FOXAs suppress the metastasis of hepatocellular carcinoma partially through matrix metalloproteinase-9 inhibition

Jian Wang, Chang-Peng Zhu, Ping-Fang Hu, Hui Qian, Bei-Fang Ning, Qing Zhang, Fei Chen, Jiao Liu, Bin Shi, Xin Zhang and Wei-Fen Xie

Department of Gastroenterology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China and 1Department of Internal Medicine, Chinese PLA 531 Hospital, Tonghua 134000, Jilin, China

*To whom correspondence should be addressed. Tel: +86 21 8188 5341; Fax: +86 21 8188 9624; Email: weifenxie@medmail.com.cn

The forkhead box transcription factor A2 (FOXA2) is a member of the hepatocyte nuclear factor family and plays an important role in liver development and metabolic homeostasis, but its role in the metastasis of hepatocellular carcinoma (HCC) has not been evaluated. In this study, we found that the expression of FOXA2 was decreased in 68.1% (49/72) of human HCC tissues compared with their paired non-cancerous adjacent tissues. Clinicopathological analysis revealed that reduced FOXA2 expression was correlated with aggressive characteristics (venous invasion, poor differentiation, high tumor node metastasis grade). FOXA2 level was even lower in portal vein tumor thrombus compared with primary tumor tissues and correlated with epithelial-mesenchymal transition in HCC cells. Overexpression of FOXA2 inhibited migration and invasion of Focus cells, whereas knockdown of FOXA2 in HepG2 showed the opposite effect. Moreover, upregulation of FOXA2 suppressed HCC metastasis to bone, brain and lung in two distinct mouse models. Finally, we proved that FOXA2 repressed the transcription of matrix metalloproteinase (MMP)-9 and exerted its antimetastasis effect partially through downregulation of MMP-9. In conclusion, our findings indicate that FOXA2 plays a critical role in HCC metastasis and may serve as a novel therapeutic target for HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumor globally, especially in China (1). Despite the clinical implementation of numerous therapeutic strategies, the 5-year survival of HCC still remains low. Metastasis is the main risk for the long-term survival for HCC patients after surgical treatment and is considered to be responsible for the high HCC mortality rate (2,3). HCC metastasis is a complex cascade of events that include multiple genetic alterations. In the last few years, epithelial-mesenchymal transition (EMT) has been thought to play a critical role in HCC metastasis (4). There are several pathways that have emerged as important regulatory signals for EMT, including the PI3-K/Akt-, Wnt-, Notch-, Hedgehog- and nuclear factor-kappaB-dependent pathways (5). However, the mechanisms underlying the metastasis of HCC have not been thoroughly understood.

The hepatocyte nuclear factor (HNF) family, which consists of HNF1, HNF3, HNF4, HNF6 and CCAAT/enhancer binding proteins, form a systemic transcriptional network critical for the development and functional maintenance of hepatocytes in the liver (6). More and more evidence has suggested that the suppression and genetic alterations of HNFs are involved in liver tumorogenesis (7–9). Our previous studies demonstrated that the expression of both HNF4α and HNF1α was markedly reduced in most of the human HCC tissues. Upregulation of either HNF4α or HNF1α induced the differentiation of hepatoma cells into more mature phenotypes, abolished their tumorigenesis and inhibited the growth of HCC xenograft in mice (10,11). Moreover, we also showed that overexpression of HNF4α could ameliorate hepatic fibrosis and liver function with the inhibition of EMT in both hepatocytes and activated hepatic stellate cells in rats (12). Interestingly, a study by Hatzapostolou et al. (13) reveals that even transient inhibition of HNF4α in hepatocytes can initiate hepatocellular transformation in vitro and in vivo through a microRNA inflammatory feedback circuit. More recently, Walsey et al. (14) demonstrated that HNF4α deletion in adult hepatocytes leads to increased progression of HCC in mice by using a tamoxifen-inducible Cre recombinase. All these data suggest that HNFs, the differentiation-determining transcription factors for hepatocytes, are central regulators of hepatocarcinogenesis and highlight HNFs as potential therapeutic targets for HCC.

The forkhead box transcription factor A (FOXA, also known as HNF3) consists of FOXA1 (HNF3α), FOXA2 (HNF3β) and FOXA3 (HNF3γ). Experiments examining embryos deficient for both FOXA1 and FOXA2 genes indicated that both genes are required for hepatic specification (15). It has also been proven that FOXA2 is essential for glucose and lipid homeostasis in the liver (16,17). Early studies showed a unchanged FOXA2 expression in mouse liver tumor (18,19) and even an increase in human HCC (9). However, FOXA2 suppression was documented in several other kinds of tumors, including lung, breast and thyroid cancers (20–22). More recently, the role of FOXA2 in tumor metastasis has also been described: loss of FOXA2 expression promoted EMT through regulation of E-cadherin and promotes metastasis of pancreatic cancer (23). More interestingly, the inactivation of FOXA2 through IKKα-mediated phosphorylation contributed to inflammation-mediated liver tumorogenesis (24). FOXA1 and FOXA2 were essential for sexual dimorphic HCC in mice (25). These data indicate that FOXA2 functions as a suppressor in HCC. Nevertheless, the expression levels of FOXA2 in human HCC have not been evaluated and the role of FOXA2 in HCC metastasis remains obscure.

In this study, we explored the expression of FOXA2 in human HCCs and its clinical significance. The role of FOXA2 in HCC metastasis and the potential mechanism were also addressed.

Materials and methods

Tissues

The human tissue specimens including HCC tissue, non-cancerous adjacent tissue and portal vein tumor thrombus (PVTT) were obtained from HCC patients undergoing curative resection in Eastern Hepatobiliary Surgery Hospital (Shanghai, China) with written informed consent. They were snap-frozen in liquid nitrogen and stored at −80°C for later RNA and protein extraction. The procedure of human sample collection was approved by the China Ethical Review Committee.

Cell culture

The human HCC cell lines including HepG2, Huh7, MHCC-LM3, MHCC-L, MHCC-H and Focus were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), where the cells were properly stored and routinely authenticated (including isoenzyme analysis, DNA fingerprinting, mycoplasma detection and cell viability analysis). After resuspension in our lab, all the cells were used no longer than 6 months. All these HCC cell lines have been used and identified widely (20–29). All cells except HepG2 were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS; Hyclone). HepG2 was cultured in modified Eagle’s medium containing 10% fetal bovine serum.

Abbreviations: EMT, epithelial–mesenchymal transition; FOXA2, forkhead box transcription factor A2; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; HNF, hepatocyte nuclear factor; Luc, luciferase; MMP, matrix metalloproteinase; mRNA, messenger RNA; PVTT, portal vein tumor thrombus; siRNA, small interfering RNA.

*These authors contributed equally to this work.
Molecular cloning and lentivirus production

The complementary DNA of the FOXA2 gene was cloned into the pSin-EF2-puro plasmid (Addgene) between the EcoRI and BamHI restriction sites. In addition, pSin-EF2-GFP-puro, a plasmid expressing green fluorescent protein (GFP), was constructed by subcloning the sequence of GFP into the EcoRI and BamHI sites of pSin-EF2-puro plasmid. For FOXA2 short hairpin RNA (shRNA) oligonucleotides encoding FOXA2 shRNA (CCCGA AGAACATGT CGTGCAGTGT CTCGA GGACGTAC GACAGCATGTCTTGTTCCTT) were inserted into the AgeI and EcoRI restriction sites of the pLKO.1 plasmid (Addgene). Constructed pSin-EF2-puro or pLKO.1 plasmids were then transfected into subconfluent HEK 293T cells together with packaging plasmid psPAX2 (Addgene) and envelope plasmid pMD2.G (Addgene) using FuGENE® 6 transfection reagent (Roche). The medium containing lentiviruses was collected 48 h later. The supernatant was centrifuged at 3000 rpm for 5 min at 4°C to remove cell debris and was passed through a 0.45 μm filter (Millipore). Filtered viruses were used directly or stored in cryovials at −80°C until use.

siRNA and cell transfection

Synthetic FOXA2-specific small interfering RNA (siRNA), matrix metalloproteinase (MMP)-9-specific siRNA and the control siRNA were purchased from GenePharma. For all the siRNA transfection experiments, Lipofectamine 2000 (Life Technologies) was used as a transfection mediator according to the manufacturer’s instructions. All siRNA sequences are provided in Supplementary Table S1, available at Carcinogenesis Online.

Real-time PCR

Total RNA was isolated according to the standard TRIZOL (Takara) method. First-strand complementary DNA was synthesized from 1 μg of total RNA using PrimeScript™ RT Master Mix (Takara). Real-time PCR was performed in an ABI StepOne Real-time PCR Detection system (Life Technologies) using SYBR Green (Takara). Primer sequences are given in Supplementary Table S2, available at Carcinogenesis Online.

Western blot analysis

Cells were harvested by scraping, washed in ice-cold phosphate-buffered saline and collected with RIPA buffer (Beyotime) containing a protease inhibitor cocktail (Roche), separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Millipore). The membrane was probed with FOXA2 (Cell Signaling Technology), MMP-9 (Cell Signaling Technology) or GAPDH (Biorad) antibody overnight at 4°C, followed by incubation with an goat-anti-mouse or goat-anti-rabbit secondary antibody IRDye 700 or IRDye 800 (LI-COR). Detection was performed using an Odyssey Imaging system (LI-COR).

Transwell migration and invasion assays

Cell migration and invasion assays were performed by using transwell chambers (BD Bioscience) with or without Matrigel according to the vendor’s instructions. Cells were plated into the well in serum-free medium. Medium containing 10% fetal bovine serum was added to the lower chamber. After a 24 or 48 h incubation at 37°C, the cells remaining in the upper chamber or on the upper membrane were removed with a cotton swab. Cells that migrated or invaded to the lower surface of the membrane were fixed with a solution containing 0.1% crystal violet and 20% methanol. Cells on the lower surface of the membrane were photographed and five fields of cells were counted to estimate cell density. The mean was calculated and data were presented as mean ± SD from three independent experiments performed in triplicate.

HCC metastatic mouse models

Six-week-old male NOD/SCID mice (30) were purchased from Shanghai Experimental Animal Center and housed under pathogen-free conditions. Procedures in this experiment were approved by the Institutional Animal Care and Use Committee at the Second Military Medical University. Focus cells were labeled with luciferase gene (Luc) by lentivirus infection. In this study, two distinct HCC metastatic mouse models were established. For one model, 2 × 10^5 Focus-Luc cells infected with lentivirus-FOXA2 or lentivirus-GFP were inoculated subcutaneously into the left armpit (five in each group). To ensure enough time for the tumor metastasis, mice were kept for 6 weeks until obvious tumors in the hypodermal tissue could be observed in both groups. For another model, 2 × 10^5 Focus-Luc cells infected with lentivirus-FOXA2 or lentivirus-GFP were injected through the tail vein (five in each group) and kept for 6 weeks. Mice were injected with 3 mg luciferin intraperitoneally under anesthesia and then organs including brain, lung, bone, liver, spleen and kidney were removed from the body quickly after the mice were killed. Metastasis nodules were monitored by the NightOWL LB 983 in vivo imaging system (Berthold Technologies).

Chromatin immunoprecipitation assay

After infection with FOXA2 lentivirus for 72 h, Focus cells were cross-linked with 1% formaldehyde at room temperature for 10 min. Cell pellets were suspended in lysis buffer with protease inhibitors (Roche). Chromatin was then sheared by sonicating on ice. Chromatin complexes were immunoprecipitated with anti-FOXA2 antibody or normal rabbit IgG (as negative control). The precipitated DNA was purified and then subjected to PCR analysis.

Reporter gene plasmid construct and luciferase assay

Two pGL3-promoter plasmids containing different predicted binding sites of FOXA2 on the MMP-9 gene were constructed. To perform the luciferase assay, 1 × 10^5 Focus cells infected with lentivirus-FOXA2 or lentivirus-GFP were transfected with 24-well plates and cultured for 24 h. Then, the reporter plasmid (0.8 μg) or empty vector (0.8 μg) was cotransfected with pRL-CMV plasmid (0.08 μg) with Lipofectamine 2000 transfection reagent (Life Technologies). The Dual-Glo Luciferase Assay System (Promega) was used according to the manufacturer’s instructions. Luminescence was measured using a Synergy™ 4 Hybrid Microplate Reader (BioTek). The relative luciferase activity was calculated by normalizing the Renilla luciferase signal to that of firefly luciferase. Each experiment was performed in triplicates and repeated three times.

Statistical analysis

All data are presented as mean ± SD. In this study, unpaired Student’s t-test and χ² test were applied for calculating statistical probability. A value of P < 0.05 was considered statistically significant and P < 0.01 was considered very significant. Statistical calculation was performed using the Statistical Program for Social Sciences software (SPSS, IBM). For all statistics, data from at least three independent samples or repeated experiments were used.

Results

Downregulation of FOXA2 correlates with metastasis of HCC

To clarify the underlying role of FOXA2 in HCC progression, we first examined the expression of FOXA2 in 72 paired human HCC specimens by real-time PCR. As shown in Figure 1A, the expression of FOXA2 was decreased in 68.1% (49/72) of the HCC tissues relative to paired non-cancerous adjacent tissues. The analysis of FOXA2 expression and clinicopathologic characteristics in HCC tissues indicated that the downregulation of FOXA2 correlated strongly with aggressive characteristics including venous invasion, poor differentiation and high tumor node metastasis grade (Table 1). Moreover, the messenger RNA (mRNA) level of FOXA2 was significantly lower in HCC with venous invasion group (labeled with HCC-VI) than that in non-venous invasion group (labeled with HCC) (Figure 1B). Consistently, western blot analysis showed the protein levels of FOXA2 were also decreased in HCC tissues with venous invasion (Figure 1C). The decrease of FOXA2 expression in tumor tissues was even more obvious in the HCC-VI group (91.4%) than that in HCC group (46.0%) when compared with respective adjacent normal tissues (Supplementary Figure S1A and B, available at Carcinogenesis Online).

Since portal vein metastasis is the most common type of HCC metastasis and PVTT always predicts poor prognosis of HCC, we further examined the expression of FOXA2 in PVTT, tumor and non-cancerous tissues by western blot. The results showed that FOXA2 expression level was even lower in PVTT tissues compared with primary tumor and non-cancerous tissues (Figure 1D). Taken together, these data suggested that FOXA2 was downregulated during HCC progression.

FOXA2 is a potent inhibitor of EMT in HCC cells

To explore the effect of FOXA2 on HCC metastasis, we first determined the expression of FOXA2 and EMT markers in a variety of human HCC cell lines by real-time PCR and western blot analysis. The result revealed that the expression level of FOXA2 was correlated with E-cadherin in hepatoma cells. The level of FOXA2 was significantly higher in epithelial-like HCC cells, including HepG2 and Huh7, in comparison with that in mesenchymal-like HCC cells including MHCC-LM3 and Focus (Figure 2A and B). We then investigated the effect of FOXA2 on EMT by upregulating FOXA2 expression in Focus cells and knockdown of FOXA2 in HepG2 cells. As expected, overexpression of FOXA2 significantly increased E-cadherin level, inhibited Vimentin expression and resulted in striking morphological changes.

Figure 1D
FOXA2 is downregulated in the progression of HCC. (A) Expression levels of FOXA2 mRNA in tumor tissue and paired non-cancerous tissue from 72 HCC patients were detected by quantitative real-time PCR. (B) Real-time PCR analysis for FOXA2 mRNA expression was performed in HCC tissues with or without venous invasion (n = 35 and n = 37, respectively). (C) Western blot showing FOXA2 protein expression in randomly selected HCC tissues with or without venous invasion. HCC, hepatocellular carcinoma without venous invasion; HCC-VI, hepatocellular carcinoma tissues with venous invasion. (D) Western blot showing the expression of FOXA2 protein in tumor tissue (T), paired non-cancerous tissue (N) and portal vein tumor thrombus (P) from four HCC patients. Data represent mean ± SD, ***P < 0.001.

Table I. Relationship between FOXA2 expression and clinicopathologic features of HCC patients

<table>
<thead>
<tr>
<th>Features</th>
<th>Relative FOXA2 expression</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤55</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>&gt;55</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>AFP (µg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤100</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>&gt;100</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>HBsAg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>33</td>
<td>35</td>
</tr>
<tr>
<td>Negative</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>No</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤5</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>&gt;5</td>
<td>33</td>
<td>31</td>
</tr>
<tr>
<td>Tumor multiplicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>No</td>
<td>26</td>
<td>30</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>III–IV</td>
<td>34</td>
<td>28</td>
</tr>
<tr>
<td>Capsular invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>27</td>
<td>21</td>
</tr>
<tr>
<td>No</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>Venous invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td>No</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Tumor staging (TNM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>III–IV</td>
<td>23</td>
<td>10</td>
</tr>
</tbody>
</table>

The median value of all 72 HCC samples was chosen as the cut-off point. AFP, α-fetoprotein; HBsAg, hepatitis B surface antigen; TNM, tumor node metastasis. *χ2 test.

change in Focus cells (Figure 2C–E). In contrast, the knockdown of FOXA2 promoted EMT in HepG2 cells (Figure 2F–H).

FOXA2 suppresses migration and invasion of HCC in vitro

Next, we examined the role of FOXA2 in the migration and invasion of HCC cells. The migratory ability of HCC cells with high FOXA2 expression levels was weaker than that with low FOXA2 expression (Figure 3A and B). We also evaluated the effect of FOXA2 overexpression or knockdown on HCC cell migration and invasion ability. In Focus cells and MHCCCLM3 cells, which both have strong metastasis capabilities, lenti-FOXA2 caused decreased migration and invasion compared with that of the control cells (Figure 3C and Supplementary Figure S2A, available at Carcinogenesis Online). In contrast, the knockdown of FOXA2 in HepG2 and Huh7, which have very weak metastatic capabilities, resulted in increased migration and invasion (Figure 3D and Supplementary Figure S2B, available at Carcinogenesis Online).

FOXA2 inhibits the metastasis of HCC in vivo

To further assess the effect of FOXA2 on the HCC metastasis in vivo, two different HCC metastasis models were established in NOD/SCID mice. In the first model, metastasis nodules were evaluated after obvious subcutaneous tumors were formed. Even though the growth of nodules from Focus-Luc cells infected with FOXA2 lentivirus was slower than the control group, the mean volume of tumors in the two groups did not differ significantly at the endpoint (2.36 ± 2.40 versus 3.90 ± 2.52 cm³, P = 0.403, Supplementary Figure S3, available at Carcinogenesis Online). However, overexpression of FOXA2 inhibited metastasis of Focus-Luc cells to bone and brain, as indicated by the luciferase signals (Figure 3E). The bone and brain metastasis rate of the GFP group was 100% (5/5) and 20% (1/5), respectively, whereas that of FOXA2 group was 20% (1/5) and 0% (0/5), respectively (Supplementary Figure S4A, available at Carcinogenesis Online). In the second model, Focus-Luc cells infected by injection through the tail vein with lenti-FOXA2 or lenti-GFP were forced to undergo metastasis. FOXA2 also inhibited Focus-Luc cells to form metastasis nodules in the lung (Figure 3F). The lung metastasis rate of the GFP group was 60% (3/5), whereas no metastasis (0/5) was detected in the FOXA2 group (Supplementary Figure S4B, available at Carcinogenesis Online).

FOXA2 represses the expression of MMP-9 transcriptionally

Since MMPs are known to be initial factors for tumor metastasis (31), we examined the levels of MMPs in HCC cells with overexpression
FOXA2 suppresses metastasis of HCC

or knockdown of FOXA2 (Supplementary Figure S5, available at Carcinogenesis Online). Interestingly, only MMP-9 expression was significantly decreased in Focus cells infected with lenti-FOXA2 and was increased in HepG2 cells treated with shFOXA2 lentivirus (Figure 4A and B). To explore the potential mechanism of FOXA2-mediated regulation on MMP-9, we used JASPAR (32) to analyze the FOXA2-binding site in the region of the MMP-9 gene from −1000 to 3000 bp relative to the transcription start site. Six putative FOXA2-binding sites were predicted (Figure 4C and Supplementary Table S3, available at Carcinogenesis Online). The effect of FOXA2 on MMP-9 transcription was then examined with reporter assay using pGL3-promoter plasmids containing putative FOXA2-binding sites. Overexpression of FOXA2 reduced the luciferase activity of pGL3-B (1–988 bp) plasmid in Focus cells, whereas no effect of FOXA2 was found in the pGL3-A (1–511 bp) plasmid (Figure 4D). This approach identified a potential FOXA2 response region between 511 and 988 bp relative to the transcription start site of MMP-9 which is mostly located in the first intron (158–958 bp). Chromatin immunoprecipitation assay confirmed the direct binding of FOXA2 in the first intron of MMP-9 (Figure 4E).

Discussion

The HNF family including HNF1, HNF3, HNF4, HNF6 and CCAAT/enhancer binding proteins is indispensable for the normal architecture and function of the liver (6). It is now well established that dysregulation of HNFs also plays a pivotal role in liver carcinogenesis (7–9). FOXA2, a key transcription factor of the HNF family, is associated with 43.5% of genes expressed in the adult liver (15,33). The roles of FOXA2 in inflammation-mediated liver tumorigenesis and sexual dimorphism of HCC have been characterized recently (24,25). However, there has been no previous study examining the role of FOXA2 in HCC metastasis. With this study, we demonstrated that FOXA2 expression was decreased in most of human HCC tissues. Moreover, clinicopathological analysis revealed that downregulation of FOXA2 was significantly associated with the HCC vascular invasion potential. More intriguingly, based on the result of the transwell assay and HCC metastasis models in mice, we demonstrated that overexpression of FOXA2 could inhibit the metastasis of HCC both in vitro and in vivo. These data clearly suggest that suppression of FOXA2 not only involves in hepatocarcinogenesis but also is associated with HCC progression.

Previous studies have shown that epigenetic silencing is a key mechanism in gene repression and the consequent tumorigenesis

Fig. 2. FOXA2 inhibits EMT in HCC cells. (A and B) The expression of FOXA2, E-cadherin and Vimentin in HCC cell lines were detected by real-time PCR and western blot. The effect of FOXA2 overexpression in Focus cells and knockdown in HepG2 cells on EMT were examined by observing the morphology changes (C and F) and measuring the expression levels of E-cadherin and Vimentin by real-time PCR (D and G) and western blot (E and H).
Fig. 3. FOXA2 inhibits HCC metastasis in vitro and in vivo. (A and B) The migration ability of HCC cell lines (Huh7, HepG2, MHCC-97L, MHCC-97H, MHCC-LM3 and Focus) was assessed in transwell assays without Matrigel and the FOXA2 expression in the six HCC cells lines was examined by western blot. (C and D) The effects of overexpression and knockdown of FOXA2 on migration and invasion ability of HCC cells in vitro were examined by transwell assay. (E) Representative images of luciferase signals show the metastatic nodules in the brain and bone (red arrow, labeled with ‘Br’ and ‘Bo’) from subcutaneous tumors of each group. (F) Representative images of luciferase signals and hematoxylin and eosin staining show the metastatic nodules in the lung (red arrow, labeled with ‘L’) of the mice injected with Focus-Luc through the tail vein. Data represent mean ± SD, *P < 0.05, **P < 0.01.
Fig. 4. FOXA2 is a potent inhibitor of MMP-9 in HCC. (A) Real-time PCR and western blot analysis of FOXA2 and MMP-9 expression in Focus cells infected with lenti-GFP or lenti-FOXA2. (B) The expression of FOXA2 and MMP-9 in HepG2 cells infected with shNC or shFOXA2 lentivirus. (C) A schematic representation of the MMP-9 gene with predicted binding sites of FOXA2 (arrow) and the fragment amplified by the designed primers for ChIP-PCR. (D) The transcriptional activity of the reporter plasmids (pGL3-A and pGL3-B) containing different predicted binding regions was assessed. (E) ChIP assay confirmed the binding of FOXA2 on the first intron of the MMP-9 gene. ChIP: chromatin immunoprecipitation.

Fig. 5. Downregulation of MMP9 contributes to the antimetastasis effect of FOXA2. (A) The expression levels of FOXA2 and MMP-9 were detected in HepG2 cells transfected with NC, siFOXA2, siMMP-9 or siFOXA2 and siMMP-9 by real-time PCR. (B) The invasive properties of HepG2 cells transfected with NC, siFOXA2, siMMP-9 or siFOXA2 and siMMP-9 were analyzed in transwell assays with Matrigel. (C) Expression levels of FOXA2 and MMP-9 in subcutaneous tumor nodules in mice were detected by real-time PCR. (D) The expression of MMP-9 in the human HCC samples with low-level or high-level expression of FOXA2 was examined by real-time PCR. Data represent mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001.
(34). Akagi et al. (22) demonstrated that the expression of FOXA2 was decreased in papillary thyroid carcinoma cell lines owing to methylation of the promoter region. Treatment with the demethylating agent 5-aza-2-deoxycytidine induced its expression. In this work, we found that both of the FOXA2 mRNA and protein levels were reduced in human HCC samples. These data indicated that FOXA2 was regulated at transcription level (possibly epigenetic silence) or through mRNA processing/stability in HCC. In addition, recent studies showed that FOXA2 could also be regulated by sumoylation in prostate cancer cells (35) and by acetylation in normal liver cells (36) in a posttranslational modification-dependent manner. The role of these posttranslational modifications of FOXA2 in HCC progression remains unclear and needs further investigation.

MMPs comprise a large family of proteins, which includes >25 members classified as collagens, gelatinases, stromelysins, matrilysins and membrane-associated MMPs (37). MMPs have been regarded as prime candidates for mediating tumor progression, which degrade and modify the extracellular matrix, thus promoting the detachment of tumor cells from the surrounding tumor mass (38). Overexpression of MMPs in tumors is closely associated with poor clinical outcomes. Consistent with MMP-9 being an important member of the MMP family, enhanced expression and activation of MMP-9 in cancerous tissues have been reported in many types of metastatic tumors (39–42). Here, we show that FOXA2 can suppress the expression of MMP-9 transcriptionally. The analysis of human HCC samples showed that the expression of FOXA2 is negatively correlated with MMP-9 level. We also documented that MMP-9 reduction partially reversed the effect of FOXA2 on the hepatoma cell invasion. All together, these results indicate that the antitumor effects of FOXA2 was at least partially attributed to MMP-9 downregulation in HCC.

It is well known that EMT play a pivotal role in tumor metastasis (4). It has been proven that FOXA2 induces an epithelial phenotype and suppresses a mesenchymal fate during mouse embryo development (43). Moreover, studies showed that FOXA2 inhibits EMT through regulation of EMT markers in tumor progression (23,44). In the current study, we also found that the level of FOXA2 was associated with the phenotype of HCC cells. FOXA2 inhibited EMT and suppressed the metastasis of HCC cells. These data confirm that the regulation of EMT by FOXA2 also contributes to the inhibition of HCC metastasis. Mounting evidence supports the view that the MMP-9-mediated EMT process is closely associated with tumor metastasis (38,45,46). Since FOXA2 was able to inhibit MMP-9 expression, it can be speculated that FOXA2 may also inhibit EMT through suppression of MMP-9 expression.

The microenvironment of the tumor is an active participant throughout the entire process of tumorigenesis, progression and metastasis (47). MMPs, the most prominent family of extracellular proteinases, exert significant effect on the tumor microenvironment (31). In addition to their role in extracellular matrix turnover, these enzymes also work in a non-proteolytic manner and represent key players in the microenvironment to their role in extracellular matrix turnover, these enzymes also represent key players in the microenvironment.

In conclusion, the current study has proven the role of FOXA2 in the metastasis of HCC. Exogenous re-expression of FOXA2 inhibited the metastasis of HCC cells through suppression of MMP-9 in vitro and in vivo. These findings provide further evidence that FOXA2 might be a promising target for HCC treatment.

Supplementary material
Supplementary Tables S1–S3 and Figures S1–S6 can be found at http://carcin.oxfordjournals.org/

Received April 11, 2014; revised August 8, 2014; accepted August 15, 2014