NK3 homeobox 1 (NKX3.1) up-regulates forkhead box O1 expression in hepatocellular carcinoma and thereby suppresses tumor proliferation and invasion

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Hepatocellular carcinoma (HCC), the leading cause of cancer-related mortality in China, and the molecular mechanism of uncontrolled HCC progression remains to be explored. NK3 homeobox 1 (NKX3.1), an androgen-regulated prostate-specific transcription factor, suppresses tumors in prostate cancer, but its role in HCC is unknown, especially in hepatocellular carcinoma. In the present study, the differential expression analyses in HCC tissues and matched adjacent noncancerous liver tissues revealed that NKX3.1 is frequently down-regulated in human primary HCC tissues compared with matched adjacent noncancerous liver tissues. We also noted that NKX3.1 significantly inhibits proliferation and mobility of HCC cells both in vitro and in vivo. Furthermore, NKX3.1 overexpression resulted in cell cycle arrest at the G1/S phase via direct binding to the promoter of forkhead box O1 (FOXO1) and up-regulation of expression. Of note, FOXO1 silencing in NKX3.1-overexpressing cells reversed the inhibitory effects of NKX3.1 on HCC cell proliferation and invasion. Consistently, both FOXO1 and NKX3.1 were down-regulated in human HCC tissues, and their expression was significantly and positively correlated with each other. These results suggest that NKX3.1 functions as a tumor suppressor in HCC cells through directly up-regulating FOXO1 expression.

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This article contains supplemental Figs. S1–S5 and Tables S1–S6.

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an important role in apoptosis, cell cycle control, autophagy, and antioxidant response (19). Forkhead box O1 (FOXO1) is a member of forkhead transcription factor family, acts as a tumor suppressor in various carcinomas including in HCC, and its expression is negatively correlated with tumor progression (20–23).

In this study, we performed functional studies to determine the role of NKX3.1 in human primary HCC. We also integrated flow cytometry analysis, luciferase reporter assay, and ChIP assay results to reveal its potential molecular mechanism in HCC. We found that NKX3.1 was down-regulated in HCC tissues compared with matched adjacent noncancerous liver tissues. NKX3.1 functions as a tumor suppressor in terms of cell proliferation and motility in HCC. It is also the first time to characterize FOXO1 as a direct and functional binding target of NKX3.1 in HCC cells.

**Results**

**NKX3.1 expression is down-regulated in HCC tissues**

As the suppressor role of NKX3.1 in prostate cancer, the association between NKX3.1 and HCC is still unknown. To address this, we tested NKX3.1 mRNA expression in 60 pairs of human primary HCC tissues and matched adjacent noncancerous liver tissues by quantitative (q) RT-PCR. The results showed that NKX3.1 mRNA expression was frequently down-regulated in HCC tissues compared with matched adjacent noncancerous liver tissues (p < 0.001; Fig. 1A). This is in agree-
NKX3.1 acts as a tumor suppressor in HCC

Western blotting analyses were also performed and showed that the NKX3.1 protein level was markedly higher in noncancerous liver tissues (Fig. 1C). In addition, NKX3.1 expression levels are inversely correlated with serum AFP levels, histological grade, and pathological stage in TCGA cohort (supplemental Table S5). Therefore, these findings indicate that lower expression levels of NKX3.1 are associated with the malignant progression of HCC and it is seriously possible that NKX3.1 plays a key role in the development of HCC.

**Overexpression of NKX3.1 inhibits HCC cell proliferation and tumorigenesis in vitro and in vivo**

We thus measured endogenous mRNA and protein expressions of NKX3.1 in HCC cell lines and normal hepatocyte L02 (Fig. 2A and supplemental Fig. S1). NKX3.1 mRNA and protein expressions were barely detected in most HCC cell lines,
whereas the expression level was higher in immortalized normal hepatocyte L02. To determine the biological function of NKX3.1 in HCC, we selected SMMC-7721, HCC-LY10, and PLC/PRF/5 to infect lentiviral vector containing complete ORF of NKX3.1 and successfully established stable HCC cell lines with NKX3.1 overexpression (Fig. 2B), which were then used in functional experiments. As a control, cells were infected with empty vector pWPXL in the meantime. The plate colony formation assay and MTT assay for cell viability indicated that overexpression of NKX3.1 inhibits HCC cell proliferation in vitro (Fig. 2, C and D).

We then tested whether NKX3.1 displayed inhibitory ability of tumorigenesis in vivo. For this, stable SMMC-7721 and HCC-LY10 cells with NKX3.1 overexpression were, respectively, transplanted into the livers of non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice orthotopically. Cells transfected with empty vector pWPXL served as a control. The livers were separated from the mice after 4 weeks. Our results indicated that the average volume of tumors and weight of livers with xenografts were significantly decreased in NKX3.1-overexpressing mice (Fig. 2E). Meanwhile, the tissues of xenograft overexpressing NKX3.1 still maintained a high expression level of NKX3.1 (Fig. 2E). These results suggest that NKX3.1 represses cell proliferation in vitro, consistent with the in vivo results.

**Overexpression of NKX3.1 suppresses HCC cells mobility in vitro and metastasis in vivo**

Next, we explored the effect of NKX3.1 expression on HCC cells migration and invasion. The results of the in vitro wound-healing assay revealed NKX3.1 overexpression decreased migration of the HCC cells compared with the control (Fig. 3A). The in vitro invasion assay indicated that the invasive capacities of HCC cells were dramatically suppressed by NKX3.1 transfection compared with the control cells (Fig. 3B). To further investigate the role of NKX3.1 in HCC metastasis in vivo, paraffin sections were prepared with the liver tissues and lung tissues from the mice injected with HCC-LY10-pWPXL/NKX3.1 cells orthotopically, and histological examination was performed. The results showed that mice in the NKX3.1-overexpressing group exhibited fewer intrahepatic and lung metastasis nodules than the control mice (Fig. 3, C and D). In addition, all of the nine mice in the control group developed lung metastasis (100%), whereas only five of the nine mice in the NKX3.1-overexpressing group developed lung metastasis (55.56%). Collectively, these findings indicated that overexpression of NKX3.1 markedly inhibits the mobility of HCC cells in vitro and in vivo.

**Knockdown of NKX3.1 promotes HCC cell proliferation and mobility in vitro**

To determine the function of endogenous NKX3.1, three lentiviral vectors expressing effective shRNAs targeting NKX3.1 were used to knock down endogenous NKX3.1 (Fig. 4A). As expected, knockdown of NKX3.1 in MHCC-97L markedly promoted cell growth and colony formation (Fig. 4, B and C). Results from the wound-healing assay (Fig. 4D) and in vitro invasion assay (Fig. 4E) showed that NKX3.1 down-regulation increased the cell migration and invasion capacities of MHCC-97L. The results validate the conclusions drawn by overexpression of NKX3.1.

**Overexpression of NKX3.1 induces G1/S phase arrest in HCC cells through up-regulation of FOXO1**

To further investigate the inhibitory effect of NKX3.1 on HCC cell proliferation, the cell cycle distributions among SMMC-7721 and HCC-LY10 cells was evaluated by flow cytometry (Fig. 5A). After treatment of 2 μM thymidine for 24 h to synchronize cells at the G1/S phase border, cells were collected at 0, 12, and 24 h. Flow cytometry analysis showed that the percentage of cells at the G1 phase was significantly higher in the SMMC-7721–NKX3.1 and HCC-LY10–NKX3.1 cells than the control cells after the thymidine treatment (Fig. 5B and Table 1). Cells synchronized at the G1/M phase boundary with 0.3 μM nocodazole displayed similar results (supplemental Fig. S2 and supplemental Table S6). We next detected the expression of essential molecules that regulate the G1/S phase transition in NKX3.1-overexpressing cells and control cells. Upon NKX3.1 overexpression, protein levels of FOXO1 and its downstream molecules p21cip1 and p27kip1 increased notably, whereas protein levels of CDK2, Cyclin E, and phosphorylated RB (Ser-807/811) were decreased (Fig. 5C). We then detected these proteins after treatment of thymidine for 0 and 24 h. As time goes on, expression of FOXO1, p21cip1, and p27kip1 increased in NKX3.1-overexpressing cells at 24 h, whereas expression of CDK2, Cyclin E, and phosphorylated RB (Ser-807/811) were decreased compared with control pWPXL cells (Fig. 5D). This implied that overexpression of NKX3.1 results in G1/S phase arrest through up-regulating FOXO1 in HCC cells.

**FOXO1 is a direct target of NKX3.1**

Given the results that FOXO1 expression was up-regulated in NKX3.1 overexpression HCC cells, we then explored whether FOXO1 is the direct target of NKX3.1. The JASPAR (jaspar.genereg.net) database was used to analyze the potential NKX3.1-binding site in the FOXO1 promoter (Fig. 6A). The promoter of the FOXO1 gene, which spans a 1794-bp sequence upstream of the first ATG, was cloned and linked to pGL3 vector. The results of the luciferase assay showed that relative luciferase activity markedly increased by overexpression of NKX3.1 (Fig. 6B). We then deleted the sequences that span the region from −1010 and −550 bp to the first ATG relative to the enhanced luciferase activity markedly in −550 bp (Fig. 6C). According to the sequence logo of the NKX3.1 potential binding site in JASPAR (jaspar.genereg.net), we mutated the high-score base to the low-score base at the predicted NKX3.1-binding site (−230 to −239 bp), as shown in Fig. 6D. Enhanced luciferase activity was reversed by transfection of the mutant (Fig. 6E). Chromatin immunoprecipitation assay further verified that the site was the NKX3.1-binding site on the FOXO1 promoter (Fig. 6F). Taken together, FOXO1 promoter is a direct transcriptional target of NKX3.1.

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FOXO1 is involved in the NKX3.1-induced suppressive effect of HCC cells

To explore whether FOXO1 up-regulation by NKX3.1 is responsible for the tumor suppressive function in HCC, FOXO1 expression was knocked down in SMMC-7721, HCC-LY10, and PLC/PRF/5 cells with stable overexpression of NKX3.1 (Fig. 7A). We use two lentiviral vectors expressing effective short hairpin RNAs designed to knock down FOXO1 (shFOXO1-1 and shFOXO1-2). We also tested the expression changes of cell cycle-related proteins (FOXO1, p21\(^{\text{KIP1}}\), p27\(^{\text{KIP1}}\), CDK2, Cyclin E, phosphorylated RB, and total RB) in NKX3.1-overexpressing HCC cells after knockdown of FOXO1. Upon FOXO1 knockdown, expression levels of p21\(^{\text{KIP1}}\) and p27\(^{\text{KIP1}}\) decreased, whereas expression levels of CDK2, Cyclin E, and phosphorylated RB increased. Thus, FOXO1 knockdown can rescue the NKX3.1 overexpression induced by the expression changes of cell cycle-related proteins. MTT and colony assays in vitro revealed that the decrease of FOXO1 significantly reversed NKX3.1-induced inhibition of cell proliferation (Fig. 7, B and C). Moreover,
The inhibited invasion ability by NKX3.1 was reversed after FOXO1 interference (supplemental Fig. S3). Therefore, these data demonstrated that FOXO1 is a direct and functional target for NKX3.1 and is also responsible for the suppressive effects of NKX3.1 in HCC cells.

**The expression levels of NKX3.1 and FOXO1 show positive correlation in HCC tissues**

To further investigate the correlation between expression levels of NKX3.1 and FOXO1 in human primary HCC tissues, we detected FOXO1 mRNA expression levels in 60 pairs of HCC tissues and matched adjacent noncancerous liver tissues and protein expression levels in 20 pairs of matched HCC/non-cancerous liver tissues. FOXO1 mRNA expression was down-regulated significantly in HCC tissues compared with noncancerous liver tissues (supplemental Fig. S4A). The result was in common with the analysis in TCGA cohort (supplemental Fig. S4B). Moreover, protein expression of FOXO1 was frequently down-regulated in HCC tissues (supplemental Fig. S4C). Analysis of the relative protein expression levels (tumor/non-tumor) of NKX3.1 and FOXO1 together showed that there was a positive correlation between NKX3.1 and FOXO1 protein expression levels.
NKX3.1 acts as a tumor suppressor in HCC

A

DNA content

Cell number

No treatment

Mock

pWPXL

NKX3.1

Mock

pWPXL

NKX3.1

G1: 46.82%
S: 31.39%
G2: 21.79%

G1: 46.84%
S: 30.36%
G2: 22.84%

G1: 56.71%
S: 24.22%
G2: 19.07%

G1: 47.92%
S: 26.42%
G2: 25.65%

G1: 46.23%
S: 20.32%
G2: 33.48%

G1: 55.87%
S: 21.97%
G2: 22.17%

B

DNA content after Thymidine treatment

Cell number

0 h

Mock

pWPXL

NKX3.1

Mock

pWPXL

NKX3.1

G1: 72.16%
S: 27.65%
G2: 1.19%

G1: 71.20%
S: 28.25%
G2: 0.49%

G1: 72.09%
S: 27.34%
G2: 0.66%

G1: 68.34%
S: 30.59%
G2: 1.08%

G1: 68.33%
S: 21.44%
G2: 0.50%

G1: 68.62%
S: 31.44%
G2: 0.50%

12 h

Mock

pWPXL

NKX3.1

Mock

pWPXL

NKX3.1

G1: 67.26%
S: 22.39%
G2: 10.35%

G1: 66.62%
S: 11.15%
G2: 23.06%

G1: 70.72%
S: 10.07%
G2: 19.11%

G1: 78.40%
S: 12.12%
G2: 12.12%

G1: 78.80%
S: 10.35%
G2: 10.85%

G1: 81.40%
S: 9.44%
G2: 8.16%

24 h

Mock

pWPXL

NKX3.1

Mock

pWPXL

NKX3.1

G1: 67.18%
S: 11.15%
G2: 21.64%

G1: 66.24%
S: 11.15%
G2: 23.06%

G1: 70.72%
S: 10.07%
G2: 19.11%

G1: 78.40%
S: 12.12%
G2: 12.12%

G1: 78.80%
S: 10.35%
G2: 10.85%

G1: 81.40%
S: 9.44%
G2: 8.16%

C

SMMC-7721 HCC-LY10

pWPXL NKX3.1 pWPXL NKX3.1

NKX3.1

FOXO1

p21

p27

CDK2

Cyclin E

p-Rb
(Ser 807/811)

Rb

β-actin

D

Thymidine treatment

Mock pWPXL NKX3.1 pWPXL NKX3.1 Mock pWPXL NKX3.1 pWPXL NKX3.1

NKX3.1

FOXO1

p21

p27

CDK2

Cyclin E

p-Rb
(Ser 807/811)

Rb

β-actin

SMCC-7721 HCC-LY10

0 h

24 h

0 h

24 h

-35 kD

-70 kD

-20 kD

-25 kD

-35 kD

-55 kD

-100 kD

-100 kD

-40 kD
expression in HCC and matched noncancerous liver tissues \((r = 0.677, p = 0.001; \text{Fig. } 8, \text{A}–\text{D})\). Of note, FOXO1 mRNA expression was also positively associated with NKX3.1 expression in HCC tissues (Fig. 8, E and F). In addition, we measured mRNA and protein expression levels of FOXO1 in tissue samples of xenografts mentioned in Fig. 3E. FOXO1 is expressed highly in NKX3.1-overexpressing samples (supplemental Fig. S5, A and B). Taken together, these results suggest that the expression levels of NKX3.1 and FOXO1 show positive correlation in HCC tissues and are promising prognostic indicators for HCC patients in the clinic.

**Discussion**

NKX3.1 plays an important role in prostate development, proliferation of the prostate glandular epithelium, formation of prostatic ducts, and regulation of prostate epithelial differentiation (8, 9). NKX3.1 expression in androgen-deprived prostate epithelial cells is a luminal cell population that appears as stem properties during prostate regeneration (24). Numerous reports have shown that NKX3.1 acts as a tumor suppressor in prostate. Mouse models suggest that the phosphatase and tensin homolog deleted on chromosome 10 (Pten) loss could drive invasion and metastasis in cooperation with loss of NKX3.1 (11, 25). Moreover, loss of NKX3.1 expression is strongly related to the advanced tumor stage in prostate cancer (26). It has been reported that the loss of NKX3.1 protein is mediated by several mechanisms, including gene methylation, post-translational modification, and genetic loss (27). Inflammation that releases cytokine TNF-α and IL1-β could destabilize the NKX3.1 protein by phosphorylating at serine 196 and decreasing its half-life (28). Phosphorylation at serine 185 is associated with ubiquitination signaling and degradation of the NKX3.1 protein in the proteasome (29). Song and colleagues (30) identified DYRK1B kinase as a target for enzymatic inhibition to increase cellular NKX3.1 expression in the prostate. Thus, searching for potential candidate targets mediated by the enhancing NKX3.1 expression level in HCC is meaningful for treatment.

However, the function and the role of NKX3.1 in other cancers were rarely reported. In the current study, we found that NKX3.1 was overexpressed in noncancerous liver tissues compared with HCC tissues. Analysis of the relationship between NKX3.1 expression level and clinicopathological features in the TCGA cohort revealed that lower expression levels of NKX3.1 are related to the malignant progression of HCC. These results suggest that NKX3.1 may play a negative regulating role in HCC progression. For the reason that endogenous NKX3.1 expression was at a low level, its stable overexpression cell lines were established to explore the function of NKX3.1 in HCC. We confirmed for the first time that NKX3.1 suppresses HCC cell proliferation and metastasis in vitro and in vivo. Knockdown of NKX3.1 was also performed to validate the function of NKX3.1 in HCC.

Based on previous documents, FOXO1 acts as a tumor suppressor in HCC, the underlying molecular mechanism of the anticancer effects of FOXO1 has been widely reported as well (31–35). Deletion of Aurora A kinase up-regulated FOXO1 in a p53-dependent manner and induces cell cycle arrest at the G(2)/M phase (36). FOXO1 is downstream of the PI3K–Akt signaling. AQP9 overexpression in liver cancer cells inhibits PI3K/Akt signaling and subsequently increased FOXO1 protein expression, resulting in cell cycle arrest and apoptosis (37). However, the relationship between NKX3.1 and FOXO1 is still unknown in HCC. The data presented in our study showed that overexpression of NKX3.1 up-regulates expression of FOXO1 and p21, resulting in cell cycle arrest at the G(1)/S phase. We report that NKX3.1 inhibits the HCC progression by up-regulating FOXO1 expression via directly binding to the promoter of FOXO1. In terms of invasion, we found that inhibited invasion by NKX3.1 could be reversed after FOXO1 interference. NKX3.1 could suppress cell invasion via FOXO1. There are
Figure 6. NKX3.1 up-regulates FOXO1 expression through directly binding to FOXO1 promoter. A, potential NKX3.1-binding sites in the FOXO1 promoter identified with the JASPAR database (http://jaspar.genereg.net/).4 B, relative activities of the FOXO1 promoter after transfection of NKX3.1 and pWPXL into HEK 293T and SMMC-7721 cells analyzed by luciferase assay. C, relative activities of FOXO1 promoter deletion mutants in HEK 293T and SMMC-7721 cells by luciferase assay. D, the top panel is the sequence logo of NKX3.1 potential binding site in JASPAR (http://jaspar.genereg.net/).4 The bottom panel is the diagram of mutant sites in the FOXO1 promoter. E, relative activities of the FOXO1 promoter and the mutant promoter after transfection of NKX3.1 and pWPXL. Data are mean ± S.D. from experiments with three replicates. **, p < 0.01. F, binding of NKX3.1 to the FOXO1 promoter was performed by ChIP using the antibody against NKX3.1 and negative control (IgG) in 293T and NKX3.1-overexpressing SMMC-7721 cells.
**A**

|          | SMMC-7721 |          | HCC-LY10 |          | PLC/PRF/5 |
|----------|------------|----------|----------|----------|-----------|
| pWPXL    | +          | -        | -        | -        | -         |
| NKK3.1   | -          | +        | +        | +        | +         |
| shNC     | +          | +        | -        | -        | +         |
| shFOXO1  | -          | -        | +1       | +2       | +1        |
| FOXO1    |            |          |          |          |           |
| p21      |            |          |          |          |           |
| p27      |            |          |          |          |           |
| CDK2     |            |          |          |          |           |
| Cyclin E |            |          |          |          |           |
| p-Rb (Ser 807/811) |            |          |          |          |           |
| Rb       |            |          |          |          |           |
| β-actin  |            |          |          |          |           |

**B**

**C**
studies reported that FOXO1 inhibits cell migration and invasion in HCC and prostate cancer as well (38–40). In HCC, FOXO1 expression levels are inversely correlated with epithelial-to-mesenchymal transition inducers and it directly inhibits ZEB2 expression to suppress epithelial-to-mesenchymal transition (41). It is worthy to explore whether NKX3.1 suppresses cell invasion and metastasis through inhibiting epithelial-to-mesenchymal transition in HCC.

NKX3.1 exerts tumor suppressive effects in prostate cancer partly by activating the cellular response to oxidation and accelerating DNA repair. It is reported that NKX3.1 has an important role in regulating the DNA damage response by interacting with ataxia telangiectasia-mutated kinase and by activation of topoisomerase I (16, 42–44). Early molecular events in prostate cancer often include gene rearrangement of ETS family members and loss of NKX3.1 expression (45).

Figure 7. Knockdown of FOXO1 after NKX3.1 overexpression rescues NKX3.1-induced suppressive effect of HCC cells. A, Western blot analysis of expression levels of FOXO1 and cell cycle-related proteins shown in Fig. 4 in NKX3.1-overexpressing HCC cells after knockdown of FOXO1. β-Actin was used as a loading control. B, knockdown of FOXO1 reversed the inhibitory effect of NKX3.1 on cell proliferation in vitro by MTT assay. C, knockdown of FOXO1 reversed the inhibitory effect of NKX3.1 on cell colony formation in vitro. Data are mean ± S.D. from experiments with three replicates. *, p < 0.05; **, p < 0.01.

Figure 8. Positive correlation between FOXO1 and NKX3.1 expression in human HCC tissues. A, representative images of NKX3.1 and FOXO1 protein levels in human HCC tissues (T) and the corresponding adjacent non-cancerous liver tissues (N). β-Actin was used as a loading control. Images were taken from Fig. 1C and supplemental Fig. S4C. B and C, the fold-change of NKX3.1 (B) and FOXO1 (C) protein levels in 20 paired HCC/non-cancerous liver tissues. Data were normalized to β-actin and presented as log2 fold-change. Red bar in the waterfall plot represents a patient with a higher expression of NKX3.1 or FOXO1 in HCC tissues than paired non-cancerous liver tissue; the gray bar represents a patient with a lower expression of NKX3.1 or FOXO1 in HCC tissues than paired non-cancerous liver tissue. D, the correlation between NKX3.1 and FOXO1 protein levels in 20 paired HCC/non-cancerous liver tissues. E and F, the correlation between NKX3.1 and FOXO1 mRNA levels in HCC tissues of 60 patients (E) and 373 patient TCGA cohort (F). The Pearson correlation coefficients (r) and p value were indicated.
HCC treatment. That NKX3.1 may serve as a promising therapeutic target for serum (FBS, Gibco), 100 units/ml of penicillin (Sigma) in a humidified 37 °C incubator.

All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco). The medium contained 10% fetal bovine serum (FBS, Gibco), 100 μg/ml of streptomycin, and 100 units/ml of penicillin (Sigma) in a humidified 37 °C incubator with 5% CO₂.

**Experimental procedures**

**Human liver specimens and TCGA cohort**

Sixty paired human primary HCC/matched adjacent noncancerous liver tissue specimens were obtained from Qidong Liver Cancer Institute. Informed consent was obtained from all patients, and the collection of tissue specimens for clinical analysis was approved by the University Ethical Committee. TCGA data were accessed from the website (https://tcga-data.nci.nih.gov/tcga/). We downloaded LIHC gene expression (Illumina-HiSeq percentile) data including all of the 373 liver cancer patients (mRNA expression data of 50 paired cancer/noncancerous tissues were available) to analyze the mRNA expression levels of NKX3.1 and FOXO1. The tests were performed on matched samples.

**Cell lines and culture**

The human hepatocellular carcinoma cell lines SMMC-7721 and Li7 were purchased from the Riken Cell Bank. MHCC-LM3 and MHCC-97L cells were kindly provided by the Liver Cancer Institute of Zhongshan Hospital at Fudan University (Shanghai, China). The HCCC-LY10 cell line was established in our laboratory. The PLC/PRF5 and SK-Hep1 cell lines, immortalized normal hepatocyte L02, and human embryonic kidney 293T cell line were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Huh7 cells were obtained from the Cell bank of the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The full-length human NKX3.1 open read frame cDNA sequence was generated and cloned into the lentiviral vector pWPXL (Addgene, Cambridge, MA). The FOXO1 promoter, which spans a 1794-bp sequence upstream of the first ATG was amplified. The deleted sequences span the region from −1010 and −550 bp to the first ATG. The mutant was generated by mutating the DNA-binding site (−230 to −239 bp). All promoter sequences were cloned into the pGL3 vector (Promega, Madison, WI). The primers for cloning are provided in **supplemental Table S3**. shRNAs targeting NKX3.1, FOXO1, and a negative control (shNC) were obtained from Genechem (Shanghai, China). Target sequences are listed in **supplemental Table S4**. Lentivirus production and cell transfection were performed as described previously (47).

**Cell proliferation assay (MTT assay)**

A total of 1000 cells were seeded into 96-well culture plates per well and incubated for 7 days. MTT reagent (5 mg/ml, Sigma) was added to each well and incubated at 37 °C. After 4 h, the formazan was dissolved in 100 μl of DMSO and the absorbance value was measured at 570 nm. Each experiment was performed in triplicate.

**Flow cytometry analysis**

The cell cycle distribution was detected using flow cytometry analysis. Cells were plated into 6-well culture plates at a density of 1 × 10⁶ cells per well for 24 h. They were treated with 2 mM thymidine or 0.3 μM nocodazole (Sigma) for 24 h to be synchronized at the G1/S and G2/M boundaries. Cells were then harvested by trypsin after 0, 12, and 24 h, centrifuged, and washed with PBS twice and fixed with 70% ethanol at −20 °C overnight. Before analysis by flow cytometry, cells were washed twice again with PBS and resuspended with 400 mg/ml of propidium iodide, 10 mg/ml of RNase (Sigma), and 0.1% Triton X-100 in 500 μl of PBS at 4 °C for 30 min. DNA content was quantified using ModFit 3.2 software. Each experiment was performed in triplicate.

Proteins extracted from HCC tissues and cells were lysed by RIPA buffer (Thermo Scientific) containing protease inhibitor mixture and phosphatase inhibitor (Roche, Welwyn Garden, Swiss), separated by 10% SDS-PAGE, and transferred to 0.45-μm polyvinylidene difluoride membranes (Merck Millipore). After blocking with 5% nonfat milk solution for 1 h at room temperature, the membranes were incubated with primary antibody at 4 °C overnight and probed with HRP-conjugated secondary antibody for 1.5 h at room temperature. The antibody information is listed in **supplemental Table S2**.

Plasmid constructs, lentivirus production, and cell transfection

The full-length human NKX3.1 open read frame cDNA sequence was generated and cloned into the lentiviral vector pWPXL (Addgene, Cambridge, MA). The FOXO1 promoter, which spans a 1794-bp sequence upstream of the first ATG was amplified. The deleted sequences span the region from −1010 and −550 bp to the first ATG. The mutant was generated by mutating the DNA-binding site (−230 to −239 bp). All promoter sequences were cloned into the pGL3 vector (Promega, Madison, WI). The primers for cloning are provided in **supplemental Table S3**. shRNAs targeting NKX3.1, FOXO1, and a negative control (shNC) were obtained from Genechem (Shanghai, China). Target sequences are listed in **supplemental Table S4**. Lentivirus production and cell transfection were performed as described previously (47).

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**Cell lines and culture**

The human hepatocellular carcinoma cell lines SMMC-7721 and Li7 were purchased from the cell bank of the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Huh7 cells were obtained from the Riken Cell Bank. MHCC-LM3 and MHCC-97L cells were kindly provided by the Liver Cancer Institute of Zhongshan Hospital at Fudan University (Shanghai, China). The HCCC-LY10 cell line was established in our laboratory. The PLC/PRF5 and SK-Hep1 cell lines, immortalized normal hepatocyte L02, and human embryonic kidney 293T cell line were obtained from the American Type Culture Collection (Manassas, VA). All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco). The medium contained 10% fetal bovine serum (FBS, Gibco), 100 μg/ml of streptomycin, and 100 units/ml of penicillin (Sigma) in a humidified 37 °C incubator with 5% CO₂.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA from human primary HCC tissues and cell lines were extracted using TRIzol reagent (Invitrogen). Complementary DNA from reverse transcription was performed using the PrimeScript™ RT Reagent Kit (TaKaRa Bio, Japan). The real-time PCR using SYBR Green Master Mix (TaKaRa Bio, Japan) was performed with an Applied Biosystems 7500 Software version 2.0.5 real-time PCR system (Thermo Scientific). The primer sequences are listed in **supplemental Table S1**. The expression data were normalized to the mean of the housekeeping gene GAPDH.
**NKK3.1 acts as a tumor suppressor in HCC**

**In vitro wound-healing assay**

Cells were plated into 6-well culture plates equally and grown to about 90% confluence in 24 h. We used the fine end of a 200-μl pipette tip to create an artificial homogenous wound in the monolayer. After scratching, the cells were cultured in DMEM containing 2% FBS and 1 mM thymidine (Sigma). Before taking pictures, cells were washed twice with serum-free DMEM. Images of cells migrating into the wound were photographed at 0, 24, and 48 h using an inverted fluorescence microscope (Axiovert 200, HAL 100, Carl ZEISS, Oberkochen, Germany) under a ×10 objective lens. Each experiment was performed in triplicate.

**In vitro invasion assay**

A total of 1 × 10^5 cells were seeded into the top chamber of a transwell (BD Biosciences) in serum-free DMEM, whereas the bottom chamber was filled with DMEM containing 10% FBS. The transwell chambers contained Matrigel-coated membrane filter inserts. After 24 h incubation, cells adhering to the lower membrane of the inserts were fixed in 10% neutral phosphate-buffered formalin for 30 min, stained with Giemsa solution, and counted in five randomly selected fields.

**Luciferase assay**

Cells were plated in 96-well culture plates for 24 h and grown to about 90% confluence in 24 h. Cells were then transfected with the relevant reporter plasmids and the PRL-TK reporter construct with Lipofectamine 2000 (Invitrogen). Forty-eight hours later, firefly luciferase activity and Renilla activity were determined according to the manufacturer’s instructions (Promega).

**Chromatin immunoprecipitation (ChIP)**

ChIP assay was performed in SMMC-7721–pWPXL and SMMC-7721–NKK3.1 cells. Cells were cross-linked with 10% formaldehyde and reversed with 1 M glycine. After washing with PBS buffer, cells were harvested in Tissue Protein Extraction Reagent (Thermo Scientific) for 5 min on ice and centrifuged at 2,000 × g for 5 min. The precipitants were suspended in nuclei lysates buffer and DNA was crushed into fragments by sonication. Mouse anti-NKK3.1 (Invitrogen) or mouse IgG with protein A/G-agarose beads (Sigma) were added and incubated overnight at 4°C to immunoprecipitate DNA-containing complexes. After washing, DNA was isolated and used for PCR analysis. Primers for the FOXO1 promoter were: forward, 5′-cactgagaaagggagagaatc-3′ and reverse, 5′-ggttttccagggagggc-3′.

**Tumor xenograft models**

Six to 8-week-old male NOD/SCID mice were divided into groups randomly. For the liver orthotopic model, 2 × 10^6 SMMC-7721 cells or HCC-LY10 cells stably expressing NKK3.1 and control pWPXL were suspended in serum-free DMEM with Matrigel (1:1, BD Biosciences) for each mouse. After 4 to 6 weeks, all mice were sacrificed. Xenograft tumors were weighted, collected livers and lungs were fixed in 10% neutral phosphate-buffered formalin. The samples were embedded in par-}

affin and stained with hematoxylin and eosin. All animal experimental protocols were approved by the Shanghai Medical Experimental Animal Care Commission.

**Statistical analysis**

The data were presented as the mean ± S.D. Statistical analysis (comparisons between two groups) was performed using Student’s t test. The Pearson correlation was performed to analyze the correlation between expression levels of NKK3.1 and FOXO1, p < 0.05 was considered statistically significant (*, p < 0.05; **, p < 0.01). Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) 19.0 software and GraphPad Prism 5.

**Author contributions**—J. Y. J. and J. L. designed the study and analyzed the data. J. Y. J. and H. L. performed the in vitro function of NKK3.1-overexpression and molecular mechanism experiments. Z. L. performed the in vitro functional experiments of NKK3.1-knockdown. C. C. extracted the plasmids of shRNAs targeting NKK3.1. F. Y. Z. and M. Y. performed the in vivo experiments. C. G. performed the histopathological examination. T. Y. C collected the primary HCC tissue samples and clinical significant survey and analysis. J. Y. J. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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