Supplemental Information

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

Xuanyu Chen, Xuegang Wang, Anming Ruan, Weiwei Han, Yan Zhao, Xing Lu, Pei Xiao, Hangchuan Shi, Rong Wang, Li Chen, Shaoyong Chen, Quansheng Du, Hongmei Yang, and Xiaoping Zhang

Supplemental Experimental Procedures

Sequence of primers used in qRT-PCR and RT-PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward Primer(5'-3')</th>
<th>Reverse Primer(5'-3')</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>EphA2</td>
<td>GCCCACATGAACTACACCT</td>
<td>ACCTCGTACTCCACACCTCG</td>
<td>204</td>
</tr>
<tr>
<td>ZEB2</td>
<td>TGGGCAAGAAGAAAAATGACCTGCC</td>
<td>TCGTGCGGTACTTTGTGCT</td>
<td>129</td>
</tr>
<tr>
<td>TGFB2</td>
<td>CCACTCCGCCCCACTTTCTAC</td>
<td>AGCTCAATCCGGTTCAGG</td>
<td>154</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>TCCCACCACGTACAGGGTCAG</td>
<td>TCCACGCTGGGTATTGGGG</td>
<td>102</td>
</tr>
<tr>
<td>JAG-1</td>
<td>CCAATCTCTAATCATTCAAC</td>
<td>AGATACGCGATAACCATTACCAAA</td>
<td>153</td>
</tr>
<tr>
<td>HES-1</td>
<td>ACAGCACAGCGATTAACCAA</td>
<td>GCCGCGAGCTCTTCTCTCA</td>
<td>150</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGTGAAGGTGGAGTGCAACGG</td>
<td>GAGGGCAATGAAGGGTGTTTG</td>
<td>112</td>
</tr>
</tbody>
</table>

Protein extraction and western blot (WB)

WB analysis was done as previously described (1, 2). Briefly, cell and tissue proteins were isolated using RIPA buffer (Beyotime Institute of Biotechnology, Jiangsu, China) supplemented with a protease inhibitor mixture stock solution (Roche Molecular Biochemicals, Mannheim, Germany) and phenylmethanesulfonyl fluoride (PMSF). Equivalent amounts of protein were resolved by 10% SDS polyacrylamide gels and transferred to PVDF membranes, which then was blocked with 5% (w/v) nonfat dried-milk and incubated with the indicated primary antibody in
Tris-buffered saline overnight at 4°C. Primary antibodies were EphA2 (Genetex, San Antonio, TX), FAK (Epitomics, Burlingame, CA), FAK phosphor (pY576/577) (Epitomics), MMP-2 (Epitomics), MMP-9 (Epitomics), AKT (Cell Signalling Technology, CST, Beverly, MA), p-Akt (S473) (Bioworld Technology, Louis Park, MN), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The blots then were stained with the appropriate horseradish peroxidase-conjugated secondary antibody, goat-anti-rabbit (ProSci Inc., Poway, CA) and goat-anti-mouse (Santa Cruz Biotechnology). Bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific Inc., Waltham, MA) using the Chemidoc XRS+ system (Bio-Rad, Hercules, CA).

**Immunohistochemistry (IHC)**

IHC was performed as previously described (2). Sections were incubated overnight at 4°C in a humidified chamber with EphA2 antibody (Genetex, San Antonio, TX) at a 1:100 dilution. Staining of the protein was visualized by incubating sections with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and lightly counterstaining them with hematoxylin.

**Legends**

Table S1. Statistic characterization of ccRCC patients and tumors for molecular signature analyses (2008-2013).

Table S2. Differentially expressed miRNAs in ccRCCs as compared to the matched adjacent normal tissues.
**Fig. S1 Statistical analyses of the clinical significance of miR-141 in kidney tumors.**

A and B, relative expression of miR-141 in ccRCC tissues with different TNM stage (A) and Fuhrman grade (B). P values are calculated by the Kruskal-Wallis test. Each dot represents a sample. Data were normalized to U6.

C, the correlation between the levels of miR-141 expression and tumor size of ccRCC based on Pearson’s correlation analysis.

D, miR-141 is downregulated in kidney tumors. P values are determined by Mann–Whitney test. Data were normalized to U6.

**Fig. S2 Validation of miR-141 overexpression in two RCC cell lines 786-O and SN12-PM6 infected with lentiviruses.**

A and B, phase contrast and fluorescent images of 786-O (A) and SN12-PM6 (B) cells transduced with lentiviruses containing GFP and miR-141, versus the control (miR-NC).

C and D, the expression of mature miR-141 and miR-16 in 786-O (C) and SN12-PM6 (D) cells stably overexpressing miR-141 versus miR-NC constructs was confirmed by qRT-PCR. Data were normalized to U6.

E and F, the level of extracellular miR-141 was dramatically lower in the conditioned media (CM) than that in the parental cells. Ct values of cellular and extracellular miR-141 and miR-16 expression were based on qRT-PCR analysis in 786-O (E) and SN12-PM6 (F) cells stably overexpressing miR-141. ***, p<0.001, t -test.
**Fig. S3** Related to Figure 2 and 5. miR-141 acts as a tumor-suppressor in RCC.  

A and B, representative images of cell cycle (A) and transwell migration and invasion (B) analyses of 786-O and SN12-PM6 cells infected with miR-141 expression or control lentiviruses.  

C, representative images of transwell migration and invasion analyses of 786-O and SN12-PM6 cells stably expressing miR-NC upon pretreatment with the CM of miR-141 versus miR-NC cells.  

D, representative images of migration and invasion in RCC cells co-transfected with 50 nM siRNA duplexes (siRNA against EphA2 or negative control) and 50 nM miRNA inhibitors (miR-141 or negative control).

**Fig. S4** Overexpression of miR-141 leads to a significant decrease of multiple targets.  

The mRNA levels of ZEB2, TGFβ2, JAG1, HES1 and E-cadherin were quantified by qRT-PCR in RCC cells stably overexpressing miR-141 versus control (miR-NC). Data were normalized to GAPDH. *, p<0.05; **, p<0.01; ***, p<0.001, t -test.

**Fig. S5** Related to Figure 6. EphA2 protein is upregulated in clinical ccRCC tissues.  

Analysis of EphA2 protein expression in paired normal and ccRCC tissues by IHC. Original magnification was ×400.

**References**
