Reciprocal Activation Between ATPase Inhibitory Factor 1 and NF-κB Drives Hepatocellular Carcinoma Angiogenesis and Metastasis

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Hepatocellular carcinoma (HCC) is a highly vascularized tumor with frequent extrahepatic metastasis. Active angiogenesis and metastasis are responsible for rapid recurrence and poor survival of HCC. However, the mechanisms that contribute to tumor metastasis remain unclear. Here we evaluate the effects of ATPase inhibitory factor 1 (IF1), an inhibitor of the mitochondrial H(+)-adenosine triphosphate (ATP) synthase, on HCC angiogenesis and metastasis. We found that increased expression of IF1 in human HCC predicts poor survival and disease recurrence after surgery. Patients with HCC who have large tumors, with vascular invasion and metastasis, expressed high levels of IF1. Invasive tumors overexpressing IF1 were featured by active epithelial-mesenchymal transition (EMT) and increased angiogenesis, whereas silencing IF1 expression attenuated EMT and invasion of HCC cells. Mechanistically, IF1 promoted Snai1 and vascular endothelial growth factor (VEGF) expression by way of activating nuclear factor kappa B (NF-κB) signaling, which depended on the binding of tumor necrosis factor (TNF) receptor-associated factor 1 (TRAF1) to NF-κB-inducing kinase (NIK) and the disruption of NIK association with the TRAF2-cIAP2 complex. Suppression of the NF-κB pathway interfered with IF1-mediated EMT and invasion. Chromatin immunoprecipitation assay showed that NF-κB can bind to the Snai1 promoter and trigger its transcription. IF1 was directly transcribed by NF-κB, thus forming a positive feedback signaling loop. There was a significant correlation between IF1 expression and pp65 levels in a cohort of HCC biopsies, and the combination of these two parameters was a more powerful predictor of poor prognosis. Conclusion: IF1 promotes HCC angiogenesis and metastasis by up-regulation of Snai1 and VEGF transcription, thereby providing new insight into HCC progression and IF1 function. (HEPATOLOGY 2014;60:1659-1673)
mechanisms responsible for HCC progression and metastasis is far from being clear at present. It is believed that the elucidation of molecular mechanisms underlying HCC progression and metastasis is important for the development of novel treatments and a possible cure. The primary cause of cancer development including HCC was initially attributed to genetic mutations. However, some properties of liver cancer cells cannot be explained only by mutations. And it has been accepted that various risk factors have been associated with HCC invasion and metastases.

Although the role of oxidative stress as a consequence of mtDNA mutations and/or altered mitochondrial functions has been demonstrated in carcinogenesis, a causative role of mitochondria in promoting cancer metastasis has only been demonstrated recently, which stresses the relevance of mitochondrial dysfunction as a central player of tumor metastases. The endogenous inhibitor of the ATPase, IF1, is a small, basic, heat-stable protein composed of 80-84 amino acids (∼10 kDa) in mammals and predominantly compartmentalized inside the mitochondrial matrix. IF1 has the unique capacity to inhibit the adenosine triphosphate (ATP)-hydrolyzing activity of the F1Fo-ATP synthase without affecting the synthesis of ATP during oxidative phosphorylation. At low pH (pH <6.7), IF1 becomes active as a dimer, which is critical for the inhibition of the F1Fo-ATP synthase. IF1 expression is greatly increased in a number of human cancers, and recently it has been proposed to interact with the canonical nuclear factor kappa B (NF-κB) prosurvival pathway to prime an adaptive response of cancer cells. However, the biological relevance of IF1 in the HCC field is largely unknown, and specifically little is known about how the expression of IF1 is regulated in HCC.

Numerous studies have found strong similarities between key features of inflammatory and tumor development, including stem cell activation, cell proliferation, and neoangiogenesis, and NF-κB is believed to link inflammation to cancer development and progression. Two distinct NF-κB activation pathways have been described that are referred to as the canonical and noncanonical pathways. RelA/p50 dimers represent the classical (canonical) NF-κB1 and RelB/p52 dimers the alternative (noncanonical) NF-κB2 complex. The noncanonical pathway is primarily activated by receptor activator of NF-κB ligand, B-cell activating factor, dsRNA, reactive oxygen species (ROS), and CD40 ligands. The rate-limiting step in noncanonical NF-κB pathway activation is mediated by NF-κB-inducing kinase (NIK), an upstream MAP kinase activating IkB kinase. In resting cells, NIK is rapidly degraded by cellular inhibitor of apoptosis proteins (cIAPs), which depends on its association with tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) because TRAF3 recruits NIK to the TRAF2/cIAP2 ligase complex. In contrast, in response to noncanonical stimuli, cIAP2 ubiquinates and degrades TRAF3. NIK is thereby released from negative regulation leading to its stabilization, which causes its activation by concentration-dependent oligomerization.

Recently, NIK has been demonstrated to play a crucial role in hepatocarcinogenesis. However, little is known about the effect of IF1 on NIK in HCC.

In this study we sought to elucidate the role of IF1, and addressed the mechanisms by which IF1 could participate in the biology of liver cancer cells. Remarkably, we reveal what we believe to be the novel mechanism involved in IF1-mediated angiogenesis and metastasis of HCC. We demonstrate that IF1 induces EMT and angiogenesis by up-regulation of Snai1 and...
vascular endothelial growth factor (VEGF) expression through NF-κB-mediated transcriptional regulation. Furthermore, we identify NF-κB as the first transcription factor directly controlling the expression of IF1 in HCC. These findings suggest that IF1 promotes HCC metastasis and angiogenesis through the NF-κB /Snai1 and VEGF pathway.

Materials and Methods

Antibodies and Reagents. Commercial primary antibodies against the following proteins were purchased from the sources: NIK (Abcam, ab7204), NF-κB p50 (Millipore, 06-886), NF-κB p65 (C-20) (Santa Cruz Biotechnology, sc-372), NF-κB (Sigma, N8523-2ML), ATPase inhibitory factor 1 (SE2D7) (Abcam, ab110277), vimentin (Cell Signaling, #3932), antibody (E-cadherin (Cell Signaling, #3195), β-catenin (Cell Signaling, #9587), or fibronectin (Santa Cruz Biotechnology, sc-71112), Snai1 (Abcam, ab117866), VEGF (Abcam, ab52917), β-actin (Cell Signaling Technologies, #5125), α-Tubulin (Cell Signaling Technology, #2144), TRAF1 (Abcam, ab37410), TRAF2 (Abcam, ab37118), clAP2 (Abcam, ab32059). IMD-0354 (I3159) and dimethyl sulfoxide (DMSO, D2650) were purchased from Sigma.

Coimmunoprecipitation. Cells were lysed in coimmunoprecipitation buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM CaCl₂, 2 mM MnCl₂, 1.2% Triton X-100) supplemented with protease inhibitors cocktails (Sigma-Aldrich). Lysates were centrifuged for 20 minutes at 14,000g, and the resulting supernatant was precleared by incubation with immobilized Protein A/G beads (20 μL; Santa Cruz) for 1 hour at 4°C. The precleared supernatant was subjected to overnight immunoprecipitation using the indicated antibodies or control IgG antibodies at 4°C. The next day, protein complexes were collected by incubation with 35 μL of immobilized Protein A/G beads for 1 hour at 4°C. The collected protein complexes were washed 5 times with coimmunoprecipitation buffer and eluted by boiling in protein sample buffer under reducing conditions, after which proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blot.

HCC Tissue Collection. Two independent cohorts totaling 232 HCC patients were enrolled in this study (cohort 1, n = 102; cohort 2, n = 130). Archival HCC tissues were collected from patients undergoing HCC resection from January 2006 to May 2012 at the First Affiliated Hospital of Harbin Medical University. HCC diagnosis was based on World Health Organization criteria. The clinical typing of tumors was determined according to the International Union against Cancer TNM classification system. The liver function was assessed by Child-Pugh score system. Tumor differentiation was defined according to the Edmondson grading system. Ethical approval for the use of human subjects was obtained from the Research Ethics Committee of the First Affiliated Hospital of Harbin Medical University with the informed consent of patients. Detailed characteristics of all patients were summarized in Supporting Tables 1 and 2.

For details of additional experimental materials and procedures, please see the Supplementary Materials and Methods.

Results

IF1 Expression Positively Correlates With Poor Prognosis of Patients With HCC. To determine the clinical significance of IF1 expression in patients with HCC, we analyzed samples from a cohort of 102 human patients with HCC using immunohistochemical (IHC) staining (Fig. 1A, upper panel) and western blot (Fig. 1A, lower panel) with an antibody against IF1. IF1 expression was detectable in normal liver tissue. Strong IF1 staining was observed in the cytoplasm in advanced-stage liver cancer. In contrast, weak IF1 staining was observed in early-stage liver cancer. IF1 expression positively correlated with advanced-stage tumor status, and lymph node status (Fig. 1B). Additionally, we found that IF1 mRNA expression was lower in primary liver tumors than in lung metastatic HCC (Fig. 1C). Most important, a patient who has high IF1 expression had shorter overall survival (OS) and disease-free survival (DFS) (Fig. 1D). Taken together, these results indicate that IF1 positively correlates with poor prognosis in patients with HCC.

IF1 Promotes HCC Cell Migration, Invasion, and Lung Metastasis. To explore the effects of IF1 on the invasiveness of HCC cells, we examined the expression of IF1 in a panel of HCC cell lines which exhibited increasingly invasive behavior. We found that IF1 mRNA and protein levels increased progressively from healthy liver cells to HCC cells with low metastatic potential and, finally, to HCC cells with high metastatic potential (Fig. 2A). To further examine the effect of IF1 on the motility and invasiveness of HCC cells, we transfected IF1-expressing vectors or control vectors into less invasive SMMC7221 and Huh7 cells. Overexpression of IF1 significantly increased the migratory and invasive capabilities of SMMC7221 and Huh7...
cells, as assessed by transwell migration and Matrigel invasion assays (Fig. 2B,C). In contrast, knockdown of IF1 in the highly invasive HCCLM3 and MHCC-97H cells decreased their migratory and invasive behaviors (Fig. 2B,C). Cell motility was also examined using a wound-healing assay (Supplemental Fig. 1). The results showed that IF1 significantly promotes HCC cell migration and invasiveness.

To further investigate the role of IF1 in an in vivo model, we injected stable transfected cell lines...
(SMMC7221-IF1 and HCCLM3-shRNA-IF1) through the tail vein of nude mice and detected the lung metastatic nodules. Mice injected with SMMC7221-IF1 had more and bigger lung metastases compared to those in the control group; however, HCCLM3-shRNA-IF1 cells had less and smaller lung metastases than those injected with HCCLM3-shRNA-control cells, respectively (Fig. 2D, E). Histologic analyses (Fig. 2F) of lung dissected from each mouse further confirmed lung metastases. These results demonstrate that IF1 is crucial for promotion of HCC invasion and metastasis.

**IF1 Induces EMT in HCC Cells.** EMT plays an important role in metastasis, because EMT induces tumor-associated epithelial cells to obtain mesenchymal features, which leads to reduced cell-cell contact and increased migration. Thus, we next wanted to identify how IF1 regulates the migratory and invasive phenotypes of HCC cells. In phase-contrast images, we found that the HCCLM3-shRNA-IF1 cells appeared round, with a more epithelial morphology than that of HCCLM3-shRNA-control cells (Fig. 3A). In contrast, SMMC7221-IF1 cells showed a spindle-like, mesenchymal morphology, one of the major characteristics of...
EMT (Fig. 3A). Similar results were also found in MHCC-97H and Huh7 cells (data not shown). We further investigated the expression of EMT markers using western blot analysis. Our data showed that IF1 repressed the expression of the epithelial markers E-cadherin and β-catenin, and increased the expression of mesenchymal markers (fibronectin and vimentin) in HCC cell lines, as evidenced by immunofluorescence (IF) and real-time polymerase chain reaction (PCR) (Fig. 3A-C). However, after IF1 knockdown in HCCLM3 cells, expression of epithelial markers was significantly increased and expression of mesenchymal markers was markedly decreased. These findings suggested that IF1 induced EMT in HCC cells.
**IF1 Enhances EMT Through the Transactivation of Snail Expression.** To uncover the mechanism involved in IF1-mediated enhancement of invasion and metastasis of HCC, we focus on the expression of E-cadherin, which is thought to be a repressor of invasion and metastasis, and one of the hallmarks of EMT in cancer is the down-regulation of E-cadherin.13 The Zeb1, Slug, Snail1, Twist, and SIP1 families have been found to act as oncogenic transcription factors by suppressing E-cadherin expression.14 We investigated whether IF1 promoted EMT by regulating the expression of these repressors. Real-time PCR analysis showed that IF1 markedly increased Snail1 expression, but had no significant effect on mRNA levels of Twist, Slug, Zeb1, or SIP1 (Fig. 4A). Moreover, IF1 upregulated Snail1 expression and decreased E-cadherin expression in SMMC7721 cells, whereas the inhibition of Snail1 expression using sh-Snail1 significantly decreased the loss of E-cadherin expression induced by IF1 (Fig. 4B). In contrast, knockdown of IF1 decreased Snail1 expression and increased E-cadherin expression in HCCLM3 cells, whereas overexpression of Snail1 markedly inhibited the increase in E-cadherin expression in HCCLM3-shIF1 cells. Similar results were also observed in Huh7 and MHCC-97H cells (data not shown). We further investigated the expression of E-cadherin by immunofluorescence and morphology change, as shown in Fig. 4C. Thus, Snail1 possibly is critical for IF1-induced EMT in HCC cells.

As expected, we found that the migratory and invasive capabilities of HCC cells were repressed by knockdown of Snail1 in SMMC7721-IF1 cells (Fig. 4D). In contrast, shRNA-IF1-induced migratory and invasive capabilities were restored by ectopic expression of Snail1 (Supporting Fig. 2). To determine whether these results were reproducible in metastasis in vivo, the luciferase-expressing indicated HCC cells were injected by way of the tail vein into the nude mice. Tumor metastasis was monitored by bioluminescence imaging. It was shown that tumor lung metastasis was significantly decreased by shRNA-IF1 and significantly recovered by Snail1 overexpression (Fig. 4E). In contrast, the metastatic capabilities of SMMC7721-IF1 cells were reduced by knockdown of Snail1 (Fig. 4E). Taken together, these results indicate that Snail1 is an important downstream effector of IF1-mediated promotion of HCC cell invasiveness and metastasis.

**IF1 Induces VEGF Expression and Promotes Angiogenesis In Vitro and In Vivo.** To investigate whether dysregulation of IF1 is able to affect HCC-induced angiogenesis, a xenograft tumor-induced angiogenesis model was used. Indicated HCC cells were respectively mixed with Matrigel and injected into the flanks of nude mice. We found that HCCLM3-sh-control and SMCC7221-IF1 cells profoundly induced tumor mass formation. However, knockdown of IF1 reduced tumor growth in mice (Fig. 5A, I-III; Supporting Fig. 3). On the other hand, we also quantified the level of angiogenesis by determining the hemoglobin content of the plugs, and found that reducing IF1 expression diminished HCC-induced angiogenesis in vivo (Fig. 5A, IV). Blood vessels also were stained using CD31 antibody, and random photographs were used for quantification. The results demonstrated a marked decrease of blood vessel density in IF1 knockdown tumors (Fig. 5B). Overall, these results suggest that IF1 promotes angiogenesis in vivo.

In angiogenic processes, endothelial cells must undergo migration, proliferation, and tube formation to form new blood vessels.15 We then examined whether IF1 expression induced angiogenesis by using a human umbilical vein endothelial cells (HUVECs) model in vitro. We found that the conditioned medium (CM) from transfected HCC cells altered HUVECs migration and tube formation (Fig. 5C). To elucidate whether VEGF (an important angiogenic factor) in the conditioned medium plays an important role in IF1-induced angiogenesis, the VEGF antibody was used. As shown in Fig. 5D, pretreatment of SMCC7221-IF1 cells with VEGF antibody reduced IF1-induced migration and tube formation in HUVECs. Next, we directly transfected different doses of IF1 plasmid to HCC cell lines and examined the expression of VEGF. The results showed that IF1 increased not only VEGF mRNA expression, but also led to an increase in the protein expression of VEGF (Fig. 5E) and secretion (data not shown). These data indicated that IF1-dependent VEGF expression promotes angiogenesis in human HCC.

**IF1 Exerts Dual Functions by Activating NF-κB Signaling.** The role of NF-κB in IF1-mediated phenotypes was then evaluated. First, our experiments showed that IF1 overexpression enhanced, whereas IF1 knockdown suppressed, the activity of NF-κB luciferase reporter in HCC cells (Fig. 6A), indicating that IF1 is implicated in activation of the NF-κB pathway in HCC.

Next we examined whether blockage of NF-κB could mimic the effect of IF1 knockdown. As expected, HCC cells or CM that was preincubated with NF-κB inhibitor IMD0354 resulted in down-regulation of VEGF (Fig. 5E) and displayed a decreased capacity to promote
Fig. 4. Snai1 is critical for IF1-enhanced HCC invasion and metastasis. (A) Effect of IF1 on expression of Snai1, Twist, Slug, Zeb1, and SIP1. (B) Snai1 is critical for IF1-induced reduction of E-cadherin expression. Real-time PCR and western blotting were used to detect expression of IF1, Snai1, and E-cadherin. Knockdown of Snai1 expression using LV-shSnai1 significantly attenuated the loss of E-cadherin expression induced by IF1. In contrast, up-regulation of Snai1 using LV-Snai1 markedly inhibited the increase in E-cadherin expression in HCCLM3-shIF1 cells. (C) IF staining was used to detect expression of epithelial markers (E-cadherin) and expression of mesenchymal markers (vimentin) in HCC. (D) Snai1 knockdown significantly decreased IF1-enhanced cell migration and invasion. After SMMC7721-IF1 cells were infected with the lentivirus, LV-shSnai1, migration, and invasion abilities of the cells were detected using transwell assays. (E) In vivo metastatic assay. Eight luciferase-expressing cell lines (SMMC7721-IF1 and SMMC7721-IF1 plus LV-shSnai1; HCCLM3-shIF1 and HCCLM3-shIF1 plus Snai1; and their control cells) were injected by way of the tail vein into the nude mice. Tumor metastasis was monitored by bioluminescence imaging. Bioluminescent imaging showed the presence of lung metastases in mice implanted with indicated cells (upper panel). Incidence of lung metastases observed in the different experimental groups of nude mice (lower, left panel). (Lower, right panel) Images showing representative H&E staining of lung tissue samples from the different experimental groups. Data are expressed as mean ± SD. The results are representative of three independent experiments.
Fig. 5. IF1 induces VEGF expression and promotes angiogenesis in vitro and in vivo. (A) Indicated stable HCC cell lines were injected subcutaneously into flanks of nude mice (n = 9/group). Excised tumors derived from each cell line (I). Tumor volume (II) and tumor weight (III) were measured at the experiment endpoint. (IV) Hemoglobin content of tumors from groups of mice was quantified by using QuantiChrom Hemoglobin Assay Kit. (B) Tumor microvessel density was assessed by IHC staining for the endothelial cell marker CD31 (upper panel), and the number of blood vessels per field was quantified (lower panel). (C) The effect of IF1 on the capillary-like tube formation of HUVECs. HUVECs (1.50 × 10^5 cells/well) were seeded onto Matrigel-coated 24-well plates and incubated for 16 hours. Representative appearance of HUVECs tube formation (upper panel). The tube formation was quantified by counting the number of branch points of the capillary network (middle panel). HUVECs migration assay also was done as described above (lower panel). (D) Conditioned media from indicated HCC cells induced migration of endothelial cells and tube formation. (E) The mRNA and protein levels of VEGF in indicated HCC cells were analyzed by qRT-PCR and western blot. Data are expressed as mean ± SD. The results are representative of three independent experiments.
Fig. 6. IF1 activates the transcription factor NF-κB to promote EMT in HCC by way of a Snai1-dependent mechanism. (A) HCC cells expressing control (scrambled) shRNA, NIK-shRNA, NIK, IF1, or IF1-shRNA were additionally transfected with NF-κB-luciferase and renilla luciferase reporters. Sixteen hours after transfection, reporter gene luciferase assays were performed. (B) Schematic diagram of the Snai1 proximal promoter. The nucleotide positions and sequences of the putative NF-κB binding site located −444/−435 bp upstream of the transcriptional start site in the Snai1 promoter. (C) ChIP assays in tumor tissues produced from clinical samples from patients with HCC (P#43 and P#72) and HCC cell lines (MHCC-97H and HCCLM3). PCR was performed with primers specific for binding site and the negative site (nonbinding site of NF-κB). (D) HCC cells were transfected as described above, and proteins were immunoprecipitated with indicated antibodies. Interacting NIK was measured by western blot. (E,F) Migration and invasion in indicated cells were assayed as described above. Data are expressed as mean ± SD. The results are representative of three independent experiments.
tube formation of HUVECs (Fig. 5D). Also, HCCLM3 cells treated with this inhibitor display less invasive activity (Supporting Fig. 4A,B); these phenocopied those of IF1 knockdown.

Having documented that Snai1 mediated IF1-induced EMT, we further elucidated the mechanism by which Snai1 expression was regulated in HCC. Bioinformatics analysis demonstrated the presence of one candidate NF-κB binding site in the Snai1 promoter located −444/−435 bp upstream of the transcriptional start site (Fig. 6B). Chromatin immunoprecipitation (ChIP) experiments were thus performed with tumor samples from clinical HCC tissues (P#43 and P#72) and HCC cell lines to determine whether NF-κB binds to this site. As shown in Fig. 6C, NF-κB bound to the site in the Snai1 promoter. Overexpression of NF-κB up-regulated Snai1 in SMCC7722 cells at both the mRNA and protein levels compared with control (Supporting Fig. 4C). However, decreased NF-κB levels resulted in the down-regulation of Snai1 expression in HCCLM3 cells at both the mRNA and protein levels compared with control (Supporting Fig. 4D). These data strongly suggest that NF-κB directly up-regulates Snai1 expression in HCC and define Snai1 as a direct target of the NF-κB transcription factor in HCC cells.

Third, we sought to determine how IF1 activates the NF-κB pathway in HCC. Our data showed that IF1 overexpression in HCC cells increases the binding of TRAF1 to NIK and stabilizes it by preventing its interaction with the TRAF2-cIAP2 complex (Fig. 6D). By contrast, IF1 knockdown in HCC cells decreases the binding of TRAF1 to NIK and degrades it by promoting its interaction with the TRAF2-cIAP2 complex. Moreover, siRNA-mediated silencing or overexpression of NIK in HCC cells demonstrates the requirement for NIK in the noncanonical pathway activation and IF1-induced invasion and angiogenesis in HCC (Fig. 6A,E,F; Supporting Fig. 5).

Additionally, we also investigated whether IF1 affected metabolic changes in HCC. As shown in Supporting Fig. 6A, a significant decline in oxygen consumption rate (OCR) was observed in IF1 overexpression cells. Moreover, overexpression of IF1 in HCC cells that express low levels of IF1 triggered the up-regulation of aerobic glycolysis and an increase of lactate production, which was accompanied by a marked increase in HIF-1α protein levels (Supporting Fig. 6B). Conversely, IF1 knockdown in cells that express high levels of IF1 promoted the down-regulation of aerobic glycolysis and a decrease in lactate production and HIF-1α protein levels (Supporting Fig. 6B). Together, these findings indicated that the deregulated expression of IF1 in HCC controlled the activity of oxidative phosphorylation mediating the shift of cancer cells to an enhanced aerobic glycolysis.

**IF1 Is a Direct Transcriptional Target of NF-κB.** Next we determined what factors act upstream of IF1 in HCC. To test the effect of NF-κB on IF1, we treated HCCLM3 and MHCC-97H cells with NF-κB inhibitor IMD0354 and examined the expression level of IF1. Interestingly, suppression of NF-κB function decreased IF1 protein and mRNA levels (Fig. 7A). Luciferase reporter carrying IF1 promoter sequence was activated in HCCLM3 and MHCC-97H cells that expressed endogenous NF-κB, whereas suppression of NF-κB function by IMD0354 decreased the luciferase signal (Fig. 7B).

Based on these results, the above experiments were repeated with HCC cell lines stably expressing the IκBz-SR. Unlike control cells, IF1 induction was blocked in IκBz-SR cells (Supporting Fig. 7A-C), which further supports the notion that NF-κB can induce IF1 expression in HCC cells.

We then analyzed the IF1 promoter sequence by the PROMO algorithm and found one putative NF-κB binding site in the region −626 to −616 upstream of the transcription starting site (Fig. 7C). We hypothesized that NF-κB may directly bind to the IF1 promoter at this candidate site. To this end, we expressed recombinant NF-κB subunit p65 protein and mutated the three key nucleotides in candidate site as mutant probe. We employed an electrophoretic mobility shift assay (EMSA) to examine their binding capabilities. As we expected, purified NF-κB subunit p65 protein bound to wild-type probe containing the candidate NF-κB binding sequence (Fig. 7D). However, mutant probe has no binding capacity to NF-κB.

We also performed a ChIP assay to test the binding of NF-κB to IF1 promoter in vivo. Consistently, the promoter region of IF1 was amplifiable from the DNA recovered from the immunoprecipitation complex using a specific antibody for NF-κB (Fig. 7E, left panel). In addition, ChIP assay also suggested that IF1 promoter is bound to p65 but not to p50 (Fig. 7E, right panel).

To further examine whether the candidate binding sequences are the key sequences of NF-κB binding to the IF1 promoter, we examined the effect of NF-κB on transcriptional activity of mutated type IF1 promoters mP(−1,500/+49) and mP(−700/+49). As demonstrated in Fig. 7F, the activation ability of mutated IF1 promoters by NF-κB was markedly
Fig. 7. NF-κB directly binds to and activates IF1 promoter. (A) HCCLM3 and MHCC-97H cells were treated with the NF-κB inhibitor IMD0354 or DMSO as control, and the protein levels of IF1 were examined by western blot. The β-actin protein was included as a loading control (lower panel). The mRNA level of IF1 was also quantified by reverse-transcription quantitative PCR. The 18S rRNA level was used for normalization (upper panel). (B) Luciferase reporter assay carrying IF1 promoter sequence was transfected in SMMC7721 and Huh7 cells that expressed endogenous NF-κB. The IMD0354 compound was used to inhibit NF-κB, and the luciferase activity was quantified as the ratio of firefly/renilla. Bar plots indicate mean with 95% confidence interval. (C) The nucleotide sequences of the 5′-flanking region (−21,000/−149) of the IF1 gene. The candidate NF-κB binding site is boxed. (D) DNA binding activity assay of purified NF-κB subunit p65 protein to 30-bp annealed oligonucleotide probes encompassing the NF-κB binding motifs in the IF1 promoter and its mutant was detected by EMSA. Arrow indicates specific band of purified NF-κB protein binding to the oligonucleotides. (E) ChIP assay showing the binding of NF-κB to IF1 promoter in vivo. The promoter region of IF1 was amplified from the DNA recovered from the immunoprecipitation complex using a specific antibody for NF-κB. The input DNA and ChIP yield using nonspecific immunoglobulin G (IgG) are included as controls (left panel). ChIP assay using antibodies for p50 and p65 subunits of NF-κB. The promoter of IF1 was amplified from ChIP product of p65 (lane 5) but not of p50 (lane 6). The input amounts of DNA are shown in lanes 1-3. The ChIP assay using nonspecific IgG is included as a negative control (lane 4, right panel). (F) The effect of NF-κB protein on transcriptional activity of mutated IF1 promoters. Activity of IF1 promoters [P(−1,500/+49), P(−700/+49)], and their mutated types [mP(−1,500/+49), mP(−700/+49)] was measured by luciferase reporter assays. Data are expressed as mean ± SD, The results are representative of three independent experiments.
reduced compared with that of wild-type promoters. Taken together, these data suggest that NF-κB can directly bind to the IF1 promoter, and the key sequences of binding site are GGGGTTCCTCC.

**Combination of IF1 and pp65 Levels Have Better Prognostic Value for HCC.** Given the reciprocal relationship between IF1 and NF-κB described above, we further analyzed the expression levels of IF1 and pp65 in clinical HCC samples. IHC analysis of 130 patient specimens revealed a strong correlation of IF1 expression with pp65 levels \( r = 0.5316, P < 0.0001 \) (Fig. 8A,B). Moreover, patients whose tumors expressed above-average levels of IF1 or pp65 exhibited a significantly decreased trend in any of the prognostic indicators, including time to DFS and OS due to HCC-related death (Fig. 8C,D). For patients whose tumors had above-average levels of both IF1 and pp65, adverse outcomes were exacerbated. Using the combination of these two parameters increased the prognostic value, as compared to IF1 or pp65 overexpression alone.

**Discussion**

In this study we determined the significance and underlying mechanism for IF1 overexpression in HCC cell lines and clinical samples. The IF1 content was low in normal hepatocytes, increased in noninvasive and primary HCC cells, and reached the highest level in invasive HCC cells. This progressively increased expression profile paralleled deterioration of the disease, indicating that IF1 could be used as a potential marker for HCC. The specific inhibition of IF1 expression in vivo reduced HCC angiogenesis and metastasis, indicating that IF1 could be used as a therapeutic target. Moreover, IF1 was found to be transcribed directly by NF-κB in HCC, indicating that IF1-NF-κB exerted a positive feed-forward regulatory loop which reinforces,
stabilizes, and has sustained effects on the IF1-regulated oncogenic program.

Although IF1 has recently been shown to play a crucial role in carcinogenesis, our study also raises many critically new questions. What is the biological relevance of IF1 in the HCC field? What are the IF1-mediated signaling networks in HCC? How does IF1 activate these signaling networks? Finally, how is IF1 expression regulated in HCC?

Previous findings support that the mitochondrial content of IF1 controls the activity of oxidative phosphorylation mediating the shift of cancer cells to an enhanced aerobic glycolysis, thus supporting an oncogenic role for the deregulated expression of IF1 in cancer. Here we uncovered a new function of IF1 in HCC. The effect of IF1 on tumor invasion and metastasis was directly demonstrated in our in vitro and in vivo studies. Both subcutaneous and orthotopic xenografts, and a model established by the tail vein injection, IF1 overexpression generated larger primary tumors, more lung metastasis foci, and angiogenesis. Moreover, IF1 knockdown led to severe suppression of angiogenesis and lung metastasis of HCC in mice. To our knowledge, this is the first report that IF1 expression is critical for HCC metastasis and angiogenesis, in addition to tumor proliferation.

Additionally, we found that Snai1 is critically involved in IF1-driven EMT. Moreover, IF1 modulated NF-κB hyperactivation correlated with Snai1 up-regulation. Thus, our data suggest a requirement for NF-κB in IF1-driven EMT. An increase in NF-κB signal is a key tumor survival mechanism, and promotes tumor metastatic processes including EMT, resistance to apoptosis, and angiogenesis. Previous studies have demonstrated that activated NF-κB plays a crucial role in lung metastasis in an orthotopic implantation model of HCC. Our current in vitro and in vivo studies suggest that NF-κB/Snai1 axis is responsible for IF1-mediated metastasis. We also observed a role of NF-κB in IF1-regulated VEGF expression, consistent with previous reports that NF-κB can up-regulate VEGF and Snai1. Taken together, this study clearly demonstrates a crucial role for IF1 in induction of EMT and angiogenesis through regulation of Snai1 and VEGF expression in HCC.

Furthermore, we found that IF1 overexpression promotes the binding of TRAF1 to NIK, and disrupts the interaction between NIK and TRAF2-cIAP2 complex, resulting in NIK stabilization, NF-κB activation, and an increase of Snai1 and VEGF activity in HCC cells, which was supported by a recent report that IF1 positively regulates the ROS/NF-κB pathway for activation of AKT. Given complex NF-κB pathways, whether other upstream regulators are involved in IF1 signal remains to be further determined. Remarkably, the predictive range of IF1 expression levels combined with pp65 signal was more sensitive than that of IF1 alone for OS and cumulative recurrence, strongly suggesting that the concerted activities of IF1 and pp65 detected in our experiments are recapitulated in clinical patients with HCC. Identification of tumor IF1 alone or combined evaluation of IF1/pp65 levels as a new prognostic marker in patients with HCC is important because they provide not only a new criterion for prognosis, but also a potential therapeutic target. In conclusion, evaluation of both IF1 expression and pp65 signal is a powerful predictor of poor prognosis, further supporting a model of IF1 activation of NF-κB/Snai1 and VEGF signaling axis and NF-κB positive feedback of IF1, resulting in EMT occurrence and angiogenesis, thus metastases of HCC cells (Fig. 8E).

Much less is known about the pathways and transcription factors that regulate IF1 expression in the liver. Several studies have shown that NF-κB is up-regulated in a large proportion of human HCCs. Thus, we analyzed and cloned the IF1 regulatory region and characterized its transcriptional regulation. We found that p65/NF-κB is a direct transcriptional regulator of IF1 expression in HCC. Together, NF-κB is the first transcription factor identified for IF1 so far, and these findings define a novel cellular mechanism regulated by IF1, which controls the angiogenesis and EMT phenotype in HCC.

In summary, we have identified IF1 as a key regulator that controls multiple facets essential for HCC development and metastasis. In particular, our investigation identifies p65/NF-κB as a direct transcriptional regulator of IF1 expression and link IF1 up-regulation with the activation of the NF-κB, Snai1, and VEGF expression, metastasis, and angiogenesis in HCC. Thus, the data led us to propose that IF1 or a combination of IF1 with pp65 is a novel marker in the prognosis of HCC and a potential therapeutic target. Because IF1 is also overexpressed in other types of cancers, including lung, colon, ovarian carcinoma, and breast cancer, we believe that this oncoprotein may be widely involved in tumorigenesis and progression in human cancers.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website.