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CCR7 preservation via histone deacetylase inhibition promotes epithelial-mesenchymal transition of hepatocellular carcinoma cells

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Abstract

The effects of Histone deacetylase (HDAC) inhibition on epithelial-mesenchymal transition (EMT) differs in various types of cancers. However, its function in hepatocellular carcinoma (HCC) is not well-explored. In this study, we investigated the effect of HDAC inhibition on EMT in HCC cells by using trichostatin A (TSA) and valproic acid (VPA). The results showed that TSA/VPA significantly induced EMT phenotype, as demonstrated by the decreased level of E-cadherin, increased level of N-cadherin, vimentin, Twist and snail, and enhanced capacity of cell migration and invasion. In addition, CCR7 was speculated and confirmed as a function target of HDAC inhibition. CCR7 promotes the progression of HCC and is associated with poor survival. Knockdown of CCR7 significantly attenuated the effect of TSA on EMT. Moreover, our results demonstrated that HDAC inhibition up-regulates CCR7 via reversing the promoter hypoacetylation and increasing CCR7 transcription. Taken together, our study has identified the function of HDAC in EMT of HCC and suggested a novel mechanism through which TSA/VPA exerts its carcinogenic roles in HCC. HDAC inhibitors require careful caution before their

[1] Lingling Yang and Yanxiang Chang contribute equally to this work.

application as new anticancer drugs.

Keywords: Histone deacetylase; CCR7; Hepatocellular carcinoma; Epithelial-mesenchymal transition

Introduction

Hepatocellular carcinoma (HCC) is one of the most deadly cancers worldwide, and ranks the third leading cause of cancer deaths [1]. It has been reported that infection with the hepatitis B or C viruses, non-alcoholic fatty liver disease and alcohol abuse are the major contributors for HCC [2]. Epithelial-mesenchymal transition (EMT) is critical for carcinogenesis, tumor invasion, metastasis, chemo-resistance and acquisition of stem cell properties [3]. The EMT phenotype is indicative of a poor prognosis in HCC [4]. Therefore, it is an extreme urgency to elucidate the specific mechanisms underlying the EMT of HCC.

Histone modification has been shown to play a crucial role in controlling EMT. Protein acetylation modifications of histone or non-histone proteins, catalyzed by two groups of enzymes of opposite functions- histone acetyl-transferase (HAT) and histone deacetylases (HDAC) [5]. It was reported that HDAC inhibition could reverse or attenuate EMT through up-regulating E-cadherin in different cancers such as esophageal cancer [6], breast cancer [7], and ovarian cancer [8]. In addition, HDAC inhibition attenuates TGF-β1-induced EMT in various cells [9, 10], suggesting that HDAC inhibition may have therapeutic roles to alleviate EMT. However, recent studies have also demonstrated that HDAC inhibition promotes EMT, reverse stem cell properties and facilitate metastasis in different cancers, such as nasopharyngeal carcinoma [11], squamous cell carcinoma [12] and prostate cancer [13]. The effect of HDAC inhibition on EMT of HCC has not well-explored.

Chemokines and their receptors are key mediators of tumor invasion and metastasis [14-16]. CCR7 is a G protein-coupled seven transmembrane domain receptor with two high-affinity ligands, secondary lymphoid chemokine (SLC) and EB11-ligand chemokine (ELC). CCR7 activates adaptive immune system by regulating T cell and dendritic cell (DC) migration to lymph nodes [17]. However,

CCR7 expression by tumor cells promotes lymphatic invasion and lymph node metastasis and increases the likelihood metastasis in colon cancer, squamous cell carcinoma, breast cancer and HCC [18-21]. Recent studies have reported that CCR7 enhances TGF-β1-induced EMT in gastric cancer, malignant glioma and breast cancer [22-24], indicating that CCR7 may act as an EMT initiator in human cancers.

In the present study, we investigated the effect of HDAC inhibition on EMT of HCC by using two inhibitors, trichostatin A (TSA) and valproic acid (VPA). We found that HDAC inhibition promoted the EMT of HCC. Mechanically, histone acetylation induced EMT via CCR7 preservation.

Materials and methods

Reagents

TSA, VPA, vorinostat (VOR) and entinostat (ENT) were purchased from Selleckchem (Houston, TX); Antibodies to E-cadherin, N-cadherin and Vimentin were purchased from Cell Signaling Technology (Danvers, MA); Antibodies to Twist, Snail, CCR7 and acetyl-histone H3 were purchased from Abcam (Cambridge, MA); Antibody to β-actin and HRP-conjugated secondary antibodies were purchased from Vazyme (Nanjing, China).

Patients

HCC tissues were obtained from 39 patients (one in stage III and others in stage I) undergoing surgery for HCC at The First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). Histological diagnosis and differentiation were evaluated independently by three pathologists according to the WHO classification system. After surgery, Patients in stage I were further followed up per month. Informed consents were obtained from all patients, and the study was approved by the Research Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University.

Cell culture

Huh7 and HepG2 cells were purchased from ATCC (Manassas, USA). All cells were grown in DMEM complemented with 10% FBS (Life Technologies, Grand Island, USA) at 37°C in a 5% CO_2 incubator.

Western blot

Cells were lysed with RIPA Lysis Buffer (Thermo Scientific, USA). Equivalent amounts of protein sample were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, USA). After incubation with 5% non-fat dry milk, the membranes were probed with respectively primary antibodies at 4℃ and then were incubated with HRP-conjugated secondary antibodies at room temperature. Finally, the blots were visualized by ECL and detected using a ChemiDoc XRS imaging system. β-actin was used as endogenous reference.

Wound healing assay

Confluent cell monolayer was cultured in a 24-well chamber. Then cells were stroked with a plastic pipette tip and were washed to remove detached and damaged cells. After treated with TSA or VPA for 24 h, the cell migrations were monitored microscopically and the migration distance was measured by ImageJ software.

Transwell invasion assay

The invasion capacity of Huh7 and HepG2 cells was examined using Transwell invasion assay. Briefly, cells treated with TSA or VPA were seeded in the upper chamber with serum-free medium. And then, 20% FBS was added to the medium in the lower chamber. After 24 h incubation, non-invading cells were removed from the top well with a cotton swab, while the bottom cells were fixed in 95% ethanol and stained with hematoxylin. The invaded cell numbers were counted under a microscope on 10 random fields.

Immunohistochemistry

Tissue sections were prepared essentially by standard protocol. After the sequential processes of rehydration and antigen retrieval, the sections were incubated with antibody against CCR7 overnight, and subsequently incubated with secondary antibody for 30 minutes. Diaminobenzidine (DAB) was used for performing color reactions. Sections were visualized with a fluorescent microscope.

CCR7 knockdown

siRNA against CCR7 (si-CCR7) and si-control were obtained from RIBOBIO (Guangzhou, China). siRNA transfection was performed by using Lipofectamine 3000 reagent (Invitrgen, Carlsbad, USA) according to the manufacturer's protocol.

Luciferase assay

The promoter of CCR7 was PCR-amplified and inserted into pGL3 vector (pGL3-CCR7prom). For luciferase reporter assay, pGL3-CCR7prom was transfected into HepG2 by Lipofectamine 3000 reagent. After various treatments, the luciferase activities were measured with a dual luciferase reporter assay system (Promega, Madison, USA) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP)

ChIP was carried out with a ChIP assay kit (Millipore, Billerica, MA) according to the manufacturer's instructions. The immunoprecipitation was performed with anti-acetyl histone H3 antibody or an isoform-matched IgG as control. Immunoprecipitated DNA was further PCR-amplified using primer of CCR7 promoter and the PCR products were analyzed on 1.5% agarose gel and visualized under UC light.

Statistical analysis

Data were analyzed by GraphPad Prism software and the results were expressed as mean ± standard deviation (SD). The statistical significance of the studies was analyzed using one way ANOVA. Cumulative overall survival was evaluated by Kaplan-Meier method with the log-rank test applied for comparison. The difference was considered significant at $P < 0.05$.

Results

HDAC inhibition induces EMT in HCC cells

To gain insight into the function of HDAC in HCC, TSA and VPA were used for HDAC inhibition. The results showed that TSA and VPA significantly decreased the expression of E-cadherin and increased the expression of N-cadherin, Vimentin, Twist and Snail in Huh7 and HepG2 cells (Fig.1A-B). In addition, TSA and VPA notably promoted the migration and invasion capacity of Huh7 and HepG2 cells (Fig.1C-D, data of Huh7 cell not shown). To verify the EMT-promoting effect of HDAC inhibition, two FDA approved HDAC inhibitors – VOR and ENT were used. HepG2 cells were treated with or without 2.5 μM VOR (Selleckchem) or 1μM ENT (Selleckchem). The results showed that VOR and ENT significantly decreased the

expression of E-cadherin and increased the expression of N-cadherin, Vimentin, Twist and Snail in HepG2 cells (supplementary file.1). In addition, VOR and ENT obviously stimulated the migration and invasion capacity of HepG2 cells (supplementary file.1). These results suggest that HDAC inhibition promotes EMT in HCC cells.

CCR7 promotes the progression of HCC and is associated with poor survival

Since CCR7 play an important role in EMT, the expression of CCR7 in HCC tissues was examined by immunohistochemistry. As shown in Fig.2A, the expression of CCR7 in tumor from stage III patient was much higher than that from stage I patient. We also followed the survival status of 38 patients, and the Kplan-Meier survival analysis showed that sufferers with high CCR7 expression had a shorter survival than those with low CCR7 expression (Fig.2B). These results indicate that high CCR7 expression may be associated with the progression of HCC.

CCR7 is essential in TSA-induced EMT

We next examined the expression of CCR7 after HDAC inhibition. The results showed that TSA- or VPA-mediated HDAC inhibition significantly up-regulated the expression of CCR7 in Huh7 and HepG2 cells (Fig.3A and B). To determine whether CCR7 up-regulation contributes to TSA-induced EMT, si-CCR7 was used for CCR7 knockdown. As shown in Fig.3C, si-CCR7 significantly decreased the expression of CCR7 in HepG2 cells. Interestingly, CCR7 knockdown partly reversed TSA-induced changes of EMT-related proteins (E-cadherin, N-cadherin and Snail), indicating that CCR7 is essential in HDAC inhibition-induced EMT.

HDAC inhibition up-regulates CCR7 via reversing the promoter hypoacetylation and increasing CCR7 transcription

To gain further insight into the molecular basis of TSA/VPA activation of CCR7, the mRNA level of CCR7 was detected by qRT-PCR. As shown in Fig.4A and B, TSA or VPA treatment dose-dependently increased the mRNA expression of CCR7, suggesting that HDAC inhibition up-regulated CCR7 at the transcription or post-transcription level. To verify this conclusion, we then tested the role of TSA/VPA on a CCR7 promoter reporter plasmid in a transient transfection assay. TSA or VPA

significantly increased the CCR7 promoter-driven luciferase activity (Fig.4C and D). Further we examined the histone acetylation status around CCR7 promoter by ChIP assay. We precipitated the protein-cross-linked genomic DNA with a specific antibody to acetylated histone3, and then measured the amount of DNA associated with acetylated histone 3 by PCR using a primer set specific for CCR7 promoter. The results showed that TSA or VPA treated cells displayed hyperacetylated histone 3 on CCR promoter (Fig. 4E and F). Taken together, these results suggest that HDAC inhibition up-regulates CCR7 by increasing CCR7 promoter acetylation and its transcription.

Discussion

Many studies have reported that HDAC inhibition reverses or attenuates EMT in different cancers and attenuates TGF-β-induced EMT in normal epithelial cells [6-10], suggesting that HDAC inhibition plays therapeutic roles in cancers and fibrotic disorders by inhibition of EMT. However, several studies have reported opposite results that HDAC inhibition can induce EMT [11-13]. They suggest that HDAC inhibitors promote the progression of EMT through activating the promoters of Snail, Slug and Twist1. TSA, a hydroxamate, has the highest efficiency against HDAC1, 3 and 8 [25]. TSA was found to induce the EMT phenotype, including increased expression of vimentin, N-cadherin and SOX2, which is associated with cancer stem-cell characteristics in prostate cancer cells [13]. TSA-mediated HDAC inhibition also induced EMT in squamous cell carcinomas through activating BMI-1 and vimentin and enhancing tumor aggressiveness [12]. VPA is a short chain fatty acid, and has the highest activity against HDAC1 and 2, but appears also to affect HDAC3, 4, 5 and 7 at higher doses [26]. Ji et al. have demonstrated that VPA induced mesenchymal phenotype in the colon carcinoma cells and the effect was augmented by TGF-β1 [27]. A recent study showed another HDAC inhibitor, SAHA (suberoylanilidehydroxamic acid) induced EMT during human embryo implantation [28]. In the present study, we first demonstrated that TSA- or VPA-mediated HDAC inhibition promoted the progression of EMT in HCC cells.

EMT is a developmental cellular process that occurs in primary mesenchyme,

wound healing and cancers [29]. A hallmark of EMT is that polarized epithelial cells lose epithelial properties and cell-cell adhesions, and gian mesenchymal characteristics [30], which allows the cancer cell to become more invasive, metastatic and form a secondary metastasis [31]. E-cadherin is required for the formation of stable adherens junction. During EMT, epithelial cells lose E-cadherin and convert into spindle shaped mesenchymal cells by acquiring N-cadherin [32]. It was reported that E/N-cadherin switch play a crucial role in cancer progression [33]. Vimentin is a mesenchymal marker which is overexpressed in cancer cells [34]. Twist promotes EMT and simultaneously regulates various genes involved in angiogenesis, inflammation, and the anti-tumor immune response [35, 36]. Snail is a family of transcription factors that promote the repression of the adhesion molecule E-cadherin to regulate EMT during embryonic development [37]. In the present study, we found that TSA/VPA treatment induced mesenchymal phenotype in HCC cells, as demonstrated by the decreased expression of E-cadherin and increased expression of N-cadherin, vimentin, Twist and Snail. Moreover, our results also showed that TSA/VPA treatment significantly increased the migration and invasion capacity of HCC cells.

CCR7, a G protein-coupled chemokine receptor, exerts its biological function by binding CCL21, leading to alteration of cell skeleton rearrangement and cell migration [38]. Recent years, numerous studies have indicated that high expression of CCR7 is associated with tumor metastases and poor clinical outcome in many kinds of cancer, including esophageal cancer [39], lung cancer [40], and epithelial ovarian carcinomas [41]. Some other studies have reported that CCR7 promoted angiogenesis and lymphangiogenesis [42]. Furthermore, CCR7 was accompanied with EMT in human cancers. For example, Li et al. have demonstrated that CCR7 regulates Twist to induce the EMT in pancreatic ductal adenocarcinoma [43]. Xu et al. have reported that CCR7 mediates human breast cancer cell invasion, migration by inducing EMT and suppressing apoptosis through AKT pathway [44]. In lung and breast cancers, CCR7 functions with other molecules, including CCRL1, VEGF-C, COX-2 and microRNA let-7a, to regulate the metastatic activities of tumor cells [45-47]. However,

the biological function of CCR7 in HCC is not well-explored. In the present study, we found that CCR7 expression was positively related with the progression of human HCC. High expression of CCR7 was associated with poor survival in patients with HCC. Further studies demonstrated that CCR played a vital role in HDAC inhibition-induced EMT. Mechanically, HDAC inhibition up-regulates CCR7 via promoting the promoter acetylation and increasing CCR7 transcription.

Conclusions

Taken together, in this study we first reported that HDAC inhibition promoted EMT in HCC cells. CCR7 acted as an oncogene in HCC by mediating HDAC inhibition-induced EMT. Our study not only provided a new insight into the role of histone acetylation and CCR7 expression in HCC, but also elaborated a novel mechanism by which HDAC inhibitors regulate CCR7 activation.

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Figure legends

Fig.1 HDAC inhibition induces EMT in HCC cells. Huh7 and HepG2 cells were treated with TSA (100 ng/ml) or VPA (1 mM) for 24 h. (A-B) The expression of E-cadherin, N-cadherin, Vimentin, Twist and Snail were detected by Western blot. (C) Cell migration of HepG2 cells was measured by wound healing assay. (D) Cell invasion of HepG2 cells was measured by transwell invasion assay. All the experiments were repeated three times. $*P < 0.05$ versus control.

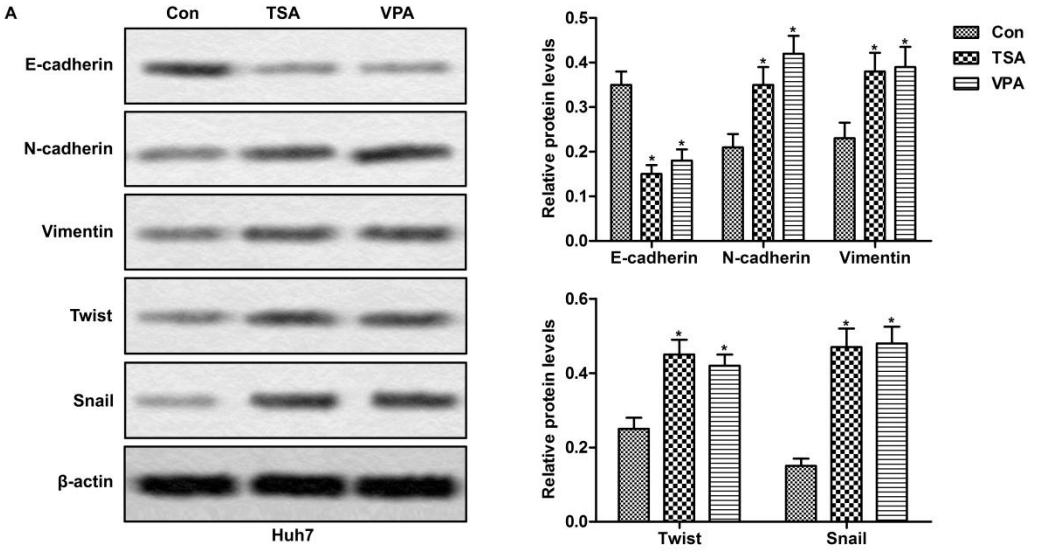
Fig.2 CCR7 promotes the progression of HCC and is associated with poor survival. (A) The expression of CCR7 in tumor tissues from patients with stage I and stage III HCC was detected by immunohistochemistry. (B) Kplan-Meier survival analysis of patients with stage I HCC was performed by GraphPad Prism Software. Low expression and high expression of CCR7 were defined by using the median values of CCR7 expression levels in 38 HCC tissues. Immunohistochemistry was repeated three times. *$P < 0.05$ versus low CCR7 expression.

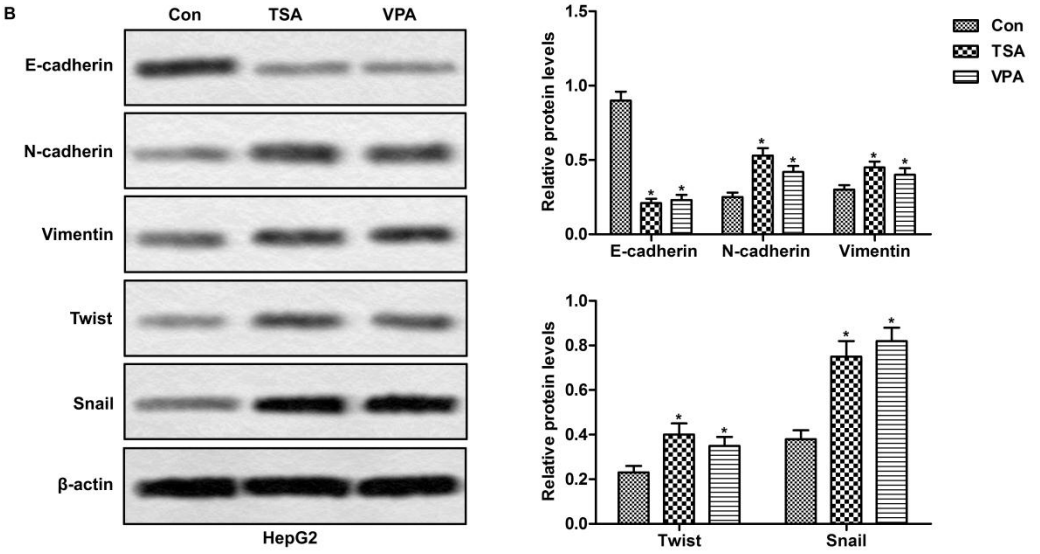
Fig.3 CCR7 is essential in TSA-induced EMT. (A-B) Huh7 and HepG2 cells were treated with TSA (100 ng/ml) or VPA (1 mM) for 24 h. The expression of CCR7 was detected by Western blot. (C) HepG2 cells were pre-transfected with si-CCR7, followed by treated with TSA (100 ng/ml) for 24 h. The expression of CCR7, E-cadherin, N-cadherin and Snail were measured by Western blot. All the experiments were repeated three times. *$P < 0.05$ versus control. $^{\#}P < 0.05$ versus TSA treated alone.

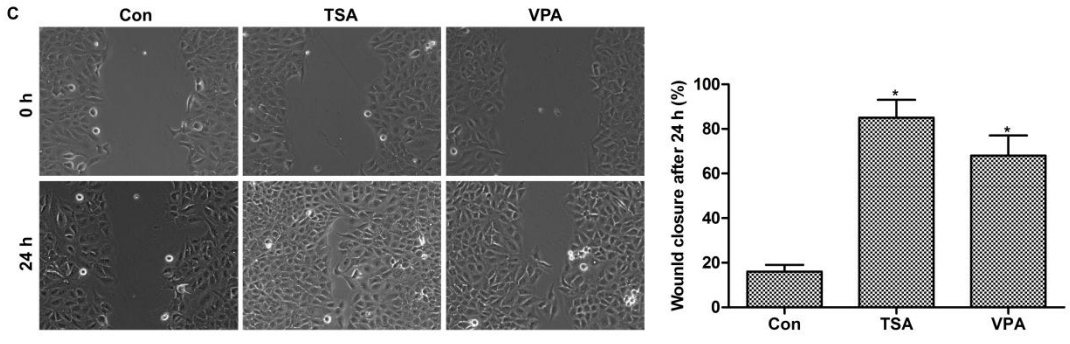
Fig.4 HDAC inhibition up-regulates CCR7 via reversing the promoter hypoacetylation and increasing CCR7 transcription. (A-B) HepG2 cells were treated with series doses of TSA (0-100 ng/ml) or VPA (0-1 mM) for 24 h. The mRNA expression of CCR7 was detected by qRT-PCR. (C-D) Luciferase assay. HepG2 cells were transfected with the CCR7 promoter reporter plasmid plus a renilla luciferase control plasmid by Lipofectamine 2000, followed by treatment with series doses of TSA (0-100 ng/ml) or VPA (0-1 mM) for 24 h. Cell lysates were assayed for luciferase activity. (E) ChIP assay. HepG2 cells were treated with TSA (100 ng/ml) for 24 h. Cell lysate were cross-linked and immune-precipitated with an anti-acetylated Histone3 antibody. The immune-precipitated DNAs were further PCR-amplified with primer sets specific for CCR7 promoter. The genomic DNAs served as input control. The PCR products were analyzed on a 1.5% agarose gel and visualized under UC light. Representative results were shown. (F) Quantification of Fig.4E. All the experiments were repeated three times. *$P < 0.05$ versus control.

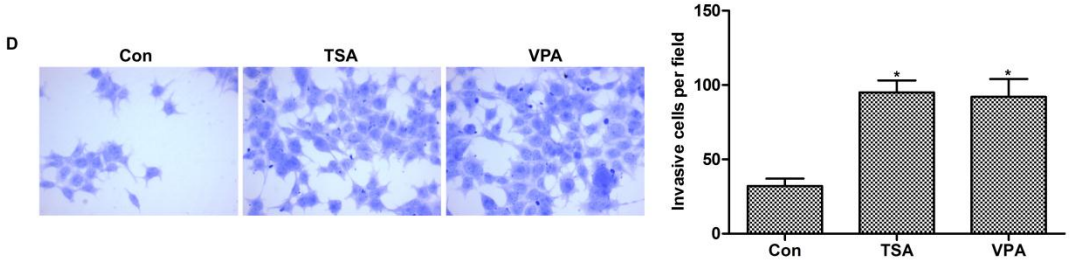
Supplementary file.1 VOR and ENT induces EMT in HepG2 cells. HepG2 cells were treated with VOR(2.5 μM) or ENT(1 μM) for 24 h. (A-B) The expression of

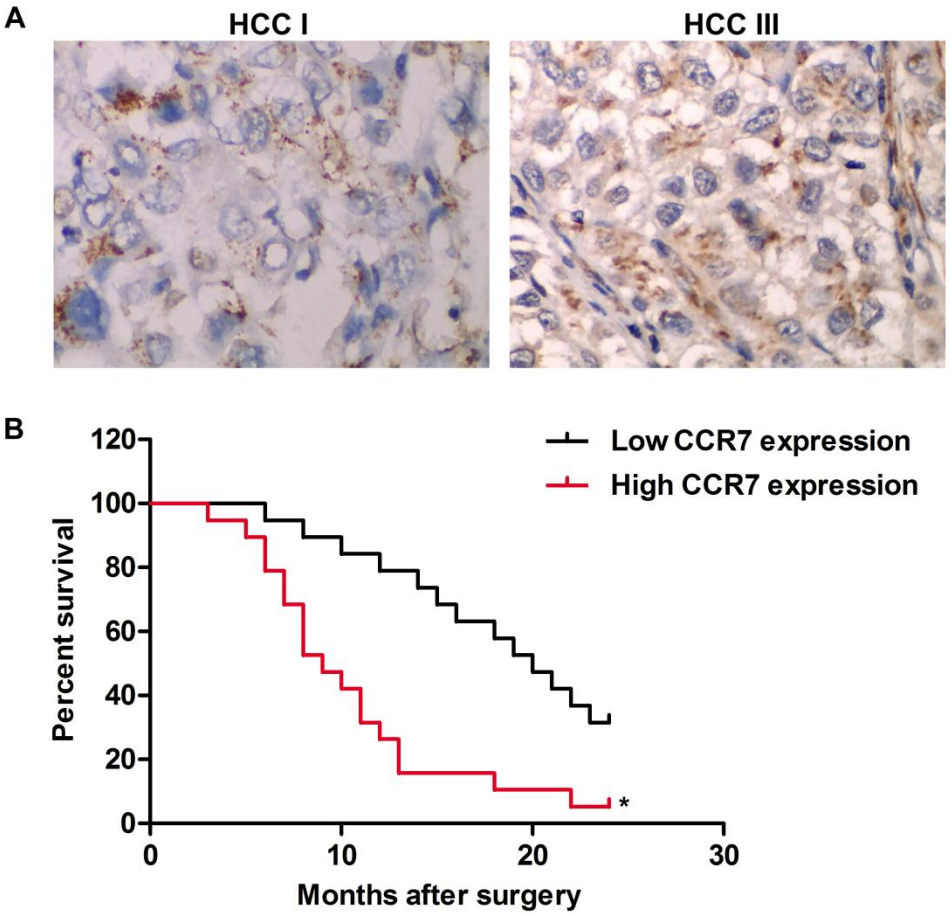
E-cadherin, N-cadherin, Vimentin, Twist and Snail were detected by Western blot. (C) Cell migration of HepG2 cells was measured by wound healing assay. (D) Cell invasion of HepG2 cells was measured by transwell invasion assay. All the experiments were repeated three times. *$P < 0.05$ versus control.

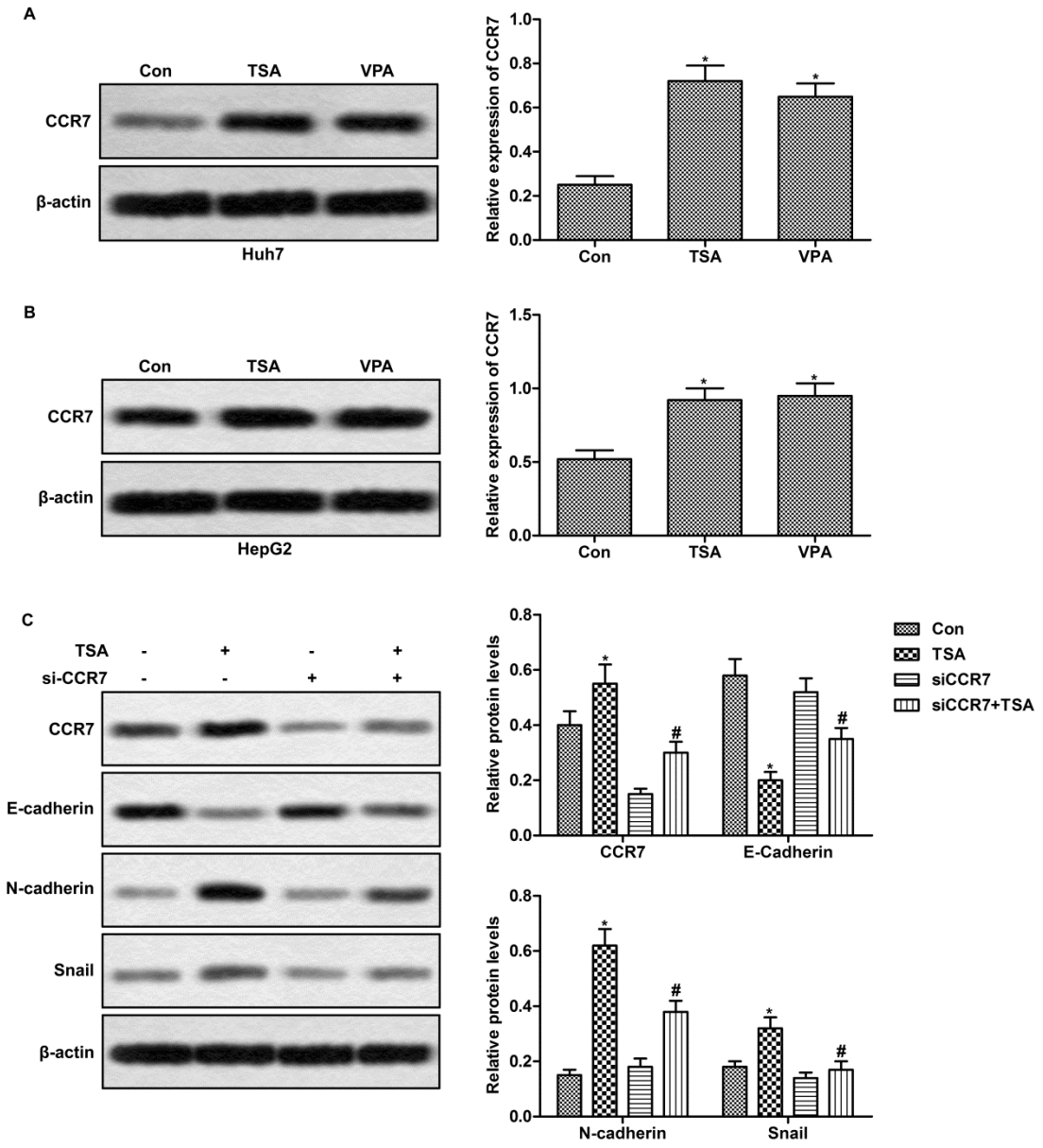


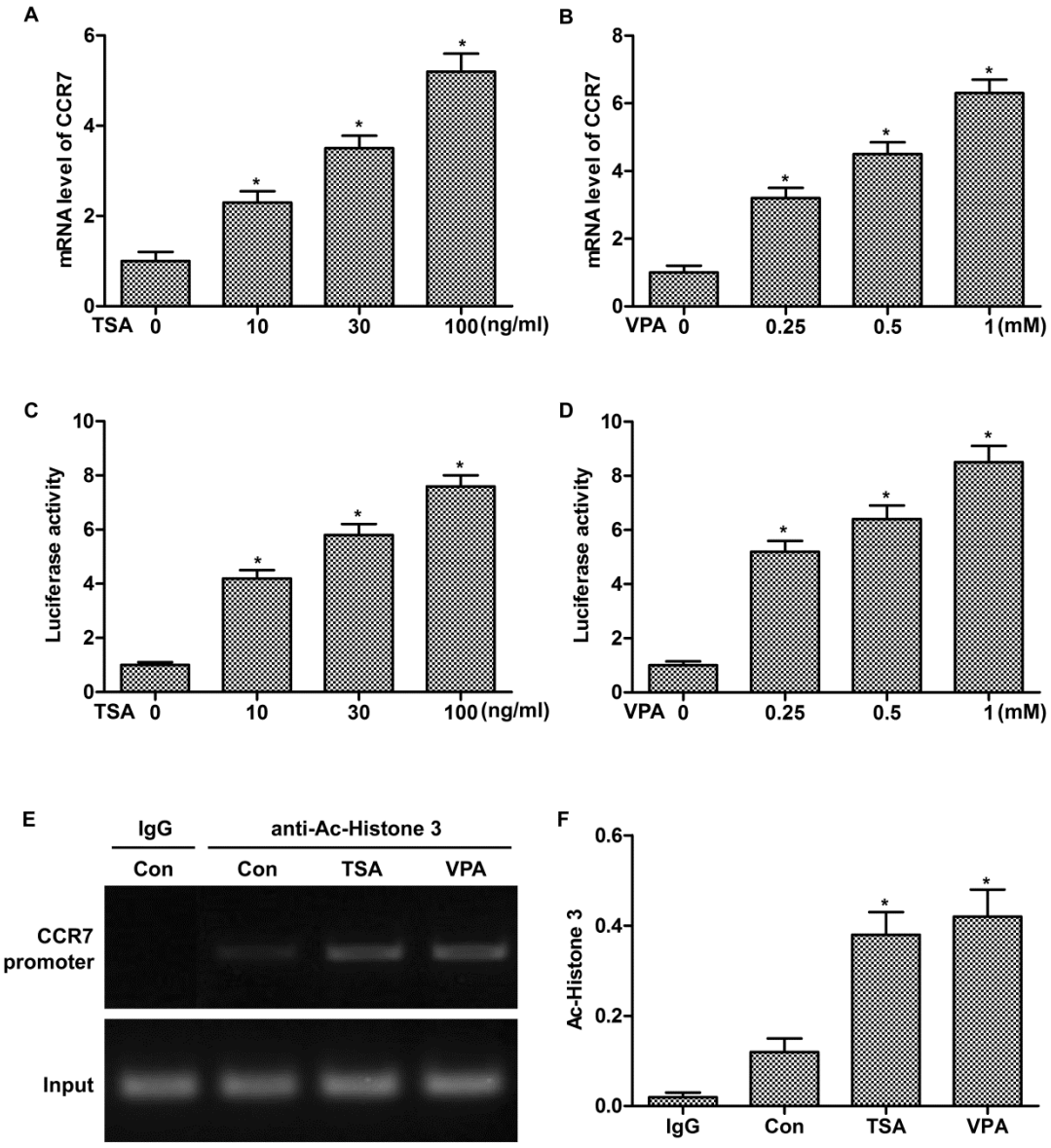












Highlights:

- CCR7 promotes the progression of HCC and is associated with poor survival.
- HDAC inhibition induces EMT in HCC cells via up-regulating CCR7.
- HDAC inhibition up-regulates CCR7 via reversing the promoter hypoacetylation and increasing CCR7 transcription.