Up-Regulated MicroRNA-143 Transcribed by Nuclear Factor kappa B Enhances Hepatocarcinoma Metastasis by Repressing Fibronectin Expression

Xiaoying Zhang, Shanrong Liu, Tingsong Hu, Shupeng Liu, Ying He, and Shuhan Sun

It is increasingly clear that hepatocellular carcinoma (HCC) has a distinct microRNA (miRNA) expression profile that is involved in malignancy; however, little is known about how functional miRNA modulates the metastasis of hepatitis B virus (HBV)-related HCC (HBV-HCC). In the present study, we demonstrate that the levels of miRNA-143 (miR-143) are dramatically increased in metastatic HBV-HCC of both p21-HBx transgenic mice and HCC patients. Moreover, we show that overexpression of this miRNA is transcribed by nuclear factor kappa B (NF-κB) and favors liver tumor cell invasive and metastatic behavior. Intratumoral administration of miR-143 shows that high levels of miR-143 can significantly promote HCC metastasis in an athymic nude mouse model. An in vivo study that used p21-HBx transgenic mice also showed that local liver metastasis and distant lung metastasis are significantly inhibited by blocking miR-143. Additionally, fibronectin type III domain containing 3B (FNDC3B), which regulates cell motility, was identified as the direct and functional target of miR-143 both in vivo and in vitro. Conclusion: Up-regulation of miR-143 expression transcribed by NF-κB in HBV-HCC promotes cancer cell invasion/migration and tumor metastasis by repression of FNDC3B expression. The present study provides a better understanding of the specificity of the biological behavior and thus may be helpful in developing an effective treatment against HBV-HCC. (HEPATOLOGY 2009;51:490-499.)

Hepatocellular carcinoma (HCC) is a common and aggressive cancer that is strongly associated with chronic infection by the hepatitis B virus (HBV).1 Poor prognosis and patient survival with HCC are largely due to invasion/metastasis and postsurgical recurrence.2 The HBV X protein (HBx), a protein encoded by HBV, is thought to play a key role in the molecular pathogenesis of HBV-related HCC (HBV-HCC).2,3 Invasion and metastasis are fundamental properties of HBV-HCC, which has a very high mortality rate. Alteration of some adhesion molecules in HBV-HCC has been described, including up-regulation of matrix metalloproteinases 1-3 and down-regulation of E-cadherin. Both of these changes indicate that HBx contributes to the metastatic spread of liver tumors.2,4 Metastasis is a complex cascade, however, and the underlying molecular mechanisms are far from being fully understood.

MicroRNAs (miRNAs) are evolutionarily endogenous regulatory noncoding RNAs that play critical roles in gene regulation.5 Recent studies implicate miRNAs in several cancers, and altered miRNA levels can result in aberrant expression of gene products that may contribute to cancer biology, including tumor metastasis.5-8 These findings suggest that expression profiling of miRNAs is an alternative method for cancer subtype classification, prognostication, and treatment.5,7,8

It is known that HCC develops several years after HBV infection.2,9 A p21-HBx transgenic mouse model was established by introducing the HBx gene into the p21 locus. About 60% of p21-HBx transgenic mice develop hepatocarcinoma (HCC) with a distinct microRNA (miRNA) expression profile that is involved in malignancy; however, little is known about how functional miRNA modulates the metastasis of hepatitis B virus (HBV)-related HCC (HBV-HCC). In the present study, we demonstrate that the levels of miRNA-143 (miR-143) are dramatically increased in metastatic HBV-HCC of both p21-HBx transgenic mice and HCC patients. Moreover, we show that overexpression of this miRNA is transcribed by nuclear factor kappa B (NF-κB) and favors liver tumor cell invasive and metastatic behavior. Intratumoral administration of miR-143 shows that high levels of miR-143 can significantly promote HCC metastasis in an athymic nude mouse model. An in vivo study that used p21-HBx transgenic mice also showed that local liver metastasis and distant lung metastasis are significantly inhibited by blocking miR-143. Additionally, fibronectin type III domain containing 3B (FNDC3B), which regulates cell motility, was identified as the direct and functional target of miR-143 both in vivo and in vitro. Conclusion: Up-regulation of miR-143 expression transcribed by NF-κB in HBV-HCC promotes cancer cell invasion/migration and tumor metastasis by repression of FNDC3B expression. The present study provides a better understanding of the specificity of the biological behavior and thus may be helpful in developing an effective treatment against HBV-HCC. (HEPATOLOGY 2009;51:490-499.)

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miRNA-143 (miR-143) attracted attention due to three factors. First, all other miRNAs examined exhibited similar high levels (Supporting Fig. 1). Thus, the p21-HBx transgenic mouse is an ideal model to screen candidate miRNAs involved in liver cancer derived from HBV infection. Furthermore, in mice, p21 deficiency does not directly increase the susceptibility to HCC. Given that the elevated expression of HBx is out of physiological context in the transgenic mouse, we assessed the expression levels of HBx in liver samples from p21-HBx transgenic mice and human HBx-HCC, both of which exhibited similarly high levels (Supporting Fig. 1). Thus, the p21-HBx transgenic mouse is an ideal model to screen the candidate miRNAs involved in liver cancer derived from HBV infection. Although HCC containing HBx protein shows enhanced invasive potential, the question remains whether specific miRNAs are involved in its invasion/metastasis. We screened specific candidate miRNA molecules in the livers of p21-HBx transgenic mice using an miRNA array and real-time polymerase chain reaction (PCR) (Supporting Information). Of note, miR-143 (miR-143) attracted attention due to three factors. First, all other miRNAs examined exhibited similar expression patterns in the livers of 10-month old transgenic mice compared with pattern after HCC developed with or without metastasis of varying extent. miR-143, however, was significantly down-regulated in the livers of 10-month old transgenic mice and then dramatically up-regulated after HCC developed especially when accompanied by tumor metastasis to the lung. Second, miR-143 is inconsistent in cancer expression profiles. Third, after examining liver samples from 241 patients, largely with cirrhosis due to hepatitis B, Budhu et al.6 found that miR-143 expression did not change significantly. Murakami et al.14 found that miR-143 was down-regulated in chronic hepatitis and up-regulated in liver cirrhosis. To date, however, the role of miR-143 in regulating HBV-HCC remains unknown.

Based on these findings, we tested whether miR-143 contributes to the cancer biology of HBV-HCC. We characterized the function of miR-143 in vitro with the HCC cell lines and in vivo using athymic nude mice and p21-HBx transgenic mice. Our results show that up-regulated miR-143 transcribed by nuclear factor kappa B (NF-κB) promotes the invasion of HBV-HCC by repressing expression of fibronectin type III domain containing 3B (FNDC3B).

Material and Methods

Samples. Female athymic nude mice (3 weeks old) were purchased from the Chinese Academy of Sciences (Shanghai, China). p21-HBx transgenic mice were a gift from Xiao Yang (Genetic Laboratory of Development and Diseases, Institute of Biotechnology, Beijing, China). All mice were bred and maintained in a pathogen-free facility and were used in accordance with the institutional guidelines for animal care. Human hepatic tissues were obtained with informed consent from patients in Changhaidai Hospital (Second Military Medical University, Shanghai, China). The study was performed in accordance with the guidelines of the Institutional Review Board of the Liver Cancer Institute. HepG2 and Huh7 cells were cultured in minimum essential medium (Gibco-BRL) with 10% fetal bovine serum (Gibco-BRL). Cells were maintained in a humidified 37°C incubator with an atmosphere of 5% CO2.

Reverse Transcription Reaction and Quantitative Real-Time PCR. Total RNAs were purified with the Absolutely RNA Nanoprep kit (Stratagene). Reverse-transcription reactions were performed as described.15 Real-time PCR was performed using a standard SYBR Green PCR kit protocol on a Rotor-Gene RG-3000A (Corbetter Research) thermocycler. The relative expression of miRNA compared with u6 was calculated using the 2−ΔΔCt method.16,17 Real-time PCR for fndc3b has been described.15 Primers are listed in Table 1.

Construction. Myc-tagged NF-κB was a gift from Jie Yang (School of Public Health, Norman Bethune University of Medical Science, Changchun, China). The pEGFP-HBx vector,17 pGL3-FNDC3B (firefly luciferase reporter vector), p-miR-143 (Hsa-miR-143 gene expressing vector), p-miR-mock, PLKO-anti-mock, and PLKO-anti-miR-143 were constructed in our laboratory (Supporting Information). All of the oligonucleotide sequences are shown in Table 1.

Small Interfering RNA Synthesis. Three small interfering RNA (siRNA) sequences (adr 1-3) were designed to
target regions of fndc3b (1, 627-645 bp; 2, 1,813-1,831 bp; 3, 3,91-4,009 bp) as described \(^1\) and were synthesized by GenePharma.

**Transient Transfection.** Transfections were performed using a Lipofectamine 2000 kit (Invitrogen) according to the manufacturer’s instructions and our previous report.\(^2\) Cells (1-3 \( \times \) \( 10^6 \)) grown to a confluency of 50%-60% in 10-cm petri dishes were transfected with different plasmids (24 \( \mu \)g), double-stranded miR-143 mimics (600 pmol, GenePharma), siRNA sequences (600 pmol) or their relative mock sequences, and the cells were harvested 48 hours after transfection.

**Western Blot Analysis.** Total soluble proteins (100 \( \mu \)g) extracted from the samples were resolved on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred electrophoretically to a polyvinylidene fluoride membrane. Blots were blocked with 5% skim milk, followed by incubation with antibodies specific for either FNDCC3B (Sigma) or \( \beta \)-actin (Cell Signaling). Blots were then incubated with goat anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence.

**Measurement of Cell Proliferation.** HepG2 or Huh7 cells transfected with miR-143 (1 \( \times \) \( 10^4 \)) were plated in 96-well plates, and the cells’ proliferation was measured with a proliferation kit (XTT [(2-3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium hydroxide)]) II, Boehringer

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**Table 1. Oligonucleotide Sequences for miR-143 and Plasmids**

| Name          | Sequence                                                                 |
|---------------|--------------------------------------------------------------------------|
| miR-143       | Stem-loop: 5'-GCTCAGACGGGTTCATGTAAGTTGGTGCTTCGTGGTTGCAATCATTAGATCAGCT-3' |
| Primer S      | 5'-AGCAGGGCGGCTGTAATGAGCTCATTCGACGCTAGCAGAAGCTGAG-3'                     |
| Primer A      | 5'-GGCCTGAGATGAGGAGAGCTATAGTGCTGGTTCGTGGAGATCCAGAAGCAGCAGCTG-3'          |
| U6            | Primer S: 5'-GCTTTCCGAGAGACATATAATACAAAATAC-3'                           |
| Primer A      | 5'-GCCCTCAGACGGGTTCATGTAAGTTGGTGCTTCGTGGTTGCAATCATTAGATCAGCT-3'          |
| fndc3b        | Primer S: 5'-GGGAGACAGACCCGTTTTTGGA-3'                                  |
| Primer A      | 5'-GGTGTGGGAGAGAGCTATAGTGCTGGTTCGTGGAGATCCAGAAGCAGCAGCTG-3'             |
| \( \beta \)-actin | Primer S: 5'-GCACACACAGCTTCTCAGAATG-3'                                  |
| Primer A      | 5'-AGACGCTGAGATGAGGAGAGCTATAGTGCTGGTTCGTGGAGAAGCT-3'                    |
| pGL3-FNDC3B   | Primer S: 5'-GCTCAGACGGGTTCATGTAAGTTGGTGCTTCGTGGTTGCAATCATTAGATCAGCT-3' |
| Primer A      | 5'-GGCCTGAGATGAGGAGAGCTATAGTGCTGGTTCGTGGAGATCCAGAAGCAGCAGCTG-3'          |
| PLKO-anti-143 | Primer S: 5'-CGGTTGAGCTACAGTGCTCATCTCAGTTTTTG-3'                        |
| Primer A      | 5'-AATTCAAAAATGAGATGAGCACTGAGTGCATACAACTACA-3'                         |
| PLKO-anti-mock| Primer S: 5'-CGGTTCACTCTTCTTTAGAGGTGCTGTGTGTGTGATTTTTG-3'              |
| Primer A      | 5'-AATTCAAAAATGAGATGAGCACTGAGTGCATACAACTACA-3'                         |
| miRNA mimics  | Has-miR-143 Sense: 5'-UGAGAAGAAGACGUGACUUCUU-3'                        |
|               | Anti-sense: 5'-GACGUACAGUGUUCUCAIUAAU-3'                                |
| miR-mock      | Has-miR-143 AS Sense: 5'-UUCUCGAGGCUACAGUGUCAGUTT-3'                    |
|               | Anti-sense: 5'-ACGGUAGCGCUGUUCGAGAAATT-3'                               |
| Chip-PCR primer | Site 1 Primer-S: 5'-AAATTAGCCGAGCCATGTTG-3'                              |
|               | Primer A: 5'-GACTGTGCTGTTCCCCGCTGCTG-3'                                 |
|               | Site 2 Primer-S: 5'-ACCCATCGCCCTCGACAGATT-3'                             |
|               | Primer A: 5'-CTTTACAGGTGAGCCGAGAAA-3'                                   |
|               | Site 3 Primer-S: 5'-TGAGACATCCAGCGTAGAGTTT-3'                           |
|               | Primer A: 5'-TTTGTCGCTGTGAATATAATCATGTTG-3'                             |
|               | Site 4 Primer-S: 5'-TGCTTGAGCCAGACAGAGGACGACGACGAGGAGAGGAGGACGAGG-3'    |
|               | Primer A: 5'-TCTATGCTCGAAGGGGAAGTGC-3'                                  |
|               | Site 5 Primer-S: 5'-AGAGCCAAGAGGGGCGCTGTGTTT-3'                         |
|               | Primer A: 5'-GGCTGTGCTAGTGGCGCAGCTG-3'                                  |
|               | Site 6 Primer-S: 5'-TGAAAGGAGAGAGTGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG-3'|
|               | Primer A: 5'-TGAGAGCAGAGAGAGTGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG-3' |
|               | Site 7 Primer-S: 5'-TTGGTAAGGAGGAGACGACCCCA-3'                          |
|               | Primer A: 5'-TGAGATCCCAAAGCTTCTTACCTG-3'                                |
|               | Site 8 Primer-S: 5'-AATGAGATGAGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG-3'|
|               | Primer A: 5'-TTGAGAGCAGAGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG-3' |
|               | Site 9 Primer-S: 5'-ACAGGGGGTCTTCCGGAGAGA-3'                            |
|               | Primer A: 5'-TTGAGAGCAGAGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG-3' |
|               | Negative Primer-S: 5'-CATAAGACATGGGAAGGAGGAGA-3'                        |
|               | Primer A: 5'-GAAAGGAGGACAGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG-3' |
Mannheim, Germany). Optical density was read with a microplate reader (BIO-RAD).

**Flow Cytometric Analysis.** HepG2 or Huh7 cells transfected with miR-143 (5 × 10^5) were plated in 6-well plates and apoptosis inducers A and B (1:1,000, Beyotime) were added to the culture. After a 24-hour incubation, the cultures were stained with annexin V–fluorescein isothiocyanate and apoptosis rates were analyzed using a flow cytometer (FACSCalibur, BD Biosciences).

**Transwell Invasion Assay.** HepG2 or Huh7 cells transfected with miR-143 or siRNA against fndc3b (1 × 10^5) were plated in medium without serum in the top chamber of a transwell (24-well insert; pore size, 8 mm; BD Biosciences). The invasive activity of these two cell lines was then analyzed as described.

**Evaluation of NF-κB Activity.** Nuclear extracts from cells and livers were prepared by using the Nuclear Extraction Kit (Active Motif) according to the manufacturer’s instructions. NF-κB activation was quantified by way of enzyme-linked immunosorbent assay using the TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif) according to the manufacturer’s instructions.

**Luciferase Reporter Assay.** HepG2 cells (5 × 10^4 per well) were seeded in a 24-well plate the day before transfection and transfected with pGL3-FNDC3B (500 ng), Renilla luciferase control vector (50 ng, pRL-TK-Pro-mega), and miR-143 or mock (15 pmol miRNA mimics, GenePharma). Assays were performed 48 hours after transfection using the dual luciferase reporter assay system (Promega).

**Chromatin Immunoprecipitation Assay.** Chromatin immunoprecipitation (ChIP) assay was performed with HepG2 cells transfected with a vector expressing pEGFP-HBX or pEGFP alone using a ChIP assay kit (Agilent) according to the manufacturer’s instructions. Protein–DNA complexes were precipitated with and without anti–NF-κB p65 (Millipore) at 4°C overnight with rotation. PCR was performed with primers specific for human mir-143 (Table 1).

**Athymic Nude Mouse Surgery, Necropsy, and Histopathology.** An orthotopic transplantation nude mouse model was established using HepG2 cells as described. The transplanted nude mice were randomly divided into two groups (n = 15 each). p-miR-mock or p-miR-143 was directly injected into the implanted tumor, thrice weekly (1 μg each) for 3 months. After 3 months, the mice were sacrificed, and liver tumors and lung tissues were analyzed using histopathology.

**p21-HBx Transgenic Mouse Treatment, Necropsy, Histopathology, and Immunohistochemistry.** Seventeen-month-old male p21-HBx transgenic mice were used in the present study. Mice were injected with PLKO-anti-miR-143 (n = 15) or PLKO-anti-mock (n = 15) via the tail vein twice weekly (35 μg each). After 6 months of injections, the mice were euthanized. The livers and lungs were analyzed using histopathology and immunohistochemistry.

**Statistical Analysis.** The Student t test was used to compare two groups unless otherwise indicated (χ^2 test). The difference was deemed statistically significant at P < 0.05.

**Results**

**miR-143 is Significantly Up-Regulated in HBV-Related HCC.** First, we assessed the aberrant expression of miR-143 in p21-HBx transgenic mice as described (Supplementary Information). The livers in 10-month-old transgenic mice lacked neoplastic changes and exhibited significantly reduced miR-143 expression levels compared with wild-type mice (Fig. 1A). Surprisingly, miR-143 increased approximately two-fold in livers with tumors but without lung metastasis and approximately six-fold in livers with tumors and lung metastasis, compared with livers without tumors from 22-month-old transgenic mice.

Next, we determined whether miR-143 was expressed differentially in human HCC. Twenty-five pairs of primary HCC and adjacent noncancerous hepatic tissue samples were collected from clinical patients (Supporting Table 1): HBV-negative HCC without metastasis (n = 5); HBV-negative HCC with lung metastasis (n = 7); HBV-HCC without metastasis (n = 5); and HBV-HCC with metastasis to lung (n = 8). Compared with the adjacent noncancerous hepatic tissues, miR-143 was significantly up-regulated by approximately four-fold among the HBV-HCC samples without lung metastasis and eight-fold among the HBV-HCCs with lung metastasis. Very slight up-regulation of miR-143 was shown in HBV-negative HCC with or without metastasis (Fig. 1B).

In order to verify whether this up-regulation correlated with HBx expression, HepG2 cells were transiently transfected with pEGFP-HBx and pEGFP. Compared with pEGFP transfected cells (pEGFP), miR-143 was up-regulated in HBx-HepG2 cells (Fig. 1C).

**MiR-143 Promotes Cell Migration and Invasion In Vitro.** Next, we wanted to determine whether miR-143 up-regulation would affect cancer biology. The proliferation potential and induced apoptosis of cells transfected with miR-143 or its mock were analyzed. miR-143 overexpression had no significant effect on cell proliferation or
apoptosis in vitro (Fig. 2A,B). Therefore, we evaluated whether miR-143 contributed to the metastasis of HBV-HCC. Compared with the blank or mock group, cell migration was significantly increased in cells transfected with miR-143 mimics (Fig. 2C,D). These results indicate that miR-143 up-regulation could promote cell migration. In order to demonstrate whether miR-143 is involved in the enhanced invasion capability elicited by HBx, cells were then either transfected with pEGFP-HBx (HBx) or cotransfected with miR-143 antisense (AS) oligonucleotides (HBx/H11001miR-143 AS) or with mock AS (HBx + mock AS). As shown in Fig. 2C and 2D, transfection of HBx dramatically enhanced invasive activity compared with the EGF vector group. Remarkably, the invasion of HBx-expressing cells was markedly inhibited by the transfection of miR-143 AS, whereas migration was not altered significantly in cells that were cotransfected with mock AS. This result strongly suggests that HBx promotes migration and invasiveness of the cells, at least in part by increasing the level of miR-143.

miR-143 Is Directly Regulated by the Transcription Factor NF-κB. We wanted to determine how transcription of miR-143 was controlled under our experimental conditions. HBx does not bind DNA directly; its multi-regulative functions are mediated by its interactions with host factors, such as activator protein-1 and -2, NF-κB, and ATF2.9 NF-κB transcriptional activity was significantly enhanced through interacting with HBx in HBV-HCC.21 Under our experimental set-up, NF-κB transcriptional activity dramatically increased in vitro (Fig. 3A) and in vivo (Fig. 3B). We performed a computational screen (http://microrna.sanger.ac.uk; http://jaspar.genereg.net) and found that NF-κB localizes within the mir-143 gene transcriptional element. We further
found that transfection of the NF-κB encoding sequence into HepG2 cells resulted in significantly increased miR-143 levels (Fig. 3C). This result strongly suggests that miR-143 up-regulation is due to the enhanced transcriptional activity of NF-κB. To verify this hypothesis, we performed ChIP assays to determine whether NF-κB controls miR-143 expression by binding directly to the mir-143 gene transcriptional element. We examined the 10-kb genomic sequence upstream of the human mir-143 stem-loop and identified nine putative binding sites (Fig. 3D). We designed nine PCR amplicons to check for the presence of these putative binding sites in the chromatin immunoprecipitates (Table 1). NF-κB bound to site 5 (5.06-kb upstream), which means miR-143 can be directly regulated by specific binding of NF-κB to its putative promoter (Fig. 3E). Together, these data provide strong evidence that miR-143 is directly transcribed by NF-κB specifically binding of NF-κB to its putative promoter.

**miR-143 Represses FNDC3B Expression.** To elucidate the mechanisms by which miR-143 induces tumor cell migration, we performed a TargetScan (Release 4.2, April 2008; http://www.targetscan.org/) to help identify miR-143 targets. Among the approximately 250 candidate genes (Supporting Table 2), FNDC3B was one of the high-scoring candidates. FNDC3B is a member of the fibronectin family, which was previously reported to be distinctly down-regulated in tumor cells with high metastatic potential and thus attracted our interest. As shown in Fig. 4A (left), the FNDC3B-encoded messenger RNA (mRNA) contains a 3′ untranslated region (UTR) element that is partially complementary to miR-143, indicating that miR-143 would directly target this site. In order to verify this finding, a luciferase reporter assay was performed. miR-143 can effectively inhibit luciferase activity of pGL3-FNDC3B, whereas no significant reduction in luciferase reporter activity was observed when cells were cotransfected with miR-152 (noncognate miRNA) or mock (Fig. 4A, right). Finally, we tested FNDC3B mRNA and protein levels in HepG2 with and without
miR-143 mimic transfection by way of reverse-transcription polymerase chain reaction (RT-PCR) and western blotting to determine how miR-143 acts through mRNA degradation or translational repression. As shown in Fig. 4B, fndc3b mRNA levels were not significantly affected, whereas protein levels decreased substantially after treatment with miR-143. These results strongly indicate that FNDC3B gene expression is directly translationally suppressed by miR-143.

### Down-Regulation of FNDC3B Enhances the Migration and Invasion Capability of Hepatocarcinoma Cells In Vitro

Having documented that FNDC3B was the target of miR-143, we next sought to determine the effect of FNDC3B on hepatocarcinoma cell migration and invasion in vitro. We first detected FNDC3B expression in HepG2 carcinoma cells through immunohistochemical staining. As indicated in Fig. 4C, FNDC3B was expressed at high levels in HepG2 cells. Subsequently, we transfected three different siRNA sequences against human fndc3b (adr1-3) into HepG2 cells. Forty-eight hours later, the level of fndc3b mRNA in HepG2 was quantified using real-time PCR and shown to be reduced by 35%, 84%, and 48%, as compared with mock (Fig. 4D, left). The reduction in FNDC3B protein expression mediated by adr 2 was further confirmed through western blotting (Fig. 4D, right), which was used in the following in vitro analyses. A Matrigel invasion assay was performed as described above to assess the effect on the migration and invasion capabilities of HepG2 cells. In comparison with adr-mock (mock), the silencing of fndc3b by adr 2 increased the invasive properties of these cells by more than five-fold (Fig. 4E). Furthermore, FNDC3B was greatly down-regulated in human HCC, especially in metastatic HCC (Fig. 4F), which coincided with miR-143 expression (Fig. 1B).

### Intratumoral Administration of miR-143 Promotes Tumor Invasion In Vivo

Having documented that high levels of miR-143 significantly enhance tumor cell migration activity in vitro, we next determined whether overexpression of miR-143 could promote tumor invasion in vivo. HepG2 cells, which show weak metastasis activity, were used in an orthotropic transplantation nude mouse model to study the role of miR-143 in tumor metastasis. Following treatment with p-miR-143, there were seven cases of intrahepatic metastasis and two cases of lung metastasis (47% and 13%, respectively) (Fig. 5A). The nude mice exhibited regional, intrahepatic, and pulmonary metastasis. These metastases comprised small visible metastatic colonies that were distributed around local tumors and a few dominant multiple metastases (Fig. 5B,C). In contrast, there were no metastatic cases following intratumoral administration of p-miR-mock. To determine whether miR-143 negatively regulated FNDC3B expression in the orthotopic liver tumors in vivo, FNDC3B protein levels were detected by way of western blotting. As shown in Fig. 5D, FNDC3B expression was much stronger in the nude mice treated with p-miR-mock compared with those treated with p-miR-143. Taken together, these observations indicate that high levels of miR-143 expression can drive tumor invasion and metastasis.
metastasis by repressing FNDC3B expression in HCC in vivo.

**Blocking miR-143 Inhibits HBV-HCC Metastasis in p21-HBx Transgenic Mice.** Having demonstrated that miR-143 promotes metastatic behavior of cancer cells by negatively regulating FNDC3B expression in vitro and in vivo, we then determined whether blocking miR-143 could prevent tumors that overexpress miR-143 from metastasizing in vivo. Because miR-143 was significantly up-regulated in developed hepatocarcinoma in p21-HBx transgenic mice, this model should exhibit multifocal tumors (local metastasis) and lung tumors (distant metastasis) at old age. This model is therefore used for in vivo analysis of the treatment effect on HCC metastasis of blocking miR-143 expression. To this end, PLKO-anti-mock and PLKO-anti–miR-143 were administered to p21-HBx transgenic mice. Six months later, all mice were sacrificed. First, the levels of miR-143 expression in the livers were measured by RT-PCR. As shown in Fig. 6A, miR-143 was expressed at high levels in the livers of mice with the anti-mock injection, which was consistent with the real-time PCR results shown in Fig. 1A. In contrast, miR-143 was dramatically reduced in the livers of mice treated with anti–miR-143. In parallel, FNDC3B expres-

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**Fig. 5.** Intratumoral administration of miR-143 promoted HCC metastasis in vivo. (A) Incidence of local and distant metastasis in transplanted nude mice treated with p-miR-143 or p-miR-mock. (B) Histopathological analysis of liver tissues from transplanted nude mice treated with p-miR-143 or p-miR-mock. (C) Histopathological analysis of lung tissues from transplanted nude mice treated with p-miR-143 or p-miR-mock. Bars represent 100 μm. Arrows indicate HCC nodules. (D) Western blotting of FNDC3B in the livers of transplanted nude mice treated with p-miR-143 or p-miR-mock.

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**Fig. 6.** Blocking miR-143 inhibited HCC metastasis in vivo. (A) RT-PCR of miR-143 in p21-HBx transgenic mouse liver tissues treated with PLKO-anti-miR-143 (anti–miR-143) or PLKO-anti-mock (anti-mock). (B) Immunohistochemical staining of liver tissues from 22-month-old p21-HBx transgenic mice treated with anti–miR-143 or anti-mock to detect FNDC3B expression. Bars represent 20 μm. (C) Western blotting of FNDC3B in the livers of 22-month-old p21-HBx transgenic mice treated with anti–miR-143 or anti-mock. (D) Incidence of local and distant metastasis in p21-HBx transgenic mice treated with anti–miR-143 or anti-mock.
sion was detected through immunohistochemical staining. In the anti-mock group, the level of FNDC3B in the livers was negligible; when miR-143 was reduced by anti–miR-143 injection, FNDC3B expression was markedly up-regulated (Fig. 6B). This result was also confirmed through western blotting (Fig. 6C). These results show that high levels of miR-143 were successfully reduced by administration of anti–miR-143.

In addition, necropsy was performed on all sacrificed mice. In the anti-mock group, nine out of 15 transgenic mice developed HCC tumors. Furthermore, 100% of mice (9/9) had liver metastases, and 78% of mice (7/9) had lung metastases. In the anti–miR-143 group, eight of 15 transgenic mice developed HCC. The HCC tumors, however, were mostly single-focal, with only 25% of mice (2/8) displaying liver metastasis and 12.5% of mice (1/8) displaying lung metastasis (Fig. 6D).

Collectively, these results show that repression of miR-143 levels by in vivo anti–miR-143 administration can effectively inhibit metastasis for tumors that overexpress miR-143.

**Discussion**

miR-143 was dramatically up-regulated in HBV-HCC, especially in those with metastasis. This result is inconsistent with some reports that showed down-regulation of miR-143 in the development of cancers. Some reports found that miR-143 was overexpressed in cancer samples. Akao et al. found that hsa-miR-143 expression levels were extremely reduced in the colon cancer cell lines DLD-1 and SW480. However, Bandres et al. reported that hsa-miR-143 expression was not detected in any of 15 tested colorectal carcinoma cell lines, including DLD-1 and SW480. Paradoxically, Nakajima et al. claimed that the hsa–miR-143 expression levels were not significantly altered in Japanese clinical colorectal tumor samples. As for the miR-143 expression profile in liver samples, Budhu et al. did not find that miR-143 was down-regulated in the colon cancer cells DLD-1 and SW480. However, Murakami et al. found miR-143 to be down-regulated in chronic hepatitis and up-regulated in liver cirrhosis. Several factors could lead to these inconsistent results. First, the expression of certain miRNAs in tumor specimens may differ from each other in vivo. Second, there were discrepancies in microarray analysis for sample origins, hybridization conditions, and so forth. Third, this discrepancy could be due to the multifunctional nature of microRNAs. Aside from its involvement in cancer behavior, miR-143 was once reported to play a critical role in adipocyte differentiation through the target gene ERK5. miR-17-5p, another well-known miRNA, could target pro- and antiproliferative genes and act as both an oncogene and a tumor suppressor in different cellular contexts. Taken together, these findings indicate that miR-143 has the potential to modulate cell biology by regulating the expression of different targeting genes.

We found that there is a pathway mediated by miR-143 that promotes the metastasis of HCC derived from HBV infection. A specific complex network, which contains several key molecules, controls the metastasis of HBV-HCC (Fig. 7). NF-κB is a critical member of this network. NF-κB can be significantly induced by HBx and is critically involved in tumor progression due to its transcriptional regulation of invasion-related factors such as matrix metalloproteinase 9 and vascular endothelial growth factor. In addition, fibronectin is regulated by NF-κB through miR-143. Cell attachment on fibronectin activates NF-κB, and fibronectin type III promotes cell type–specific adhesion. miR-143 is controlled by NF-κB, but other molecules such as HBx and Raf kinase inhibitor protein could also influence the levels of miR-143 by modulating the transcriptional activity of NF-κB. The novel HBx/NF-κB/miR-143/FNDC3B pathway is an important complement to the network. The distinct role of high levels of miR-143 involved in metastasis elucidates the HBV-HCC invasion–metastasis cascade and sheds new insight on treatment of HBV-HCC.

The present work indicates that high miR-143 levels could promote tumor metastasis through FNDC3B in vitro and in vivo. Although this was first identified in HCC developed in p21-HBx transgenic mice and in patients with HBV infection, we cannot conclude that this pathway is specifically involved in the metastasis of HBV-HCC. Under our experimental conditions, miR-143 was directly controlled by NF-κB transcriptional activity. Overexpression of NF-κB is always observed in cancers (including colorectal carcinoma), varying levels of miR-
499

143 expression may be due to the epigenetic modifications.

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