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Original Article

Down-regualtion of miR-106b induces epithelial-mesenchymal transition but suppresses metastatic colonization by targeting Prrx1 in colorectal cancer

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Abstract: Accumulating evidence identified that epithelial-mesenchymal transition (EMT) is acquired during progression to metastatic, but whether it is an absolute requirement is still controversial. MiR-106b has been confirmed to promote cancer cell proliferation; however few studies are available on its functions in EMT and metastasis in colorectal cancer (CRC). In this study, we found that knocking down miR-106b induced EMT conferring migratory and invasive properties. MiR-106b knockdown induced cytoskeletal reorganization through staining intracellular F-actin. The expression of Rho GTPases (Rac1 and Cdc42) and Tiam1 was significantly enforced after miR-106b down-regulation. However, miR-106b knocking down could suppress metastatic colonization in vivo. Correspondingly, over expression of miR-106b obtained an opposite effect. We identified Prrx1 was a direct target of miR-106b through using target prediction algorithms and dual-Luciferase reporter assay. Moreover, Moreover, we also found TGF-β1 could down-regulate miR-106b, and simultaneously miR-106b also influences the expression of TGF-β1, establishing a negative feedback loop to regulate the expression of Prrx1 together. Taken together, these findings demonstrated that miR-106b knockdown could induce EMT which conferring cells migratory and invasive properties but could not accomplish distant metastatic colonization efficiently.

Keywords: Prrx1, miR-106b, EMT, metastasis, colorectal cancer

Introduction

Every year, over 1 million individuals are diagnosed with CRC, the prognosis is poor and mortality is very high in the whole world [1]. Metastasis is inseparable associated with the poor prognosis and it is a complex process with many steps. To form distant metastasis, cancer cells first separate from the primary tumor, invade adjacent tissues, and then intravasate into lymphatic and blood vessels to colonize distant organs, however little is known about the underlying mechanisms of the metastasis. Recent studies have proved that EMT is reactivated many diseases including cancer metastasis [2].

EMT was first recognized as a central differentiation process in early embryogenic morphogenesis [3]. Accumulating evidence confirmed that carcinoma cells could undergo EMT, which enables cells to invade, intravasate, and navigate through a network of thin vessels that enhances the detachment of cancer cells from primary tumors [4, 5]. However, it is still not sure whether individual mesenchymal cells can form metastatic colonization completely in vivo. Takanori Tsuji and his collaborators found EMT cells could be detectable in the blood stream, but no lung metastasis formed [6]. MET, the reverse process of EMT, indeed occurs during tumor progression and metastatic colonization and has been postulated as part of the process

of metastatic tumor formation which also can explain the histopathological similarity between primary and metastatic tumors [7, 8].

There are mounting evidences suggesting that miRNAs is associated with the pathogenesis classification, diagnosis, and prognosis and progression of cancer [9, 10]. MiR-106b, as a member of miR-106b-25 cluster, has been confirmed to promote cancer cell proliferation in human various cancers [11-13]. WANG YX and his collaborators have been previously demonstrated that miR-106b was up-regulated in CRC with lymph node metastasis but few studies are available on its functions in CRC metastasis [14].

Our studies showed that miR-106b promoted cell migratory and invasive through regulating EMT and cytoskeletal reorganization, while suppressed metastatic colonization in vivo. Taken together, we confirmed miR-106b inducing EMT by targeting Prrx1 which conferring CRC cells migratory and invasive properties but insufficiently accomplish distant metastatic colonization.

Material and methods

Clinical specimens

CRC tissue of patients were collected from fresh surgical specimens, frozen in liquid nitrogen, and stored at -80°C until further analysis. All tissues had been histologically confirmed to be adenocarcinoma. The research protocol was approved by the Ethics Committee at Nanfang Hospital, and written consent was obtained from all patients for the use of their tissues.

Cell culture

Human CRC cell lines HT29, SW480, SW620 and LOVO were obtained from American Type Culture Collection (ATCC). Additionally, a human CRC cell subline from SW480 with high metastatic potential, designated as SW480/SCP51 and SW480/SCP17, was established in our laboratory [15]. Cell lines were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 IU/ml penicillin and 100 g/ml streptomycin in humidified 5% CO_2 at 37°C.

In vitro cell growth assay, immunofluorescence, in vitro migration analysis, protein isolation and western blotting

There experiments were performed as previously described [16].

RNA isolation, reverse transcription, and qRT-PCR

Total RNA extracted from CRC cell lines was performed as previously described [17]. For detection of miR-106b expression, stem-loop reverse transcription-polymerase chain reaction (RT-PCR) was performed using All-in-One TM miRNA quantitative RT-PCR (qRT-PCR). The sequence-specific forward primers for mature miR-106b and U6 internal control were 5-TGTAAAGTGCTGACAGTGCA-3 and 5-CTCGCTTCGGCAGCACA-3, respectively. Relative expression was calculated via the comparative cycle threshold (Ct) method using the expression of U6 small nuclear RNA as the reference. The qRT-PCR for the analysis of mRNA expression was performed on a Stratagene ABI PRISM7500 Fast Real-time PCR system by using the SYBR Green qRT-PCR master mix (TaKaRa) and GAPDH for internal control. Data were processed using $2^{-\Delta\Delta CT}$ method.

MicroRNA mimics, siRNA transient transfection

MicroRNA-106b mimics (sense 5'-UAAAGUGCUGACAGUACAGUGCAGAU-3' and anti-sense 5'-AUUUCACGACUGUCACGACUA-3'), miR-106b inhibitor (5'-AUUUCACGACUGUCACGACUA-3'), the negative control (5'-CAGUACUUUGUGUAGUACAA-3'), were purchased from Gene Pharma (Shanghai, China). The transfection was performed using Lipofectamine™ 2000 (Invitrogen, USA) according to the instructions provided by the manufacturer. 24 or 48 hours after transfection, cells were harvested for further experiments.

In vivo metastasis assays

In vivo metastasis assays was performed as previously described [18].

Tumor sphere formation assay

Cells were digested with 0.25% trypsin (Sigma, St. Louis, MO, USA), washed twice with calci-

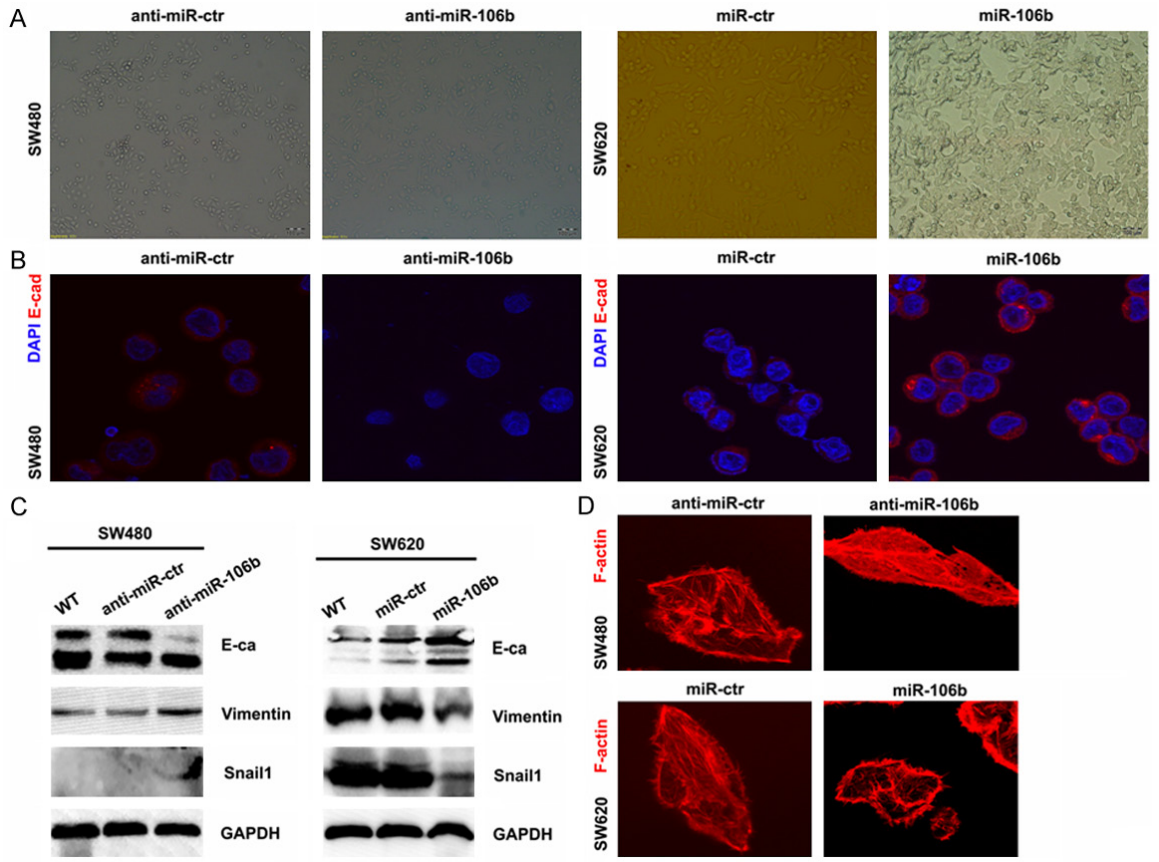

Figure 1. Knocking down miR-106b induced EMT. A. phase-contrast images of cell morphology after miR-106b down-regulation or over expression. B. The expression of epithelial marker E-cadherin after miR-106b down-regulation or overexpression examined by immunofluorescence. Cells were stained with E-cadherin (E-Cad) antibodies, and counter stained with DAPI. C. The expression levels of E-cadherin, Vimentin and snail1 in SW480 cells after inhibition of miR-106b or in SW620 cells overexpression of the same miRNA were detected by western blot. D. Immunofluorescence showing the morphology of cells after miR-106b down-regulation or over expression. Cells were stained with phalloidin for F-actin antibodies.

um/magnesium-free PBS, suspended in sphere formation medium (DMEM-F12 50 ml + 100 g/ml EGF + 100 g/ml bFGF + B27 supplement 1 ml), and seeded in 6cm or 6-well plate (3000 cells/ml). The cells were cultivated for 5~7 days (depending on the cell type), and the spheres were then counted under a microscope.

Dual-Luciferase reporter assay

The 3'UTR of the human Prrx1 (primers: Fw, 5'-ACACACCCTCACCACTGTTC-3'; Rw, 5'-TGCTGTTCAGCAAGCCTACA-3'), and 3'UTR mut-Prrx1 genes (primers-1: Fw, 5'-GCTACTTTCACGTGGACATACAGAGAATACAGGAAAG-3'; Rw, 5'-GTCCACGTGAAAGTAGCCATGGGAGGAAAGGGACTAT-3') were PCR amplified. They were then cloned downstream of the Firefly Luciferase stop codon in pPLuc control vector (Promega). All constructs were verified by DNA sequencing. Cells were seeded in 48-well plates and dual-Luciferase reporter assay was performed as previously described [17].

Viral Vectors

The Viral Vectors was purchased from GENECHEM company Shanghai of china.

Statistical analysis

One-way ANOVA was performed to assay the expression of miR-106b in six human colorectal carcinoma cell lines, and the others experiments data were assayed by student t-test. Results of all experiments are expressed as mean ± standard deviation SD of 3 independent experiments. (*P*-values, <0.05 were considered statistically significant. *P<0.05, **P<0.01, ***P<0.001.)

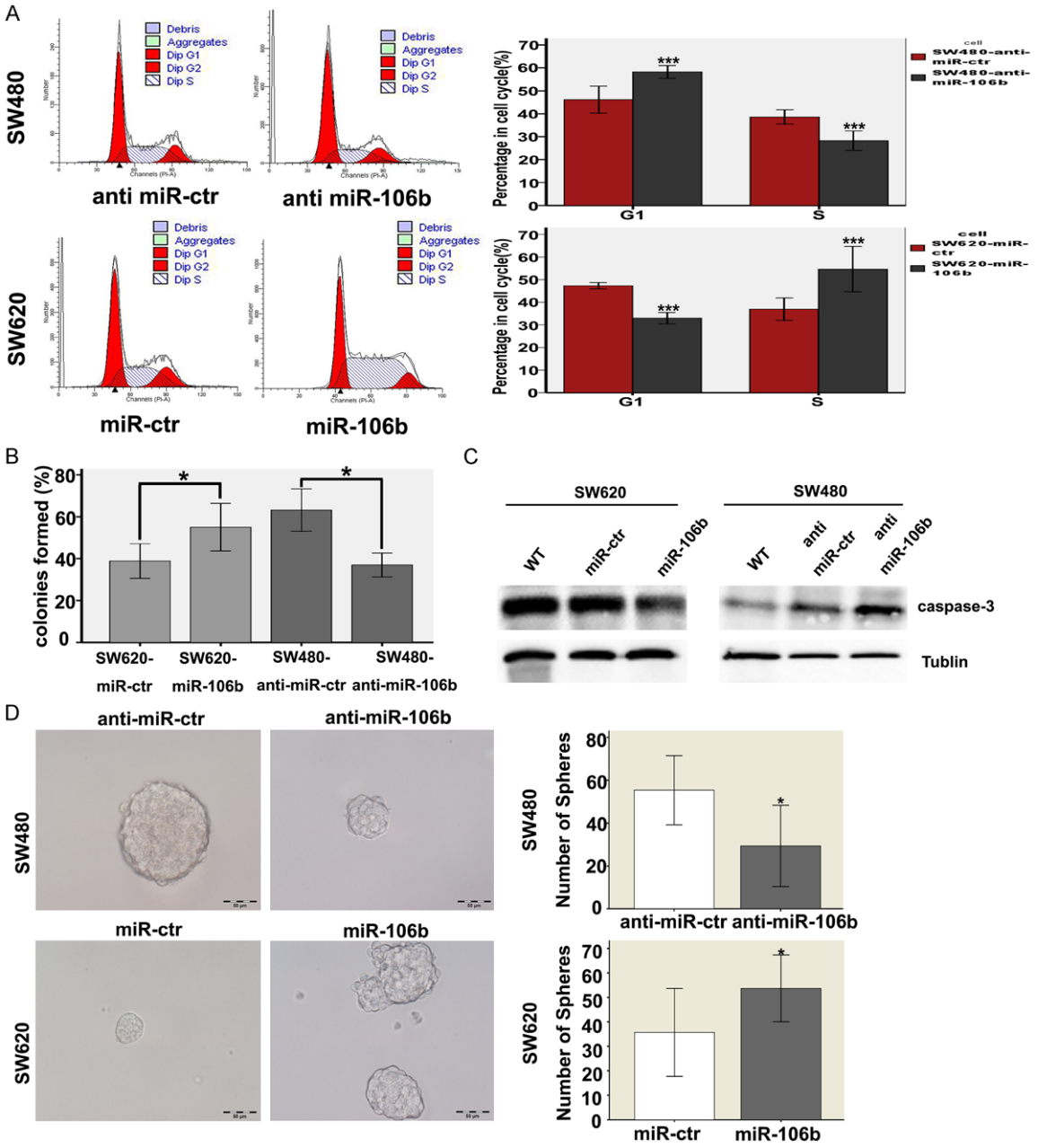

Figure 2. Knocking down miR-106b weakened the proliferation and stemness capacity. A. Flow cytometry analysis, comparison of G1/S fractions after miR-106b over expression in SW620 cells or down-regulation in SW480 cells. The percentage of cells in G1 and S phases and the statistic analysis are shown in the right panel. ***P<0.001. B. Impact of miR-106b on cell colonies formed. Cells colonies were shown 2 weeks after plating. The panel showed the relative colony formation ratio. *P<0.05. C. Apoptosis evaluated by caspase-3 after miR-106b over expression in SW620 cells or down-regulation in SW480 cells, the protein levels of caspase-3 were examined by Western blot. D. Knocking down mir-106b restrained stem cell-like self-renewal properties. Sphere sizes are shown in the left panels, and the numbers of spheres is shown in the right panels. Scale bars represent 50 mm. *P<0.05.

Results

The expression of miR-106b in CRC cell lines and tissues

We detected the expression of miR-106b in six human colorectal carcinoma cell lines by quantitative reverse transcriptase-PCR (qRT-PCR), and found miR-106b was relatively higher in CRC cell lines SW480 and HT29 with lower migratory and invasive properties than in high migratory and invasive properties cell lines SCP17, SCP51, LOVO and SW620 (Supplementary Figure 1A).

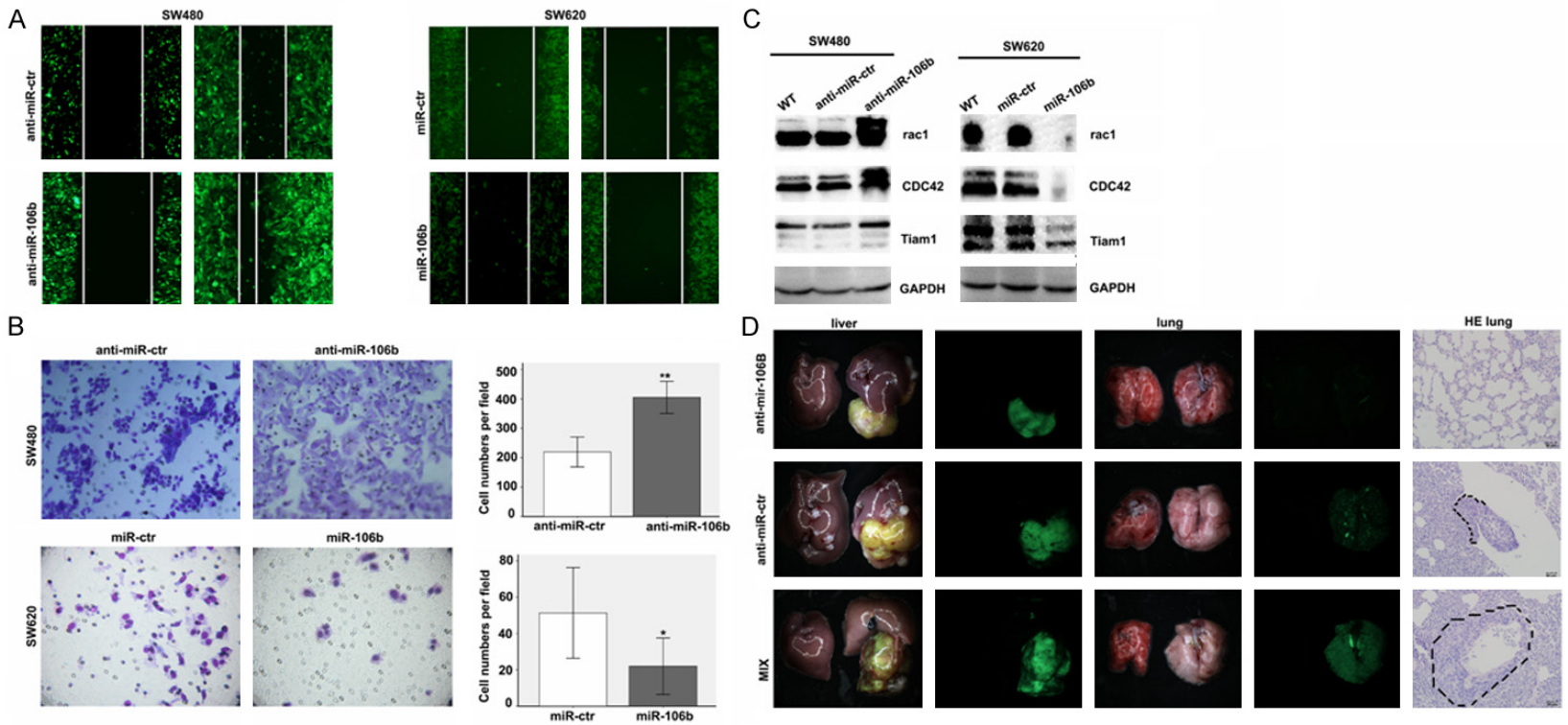

Figure 3. Knocking down miR-106b promoted migration but suppresses metastatic colonization. A. The migratory properties of cells after miR-106b down-regulation or over expression were tested by wound healing assays. B. Transwell assay was employed to migratory properties of cells after miR-106b down-regulation or over expression. **P*<0.05, ***P*<0.01. C. Rho GTPases (Rac1 and Cdc42) and Tiam1 were examined by western blot after miR-106b down-regulation or overexpression. D. External whole-body fluorescence images of lung and liver were obtained 60 days after implanted into the left lobes of the livers of the nude mice. Metastatic cancer tissue (H&E staining, magnification, ×100). Arrows indicate metastatic nodes in the lung.

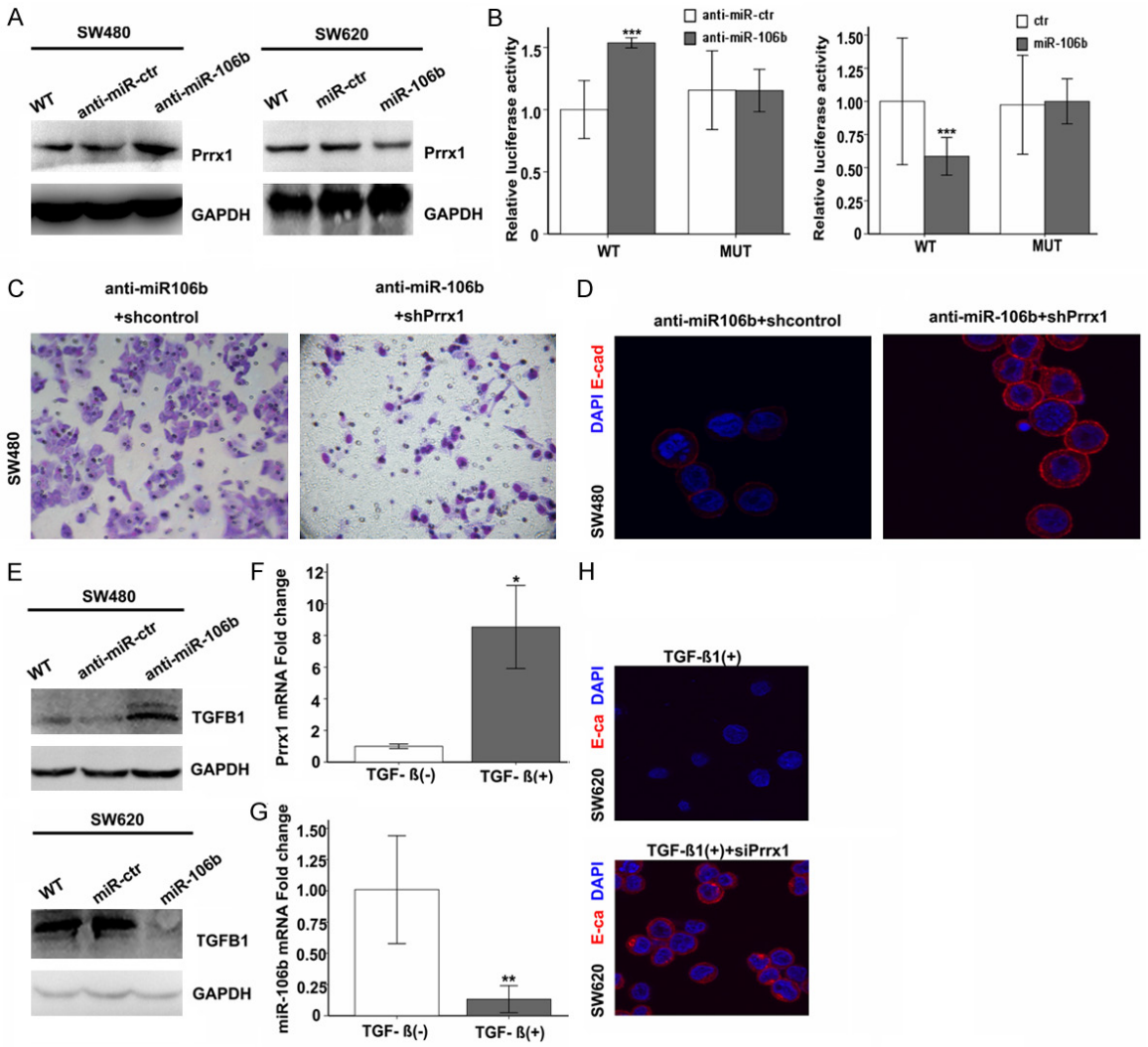

Figure 4. miR-106b directly target Prrx1 and formed a negative feedback loop with TGF-ß1 co-regulated Prrx1. A. The expression levels of Prrx1 in SW480 cells after inhibition of miR-106b or in SW620 cells overexpression of the same miRNAs were detected by western blot. B. Prrx1 3'UTRs are targets of miR-106b. PLuc-Prrx1 containing a wild-type or mutated Prrx1 3'UTRs (indicated as WT or MUT on the X-axis), were transfected into SW620 or SW480 cells. Relative repression of firefly Luciferase expression was standardized to a transfection control. The reporter assays were performed three times with essentially identical results. ***P<0.001. C. The cells migration capacity was measured by transwell in SW480 cells co-transfected with anti-miR-106b and siRNA/Prrx1. D. E-cadherin was examined by immunofluorescence in SW480 cells co-transfected with anti-miR-106b and siRNA/Prrx1. E. TGFB1 was examined by western blot after miR-106b over expression or down-regulation. F. The expression of Prrx1 was analyzed by a qRT-PCR array after the cells treatment with TGF-ß1. *P<0.05. G. The expression of miR-106b was analyzed by a qRT-PCR array after the cells treatment with TGF-ß1. **P<0.01. H. The expression of epithelial marker E-cadherin after co-treated with TGF-ß1 and siPrrx1 in SW620-lenti-miR-106b cells was examined by immunofluorescence. Cells were stained with E-cadherin (E-Cad) antibodies, and counter stained with DAPI.

To further investigate the clinic pathological significance of miR-106b levels in patients with CRC, the levels of miR-106b in 22 CRC tissues were examined by qRT-PCR. An analysis showed that lower-level expression of miR-106b in CRCs was significantly associated with a more aggressive tumor phenotype (Supplementary Figure 1B).

Knocking down miR-106b induced EMT in CRC cell lines

For further studying the function of miR-106b in CRC cell lines, we established the stable miR-106b over expression or knockdown cell lines by lentiviral transduction. Modulating the expression of miR-106b in CRC cell lines showed

a concomitant morphology transformation. The morphology of miR-106b-reduced cells SW480 exhibited more spindle-like fibroblastic structures without tight cell-cell contacts, while miR-106b-overexpressed cells SW620 exhibited more epithelial characteristics such as polygonal structures and cell-cell contacts (**Figure 1A**). E-cadherin is one of the most important genes for MET process, immunofluorescence validated that miR-106b down-regulation in SW480 dramatically decreased the induction of E-cadherin expression, while miR-106b up-regulation SW620 cells exhibited an opposite effect (**Figure 1B**). The expression of E-cadherin was also verified by western blot (**Figure 1C**). Other EMT markers, such as vimentin and snail1 were analyzed by Western blot and showed similar trends (**Figure 1C**). We also found that the loss of miR-106b was indeed sufficient for SW480 cells to revert MET, undergoing an EMT in culture, evidenced by the changes in actin filament reorganization (**Figure 1D**).

Knocking down miR-106b weakened the proliferation and colon sphere formation

MTT assay confirmed knocking down miR-106 could inhibit cell proliferation (Supplementary Figure 2). Cell cycle assays proved miR-106b exerted internally effects on the G1/S checkpoint: inhibition of miR-106b induced cell cycle G1 arrest, while over expression of miR-106b promoted G1 to S transition (**Figure 2A**). In addition, colony formation assay was administered to assess the long-term impact of miR-106b on cell growth and data showed down-expression miR-106b could inhibit cell colony formation (**Figure 2B**). Apoptosis-related gene caspase-3 protein level was negative correlated with miR-106b expression levels (**Figure 2C**).

To further characterize the impact of miR-106b, we examined the effect on the colon sphere formation. We found that miR-106b inhibition cells significantly restrained colon sphere formation capacity showing a smaller number and spheres sizes compared with the vector controls cells, whereas over expression of miR-106b promoted the colon sphere formation capacity under these conditions, (**Figure 2D**).

Knocking down miR-106b promoted migration but suppressed metastatic colonization

EMT is a morphogenetic process in which cells lose their epithelial characteristics like cell polarity, cell-cell contact, and gain mesenchymal properties which could increase motility [19]. To investigate whether miR-106b regulated CRC cell migration and invasion, we performed a series of in vitro assays. Cell heal wound and migration analysis demonstrated that depletion of miR-106b strongly enhanced cell migration ability (**Figure 3A** and **3B**).

Then we detected the expression of migration and metastastic-related genes Rho GTPases (RhoA, Rac1 and Cdc42) and Tiam1, and we found Rac1 and Cdc42 and Tiam1 were significantly up-regulated when down-regulated miR-106b (**Figure 3C**), while RhoA showed no significantly change (data not shown).

The effect of miR-106b on cancer metastatic was assessed in vivo in nude mice. Two months after injection, miR-106b-knockdown cells SW-480 formed much fewer and smaller metastatic nodules in lungs, while control group showed a relatively higher capability of metastatic colonization, however, injection of a mixture of EMT and non-EMT cells showed the highest capability in the formation of lung metastasis (**Figure 3D**).

The results indicated that knocking down miR-106b could promote migration but weakened capability of metastatic colonization in CRC cells.

miR-106b directly target Prrx1 and formed a negative feedback loop with TGF-ß1 co-regulated Prrx1

We performed a bioinformatics search (Targetscan http://www.targetscan.org) and found Prrx1 3'-UTR contained region that matched the seed sequences of miR-106b (Supplementary Figure 3). The increase in endogenous miR-106b levels lentiviral transduction significantly decreased Prrx1 protein expression, whereas miR-106b inhibition increased it expression which was determined by western blot (**Figure 4A**). Prrx1 mRNA expression showed no significant change (data not shown).

To verify that Prrx1 is a direct target of miR-106b, Prrx1 3'-UTR, containing the miR-106b binding sites were cloned into downstream of the Luciferase open reading frame. Co-transfected miR-106b mimics and Prrx1-3'-UTR-wild vector into SW620 (PLuc-Prrx1-3'-UTR) significantly decreased the Luciferase activity, Conversely, when we performed Luciferase assays

using a plasmid harboring the 3'UTR of Prrx1, where the binding sites for miR-106b were inactivated by site-directed mutagenesis, we observed no effect on Luciferase activity. While transfection with miR-106b inhibitor into SW-480 increased the Luciferase reporter activity (**Figure 4B**).

Next we resuced the expression of Prrx1 in SW480-Lenti-anti-106b cells and explored the change of E-cadherin expression and migration capacity. We found knocking down Prrx1 in SW480-Lenti-anti-miR-106b by transfecting siRNA/Prrx1 could restore E-cadherin expression. And restrained the cells migration capacity (**Figure 4C** and **4D**).

All above results indicated Prrx1 is a direct target of miR-106b and could rescue the functions of miR-106b in CRC cell lines. TGF-ß1 is an inducer of EMT transcription factors in epithelial cells [20]. We wondered whether the miR-106b could alter the expression of TGF-ß1. Western blot analysis validated that miR-106b knockdown cells SW480 could up-regulate TGF-ß1 expression, while miR-106b-overexpressed cells SW620 exhibited an opposite effect (**Figure 4E**).

We also found SW480 cells showed a significantly higher Prrx1 expression after treatment with TGF-ß1, while the expression of miR-106b showed an opposite trend (**Figure 4F** and **4G**). Prrx1 knockdown in SW620 could rescue EMT induced by TGF-ß1 (**Figure 4H**).

These findings showed that TGF-ß1 and miR-106b could be regulated by each other establishing a negative feedback loop to regulate the expression of Prrx1 together, playing a key role in EMT induced by TGF-ß1 and miR-106b.

Discussion

Mounting experimental data supports that EMT plays an important role in tumor metastasis [19]. It has confirmed that cells undergoing EMT induced by Snail1 gained the capacity of antiproliferative [21]. Previous studies also confirmed that carcinoma metastases usually exhibited a well-differentiated epithelial phenotype and mesenchymal-to-epithelial have been confirmed to occur and involve in the tumor progression and metastatic colonization [7, 8]. This maybe one of the reasons why pluripotency accompanied by the epithelial phenotype more than the mesenchymal phenotype, is a major driver of metastatic potential [22].

Transient EMT, such as that induced by the cooperation between epithelial and mesenchymal tumor subpopulations, could enhance the local invasiveness of the epithelial subpopulation, thus contributing to the overall metastatic potential of a tumor in which heterogeneous epithelial and mesenchymal subpopulations coexist [21]. However, injection of a mixture of EMT and non-EMT cells showed a highest capability in the formation of lung metastasis, there results suggest that EMT cells and non-EMT cells cooperated to accomplish distant metastatic colonization. Taken together, the present papers suggest that both mesenchymal and epithelial phenotypes are required for metastatic competence.

Our laboratory have identified that Tiam1 regulates migration, invasion of colon cancer cells [23]. Here we found Tiam1 was up-regulated during miR-106b-mediated EMT and conferred migratory and invasive properties as well as GTPases (Rac1 and Cdc42). The Rho family of low-molecular-weight G proteins consists of the Rho, Rac and Cdc42 subfamilies that mediate the distinct changes in the actin cytoskeleton required for transformation [24]. Recent studies showed Rac1-specific activator Tiam1 is one of the components of beta-catenin/TCF complexes at Wnt-responsive promoters, within these complexes serves to enhance target gene transcription in CRC cells [25]. We guessed Tiam1 may interact with Rac1 and other Rho family of low-molecular-weight G proteins during miR-106b-mediated cytoskeletal reorganization, migratory and EMT.

Knocking down miR-106b could induce EMT through regulating a series of migration and metastatic-related genes directly (Prrx1) or indirectly (Rac1 and Cdc42 and Tiam1) but weaken cell proliferation capacity. We guessed miR-106b knockdown weakened the cells capacity of distant metastatic colonization through restraining proliferation, although conferring the cells migratory and invasive properties.

Identifying targets is important to understand the functional mechanism of microRNAs. MiR-106b-25 could induce EMT and tumor initiating cell by targeting Smad7 in human breast can-

cer [26]. It also has been showed that Smad7 could induce hepatic metastasis in CRC [27]. But we found the expression of Smad7 showed no changes when altering the expression of miR-106b (data not shown). We guessed that miR-106b-mediated EMT and conferring migratory and invasive properties in CRC by targeting other genes but not Smad7.

Prrx1 is expressed in undifferentiated mesenchymal cells [28], and it had previously been implicated in several aspects of fibroblast migration [29]. We confirmed miR-106b inducing EMT by targeting Prrx1 which conferring CRC cells migratory and invasive properties but insufficiently accomplish distant metastatic colonization.

It is not contradictory EMT and distant metastatic colonization is uncoupled. Tsai and coworkers also confirmed Twist1 induces EMT and promotes tumor cells transit into the bloodstream, however, metastases did not form if the cancer cells could not switch Twist1 off after disseminating [30]. Down-regulation of ZO-1 leads to increased motility and ZO-1 is up-regulated in melanoma cells, up-regulation of ZO-1 contributes to the oncogenic behavior of this tumor [31]. Ocana and his collaborators have reported that Prrx1 is an EMT inducer and conferred the cells migratory and invasive properties, while suppressed metastatic colonization [32]. This suggests that halting EMT, and thereby allowing MET, is required for completion of the metastatic process. Prrx1 is a target of miR-106b, this maybe one of the reasons why down-regulation of miR-106b promotes EMT but suppresses metastatic colonization.

Taken together our studies showed that knocking down miR-106b could induce EMT but suppressed metastatic colonization capacity. We also found the expression of miR-106b was relatively lower in primary CRC tissues with lymph node or distant metastasis than those without lymph node or distant metastasis. We guess miR-106b knockdown is a necessary but not sufficient condition in distant metastasis. Our data highlights a key role for miR-106b in the regulation of invasion and metastasis in the molecular etiology of CRC.

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Disclosure of conflict of interest

None.

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References

[1] Parkin DM, Bray F, Ferlay J and Pisani P. Global cancer statistics. CA Cancer J Clin 2005; 55: 74-108.

[2] Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED and Thompson EW. Epithelial-mesenchymal and mesenchymal-epithelial transitions in carcinoma progression. J Cell Physiol 2007; 213: 374-383.

[3] Shook D and Keller R. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. Mech Dev 2003; 120: 1351-1383.

[4] Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J and Weinberg RA. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008; 133: 704-715.

[5] Morel AP, Lievre M, Thomas C, Hinkal G, Ansieau S and Puisieux A. Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS One 2008; 3: e2888.

[6] Tsuji T, Ibaragi S, Shima K, Hu MG, Katsurano M, Sasaki A and Hu GF. Epithelial-mesenchymal transition induced by growth suppressor p12CDK2-AP1 promotes tumor cell local invasion but suppresses distant colony growth. Cancer Res 2008; 68: 10377-10386.

[7] Chaffer CL, Brennan JP, Slavin JL, Blick T, Thompson EW and Williams ED. Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2. Cancer Res 2006; 66: 11271-11278.

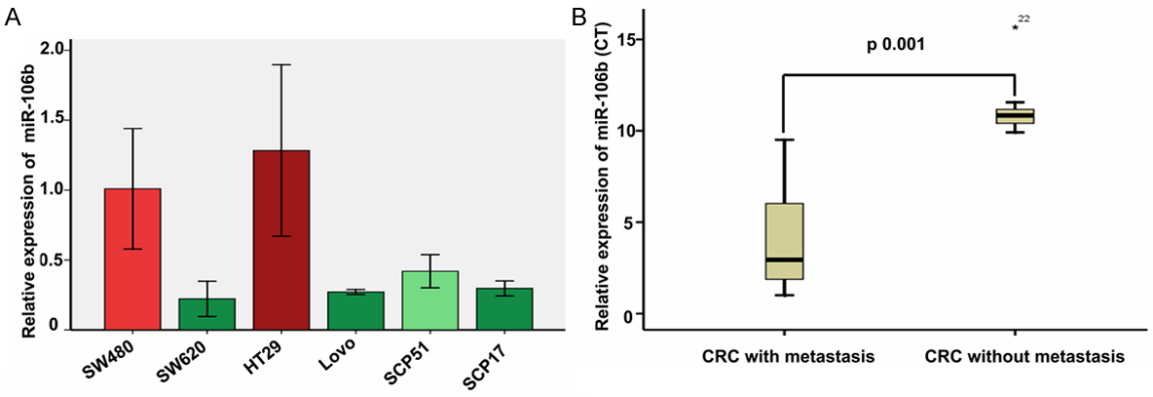
[8] Dykxhoorn DM, Wu Y, Xie H, Yu F, Lal A, Petrocca F, Martinvalet D, Song E, Lim B and Lieberman J. miR-200 enhances mouse breast cancer cell colonization to form distant metastases. PLoS One 2009; 4: e7181.

[9] Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR and Golub TR. MicroRNA expres-

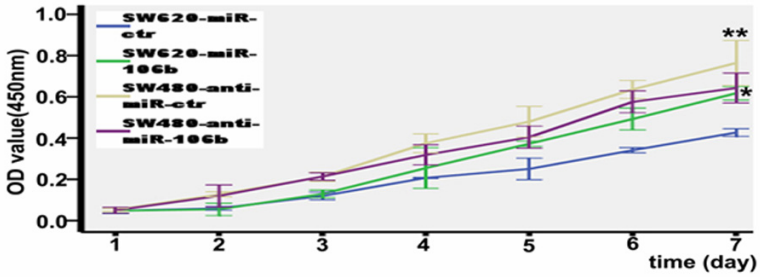
sion profiles classify human cancers. Nature 2005; 435: 834-838.
[10] Slaby O, Krekac D, Hrstka R, Svoboda M and Vyzula R. [Involvement of microRNAs in cancer biology and possibilities of their application to diagnostic and predictive oncology]. Cas Lek Cesk 2008; 147: 25-31.
[11] Ivanovska I, Ball AS, Diaz RL, Magnus JF, Kibukawa M, Schelter JM, Kobayashi SV, Lim L, Burchard J, Jackson AL, Linsley PS and Cleary MA. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Mol Cell Biol 2008; 28: 2167-2174.
[12] Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, de Martino I, Iliopoulos D, Pilozzi E, Liu CG, Negrini M, Cavazzini L, Volinia S, Alder H, Ruco LP, Baldassarre G, Croce CM and Vecchione A. E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell 2008; 13: 272-286.
[13] Yau WL, Lam CS, Ng L, Chow AK, Chan ST, Chan JY, Wo JY, Ng KT, Man K, Poon RT and Pang RW. Over-expression of miR-106b promotes cell migration and metastasis in hepatocellular carcinoma by activating epithelial-mesenchymal transition process. PLoS One 2013; 8: e57882.
[14] Wang YX, Zhang XY, Zhang BF, Yang CQ, Chen XM and Gao HJ. Initial study of microRNA expression profiles of colonic cancer without lymph node metastasis. J Dig Dis 2010; 11: 50-54.
[15] Hao JM, Chen JZ, Sui HM, Si-Ma XQ, Li GQ, Liu C, Li JL, Ding YQ and Li JM. A five-gene signature as a potential predictor of metastasis and survival in colorectal cancer. J Pathol 2010; 220: 475-489.
[16] Zhao L, Wang H, Liu C, Liu Y, Wang X, Wang S, Sun X, Li J, Deng Y, Jiang Y and Ding Y. Promotion of colorectal cancer growth and metastasis by the LIM and SH3 domain protein 1. Gut 2010; 59: 1226-1235.
[17] Zhou C, Liu G, Wang L, Lu Y, Yuan L, Zheng L, Chen F, Peng F and Li X. MiR-339-5p regulates the growth, colony formation and metastasis of colorectal cancer cells by targeting PRL-1. PLoS One 2013; 8: e63142.
[18] Zheng F, Liao YJ, Cai MY, Liu YH, Liu TH, Chen SP, Bian XW, Guan XY, Lin MC, Zeng YX, Kung HF and Xie D. The putative tumour suppressor microRNA-124 modulates hepatocellular carcinoma cell aggressiveness by repressing ROCK2 and EZH2. Gut 2012; 61: 278-289.
[19] Berx G, Raspe E, Christofori G, Thiery JP and Sleeman JP. Pre-EMTing metastasis? Recapitulation of morphogenetic processes in cancer. Clin Exp Metastasis 2007; 24: 587-597.
[20] Thiery JP, Acloque H, Huang RY and Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell 2009; 139: 871-890.
[21] Vega S, Morales AV, Ocana OH, Valdes F, Fabregat I and Nieto MA. Snail blocks the cell cycle and confers resistance to cell death. Genes Dev 2004; 18: 1131-1143.
[22] Celia-Terrassa T, Meca-Cortes O, Mateo F, de Paz AM, Rubio N, Arnal-Estape A, Ell BJ, Bermudo R, Diaz A, Guerra-Rebollo M, Lozano JJ, Estaras C, Ulloa C, Alvarez-Simon D, Mila J, Vilella R, Paciucci R, Martinez-Balbas M, de Herreros AG, Gomis RR, Kang Y, Blanco J, Fernandez PL and Thomson TM. Epithelial-mesenchymal transition can suppress major attributes of human epithelial tumor-initiating cells. J Clin Invest 2012; 122: 1849-1868.
[23] Liu L, Zhang Q, Zhang Y, Wang S and Ding Y. Lentivirus-mediated silencing of Tiam1 gene influences multiple functions of a human colorectal cancer cell line. Neoplasia 2006; 8: 917-924.
[24] Hall A. Rho GTPases and the actin cytoskeleton. Science 1998; 279: 509-514.
[25] Buongiorno P, Pethe VV, Charames GS, Esufali S and Bapat B. Rac1 GTPase and the Rac1 exchange factor Tiam1 associate with Wnt-responsive promoters to enhance beta-catenin/TCF-dependent transcription in colorectal cancer cells. Mol Cancer 2008; 7: 73.
[26] Smith AL, Iwanaga R, Drasin DJ, Micalizzi DS, Vartuli RL, Tan AC and Ford HL. The miR-106b-25 cluster targets Smad7, activates TGF-beta signaling, and induces EMT and tumor initiating cell characteristics downstream of Six1 in human breast cancer. Oncogene 2012; 31: 5162-5171.
[27] Halder SK, Rachakonda G, Deane NG and Datta PK. Smad7 induces hepatic metastasis in colorectal cancer. Br J Cancer 2008; 99: 957-965.
[28] Leussink B, Brouwer A, el Khattabi M, Poelmann RE, Gittenberger-de Groot AC and Meijlink F. Expression patterns of the paired-related homeobox genes MHox/Prx1 and S8/Prx2 suggest roles in development of the heart and the forebrain. Mech Dev 1995; 52: 51-64.
[29] McKean DM, Sisbarro L, Ilic D, Kaplan-Alburquerque N, Nemenoff R, Weiser-Evans M, Kern MJ and Jones PL. FAK induces expression of Prx1 to promote tenascin-C-dependent fibroblast migration. J Cell Biol 2003; 161: 393-402.
[30] Tsai JH, Donaher JL, Murphy DA, Chau S and Yang J. Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. Cancer Cell 2012; 22: 725-736.

[31] Smalley KS, Brafford P, Haass NK, Brandner JM, Brown E and Herlyn M. Up-regulated expression of zonula occludens protein-1 in human melanoma associates with N-cadherin and contributes to invasion and adhesion. Am J Pathol 2005; 166: 1541-1554.

[32] Ocana OH, Corcoles R, Fabra A, Moreno-Bueno G, Acloque H, Vega S, Barrallo-Gimeno A, Cano A and Nieto MA. Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prrx1. Cancer Cell 2012; 22: 709-724.



Supplementary Figure 1. The expression levels of miR-106b in CRC cell lines. A. The expression of miR-106b in CRC cell lines with different metastatic ability. The relative expression of miR-106b was normalized to the endogenous control U6. B. The expression of miR-106b in primary CRC tissues with or without lymph node or distant metastasis. The expression of miR-106b was relative lower in primary CRC tissues with lymph node or distant metastasis than those without lymph node and distant metastasis. ***P<0.001.



Supplementary Figure 2. The vitality of SW620 cells over expression of miR-106b or SW480 cells after inhibition of the same miRNAs was detected using the MTT assay. Values at the indicated time points were provided as the mean absorbance with an SD of three wells. *P<0.05, **P<0.01.

```
3'      UAGACGUGACAGUCGUGAAAU      hsa-miR-106b
                  |||||||
5' ...UUCUCUGUAUGUCCAGCACUUUG...   PRRX1 3' UTR  WT
5' ...UUCUCUGUAUGUCCACGUGAAAG...   PRRX1 3' UTR  Mut
```

Supplementary Figure 3. Prrx1 3'UTRs contain predicted miR-106b binding site.