miR-141 is A Key Regulator of Renal Cell Carcinoma Proliferation and Metastasis by Controlling EphA2 Expression

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No potential conflicts of interest were disclosed.

Other Notes

Our manuscript contains 4,879 words, one table and seven figures. The supplemental information is composed of supplemental experimental procedures, two tables and five figures.
**Translational Relevance**

Renal cell carcinoma (RCC) is a particularly aggressive and chemo-resistant malignancy. Current targeted therapies based on the discovery of the VHL/HIF/VEGF pathway have changed the treatment landscape for patients with metastatic RCC, whereas these therapies have not lived up to their initial promise. A central role of microRNAs (miRNAs) in the initiation and progression of cancers has begun to emerge. Here we screened and identified that miR-141 was the most significantly downregulated in clear cell RCC (ccRCC) tissues and cell lines, and a promising biomarker for discriminating ccRCC and normal renal tissues. Furthermore, miR-141 overexpression effectively suppressed tumor growth, local invasion and metastatic colonization in human RCC orthotopic xenografts by decreasing a direct functional effector EphA2. Because our findings are based on clinical RCC samples and a good animal model, miR-141’s ability to suppress of tumorigenesis and metastasis may prove to be clinically useful.
Abstract

Purpose: Although microRNAs (miRNAs) have been revealed as crucial modulators of tumorigenesis, our understanding of their roles in renal cell carcinoma (RCC) is limited. Here we sought to identify human miRNAs that act as key regulators of renal carcinogenesis.

Experimental Design: We performed microarray-based miRNA profiling of clear cell RCC (ccRCC) and adjacent normal tissues and then explored the roles of miR-141 both in vitro and in vivo, which was the most significantly downregulated in ccRCC tissues.

Results: 74 miRNAs were dysregulated in ccRCC compared with normal tissues. miR-141 was remarkably downregulated in 92.6% (63/68) ccRCC tissues and would serve as a promising biomarker for discriminating ccRCC from normal tissues with an AUC of 0.93. Overexpression of miR-141 robustly impaired ccRCC cell migratory and invasive properties and suppressed cell proliferation by arresting cells at G0/G1 phase in vitro and in human RCC orthotopic xenografts. Significantly, the anti-tumor activities of miR-141 were mediated by its reversal regulation of EphA2, which then relayed a signaling transduction cascade to attenuate the functions of focal adhesion kinase (FAK), AKT, and MMP2/9. In addition, a specific and inverse correlation between miR-141 and EphA2 expression was obtained in human ccRCC samples. Finally, miR-141 could be secreted from the ccRCC donor cells, and be taken up and function moderately in the ccRCC recipient cells.

Conclusion: miR-141 serves as a potential biomarker for discriminating ccRCC from normal tissues and a crucial suppressor of ccRCC cell proliferation and metastasis by modulating the EphA2/p-FAK/p-AKT/MMPs signaling cascade.
Introduction

Renal cell carcinoma (RCC) accounting for 3% of adult malignancies is the most lethal urological malignancy, with about 65,150 new cases and 13,680 deaths estimated for 2013 in the United States (1). RCC is heterogeneous and comprises several histological subtypes according to the differences in genetics, biology and behavior. The most common and aggressive RCC subtype is clear cell RCC (ccRCC) with the highest rates of local invasion, metastasis, mortality and refractory to current treatments. Recently, advancements in understanding of the VHL gene pathway in ccRCC have produced pharmacueutic outcomes based on specific molecular targets that have changed the treatment landscape for patients with metastatic RCC (mRCC) (2). Unfortunately, the vast majority of treated patients with mRCC eventually develop progressive disease due to acquired resistance or other reasons. Hence, a better understanding of the mechanisms involved in the pathogenesis of ccRCC and more effective therapeutic approaches are urgently required.

MicroRNAs (miRNAs), a group of small non-coding RNAs of about 22 nucleotides in length, negatively regulate gene expression, primarily through interaction with the 3′untranslated region (3′UTR) of target mRNAs (3). miRNAs are known to contribute to multiple tumorigenic steps in human cancers, including RCC. Recent studies have identified regulatory activities of miRNAs in ccRCC cell growth (4-8), apoptosis (5, 6, 8, 9), migration and invasion (5-8). A predominant and systemic alteration in miRNA expression during renal carcinogenesis has been indicated by present studies of miRNA expression profiling (10-17). Consistently, the miR-200 family members (miR-200s) that comprise miR-141, 200c, 200a, 200b and 429 are among the most markedly decreased miRNAs in ccRCC. These miRNAs were previously found to be closely
associated with cell motility by modulating epithelial-mesenchymal transition (EMT), the formation of cancer stem cells, sensitivity to chemotherapeutic agents, and apoptosis (9, 18-23). However, how miR-200s function in ccRCC pathogenesis remains largely unknown. Growing evidences reveal that extracellular miRNAs are chemically stable and can be detected in a broad range of clinical samples and hence, have diagnostic and prognostic values (24-26). Furthermore, certain secreted miRNAs from living cells (for example, miR-146a and miR-223), are transferable and can affect surrounding and remote cells by regulating target mRNAs and proteins, implying functions of miRNAs in intercellular communication (27-29). Given their small size and substantial effects in a wide range of pathologies, miRNAs can be potentially utilized in ccRCC therapy.

Here, we monitored miRNA expression profiles of ccRCC and identified miR-141 as one of the most significantly downregulated miRNAs in ccRCC tissues and cells and a critical suppressor of ccRCC cell growth and metastasis both in vitro and in vivo. We further demonstrated that the attenuation of miR-141 in RCC is associated with the amplification of EphA2 and additional downstream pathways. Moreover, our results based on clinical RCC samples showed that miR-141 might be a potential biomarker for discriminating between ccRCC and normal tissues. Finally, we found that miR-141 can be secreted from the ccRCC donor cells, and be taken up and function moderately in the ccRCC recipient cells.

Materials and Methods

Human samples
Surgical specimens (paired normal and cancerous tissues) were obtained from 78 patients with kidney tumors in Department of Urology, Union Hospital, Tongji Medical College (Wuhan, China), freshly frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction. Informed consent was obtained from patients and the study was approved by the Institutional Review Board of Huazhong University of Science and Technology. Relevant clinical and pathological information based on patient records was collected and listed in the Supplementary Table S1.

**miRNA microarray**

The miRNA expression profiling was conducted using the commercially available G4471A Human miRNA Microarray (Agilent Technologies, Palo Alto, CA), which consists of 961 probes for 851 human miRNAs, based on Sanger miRBase release 12.0. The arrays were washed and scanned with a laser confocal scanner (G2565BA, Agilent Technologies, Palo Alto, CA) according to the manufacturer’s instructions. The intensities of fluorescence were calculated by Feature extraction software (Agilent Technologies). Differentially expressed miRNAs were identified by arbitrarily setting the threshold at a fold change of 2.0 or above combined with p < 0.05 (ANOVA).  

**Cell culture, infection, transfection and preparation of conditioned media (CM)**

Human RCC cell lines 786-O and A498 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The RCC cell line SN12-PM6 was supplied by Dr. I.J. Fidler (MD Anderson Cancer Center, Houston, TX). All cells were cultured in Dulbecco’s modified...
Eagle’s media plus 10% fetal bovine serum (FBS) with 1% penicillin-streptomycin at 37°C in 5% CO₂. Lentiviruses containing vector pGCSIL-GFP and pGCSIL-GFP-miR-141 were constructed by GENECHEM (Shanghai, China) and used to infect 786-O or SN12-PM6 cells at a multiplicity of infection (MOI) of 10 or 50, respectively, according to the manufacturer’s instructions. miR-141 inhibitor (Inh-miR-141) and its negative control (Inh-NC) were designed and synthesized by Ribobio (Guangzhou, China). Short interfering RNA (siRNA) against EphA2 (si-EphA2) and negative control (si-NC) with non-specific sequences was synthesized by Genepharma (Shanghai, China). Sequence of EphA2 siRNA is as following:

5’-UGACAUGCCGGAUCUACAUGdTdT-3’ (sense), 5’-CAUGUAGAUCGGCAUGUCAdTdT-3’ (antisense) (30). Cells were seeded in media without antibiotics approximately 24 hours before transfections. Oligonucleotide transfection of a final concentration of 50 or 100 nM was performed with Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Cells were used for experiments after siRNA transfection for 48 hours.

To prepare CM, cells infected with lentiviruses were cultured in fresh complete media. After incubation for 0, 24, 48, 72 hours, respectively, media were collected and centrifuged at 2,000g for 15 minutes at 4°C to remove living cells and centrifuged again at 12,000g for 35 minutes at 4°C to thoroughly remove cellular debris (27). Then the CM were transferred to an RNase/DNase-free 1.5 ml tube and used for RNA extraction. In addition, the media from miR-141 or miR-NC cells after incubation for 48 hours were used to culture miR-NC cells for another 48 hours.

RNA extraction, quantitative real-time PCR (qRT-PCR) and semiquantitative reverse-transcription PCR (RT-PCR)
Total RNA of tissues and cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA in CM was isolated by using TRI Reagent BD (Molecular Research Centre, Cincinnati, OH) according to the manufacturer’s protocol with modification. In brief, 0.2 ml of CM was added to 0.75 ml of TRI Reagent BD supplemented with 20 µl of acetic acid (5 mol/L). 25 fmol of synthetic C. elegans miRNA cel-miR-39 (Qiagen, Hilden, Germany) was spiked-in as a normaliser before chloroform extraction, and then the RNA was precipitated in isopropanol overnight at -20°C. Finally the pellet was dissolved in 15 µl of DEPC-treated water. Reverse transcription of miRNA and mRNA was done using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) and a reverse transcript primer from RiboBio (Guangzhou, China). qRT-PCR analysis was performed with the Platinum SYBR Green qPCR Supermix UDG kit (Invitrogen, Carlsbad, CA) using synthesized primers from RiboBio (Guangzhou, China) or Sangon biotech (Shanghai, China). Mature miRNAs and mRNAs were measured in accordance with the manufacturer's instructions (LightCycler® 480II, Roche, Mannheim, Germany). RT-PCR for EphA2 was performed with 2 x Taq PCR MasterMix (Tiangen Biotech, Beijing, China) according to the manufacturer’s protocol. The primers were listed in the Supplemental Experimental Procedures. Samples were normalized to RNU6B (U6) or GAPDH. Relative expression was calculated using the power formula: $2^{-\Delta \Delta Ct} (\Delta \Delta Ct = Ct_{miR-141} - Ct_{U6})$.

**Cell viability, drug sensitivity, cell cycle, migratory and invasion assays**

Cell viability was assessed at 24, 48, 72 and 96 hours upon treatments by the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) method (Sigma, USA) as previously described (31). For drug sensitivity assay, cells were treated with various
concentrations of cisplatin (DDP), 5-fluorouracil (5-FU) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) for 24 or 48 hours, then measured by above MTT assay. Fluorescence-activated cell-sorting (FACS) (BD, USA) analysis was done using propidium iodide (PI) stains for cell-cycle analysis according to the manufacturer's protocol. The 24-well transwell plate with 8 µm pore polycarbonate membrane inserts (Corning, New York, USA) was used to analyze the migration and invasive potential of cells according to manufacturer's protocol. For invasion assay, the membrane was coated with the matrigel (200 ng/ml) (BD Biosciences, Bedford, MA). After 24 hours of incubation, cells invading into the lower surface of the membrane insert were fixed in 100% methanol, stained with 0.05% crystal violet, and quantified by counting in 10 random fields. To evaluate the effects of secreted miR-141 on cell migration and invasion, cells overexpressing miR-141 or miR-NC were cultured in the lower chambers for 24 hours and then control (miR-NC) cells were placed in the upper chamber for another 24 hours.

Xenograft orthotopic implantations

For in vivo studies, 1 x 10^6 SN12-PM6 cells stably expressing miR-141 or miR-NC were injected into the left kidney of male BALB/c nude mice at 4-5 weeks of age as previously described (32). The green fluorescence intensity of the xenografts was monitored periodically using the Lumazone FA 2048 system (Roper Scientific, USA). 6 weeks and 8-9 weeks after the implantation of the xenografts, animals were euthanized and xenografts were harvested, and assessed for tumor weight, local invasion and distant metastasis. Renal tumors from xenografts were flash frozen in liquid nitrogen and stored at -80°C for RNA extraction. Formalin-fixed, paraffin-embedded RCC xenografts were assessed by hematoxylin and eosin (HE) staining and
evaluated for EphA2 expression. All experiments were approved by the Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology.

**Luciferase assays**

Wild-type and mutant EphA2 3’UTR reporter and control construct were purchased from GENECHEM (Shanghai, China). Tumor cells overexpressing miR-141 and miR-NC cultured in 48-well plates were cotransfected with 1.5 μg of firefly luciferase reporter and 0.35 ng Renilla luciferase reporter with Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA). 24 hours posttransfection, firefly luciferase activities were measured using the Dual Luciferase Assay (Promega, Madison, WI) and the results were normalized with Renilla luciferase according to the manufacturer’s protocol.

**Statistical analysis**

Results are expressed as mean±SEM from at least three independent experiments. Using the GraphPad Prism statistical program, data were analyzed using Student’s t- test unless otherwise specified (Mann–Whitney test, ROC, Pearson’s correlation, etc.). The 2-tailed P values <0.05 were considered significant.

**Supplemental information**

The supplemental information includes supplemental methods, two tables, and five figures.

**Results**
**Loss of miR-141 expression in ccRCC tissues and cells**

Using a microarray platform which contains 851 human miRNAs to study miRNA profiles in paired primary tumor and adjacent normal tissues (NTs) from 5 patients with the mean tumor size of 4.7 cm (range, 2.3-6.0 cm), we identified 74 miRNAs dysregulated in ccRCCs compared with NTs (fold change $>2.0$ and $p<0.05$), among which 44 were significantly downregulated and the other 30 were upregulated (Supplementary Table S2). Notably, four members (miR-141, 200b, 200c and 429) of miR-200s were all markedly reduced in ccRCC tissues. Our miRNA expression pattern was similar with previous studies identified by microarray, qRT-PCR or deep-sequencing, which covered less miRNAs (10-14, 17). Based on these findings, we reasoned that miR-200s may contribute to the development of ccRCC. Among these differentially expressed miRNAs in ccRCC, miR-141 was one of the most remarkably downregulated miRNAs (104-fold) and its roles in RCC have not been fully documented yet (10-14, 17). Thus, we chose miR-141 as a representative to substantiate the functions of miR-200s in ccRCC.

Next, we validated the clinical significance of miR-141 by analyzing its expression in 68 pairs of ccRCCs and NTs by qRT-PCR. In accordance with microarray results, qRT-PCR showed that miR-141 was significantly downregulated in 92.6% (63/68) ccRCCs ($p<0.0001$) (Fig. 1A and B). Receiver operating characteristics (ROC) analysis revealed that miR-141 might serve as a useful biomarker for discriminating ccRCC from normal tissues with an area under the ROC curve (AUC) of 0.93 (95% CI, 0.881 to 0.981) (Fig. 1C). At a threshold of 0.00005 for its relative expression, the sensitivity was 86.76% and the specificity was 97.06%. As demonstrated, miR-141 expression
was not associated with tumor stage, grade or size, while its expression gradually decreased during tumor progression (Supplementary Fig. S1A-C). Notably, miR-141 was barely expressed in ccRCC cell lines, including 786-O, SN12-PM6 and A-498 cells (Fig. 1D).

We then addressed whether miR-141 could be a potential marker to differentiate malignant tumors from benign tumors. The levels of miR-141 in 2 chromophobe RCCs (chRCCs), 1 sarcoma RCC, 7 renal angiomyolipomas (AMLs) and their NTs were analyzed by qRT-PCR. Unexpectedly and significantly, miR-141 was also markedly decreased in these tumors, although there was no difference between ccRCCs and AMLs (Fig. 1B and Supplementary Fig. S1D). The results imply that miR-141 contributes to the development of tumors originated from kidney independent of specific subtypes.

**Overexpression of miR-141 attenuates ccRCC cell proliferation and motility**

To explore the biological significance of miR-141, we stably overexpressed miR-141 in two ccRCC cell lines 786-O and SN12-PM6 with lentiviruses carrying miR-141 and its control (miR-NC) (Supplementary Fig. S2A and B). The efficacy of infection was tested by qRT-PCR (Supplementary Fig. S2C and D). miR-141 expression was elevated up to 400- and 2400-fold in 786-O and SN12-PM6 cells, respectively. An increase in the control miR-16 expression was not observed, suggesting that lentiviruses specifically increased miR-141 expression. There were no marked cellular morphologic changes in the miR-141-overexpressed cells (Supplementary Fig. S2A and B), however, the cellular proliferation (MTT and FACS) analyses showed that overexpression of miR-141 suppressed ccRCC cell proliferation (p<0.05) (Fig. 2A), caused cell cycle arrest at G0/G1 phase and decreased the S phase population (Fig. 2B and Supplementary Fig.
More importantly, overexpression of miR-141 markedly impaired ccRCC cell migration and invasiveness compared to miR-NC (p<0.001) (Fig. 2C and Supplementary Fig. S3B).

Additionally, miR-141 overexpression did not directly induce apoptosis in the two tested ccRCC cell lines, with no pronounced alterations in the responsiveness to DDP, 5-FU and TRAIL (data not shown). These findings indicate that miR-141 acts as a tumor-suppressor in RCC by inhibiting cell proliferation and motility.

Extracellular miR-141 modulates cell motility of recipient cells

Mounting evidence indicate certain miRNAs released from the donor tumor cells can be taken up by the recipient tumor cells to function in cell-cell communications during cancer progression (29). To determine whether miR-141 had similar features in the ccRCC cell lines, we first prepared conditioned media (CM) from miR-141-overexpressed 786-O and SN12-PM6 cells, as well as control cells (miR-NC). The levels of miR-141 in the CM from miR-141 and miR-NC cells were measured at several time points. Similar to previous reports (27, 28), extracellular miR-141 in CM of miR-141 cells was significantly higher than that of miR-NC cells (Fig. 2D). As a control, extracellular miR-16 expression remained unchanged (Fig. 2D). In addition, the ratios of extracellular/intracellular miR-141 in the 786-O and SN12-PM6 cells were less than 2.2% and 0.01%, respectively (Supplementary Fig. S2E and F). To test whether extracellular miR-141 was taken up in the recipient ccRCC cells, CM from miR-141 or miR-NC cells were used to culture miR-NC cells. As shown in Fig. 2E, levels of intracellular miR-141 in the miR-NC cells was increased for about 10 or 7-fold after adding CM of the miR-141 cells. We next assessed whether extracellular miR-141 can make an impact on cell motility using transwell assay. The data showed
that co-culture with CM of the miR-141 cells decreased migration and invasion of the recipient miR-NC cells (Fig. 2F and Supplementary Fig. S3C). However, CM of miR-141 cells did not impact cell proliferation and cell cycle of miR-NC cells (data not shown). The above data demonstrated that miR-141 can be excreted into extracellular environment and plays active biological functions in the recipient cells. However, the level of extracellular miR-141 was markedly lower than its intracellular counterpart in the parental cells, suggesting that more predominant miRNA activities reside intracellularly. Accordingly, we next focused on the functions of intracellular miR-141.

**miR-141 suppresses tumorigenesis and metastasis in human RCC orthotopic xenografts**

To identify antitumorigenic roles of miR-141 in RCC in vivo, SN12-PM6 cells overexpressing miR-141 versus miR-NC were injected into the left kidney of nude mice and then tumor growth and metastasis were evaluated. Our previous results have demonstrated that the renal orthotopic xenografts can develop primary renal tumors and give rise to metastases in multiple organs (32). Tumor growth was surveilled by detecting GFP expression using the automated fluorescence imaging system. As shown in Fig. 3A, fluorescence imaging showed a significant reduction of tumor growth in miR-141-overexpressed cells at as early as 3rd week. Meanwhile, no difference of tumor incidence was observed between miR-141 and miR-NC tumors (Table 1). Over a period of 6 weeks and 8-9 weeks postimplantation, there was a more significant decrease in tumor weight and size upon expression of miR-141 (p<0.05) (Table 1 and Fig. 3B). More specifically, tumors with miR-141 overexpression were commonly encapsulated and confined to kidney parenchyma, whereas tumors of control cells presented more aggressive growth (Fig. 3B and C). In addition, no
Macroscopic metastases were found in tumors with miR-141 overexpression. In contrast, miR-NC tumors extensively infiltrated the kidney fascia, especially at 8th-9th week after implantation (Table 1 and Fig. 3B). Compared with miR-141 tumors, the miR-NC tumors were prone to local invasion and metastasis. As shown in Fig. 3D, the miR-NC tumors metastasized to various sites, including lung, liver, lymph nodes, cecum, musculus diaphragm and peritoneum ((Table 1 and Fig. 3D). These data further demonstrated that miR-141 functions as a critical tumor-suppressor in RCCs by suppressing tumorigenesis, local invasion and metastatic colonization.

**EphA2 is a novel direct target of miR-141**

Our aforementioned work demonstrated that miR-141 plays a critical role in modulating migration and invasion in ccRCC cells, which promoted us to identify specific miR-141 targets with potential relevance in the regulation of metastasis. Recent work on miR-141 mostly focused on the association with the process of EMT by downregulating ZEB2, TGFβ2 and upregulating E-cadherin in different cancers (18-21). Several reports also showed miR-141 regulated Notch signaling pathway by targeting JAG1 (33, 34). Consistent with previous observations, 786-O and SN12-PM6 cells with overexpression of miR-141 showed decreased levels of ZEB2, TGFβ2, JAG1, HES1 and increased E-cadherin expression (Supplementary Fig. S4). However, the alterations just based on EMT cannot fully recapitulate its profound effects on tumor progression. Next, we used different miRNA target-predicting algorithms such as TargetScan, Pictar, miRanda, miRDB and miRwalk to identify potential effector (s) of miR-141 and found conserved miR-141 sites at the 3’UTR of EphA2. As EphA2 has been reported to be highly expressed in RCC cells and tissues and associated with advanced stage disease and poor prognosis (35, 36), we reasoned...
that the loss of miR-141 might be an important factor of RCC malignancy by upregulating EphA2 expression.

To prove the above hypothesis, we examined the correlation between miR-141 and EphA2 expression in both in vitro and in vivo studies. As shown in Fig. 4A and B, enforced miR-141 expression led to a decrease in EphA2 mRNA and protein expression in both 786-O and SN12-PM6 cells. Consistently, evaluation of primary tumors in the renal orthotopic xenografts models showed an inversed correlation between miR-141 expression and EphA2 mRNA and protein levels (Fig. 4C and D).

To further validate EphA2 as a direct target of miR-141, a 208 bp fragment from the EphA2 3’UTR containing the putative miR-141 target sites was cloned into a luciferase reporter construct. As shown in Fig. 4E, the miR-141 binding site in EphA2 mRNA is a broadly conserved element among vertebrates and locates in the vicinity of 753 to 759 bp of the EphA2 3’UTR. Dual-luciferase reporter assays were performed in 786-O and SN12-PM6 cells with overexpression of miR-141 versus miR-NC to assess the functions of this potential miR-141 target site. Significantly, miR-141 overexpression substantially repressed activity of the reporter that carried the wild-type but not mutant 3’UTR of EphA2 (Fig. 4F), suggesting the regulation is mediated in sequence-specific manner and the tested region is a bona fide miR-141 targeting site.

miR-141-repressed tumor proliferation and aggressive behavior is mediated by EphA2

The above findings indicate that EphA2 is a candidate effector to mediate biological functions of miR-141. We next examined whether knockdown of EphA2 could recapitulate the inhibitory effects of miR-141 on ccRCC cell proliferation and progression. 786-O and SN12-PM6 cells were
transfected with si-EphA2 versus si-NC. RT-PCR and WB analysis confirmed the EphA2 siRNA markedly and specifically decreased EphA2 expression (Fig. 5A and B). Significantly, knockdown of EphA2 in ccRCC cells attenuated cell growth, induced G0/G1 cell-cycle arrest, and suppressed cell migration and invasion (Fig. 5C-E), similarly to the phenotypic alterations upon miR-141 overexpression. To further determine whether EphA2 is the direct and functional mediator of miR-141-repressed cell migration and invasion, we performed a rescue experiment by co-transfecting with EphA2 siRNA (versus the negative control) and miR-141 inhibitor (versus the negative control) into 786-O and SN12-PM6 cells. Transient transfection of miR-141 inhibitor led to downregulation of miR-141 and upregulation of EphA2 as determined by qRT-PCR and WB (Fig. 5F). Importantly, the enhancement in ccRCC cell migration and invasion induced by miR-141 inhibitor was effectively reversed by EphA2 attenuation (Fig. 5G and Supplementary Fig. S3D). Collectively, these findings indicate that EphA2 is an essential functional effector of miR-141 in ccRCC.

**EphA2 protein is frequently upregulated in ccRCC tissues**

We then further investigate based on clinical samples whether miR-141 is involved in the pathogenesis of human ccRCC through EphA2. The expression of EphA2 mRNA and protein from 20 pairs of ccRCC and their NTs were analyzed by qRT-PCR, WB and IHC, respectively. These 20 pairs of samples have showed a statistically significant decrease in miR-141 in the ccRCC tissues (Fig. 1A). Unexpectedly, no remarkable difference of EphA2 mRNA was observed between ccRCC and NTs (Fig. 6A). Anyhow, Pearson’s correlation analysis showed a good reverse correlation between levels of miR-141 and EphA2 mRNA in ccRCC tissues ($R^2=0.3661,$
p=0.0047) (Fig. 6B). Specifically, lower miR-141 levels were associated with higher EphA2 mRNA expression, and vice versa (Fig. 6C). Notably, compared to normal tissues, meaningful overexpression of EphA2 protein was observed in 17 of 20 and decreased expression of EphA2 was showed in 1 of 20 ccRCC cases by WB analysis (Fig. 6D). A similar result was obtained in IHC analysis (Supplementary Fig. S5), further supporting that the increased level of EphA2 is, at least in part, attributed to loss of miR-141 in ccRCC. Collectively, these findings clearly substantiate that EphA2 is a direct and functional target of miR-141.

**miR-141-EphA2 modulates FAK and AKT phosphorylation**

Next, we extended the studies on the miR141-EphA2 module to further downstream, based on reported EphA2 signaling to the FAK and AKT pathways (28, 35-37). For this purpose, we monitored whether p-FAK and p-AKT were the downstream effectors of the miR-141-EphA2 module. Expectedly, overexpression of miR-141 adequately led to compromised p-FAK and p-AKT in 786-O and SN12-PM6 cells (Fig. 7A). Suppression of EphA2 also resulted in an attenuation of p-FAK and p-AKT (Fig. 7B). Moreover, the rescue experiment showed that upregulation of EphA2 expression following miR-141 inhibition led to increased phosphorylation of FAK and AKT, while knockdown of EphA2 rescued the stimulatory effects of miR-141 inhibitor on p-FAK and p-AKT (Fig. 7C). These results suggest that miR-141 may act as a tumor-suppressor through negatively regulating the EphA2/p-FAK/p-AKT pathway.

It has been reported that FAK and AKT promote cancer cell migration and invasion by elevating MMP-2/9 expression, which have been implicated in the aggressiveness of RCC (37, 38). Accordingly, we further pursued whether miR-141-EphA2 mediated tumorigenesis and
progression in ccRCC was correlated with MMP-2/9. WB analysis showed that stable overexpression of miR-141 or EphA2 knockdown in 786-O and SN12-PM6 cells led to a marked reduction of MMP-2 (Fig. 7A and 7B). However, a significant downregulation of MMP-9 was only observed in SN12-PM6 cells but not in 786-O cells. Together, these data imply that miR-141-EphA2 may contribute to ccRCC metastasis through that p-FAK/p-AKT/MMPs cascade.

Discussion

Identification of additional and essential molecular determinant(s) is imminent to designate alternative strategies to overcome resistance in RCC therapy. Recent advances have established dysregulation of miRNAs as a common event in cancers. Others and our groups have identified that global downregulation of miRNA expression is a rising feature in ccRCC (10-14, 16, 17). Among these miRNAs, four members (miR-141, 200c, 200b and 429) of miR-200s were all markedly reduced in ccRCC tissues. However, how miR-200s functions in ccRCC pathogenesis are still not well understood. Despite of our globe miRNA screen in the small sample size (N=5), miR-141 has been reported to be one of the most significantly down-regulated in ccRCC tissues and cells (10-14, 16, 17). Thus, we chose miR-141 as a representative to study the roles of miR-200s. We demonstrated that miR-141 serves as a potential biomarker for discriminating ccRCC from normal tissues and a crucial suppressor of ccRCC cell proliferation and metastasis by modulating the EphA2/p-FAK/p-AKT/MMPs signaling cascade. This is the first in vivo study on the functional characterization and mechanistic investigation of miR-141 in RCC.
Our findings indicated that miR-141 can be a potential biomarker in RCC diagnosis. Similar to Jung’s reports (11, 15), our study showed that miR-141 measurement yielded 92.6% accuracy in discriminating ccRCC tissues from normal kidney tissues. In consistence with previous studies (15, 39), no significant correlation was identified between miR-141 expression and RCC tumor stage, grade, or size. Recent reports have also showed the ability of miRNAs to distinguish between RCC subtypes with an accuracy of about 90%, including ccRCC, papillary RCC (pRCC), chRCC and the closely related benign tumor oncocytoma (40, 41). Here, miR-141 expression was equally decreased in malignant (ccRCC, chRCC and sarcoma RCC) and benign renal tumors (AML). Considering that these results come from the relatively small number of clinical samples and SYBR Green dye-based assays, the clinical significance of miR-141 remains to be investigated by TaqMan Assay with higher specificity in a large cohort of RCC patients. Another limitation of present study is that benign renal lesion oncocytomas, which is histologically quite similar to chRCC, have not been included in our study. The comparison among oncocytomas, AML, chRCC and ccRCC may further determine the role of miR-141 as a potential biomarker.

It has been well documented that a reciprocal repression between ZEB1/2 and miR-200s induces EMT, which confers invasive properties to tumors (18-21). However, the effects of miR-141 on ZEB1/2 mediated EMT are less efficient compared with other miR-200s members (18-21, 23), indicating the additional biological functions of miR-141 in malignancy. Consistently, our in vitro data suggested that miR-141 overexpression dramatically attenuated ccRCC cell migration and invasiveness, suppressed cell proliferation and induced cell cycle arrest at the G0/G1 phase. Moreover, our in vivo studies based on orthotopic xenograft model of human RCC cells indicated that suppression of miR-141 led to a pronounced increase in renal tumor weight, local
invasion and metastasis rate. These data suggest that miR-141 is involved in RCC proliferation and progression. However, similar correlation was not obtained in clinical ccRCC cohort (15, 39, 42). The inconsistency may be due to limited samples, improvement of early-diagnosis of clinical RCC and genetic intratumor heterogeneity (variable levels of miR-141 expression within different regions of one tumor sample) (39).

Most importantly, our results established EphA2 as a direct functional effector of miR-141 in RCC. EphA2, a member of the erythropoietin-producing hepatocellular (Eph) tyrosine kinases receptor family, is an emerging target for cancer therapeutics (43). High EphA2 expression has been correlated with cancer progression and metastasis in many cancers, including RCC (35, 36). Downregulation of EphA2 expression with various approaches has been shown to inhibit malignant behavior \textit{in vitro} and \textit{in vivo} (43). Up to now, knowledge of functional roles and regulatory mechanism of EphA2 in RCC is still missing. Here we showed that EphA2 protein was significantly upregulated in the vast majority of clinical ccRCC tissues and EphA2 mRNA expression was inversely correlated with miR-141 levels in ccRCC tissues and cells. The restoration of miR-141 in ccRCC cells markedly downregulated EphA2 expression through direct interaction with the 3'UTR of EphA2 mRNA, and vice versa. Our results also indicated that EphA2 knockdown suppressed RCC cell growth by inducing G0/G1 cell-cycle arrest, migration and invasion, which phenocopied the effects of miR-141 overexpression \textit{in vitro}. Knockdown of EphA2 rescued the promoting effects of miR-141 inhibitor on RCC cell migration and invasion. These data clearly demonstrated that EphA2 contributes to cell growth and migration in RCC and is a direct and functional target of miR-141.
Two prominent oncogenic pathways, FAK and AKT, have been associated with EphA2 overexpression (30, 44-46). FAK pathway has been reported to be inversely correlated with miR-200a expression in ovarian tumors, the seed sequence of which is the same as that of the miR-141 (23). In our study, either EphA2 knockdown or miR-141 overexpression suppressed the phosphorylation of FAK and AKT in ccRCC cells. In contrast, inhibition of miR-141 led to increased phosphorylation of FAK and AKT. Additional downstream mediators of FAK/AKT such as MMPs (MMP-2/9) that have been implicated in the aggressiveness of RCC (37, 38) are known to contribute to FAK/AKT-mediated cell proliferation and progression (47-50), which is further supported by our study on the functional activities of miR-141 and EphA2. However, further investigation of EphA2/p-FAK/p-AKT/MMP pathway regulation in RCC is mandatory.

In conclusion, our identification of the remarkable alterations of miR-141 in RCC and its specific functional mediators may provide novel mechanism (s) in tumor progression and additional diagnostic and/or therapeutic target(s). The specification of the miR-141-EphA2-FAK/Akt-MMP (2/9) pathway also has fundamental importance in basic research.

Acknowledgment

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References


Legends
Table 1. Incidence of renal tumor, tumor weight, invasion and metastasis in orthotopic xenografts after 6 and 8-9 weeks.

Figure 1. miR-141 is frequently attenuated in kidney tumor tissues and ccRCC cell lines.

A, relative miR-141 expression was determined by qRT-PCR in paired normal and ccRCC tissues and analyzed with Mann–Whitney test. Each dot represents one sample.

B, relative miR-141 expression levels in kidney tumors are presented as fold change = $2^{\Delta \Delta Ct}$ of tumor versus matched normal tissues. The 0.5-fold-change threshold was defined as differentially expressed. The relative miR-141 expression levels in ccRCC samples were summarized in the table.

C, receiver operator characteristic (ROC) analysis of the cohort (A) to assess the specificity and sensitivity of miR-141 to differentiate between ccRCC and normal tissues.

D, qRT-PCR analysis of miR-141 expression levels in pools of normal and ccRCC tissues, and ccRCC cell lines. miR-141 level of normal tissues was set as 1. Data were normalized to U6 and represented as mean±SEM.

Figure 2. miR-141 acts as a tumor-suppressor in RCC.

A-C, overexpression of miR-141 inhibits ccRCC cell proliferation, migration and invasion, and induces cell-cycle arrest at G0/G1 in vitro. 786-O and SN12-PM6 cells infected with miR-141 expression or control lentiviruses were monitored for cell proliferation (A), cell-cycle (B) and transwell migration and invasion (C).
D-F, released miR-141 from the donor tumor cells functions in the recipient tumor cells. D, time-course expression of miR-141 and miR-16 in the CM from ccRCC cells stably overexpressing miR-141 versus miR-NC. miR-16 expression was used as a control for exported miRNA. Samples were measured by qRT-PCR and data were normalized to cel-miR-39. E, expression of miR-141 and miR-16 in the ccRCC cells stably expressing miR-NC. Cells were cultured with the CM of miR-141 or miR-NC cells for 48 hours, followed by assessing miR-141 and miR-16 expression using qRT-PCR. Data were normalized to U6. F, migration and invasion of ccRCC cells stably expressing miR-NC were analyzed by transwell assay upon pretreatment with the CM of miR-141 versus miR-NC cells. The results were derived from at least three independent experiments and analyzed with Student’s t-test. Data are represented as mean±SEM. *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 3. Overexpression of miR-141 attenuates the tumor growth, migratory and invasive properties of SN12-PM6 cells in vivo.

A, representative fluorescence images with primary tumors in the left kidney of nude mice after orthotopic injections of SN12-PM6 cells stably overexpressing miR-141 or miR-NC for three weeks.

B, macroscopic appearance of the tumor xenograft (arrows) in nude mice from the 6th-week and 8th-9th-week group. L, left kidney; R, right kidney.

C, HE staining of the tumor xenograft. N, normal renal tissues; T, primary renal tumors. Original magnification was ×100.

D, SN12-PM6 cells stably overexpressing miR-NC metastasized to multiple organs (arrows).
Figure 4. miR-141 downregulates EphA2 expression through specifically targeting its 3’UTR.

A and B, the mRNA (A) and protein (B) levels of EphA2 were examined by qRT-PCR and WB in 786-O and SN12-PM6 cells stably overexpressing miR-141 versus the control (miR-NC), respectively. mRNA data were normalized to GAPDH and β-actin was used as loading control in WB.

C, the expression of miR-141 and EphA2 mRNA was assessed by qRT-PCR in renal tumor xenografts. Data were normalized to U6 and GAPDH, respectively.

D, analysis of EphA2 protein expression in orthotopic renal tumors by IHC. Original magnification was ×400.

E, sequence alignment of the EphA2 3’UTR with wild-type (WT) versus mutant (mut) potential miR-141 targeting sites.

F, luciferase reporter assays showing decreased reporter activity after transfection of wild-type EphA2 3’UTR reporter construct in the 786-O and SN12-PM6 cells overexpressing miR-141. The EphA2 3’UTR mutant and control constructs had no effect on reporter activity. A renilla luciferase construct was co-transfected into cells as internal control. The normalized luciferase activity of control construct in each experiment was set as 1. Data are represented as mean±SEM. *, p<0.05; ***, p<0.001.

Figure 5. Knockdown of EphA2 in ccRCC cells significantly inhibits cell growth, induced G0/G1 cell-cycle arrest, and suppressed cell migration and invasion.
A and B, the levels of EphA2 mRNA (A) and protein (B) in 786-O and SN12-PM6 cells were assessed by RT-PCR and WB, respectively. Cells were transfected with 100 nM EphA2 siRNA versus non-specific control for 48 hours before detection. GAPDH and β-actin served as internal controls for mRNA and protein loading, respectively.

C-E, cell proliferation (C), cell-cycle (D), transwell migration and invasion (E) assays of 786-O and SN12-PM6 cells were performed after transfection with EphA2 siRNA (si-EphA2) versus control (si-NC) for 48 hours.

F, after co-transfection with 50 nM siRNA duplexes (siRNA against EphA2 or negative control) and 50 nM miRNA inhibitors (miR-141 or negative control), the levels of miR-141 and EphA2 mRNA were analyzed by qRT-PCR analysis (top) and the levels of EphA2 protein were measured by WB (bottom) in both 786-O and SN12-PM6 cells.

G, cell migration and invasion in 786-O and SN12-PM6 cells were analyzed by transwell assays. Cells were co-transfected with 50 nM siRNA duplexes (siRNA against EphA2 or negative control) and 50 nM miRNA inhibitors (miR-141 or negative control). Data are represented as mean±SEM.

*, p<0.05; **, p<0.01; ***, p<0.001.

Figure 6. miR-141 expression is reversely correlated with expression of EphA2 mRNA and EphA2 protein is frequently upregulated in clinical ccRCC tissues.

A, EphA2 mRNA expression was examined by qRT-PCR in 20 pairs of normal and ccRCC tissues and analyzed with Mann–Whitney test. Each dot represents a sample. GAPDH served as internal control.

B, correlation between levels of miR-141 and EphA2 mRNA in ccRCC tissues. Data was analyzed
with Pearson’s correlation analysis.

**C**, ccRCC tissues were divided into two groups according to their miR-141 expression. ccRCC tissues with lower miR-141 expression displayed higher EphA2 level, and vice versa.

**D**, the expression of EphA2 protein was determined by WB in 20 pairs of normal (N) and ccRCC (T) tissues. An increase in EphA2 protein was detected in 17 of 20 RCC cases, with the exception of 3 pairs of ccRCCs (*Italics*).

**Figure 7.** Ectopic miR-141 expression and knockdown of EphA2 suppress activated focal adhesion kinase (FAK) and AKT (p-FAK and p-AKT) and MMP2/9 expression.

**A**, WB showing decreased levels of p-FAK, p-AKT, MMP2/9 in miR-141-overexpressed 786-O and SN12-PM6 cells, as compared to the miR-NC counterparts.

**B**, WB for FAK/p-FAK, AKT/p-AKT, MMP2/9 protein expression in 786-O and SN12-PM6 cells following transfection with EphA2 siRNA (si-EphA2) versus control (si-NC).

**C**, the protein levels of FAK/p-FAK, AKT/p-AKT were determined by WB after co-transfection with miR-141 inhibitor (versus control) and EphA2 siRNA (versus control) in 786-O and SN12-PM6 cells. β-actin served as an internal control.
Table 1. Incidence of renal tumor, tumor weight, invasion and metastasis in orthotopic xenografts after 6 and 8-9 weeks.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Tumor incidence (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tumor weight (mg) (mean ± SEM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Beyond renal fascia rate (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Metastasis rate (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>6th week</td>
<td>8th-9th week</td>
<td>6th week</td>
<td>8th-9th week</td>
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<tr>
<td>miR-NC</td>
<td>10</td>
<td>5/5 (100)</td>
<td>5/5 (100)</td>
<td>124.7 ± 29.6</td>
<td>368.6 ± 131.1</td>
</tr>
<tr>
<td>miR-141</td>
<td>10</td>
<td>5/5 (100)</td>
<td>5/5 (100)</td>
<td>38.7 ± 8.1</td>
<td>52.9 ± 6.2</td>
</tr>
<tr>
<td>P value</td>
<td>NS&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.023</td>
<td>0.043</td>
<td>0.011</td>
<td>0.033</td>
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</table>

Note: <sup>a</sup>P values were determined by Fisher’s exact test.

<sup>b</sup>Tumor weight was calculated by subtracting the weight of the right kidney (normal) from the weight of the left kidney (implanted with tumor). If the tumor xenograft weight was small and the left kidney was just slightly heavier than the right, the weight of the tumor was recorded as zero.

<sup>c</sup>Not significant.
Figure 1.
Figure 2.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 5.
Figure 6.

(A) Relative EphA2 expression

(B) EphA2 (log2) vs. miR-141 (log2)

(C) Relative EphA2 expression

(D) Western blot analysis of EphA2 and β-actin in Normal (n=20) and ccRCC (n=20) samples.
Figure 7.
miR-141 is A Key Regulator of Renal Cell Carcinoma Proliferation and Metastasis by Controlling EphA2 Expression

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