MicroRNAs (miRNAs) have been reported to be associated with the development of cancers. However, the function of miRNAs in human hepatocellular carcinoma (HCC) remains largely undefined. Here we found that overexpression of miR-10a promoted the migration and invasion of QGY-7703 and HepG2 cells in vitro but suppressed metastasis in vivo. Cell adhesion assays showed that miR-10a suppressed HCC cell-matrix adhesion, which could explain the results of the in vivo animal experiments. The Eph tyrosine kinase receptor, EphA4, was identified as the direct and functional target gene of miR-10a. Knockdown of EphA4 phenocopied the effect of miR-10a and ectopic expression of EphA4 restored the effect of miR-10a on migration, invasion, and adhesion in HCC cells. We further demonstrated that miR-10a and EphA4 regulated the epithelial-mesenchymal transition process and the β1-integrin pathway to affect cell invasion and adhesion.

Conclusion: Our findings highlight the importance of miR-10a in regulating the metastatic properties of HCC by directly targeting EphA4 and may provide new insights into the pathogenesis of HCC. (Hepatology 2013;57:667-677)
involves interactions between tumor cells and the local microenvironment at the secondary site, such as cell-matrix adhesion.\(^{18}\) Epithelial-mesenchymal transition (EMT) is the key process that drives cancer metastasis and it is characterized by loss of the epithelial marker E-cadherin, increased expression of the mesenchymal marker vimentin, and enhanced migratory and invasive behaviors.\(^{19}\) Barrios et al.\(^{20}\) indicated that Eph tyrosine kinase receptor A4 (EphA4) regulates the mesenchymal-to-epithelial transition (MET) of the paraxial mesoderm during somite morphogenesis.

The Eph receptors represent the largest family of receptor protein tyrosine kinases and they interact with their ligands, ephrins. Most recently, the genes for Eph receptors and ephrins have been demonstrated to be differentially expressed in various human tumors.\(^{21-27}\) EphA4 is a member of the Eph receptor tyrosine kinase family and has been reported to play roles in different types of human cancers. EphA4 promotes cell proliferation and migration through an EphA4-FGFR1 signaling pathway in the human glioma U251 cell line.\(^{28}\) Overexpression of the EphA4 gene and reduced expression of the EphB2 gene correlate with liver metastasis in colorectal cancer.\(^{29}\) However, EphA4 has never been described in association with HCC.

In this study we found that miR-10a promoted the migration and invasion of the human HCC cell lines QGY-7703 and HepG2 but suppressed the metastasis of HCC cells in \textit{in vivo} metastasis assays. We identified EphA4 as a direct target of miR-10a. Furthermore, we found that miR-10a and EphA4 regulated the EMT process to influence cell migration and invasion; they also affected cell adhesion by regulating the β1-integrin pathway, which may explain the promotion of invasion from the primary site and the reduction of homing to metastasis loci. Together, these data contribute to the characterization of the molecular mechanisms of HCC metastasis, and might provide new potential biomarkers for HCC.

**Materials and Methods**

**Cell Culture, Transfection, and RNA Extraction.** The human HCC cell lines HepG2, PLC-PRF-5, and QGY-7703 were cultured in MEM-\(x\) or RPMI 1640 media (Gibco, Gaithersburg, MD), respectively. Hep3B was cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA). Transient transfection was performed using the Lipofectamine 2000 reagent (Invitrogen). Total RNA was extracted using the Trizol reagent (Invitrogen), and miRNAs were obtained using the mirVana miRNA isolation kit (Ambion, Austin, TX).

**Clinical HCC Specimens.** Forty paired human HCC and adjacent nontumor liver tissues were collected from the cancer center of Sun Yat-sen University. Among them, 30 paired specimens were from male and 10 paired specimens were from female patients. At collection, six patients were defined as stage I, 20 patients were defined as stage II, and 14 patients were classified as stage III (TNM classification). Venous invasion or tumor microsatellite formation was observed in 22 patients. Informed consent was obtained from each patient and ethics approval was granted by the Ethics Committee of Sun Yat-sen University.

**Vector Constructions.** The vector constructions used in this study are shown in the Supporting Materials and Methods.

**miRNA Target Prediction.** The analysis of miR-10a predicted targets was performed using the TargetScan, PicTar, miRanda algorithms and the complementary DNA (cDNA) microarray results of colon carcinoma in our previous study. The related functions of the targets were also considered.

**Enhanced Green Fluorescent Protein (EGFP) Reporter Assay.** QGY-7703 cells were transiently cotransfected with EGFP reporter plasmid and pcDNA3-pri-10a, pcDNA3, ASO-miR-10a, and ASO-NC. The RFP expression vector, pDsRed2-N1, was used as the internal control. The intensities of EGFP and RFP fluorescence were detected with an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and Western Blot Analysis.** Target genes and controls were analyzed by qRT-PCR using SYBR Premix Ex TaqTM (TaKaRa, Dalian, China). Details and antibodies used in western blot are in the Supporting Materials and Methods.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) and Colony Formation Assays.** Cell viability and proliferation were determined by MTT and colony formation assays as described in the Supporting Information.

**Migration and Invasion Assays.** The 24-well Boyden chamber with 8-μm pore size polycarbonate membrane (Corning, Cambridge, MA) was used to analyze the migration and invasion of tumor cells. For invasion assay, the membrane was coated with Matrigel to form a matrix barrier. Details are in the Supporting Information.

**Cell Adhesion Assay.** Transfected QGY-7703 (2.5 × 10\(^5\)/well) or HepG2 (5 × 10\(^5\)/well) cells were added into 96-well plates coated with Matrigel (0.2 mg/mL). Cells were allowed to adhere at 37°C for 30 minutes,
60 minutes, and 90 minutes, then washed three times
with phosphate-buffered saline (PBS). MTT was added
to each well and incubated for another 4 hours. The
number of adherent cells was estimated by reading the
absorbance at a wavelength of 570 nm.30

**In Vivo Metastasis Assays.** For in vivo metastasis
assays, 5 × 10⁶ QGY-7703 and 1 × 10⁷ HepG2 cells
(stably transfected with pcDNA3-pri-10a, pRNAT-
U6.2/Lenti-anti-miR-10a, and their control vectors)
were suspended in 40 μL of serum-free RPMI 1640 /
Matrigel (1:1) for each mouse. Each nude mouse (10 in
each group, female BALB/c-nu/nu at 5-6 weeks of age)
was inoculated in the upper pole of the spleen with a
microsyringe under anesthesia. After 6 or 8 weeks mice
were sacrificed and their spleens and livers were har-
vested and fixed with phosphate-buffered neutral forma-
lin and prepared for standard histological examination.
All studies were performed under the American Associa-
tion for the Accreditation of Laboratory Animal Care
guidelines for humane treatment of animals and
adhered to national and international standards.

**Immunohistochemical and Immunofluorescence
Staining.** In these two assays, polyclonal rabbit anti-
human EphA4 and E-Cadherin (Saierbio, Tianjin,
China) were used. Details are in the Supporting
Information.

**Statistical Analysis.** Data are presented as the mean ±
standard deviation (SD). Statistical analyses were
performed using a paired t test to compare data. P <
0.05 was considered statistically significant.

**Results**

**miR-10a Promotes HCC Cell Migration and Inva-
sion In Vitro but Suppresses HCC Metastasis In Vivo.**

To determine whether miR-10a had an effect on the
malignant phenotype of HCC cells, we constructed an
miR-10a expression plasmid (pcDNA3-pri-10a, pri-
miR-10a) and validated the efficiency of pri-miR-10a
and ASO-miR-10a (Supporting Fig. 1). QGY-7703
and HepG2 cells were then transfected with them or
their respective controls to explore their effects on the

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**Fig. 1.** miR-10a promotes HCC cell migration and invasion in vitro but suppresses HCC metastasis in vivo. (A) Transwell migration assay of
QGY-7703 and HepG2 cells. (B) Transwell invasion assay of QGY-7703 and HepG2 cells. Cells in five random fields of view at 100 × magnifica-
tion were counted and expressed as the average number of cells per field of view. (C) The number of mice in which primary tumors and intrahe-
patic metastatic nodules were formed is shown in the table. A representative picture of tumor nodules in primary sites (spleen) and metastatic
sites (liver) at the sixth or eighth week after spleen transplantation is shown on the left. Quantification of the metastatic ability of the pcDNA3-
pri-10a vector or control vector is shown on the right. The numbers of intrahepatic metastatic nodules in each mouse were counted. Black arrows
indicate the location of primary tumors and metastatic nodules. *P < 0.05, **P < 0.01.
Fig. 2. miR-10a targets the 3'-UTR of the EphA4 transcript and down-regulates its expression. (A) The sequences of the predicted miR-10a binding site and the EphA4 3'-UTR segments containing the wildtype or mutant binding site are shown. (B) qRT-PCR was performed to detect EphA4 mRNA levels in transfected QGY-7703 cells. (C) Western blot assay was used to detect the protein expression levels of EphA4 in transiently transfected QGY-7703 cells. (D) Relative EGFP activity was analyzed after the wildtype or mutant 3'-UTR reporter plasmids were cotransfected with miR-10a or ASO-miR-10a in QGY-7703 cells. The histogram shows the mean ± SD of the normalized EGFP intensity from three independent experiments. (E,F) qRT-PCR was used to detect the mRNA levels of miR-10a and EphA4 in HCC clinical specimens. Boxplot lines represent medians and interquartile ranges of the normalized threshold values; whiskers and spots indicate 10-90 percentiles and the remaining data points. The miR-10a abundance was normalized to U6. Immunohistochemical (IHC) staining of EphA4 in HCC (right) and noncancerous liver tissues (left) is also shown. *P < 0.05, **P < 0.01, ***P < 0.001.
cancer cells. MTT or colony formation assays showed no significant differences in cell viability or proliferation when miR-10a was overexpressed or blocked (Supporting Fig. 2). However, in transwell assays the migration (Fig. 1A) and invasion (Fig. 1B) capacities of QGY-7703 and HepG2 cells transfected with pri-miR-10a were increased by ~1.6- to 2.5-fold. ASO-miR-10a reduced these capacities by ~50%-70% when compared with the controls. The representative images are shown in Supporting Fig. 3. These data indicated that miR-10a promoted both the migration and invasion of HCC cells. We also detected the expression level of miR-10a in HCC cell lines, QGY-7703, HepG2, PLC-PRF-5, and Hep3B (Supporting Fig. 4), and found that the expression of miR-10a was highest in HCC cell PLC-PRF-5, whereas it was lowest in the low-invasive cell line Hep3B. The expression level of miR-10a in QGY-7703 was higher than in HepG2 cells. This result suggested that miR-10a was positively related to the invasion of HCC cells. We next explored the role of miR-10a in HCC metastasis in vivo. By G418 screening we obtained QGY-7703 and HepG2 pooled clones that stably expressed higher or lower levels of miR-10a (Supporting Materials and Methods). qRT-PCR showed that the establishment of the clones was successful (Supporting Fig. 5). The growth, migration, and invasion capabilities of QGY-7703 pooled clones were also assessed and were significantly increased compared to the controls. These findings suggested that miR-10a was involved in HCC metastasis in vivo.

Fig. 3. EphA4 can suppress HCC cell migration and invasion in vitro. (A) EphA4 protein level in QGY-7703 and HepG2 cells transfected with pSilencer/shR-EphA4 or pSilencer plasmids. (B) Transwell migration assay of QGY-7703 and HepG2 cells. (C) Transwell invasion assay of QGY-7703 and HepG2 cells. Cells in five random fields of view at 100× magnification were counted. **P < 0.01.

Fig. 4. miR-10a and EphA4 regulate HCC cell migration and invasion by way of blockage of the EMT process. (A) The protein level of EphA4 when miR-10a expression was blocked or EphA4 was overexpressed in QGY-7703 cells. (B,C) The protein expression levels of E-cadherin, vimentin, and ICAM-1 when miR-10a expression was blocked or when EphA4 was overexpressed. **P < 0.01.
consistent with those of transiently transfected cells (Supporting Fig. 6). Next, QGY-7703/miR-10a, QGY-7703/anti-miR-10a, HepG2/miR-10a, and control pooled clones were transplanted into the upper pole of the spleen of nude mice. After 6 or 8 weeks the spleens and livers were harvested. Surprisingly, in the respective 10 nude mice local cancers developed in the spleens of all mice. Intrahepatic metastasis nodules were detected in eight mice of the QGY-7703/miR-10a group but in nine mice of the QGY-7703/anti-miR-10a group (Supporting Fig. 7). The average number of metastatic nodules in the liver was dramatically decreased by 52% in groups with ectopic expression of miR-10a (Fig. 1C) and increased about 2.4-fold in the anti-miR-10a group as compared with control groups (Supporting Fig. 7). Similar results were observed in HepG2 cells (Fig. 1C). Taken together, these data indicated that miR-10a could suppress the metastasis of HCC in vivo, but it enhanced the migration and invasion of HCC cells in vitro.

miR-10a Targets the 3'-UTR of EphA4 Transcripts and Down-regulates EphA4 Expression. To explore the molecular mechanism through which miR-10a exerts its function we predicted and identified the candidate target genes of miR-10a. First, various algorithms mentioned above were used to determine the
potential target gene(s), the sequence of the predicted miR-10a binding site and the EphA4 3'-UTR segments containing the miR-10a complementary sequence (Fig. 2A). qRT-PCR and western blot analysis further demonstrated that overexpression of miR-10a dramatically suppressed the endogenous mRNA and protein levels of EphA4 by 78% and 29%, respectively, whereas the inhibition of miR-10a increased the expression of EphA4 by 3.1- and 1.3-fold in QGY-7703 cells (Fig. 2B,C). The miR-10a or ASO-miR-10a constructs were then cotransfected with the EGFP reporter vector into QGY-7703 cells. Interestingly, EGFP intensities were reduced by almost 31% by miR-10a when the wildtype 3'-UTR of EphA4 was present, and when miR-10a expression was blocked the EGFP intensities were enhanced by approximately 1.2-fold (Fig. 2D). In contrast, the reporter vector carrying the mutated EphA4 3'-UTR could restore EGFP activity when this construct was cotransfected with miR-10a (Fig. 2D), indicating that miR-10a might suppress gene expression through the miR-10a-binding sequence in the 3'-UTR of EphA4.

The mRNA levels of EphA4 and miR-10a were also measured in 40 paired clinical specimens. Compared with the adjacent noncancerous tissues, EphA4 mRNA was significantly down-regulated in 35 HCC tissues and this down-regulation was strongly correlated with the up-regulation of miR-10a (Fig. 2E,F). Additionally, the protein levels of EphA4 in 20 paired tissues were analyzed using immunohistochemical staining. Strong staining of EphA4 was observed in noncancerous tissues (Fig. 2F). These observations suggested that EphA4 expression was reduced in HCC tissues and was inversely correlated with miR-10a levels. Furthermore, the relationship between the expression of miR-10a and the metastatic status of HCC patients was analyzed, which showed that miR-10a expression is lower in HCC patients with tumor metastasis (venous invasion or tumor microsatellite formation) (n = 22) than in those without (n = 18) (Supporting Fig. 8).

**EphA4 Suppresses the Migration and Invasion of HCC Cells In Vitro.** Because we identified EphA4 as a direct target of miR-10a, we next investigated whether EphA4 was involved in miR-10a-mediated migration and invasion by examining whether the down-regulation of EphA4 could mimic the effect of miR-10a overexpression. As expected, knockdown of
endogenous EphA4 expression by small interfering RNA (siRNA) in QGY-7703 and HepG2 cells (Fig. 3A) resulted in a significant increase in cell migration by 2.1-fold (Fig. 3B) and an increase in invasion by 17.2- and 48-fold, respectively (Fig. 3C). The representative images are shown in Supporting Fig. 9. However, cell viability and proliferation were not obviously affected (Supporting Fig. 10).

**miR-10a and EphA4 Affect HCC Cell Migration and Invasion by Way of Regulation of the EMT Process.** As miR-10a could promote HCC cell migration and invasion and we confirmed that EphA4 was a direct target of miR-10a, we investigated the pathway by which miR-10a and EphA4 mediated the regulation of migration and invasion of HCC cells in vitro.

In this study we noticed a striking change in cellular shape due to the inhibition of miR-10a or the overexpression of EphA4; an initial spindle- and fibroblast-like morphology was observed to switch to the cobblestone-like appearance of epithelial cells (Supporting Fig. 11). To determine whether the typical molecular alterations of EMT occurred, we examined the localization of the adherent and tight junction marker E-cadherin in transfected QGY-7703 cells. Immunofluorescence analysis showed that E-cadherin was strongly up-regulated when miR-10a expression was blocked or when EphA4 was overexpressed (Supporting Fig. 11). The protein levels of E-cadherin, vimentin, and intercellular adhesion molecule 1 (ICAM-1; another mesenchymal marker) were also assessed. Interestingly, E-cadherin protein expression was up-regulated by ~1.3-fold, whereas vimentin and ICAM-1 were down-regulated by 30.6% and 21.9%, respectively, following the inhibition of miR-10a (Fig. 4B). Additionally, similar results were observed when EphA4 was overexpressed (Fig. 4C). These data suggested that miR-10a and EphA4 influenced the migratory and invasive behavior of HCC cells by way of regulation of the EMT process.

**miR-10a and EphA4 Can Affect HCC Cell Adhesion by Regulating β1-Integrin, Which Mediates Cell-Matrix Adhesion.** The results above suggested a “conflict” in that miR-10a exerted different functions in vitro and in vivo. This led us to further explore the mechanism by which miR-10a regulated the malignant properties of HCC. Tumor metastasis is a complex process that encompasses cell mobility, cell migration, cell invasion, and cell adhesion to target tissues. Among these features, cell-matrix adhesion is an essential process for metastatic homing to tissues. We hypothesized that cell adhesion might explain the miR-10a conflict. Thus, cell adhesion was measured in cells with alterations in miR-10a or EphA4 expression. Interestingly, expression of miR-10a suppressed cell adhesion by 31%, 28%, and 26% at 30, 60, and 90 minutes, respectively, in QGY-7703 cells. Inhibition of miR-10a enhanced cell adhesion by ~1.17-, 1.18-, and 1.15-fold at the respective times listed above (Fig. 5A). Similar results were observed in HepG2 cells (Fig. 5B). In addition, knockdown of EphA4 phenocopied miR-10a overexpression in both QGY-7703 and HepG2 cells (Fig. 5C.D).

Next, we asked how miR-10a and EphA4 regulated cell adhesion. It has been reported that β1-integrin is associated with cell-matrix adhesion, and recent studies have indicated crosstalk between EphA4 and the β1-integrin signaling pathway. Therefore, we studied the relationship between miR-10a or EphA4 expression and β1-integrin expression. The protein expression level of β1-integrin was increased by 3.9-fold when miR-10a was blocked and enhanced by 2.1-fold when EphA4 was overexpressed in QGY-7703 cells (Fig. 5E). These data suggested that miR-10a and EphA4 affected cell adhesion through the β1-integrin signaling pathway.

**Restoration of EphA4 Inhibits miR-10a-Mediated HCC Cell Migration and Invasion but Promotes Cell Adhesion.** The above observations indicated that the knockdown of EphA4 could mimic the overexpression of miR-10a and that miR-10a could regulate the expression of EphA4 both at the mRNA and protein levels by directly binding to its 3′-UTR. We hypothesized that down-regulation of EphA4 directly mediated miR-10a-initiated HCC migration, invasion, and adhesion. To further confirm this hypothesis, QGY-7703 cells were cotransfected with miR-10a and pA3M1-EphA4, which encoded the entire EphA4 coding sequence but lacked the 3′-UTR of EphA4 to avoid the influence of the miRNA. As expected, the restoration of EphA4 inhibited the miR-10a-mediated migration and invasion and rescued miR-10a-suppressed cell adhesion (Fig. 6). The representative images are shown in Supporting Fig. 12. Taken together, our results suggested that miR-10a exerted its function by way of regulation of EphA4 expression.

**Discussion**

Invasion and metastasis are the lethal factors promoting malignant cancers, especially in HCC. Due to the unpredictability of these two factors, HCC therapy is still faced with tremendous obstacles. Recent studies have shown that miRNAs play a fundamental role in the invasion and metastasis of HCC. miR-193b has
been shown to regulate proliferation, migration, and invasion in HCC cells, and miR-125b has been found to suppress HCC cell proliferation and metastasis by directly targeting the oncogene LIN28B2. In this study, miR-10a, a new metastatic regulator of HCC, was shown to promote HCC cell migration and invasion. The expression level of miR-10a in different HCC cell lines suggested that miR-10a was positively related to the invasion ability of HCC cells. In addition, we used the metastatic model of HCC in nude mice to determine the effect of miR-10a on metastasis of HCC in vivo. Interestingly, the number of intrahepatic metastatic nodules was dramatically decreased when miR-10a was overexpressed, whereas it was obviously increased when miR-10a was inhibited. Our findings are the first to suggest that miRNA plays different roles in that it promotes migration and invasion but suppresses the homing at metastatic foci in metastatic processes.

Tumor metastasis occurs by a complex series of events, including invasion, adhesion, proliferation, and vessel formation. Invasion of tumor cells involves multiple processes and depends on specific cell-to-cell and cell-to-extracellular matrix (ECM) interactions. It has been suggested that blocking adhesion is an effective strategy for metastasis inhibition. Based on these previous studies, we hypothesized that miR-10a suppressed the metastasis of HCC in vivo because of its effect on cell adhesion. The cell adhesion assays confirmed our hypothesis. miR-10a significantly suppressed the cell-matrix adhesion both in QGY-7703 and HepG2 cells. Such an activity may diminish the migration and invasion of HCC cells from primary loci and may also result in decreased numbers of HCC cells that colonize target tissues. Accordingly, the metastasis of HCC is suppressed by miR-10a in vivo.

To further explore the mechanism by which miR-10a exerts its function, the determination of its functional target gene is essential. More than 200 genes are predicted to be the potential targets of miR-10a using TargetScan, PicTar, and miRanda algorithms. Combining the functions of these genes and the effect of miR-10a on HCC cells, we chose EphA4 as the interesting gene in further study. Our data clearly indicate that miR-10a promotes invasion and suppresses metastasis of HCC by directly targeting EphA4. This conclusion is based on several pieces of evidence. First, miR-10a overexpression significantly decreases the expression of EphA4 both at the mRNA and protein levels in HCC cells. Second, the EGFP reporter assay showed that miR-10a could bind the 3′-UTR of the EphA4 transcript. Third, EphA4 expression is down-regulated in HCC tissues, and this down-regulation is strongly correlated with the up-regulation of miR-10a. Fourth, knockdown of EphA4 phenocopies the effect of miR-10a expression, whereas restoration of EphA4 antagonizes the function of miR-10a. These results indicate that miR-10a targets EphA4 and down-regulates its expression in HCC.

EphA4 belongs to the Eph receptor tyrosine kinase family. The Eph receptors and their ligands, ephrins, are divided into two subclasses, A and B, based on their homologies, structures, and binding affinities. EphA4 is the only receptor that can interact with both ephrin-A and ephrin-B ligands. The Eph-ephrin system comprises a direct cell-cell contact-mediated, bidirectional signaling pathway. Eph-ephrin signaling mainly affects cell shape and motility by regulating cytoskeletal organization and cell adhesion and also influences cell proliferation and cell-fate determination. In our research, we found that EphA4 suppressed cell migration and invasion but promoted cell adhesion, which was the inverse of the functions of miR-10a in HCC cells.

As described above, EMT is a process that plays important roles in both development and oncogenesis. During EMT, epithelial cells acquire a mesenchymal phenotype that is characterized by the loss of intercellular junctions and increased cell migration. A previous study has also indicated that EphA4 participates in the MET process, and the morphology of the QGY-7703 cells changed after alteration of miR-10a or EphA4 expression (Supporting Fig. 11). We speculated that miR-10a and EphA4 played roles in the EMT process in HCC. Usually, the loss of intercellular junctions and the increased cell migration during EMT are evidenced by increasing expression of vimentin and decreasing expression of E-cadherin. To test our hypothesis, we examined the expression of the epithelial marker E-cadherin and the mesenchymal markers vimentin and ICAM-1. As expected, down-regulation of miR-10a or up-regulation of EphA4 suppressed the EMT phenotype. In other words, miR-10a can increase, whereas EphA4 can suppress HCC cell migration and invasion by mediating the EMT process. Furthermore, Xiang et al. indicated that tumor cells with an epithelial phenotype have a growth advantage in the tissue environment when compared with those with a mesenchymal phenotype. When miR-10a is up-regulated, the expression level of EphA4 is accordingly down-regulated, and the blockage of the EMT process is relieved. HCC cells with enhanced miR-10a expression reacquire the mesenchymal phenotype, which may impair the proliferation capacity in the liver, resulting in decreased intrahepatic metastatic nodules.
Although EphA4 is the direct target of miR-10a, we further explored the pathway by which miR-10a and EphA4 affected cell adhesion. Bourgin et al.\textsuperscript{32} reported that EphA4 regulates dendritic spine remodeling by affecting β1-integrin signaling pathways. Davy and Robbins\textsuperscript{40} also suggested that Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner, and previous studies have indicated that EphA4 can interact with Ephrin-A5 and participate in signal transduction. Integrin is an αβ heterodimeric membrane protein that mediates the adhesion of cells to components of the ECM. The integrin β1 subunit is crucial for adhesion to fibronectin (FN),\textsuperscript{41} which is one important component of the ECM. We measured the protein level of β1-integrin and found that it was up-regulated by miR-10a inhibition or EphA4 overexpression. These observations suggest that miR-10a and EphA4 regulate cell adhesion by mediating the β1-integrin signaling pathway.

In summary, we discovered that miR-10a can promote HCC migration and invasion in vitro but suppresses the metastasis of HCC in vivo. These functions are enacted by targeting EphA4, thereby regulating EMT and cell adhesion. Our research thus provides new insight into the mechanism of the pathogenesis of HCC and suggests that miR-10a and EphA4 play an important role in cancerogenesis.

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References

1. Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basuyuk E, et al. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 2005;309:1573-1576.
2. Zamore PD, Haley B. Ribo-gnome: the big world of small RNAs. Science 2005;309:1519-1524.
3. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 2005;102:13944-13949.
4. Johnson SM, Groshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. Ras is regulated by the let-7 microRNA family. Cell 2005;120:635-647.
5. O'Donnell KA, Wenzel EA, Zeller KJ, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 2005;435:839-843.
6. Akeakaya P, Ekelund S, Kolosenko I, Caramuta S, Ozata DM, Xie H, et al. mir-185 and mir-133b deregulation is associated with overall survival and metastasis in colorectal cancer. Int J Oncol 2011;39:311-318.
7. Fang JH, Zhou HC, Zeng C, Yang J, Liu Y, Huang X, et al. MicroRNA-29b suppresses tumor angiogenesis, invasion, and metastasis by regulating matrix metalloproteinase 2 expression. HEPATOLOGY 2011;54:1729-1740.
8. Kong D, Li Y, Wang Z, Banerjee S, Ahmad A, Kim HR, et al. miR-200 regulates PDGF-D-mediated epithelial-mesenchymal transition, adhesion, and invasion of prostate cancer cells. Stem Cells 2009;27:1712-1721.
9. Penna E, Orso F, Cimino D, Tenaglia E, Lembo A, Quaglini E, et al. microRNA-214 contributes to melanoma tumour progression through suppression of TFA2C. EMBO J 2011;30:1990-2007.
10. Ding J, Huang S, Wu S, Zhao Y, Liang L, Yan M, et al. Gain of miR-151 on chromosome 8q24.3 facilitates tumour cell migration and spreading through downregulating RhoGDIa. Nat Cell Biol 2010;12:390-399.
11. Ji J, Zhao L, Budhu A, Forgues M, Jia HL, Qin LX, et al. Let-7g targets collagen type I alpha2 and inhibits cell migration in hepatocellular carcinoma. J Hepatol 2010;52:690-697.
12. Li N, Fu H, Tie Y, Hu Z, Kong W, Wu Y, et al. miR-34a inhibits migration and invasion by down-regulation of c-Met expression in human hepatocellular carcinoma cells. Cancer Lett 2009;275:44-53.
13. Yang F, Yin Y, Wang F, Wang Y, Zhang L, Tang Y, et al. miR-17-5p promotes migration of human hepatocellular carcinoma cells through the p38 mitogen-activated protein kinase-hex shock protein 27 pathway. HEPATOLOGY 2010;51:1614-1623.
14. Yao J, Liang L, Huang S, Ding J, Tan N, Zhao Y, et al. MicroRNA-30d promotes tumor invasion and metastasis by targeting Gai alpha2 in hepatocellular carcinoma. HEPATOLOGY 2010;51:846-856.
15. Ma L, Teruya-Feldstein J, Weinberg RA. Tumor invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 2007;449:682-688.
16. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74-108.
17. Thorgerisson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002;31:339-346.
18. Chau I, Normann AR, Cunningham D, Waters JS, Oates J, Ross PJ. Multivariate prognostic factor analysis in locally advanced and metastatic esophageal-gastric cancer—pooled analysis from three multicenter, randomized, controlled trials using individual patient data. J Clin Oncol 2004;22:2395-2403.
19. Kraljevic Pasic S, Sedic M, Bosnjak J, Spaventi S, Pavelic K. Metastasis: new perspectives on an old problem. Mol Cancer 2011;10:22.
20. Barrios A, Poole RJ, Darbin L, Brennan C, Holder N, Wilson SW. Eph/Ephrin signaling regulates the mesenchymal-to-epithelial transition of the paraxial mesoderm during somite morphogenesis. Curr Biol 2003;13:1571-1582.
21. Easty DJ, Hill SP, Hsu MY, Fallowfield ME, Floresen VA, Herlyn M, et al. Up-regulation of ephrin-A1 during melanoma progression. Int J Cancer 1999;84:494-501.
22. Giongosawa E, Takai S, Tanaka M, Iwase T, Suzuki M, Xiang YY, et al. Overexpression of ERK, an Eph family receptor protein tyrosine kinase, in various human tumors. Cancer Res 1994;54:3645-3650.
23. Miyazaki T, Kato H, Fukuchi M, Nakajima M, Kusano H. EphA2 overexpression correlates with poor prognosis in esophageal squamous cell carcinoma. Int J Cancer 2003;103:657-663.
24. Suraweera H, Ma PC, Salgia R. The role of ephrins and Eph receptors in cancer. Cytokine Growth Factor Rev 2004;15:419-433.
25. Tang XX, Zhao H, Robinson ME, Cohen B, Cnaan A, London W, et al. Implications of EPHB6, EFNB2, and EFNB3 expressions in human neuroblastoma. Proc Natl Acad Sci U S A 2000;97:10936-10941.
26. Vogt T, Stolz W, Welsh J, Jung B, Kerbel RS, Kobayashi H, et al. Overexpression of Lerk-5/Eplg5 messenger RNA: a novel marker for increased tumorigenicity and metastatic potential in human malignant melanomas. Clin Cancer Res 1998;4:791-797.
27. Walker-Daniel J, Coffman K, Azimi M, Rhim JS, Bostwick DG, Snyder P, et al. Overexpression of the EphA2 tyrosine kinase in prostate cancer. Prostate 1999;41:275-280.
28. Fukai J, Yokote H, Yamanaka R, Arao T, Nishio K, Itakura T. Overexpression of EphA4-FGRF1 signaling pathway in the human glioma U251 cell line. Mol Cancer Ther 2008;7:2768-2778.
EphB2 gene correlates with liver metastasis in colorectal cancer. Int J Oncol 2008;33:573-577.

30. Xiao CL, Tao ZH, Guo L, Li WW, Wan JL, Sun HC, et al. Isomaltooligosaccharide sulfate inhibits tumor growth and metastasis of hepatocellular carcinoma in nude mice. BMC Cancer 2011;11:150.

31. Piao Y, Lu L, de Groor J. AMPA receptors promote perivascular glioma invasion via beta1 integrin-dependent adhesion to the extracellular matrix. Neuro Oncol 2009;11:260-273.

32. Bourgin C, Murai KK, Richter M, Pasquale EB. The EphA4 receptor regulates dendritic spine remodeling by affecting beta1-integrin signaling pathways. J Cell Biol 2007;178:1295-1307.

33. Xu C, Liu S, Fu H, Li S, Tie Y, Zhu J, et al. MicroRNA-193b regulates proliferation, migration and invasion in human hepatocellular carcinoma cells. Eur J Cancer 2010;46:2828-2836.

34. Liang L, Wong CM, Ying Q, Fan DN, Huang S, Ding J, et al. MicroRNA-125b suppressed human liver cancer cell proliferation and metastasis by directly targeting oncogene LIN28B2. Hepatology 2010;52:1731-1740.

35. Bogenrieder T, Herlyn M. Axis of evil: molecular mechanisms of cancer metastasis. Oncogene 2003;22:6524-6536.

36. Zhang C, Liu Y, Gao Y, Shen J, Zheng S, Wei M, et al. Modified heparins inhibit integrin alpha(IIb)beta(3) mediated adhesion of melanoma cells to platelets in vitro and in vivo. Int J Cancer 2009;125:2058-2065.

37. Murai KK, Pasquale EB. ‘Eph’ective signaling: forward, reverse and crosstalk. J Cell Sci 2003;116:2823-2832.

38. Pasquale EB. Eph receptor signalling casts a wide net on cell behaviour. Nat Rev Mol Cell Biol 2005;6:462-475.

39. Xiang X, Zhuang X, Ju S, Zhang S, Jiang H, Mu J, et al. miR-155 promotes macroscopic tumor formation yet inhibits tumor dissemination from mammary fat pads to the lung by preventing EMT. Oncogene 2011;30:3440-3453.

40. Davy A, Robbins SM. Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner. EMBO J 2000;19:5396-5405.

41. Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. J Cell Sci 2006;119:3901-3903.