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MiR-302 a/b/c suppresses tumor angiogenesis in hepatocellular carcinoma by targeting MACC1

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Abstract. – OBJECTIVE: Hepatocellular carcinoma (HCC) is a hypervascularized tumor. Aberrant angiogenesis is the main cause, which results in cancer growth and progression. It has been showed that microRNA-302 cluster (miR-302) may be associated with angiogenesis. Here, we aimed to identify the role of miR-302a/b/c in the regulation of cell angiogenesis in HCC.

PATIENTS AND METHODS: MRNA expression of miR-302a/b/c and MACC1 was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The protein of MACC1 was measured using Western blot. Cells proliferation, migration, and invasion abilities were investigated *via* Cell Counting Kit-8 (CCK-8) assay or transwell assay, respectively. Tube formatting assays were used to explore the tube formation capacity. The interaction among miR-302a/b/c was analyzed by luciferase assay.

RESULTS: The expression of miR-302a/b/c was greatly reduced while MACC1 expression, whether mRNA or protein was conspicuously elevated in HCC tissues and cells. Then, functional experiment results showed miR-302a/b/c overexpression and MACC1 down-regulation inhibited the proliferation, migration, invasion ability, and tube formation capacity of HUVECs. In addition, we detected that miR-302a/b/c directly targeted MACC1 and suppressed MACC1 expression, and miR-302a/b/c could suppress tumor angiogenesis in HCC by targeting MACC1.

CONCLUSIONS: MiR-302a/b/c may function as a potential suppressor of tumor angiogenesis in HCC by targeting MACC1, indicating a promising target for HCC therapy.

Key Words:
MiR-302, MACC1, Angiogenesis, Invasion, Metastasis, HCC.

Abbreviations

HCC = Hepatocellular carcinoma; VEGF = vascular endothelial growth factor; PDGF = platelet derived growth factor; FGF = fibroblast growth factor; EGF = epidermal growth factor; hESCs = human embryonic stem cells; ECs = endothelial cells, HUVECs = umbilical vein endothelial cells; TCM = tumor-conditioned medium; qRT-PCR = quantitative Real Time-Polymerase Chain Reaction; FBS=fetal bovine serum.

Introduction

Hepatocellular carcinoma (HCC), a leading cause of cancer-related death worldwide, is the most common type of primary liver cancer[1]. HCC is a hypervascularized tumor, and most tumors above 20 mm are arterially hypervascularized[2]. Angiogenesis has an important effect on the pathophysiology of chronic liver disease[3]. Aberrant angiogenesis is the main cause, which results in cancer growth and progression, and the angiogenic switch is pivotal for HCC dedifferentiation and progression. Secretion of proangiogenic factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), etc., and suppression of antiangiogenic factors, such as angiostatin, endostatin, etc., stimulate endothelial cells to generate new blood vessels (sprouting) and facilitate tumor growth and aggressiveness[2,4]. Therefore, antiangiogenic drugs are a prerequisite.

MicroRNAs (miRNAs) have emerged as critical regulators of gene expression *via* post-transcriptional inhibition and RNA decay, and have been involved in the regulation of cellular differentiation, proliferation, metastasis, apoptosis, and angiogenesis[5,6]. The miR-302/367 cluster, mainly composed of five members, miR-302a, miR-302b, miR-302c, miR-302d, and miR-367, is ubiquitously distributed in vertebrates and occupies an in-

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tragenic cluster located in intron 8 of Larp7 gene[7]. MiR302/367 is highly expressed in embryonic stem cells and plays an important role in diverse biological processes, such as the pluripotency of human embryonic stem cells (hESCs), self-renewal, and reprogramming[8,9]. Pi et al[10] recently demonstrated that miR302/367 regulation of an Erk1/2-Klf2-S1pr1 pathway could restrict tumor development and angiogenesis, improve vascular stability, and potently retard tumor growth in an endothelial cells (ECs) autonomous manner. MiR-302 cluster can inhibit angiogenesis and growth of K562 leukemia cells by targeting VEGFA[11]. However, the role of miR-302 and the underlying mechanisms in HCC angiogenesis have not yet been elucidated.

Metastasis-associated in colon cancer-1 (MACC1), located on chromosome 7 at position 7p21.1, was initially identified by genome-wide expression analysis in primary and metastatic tissues of human colon cancer[12]. The effects of deregulation of MACC1 on the progression of cancers have been revealed, such as MACC1 induces autophagy to regulate proliferation, apoptosis, migration, and invasion of squamous cell carcinoma through the underlying signaling pathway[13,14]. MACC1 may regulate the expression of HGF, MMP-2, and MMP-9 in HSCs, and may thus promote migration and invasion of gastric cancer[15]. MACC1 downregulation significantly induced G1 phase arrest, suppressed endometrial carcinoma cell proliferation, migration, and invasion[16]. MACC1 suppressed cell apoptosis in hepatocellular carcinoma by targeting the HGF/c-MET/AKT pathway[17]. Thus, MACC1 may be a useful target for developing cancer treatment strategies.

Here, we took miR-302a/b/c as the research target, and we found miR-302a/b/c might function as a potential suppressor of tumor angiogenesis in HCC by targeting MACC1, indicating a novel strategy to improve HCC therapy.

Patients and Methods

Patients and Specimens

In this study, a total of 33 patients undersetting hepatectomy for HCC at the Yanbian University Hospital were collected. All patients were diagnosed with primary HCC, and none of them had received any preoperative cancer treatment. Tumor specimens were immediately stored at –80°C until further study. Informed consent had been obtained from patients and hospital, and the work was approved by the Ethics Committee of Yanbian University Hospital.

Cell Culture and Transfection

The human hepatoma cell lines (Huh7 and PLC/PRF/5) and normal human hepatoma cell line THLE-2 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The umbilical vein endothelial cells (HUVECs) were purchased from AllCells (Shanghai, China). Huh7 and PLC/PRF/5 cells were incubated with Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). HUVECs and THLE-2 cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) containing 10% phosphate-buffered saline (PBS). All cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C.

The miRNA mimic (miR-302a/b/c) and corresponding negative control (miR-NC) were purchased from RiboBio (Guangzhou, China). Small interfering RNA (siRNA) targeting MACC1 (si-MACC1), siRNA negative control (si-NC), pcDNA3.1, and pcDNA3.1-MACC1 overexpression vector (MACC1) were synthesized by Genepharma (Shanghai, China). These oligonucleotides or vectors were transfected into Huh7 and PLC/PRF/5 cells using Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Cells were harvested at 24 h for the following experiments.

Collection of the Tumor-Conditioned Medium (TCM)

Huh7 and PLC/PRF/5 cells were transfected with or without corresponding miRNA mimic, siRNAs, or plasmids or their matched control, alone or in combination for 24 h, and then maintained in DMEM supplemented with serum-free medium. After culture for further 14 h, the supernatant was collected and sequentially centrifuged at 500 g and 12,000 g to remove cell debris at 4°C for 15 min each. Then, aliquots of the TCM were stored at –80°C until needed.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Once transfected under various conditions, total RNA was extracted from the Huh7 and PLC/

PRF/5 cells, which were lysed with TRI reagent (Sigma-Aldrich, St. Louis, MO, USA). Then, the cDNA was synthesized using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. SYBR Green qPCR Master Mix kit (Qiagen, Hilden, Germany) was used to estimate the relative expression of mRNA following the manufacturer's instruction. The expression levels were normalized using U6 for miR-302/b/c and β-actin for MACC1. Fold change was analyzed using the $2^{-\Delta\Delta Ct}$ equation. The specific primers were listed as follows: MACC1 Forward 5'-CTTGCGGAGGTCACCATAGC-3' and Reserve 5'-GATTTCCAACAACGGGCTCA-3'; β-actin Forward 5'-GGGAAATCGTGC- GTGACAT-3' and Reserve 5'-CTGGAAGGTGGACAGCGAG-3'.

Cells Viability

Cell proliferation analyses were conducted using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). HUVECs cells were seeded in 96-well plates at a density of $5x10^3$ cells per well at 6 h. Next, cells were incubated with the TCM gathered from different background of miR-302a/b/c, or MACC1 expression for the indicated times (0, 24, 48, and 72 h). Subsequently, 10 μl of CCK-8 reagents were added to each well for further 2 h incubation at 37°C. Finally, the absorbance at 450 nm value of each test well was measured using Microplate reader (Bio-Rad, Hercules, CA, USA).

Tube Formation

HUVECs at the density of 2×10^4 were maintained in the absence or presence of 100% TCM for 24 h at 37°C in a 96-well plate pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The formation of capillary-like structures was analyzed under an inverted microscope. The branch points of the formed tubes were counted to evaluate tube formation.

Transwell Assay

Cell migration and invasion were evaluated by transwell assays. Briefly, HUVECs in serum-free medium were seeded on the upper chamber, and then the TCM collected from different background of miR-302a/b/c or MACC1 expression, was added into the lower insert. After 12 h of incubation, cells on the upper chamber were removed gently, and the migrated and invaded cells were fixed with methanol for 20 min and stained by 0.1% crystal violet 30 min. Five fields randomly selected were photographed and counted using a microscope.

Western Blot

Cells from transfection HCC cells lines were solubilized using Radio Immunoprecipitation Assay (RIPA) Lysis (Beyotime, Shanghai, China), and the extracted protein concentration was quantified using the bicinchoninic acid (BCA) assay (Beyotime, Shanghai, China). Then, about 50 μg of proteins was subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and followed by the transfer to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membrane was incubated with primary antibodies against MACC1 and β-actin (CST, Danvers, MA, USA) at 4°C overnight. Subsequently, horseradish peroxidase (HRP)-conjugated secondary antibodies were added for further incubation at room temperature for 2 h. Band signals were visualized *via* an enhanced chemiluminescence (ECL) kit (Pierce, Minneapolis, MN, USA).

Luciferase Reporter Assay

The MACC1 UTR wild-type or mutant with miR-302a/b/c binding sequences were cloned into the pGL3 luciferase reporter vector (Promega, Madison, WI, USA) to synthesize MACC1-Wt or -Mut luciferase reporter vector (pGL3-MACC1-Wt or -Mut). Then, Huh7 and PLC/PRF/5 cells were co-transfected with corresponding Wt or Mut luciferase reporter vectors and miR-302a/b/c mimics or mimic control using Lipofectamine 3000. After transfection for 48 h, luciferase activities were monitored using Dual-Luciferase assay kit (Biotek Synergy, Winooski, VT, USA).

Statistical Analysis

All the data are expressed as the mean ± SD. All statistical analysis was conducted using GraphPad Prism 7.0 (GraphPad Inc., San Diego, CA, USA). All experiments were repeated three times. The correlation analysis was analyzed by Spearman's correlation. Statistical analysis was evaluated using Student's *t*-test or one-way ANOVA as appropriate. Tukey's test was used for pairwise comparisons. $p< 0.05$ was considered statistically significant.

Results

The Expression of MiR-302 a/b/c Is Reduced in HCC Tissues and Cells

The expression of miR-302 a/b/c was measured in HCC tissues and cell lines. The results showed that the expression level of miR-302 a/b/c was significantly decreased in HCC tissues compared with normal group (Figure 1A-C). Moreover, we found a great reduction of miR-302 a/b/c in HCC cell lines (Huh7 and PLC/PRF/5) compared to the human normal hepatocytes THLE-2 (Figure 1D-F).

MiR-302 a/b/c Evidently Inhibits Tube Formation, Proliferation, Migration, and Invasion of HUVECs

To explore the biological role of miR-302a/b/c in HCC angiogenesis, its effects on cell tube formation, proliferation, migration, and invasion of HUVECs were investigated. After transfected with miR-NC or miR-302a/b/c mimic, a significant increase of miR-302a/b/c expression in Huh7 or PLC/PRF/5 cells was detected (Figure 2A). Subsequently, the tube formation assay substantiated incubation with TCM from miR-302a/b/c-overexpressed Huh7 or PLC/PRF/5 cells remarkably inhibit the capillary tube formation of HUVECs (Figure 2B). Additionally, a significant suppression of HUVECs cells proliferation was verified when they were grown in TCM obtained from miR-302a/b/c-overexpressed Huh7 or PLC/PRF/5 cells (Figure 2C, D). Furthermore, TCM from miR-302a/b/c-overexpressed Huh7 or PLC/PRF/5 cell notably attenuated cell migration and invasion of HUVECs (Figure 2E, F). All the data showed that miR-302 a/b/c could antagonize tube formation, proliferation, migration, and invasion of HUVECs.

MiR-302a/b/c Directly Target MACC1 and Suppress MACC1 Expression

To investigate the underlying mechanism of miR-302a/b/c involved in the angiogenesis inhibition, bioinformatics analysis was performed to predict the potential target of miR-302a/b/c, and MACC1 was identified as a potential target with a putative binding site of miR-302a/b/c (Figure 3A). Then, luciferase reporter assay was conducted, and the result indicated luciferase activity was noticeably abolished following co-transfection of miR-302a/b/c with MACC1-Wt vector, but not in MACC1-Mut groups (Figure 3B, C) in Huh7 and PLC/PRF/5 cells. Meanwhile, we found that miR-302a/b/c mimic transfection markedly inhibited the expression of MACC1, whether protein or mRNA, both in Huh7 and PLC/PRF/5 cells (Figure 3D, E). Moreover, the expression of MACC1 in HCC tissues and cells was examined, and the cor-

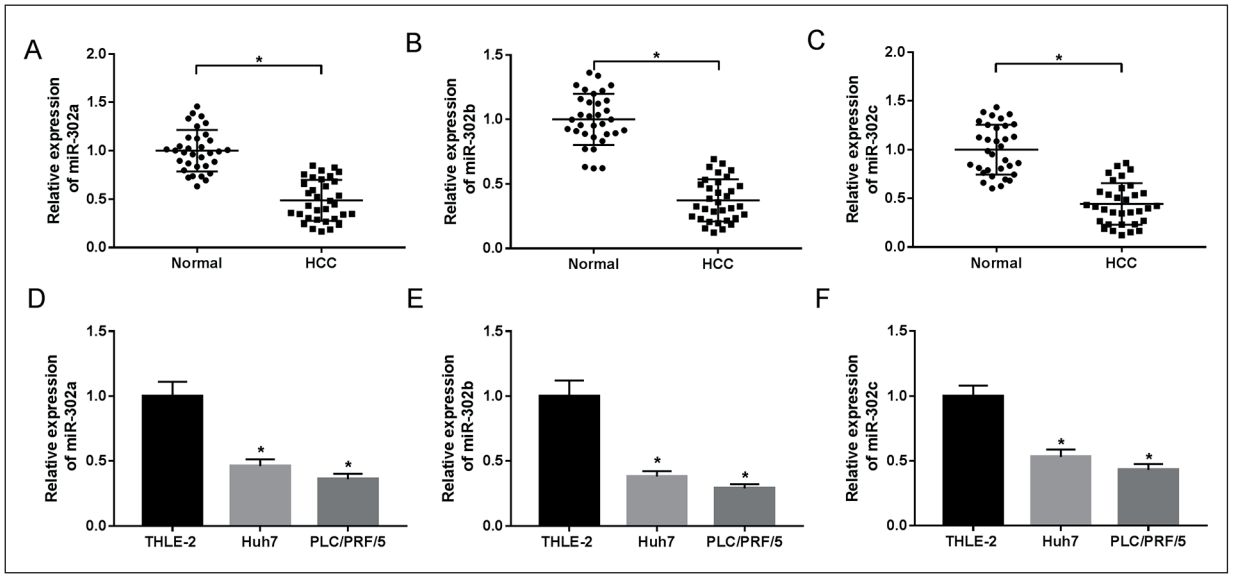

Figure 1. Expression patterns of miR-302a/b/c in HCC tissues and cells. The expression of miR-302a/b/c was detected using qRT-PCR in HCC, normal tissues **(A-C)** and HCC cell line (Huh7 and PLC/PRF/5) and human normal hepatocytes THLE-2, respectively **(D-F)**. *$p < 0.05$.

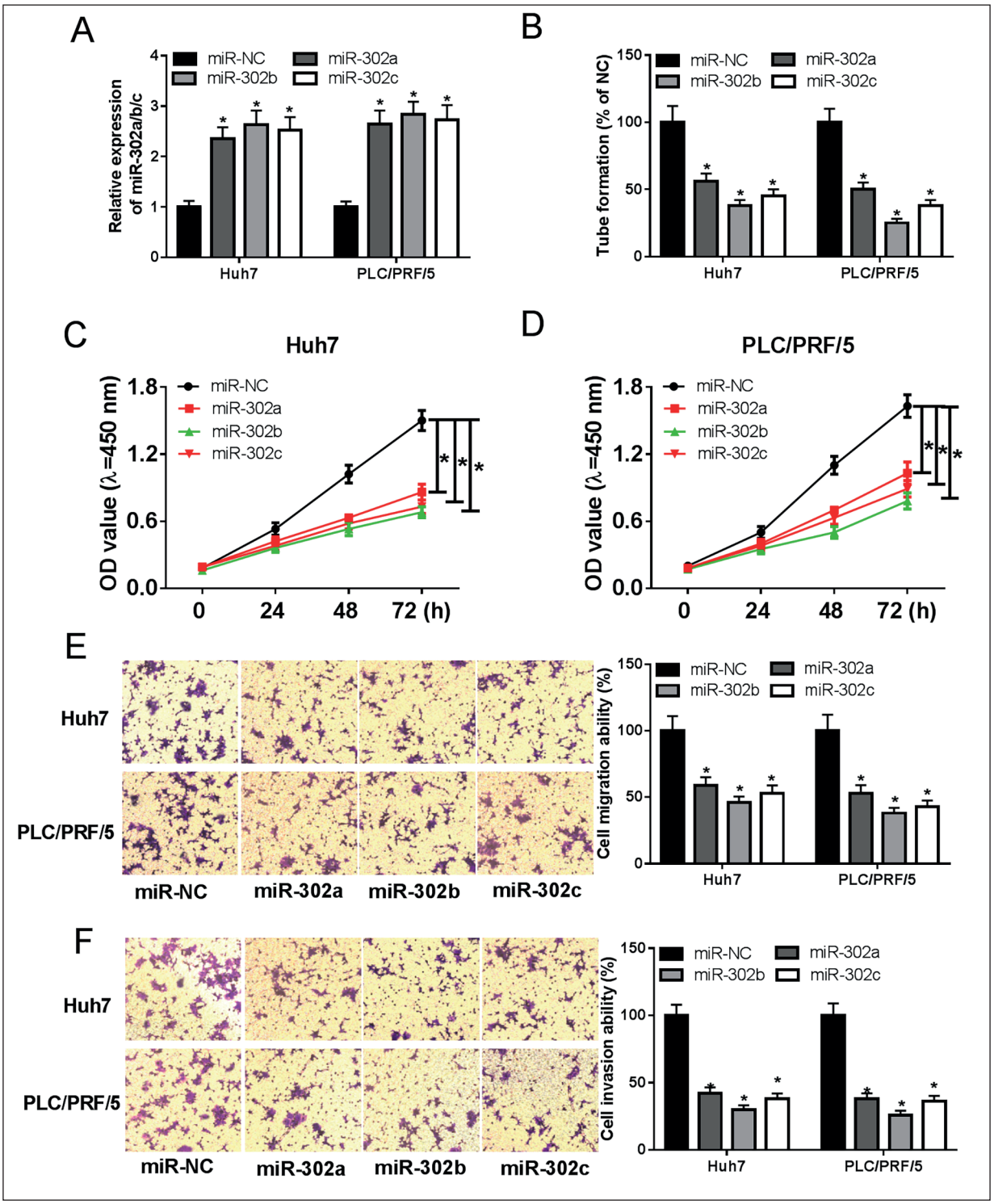

Figure 2. Effect of miR-302a/b/c on angiogenesis of HCC cells. **A,** Transfection efficiency was measured by qRT-PCR after transfected with miR-NC or miR-302a/b/c mimic. Then, after incubation with TCM from Huh7 or PLC/PRF/5 cells transfected with miR-302a/b/c or miR-NC, **(B)** the functions in cell capillary tube formation, **(C-D)** proliferation, **(E)** migration and invasion **(F)** were examined. *$p < 0.05$.

relation among miR-302a/b/c and MACC1 was analyzed. Then, the data exhibited a significant increase of MACC1 expression in HCC tissues and cells compared to the normal controls (Figure 4A-C). A perfect negative correlation among miR-302a/b/c and MACC1 was dis-

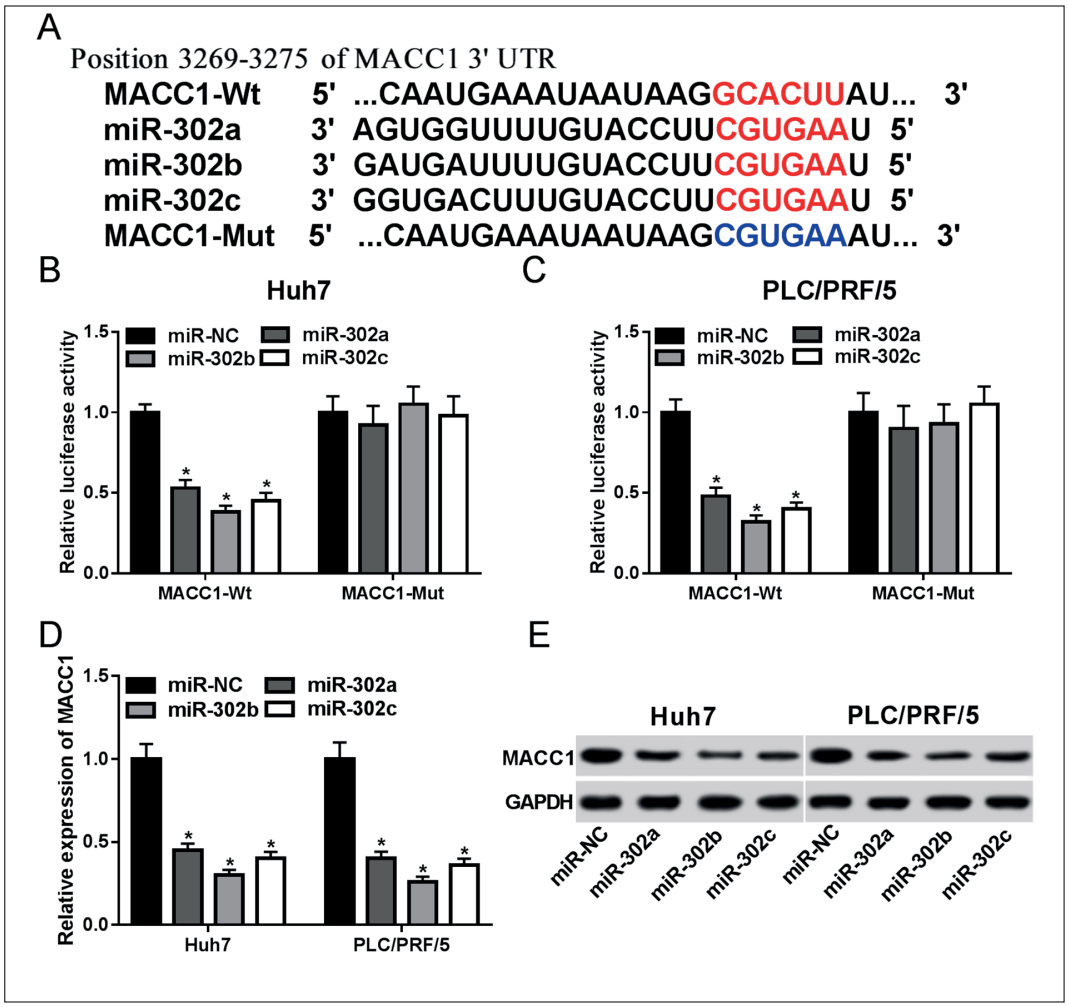

Figure 3. MACC1 was a direct target of miR-302a/b/c. **A,** Putative complementary sites between MACC1 and miR-302a/b/c. **B-C,** Luciferase activity was detected in Huh7 and PLC/PRF/5 cells co-transfected with MACC1-WT or MACC1-Mut and miR-302a/b/c or miR-NC. **D-E,** Level of MACC1 mRNA or protein was measured in Huh7 and PLC/PRF/5 cells transfected with miR-NC or miR-302a/b/c by qRT-PCR or Western blot, respectively. *$p < 0.05$.

covered (Figure 4D-F). Thus, based on these results, we know that miR-302a/b/c targetedly suppressed MACC1 expression.

Knockdown of MACC1 Antagonizes Tube Formation, Proliferation, Migration, and Invasion of HUVECs

To explore the effects of MACC1 on HCC angiogenesis, the tube formation, proliferation, migration, and invasion abilities of HUVECs were determined. After knockdown of MACC1, a significant reduction of MACC1 was detected (Figure 5A). Subsequently, a notable inhibition of HUVECs cells tube formation, proliferation, migration, and invasion was verified in the presence of TCM obtained from MACC1-depleted Huh7 or PLC/PRF/5 cells (Figure 5B-F). These data revealed that MACC1 induced tube formation, proliferation, migration, and invasion of HUVECs, while knockdown of MACC1 could hinder the angiogenesis in HCC cells.

Overexpression of MiR-302a/b/c Attenuates the Promotion Effect of MACC1 on HUVEC Tube Formation, Proliferation, Migration, and Invasion

Based on the previous results, cells were transfected with pcDNA or MACC1, or MACC1 and miR-NC, or MACC1 and miR-302a/b/c to investigate the interaction among miR-302a/b/c and MACC1 on angiogenesis in HCC cells. The

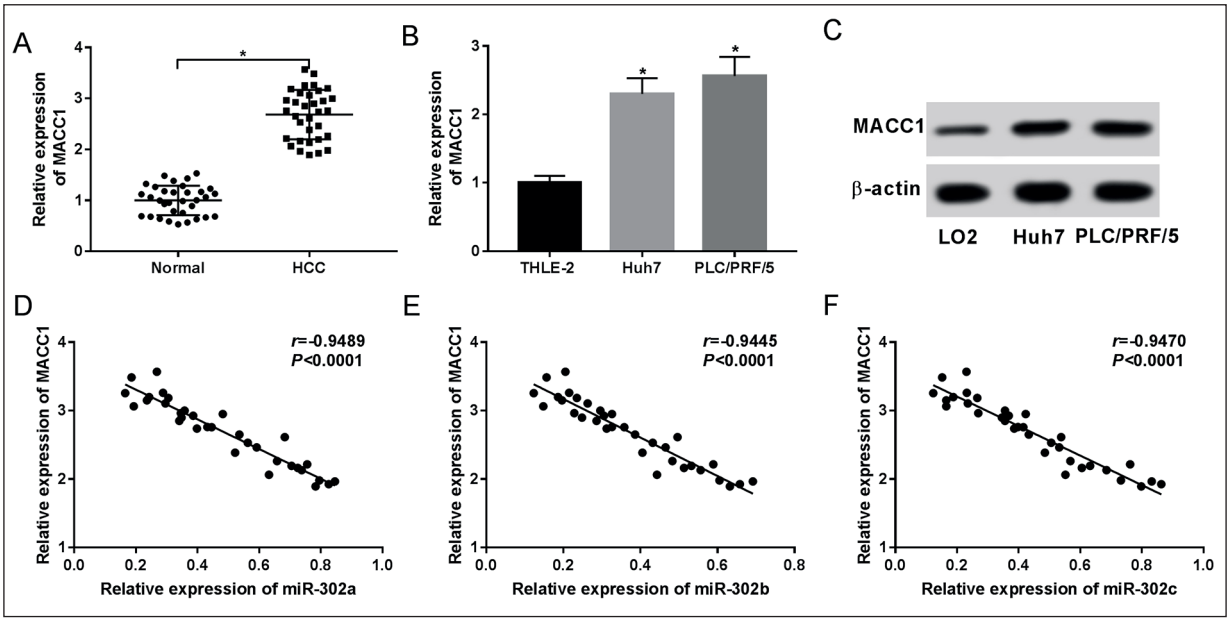

Figure 4. The correlation between miR-302a/b/c and MACC1. **A,** Expression of MACC1 mRNA was detected using qRT-PCR in HCC and normal tissues **(A)** and HCC cell line (Huh7 and PLC/PRF/5) and human normal hepatocytes THLE-2, respectively **(B)**. The protein of MACC1 was examined using Western blot **(C)**. The correlation among miR-302a/b/c and MACC1 was analyzed *via* Spearman's correlation **(D-F)**. *$p < 0.05$.

transfection efficiency was measured (Figure 6A). Subsequently, we found overexpression of miR-302a/b/c attenuates the promotion effect of MACC1 on HUVECs tube formation (Figure 6B), proliferation (Figure 6C, D), migration, and invasion (Figure 6E, F). These indicated that overexpression of miR-302a/b/c could attenuate the angiogenesis effect of MACC1 on HUVEC.

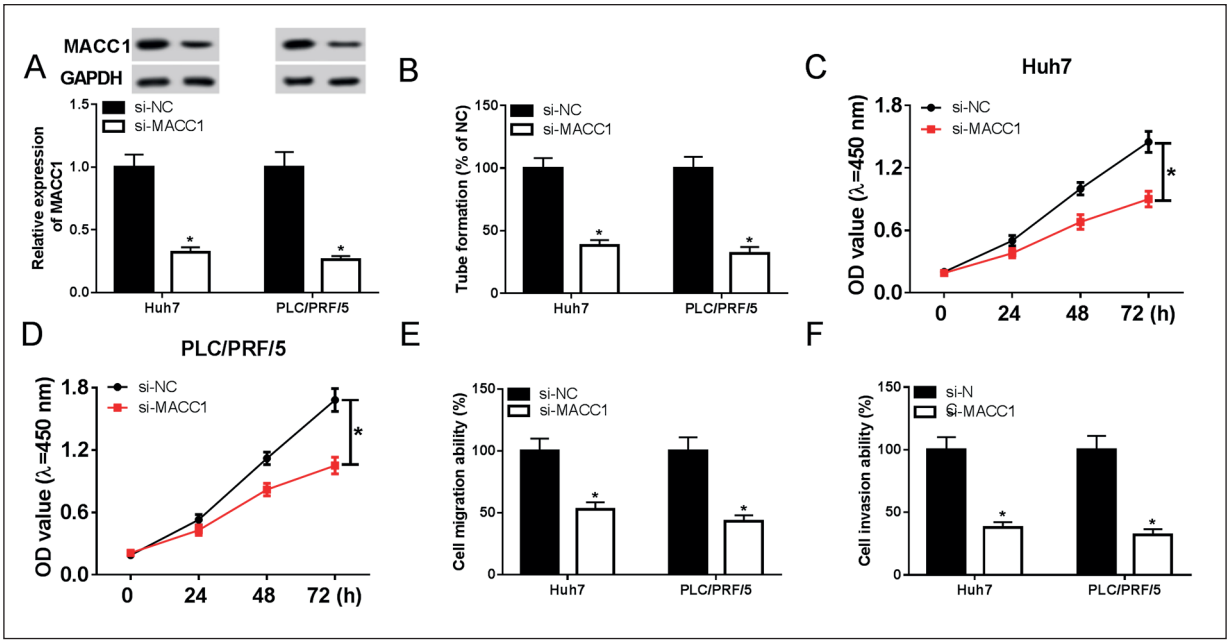

Figure 5. Effect of MACC1 on angiogenesis of HCC cells. **A,** Transfection efficiency was determined by qRT-PCR after transfected with si-MACC1 or si-NC. After incubation with TCM from Huh7 or PLC/PRF/5 cells transfected with si-MACC1 or si-NC, **(B)** the functions in cell capillary tube formation, **(C, D)** proliferation, **(E)** migration and invasion **(F)** were investigated. *$p < 0.05$.

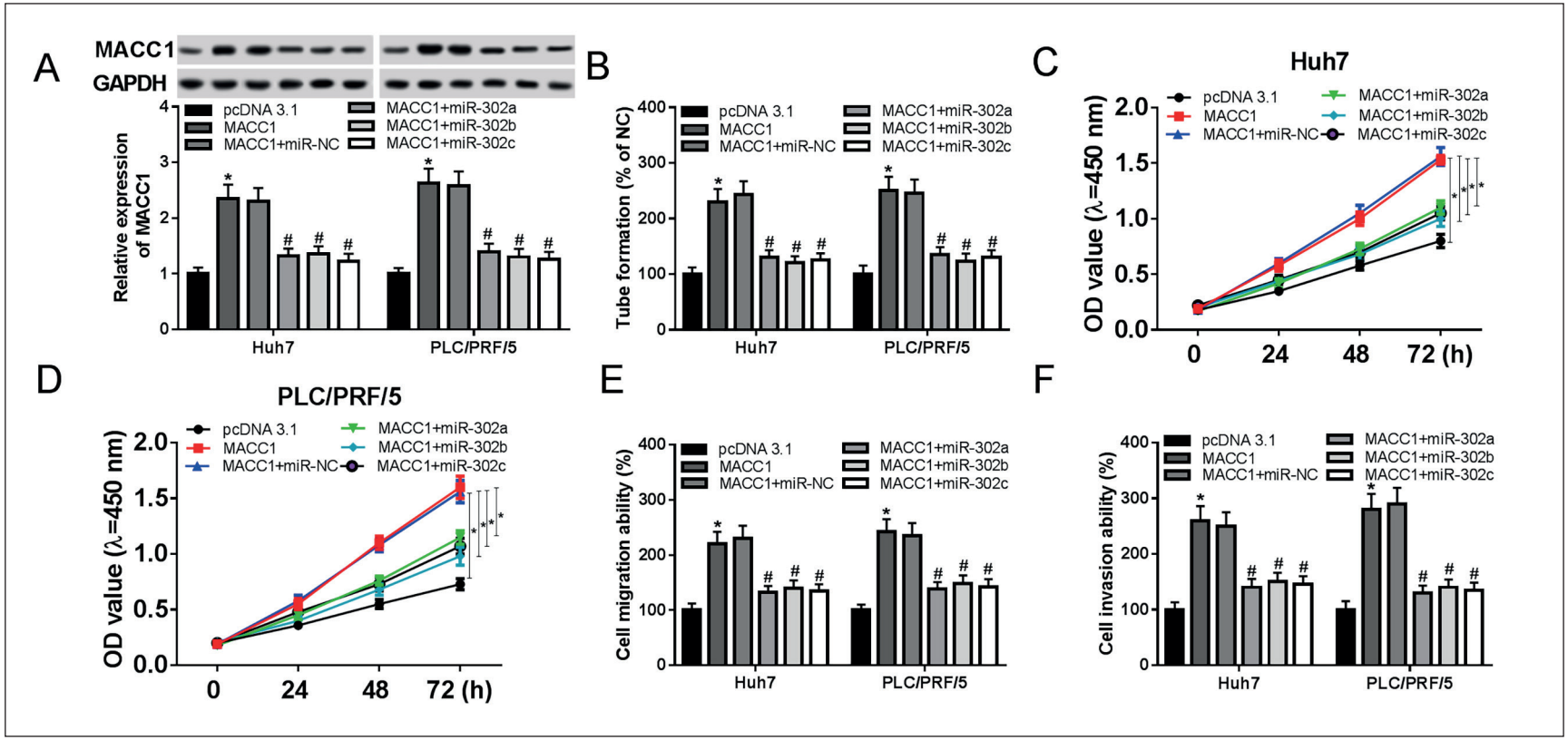

Figure 6. The interaction among miR-302a/b/c and MACC1 on the angiogenesis of HCC cells. **A,** Transfection efficiency was determined by qRT-PCR after transfected with pcDNA or MACC1 or MACC1 and miR-NC or MACC1 and miR-302a/b/c. Then after incubation with TCM from Huh7 or PLC/PRF/5 cells transfected with pcDNA or MACC1 or MACC1 and miR-NC or MACC1 and miR-302a/b/c, **(B)** the functions in cell capillary tube formation, **(C-D)** proliferation, **(E)** migration and invasion **(F)** were demonstrated. *$p < 0.05$.

Discussion

Hepatocarcinogenesis is a multistep process involving genetic and environmental changes, permitting the hepatocyte to escape normal control mechanisms in cell differentiation, proliferation, migration, growth, and death, leading to the development of malignant diseases[18]. Angiogenesis is a vital biological process in HCC development and progression due to HCC, which is one of the most known vascular solid tumors[19]. At present, antiangiogenic treatments, which can inhibit blood vessel formation, have been reported to be a promising effective treatment for HCC[20]. Several antiangiogenic drugs have been indicated, such as sorafenib, regorafenib, lenvatinib, cabozantinib, etc. However, the safety and efficacy of antiangiogenic therapies are still controversial. Up to date, sorafenib monotherapy is the only global standard of antiangiogenic care for advanced HCC patients. None of the other antiangiogenic drugs or combinations tested to date have improved survival compared with sorafenib monotherapy[4,21]. Therefore, we should take significant efforts to advance knowledge of the molecular mechanisms of HCC angiogenic initiation and progression.

MiRNAs, with 21-23 nucleotides in length, are noncoding, single-stranded RNA molecules generated endogenously. Recently, miRNAs have been discovered to modulate various stages of angiogenesis[22]. MiRNAs can regulate the function of ECs *via* non-autonomous and autonomous manner during tumor angiogenesis, and miRNAs modulate the expression of pro- or anti-angiogenic factors in tumor cells, thus regulating the proliferation and metastasis of ECs in a paracrine manner[23]. Malformation of various miRNAs mediated processes of angiogenesis has been found in diverse diseases like cardiovascular diseases[24], non-small cell lung cancer[25], multiple sclerosis, type 1, and type 2 diabetes, diabetic retinopathy[22], including HCC[26,27]. Some miRNAs, such as miR-17-92 cluster, miR-128, miR-497, miR-296, miR-125b, etc., targeted the genes to inhibit or promote ECs tube formation[23,28,29]. Therefore, the regulation of angiogenesis by microRNAs in HCC may be a novel therapeutic approach. In this study, the expression of miR-302 a/b/c was measured in HCC tissues and cell lines. We found a significant decrease of miR-302a/b/c expression in HCC tissues and cell lines. Then, HUVECs cells were incubated with TCM from miR-302a/b/c-overexpressed Huh7 or PLC/PRF/5 cells. The proliferation, migration, invasion abilities, and tube formation capacity of HUVECs were detected, and the results suggested that overexpression of miR-302a/b/c could attenuate the angiogenesis effect of HUVEC in HCC cell lines.

To investigate the underlying molecular mechanism of miR-302a/b/c on the angiogenesis of HUVECs, bioinformatics analysis and luciferase assay were conducted, and MACC1 was identified as a potential target with a putative binding site of miR-302a/b/c. MACC1 was reported to be an independent prognostic biomarker of overall survival for several digestive system neoplasms (e.g., pancreatic cancer, colorectal cancer, esophageal cancer, gastric cancer, and hepatocellular carcinoma). High MACC1 expression markedly associated with poorer overall survival, as well as poorer relapse-free survival[30,31]. In the angiogenesis, MACC1 was discovered to promote vascular formation in cholangiocarcinoma, gastric cancer, hepatocellular carcinoma, and cervical cancer[27,32-34]. In the present study, a significantly elevated MACC1 mRNA and protein expression in HCC tissues and cells were determined. Furthermore, after incubated with TCM obtained from MACC1-depleted Huh7 or PLC/PRF/5 cells, the inhibition of HUVECs proliferation, migration, invasion abilities, and tube formation was identified consistently with previous researches. Additionally, we detected that miR-302a/b/c directly targets MACC1 and suppresses MACC1 expression, and that a perfect negative correlation among miR-302a/b/c and MACC1 was demonstrated. Meanwhile, we also illustrated that miR-302a/b/c could antagonize the angiogenesis effect of MACC1 on HUVEC.

Conclusions

MiR-302a/b/c regulates MACC1 suppression to counterwork angiogenesis in HUVECs. We first verified the miR-302/MACC1 signaling pathway in HCC, indicating a promising target for the evolvement of anti-angiogenesis drugs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Yanbian University Hospital. The methods used in this study were performed in accordance with the relevant guidelines and regulations.

Consent for Publication

All authors about this work consent this manuscript to be published.

Availability of Data and Materials

All original data and materials are available from the corresponding author upon request.

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