Ubiquitin-like Protein FAT10 Promotes the Invasion and Metastasis of Hepatocellular Carcinoma by Modifying β-Catenin Degradation

Rongfa Yuan1,2, Kai Wang1,2, Junwen Hu1, Chen Yan1, Ming Li1, Xin Yu1,2, Xiuxia Liu1,3, Jun Lei1,2, Wuhua Guo4, Linquan Wu1,2, Kui Hong3, and Jianghua Shao1,2,3

Abstract

The ubiquitin-like protein FAT10 and the homeobox protein HOXB9 each promote metastatic progression in hepatocellular carcinoma (HCC). In this study, we investigated the clinicopathologic significance of FAT10 and HOXB9 in HCC and investigated a mechanistic role for FAT10 in HOXB9-mediated invasiveness and metastasis. Relative to adjacent normal tissues, FAT10 and HOXB9 were markedly overexpressed in HCC, where a positive correlation in their expression and associated malignant characteristics were found. RNAi-mediated silencing of FAT10 decreased HOXB9 expression and inhibited HCC invasion and metastasis in vitro and in vivo. The effects of FAT10 silencing were reversed by HOXB9 overexpression, whereas RNAi-mediated silencing of HOXB9 decreased HCC invasion and metastasis driven by FAT10 overexpression. Mechanistically, FAT10 regulated HOXB9 expression by modulating the β-catenin/TCF4 pathway, directly binding to β-catenin and preventing its ubiquitination and degradation. Together, our results identified a novel HCC regulatory circuit involving FAT10, β-catenin/TCF4, and HOXB9, the dysfunction of which drives invasive and metastatic character in HCC.

Tumor and Stem Cell Biology

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies and leading causes of death worldwide (1). Although some recent progress has been made with regard to the clinical detection of and treatment methods for HCC, the metastasis and recurrence rates after radical resection of HCC remain high (2, 3). The elucidation of the mechanisms involved in HCC progression and metastasis is important for the development of HCC therapeutic agents.

HLA-F locus adjacent transcript 10 (FAT10) is a member of the ubiquitin-like protein family (Ubl), which covalently modifies targets substrates by binding via their C-termini containing conserved Gly–Gly motifs (4, 5). FAT10 has been implicated in cell growth and survival; the deregulation of FAT10 could induce abnormal alterations in the cell cycle, apoptosis, and immune response (5–7). Recently, there have been an increasing number of studies on FAT10 in malignant tumors. The FAT10 gene is overexpressed in various cancers, such as gastrointestinal cancer, HCC, pancreatic ductal adenocarcinoma, and human glioma (8–12). In the liver, FAT10 overexpression is an epigenetic marker for liver neoplasia (13). In addition, FAT10 overexpression in HCC tissues is an independent poor prognostic factor in patients with HCC (14). These studies have suggested that FAT10 may play an important role in the tumorigenesis and progression of HCC. However, its specific mechanism of action in the process of HCC invasion and metastasis is still unclear.

Homeobox B9 (HOXB9), a member of the class I homeobox (HOX) genes, is critical for embryonic segmentation and limb patterning (15). Recently, emerging evidence links the biologic function of HOXB9 to tumor metastasis. For example, the overexpression of HOXB9 in breast cancer could increase angiogenesis and distal metastasis, and the knockdown of HOXB9 significantly decreased the ability of lung cancer cells to form bone and brain metastases (16, 17). Previously, we used cDNA microarray analysis to determine that the knockdown of FAT10 could inhibit HOXB9 expression in HCC cells (4,912-fold; see Supplementary Table S1). Thus, we presumed that FAT10 may play an important role in the tumorigenesis and progression of HCC. Furthermore, its specific mechanism of action in the process of HCC invasion and metastasis is still unclear.
Figure 1. FAT10 overexpression correlates with HOXB9 expression in human HCCs. A, qRT-PCR analysis of FAT10 and HOXB9 mRNA expression in 102 HCC tumors and peritumoral liver tissues (**, P < 0.01; paired Student t test). B, representative IHC staining of FAT10 and HOXB9 in HCC tissues (magnification: ×100; inset magnification: ×400). C, representative Western blotting analysis of FAT10 and HOXB9 protein expression (T, tumor; NT, nontumoral tissues). D, quantification of FAT10 and HOXB9 protein expression using Western blot analysis in 102 paired HCCs and their adjacent nontumoral livers. β-Actin protein expression was used as an internal control (**, P < 0.01). E, scatter plots show a positive correlation between FAT10 and HOXB9 at the mRNA and protein level in 102 HCCs (r = 0.399, P < 0.001 and r = 0.341, P < 0.001).
carcinogenesis and metastasis (19, 20). It has been reported that β-catenin is a central effector of the Wnt pathway, and its stabilization is the key event in transduction of the Wnt signal (21, 22). It is also known that β-catenin is targeted to ubiquitin-proteasome-mediated degradation (23). FAT10 is a ubiquitin-like protein, and it functions similarly to polyubiquitin as a tag for proteasome targeting (24, 25). However, whether FAT10 is involved in regulating the degradation of β-catenin has not been clarified.

In this study, we found that FAT10 and HOXB9 were aberrantly upregulated in HCC tissues and demonstrated a significantly positive correlation between the expression levels of these genes. The clinical significance of FAT10 and HOXB9 overexpression was also addressed. Furthermore, the functional studies provided the first evidence that FAT10 facilitates HCC invasion in vitro and metastasis in vivo by upregulating HOXB9 expression. FAT10 regulated HOXB9 through the β-catenin/TCF4 signaling pathway. Further investigations indicated that FAT10 could directly interact with and stabilize β-catenin expression by modulating the ubiquitination and degradation of β-catenin.

**Materials and Methods**

**Human tissue specimens**

Human HCC specimens were collected from 102 patients who underwent HCC resection at the Second Affiliated Hospital of Nanchang University between January 2008 and June 2013. Informed consent was obtained from each patient, and the study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University.

**Table 1. Correlation between FAT10 or HOXB9 and clinicopathologic characteristics in 102 HCC cases**

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<th>FAT10 Nonoverexpression (number of cases)</th>
<th>P value</th>
<th>HOXB9 Overexpression (number of cases)</th>
<th>HOXB9 Nonoverexpression (number of cases)</th>
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**NOTE:** The P values represent probabilities for FAT10 or HOXB9 expression levels between variable subgroups determined by a χ² test. Abbreviations: AFP, α-fetoprotein; HBsAg, hepatitis B surface antigen; TNM, tumor-node-metastasis.
Figure 2. Stable knockdown of FAT10 reduced HOXB9 expression and inhibited HCC invasion and metastasis in vitro and in vivo. A, qRT-PCR and Western blot analysis of FAT10 and HOXB9 expression in human normal hepatocyte and HCC cell lines. B, qRT-PCR and Western blot analyses were used to detect FAT10 and HOXB9 expression in MHCC97H and Huh-7 cells stably transfected with the shNC vector or the shFAT10 plasmid. C, wound healing assay. Wound closure was delayed in FAT10 stable knockdown cells compared with shNC control at both the 24- and 36-hour time points. D, Transwell migration assays of MHCC97H and Huh-7 cells with stable FAT10 knockdown (*, P < 0.01). (Continued on the following page.)
Cell culture, plasmids, and reagents
The human HCC cell lines HepG2, SMMC7721, Huh-7, Hep3B, HCCLM3, and MHCC97H were purchased from the Shanghai Institute of Cell Biology, China. The immortalized liver cell line HL-7702 was purchased from Shanghai Fu Xiang Biotechnology Co., Ltd. The cells were cultured in DMEM (Gibco) supplemented with FBS (Hyclone) to a final concentration of 10% and were exposed to antibiotics at 37°C with 5% CO2. Plasmids and reagents are included in Supplementary Materials and Methods.

PCR site-directed mutagenesis
shRNA-resistant FAT10 plasmids (nonsense mutation FAT10 gene expression vector) were constructed by site-directed mutagenesis using a QuickChange mutagenesis kit (Agilent Technologies). The plasmid pCDNA3.1(+)-FAT10 was used as a template. The mutagenic primers were shown in Supplementary Table S2. The mutant alleles were confirmed by sequencing.

qRT-PCR, Western blotting analysis and immunofluorescence, and coimmunoprecipitation
qRT-PCR, Western blotting, immunofluorescence, and coimmunoprecipitation (Co-IP) were performed as previously described (26). The specific primers used for PCR amplification were shown in Supplementary Table S2.

Wound-healing assays
The cells were grown to 80% to 90% confluence in 60-mm dishes. Artificial wounds were generated by scraping a pipette tip across the cell surface. After the removal of the detached cells by gentle washing with PBS, the cells were fed with fresh complete medium and incubated over time to allow the cells to migrate into the open area. The cell movement during wound closure was measured by phase-contrast photography at 37°C for incubations of 0, 24, and 36 hours, and three randomly selected wound areas were analyzed.

In vitro migration and invasion assays
For the migration assay, 5 × 10^4 cells were resuspended in serum-free medium and placed in the upper chambers; for invasion assays, 1 × 10^5 cells were seeded in a Matrigel-coated chamber (BD Biosciences). After 24 hours (to examine migration) or 48 hours (to examine invasion) of incubation, the nonmigrated cells on the upper surface of the membrane were removed, and the cells on the lower surface were fixed and stained with 0.1% crystal violet. The cells in five random microscopic fields were counted and imaged using a light microscope with a DP70 CCD system (Olympus Corp.).

Orthotopic liver implantation in nude mice
For in vivo metastasis assays, 1 × 10^7 cells in 100 μL of phosphate-buffered saline were injected subcutaneously into the flanks of nude mice (Shanghai SLAC Laboratory Animal Co., Ltd.). Once the subcutaneous tumors reached 1 to 2 cm in diameter, they were removed and cut into pieces with a volume of approximately 1 mm³, and then the pieces were implanted in the livers of the nude mice (6 in each group, male BALB/c-nu/nu, 6–8 weeks). The mice were sacrificed 6 weeks after tumor implantation. The livers and lungs were then processed and embedded in paraffin. The animal work was approved by the Ethics Committee for Animal Experiments of the Second Affiliated Hospital of Nanchang University and was performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (revised 1985).

Hematoxylin & eosin and immunohistochemical staining
The HCC and adjacent tissues were fixed in 10% formalin, embedded in paraffin, and sectioned. Then, the tissue sections were deparaffinized. Some of the deparaffinized sections were stained with hematoxylin & eosin (H&E), which was used to detect morphologic changes, and some were rehydrated and microwave-heated in sodium citrate buffer (10 mmol/L, pH 6.0) for antigen retrieval. The sections were then incubated with 0.3% hydrogen peroxide/phosphate-buffered saline for 30 minutes and blocked with Super Blocking solution (Pierce). The slides were first incubated with the antibody for FAT10 and HOXB9 at 4°C overnight with optimal dilution. Then, they were labeled by EnVision HRP kits (DAKO) at room temperature for 30 minutes, incubated with DAB substrate liquid (DAKO), and counterstained with Mayer’s hematoxylin (DAKO). All of the sections were observed and photographed with a light microscope with a DP70 CCD system (Olympus Corp.). The staining intensity and percentage of positive cells were scored semiquantitatively by two pathologists who were blind to the clinical parameters. The protein expression levels were subjectively graded as a function of relative nuclear staining intensity: no or low staining (0–1+) and moderate or high staining (2–3+).

Luciferase reporter gene assay
The cells were seeded at a density of 1 × 10^5 cells per well in 6-well plates and incubated for 24 hours before transfection with wild-type TOP-flash (2 μg) or mutant FOP-flash (2 μg) using Lipfectamine™ LTX according to the manufacturer’s instructions. The pRL-SV40 expression vector (0.3 μg) was added to each transfection system to normalize the transfection efficiency. The reporter assays were performed according to the manufacturer’s instructions. The TCF4 transcriptional activity was calculated as the ratio of specific TOP-flash over basement membrane.

(Continued.) E, Transwell invasion assays of MHCC97H and Huh-7 cells transfected with FAT10-shRNA (***, *P < 0.01). F, H&E–stained sections of orthotopic primary liver tumors and distal metastatic nodules in the lung formed by MHCC97H-shFAT10 or shNC (left). Magnification: a and b, ×100; c and d, ×400. Summary of the pathologic analysis of MHCC97H-shNC– and shFAT10–derived tumors in the livers of nude mice (right). G, representative H&E–stained sections of the lung tissues collected from shNC and shFAT10 groups are shown in the left. Magnification: a and b, ×100; c and d, ×400. A total of 10 random visual fields were chosen from different lung sections of each group, and pulmonary metastatic foci were quantified as the average number across the 10 visual fields per group (right; ***, *P < 0.01).
nonspecific FOP-flash relative renilla luciferase units. The mean values of the normalized ratios were compared.

**In vivo ubiquitination assay**

HepG2 and SMCC7721 cells were transfected with His–Ub and HA–β-catenin expression plasmids with or without combination of the plasmid encoding FAT10. The transfected cells were incubated for 30 hours and then exposed to MG132 (15 μmol/L) treatment for 4 hours before harvesting. The cell lysate was immunoprecipitated with anti-HA antibody, and the ubiquitination of β-catenin was detected with anti-His antibody.

**Statistical analysis**

All data were analyzed using SPSS 16.0 (SPSS, Inc.). The results were presented as the mean ± SD of three independent experiments. The differences between the groups were analyzed by the Student *t* test when two groups were compared or by one-way ANOVA when more than two groups were compared. Furthermore, univariate and multivariate analysis for invasion and metastasis was performed using the logistic regression model. The test results were considered significant at *P* < 0.05.

**Results**

**FAT10 and HOXB9 expression are both upregulated in HCC tissues and are closely related to HCC progression**

To explore the expression and significance of FAT10 and HOXB9 in HCC tissues, we first examined FAT10 and HOXB9 expression in 102 HCC tissue samples and the corresponding adjacent tissues by qRT-PCR, IHC, and Western blotting. The qRT-PCR revealed that the average fold change of FAT10 and HOXB9 mRNA expression in tumor tissues was significantly higher than that in paired nontumor tissues (Fig. 1A). The IHC results showed that the FAT10 protein was highly expressed in 71.57% (73 of 102) of the HCC tissue samples and in only 13.72% (14 of 102) of the adjacent tissues, whereas HOXB9 protein was highly expressed in 51.96% (53 of 102) of the HCC tissue samples and in only 6.86% (7 of 102) of the adjacent tissues (Fig. 1B). In addition, the Western blotting results showed that the FAT10 and HOXB9 protein levels were significantly elevated in the HCC tissues (Fig. 1C), which was consistent with the IHC results. These results indicate that the FAT10 and HOXB9 protein expression levels were significantly upregulated in HCC tissues (*P* < 0.01 for both; Fig. 1D). Moreover, the scatter plots showed that FAT10 and HOXB9 mRNA and protein expression levels were positively correlated in HCC tissues (both *P* < 0.001, Fig. 1E).

Next, we analyzed the correlations between FAT10 and HOXB9 protein overexpression and HCC clinicopathologic parameters. The results showed that both FAT10 and HOXB9 overexpression correlated closely with tumor size, tumor microsatellite formation, and vascular invasion (*P* < 0.05 for all; Table 1). These results indicated that FAT10 and HOXB9 overexpression is involved in HCC aggressiveness and metastasis. To further determine whether the status of FAT10 and HOXB9 cooverexpression might be an independent factor regulating invasion and metastasis in HCC cases, univariate and multivariate logistic regression models were applied. The results revealed that cooverexpression of FAT10 and HOXB9 was an independent factor for predicting tumor microsatellite formation and vascular invasion (both *P* < 0.01; Supplementary Table S3).

**Stable knockdown of FAT10 represses HOXB9 expression and inhibits HCC invasion and metastasis**

To investigate whether FAT10 regulated HOXB9 expression in HCC cells, we first examined the levels of FAT10 and HOXB9 in a variety of HCC cells by qRT-PCR and Western blotting. The results indicated that the expression of FAT10 and HOXB9 in HCC cells was higher than that in normal liver cells, and the FAT10 levels correlated with levels of HOXB9 (Fig. 2A). Second, we stably transfected a FAT10-specific short hairpin RNA (shFAT10) into MHCC97H and Huh-7 cells. qRT-PCR and Western blotting results showed that the downregulation of FAT10 could decrease HOXB9 expression levels in the MHCC97H and Huh-7 cells (*P* < 0.01). Furthermore, we observed that the cell migration ability was significantly decreased in the shFAT10 group (*P* < 0.01, Fig. 2C and D). In addition, using a Matrigel-coated Transwell chamber, we found that the stable FAT10 knockdown HCC cells invaded through the matrix slower than in the control group (*P* < 0.01, Fig. 2E). We further examined the effects of FAT10 on HCC metastasis by establishing an orthotopic liver tumor model in nude mice. The experiment included shNC and shFAT10 groups. Histologic analysis demonstrated that five cases developed intrahepatic metastasis in the shNC group. However, in the MHCC97H-shFAT10 group, there was only one case of intrahepatic metastasis (Fig. 2F). In addition, I&EE–stained serial lung sections revealed that the number of HCC lung micrometastases was significantly decreased in the shFAT10 group (*P* < 0.01; Fig. 2G). These results indicate that the stable knockdown of FAT10 can reduce HOXB9 expression and thus inhibit HCC invasion and metastasis.

To rule out possible off-target effects associated with shRNAs, we generated a FAT10 CDNA harboring silent mutations in the shRNA-targeting sequence that made the mRNA insensitive to this shRNA (Supplementary Fig. S2A). The shRNA-resistant FAT10 plasmid was transfected into stable FAT10 knockdown MHCC97H and Huh-7 cells. The results showed that the shRNA-resistant FAT10 plasmid could increase FAT10 protein expression, and the HOXB9 protein expression was also significantly rescued (Supplementary Fig. S2B). Furthermore, the shRNA-resistant FAT10 plasmid was also able to rescue the migration and invasion abilities of HCC cells with the stable knockdown of FAT10 (*P* < 0.01; Supplementary Fig. S2C and S2D). Altogether, these data show that the effects of shFAT10 on HOXB9 expression and cell invasion are sequence specific.

**HOXB9 is essential for FAT10-mediated HCC invasion and metastasis in vitro and in vivo**

To further validate that FAT10 mediated HCC invasion and metastasis by regulating HOXB9, we first increased the
Figure 3. FAT10 promotes HCC invasion and metastasis by upregulating HOXB9 expression. A, Western blotting was used to detect the expression of FAT10 and HOXB9. The upregulation of HOXB9 attenuated the loss of HOXB9 expression in MHCC97H-shFAT10 cells. B, Transwell assays showed that the upregulation of HOXB9 significantly rescued the cell migration and invasion in MHCC97H-shFAT10 cells (**, P < 0.01). C, representative H&E staining of livers and lungs from the MHCC97H-shFAT10+pcDNA3.1(+) vector and MHCC97H-shFAT10+pcDNA3.1(+)HOXB9 groups is shown. Magnification: a and b, ×100; c and d, ×400. D, summary of the incidence of liver and lung metastases in the different groups of nude mice. E, protein levels of FAT10 and HOXB9 were detected by Western blot analysis. The knockdown of HOXB9 expression dramatically inhibited the increase of HOXB9 expression in HepG2-FAT10 cells. F, Transwell assays showed that HOXB9 inhibition reduced FAT10-enhanced cell migration and invasion (**, P < 0.01). G, representative H&E staining of livers and lungs from the HepG2-FAT10+shNC and HepG2-FAT10+shHOXB9 groups is shown. Magnification: a and b, ×100; c and d, ×400. H, incidence of liver and lung metastases in the HepG2-FAT10+shNC and HepG2-FAT10+shHOXB9 groups of nude mice.
expression of HOXB9 in FAT10 knockdown HCC cells and then observed the FAT10 and HOXB9 protein expression levels and cell migration and invasion abilities by Western blotting and Transwell assay. As illustrated in Fig. 3A, the immunoblot analysis results showed that the downregulation of FAT10 decreased HOXB9 expression, whereas the upregulation of HOXB9 attenuated the loss of HOXB9 expression in FAT10 knockdown MHCC97H cells. We also found that the knockdown of FAT10 dramatically decreased the migration and invasion abilities of MHCC97H, whereas the upregulation of HOXB9 rescued the decreased migration and invasion abilities induced by FAT10 knockdown (Fig. 3B). In addition, the in vivo metastatic assay also showed that the upregulation of HOXB9 rescued the decreased incidence of intrahepatic and lung metastasis of the MHCC97H-shFAT10 group (Fig. 3C and D). Similar results were also found in Huh-7 cells (Supplementary Fig. S3A and S3B).

Second, we decreased the expression of HOXB9 in FAT10-overexpressing HCC cells and then observed the FAT10 and HOXB9 protein levels and cell migration and invasion abilities. The Western blotting results showed that overexpression of FAT10 significantly increased the expression of HOXB9, whereas the knockdown of HOXB9 expression dramatically inhibited the increase of HOXB9 expression induced by FAT10 in HepG2 cells (Fig. 3E). Meanwhile, the downregulation of HOXB9 significantly reduced FAT10-enhanced cell migration and invasion (Fig. 3F). Furthermore, the in vivo metastatic assay results showed that downregulation of HOXB9 decreased the incidence of intrahepatic and lung metastasis of the Hep2-FAT10 group (Fig. 3G and H). Thus, these results confirm that HOXB9 is essential for FAT10-mediated HCC metastasis.

**FAT10 regulates HOXB9 expression through the β-catenin/TCF4 pathway in HCC cells**

To further clarify the mechanism through which FAT10 regulates HOXB9 in HCC cells, we first observed whether there was direct interaction between the FAT10 and HOXB9 proteins. FAT10 has been reported to interact with different substrates to exert its effects (27). However, Co-IP showed that there was no direct interaction between the FAT10 and HOXB9 proteins (Fig. 4A). It has been reported that HOXB9 is a β-catenin/TCF4 target gene (18). Therefore, we speculated that FAT10 regulated HOXB9 via the β-catenin/TCF4 signaling pathway. To test this hypothesis, we first observed whether FAT10 and β-catenin directly interacted in HCC cells. Interestingly, the Co-IP study showed that FAT10 and β-catenin were detected in the immunoprecipitant, demonstrating the interaction between FAT10 and β-catenin (Fig. 4B). Co-localization by confocal microscopy was used to further confirm the FAT10/β-catenin interaction in MHCC97H and Huh-7 cells. The results indicated identical localization of both FAT10 and β-catenin, which provided further evidence of the interaction between these two proteins (Fig. 4C).

Next, to explore whether FAT10 regulated β-catenin/TCF4 signaling in HCC cells, we measured the changes in β-catenin expression in the FAT10 knockdown MHCC97H and Huh-7 cells. The Western blotting results showed that as the FAT10 expression decreased, the total and nuclear β-catenin protein expression decreased in MHCC97H cells (Fig. 4D). Next, in a TOP-Flash reporter luciferase assay, the knockdown of FAT10 in MHCC97H cells decreased the transcriptional activity of TCF4 compared with the shNC control (Fig. 4E). Studies have confirmed that HOXB9, c-Myc, and MMP-7 are target genes of β-catenin/TCF4 (18, 28, 29). We found that the knockdown of FAT10 decreased the expression of HOXB9, and the other Wnt pathway signaling components, including c-Myc and MMP-7, were also decreased in MHCC97H, whereas upregulation of β-catenin rescued the decreased expression of HOXB9, c-Myc, and MMP-7 induced by the FAT10 knockdown (Fig. 4F). In addition, we also found that upregulation of β-catenin rescued the decreased cell migration and invasion induced by the knockdown of FAT10 (Fig. 4G). Similar results were obtained with the Huh-7 cell lines (Supplementary Fig. S4).

To further verify that FAT10 regulates HOXB9 expression through the β-catenin/TCF4 pathway, we investigated the effect of FAT10 overexpression on the β-catenin/TCF4 pathway. The Western blotting results showed that upregulation of FAT10 expression significantly increased the expression levels of the total and nuclear β-catenin, and the transcriptional activity of TCF4 was also increased (Fig. 4H and I). Next, we detected the expression of HOXB9, c-Myc, and MMP-7 in HepG2-FAT10 transfected with the shβ-catenin plasmid. The results showed that the overexpression of FAT10 increased the expression of the HOXB9, c-Myc, and MMP-7 proteins, whereas the downregulation of β-catenin dramatically inhibited the increase in HOXB9, c-Myc, and MMP-7 expression in HepG2-FAT10 cells (Fig. 4J). Meanwhile, a Transwell assay showed that the downregulation of β-catenin dramatically decreased FAT10-induced cell migration and invasion (Fig. 4K). These studies confirmed that...
Figure 5. FAT10 stabilizes β-catenin protein by attenuating β-catenin ubiquitination and degradation in HCC cells. A, co-IP between endogenous β-catenin and ubiquitin in MHCC97H and Huh-7 cells. B, MHCC97H and Huh-7 cells were treated with MG132 (Z-Leu-Leu-Leu-CHO, 15 μmol/L) for the indicated times, and then the levels of β-catenin were detected. (Continued on the following page.)
FAT10 regulates HOXB9-induced HCC migration and invasion through the β-catenin/TCF4 pathway.

**FAT10 stabilizes β-catenin by regulating the ubiquitination level of β-catenin in HCC cells**

These experiments verified that FAT10 could directly bind to β-catenin and regulate HOXB9 expression via the β-catenin/TCF4 pathway. We then further assessed the mechanisms through which FAT10 regulates β-catenin. The study has reported that the β-catenin protein undergoes degradation via the ubiquitin proteasome pathway (23). To determine whether the ubiquitination and degradation of β-catenin proteins also occur in HCC cells, we first investigated the interaction between endogenous β-catenin and ubiquitin in HCC cells. The Co-IP results showed that the endogenous β-catenin and ubiquitin are directly bound in MHCC97H and Huh-7 cells (Fig. 5A). Furthermore, treatment with the proteasome inhibitor MG132 for the indicated time caused significant accumulation of the endogenous β-catenin protein in MHCC97H and Huh-7 cells (Fig. 5B). This result demonstrated that β-catenin protein is also degraded by the ubiquitin-proteasome system (UPS) in HCC cells.

Next, to determine whether FAT10 was involved in regulating the degradation of β-catenin protein, we transfected the shFAT10 and pcDNA3.1(+-)FAT10 plasmids into MHCC97H and Huh-7 cells and detected the effects of variable FAT10 on β-catenin expression, either with or without the proteasome inhibitor MG132. The results showed that reducing or increasing FAT10 had no significant effect on β-catenin expression after MHCC97H and Huh-7 cells were treated with MG132 (Fig. 5C). Moreover, the degradation dynamics assay showed that the half-life of the ectopically expressed β-catenin was significantly increased in the FAT10-overexpressing HepG2 and SMCC7721 cells compared with that in the control cells (Fig. 5D). These results suggest that FAT10 was involved in the degradation of β-catenin.

In addition, we further assessed the role of FAT10 in the process of β-catenin degradation. The results showed that the ectopic dose-dependent effect of FAT10 overexpression caused a significant accumulation of endogenous β-catenin proteins in HepG2 and SMCC7721 cells (Fig. 5E). Consistently, a single dose of HA-β-catenin and an increasing amount of Flag-FAT10 were cotransfected into HepG2 and SMCC7721 cells, and a dose-dependent effect of FAT10 overexpression on β-catenin expression was found (Fig. 5F).

These data demonstrate that FAT10 can stabilize the expression of β-catenin.

Finally, to investigate the mechanism by which FAT10 stabilizes the expression of β-catenin, we added MG132 to MHCC97H and Huh-7 cells that were transfected with the shFAT10 and pcDNA3.1(+-)FAT10 plasmids. After cell lysis, the lysates were mixed with a β-catenin antibody for immunoprecipitation, and the results showed that the knockdown of FAT10 could promote levels of β-catenin ubiquitination, whereas increased FAT10 significantly reduced the levels of β-catenin ubiquitination (Fig. 5G). In addition, plasmids encoding HA–β-catenin and His–Ub were cotransfected into the HepG2 and SMCC7721 cells with or without a combination of Flag-FAT10 overexpression. As shown in Fig. 5H, ubiquitin-conjugated β-catenin was detectable in the cells cotransfected with HA–β-catenin and His–Ub plasmids but not in the FAT10-overexpressing cells. These results suggest that FAT10 stabilizes β-catenin expression by regulating β-catenin ubiquitination.

**Discussion**

FAT10 belongs to the Ubls and is encoded in the HLA-F locus at the telomeric end of the MHC class I (30). There is accumulating evidence that FAT10 is upregulated in a variety of malignancies, including HCC, colorectal tumors, gastric tumors, and gynecological tumors (10, 31, 32). Liu and colleagues found that FAT10 overexpression can inhibit HCC cell apoptosis (14). We previously also reported that increasing the expression of FAT10 can lead to apoptosis resistance (33). These studies suggest that FAT10 plays an important role in the processes of HCC tumorigenesis.

Recently, FAT10 has been shown to be involved in regulating tumor migration and invasion. Gao and colleagues (34) reported that high FAT10 expression enhanced the invasive, migratory nature of the transformed cell line and HCT116 via the NF-κB–CXCR4/7 pathway. Another study found that FAT10 promoted the invasion of HCC cells via the Akt/GSK3β pathway (14). In this study, we reveal a novel mechanism in which FAT10 promotes HCC invasion and metastasis by upregulating HOXB9. First, our data indicated that FAT10 and HOXB9 were upregulated in HCC tissues and revealed a significantly positive correlation. Both FAT10 and HOXB9 overexpression were closely related to invasive pathological features of HCC. The logistic regression results
revealed that cooverexpression of FAT10 and HOXB9 was an independent factor for predicting HCC microsatellite formation and vascular invasion. In addition, we also found that FAT10 inhibition could reduce HOXB9 expression and decrease the invasion and metastasis of HCC in vitro and in vivo. Moreover, upregulation of HOXB9 rescued the decreased invasion and lung metastasis induced by FAT10 knockdown, whereas HOXB9 inhibition significantly decreased FAT10-enhanced invasiveness and metastasis. These results suggested that one of the mechanisms through which FAT10 promotes HCC metastasis involves the upregulation of HOXB9 expression.

HOXB9 is part of a HOX family gene cluster whose expression is normally restricted to embryogenesis (15). In recent years, studies have shown that HOXB9 is highly expressed in a variety of malignant tumors, where it plays an important role in tumor invasion and metastasis (16, 17, 35). For example, HOXB9 overexpression was confirmed to promote breast cancer angiogenesis and the distant metastasis of tumor cells (16). The knockdown of HOXB9 expression in lung cancer cells could significantly inhibit cellular invasion abilities and distant brain and bone metastases (17). In our study, we have shown that FAT10 promotes HCC invasion and metastasis by upregulating HOXB9, and the mechanism by which FAT10 regulates HOXB9 has further been investigated. First, we did not detect a direct interaction between the FAT10 and HOXB9 proteins. The FAT10 protein always functions by binding to its substrates. As such, FAT10 has been shown to interact with MAD2, NUB1L, and HDAC6 (36–38). Our previous work has also confirmed that eukaryotic translation elongation factor IA1 (eEF1A1) is a new substrate protein of FAT10 in HCC cells (39). In addition, we considered that HOXB9 is a direct target of β-catenin/TCF4 (18). Therefore, we observed whether there was a direct interaction between FAT10 and the β-catenin protein. Interestingly, we first found a direct interaction between FAT10 and the β-catenin protein in HCC cells. Furthermore, we also determined that the knockdown of FAT10 decreased the β-catenin expression, and the transcriptional activity of TCF4 was also reduced, ultimately leading to downregulation of its target genes, such as HOXB9, c-Myc, and MMP-7, whereas upregulation of β-catenin could rescue the decreased HOXB9 and other Wnt pathway signaling component expression levels induced by FAT10 knockdown. Furthermore, a Transwell assay indicated that overexpression of β-catenin significantly rescued the decreased cell migration and invasion induced by the knockdown of FAT10. Taken together, these studies demonstrate that FAT10 regulates HOXB9-mediated HCC migration and invasion through the β-catenin/TCF4 pathway.

In addition, we further investigated the mechanism by which FAT10 regulates the β-catenin/TCF4 pathway. The study has reported that β-catenin is the key transducer of Wnt signaling (40). Ubiquitin-proteasome–mediated degradation of β-catenin is the critical mechanism for the regulation of β-catenin levels in cells (41). Our results first confirmed that β-catenin is also degraded by UPS in HCC cells. Second, we found that FAT10 was involved in the degradation process of β-catenin and could function as a stabilizer for β-catenin. FAT10 overexpression has the effect of suppressing β-catenin ubiquitination and degradation. FAT10 is a member of the Ubls (30). It is generally believed that FAT10 and various other Ubls members, including NEDD8, SUMO, and ISG15, are similar to ubiquitin in that they target a protein substrate for degradation (42–46). However, many studies have reported that ubiquitin-like molecules (ISG15 and SUMO) and ubiquitin have antagonistic effects on the modification of substrate proteins (47–50). In this study, our results have suggested for the first time that the FAT10 pathway negatively regulates β-catenin protein ubiquitination and degradation. This conclusion is based on the following observations. First, FAT10 can increase the half-life of β-catenin, and a dose-dependent effect of FAT10 overexpression on enhancing the β-catenin expression was found. Second, the knockdown of FAT10 could promote levels of β-catenin ubiquitination, whereas increased FAT10 significantly reduced the levels of β-catenin ubiquitination. Third, in vivo ubiquitination assay, the levels of ubiquitin-conjugated β-catenin were significantly decreased in the presence of FAT10 overexpression. Furthermore, we also found that FAT10 and ubiquitin can competitively bind the same motifs of eEF1A1 and regulate the degradation of eEF1A1 (unpublished data). Thus, we speculate that the mechanism by which FAT10 stabilizes the β-catenin protein might be related to the fact that FAT10 and ubiquitin act antagonistically.
In summary, we demonstrated that FAT10 promotes HCC metastasis by regulating HOXB9 expression. FAT10 regulated HOXB9 expression through the β-catenin/TCF4 signaling pathway. Furthermore, we found that FAT10 stabilized the β-catenin protein by suppressing the ubiquitination and degradation of β-catenin, thereby regulating the transcriptional activity of TCF4 and HOXB9 expression (Fig. 6). The newly identified FAT10–β-catenin/TCF4–HOXB9 axis provides new insight into the invasion and metastasis of HCC and represents a valuable target for HCC therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: R. Yuan, K. Wang, K. Hong, J. Shao
Development of methodology: R. Yuan, K. Wang, J. Hu, C. Yan, M. Li, X. Yu, X. Liu, J. Lei, K. Hong, J. Shao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Yuan, K. Wang, J. Hu, C. Yan, M. Li, X. Yu, X. Liu, J. Lei, W. Guo, L. Wu, K. Hong, J. Shao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Yuan, K. Wang, J. Hu, C. Yan, M. Li, X. Yu, X. Liu, W. Guo, L. Wu, K. Hong, J. Shao
Writing, review, and/or revision of the manuscript: R. Yuan, K. Hong, J. Shao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Yuan, K. Wang, J. Hu, C. Yan, M. Li, J. Lei, W. Guo, L. Wu, K. Hong, J. Shao
Study supervision: X. Yu, X. Liu, K. Hong, J. Shao

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References


Ubiquitin-like Protein FAT10 Promotes the Invasion and Metastasis of Hepatocellular Carcinoma by Modifying $\beta$-Catenin Degradation

Rongfa Yuan, Kai Wang, Junwen Hu, et al.

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