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Original Paper

MicroRNA-197 Promotes Metastasis of Hepatocellular Carcinoma by Activating Wnt/β-Catenin Signaling

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Key Words
Hepatocellular carcinoma • MicroRNA • EMT • Metastasis • Wnt/β-catenin

Abstract
Background/Aims: MicroRNA-197 (miR-197) has been shown to play roles in epithelial-mesenchymal transition (EMT) and metastasis. The Wnt/β-catenin pathway is associated with EMT, but whether miR-197 regulatesWnt/β-catenin remains unclear. This study was to demonstrate the role of miR-197 on the Wnt/β-catenin pathway in hepatocellular carcinoma (HCC). ***Methods:*** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to detect the expression of miR-197 in 105 HCC specimens and 15 HCC cell lines. We tested the predicted target gene of miR-197 using a genetic report system. The role of miR-197 in HCC cell invasion and migration (wound healingand cell invasion and migrationby Transwell assays) and in an HCC xenograft modelwas analyzed. ***Results:*** Using a miRNA microarray analysis of HCC specimens and compared with non-metastatic HCC, miR-197 was identified as one of the most upregulated miRNAs in metastatic HCC. miR-197 expression was positively associated with the invasiveness of HCC cell lines. Metastatic HCC cells with high miR-197 expression had Wnt/β-catenin signaling activation. High levels of miR-197 expression also promoted EMT and invasionHCC cells *in vitro* and *in vivo*. miR-197 directly targeted Axin-2, Naked cuticle 1 (NKD1), and Dickkopf-related protein 2 (DKK2), leading to inhibition of Wnt/β-catenin signaling. High miR-197 expression was found in HCC specimens from patients with portal vein metastasis;high miR-197 expression correlated to the expression of Axin2, NKD1, and DKK2. ***Conclusion:*** miR-197 promotes HCC invasion and metastasis by activating Wnt/β-catenin signaling. miR-197 could possibly be used as a prognostic marker and therapeutic target for HCC.

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Introduction

Hepatocellular carcinoma (HCC) has a high incidence and poor prognosis due to high rates of intra- and extra-hepatic metastasis and postsurgical recurrence [1]. Metastasis is a hallmark of cancer and the main cause of cancer-related deaths [2]. Epithelial-mesenchymal transition (EMT) occurs in many typesof cancers (including HCC) and is the initial step of metastatic progression [3-6].EMT is characterized by the disruption of the tight junctions betweencells and by the acquisition of new functionssuch asmigration and invasion [7, 8]. EMTis a potential target for drugs that could prevent or hinderthe metastatic spread of cancers [7, 9].

Wnt/β-catenin signaling is the main mechanism involved in EMT or progression of cancer [10-12]. Sustained activation ofβ-catenin signaling is reported in about 50% ofHCC[13, 14]. When Wnt/β-catenin signaling is not activated, a destruction complex made of Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3), and casein kinase 1α (CK1α) is present in the cytoplasm. This destruction complex would normally degrade β-catenin by targeting it for ubiquitination and proteasome digestion [13-16].As a consequence of Wnt/β-catenin signaling activation, β-catenin dissociates from the destruction complex [15, 16], accumulates in the cytoplasm, translocates to the cell nucleus, and recruits to the lymphoid enhancer factor and T-cell factor (LEF/TCF), which are transcription factors that induce downstream gene transcription involved in a number of processes involved in cell proliferation and survival [17]. Wnt signaling is inhibited by the Wnt inhibitory factor-1 (WIF1), naked cuticle-1 (NKD1), and Dickkopf (DKK) [15, 16]. Because Wnt/β-catenin is associated with cancer and EMT, understanding how Wnt/β-catenin signaling is regulated is akey for the development of anti-metastatic drugs.

MicroRNAs (miRNAs) bind to the 3′-untranslated region (UTR) of their target mRNA [18, 19]. Dysregulation of miRNAs occurs in many cancers [20-24]and miRNAs play essential roles in EMT[19, 25]. miR-197 is significantly dysregulated in a wide range of cancers such as lung cancer [26], breast cancer [27], ovarian cancer [28], HCC[29, 30], colorectal cancer [31], thyroid cancer [32], head and neck carcinoma [33], and prostate cancer [34], as well as in non-alcoholic fatty liver disease [35] and primary biliary cirrhosis [36]. Furthermore, miR-197 plays a vital role in cell proliferation, apoptosis, cell differentiation, metastasis, and drug resistance [28, 29, 31, 37]. miR-197 promotes cell invasion and metastasis through directly targeting mRNAs like CD82/KAI1[38], p120 catenin [39], and nemo-like kinase (NLK)[28], and indirectly targeting mRNAs like Rac1 and ROCK [38]. Nevertheless, the role of miR-197 in EMT and Wnt/β-catenin signaling remains largely unknown. There are only two studies on the impact of miR-197 on EMT[39, 40], but the regulation mechanisms are not clear.

Considering the important role of Wnt/β-catenin signaling in cancer development (including HCC) and the involvement of miR-197 with multiple genes, the present study aimed to examine the expression of miR-197 in clinical HCC specimens, cell lines, and xenografts, and to determine the possible mechanisms.

Materials and Methods

Cell culture

The HCC cell lines QGY7703, QGY-7701, SMMC-7721, HepG2, Hep3B PLC/PRF5, Huh7, HepG2.2.15, HCCC-9810, Bel-7402,hepatoma cell line BEL-7404, HCCLM3, HCCLM6, MHCC97L, and MHCC97H were obtained from the Guangdong Provincial Key Laboratory of Liver Disease. All cell lineswere cultured in Dulbecco modified Eagle (Carlsbad, CA; Invitrogen, America) with 10% fetal bovine serum (FBS TM Hyclone; Thermo Fisher Scientific, Waltham, MA, USA). The immortalized normal liver epithelial cells THLE3 were maintained in bronchial epithelial growth medium (Clonetics Corporation, Walkersville, MD 21793), supplemented with 5 ng/ml of epithelial growth factor, 70 ng/ml of phosphoethanolamine, and 10% fetal bovine serum, at 37°C in a humidified atmosphere containing 5% CO_2.

Human liver cancer specimens

We obtained 105 paraffin-embedded HCC specimens from the Sun Yat-Sen University Cancer Center (Guangzhou, China), allfrom patients who underwent surgery between 2000 and 2005. There were 37 patients with portal vein metastasis and 68 without.Twentyfresh HCC tissue samples were obtained from HCC patients who underwent surgery at the Sun Yat-Sen University Cancer Center (Guangzhou, China). These samples were frozen and stored in liquid nitrogen. Tumor-node-metastasis (TNM) staging was performed according to the 2017 recommendations from the American Joint Committee on Cancer [41]. This study was approved by the Institutional Research Ethics Committees of Sun Yat-Sen University Cancer Center and Third Affiliated Hospital of Sun Yat-Sen University. Demographic data are summarized in Table S1 (for all supplemental material see www.karger.com/10.1159/000495242/).

Animal studies

We purchased 20 BALB/c nude mice (4 weeks of age, 12-14 g body weight) from the Institute of MateriaMedica (Chinese Academy of Sciences, Beijing, China). They were kept under pathogen-free conditions. This study was conducted in accordance with the Laboratory Animal Care and Use Guidelines by the National Institutes of Health. The Animal Care and Use Committee of Sun Yat-Sen University approved the protocol. For the lung metastasis assay, 7×10^6 Huh7-vector or Huh7-miR-197 cells [suspended in 100 μL of sterile phosphate-buffered saline (PBS)] were injected into the tail vein of eachBALB/c nude mouse. The mice were weighed weekly and sacrificed at 43 days after injection (n=5 mice per group).

We used 5×10^5 MHCC97H cells for *in vivo* antagomir administration. We injected 100 μl of miR-197 antagomir (2 mg/ml; Applied biomaterial Corporation, Richmond, Canada; in PBS) or vehicle via the tail vein every 2 days (n=5 mice per group). After 35 days, the mice were sacrificed and the lungs were removed. Surface nodules were counted and subjected for hematoxylinand eosin (H&E) staining.

Bioinformatics

The microarray data wasfrom the Gene Expression Omnibus database (GEO GSE26323 and GSE67139). DIANA-mirPath, TargetScan 3.1, and miRanda were used for bioinformatics analysis.

Plasmids

The pre-miR-197 gene was cloned into the pMSCV-puro plasmid (Clontech Laboratories Inc., Mountain View, CA, USA) to generate the pMSCV-miR-197 plasmid. We co-transfected pMSCV-miR-197 with the pIK packaging plasmid in 293FT cells and collected the supernatant after 36 h. Huh7 and PLC/PRF-5y cells were incubated in the supernatant and polybrene (2.5 μg/ml) for 24 h. After incubation, puromycin(1.5 μg/ml) was used to select stably transduced cells for 10 days. The open reading frames of NKD1, Axin2, and DKK2 were cloned into the pcDNA 3.1 vector (Invitrogen Inc. Carlsberg, CA, USA). The 3′ UTRs of NKD1, Axin2, and DKK2 were cloned in the pGL3 control vectorwith the wild type plasmid (cctttgatc; the top flash (cctttggcc) or FOP flash mutation; TCF/LEF) (Lake Village, NY, USA).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

We extracted the total RNA from the tissue samples and cultured cells with the TRIzol reagent (Invitrogen Inc., Carlsbad, CA, USA). We analyzed the miRNA levels by qRT-PCR using the TaqMan MicroRNA Assay kit (Applied Biosystems, Foster City, CA, USA). The expression of miR-197 was normalized to the expression of U6.

Western blot

Total proteins were extracted from cell samples with sodium dodecyl sulfate (SDS) lysis buffer (Sigma, St Louis, MO, USA). The lysate was diluted in SDS sample buffer (KeyGen Biotech Co., Beijing, China). The proteins wereseparatedby SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidenedifluoride (PDVF) membranes. The membranes wereblocked with skimmed milk(5%, w/v). The following primary antibodies were incubated with the membranes overnight at 4°C: anti-E-cadherin, anti-α-catenin, anti-β-catenin, anti-vimentin, and anti-fibronectin (1:1000, Pharmingen, BD Biosciences, Franklin Lake, NJ, USA); anti-NKD1 (1:1000, Santa Cruz Biotechnology, CA, USA), anti-TCF4, anti-LEF1, anti-Axin2, anti-DKK2, and GAPDH (1:1000, Abcam, Cambridge, MA, USA); and anti-EF1α (1:1000, Upstate Biotechnology, Lake Placid, NY, USA).

Nuclear protein extraction

A Nuclear Extraction Kit (Active Motif, Carlsbad, CA, USA) was used to extract the nuclear proteins, according to the manufacturer's instructions.

In vitro invasion assay

Invasion was evaluated*in vitro* using previously established methods [42] and included wound healing, cell invasion, and cell migration. For the wound healing assay, the cells were cultured in 6-well plates to 90% confluence. The monolayer was scratched with a sterile plastic micropipette tip to generate a homogenous wound. Cell migration was observed at 0, 24, and 36 h after wounding.

Cell invasion and migration were assessed using the Transwell chambers assay (Corning Inc., Corning, NY, USA). In the lower chamber of the Transwell chamber, 500 µlof DMEM with 10% FBS were added as chemoattractant. After 24 h, the cells invading into the bottom side of the topchamber were fixed with 70% ethanol for 30 min and stained with 0.1% crystal violet for 15 min. The cells were then photographed and quantified in five random fields.

Luciferase reporter assay

Cells were grown to 80% confluence and transfected with a miR-197 mimic or miR-NC in combination with WT-NKD1/Axin2/DKK2-3'-UTR or MT-NKD1/Axin2/DKK2-3'-UTR. The respective plasmids and 1 ng of pRL-TK Renilla plasmid were transfected into the cells using Lipofectamine 2000 (Life Technologies Co., Grand Island, NY, USA). The double luciferase reporter gene detection kit was used to determine luciferase activity (Promega, Madison, WI, USA).

Three-dimensional (3D) invasion assay

Briefly, 1000 Huh7, PLC/PRF-5, and MHCC97h cells were distributed on 2% Matrigel-coated 24-well plates. The growth medium was changed every 2 days. Cells spheroids were photographed.

Immunofluorescence

HCC cells (2×10^4) were cultured on cover slips and transfected with miR-197, miR-197 inhibitor, or relative controls. Forty-eight hours later, the cells were washedwith 1×PBS, fixed in 4% paraformaldehyde for 20 min, and blocked with 5% BSA(w/v) for 40 min at room temperature. The primary antibody against β-catenin was incubated overnight at 4°C. The fluorescein isothiocyanate (FITC)-conjugated secondary antibody was added and incubated for 2 h. Fluorescence images were captured using a fluorescence microscope (PRIMOSTAR-FL2; Carl Zeiss GmbH, Oberkochen, Germany).

Immunohistochemistry

Sections (2µm) from HCC paraffin-embedded specimens were used for immunohistochemistry (IHC). All sections were treated routinely with the EnVision system (Dako, Glostrup, Denmark). The primary antibodies were anti-NKD1 (Santa Cruz Biotechnology, CA, USA, 1:400), anti-Axin2, and anti-DKK2 (Abcam, Cambridge, MA, USA,1:400). IHC results were evaluated in a double-blind manner by two experienced pathologists using the 0-4scoring system. The results of IHC staining were scored 0-12. First, staining was divided into 0-3 points according to the staining strength. Then, the percentage of positive cells was evaluated: <5% was 0, 5-25% was 1, 25-50% was 2, 50-75% was 3, and >75% was 4. The two scores were multiplied. The mean score was used for analysis [43-45].

Statistical analysis

The continuous data were tested for normal distribution using the Kolmogorov-Smirnov test and were found to be normally distributed. Continuous data werepresented as the means of three independent experiments ± standard error of the mean, and analyzed using the Student t test. Statistical analysis was carried out usingSPSS 13.0 (SPSS Inc., Chicago, IL, USA). The Kaplan-Meier method was used to analyze overall survival. Differences were considered statistically significant at two-sided P-values <0.05.

Results

miR-197 was overexpressed in metastatic HCC

We assessed the differential miRNA expression in clinicalHCC specimens, including 10 specimens from patients with vascular invasion and 10 from patients without vascular invasion. By comparing published miRNA expression profiles obtained from metastatic and non-metastatic HCC specimens (NCBI/GEO/GSE67139 and GSE26323), we found that 17 miRNA were differentially expressed between metastatic and non-metastatic HCCsamples (Fig. 1A). Among these differentially expressed miRNAs, miR-197 was the most upregulated (Fig. 1B). Because of this observation and because miR-197 is relatively unstudied, we selectedmiR-197 for further analysis.

Using qRT-PCR, we found that HCC cell lines had higher miR-197 expression than the normal liver epithelial cell line THLE3 (Fig. 1C). The results suggested that miR-197 was expressed more highly in the cell lines with high metastatic ability (HCCLM3, HCCLM6, MHCC97L, and MHCC97H) than in non- or low-metastatic HCC cell lines (QGY-7703, QGY-7701 SMMC-7721, HepG2, Hep3B, PLC/PRF5, Huh7, HepG2.2.15, HCCC-9810, BEL-7402, and BEL-7404) (Fig. 1C).

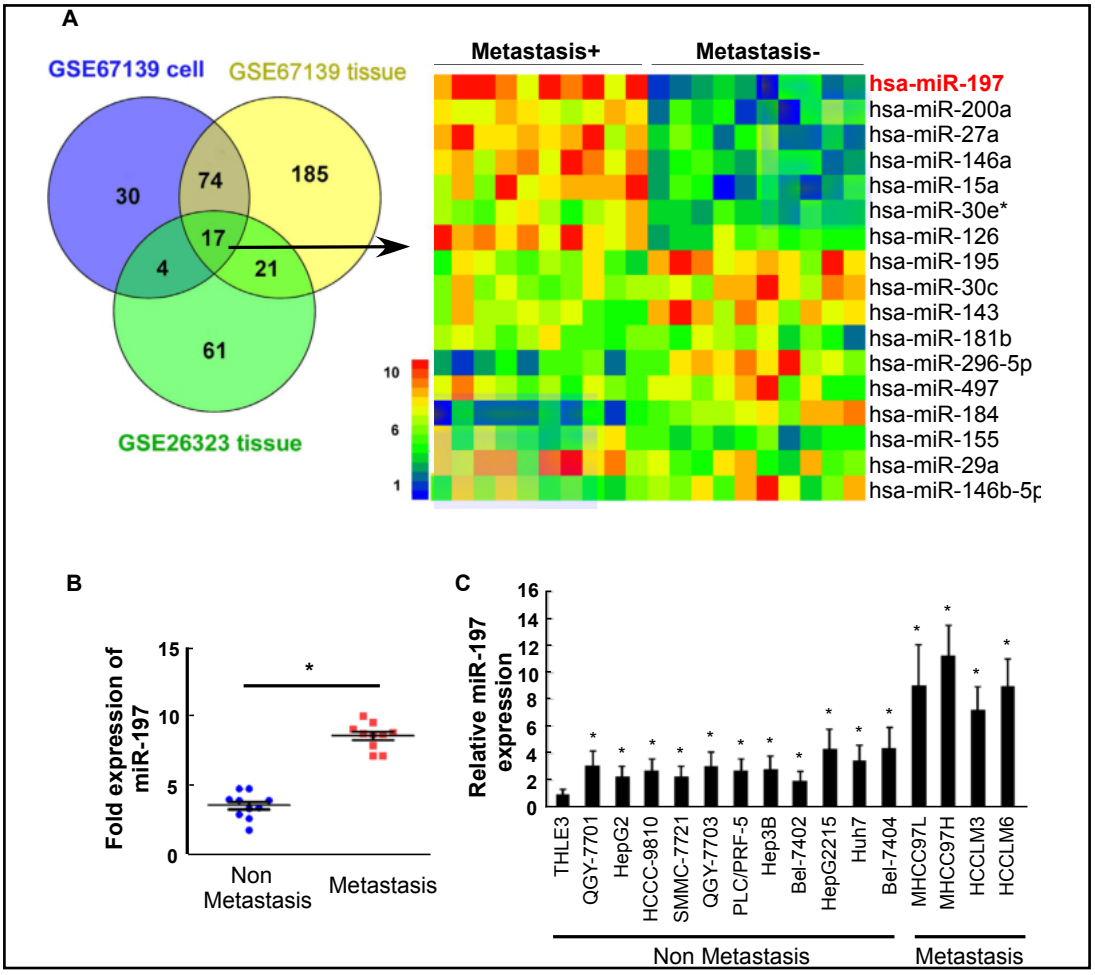

Fig. 1. miR-197 is highly expressed in metastatic hepatocellular carcinoma. (A) miRNA array analysis of differentially expressed miRNAs in HCC patients with and without lung metastasis. The colors indicate the intensity relative to the controlcells. (B) qRT-PCR analysis of miR-197 expression in 10 HCC clinical fresh tissue samples with metastasis paired with non-metastasis HCC tissues. *P<0.05. (C) qRT-PCR analysis of miR-197 expression in highly metastasis and non-metastasis HCC cell lines.

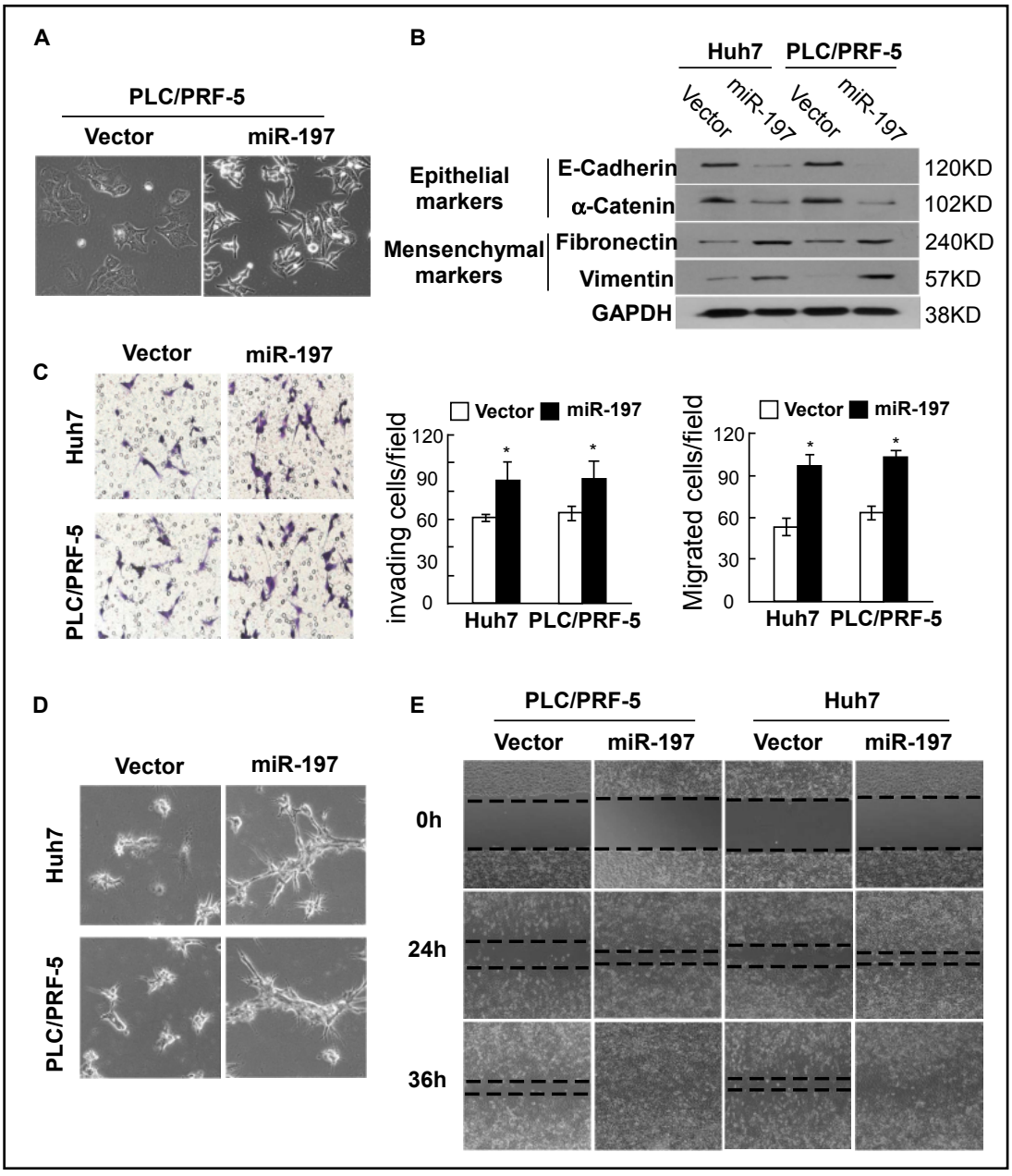

Fig. 2. The overexpression of miR-197 induces epithelial-mesenchymal transition of hepatocellular carcinoma cell lines in vitro.(A) Phase-contrast microscopy showing cell morphology change after miR-197 overexpression. (B) Western blot showing expression of EMT markers in indicated cells. (C) Representative images and quantification of cell invasion and migration assays with Huh7 and PLC/PRF-5 cells in five random fields. (D) Representative images of 3D spheroid structure of Huh7 and PLC/PRF-5 cells. (E) Images of wound healing assay with Huh7 and PLC/PRF-5 cells at 0, 24 hours and 36 hours after injury.

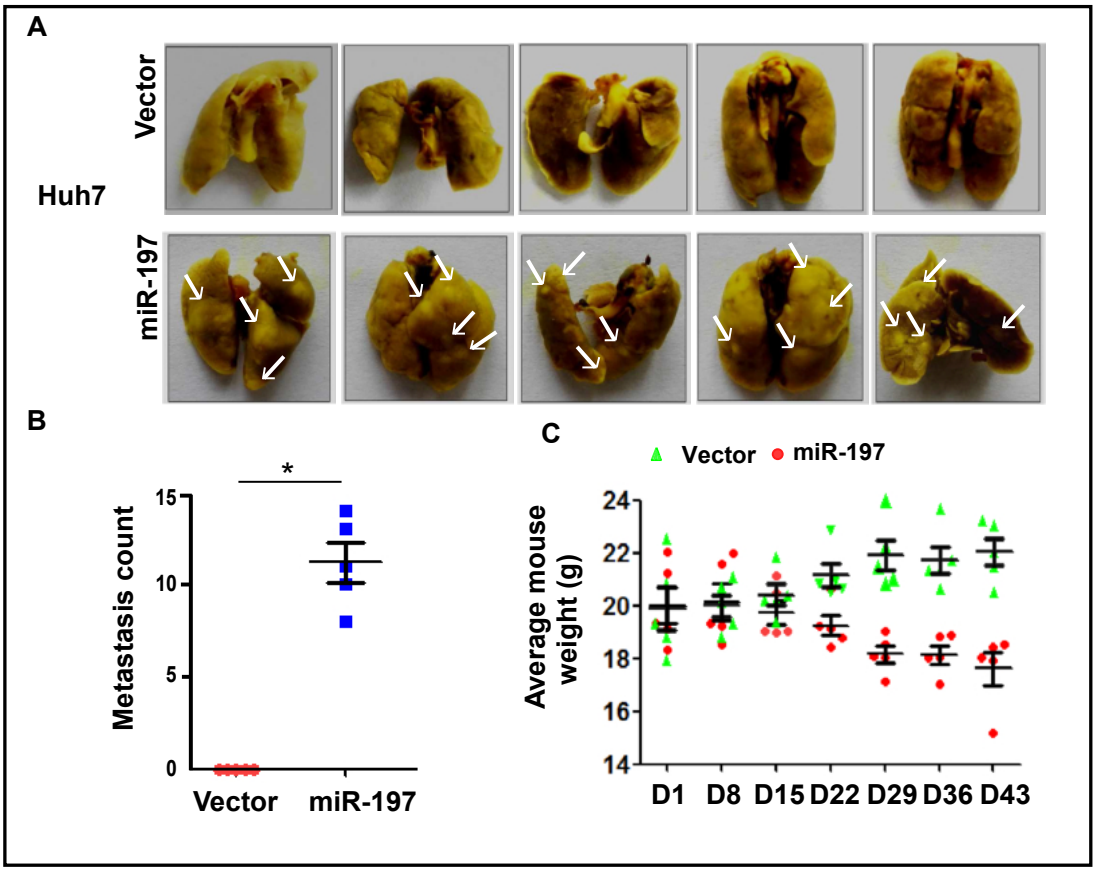

Fig. 3. miR-197 promotes hepatocellular carcinoma cell lines metastasis in vivo. (A) Bright-field imaging of lungs. On day 43, lungs of mice that received injections of Huh7/vector or Huh7/miR-197 cells were collected. Lung surface metastatic nodules are indicated by arrows. (B) Number of visible surface metastatic nodules in mice that received Huh7/vector or Huh7/miR-197 cells transplantation (n=5 mice per group). (C) Weights of mice that received injection of Huh7/vector or Huh7/miR-197 cells. (D) H&E staining of lung species in mice which received Huh7/vector or Huh7/miR-197 cells.

miR-197 promoted a pro-metastatic phenotype in vitro

To clarify the effect of miR-197 on the migration of HCC cells, we generated Huh7 and PLC/PRF-5 cells with hsa-miR-197 (miR-197 overexpression) or has-miR-NC (control miRNA). The PLC/PRF-5 cells with miR-197overexpression acquired a spindle-like morphology (Fig. 2A). Moreover, miR-197 overexpression decreased the expression of epithelial markers (E-cadherin and α-catenin) and increased the expression of mesenchymal markers (vimentin and fibronectin) (Fig. 2B). These results suggested that miR-197 induced EMT in HCC cells. Overexpression of miR-197 in PLC/PRF-5 and Huh7 HCC cells led to increased migration and invasiveness (Fig. 2C). The PLC/PRF-5 and Huh7 cells transduced with miR-197 formed more outward projections than the vector-transduced control cells (Fig. 2D). Furthermore, miR-197 overexpression increased the migration of PLC/PRF-5 and Huh7 cells compared with control cells (wound healing assay) (Fig. 2E).

To understand the effect of miR-197 on HCC metastasis, we suppressed the expression of miR-197 in the MHCC97H HCC cell line (highly metastatic cell line) (Fig. 1C). As expected, miR-197 suppression increased the expression of epithelial markers and weakened the invasive capability of MHCC97H cells (Supplementary Fig. 1A-E). These data indicated that miR-197 is involved in EMT and metastatic spread in HCC.

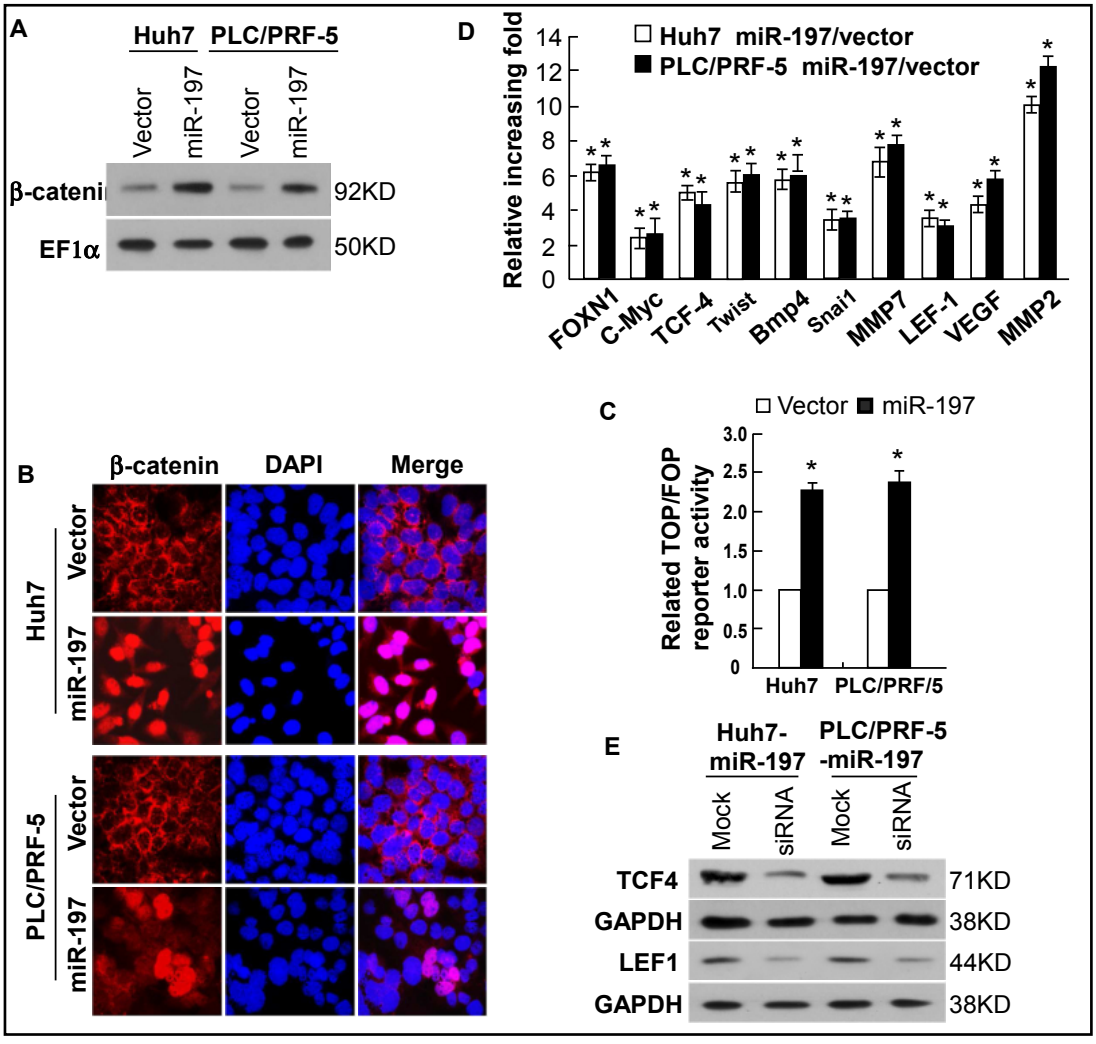

Fig. 4. miR-197 activates Wnt/β-catenin signaling. (A) Western blot was used to analyze the nuclear accumulation of β-catenin with miR-197 overexpression in Huh7 and PLC/PRF-5 cells. (B) Immunofluorescence was used to assess β-catenin subcellular distribution in Huh7 and PLC/PRF-5 cells. (C) Dual luciferase reporter system activation of Huh7 and PLC/PRF-5 cells transfected with miR-197 or vector. (D) qRT-PCR was used to analyze the expression changes of Wnt signal pathway genes after ectopic miR-197 expression. (E) Western blot was used to confirm siRNA depletion of TCF4 or LEF1 in miR-197 over expressed cells.* P<0.05.

miR-197 promoted HCC tumor metastasis in a xenograft model

To confirm the *in vitro* results in an*in vivo* model, we injected Huh7/miR-197 or a corresponding vector control into the tail vein of BALB/c nude mice. Mice that developed Huh7/miR-197 tumors had visible lung metastatic nodules compared withthe mice injected with the Huh7/vector cells (Fig. 3A-B). Notably, miR-197 overexpression resulted in only slightly decreased body weight in the mice (Fig. 3C). H&E staining was used to confirm the lung metastases (Fig. 3D).

We used antagomir-197 to inhibit the endogenous miR-197 expression in the mice. Administration of antagomir-197 inhibited lung metastasis in MHCC97H cells (Supplementary Fig. 2A-B). Histological analyses further supported that treatment with antagomir-197 inhibited the lung metastasis of MHCC97H cell in mice (Supplementary Fig. 2B-D).

Wnt/β-catenin signaling is involved in the EMT induced by miR-197 in HCC cells

Because the Wnt/β-catenin signaling plays an important role in HCC metastasis, we examined whether miR-197 expression was related to Wnt/β-catenin signaling. WB and IFH showed that miR-197-overexpressing PLC/PRF-5 and Huh7 cells had more β-catenin in the nuclear compartment, suggesting that miR-197 activates Wnt/β-catenin signaling (Fig. 4A-B). Furthermore, miR-197 overexpression increased β-catenin activity in PLC/PRF-5 and Huh7 cells (Fig. 4C-D). While, miR-197 inhibitor reduced β-catenin nuclear accumulation and subsequent TCF/LEF activity in HCC cells (Supplementary Fig. 3A-C). These results indicate that miR-197 maypromote β-catenin nuclear accumulation and, consequently, enhance the transcriptional activity of TCF/LEF.

To further test whether β-catenin activation in cells overexpressing miR-197 would enhance HCC invasion, we knocked down TCF4 or LEF1 in the PLC/PRF-5 and Huh7 cell lines transduced with miR-197 and analyzed the invasion capability of these cells. As shown in Fig. 4E, TCF/LEF knockdown reduced miR-197-induced β-catenin signaling activation. In contrast, overexpression of TCF4 or LEF1 in miR-197-inhibited MHCC97H cells led to strong invasiveness and activation of β-catenin signaling (Supplementary Fig. 3D-E). Collectively, these results demonstrate that Wnt/β-catenin signaling is involved in the EMT and metastatic spread induced by miR-197 in HCC cell lines.

miR-197targetednegative regulators of Wnt/β-catenin signaling

To understand the mechanism of miR-197 inHCC metastasis, we identified predicted target genes of miR-197 using Target Scan and evaluated the candidate targets (Fig. 5A). Among the candidates, Nkd1, Axin2, and DKK2 were related to Wnt/β-catenin signaling. WB analysis showed that miR-197 overexpression reduced the expression of NKD1, Axin2, and DKK2 (Fig. 5B), whereas miR-197 inhibition elevated the expression of these proteins (Supplementary Fig. 4A). This finding was further supported by the qRT-PCR results (Fig. 5C, 5D, Supplement Fig. 4B).

Furthermore, we performed dual luciferase reporter assays in PLC/PRF-5 and Huh7 cells expressing miR-197 with a mutated seed sequence or random sequences as controls. As shown in Fig. 5E-G, cells overexpressing miR-197 had lower luciferase activity of NKD1, Axin2, and DKK2, occurring in a miR-197 dose-dependent manner, compared to negative controlcells. Mutations of the miR-197 seed sequence abolished the suppressive effects of miR-197 overexpression on the luciferase reporter activities of the 3′ UTR of NKD1, Axin2, and DKK2. In addition, inhibition of miR-197 increased the luciferase reporter activity of the 3′ UTR of NKD1, Axin2, and DKK2 (Supplemented Fig. 4C). Taken together, these results suggest that miR-197 suppressed NKD1, Axin2, and DKK2 expression in HCC cell lines.

Silencing of NKD1, Axin2, and DKK2 increased effects associated with miR-197 overexpression

To explore the significance of NKD1, Axin2, and DKK2 function on HCC cell line invasiveness induced by miR-197, we knocked down NKD1, Axin2, and DKK2 using specific siRNAs. As shown in Fig. 6A-B, silencing NKD1, Axin2, and DKK2 in PLC/PRF-5 and Huh7 cells caused increased cell invasiveness and TCF/LEF transcriptional activity (Fig. 6C). Importantly, when we transduced miR-197-overexpressing PLC/PRF5 and Huh7 cells with NKD1, Axin2, and DKK2 (Fig. 6D), we saw that miR-197-induced cell invasion was reduced (Fig. 6E). In addition, miR-197-induced TCF/LEF transcriptional activity was abolished (Fig. 6F). Moreover, knockdown of NKD1, Axin2, and DKK2 decreased the invasiveness of MHCC97H cells in which miR-197 was suppressed (Supplementary Fig. 4D-E) and increased TCF/LEF transcriptional activity in these cells (Supplementary Fig. 4F). These results indicated that Nkd1, Axin2, and DKK2 have a functional role in miR-197-induced cell invasiveness in HCC cell lines.

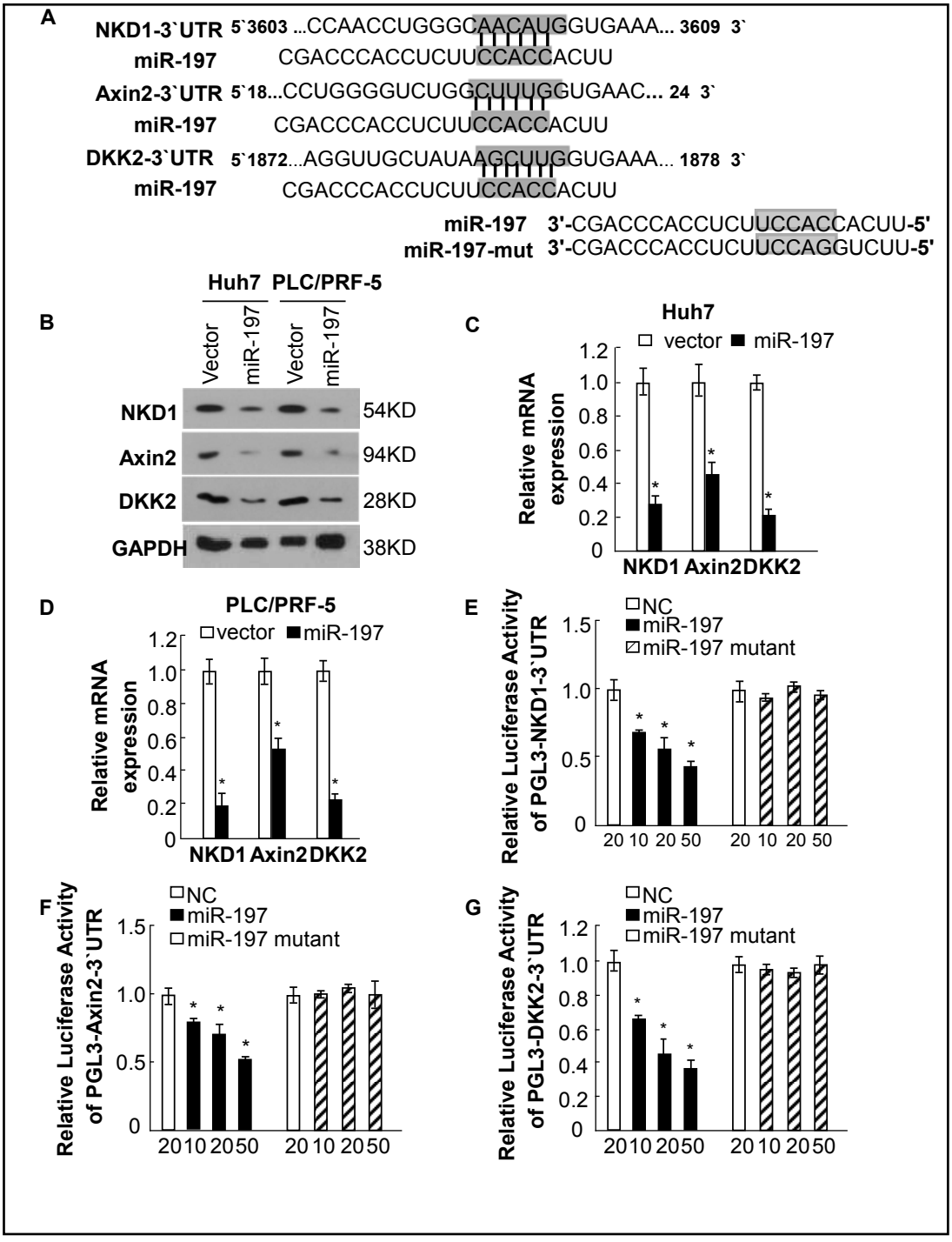

Fig. 5. miR-197 targets multiple negative regulators of β-catenin signaling. (A) Predicted binding of miR-197 to 3'-UTRs of NKD1, Axin2, and DKK2. Sequence of miR-197-mut. (B) Western blotting of NKD1, Axin2, and DKK2 expression in Huh7 and PLC/PRF-5 cells. (C-D) qRT-PCR analysis of mRNA levels of NKD1, Axin2, and DKK2 in Huh7and PLC/PRF-5 cells. (E-G) Luciferase activity of pGL3-NKD1-3'-UTR, pGL3-Axin2-3'-UTR, or pGL3-DKK2-3'-UTR reporter in Huh7 and PLC/PRF-5 cells co-transfected with the indicated oligonucleotides with different concentration.

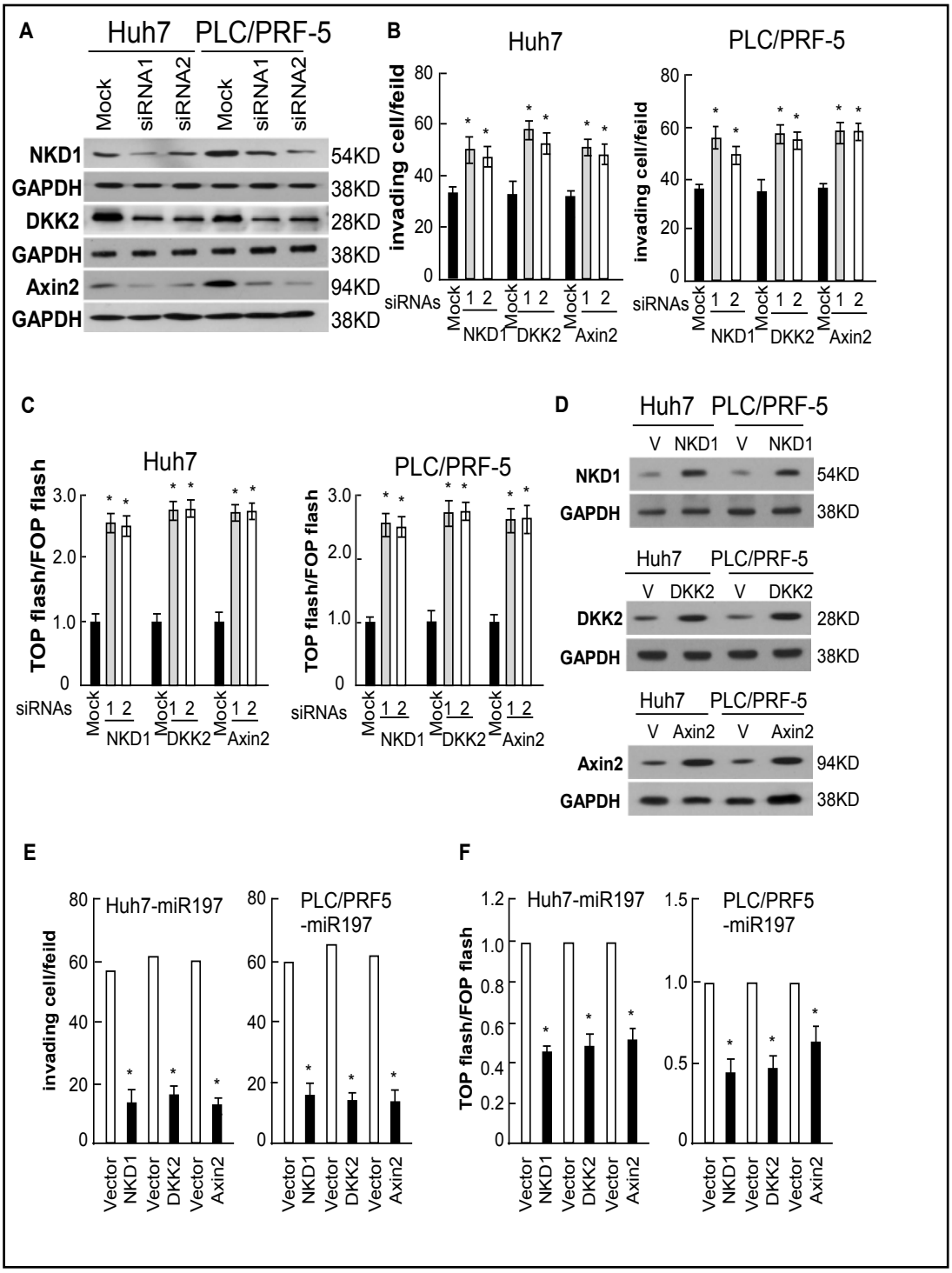

Fig. 6. NKD1, Axin2, and DKK2 play important roles in miR-197-induced cell invasion and Wnt/-catenin activation. (A) Transfection of NKD1-, Axin2-, and DKK2-targeting siRNAs in indicated cells. (B) Quantification of invading PLC/PRF-5 and Huh7 cells transfected with NKD1-, Axin2-, and DKK2-targeting siRNAs. (C) TCF/LEF transcriptional activity in Huh7 and PLC/PRF-5 cells transfected with NKD1-, Axin2-, and DKK2-targeting siRNAs. (D) Overexpression of NKD1, Axin2, and DKK2, in Huh7 and PLC/PRF-5 cells confirmed by western blot. (E) Cell invasion and migration assays in cells after overexpression of NKD1, Axin2, and DKK2. (F) TCF/LEF transcriptional activity in miR-197 highly expressed Huh7 and PLC/PRF-5 cells with or without NKD1, Axin2, and DKK2 overexpression.

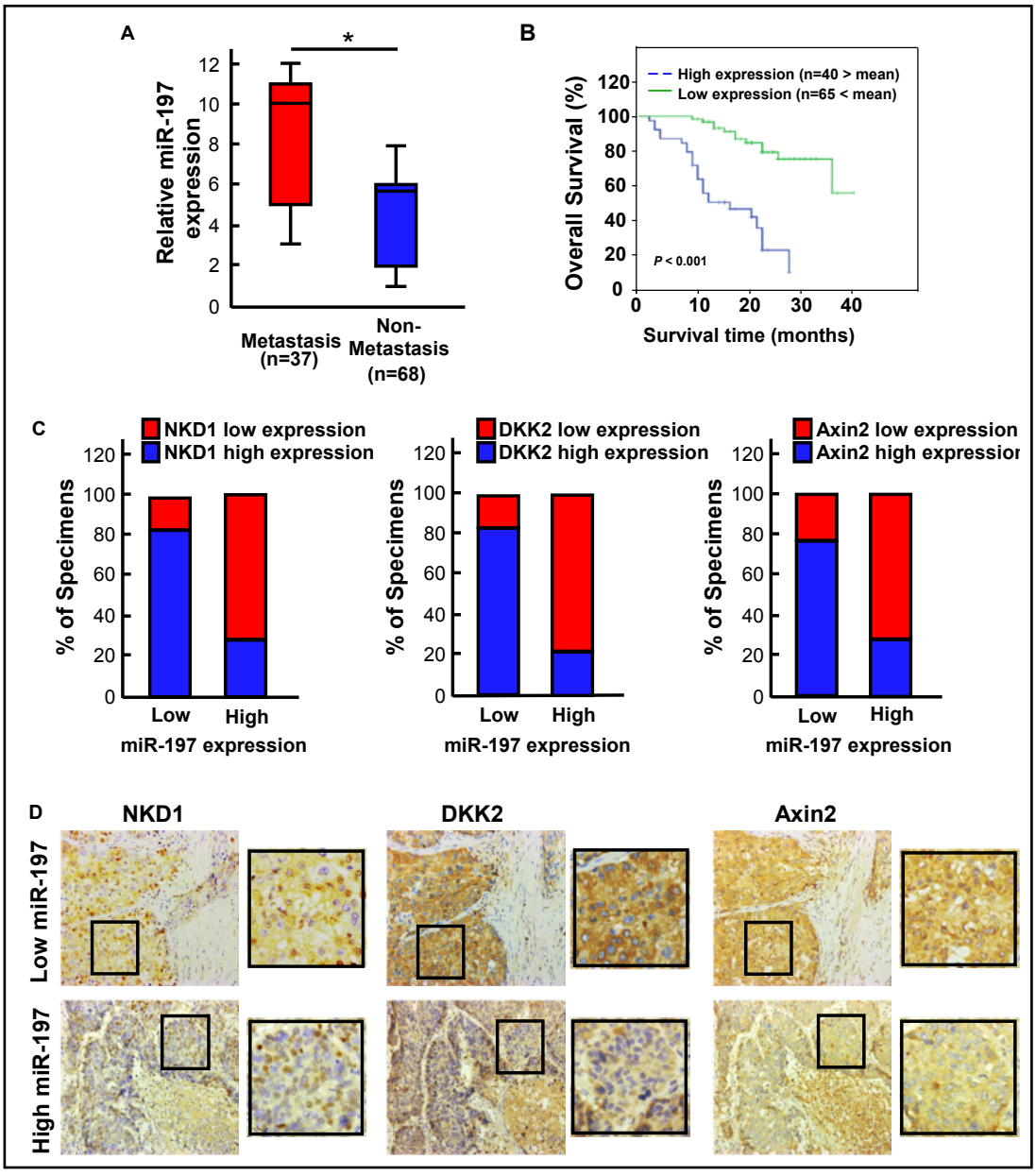

Fig. 7. Expression of miR-197 is clinically relevant to NKD1, Axin2, and Dkk2. (A) qRT-PCR analysis of miR-197 expression in HCC with or without metastasis (n=37 vs. 68). (B) Overall survival of HCC patients with high or low miR-197 expression, P<0.001. (C) Proportion of specimens showing high or low miR-197 expression in relation to the expression levels of NKD1, Axin2, and DKK2. (D) Expression of miR-197 relative to NKD1, Axin2, and DKK2 expression levels in clinical HCC tissues.

miR-197 is associated with HCC metastasis in HCC patients

To determine whether the results of the *in vitro* experiments had clinical relevance, we examined miR-197 expression in 105 HCC specimens. We first divided the specimens as withmetastasis (presence of portal vein metastasis) and without. The expression of miR-197 was higher in the 37 HCC patients with metastasis compared withthe 68 patients without (Fig. 7A). We then evaluated the association of miR-197 expression with the overall survival of the patients and found that high expression of miR-197 was associated with lower survival (Fig. 7B). Then, we analyzed the correlation of miR-197 expression with levels of the NKD1, Axin2, and DKK2proteins in clinical HCC specimens. Among the cases with high

miR-197 expression, 73% (29 cases), 73% (29 cases), and 78% (31 cases) had low levels of NKD1, Axin2, and DKK2, respectively. Among the samples with low miR-197 expression, 77% (50 cases), 83% (54 cases), and 83% (54 cases) had high expression of NKD1, Axin2, and DKK2, respectively (P<0.05) (Fig. 7C). IHC showed thatNKD1, Axin2, and DKK2 were downregulated(Fig. 7D). The expression of NKD1, Axin2, and DKK2 was inversely correlated with the upregulation of miR-197 in the clinical specimens. Since miR-197 was correlated with HCC metastasis, NKD1, Axin2, and DKK2 could also be involved in the metastatic spread of HCC, but additional studies are needed to confirm those results.

Discussion

Considering the important role of Wnt/β-catenin signaling in cancer development (including HCC) and the multiple gene targets of miR-197[28-40, 46], the present study aimed to examine the role of miR-197 in HCC specimens, cell lines, and xenografts, and to determine the mechanisms involved.The results showed that miR-197 contributes to HCC invasion and metastasis by maintaining Wnt/β-catenin signaling activation. miR-197could be a potential prognostic marker and cancer treatment target.

miRNAs play crucial roles in carcinogenesis and EMT[19, 25]. miRNAs may regulate multiple gene targets to modulate metastatic progression in various cancer types [19, 25]. We analyzed miRNA microarray databases and found 17 miRNAs to be differentially expressed in metastatic HCC tissues or cells, compared with non-metastatic HCC tissues or cells. Among these candidates, miR-197 was significantly upregulated in metastatic HCC tissues. Two studies [39, 40] examined the impact of miR-197 on EMT, but the regulation mechanisms are not clear.Moreover, we found that miR-197 overexpression induced non-metastatic PLC/PRF-5 and Huh7 cells to invade and metastasize. In addition, miR-197 suppression reduced the invasiveness and metastatic capabilities of highly metastatic MHCC97H HCC cells. Furthermore, examination of clinical HCC samples showed that levels of miR-197 expression positively correlated with metastasis in HCC patients. Consistently, miR-197 has been shown to promote pulmonary metastasis for non-small cell lung cancer [37, 46]. miR-197 also participates in the development of a number of types of cancer [26-34], including HCC[29, 30], indicating the role of miR-197 in cancer.miR-197 also participates in liver fibrosis [35, 36], in which Wnt/β-catenin signaling also plays a central role [47].

Wnt/β-catenin signaling activation is commonly activated in a wide variety of cancers [10, 12], including HCC[13, 14]. The mechanism of β-catenin activation in HCC involves mutations of the β-catenin gene,whichare noted in around 19% of all cases [48, 49]. Mutations are also reported in multiple negative regulators of β-catenin signaling, including Axin1 in about 3-16% of HCC cases [50, 51] and Axin2 in about 3% [50, 52]. Intriguingly, many cases of HCC have Wnt/β-catenin signal activation without mutations of β-catenin or Axin. Therefore, clarifying the mechanisms of β-catenin activation and understanding how it interacts with other regulatory factors may reveal the mechanisms of HCC invasion and metastasis and help identify new treatment targets.

Our data showed that NKD1, Axin2, and DKK2 contributed tothe effects of miR-197 on β-catenin activation. First, NKD1, one of most important Wnt antagonists, is often silenced in many tumor types [53-55]. NKD1 acts as a negative regulator through directly binding to Dvl and switching Dvl from Wnt/β-catenin signaling pathway toward the JNK pathway. Secondly, the DKK family of Wnt antagonists contains four members, DKK1-DKK4 [56]. DKK2 is generally considered to be a direct inhibitor of Wnt binding to the LRP5/LRP6 co-receptor of FZD[56]. In addition, decreased DKK2 expression is seen in melanoma cell lines and tumors [57, 58]. Thirdly, Axin2 has similar biochemical and cell biological properties to Axin. Axin2 is a scaffolding component in the assembly of the β-catenin destruction complex [59] and a tumor suppressor in numerous cancers [59, 60]. Axin2 mutations are present in a small percentage of HCC cases [52]. The mutation of key regulators of the Wnt/β-catenin pathway leads to HCC, but does not explain most hepatic carcinogenesis. The primary

finding of this study provides a novel insight into miR-197's role in suppressing multiple negative regulators of Wnt/β-catenin signaling to promote HCC tumor metastasis. Further investigation is necessary to clarify what factors contribute to aberrant miR-197 expression.

Conclusion

In summary, the results suggest an important role for miR-197 in HCC. This study suggests that the overexpression of miR-197 promotes metastasis in HCC by suppressing the β-catenin signaling pathway (including NKD1, Axin2, and DKK2). Our findings suggest a new mechanism for the activation of the Wnt/β-catenin pathway and HCC metastasis. Further investigation is needed to explore the development potential of miR-197-based prognostic biomarkers and therapeutic approaches to HCC invasion and metastasis.

Abbreviations

APC (adenomatous polyposis coli); CK1 (casein kinase 1); DKK (Dickkopf); Dvl (Dishevelled); EMT (epithelial-mesenchymal transition); GSK3β (glycogen synthase kinase 3β); HCC (hepatocellular carcinoma); miRNAs (microRNAs); NKD1 (naked cuticle-1); TNM (tumor-node-metastasis); UTR (untranslated region); WIF1 (Wnt inhibitory factor-1).

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Disclosure Statement

The authors declare no conflicts of interest.

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