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ORIGINAL ARTICLE

Regulation of proliferation, angiogenesis and apoptosis in hepatocellular carcinoma by miR-26b-5p

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Abstract MicroRNAs (miRNAs) play vital roles in cell proliferation, differentiation and apoptosis in hepatocellular carcinoma (HCC). miR-26b has been confirmed as an important regulator in carcinogenesis and other pathological processes. miR-26b-5p is one member of the mature miR-26 family, and its functional role in proliferation, angiogenesis and apoptosis in HCC remains unknown. Here, we demonstrate that miR-26b-5p expression was significantly decreased in HCC tissues and HCC cell lines compared with normal liver tissues and liver cells by quantitative real-time polymerase chain reaction (qRT-PCR). The relationships between miR-26b-5p and the clinical characteristics of HCC patients were further analysed, and miR-26b-5p was positively correlated with the differentiation of HCC cells. Computational searches were further used to identify the downstream targets and signalling pathways of miR-26b-5p in HCC cells. Cell viability, proliferation and tube formation abilities were assessed by scrape, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and three-dimensional culture assays to confirm that miR-26b-5p inhibited HCC cell growth and impaired the tube formation ability of the HCC cells. Both in vitro and in vivo studies showed that miR-26b-5p could suppress vascular mimicry (VM) and angiogenesis by down-regulating the expression of VE-cadherin, Snail and MMP2 and could inhibit the apoptosis of HCC cells. Using mouse models, we revealed that tumours derived from miR-26b-5p-expressing HCC cells displayed a significant decrease in microvessel density compared with those derived from control cells. Therefore, our data provide further insight into the role of miR-26b-5p as a negative regulator of proliferation, angiogenesis, and apoptosis in HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy globally and remains a difficult-to-treat cancer due to rapid recurrence and metastasis [1]. MicroRNAs (miRNAs), a class of 21- to 25-nucleotide noncoding RNAs, regulate many genes by binding to the 3′-untranslated region (UTR) of their mRNAs [2–5]. Emerging evidence has revealed that aberrant expression of miRNA is very common in HCC, and some deregulated miRNAs, including miR-429, miR-1285-3p, miR-335, miR-31, miR-26b, miR-206, miR-10a, miR-216a/217 and miR-29, have been shown to regulate

Fig. 1 The expression of miR-26b-5p in HCC tissues and HCC cell lines and the relationship between miR-26b-5p expression and rapid recurrence in HCC patients. **a** qRT-PCR was used to determine the mRNA expression of miR-26b-5p in 41 HCC tissues compared to that in 38 normal liver tissues. *$p < 0.05$. U6 was used as the internal control. **b** The mRNA level of miR-26b-5p in HCC cell lines (Bel7402, SMMC, HepG2 and PLC) compared to that in a normal liver cell line (LO2), as detected by qRT-PCR. **$p < 0.01$. U6 was used as the internal control. **c** The low level of miR-26b-5p predicts rapid recurrence in HCC patients. The average expression value obtained for miR-26b-5p from the 41 HCC samples examined with qRT-PCR was chosen as the cutoff point for analysis using the Kaplan-Meier method
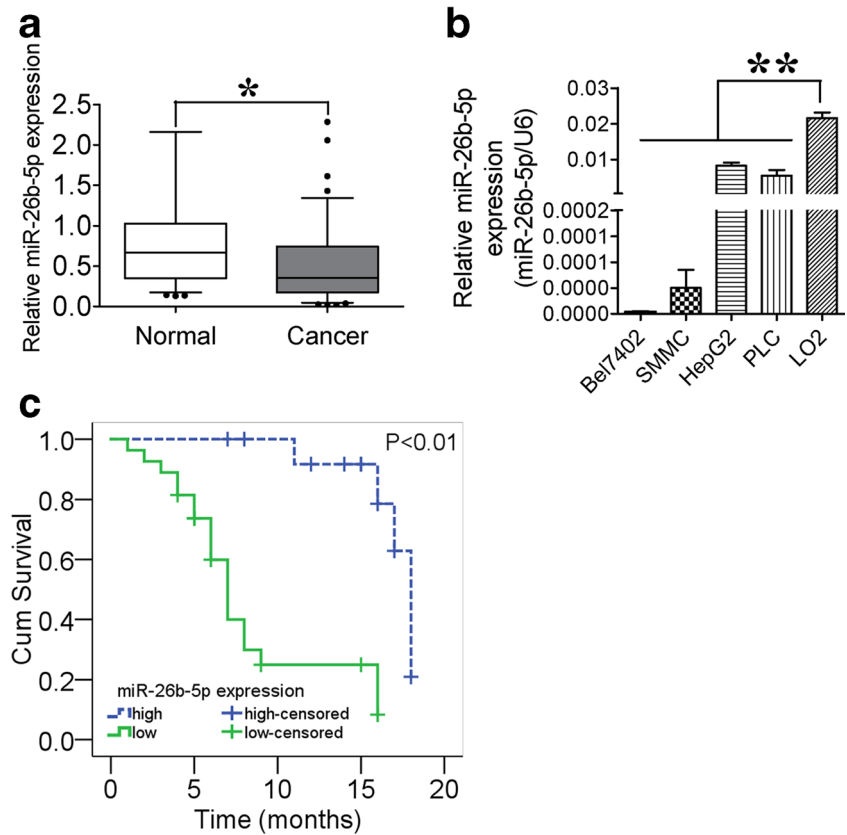
the cell cycle, apoptosis, migration, invasion, drug resistance and recurrence in HCC [6–14]. The above findings indicate that abnormal expression of miRNAs may be associated with the progression of HCC. Although many studies have recently postulated the widespread disruption of miRNAs in HCC, the pathophysiological contributions of miRNAs to HCC are still largely unknown. These miRNAs may be new prognosis predictors and therapeutic targets for HCC.

Studies from many groups have shown that the miR-26 family (miR-26a/b) is commonly down-regulated in multiple types of cancer, including HCC, breast cancer, colon cancer and melanoma. The regulatory properties of miR-26b in the

Table 1 Associations between miR-26b-5p expression and clinicopathologic characteristics in 41 patients with HCC

Characteristics	No.	%	Median expression of miR-26b-5p/U6	P
Gender				
Male	34	82.9	0.388031277	0.579
Female	7	17.1	0.413087401	
Age (year)				
<50	10	24.4	0.354797558	0.627
≥50	31	75.6	0.381888152	
Size(cm)				
≤5	27	65.9	0.381888152	0.762
>5	14	34.1	0.426271157	
Hepatic cirrhosis				
+	13	31.7	0.413087401	0.978
−	28	68.3	0.368605535	
Cell differentiation				
Well or moderately	20	48.8	0.304232788	0.011*
Poorly	21	51.2	0.279236486	

miR microRNA, *Statistical significance ($p < 0.05$)

Fig. 2 miR-26b-5p alleviates the viability of HCC cells. **a**, **b** MTT assays with (**a**) miR-26b-5p-Bel7402 and miR-26b-5p-SMMC cells and (**b**) miR-26b-5p-inhibitor-HepG2 and miR-26b-5p-inhibitor-PLC cells. **c**, **d** Scrape assays with (**c**) miR-26b-5p-Bel7402 and miR-26b-5p-SMMC cells and (**d**) miR-26b-5p-inhibitor-HepG2 and miR-26b-5p-inhibitor-PLC cells. *Error bars* represent the SD; and *p < 0.05
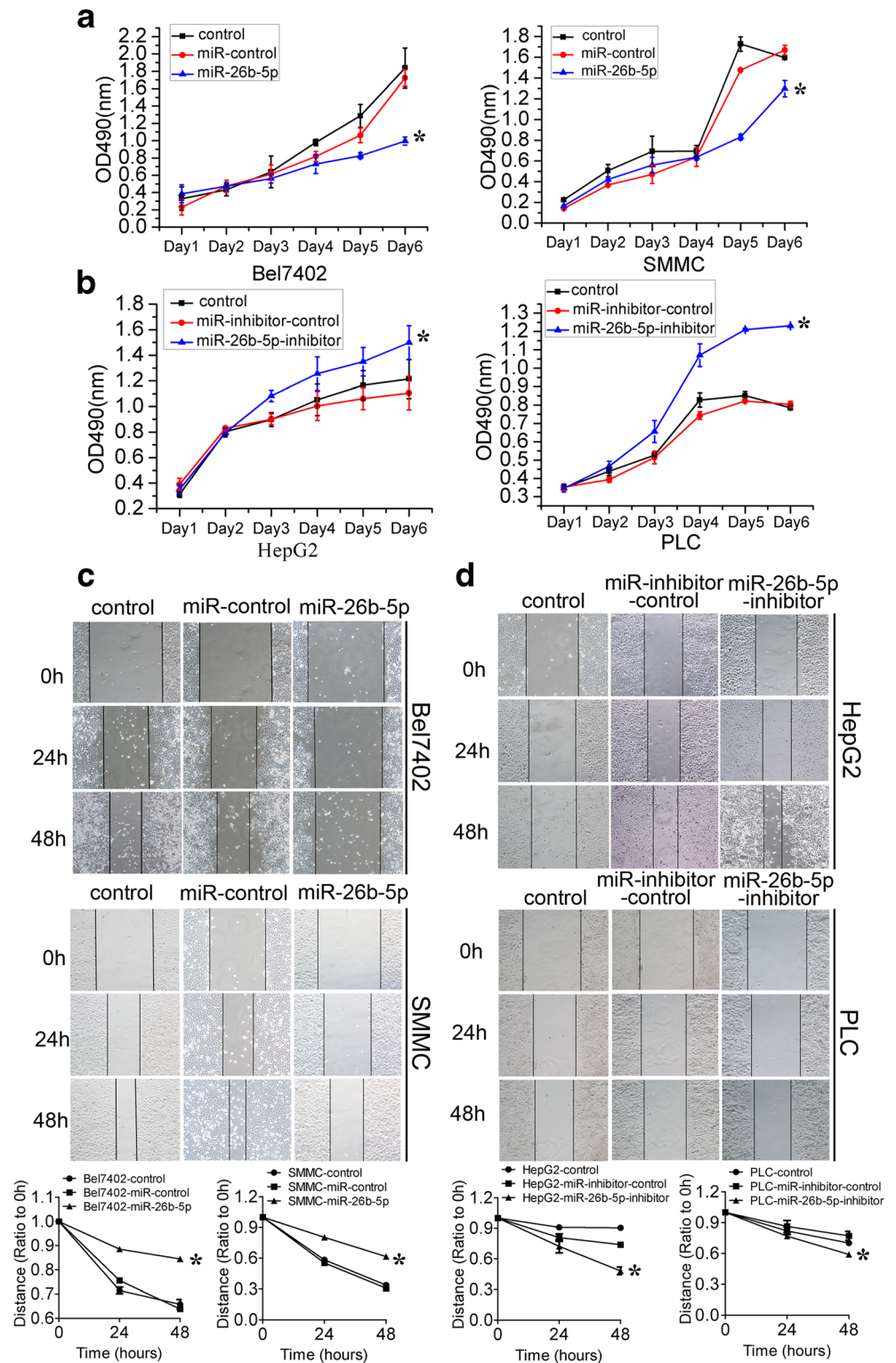
proliferation of various cancerous cells, cell differentiation, apoptosis, and chemosensitivity have been reported [10,15–18]. miR-26b-5p is one type of mature miR-26b. However, the mechanism by which miR-26b-5p regulates the development of HCC has not yet been pinpointed.

However, regardless of the results of one recent study that dissected the role of miR-26b-5p in human pulmonary cancer cells [19], the role of miR-26b-5p in the regulation of angiogenesis, cell proliferation and apoptosis in HCC remains largely unclear. Therefore, this study aimed to elucidate the

role of miR-26b-5p in HCC proliferation, angiogenesis and apoptosis.

Materials and methods

Cell culture and transfection

The Bel7402, SMMC7721 (SMMC), HepG2, PLC and LO2 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) with 10 % foetal bovine serum (Hyclone). Vectors were transfected into cells via percutaneous ethanol injection (Polysciences, Inc., Cat #23966).

Clinical HCC specimens

Through the Tumor Tissue Bank of Tianjin Cancer Hospital, 41 HCC tissue specimens and 38 noncancerous liver tissues were obtained from patients who underwent resection of HCC in 2014. The diagnoses of these HCC samples were verified by pathologists. The use of these tissue samples in this study was approved by the Institutional Research Committee.

RNA extraction and microarray analysis

Total RNA was extracted using Trizol reagent (Tiangen Biotech, Beijing, China), and miRNAs were obtained using the miRcute miRNA isolation kit (DP501) (Tiangen Biotech, Beijing, China). Microarray analysis was completed by Beijing Boao Biotechnology Company.

Vector construction

The pcDNA3-Twist1 and pcDNA3-Bcl-2 plasmids have been described [20,21]. The human pre-miR-26b-5p gene expression plasmid (referred to as miR-26b-5p), the miR-26b-5p gene expression inhibitor plasmid (referred to as miR-26b-5p-inhibitor) and the corresponding empty vector plasmids (referred to as miR-control and miR-inhibitor-control, respectively) were purchased from GeneCopoeia (USA). The stable cell line selection marker was puromycin.

Semiquantitative reverse-transcription polymerase chain reaction and quantitative real-time polymerase chain reaction

Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (qRT-PCR) were performed as previously described [22] using the primers listed in Tables S1 and S2. qRT-PCR was conducted using a 7500HT Real-Time PCR System (Applied Biosystems, Foster City, CA). A U6 or GAPDH internal control was used as an endogenous control, and fold

Fig. 3 miR-26b-5p impaired the tube formation abilities of HCC cells in vitro. **a**, **b** Effects of miR-26b-5p on the tube formation abilities of the HCC cell lines. Results for (**a**) miR-26b-5p-transfected Bel7402 and SMMC cells and (**b**) miR-26b-5p-inhibitor-transfected HepG2 and PLC cells. **c** The protein and mRNA levels of VE-cadherin and Snail in miR-26b-5p-transfected-Bel7402 and SMMC cells and in miR-26b-5p-inhibitor-transfected-HepG2 and PLC cells, as analysed by Western blotting, RT-PCR and qRT-PCR. β-actin and GAPDH were used as internal controls. **d** Gelatin zymography analysis of MMP2 expression in Bel7402 and SMMC cells treated with miR-26b-5p or in HepG2 and PLC cells treated with miR-26b-5p inhibitor. Original magnification: 100X. *Scale bar* represents 100 μm. *Error bars* represent the SD and *p < 0.05

changes were calculated via relative quantification ($2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$) [23].

For miRNA detection, a two-step quantitative RT-PCR reaction using the miRcute miRNA cDNA Synthesis Kit (KR201) and miRcute miRNA qPCR Detection Kit (SYBR Green) (FP401) (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions was used.

Western blot analysis

Protein extractions and Western blot analyses were performed as described [20]. Blots were blocked and incubated with antibodies (Table S3).

3-(4,5Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

The Bel7402, SMMC, HepG2 and PLC cells (8000 cells/well) were placed in 96-well plates. At 24 h following transient transfection, the cells were continually cultured for different periods (1, 2, 3, 4, 5, or 6 days). Subsequently, 10 μl of 0.5 mg/ml MTT was added to each well. The cells were incubated at 37 °C for another 4 h, the medium was removed and the precipitated formazan was dissolved in 100 μl of DMSO. After the solution was shaken for 10 min using an Eppendorf Mixmate (Eppendorf, GRE), the absorbance was detected at 570 nm (A570) on a BioTek ELx800 (BioTek, USA).

Scrape assays

In the scrape assays, cell motility was assessed by measuring the movement of cells into a scrape. The speed of wound closure was monitored after 24 and 48 h by measuring the ratio of the size of the wound relative to that at hour 0. Each experiment was performed in triplicate.

Three-dimensional culture

Twenty-four hours after the tumour cells were transfected, they were then trypsinised and plated onto 24-well plates that
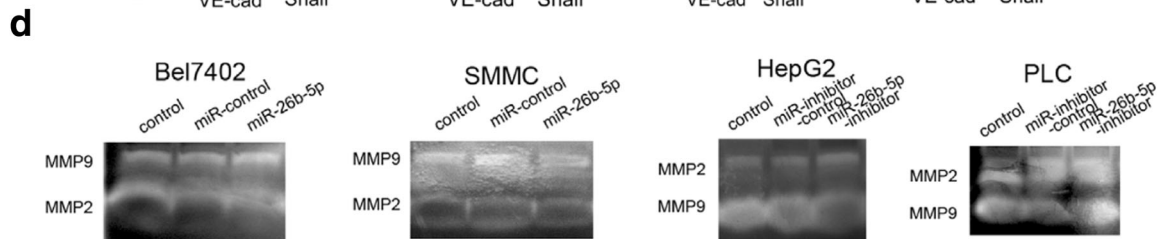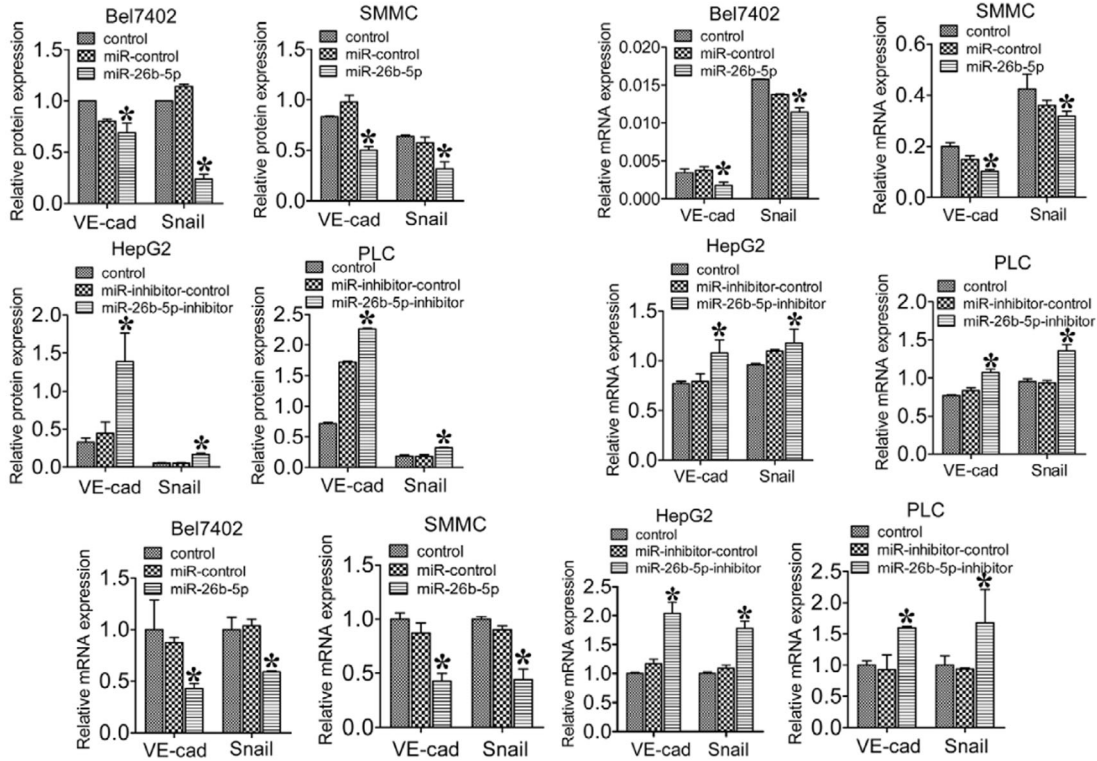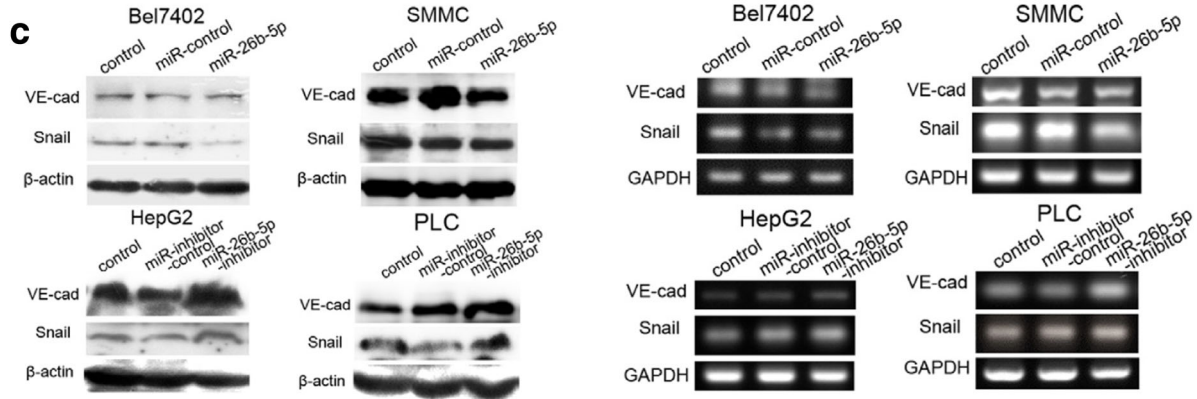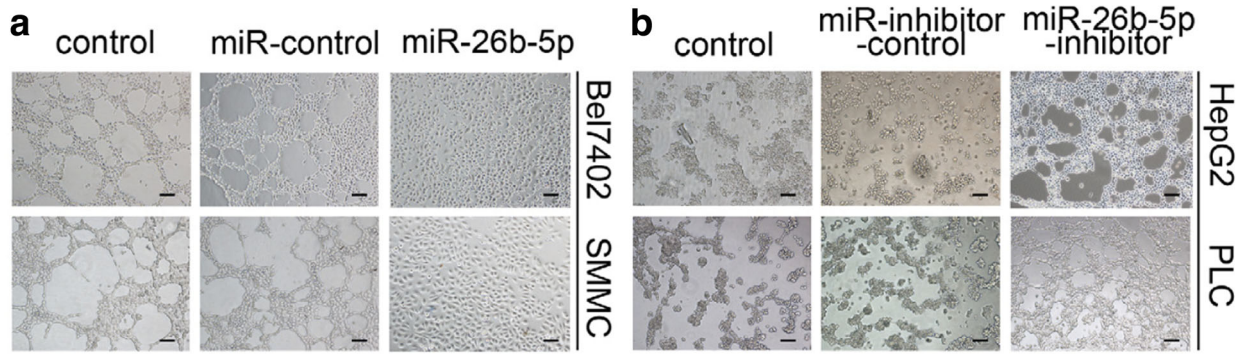
were pre-coated Matrigel (BD Biosciences), following the methods used in our previous studies [20]. After 48 h of culturing, the cells were photographed.

Gelatin zymography

For gelatin zymography, experiments were performed as previously described [20]. The gels were then stained with 0.1 % Coomassie Brilliant Blue G250 and destained until the wash buffer became clear, and the clear bands associated with matrix metalloproteinase (MMP) activity became apparent.

Apoptosis assay and hoechst 33342 staining

Cell apoptosis was analysed using a BD Accuri™ C6 flow cytometer (BD Biosciences, USA) and the Annexin V-FITC Apoptosis Detection Kit (KeyGEN BioTECH). The experiments were performed according to the manufacturer's instructions. Flow cytometric analysis of PI-Annexin V staining was repeated at least three times.

For Hoechst 33342 staining, cells were briefly trypsinized, plated onto 6-well culture plates and allowed to completely adhere in standard culture medium. The Hoechst 33342 stain (2.5 μg/ml) was then added to each well, and the cells were allowed to incubate for another 4 h. The cells were viewed under a fluorescence microscope (Nikon).

Animal studies

For the subcutaneous xenograft model, SMMC (5×10^6) and HepG2 cells (1×10^7) (stably transfected with P-miR-26b-5p, P-miR-26b-5p-inhibitor and the corresponding control vectors) were suspended in 100 μl of PBS and then subcutaneously injected into the upper right flank region of 3- to 4-week-old female BALB/c-nu/nu mice (8 in each group) with a microsyringe under anaesthesia. After 4 or 5 weeks, the mice were sacrificed, and the tumours were harvested, fixed with phosphate-buffered neutral formalin and prepared for standard histological examination. All studies were performed under the American Association for the Accreditation of Laboratory Animal Care guidelines for the humane treatment of animals and adhered to national and international standards.

Immunohistochemical and endomucin/periodic acid-Schiff double staining, microvessel density

Sections were pretreated with a microwave, blocked and incubated with a series of antibodies (Table S3). The immunohistochemical (IHC) staining system used in this study and the endomucin/periodic acid-Schiff (PAS) double staining experiments were performed as previously described [22]. The results were quantified according to the method described by Bittner and colleagues.

Fig. 4 miR-26b-5p inhibited tumour angiogenesis in vivo. **a** The ▶ expression of VE-cadherin was studied in the tissues used in Fig. 1a by qRT-PCR. **b** Tumours with a lower miR-26b-5p level displayed higher VE-cadherin expression. The median of all 41 HCC cases was chosen as the cut-off point for separating the low- from the high-miR-26b-5p-expressing tumours. **c** Pearson correlation analysis showed an inverse correlation between miR-26b-5p expression level and VE-cadherin mRNA level in the HCC specimens ($r = -0.4611$, $p = 0.0024$). **d, e** SMMC and HepG2 cells (stably transfected with P-miR-26b-5p, P-miR-26b-5p-inhibitor, and the corresponding control vectors) were subcutaneously suspended in each mouse. **d** Tumours with higher miR-26b-5p expression showed lower MVD. Original magnification: 100X. *Scale bar* represents 100 μm. **e** IHC staining of VE-cadherin and MMP2 protein expression in the control and treatment groups. **f** Endomucin/PAS double staining showed VM formation in HCC tissues. *Red arrows* indicate VM, and *black arrows* indicate endothelium-dependent vessels. Original magnification: 400X. *Scale bar* represents 50 μm. *Error bars* represent the SD; and **$p < 0.01$, ***$p < 0.001$

The microvessel density (MVD) in the mouse tumour tissues, which represents the degree of angiogenesis in vivo, was evaluated by staining for endomucin. Any discrete cluster or single cell stained for endomucin was counted as one microvessel.

Statistical analysis

All data were evaluated using SPSS 22 (SPSS Inc., Chicago, USA). All statistical analyses were performed using ANOVA or a two-tailed Student's *t* test to compare data. The survival curves were calculated using the Kaplan-Meier method. Differences were considered significant at $p < 0.05$.
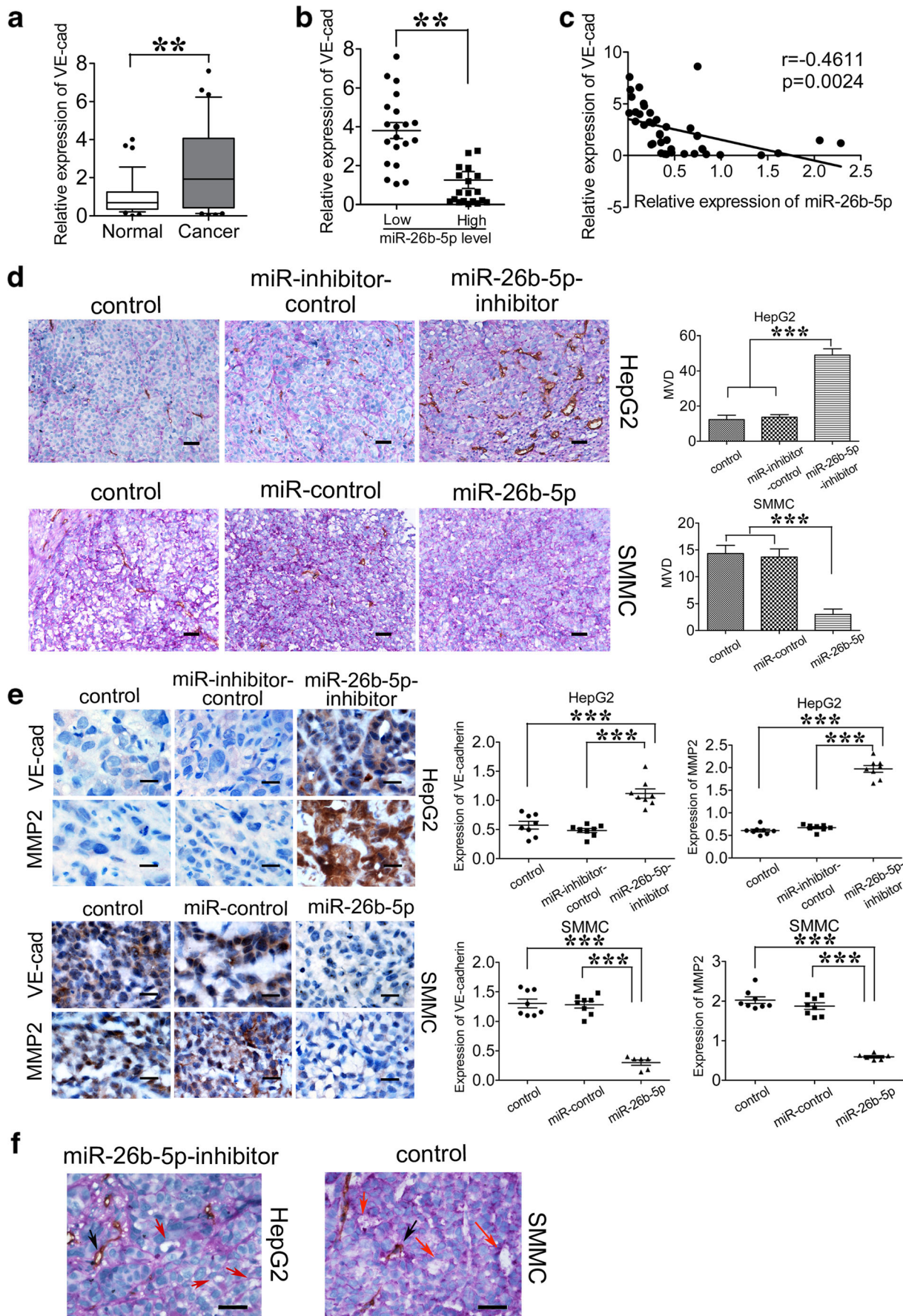
Results

Expression of miR-26b-5p is frequently down-regulated and is positively correlated with cell differentiation in HCC

Here, miR-26b-5p expression was first analysed in 41 HCC tissues and 38 normal liver tissues by way of qRT-PCR. Notably, miR-26b-5p was significantly down-regulated in the majority of the examined HCC tissues (Fig. 1a). Consistent with the results from the HCC tissues, a similar trend of a decrease in miR-26b-5p in HCC cells was observed by qRT-PCR analysis (Fig. 1b).

The relationship between the expression of miR-26b-5p and the clinical data of the HCC patients was further investigated. We discerned from the results that the tumours with decreased expression of miR-26b-5p had poor cellular differentiation (Table 1). Moreover, using the Kaplan-Meier method, we found that a low level of miR-26b-5p was inversely correlated with short-term recurrence ($p < 0.01$) (Fig. 1c).

Overall, our data suggest that a low level of miR-26b-5p in HCC may result in the poor prognosis of HCC patients by regulating cell differentiation and rapid recurrence.

miR-26b-5p inhibits HCC cell growth in vitro

The functional importance of miR-26b-5p in HCC progression was further characterized in a gain-of-function study. The effect of miR-26b-5p on the proliferation of HCC cells was first examined using the MTT assay. Bel7402 and SMMC cells that were transfected with miR-26b-5p displayed a much lower ability to promote proliferation compared with those that were transfected with empty vector or with non-transfected cells (Fig. 2a). We next examined the role of miR-26b-5p in the motility capacity of HCC cells using scrape assays. The introduction of miR-26b-5p substantially reduced the motility capacity of HCC cells (Fig. 2c). However, the motility capacity of the non-transfected cells and those that were transfected with empty vectors were similar at the end of experiment.

Loss-of-function analysis was performed using miR-26b-5p-inhibitor (anti-miR-26b-5p), which strikingly decreased the endogenous miR-26b-5p level in HepG2 and PLC cells. The suppression of cellular miR-26b-5p enhanced the proliferation and motility activity of HCC cells (Fig. 2b, d).

miR-26b-5p impaired the tube formation abilities of HCC cells in vitro and tumour angiogenesis in vivo

To explore the biological significance of miR-26b-5p in tumour angiogenesis, in vitro capillary tube formation abilities were first analysed using a three-dimensional culture assay. Bel7402 and SMMC cells that were transfected with miR-26b-5p developed fewer capillary-like structures compared with those that were not transfected or that were transfected with empty vectors (Fig. 3a). However, in the HepG2 and PLC cells that were transfected with miR-26b-5p-inhibitor, the number of branch points of capillary-like structures was dramatically increased compared with that in the corresponding controls (Fig. 3b). Vascular endothelial-cadherin (VE-cadherin) and Snail, common vascular mimicry (VM)-related transcription factors, were detected by Western blot, RT-PCR and qRT-PCR analyses. Cells that were transfected with miR-26b-5p showed lower expression of VE-cadherin and Snail than those that were not transfected or that were transfected with empty vectors. The opposite results were observed in the group transfected with miR-26b-5p-inhibitor (Fig. 3c). The overexpression of MMP-2 has been frequently reported in tumour tissues, and its importance in tumour angiogenesis is also well known. A gelatin zymography assay was used to detect the activity of MMP-2 (Fig. 3d). The results were similar to those of VE-cadherin expression.

Fig. 5 miR-26b-5p inhibited apoptosis in HCC. **a** The Annexin V-FITC ▶ Apoptosis Detection Kit was used to detect the percentage of apoptotic cells in the negative control miRNA, miR-26b-5p and miR-26b-5p-inhibitor treatment conditions. FL1-A: Annexin V; FL3-A: PI. The *bar* graph shows the mean ± SD of three independent transfection experiments. *$p < 0.05$. **b** An apoptotic phenotype was detected using Hoechst 33342 staining, as observed under a fluorescent microscope. The nuclei of the apoptotic cells are bright *blue*, whereas the nuclei of live cells are *light blue*. Original magnification: 200X. *Scale bar* represents 50 μm. *$p < 0.05$

qRT-PCR was used to analyse human HCC tissues samples from 41 HCC cases for VE-cadherin, and then the association between miR-26b-5p level and VE-cadherin expression was further analysed (Fig. 4a). Obviously, VE-cadherin was significantly down-regulated in the HCC tissues, and the level of miR-26b-5p was inversely correlated with VE-cadherin expression ($r = -0.4611$, $p = 0.0024$) (Fig. 4b, c). In addition, bioinformatics tools identified VE-cadherin as a putative target gene of miR-26b-5p. Based on these results and our previous observations, we suggest that down-regulation of miR-26b-5p may be responsible for the increased level of VE-cadherin in human HCC tissues, which in turn promotes angiogenesis in HCC.

To verify this role of miR-26b-5p, we used an in vivo model by subcutaneously injecting HCC cells into nude mice. The tumours derived from cells transfected with miR-26b-5p displayed much smaller and fewer blood vessels than those of the control groups (Fig. 4d). miR-26b-5p downregulation was significantly associated with higher MVD in the miR-26b-5p-inhibitor group (Fig. 4d). The samples from the nude xenograft model mice were immunohistochemically stained for VE-cadherin and MMP-2. Compared with those of the controls, the tumours derived from miR-26b-5p-transfected cells showed lower levels of these two indicators, while in the groups with the anti-miR-26b-5p-transfected cells, up-regulated MMP-2 activity was observed (Fig. 4e). Additionally, VM was observed in the tissues from the mice that were injected with HepG2 cells that were stably transfected with the miR-26b-5p inhibitor or with SMMC control cells (Fig. 4f). This finding suggests that miR-26b-5p inhibited the formation of VM in vivo, and this effect of miR-26b-5p is consistent with its effects in vitro.

In short, these data suggest that miR-26b-5p has a suppressive effect on tumour angiogenesis and VM.

The regulation of miR-26b-5p on the apoptosis of HCC cells

Next, the possibility of the regulation of apoptosis in HCC by miR-26b-5p was evaluated. The putative target genes of miR-26b-5p were analysed in several databases, such as TargetScan, PicTar and miRanda. These targets were
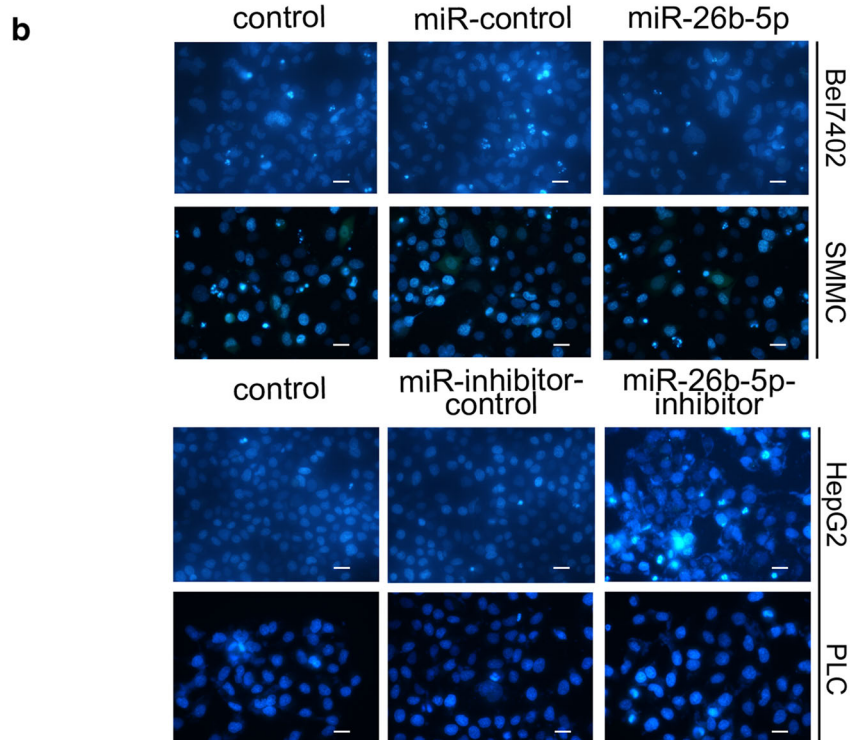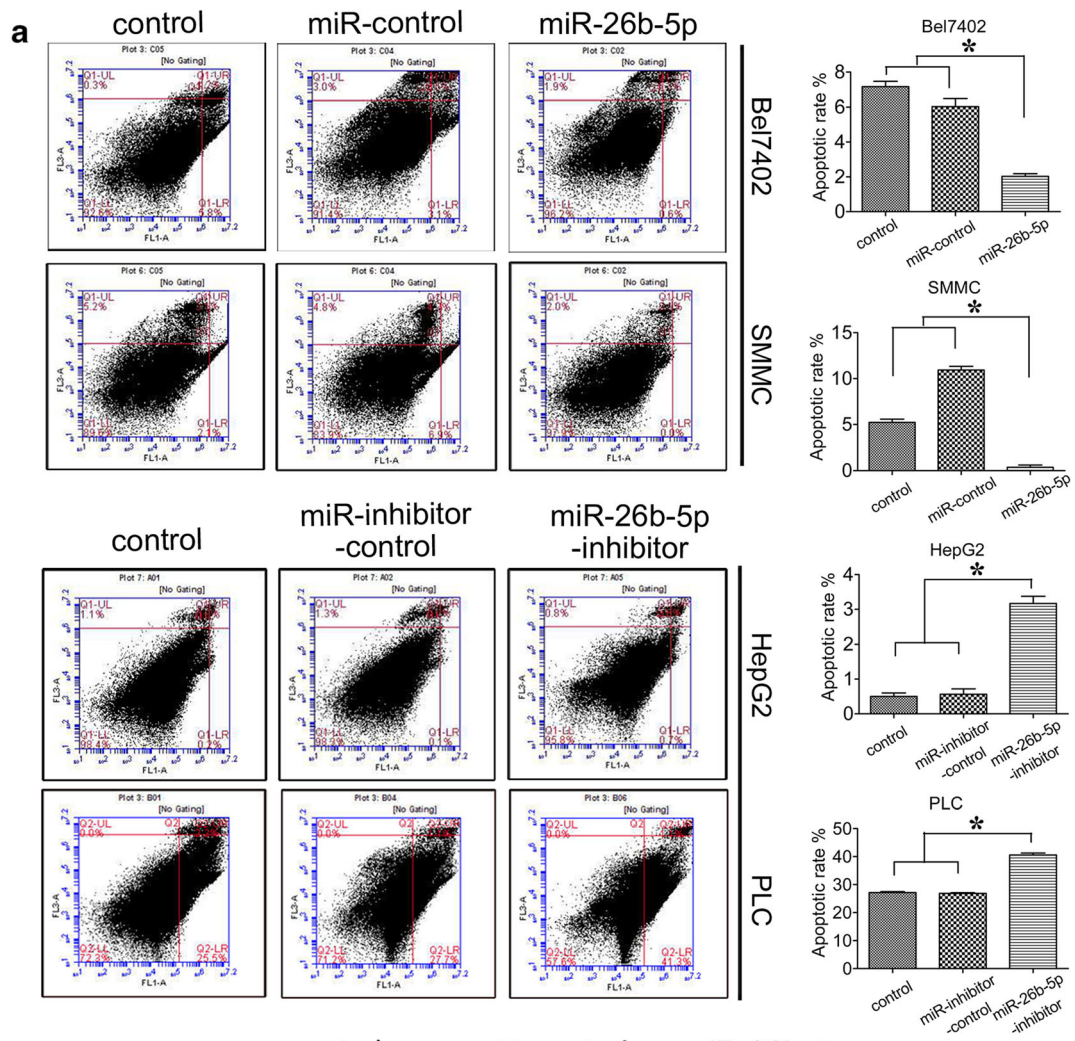
subjected to further analysis by the ToppCluster network analyser to identify its signalling pathways. Cytoscape 3.0.1. was used to generate the regulatory network of miR-26b-5p (Fig. 7c, d).

We further evaluated the effect of miR-26b-5p expression on the apoptosis of HCC cells. Bel7402 and SMMC cells were transiently transfected with negative control miRNA or miR-26b-5p, and after 2 days, the percentage of apoptotic cells was quantified using the Annexin V-FITC Apoptosis Detection Kit. The percentage of apoptotic (early and late) and dead cells was calculated. A significant change was observed in the percentage of apoptotic cells in the negative control miRNA and miR-26b-5p treatment groups. The group with miR-26b-5p upregulation showed a low percentage of apoptotic cells (Fig. 5a). Hoechst 33342, a fluorescent DNA dye, was used to detect the apoptotic phenotype. The results in the different groups were similar to those obtained with the Annexin V-FITC Apoptosis Detection Kit (Fig. 5b).

The molecular mechanisms that participate in apoptosis were also explored. Given that both anti-apoptotic Bcl-2 and pro-apoptotic Bax play crucial roles in the mitochondrial apoptotic pathway [24–26], we examined changes in Bcl-2 and Bax expression. There was a significant change in the mRNA and protein expression levels of Bcl-2 and Bax between the negative control miRNA- and miR-26b-5p-transfected Bel7402 and SMMC cells (Fig. 6a). miR-26b-5p could increase the expression of Bcl-2 and decrease the expression of Bax in HCC cells. These findings suggest that miR-26b-5p might have an inhibitory role on human HCC cell apoptosis. These results are consistent with previous reports [27,28]. However, other studies have indicated that miR-26b-5p induces apoptosis [19,29]. These conflicting results show that the apoptotic regulation by miR-26b-5p could depend on the specific cellular context.

The inhibitory effect of miR-26b-5p on apoptosis was replicated in HepG2 and PLC cells (Fig. 5a and b). Loss-of-function analysis was also carried out using inhibitors of miR-26b-5p, and the effect of miR-26b-5p-inhibitor on apoptosis was then analysed. Consistently, compared with the control groups, the groups transfected with miR-26b-5p-inhibitor displayed a notably higher apoptotic rate (Fig. 5a). The results of the Western blot, RT-PCR and qRT-PCR assays showed that the mRNA and protein expression levels of Bcl-2 and Bax between the negative control- and miR-26b-5p-inhibitor-transfected groups had opposite changes compared with those in the miR-26b-5p-transfected groups (Fig. 6a).

In HCC samples, the expression of Bcl-2 and Bax was detected by qRT-PCR. A trend toward reduced Bcl-2 and Bax levels was found in the HCC samples compared with the levels found in the normal liver tissues, and the difference reached statistical significance (Fig. 6b). HCC samples with low miR-26b-5p expression displayed a much higher Bcl-2

Fig. 6 miR-26b-5p suppressed the expression of Bcl-2 and Bax in HCC cells, and the level of miR-26b-5p was positively correlated with Bcl-2 expression in HCC specimens. **a** The protein and mRNA expression levels of Bcl-2 and Bax in miR-26b-5p-transfected Bel7402 and SMMC cells and in miR-26b-5p-inhibitor-transfected HepG2 and PLC cells, as determined by Western blotting, RT-PCR and qRT-PCR. β-actin and GAPDH were used as internal controls. **b** qRT-PCR was used to analyse the expression of Bcl-2 and Bax in the 41 HCC specimens. **c** Tumours with a lower miR-26b-5p level displayed lower Bcl-2 expression. The median of all 41 HCC cases was chosen as the cut-off point for separating low- from high-miR-26b-5p-expressing tumours. **d** SMMC and HepG2 cells (stably transfected with P-miR-26b-5p, P-miR-26b-5p-inhibitor, and the corresponding control vectors) were subcutaneously suspended in each mouse. IHC staining of Bcl-2 and Bax protein expression in the control and treatment groups. Original magnification: 400X. *Scale bar* represents 50 μm. *Error bars* represent the SD. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

level compared with those with high miR-26b-5p expression (Fig. 6c). Comparison with controls revealed that the results from the xenograft mouse model partially phenocopied the above findings (Fig. 6d).

Taken together, our data suggest that miR-26b-5p may inhibit apoptosis in HCC.

Discussion

The role of miRNA in tumourigenesis is paradoxical. One miRNA can control multiple genes, but it can also be regulated by more than one gene. For example, miR-29b can target MMP-2 and PI3K/AKT [30,31], and miR-29b can be regulated by both c-MYC and BAG3 [32,33]. Therefore, it will be important to highlight the functions of miRNA in carcinogenesis. The roles of miRNA in HCC have been investigated in previous studies [6–13]. Here, we demonstrated that miR-26b-5p was a tumour suppressor in HCC. The miR-26b-5p expression level was decreased in HCC cell lines and tissues, especially in tumour tissues of more advanced clinical stages. Using both in vitro and in vivo analyses, we showed that miR-26b-5p is capable of repressing tumour proliferation, angiogenesis and apoptosis. Our data may add a new miRNA maker in HCC.

Angiogenesis, which is defined as new vessel growth from a pre-existing vessel, was first introduced in melanoma by Warren in 1966 and has since been shown to be crucial for tumour survival and growth [34]. The concept of VM was first described in 1999 by Maniotis et al. as a process in which aggressive melanoma cells may generate vascular channels that facilitate tumour perfusion independent of endothelial cells. VM is associated with an aggressive and metastatic tumour phenotype [35]. We and others have proposed that tumours, including HCC, may develop vascularization not only through angiogenesis but also through alternative pathways, such as VM and mosaic vessels. The use of tracers and
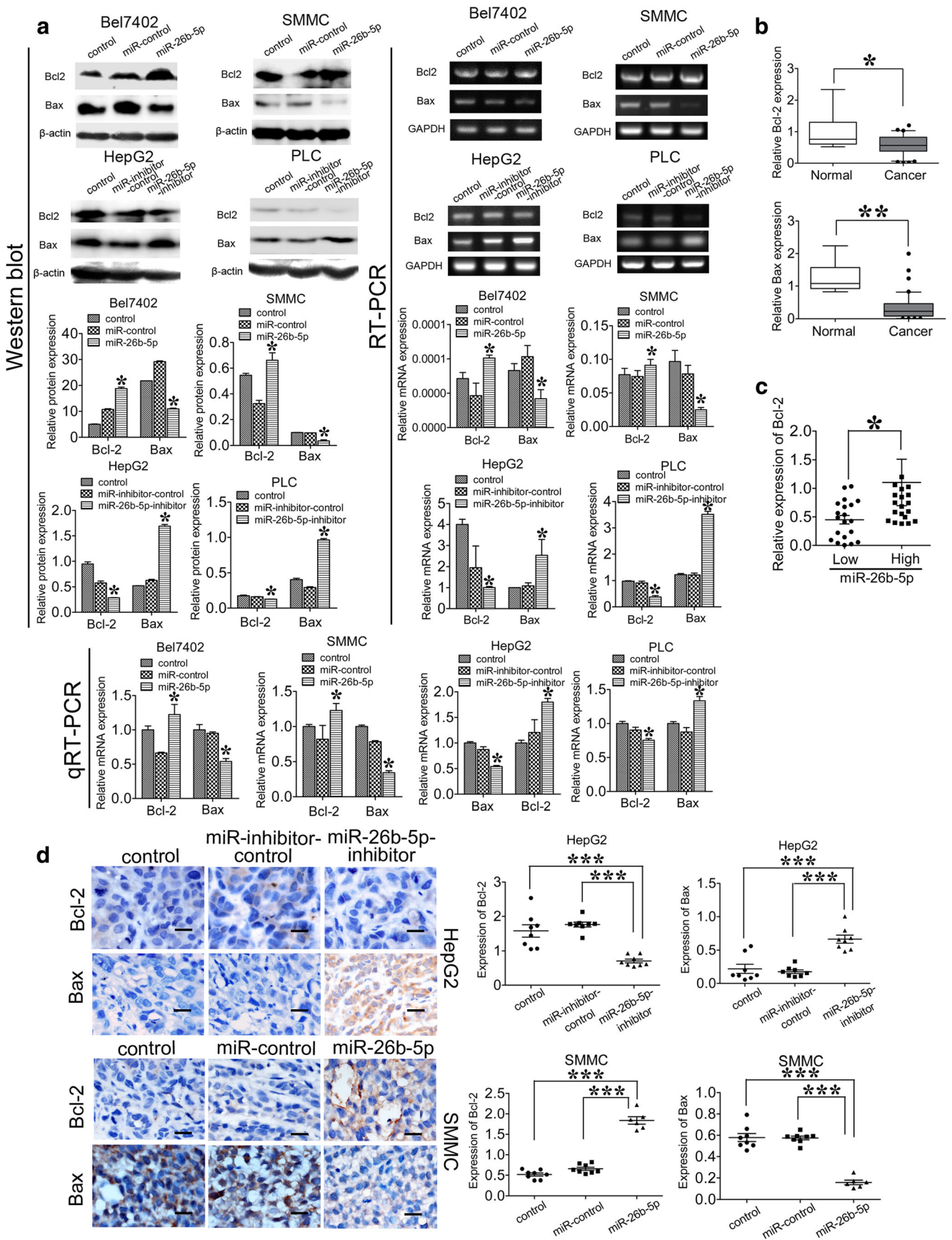
magnetic resonance imaging has confirmed the physiological connection between endothelial-lined vasculature and tumour-lined networks [36–39]. We have reported that VM formation was induced in HCC by up-regulating VE-cadherin and increasing the activity of MMP-2 [20,39]. In the present study, miR-26b-5p or miR-26b-5p inhibitor could lead to decreased or increased VE-cadherin expression at the transcriptional and translational levels, respectively, in HCC cells. The mRNA level of VE-cadherin was also inversely correlated with miR-26b-5p expression in HCC tissues. These data suggest that miR-26b-5p may have a suppressive effect on VM formation. Moreover, the results from the in vitro capillary tube formation assay and in vivo subcutaneous xenograft mouse model further confirmed the above findings.

Detecting MVD for angiogenesis in histopathology may also be a useful prognostic marker for the risk stratification of tumours [40]. Based on the xenograft mouse models, compared with the control groups, the miR-26b-5p expression groups showed much less MVD. MMP-2 is well known to be associated with angiogenesis, which can degrade basement membrane components. Its overexpression has been reported in different types of tumours, including HCC [40]. In vitro, the activity of MMP-2 was downregulated after miR-26b-5p upregulation. In vivo, the expression of MMP-2 in tumours derived from miR-26b-5p-inhibitor-transfected cells was obviously higher than that in the control groups.

Overall, our data suggest that miR-26b-5p deregulation in HCC may result in enhanced VE-cadherin and MMP-2 levels in the tumour microenvironment, which in turn induces VM formation and thereby promotes angiogenesis.

Cancer cells evolve to circumvent apoptosis, which is a major barrier to tumour progression so that they can survive under environmental conditions crucial for tumour growth, such as hypoxia [41]. miR-26 has been shown to suppress apoptosis in a hypoxic environment [27–29]. miR-26b-5p is one member of the mature miR-26 family, and to our knowledge, this is the first analysis of its effect on apoptosis in HCC. In our study, both in vitro and in vivo analyses suggested an inhibitory effect of miR-26b-5p on apoptosis in HCC. Our clinical data suggested that the expression of Bcl-2 and Bax in HCC specimens was markedly lower than that in normal liver tissues. These above results are consistent with those of previous reports [25,42]. Additionally, miR-26b-5p and Bcl-2 mRNA levels were positively correlated in the HCC samples.

To screen for miRNAs that could be involved in apoptosis in HCC, we performed miRNA expression profiling in HepG2-vector, HepG2-Bcl-2, HepG2-Twist-1/Bcl-2 (stable transfection) cells by microarray (Fig. 7a, b). Among several miRNAs, miR-26b-5p drew our attention. The expression of miR-26b-5p was significantly upregulated in the HepG2-Bcl-

Fig. 7 Microarray analyses of miR-26b-5p expression in stably ▶ transfected HepG2 cells. **a** The expression of miR-26b-5p in HepG2-vector, HepG2-Bcl-2, and HepG2-Twist-1/Bcl-2 cells, as determined by microarray. *Error bars* represent the SD. *$p < 0.05$. **b** miRNA microarray analysis revealed that miR-26b-5p was differentially expressed between HepG2- Bcl-2 cells and HepG2-vector cells and between HepG2-Twist-1/Bcl-2 cells and HepG2-vector cells. **c, d** Microarray analyses revealed the predicted targets (**c**) and signalling pathways (**d**) of miR-26b-5p

2 cell line compared with the HepG2-vector cell line ($p = 0.01777983$). The increase in miR-26b-5p expression was significantly enhanced in the HepG2-Twist-1/Bcl-2 cell line compared with the HepG2-Bcl-2 cell line ($p = 0.00121265$). Our previous study reported that Twist-1 and anti-apoptotic Bcl-2 in HCC may form a complex at the protein level in vivo and synergistically activate multiple downstream signalling pathways, including miRNAs, which lead to VM and tumour promotion [21]. These results showed that the upregulation of Bcl-2 expression may increase the expression of miR-26b-5p and that this effect of Bcl-2 was enhanced by Twist-1.

The above findings suggest that a double-positive feedback loop may exist between Bcl-2 and miR-26b-5p and that apoptosis may be regulated by this feed-forward loop involving Bcl-2 and miR-26b-5p. This hypothesis needs further evidence to be confirmed. Interestingly, our results demonstrated that miR-26b-5p could simultaneously inhibit the proliferation and apoptosis of HCC cells. These controversial results may be due to differences in experimental models (hypoxia or not) and/or the various functions of miR-26b-5p in different diseases. In addition, the low levels of miR-26b-5p and Bcl-2 in HCC are consistent with the fact that apoptosis rarely occurs in normal livers but increases in HCC, indicating that miR-26b-5p may play a very important role in regulating the apoptosis of normal liver and HCC cells.

In conclusion, our analyses identified miR-26b-5p as a tumour suppressive miRNA in HCC and that it exerts its effects partially by suppressing VM and angiogenesis. Decreased miR-26b-5p expression in HCC patients was correlated with poor cellular differentiation and rapid recurrence. Even so, downregulation of miR-26b-5p expression resulted in the induction of Bax and inactivation of the Bcl-2-related mitochondrial apoptotic signalling pathway, which promotes HCC cell apoptosis. Our data provide further evidence of the extensive role of miR-26b-5p in HCC progression and identify it as a potential molecular target for HCC therapy.

HCC, hepatocellular carcinoma; *VE-cadherin*, vascular endothelial-cadherin; *VM*, vasculogenic mimicry; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *MMP-2*, matrix metalloproteinase-2; *RT-PCR*, semiquantitative reverse-transcription polymerase chain reaction; *qRT-PCR*, quantitative real-time polymerase chain reaction; *MTT*, 3-(4, 5Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
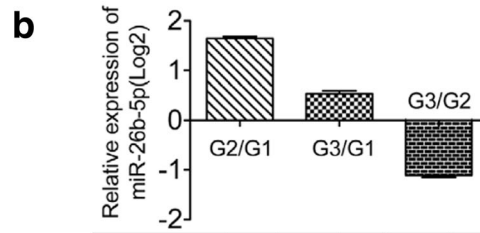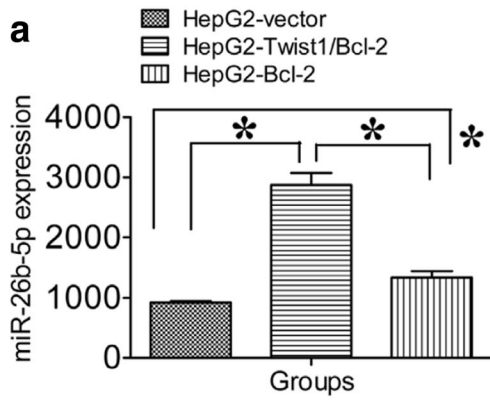
Reporter Name	Mean	StDev	Mean	StDev	Log2 (G2/G1)	p-value
hsa-miR-26b-5p	918	20	2,873	201	1.65	1.40E-03

Reporter Name	Mean	StDev	Mean	StDev	Log2 (G3/G1)	p-value
hsa-miR-26b-5p	918	20	1,334	110	0.54	1.78E-02

Reporter Name	Mean	StDev	Mean	StDev	Log2 (G3/G2)	p-value
hsa-miR-26b-5p	2,873	201	1,334	110	-1.11	1.21E-03

G1: HepG2-vector, **G2:** HepG2-Twist1/Bcl2, **G3:** HepG2-Bcl2
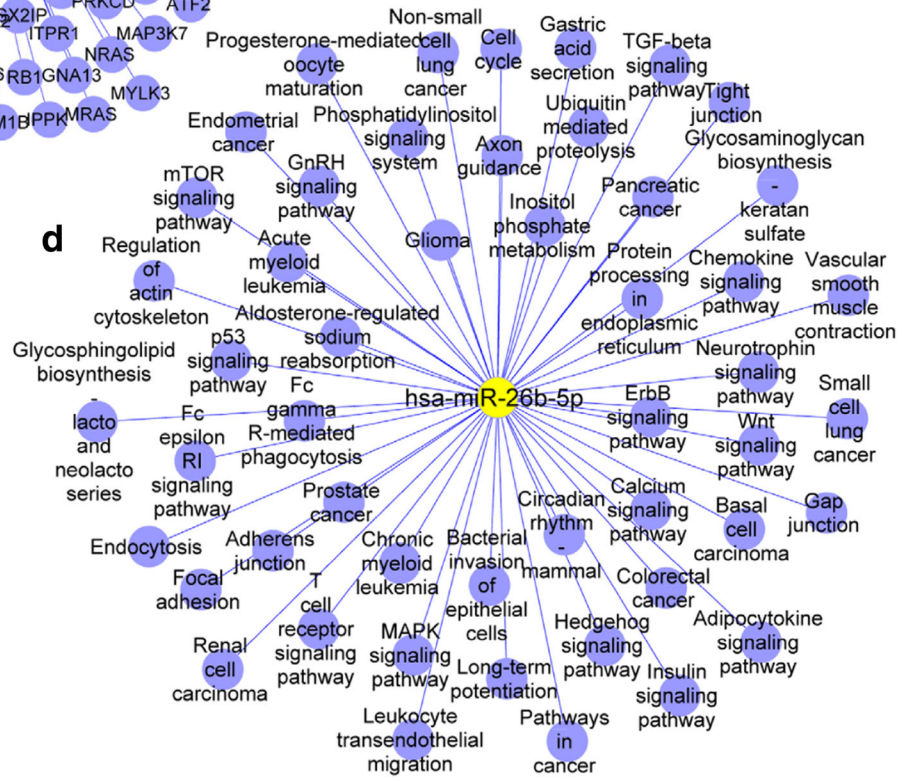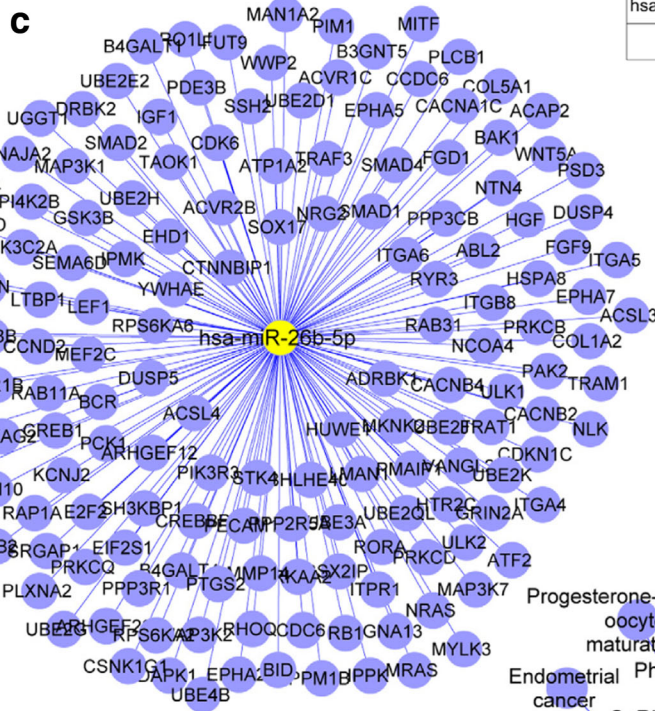
Compliance with ethical standards

Conflicts of interest   The authors declare no conflict of interest.

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