Abstract. Renal cell carcinoma (RCC) is the most common kidney malignancy, responsible for ~80% of all cases in adults. The pathogenesis of RCC is complex, involving alterations at both the genetic and epigenetic levels. Numerous signaling pathways, such as PI3K/Akt/mTOR and Wnt-β-catenin have been demonstrated to be associated with the tumorigenesis and development of RCC. Long non-coding RNAs (lncRNAs) are functional RNA molecules involved in the initiation and progression of cancer, and investigating the effects of IncRNA could facilitate the development of novel treatments. The lncRNA regulator of reprogramming (ROR) is aberrantly expressed in a variety of tumors. However, its underlying mechanisms remain largely unknown. In the present study, ROR was found to be upregulated and micro rna (miR)-206 was found to be downregulated in RCC tissues and cells. Furthermore, the knockdown of ROR inhibited the proliferation, migration and invasion of RCC cells. It was found that ROR binds to miR-206, and that ROR-induced cell proliferation and metastasis were reversed by the overexpression of miR-206. In addition, the levels of miR-206 and ROR were negatively correlated in RCC tissues. Furthermore, the overexpression of miR-206 notably suppressed the proliferation, migration and invasion of RCC cells, and these effects were enhanced by the knockdown of vascular endothelial growth factor (VEGF); cell growth and metastasis induced by miR-206 inhibitors could be reversed by the knockdown of VEGF. In addition, the expression levels of miR-206 and VEGF were inversely correlated in RCC samples. In summary, the results of the present study revealed that ROR was upregulated in RCC tissues, which promoted tumor progression by regulating the miR‑206/VEGF axis. The present findings provided a novel insight into the potential functions of ROR in RCC, and the ROR/miR-206/VEGF pathway may be a promising therapeutic target for the treatment of patients with RCC.

Introduction

Renal cell carcinoma (RCC) is the most prevalent kidney cancer in adults, accounting for ~80% of patients with kidney cancer in European countries between 2006 and 2011 (1). Although multimodal approaches for diagnosis have been previously developed, such as ultrasound and computed tomography technologies, it remains difficult to distinguish between benign and malignant tumors (2). Therefore, it is important to discover novel non-invasive diagnostic and prognostic biomarkers for RCC. In 2015, there was a ~66.8% increase in the number of new cases, and ~23.4% increase in mortality, of renal cancer reported in China (3). The most common subtype of RCC is clear cell RCC, followed by papillary RCC and chromophobe RCC (4). The pathogenesis of RCC is complex, and previous studies have reported that alterations at both the genetic and epigenetic levels contribute to the development of RCC (5-9); however, the mechanisms underlying the initiation and progression of RCC remain largely unknown.

Long non-coding RNAs (lncRNAs) are a type of RNAs that are >200 nucleotides in length (10). lncRNAs are associated with numerous biological functions, including the regulation of cell proliferation and gene expression (10-13). Accumulating evidence has revealed that IncRNAs are associated with the initiation and development of numerous types of cancer, and they may function as oncogenes or tumor suppressors (14-16). In addition, the impaired expression of IncRNAs has been detected in tumor cells, suggesting an important role of IncRNAs in carcinogenesis (16-21). Aberrant levels of IncRNAs have been reported in RCC (22-25). Therefore, investigating the effects of misregulated IncRNAs in RCC may facilitate the development of novel therapies.

The lncRNA regulator of reprogramming (ROR) is involved in carcinogenesis, and previous studies have suggested the role of ROR in cancer (26,27). ROR has been reported to regulate the initiation and progression of tumors through various signaling pathways, such as RAD18 and SOX9 (28,29). Furthermore, our previous study revealed that ROR is a promising biomarker for RCC (30); however, the molecular targets of ROR require further investigation.
MicroRNAs (miRNAs/miRs) are non-coding RNAs and are ~22 nucleotides in length, and are potential downstream targets of IncRNAs (31). Emerging evidence has revealed that the expression of miRNAs is misregulated in cancer, which consequently initiates tumorigenesis (32,33). Additionally, miRNAs, such as miR-122-5p and miR-206, are novel biomarkers for patients with RCC (34); however, the functions of miRNAs in RCC remain unclear.

Vascular endothelial growth factor (VEGF) is a soluble ligand secreted by cells that stimulates the formation of blood vessels, and it is a potent pro-angiogenic factor involved in wound healing and pathogenic processes, including carcinogenesis (35). In the present study, the function of the ROR-mediated miR-206/VEGF signaling pathway in RCC cell growth and metastasis was investigated, which may provide novel insights into the treatment of patients with RCC.

Materials and methods

Clinical samples. A total of 36 paired RCC and para-carcinoma tissues were collected from patients (16 male and 20 female, average age 46±12) who underwent radical nephrectomy in the Department of Urology (The First Affiliated Hospital of Jinzhou Medical University) between June 2014 and July 2015. None of the patients recruited in the present study had received any other treatments prior to surgery. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Jinzhou Medical University. Written informed consent was obtained from each patient. The protocols were approved by the Institutional Review Board of The First Affiliated Hospital of Jinzhou Medical University. All kidney tissues samples were immediately snap-frozen using liquid nitrogen and stored at -80˚C until further use.

Cell culture. The human RCC cell lines Caki-1 and Caki-2, and normal human kidney cells HK-2, were purchased from the American Type Culture Collection. Cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and and 100 µg/ml streptomycin (all from Gibco; Thermo Fisher the a merican Type c ulture collection. c ells were cultured

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Cell transfection. Short hairpin (sh)RNA sequences targeting ROR (sh-ROR), VEGF (sh-VEGF), negative control (sh-NC), miR-206 mimic/inhibitor and miRNA control (mir-NC) were synthesized by Guangzhou RiboBio Co., Ltd. The sequences were: sh-ROR: 5’-Gcc TcT Gca cTc TTa TGG aaG GaG Gaa Ga-3’; sh-VEGF: 5’-Gcc TcT Gca cTc TTa TGG aaG GaG Gaa Ga-3’; sh-NC: 5’-cGa GGa ccG ccT GTc cTG aTc Tc-3’. Following annealing, shrna were inte-

Western blot analysis. Total protein from tissues or cells was extracted using RIPA buffer (Beyotime Institute of Biotechnology). The protein concentration was evaluated using the bicinchoninic acid method. Equal amounts (50 µg) of protein samples were loaded on to 10% SDS-PAGE gels and transferred onto PVDF membranes. Subsequently, the membranes were blocked in TBST containing 5% skimmed milk for 2 h at room temperature. The membranes were then incubated with primary antibodies against VEGF (1:500; cat. no. MA5-13182; Invitrogen; Thermo Fisher Scientific, Inc.).
Inc.) or GAPDH (1:1,000; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.) at 4°C overnight. Following three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. sc-2371; Santa Cruz Biotechnology, Inc.) for 1 h at 37°C. Protein bands were visualised using an ECL detection kit (Pierce; Thermo Fisher Scientific, Inc) and quantified by densitometric analysis using ImageJ 1.49 software (National Institutes of Health).

Cell proliferation assays. Transfected cells were harvested 24 h post-transfection and seeded into 96-well plates at a concentration of 5,000 cells/well. Cells were then incubated at 37°C and cell proliferation was determined using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.) at day 1, 2, 3 and 4 according to the manufacturer's protocols. Briefly, 10 µl of CCK-8 solution was added into each well at the indicated time points. Following incubation at 37°C for a further 2 h, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc.).

Transwell assay. The migration and invasion of cells was evaluated using a Transwell assay. For the migration assay, a total of 2X10⁵ cells in FBS-free DMEM were seeded into the upper chamber (BD Biosciences) with an 8 µm pore size. For the invasion assay, cells were inoculated onto a Matrigel-pre-coated (room temperature for 1 h) upper chamber (Sigma-Aldrich; Merck KGaA). Subsequently, 500 µl of culture medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) was added into the lower chamber. Following overnight incubation at 37°C, cells that had not migrated/invaded were removed using a cotton swab, while the migrated/invaded cells in the lower chamber were fixed with 4% paraformaldehyde at room temperature for 10 mins and stained using 0.5% crystal violet at room temperature for 20 mins. The numbers of migratory/invasive cells were counted in five randomly selected fields using an inverted light microscope (magnification, x200; Olympus Corporation).

Bioinformatic prediction and luciferase reporter assay. TargetScan 6.2 (www.targetscan.org/) and miRanda 0.10.x (www.microrna.org/microrna/) were employed to predict the potential targets of ROR and miR-206. Wild-type (WT) fragments of the 3’ untranslated region (UTR) of ROR and VEGF containing the potential binding sites of miR-206 were synthesized by Shanghai GenePharma Co., Ltd. and were cloned into pmirGLO Dual-Luciferase miRNA Target Expression Vector using Xhol and XbaI restriction sites (Promega Corporation), according to the manufacturer's protocols. QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene; Agilent) was used to generate the ROR/VEGF-3’UTR-MUT reporter containing mutant miR-206 binding sites. The luciferase vectors were co-transfected with miR-NC or miR-206 mimics/inhibitors (50 pg/µl) into DH5α competent cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). Luciferase activity was assessed at 48 h post-transfection using a Dual Luciferase Reporter Assay System (Promega Corporation), according to the manufacturer's protocols. The level of firefly luciferase activity was normalized to that of Renilla luciferase.

Statistical analysis. SPSS 17.0 software (SPSS, Inc.) was used for statistical analysis. All experiments were repeated a minimum of three times. Data are presented as the mean ± SD and were analyzed using a Student's t-test or ANOVA. A Student-Newman-Keuls test was performed as a post-hoc test following ANOVA. The association between RNA levels was evaluated using Spearman's correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

ROR is upregulated and miR-206 is downregulated in RCC tissues and cells. The upregulation of ROR was detected in RCC compared with the adjacent normal tissues, which may be associated with poorer prognosis as described in our previous study (33). In addition, the expression of ROR was increased in RCC cells compared with non-RCC cells lines (33). In the present study, the expression level of miR-206 RNA in 36 paired RCC and para-carcinoma samples was determined using RT-qPCR. The present results indicated that miR-206 was significantly downregulated in glioma tissues compared with the control (Fig. 1A). Furthermore, miR-206 RNA was significantly decreased in aggressive RCC, suggesting that downregulation of miR-206 is associated with the development of this disease (Fig. 1B). In addition, the expression levels of ROR and miR-206 were found to be negatively correlated in RCC tissues (Fig. 1C). miR-206 was significantly downregulated in RCC cell lines in comparison with HK-2 cells (Fig. 1D). The present results suggested that the expression levels of ROR and miR-206 were upregulated and downregulated in RCC, respectively, which may be associated with the progression of this disease.

Downregulation of ROR suppresses the proliferation, migration and invasion of RCC cells. To explore the effects of ROR on the proliferation, invasion and migration of RCC cells, the expression of ROR was decreased in Caki-1 and Caki-2 cells. The transfection efficiency was determined using RT-qPCR (Fig. 2A). The results of the CCK-8 assay indicated that the proliferative ability of Caki-1 and Caki-2 cells transfected with sh-ROR was reduced compared with the control (Fig. 2B and C). In addition, Transwell assays indicated that the migration and invasion of sh-ROR-transfected cells was significantly reduced (Fig. 2D-G). These results suggested that the knockdown of ROR inhibited the proliferation, migration and invasion of RCC and may be involved in the development and progression of RCC.

miR-206 is a potential target gene of ROR in RCC cells. To determine whether ROR exerts its functions in RCC by suppressing target miRNAs, the potential binding sites of miR-206 in ROR transcripts were predicted using miRanda (Fig. 3A). Luciferase reporter vectors containing WT (WT-ROR) and mutant ROR (MUT-ROR) sequences of the predicted miR-206 binding sites were constructed. The results revealed that the overexpression of miR-206 significantly attenuated the activity of the luciferase plasmid carrying the WT binding sites, which was not observed in the MUT control (Fig. 3B). In order to further investigate the influence of ROR on the expression of miR-206, Caki-1 and Caki-2 cells were
transfected with sh-ROR. Cells transfected with sh-ROR exhibited significantly increased miR-206 expression, which was also detected in cells transfected with the miR-206 mimic (Fig. 3C and D), suggesting that miR-206 may be a novel target of ROR in RCC.

Overexpression of ROR promotes cell proliferation, migration and invasion by regulating miR-206. To investigate whether ROR suppresses the proliferation and metastasis of RCC cells by targeting miR-206, Caki-1 and Caki-2 cells were transfected with o/e-NC, o/e-ROR or co-transfected with o/e-ROR and the miR-206 mimic. The expression of ROR was significantly increased (Fig. 4A) and the level of miR-206 was decreased (Fig. 4B) in Caki-1 and Caki-2 cells transfected with o/e-ROR. Additionally, the overexpression of ROR promoted the proliferation (Fig. 4C and D), migration (Fig. 4E) and invasion (Fig. 4F) of Caki-1 and Caki-2 cells, whereas these effects were significantly reversed by the miR-206 mimic. These results suggested that ROR induced the proliferation, migration and invasion of RCC cells by downregulating miR-206.

VEGF is a target gene of miR-206 in RCC cells. Using the TargetScan database, the complementary sequence between VEGF and miR-206 was identified (Fig. 5A). To investigate whether VEGF was a potential target of miR-206, WT and MUT fragments of VEGF were cloned downstream of the firefly luciferase coding domain. The present results indicated that the overexpression of miR-206 significantly reduced the luciferase activity of the VEGF-WT reporter but not of the VEGF-MUT control (Fig. 5B). To further determine whether miR-206 regulates the expression of VEGF, Caki-1 and Caki-2 cells were transfected with the miR-206 inhibitor. The transfection efficiency was determined by evaluating the level of miR-206 (Fig. 5C). The protein level of VEGF was elevated in cells transfected with the miR-206 inhibitor (Fig. 5D). Furthermore, VEGF was upregulated in RCC tissues compared with the paired para-carcinoma controls (Fig. 5E) and VEGF expression was found to be inversely correlated with miR-206 in RCC samples (Fig. 5F), further suggesting that VEGF may be a target of miR-206 in RCC.

Downregulation of VEGF enhances the effects of miR-206 overexpression and reverses the effects of miR-206 inhibition in RCC cells. To investigate whether the effect of VEGF on the growth and metastasis of RCC cells was regulated by miR-206, Caki-1 and Caki-2 cells were transfected with miR-NC, miR-206 mimic/inhibitor or co-transfected with miR-206 mimic/inhibitor and sh-VEGF. The transfection efficiency of sh-VEGF was determined using RT-qPCR (Fig. 6A). The results revealed that the overexpression of miR-206 suppressed the proliferation (Fig. 6B and C), migration (Fig. 6F) and invasion (Fig. 6H) of Caki-1 and Caki-2 cells,
Figure 2. Downregulation of ROR suppresses the proliferation, migration and invasion of RCC cells. (A) The transfection efficiency of sh-ROR was accessed using reverse transcription quantitative PCR. The proliferation rate of (B) Caki-1 and (C) Caki-2 cells transfected with sh-ROR or sh-NC was determined using the Cell Counting Kit-8 assay. (D) The migration rate of transfected Caki-1 and Caki-2 cells was evaluated (magnification, x200) and (E) quantified using a Transwell assay. (F) The invasive abilities of Caki-1 and Caki-2 cells transfected with sh-ROR or sh-NC were determined (magnification, x200) and (G) quantified. *P<0.05 vs. sh-nc. nc, negative control; sh-, short hairpin RNA; ROR, long non-coding RNA regulator of reprogramming.
while these effects were increased by the depletion of VEGF. Additionally, the downregulation of miR-206 promoted the proliferation (Fig. 6D and E), migration (Fig. 6G) and invasion (Fig. 6I) of RCC cells, whereas these effects were abrogated following knockdown of VEGF. The present results suggested that miR-206 inhibits the growth of RCC cells by downregulating VEGF. In summary, ROR may regulate the proliferation, migration and invasion of RCC cells via the miR-206/VEGF signaling pathway.

Discussion

lncRNAs are a group of non-coding RNAs of >200 nucleotides in length. Previous studies have revealed the significance of lncRNAs, and accumulating evidence demonstrated that lncRNAs are important regulators of the growth and metastasis of cancer cells (14-17). lncRNAs act as oncogenes or suppressing factors in cancer; the dysregulation of lncRNAs is associated with the progression of numerous types of cancer including glioblastoma and astrocytoma (10,18-21). The upregulation of long intergenic non-protein coding RNA 01116 was reported to be associated with the overall survival of patients with cancer and metastasis (37). Furthermore, the downregulation of lncRNA-small nucleolar host gene 5 inhibited the proliferation and migration of gastric cancer cells through the miR-32/KLF4 axis (38). The expression level of prostate cancer upregulated lncRNA-1 (HincRNA-1) was found to be decreased in tumor tissues and the induced expression of HincRNA-1 was reported to suppress the proliferation and promote the apoptosis of breast cancer cells through the transforming growth factor-β1/D-3-phosphoglycerate dehydrogenase signaling pathway (39). A number of lncRNAs regulate gene expression by interacting with their target miRNAs. For example, the lncRNA H19 imprinted maternally expressed transcript was reported to modulate the proliferation, migration and invasion of gastric cancer cells through downstream miRNAs (40,41). Additionally, the lncRNA BC032469 was found to bind miR-1207-5p and human telomerase reverse transcriptase, inducing the proliferation of cancer cells (42). However, the roles of lncRNAs and their underlying mechanisms in cancer remain largely unknown and require further investigation.

Previous studies have revealed the impaired expression of ROR in prostate and breast cancer (26,27). Furthermore, ROR is involved in the initiation and progression of tumor by regulating numerous signaling pathways, such as RAD18 and SOX9 (28,29). The results of the present study indicated that ROR was significantly upregulated, while miR-206 was
downregulated, in RCC tissues, which may be associated with poor prognosis. In addition, the present study suggested a negative correlation between the levels of ROR and miR-206, and miR-206 and VEGF in RCC samples. Therefore, further experiments were conducted to explore the downstream targets of ROR in RCC.

The results of the present study indicated that the knockdown of ROR inhibited the proliferation, migration and invasion of RCC cells. Furthermore, the overexpression of ROR induced the proliferation, migration and invasion of RCC cells, whereas these effects were reversed by the overexpression of miR-206, suggesting that ROR promotes RCC
cell growth and metastasis in an miR-206-dependent manner. miRNAs may function as oncogenes or tumor suppressors and are potential targets of lncRNAs (31,32). Consistent with the finding of the present study, previous studies reported impaired levels of miRNAs in various cancer types, including RCC (32-34). Furthermore, it was reported that ROR was able to induce the development of osteosarcoma by regulating miR-206 (43).

In addition, luciferase reporter assay revealed that VEGF was a potential target of miR-206, and that the upregulation of miR-206 suppressed RCC cell proliferation, migration and invasion by targeting VEGF. Conversely, the downregulation of miR-206 induced the proliferation, invasion and migration of RCC cells, whereas these effects were abrogated following the depletion of VEGF. Furthermore, VEGF expression was found to be significantly upregulated in RCC tissues compared with

Figure 5. VEGF is a target gene of miR-206 in RCC cells. (A) The putative binding sites of VEGF in the miR-206 transcript. (B) The overexpression of miR-206 resulted in a significant decrease in the luciferase activity of WT-VEGF, whereas no change was observed in MUT-VEGF. (C) The expression levels of miR-206 in Caki-1 and Caki-2 cells transfected with miR-NC or miR-206 inhibitors were evaluated using reverse transcription-quantitative PCR. (D) The protein level of VEGF in transfected Caki-1 and Caki-2 cells was determined using western blot analysis. (E) The expression of VEGF was assessed in RCC and matched para-carcinoma tissues. (F) The RNA levels of miR-206 and VEGF were inversely correlated in RCC samples (r=-0.3602; P=0.0132). *P<0.05 vs. respective control. RCC, renal cell carcinoma; miR, microRNA; NC, negative control; VEGF, vascular endothelial growth factor; MUT, mutant; WT, wild-type.
Figure 6. Downregulation of VEGF enhances the effects of the overexpression of miR-206 and reverses the effects of the miR-206 inhibitor in RCC cells. (A) The transfection efficiency of sh-VEGF was determined by reverse transcription-quantitative PCR. The proliferation of (B) Caki-1 and (C) Caki-2 cells transfected with miR-NC, miR-206 mimic or co-transfected with miR-206 mimic and sh-VEGF was evaluated using the Cell Counting Kit-8 assay. The proliferation of (D) Caki-1 and (E) Caki-2 cells transfected with miR-NC, miR-206 inhibitors or co-transfected with miR-206 inhibitors and sh-VEGF was assessed. The migration of Caki-1 and Caki-2 cells transfected with (F) miR-206 mimic or (G) miR-206 inhibitor and co-transfected with sh-VEGF were determined using a Transwell assay. The invasion of Caki-1 and Caki-2 cells transfected with (H) miR-206 mimic or (I) miR-206 inhibitor and co-transfected with sh-VEGF were evaluated. *P<0.05 vs. miR-nc; #P<0.05 vs. miR-206 mimic. RCC, renal cell carcinoma; miR, microRNA; NC, negative control; VEGF, vascular endothelial growth factor; sh-, short hairpin.
the matched non-tumor controls, and was negatively correlated with the level of miR-206. VEGF is a signal protein produced by cells that stimulates the formation of blood vessels and is a potent proangiogenic factor involved in wound healing, and pathogenic processes, including carcinogenesis (35). A previous study reported that the overexpression of VEGF was associated with poor survival for patients with squamous cell carcinoma (44). Furthermore, the downregulation of miR-206 induced the development of breast and laryngeal cancer through the VEGF pathway (45,46). Consistent with these findings, the present study indicated that ROR was upregulated in RCC, which may promote the development of tumors via the miR-206/VEGF signaling pathway. However, there were some limitations to the present study, for example, markers of proliferation and apoptosis were not examined; such markers should be investigated in future studies to support the findings of the present study.

In conclusion, the present study indicated that ROR was a potential oncogene, which could increase the level of VEGF and induce RCC cell proliferation and migration through miR-206. The findings of the present study indicated the important roles of ROR and its underlying mechanisms in the proliferation, migration and invasion of RCC cells. The present study suggested that the ROR/miR-206/VEGF signaling pathway may be a novel therapeutic target for the treatment of patients with RCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

JS initiated and designed the present study. DZ, ZZ and WZ performed the experiments and interpreted the results. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Jinzhou Medical University. Written informed consent was obtained from each patient for the use of clinical tissues.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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