Hepatocellular Carcinoma Cell-Secreted Exosomal MicroRNA-210 Promotes Angiogenesis In Vitro and In Vivo

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We previously found that 19 microRNAs (miRNAs) significantly increased in the sera of hepatocellular carcinoma (HCC) patients. Here, we evaluated whether these miRNAs were secreted by HCC cells and contributed to tumor angiogenesis. High level of miR-210-3p (miR-210) was detected in the exosomes isolated from the sera of HCC patients and the conditioned media of hepatoma cells. Higher miR-210 level in serum was correlated with higher microvessel density in HCC tissues. Moreover, the HCC cell-secreted exosomes promoted in vitro tubulogenesis of endothelial cells, which was strengthened by overexpressing miR-210 in HCC cells but was attenuated by repressing miR-210 or DROSHA in HCC cells. This pro-tubulogenesis effect by HCC exosomes was also abrogated by antagonizing miR-210 in endothelial cells. Subsequent in vivo studies revealed that Matrigel plug and subcutaneous tumor xenografts treated with HCC cell-derived exosomal miR-210 displayed much more vessels. Furthermore, exosomal miR-210 could be delivered into endothelial cells and directly inhibited the expression of SMAD4 and STAT6, resulting in enhanced angiogenesis. Collectively, HCC cell-secreted exosomal miR-210 may be transferred into endothelial cells and thereby promotes tumor angiogenesis by targeting SMAD4 and STAT6. Our findings identify a novel mechanism of HCC angiogenesis and highlight the biological importance of exosomal miR-210.

INTRODUCTION
Active angiogenesis is responsible for rapid growth, early metastasis and poor survival of cancer. Much progress has been made in identifying the proteins that regulate angiogenesis. Several proteins, such as vascular endothelial growth factor (VEGF) and angiopoietin (ANG), have been demonstrated to be critical in tumor angiogenesis. Although various inhibitors of the VEGF signaling pathway have been developed and approved for cancer therapy, the efficacy and side effect of these drugs still merit careful evaluation, and more extensive exploration on the new angiogenesis regulators is required.

MicroRNAs (miRNAs) belong to a class of small non-coding RNAs. Cellular miRNAs modulate gene expression post-transcriptionally and regulate different cell activities, such as proliferation, differentiation, apoptosis, angiogenesis, etc. Emerging evidences disclose that miRNAs can be selectively packed into vesicle, particularly exosome with a diameter of 50–100 nm, and then secreted to extracellular fluid and delivered into recipient cells, where they block the translation of target genes and thereby affect the activities of recipient cells. Thus, the exosomal miRNAs may serve as a novel class of signaling molecules in cell-to-cell communication. Recent investigations have demonstrated that exosomal miRNAs play critical roles in various pathophysiologic processes, including immune response, atherosclerosis, and tumorigenesis. Although some exciting findings have been achieved, the progress is still limited due to difficulty in elucidating the functions of exosomal miRNAs in vivo.

Hepatocellular carcinoma (HCC) is a common malignancy with poor prognosis. To date, five studies have disclosed that the intercellular transfer of exosomal miRNAs modulate the growth, migration, invasion, and chemosensitivity of HCC cells. However, these studies only provide evidence and mainly focus on the intercellular communication between different HCC cells. The significance of exosomal miRNAs in HCC development is still poorly understood, especially for angiogenesis of HCC, which has not yet been reported.

In a previous study, we found that the levels of 19 miRNAs significantly increased in the sera of HCC patients. Herein, we evaluated whether these miRNAs were secreted by HCC cells and contributed to HCC angiogenesis. The results revealed that HCC cells secreted miR-210-3p (miR-210) via exosomes and the exosomal miR-210 promoted the in vitro tubulogenesis of endothelial cells and the in vivo angiogenesis of HCC by inhibiting the expression of SMAD4 and signal transducer and activator of transcription 6 (STAT6) in endothelial cells. These findings suggest that exosomal miRNAs play an important role in intercellular communication during angiogenesis.

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and also implicate that antagonism of exosomal miR-210 represents a potential therapeutic strategy for cancer therapy.

RESULTS

HCC Cells Secrete miR-210, and Serum miR-210 Level Is Associated with Microvessel Density in HCC Tissues

We first explored whether hepatoma cells secreted those 19 miRNAs that were increased in the sera of HCC patients in our previous study. As shown, only miR-29a, miR-29c, miR-145, miR-192, and miR-210 were detected in the conditioned medium (CM) of all four examined hepatoma cell lines, including QGY-7703, HepG2, SK-Hep-1, and Huh-7 (Figure S1; Table S1).

To identify the secreted miRNAs that were critical for angiogenesis, we analyzed the correlation between the microvessel density (MVD) in HCC tissues and the levels of these five miRNAs in the sera of HCC patients. Notably, the patients with higher MVD in tumor tissues showed higher levels of serum miR-210 (Figure 1A; p < 0.001), miR-145, and miR-192 (Figure S2; p < 0.05). Therefore, miR-210, the one revealing the most evidenced correlation, was chosen for detailed investigations.

The results showed that the level of miR-210 significantly increased in the sera from HCC patients (Figure 1B) and from hepatoma-bearing mice (Figure 1C) and also elevated in the serum-derived exosomes from HCC patients (Figure 1D). Furthermore, silencing of DROSHA (Figure S3A), the essential regulator required for miRNA biogenesis, reduced the level of mature miR-210 not only in HCC cells but also in HCC cell-derived CM and exosomes (Figure 1E). Knockdown of ALIX and/or HRS (Figure S3B), two critical components for exosome secretion, also decreased miR-210 level in the CM of HCC cells (Figure 1F).

These data indicate that HCC cells may secrete miR-210 via exosomes and the secreted miR-210 may be involved in HCC angiogenesis.

HCC Cell-Derived Exosomal miR-210 Promotes Tube Formation of Endothelial Cells

We next examined whether the HCC cell-secreted exosomal miR-210 affected the in vitro tubulogenesis of human umbilical vein endothelial cells (HUVECs). Exosomes that were isolated from the sera of HCC patients promoted endothelial cells to form capillary-like structures (Figure 2A), and exosomes derived from QGY-7703, HepG2, SK-Hep-1, and Huh-7 cell lines universally enhanced tubulogenesis of HUVECs (Figure 2B). Compared with control group, exosomes from DROSHA-knockdown cells had decreased ability to promote tube formation of HUVECs (Figure 2C), indicating that exosomal miRNA may promote tubulogenesis.

Subsequent investigations disclosed that, when anti-miR-210, the inhibitor of miR-210, was introduced into HUVECs, the enhanced tubulogenesis of HUVECs by the HCC cell-derived exosomes was significantly attenuated (Figure 3A). Furthermore, exosomes that were isolated from the anti-miR-210-transfected HCC cells exhibited reduced amount of miR-210 (Figure 3B) and displayed reduced activity in promoting tube formation of HUVECs (Figure 3C) compared with those from anti-negative control (NC) transfectants. The role of exosomal miR-210 was further confirmed...
using QGY-miR-210 subline that stably expressed miR-210. Compared with QGY-control group, exosomes from QGY-miR-210 subline had increased amount of miR-210 (Figure 3D) and showed stronger ability to promote tubulogenesis of HUVECs (Figure 3E). Consistently, HUVECs that were transfected with miR-210 duplex revealed increased ability to form tube structures (Figure S4).

We further exclude the possibility that exosomes induced the expression of endogenous miR-210 in HUVECs, because treatment with HCC cell-derived exosomes significantly increased the level of mature miR-210 but did not affect that of primary miR-210 in HUVECs (Figure 3F).

These data indicate that HCC cell-derived exosomal miR-210 promotes in vitro tubulogenesis of endothelial cells.

**HCC Cell-Derived Exosomal miR-210 Promotes Angiogenesis In Vivo**

The in vivo function of exosomal miR-210 was next examined. Matrigel was mixed with the exosomes derived from QGY-miR-210 or QGY-control subline and then injected subcutaneously into nude mice. Of note, Matrigel plugs did not contain any tumor cells. Compared to the Matrigel with exosomes of QGY-control, the Matrigel containing QGY-miR-210-derived exosomes showed much more vessels and higher level of endothelial cell marker CD34 (Figure 4A), suggesting that QGY-miR-210-derived exosomes recruit more endothelial cells to the Matrigel. The same results were observed in mouse xenograft model, in which exosomes from QGY-miR-210 or QGY-control subline were injected into Hepa1-6 cell-derived xenografts. Compared with control group, xenografts that were injected with QGY-miR-210-derived exosomes displayed higher MVDs (Figure 4B) and larger tumor size (Figure 4C).

We further showed that the hepatoma cell-derived exosomes were delivered into endothelial cells. In the xenografts that were originated from Hepa1-6-mCD63-GFP cell line, which stably expressed mCD63-GFP fusion protein, the signals of exosome-enriched protein CD63 were observed within endothelial cells (Figure 4D). Consistently, after HUVECs were incubated with the HCC cell-derived exosomes that were labeled with fluorescent dye DiIC<sub>16</sub> (Figure 4E) or with the exosomes from Cy3-labeled miR-210 transfectants (Figure S5), internalized fluorescent punctuate structures were observed in the cytoplasm of HUVECs.

These findings imply that the exosomal miR-210 derived from hepatoma cells may be delivered into endothelial cells and promotes angiogenesis in vivo.

**HCC Cell-Derived Exosomal miR-210 Promotes Tube Formation by Targeting SMAD4 and STAT6 in Endothelial Cells**

To explore by which mechanism exosomal miR-210 regulated angiogenesis, putative targets of miR-210 were predicted using TargetScan and miRanda databases. In total, 227 genes were identified as putative targets of miR-210 by both databases. Among them, four genes have been implicated in the suppression of angiogenesis. SMAD4 and STAT6, which have not been...
Figure 3. HCC Cell-Derived Exosomal miR-210 Promotes Tubulogenesis of Endothelial Cells

(A) Antagonism of miR-210 in HUVECs attenuated the tubulogenesis promoted by HCC cell-derived exosomes. HUVECs without transfection (untransfected) or transfected with anti-NC control or anti-miR-210 were cultured in the absence (indicated as –) or presence (+) of QGY-7703-derived exosomes. (B) The level of cellular and exosomal miR-210 decreased in anti-miR-210-transfected QGY-7703 cells. (C) Exosomes from anti-miR-210-transfected QGY-7703 cells displayed reduced activity in promoting tubulogenesis. In (B) and (C), QGY-7703 cells were reversely transfected with 100 nM anti-NC (negative control) or anti-miR-210, and their exosomes were subjected to qPCR assay (B) and to HUVEC culture (C). (D) The level of cellular and exosomal miR-210 increased in QGY-miR-210 subline. In (B) and (D), the levels of exosomal miR-29a and miR-29c (negative control) didn’t change. (E) Exosomes from QGY-miR-210 subline showed enhanced activity in promoting tubulogenesis. In (D) and (E), exosomes of QGY-miR-210 and QGY-control sublines were subjected to qPCR assay (D) and to HUVEC culture (E). (F) HCC cell-derived exosomes increased the level of mature miR-210, but not primary miR-210, in endothelial cells. HUVECs were cultured in 1% FBS-containing SFM without (–) or with the exosomes (+) from the indicated cell lines and then subjected to qPCR assay for mature miR-210 (upper panel) and RT-PCR assay for pri-miR-210 (lower panel). The scale bar represents 50 μm. Data are presented as mean ± SEM in (A)–(F). *p < 0.05; **p < 0.01; ***p < 0.001. ns, not significant. See also Figure S4.
reported as target genes of miR-210, were chosen for further validation.

A dual-luciferase reporter assay revealed that co-transfection of miR-210 significantly inhibited the activity of firefly luciferase reporter containing wild-type 3' UTR of SMAD4 or STAT6, whereas this effect was abrogated when the predicted binding site in 3' UTR was mutated (Figure 5A). Compared with control group, treatment with exosomes that were derived from serum of HCC patient or from CM of QGY-miR-210 cells reduced the levels of SMAD4 and STAT6 proteins in HUVECs (Figure 5B). Furthermore, compared with untreated control, treatment with exosomes derived from anti-NC-transfected QGY-7703 cells, which exhibited very low amount of miR-210 (Figure 3B), did not affect the levels of SMAD4 and STAT6 proteins in HUVECs (Figure 5C).

Subsequent investigations further showed that knockdown of either SMAD4 or STAT6 in HUVECs (Figure S6A) significantly promoted tube formation, which mimicked the effect of miR-210 transfection (Figure 5D). Consistently, overexpression of SMAD4 or STAT6 in HUVECs (Figure S6B) inhibited tube formation (Figure 5E).

These data indicate that the exosomal miR-210 from HCC cells may promote tumor angiogenesis by inhibiting SMAD4 and STAT6 in endothelial cells.

DISCUSSION

In the present study, we revealed that hepatoma cell-secreted exosomal miR-210 could be delivered into endothelial cells and thereby exerted its pro-angiogenesis effects by targeting SMAD4 and STAT6. These findings support the hypothesis that secreted miRNAs play an important role in the fine-tuned complex interactions between cancer cells and stroma cells. To our knowledge, this is the first attempt to circumstantiate the communication between HCC cells and endothelial cells via exosomal miRNA.
Exosomes, the small endosome-derived vesicles that are actively secreted via exocytosis, contain functional proteins, miRNAs, and mRNAs. Emerging evidences disclose that exosomal miRNA may serve as a novel class of signaling molecules in cell-to-cell communication. It has been shown that the HCC cell-derived exosomes can deliver the miRNAs into recipient HCC cells and thereby promote the growth, migration, and invasion of HCC cells. On the other hand, the Huh-7 cell-secreted exosomal miR-122 can be taken by HepG2 cells and effectively inhibits the growth and proliferation of HepG2 cells. Additionally, transfer of miRNAs from macrophages into HCC cells results in reduced proliferation of cancer cells. To date, the intercellular shuttle of exosomal miRNAs between HCC cells and endothelial cells and the role of exosomal miRNAs in HCC angiogenesis have not been reported. Here, we presented the following evidences. (1) There is significant association between the number of microvessels in HCC tissues and the serum miR-210 level of HCC patients. (2) HCC cell-derived exosomes were delivered into endothelial cells in vitro and in vivo. (3) Exposure to the HCC cell-derived exosomes increased the level of mature miR-210, but not that of primary miR-210, in endothelial cells. (4) Treatment with the HCC cell-derived exosomes promoted the tubulogenesis of endothelial cells, and this effect was enhanced when miR-210 was overexpressed in HCC cells but was attenuated when DROSHA was silenced in HCC cells or when anti-miR-210 was introduced into either HUVECs or HCC cells. Consistently, the in vivo Matrigels or the tumor xenografts that were treated with the QGY-miR-210-overexpressed exosomes displayed many more vessels. (5) Treatment of endothelial cells with exosomes that were derived from the serum of HCC patients or from the miR-210-overexpressing HCC cells reduced the expression of SMAD4 and STAT6 in endothelial cells, whereas this effect was not observed in the endothelial cells treated with the exosomes derived from the anti-miR-210-transfected HCC cells. Furthermore, knockdown of either SMAD4 or STAT6 in HUVECs promoted tubulogenesis, whereas overexpression of SMAD4 or STAT6 in HUVECs repressed tubulogenesis.
our data suggest that exosomal miR-210 may be transferred from HCC cells to endothelial cells and thereby promotes angiogenesis by targeting SMAD4 and STAT6 in endothelial cells.

A few studies have explored the significance of cellular miR-210 in HCC. miR-210 is upregulated in HCC and enhances the hypoxia-induced migration and invasion of HCC cells by targeting VMPI122 or by suppressing TIMP2 expression via HIF-1α/miR-210/HIF-3α regulatory feedback circuit.23 miR-210 also promotes proliferation and reduces apoptosis of HCC cells.24,25 However, the role of miR-210, especially exosomal miR-210, in the angiogenesis of HCC has not been reported. Our data reveal the pro-angiogenesis effect of exosomal miR-210, which may be applied as a potential therapeutic target in anti-HCC therapy.

miRNAs can regulate different cell activities by inhibiting multiple target genes. Here, we demonstrated that exosomal miR-210 promoted angiogenesis by targeting SMAD4 and STAT6. It has been shown that the supernatant of SMAD4-negative pancreatic cells strongly stimulates migration of endothelial cells and induces neovascularization, whereas the conditioned medium of SMAD4-expressing cells has opposite effect.26 However, the impact of SMAD4 on endothelial cells is still unknown. On the other hand, depletion of STAT6 by small interfering RNAs (siRNAs) impairs the inhibitory effects of interleukin-13 (IL-13) on the migration and tube formation of human coronary artery endothelial cells.27 These data suggest that SMAD4 and STAT6 may act as negative regulators for angiogenesis. In our study, both gain- and loss-of-function analyses revealed that SMAD4 and STAT6 inhibited tubulogenesis of endothelial cells. Taken together, exosomal miR-210 may exert its pro-angiogenesis effect by targeting multiple negative regulators of angiogenesis.

To date, the underlying mechanisms of biogenesis, packaging, and release of exosomal miRNAs remain unclear. In previous study,20 we found that 19 miRNAs increased significantly in the sera of HCC patients. However, only miR-29a, miR-29c, miR-145, miR-192, and miR-210 could be detected in the conditioned media and exosomes of all four hepatoma cell lines, whereas the rest of the miRNAs were undetectable or only detected in the conditioned media of some cell lines, implying that certain miRNA may be uniquely packed into exosomes. Alternatively, those miRNAs increased in the sera of HCC patients were not secreted by HCC cells. Further studies are necessary to unveil how miR-210 is sorted into exosomes and why exosomal miR-210 is increased during HCC development.

Active angiogenesis is responsible for rapid growth, early metastasis, and poor survival of cancers.1 Tumor angiogenesis is a complex process modulated by multiple regulators, and the roles of secreted proteins in tumor angiogenesis have been extensively explored. Although exosomal miRNAs have been shown to enhance angiogenesis in lung cancer13 and renal cell carcinoma,14 the function of secreted miRNAs in HCC angiogenesis remains unclear. The present study extends our understanding of how this process is regulated by tumor-derived exosomal miRNAs, which represent a new class of mediators in the interaction between HCC cells and endothelial cells. In conclusion, our findings highlight the importance of secreted miRNA in tumor angiogenesis and implicate exosomal miR-210 as a potential therapeutic target for HCC.

MATERIALS AND METHODS
Details are in the Supplemental Information.

Human Tissues and Serum Specimens
Human tumor tissues and serum samples were obtained from 104 histologically confirmed HCC patients (age, mean ± SD: 49.42 ± 10.97 years) who underwent tumor resection at Cancer Center of Sun Yat-sen University (SYSU). All patients did not receive any anticancer treatments prior to the surgery. Sera from 60 healthy individuals (age, mean ± SD: 44.35 ± 11.68 years) were also collected from Cancer Center of SYSU. The study was approved by the Institutional Ethics Committee at the Cancer Center of SYSU. Informed consent was obtained from the participants.

RNA Oligoribonucleotides and Vectors
All RNA oligoribonucleotides, including miR-210 mimic, and siRNAs were from GenePharma (Shanghai, China). siALIX, siDROSHA, siSMAD4, and siSTAT6 targeted the mRNAs of human ALIX (NM_001162429), HRS (NM_004712), DROSHA (NM_013235), SMAD4 (NM_005359), and STAT6 (NM_001178078), respectively. The negative control RNA duplex (NC) for both miRNA mimic and siRNA was non-homologous to any human genome sequence. The sequence-specific miR-210 inhibitor (anti-miR-210) and its negative control (anti-NC) were from Ribobio (Guangzhou, China).

For construction of lentiviral vector expressing miR-210, SMAD4, or STAT6, the corresponding fragments were subcloned into the pCDH-CMV-MCS-EF1-copGFP vector (pCDH; System Biosciences, CA, USA), and the resulting vectors were designated as pCDH-miR-210, pCDH-SMAD4, and pCDH-STAT6, respectively.

For construction of the retroviral vector expressing CD63-GFP fusion protein, the coding sequences of mouse Cd63 and gfp linked by a (GGGGS)3 linker were subcloned into a retroviral expression vector (named pBP-vec), which was created based on pBABE-puro (Cell Biolabs, San Diego, CA, USA). The resulting vector was designated as pBP-mCD63-GFP (Figure S7).

For construction of firefly luciferase reporter plasmids, a fragment of wild-type or mutant 3' UTR of target genes were inserted after the stop codon of firefly luciferase in pGL3cm.30 All sequences of oligonucleotides are listed in Table S2.

Cell Lines
Hepatoma cell lines from human (QGY-7703, HepG2, SK-Hep-1, and Huh-7) and mouse (Hepa1-6) and transformed HEK293T were...
maintained in DMEM (Invitrogen, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone; Thermo Fisher Scientific, Austria), 100 U/mL penicillin, and 100 μg/mL streptomycin.

The following sublines were established: QGY-7703 subline that stably expressed miR-210 (QGY-miR-210) and its control subline (QGY-control) and the Hepa1-6 subline (Hepa1-6-mCD63-GFP), which stably expressed mouse CD63 and GFP fusion protein.

As described,31 HUVECs were isolated and cultured in serum-free medium for endothelial cells (SFM) (Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS and 0.03 mg/mL of endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY, USA).

**Cell Transfection and Infection**

RNA oligoribonucleotides were reversely transfected into cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). A final concentration of 50 nM RNA duplex or 200 nM miRNA inhibitor was used for each transfection unless otherwise specified. Transfection of plasmid DNA was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or by calcium phosphate precipitation.

**Isolation, Identification, and Quantification of Exosomes**

To isolate exosomes, human hepatoma cell lines (4 × 10^6) were plated on a 10-cm dish. Twenty-four hours after seeding, cells were washed with 1 × PBS twice and refreshed with 6 mL serum-free DMEM. The conditioned medium was collected 24 hr later and centrifuged at 500 g, room temperature (RT) for 10 min to remove cells and then at 16,000 g, 4°C for 30 min to remove any cell debris. The supernatant or serum samples were further ultracentrifuged at 120,000 g, 4°C for 120 min to collect extracellular vesicles. The pellets were washed by resuspending in 1 × PBS and ultracentrifuged at 120,000 g for 60 min and then dissolved in 50–100 μL 1 × PBS and stored at −80°C until use.

The extracellular vesicles were identified as exosomes by electron microscopy (Figure S8A) using JEM-100CX-II transmission electron microscope (JEOL, Japan) and by western blotting for exosome-enriched marker CD63 (Figure S8B). The protein yield of exosomes was quantified with bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

**RNA Isolation**

Isolation of RNA was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Prior to isolation of extracellular RNA, a C. elegans miRNA, cel-miR-67 (NC67), was spiked into the human serum samples (final concentration: 0.2 nM) and used as a reference as reported,20 whereas a commercial spike-in (cat. 203300; Exiqon, Vedbaek, Denmark) was added to the samples of mouse serum, culture medium, or exosome (final concentration: 0.3 pM) and used as a reference. During isopropanol precipitation, glyogen (cat. AM9510; Ambion/Applied Biosystems, Foster City, CA, USA) was added as a co-precipitant (final concentration: 100 μg/mL) to enhance the yield of extracellular RNA.

**Analysis of Gene Expression**

The level of target gene was assessed by semiquantitative RT-PCR, real-time qRT-PCR, or western blotting. The primers used are provided in Table S2.

**Cellular Internalization of HCC Cell-Derived Exosomes**

To prepare DiIC16-labeled exosomes, QGY-7703 cells were washed twice with 1 × PBS 24 hr after seeding, followed by incubation for 48 hr in the serum-free DMEM containing DiIC16 (cat. D384; Invitrogen, Carlsbad, CA, USA) at a final concentration of 1 μg/mL. The DiIC16-containing medium that was incubated without QGY-7703 cells was used as a control for detecting any carryover of free DiIC16. The medium was then applied to sequential ultracentrifugation to collect exosomes as described above.

To prepare exosomes from Cy3-labeled miR-210 transfectants, QGY-7703 cells transfected with 50 nM Cy3-labeled miR-210 (Cy3-miR-210) were washed as above, followed by replacement with 10 mL 10% FBS-containing DMEM for 48 hr. The exosomes were collected by sequential ultracentrifugation as described above.

HUVEC cells were incubated with exosomes in 1% FBS-containing SFM for 24 hr and then washed with 1 × PBS thrice before imaging under a fluorescence microscope.

**Capillary Tube Formation Assay**

HUVECs were transfected with RNA duplex or treated with exosomes for 48 hr. Thereafter, HUVECs cells were resuspended in SFM containing 1% FBS, added to a plate coated with Matrigel (cat. 3432-005-01; R&D Systems, MN, USA), and incubated for 6 hr. The formation of capillary-like structures was captured under a light microscope. The branch points of the formed tubes, which represented the degree of angiogenesis in vitro, were quantitated in whole field.

**Immunohistochemical and Immunofluorescent Staining**

Tissues embedded in paraffin or Tissue-Tek OCT (Miles, Elkhart, IN, USA) were cut into 5-μm sections and applied to immunohistochemical or immunofluorescent staining as described.22 Antibodies used included mouse monoclonal antibodies (mAbs) against human CD34 (cat. sc-52312; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rat mAb against mouse CD34 (cat. 119302; BioLegend, San Diego, CA, USA), and rabbit polyclonal antibodies (pAb) against GFP (cat. LS-C154219; LifeSpan Biosciences, Seattle, WA, USA).

The MVD, which represented the degree of angiogenesis in vivo, was evaluated by immunohistochemical staining in tumor tissues for human or mouse CD34, a marker for endothelial cells. Ten and five random fields (200×) were screened for human tissues and mouse xenografts, respectively. Any CD34-stained discrete cluster or single cell...
was counted as one microvessel. The average number of microvessels per field was presented as MVD.

Luciferase Reporter Assay
HEK293T cells in a 48-well plate were co-transfected with 50 nM of miR-210 or NC duplex, 2 ng of pRL-CMV (expressing Renilla luciferase), and 50 ng of firefly luciferase reporter plasmid that contained either wild-type or mutant 3’ UTR of target gene. Forty-eight hours after transfection, cell lysates were applied to luciferase assay as described.  

Mouse Studies
All procedures for animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications nos. 80–23, revised 1996) and according to Sun Yat-sen University Institutional Ethical Guidelines for animal experiments.

For orthotopic liver xenograft model, Hepa1-6 (106) was resuspended in 25 μL of 1× PBS/Matrigel (1:1) and inoculated under the capsule of the left hepatic lobe of male C57BL/6 mice at 4 or 5 weeks of age. Tumor tissues of Hepa1-6-mCD63-GFP were embedded in TissueTek OCT. The sera were collected from tumor-bearing mice with implantation of Hepa1-6 cells.

Statistical Analysis
Data were presented as mean (SEM) or median (interquartile ranges [IQRs]). The differences between the groups were analyzed by Student’s t test or Mann-Whitney U test. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). All statistical tests were two sided, and p < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Materials and Methods, eight figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.02.014.

AUTHOR CONTRIBUTIONS
S.-M.Z. and X.-J.L. conceived and designed the project. X.-J.L. and J.-H.F. performed the experiments, analyzed and interpreted the data, and wrote the manuscript. X.-Y. and C.Z. did the experiments and analyzed and interpreted the data. Y.Y. provided human samples and interpreted clinical data. L.Z. advised on the conception and design of the study and interpreted the data. S.-M.Z. supervised the project, conceptualized the study, analyzed and interpreted data, and wrote the paper. All authors approved the final version and agreed to publish the manuscript.

CONFLICTS OF INTEREST
The authors declare no conflict of interest.

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