Leptin stimulates the epithelial-mesenchymal transition and pro-angiogenic capability of cholangiocarcinoma cells through the miR-122/PKM2 axis

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Abstract. Leptin is an adipokine minimally known for its activities or underlying mechanisms in cholangiocarcinoma. The present study explored the effects of leptin on the epithelial-mesenchymal transition (EMT) and pro-angiogenic capability of cholangiocarcinoma cells, and investigated the underlying mechanisms. Cholangiocarcinoma cells were treated with leptin, and their migration and invasion rates were investigated using Transwell assays. Furthermore, conditioned medium was collected from cholangiocarcinoma cells following leptin treatment and applied to human umbilical vein endothelial cells to assess tube formation. The expression of EMT and pro-angiogenic factors was examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses. Mechanistically, the function of pyruvate kinase muscle isozyme M2 (PKM2) was assessed in leptin-induced phenotypes using siRNA targeting PKM2 (si-PKM2). Bioinformatics screening and luciferase reporter assays were used to reveal microRNA (miR)-122 as the potential mediator between leptin and PKM2. Finally, the associations between leptin and miR-122 or PKM2 levels in patients with cholangiocarcinoma were assessed by ELISA and RT-qPCR. Leptin significantly increased the EMT and pro-angiogenic capability of cholangiocarcinoma cells, visibly inhibited endogenous miR-122 expression, and upregulated PKM2. Furthermore, si-PKM2 inhibited leptin-induced migration, invasion, EMT-associated marker expression levels and the pro-angiogenic capability in cholangiocarcinoma cells. In addition, miR-122 negatively regulated the expression of PKM2. When applied together with leptin, miR-122 was sufficient to reverse the multiple malignancy-promoting effects of leptin. Consistently, the serum leptin level positively correlated with that of PKM2, but negatively with that of miR-122 in patients with cholangiocarcinoma. Leptin, by downregulating miR-122 and elevating PKM2 expression, acts as a pleiotropic pro-malignancy cytokine for cholangiocarcinoma. Therefore, increasing miR-122 expression and inhibiting PKM2 may be future approaches for cholangiocarcinoma treatment.

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Introduction

Cholangiocarcinoma, also known as bile duct cancer, is a highly aggressive and metastatic cancer derived from epithelial cells lining the intrahepatic or extrahepatic bile ducts (1). Due to the presence of metastasis at diagnosis for the majority of patients, rendering the tumour inoperable, the overall prognosis for cholangiocarcinoma is poor, and the five-year survival is 5-10% (2). Intensive efforts have been dedicated to developing effective therapies, particularly those targeting the metastatic spread of cholangiocarcinoma.

Cancer metastasis is a multi-step process that involves the local migration and invasion of tumour cells followed by distant dissemination of these cells through blood and lymphatic vessels. Metastatic phenotypes gained by tumour cells, coupled with enhanced angiogenesis, predispose malignant cancer development (3). Epithelial-mesenchymal transition (EMT) is a biological process through which epithelial cells acquire morphological, structural, and functional features of mesenchymal cells, characterized by the downregulation of cell-cell junction components, including E-cadherin and β-catenin, the upregulation of mesenchymal markers, including N-cadherin and catenin, and enhanced migratory and invasive capacities (4,5). EMT has been well demonstrated to be essential for the malignant growth and the metastatic spread of multiple cancer types, including cholangiocarcinoma (6,7). Therefore, characterizing and targeting the molecular mechanisms underlying EMT and angiogenesis may be beneficial for developing treatment strategies against cholangiocarcinoma.

Leptin is an adipocyte-derived cytokine (adipokine) that signals through specific leptin receptors, activates various downstream signalling pathways, and serves multiple roles in human physiology and pathology (8). The well-documented

association between obesity and cancer has directed attention towards leptin. Studies over the past decade have reported a positive association between serum leptin levels and multiple cancer types, including breast (9), lung (10), pancreatic (11), and gastrointestinal cancer (12). Furthermore, the signalling mechanisms and functional significance of leptin during the development of multiple cancer types have been demonstrated (13). The primary signal transduction pathways downstream of leptin receptors include Janus kinase 2 (JAK2), phosphoinositide 3-kinase (PI3K), SH2-containing protein tyrosine phosphatase 2/mitogen-activated protein kinase (SHP2/MAPK), 5'-AMP-activated protein kinase/acetyl-CoA carboxylase (AMPK/ACC), and mammalian target of rapamycin (mTOR) kinase (14). A recent study on multiple breast cancer cell lines demonstrated that leptin, through the activation of the PI3K/AKT signalling pathway, upregulates pyruvate kinase muscle isozyme M2 (PKM2), a pyruvate kinase isoform specific to embryonic development and absent in the majority of adult tissues (15). PKM2 is overexpressed in breast cancer cells and essential for leptin-induced EMT in these cells (15). Similarly, PKM2 is upregulated in other cancer types, and functionally important for multiple phenotypes, including cancer metabolism, tumour growth and EMT (16,17). In addition to acting on cancer cells, leptin increases the expression of vascular endothelial growth factor (VEGF) and promotes angiogenesis of endothelial cells (18). Therefore, leptin is an attractive target for cancer therapy.

Despite the abundant evidence regarding the association between leptin and numerous cancer types, few studies have assessed its significance in cholangiocarcinoma. Fava *et al* (19) reported that, by activating PI3K/JAK signalling, leptin stimulates the growth and migration of cholangiocarcinoma cells. However, whether leptin is essential for the malignant phenotypes of cholangiocarcinoma and, if so, which mechanisms are responsible remain unclear. To address these questions, the present study focused on EMT and angiogenesis, assessed the significance of leptin in regulating these biological behaviours, and explored the underlying molecular mechanisms.

Materials and methods

Cell culture and treatments. The human cholangiocarcinoma cell lines SK-ChA-1 and TFK-1 and the human umbilical vein endothelial cells (HUVECs) were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Medical Sciences. The SK-ChA-1 cells were cultured in Dulbecco's modified Eagle medium (DMEM) and TFK-1 cells were cultured in RPMI-1640 (both from Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% foetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 mg/ml streptomycin. HUVECs were cultured in endothelial cell growth medium (PromoCell GmbH). All cell cultures were maintained at 37˚C in a humidified atmosphere with 5% CO_2.

For leptin treatment, cells in the log phase were treated with leptin (R&D Systems, Inc.) at 0, 50, 100 or 200 ng/ml, for 0, 24 or 48 h. The morphology of cells was imaged under a light microscope (magnification, x400).

Synthesis and transfection of RNA oligoribonucleotides. Negative control siRNA (si-NC; 5'-GGCCAGACTGGGAAGAAAA-3'), siRNA targeting PKM2 (si-PKM2; 5'-CUUGUCCGAUGUUACCCAATT-3'), miRNA mimics negative control (miR-NC; sense 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense 5'-ACGUGACACGUUCGGAGAATT-3'), miR-122 mimics (sense 5'-UGGAGUGUGACAAUGGUGUUUG-3' and antisense 5'-AACACCAUUGUCACACUCCAUU-3'), miRNA inhibitor negative control (inhibitor-NC; 5'-CAGUACUUUUGUGUAGUACAA-3') and inhibitor miR-122 (5'-CAAACACCAUUGUCACACUCCA-3') were designed and synthesized by Shanghai GenePharma Co., Ltd.

Transfection of siRNA, miRNA mimics or inhibitor was performed using Lipofectamine 2000 reagents (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. In brief, SK-ChA-1 and TFK-1 cells were seeded on 6-well plates ($5x10^5$ cells/well) until ~75% confluency was achieved. si-PKM2 (100 nM), miR-122 mimics (50 nM), miR-122 inhibitor (100 nM) or the corresponding scrambled sequence (100 nM; si-NC, miR-NC and inhibitor NC, respectively) that served as negative control were transfected into the cells in serum-free medium using Lipofectamine 2000. At 48 h post-transfection, the levels of PKM2 and miR-122 were quantified using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to evaluate the effect of transfection.

MTT assay. To assess the effects of leptin on cytotoxicity and cell proliferation, TFK-1 cells were seeded into 96-well plates (Corning Incorporated) in triplicate at $5x10^5$ cells/100 μl/well. Upon treating the cells with leptin at 0, 100 or 200 ng/ml for 24 h or 48 h, 20 μl of MTT reagent (5 mg/ml) was added to each well and incubated at 37˚C for a further 4 h. Following centrifugation at 250 x g for 5 min at room temperature, the supernatant was discarded from each well. DMSO (150 μl/well) was added to each well to dissolve the formazan crystals. The absorbance was measured using a microplate reader at 490 nm.

Transwell migration and invasion assays. Transwell chambers coated with Matrigel matrix (BD Biosciences) and uncoated Transwell chambers (8-μm pore; Corning Incorporated) were used for the invasion and migration assays, respectively. Briefly, $1x10^5$ SK-ChA-1 or TFK-1 cells were seeded into the top chamber, and 600 μl of medium containing leptin was added to the lower chamber. After 24 h, cells that did not invade through the membrane were removed using a cotton swab. Cells that invaded the membrane and attached to the bottom side of the inserted membrane were stained with 1% crystal violet at room temperature for 10 min, imaged and counted under a light microscope (magnification, x100).

Extraction of total RNA and RT-qPCR. Total RNA was extracted from SK-ChA-1 or TFK-1 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was then synthesized with M-MLV reverse transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Briefly, 1 μg total RNA and 1 μl dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP) were mixed and incubated at 65˚C for 5 min. Then, the reaction mixtures, including 4 μl 5X first-strand buffer, 2 μl 0.1 mol DTT and 40 U of ribonuclease inhibitor were incubated at 37˚C for 2 min, followed by incubation with 1 μl M-MLV RT at 37˚C for 2 min, 50 min at 37˚C and

heat inactivation at 70˚C for 15 min. qPCR was performed using SuperScript® III Platinum® SYBR Green One-Step qRT-PCR kit (Invitrogen; Thermo Fisher Scientific, Inc.) on the ABI PRISM 7300 Fast Real-Time PCR system (Ambion; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. The thermocycling conditions were as follows: Initial 3 min denaturation step at 95˚C; followed by 40 cycles at 95˚C 3 sec for denaturation; and at 60˚C for 30 sec for annealing and extension. Primer sequences are listed in Table I. Each reaction was performed in triplicate, and the relative expression levels of each target gene were calculated as a ratio to that of GAPDH, the internal control, using the $2^{-\Delta\Delta Cq}$ method (20).

Western blot analysis. SK-ChA-1 or TFK-1 cells were collected and lysed using cell lysis buffer (Beyotime Institute of Biotechnology). Protein concentration was determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Subsequently, 30 μg total protein/lane were boiled at 95-100˚C for 5 min and separated on a 12% SDS-PAGE gel, and then proteins were transferred onto a polyvinylidene difluoride membrane. Upon blocking the membrane in TBST buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween-20) containing 5% non-fat milk at room temperature for 1 h, the target protein was probed with one of the following primary antibodies at 4˚C overnight: Rabbit anti-human E-cadherin, (1:1,000; cat. no. A3044), rabbit anti-human β-catenin (1:1,000; cat. no. A10834), rabbit anti-human N-cadherin (1:1,000; cat. no. A0433), rabbit anti-human vimentin (1:1,000; cat. no. A11952), rabbit anti-human VEGFA (1:1,000; cat. no. A0280), rabbit anti-human angiotensin 1 (Ang1; 1:1,000; cat. no. A945; all from ABclonal Biotech Co., Ltd.), anti-PKM2 (1:1,000; cat. no. 4053; Cell Signaling Technology, Inc.), or rabbit anti-human GAPDH (internal control; 1:5,000, AC001, ABclonal Biotech Co., Ltd.). The membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) at room temperature for 2 h. The signal was developed using the Pierce™ Enhanced Chemiluminescence system (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The signal density was analysed using ImageJ software (version 1.51q; National Institutes of Health), and the relative protein level was calculated as the density ratio of the target protein to GAPDH (internal control).

Tube formation assay. To assess *in vitro* angiogenesis, conditioned medium was collected from cholangiocarcinoma cells. Briefly, $2x10^5$ cells were seeded into 10-cm tissue culture plates. Following an overnight culture, cells were treated with 0 or 200 ng/ml leptin for 24 h. Following three washes with DMEM, cells were cultured in serum-free DMEM for a further 24 h. The conditioned medium was collected and centrifuged at 2,000 x g for 10 min at 4˚C to remove any cell debris. A 24-well plate was coated with Matrigel. Upon gel solidification, HUVECs ($1x10^5$ cells/well) were seeded on top of the Matrigel in triplicate, treated with a mixture of conditioned medium:EGM2 medium (volume ratio 2:1), and incubated at 37˚C with 5% CO_2 for 6 h. Each well was imaged under an Olympus DP71 microscope (Olympus Corporation) at x100 magnification, and the quantification of branching points was performed using ImageJ software (version 1.51q).

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Sequence (5'-3')
hPKM2	
Forward	GAGTACCATGCGGAGACCAT
Reverse	GCGTTATCCAGCGTGATTTT
hsa-miR-122	
Forward	AGCGTGGAGTGTGACAATGG
Reverse	CGGCCCAGTGTTCAGACTAC
hβ-catenin	
Forward	GAACTCTCGAAGGACCCAGC
Reverse	TAAATCCCAAGAGGCCCTGC
hE-cadherin	
Forward	TCACATCCTACACTGCCCAG
Reverse	AGTGTCCCTGTTCCAGTAGC
hAng1	
Forward	GGGGGAGGTTGGACTGTAAT
Reverse	GAATAGGCTCGGTTCCCTTC
hVEGFA	
Forward	CACCAAGGCCAGCACATAGG
Reverse	AGGGAGGCTCCAGGGCATTA
hN-cadherin	
Forward	AGGGGACCTTTTCCTCAAGA
Reverse	TCAAATGAAACCGGGCTATC
hVimentin	
Forward	GGACCAGCTAACCAACGACA
Reverse	AAGGTCAAGACGTGCCAGAG
GAPDH	
Forward	GCTGTAGCCAAATCGTTGT
Reverse	CCAGGTGGTCTCCTCTGA

PKM2, pyruvate kinase muscle isozyme M2; h, human; miR-122, microRNA-122; Ang1, angiopoietin 1; VEGFA, vascular endothelial growth factor A.

Luciferase reporter assay. starBase (http://starbase.sysu.edu.cn/) and miRDB (http:// mirdb.org) software tools were used to identify potential miRNAs that may bind to the 3'-untranslated region (3'-UTR) of the human PKM2 gene (21,22). For the luciferase reporter assay, the 3'-UTR sequence of the PKM2 gene containing the potential miR-122-binding site was cloned into a psiCHECK-2 luciferase reporter plasmid (Promega Corporation) and transfected into TFK-1 cells using Lipofectamine 2000 according to the manufacturer's protocol. Upon treating the cells with vehicle control (PBS only), or transfecting miR-122 mimics or miR-122 inhibitor for 36 h, luciferase activity was detected using the Dual Luciferase Reporter assay system (Promega Corporation) according to the manufacturer's protocol. *Renilla* luciferase activity was normalized to firefly luciferase activity in cells.

Collection of blood samples and ELISA. The present study was approved by the Institutional Review Board of Hunan Provincial

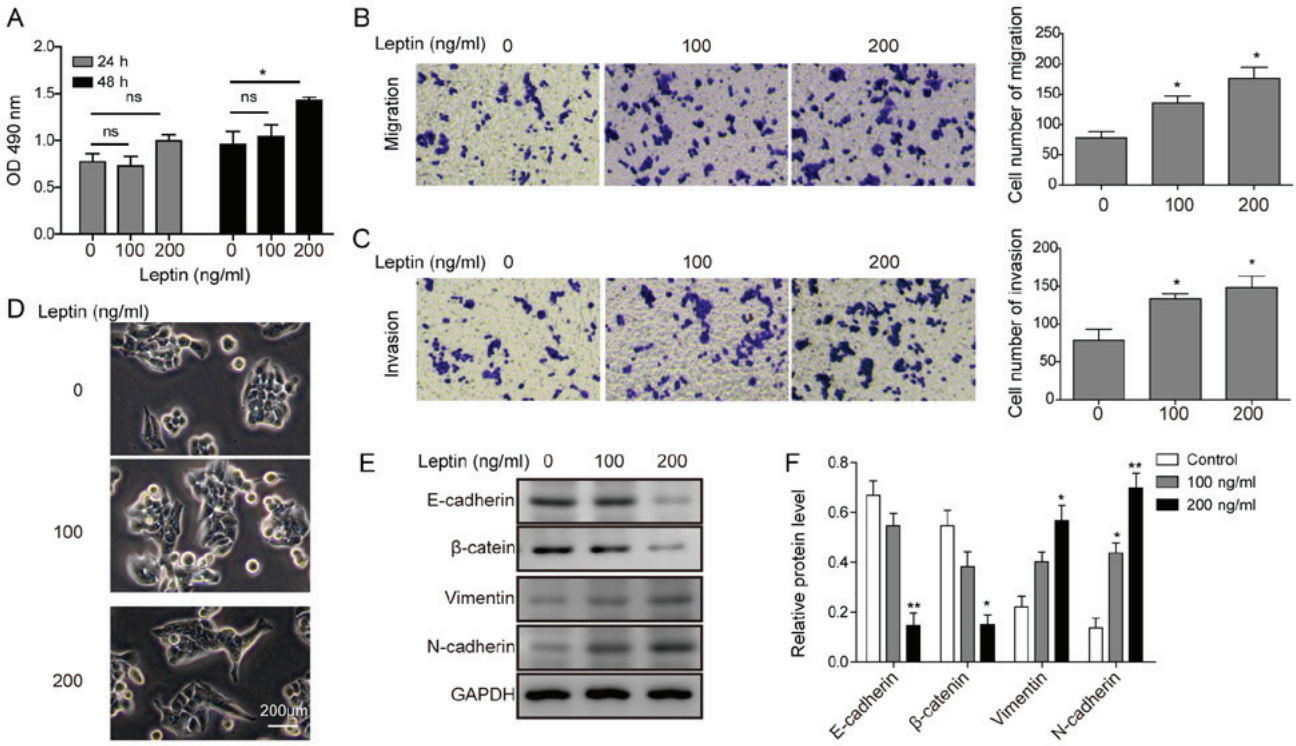

Figure 1. Leptin induces EMT in cholangiocarcinoma cells. (A) TFK-1 cells were exposed to different treatments for 24 or 48 h, and cell viability was detected by MTT assay. (B) TFK-1 cell migration and (C) invasion were examined with leptin treatment at the indicated concentrations. After 24 h, the migrated or invaded cells were stained with crystal violet, imaged (left panels), and counted under the microscope (right panel). Each condition was tested in triplicate. (D) Morphological changes of TFK-1 cells following treatment with 100 and 200 ng/ml leptin. Scale bar, 200 μm. (E) TFK-1 cells were treated with leptin at the indicated concentrations for 24 h. Protein expression levels of E-cadherin, β-catenin, N-cadherin, and vimentin were examined by western blot analysis. GAPDH was detected as the internal control. (F) Quantification of protein levels (relative to GAPDH) from D. Data are presented as the mean ± standard deviation from at least three independent experiments. *P<0.05 and **P<0.01. EMT, epithelial-mesenchymal transition; OD, optical density; ns, not significant.

People's Hospital, and written informed consent was obtained from all participants. A total of 15 patients with cholangiocarcinoma admitted to the Hunan Provincial People's Hospital between January 2016 and December 2016 were recruited for the present study. The diagnosis for cholangiocarcinoma was established by combining clinical symptoms and examination with ultrasound, computed tomography, and magnetic resonance imaging. Fifteen sex- and age-matched healthy individuals were also recruited as controls. Venous blood was collected from each participant (total 15 patients), and the serum concentration of leptin (cat. no. RAB0333; Sigma-Aldrich; Merck KGaA) and plasma concentration of PKM2 (cat. no. LS-F29732-1; LifeSpan BioSciences, Inc.) were measured by ELISA kit, following the manufacturer's protocols.

Statistical analysis. All data were analysed using SPSS 13.0 software (SPSS, Inc.) and presented as the mean ± standard deviation. The difference between two groups was analysed using an unpaired two-tailed Student's t-test. One-way analysis of variance was used for comparison among multiple groups and multiple comparisons were further performed using the post hoc Tukey's test. P≤0.05 was considered to indicate a statistically significant difference.

Results

Leptin induces EMT in cholangiocarcinoma cells. Accumulating evidence suggests that leptin is a pleiotropic cytokine involved in cancer development (23,24). However, in cholangiocarcinoma, few studies have investigated the functions or molecular mechanisms of leptin (19). To examine the biological significance of leptin, its biosafety and effect on the proliferation of the human TFK-1 cholangiocarcinoma cell line was first assessed using an MTT assay. As shown in Fig. 1A, no significant decreases in cell viability were observed when the cells were treated with 100 or 200 ng/ml leptin for 24 h, and leptin at 200 ng/ml promoted TFK-1 cell proliferation when detected at 48 h, suggesting that leptin is not toxic to TFK-1 cells. Next, the effect of leptin on the migratory and invasive behaviours of TFK-1 cells was examined. In response to increasing concentrations of leptin (0, 100 and 200 ng/ml), leptin significantly stimulated the migration and the invasion of cells in a dose-dependent manner, as indicated by Transwell assay (Fig. 1B and C). Monitoring the changes in cell morphology revealed that leptin transformed the cholangiocarcinoma cells from cuboidal shaped cells to elongated spindle fibroblast-like cells (Fig. 1D), resembling the changes associated with EMT. To follow up, the levels of several EMT promoters, including E-cadherin, β-catenin, N-cadherin and vimentin were measured in cell lines following leptin treatment. As shown in Fig. 1E and F, leptin treatment significantly reduced the protein expression of epithelial markers E-cadherin and β-catenin, while enhancing that of mesenchymal markers N-cadherin and vimentin in a dose-dependent manner. Collectively, these data support that leptin promotes EMT in cholangiocarcinoma cells.

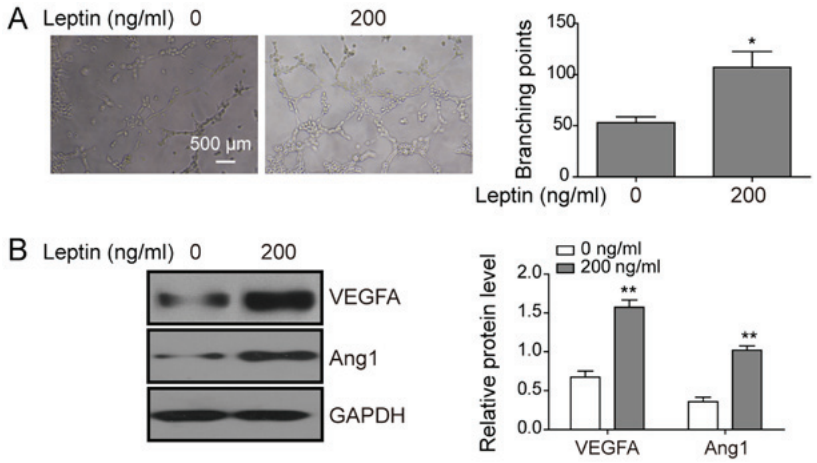

Figure 2. Leptin stimulates the pro-angiogenic capability of cholangiocarcinoma cells. (A) Cholangiocarcinoma cells were treated with leptin at indicated concentrations. The conditioned medium was collected and applied to HUVECs on Matrigel to assess angiogenesis. Representative images from each group were presented on the left and the quantification of branching points on the right. Scale bar, 500 μm. (B) Cholangiocarcinoma cells were treated with leptin at the indicated concentrations for 24 h. Expression of VEGFA and Ang1 was examined by western blot analysis, with representative images presented on the left and the quantification of relative protein levels on the right. GAPDH was detected as the internal control. Data are presented as the mean ± standard deviation from at least three independent experiments. *P<0.05 and **P<0.01. HUVECs, human umbilical vein endothelial cells; VEGFA, vascular endothelial growth factor A; Ang1, angiopoietin 1.

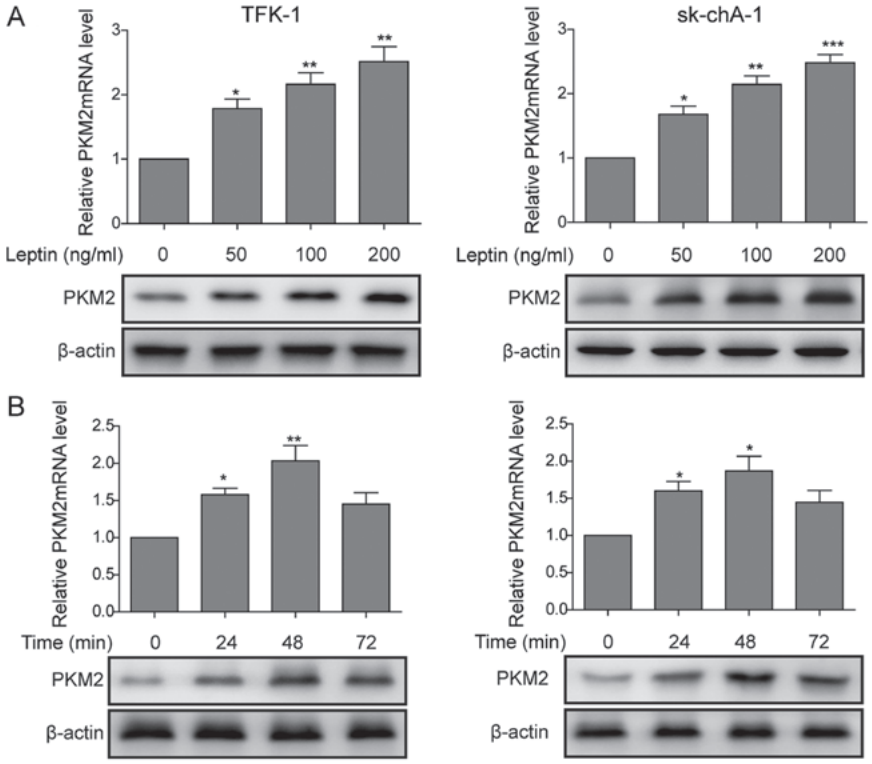

Figure 3. Leptin increases the expression of PKM2 in cholangiocarcinoma cells. TFK-1 (left panels) or SK-CHA-1 (right panels) cells were treated with leptin at (A) indicated concentrations for 24 h or at (B) 100 ng/ml for the time periods indicated. The relative expression of PKM2 on the steady-state mRNA land protein levels was examined by reverse transcription-quantitative PCR and western blot analysis, respectively. β-actin was used as the internal control. Data are presented as the mean ± standard deviation from at least three independent experiments. *P<0.05, **P<0.01 and ***P<0.001. PKM2, pyruvate kinase muscle isozyme M2.

Leptin stimulates the pro-angiogenic capability of cholangiocarcinoma cells. To examine the activity of leptin in angiogenesis, TFK-1 cells were treated with 0 or 100 ng/ml leptin and the conditioned medium was collected from these cells. By applying the conditioned medium to HUVECs, monitoring their angiogenesis (Fig. 2A, left), and measuring the number of branching points (Fig. 2A, right), conditioned medium from leptin-treated cholangiocarcinoma cells was demonstrated to induce tubular formation of HUVECs. Concomitantly, leptin significantly increased the protein expression of VEGFA and Ang1 (Fig. 2B), which are known pro-angiogenic factors in cholangiocarcinoma cells, suggesting that leptin stimulates the pro-angiogenic capability of cholangiocarcinoma cells.

Leptin increases the expression of PKM2 in cholangiocarcinoma cells. To explore the molecular mechanisms underlying

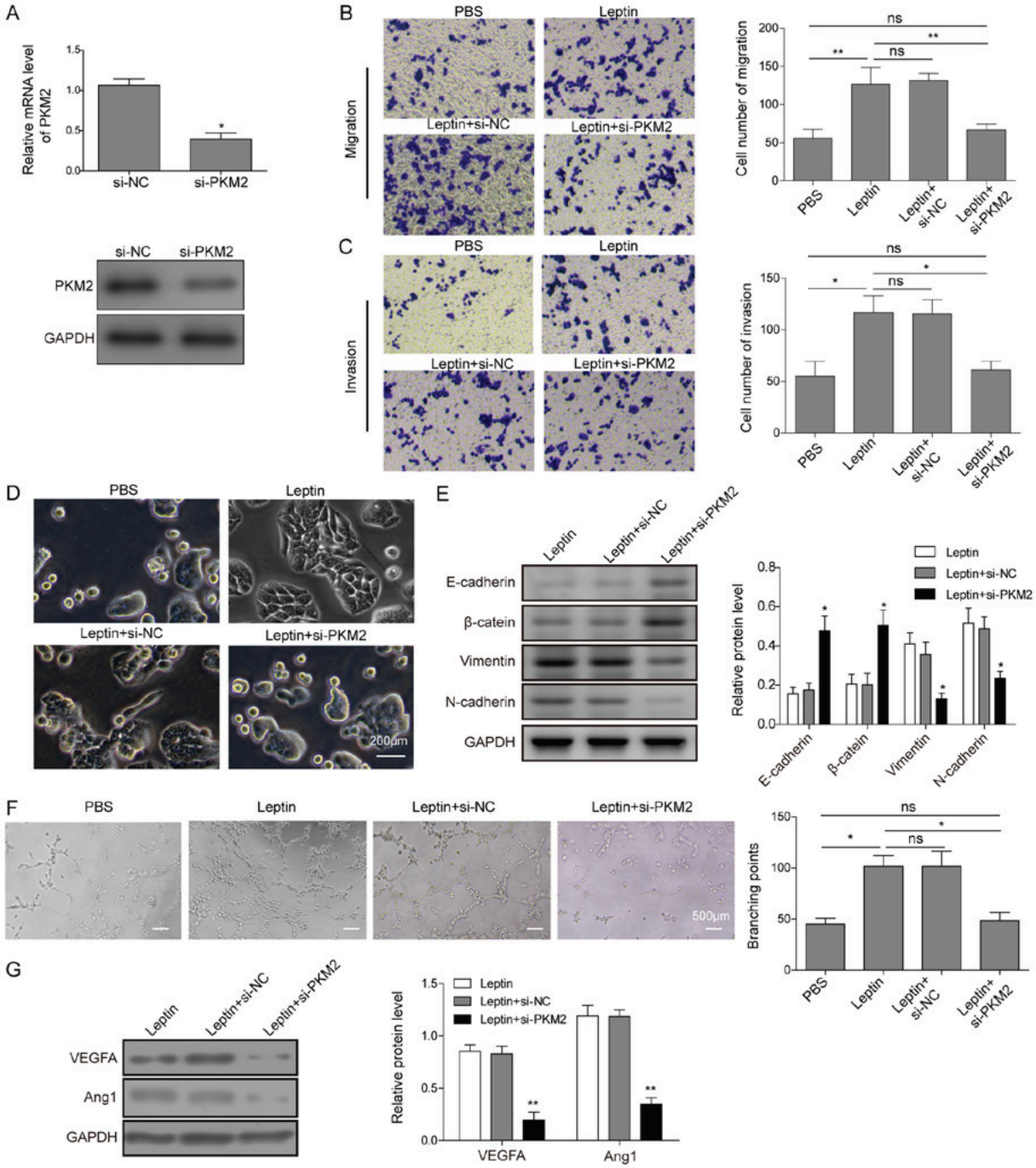

Figure 4. PKM2 is essential for leptin-induced EMT and angiogenesis. (A) TFK-1 cells were transfected with either negative control siRNA (si-NC) or si-PKM2, and the expression of PKM2 was examined by reverse transcription-quantitative PCR and western blot analysis. (B) TFK-1 cells not transfected, transfected with negative control siRNA (si-NC), or si-PKM2 were treated with vehicle control (PBS) or with leptin (100 ng/ml) for 24 h. Migration and (C) invasion were examined by Transwell assays in triplicate, with representative images of migrated or invaded cells presented on the left and the quantification on the right. (D) Cell morphology was imaged under a light microscope. Scale bar, 200 μm. (E) Expression of E-cadherin, β-catenin, N-cadherin, and vimentin was examined by western blot analysis. GAPDH was detected as the internal control (left panels). Quantification of protein levels (relative to GAPDH) is presented (right panels). (F) Cholangiocarcinoma cells not transfected, and transfected with negative control siRNA (si-NC) or si-PKM2 were treated with leptin (100 ng/ml), and the conditioned medium was collected. The tubulogenesis assay was performed on HUVECs growing on Matrigel upon treatment with the conditioned medium. Representative images from each group are presented on the left and the quantification of branching points on the right. Scale bar, 500 μm. (G) Expression of VEGFA and Ang1 in indicated cholangiocarcinoma cells was examined by western blot analysis, with representative images presented on the left and the quantification of relative protein levels on the right. GAPDH was detected as the internal control. Data are presented as the mean ± standard deviation from at least three independent experiments. *P<0.05 and **P<0.01. PKM2, pyruvate kinase muscle isozyme M2; EMT, epithelial-mesenchymal transition; si, small interfering; HUVECs, human umbilical vein endothelial cells; VEGFA, vascular endothelial growth factor A; Ang1, angiopoietin 1; ns, not significant.

the EMT- and angiogenesis-stimulating activities of leptin, PKM2, a pyruvate kinase essential for leptin-induced EMT in breast cancer, was examined (15). By measuring PKM2 on the steady-state mRNA and the protein level in TFK-1 and sk-chA-1 cells, leptin was revealed to significantly upregulate the expression of PKM2 in a dose-dependent manner (Fig. 3A).

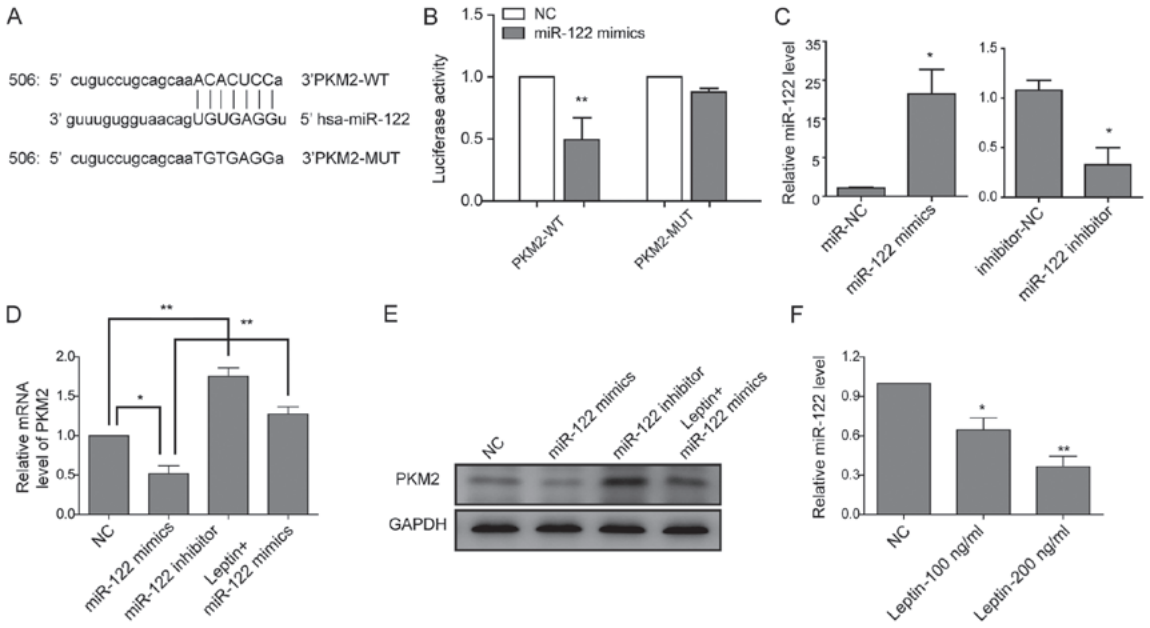

Figure 5. miR-122 mediates the control of leptin on PKM2. (A) Sequence alignment showing the potential binding site for PKM2 and the mutagenesis design for the reporter assays. (B) Luciferase activity in TFK-1 cells co-transfected with miR-122 mimics and luciferase reporters containing PKM2 wild-type (PKM2-WT) or PKM2 mutant transcripts (PKM2-MUT). Data are presented as the relative ratio of firefly luciferase activity to *Renilla* luciferase activity. (C) TFK-1 cells were transfected with either miR-122 mimics vs. miRNA-NC or miR-122 inhibitors vs. inhibitor NC. The levels of miR-122 were determined by RT-qPCR. The expression of PKM2 was measured in the steady-state mRNA (D) and the protein level (E) in TFK-1 cells not treated or treated with miR-122 mimics, miR-122 inhibitors, or miR-122 mimics+leptin. (F) TFK-1 cells were treated with leptin at indicated concentrations, and the level of miR-122 was measured by RT-qPCR. Data are presented as the mean ± standard deviation from at least three independent experiments. *P<0.05 and **P<0.01. PKM2, pyruvate kinase muscle isozyme M2; WT, wild-type; MUT, mutant; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR.

The time-course study revealed that PKM2 mRNA and protein (Fig. 3B) peaked at 24 h after leptin treatment, and gradually reduced thereafter in TFK-1 and sk-chA-1 cells. Taken together, these results suggest that leptin increased the expression of PKM2 in cholangiocarcinoma cells.

PKM2 is essential for leptin-induced EMT and pro-angiogenesis. To assess the significance of upregulated PKM2 in leptin-mediated EMT or pro-angiogenesis of cholangiocarcinoma cells, the expression of endogenous PKM2 was targeted with siRNA (si-PKM2). RT-qPCR and western blot analysis demonstrated that si-PKM2 significantly and specifically knocked down the level of PKM2 in TFK-1 cells (Fig. 4A). When compared with vehicle control (PBS only) treatment, leptin alone or leptin together with si-NC, si-PKM2 completely abolished the effects of leptin on cell migration (Fig. 4B), invasion (Fig. 4C), and mesenchymal morphology (Fig. 4D). Then, the expression of EMT markers E-cadherin, β-catenin, N-cadherin, and vimentin were detected by western blot analysis, which demonstrated that si-PKM2 abolished the effects of leptin (Fig. 4E). Furthermore, conditioned medium from si-PKM2-treated cholangiocarcinoma cells abolished tubular formation of HUVECs (Fig. 4F) and inhibited the expression of VEGFA or Ang1 (Fig. 4G) in cholangiocarcinoma cells. Taken together, these data suggest that PKM2 is an essential signalling molecule for leptin-induced EMT and pro-angiogenesis of cholangiocarcinoma cells.

miR-122 mediates the regulation of leptin on PKM2. To identify the molecules controlling leptin-mediated regulation on PKM2, a bioinformatics search was performed, and miR-122 was predicted as a potential miRNA that interacts with the 3'-UTR of PKM2 (Fig. 5A). The luciferase reporter assay demonstrated that miR-122 mimics significantly reduced the luciferase activity of the reporter carrying the 3'-UTR of PKM2. In contrast, a mutated luciferase reporter was developed by site-directed mutagenesis, and the results suggested that mutations on the binding sites successfully abolished the suppressive effect of miR-122 (Fig. 5B). Next, miR-122 mimics and miR-122 inhibitors were used for gain-of-function and loss-of-function experiments, respectively. RT-qPCR analysis revealed that miR-122 mimics elevated the miR-122 level to ~23-fold of that in cells transfected with miR-NC, while miR-122 inhibitor significantly reduced the level of miR-122 when compared with the expression in cells transfected with inhibitor NC (Fig. 5C). Consistently, miR-122 mimics significantly reduced, while miR-122 inhibitors potently increased the endogenous mRNA (Fig. 5D) as well as the protein level of PKM2 (Fig. 5E) in cholangiocarcinoma cells. The addition of leptin reduced the inhibitory effect of miR-122 mimics on PKM2 expression (Fig. 5D and E). Furthermore, leptin significantly reduced the miR-122 level in a dose-dependent manner (Fig. 5F). Collectively, these data suggest that leptin may target miR-122, thus releasing miR-122-mediated inhibition on PKM2.

miR-122 mimics reverse leptin-induced EMT and are pro-angiogenesis. To analyse the functional significance of miR-122 as a negative regulator for leptin-mediated expression of PKM2, cholangiocarcinoma cells were treated with leptin together with miR-122 mimics. As shown in Fig. 6A and B, miR-122 mimics were sufficient to reduce leptin-induced

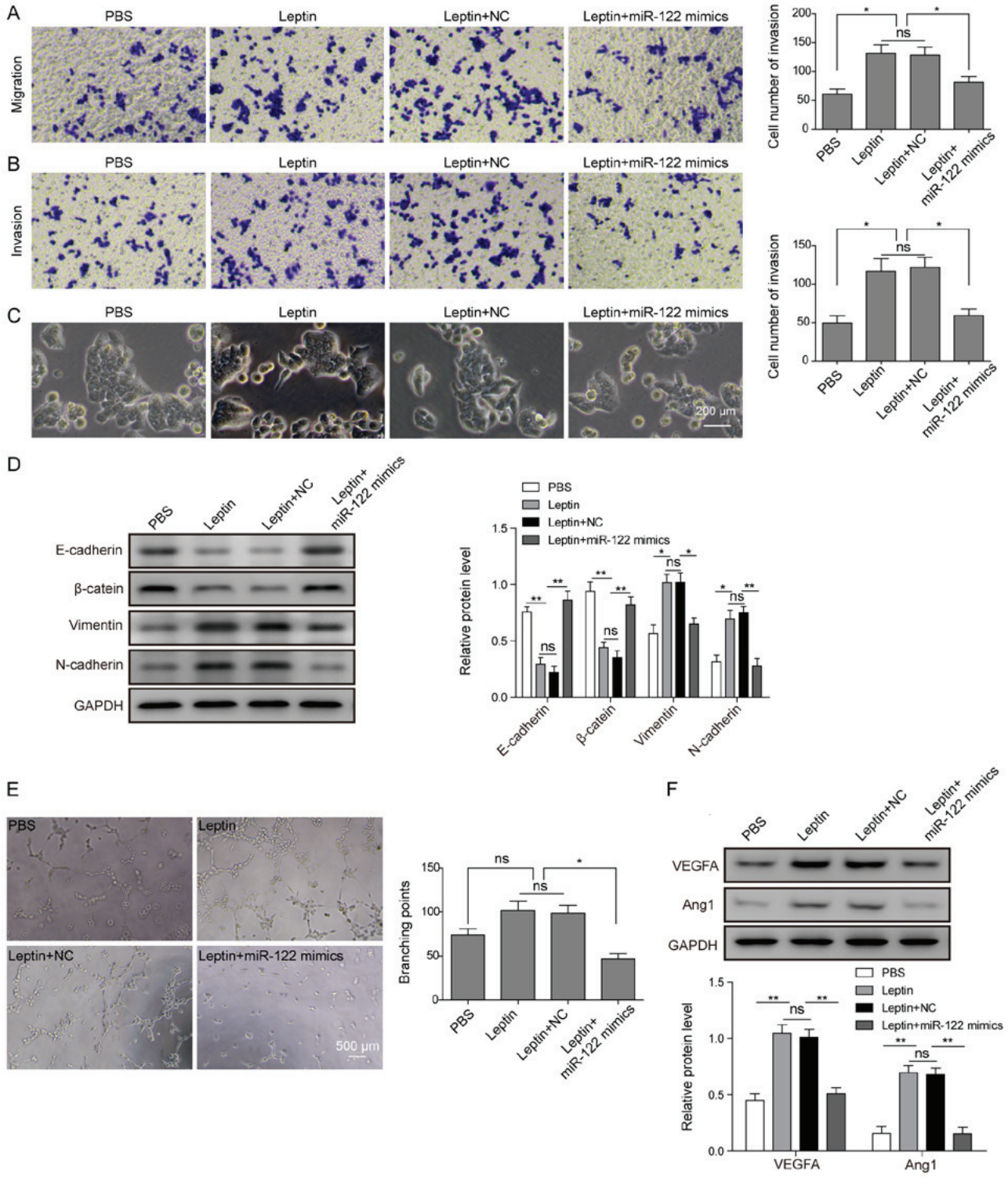

Figure 6. miR-122 mimics reverses leptin-induced EMT and angiogenesis. (A) TFK-1 cells were treated with PBS, leptin (100 ng/ml), leptin+NC, or leptin+miR-122 mimics. Cell migration and (B) invasion were examined by Transwell assays in triplicate, with representative images of migrated or invaded cells presented on the left and the quantification on the right. (C) Cell morphology was imaged under a light microscope. Scale bar, 200 μm. (D) Expression of E-cadherin, β-catenin, N-cadherin, and vimentin was examined by western blot analysis. GAPDH was detected as the internal control. (E) Cholangiocarcinoma cells were treated with PBS, leptin (100 ng/ml), leptin+NC, or leptin+miR-122 mimics, and the conditioned medium was collected. The tube formation assay was performed on HUVECs growing on Matrigel upon treatment with the conditioned medium. Representative images from each group are presented on the left and the quantification of branching points on the right. Scale bar, 500 μm. (F) Expression of VEGFA and Ang1 in cholangiocarcinoma cells was examined by western blot analysis. Representative images and quantification of relative protein levels are shown. GAPDH was detected as the internal control. Data are presented as the mean ± standard deviation from at least three independent experiments. *P<0.05 and **P<0.01. EMT, epithelial-mesenchymal transition; NC, negative control; HUVECs, human umbilical vein endothelial cells; VEGFA, vascular endothelial growth factor A; Ang1, angiopoietin 1.

migration and invasion of cholangiocarcinoma cells to the basal level (when cells were treated with PBS) and a level significantly lower compared with that in cells treated with leptin alone or leptin with vehicle control (leptin+NC). In addition,

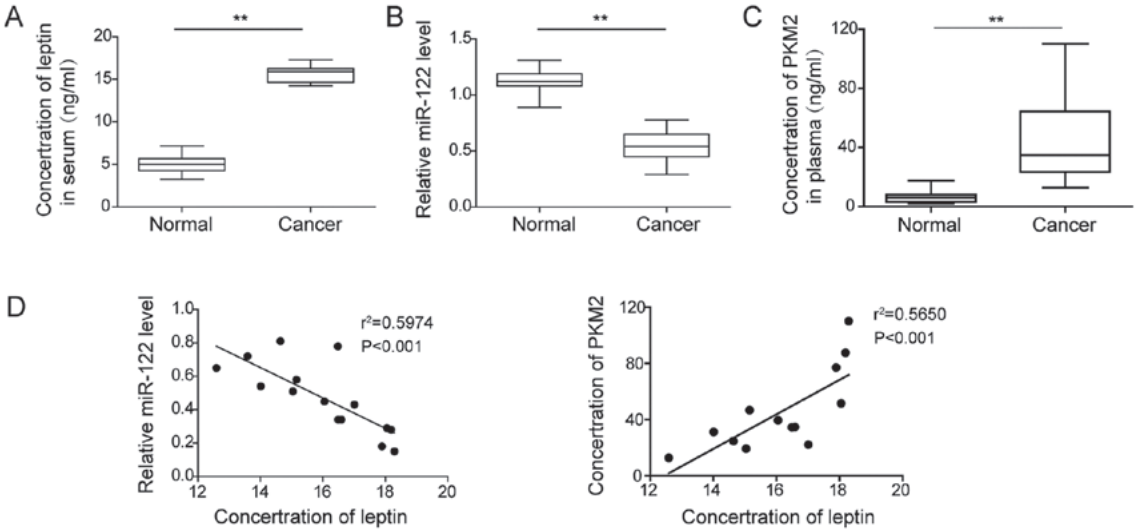

Figure 7. Serum levels of leptin correlate positively with PKM2, but negatively with miR-122 in cholangiocarcinoma patients. Serum was collected from patients with cholangiocarcinoma and healthy controls (n=15/group). (A) The concentration of leptin, (B) the levels of miR-122 in the serum and (C) the concentration of PKM2 in the plasma were measured and compared between normal controls and cancer patients. (D) The correlation between serum leptin levels and serum miR-122 levels or serum PKM2 levels was examined using the Pearson's correlation test. **P<0.01. PKM2, pyruvate kinase muscle isozyme M2.

miR-122 mimics significantly suppressed the changes in cell morphology (Fig. 6C) and gene expression (Fig. 6D) associated with leptin-induced EMT. The conditioned medium collected from cholangiocarcinoma cells treated with leptin+miR-122 mimics presented similar activities as the medium from cells treated with leptin+si-PKM2 (Fig. 5E), disrupting tubular formation of HUVECs (Fig. 6E), which was associated with the reduced expression of VEGFA and Ang1 (Fig. 6F). Taken together, these results suggested that increasing miR-122 activity is sufficient to reverse leptin-induced EMT as well as the pro-angiogenic capability of cholangiocarcinoma cells.

Serum level of leptin is positively correlated with PKM2, but negatively correlated with miR-122 in cholangiocarcinoma patients. The data presented above support the essential role of the leptin/miR-122/PKM2 axis in regulating EMT and angiogenesis of cholangiocarcinoma. To analyze the significance of this axis in the clinical setting, the serum levels of leptin and miR-122, and plasma level of PKM2 from patients with cholangiocarcinoma and age- and sex-matched healthy individuals were measured. As shown in Fig. 7A-C, serum leptin and PKM2 levels were significantly higher, while that of miR-122 was significantly reduced in patients when compared with the corresponding levels in healthy controls. Further correlation analysis revealed that in the patients, the serum level of leptin positively correlated with that of PKM2, but negatively with that of miR-122 (Fig. 7D).

Discussion

In the present study, it was demonstrated that leptin is a potent cytokine capable of inducing EMT and stimulating the pro-angiogenic capability of cholangiocarcinoma cells. Furthermore, the effects of leptin on cholangiocarcinoma cells were indicated to be mediated through the leptin/miR-122/PKM2 axis as the upregulation of miR-122 or downregulation of PKM2 was sufficient to inhibit leptin-induced phenotypes. Consistently, the serum level of leptin was significantly higher in patients with cholangiocarcinoma compared with healthy controls, and positively correlated with serum PKM2 levels, but negatively correlated with serum miR-122 levels. The results of the present study support the notion that leptin is an ideal therapeutic target for cholangiocarcinoma since antagonizing leptin was able to simultaneously target multiple phenotypes responsible for the malignant progression of the disease.

An increasing body of evidence supports the association between obesity and a higher risk of developing different types of cancer (25,26). In a study on 30 patients with colorectal, breast, lung, testicular or gastric cancer, no significant differences were observed in the serum leptin level at baseline or in response to chemotherapy between patients and matched healthy controls, although leptin was identified to be positively associated with the body mass index of patients (27). By contrast, when focusing on a specific type of cancer, several studies suggest a positive association between higher serum leptin level and cancers (28,29), while some suggest a negative correlation (11,30). Meta-analysis revealed that obesity is a risk factor for cholangiocarcinoma (31). Consistently, the results of the present study demonstrated that the baseline level of serum leptin was significantly higher in cholangiocarcinoma patients compared with that in healthy individuals, indicating that leptin is functionally important for the development of cholangiocarcinoma.

Aggressive development and early metastatic spread constitute the major obstacles for effective treatment of cholangiocarcinoma. Therefore, understanding and targeting mechanisms responsible for the malignancy of the disease may aid in developing novel therapies for cholangiocarcinoma. Previous studies have presented convincing evidence that EMT is present in cholangiocarcinoma development and progression, but also associated with poor prognosis of patients (32,33). In the present study, leptin was revealed to be functionally capable of inducing EMT phenotypes in cholangiocarcinoma cells: Stimulating their migration and invasion; changing tumour cells from the cuboidal epithelial morphology to a spindle

mesenchymal shape; downregulating junction molecules, E-cadherin and β-catenin; and upregulating mesenchymal markers, N-catenin and vimentin. Although, to the best of our knowledge, this is the first study to demonstrate that leptin is capable of inducing EMT in cholangiocarcinoma, EMT is not a novel functional phenotype for leptin, as has been well documented in multiple cancer types, including breast (34), lung (35), and ovarian (36) cancer. Several signalling pathways have been identified to mediate leptin-induced EMT, including PI3K/AKT/PKM2 (15), TGF-β (35), AKT/GSK3β and MTA1/Wnt1 axes (34). In the present study, leptin increased the expression of PKM2 in cholangiocarcinoma cells, and siRNA-mediated downregulation of PKM2 was sufficient to abolish multiple leptin-induced EMT phenotypes.

Notably, through bioinformatic miRNA screening, miR-122 was identified as a negative regulator between leptin and PKM2, whereby leptin, through the suppression of miR-122, reversed the inhibitory effects of miR-122 on PKM2 in cholangiocarcinoma cells. The essential role of miR-122 in leptin-PKM2 regulation was further corroborated by the findings that miR-122 mimics were sufficient to downregulate endogenous PKM2, while miR-122 inhibitors significantly increased the expression of PKM2. Karakatsanis *et al* (37) reported the downregulation of miR-122 in intrahepatic cholangiocarcinoma. Wu *et al* (38) demonstrated that, by inhibiting p53 expression, miR-122 was necessary and sufficient to reduce the proliferation, and suppress the migration and invasion of cholangiocarcinoma cells. Although these studies support the idea that miR-122 functions as a tumour suppressor, they did not explore the mechanism by which miR-122 inhibited the aggressive and metastatic behaviours, specifically EMT of cholangiocarcinoma.

In addition to tumour cells, other mesenchymal components within the tumour microenvironment, including endothelial cells, fibroblasts, and macrophages are also essential for the malignant progression of cholangiocarcinoma (39). Studies have established leptin as a potent angiogenic factor and revealed multiple underlying mechanisms: Activating P38 MAPK/AKT/COX-2 signalling or JAK/STAT and AKT signalling pathways; and increasing the production of VEGF, fibroblast growth factor-2 (FGF-2) and matrix metalloproteinases from endothelial cells (40). In the present study, the leptin/miR-122/PKM2 axis was demonstrated to regulate the pro-angiogenic capability of cholangiocarcinoma cells *in vitro*. Conditioned medium from tumour cells treated with miR-122 mimics and si-PKM2 presented comparable effects in disrupting tubular formation associated with the downregulation of VEGF and Ang1 expression in cholangiocarcinoma cells. Although Wang *et al* (41) suggested that miR-122 directly targeted VEGF through post-transcriptional regulation, the findings of the present study indicate that it may indirectly target VEGF expression via PKM2. Consistently, Azoitei *et al* (42) demonstrated that PKM2 promoted HIF-1α accumulation and stimulated the subsequent secretion of VEGF from pancreatic cancer cells by activating NF-κB. Li *et al* (43) reported that PKM2 increased endothelial cell proliferation, migration and adhesion to the extracellular matrix. As the angiogenesis assays were performed on endothelial cells using the conditioned medium from cholangiocarcinoma cells in the present study, the possibility that the leptin/miR-122/PKM2 axis may directly act on endothelial cells to stimulate angiogenesis in an autocrine manner cannot be excluded.

In conclusion, the results of the present study provided clinical relevance and demonstrated the pleiotropic activities of leptin in promoting the malignant transformation of cholangiocarcinoma. Leptin not only stimulates multiple EMT phenotypes, but also enhances the pro-angiogenic activity of cancer cells. Therefore, targeting leptin may aid in supressing a multitude of malignant behaviours of cholangiocarcinoma and achieve superior anticancer effects. Molecularly, the actions of leptin in cholangiocarcinoma cells appear to be mediated through the miR-122/PKM2 axis, allowing for the development of novel anticancer therapies that enable the fine-tuning of leptin activities. As this study was primarily performed using cells cultured *in vitro*, it is essential to follow up this study with properly established *in vivo* cholangiocarcinoma models.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BJ substantially contributed to the conception of this study, as well as edited the manuscript. CP was involved in the study conception and design. ZS performed the literature research and experimental studies. WY performed the clinical studies. OL performed data acquisition and edited the manuscript. ZT and BJ performed data and statistical analysis. CG was involved in drafting the manuscript and revising it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Hunan Provincial People's Hospital, and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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