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Midline 1 interacting protein 1 promotes cancer metastasis through FOS-like 1-mediated matrix metalloproteinase 9 signaling in HCC

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Abstract

Background and Aims: Understanding the mechanisms of HCC progression and metastasis is crucial to improve early diagnosis and treatment. This study aimed to identify key molecular targets involved in HCC metastasis.

Approach and Results: Using whole-transcriptome sequencing of patients' HCCs, we identified and validated midline 1 interacting protein 1 (MID1IP1) as one of the most significantly upregulated genes in metastatic HCCs, suggesting its potential role in HCC metastasis. Clinicopathological correlation demonstrated that MID1IP1 upregulation significantly correlated with more aggressive tumor phenotypes and poorer patient overall survival rates. Functionally, overexpression of MID1IP1 significantly promoted the migratory and invasive abilities and enhanced the sphere-forming ability and expression of cancer stemness-related genes of HCC cells, whereas its stable knockdown abrogated these effects. Perturbation of MID1IP1 led to significant tumor shrinkage and reduced pulmonary metastases in an orthotopic liver injection mouse model and reduced pulmonary metastases in a tail-vein injection model *in vivo*. Mechanistically, SP1 transcriptional factor was found to be an upstream driver of MID1IP1 transcription. Furthermore, transcriptomic sequencing on MID1IP1-overexpressing HCC cells identified

Abbreviations: CCL2, C-C motif chemokine ligand 2; ChIP, chromatin immunoprecipitation; ECM, extracellular matrix; EV, empty vector; FOSL1, FOS-like 1; FRA1, FOS-like 1; GSEA, gene set enrichment analysis; HKU-QMH, The University of Hong Kong-Queen Mary Hospital; JUNB, junB proto-oncogene; JUND, junD proto-oncogene; KLF4, Kruppel-like factor 4; OE, overexpression; LIF, LIF interleukin 6 family cytokine; MID1IP1, midline 1 interacting 1; MMP, matrix metalloproteinase; MMP9, matrix metalloproteinase 9; NESTIN, nestin; NFATC2, nuclear factor of activated T-cells 2; NOTCH1, notch receptor 1; NTC, no template control; NTL, nontumoros liver; OCT4, POU class 5 homeobox 1; RT-PCR, reverse-transcriptase PCR; shRNA, short-hairpin RNA; siRNA, small interfering RNA; SMO, smoothened, frizzled class receptor; SP1, Sp1 transcription factor; TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma; TFs, transcription factors; TSS, transcription start site; TXLNG, taxilin gamma.

Yung-Tuen Chiu and Abdullah Husain contributed equally to this study.

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FOS-like 1 (FRA1) as a critical downstream mediator of MID1IP1. MID1IP1 upregulated FRA1 to subsequently promote its transcriptional activity and extracellular matrix degradation activity of matrix metalloproteinase MMP9, while knockdown of FRA1 effectively abolished the MID1IP1-induced migratory and invasive abilities.

Conclusions: Our study identified MID1IP1 as a regulator in promoting FRA1-mediated-MMP9 signaling and demonstrated its role in HCC metastasis. Targeting MID1IP1-mediated FRA1 pathway may serve as a potential therapeutic strategy against HCC progression.

INTRODUCTION

HCC is the third most common cause of cancer-related death worldwide.[1] HCC patients with early-stage solitary tumors qualify for undergoing surgical resection and demonstrate the longest survival rates.[2] However, the majority of HCC cases are diagnosed at unresectable intermediate or advanced stages, when the disease has progressed into forming multinodular tumors, with vascular invasion and extrahepatic spread.[3] This stepwise progression of disease and the corresponding therapeutic options are strongly tied with the likelihood of patients having metastatic spread of the disease. Although the repertoire of therapeutic regimens against advanced HCCs has been significantly expanded in recent years, the survival benefit of patients remains modest.[4] High relapse rates from residual or disseminated metastatic cells is the major unovercome challenge in achieving better survival benefit outcomes in patients.

Metastasis is known to be the primary determinant for survival rates of cancer patients.[5] The journey of tumor cells from the primary site to the colonization of a secondary site encompasses a number of bottlenecks resulting in a small fraction of disseminated cells being able to successfully initiate tumors in secondary sites.[6] Abilities to modulate extracellular matrix (ECM), rearrange the cytoskeleton, metabolic plasticity, and cancer stemness are key cell intrinsic properties that directly contribute to their metastatic success rate.[7] The lack of effective therapies against this phenomenon warrants the need for better clinically relevant mechanistic understanding of the regulator elements in this process. Investigation of the tie between advanced tumor stage and higher metastatic potential of HCC cells can provide useful and actionable insights.

Herein, we utilized vascular invasion status, a distinguishing characteristic that strongly correlates with metastatic potential, to classify primary tumors into metastatic or nonmetastatic HCCs and identified midline 1 interacting protein 1 (MID1IP1) as a key proponent of HCC metastasis. Previous studies have suggested that MID1IP1 cooperates with MID1 to exert its functions in microtubule stabilization and is therefore implicated in cellular processes that are affected by the stability of microtubules, such as cell division and migration.[8] In addition, MID1IP1 is involved in hepatic lipogenesis through the activation of acetyl-coenzyme A carboxylate and liver X receptor.[9,10] However, the relevance and role of MID1IP1 in cancer remain largely uncharacterized. In this study, we pinpointed the protumorigenic role of MID1IP1 as a key inducer of aggressive phenotypes in HCC and provide a mechanistic explanation for these cellular phenotypes. We also demonstrated that SP1, a well-known tumor-promoting transcriptional factor, to be a primary upstream driver of MID1IP1 overexpression in human HCCs. Furthermore, MID1IP1 was found to exert its tumor-promoting function by modulating the expression of genes downstream of FOS-like 1 (FRA1), a key regulator of HCC stemness.[11] We also pinpointed matrix metalloproteinase 9 (MMP9), a regulator of (ECM degradation in liver cancer, as one of the important effector molecules downstream of FRA1.[12–14] Taken together, our study identified MID1IP1 as a regulator in promoting FRA1-mediated-MMP9 signaling and demonstrated its crucial role in HCC metastasis.

MATERIALS AND METHODS

Clinical samples

Human HCCs and their corresponding nontumoros liver tissues from patients with liver resection for HCC between 1991 and 2001 at Queen Mary Hospital, Hong Kong were randomly selected. Of the patients, 85% were HBsAg positive in this cohort, with a ratio of male to female patients of 4 to 1. The age of patients ranged from 24 to 73 years. All specimens were snap-frozen in liquid nitrogen and kept at −80 °C. Use of human samples was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW 17-056) and informed consent was obtained in writing from all patients. All

research conducted in this work was performed in accordance with the Declaration of Helsinki and the Declaration of Istanbul.

Cell lines

HCC cell lines PLC/PRF/5, Hep3B, and MHCC97L, and the hepatoblastoma cell line HepG2 were obtained from American Type Culture Collection (ATCC). MHCC97L was authenticated to have no contamination (see Supplemental Materials and Methods http://links.lww.com/HEP/A826 and Supplemental Figure 6, http://links.lww.com/HEP/D711). HCC cell line Huh7 was a gift from Dr. Hidekazu Nakabayashi of Hokkaido University School of Medicine. Human immortalized normal liver cell line, MIHA, was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Human embryonic kidney cell line HEK293FT was obtained from Invitrogen (Carlsbad, USA).

Transcriptome sequencing

Total RNA was isolated from samples according to the protocol described previously.[2] Agilent 2100 Bioanalyzer was used for quality check of isolated RNA. Transcriptome sequencing of both HCC and the corresponding NTL tissue samples was performed by Axeq (Seoul, Korea), using 100 bp pair-end sequencing on the HiSeq. 2000 platform. On average, 10 million reads per sample were generated.[2] Sequencing data is deposited in the Sequence Read Archive (SRA) with ID SRP062885. For the transcriptome sequencing of cell line, TruSeq stranded mRNA sample Prep kit (Illumina) was used for PolyA+mRNA library preparation. This was followed by a 2×150 bp read length with 50 million paired-end reads sequencing in NovaSeq. 6000 (Illumina) by the Centre for PanorOmic Science, The University of Hong Kong. FPKM output was obtained via TopHat-Cufflinks pipeline.

For the identification of differentially expressed genes in the University of Hong Kong-Queen Mary Hospital (HKU-QMH) cohort, paired *t* test for 16 paired HCC and nontumoros livers (NTL) was performed, and a 2-fold increase was used as a cutoff threshold. HKU-QMH cohort was used as the discovery cohort where cases were stratified into 2 groups, that is with (n = 7) or without (n = 9) venous invasion, and genes with a > 2-fold enrichment in the expression for cases with venous invasion were considered for further validation. The Cancer Genome Atlas-Liver Hepatocellular Carcinoma (TCGA-LIHC) cohort, where cases with (n = 109) or without (n = 206) venous invasion, was used as the validation cohort.

Detailed materials and methods can be found in Supporting Materials and Methods and Supplementary Table 1 (http://links.lww.com/HEP/A826).

RESULTS

Identification of MID1IP1 as a clinically relevant genetic factor in human metastatic HCCs

To identify key molecular targets involved in HCC metastasis, transcriptome sequencing of 16 pairs of HCCs and its corresponding NTLs in a HKU-QMH cohort was performed. We identified 213 genes that were upregulated by ≥ 2-folds in HCC as compared with NTLs. Within these, 25 genes were further upregulated by ≥ 2-folds in HCC with the presence of venous invasion compared with those without. Among these upregulated genes, MID1IP1 ranked among the top 10 most significantly upregulated in metastatic HCC (Figure 1A). Moreover, it was found to be the only protein-coding gene with a higher expression for cases with vascular invasion in HCCs in TCGA-LIHC cohort, where the higher expression of MID1IP1 was more prominent in cases with macro-vascular invasion (Figure 1B and Supplemental Figure 1a, http://links.lww.com/HEP/D711). Further validations were carried out by examination of the mRNA expression of MID1IP1 in an expanded clinical sample cohort (HKU-QMH) (n = 89) by real-time PCR, where MID1IP1 was significantly overexpressed in HCCs with 47% of cases showing ≥ 2-fold overexpression. Concordantly, significant upregulation in HCCs with 50% of cases having ≥ 2-fold upregulation was observed in paired HCC cases of the TCGA cohort (n = 50 pairs) (Figure 1C). Western blotting and immunohistochemical staining also showed that MID1IP1 was significantly overexpressed at the protein level in human HCCs compared with NTLs (Figure 1D). In liver cell lines, MID1IP1 mRNA and protein expression were significantly higher in HCC cell lines (Hep3B, Huh7, PLC/PRF/5, and MHCC97L (authenticated to have no contamination; see Supplemental Information http://links.lww.com/HEP/A826 and Supplemental Figure 8, http://links.lww.com/HEP/D711) in comparison to nontumorigenic liver cells (MIHA) (Supplemental Figure 1b and c, http://links.lww.com/HEP/D711). It is noteworthy that MID1IP1 is also significantly overexpressed in several other cancer types such as breast cancer, cholangiocarcinoma, etc (Supplemental Figure 1d, http://links.lww.com/HEP/D711). Clinicopathological correlation analysis revealed that upregulation of mRNA expression of MID1IP1 by ≥ 2 folds was significantly associated with more aggressive HCC phenotypes including the presence of venous invasion, tumor microsatellite formation, direct liver invasion, absence of tumor encapsulation, and more advanced tumor stage (Figure 1E). Poorer overall survival was also observed in HCCs with higher than median MID1IP1 expression in both HKU-QMH and TCGA cohorts (Figure 1F). Taken together, these results suggest that MID1IP1 is frequently overexpressed in human HCCs, and its overexpression is associated with metastatic features and an aggressive phenotype.

Upregulation of MID1IP1 augments metastatic potential of HCC by enhancing its migratory and invasive abilities *in vitro* and *in vivo*

As mentioned earlier, we identified MID1IP1 as a potentially key molecular target that was clinically associated with metastatic HCC (Figure 1). Therefore, we assessed the tumor-promoting functions of MID1IP1 in HCC cells by stable knockdown of MID1IP1 with shRNA (shMID1IP1) in MHCC97L and Hep3B cells and its stable overexpression (MID1IP1 OE) in PLC/PRF/5 cells. As shown in Figure 2A, the mRNA and protein expression levels were significantly attenuated upon shMID1IP1 as compared with the nontarget control (NTC), whereas MID1IP1 was ectopically expressed in MID1IP1 OE cells as compared with empty vector (EV) control (Figure 2B). Fewer cells for both MHCC97L and Hep3B shMID1IP1, that is sh#3 and sh#5 were able to migrate and invade in our transwell assays, indicating a significant reduction in their migratory and invasive abilities in HCC. In line with this, MID1IP1 OE PLC/PRF/5 cells demonstrated a significant induction in their migration and invasion rates (Figure 2C, D).

We further tested the effect of manipulating MID1IP1 expression on the metastatic potential of HCC cells *in vivo* using an orthotopic liver injection model. We observed significantly fewer pulmonary metastases in BALB/cAnN-nu (Nude) mice with shMID1IP1 MHCC97L-luc liver orthotopic xenografts at week 5. This was further supported by increased pulmonary metastases for MID1IP1 OE PLC/PRF/5-luc liver orthotopic xenograft model in nude mice compared with EV controls (Figure 2F). In addition, histological assessment showed that MID1IP1 OE led to a substantial increase in the metastatic features including presence of vascular invasion and tumor microsatellites within the liver and in the invasive feature of irregular tumor growth front (Supplemental Figure 3c, http://links.lww.com/HEP/D711). Importantly, knockdown of MID1IP1 could significantly suppress the metastatic success rates of MHCC97L cells as assessed by lung colonization levels in the tail-vein injection model (Supplemental Figure 3b, http://links.lww.com/HEP/D711).These functional observations were in concordance with the clinical association of MID1IP1 overexpression in invasive HCC. They strongly suggest that MID1IP1 is a key modulator of migratory and invasive abilities in HCC cells and its upregulation drives tumor metastasis in HCC.

Depletion of MID1IP1 diminishes the self-renewal ability, proliferation, and *in vivo* tumor growth of HCC cells

Since a large proportion of HCC cases showed MID1IP1 upregulation in HKU-QMH and TCGA cohorts, we investigated its contribution in other oncogenic properties in addition to metastasis. First, our sphere formation assay showed that MHCC97L and Hep3B cells were able to form fewer spheres upon knockdown of MID1IP1 cells, while MID1IP1 OE PLC/PRF/5 cells demonstrated an enhanced ability in forming spheres (Figure 3A). These indicate that MID1IP1 positively regulates the self-renewal ability, a defining feature of cancer stemness, of HCC cells. The significant suppression of expression of stemness-related genes including Notch1, Oct4, KLF4, SMO, and Nestin in shMID1IP1 MHCC97L and Hep3B cells further substantiated its role in regulating HCC stemness (Figure 3B). Moreover, knockout of MID1IP1 in Hep3B cells echoed these results (Supplemental Figure 2 a–e http://links.lww.com/HEP/D711). Within the TCGA-LIHC cohort, cases with high MID1IP1 expression showed a significantly higher expression for HCC markers CD133 and EpCAM (Supplemental Figure 3e, http://links.lww.com/HEP/D711). These suggest that MID1IP1 may play a functional role in tumor initiation. Next, the proliferation rates of the shMID1IP1 MHCC97L and Hep3B were assessed by measuring the number of cells for 5 or 6 consecutive days (Figure 3C). A marked reduction in proliferation rates was observed for shMID1IP1. Similarly, the colony-forming ability of HCC cells was also reduced in shMID1IP1 MHCC97L and Hep3B cells, which formed fewer numbers of colonies 20 days after seeding as compared with NTC (Figure 3D). In addition, suppression of MID1IP1 expression significantly reduced the tumor volume of xenografts, as indicated also by the luciferase intensity (total flux P/s) *in vivo* as demonstrated in the shMID1IP1 MHCC97L-luc liver orthotopic xenografts in BALB/cAnN-nu (Nude) 4 weeks after injection (Figure 3E). A significant decrease in proliferative index was also observed in shMID1IP1 xenograft tumors (Supplemental Figure 3a, http://links.lww.com/HEP/D711). Conversely, MID1IP1 OE significantly induced tumor growth rate in PLC/PRF/5-luc liver orthotopic xenografts compared with the EV control (Figure 3F). Several HCC stemness markers were also increased in MID1IP1 OE xenografts by qPCR (Supplemental Figure 3d, http://links.lww.com/HEP/D711). Together, these results demonstrated the oncogenic role of MID1IP1 in promoting the tumor formation and progression in HCC.

SP1 transcription factor is an upstream driver regulator of MID1IP1 overexpression in HCC

To find the upstream regulator that contributed to the upregulation of MID1IP1 in HCC, we used ENCODE integrative analysis to predict potential transcriptional factors (TFs) that bind to the MID1IP1 promoter region. POLR2A, TAF1, EP300, SP1, and TBP were the top 5 TFs with the highest probability of interacting with the MID1IP1 promoter based on the number of previous

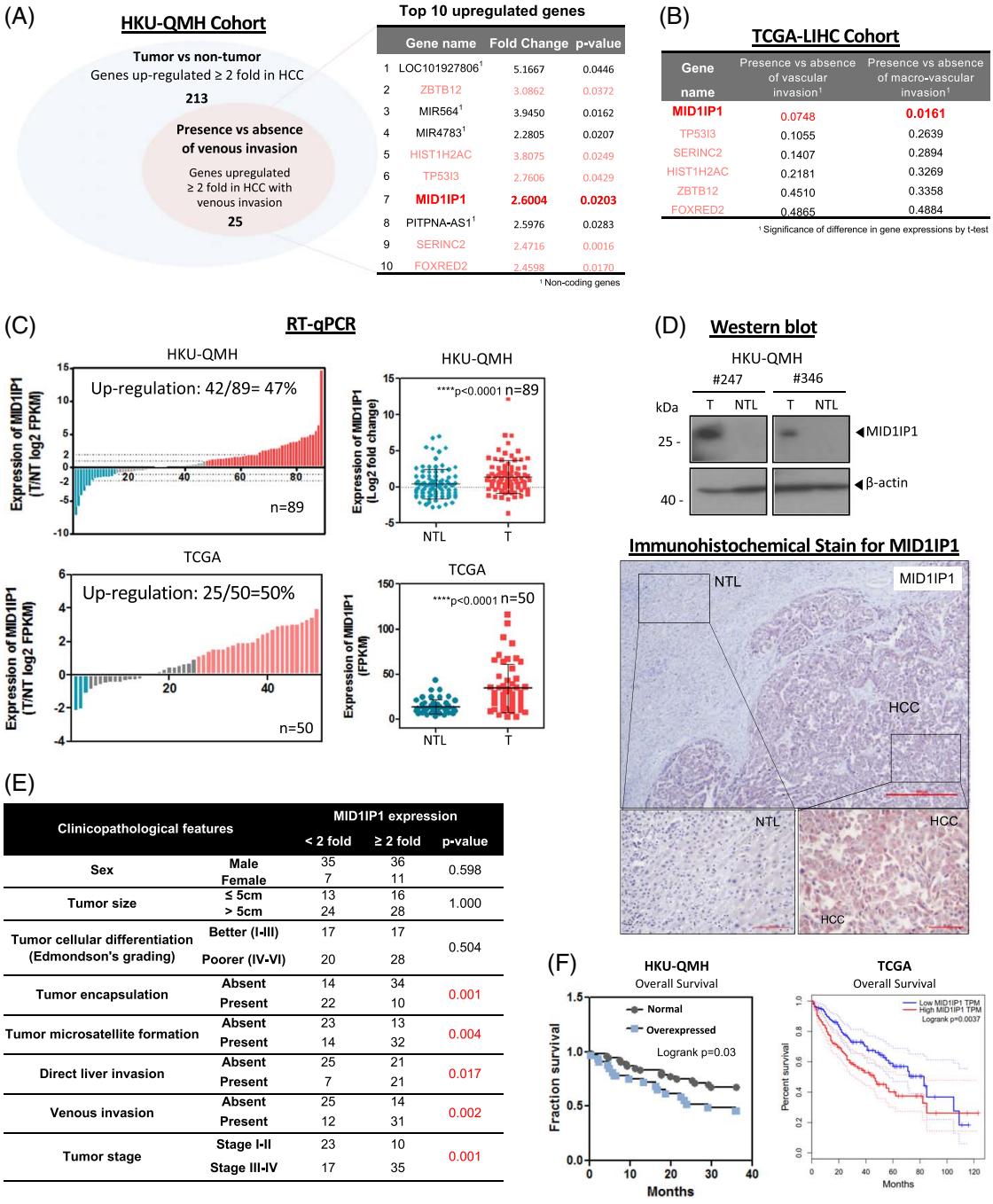

Figure 1 Frequent upregulation of MID1IP1 in HCC is associated with more aggressive clinicopathological features. (A) Schematic Venn diagram representing the number of genes upregulated ≥2-fold in HCC with venous invasion. MID1IP1 identified as one of the top 10 upregulated genes in transcriptome sequencing dataset in HKU-QMH cohort (n = 16). (B) List of protein-coding genes ranked according to their significant increase in expression within cases with vascular or macro-vascular invasion compared with the absence of vascular invasion in HCC cases of TCGA cohort. (C) Expression of MID1IP1 in paired HCCs and NTLs from HKU-QMH cohort (n = 89) by real-time PCR and the TCGA database (n = 50) (****$p < 0.0001$, *t* test). (D) The protein expression of MID1IP1 of HCC compared with the NTL counterpart examined by Western blotting and immunohistochemical staining. (E) Summary table of clinicopathological correlation analysis for the overexpression of MID1IP1 (2-fold) in the HKU-QMH cohort (Fisher exact test). (F). Kaplan-Meier plots for overall survival of HCC patients in HKU-QMH and TCGA cohorts stratified by MID1IP1 expression (*$p < 0.05$, log-rank test). Abbreviations: HKU-QMH, The University of Hong Kong-Queen Mary Hospital; NTL, nontumorous liver; TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma.

experiments that supported its TF binding (Figure 4A). Among these candidate TFs, only the expression of SP1 was significantly and positively associated with the expression of MID1IP1 in both HKU-QMH and TCGA cohorts (Figure 4B and Supplemental Figure 7d, http://links.lww.com/HEP/D711). This association was absent in NTL tissues of the TCGA cohort, suggesting that the observed association between MID1IP1 and SP1 is

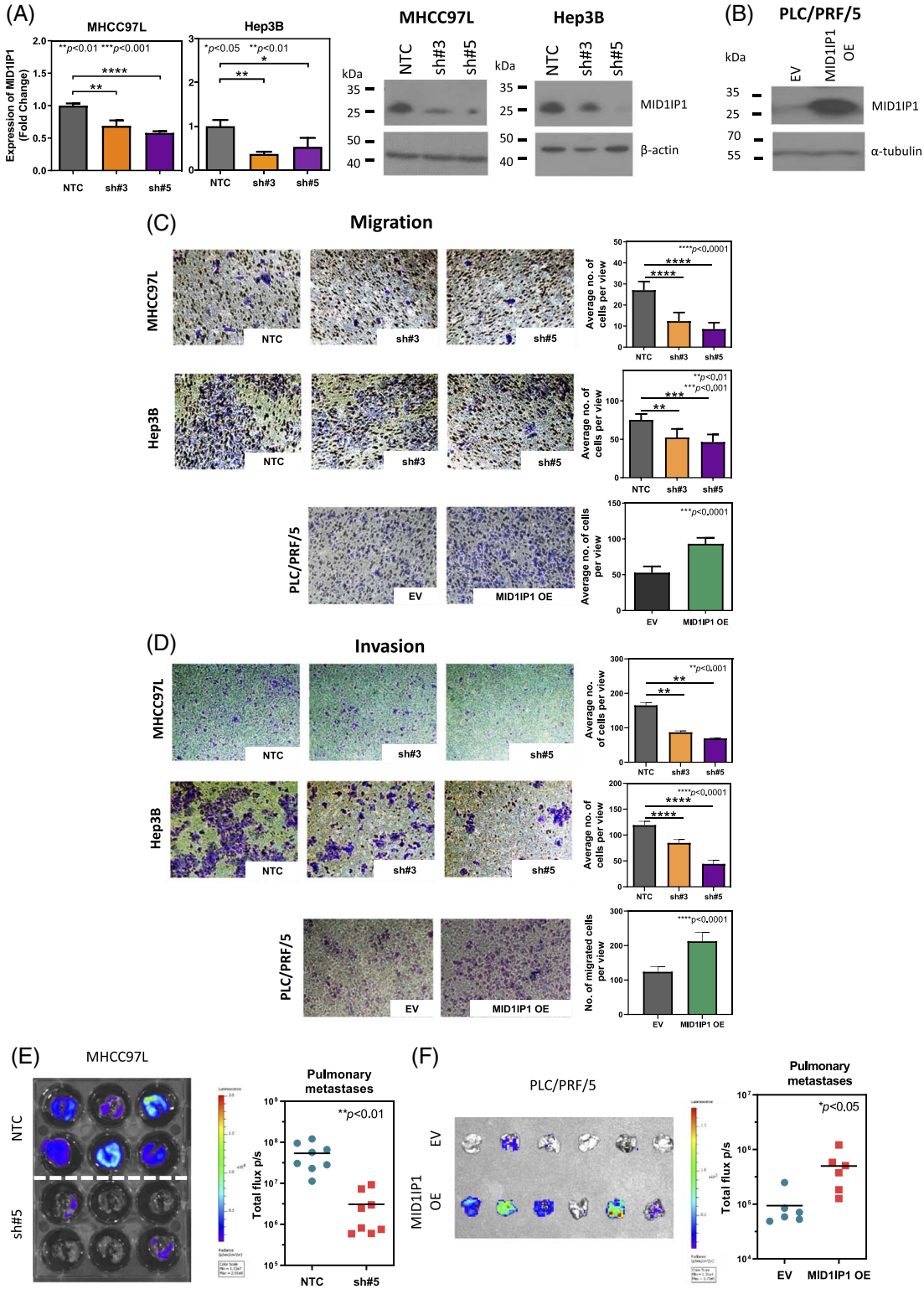

Figure 2 MID1IP1 promotes migratory and invasive abilities of HCC cells. (A) Knockdown efficiencies of MID1IP1 in MHCC97L and Hep3B by RT-PCR and Western blotting (n = 3, data displayed as ± SD). (B) Protein expression level of MID1IP1 in MID1IP1-overexpressing (MID1IP1 OE) PLC/PRF/5 cells. (C) Migration and (D) invasion assays to measure relative migratory and invasive abilities of HCC cells, respectively, in shMID1IP1 MHCC97L and Hep3B cells or MIDIP1 OE PLC/PRF/5 cells (n = 3, data displayed as ± SD). Bioluminescence imaging and quantification for distant pulmonary metastases in BAL/cAnM-nu (nude) mice in orthotopic liver xenografts for (E) shMID1IP1 MHCC97L-luc and (F) MID1IP1 OE PLC/PRF/5-luc cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ***$p < 0.0001$, *t* test). Abbreviations: EV, empty vector; OE, overexpression; NTC, no template control.

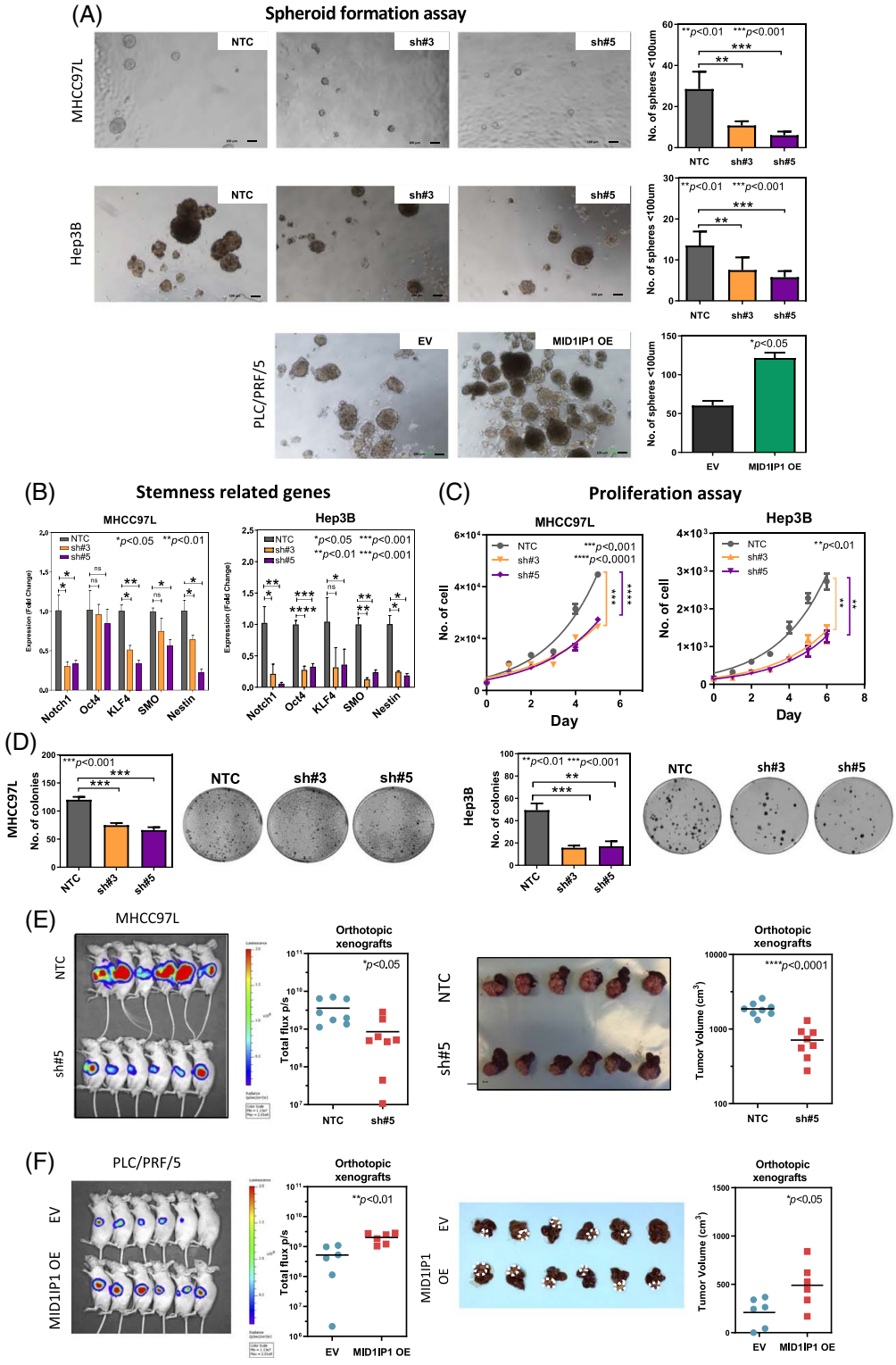

Figure 3 MID1IP1 contributes to self-renewal ability, proliferation, and tumor growth rates of HCC cells. (A) Relative sphere-forming ability of shMID1IP1 MHCC97L and Hep3B cells or MID1IP1 OE PLC/PRF/5 cells by sphere formation assay (n = 3, data displayed as ± SD). (B) Relative mRNA expression levels of stemness-related genes (Notch1, Oct4, KLF4, SMO, and Nestin) examined by real-time PCR in shMID1IP1 MHCC97L and Hep3B cells (n = 3, data displayed as ± SD). (C) The proliferation curves of shMID1IP1 MHCC97L and Hep3B cells (n = 3, data displayed as ± SD). (D) Relative numbers of colonies formed by shMID1IP1 MHCC97L and Hep3B cells at day 20. Colonies with cells > 30 were counted (n = 3, data displayed as ± SD). Representative images and graphical representation of relative bioluminescence signal intensity and tumor volume of liver orthotopic xenografts of (E) shMID1IP1 and (F) MID1IP1 OE MHCC97L-luc in BALB/cAnN-nu (Nude) mice 5 weeks after inoculation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{***}p < 0.0001$, *t* test. Abbreviations: EV, empty vector; OE, overexpression; NOTCH1, notch receptor 1; NTC, no template control.

tumor-specific (Supplemental Figure 7c, http://links.lww.com/HEP/D711). To test the regulatory role of SP1 on MID1IP1 expression, we generated stable knockdown models for SP1 (shSP1) in MHCC97L and PLC/PRF/5 cells. Both mRNA and protein expression of MID1IP1 were significantly suppressed upon shSP1 (Figure 4C). SP1 was validated to directly bind to the promoter of MID1IP1 by chromatin-immunoprecipitation (ChIP) assay, as a significant enrichment of MID1IP1 promoter for regions −164 to −22nt and −904 to −554nt transcription start site (TSS) was found in the anti-SP1 immunoprecipitation (Figure 4D). The binding site of SP1 on MID1IP1 promoter was further predicted using the INSECT 2.0 tool. MID1IP1 transcription start site (TSS) −129 to −139 nt and −40 to −50 nt within its promoter region showed the highest score of the position weight matrix for the SP1 binding. A number of mutant promoter clones were generated to study its activity levels in relation to SP1 (Figure 4E). First, we assessed the promoter activity of TSS −180 to −22nt Del truncated MID1IP1 promoter constructs in the SP1 overexpressing (OE) PLC/PRF/5 by the luciferase reporter assay as compared with the EV control. The SP1-induced MID1IP1 promoter activity was abolished in the −180 to −22nt Del MID1IP1 promoter, suggesting TSS −40 to −50 nt, 1129 to −139 nt, and −150 to −160 nt of the MID1IP1 promoter, which were the potential binding sites of SP1 (Figure 4F). To further pinpoint the binding sites of SP1 on MID1IP1 promoter, −160 −139 −50 Mut and −139 −50 mut MID1IP1 promoters, that is the potential SP1-binding sites within this −180 to −22nt, were mutated and tested for the promoter activity upon SP1 OE. Moreover, −835 −676 Mut and 5x Mut (ie, −835 –676 −160 −139 −50 Mut) constructs were also generated, as these additional sites were also predicted to harbor SP1-biding sites in our analysis using the INSECT 2.0 tool. Similarly, the SP1-induced MID1IP1 promoter activity was significantly suppressed in all mutant promoter constructs generated, suggesting that SP1 bound to these sites to promote the transcription of MID1IP1 mRNA expression. These results indicate that SP1 is the upstream transcriptional regulator of MID1IP1 promoter and a key modulator for the upregulation of MID1IP1 in HCC.

MID1IP1 modulates FRA1 to regulate its transcriptional pathway

To further dissect the underlying mechanisms of MID1IP1 conferring the oncogenic and metastatic potential in HCC, transcriptome sequencing was performed on PLC/PRF/5 MID1IP1 OE cells and compared with EV as the corresponding control (Figure 5A). We performed the Gene Set Enrichment Analysis (GESA) and identified PID_FRA_PATHWAY as a potential downstream functional pathway for MID1IP1. It ranked as one of the top 20 canonical pathways, which are significantly and positively enriched (NES = 1.4284, Nom *p*-value = 0.0321) in MID1IP1 OE HCC cells (Figure 5B and Supplemental Figure 4a, b, http://links.lww.com/HEP/D711). We further utilized the TCGA cohort to assess the correlation of expression between MID1IP1 and target genes within the gene set. The expression of multiple target genes, particularly LIF, MMP9, CCNA2, TXLNG, and NFATC2, showed significant positive correlation and strongest association with MID1IP1 expression (Supplemental Figure 4c, http://links.lww.com/HEP/D711). It was noteworthy that MMP9 and MMP2, which are known contributors to metastasis of HCC, were among the target genes within this gene set.[12,13] We next quantified the transcript levels of LIF, MMP9, TXLNG, NFATC2, FOSL1, JUNB, JUND, and CCL2 by real-time PCR to assess the transcriptional activity of FRA-transcription factors upon manipulation of MID1IP1 expression. In concordance with our transcriptomics experiment, it showed that most of the FRA-target genes were upregulated with MID1IP1 overexpression in MHCC97L and PLC/PRF/5 cells (Figure 5C). A reduction of these FRA-targeted genes was observed in shMID1IP1 MHCC97L cells (Figure 5C). The PID_FRA_PATHWAY comprises target genes of 2 TFs, that is FRA1 and FRA2. To assess the relevance of these 2 TFs with MID1IP1, we checked their association with the target genes that were correlated with the expression of MID1IP1. LIF and MMP9 genes having the strongest correlation with MID1IP1 expression showed a stronger association with FOSL1 (or FRA1) than FOSL2 (or FRA2) (Supplemental Figure 4d, http://links.lww.com/HEP/D711). Moreover, we had previously seen that FRA1 plays a critical tumor-promoting role in HCC[11] and hence reasoned that FRA1 was likely the TF associated with MID1IP1. To this end, we examined the protein levels of FRA1 and observed that MID1IP1 positively regulated protein levels of FRA1. PLC/PRF/5 and MHCC97L cells overexpressing MID1IP1 showed higher FRA1 protein levels, and shMID1IP1 MHCC97L cells showed a lower level of FRA1 by Western blot (Figure 5D). Immunohistochemical staining for MID1IP1 and FRA1 on the liver orthotopic xenografts in consecutive sections showed that knockdown of MID1IP1 suppressed the abundance and nuclear localization of FRA1 *in vivo* (Figure 5E). Immunohistochemical staining on a clinical case also demonstrated correspondent staining of the 2 proteins in HCC tumors (Figure 5F). These results suggest that MID1IP1 causes FRA1 to increase in abundance, which subsequently results in the induction of the transcription of downstream target genes.

MID1IP1 regulates the expression of MMP9 and extracellular matrix degradation activity of HCC cells

As described above, we showed that MID1IP1 is clinically and functionally associated with the metastatic

(A)

Transcriptional factors bind to MID1IP1 promoter

TF	No. of experiments that support TF binding	No. of experiments in total
POLR2A	64	82
TAF1	7	11
EP300	7	26
SP1	**5**	**9**
TBP	5	5
YY1	5	13
CTCF	5	180
REST	5	20
MYC	4	10
HDAC2	4	9

ENCODE integrative analysis

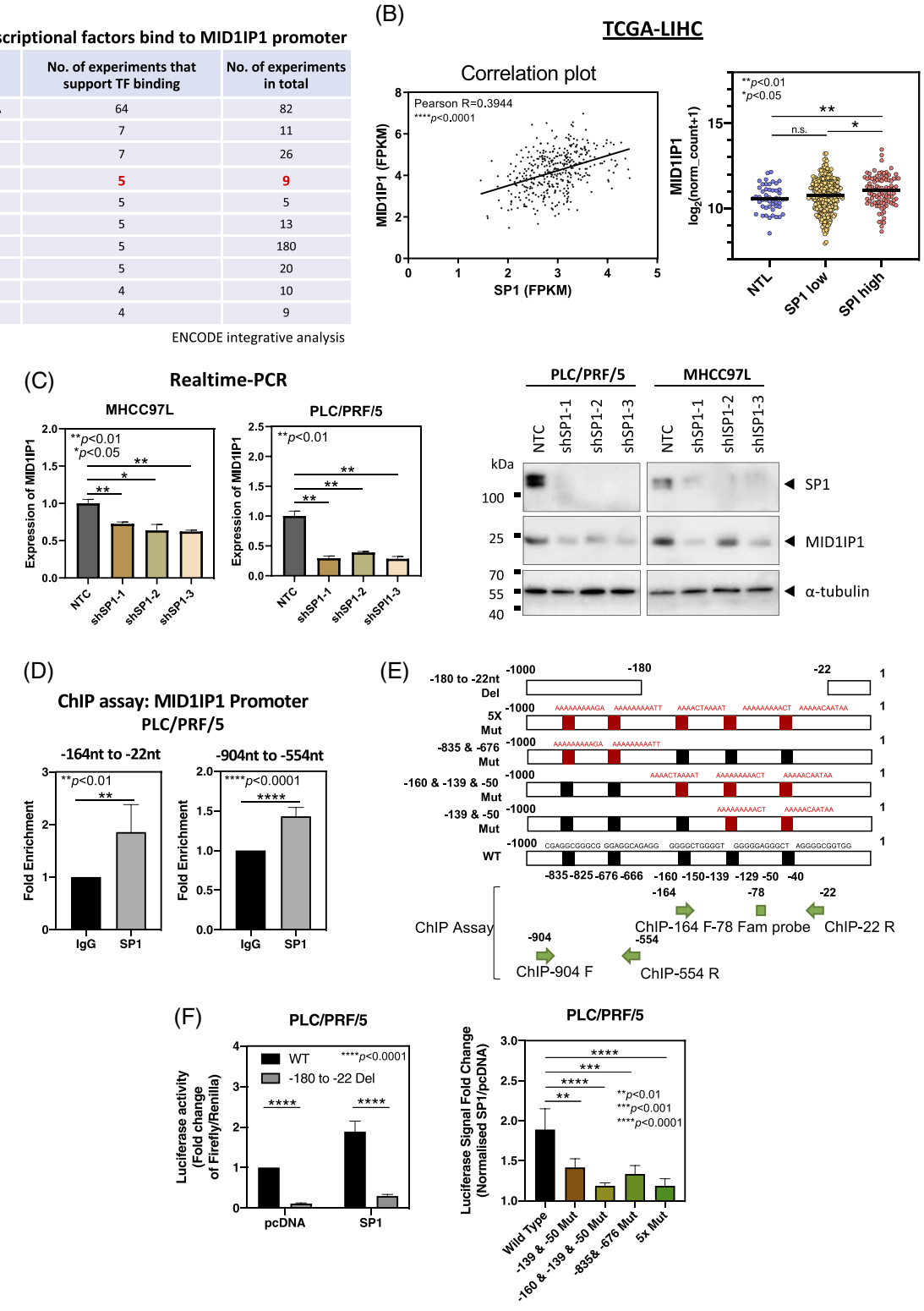

Figure 4 SP1 transcription factor is an upstream regulator for MID1IP1 overexpression in HCC. (A) List of transcription factors predicted to bind to MID1IP1 promoter by ENCODE integrative analysis. (B) Scatterplots showing correlation of SP1 and MID1IP1 mRNA expression in HCC patients and differential expression of MID1IP1 in NTL samples compared with HCC cases stratified according to SP1 expression (using upper quartile as a cutoff threshold) in TCGA cohort. (C) RT-PCR and Western blot analysis for MID1IP1 expression upon knockdown of SP1 in PLC/PRF/5 and MHCC97L cells (n = 3, data displayed as ± SD) (*$p < 0.05$, **$p < 0.01$, *t* test). (D) ChIP assay significantly enriched MID1IP1 promoter regions to −164 to −22nt and −904 to −554nt TSS by immunoprecipitation of SP1 in PLC/PRF/5 cells (**$p < 0.01$, ***$p < 0.001$, *t* test) (n = 5, data displayed as ± SD). (E) Schematic diagram of wild-type (WT) and designed mutants (Mut and Del) of the MID1IP1 promoter region. (F) Relative activity of MID1IP1 promoter upon overexpression of WT SP1 or SP1 mutants (−180 to −22nt Del, −139 and −50 Mut, −160, −139 and −50 Mut, −835 and −676 Mut and 5x Mut) by luciferase reporter assay (n = 6, data displayed as ± SD) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, *t* test). Abbreviations: ChIP, Chromatin-immunoprecipitation; TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma.

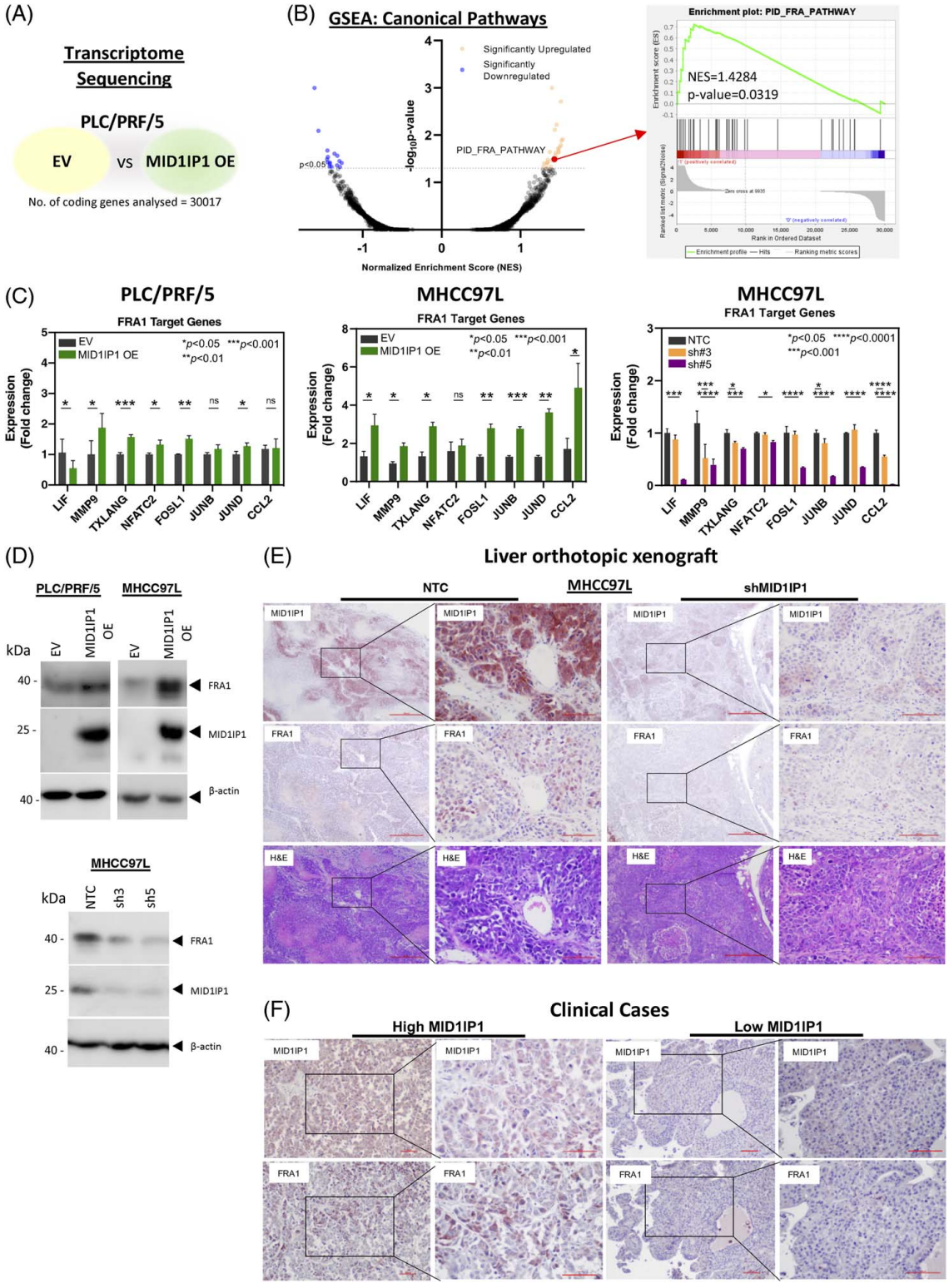

Figure 5 MID1IP1 promotes FRA1-mediated transcription. (A) Schematic diagram of transcriptome-sequencing strategy performed on PLC/PRF/5 HCC cells with either empty vector control (EV) or MID1IP1 OE. (B) Volcano-plot of GSEA and enrichment plot revealed PIDFRA_PATHWAY as one of the topmost significantly enriched pathways with MID1IP1 overexpression (NES = 0.428, NOM *p*-value = 0.0321) in the transcriptome sequencing data. (C) Relative mRNA expression of FRA1 targeted genes (LIF, MMP9, TXLNG, NFATC2, FOSL1, JUNB, JUND, and CCL2) in PLC/PRF/5 or MHCC97L EV/MID1IP1 OE and MHCC97L NTC/shMID1IP1 by RT-PCR (n = 3, data displayed as ± SD) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, *t* test). (D) Western blot of FRA1 protein expression level PLC/PRF/5 or MHCC97L MID1IP1 OE cells and shMID1IP1 MHCC97L cells. Relative MID1IP1 and FRA1 protein expression levels in (E) NTC/shMID1IP1 MHCC97L liver orthotopic xenograft models and (F) MID1IP1 high/low clinical HCC specimens by immunohistochemical staining. Abbreviations: GSEA, gene set enrichment analysis; EV, empty vector; OE, overexpression; NTC, no template control.

properties of HCC and regulates FRA1-signaling pathway. To identify the key downstream functional mediator of MID1IP1/FRA1, we utilized the TCGA-LIHC cohort. Among the genes most strongly correlated with the expression of MID1IP1, MMP9 was the only gene which had a higher expression in HCCs, correlated with poorer survival of patients, and showed a significantly positive correlation with the expression of FRA1 (Supplemental Figure 5a–d, http://links.lww.com/HEP/D711). In addition to MMP9, MMP2 was also found to be upregulated in our GSEA analysis. Matrix metalloproteinases (MMPs) are gelatinases that are implicated in HCC progression with their roles in ECM degradation and cancer cell invasion[14–16.] Therefore, we hypothesized that MID1IP1 regulated the metastatic properties of HCC cells through modulation of the expression of MMPs). In our transcriptome sequencing data, we observed that the majority of MMP family members were upregulated in MID1IP1 OE PLC/PRF/5 cells, with MMP23B, MMP9, and MMP26 having >2-fold increase in expression (Figure 6A). Among these, only MMP9 was found to have a significant association with the expression of MID1IP1 in both HKU-QMH cohort and TCGA (Figure 6B). To further confirm if MID1IP1 modulated the expression of MMP9, we examined the mRNA (Figure 6C) and protein levels (Figure 6D) of MMP9, and MMP9 promoter activity (Figure 6E) in shMID1IP1 and MID1IP1 OE HCC cells. MMP9 mRNA, protein, and promoter activity were consistently suppressed upon shMID1IP1 in MHCC97L and, conversely, induced with MID1IP1 OE MHCC97L and/or PLC/PRF/5 cells. MMP9, also known as gelatinase B, belongs to a class of enzymes that are involved in the breakdown of extracellular matrix in normal physiological processes, angiogenesis, cell migration, and in pathological processes such as metastasis.[17,18] Subsequently, we found that the MID1IP1 promoted the gelatinase activity in HCC cells by gelatin degradation assay. The number of gelatin degraded foci was significantly less in shMID1IP1 MHCC97L and higher in MID1IP1 OE MHCC97L or PLC/PRF/5 cells (Figure 6F). Taken together, our data implicates that MID1IP1 regulates the expression of MMP9 and the gelatinase activity, which contributes to the migratory and invasive abilities in HCC.

FRA1 is the key mediator of upregulation of MMP9 expression and metastatic potential by MID1IP1 in HCC

As described above, we demonstrated that MID1IP1 promoted migration and invasion through upregulation of MMP9 expression in HCC. To verify FRA1 as the key mediator of induction, FRA1 expression was suppressed by siRNA (siFRA1) in combination with overexpression of MID1IP1 in MHCC97L and PLC/PRF/5 cells. The number of migrated and invaded cells was examined by transwell assays. The induced migratory and invasive abilities of HCC cells upon overexpression of MID1IP1 were abolished when combined with siFRA1 in both cell lines (Figure 7A, B). In addition to functional validation, we also observed abolishment of the MID1IP1-induced upregulation of mRNA (Figure 7C) and protein expression (Figure 7D) of MMP9 in the MID1IP1 OE HCC cells when treated with siFRA1. Furthermore, knockdown of MMP9 abolished MID1IP1 OE-induced migratory and invasive abilities of PLC/PRF/5 and MHCC97L cells, confirming its functional role in the pathway (Supplemental Figure 6a–d, http://links.lww.com/HEP/D711). These results further substantiate that FRA1 is the critical functional mediator in the MI1D1IP1-MMP9 pathway and subsequently leads to the MID1IP1-regulated migratory and invasive abilities of HCC cells.

DISCUSSION

Reports on the implications of MID1IP1 in cancers are very scanty. In this study, we have identified MID1IP1 as a clinically relevant contributing gene, demonstrated its tumor- promoting function, and uncovered the underlying mechanism for its role in promoting metastatic potential of HCCs. Here, we also identified a novel regulatory role of MID1IP1 on FRA1-MMP9 signaling as a contributing factor in liver cancer metastasis. Importantly, the significant clinical correlation between the overexpression of MID1IP1 in human HCCs and more aggressive and invasive features indicate that the mechanism described herein may be a key pathway that endows HCC cells with higher metastasis success rates.

To the best of our knowledge, this is the first study to comprehensively characterize the implications of MID1IP1 as a potent tumor-promoting gene in cancers. Previous studies of MID1IP1 have been limited to its implications in metabolic pathways such as lipogenesis and glucose metabolism in various cell types including liver cells.[19,20] Although these pathways are important in cancer cell metabolism, its direct relevance and functional role on tumor development and progression were not clearly elucidated. Scattered and brief studies touching on the relevance of MID1IP1 in cancer remain inconclusive on its functional role in tumors. For example, Jung et al.[21] illustrated the colocalization of MID1IP1, and c-Myc can affect the liver cancer growth through CCR4-NOT transcription complex subunit 2 (CNOT2). However, in a different study, they demonstrated the induction of apoptosis requiring MID1IP1 for colorectal cancer, suggesting a paradoxical role for the gene.[22] Herein, we report that MID1IP1 is predominantly overexpressed in different cancer types within the TCGA cohort in addition to HCC, such as breast carcinoma, cholangiocarcinoma, and colon adenocarcinoma (Supplemental

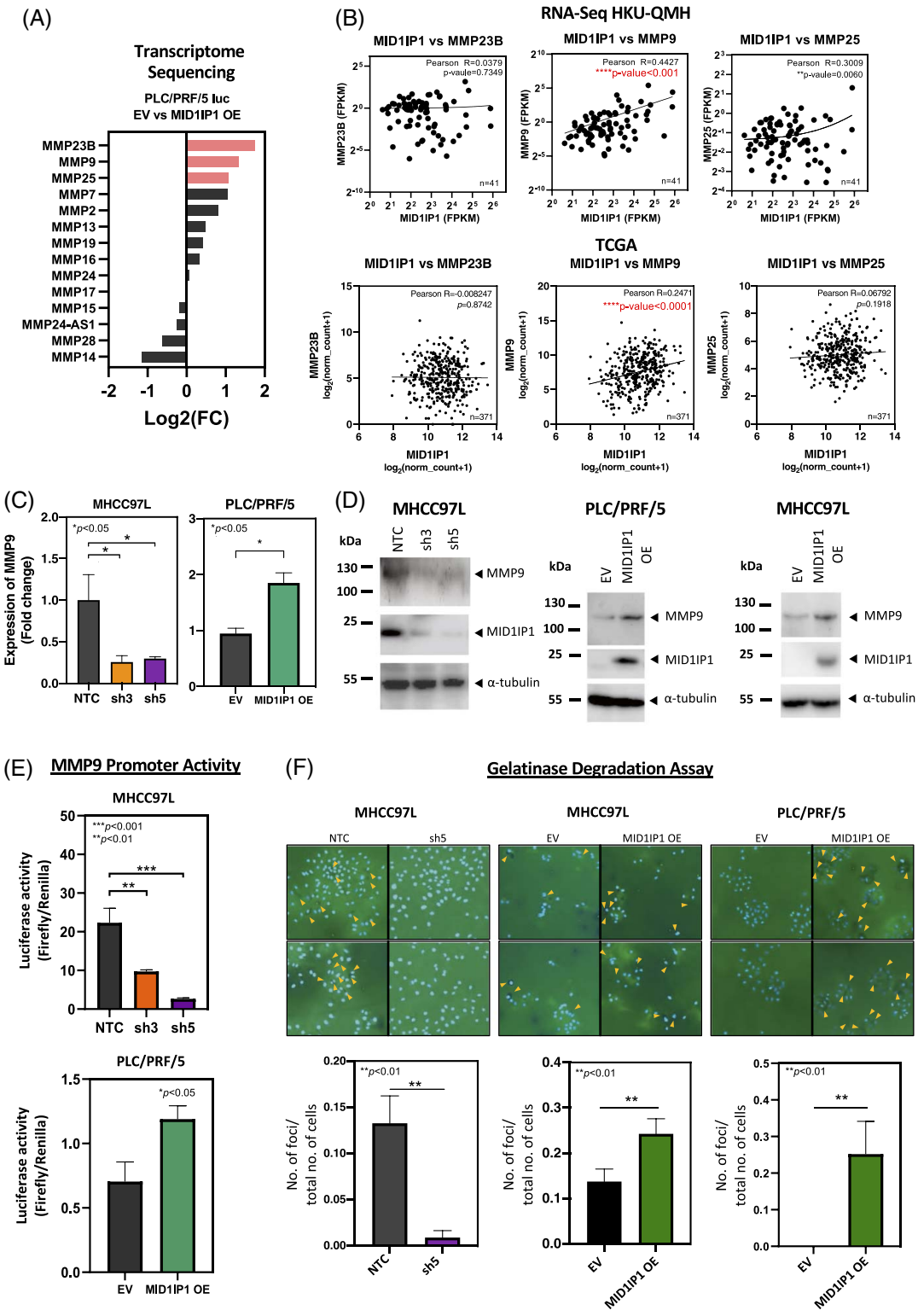

Figure 6 MID1IP1 upregulates the expression of MMP9 to induce extracellular matrix degradation activity in HCC. (A) Relative fold change in the expression of MMP family members upon overexpression of MID1IP1 in PLC/PRF/5 cells from RNA-seq data. (B) Scatterplots for correlation analysis of MID1IP1 against MMP23B, MMP9, or MMP25 transcript expressions in HCC patients of HKU-QMH cohort (upper panel) and TCGA cohort (lower panel) (*$p < 0.05$, ****$p < 0.0001$, Pearson correlation test). (C) Relative mRNA expression of MMP9 in shMID1IP1 MHCC97L cells and MID1IP1 OE PLC/PRF/5 cells by RT-PCR (n = 3, data displayed as ± SD). (D) Relative protein expression of MMP9 in shMID1IP1 MHCC97L cells and MID1IP1 OE PLC/PRF/5 or MHCC97L cells by Western blot. (E) Relative MMP9 promoter activity levels in shMID1IP1 MHCC97L cells and MID1IP1 OE PLC/PRF/5 by luciferase reporter assay (n = 3, data displayed as ± SD). (F) Relative extracellular matrix degradation activity of shMID1IP1 or MID1IP1 OE in MHCC97L cells and MID1IP1 OE in PLC/PRF/5 cells by gelatine degradation assay (n = 3, data displayed as ± SD). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, *t* test. Abbreviations: EV, empty vector; HKU-QMH, The University of Hong Kong-Queen Mary Hospital; OE, overexpression; NTC, no template control; TCGA, The Cancer Genome Atlas.

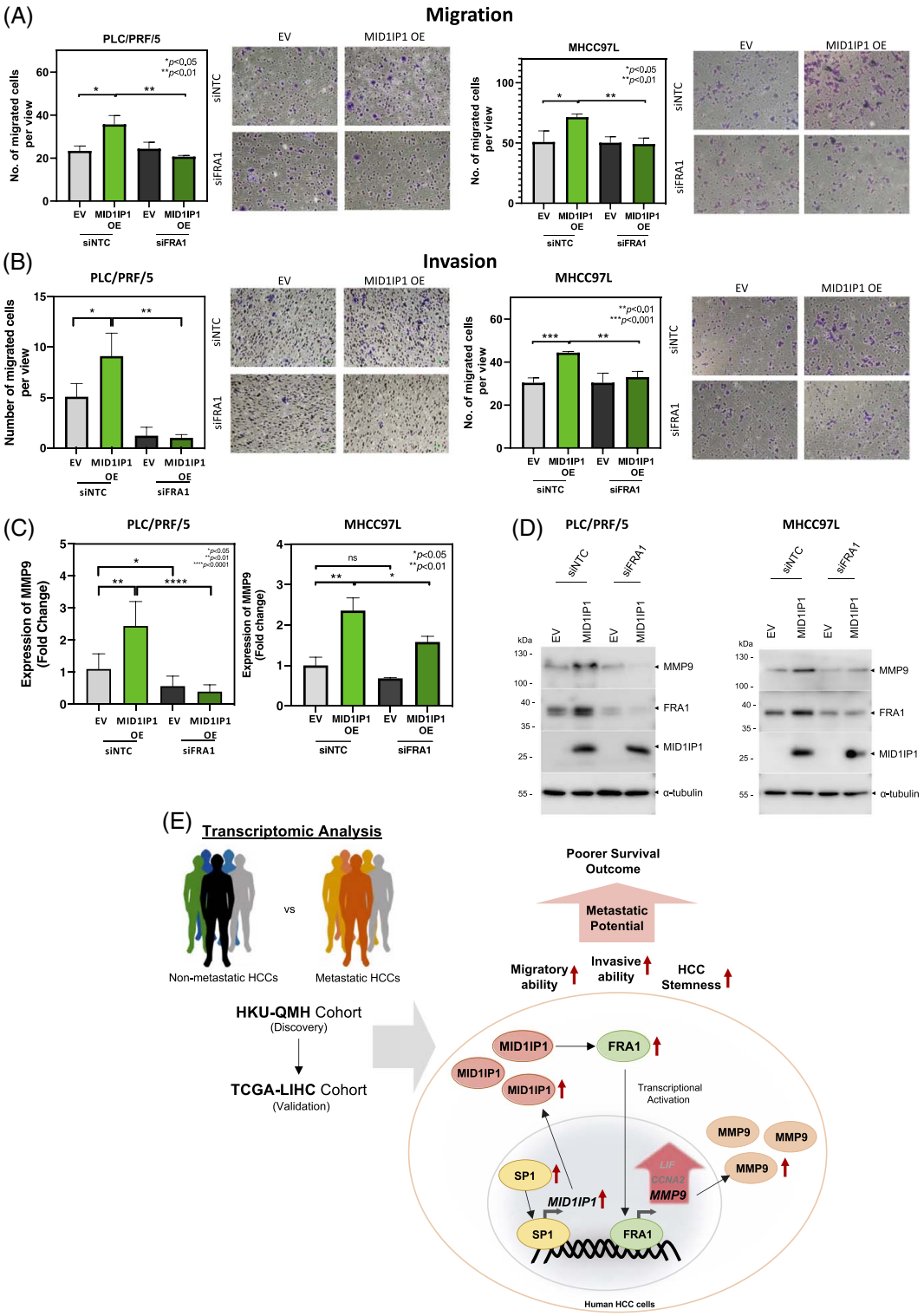

Figure 7 FRA1 mediates MID1IP1-induced migratory and invasive ability and MMP9 expression in HCC. Relative changes in (A) migratory and (B) invasive abilities of HCC cells upon overexpression of MID1IP1 with or without knockdown of FRA1 in PLC/PRF/5 and MHCC97L cells by transwell migration and invasion assay, respectively. Relative (C) mRNA and (D) protein expression of MMP9 in MID1IP1 OE MHCC97L and PLC/PRF/5 cells with or without knockdown of FRA1 by RT-PCR and Western blot, respectively (n = 3, data displayed as ± SD). (E) A graphical summary of the findings. MID1IP1 is frequently overexpressed in human HCCs and plays a contributory role in several tumor-promoting functional phenotypes. It drives cancer metastasis by promoting FRA1-mediated MMP9 pathway, which leads to higher migratory and invasive properties of HCC cells and consequentially poorer survival of patients (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, *t* test). Abbreviations: EV, empty vector; HKU-QMH, The University of Hong Kong-Queen Mary Hospital; OE, overexpression; TCGA, The Cancer Genome Atlas.

Figure 1d, http://links.lww.com/HEP/D711). Importantly, tissue samples of metastases of cutaneous melanoma in TCGA (TCGA-SKCM) have a significantly higher expression of MID1IP1, suggesting its tumor-promoting role in cancer metastasis. In our study, exogenous overexpression of MID1IP1 in HCC cells was sufficient to enhance the functional features essential for metastatic success, that is their migratory, invasive, and self-renewal abilities (Figures 2, 3 and Supplemental Figures 2f–h, http://links.lww.com/HEP/D711). These highlight the importance of MID1IP1 in modulating the metastatic potential of HCC cells. Since MID1IP1 has been shown to be involved in lipid and glucose metabolism, the intersection of cellular energy homeostasis with FRA1-MMP9 signaling could reveal further insight into its complex regulatory role in tumorigenesis.

Given the strong clinical association and functional role of MID1IP1 in metastatic properties of HCCs, it is important to understand the underlying mechanism for this phenotype. Transcriptomic sequencing led us to identify FRA1 signaling as a critical downstream effector for MID1IP1 (Figure 5). FRA1 is a member of the FOS family (c-FOS, FOSB, FRA1, and FRA2), which binds to the JUN-family proteins (c-JUN, JUNB, and JUND) to form the AP-1 TF complex. We previously reported the identification of the c-Met/FRA1/HEY1 signaling cascade as an inducer for cancer-associated fibroblast-derived HGF-mediated regulation of HCC stemness.[11] To the best of our knowledge, this is the only comprehensive report investigating the role of FRA1 in HCC till date. Hence, this study provides an additional insight into the oncogenic roles of FRA1 in HCC. The contribution of FRA1 in metastasis was further exemplified by its indispensable role in the ability of MID1IP1 to regulate the expression of MMP9, a well-studied metastasis-related gene. This echoed with the observation that modulation of MID1IP1 was able to regulate the ECM degradation properties of HCC cells, a functional phenotype for MMP9 (Figures 6, 7). This is further supported by the significant association between the expression of MMP9 and MID1IP1 in clinical liver cancer tissues (Figure 6B). In addition, we also examined the various other mechanisms highly involved in cancer metastasis. We found that modulation of MID1IP1 did not alter the epithelial–mesenchymal transition markers, that is E-cadherin, N-cadherin, and vimentin status in HCC cells (data not shown). We had also examined the structure of the actin cytoskeleton of the cells with staining of phalloidin (data not shown). Similarly, no significant difference was observed with MID1IP1 induction. These suggested that the downstream cascade of MID1IP1 was independent of these cellular characteristics. Taken together, MID1IP1 was found to regulate the metastatic potential of HCC cells, primarily through modulating ECM degradation ability through the FRA1-MMP9 pathway.

MID1IP1 was found to be frequently overexpressed in HCC. This was despite a fraction of cases having shallow deletion (Supplemental Figure 7a, http://links.lww.com/HEP/D711); however, samples with copy number deletion of MID1IP1 showed similar levels of expression compared with diploid samples, highlighting the importance of its expression at the transcriptional level. Little has been reported on the transcriptional regulation of MID1IP1 expression. So far, only carbohydrate response element-binding protein, a transcriptional factor, and liver X receptor, a nuclear receptor, were reported to promote the expression of MID1IP1 in beta cells and HCC cells, respectively.[19,23] However, neither of these are significantly correlated with MID1IP1 expression in HCC samples within the TCGA cohort (Supplemental Figure 7b, http://links.lww.com/HEP/D711). We identified the transcriptional regulatory role of SP1 on MID1IP1 promoter as the predominant upstream regulator driving its overexpression in human HCCs. Elevated expression of SP1 has previously been demonstrated in different types of cancers including HCC.[24–26] Various key cancer-related genes such as VEGF, HELicase, lymphoid-specific, SET domain, and bifurcated 1 are also regulated by SP1.[27–29] In our findings, expression of SP1 was significantly correlated with that of MID1IP1 in both QMH-HKU and TCGA-LIHC cohorts. The correlation was specific to HCC tumors only and not observable in non-tumorous liver samples (Figure 4B and Supplemental Figure 7c, http://links.lww.com/HEP/D711). Our data suggests that MID1IP1/FRA1/MMP9 is a clinically relevant functional downstream pathway of SP1 in human HCCs.

In summary, we have identified MID1IP1 as a driver gene of cancer metastasis in human HCCs. Our study defines MID1IP1 as a critical player which relays pro-tumorigenic signals between SP1 and FRA1, which are two important tumor-promoting genes in cancers. We describe MID1IP1-mediated FRA1/MMP9 signaling as a novel pathway that acts as a proponent of cellular phenotypes essential for metastasis in patients with primary HCCs (Figure 7E). Our results also provided a better understanding and revealed MID1IP1/FRA1/MMP9 cascade as a potential key molecular pathway that may be used for the development of novel diagnostic markers and effective therapeutic treatment in metastatic HCC.

AUTHOR CONTRIBUTIONS

Yung-Tuen Chiu, Abdullah Husain, and Irene Oi-Lin Ng provided the study concept and design; interpreted and analyzed the data. Yung-Tuen Chiu, Abdullah Husain, Karen Man-Fong Sze, Daniel Wai-Hung Ho, Xia Wang, Eliana Mary Senires Suarez, Eva Lee, Hoi-Tang Ma, and Lo-Kong Chan performed the experiments. Eva Lee and Joyce Man-Fong Lee collected the patients' samples. Yung-Tuen Chiu, Abdullah Husain, and Irene

Oi-Lin Ng wrote the manuscript. All authors approved the final version of the manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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