Circular RNA circSFMBT2 downregulation by HBx promotes hepatocellular carcinoma metastasis via the miR-665/TIMP3 axis

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Hepatitis B virus X protein (HBx) is considered as an oncogene in tumorigenesis and progression of hepatocellular carcinoma (HCC). In recent years, the important role of circular RNAs (circRNAs) in HCC has been increasingly demonstrated. However, the regulatory mechanisms of HBx on circRNAs remains largely unknown. In this study, we identified that a novel circRNA, circSFMBT2, was markedly downregulated by HBx. Low expression of circSFMBT2 was correlated with poor prognosis and vascular invasion. Functionally, overexpression of circSFMBT2 significantly inhibited HCC metastasis both in vitro and in vivo. The mechanism of circSFMBT2 was to act as a sponge of miR-665, which is a negative regulator of tissue inhibitor of metalloproteinases 3 (TIMP3). However, HBx downregulated circSFMBT2 via the interaction with DExH-box helicase 9 (DHX9), which binds to flanking circRNA-forming introns. In conclusion, circSFMBT2, which is downregulated by HBx, acts as a tumor suppressor to inhibit tumor metastasis through the miR-665/TIMP3 axis. Our study suggests that circSFMBT2 could be a potential prognostic biomarker and therapeutic target for HCC.

INTRODUCTION
Hepatocellular carcinoma (HCC) is the most common pathological type (75%–85%) of primary liver cancer and the fourth leading cause of cancer-related death worldwide.1 However, the 5-year survival rate is approximately 18%,2 and the recurrence rate after surgery is as high as 65%–80%.3 The poor prognosis of HCC is closely associated with intrahepatic metastasis,4 vascular invasion,5 hepatitis B virus (HBV) infection,6 and so on. Postoperative metastasis and recurrence are important factors that restrict the long-term survival of HCC patients. Therefore, novel targets to reduce HCC metastasis need to be developed.

HBV is responsible for more than half of HCC cases worldwide6 and serves as a crucial aspect of the progression of HCC with its X gene-coded protein (HBx),7 which has been implicated as an oncogene in both genetic regulation and epigenetic modification.8 Our team has been focusing on the role and mechanism of the oncogenic protein HBx, which is involved in promoting the occurrence and development of HCC.9–12 Increasing evidence has shown that HBx contributes to the metastasis of HCC, including the regulation of noncoding RNA (ncRNA) expression,13 activation or degradation of proteins,14–16 translocation of transcription factors,17 etc.18 To date, there are many studies on HBx-regulated microRNAs (miRNAs) and long noncoding RNAs (lncRNAs),19 However, circular RNAs (circRNAs) regulated by HBx are entirely unknown.

CircRNA is a novel ncRNA with a highly conserved and stable covalently closed structure.19,20 CircRNA functions in HCC were recently revealed20 and include competing endogenous RNAs (ceRNAs) or miRNA sponges,21–26 interactions with RNA-binding proteins27 and translating proteins.28 Because studies on the regulatory role of circRNAs by HBx in HCC have not been reported, it is still promising to study the functions of circRNAs and the regulatory mechanism of HBx on circRNAs in HCC.

Therefore, the principal purpose of our study was to reveal the significant role of circSFMBT2, which was validated not only to be downregulated by HBx in HCC but also to have low expression in HCC. Low expression of circSFMBT2 was associated with poor prognosis and vascular invasion. The function and mechanism of circSFMBT2

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Figure 1. Identification of HBx-downregulated circSFMBT2
(A) Volcano plot of differentially expressed circRNAs identified from RNA-seq. (B) Combinational analysis of circRNAs detected in the Salzman2013 dataset and differentially expressed circRNAs in our data. (C and D) Relative expression of circRNAs was verified in HBx-overexpressing cells versus control cells (C) and validated in HepG2.2.15 versus HepG2 cells using qRT-PCR assays (D). (E) Formation of circSFMBT2. Black arrows represent the divergent primers, and the yellow arrow indicates the back-splicing junction detected by Sanger sequencing. (F) The qRT-PCR products amplified by divergent and converge primers of SFMBT2 and GAPDH were examined using 2.5%
were verified to inhibit cell migration and invasion in vitro and in vivo through the miR-665/tissue inhibitor of metalloproteinases 3 (TIMP3) axis. In addition, HBx downregulated circSFMBT2 by interacting with DExH-box helicase 9 (DHX9), which could bind more flanking Alu elements and suppress circSFMBT2 forming when HBx was overexpressed. The aim of our study was to provide a potential prognostic biomarker for prognostic evaluation or a therapeutic target for the clinical treatment of HCC.

RESULTS

Identification of HBx-downregulated circSFMBT2 in HCC cell lines
To investigate the regulatory effect of HBx on circRNAs, SK-HEP-1 cells were first modified to overexpress HBx after infection with HBx-overexpressing lentivirus (Figure S1A). RNA sequencing (RNA-seq) was performed in three pairs of SK-HEP-1-HBx/pMSCV cells, and there were nine differentially expressed circRNAs (Figure 1A). We further screened the circRNAs that could be detected in HepG2 cells of Salzman et al.,29 a circBase dataset of Homo sapiens,30,31 and selected four candidate circRNAs for further validation. Among them, two circRNAs were downregulated by HBx, while two circRNAs were upregulated (Figure 1B). Another two HBx-overexpressing HCC cell lines (MHCC97H-HBx/pMSCV and HepG2.2.15/HepG2) were also involved for the validation of circRNAs expression (Figure S1B). The qRT-PCR results of three HCC cell lines both showed that hsa_circ_0004073 and hsa_circ_0000211 were markedly downregulated in HBx-overexpressing cells (Figures 1C and 1D). Because the downregulations of hsa_circ_0000211 by HBx were more significant than hsa_circ_0004073 in two-thirds of cell lines, we chose hsa_circ_0000211 (circSFMBT2) for further identification.

CircSFMBT2 was derived from the genomic locus of scm-like with four mbt domains 2 (SFMBT2) in the antisense strand of chromosome 10. It was a back-splicing product and forms from the fifth to seventh exons of the SFMBT2 gene. To identify the stable expression of circSFMBT2, we designed divergent primers on both flanks of the back-splicing junction and verified the specificity of the qRT-PCR product by Sanger sequencing and 2.5% agarose gel electrophoresis (Figures 1E and 1F). CircSFMBT2 was less amplified when oligo dT primers were used, suggesting that circSFMBT2 lacked a poly-A tail (Figure 1G). According to the degradation effect of ribonuclease R (RNase R) on linear RNA and the inhibitory effect of actinomycin D on RNA transcription, the degradation of linearSFMBT2 was significantly faster than that of circSFMBT2, indicating that the stability of circSFMBT2 was increased because of its circular structure (Figures 1H and 1I). Moreover, the intracellular localization of circSFMBT2 was examined by nuclear and cytoplasmic separation RNA extraction assays and fluorescence in situ hybridization (FISH) assays (Figures 1J and 1K). The results both identified that circSFMBT2 was located mainly in the cytoplasm. Finally, the probe on back-splicing junction was used to perform FISH assays in human tissues (Figures S2A and S2B). According to the staining score standard, we found that the downregulation of circSFMBT2 was more significant in HBV positive HCC tissues (Figure 1L).

CircSFMBT2 is expressed at low levels in HCC and correlates with poor prognosis and invasion
First, the expressions of circSFMBT2 in HCC cell lines and normal liver cell line LO2 were evaluated using qRT-PCR assays, showing that circSFMBT2 was markedly downregulated in HCC cell lines (Figure 2A). In addition, circSFMBT2 expression was detected in 86 pairs of tumor and adjacent tissues from HCC patients. The medians of circSFMBT2 expression in tumor and adjacent tissues were calculated respectively, which showed a lower expression level in tumorous tissues than in adjacent nontumorous tissues (Figure 2B). Comparing circSFMBT2 expression between tumor and adjacent tissue for each patient, 48 patients (55.81%) showed that circSFMBT2 was downregulated in tumor samples (Figure 2C). After using X-tile to find the best cut-off value, 86 patients were divided into the low-circSFMBT2 expression group (n = 55) and the high-expression group (n = 31). We found that low expression of circSFMBT2 was associated with worse overall survival (OS) and disease-free survival (DFS) by Kaplan-Meier analysis (Figures 2D and 2E). In addition, the correlations between circSFMBT2 expression and clinical characteristics were further analyzed using the chi-square test (Table 1), which showed that low circSFMBT2 expression was positively correlated with vascular invasion and capsule invasion (Figures 2F and 2G). Therefore, we concluded that circSFMBT2 was expressed at low levels in HCC and correlated with poor prognosis and invasion.

CircSFMBT2 inhibits HCC metastasis in vitro and in vivo
SK-HEP-1 and MHCC97H cells were infected with circSFMBT2-overexpressing lentiviruses to overexpress circSFMBT2 and were transfected with a specific small interfering RNA (siRNA) targeting the back-splicing junction to knock down circSFMBT2 (Figures 3A and 3B). The efficiency of was observed using qRT-PCR assays, and linearSFMBT2 expression was not affected. Initially, we conducted CCK-8 assays in SK-HEP-1 and MHCC97H cells, but it was found that the overexpression of circSFMBT2 had no effect on the proliferation of HCC (Figure 3C). As circSFMBT2 has been previously verified to be correlated with tumor invasion in our study, we were convinced that circSFMBT2 might affect the metastatic ability of HCC. For the in vitro experiments, we used Transwell migration and invasion assays to verify that the overexpression of circSFMBT2
inhibit migration and invasion in SK-HEP-1 and MHCC97H cells (Figure 3D). Wound-healing assays confirmed that the migration ability of cells in the circSFMBT2-overexpressing group was weaker than that of cells in the empty vector (EV) group (Figure 3E). In addition, loss-of-function assays were also performed to validate that knockdown of circSFMBT2 promotes the migration and invasion of HCC cells in vitro (Figure 3F). The wound-healing assays also confirmed a similar conclusion (Figure 3G). Moreover, we established in vivo models of pulmonary metastasis in nude mice by tail vein injections of SK-HEP-1 cells. The results of the in vivo imaging system revealed that both the extent of the tumors and the intensity of bioluminescence were significantly less in the circSFMBT2-overexpressing group than in the EV group (Figure 3H). It was also found that the number of tumors was significantly reduced in the overexpression group by H&E staining of lung tissue sections (Figure 3I). Accordingly, we concluded that circSFMBT2 acts as a tumor suppressor to inhibit HCC metastasis both in vitro and in vivo. Therefore, we could further search the molecular mechanism of circSFMBT2 in HCC.

**CircSFMBT2 acts as a sponge of miR-665**

Above all, circRNAdb32 was involved in predicting the open reading frame of circSFMBT2 and showed that the possibility of encoding a protein was relatively low. According to the cytoplasmic localization of circSFMBT2, we hypothesized that it was more likely to play an important role as a miRNA sponge. After combinational analysis of three databases (circBank, CircInteractome, and starBase) used for screening possible binding miRNAs, we found that only miR-665 could be predicted in all databases, while the other four miRNAs (miR-188-3p, miR-582-3p, miR-107, and miR-103a-3p) could be predicted in two different databases (Figures 4A and 4B, Table S3). To further screen out the candidate miRNAs, dual-luciferase reporter plasmids containing full-length sequences of circSFMBT2 were cotransfected into SK-HEP-1 and MHCC97H cells with potential miRNA mimics. The relative Renilla luciferase activity of miR-655 was found to be the weakest among them in both cell lines, suggesting that miR-655 might have a higher possibility of binding to circSFMBT2 than the other four miRNAs (Figure 4C). Then, to determine the precise complementary binding site between both sequences, circSFMBT2 fragments with wild-type or mutant complementary binding sites were inserted into luciferase reporter plasmids (Figure 4D). The relative Renilla luciferase activity was indeed reduced in the wild-type group, but the effect of miR-665 on the relative Renilla luciferase activity was not obvious in the mutant group, indicating that the complementary binding site of
circSFMBT2 and miR-665 was at the predicted position (Figure 4E). In addition, we further designed a biotin-labeled probe targeting the back-splicing junction of circSFMBT2 and an oligo probe for RNA pull-down assay. As shown in Figure 4F, circSFMBT2 probes showed better pull-down efficiencies than oligo probes in both SK-HEP-1 and MHCC97H cells. The circSFMBT2 probes pulled down more miR-665 than the oligo probes, indicating that circSFMBT2 directly bind to miR-665 (Figure 4G). Moreover, we used biotin-labeled miR-665 mimics to reversely pull down circSFMBT2. CircSFMBT2 precipitation was more enriched in the circSFMBT2-overexpressing group (Figure 4H). Biotin-labeled miR-665 mimics could also pull down more circSFMBT2 standardized to biotin-labeled negative control (NC) mimics (Figure 4I).

To validate that circSFMBT2 could only act as a sponge of miR-665 but not influence the expression of miR-655, qRT-PCR assays were performed. The miR-665 expression showed no statistically significant difference between the circSFMBT2-overexpressing group and the EV group (Figure S3A). However, miR-665 did not affect the expression of circSFMBT2 and HBx (Figures S3B and S3C).

MiR-665 mimics and inhibitors were transfected to overexpress and interfere miR-655 exogenously in SK-HEP-1 and MHCC97H cells (Figures S3D and S3E). Transwell migration and invasion assays were performed to validate that miR-665 could promote the migration and invasion of HCC cells (Figures S3F and S3G). This suggested that miR-665 could actually act as an oncogenic miRNA in HCC. We further performed a “rescue” experiment to examine the functional interaction of circSFMBT2 and miR-665. Transwell migration and invasion assays revealed that the cell migration and invasion abilities were also strengthened by miR-665 mimics but were impaired when circSFMBT2 was overexpressed in SK-HEP-1 and MHCC97H cells (Figure 4K). We continued to analyze the downstream genes of circSFMBT2 that were targeted by miR-665.

### CircSFMBT2 regulates HCC metastasis through the miR-665/TIMP3 axis

After RNA-seq was performed in circSFMBT2-overexpressing cells and control cells, there were eight differentially expressed genes (DEGs) that might be regulated by circSFMBT2 and targeted by miR-665 (predicted by TargetScan36 and miRDB37) (Figure 5A, Table S4). Among them, TIMP3 was the only downregulated DEG that was discovered in RNA-seq of HBx-overexpressing cells (Figure 5B). And TIMP3 was reported as a classical suppressor for tumor metastasis, so we examined the regulation of TIMP3 expression and verified that circSFMBT2 could upregulate TIMP3 expression, while miR-665 downregulated TIMP3 (Figures 5C and 5D). In addition, there were three potential complementary sites between the 3′ untranslated region (UTR) sequence of TIMP3 and miR-665, so we constructed UTR segments containing different complementary sites into luciferase reporter plasmids. The results of the luciferase reporter assay revealed that miR-665 had better complementary pairing with UTR #3 (Figure 5E). Moreover, relative Renilla luciferase activity of UTR #3 could be enhanced by circSFMBT2, but the enhancement of Renilla luciferase activity was offset when miR-665 mimics were cotransfected (Figure 5F). This confirmed that miR-665 could actually bind to TIMP3 and act as a negative regulator to downregulate its expression.

Western blotting analysis showed that the level of the TIMP3 protein was downregulated after overexpression of miR-665, but the effect was partially eliminated by overexpression of circSFMBT2 (Figure 5G). Thus far, our study discovered a molecular mechanism by

### Table 1. Correlation between circSFMBT2 expression and clinical characteristics

| Characteristics | circSFMBT2 expression | p value |
|-----------------|-----------------------|---------|
| | Low (n = 55) | High (n = 31) |
| Sex | | |
| Female | 5 | 4 | 0.579 |
| Male | 50 | 27 | |
| Age, y | | |
| <60 | 38 | 25 | 0.245 |
| ≥ 60 | 17 | 6 | |
| Tumor size, cm | | |
| ≤ 5 | 15 | 13 | 0.164 |
| >5 | 40 | 18 | |
| TNM stage | | |
| I or II | 17 | 13 | 0.303 |
| III or IV | 38 | 18 | |
| Pathological grade | | |
| I | 20 | 9 | 0.605 |
| II | 20 | 10 | |
| III | 15 | 12 | |
| Vascular invasion | | |
| No | 24 | 22 | 0.015a |
| Yes | 31 | 9 | |
| Capsular invasion | | |
| No | 31 | 27 | 0.003b |
| Yes | 24 | 4 | |
| Bile duct invasion | | |
| No | 48 | 29 | 0.361 |
| Yes | 7 | 2 | |
| AFP, ng/mL | | |
| <400 | 24 | 20 | 0.063 |
| ≥ 400 | 31 | 11 | |
| HBV infection | | |
| No | 7 | 5 | 0.667 |
| Yes | 48 | 26 | |

*p < 0.05 and *p < 0.01 (chi-square test).
which circSFMBT2 regulated TIMP3 expression by sponging miR-665.

We further validated that TIMP3 was significant downregulated by HBx (Figure 5H), while TIMP3 downregulation by HBx could also be repaired by circSFMBT2 overexpression (Figure 5I).

**HBx facilitates HCC metastasis through circSFMBT2/miR-665/TIMP3 pathways**

To investigate whether the effect of HBx could be rescued by circSFMBT2, we transfected circSFMBT2-overexpressing plasmids into SK-HEP-1 and MHCC97H cells that stably overexpressed HBx. Transwell migration and invasion assays demonstrated that the migration and invasion abilities enhanced by HBx could be reduced by circSFMBT2 (Figures S4A and S4B). In addition, short hairpin RNAs (shRNAs) targeting TIMP3 mRNA and TIMP3-overexpressing vector were designed to disrupt the endogenous expression of TIMP3 (Figures S5A and S5B). A rescue experiment was conducted to show that the inhibitory effect of circSFMBT2 overexpression on the migration and invasion abilities of HCC cells could be abolished by knocking down TIMP3 expression (Figure 6A), indicating that circSFMBT2 plays an antitumor role in suppressing tumor metastasis through the miR-665/TIMP3 axis in HCC. Another rescue experiment was performed to verify that the tumor metastasis promotion of HBx could be impaired by overexpressing TIMP3, indicating that HBx could facilitate HCC metastasis through circSFMBT2/miR-665/TIMP3 pathways (Figure 6B).

**HBx downregulates circSFMBT2 via interacting with DHX9 and promoting DHX9 to bind flanking Alu elements**

To explore whether HBx downregulates circSFMBT2 by initiating transcription from genome or effecting alternative splicing of RNA precursor, we first performed qRT-PCR assays to validate that circSFMBT2 precursor (preSFMBT2) was not regulated by HBx (Figure 7A). We hypothesized that HBx might influence the alternative splicing of preSFMBT2 through a RNA-binding protein (RBP). Therefore, co-immunoprecipitation (co-IP) and silver staining assay were performed in SK-HEP-1-HBx/pMSCV cells, followed by liquid mass spectrometry of desired band to screen out which RBPs could interact with HBx (Figure S6A). We focused on one of 13 predicted RBPs, DHX9, which was reported to bind the flanking Alu elements, unwind double-stranded RNA, and act as a circRNA-forming suppressor. DHX9 was upregulated in HCC cell lines and TCGA (The Cancer Genome Atlas) LIHC tumor samples (Figures S6B and S6C), while high DHX9 expression was associated with poor OS and DFS (Figures S6D and S6E). The HBx predicted binding domains of DHX9 did not compete with the RNA-binding domain (Figure S6F). Hence, we reconfirmed the interaction between HBx and DHX9 by co-IP assays (Figure 7B) and verified that DHX9 expression was not influenced by HBx (Figures S6G and S6I). Then, we interfered DHX9 expression with siRNAs (Figure S6H), leading to the upregulation of circSFMBT2 expression (Figure S6J). Knockdown of DHX9 could also reverse circSFMBT2 downregulation by HBx (Figure 7C), so we hypothesized that HBx might enhance the alternative splicing effect of DHX9 on preSFMBT2 to downregulate circSFMBT2 expression through interacting with DHX9.

Alu element was a family of short interspersed elements that was reported to occur complementary base pairing and facilitate circRNAs forming. Therefore, we further searched for the inverted complementary sequences in the flanking introns closed to circSFMBT2 and found out two reversed Alu elements, AluSg7 (Alu1) and AluSj7 (Alu2) in upstream intron of preSFMBT2 and one forward Alu elements AluS5 (Alu3) in downstream intron. Alu1 and Alu2 both had high identities (73% and 77%) with Alu3 after nucleotide alignment (Figure S7A). To test whether circSFMBT2 expression was promoted by Alu elements, full length of circSFMBT2 exons and flanking introns and a series of Alu-deleted sequences were cloned into pZW1-eGFP vectors that were transfected into 293T cells (Figure 7D). The images of green fluorescence and relative circSFMBT2 levels illustrated that Alu1 and Alu3 were more important than Alu2 for circSFMBT2 forming (Figures 7E–7G), while the result of qRT-PCR assay also confirmed a similar conclusion (Figure S7B). To investigate whether DHX9 could bind to paired Alu elements, unwind double-stranded RNA, and suppress circSFMBT2 forming, we performed RNA immunoprecipitation (RIP) assays and validated that Alu1–3 could be captured by DHX9. Furthermore, the Alu elements that captured by DHX9 were more abundant when HBx was overexpressed (Figures 7H and 7I), and the relative enrichment of Alu1 and Alu3 were more remarkable than Alu2. Then, we considered that DHX9 could bind more frequently to AluSg7 and AluS5 to suppress circSFMBT2 forming through the interaction with HBx.

In conclusion, circSFMBT2 was downregulated by HBx via interacting with DHX9 and promoting DHX9 to bind flanking Alu elements as well as suppress circSFMBT2 forming, while downregulation of...
circSFMBT2 would release more free miR-655 to inhibit TIMP3 expression, following with HCC metastasis.

DISCUSSION

HCC is the fourth leading cause of cancer-related death among both men and women in China. HBV infection contributes to 70%–80% of HCC cases in highly HBV endemic regions, including China. A high HBV viral load may affect the prognosis of HBV-related HCC patients. HBV contributes to HCC development through direct oncogenic effectors, such as HBx, or indirect mechanisms, such as gene mutations, epigenetic modifiers and ncRNAs. There have been many studies on ncRNAs, including miRNAs or lncRNAs, regulated by HBx in HCC. Nevertheless, to date, there has been no research on circRNAs regulated by HBx. Therefore, it could be considered a highlight of our study, as we identified a circRNA, circSFMBT2, regulated by HBx for the first time. However, differentially expressed circRNAs were identified using RNA-seq from three pairs of biologically repetitive cell lines but not from HCC tissues and adjacent nontumorous tissues. Although overexpression of HBx could not completely simulate the intracellular environment after HBV infection, it could highlight the regulatory effect of HBx on downstream circRNAs.

Two studies of hsa_circ_0000211 or circSFMBT2 were published recently. Hsa_circ_0000211, which was reported in lung adenocarcinoma played a role in promoting migration and invasion by sponging miR-655. In addition, hsa_circ_0017639, also called circSFMBT2, was derived from the fifth to eighth exons of SFMBT2 and was first identified to promote cell proliferation by sponging miR-182-5p in gastric cancer. The mechanisms of these studies were both miRNA sponges, which made the mechanism of our study more convincing. Both hsa_circ_0000211 and circSFMBT2 acted as tumor promoters. Nevertheless, we considered that the function of circRNA that acted as a sponge of miRNA was dependent on the function of miRNA in different cancer types. In our study, we identified that circSFMBT2 acts as a tumor suppressor to sponge the oncogenic miRNA miR-665. In our study, dual-luciferase reporter assay, RNA pull-down, and FISH were applied to confirm that circSFMBT2 could target miR-665. However, there are still several methods that could be involved to more directly confirm the interaction between circSFMBT2 and miR-665, such as RIP assay by AGO2 antibody, prediction of chemical binding energy by base pairing, construction of the secondary structure or tertiary structure of RNA, prediction of nucleic acid docking, etc. As the experimental conditions cannot be fully met, we plan to try our best to improve the above experiments in the further RNA-related studies.

In addition to DHX9, there were many RBPs acting on circRNA alternative splicing, such as quaking, adenosine deaminase RNA specific B2 (ADARB2), ADAR1, and so on. Among them, ADARB2 was a circRNA-forming promoter and a downregulated DEG of SK-HEP-1-HBx in RNA-seq data. However, its downregulation by HBx could not be validated in cell lines (data not shown). Therefore, we hypothesized that HBx might influence RBPs through protein interaction rather than transcriptional regulation. Through a series of experiments, DHX9 was identified as an important RBP that mediates HBx regulation of circSFMBT2. Finally, we verified that DHX9 could capture more Alu elements in HBx-overexpressing background. However, more experiments are suitable to perform and reconfirm the binding of DHX9 to preSFMBT2. For example, we could directly interfere with endogenous circSFMBT2 formation by knocking out Alu elements, reverse pull down DHX9 by specific Alu sequence probes, predict the specific binding sites of DHX9 and Alu elements by protein-RNA docking, verify the predicted binding sites after mutation, etc. In this study, we aimed to illustrate the role of circSFMBT2 in HCC metastasis, and we will focus on the mechanism that promotes the function of DHX9 combined with HBx in a future study.

It is very important to us that the circRNA identified in our study will successfully achieve clinical transformation and application. As a tumor suppressor, circSFMBT2 is expressed at a lower level in HCC. It possesses the potential to be a prognostic biomarker or a therapeutic target for HCC. The role of the prognostic biomarker lies in the positive correlations between the low expression of circSFMBT2 and poor prognosis, vascular invasion, and capsular invasion, suggesting that detecting low expression of circSFMBT2 in postoperative patients may predict a worse prognosis and lead to more positive treatment. However, we considered circSFMBT2 to be a more likely therapeutic target because of its conserved and stable structure. If in vitro transcription of circSFMBT2 could be accomplished and applied in vivo, its stable structure could help slow down the process of degradation and thus play a better role as a therapeutic target.

Conclusions

We identified that circSFMBT2, which was downregulated by HBx, acted as a tumor suppressor to inhibits tumor metastasis through
Figure 5. CircSFMBT2 regulates TIMP3 expression through sponging miR-665

(A) The downstream genes intersection of RNA-seq, target genes predicted by TargetScan and miRDB. (B) Relative expression of eight candidate genes in RNA-seq of SK-HEP-1-HBx versus pMSCV. (C and D) Relative expression and western blotting analysis of TIMP3 after overexpressing circSFMBT2 (C) or miR-665 (D) in SK-HEP-1 and MHCC97H cells. Tubulin was used as reference. (E) Relative Renilla luciferase activities of UTR #1–3 cotransfected with NC mimics or miR-665 mimic. (F) Relative Renilla luciferase activities of UTR#1–3 cotransfected with NC mimics or miR-665 mimic. (G) Relative Renilla luciferase activities of UTR#1–3 cotransfected with NC mimics or miR-665 mimic. (H) Relative Renilla luciferase activities of UTR#1–3 cotransfected with NC mimics or miR-665 mimic. (I) Relative Renilla luciferase activities of UTR#1–3 cotransfected with NC mimics or miR-665 mimic.

(legend continued on next page)
the miR-665/TIMP3 axis in HCC. In other words, HBx could facilitate HCC metastasis by downregulating circSFMBT2. HBx interacted with DHX9 and promoted DHX9 to bind to more flanking Alu elements of circSFMBT2 and suppress circSFMBT2 forming. Furthermore, we found that decreased circSFMBT2 expression was associated with poor prognosis and invasion in HCC patients. Our study suggested that circSFMBT2 could be a potential prognostic biomarker and therapeutic target for HCC.

MATERIALS AND METHODS

Tissues and cell lines

All tumorous tissues and adjacent nontumorous tissues were collected from HCC patients who underwent heptatectomies at Sun Yat-sen Memorial Hospital, Sun Yat-sen University, between 2009 and 2013. Tumorous tissues and paired adjacent nontumorous tissues from 86 HCC patients were stored at −80°C and used for RNA extraction. The human materials were obtained with the consent of patients and approved by the ethics committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University. Detailed clinical characteristics are listed in Table S1.

The HCC cell lines (SK-HEP-1, MHCC97H, PLC/PRF/5, BEL7402, HepG2, and HepG2.2.15), human immortalized liver cell line L02, while the short hairpin RNAs of TIMP3 were also purchased from Genechem Co. Before transfection, SK-HEP-1 and MHCC97H cells were seeded at 3 × 10^5 cells per well in 6-well plates. For transient transfection, 3 × 10^5 cells were transfected with 5 μL Lipofectamine 3000 and 6 μL Lipofectamine 2000 (Thermo Fisher Scientific), while BEL7402, HepG2.2.15, and L02 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Every Green) and 1% penicillin/streptomycin (Gibco) in a humidified atmosphere of 5% CO₂ at 37°C.

Plasmid construction and transfection

To construct overexpression plasmids of HBx, HBx-HA was synthesized and cloned into the pMSCV-eGFP vector as described in our previous study.44 CircSFMBT2 cDNA was inserted into the plenti-cir-GFP-T2A vector, and the coding sequence of TIMP3 was cloned into the pCDH-CMV-MCS-EF1 vector (IGE Biotech Co.). CircSFMBT2 exons and flanking introns were construct into the pZW1-eGFP vector, while the short hairpin RNAs of TIMP3 were also purchased from Genechem Co. Before transfection, SK-HEP-1 and MHCC97H cells were seeded at 3 × 10^5 cells per well in 6-well plate. For transient transfection, 3 μg plasmids were transfected with 6 μL Lipofectamine 3000 and 6 μL Lipofectamine 3000 (Thermo Fisher Scientific), while 5 μL small interfering RNAs, miRNA mimics, and miRNA inhibitors (GenePharma) were transfected with 5 μL Lipofectamine 3000. For stable transfection, cells were infected with the packaged lentivirus and selected with 2 μg/mL puromycin for 3 days. Surviving cells were then used to confirm overexpression efficiency.

RNA preparation and subcellular isolation

Total RNA from tumorous tissues and adjacent nontumorous tissues was extracted using RNAiso Plus (TaKaRa). Total RNA from different cell lines was extracted using an RNA-Quick Purification Kit (ESscience). RNA isolation of nuclear and cytoplasmic fractions was performed using an RNA Subcellular Isolation Kit (Active Motif). All experiments were accomplished according to the manufacturers’ protocols.

Reverse transcription and qRT-PCR assay

CircRNAs and mRNAs were reverse-transcribed using PrimeScript RT Reagent Kit (TaKaRa). Reverse transcription for miRNAs was performed using a Mir-X miRNA First-Strand Synthesis Kit (TaKaRa). qRT-PCR assays were performed using 2x SYBR Green qPCR Master Mix (Bimake). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for circRNAs and mRNAs. The expression of miRNAs was normalized to that of U6.

RNA-seq

Total RNA was isolated from 3 repetitive pairs of HBx-overexpressing cells and pMSCV vector control cells (SK-HEP-1-HBx and SK-HEP-1-pMSCV) by RNAiso Plus (TaKaRa). Whole-transcriptome sequencing was accomplished by Novelbio Company (Shanghai, China), and DEGs and circRNAs were filtered by a fold change (FC) > 2 and a false discovery rate (FDR) < 0.05. Transcriptome sequencing of SK-HEP-1-circSFMBT2 and SK-HEP-1-EV was performed by ANOROAD Company (Beijing, China), and DEGs were filtered by a FC > 1.5 and an adjusted p value of <0.05.

RNase R treatment

For RNase R treatment, 2 μg total RNA extracted from SK-HEP-1 cells was mixed with 0.2 μL RNase R (20 U/μL) (Epicenter Technologies) and 0.6 μL 10× RNase R Reaction Buffer; for the control treatment, 0.2 μL RNase-free water was used instead of RNase R in the same system. Then, the samples were incubated at 37°C for 30 min before reverse transcription. GAPDH in the control group was used as an internal control.

Actinomycin D assay

SK-HEP-1 cells were seeded at 2 × 10^5 cells per well in a 6-well plate. After adherence, the cells were exposed to 2 μg/mL actinomycin D (Sigma-Aldrich) for 4, 8, and 12 h. The cells were harvested at the indicated time points, and the stabilities of circSFMBT2 and linear SFMBT2 were analyzed using qRT-PCR assays.

FISH

MHCC97H cells were seeded in confocal dishes and hybridized with Cy3-labeled circSFMBT2 probes (GenePharma) or Cy5-labeled miR-665 probes (GenePharma) at 37°C overnight according to the protocol of the RNA FISH Kit (GenePharma). Nuclei were stained with DAPI.
with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The fluorescence signals were detected using a Zeiss LSM800 confocal microscope (Carl Zeiss AG).

**Cell proliferation assay**

SK-HEP-1 and MHCC97H cells stably overexpressing circSFMBT2 were seeded into 96-well plates (1 × 10^3 cells per well). After incubating with cell counting kit-8 (CCK-8; APExBIO) for 2 h, the cell vitality of each well was evaluated as the absorbance read at a wavelength of 450 nm using a SPARK10 M spectrophotometer (Tecan).

**Transwell migration and invasion assay**

Cells suspended in 200 μL serum-free DMEM (4 × 10^4 cells/well for migration, 6 × 10^4 cells/well for invasion) were added to the cell culture inserts (Falcon), and DMEM supplemented with 20% FBS was applied to the wells of the companion plate (Falcon). Cell culture inserts with or without precoated Matrigel (BD Biosciences) were used to detect cell migratory or invasive abilities according to the manufacturer’s protocol. After incubating the cells for 24 h (for SK-HEP-1) and 48 h (for MHCC97H) at 37°C and 5% CO₂, the cells that migrated to the lower membrane surface were fixed with 4% paraformaldehyde and stained with 1% crystal violet (Beyotime). The migrated and invaded cells were counted in 3 randomly selected fields.

**Wound-healing assay**

SK-HEP-1 and MHCC97H cells were seeded in 6-well plates and cultured to 100% confluence. Straight scratches were made with a 200 μL pipette tip. The images were captured under a microscopic magnification of 100× using a Zeiss LSM800 confocal microscope (Carl Zeiss AG).
200 μL pipette tips, and images were captured 0 and 24 h after the scratch was made.

**Animal experiments**

All animal care and experiments were performed according to the guidelines of the National Institutes of Health and were approved by the Animal Ethics Committee of South China University of Technology. SK-HEP-1 cells were infected with luciferase-overexpressing lentivirus before injection. A total of 1.5 × 10⁶ cells were injected into the tail veins of 4-week-old male BALB/c nude mice as lung metastasis models. Six weeks later, lung metastases were captured using an *in vivo* bioluminescence imaging system (Bruker). Subsequently, lung tissues were excised from sacrificed mice and were fixed using formalin and further used for paraffin sections and H&E staining.

**Dual-luciferase reporter assay**

The full-length sequence of circSFMBT2 was cloned into a psicheck2 vector between the XhoI and NotI sites. A total of 3 × 10⁴ cells were individually seeded in a 24-well plate. After transfection with 300 ng luciferase mimics or negative control sequences for another 24 h, the Renilla luciferase activity, which was normalized to the luminescence of firefly luciferase, was measured with a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol.

**RNA pull-down assay**

First, 10 μL biotin-labeled circSFMBT2 probes (10 μM) or oligo probes (GenePharma) was incubated with 50 μL Streptavidin-Dynabeads M-280 (Thermo Fisher Scientific) at room temperature for 2 h. Second, approximately 1 × 10⁶ cells were cultured in 10 cm
dishes, fixed with 1% formaldehyde, lysed with lysis buffer, and incubated with probe-coated beads at 4°C overnight. Then, the RNA complexes washed from the mixture were extracted by mRNAiso Plus and detected using a qRT-PCR assay.

**Western blotting analysis**

Total proteins were extracted using cell lysis buffer for western blotting and immunoprecipitation (Beyotime) with protease and phosphatase inhibitors (Selleck). Equal amounts of total proteins (30 μg) were separated using 10% SDS-PAGE gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies specific for HBx (1:500 dilution; Abcam), DHX9 (1:1,000 dilution; Abcam), TIMP3 (1:500 dilution; ImmunoWay) and tubulin (1:1,000 dilution; Cell Signaling Technology) at 4°C overnight, followed by incubation with secondary antibodies (1:10,000; Cell Signaling Technology) at room temperature at 1h. Signals were detected using West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific), and the images were acquired using an Optimax X-ray Film Processor (Protec).

**Silver staining**

The gel was washed, fixed, and incubated with buffers in the Pierce Silver Stain Kit (Thermo Fisher Scientific). The desired band was cut down and re-dissolved for liquid mass spectrometry. All experiments were accomplished according to the manufacturers’ protocols.

**RNA IMMUNOPRECIPITATION**

AR total of 5 μg antibody was incubated with 200 μL protein A+G beads at 4°C for 2 h. IgG was used as a negative control. The lysis of SK-HEP-1 (2 × 10^7 cells) were further incubated with antibody-linked beads at 4°C overnight. Protein and RNA of the input, IgG, and RIP samples were finally purified respectively according to the protocol of RNA Immunoprecipitation Kit (Geneseed).

**Sequences of this study**

The sequences of the primers, probes, siRNAs, miRNA mimics, miRNA inhibitors and shRNAs used in this study are listed in Table S2.

**Statistical analysis**

All statistical analyses were performed using SPSS Statistics version 20.0 (IBM). Correlations between circSFMBT2 expression and the clinicopathological characteristics were analyzed using a chi-square test. Two-tailed Student’s t test, two-way ANOVA, the Mann-Whitney U test, and the Wilcoxon signed-rank test were used to compare two experimental groups. Kaplan-Meier analysis was performed to calculate OS and DFS. Results are presented as mean ± SD for parametric tests and median ± interquartile range for nonparametric tests. p values < 0.05 were considered to indicate statistical significance.

**DATA AVAILABILITY**

The data supporting the findings in the present study are available from the corresponding author on reasonable request.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.08.008.

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**AUTHOR CONTRIBUTIONS**

H. Liu, Y.Y., and Z.X. conceived and designed the study. K.M., C.H., and H. Liu, Y.Y., and Z.X. performed the experiments. H. Liu, H. Li, and Z.Z. performed statistical analyses. H. Liu, Y.Y., and C.H. wrote the manuscript. J.W., K.M., and Z.X. revised the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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