miR-122 promotes metastasis of clear-cell renal cell carcinoma by downregulating Dicer

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Although overall downregulation of microRNAs (miRNAs) is a general feature of clear-cell renal cell carcinoma (ccRCC), several miRNAs are consistently upregulated, among which miR-122 was markedly increased in ccRCC tissues. Our study aims to determine the functional importance and underlying mechanism of miR-122 in ccRCC metastasis. Here, we demonstrate that the expression of miR-122 increased in ccRCC tissues, and higher miR-122 expression was found in ccRCC tissues with metastatic disease than in those without metastasis. The increased miR-122 levels were associated with poor metastasis-free survival in ccRCC patients with localized disease. Dicer was validated as a direct functional target of miR-122. Overexpression of miR-122 promoted migration and invasion of ccRCC cells in vitro and metastatic behavior of ccRCC cells in vivo. Inhibition of miR-122 attenuated this metastatic phenotype in vitro. Importantly, miR-122 exerted its pro-metastatic properties in ccRCC cells by downregulating Dicer and its downstream effector, the miR-200 family, thereby inducing epithelial–mesenchymal transition (EMT). Our results suggest an important role of the miR-122/Dicer/miR-200s/EMT pathway in ccRCC metastasis. Furthermore, miR-122 may serve as a biomarker for discriminating ccRCC with metastatic potential.

Introduction
Renal cell carcinoma (RCC), accounting for 2–3% of all human malignancies, is the most lethal urological malignancy, and clear-cell RCC (ccRCC) represents the most common subtype of RCC.1 ccRCC is a highly aggressive disease, with approximately one-third of patients presenting metastasis at initial diagnosis and another one-third of patients diagnosed with localized disease eventually developing metastasis.2,3 Although nephrectomy remains the gold standard treatment for localized ccRCC, the prognosis of metastatic ccRCC is usually very poor owing to its refractory nature toward current systematic treatment strategies.4 Therefore, further understanding of the mechanisms underlying ccRCC metastasis is crucial to identify novel biomarkers for surveillance of ccRCC and more effective therapies.

MicroRNAs (miRNAs) are small noncoding RNAs with lengths of 18–24 nucleotides; miRNAs function as important regulators of gene expression.5 Moreover, miRNAs can bind to the 3’-untranslated region (3’-UTR) of target mRNAs, leading to translational suppression or mRNA degradation.5 Aberrant expression patterns of miRNAs have been observed in various human cancers, and the differential expression of certain miRNAs in specific cancers may help in the diagnosis and treatment of malignancies.6,7 Recent studies have demonstrated the differential expression profiles of miRNAs in renal cancers compared to adjacent normal tissues,8–11 suggesting an important role of miRNAs in ccRCC progression. Consistent with our previous miRNA expression profiling results (unpublished), miR-122 is among the most markedly upregulated miRNAs in ccRCC. In particular, miR-122 is known as the most abundant miRNA in the liver and has been described as a liver-specific miRNA.12,13 Loss of miR-122 expression resulted in suppression of hepatic phenotype and acquisition of invasive properties in liver cancer.14 By contrast, increased miR-122 expression was observed in the advanced stage of cutaneous T-cell lymphoma and overexpression of
miR-122 decreased the sensitivity to chemotherapy-induced apoptosis. Thus, miR-122 may function as either a tumor suppressor gene or an oncogene in a tissue-specific manner. However, to our knowledge, the functional implication and prognostic significance of miR-122 in ccRCC remain largely unknown.

In our study, we determined that miR-122 was upregulated in ccRCC, and the increased expression of miR-122 was associated with ccRCC metastasis. We also determined that miR-122 promoted the metastatic properties of ccRCC cells by targeting Dicer both in vitro and in vivo. Finally, we revealed that miR-122 induced the malignant phenotype of ccRCC by stimulating epithelial–mesenchymal transition (EMT), which was regulated by the Dicer-mediated miR-200 family.

**Material and Methods**

**Patients and tissue samples**

A total of 148 ccRCC tissue samples along with 60 adjacent normal renal tissue samples were obtained from patients who underwent nephrectomy at the Urology Department of Chinese People’s Liberation Army (PLA) General Hospital (Beijing, China) from 2009 to 2013. All specimens were immediately snap frozen in liquid nitrogen after resection, and the pathologic diagnosis of ccRCC was confirmed by two senior pathologists. The TNM stages of the specimens were assigned in accordance with the 2009 TNM staging classification system, and the nuclear grades were determined using the Fuhrman nuclear grading system. Patients’ characteristics are summarized in Supporting Information Table S1. All 128 ccRCC patients with localized disease at initial diagnosis were followed up for a median period of 41 months (range, 2–53 months), and 34 of them developed metastasis at the end-point. Our study was approved by the Ethics Committee of Chinese PLA General Hospital, and written informed consent was obtained from all included patients.

**Cell lines and cell culture**

Human ccRCC cell lines OS-RC-2, 769-P, A498, 786-O and Caki-2 and human renal proximal tubular epithelial cell lines HKC and HK-2 were obtained from the National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) and maintained at 37°C in an incubator with 5% CO₂.

**Plasmids and viral infections**

For ectopic expression of miR-122, an artificial miR-122 hairpin precursor sequence was cloned into a pLVTHM lentiviral vector (Addgene, USA) as previously described. For ectopic expression of Dicer, a Dicer open reading frame was cloned into a pLVX-Puro lentiviral vector (Clontech, USA). For knockdown of Dicer, annealed DNA oligonucleotides targeted against Dicer were cloned into a pLVTHM lentiviral vector (Addgene). Viral particle generation and infection were performed as previously described.

**Transfection of small interfering RNAs (siRNAs) and miRNA mimics or inhibitors**

siRNAs targeting Dicer, miR-122, and miR-200b mimics or inhibitors were chemically synthesized by GenePharma (Shanghai, China). Transfection was performed using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s instructions. The siRNA sequences of Dicer are listed in Supporting Information Table S2.

**Quantitative real-time PCR (qRT-PCR)**

Total RNAs were extracted using TRIzol reagent (ComWin Biotech, Beijing, China). cDNA synthesis of regular genes was performed using TransScript First-Strand cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China) in accordance with the manufacturer’s instructions. miRNAs were reverse transcribed through a specific stem-loop RT-PCR as previously described. Afterward, qRT-PCR was conducted using TransStart Green qPCR SuperMix (TransGen Biotech, Beijing, China) on an Applied Biosystems 7500 Detection System. The relative mRNA expression was normalized to peptidylprolyl isomerase A (PPIA), and the relative miRNA expression was normalized to small nuclear RNA U6 by using the 2^ΔΔCT method. The primers used for this set of experiments are listed in Supporting Information Tables S3 and S4.

**Western blot analysis**

Total proteins extracted from tissues or cells were fractionated using 10% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Billerica,
MA). After blocking with 5% nonfat milk for 1 hr, the membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with the corresponding secondary antibodies (ZSGB-BIO, Beijing, China) for 1 hr at room temperature (RT). Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Thermo, USA), and signal quantification was normalized to β-actin. The primary antibody sources are listed in Supporting Information Table S5.

**Immunofluorescence analysis**

At 48 hr after transfection, the treated cells were seeded and grown on coverslips. After fixation with 4% paraformaldehyde-PBS for 10 min at RT, cells were permeabilized in 0.5% Triton X-100 in PBS for 10 min at RT and blocked with 3% bovine serum albumin for 30 min at RT. The coverslips were incubated with primary antibodies against Vimentin (Cell Signaling, USA) or E-cadherin (Cell Signaling) for 1 hr at 37°C and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibodies at 1:200 dilution for 1 hr at 37°C. Nuclei were counterstained with 0.2 mg/mL DAPI for 15 min at 37°C. Stained cells were visualized under an Olympus confocal microscope, and captured images were assembled using OLYMPUS FluoView FV1000 (version 1.6). The primary antibody information is presented in Supporting Information Table S5.

**Luciferase reporter assay**

The 3’-UTR of Dicer containing the miR-122 binding site with the wild-type or mutated seed sequence was cloned into a psiCHECK-2 dual-luciferase vector (Promega, Madison, WI). To test the effect of miR-122 on luciferase activity, OS-RC-2 cells were co-transfected with luciferase reporter of wild-type or mutated 3’-UTR and miR-122 mimics or control, whereas A498 cells were co-transfected with luciferase reporter of wild-type or mutated 3’-UTR and miR-122 inhibitors or control. Firefly and Renilla luciferase activities were measured 48 hr after transfection in accordance with the Dual-Luciferase Assay Manual. The relative luciferase activity for each group was based on the Renilla luciferase signal normalized to the firefly luciferase signal.

**Cell migration and invasion assays**

Cell migration and invasion assays were performed in 24-well plates by using Boyden chambers containing Transwell (Corning, NY) membrane filter inserts with a pore size of 8 μm. For invasion assay, the membrane was coated with Matrigel (200 ng/mL; BD Biosciences, USA). At 48 hr after transfection, 1 × 10^5 treated cells in serum-free medium were added to the upper chambers, and the lower chambers were filled with 500 μL of medium containing 10% FBS as chemoattractant. After 12 hr migration or 24 hr invasion at 37°C, cells on the upper surface of the Transwell were gently scraped. Migrated and invaded cells were fixed in methanol and stained with 0.1% crystal violet. Cells were quantified by counting under a microscope in five random fields on each plate.

**Wound healing assay**

Wound healing assay was performed in six-well plates. At 48 hr after transfection, treated cells grown to confluence were serum-starved and scratched using a sterile 200 μL pipette tip. The coverage of the scratch area was measured from images photographed under a microscope in five random fields on each plate at different time points (0, 12 and 24 hr after scratching).

**In vivo metastasis assay**

The animal experiments were approved by the Animal Ethical Committee of Chinese PLA General Hospital. Male BALB/c nude mice aged 4–6 weeks old were used to establish in vivo metastasis models. A total of 5 × 10^5 Luc-labeling OS-RC-2 cells stably transfected with sh-Scramble, miR-122, sh-Dicer or miR-122 plus Dicer open reading frame were injected into the lateral tail vein of nude mice. Lung metastasis was monitored using an in vivo imaging system (Night-Owl II LB 983) as previously described. All mice were sacrificed at 4 weeks after injection. Lungs were initially assessed for gross metastatic nodules and then fixed in 10% formalin, embedded in paraffin, and subsequently assessed by hematoxylin and eosin (H&E) staining.

**Statistical analysis**

All data were presented as mean ± SEM of at least three independent experiments and analyzed by Student’s t-test unless otherwise stated. A value of p < 0.05 was considered statistically significant.

**Results**

**miR-122 is upregulated in ccRCC tissues, and increased miR-122 levels are associated with ccRCC metastasis**

To validate the miRNA expression profiling results and analyze the expression patterns of miR-122 in ccRCC tissues, 60 ccRCC tissue samples, including 20 cases with distant metastasis at initial diagnosis, and their matched adjacent normal renal tissues were detected by qRT-PCR. The results revealed that miR-122 was upregulated in ccRCC tissues, in both localized disease (p < 0.001) and metastatic disease (p < 0.001) (Fig. 1a), compared to adjacent normal renal tissues. More importantly, the expression of miR-122 was greater in ccRCC tissues with metastatic disease at initial diagnosis (p < 0.001) (Fig. 1a) than in that with localized disease. The miR-122 expression pattern in ccRCC tissues suggested a possible link between miR-122 expression and ccRCC metastasis.

To investigate whether miR-122 expression levels correlate with ccRCC metastasis, we followed up 128 ccRCC patients with localized disease for 2–53 months (median, 41 months) post-operatively. After analyzing the miR-122 levels of 128 ccRCC tissue samples, we ranked the entire ccRCC dataset and selected the median of the dataset as the cutoff point...
Increased miR-122 levels are associated with ccRCC metastasis, and Dicer is a direct target of miR-122. (a) qRT-PCR analysis of miR-122 levels in ccRCC tissues with localized disease (n = 40) or metastatic disease (n = 20) at initial diagnosis, compared to adjacent normal renal tissues (n = 60). Data were presented as mean ± SEM. (b) Kaplan–Meier analysis of high miR-122 group (n = 64) and low miR-122 group (n = 64) of localized ccRCC patients at initial diagnosis with regard to metastasis-free survival (p = 0.0001; log-rank test), and the median of the dataset is selected as the cutoff point between “High miR-122” and “Low miR-122.” (c) Schematic of the potential binding site of miR-122 in the Dicer 3′-UTR and its wild-type (WT) or mutated (MUT) sequence within the luciferase reporter vector. (d) Correlation between Dicer mRNA levels and miR-122 levels in ccRCC tissues (n = 60). (e) qRT-PCR analysis of miR-122 levels in normal renal cell lines and ccRCC cell lines and Western blot for Dicer in these cell lines showed inverse correlation between Dicer protein levels and miR-122 levels. (f, g) Protein levels (f) and mRNA levels (g) of Dicer were examined by Western blot and qRT-PCR, respectively, in OS-RC-2 cells transfected with miR-122 mimics compared to control or miR-NC cells. (h, i) Protein levels (h) and mRNA levels (i) of Dicer were examined by Western blot and qRT-PCR, respectively, in A498 cells transfected with miR-122 inhibitors compared to control or Inh-NC cells. (j, k) Luciferase activity of OS-RC-2 cells transfected with Dicer 3′-UTR luciferase reporter vector with intact or mutated seed sequence in the presence of miR-122 mimics or miR-NC, respectively. (l) Luciferase activity of A498 cells transfected with Dicer 3′-UTR luciferase reporter vector with intact or mutated seed sequence in the presence of miR-122 inhibitors or Inh-NC, respectively. Control = untreated. Inh = inhibitors. NC = negative control. Data were presented as mean ± SEM (n ≥ 3). ***p < 0.001 (Student’s t-test) [Color figure can be viewed at wileyonlinelibrary.com]
between the high miR-122 group and the low miR-122 group ($n = 64$ per group). Kaplan–Meier analysis illustrated that patients with high miR-122 levels demonstrate poorer metastasis-free survival rates than those with low miR-122 levels ($p = 0.000001$) (Fig. 1b). In addition, univariate analysis indicated high miR-122 level as a poor prognostic factor for metastasis (hazard ratio, HR = 7.324; 95% confidence interval, CI = 2.831–18.948; $p < 0.001$) (Table 1). Multivariate analysis was also performed, and the results indicated high miR-122 level as an independent prognostic factor (HR = 4.705; 95% CI = 1.730–12.797; $p = 0.002$) from other clinicopathological parameters, such as gender, age, body mass index (BMI), tumor size, overall TNM staging and Fuhrman grade (Table 1). Overall, the results indicated that increased miR-122 levels are highly associated with ccRCC metastasis.

**Dicer is a direct target of miR-122**

A miRNA usually exerts its function by downregulating the expression of target genes. Thus, we performed *in silico* analysis to explore the targets of miR-122. Among the predicted genes that might be the targets of miR-122, Dicer was of particular interest because miR-122 was present in seven evolutionarily conserved binding sites in the Dicer $3'$-UTR sequence (Fig. 1c) and our previous work had demonstrated that Dicer was downregulated in ccRCC tissues, whose expression pattern was directly opposite to miR-122. To further confirm the association of miR-122 and Dicer in ccRCC, we analyzed the miR-122 levels and Dicer mRNA levels in ccRCC tissues ($n = 60$), and the results indicated that the miR-122 levels were inversely correlated with the Dicer mRNA levels ($r = -0.531$, $p = 0.00001$) (Fig. 1d). Similar to ccRCC tissues, miR-122 levels increased from normal renal cell lines (HKC and HK-2) to ccRCC cell lines (OS-RC-2, 769-P, A498, 786-O and Caki-2); Western blot for Dicer in these cell lines showed a good inverse correlation between Dicer protein levels and miR-122 levels (Fig. 1e).

On the basis of the observed miR-122 levels in the ccRCC cell lines, OS-RC-2 cells with relatively low miR-122 levels and A498 cells with relatively high miR-122 levels were selected for the following experiments. To determine whether miR-122 can regulate Dicer, we transfected miR-122 mimics into OS-RC-2 cells and found that the protein and mRNA levels of Dicer significantly decreased compared to those in the control groups ($p < 0.001$) (Figs. 1f and 1g). To confirm the effect of miR-122 on Dicer, we transfected miR-122 inhibitors into A498 cells and found that the protein and mRNA levels of Dicer significantly increased compared to those in the control groups ($p < 0.001$) (Figs. 1h and 1i). These results indicated that miR-122 considerably inhibits Dicer expression in ccRCC cells.
To determine whether miR-122 can directly target its binding sites in the Dicer 3′-UTR, we generated luciferase reporter vectors in which the 3′-UTR sequence of Dicer containing either wild-type or mutant miR-122 binding sites (Fig. 1c) was cloned downstream of the luciferase open reading frame. The reporter vectors were co-transfected with miR-122 mimics or inhibitors into OS-RC-2 or A498 cells, respectively. The findings showed that overexpression of miR-122 caused significant inhibition of the wild-type reporter activity in OS-RC-2 cells (p < 0.001) (Fig. 1j), whereas inhibition of miR-122 caused significant increase in the wild-type reporter activity in A498 cells (p < 0.001) (Fig. 1k). By contrast, the mutant reporter activity was not affected by miR-122 in both cell lines (Figs. 1j and 1k). Overall, these results indicated that Dicer is a direct target of miR-122.

miR-122 promotes migration and invasion of ccRCC cells in vitro by downregulating Dicer

Given the prior evidence, we next explored whether miR-122 can promote metastatic capacity of ccRCC cells in vitro. According to the results of Transwell assays, overexpression of miR-122 markedly enhanced the cell migration and invasion of OS-RC-2 cells compared to those transfected with miR-NC (p < 0.001 and p < 0.001, respectively) (Figs. 2a and 2b), and inhibition of miR-122 markedly attenuated the cell migration and invasion of A498 cells compared to those transfected with Inh-NC (p < 0.001 and p < 0.01, respectively) (Figs. 2c and 2d). Moreover, silencing Dicer significantly promoted the cell migration and invasion of OS-RC-2 cells (p < 0.001 and p < 0.001, respectively) (Figs. 2a and 2b), whereas overexpression of Dicer significantly attenuated the cell migration and invasion of A498 cells (p < 0.001 and p < 0.01, respectively) (Figs. 2a and 2b), compared to the corresponding control cells, which phenocopied the effects of miR-122 on ccRCC cells. To further determine the causal role of the miR-122/Dicer pathway in the migration and invasion capacities of ccRCC cells, we performed a rescue experiment by co-transfecting miR-122 mimics and Dicer open reading frame into OS-RC-2 cells or co-transfecting miR-122 inhibitors and Dicer siRNA into A498 cells. The results showed that restoration of Dicer expression can reverse the miR-122-induced phenotypes in these assays (Figs. 2ad).

Table 1. Univariate and multivariate analysis of clinicopathologic parameters and miR-122 levels of 128 localized ccRCC patients with regard to metastasis-free survival

| Variables            | Univariate analysis |          | Multivariate analysis |          |
|----------------------|---------------------|----------|-----------------------|----------|
|                      | HR                  | 95% CI   | p-Value               | HR                  | 95% CI   | p-Value               |
| Gender               |                     |          |                       |                     |          |                       |
| Male                 | 1                   |          | 1                     | 1                   |          |                       |
| Female               | 0.783               | 0.354–1.729 | 0.544                | 0.810               | 0.352–1.866 | 0.621                |
| Age (years)          |                     |          |                       |                     |          |                       |
| <60                  | 1                   |          | 1                     | 1                   |          |                       |
| ≥60                  | 1.713               | 0.874–3.356 | 0.117                | 1.695               | 0.841–3.416 | 0.140                |
| BMI                  |                     |          |                       |                     |          |                       |
| <25                  | 1                   |          | 1                     | 1                   |          |                       |
| ≥25                  | 0.829               | 0.423–1.624 | 0.584                | 0.883               | 0.415–1.878 | 0.746                |
| Tumor size (cm)      |                     |          |                       |                     |          |                       |
| <7                   | 1                   |          | 1                     | 1                   |          |                       |
| >7                   | 5.293               | 2.681–10.449 | <0.001              | 3.044               | 1.410–6.572 | 0.005                |
| Overall TNM staging  |                     |          |                       |                     |          |                       |
| I=II                 | 1                   |          | 1                     | 1                   |          |                       |
| II+III               | 8.447               | 3.176–22.468 | <0.001              | 2.261               | 0.695–7.359 | 0.175                |
| Fuhrman grade        |                     |          |                       |                     |          |                       |
| 1+2                  | 1                   |          | 1                     | 1                   |          |                       |
| 3+4                  | 3.526               | 1.795–6.925 | <0.001              | 2.133               | 1.050–4.333 | 0.036                |
| miR-122 levels       |                     |          |                       |                     |          |                       |
| Low                  | 1                   |          | 1                     | 1                   |          |                       |
| High                 | 7.324               | 2.831–18.948 | <0.001              | 4.705               | 1.730–12.797 | 0.002                |

Abbreviations: BMI, body mass index; HR, hazard ratio; CI, confidence interval.

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Figure 2. miR-122 downregulates Dicer to promote migration and invasion of ccRCC cells in vitro. (a, b) Representative images (a) and cell quantifications (b) of Transwell migration and invasion assays in OS-RC-2 cells transfected with miR-NC, miR-122 mimics, si-Dicer or miR-122 mimics plus Dicer open reading frame. (c, d) Representative images (c) and cell quantifications (d) of Transwell migration and invasion assays in A498 cells transfected with Inh-NC, miR-122 inhibitors, Dicer open reading frame or miR-122 inhibitors plus si-Dicer. (e, f) Representative images (e) and scratch covered percentage (f) at indicated time points of wound healing assay in OS-RC-2 cells transfected with miR-NC, miR-122 mimics, si-Dicer or miR-122 mimics plus Dicer open reading frame. (g, h) Representative images (g) and scratch covered percentage (h) at indicated time points of wound healing assay in A498 cells transfected with Inh-NC, miR-122 inhibitors, Dicer open reading frame or miR-122 inhibitors plus si-Dicer. Inh = inhibitors. NC = negative control. Data were presented as mean ± SEM (n ≥ 3). **p < 0.01; ***p < 0.001 (Student’s t-test) [Color figure can be viewed at wileyonlinelibrary.com]
expression (Figs. 2e and 2f). Similar results were obtained by wound healing assay in the loss-of-function effect of miR-122 on A498 cells (Figs. 2g and 2h). Collectively, these findings indicated that miR-122 promotes the migration and invasion of ccRCC cells in vitro through downregulation of Dicer.

**miR-122 promotes metastatic behavior of ccRCC cells in vivo by targeting Dicer**

To further investigate whether the miR-122/Dicer connection can affect tumor metastasis of ccRCC cells in vivo, we established nude-mouse metastasis models by injection through the tail vein with Luc-labeling OS-RC-2 cells stably expressing sh-Scramble (control), miR-122, sh-Dicer or miR-122 plus Dicer open reading frame; lung metastasis was monitored by bioluminescence imaging. At 4 weeks after injection, tumor signals in the lungs were significantly higher in the miR-122 group than in the sh-Scramble group (p < 0.001) (Figs. 3a and 3b), and the effect of miR-122 was phenocopied by knockdown of Dicer (p < 0.001) (Figs. 3a and 3b). Moreover, rescuing the Dicer expression counteracted the metastasis-enhancing effect of miR-122 (p < 0.001) (Figs. 3a and 3b).

To confirm the bioluminescence imaging results, all mice were sacrificed and assessed for the presence of metastatic nodules in the lungs. As shown in macroscopic view and histological analysis, the number of metastatic nodules in each group was consistent with the tumor signals detected by bioluminescence imaging (Figs. 3c and 3d). These data further demonstrated that miR-122 promotes the metastatic behavior of ccRCC cells in vivo by targeting Dicer.

**miR-122 promotes EMT of ccRCC cells by inhibiting Dicer**

Considering the causal role of the miR-122/Dicer pathway in ccRCC metastasis in vitro and in vivo, we next explored the mechanism underlying this behavior. As shown in Fig. 4a, OS-RC-2 cells in the miR-NC-treated group (control) showed...
miR-122 changed the cellular morphology to spindle fibroblast-like shape. This morphological transition caused by overexpression of miR-122 was phenocopied by Dicer knockdown and reversed by rescuing the Dicer expression (Fig. 4a).

These changes are characteristics of the EMT process, which is an important mechanism for tumor metastasis. To determine the alteration of EMT markers, we performed immunofluorescence analysis of OS-RC-2 cells from different treated groups. The results showed the upregulation of the
mesenchymal marker vimentin and the downregulation of the epithelial marker E-cadherin in miR-122 overexpression cells compared to control cells; these changes were also phenocopied by Dicer knockdown and abolished by rescuing the Dicer expression (Fig. 4b).

To further validate the molecular levels of EMT markers, we detected the protein and mRNA levels of related genes by Western blot and qRT-PCR. After overexpression of miR-122 in OS-RC-2 cells, the protein and mRNA levels of mesenchymal markers vimentin, N-cadherin and α-SMA significantly increased, whereas the expression of epithelial marker E-cadherin significantly decreased compared to control cells (Figs. 4c and 4e). Similarly, decreasing Dicer levels phenocopied the gene expression alterations caused by miR-122 overexpression, and rescuing the Dicer expression can reverse this change (Figs. 4c and 4e). For the loss-of-function effect of miR-122 on EMT-related genes in A498 cells, miR-122 inhibition reduced the expression of mesenchymal markers but increased the expression of epithelial markers (Figs. 4d and 4f). This effect was phenocopied by overexpression of Dicer and reversed by restoration of the Dicer expression (Figs. 4d and 4f). Overall, these results indicated that miR-122 promotes EMT of ccRCC cells by inhibiting Dicer.

miR-122 promotes EMT of ccRCC cells by regulating the Dicer-mediated miR-200 family

Given that Dicer is the key enzyme in the miRNA processing machinery, the connection between miR-122/Dicer axis and EMT is likely ascribed to the regulation of Dicer-mediated miRNAs. The miR-200 family has been previously implicated in the regulation of the transcription factors ZEB1 and ZEB2, which can repress E-cadherin and stimulate EMT.21–25 Consequently, we aimed to determine whether the miR-200 family was downstream of the miR-122/Dicer pathway. We found that both miR-122 overexpression and Dicer knockdown significantly downregulated the miR-200 family members (miR-200a, miR-200b, miR-429, miR-200c and miR-141) and upregulated ZEB1 and ZEB2 in OS-RC-2 cells; the effects caused by miR-122 overexpression were potently reversed by the reintroduction of Dicer (Figs. 5a and 5c). For A498 cells, miR-200 levels were increased, whereas ZEB1 and ZEB2 levels were reduced by both miR-122 inhibition and Dicer overexpression (Figs. 5b and 5d). In agreement with the above findings, the effects caused by miR-122 inhibition were abolished by Dicer knockdown (Figs. 5b and 5d). Therefore, the miR-200 family expression levels were potently regulated by the miR-122/Dicer axis.
Figure 5. miR-122 promotes EMT of ccRCC cells by regulating the Dicer-mediated miR-200 family. (a) qRT-PCR analysis of the expression of the miR-200 family members in OS-RC-2 cells transfected with miR-NC, miR-122 mimics, si-Dicer or miR-122 mimics plus Dicer open reading frame. (b) qRT-PCR analysis of the expression of the miR-200 family members in A498 cells transfected with Inh-NC, miR-122 inhibitors, Dicer open reading frame or miR-122 inhibitors plus si-Dicer. (c) qRT-PCR analysis of the expression of the miR-200 family direct targets ZEB1 and ZEB2 in OS-RC-2 cells transfected with miR-NC, miR-122 mimics, si-Dicer or miR-122 mimics plus Dicer open reading frame. (d) qRT-PCR analysis of the expression of the miR-200 family direct targets ZEB1 and ZEB2 in A498 cells transfected with Inh-NC, miR-122 inhibitors, Dicer open reading frame or miR-122 inhibitors plus si-Dicer. (e, f) Protein levels (e) and mRNA levels (f) of the mesenchymal marker vimentin and the epithelial marker E-cadherin were examined by Western blot and qRT-PCR, respectively, in OS-RC-2 cells transfected with miR-NC, miR-122 mimics or miR-122 mimics plus miR-200b mimics. (g, h) Protein levels (g) and mRNA levels (h) of the mesenchymal marker vimentin and the epithelial marker E-cadherin were examined by Western blot and qRT-PCR, respectively, in A498 cells transfected with Inh-NC, miR-122 inhibitors or miR-122 inhibitors plus miR-200b inhibitors. (i, j) Cell quantifications of Transwell migration and invasion assays in OS-RC-2 cells transfected with miR-NC, miR-122 mimics or miR-122 mimics plus miR-200b mimics. (k, l) Cell quantifications of Transwell migration and invasion assays in A498 cells transfected with Inh-NC, miR-122 inhibitors or miR-122 inhibitors plus miR-200b inhibitors. (m, n) Scratch covered percentage at indicated time points of wound healing assay in OS-RC-2 cells transfected with miR-NC, miR-122 mimics or miR-122 mimics plus miR-200b mimics. Inh = inhibitors. NC = negative control. Data were presented as mean ± SEM (n ≥ 3). *p < 0.05; **p < 0.01; ***p < 0.001 (Student’s t-test)
To further confirm the functional importance of the miR-200 family in miR-122-induced EMT, we co-transfected miR-122 mimics and miR-200b mimics into OS-RC-2 cells. The results revealed that miR-200b overexpression reversed the EMT-related gene alterations in both protein and mRNA levels (Figs. 5e and 5f). The enhanced migration and invasion abilities of OS-RC-2 cells in vitro caused by miR-122 overexpression were also reversed by miR-200b overexpression, as shown by Transwell assay and wound healing assay (Figs. 5i and 5k and Supporting Information Figs. S1a and S1c). Similarly, the loss-of-function effect of miR-122 on A498 cells was reversed by miR-200b inhibition, as assayed by EMT-related gene expression and cellular function assays in vitro (Figs. 5g–j and S1 and Supporting Information Figs. S1b and S1d). Overall, these results indicated that miR-200b promotes EMT of ccRCC cells by regulating the Dicer-mediated miR-200 family.

Discussion
Recent studies have suggested a causal role of miRNAs in ccRCC and other human malignancies. Although overall downregulation of miRNAs is a general feature of ccRCC, several miRNAs are consistently upregulated, among which miR-122 was markedly increased in ccRCC tissues.8–11 As a liver-specific miRNA, miR-122 plays an important role in lipid and cholesterol metabolism.26,27 Reduced miR-122 expression was found associated with hepatocellular carcinoma (HCC) in most studies.4,28–31 However, miR-122 is reported to play a positive role in hepatitis C virus (HCV) infection or HCV-related HCC.32,33 This differential role of miR-122 in HCC may be ascribed to the molecular heterogeneity of HCC in terms of different liver carcinogens, viruses and genetic background.35 In our study, we validated the upregulation of miR-122 in ccRCC tissues compared to matched adjacent normal renal tissues. Importantly, miR-122 levels were elevated to a much greater extent in ccRCC tissues with metastasis at initial diagnosis, and the increased miR-122 levels in localized ccRCC were associated with poor metastasis-free survival in this fraction of patients. Similar to our findings, miR-122 was upregulated in advanced-stage cutaneous T-cell lymphoma,15 and increased levels of circulating miR-122 specifically predicted metastatic recurrence in stages II–III breast cancer patients.34 These reports indicated that miR-122 showed its expression pattern in a tissue-specific manner and can function as an oncogene in certain human cancers, including ccRCC. The different effects of miR-122 in different cell types can be explained by the fact that a certain miRNA can modulate various target genes in different background, and our study demonstrates that miR-122 can target Dicer and affect its downstream miR-200 family and its EMT target genes, which may play a causal role in metastasis of ccRCC.

The prognosis of patients with metastatic ccRCC is considerably different from that of patients with localized ccRCC.35 Considering the ccRCC’s resistance to traditional systemic therapies and inconsistent response to novel targeted therapies, metastasis is the main cause for treatment failure and death from this disease.36,37 In our study, we demonstrated that increased miR-122 level is an independent prognostic factor for ccRCC metastasis, as confirmed by multivariate analysis. Given the strong implication of miR-122 in ccRCC metastasis, we further explored the functional importance and underlying mechanism of miR-122 expression in ccRCC cells. Consistently, gain- and loss-of-function analyses of miR-122 in vitro suggested that miR-122 overexpression significantly enhanced the migrative and invasive abilities of ccRCC cells, which are two key features of metastatic properties. Moreover, our in vivo study based on a mouse metastasis model of ccRCC cells indicated that miR-122 overexpression markedly increased metastatic colonization in the lungs. These findings confirmed that miR-122 plays a positive role in ccRCC metastatic behavior. However, Coulouarn et al.14 showed that loss of miR-122 expression results in suppression of the hepatic phenotype and the acquisition of invasive properties, whereas restoration of miR-122 reversed this phenotype. The inconsistency regarding the miR-122 effect may be ascribed to the different dominant downstream pathways of miR-122, favoring and opposing the same process in different tissue backgrounds.

In our study, we identified Dicer as a direct functional target of miR-122 in ccRCC, and Dicer knockdown can phenocopy the miR-122 overexpression effect in vitro and in vivo. Dicer, which belongs to the RNase III family of double-stranded RNases and key enzymes controlling the maturation of miRNAs in the cytoplasm, is most commonly known as a haploinsufficient tumor suppressor gene.38,39 In addition, decreased expression of Dicer is associated with poor clinical outcomes in many cancers.40–43 We previously confirmed the downregulation of Dicer in ccRCC.20 However, the regulatory mechanism of Dicer in ccRCC is not well understood. Considering that miR-122 is also processed by Dicer, our report on miR-122 targeting Dicer indicated the existence of a novel feedback loop, in which miR-122 may work as a key miRNA to control various mature miRNAs through Dicer modulation. This mutual feedback relationship between miRNAs and Dicer has also been reported elsewhere, such as on the let-7-Dicer axis and miR-103/107-Dicer axis.44,45 With our newly found miR-122-Dicer axis, the feedback loops may work together for tuning the overall mature miRNA expression profiles in human cancers, including ccRCC. Lian et al.46 suggest that miR-122 influences PI3K/Akt signaling in ccRCC-derived cell lines. Combined with our results, it can be explained by that certain miRNAs, which target PI3K/Akt signaling, are downstream to miR-122-Dicer axis, and downregulation of these miRNAs can activate PI3K/Akt signaling, thus promoting ccRCC tumor growth.

EMT is an important mechanism of metastasis. EMT can transform the epithelial state of tumor cells and confer mesenchymal traits facilitating dissemination of tumor cells, leading to metastasis.47 In our study, we demonstrated that miR-
122 induced the acquisition of mesenchymal characteristics by downregulating Dicer. This significant finding suggests that the association between the miR-122/Dicer axis and EMT may represent a leading mechanism by which miR-122 promotes ccRCC metastasis. Moreover, we found that the miR-122-induced mesenchymal phenotype in ccRCC was negatively controlled by the Dicer-mediated miR-200 family. The literature clearly shows that a reciprocal repression between the miR-200 family and ZEB1/ZEB2 induces EMT. Dicer knockdown is also implicated in promoting EMT through the miR-200 family in breast cancer. Therefore, the existence of the miR-122/Dicer/miR-200 sec/EMT pathway may largely explain the metastatic properties induced by miR-122 overexpression in ccRCC.

In conclusion, we demonstrated that miR-122 expression is significantly correlated with tumor progression in ccRCC, and increased miR-122 level provides an independent prognostic value on ccRCC metastasis. Overexpression of miR-122-induced EMT by downregulating Dicer and its mediated miR-200 family, which facilitated the metastatic behavior of ccRCC cells in vitro and in vivo. Therefore, miR-122 may be considered as a novel predictive biomarker and therapeutic target for metastatic ccRCC.

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