 sense primer 5′-GTCTACGGGATCAGCACTTGGGACGACAGGTACCCTGTGAGACTTTGGCTCC−3′, MORC2 antisense primer 5′-CGCCGATCCAGGAGA−3′, MORC1 sense primer 5′-GTCTACGGGATCAGCACTTGGGACGACAGGTACCCTGTGAGACTTTGGCTCC−3′, MORC1 antisense primer 5′-CGGCCATGCCAGGAGA−3′, MORC3 sense primer 5′-GTCTACGGGATCAGCACTTGGGACGACAGGTACCCTGTGAGACTTTGGCTCC−3′ and MORC3 antisense primer 5′-CGCCGATCCAGGAGA−3′. The amplified MORC2 fragment was digested with KpnI/XhoI and inserted into pcDNA3.1/HisA plasmid (Invitrogen). The amplified MORC1 and MORC3 fragments were digested with BamHI/XhoI 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-32P] 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′-GAAAGCAGAGCAGTACGCAA GGATT-3′ and 5′-GAAAGCAGAGCAGTACGCAA 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′-AGATGAGAAAGGCACGACGAA-3′ (sense) and 5′-GAAGTGCATATGACAGGAG-3′ (antisense). Primer pairs for detection of MORC2 mRNA expression were synthesized as 5′-TCGGAAGGCGAGTGC-3′ (sense) and 5′-CGTGCAGCCTTTATCT-3′ (antisense). The primers used for analysis of β-actin mRNA were: 5′-TCGTGCCTCATTAAGGAG-3′ (sense) and 5′-ATG CCAAGGGTACATGTTGTT-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 immunoprecipitated 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 CCGCCACCCTACTCC-3′ (CAIX promoter sense), 5′-G GGTGTGTCCGCCAGC-3′ (CAIX promoter antisense), 5′-CATATCGTGGCGAGCAGTG-3′ (upstream sense), 5′-CCAGGAAGCAGTAAAGGTT-3′ (upstream antisense), 5′-TCTGCGTTTTGTCGACATGT-3′ (down- stream 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 × 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 2x 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 5’0 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 RNA was isolated and 20μg 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 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 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 20μg 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 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.
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 of MORC2 increased CAIX expression. Figure 2.
of MORC2 increased CAIX expression. BGC-823 cells were transfected with siRNAs targeting MORC2 or non-silencing control. After 24 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. After 30 h of transfection, the protein levels of MORC2 and CAIX were measured by western blot analysis. (A) 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). (B) 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). (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). 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.

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 showed the specific knockdown of MORC2, the promoter activity was 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...
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 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
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
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
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 suppressing the transcriptional activity of the CAIX promoter by ChIP assays (Figure 6A and B). We also 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 (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).

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(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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**REFERENCES**

1. Robertson, N., Potter, C. and Harris, A. L. (2004) Role of carbonic anhydrase IX in human tumor cell growth, survival and invasion. *Cancer Res.*, 64, 6160–6165.

2. Potter, C. and Harris, A. L. (2004) Hypoxia inducible carbonic anhydrase IX, marker of tumor hypoxia, survival pathway and therapy target. *Cell Cycle*, 3, 164–167.

3. Gut, M. O., Parkkila, S., Vernerová, Z., Rohde, E., Zaváda, J., Höcker, M., Pastorek, J., Jentzen, T., Gibadulinová, A., Zavádová, Z. et al. (2002) Gastric hyperplasia in mice with targeted disruption of the carbonic anhydrase gene Car9. *Gastroenterology*, 123, 1889–1903.

4. Driessen, A. J., Landuyt, W., Pastorekova, S., Moons, J., Goethals, L., Haustermans, K., Naef, P., Penninckx, F., Geboes, K., Lerut, T. et al. (2006) Expression of Carbonic Anhydrase IX (CA IX), a hypoxia-related protein, rather than Vascular-Endothelial Growth Factor (VEGF), a pro-angiogenic factor, correlates with an extremely poor prognosis in esophageal and gastric adenocarcinomas. *Am. J. Surg.*, 243, 334–340.

5. Airley, R. E., Loncaster, J., Raleigh, J. A., Harris, A. L., Davidson, S. E., Hunter, R. D., West, C. M. and Stratford, I. J. (2003) GLUT-1 and CAIX as intrinsic markers of hypoxia in carcinoma of the cervix: relationship to pimonidazole binding. *Int. J. Cancer*, 104, 85–91.

6. Liao, S. Y., Aurelio, O. N., Jan, K., Zavada, J. and Stanbridge, E. J. (1997) Identification of the MN/CA9 protein as a reliable diagnostic biomarker of clear cell carcinoma of the kidney. *Cancer Res.*, 57, 2827–2831.

7. Chai, S. K., Wykoff, C. C., Watson, P. H., Han, C., Leek, R. D., Pastorek, J., Gatter, K. C., Ratcliffe, P. and Harris, A. L. (2001) Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. *J. Clin. Oncol.*, 19, 3660–3668.

8. Tan, E. Y., Yan, M., Campo, L., Han, C., Takano, E., Turley, H., Candiloro, J., Pezzella, F., Gatter, K. C., Millar, E. K. et al. (2009) The key hypoxia regulated gene CAIX is upregulated in basal-like breast tumours and is associated with resistance to chemotherapy. *Br. J. Cancer*, 100, 405–411.

9. Schmitt, A., Barth, T. F., Beyer, E., Borchert, F., Rojewski, M., Chen, J., Guillaume, P., Gronau, S., Greiner, J., Möller, P. et al. (2009) The tumor antigens RHAMM and G250/CAIX are...
expressed in head and neck squamous cell carcinomas and elicit specific CD8+ T cell responses. *Int. J. Oncol.*, 34, 629–639.

10. Beasley, N.J., Wykoff, C.C., Watson, P.H., Leck, R., Turley, H., Gatter, K., Pastorek, J., Cox, G.J., Ratcliffe, P. and Harris, A.L. (2001) Carbonic anhydrase IX, an endogenous hypoxia marker, expression in head and neck squamous cell carcinoma and its relationship to hypoxia, necrosis, and microvessel density. *Cancer Res.*, 61, 5262–5267.

11. Swinson, D.E., Jones, J.L., Richardson, D., Wykoff, C., Turley, H., Pastorek, J., Taub, N., Harris, A.L. and O’Byrne, K.J. (2003) Carbonic anhydrase IX expression, a novel surrogate marker of tumor hypoxia, is associated with a poor prognosis in non-small-cell lung cancer. *J. Clin. Oncol.*, 21, 473–482.

12. Proescholdt, M.A., Mayer, C., Kubitzka, M., Wykoff, C., Turley, H., Pastorek, J., Taub, N., Harris, A.L. and O’Byrne, K.J. (2005) Expression of hypoxia-inducible carbonic anhydrases in brain tumors. *Neuro-Oncology*, 7, 465–475.

13. Kaluz, S., Kaluzova, M., Liao, S.Y., Lerman, M. and Stanbridge, E.J. (2009) Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: a one transcription factor (HIF-1) show? *Biochim. Biophys. Acta*, 1795, 162–171.

14. Leppilampi, M., Saarnio, J., Karttunen, T.J., Pastorek, J.K.S.P.J., Waheed, A., Sly, W.S. and Parkkila, S. (2003) Carbonic anhydrase isozymes IX and XII in gastric tumors. *World J. Gastroenterol.*, 9, 1398–1403.

15. Grassl, T.A., Gabriel, P., Grabmaier, K., de Weijert, M.C., Verhaegh, G.W., Schalken, J.A., Pastorekova, S., Malfertheiner, P. and Ebert, M.P. (2005) Expression of carbonic anhydrases. *Biophys. Acta*, 162–172.

16. Grabmaier, K., de Weijert, M.C., Verhaegh, G.W., Schalken, J.A., and Oosterwijk, E. (2004) Strict regulation of CAIX (G250/MN/CA9) promoter proximal region: a role for specificity protein (SP) and activator protein 1 (AP1) factors. *Biochem. J.*, 359, 669–677.

17. Kaluzova, M., Pastorekova, S., Svastova, E., Pastorek, J., Stanbridge, E.J. and Kaluz, S. (2001) Characterization of the MN/CA9 promoter proximal region: a role for specificity protein (SP) and activator protein 1 (AP1) factors. *Biochem. J.*, 359, 669–677.

18. Wykoff, C.C., Beasley, N.J., Watson, P.H., Turner, K.J., Pastorek, J., Sibatia, A., Wilson, G.D., Turley, H., Talks, K.L., Maxwell, P.H. et al. (2000) Hypoxia-inducible expression of tumor-associated carbonic anhydrases. *Cancer Res.*, 60, 7075–7083.

19. Kaluzova, M., Pastorekova, S., Svastova, E., Pastorek, J., Stanbridge, E.J. and Kaluz, S. (2001) Characterization of the MN/CA9 promoter proximal region: a role for specificity protein (SP) and activator protein 1 (AP1) factors. *Biochem. J.*, 359, 669–677.

20. Kaluz, S., Kaluzova, M. and Stanbridge, E.J. (2003) Expression of the hypoxia marker carbonic anhydrase IX is critically dependent on SP1 activity. Identification of a novel type of hypoxia-responsive enhancer. *Cancer Res.*, 63, 917–922.

21. Grabmaier, K., de Weijert, M.C., Verhaegh, G.W., Schalken, J.A. and Oosterwijk, E. (2004) Strict regulation of CAIX (G250/MN/CA9) by HIF-lalp6 in clear cell renal cell carcinoma. *Oncogene*, 23, 5624–5631.

22. Perry, J. and Zhao, Y. (2003) The CW domain, a structural module shared amongst vertebrates, vertebrate-infecting parasites and higher plants. *Trends Biochem. Sci.*, 28, 576–580.