HDAC2 Inhibits EMT-Mediated Cancer Metastasis by Downregulating the Long Noncoding RNA H19 in Colorectal Cancer

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Abstract

Background: Epithelial to mesenchymal transition (EMT) is a critical step for cancer metastasis, which is regulated by epigenetic mechanisms. The role of HDACs-mediated deacytelation remains unclear in colorectal cancer (CRC) metastasis. Here, we revealed the biological role and underlying mechanism of histone deacetylase2 (HDAC2) in EMT-mediated CRC metastasis.

Methods: The expression of HDACs in CRC was analyzed using the public database Oncomine and TCGA, human colorectal tumor primary sites and paired metastatic tissues, high or low metastatic CRC cell lines (DLD1, HCT116, SW480 and SW620). Microarray analysis was used to detect the gene expression changes in HDAC2 knock out CRC cells. Epithelial-mesenchymal transition (EMT)-related proteins were determined using western blot and immunofluorescence. CRC cell metastasis was assessed by transwell assay. To study the underlying mechanism of HDAC2 in EMT-mediated CRC metastasis, we performed chromatin immunoprecipitation, promoter activity, co-immunoprecipitation and RNA-binding protein immunoprecipitation assays through using CRC cells and specific siRNAs targeting H19, SP1 and MMP14. Finally, CRC metastasis in vivo was performed using a tail vain injection model.

Results: Our data showed that the expression of HDAC2 was reduced in CRC metastatic tissues and and low HDAC2 expression predicted a poor clinical prognosis in CRC patients. HDAC2 deletion or knock down in CRC cells induced EMT and metastasis by upregulating the long noncoding RNA H19 (LncRNA H19). HDAC2 decreased histone H3K27 acetylation at the promoter of LncRNA H19 and its expression via a SP1-dependent mechanism. LncRNA H19 functioned as a miR-22-3P sponge to increase the expression of MMP14. Finally, using a tail vain injection model, we showed the HDAC2 loss strongly induces lung metastasis, which is suppressed by LncRNA H19 knockdown.

Conclusion: Our study proved that HDAC2 is a negative regulator of EMT-mediated CRC metastasis through regulating H19 and MMP14 expression.

Background

Colorectal cancer (CRC) is one of the most common malignancies worldwide [1]. Although encouraging progress in its diagnosis and therapy has been achieved in the past decade, the overall survival of CRC patients remains unfavorable [2]. CRC treatment failure is largely due to metastasis. Metastasis is a complex process, and many cell-intrinsic identities and extrinsic microenvironment factors influence the metastatic potential of CRC cells [3]. Fully understanding the molecular mechanisms of tumor metastasis may promote the development of effective metastasis-targeted therapy and improve the overall survival of patients with CRC.

Epithelial to mesenchymal transition (EMT) is a process characterized by the loss of cell-cell adhesion and the gain of migratory and invasive traits[4]. EMT has been reported to play important roles in many physiological and pathological processes[5]. Under pathological conditions, epithelial to mesenchymal

transition frequently occurs at the initial stage of cancer metastasis. During epithelial to mesenchymal transition, epithelial cells destroy extracellular constraints, which consist of cell adhesion molecules, such as E-cadherin. Meanwhile, the expression of mesenchymal markers, such as fibronectin and ITGα5, increases and hence contributes to cell motility[6]. Many researchers have highlighted EMT as the initialization step for cancer metastasis, and EMT-related molecules may act as novel targets for clinical cancer prognosis and therapy[7]. Clarifying the regulatory mechanism of EMT is of great significance in restraining tumor metastasis.

Histone deacetylases (HDACs) are enzymes that catalyze the removal of acetyl groups from lysine residues in both histones and other proteins to repression gene transcription. Eighteen types of HDACs, named HDAC1-11 and SIRT1-7, have been identified in mammals[8]. HDACs are involved in different stages of cancer through the deacetylation of histone and non-histone substrates. The aberrant expression of classical (class I, II, IV) HDACs has been linked to the initiation and progression of a variety of malignancies [8]. In most cases, a high level of HDACs is associated with advanced disease and a poor outcome in patients[9–11]. Therefore, HDACs are considered valid targets for cancer prevention and therapy, and many HDAC inhibitors have been developed. Through hyperacetylating histone and nonhistone targets, HDAC inhibitors (HDACis) enable the reestablishment of cellular acetylation homeostasis and restore the normal expression and function of numerous proteins that may reverse cancer initiation and progression[8]. However, HDACis have low efficacy because of the feedback-mediated activation of tumor protection signaling in malignant solid tumor monotherapy[12], and in some cancers, the opposite results have been found; the genetic inactivation of HDACs might have tumorigenic effects. HDAC1 somatic mutations were detected in 8.3% of dedifferentiated liposarcomas, and HDAC4 homozygous deletions occurred in 4% of melanoma[13, 14]. Low HDAC10 expression is associated with poor prognosis in lung and gastric cancer patients [15, 16]. HDAC6 is downregulated in human hepatocellular carcinoma (HCC) tissues, and low HDAC6 expression is associated with poor prognosis in liver transplantation patients. HDAC6 knockdown promotes angiogenesis in HCC [17]. Truncating mutations and the loss of HDAC2 protein expression have been observed in human epithelial cancers with microsatellite instability[18]. Furthermore, the ectopic expression of HDAC2 in mutant cancer cells induces reduced tumor cell growth in-vitro and *in vivo*[19], suggesting a putative tumor suppressor role for HDAC2 in this cellular setting, but its mechanism remains unclear. This evidence reveals the dual role for HDACs in cancer initiation and maintenance, but their regulatory mechanisms still need to be uncovered.

HDACs also play a dual role in EMT and cancer metastasis, and the mechanisms by which individual HDACs regulate cancer metastasis are quite diverse. Junyi He found that HDAC1 could promote EMT progression in gallbladder cancer by binding with TCF12[20]. Furthermore, Ali Aghdassi found that HDAC1 and HDAC2 could be recruited by the transcriptional repressor ZEB1 and downregulate E-cadherin expression in pancreatic cancer[21]. In addition, SIRT1, another HDAC, was found to induce EMT and enhance prostate cancer cell migration and metastasis by cooperating with the EMT transcription factor ZEB1[22]. In contrast, however, many studies have also shown that HDAC inhibitors induce epithelial to

mesenchymal transition and cancer metastasis. Cell migration was dramatically enhanced by various classes of HDACi treatments in 13 of 30 examined human breast, gastric, liver, and lung cancer cell lines in a dose-dependent manner[23]. Tumor metastasis was also enhanced through activating multiple PKCs and downstream substrates along with upregulated proapoptotic p21 expression in HDACi-treated mice[23]. The HDAC inhibitors trichostatin A (TSA) and valproic acid (VPA) induced mesenchymal features in colon carcinoma cells by a decrease in E-cadherin and an increase in vimentin expression at the mRNA and protein levels[24]. However, to our knowledge, the role of HDACs in CRC metastasis remains unclear.

In this study, we found that the expression of HDAC2 was low in metastatic colorectal cancer tissues, which was associated with poor survival in CRC patients. Furthermore, we found that HDAC2 deletion induced EMT-mediated colorectal cancer metastasis *in vivo* and in-vitro. Mechanistically, HDAC2 regulated EMT and colorectal cancer metastasis *via* the HDAC2/H19/MMP14 axis. Taken together, the results of this study have uncovered for the first time a key role for HDAC2 as a negative regulator of CRC metastasis and suggest H19 and MMP14 as targets of combination inhibition for metastatic CRC therapy.

Materials And Methods

Dataset

Data on the relative expression of HDAC1, HDAC2, HDAC3 and HDAC8 were downloaded from the Oncomine public database (www.oncomine.org). The dataset contains 330 primary sites and 43 metastatic CRC tissue samples.

Survival analysis of HDAC1, HDAC2, HDAC3 and HDAC8

We labeled TCGA samples as “high” or “low” according to whether the expression of HDAC1, HDAC2, HDAC3 and HDAC8 was higher or lower than the corresponding median value among all samples. The log-rank test was used to measure whether the survival time was significantly different between the “high” and “low” expression groups. Kaplan-Meier plots were made by Gene Expression Profiling Interactive Analysis (GEPIA: http://gepia.cancer-pku.cn/).

CRC tissue sample and immunochemistry

Commercially available tissue microarray (TMA) slides (HLin-Ade075Met-01, Shanghai Biochip Co., Ltd., Shanghai, China) containing histologically confirmed tissues from CRC patients were purchased for immunohistochemistry (IHC) analysis. Specific primary antibodies against HDAC2 (Cell Signaling Technology) were used for IHC with a 2-step protocol.

Cell culture

DLD1, HCT116, SW480 and SW620 cells were obtained from ATCC. DLD1 and HCT116 cells were cultured in RPMI 1640 medium, while SW480 and SW620 were cultured in high glucose DMEM. All media

were supplemented with 10% FBS, 100 μg/m L penicillin and 100 U/m L streptomycin. The DLD1HDAC2 KO cell line was constructed in the lab of Professor Run-lei Du, who is our collaborator in this study[25].

Microarray analysis

The total RNA from DLD1 and DLD1HDAC2 KO cells was prepared for microarray analysis (n = 3 each). The Affymetrix microarray was used to detect mRNA and long noncoding RNA expression profiles. Microarray data were normalized using the RAM (robust multiple-array average) normalization method. The differentially expressed genes were determined with a threshold cutoff of 2-fold ($p < 0.01$).

Transwell migration assays

Tumor cell migration assays were performed according to the manufacturer's instructions. Briefly, cells were harvested and resuspended in serum-free medium and then seeded onto Transwell inserts at a density of 100,000 cells/well. Then, the inserts were placed in a lower chamber filled with 600 μl culture media containing 10% FBS. Transwells were incubated for 24 h at 37°C. Cells on the inside of the Transwell inserts were removed with a cotton swab; then, cells that migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Photographs were taken from five random fields, and the cells were counted to calculate the average number of cells that had transmigrated.

Vector construction and luciferase reporter assay

The H19 promoter containing intact SP1 recognition sequences was PCR-amplified and subcloned into the KpnI and HindIII sites of the pGL-3-basic vector, and the vector was named pGL-3-H19. The pGL3-H19 vector with point mutations in the SP1 binding sites was synthesized by GenScript (Nanjing, China) and named pGL3-H19-mut (SP1). For the luciferase reporter assay, HEK293 cells cultured in 24-well plates were cotransfected with luciferase reporter plasmids and HDAC2 plasmids. Twenty-four hours posttransfection, HEK293 cells were lysed in lysis buffer. After centrifugation at 12,000 rpm for 5 min, the supernatant was transferred to a new tube. The luciferase activity was monitored by mixing 10 μl supernatant with 30 μl luciferase assay buffer and using a GloMax 20/20 Luminometer (Promega).

siRNA and gene transfection

The siRNAs were synthesized by RiboBio Company (Guangzhou, China). Oligonucleotide transfection was conducted by using Lipofectamine™ RNAiMAX transfection reagent (Life, USA) following the protocol recommended by the manufacturer. After 48 h posttransfection, the cells were collected and used for further investigations.

Chromatin immunoprecipitation (ChIP) and q PCR

ChIP was performed using a SimpleChIP® Enzymatic Chromatin IP Kit (CST) following the manufacturer's instructions. Briefly, genomic DNA-protein complexes were immunoprecipitated using anti-HDAC2

antibody or normal rabbit IgG as a control. After enzyme digestion and sonication, the precipitated DNA was amplified by SYBR Green-based quantitative real-time PCR using primers encompassing the promoter regions of the H19 gene. The ChIP PCR primers used were (the numbers in parentheses indicate the sequence regions corresponding to GenBank ID AF125183):

Primer 1: 5’-CCAGCCATGTGCAAAGTATG-3’ (9747-9766)

Primer 2: 5’-CCATCCTGGAATTCTCCAAA-3’ (9939-9920)

Primer 3: 5’-GCGGTCTTCAGACAGGAAAG-3’ (9468-9487)

Primer 4: 5’-CACGTTCCTGGAGAGTAGGG-3’ (9673-9654)

Co-immunoprecipitation

A co-immunoprecipitation assay was performed in the following steps. Briefly, the cells were washed with ice-cold PBS, lysed in NP-40 buffer containing cocktail and then centrifuged for 10 min at 12000 rpm and 4°C. Anti-SP1/HDAC2 antibody or normal rabbit IgG was added to the cell lysate and incubated at 4°C overnight. Then, 15 μl of protein A/G agarose beads was added to each tube, incubated at room temperature for 3 h and centrifuged for 3 min at 4000 rpm at 4°C. A total of 30 μl of 2× SDS-loading buffer was added to the antigen-antibody-protein A/G agarose bead complex, which was boiled for 10 min. The sample was collected for subsequent SDS-PAGE and Western blotting.

RNA-binding protein immunoprecipitation

The anti-Ago2 RIP assay was performed using a Magan RIP^{TM} RNA-Binding protein Immunoprecipitation Kit（Milipore）following the manufacturer’s instructions. Briefly, DLD1 HDAC2 KO cells were washed with cold PBS and lysis by RIP Lysis Buffer. After that, the cell lysates were incubated with antibody against Ago2 (Milipore, USA). The normal Mouse IgG was used as negative control. For RNA immunoprecipitation, the supernatant was incubated with the antibody-coated Sepharose beads overnight. The RNA bound to Ago2 antibody was extracted with TRIzol reagent (Invitrogen, USA) and detected by qRT-PCR.

Fluorescence in Situ Hybridization (FISH)

The sequence of the H19 probe was 5’-FAM/GCTGCTGTTCCGATGGTGTCTTTGATGTTGGGC/FAM-3’; this probe was used for FISH of H19 from B-NDG mouse pulmonary metastases. After the metastatic lung tissues were removed and cleaned, they were immediately fixed in an ISH fixation solution (DEPC water preparation) for 12 h. After tissue fixation, they were dehydrated by gradient alcohol and then embedded in paraffin. The tissue sections were used for H19 probe hybridization, and the nuclei were restained by DAPI. The sections were observed under a fluorescence microscope, and images were collected. The H19 probe is shown in green, while DAPI staining is shown in blue.

Animal study

B-NDG immunodeficient mice (with T cell, B cell and NK cell defects) were obtained from Beijing Biocytogen Biotechnology Co., Ltd. The B-NDG mice were randomly divided into two groups. A total of 5×10^6 luciferase-labeled CRC cells were injected intravenously into B-NDG mice via the tail vein. Four weeks later, the mice were anesthetized and injected intraperitoneally with fluorescein potassium salt (150 mg/kg), and 10 min later, the metastatic tumor was detected and photographed by a bioluminescent *in vivo* imager (VILBER Fusion FX7, France). The mice were sacrificed, and their lung tissues were removed for H&E staining, immunohistochemistry and FISH. The mean number of metastatic nodules in B-NDG mice with lung metastasis was calculated.

Statistical analysis

All experiments were repeated no fewer than 3 times. Experimental results are presented as the mean±S.E.M. The statistical significance of comparisons between two groups was determined with a two-tailed Student's t-test. P□0.05 indicated statistical significance.

Additional Materials and Methods

More details regarding the materials and methods can be found in the Supplementary information.

Results

HDAC2 is frequently downregulated in metastatic colorectal cancer tissues

To investigate the roles of HDACs in colorectal cancer metastasis, we queried the expression of HDACs in CRC from the public database Oncomine (www.oncomine.org). We found that, compared with primary sites, the expression of HDAC1 and HDAC2 was significantly lower in metastases (Fig. 1a). Factors that affect tumor metastasis usually affect patient survival. Therefore, we further analyzed the relationship between the expression of HDACs and the overall survival of patients with colorectal cancer. Notably, Kaplan-Meier analyses indicated that low HDAC2 expression was correlated with poor survival for CRC patients (Fig. 1b). Therefore, we focused on HDAC2 in our further research. We collected 22 paired clinical colorectal cancer specimens containing primary site and matched metastatic tissues, and detected the expression of HDAC2 in these samples by IHC (Fig. 1e and Fig. S1a). The expression of HDAC2 was significantly lower in 12 cases (M *vs* P) (Fig. 1c and 1d). Besides, by consulting the public starBase database□http://starbase.sysu.edu.cn/□,we found that the expression of CDH1(E-cadherin), which is generally accepted as a hallmark of cancer metastasis, is positively correlated with the expression of HDAC2 in CRC(including 471 COAD samples and 167 READ samples, Fig.1f and 1g).In addition, we found that HDAC2 was expressed at low levels in highly invasive CRC cells (HCT116 and SW620) (Fig. 1h and Fig. S1b, S1c). Collectively, these findings indicate that HDAC2 is significantly downregulated in metastatic CRC tissues and may be involved in the regulation of cancer metastasis.

Low HDAC2 expression induces epithelial to mesenchymal transition in CRC cells

To explore whether HDAC2 plays a role in the regulation of CRC metastasis, we knocked out HDAC2 in DLD1 cells as previously described[25]. We then used microarray analysis to detect the expression changes of genes in HDAC2 KO cells and found that the expression of 1555 mRNAs changed significantly (Fig. 2a and Supplementary Table 1). Among the 1555 mRNAs, 47 mRNAs were reported to be involved in EMT (Fig. 2b). Among those 47 mRNAs, we found that the epithelial marker CDH1 was downregulated, while the mesenchymal markers fibronectin and ITGA5 were upregulated (Fig. 2c). These results indicated that HDAC2 deletion led to an EMT gene expression signature.

Moreover, we found that HDAC2 deletion resulted in the conversion of epithelial features to mesenchymal features in DLD1 cells. As shown in Figure S2a, DLD1 HDAC2 KO cells displayed a non-polarized and spindle-shaped morphology. Consistent with these morphological changes, decreases in E-cadherin and increases in fibronectin and ITGA5 were observed at the protein level (Fig. 2f, 2j and 2k). In addition, we found that the migration ability of KO cells was increased (Fig. 2d and 2e). The increased migration ability of DLD1 KO cells were confirmed by RTCA (Fig. S2b).What's more, we found that knock down HDAC2 in SW480 also led to the conversion of epithelial features to mesenchymal features, including increased migration ability (Fig. 2g and 2h)and up-regulated fibronectin and ITGA5((Fig. 2i).Collectively, these data show that HDAC2 negatively regulates EMT and CRC cells migration.

Low HDAC2 expression induces EMT by upregulating the lncRNA H19

Noncoding RNAs have been reported to be important regulators for many biological processes. Recently, a series of noncoding RNAs were found to modulate the expression of genes involved in EMT and tumor metastasis[26]. To understand the mechanism of HDAC2 in regulation EMT in CRC, we used microarray analysis to evaluate expression changes of noncoding RNAs in DLD1 HDAC2 KO cells. Transcriptome profiling identified 1039 ncRNA transcripts that were differentially expressed in HDAC2 KO cells (Fig. 3a and Supplementary Table 2). Among the top five differentially expressed noncoding RNAs (Fig. 3b), a novel lncRNA (accession Number: n333410) drew our attention in particular. We downloaded the sequence of n333410 from the NONCODE database (http://www.noncode.org/) and aligned it with the sequence of H19 (GenBank: AF125183.1). Results showed that the two sequences were 100% identical (Fig. S3a). Q-PCR assays using gene-specific primers for H19 confirmed that the lncRNA H19 was significantly upregulated in DLD1 HDAC2 KO cells (Fig. 3c). HDAC2 knockdown in SW480 cells by shRNA also increased the expression of H19 (Fig. 3d). Moreover, we found that the expression of H19 in colorectal cancer cells with low HDAC2 expression was also higher than that in cells with high HDAC2 expression (Fig. S3b). These findings indicate that HDAC2 is a negative regulator of H19 in CRC.

Some reports has showed that H19 is an important regulator in cancer metastasis[27, 28], so we speculate that H19 is the downstream target of HDAC2 regulating EMT in CRC. To test our hypothesis, we synthesized siRNA targeting H19 (Fig. 3e) and transfected it into DLD1 HDAC2 KO and SW620 cells. We found that the EMT processes in the DLD1 HDAC2 KO and SW620 cells were reversed. H19 knockdown

by siRNA significantly reduced the migration ability of KO (Fig. 3f ,3g and S3c) and SW620 cells (Fig. 3i and 3j). Additionally, an increase in E-cadherin and a decrease in fibronectin and ITGA5 were observed in KO and SW620 cells after H19 knockdown (Fig. 3h and 3k). Collectively, these findings support H19 as the downstream target of HDAC2 and that low HDAC2 expression induces EMT by upregulating H19 in CRC.

HDAC2 inhibits the expression of H19 by interacting with the transcription factor SP1 and catalyzing H3K27 deacetylation

As indicated by the above results, H19 is the downstream target of HDAC2. To understand how HDAC2 regulates the expression of H19 in CRC, we performed chromatin immunoprecipitation experiments and luciferase reporter assays. We found that HDAC2 could bind to the H19 promoter (Fig. 4a). As we know, HDAC2 is an enzyme that catalyzes the removal of acetyl groups from lysine residues in histones to repress gene transcription. Therefore, we detected changes in the acetylation of different histones in DLD1HDAC2 KO cells by Western blot. An increase in H3K27 acetylation was observed in KO cells (Fig. 4b). In addition, ChIP experiments confirmed that the level of acetylated histone H3K27 at the H19 promoter increased after HDAC2 knockout (Fig. 4c). Collectively, these results indicate that HDAC2 could bind to H19 promoter and catalyze H3K27 deacetylation.

Are any transcription factors involved in the regulation of H19 expression by HDAC2? We analyzed the DNA sequence of the H19 promoter (Fig. S4a). Three binding sites for the transcription factor SP1 were found in the H19 promoter (Fig. 4d). We inferred that SP1 may be involved in the HDAC2-mediated regulation of H19. Therefore, we mutated the SP1 recognition sites (Fig. 4d) and found that the effects of HDAC2 on H19 promoter activity were attenuated (Fig. 4e and 4f). Moreover, we found a direction interaction between HDAC2 and SP1 by immunoprecipitation in both DLD1 cells (Fig. 4g left) and SW480 cells (Fig. 4g right). In addition, when we knocked down SP1 by siRNA in DLD1 and SW480 cells, the H19 expression increased significantly (Fig. 4h and 4i). Additionally, a increased migration ability (Fig. S4b and 4c), a decrease in E-cadherin and an increase in fibronectin and ITGA5 were observed in DLD1 SP1 RNAi cells (Fig. S4d). Together, these results suggest that HDAC2 inhibits the expression of H19 and EMT through interacting with the transcription factor SP1 and catalyzing H3K27 deacetylation.

H19 promotes EMT by upregulating MMP14

How does the lncRNA H19 promotes epithelial to mesenchymal transition in colorectal cancer？A report showed that the H19 promotes epithelial to mesenchymal transition by sponging miRNA-138 and miR-200a？and then upregulating their downstream targets Vimentin？ZEB1and ZEB2 in colorectal cancer[29]. But？We did not find an increase of Vimentin？ZEB1and ZEB2 in the DLD1 HDAC2 KO cells in our microarray data. Therefore, we speculate that H19 may have other mechanisms to promote EMT in colorectal cancer. When analyzing our microarray data, we noticed that some MMPs changed significantly. Given the fact that MMPs are important stimulating factors of EMT[30, 31].we speculated that MMPs might be the downstream target of H19. Q-PCR showed that MMP14 were the most up-regulated

MMPs in KO cells（Fig.5a）. Western blot confirmed the increased expression of MMP14 in CRC HDAC2 KO and knock down cells (Fig.5b).But the expression of MMP14 was decreased when we knocked down H19 in DLD1 HDAC2 KO cells(Fig.5c left) and increased when knocked down SP1 in DLD1 WT cells(Fig.5c right).Besides, we found the EMT process in DLD1 HDAC2 KO cells was reversed when we knocked down MMP14 by siRNA(Fig.5d,5e and 5f). These findings indicate that H19 promotes EMT by upregulating MMP14 in CRC.

But how does H19 upregulate MMP14? Many studies have showed that lncRNA can positively regulate gene expression by functioning as miRNAs sponge[32]. By consulting the public database, we found that the expression of H19 is positively correlated with the expression of MMP14 in CRC(Fig.5g and 5h).Also， we found that the expression of MMP14 in colorectal cancer cells with higher H19 expression was also higher (Fig. 5i).Besides，we found two binding sites between H19 and microRNA-22-3P（Fig.5j）and two recognition sites of microRNA-22-3P in the 3'-UTR region of MMP14（Fig.5k）. These findings support that H19 may up-regulate MMP14 by sponging miR-22-3P and then release its inhibitory effect on MMP14. As expected，anti-Ago2 RIP assay（Fig.5l） showed that miR-22-3P could directly bind to the lncRNA H19 （Fig.5m）.Besides, when we transfected miR-22-3P mimics to the DLD1 HDAC2 KO cells, the expression of MMP14 decreased significantly（Fig.5q） and the EMT process was repressed （Fig.5o-5q）,while the expression of H19 did not change significantly（Fig.5n）.These results show that H19 promotes EMT by binding to miR-22-3P and then upregulate MMP14.

Low HDAC2 expression promotes colorectal cancer metastasis *in vivo*

At last，we evaluated the *in vivo* biological functions of HDAC2 in CRC, we inoculated nude mice with luciferase-labeled DLD1 WT, HDAC2 KO and KO shH19 cells *via* the caudal vein. Compared with the WT group, the luciferase signal intensities of mice in the KO group were much stronger, but they were weaker when H19 was knocked down (Fig. 6a). In addition, we found more lung metastases in the KO group than in the WT group, but when we knocked down H19, lung metastases decreased significantly (Fig. 6b, 6c and 6d). These results indicated that more KO cells were transferred to the lung and that when H19 was knocked down, the metastatic ability of the KO cells was decreased. Additionally, we found an increase of H19 and MMP14 and a decrease of E-cadherin the lung metastases of mice in the KO group (Fig. 6e and 6f). However, these were restored when H19 was knocked down (Fig. 6e and 6f). We also inoculated luciferase-labeled SW480 and SW620 cells into nude mice. Consistent with our previous hypothesis and results, the SW620 group mice show more metastases in the lung(Fig. S5a-5c).Besides, a decrease of HDAC2 and E-cadherin and an increased MMP14 expression was found in the lung metastases of mice in the SW620 group (Fig.S5d).Collectively, these results indicated that low HDAC2 expression promotes EMT-mediated CRC metastasis *in vivo* by upregulating H19 and MMP14.

Discussion

Here, we reported a novel function of HDAC2 as a negative regulator of CRC metastasis. The expression of HDAC2 was low in colorectal cancer metastasis tissues, which was associated with poor survival for

CRC patients. Low HDAC2 expression promoted EMT-mediated CRC metastasis. This is the only study to our knowledge to reveal the role of HDAC2 in CRC metastasis. We also reported that HDAC2 regulates EMT-mediated CRC metastasis *via* the HDAC2/H19/MMP14 *axis* (Fig. 6g).

HDACs have been reported to be highly expressed in many tumors, and the development of cancer drugs targeting HDACs has been carried out for many years[33]. Unfortunately, clinical trials have shown that HDAC inhibitors do not benefit patients with solid tumors, such as colorectal cancer[34]. Until now, no HDAC inhibitor has been approved as a drug for solid tumors. Moreover, some HDAC inhibitors were reported to induce EMT and cancer metastasis[24, 35]. The internal reasons for this effect are still not clear. In our study, we first found that HDAC2, a member of the class I HDACs, was expressed at low levels in metastatic colorectal cancer tissues and confirmed that HDAC2 negatively regulated CRC metastasis. Our study may partly explain why the clinical efficacy of HDAC inhibitors in solid tumors is unsatisfactory and why the use of HDAC inhibitors has been shown to promote tumor EMT and metastasis.

As mentioned in the introduction part, some HDACs have been reported to play a role in EMT and cancer metastasis. Most HDACs were found to promote EMT and metastasis and usually the mechanism is that HDACs bind to EMT-related transcription factors and directly inhibit the expression of the epithelial marker E-cadherin. In this study, we found a novel function and mechanism of HDAC2 in the regulation of cancer metastasis. On the one hand, we found that HDAC2 negatively regulates CRC metastasis. On the other hand, we found that HDAC2 regulates EMT and metastasis in an indirect way by the intermediate molecule H19. The differences between our study and previous studies may be due to different HDACs playing different roles in different tumors.

As the first detected human lncRNA, H19 has been reported to play important roles in cancer initiation, progression and metastasis[36], which is one of the reasons why we chose and verified H19 as a possible downstream target gene of HDAC2. In addition, our microarray results also showed that H19 was overexpressed in DLD1 HDAC2 KO cells. H19 plays important roles in the development of cancer, but its regulatory mechanism has only been reported on sporadically[37, 38]. This study found that HDAC2 could inhibit the expression of H19 by interacting with SP1, which deepens people's understanding on the regulatory networks of H19.

Many reports revealed the mechanisms of how H19 promotes EMT and cancer metastasis. Shi [27]found that the lncRNA H19 promotes glioma cell invasion by deriving miR-675. Liang[29] found that the lncRNA H19 promotes epithelial to mesenchymal transition by functioning as miRNAs sponge in colorectal cancer. Luo[28] found that the lncRNA H19 increases bladder cancer metastasis by associating with EZH2 and inhibiting E-cadherin expression. In our study, we found that H19 can also promote EMT and CRC metastasis by sponging miR-22-3P and upregulating MMP14. Our study broadens the molecular mechanism of H19 in regulating tumor metastasis.

There are 18 kinds of HDACs in mammals. Due to limited time and money, we studied the relationship between only class I HDACs and metastasis in colorectal cancer. We may explore other HDACs in our

future work. Many genes are involved in EMT[39]. In this study, we selected E-cadherin, fibronectin and ITGA5 as markers to identify EMT. This was because they are typical markers for EMT, and these genes were differentially expressed in our microarray results.

Conclusion

In this study, we discovered a novel role for HDAC2 in CRC metastasis. HDAC2 was downregulated in metastatic colorectal cancer tissues and cells, which predicted a poor clinical prognosis. Mechanismly, HDAC2 inhibits EMT-mediated cancer metastasis by associating with SP1 and inhibiting H19 expression, while reduced HDAC2 expression promotes CRC metastasis via the LncRNAH19/MMP14 axis, indicating that H19 and MMP14 could serve as novel targets for combination therapy in colorectal cancer.

Abbreviations

CRC: colorectal cancer；EMT: Epithelial to mesenchymal transition; LncRNA: Long non-coding RNA; HDAC2: Histone deacetylases

Declarations

Ethics approval：The study was approved by the Ethics Committee of the Daping Hospital of Amy Medical University. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animal by International Committees. Every effort was made to minimize the numbers and suffering of the included animals.

Consent for publication Not applicable

Availability of data and materials：The datasets generated and/or analysed during the current study are available from the supplementary information or the corresponding author upon reasonable request.

Competing interests：The authors declare that they have no conflicts of interest.

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Authors' contributions: Xue-ting Hu and Wei Xing conceived and coordinated the study. Xue-ting Hu and Xiang Xu wrote the paper. Rong-Sen Zhao, Yan Tang, Xiao-feng Wu, Xiang Ao, Luo-quan Ao, Zhan Li, Men-wei Yao, Mu Yuan and Wei Guo performed, and analyzed the experiments. Sheng-Ze li and Jian Yu offered technical or material support for the experiments. All authors reviewed the results and approved the final version of the manuscript.

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Figures

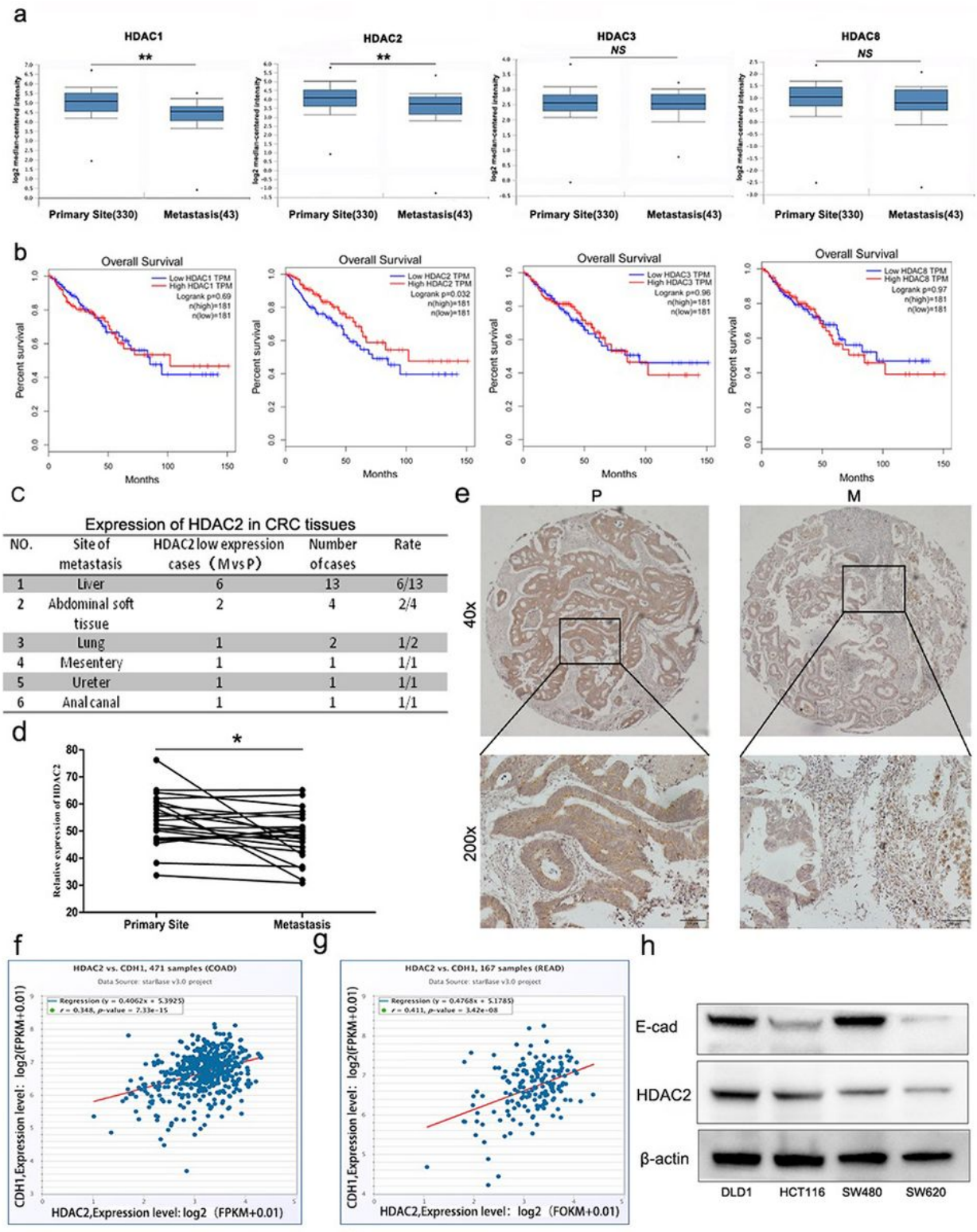

Figure 1

HDAC2 is frequently downregulated in human metastatic colorectal tissues. (a) Relative expression of HDAC1, HDAC2, HDAC3, and HDAC8 in CRC tissues. Data were collected from Oncomine, including 330 cases of primary site and 43 cases of metastasis. (b) Kaplan-Meier analyses of the correlations between HDAC1, HDAC2, HDAC3, and HDAC8 expression levels and overall survival in 362 patients with CRC. Data were collected from TCGA. We labeled TCGA samples as “high” or “low” according to whether the

expression of HDAC1, HDAC2, HDAC3 and HDAC8 was higher or lower than the corresponding median value among all samples. (c, d) Expression of HDAC2 in 22 paired CRC samples. (e) Representative immunohistochemical staining of a tissue array containing CRC samples with anti-HDAC2 antibody. Magnification: 40× (upper) and 200× (lower). (f, g) Expression correlation between HDAC2 and E-cadherin in CRC samples. Data were collected from TCGA, including 471 COAD samples and 167 READ samples.(h) Expression of E-cadherin and HDAC2 in CRC cell lines detected by Western Blot. **P <0 .01; *P <0.05. The data are representatives and are presented as mean ±standard error of the mean of 3 assays.

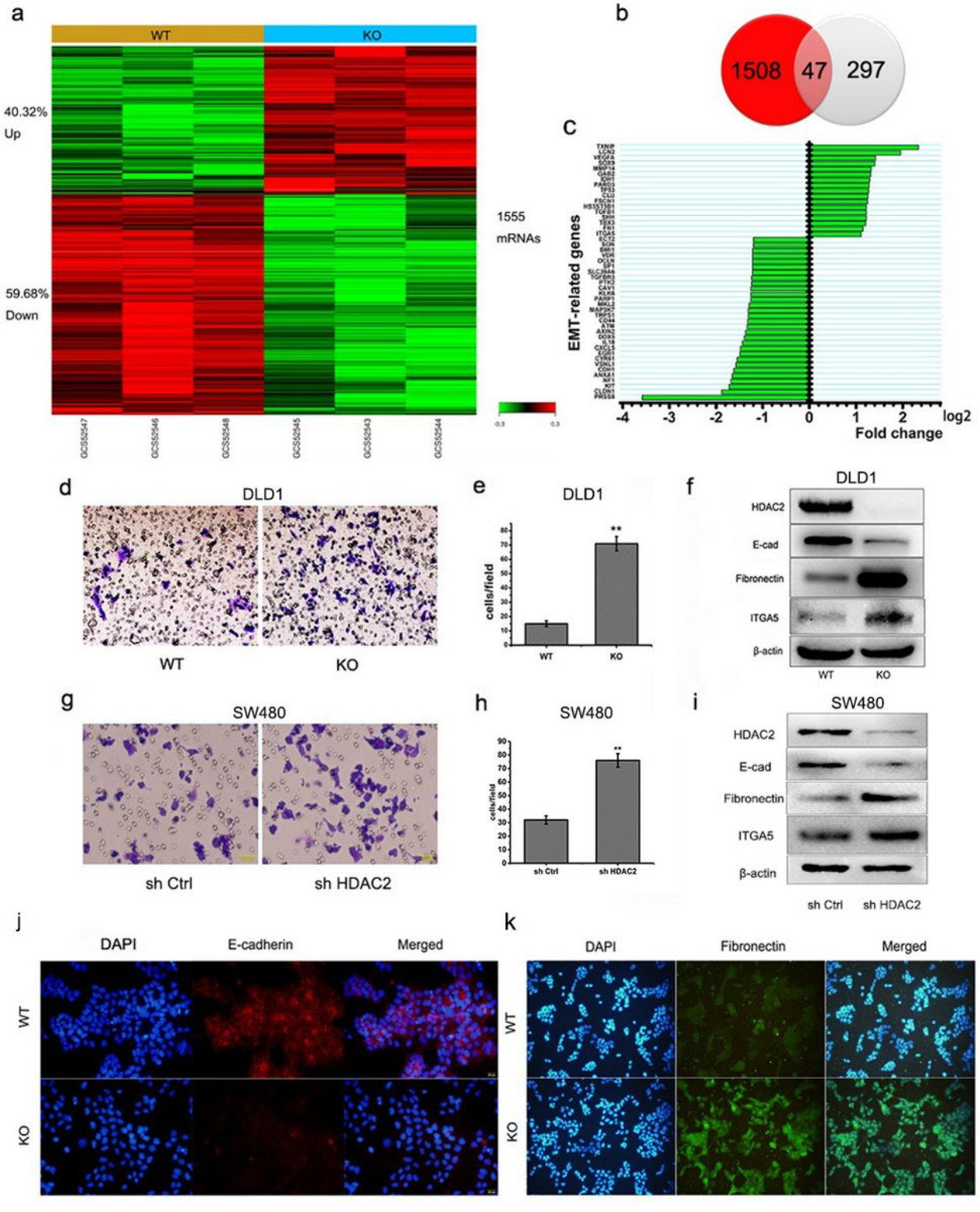

Figure 2

Reduced HDAC2 induces EMT in CRC. (a) Altered mRNA expression in DLD1 HDAC2 KO cells. (b) Merged mRNAs for EMT-related genes and those identified in our microarray. (c) Fold changes of EMT-related gene expression in our microarray.(d, e) Migration ability of DLD1 HDAC2 KO cells detected by transwell. (f ,j, k) EMT markers were analyzed by immunoblotting and IF with the indicated antibodies in DLD1 HDAC2 WT and KO cells. (g,h) Migration ability of SW480 HDAC2 RNAi cells detected by transwell . (i) EMT markers were analyzed by immunoblotting with the indicated antibodies in SW480 HDAC2 RNAi cells.**P <0 .01; *P <0.05. The data are representatives and are presented as mean ±standard error of the mean of 3 assays.

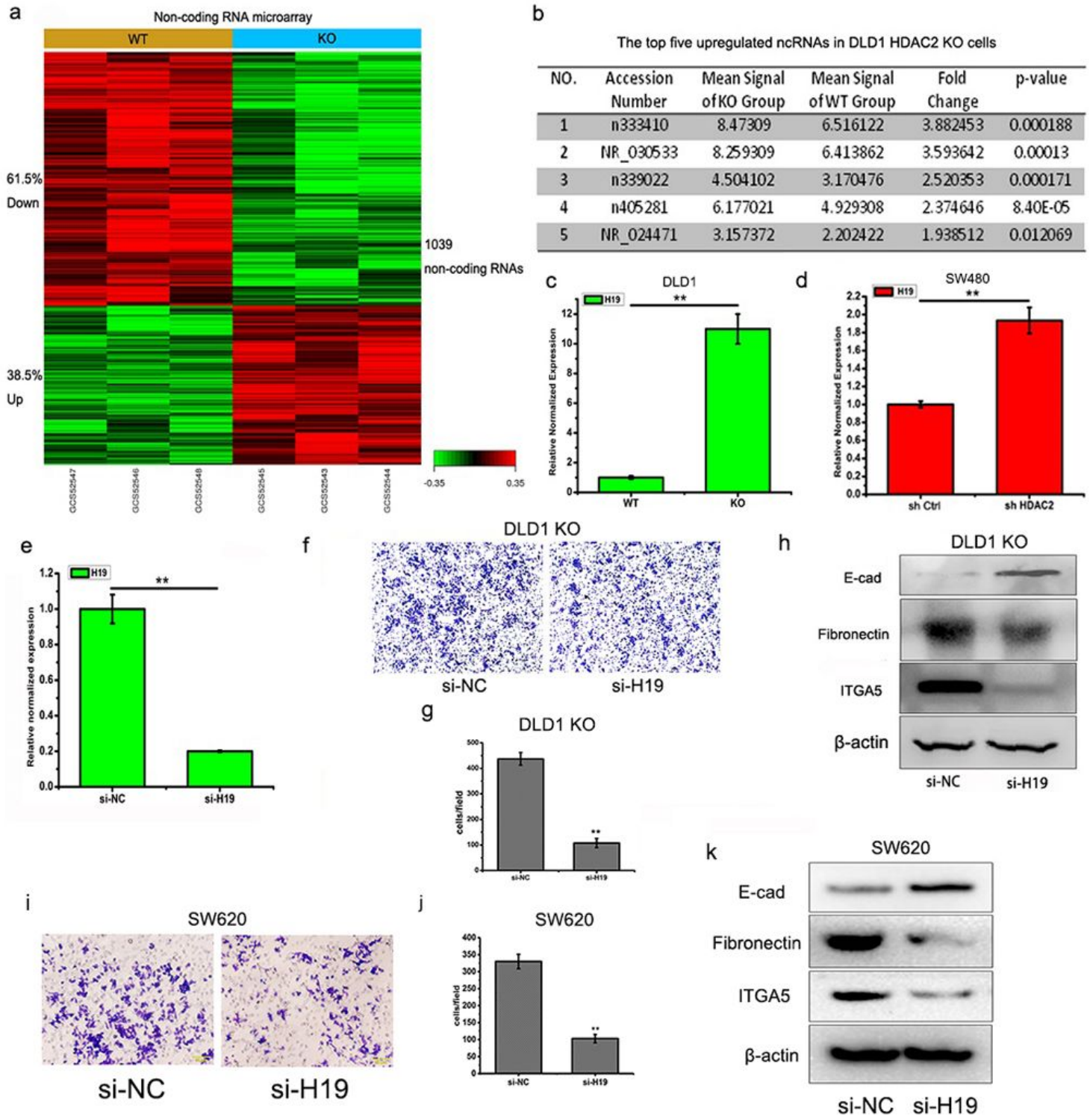

Figure 3

Reduced HDAC2 induces EMT in CRC by upregulating H19.(a) Altered non-coding RNAs expression in DLD1 HDAC2 KO cells. (b) The top five upregulated noncoding RNAs in HDAC2 KO cells. (c) Expression of H19 in DLD1 HDAC2 KO cells.(d) Expression of H19 in SW480 HDAC2 RNAi cells. (e. f. g) Migration ability of DLD1 HDAC2 KO H19 RNAi cells detected by transwell. (h) Detection of EMT markers by Western Blot in DLD1 HDAC2 KO H19 RNAi cells. (i, j) Migration ability of SW620 H19 RNAi cells detected by transwell.

(h) Detection of EMT markers by Western Blot in SW620 H19 RNAi cells.**P <0 .01; *P <0.05. The data are representatives and are presented as mean ±standard error of the mean of 3 assays.

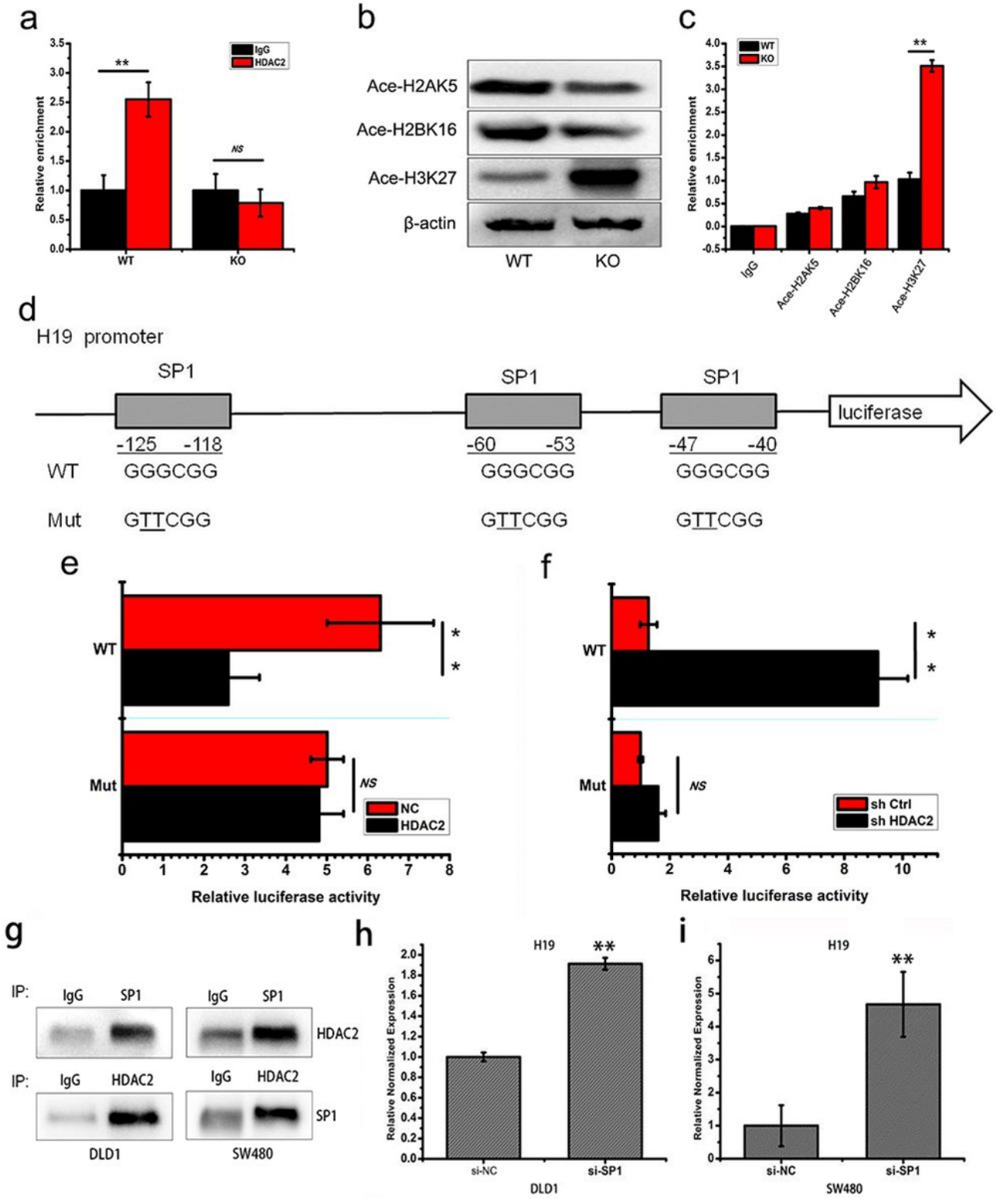

Figure 4

HDAC2 inhibits the expression of H19 by interacting with the transcription factor SP1 and catalyzing H3K27 deacetylation. (a) ChIP analysis of HDAC2 enrichment in the promoter of H19 gene in DLD1 WT or HDAC2 KO cells. (b) Detection of acetylated histone H2AK5、H2BK16 and H3K27 in DLD1 WT or HDAC2

KO cells. (c) ChIP analysis of acetylated histone H2AK5□H2BK16 and H3K27 enrichment in the promoter of H19 gene in DLD1 WT or HDAC2 KO cells. (d) A schematic of the H19 promoter-luciferase construct is depicted with the locations of the SP1 binding sites and the sequences of the point mutations. (e, f) Dual luciferase assay of 293 cells cotransfected with the H19 promoter reporter constructs (wild-type or mutants at three SP1 binding sites) and the shHDAC2 or HDAC2 plasmids. (g) Interaction between HDAC2 and SP1 displayed by Co-IP. (h,i) Expression of H19 in SP1 RNAi CRC cells. **P <0 .01; *P <0.05. The data are representatives and are presented as mean ±standard error of the mean of 3 assays.

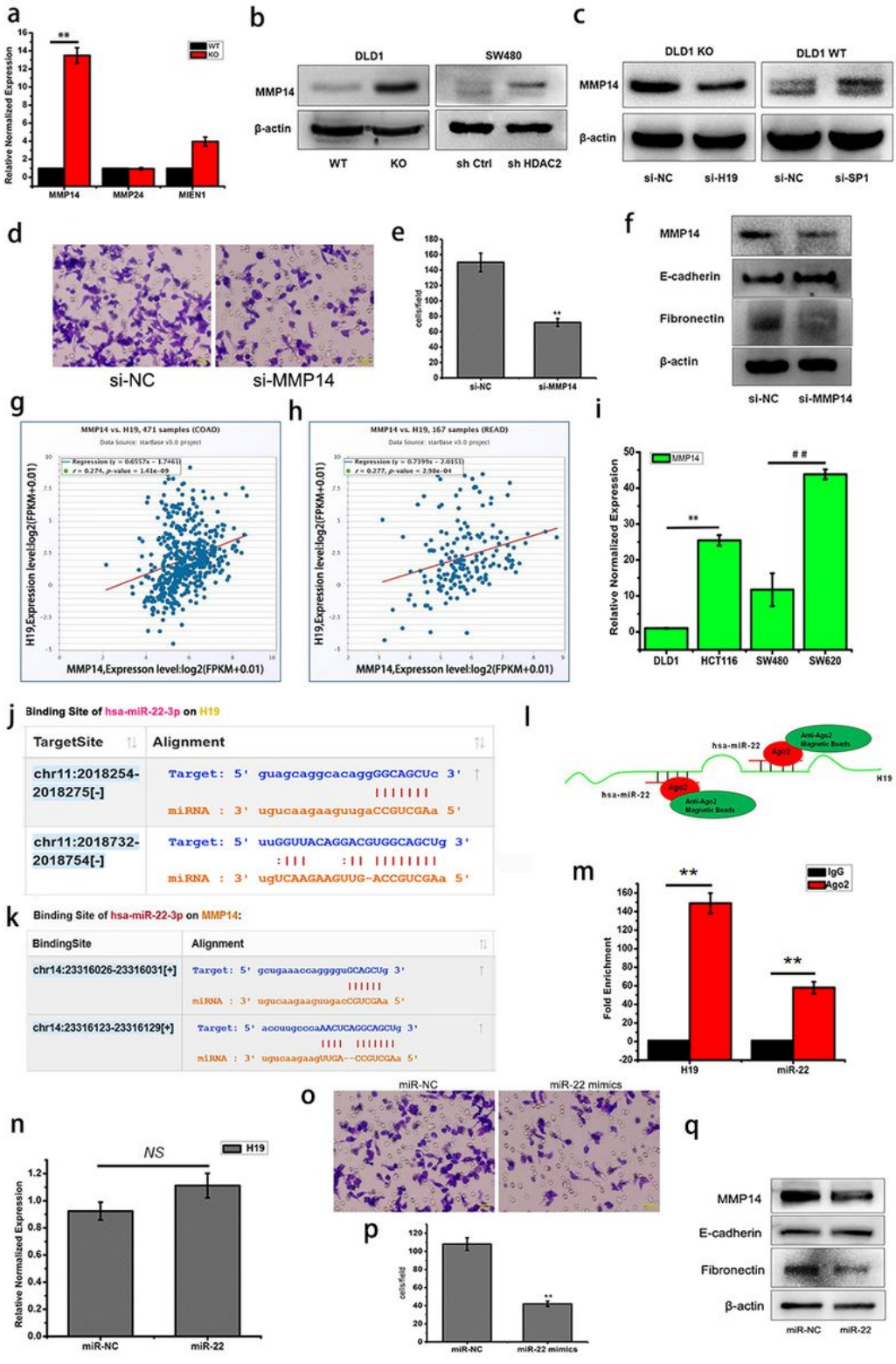

Figure 5

H19 promotes EMT by sponging miR-22 and upregulating MMP14. (a) Expression of MMPs in DLD1 HDAC2 KO cells. (b,c) Detection of MMP14 by Western blot. (d,e) Migration ability of DLD1 HDAC2 KO MMP14 RNAi cells detected by transwell. (f) Detection of EMT markers by Western blot in DLD1 HDAC2 KO MMP14 RNAi cells.(g, h) The correction between H19 and MMP14 expression in colorectal cancer samples. Data were collected from TCGA including 471 cases of COAD and 167 cases of READ. (i) Expression of MMP14 in CRC cells. (j) Schematic diagrams of the mutual interactions between miRNA-22-3P and H19. (k) Schematic diagrams of the mutual interactions between miRNA-22-3P and 3'UTR of MMP14. l,m The interactions between miRNA- 22-3P and H19 detected by anti-Ago2 RIP in DLD1 HDAC2 KO cells . n Expression of H19 in DLD1 HDAC2 KO cells transfected with miRNA- 22-3P mimincs. o,p Migration ability of DLD1 HDAC2 KO cells transfected with miRNA- 22-3P mimincs detected by transwell. q Detection of EMT markers and MMP14 by Western blot in DLD1 HDAC2 KO cells transfected with miRNA- 22-3P mimincs. **P <0 .01; *P <0.05. The data are representatives and are presented as mean ±standard error of the mean of 3 assays.

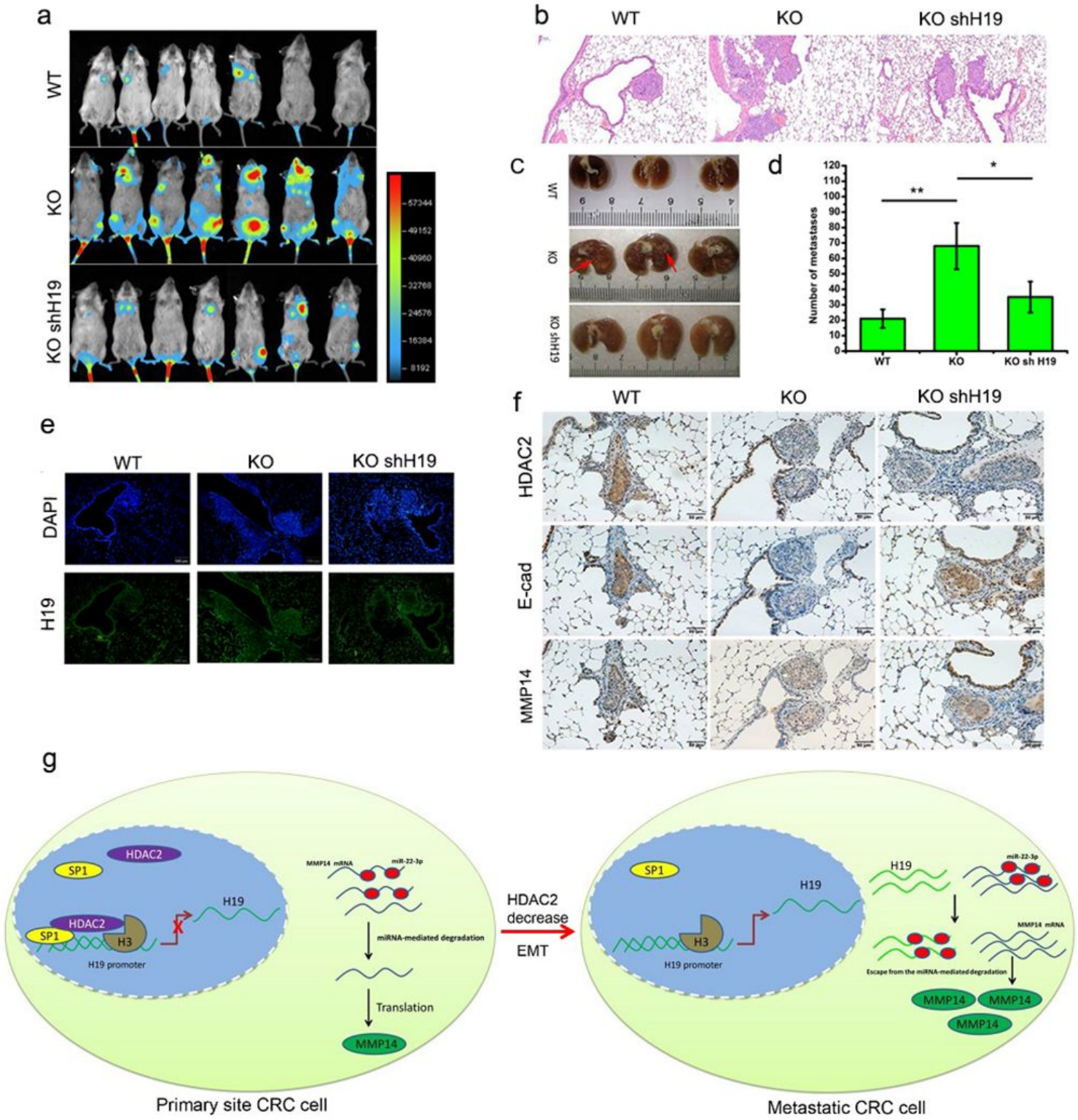

Figure 6

Low HDAC2 expression promotes colorectal cancer metastasis in vivo.(a) Metastatic tumors were detected and photographed by a bioluminescent in vivo imager, n=6. (b) Hematoxylin and eosin-stained images of mouse lung tissues. (c) Photos of mice lungs gained from WT,KO and KO shH19 group.(d) The average number of metastatic nodules in the lungs. (e) The expression of H19 in metastatic lung nodules determined by FISH. (f) The expression of HDAC2, E-cadherin and MMP14 determined by IHC staining in

metastatic nodules in the lungs.(g) Schematic depicting mechanism of HDAC2 regulating CRC metastasis. **P <0 .01; *P <0.05. The data are presented as mean ±standard error of the mean.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarytable2noncodinggene.xlsx
- supplementarytable1codinggene.xlsx
- HDAC2supplementaryinformationJECCR.doc