HIC1 modulates prostate cancer progression by epigenetic modification

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No potential conflicts of interest were disclosed
Statement of Translational Relevance

This study aimed to further our understanding of the role that hypermethylated in cancer 1 (HIC1) plays in prostate cancer (PCa) progression. Our data showed that HIC1 promoter hypermethylation was presented in cell lines, tissues and plasma of PCa patients. *In vitro* assays, restoring HIC1 expression in PCa cells by lentivirus vector or 5-Aza-2'-deoxycytidine (Decitabine) treatment markedly inhibited proliferation, migration, and invasion and induced the apoptosis in these cells. Moreover, *In vivo* assays showed that the mice bearing PCa-restoring HIC1 cells had a marked effect on reducing tumor growth, multiple tissue metastases and bone destruction. These findings suggest that therapies targeting epigenetic events regulating HIC1 expression may provide a more effective strategy for PCa treatment.
Abstract

Purpose:
Prostate cancer (PCa) is the second leading cause of cancer deaths among men in Western counties, which has been also occurred in Chinese male with markedly increasing incidence in recent years. Although the mechanism underlying its progression still remains unclear, epigenetic modifications are important ethological parameters. The purpose of this study is to determine the methylation status and function of hypermethylated in cancer 1 (HIC1) in PCa progression.

Experimental Design:
The methylation status of HIC1 promoter were assayed in cell lines, tissues and plasma of PCa patients by using MSP-PCR and bisulfate sequencing (BSP). The ability of HIC1 to regulate proliferation, migration and invasion was assessed by MTT, scratch healing assay and reconstituted extracellular matrices in porous culture chambers. Tumorigenesis, metastases and bone destruction were analyzed in mice bearing PCa cells restoring HIC1 by using Xenogen IVIS with radiographic system and small-animal PET-CT images. Microarrays were searched for genes that had correlated expression with HIC1 mRNA. Reporter gene assays were used to determine whether HIC1 affected the expression of CXCR7, and chromatin immunoprecipitation (CHIP) was used to determine whether HIC1 bound to CXCR7 promoters. All \( p \) values were determined using two-sided tests.

Results:
The methylation status of 11 CpG sites within HIC1 promoter were abundantly methylated in cell lines, tissues and plasma of PCa patients compared with those of respective normal controls. Restoring HIC1 expression in PCa cells markedly inhibited proliferation, migration, and invasion and induced the apoptosis in these cells. Moreover, mice bearing PCa-restoring HIC1 cells had a marked effect on reducing tumor growth, multiple tissue metastases and bone destruction. Notably, we also identified that the chemokine receptor CXCR7 is a direct downstream target gene of HIC1. Finally, we demonstrated that CXCR7 promoter in PCa cells is negatively regulated by HIC1, which may be responsible for PCa progression.
Conclusions:

Our data show for the first time that hypermethylation of HIC1 promoter results in loss of its repressive function, responsible for PCa progression and invasion. These findings suggest that therapies targeting epigenetic events regulating HIC1 expression may provide a more effective strategy for PCa treatment.
Introduction

Prostate cancer (PCa) is a common neoplasm and is responsible for 240,890 American men diagnosed with this disease and 33,720 deaths in 2011 (1). In China, although accurate epidemiologic data of the whole country was not available, PCa incidence rates in developed areas (such as Shanghai, Beijing, Guangzhou etc.) have been increasing dramatically during recent ten years due in part to the wide use of screening test and adaptation of a western style diet (2). Identifying new biomarkers responsible for PCa progression may prove beneficial to prevent progression and metastasis and to provide an effective therapeutic strategy for this disease.

HIC1 was originally isolated as a new candidate tumor suppressor gene because it is hypermethylated in CpG islands in many types of human cancers (3-7). Many studies have observed HIC1 and p53 function by a regulatory feedback loop in which HIC1 directly represses the transcription of SIRT1 which deacetylates and thereby inactivates p53 (8, 9). Therefore, inactivation of one allele of HIC1 results in the de-repression of SIRT1 causing decreased p53 mediated transactivation of the remaining HIC1 allele. These findings suggest that HIC1-SIRT1-p53 regulatory loop is an essential pathway through which HIC1 may function as a tumor suppressor gene and cooperate with p53, as shown by the double heterozygote model (10). To date, given many potential physiological roles of HIC1, the number of HIC1-characterized target genes appears to be a small amount.

Although homozygous disruption of HIC1 impairs development and leads to embryonic and perinatal lethality (11), mice with heterozygous HIC1 inactivation (Hic1+/−) have the propensity to form spontaneous tumors (12). The presence of HIC1 silence is associated with pre-neoplastic conditions such as smoker’s lung, colonic polyps and cirrhotic liver (13). It has been reported that HIC1 is not mutated in cancers but epigenetically mediated loss of function may help drive key stages of human cancer tumorigenesis and progression. Increasing evidence suggests that HIC1 promoter was hypermethylated, including in breast (5, 14), brain (15), gastric (6), liver (7), and colorectal (16) tumors. In addition, a possible role for HIC1 in leukaemogenesis is suggested by the fact that the distal arm of chromosome 17p is often altered in advanced stages of chronic myeloid leukaemia (CML) (17). Finally, the D17S5 microsatellite marker which allowed the positional cloning of HIC1 (3, 4) has been shown to be methylated in PCa (4). However, the status and role of HIC1 by epigenetic modification in PCa has never been analyzed in details and thus still remains unsettled.
Here, we found that HIC1 promoter was hypermethylated in PCa. *In vitro* and *in vivo* functions of HIC1 in PCa are further investigated. We also identified that chemokine receptor CXCR7 is a potential downstream target gene of HIC1. These findings for the first time indicate that HIC1 by hypermethylation may play a critical role in facilitating PCa progression.
Materials and Methods

Some reagents, antibodies, and plasmids are listed in supplementary methods.

Cell cultures

The LNCaP, its metastatic subline C4-2B, and PC3 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI Medium 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp, Carlsbad, CA). The human prostate epithelial cell line (PrEC) (PriCells Co., LTD, Wuhan, China) was cultured in keratinocyte medium supplemented with 5 ng/ml human recombinant epithelial growth factor and 50 µg/ml bovine pituitary extract (Invitrogen Corp, Carlsbad, CA).

Tissues and plasma of PCa patients

PCa tissues and the respective peripheral blood were collected from patients and healthy controls at Shanghai Ruijin Hospital, Shanghai Jiao Tong University, and Shanghai the Tenth People's Hospital of Tong Ji University after the subjects’ informed consent and with institutional review board approval of the hospitals. All patients obtained a confirmed diagnosis of PCa after resection.

Construction of lentiviral vectors

For restoring expression of HIC1 in PC3 and C4-2B cell lines, human full length HIC1 cDNA was inserted into lentivirus vector pHR-SIN-CSIGW (gift from Jun Mi, Shanghai Jiaotong University, Shanghai, China) under the control of SFFV promoter for stable expression. The fragment of HIC1 was inserted between SpeI and NdeI restriction sites according to the protocol. The construction was verified by sequencing. Supernatants were collected 36–48h after transfection, filtered through a 0.4 µm filter, and used directly to infect tumor cells. Two rounds of infection 8 h apart were usually sufficient to infect >90% of cells. Transduced cells with high GFP levels were then isolated by fluorescence-activated cell sorting (FACS). The restored expression HIC1 in PCa lines were respectively noted as PC3HIC1 and C4-2BHIC1 cells, and the respective controls were noted as C4-2BGFP and PC3GFP cells.

Methylation analysis and 5-Aza-2′-deoxycytidine treatment
Methprimer software was used to forecast CpG island of upstream 1,000bp promoter of transcription start site (TSS) in HIC1 gene subtype1 and obtained MSP and BSP primers in CpG island. Genomic DNA from cell lines (treated with without 5-Aza-2′-deoxycytidine), prostate cancer (PCa) tissues and plasma (0.2 ml) were purified using DNAzol (Invitrogen Corp, Carlsbad, CA) and QIAMP DNA Blood Mini Kit (QIAGEN GmbH, Hilden, German), treated with sodium bisulfite (ZYMO Research Corp, California, USA), and then analyzed by methylation-specific PCR (MSP) or bisulfite-sequencing PCR (BSP) (Supplementary methods).

**Quantitative real-time PCR**

Total RNA was extracted from cells using Trizol (Invitrogen Corp, Carlsbad, CA) and was reversely transcribed using the reverse transcriptase cDNA synthesis kit (Fermentas, St Leon-Rot, Germany). cDNA was then used as template in the quantitative real-time PCR (qRT-PCR) (Supplementary methods).

cDNA microarray analysis

Agilent-014850 Whole Human Genome Microarrays 4x44K G4112F were used in this study. This Chip targets > 20,000 genes with >40000 probes derived from a broad survey of well known sources such as RefSeq, Goldenpath, Ensembl, Unigene and others. The resulting view of the human genome covers 41K unique genes and transcripts which have been verified and optimized by alignment to the human genome assembly and by Agilent's Empirical Validation process. Total RNA (>300 ng) was extracted from four independent cultures of PC3\textsuperscript{GFP}, PC3\textsuperscript{HIC1} cells and C4-2B\textsuperscript{GFP}, C4-2B\textsuperscript{HIC1} cells (Supplementary methods).

Western blots analyses

PCa cell lines PC3, C4-2B were treated with and without 10 mM of 5-Aza-2′-deoxycytidine (5-Aza-dC; Sigma, St Louis, MO, USA) in 6.0 cm diameter plates (80-90% confluence), and washed three times by PBS. Cells were lysed for 10 min on ice in RIPA buffer (Thermo Scientific, Waltham, MA) containing an anti-protease mixture (Roche, Mannheim, Germany), and protein concentration was measured by BCA assays (Thermo Scientific, Waltham, MA). The protein fractions
were resuspended in loading buffer and denatured at 100°C for 10min. An aliquot of 5-15 μg of proteins was separated on 10% SDS polyacrylamide gels and transferred to PVDF membranes. The membranes were then blocked in 5% fat-free milk TBST buffer (0.1% Tween-20) for 2 hours at room temperature. For detection of GAPDH, HIC1, the membranes were either blocked in 5% fat-free milk TBST buffer (0.1% Tween-20), and 0.2 μg/ml of rabbit anti-human HIC1 polyclonal antibody (Catalog No: H8539, Sigma, St Louis, MO, USA) were used in conjunction with 0.4 μg/ml of anti-species conjugated horseradish peroxidase (Upstate, Lake Placid, NY) and detected by chemiluminescence (Amersham Pharmacia Inc, Piscataway, NJ). MMP2/3 was performed respectively similarly using mouse monoclonal antibodies (0.2 μg/ml; sc-10736, Santa Cruz, Delaware Ave, USA) and rabbit polyclonal antibodies (0.2 μg/ml; sc-21732, Santa Cruz, Delaware Ave, USA).

Subcutaneous, intracardiac, intratibial, and tail vein injections.

Subcutaneous tumors were established as described previously. Intracardiac, intratibial and tail vein injections were performed to determine the role that HIC1 plays in metastasis. After 4 weeks, bioluminescence was utilized to follow the PCa-derived tissue metastases as the primary outcome. The mice were injected intraperitoneally with luciferin (200 μl at 15 mg/ml in PBS) prior to Xenogen IVIS™ 15 (Supplementary methods).

Small animal PET-CT scanning

For the same purpose as above, the above mice injected PC3HIC1Luc and PC3GFPLuc in 2 months were underwent 18F-FDG small-animal PET and micro-CT in Shanghai Ruijin Hospital. PET/CT imaging was performed on an Inveon MM Platform (Siemens Preclinical Solutions, Knoxville, Tennessee, USA) with a computer-controlled bed and 8.5 cm transaxial and 5.7 cm axial fields of view (FOV) (Supplementary methods).

CXCR7 promoter luciferase assays

CXCR7 promoter truncation constructs have been previously described (18). The CXCR7 promoter region -813/+188, -386/+164, -191/+164, and -26/+164 constructs and the CXCR7 promoter -863/+168 △XI region containing -191/+164 and -26/+164
XI mutants were cloned into the pGL3 basic reporter gene vector to generate some CXCR7 promoter truncation constructs (Supplementary methods).

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) was conducted using an EZ-ChIP kit (Millipore Upstate, New York, USA). The dissociated DNA from immunoprecipitated protein/DNA complex was used for PCR assay (Supplementary methods).

**Statistical analysis**

qRT-PCR and cell proliferation were analyzed using Student's t test, 2-tailed, with Excel software. The Kaplan-Meier log-rank test was used for analysis of mouse tumor development and survival data with GraphPad Prism 5 software. Statistical significance was defined as $p < 0.05$. 
Results

Methylation status of HIC1 in PCa

Emerging evidence indicates that HIC1, as a suppressor gene, is silenced by hypermethylation in many types of prevalent human tumors. Surprisingly, however, the promoter hypermethylation of HIC1 in prostate tumors has not yet been studied in details. Using Methprimer software indicated 3 CpG islands of upstream 1,000bp promoter of TSS in HIC1 gene subtype1, we obtained the suitable MSP and BSP primers of -495 to -624bp in CpG island 2 containing 11 CpG sites (Supplementary Fig.1A). To explore whether the HIC1 promoter is methylated in PCa, we analyzed its methylation status in PCa tissues and cell lines by MSP-PCR and bisulfite sequencing (BSP), which covered the above promoter regions. As shown in Fig.1A, two core promoter regions were markedly methylated in C4-2B and PC3 PCa cells compared with the normal prostate epithelia cells (PrECs) by MSP analyses. C4-2B, PC3 and PrECs were treated with 5 μM 5-Aza-2′-deoxycytidine (5-Aza-dC) for 4 days. PCR and Western blot assays indicated that the expression of HIC1 mRNA and protein was restored only in PCa cells, but not effect in PrECs (Fig. 1B). Next, the methylation status of 11 CpG sites within the CpG island 2 of HIC1 by sequencing PCR products after bisulfite treatment were further assayed (Supplementary Fig. 1B). The results showed that the mean methylated percentage among 11 CpG sites of HIC1 promoter was greatly higher in C4-2B and PC3 cells than PrECs (Fig. 1C). Moreover, the levels of methylated HIC1 promoter in 36 primary Chinese PCa tissues compared with the respective healthy control cases were 80.3% ± 8.7 vs. 31.56% ± 11.3 (Fig.1D, Supplementary Table1). Similar results were observed in 20 plasma DNAs from these patients (Supplementary Fig.1C). These findings demonstrate that HIC1 promoter region is hypermethylated in PCa.

Functional analysis after restoring HIC1 expression in PCa cells

Due to silencing of HIC1 expression in PCa cells by hypermethylation, we therefore restored its expression in C4-2B and PC3 cells by using lentivirus vector. Fig.2A showed that restored HIC1 expression was confirmed by Western blot assays in both
cells (noted as PC3HIC1, C4-2BHIC1), which resulted in a proliferation arrest starting around 3 day compared with the controls (Fig.2B). One possible explanation of the inhibited PCa proliferation observed after having been infected with HIC1 is that the gene may induce apoptosis in these cells. The loss of cellular membrane integrity as a reflection of cells undergoing apoptosis was determined by staining the cells for annexin-V. Indeed, HIC1 expression induced the apoptotic fraction in both cells compared with the controls (27.4 versus 11.8 % and 15.4 versus 8.18 %) (Fig. 2C). The total number of dead or dying cells (54.7 % C4-2BHIC1 versus 15.3 % C4-2BGFP, 32.6 % PC3HIC1 versus 16.4 % PC3GFP) was increased as well.

The ability of HIC1 to regulate migration and invasion was assessed by scratch healing assay and using reconstituted extracellular matrices in porous culture chambers. The results showed that the empty GFP vector transfected cells nearly closed the wound at 24 hour after scratch, whereas HIC1-transfected cells were almost unable to heal the wound. The mean wound distances of the HIC1-transfected cells and the control cells at 24 hour were significantly different (435.231 ± 53.06 μm versus 125.28 ± 6.18 μm; p< 0.001) (Fig. 2D). In line with this finding, PC3HIC1 and C4-2BHIC1 cells resulted in less invasive capacity compared with the respective controls (Fig. 2D). As shown in Fig. 2A, the effects may be ascribed to inhibiting MMP2/3 expressions after HIC1 expression, consistent with other group findings that MMP2/3 was responsible for tumor invasion by participating in some signal transduction pathways (19, 20). However, HIC1 knockdown in PC3 cells by siRNAs (Supplementary Fig. 2A) was capable of greatly increasing ability of its migration and invasion as compared with the scrambled control (Supplementary Fig.2B and 2C).

**Tumorigenicity and metastasis by HIC1 restoring expression in mice**

To evaluate that HIC1 plays a role in tumor growth, we transplanted PC3HIC1, C4-2BHIC1, and respective control cells to SCID mice by subcutaneous injection. As shown in Fig.3A and 3B, tumors burden from PC3HIC1 were significantly reduced compared with the control. Similar results were observed in mice from C4-2BHIC1 cells, albeit the effect was not as robust as the above. To further explore whether HIC1 modulate tumor metastasis, we firstly tagged PC3HIC1 cells and its control with luciferase gene by retrovirus infection. These tagged cells were then transplanted to
BALB/c Nude mice by intracardiac injection. The results showed that PC3\textsuperscript{HIC1} cells significantly reduced tissue metastases compared with PC3\textsuperscript{GFP} cells regardless of whether the animal was imaged from ventral surface (Fig. 3C) and the dorsal surface (Supplementary Fig.3A) in 4 weeks or from ventral surface (Supplementary Fig. 3B) and the dorsal surface in 8 weeks (Supplementary Fig. 3C). Examination of individual sites of bone metastasis also showed that HIC1 expression significantly reduced the total luminescent signal (i.e., total tumor burden) by radiographic analysis (Fig.3C; Supplementary Fig. 3A, 3B and 3C). Moreover, representative fused small-animal PET and micro-CT images and immunohistochemical staining further demonstrated that HIC1 expression markedly inhibited multiple metastatic sites to liver, intestine, rhizine of thigh, pleural cavity, et al in 8 weeks compared with the controls (Fig. 3D and Supplementary Fig. 3F). Similar results were also observed in nude mice bearing PC3\textsuperscript{HIC1} cells by tail vein injection in 4 weeks (Supplementary Fig. 3D) and 8 weeks (Supplementary Fig. 3E).

Bone destruction is the main cause of morbidity of PCa patients, which prompted us to assay the effect of HIC1 expression in bone microenvironment. Intratibial injection was therefore performed in SCID mice using PC3\textsuperscript{HIC} cells and the control PC3\textsuperscript{GFP} cells. After 40 days, a radiographic and histologic analyses of the resulting tumors displayed extensive bone destruction in the control groups. In comparison, animals receiving PC3\textsuperscript{HIC1} cells had smaller lesions with less osteolytic damage (Supplementary Fig. 4A). Quantitative histomorphometry by Tartrate Resistant Acid Phosphatase (TRAP) staining confirmed that the number of osteoclasts/mm of bone surface were lower in tissues resulting from the PC3\textsuperscript{HIC1} versus PC3 \textsuperscript{GFP} cells (2.26 ± 0.4 versus 3.93 ± 0.2/ mm) (Supplementary Fig.4B, 4C). In brief, these findings strongly suggest that HIC1 expression had a significant effect on reducing osseous destruction by PCa cells.

**Identification of downstream genes by HIC1**

To explore potential downstream targets induced by HIC1, we analyzed the genome-wide transcriptome profile of HIC1-restoring PC3 and C4-2B cells and the respective control cells by Agilent Whole Human Genome Microarrays. The microarray data set has been deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42336). According to fold-change (X 2.0) screening between expression of HIC1 and its respective control
PCa cells, 310 up-regulated genes and 267 down-regulated genes were commonly observed in PC3 and C4-2B cells. As shown in Fig. 4A, the picked up 36 cancer-associated genes for cluster mapping on the MeV microarray analysis platform (www.tm4.org/mev.heml). We also identified genes related to molecular function and picked up the top 36 gene sets that overlapped with different function-clusters for exhibition (Fig. 4B and Supplementary Table 2).

**CXCR7 expression modulated by HIC1 in PCa**

Among the genes markedly regulated in two PCa cells with HIC1 expression, we paid attention to CXCR7 because our previous (21) and recent studies (22) demonstrated that CXCR7 expression is closely associated with PCa, bladder cancer, breast cancer progression. In addition, CXCR7 has been described as a direct HIC1 target gene in U2OS cells overexpressing HIC1 and the regulatory region of CXCR7 promoter contained several functional HIC1 consensus binding sites (HiRE, HIC1 responsive element) (18). In this regard, we set out to explore the mechanisms through which CXCR7 expression is modulated by HIC1 in PCa cells. Firstly, using qRT-PCR and FACS analysis confirmed that HIC1 expression markedly inhibited CXCR7 level in PC3 and C4-2B cells (Fig.5A, 5B), consistent with the above microarray assays. Similar result was also observed in histological staining of subcutaneous tumors with HIC1 expression (Supplementary Fig. 5A). Moreover, expression of CXCR7 mRNA and protein level was down-regulated in term of restoring expression of HIC1 mRNA and protein by 5-AZa treatment only in PCa cells (Fig. 5C, 1B and 5D), but not in PrECs. In contrast, knockdown of HIC1 expression by siRNA1 and siRNA3 enhanced CXCR7 level in PC3 cells and PrECs (Supplementary Fig.5B). Finally, high density tissue microarrays were stained with both an anti-human CXCR7 and HIC1. Representative images are shown in Fig. 6A, indicating that expression of CXCR7 in cancer tissues was higher than the normal and PIN tissues as reported previously (21). In contrast, HIC1 expression was markedly elevated in the normal and PIN tissues compared with the cancer tissues. Quantitative analysis confirmed the similar observations (Fig. 6B).

**CXCR7 is a direct HIC1 target gene**

Based on the above assays, these findings implied that CXCR7 may be reversibly modulated by HIC1. To determine the underlying mechanism, we utilized a series of CXCR7 truncated promoter/reporter fusion plasmids containing progressive 5’
deletions, which allowed us to perform luciferase promoter-reporter assays. These constructs were then transfected alone or with the pcDNA3-FLAG-HIC1 expression vector into PC3, C4-2B cells and promoter activities were therefore measured in the absence or presence of HIC1. The data showed that, compared with these constructs transfection alone, transient transfection of HIC1 in PC3 cells markedly inhibited the CXCR7 promoter activity in the -813/+168, -386/+164 and -191/+164 constructs, even in the smaller construct -26/+164. The similar inhibitory effect was also shown in C4-2B cells (Fig. 6C). These results suggest that the regulatory region primarily involved in the HIC1-mediated repression of CXCR7 is located in the -26/+164 upstream region of the promoter that contains one of the two phylogenetically conserved and adjacent HIC1 responsive element (HiRE), such as sites IX and XI, as assayed by Van Rechem et al (18). To verify the possibility, we used the ΔXI constructs in XI HiRE site with a mutation (TGC into CAT) to abolish the ability of HIC1 binding. In line with the previous findings (18), the -813/+168, -191/+164 and -26/+164 constructs both display a high basal activity repressed by HIC1 expression. Notably, the mutated ΔXI constructs that the most significantly decreased repression ration by HIC1 was observed in the longest promoter construct -813/+168, but not in -191/+164 and -26/+164 constructs (Fig. 6D). These findings imply that the CXCR7 promoter is negatively regulated by HIC1.

To determine that CXCR7 is indeed a direct target gene of HIC1, we performed ChIP assays using 100kD molecular weight of HIC1 antibody (Sigma, St Louis, MO, USA) and then analyzed the pull-down DNA. The primers were designed to amplify the region mediating the repressive effects of HIC1 on the -26/+164 site of CXCR7 promoter and containing adjacent HiREs as shown in the above assays (Supplementary Fig. 6A). As assay procedure control, we used primers located in the GAPDH promoter (Supplementary Fig. 6B). As shown in Supplementary Fig.6A, CXCR7 was markedly amplified from the HIC1-immunoprecipitated PC3 and C4-2B chromatins compared with PrEC chromatins, but not from chromatin immunoprecipitated by the control rabbit IgG. Taken together, these results demonstrate that endogenous HIC1 proteins are highly recruited onto the CXCR7 promoter in vivo both in PC3, C4-2B cells.
Discussion

HIC1 is a central transcriptional regulator of a few key genes controlling cell growth as well as cell death in response to p53-dependent apoptotic DNA damage through binding to SIRT1 promoter (8). Emerging evidence suggests that by combining with PATCHED, HIC1 may play an inhibitory role in Hedgehog pathway for medulloblastoma and capable of regulating Wnt pathway involved in function of stem cell (23). In fact, HIC1 is frequently hypermethylated as a result of silence or low-level in a variety of solid tumors and leukemia (13, 24), therefore making it a new therapeutic target for DNA methyltransferase inhibitors such as 5-Aza-2'-deoxycytidine (Decitabine) (25).

This study aimed to further our understanding of the role that HIC1 plays in PCa progression. Our data showed that HIC1 promoter hypermethylation was presented in Chinese PCa patients, potentially responsible for its progression, which has not been previously reported to our knowledge. Indeed, our study showed that restoring HIC1 expression in PCa cells markedly inhibited proliferation, migration, and invasion and induced the apoptosis in these cells.

In vivo experiments indicated that restoring HIC1 expression had a marked effect on reducing tumor growth, multiple tissue metastases and osseous destruction in bone microenvironment. Microarray analysis and experiments in vitro identified chemokine receptor CXCR7 target gene as immediately downstream of HIC1.

CXCR7, formerly known as RDC1, has been shown to be a second receptor, in addition to CXCR4, for the chemokine CXCL12 (SDF-1, stoma cell-derived factor 1 (21, 26). In the vasculature, the expression of CXCR7 is elevated in endothelial cells associated with tumors, and overexpression of CXCR7 in NIH 3T3 cells strongly supports a role for the receptor in tumorigenesis (27). More recently, CXCR7 expression has been shown to be elevated in endothelial cells associated with tumors. Membrane associated CXCR7 is expressed on many tumor cell lines, on activated endothelial cells, and on fetal liver cells (26). Miao et al. (28) and our group further confirmed a critical role for CXCR7 in tumor vascular formation, angiogenesis, and promotion of the growth of breast (29), PCa (21), and bladder cancer (22) cancer in vivo. Targeting CXCR7 by various strategies such as shRNA interferences, specific high affinity small molecule antagonist or intrakines severely reduces proliferation of
carcinoma cells in vitro (22, 26) as well as in vivo tumor growth in animal models (30). These characteristics suggest that CXCR7 plays a role in regulating immunity, angiogenesis, stem cell trafficking, and mediating organ-specific metastases of cancer (26-30). However, the mechanism underlying CXCR7 expression modulated by immediately upstream targets still remains unsettled.

In this study, we further demonstrated that endogenous HIC1 could bind to the promoters of CXCR7, thus resulting in inhibiting its effect. Interestingly, the mutated ΔXI constructs that the most significantly decreased repression ration by HIC1 was only observed in the longest promoter construct - 813/+168, but not in -26/+164 constructs. To be fully understood, the mechanism underlying the effect of HIC1 in CXCR7 expression is warranty investigated. However, these findings imply that the CXCR7 promoter is negatively regulated by HIC1. Indeed, CXCR7 expression in PCa tissue is negatively modulated by HIC1, as a critical upstream of CXCR7, which may participate in PCa progression.

Notable, our initial findings suggest that although HIC1 expression levels decreased by epigenetic modification during the PCa development, which inhibited CXCR7 influence on PCa progression. But hypermethylation did not consistently correlate significantly with HIC1 expression levels in breast cancer cells, non-small cell lung cancer cells compared with the corresponding noncancerous tissue, indicating that other inhibitory mechanisms other than hypermethylation of the HIC1 promoter may exist, e.g. SUMOylation or acetylation modification of the promoter, mutations/inactivation of the positive HIC1 regulator p53, or aberrant expression of not yet identified HIC1 inhibitors (31, 32). However, to be fully understood, the function of HIC1 in cancer biology is warranty investigated.

In summary, here we present that HIC1 by epigenetic modification may participate in PCa progression, suggesting that therapies targeting epigenetic events regulating HIC1 expression may provide a more effective strategy for PCa treatment.
Figure Legends

Figure 1. Methylation status of HIC1 CpG islands and the effects of 5-Aza-dC on HIC1 expression.

A) The core promoter region of HIC1 gene was methylated in two PCa cell lines PC3, C4-2B (marked as M, 1 and 2 were both repetition sample hole), but unmethylated in the PrEc cell line (marked as U, 1 and 2 were both repetition sample hole) by MSP analysis.

B) Expression of HIC1 at mRNA (upper) and protein (lower) level was restored in PC3, C4-2B cells after 5 μM of 5-Aza-dC treatment for 4 days.

C) Schematic summary of 11 CpG sites in the HIC1 promoter region from -495bp to -624bp. Methylation analysis was performed in 10 clones from each cell line. Each row of circles represents a single clone, and each circle represents a single CpG site. Open circle represents unmethylated cytosine; filled circle represents methylated cytosine. The PCa cell lines showed higher methylation levels compared with the PrEc cell line as the control. The methylation ratios of 10 clones from each cell lines are summarized in the lowest bar chart.

D) Methylation status of 11 CpG sites in the HIC1 promoter from 36 PCa tissues and the normal prostate tissues. Each row of circles represents occurred methylation status from 5 clones of a sample, and each circle represents a single CpG site. Open circle represents unmethylated cytosine; filled circle represents methylated cytosine. The mean methylated level of cancer tissues compared with the respective healthy control cases were 80.3% ± 8.7 versus 31.56% ± 11.3.

Figure 2. Effects after restoring HIC1 expression in PCa cells

A) Restoring HIC1 expression in PC3 and C4-2B cells (termed as PC3HIC1 and C4-2BHIC1 cells) was confirmed by Western blot. Whole cell lysates were immunoblotted with antibody to HIC1. The blots were stripped and reprobed with GAPDH antibody to confirm equal protein loading.

B) Proliferation of PC3 and C4-2B cells in response to restoring HIC1 expression. After a 24 h serum withdrawal, PCa cells were digested and washed three times in PBS, and 1×10^4 cells were plated in into 96-well flat-bottomed tissue culture plates in 0.1
ml in complete growth medium. Proliferation was evaluated by XTT assay over a 4-day period. * denotes significant difference from controls \((p < 0.05, \text{ANOVA})\) for means ± S.E. of \(n = 5\) samples per condition.

C) Apoptosis profile of PC3 and C4-2B cells in response to restoring HIC1 expression. The role of HIC1 in promoting apoptosis in PCa cells was evaluated culturing equal numbers of cells in complete medium for 24 hour. Annexin-V-fluorescein staining and propidium iodide were used to identify apoptotic and necrotic cells by FACS analysis.

D) Left, restoring expression of HIC1 severely impaired cell migration. Serum was withdrawn before analysis to avoid effect of cell proliferation. The migration status was assessed by measuring the movement of cells into a scraped area created by a 10 µl pipette tube, and the spread of wound closure was observed at indicated times after scratching the surface of a confluent layer of cells. Scale bar: 100 µm. This experience was performed three times. Right, HIC1 regulates PCa cell invasion. PCa cells were placed in the top chamber of invasion plates containing a reconstituted extracellular matrix in serum-free medium. Invasion was determined at 48 hour by MTT staining and the data were read on a multiwell scanning spectrophotometer (Thermo Scientific, Massachusetts, USA) at A580 and presented as % invasion binding ± standard deviation for \(n=5\). * denotes significant difference from controls \((p < 0.05, \text{ANOVA})\).

Figure 3. Tumorigenicity and metastasis by HIC1 restoring expression in vivo

A) Effect of HIC1 on PC3 and C4-2B cells growth in vivo. 6-week-old SCID mice were implanted subcutaneously with \(5 \times 10^5\) PC3 and C4-2B cells restoring HIC1 expressing. Tumor volume (mm\(^3\)) was evaluated at indicated times. * indicates significant difference from the controls \((p < 0.05)\).

B) Effect of HIC1 on tumor weight. Representative macroscopic appearance of tumor growth was assayed in PC3\(^{\text{GFP}}\) and PC3\(^{\text{HIC1}}\), C4-2B\(^{\text{GFP}}\) and C4-2B\(^{\text{HIC1}}\). Tumor weight was measured at sacrifice. The data are presented as mean ± S.D. for triplicate determinations. N=8 in groups. Representative 6 tumors are shown. * indicates
significant difference from the controls ($p < 0.05$).

C) Restoring HIC1 expression inhibits PCa metastasis in vivo. PC3\textsuperscript{HIC1Luc} and PC3\textsuperscript{GFPLuc} cells were respectively injected into the left cardiac ventricle of BALB/c Nude mice. At 4 weeks, imaging was performed with a Xenogen IVIS imaging system. Luciferase signals from the ventral surface of the representative mice are shown. Quantification of tumor burdens was identified by bioluminescence imaging. The data showed that the tumor growth and metastasis from PC3\textsuperscript{HIC1Luc} group (N=10) were greatly inhibited compared with the control PC3\textsuperscript{GFPLuc} group (N=10). *, $p < 0.05$, significant difference from controls.

D) Small animal PET-CT scanning performed at 8 weeks showed the tumor growth and organs metastasis from PC3\textsuperscript{HIC1Luc} and PC3\textsuperscript{GFPLuc} group. Representative images indicated that restoring HIC1 expression markedly inhibited multiple metastatic sites to liver, spleen, rhizine of thigh et al, compared with the control. *, $p < 0.05$, significant difference from controls.

Figure 4. Target genes identified by global microarray analysis.

A) Agilent Whole Human Genome Microarrays were used to assay the potential target genes. Clustering map of differentially expressed genes overlapped with cancer-associated genes set in the Molecular Signatures Database. Row represents gene, column represents experimental cells. The first and second columns are the control PC3\textsuperscript{GFP}, C4-2B\textsuperscript{GFP} cells, the third and forth columns are the restoring HIC1 expression PC3\textsuperscript{HIC1} and C4-2B\textsuperscript{HIC1} cells. Upregulated genes are shown in red and downregulated genes in green.

B) Fold-change map overlapped with molecular function-related genes set in the Molecular Signatures Database. Both upregulated genes in C4-2B and PC3 are listed on the upper; and both downregulated genes are listed on the lower. Part of detailed data was set in Supplemental Table2.

Figure 5. CXCR7 expression regulated by HIC1 in PCa cells

A) CXCR7 mRNA expression was confirmed by quantitative real time-PCR in PC3\textsuperscript{HIC1} and C4-2B\textsuperscript{HIC1} cells.
B) CXCR7 protein expression was evaluated by FACS analysis in PC3^{HIC1} and C4-2B^{HIC1} cells using mouse anti-human CXCR7 antibody, and mouse anti-human IgG served as isotype control.

C) CXCR7 expressions was decreased in PCa cells treated with 5-Aza-CdR. Using PCR expression of CXCR7 mRNA was assayed by restoring expression of HIC1 mRNA in presence of 5-AZA treatment at 8h.

D) CXCR7 expression was decreased in PCa cells treated with 5-Aza-CdR. Using FASC analysis, expression of CXCR7 protein level was assayed by restoring expression of HIC1 protein in presence of 5-AZA treatment at 8h.

Figure 6. CXCR7 is direct HIC1 target genes

A) Tissue microarrays and immunohistochemical staining. High-density tissue microarrays were constructed by US Biomax, Inc (Catalog No: PR2085a; Rockville, MD) with clinical samples obtained from a cohort of 114 patients containing 10 benign, 10 prostate intraepithelial neoplasia of disease(PIN) and 94 prostate cancer patients. Formalin-fixed paraffin-embedded tissues were incubated overnight at room temperature with rabbit anti-human HIC1 antibody (Sigma, St Louis, MO, USA) and rabbit anti-human CXCR7 antibody (Abcam Inc, Cambridge, MA). MBA171 (IgG2a) was used as a negative control. Representative micrographs were taken at an original magnification 20×, where the black bars represent 100 µM.

(B) Quantitative histological evaluation of HIC1 and CXCR7 expression. HIC1 and CXCR7 expression intensity was scored by a genitourinary pathologist on a four point scale as negative, weak, moderate, or strong. Mean expression scores multiplied by percent positive cells in the field using software I-SOLUTION™ (IMT I-Solution Inc, New York, USA) are presented for benign, pin, and cancer cases in a graphic format using error bars with 95% confidence intervals (CI). *, # presents statistically significant differences between normal and cancer, \( p < 0.001 \).

C) Various fragments of the human CXCR7 noncoding exon 5’ sequences subcloned firstly in the luciferase reporter plasmid pGL3 basic. These reporter constructs were then transfected in triplicate into PC3 and C4-2B cells to assay for luciferase activity.
Repression of transcription of each construct by HIC1 was calculated by first dividing luciferase activity in the absence of HIC1, and comparison with the activity in the presence of HIC1. The value obtained for each construct was then divided by the repressive effect elicited by HIC1 on the empty pGL3 basic vector to obtain the final fold of activation. Results, expressed relative to a value of 1.0 for cells transfected with the pGL3 empty vector, are expressed as the mean of three different experiments, and error bars represent standard deviations.

**D)** The mutated conserved HiRE XI (ΔXI) decreased the transcriptional activity of CXCR7 impeded by HIC1. These reporter wild-type and respective ΔXI constructs were transfected in triplicate into PC3 and C4-2B cells and assayed for luciferase activity. Luciferase activity is shown in the right panel (grey boxes). Repression of transcription of each construct by HIC1 was calculated exactly as detailed the above. Results, expressed relative to a value of 1.0 for cells transfected with the pGL3 empty vector, are expressed as the mean of four different experiments and error bars represent standard deviations. The p values are indicated ( *, p values < 0.05).
Acknowledgements

We thank Dr. Guohong Hu and Ms. Xinyi Liu for technical supports and Dr. Dominique Leprince for providing CXCR7 truncated vectors. Research in the authors’ laboratory is supported by the National Natural funding of China (81071747, 81272404), National key program (973) for Basic Research of China (2011CB510106, 2011CB504300), Shanghai Education Committee Key Discipline and Specialties Foundation (J50208), the Program for Professor of Special Appointment (Eastern Scholar to J, Wang) at Shanghai Institutions of Higher Learning, and Shanghai Pujiang Program (10PJ1406400), Shanghai Committee of Science and Technology (11DZ2260200) and Program of Shanghai Municipal Health Bureau Subject Chief Scientist (XBR20110052).
References


Zheng et al. Figure 1
A

PC3GFP  PC3HIC1  C4-2BGFP  C4-2BHIC1

HIC1
MMP3
MMP2
GAPDH

B

PC3GFP  C4-2BGFP  C4-2BHIC1  PC3HIC1

XTT(O.D560/690)

0  0.1  0.2  0.3  0.4  0.5  0.6

1d  2d  3d  4d  5d

C

PC3GFP  PC3HIC1

D

PC3GFP  PC3HIC1

MTT(O.D450/690)

0h  24h

C4-2BGFP  C4-2BHIC1

Zheng et al. Figure 2
Zheng et al. Figure 4

Up-regulated

- LRRC4
- HKDC1
- SERPIND1
- RNF180
- GPR162
- DNAH1
- PALM3
- PEG10
- NHEDC1
- IL12A
- FAM177A1
- ANKLE1
- CDH1
- KCNK3
- HIC1

Down-regulated

- GEFT
- AHSP
- DUSP5
- HAVCR1
- EDN1
- CXCR7
- TNFRSF11B
- CFTR
- NPNT
- C11orf86
- PXDN
- ITGA8
- HS6ST3
- FAM133A
- WNT8A
- FGFBP1
- LCN2
- NRG1
- ZBED2
- ZCCHC12
- MYO16

PC3HCl/PC3GFP

C4-2BHCl/C4-2BGFP
Zheng et al. Figure 5
A

Benign

PCa (Gleason 4)

B

95% CI Staining Intensity

CXCR7 Staining
HIC1 Staining

Benign (N=10) PIN (N=10) PCA (N=10)

C

PC3

CXCR7
HIC1+CXCR7

-26/+164
-191/+164
-386/+164
-863/+168

C4-2B

CXCR7
HIC1+CXCR7

-26/+164
-191/+164
-386/+164
-863/+168

Luciferase activity

D

PC3

HIC1+CXCR7
HIC1+CXCR7(HiRE XI mutation)

-26/+164
-191/+164
-386/+164
-863/+168

C4-2B

HIC1+CXCR7
HIC1+CXCR7(HiRE XI mutation)

-26/+164
-191/+164
-386/+164
-863/+168

Luciferase activity

Zheng et al. Figure 6
Clinical Cancer Research

HIC1 modulates prostate cancer progression by epigenetic modification
Jianghua Zheng, Jinglong Wang, Xueqing Sun, et al.

Clin Cancer Res  Published OnlineFirst January 22, 2013.

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doi:10.1158/1078-0432.CCR-12-2888

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