

miR-148a increases the sensitivity to cisplatin by targeting Rab14 in renal cancer cells

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Abstract. MicroRNA (miR) can exert various biological functions by targeting oncogenes or tumor suppressor genes in numerous human malignancies. Recent evidence has shown that miR-148a increases the drug sensitivity of various cancer cells. Herein, we show that ectopic expression of miR-148a induces apoptosis, reduces clonogenicity, and increases the sensitivity to TRAIL and cisplatin in renal cancer cells. The luciferase reporter assay showed that miR-148a negatively regulated ras-related protein 14 (Rab14) expression by binding to the miR-148a binding site in the 3' untranslated region (3'UTR) of Rab14. Rab14-specific siRNA-induced down-regulation of Rab14 increases the sensitivity to cisplatin, while forced expression of Rab14 lacking 3'-UTR abrogated the pro-apoptotic function of miR-148a in renal cancer cells. These findings suggest that miR-148a acts as a tumor suppressor and holds great potential for renal cancer therapy by directly targeting Rab14.

Introduction

MicroRNAs (miRs), which are 18-25-nucleotide-long small non-coding RNAs, can cause posttranscriptional repression by directly binding to the 3'-untranslational region (UTR) of mRNAs (1). Previous studies have shown that miRs play important roles in many pivotal biological processes such as cell growth, proliferation, and death (2,3).

Renal cell carcinoma (RCC) is one of the lethal urological malignancies in adults, with a high mortality rate of >40% (4,5). Approximately, 30% patients with localized RCC develop metastatic recurrence, even after radical resection of

the diseased kidney (6,7). Despite tremendous development in RCC therapy, patients with locally advanced and metastatic RCC still have poor prognosis (8). Therefore, there is an urgent need to improve the prognosis for patients with RCC and to identify novel therapeutic targets for controlling the metastatic potential of RCC and modulating apoptotic pathways in RCC. Since miRs are important genetic regulators modulating their target genes, miRs could be good candidates for regulating RCC progression and development as well as for enhancing cell death. For example, miR-148b enhances proliferation and apoptosis in human renal cancer cells by directly targeting MAP3K9 (9). In addition to the tumor-suppressive effects exerted by the miR, several miRs sensitized renal cancer cells to anticancer drugs such as sorafenib, imatinib, and 5-FU by targeting apoptosis-regulating genes (10-12).

In recent years, miR-148a was found to be aberrantly expressed in various cancers and has been demonstrated to act as an oncogene or tumor suppressor with crucial roles in the molecular mechanisms underlying oncogenesis (13-16). In addition, the ectopic expression of miR-148a attenuated the paclitaxel resistance of prostate cancer cells by suppressing the expression of mitogen- and stress-activated kinase 1 (MSK1) (17). Unfortunately, the miR-148-involving molecular mechanisms associated with the regulation of renal cancer cell proliferation and drug sensitivity are still unknown. Therefore, we investigated the role of miR-148a in apoptosis and chemosensitivity of human renal cancer cells by targeting the Ras-related protein 14 (Rab14).

Materials and methods

Cells and materials. Caki cells (human renal cancer cells) were purchased from the American Type Culture Collection (Rockville, MD, USA), and maintained in RPMI-1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum. Anti-caspase-3 antibody was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Anti-PARP antibody was purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). Rab14 and actin antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Cisplatin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The recombinant human TRAIL was purchased from KOMA Biotech (Seoul, Korea). The miR-148a

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mimics and miR-148a inhibitors were purchased from Ambion (Austin, TX, USA).

Western blotting. Cellular lysates were prepared by suspending 0.5×10^6 cells in 100 μ l of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% TritonX-100, 25 mM MOPS, 100 μ M phenyl-methylsulfonyl fluoride, and 20 μ M leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. The lysate containing proteins was quantified using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The proteins were electro-transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). Detection of specific proteins was carried out with an ECL Western blotting kit (Millipore Corp.), according to the manufacturer's instructions.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from cells using eazyBlue reagent (Intron Biotechnology, Seongnam-si, Gyeonggi-do, Korea). cDNA was synthesized from 2 μ g of total RNA by using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA). The cDNA for Rab14 and actin were amplified by PCR with specific primers. For Rab14, the sense and anti-sense primers were 5'-ATGGCAACTGCACCATACAA-3' and 5'-GCCACAGCAAAGAGGTCACT-3', respectively. PCR products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide.

Flow cytometry-based analysis. Approximately 0.5×10^6 MDA-MB-231 cell were suspended in 100 μ l of phosphate-buffered saline (PBS), and 200 μ l of 95% ethanol was added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS, and resuspended in 250 μ l of 1.12% sodium citrate buffer (pH 8.4), with 12.5 μ g of RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250 μ l of propidium iodide (PI, 50 μ g/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescence-activated cell sorting (FACS) on a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) for relative DNA content based on red fluorescence. Cell undergoing apoptosis will be a part of the DNA (due to DNA fragmentation during later stages of apoptosis). These cells may be detected as a 'sub-G1' population. Cells were further analyzed by flow cytometry using BD FACS Canto II flow cytometer (BD Biosciences), and a PI/Annexin staining kit (BD Biosciences).

Luciferase reporter assays. For the basic 3-UTR luciferase reporter assay, Caki cells were transfected with the Rab14 3'-UTR-pmirGLO Dual-Luciferase reporter plasmid (Promega, Madison, WI), miR-cont, miR-148a, or anti-miR-148a using Lipofectamine 2000. Luciferase activity assays were then performed and normalized to *Renilla* luciferase activity. The experiments were repeated three times.

4',6'-Diamidino-2-phenylindole staining for nuclear condensation and fragmentation. To examine cellular nuclei, the cells were fixed with 1% paraformaldehyde on glass slides

for 30 min at room temperature. After fixation, the cells were washed with PBS and 300 nM 4',6'-diamidino-2-phenylindole solution (Roche, Mannheim, Germany) was added to the fixed cells for 5 min. After the nuclei were stained, cells were examined by fluorescence microscopy.

Statistical analysis. Three or more separate experiments were performed. Statistical analysis was conducted with the paired Student's t-test. $P < 0.05$ was considered as a significant difference between the experimental and control groups.

Results

miR-148a inhibits renal cancer cell proliferation and promotes apoptosis. To examine the functional significance of miR-148a in RCC, renal cancer cells were transfected with miR-148a. The percentage of sub-G1 population markedly increased in response to miR-148a transfection, compared to miRNA-cont transfection of Caki cells 24 h after transfection (Fig. 1A). We next examined whether transfection with miR-148a resulted in the activation of caspases in Caki cells. Forced expression of miR-148a in Caki cells led to a significant decrease in the protein levels of procaspase-3 precursors at 48 h after transfection (Fig. 1C). Similarly, transfection with the miR-148a mimics resulted in the activation of caspase pathway, compared to miRNA-cont-transfected cells (Fig. 1B).

miR-148a sensitizes renal cancer cells to TRAIL-induced apoptosis. To examine the functional role of miR-148a in drug-mediated apoptosis in Caki cells, miR-148a-transfected cell lines were treated with TRAIL and cytotoxicity were examined using FACS. As shown in Fig. 2A and B, transfection with miR-148a caused a significant increase in the fraction of cells in the sub-G1 phase compared to the miRNA-cont-transfected cells following TRAIL treatment. As shown in Fig. 2C, treatment of Caki/miR-148a cells with TRAIL resulted in the cleavage of PARP and procaspase-3. Treatment with TRAIL decreased the clonogenicity of Caki/miR-148a cells compared to Caki/miRNA-cont cells (Fig. 2D).

miRNA-148a sensitizes renal cancer cells to cisplatin-induced apoptosis. We next investigated whether miR-148a could increase the sensitivity of renal cancer cells to anticancer drugs such as cisplatin. Cisplatin treatment of miR-148a-transfected cells caused a marked increase in the fraction of cells in the sub-G1 phase compared to the cells expressing miRNA-cont, as well as activation of caspase pathways (Fig. 3A and B). As shown in Fig. 3C, cisplatin treatment of miR-148a-transfected cells led to a decrease in the protein levels of procaspase-3, with the concomitant cleavage of PARP protein. In addition, treatment with cisplatin decreased the clonogenicity of Caki/miR-148a cells compared to Caki/miRNA-cont cells (Fig. 3D). As shown in Fig. 3E, miRNA-148 plus cisplatin treatment enhanced the number of TUNEL-positive cells. These results indicate that the miRNA-148 plus cisplatin-induced apoptosis were involved in the activation of caspase-dependent apoptotic pathways.

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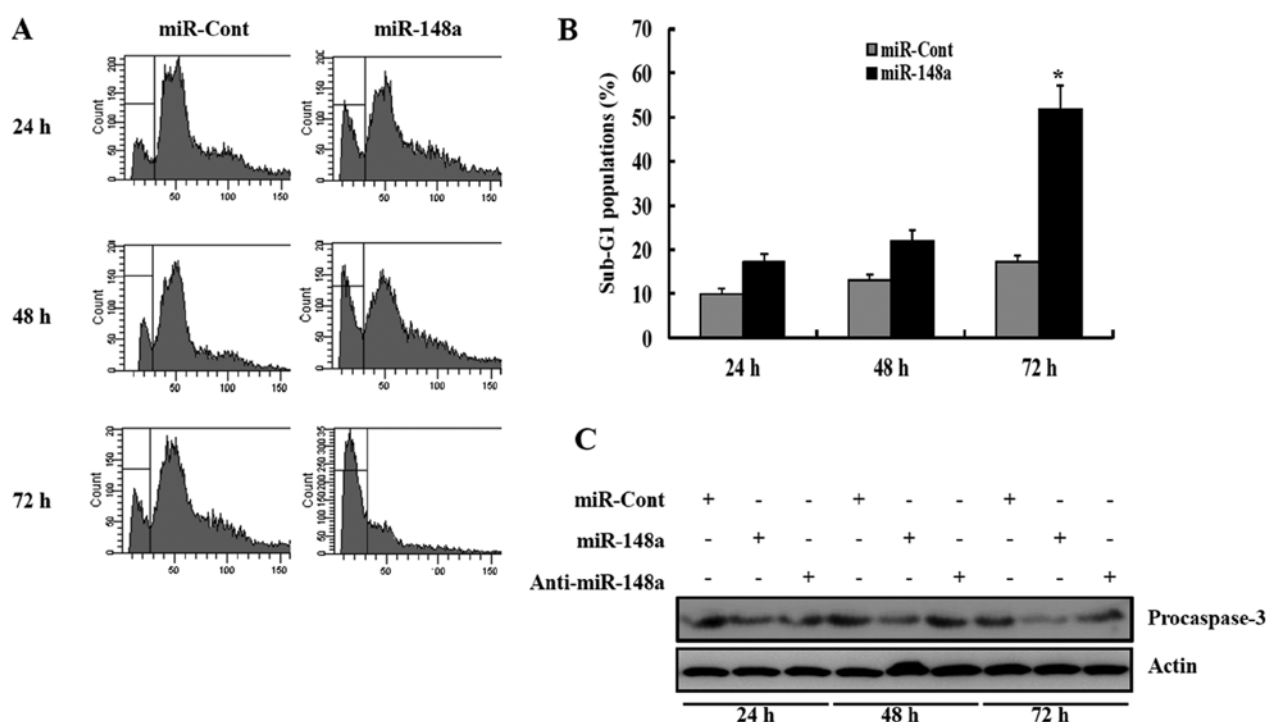


Figure 1. miR-148a promotes apoptosis. (A and B) Caki cells were transfected with miR-148a or miRNA-cont for 24, 48, and 72 h. Apoptosis was analyzed as the sub-G1 fraction obtained upon FACS (Histogram, A). Flow cytometry-based analysis of apoptotic cells (Graph, B). Data are reported as the mean \pm SD (n=3). *P<0.05, with respect to miRNA-cont-transfected cells. (C) Transfection with miR-148a induced the cleavage of procaspase-3 after 24 h. Equal amount of cell lysates (40 μ g) was subjected to electrophoresis and analyzed by western blotting with caspase-3 antibodies. Actin was used as the loading control in all immunoblots.

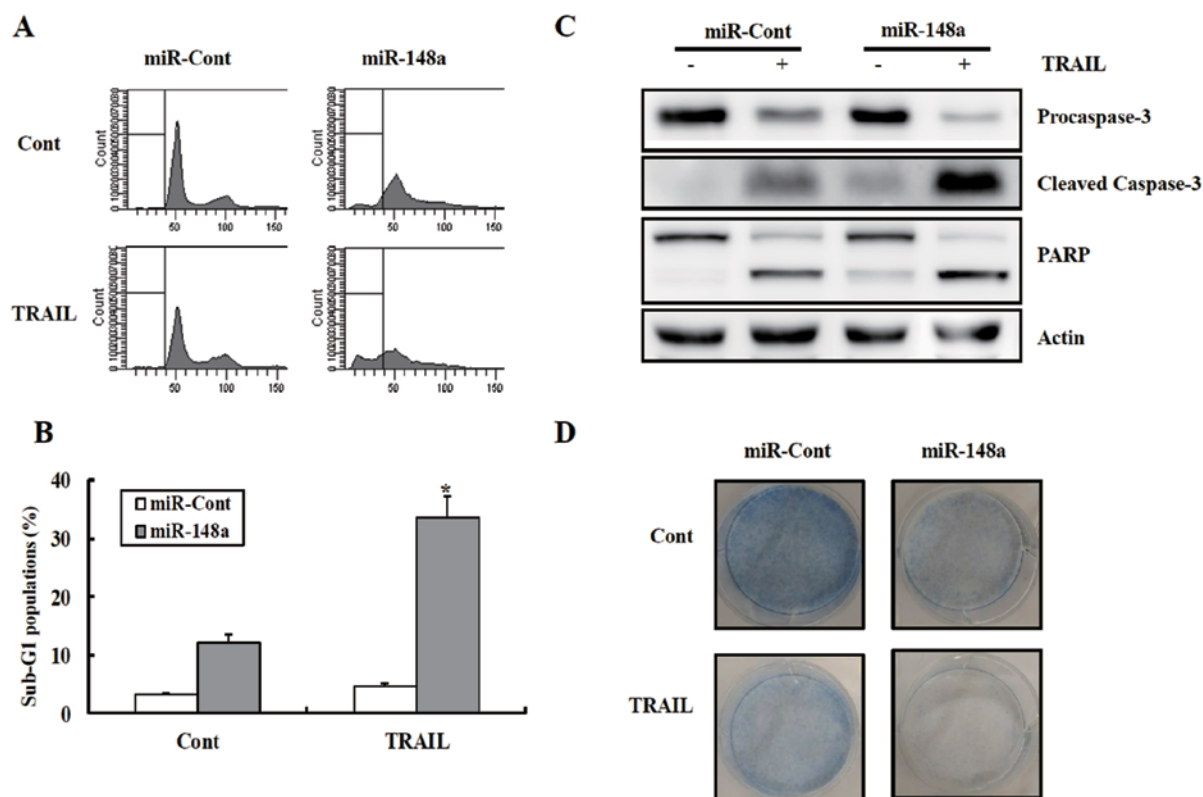


Figure 2. miR-148a sensitizes renal cancer cells to TRAIL-induced apoptosis. (A and B) After Caki cells were transfected with miR-148a or miRNA-cont for 24 h, both cells were treated with TRAIL for 24 h. Apoptosis was analyzed as the sub-G1 fraction obtained upon FACS (Histogram, A). Flow cytometry-based analysis of apoptotic cells (Graph, B). Data are reported as the mean \pm SD (n=3). *P<0.05, with respect to miRNA-cont-transfected cells. (C) Equal amount of cell lysates (40 μ g) was subjected to electrophoresis and analyzed by western blotting with anti-caspase-3 and anti-PARP antibodies. Actin was used as the loading control in all immunoblots. (D) Caki/miR-148a and Caki/miRNA-cont cells were treated with TRAIL for 24 h. Clonogenic assay was performed as described in Materials and methods.

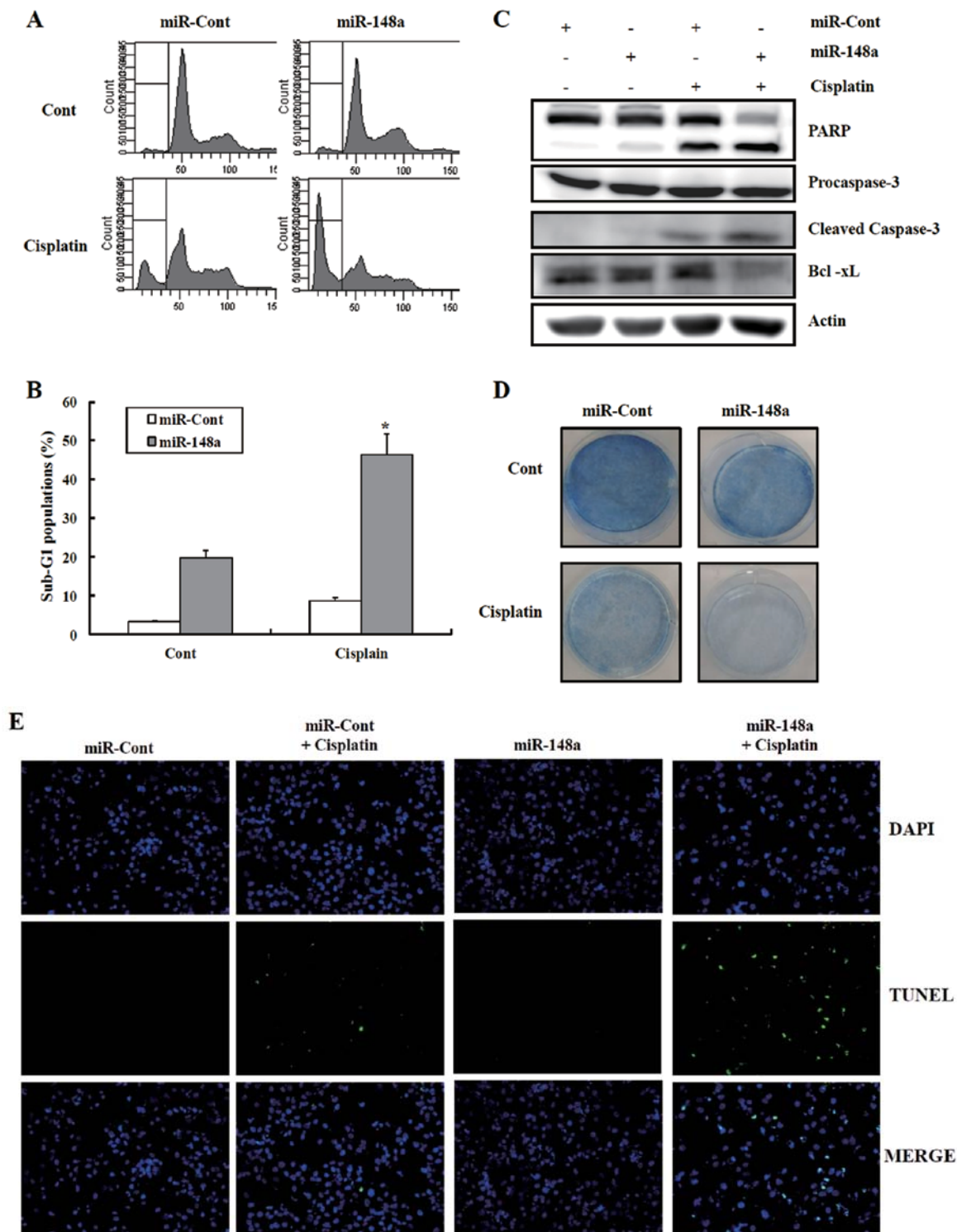


Figure 3. miR-148a sensitizes renal cancer cells to cisplatin-induced apoptosis. (A and B) After Caki cells were transfected with miR-148a or miRNA-cont for 24 h, both cells were treated with cisplatin for 24 h. Apoptosis was analyzed as the sub-G1 fraction obtained upon FACS (Histogram, A). Flow cytometry-based analysis of apoptotic cells (Graph, B). Data are reported as the mean \pm SD (n=3). *P<0.05, with respect to miRNA-cont-transfected cells. (C) Equal amount of cell lysates (40 μ g) was subjected to electrophoresis and analyzed by western blotting with anti-caspase-3 and anti-PARP antibodies. Actin was used as the loading control in all immunoblots. (D) Caki/miR-148a and Caki/miRNA-cont cells were treated with cisplatin for 24 h. Clonogenic assay was performed as described in Materials and methods. (E) Caki/miR-148a and Caki/miRNA-cont cells were treated with cisplatin, TUNEL-stained, and observed by fluorescence microscopy at x200. TUNEL-positive cells are shown in green.

This study next examined whether the activation of caspase pathway plays a critical role in miRNA-148 plus cisplatin-induced apoptosis. miR-148a plus cisplatin-induced apoptosis was completely prevented by pretreatment with the general and

potent inhibitor of caspases, the z-VAD-fmk, as determined by FACS analysis (Fig. 4A and B). In addition, z-VAD-fmk treatment completely prevented these caspase-related events such as cleavage of procaspase-3 and PARP (Fig. 4C).

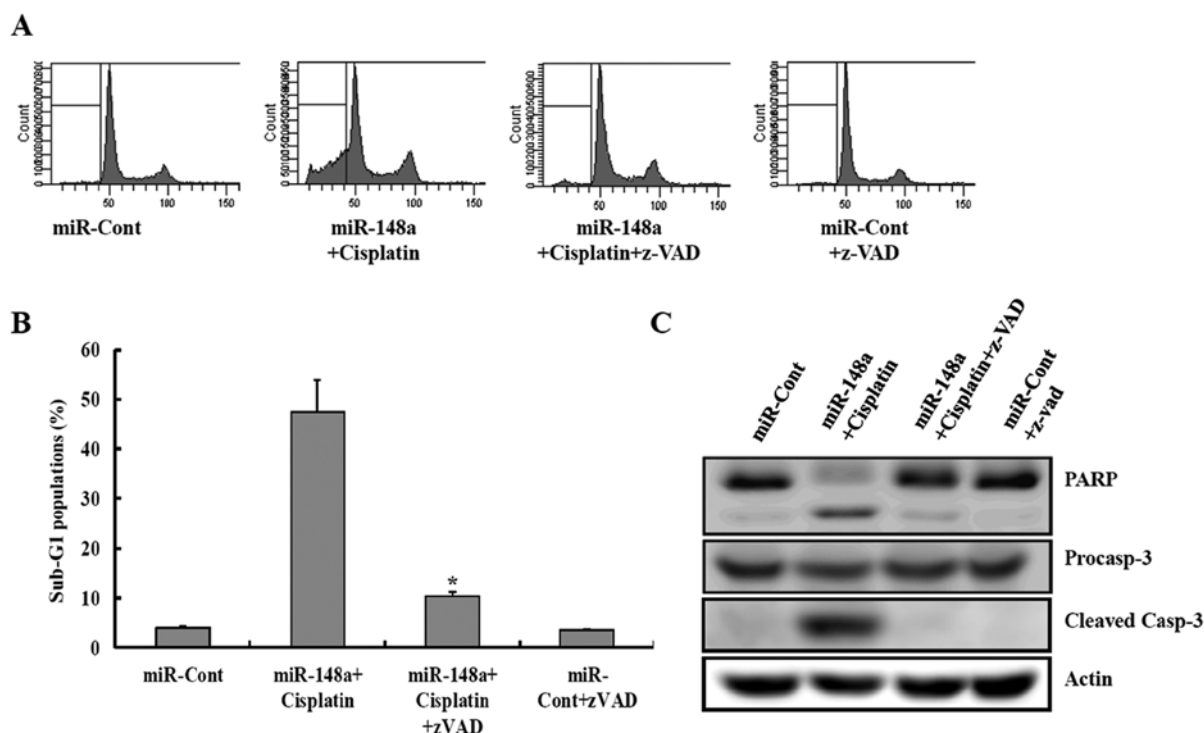


Figure 4. miRNA-148 plus cisplatin-induced apoptosis was involved in the activation of caspase-dependent apoptotic pathways. (A and B) Caki/miR-148a cells were incubated with 50 μ M z-VAD-fmk or solvent for 1 h before treatment with cisplatin for 24 h. Apoptosis was measured as the sub-G1 fraction obtained upon FACS. Data are the mean value obtained from three independent experiments; the bars represent the standard deviation. * $P < 0.05$ vs. MGO-treated cells. (C) Equal amounts of cell lysates (40 μ g) were subjected to electrophoresis and analyzed by western blotting for caspase-3 and PARP. Actin was used as the loading control.

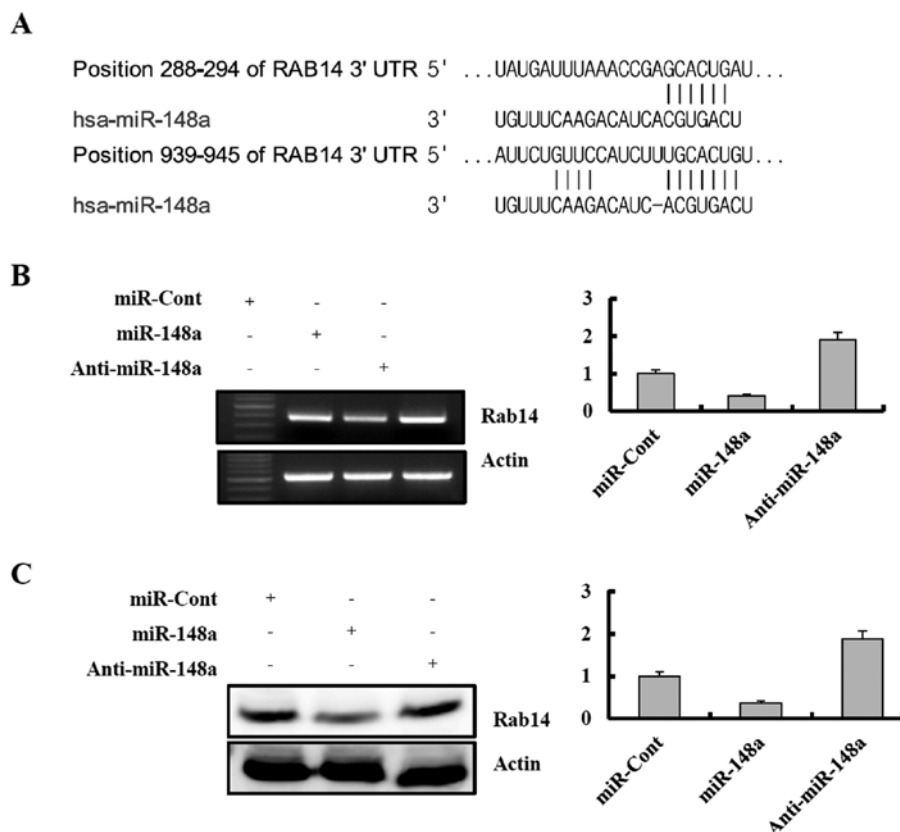


Figure 5. Identification of a novel gene regulated by miR-148a in renal cancer cells. (A) miR-148a-binding sites in the 3'-UTR of Rab14 mRNA. (B) RT-PCR analysis of Rab14 mRNA expression in transfected Caki cells. The relative levels of each Rab14 are expressed as the ratio of the densitometric value of each band to that of actin (right panel). (C) Immunoblots for Rab14 protein in the transfected Caki cells. Actin was used the loading control. The relative levels of each Rab14 in miRNA-transfected cells are expressed as the ratio of the densitometric value of each band to that of actin (right panel).

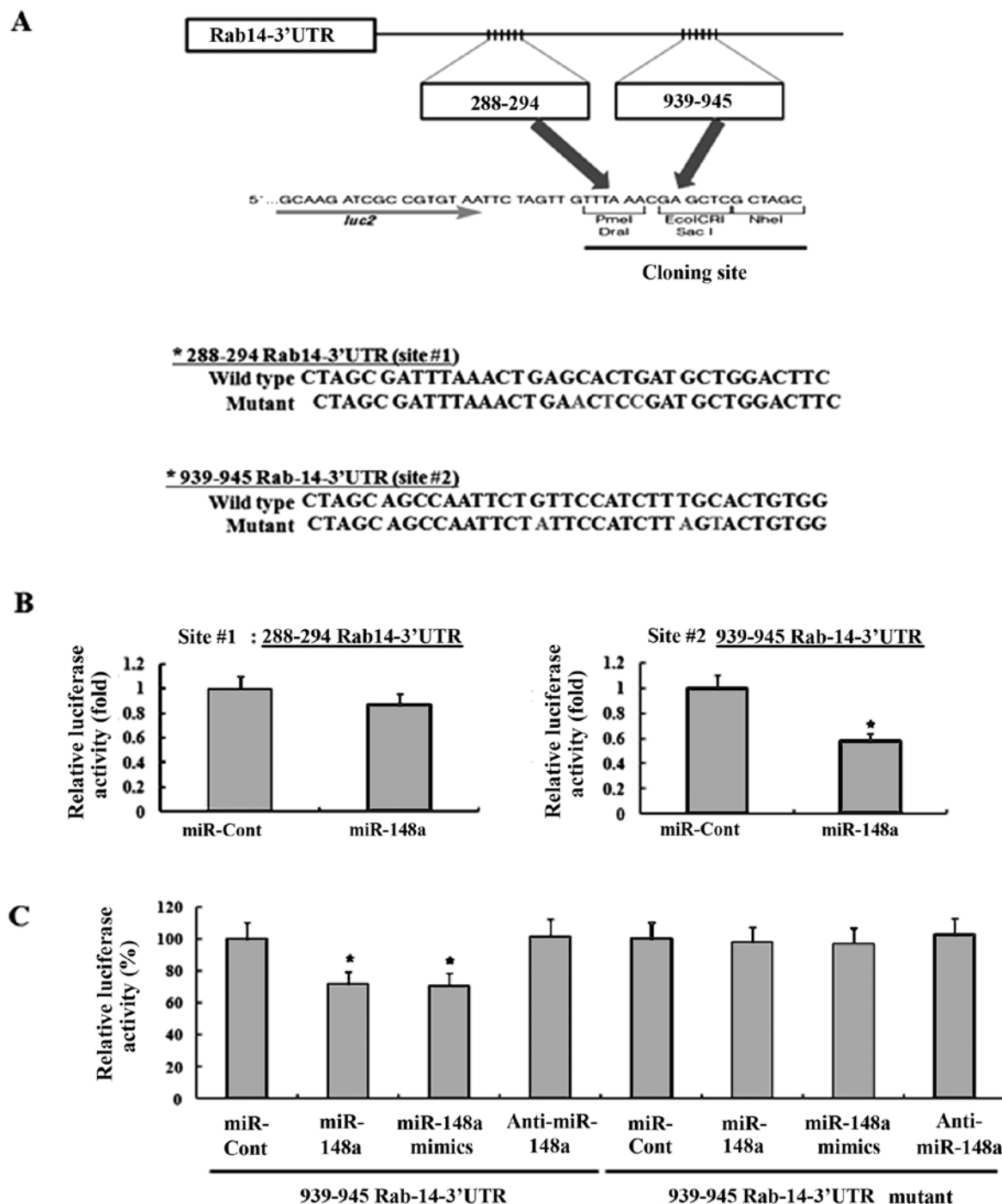


Figure 6. Rab14 is a functional target of miR-148a in renal cancer. (A) Schematic representation of Rab14 showing putative miR-148a target sites (left panel). The mutated nucleotides in the Rab14 3'-UTR mutant (right panel). (B) Luciferase activity assay with the corresponding wild-type luciferase constructs containing two different miR-148a target sequences (site #1 or site #2) transfected with miRNA-cont or miR-148a. For all luciferase activity assays, firefly luciferase values were normalized to *Renilla* luciferase activity, and plotted as the relative luciferase activity. Data are reported as the mean \pm SD (n=3). Student's t-test for unpaired values was used. *P<0.05, with respect to miRNA-cont-transfected cells. (C) Luciferase assays showing the repression of wild-type UTR (Rab14 3'-UTR) or mutant UTR (Rab14 3'-UTR-mutant), following the transfection of miR-148a, miR-148a mimics, miR-148a-708, and miRNA-cont. Data are shown as the mean \pm SD (n=3). *P<0.05, with respect to miRNA-cont-transfected cells.

miR-148a post-transcriptionally reduces *Rab14* expression by directly targeting its 3'-UTR. A bioinformatic analysis program, TargetScan, was used to identify putative protein-coding gene targets of miR-148a. The TargetScan miRNA target predictions showed that Rab14 3'-UTR contained two potential binding sites for miR-148a at the nucleotides 288 and 939 (Fig 5A, http://www.targetscan.org/cgi-bin/targetscan/vert_61/

[view_gene.cgi-taxid=9606&rs=NM_016322&members=miR-148ab-3p/152&showcnc=0&shownc=0](http://www.targetscan.org/cgi-bin/targetscan/vert_61/view_gene.cgi-taxid=9606&rs=NM_016322&members=miR-148ab-3p/152&showcnc=0&shownc=0)). To determine whether exogenous miR-148a could repress Rab14 expression, Caki cells were transiently transfected with premature miR-148a or a control miRNA (miRNA-cont) for 24 h. Rab14 expression was analyzed by RT-PCR and western blotting. As shown in Fig. 5B and C, ectopic expression of miR-148a inhibited Rab14

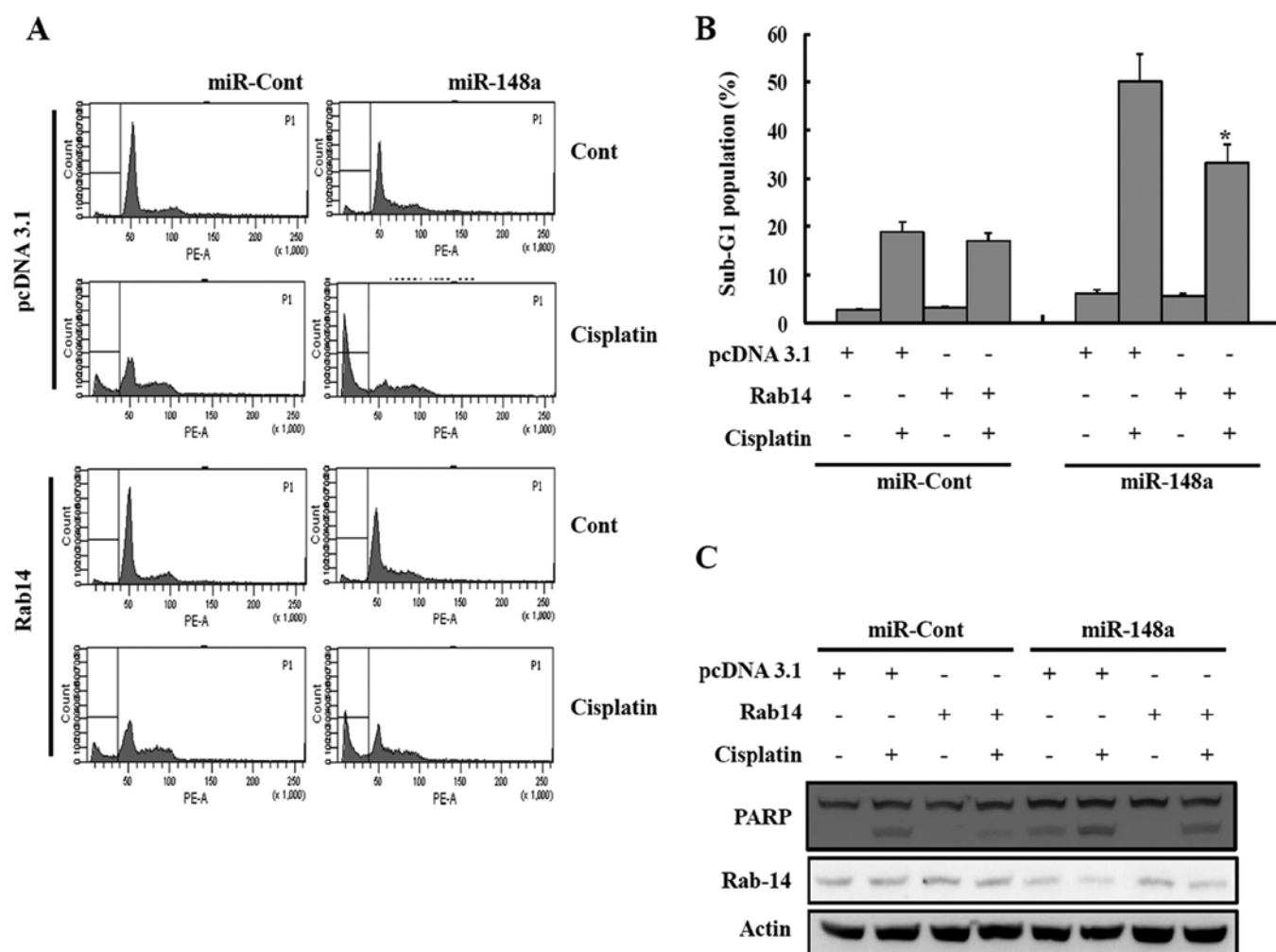


Figure 7. miR-148a-mediated upregulation of Rab14 decreases the sensitivity to cisplatin. (A) The control pcDNA3.1 or Rab14 expression vectors were transiently cotransfected with miR-148a and treated with cisplatin in Caki cells for 24 h. The cells were harvested and analyzed by FACS. (B) The control pcDNA3.1 or Rab14 expression vectors were transiently cotransfected with miR-148a and treated with cisplatin in Caki cells for 24 h. Data are reported as the mean \pm SD (n=3). *P<0.05 with respect to the drug-treated vector cells transfected with pcDNA3.1. (C) Western blotting was performed using anti-Rab14 and actin antibodies to confirm the efficiency of transfection.

mRNA and protein expression in a dose-dependent manner. In contrast, transfection with anti-miR-148a resulted in an increase in Rab14 expression in Caki cells (Fig. 5B and C).

Next, it was investigated whether the 3'-UTR of Rab14 was a functional target of miR-148a in RCC. As miR-148a could bind to two different regions of the 3'-UTR of Rab14 mRNA (Fig. 5A), we investigated which of the two regions was involved in miR-148a binding. The predicted miRNA-binding sequences of Rab14 (sites 1 and 2) were cloned into the downstream region of a luciferase reporter construct (pmirGLO-Rab14 #1 and pmirGLO-Rab14 #2, Fig. 6A). Caki cells were transiently transfected with these constructs in the presence of either pre- miR-148a or miRNA-cont. As shown in Fig. 6B, miR-148a markedly reduced the luciferase activity of pmirGLO-Rab14#2 compared to miRNA-cont, but miR-148a slightly decreased the luciferase activity of pmirGLO-Rab14 #1. These data suggested that miR-148a specifically bound to the 3'-UTR of Rab14 at nucleotide 939 and impaired Rab14 expression. In addition, miR-148a mimics significantly reduced the luciferase activity, compared to miRNA-cont. In contrast, the luciferase activity of the reporter

vector containing a mutated 3'-UTR in Rab14 was unaffected by miR-148a (Fig. 6C).

Overexpression of Rab14 decreases the sensitivity to cisplatin. As miR-148a can inhibit Rab14 expression by directly inhibiting the Rab14 transcript, it was investigated whether an increase of Rab14 expression could reduce the sensitivity to cisplatin. Therefore, miR-148a was ectopically expressed in Caki cells, together with a construct containing the Rab14-coding sequence but lacking the 3'-UTR of the Rab14 mRNA or an empty vector. After treatment with cisplatin, the accumulation of the sub-G1 population was lower in the Caki/Rab14 cells compared to the Caki/vector cells, indicating that the restoration of Rab14 counteracted the effects of miR-148a on the sensitivity to cisplatin in renal cancer cells (Fig. 7).

siRab14-mediated downregulation of Rab14 enhances the sensitivity to various apoptotic stimuli. To determine whether the anticancer effects of miR-148a in renal cancer cell lines were due to Rab14 inhibition or interaction with another gene, Caki cells were transiently transfected with a small interfering

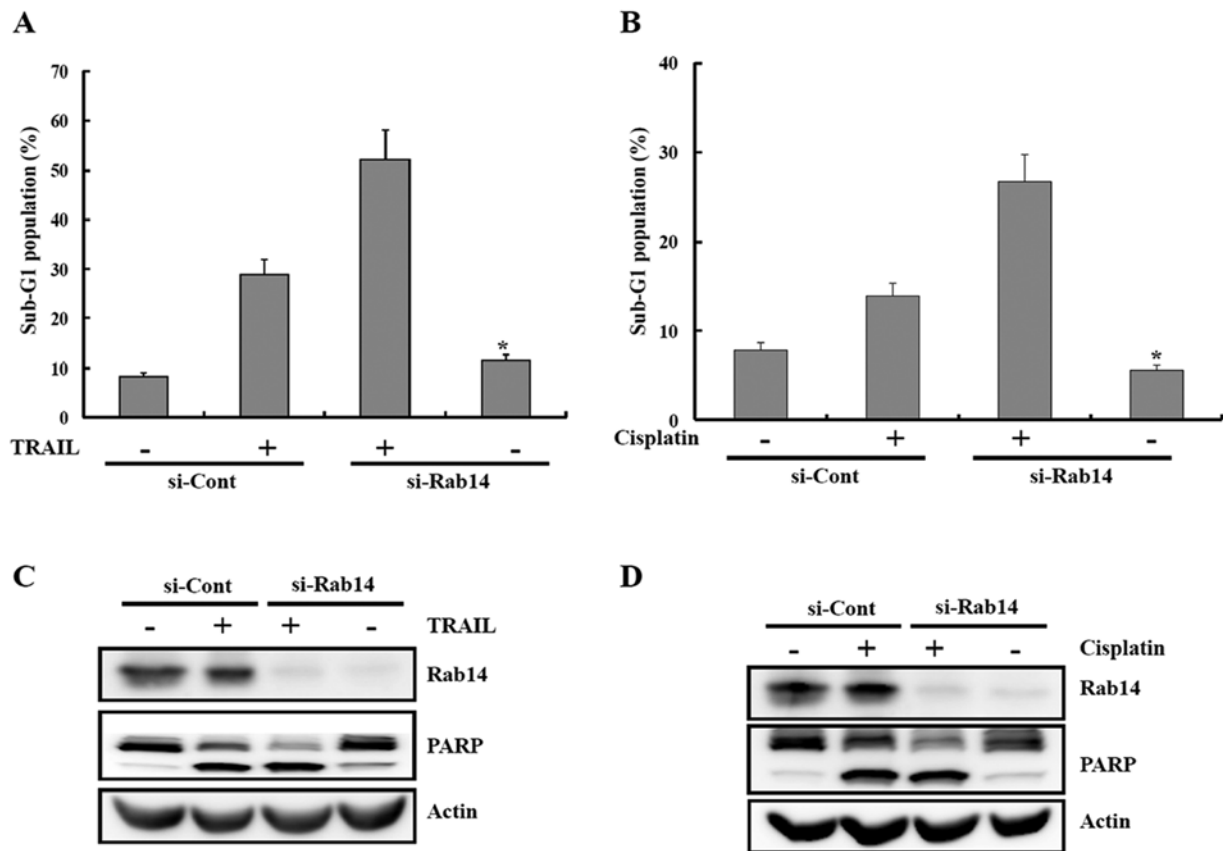


Figure 8. siRab14-mediated downregulation of Rab14 enhanced the sensitivity to various apoptotic stimuli. (A) Caki cells were transfected with siCont or siRab14. At 24 h after transfection, the cells were treated with TRAIL (A) and cisplatin (B) for 24 h. Apoptosis was analyzed as the sub-G1 fraction obtained by FACS-analysis. * $P < 0.05$, compared to each drug-treated siCont-transfected cells. (C and D) Immunoblots for Rab14 and actin antibodies.

RNA (siRNA) specific to Rab14 (siRab14) or a scrambled siRNA negative control (siCont). The si-Rab14 was able to knock down the expression of Rab14 (Fig. 8C and D). Depletion of Rab14 by siRNA significantly increased the sensitivity of the cells to apoptosis-inducing drugs, including TRAIL and cisplatin (Fig. 8).

Discussion

The present study showed that miR-148a resulted in apoptosis of human renal cancer cells via activating the caspase pathway. Moreover, ectopic expression of miR-148a enhanced the anticancer drug sensitivity of renal cancer cells. Rab14 was identified as a direct and functional target of miR-148a, and Rab14 expression was negatively regulated at the posttranscriptional level by miR-148a in renal cancer cells. Finally, we found that the anticancer effect of miR-148a on renal cancer is, at least partly, via the suppression of Rab14 expression.

Downregulation of miR-148a has been identified in various types of human cancer, including gastric cancer, breast cancer, hepatocellular carcinoma, and pancreatic ductal adenocarcinoma, and is therefore considered a tumor-suppressive miRNA (18-21). Moreover, miR-148a overexpression sensitized the cancer cells to anticancer drugs. For example, ectopic expression of miR-148a sensitized the cells to TRAIL via the down-modulation of matrix metalloproteinase 15 (MMP15) and Rho-associated kinase 1 (ROCK1) in non-small cell lung cancer (22). In addition, enforced expression of miR-148a

promotes paclitaxel-induced apoptosis of ovarian cancer cells by targeting PDIA3 (23). miR-148a was found to induce apoptosis and activate the caspase-dependent pathway, indicating that it might function as a tumor suppressor in renal cancer cells. Next, we investigated the effects of miR-148a on the sensitivity to apoptotic stimuli such as TRAIL and cisplatin. Introduction of miR-148a increased the sensitivity of Caki cells to apoptotic stimuli, indicating that miR-148a can promote the sensitivity of renal cancer cells to cisplatin or TRAIL.

Previous studies have shown that the direct targets of miR-148a include MSK1, TGIF2, DNMT3, and PXR (17,24-26). The present study showed that Rab14 is a direct target of miR-148a in renal cancer Caki cells and that some of the tumor-suppressive effects of miR-148a might be mediated through the downregulation of Rab14 expression. Rab14 is a member of the RAS oncogene family of small GTPases involved in human oncogenesis (27,28). These studies suggest that Rab14 dysfunction might be involved in human cancers and other diseases. Rab14 has been reported to play a vital role in human non-small cell lung cancer (29). Therefore, it is necessary to identify the upstream regulators of Rab14 in order to suppress tumor growth and increase drug susceptibility.

Previous studies have shown that ectopic expression of miRNAs such as miRNA-451 and miR-338-3p induces growth inhibition and enhances apoptosis by inhibiting Rab14 expression in lung cancer (29,30). Our data also showed that miR-148a directly targets Rab14 by interacting with the second binding site in the 3'-UTR, which is involved

in miR-148a-induced apoptosis, and enhancing the sensitivity to TRAIL or cisplatin in renal cancer cells. The inhibition of Rab14 by siRab14 was also found to be associated with an increase in the drug susceptibility of Caki cells. Rab14 overexpression could partially block the effects induced by miR-148a in Caki cells. These results indicated that Rab14 might work as an oncogenic factor in renal cancer cells.

In conclusion, the present study showed that Rab14 was a direct target of miR-148a, and miR-148a/Rab14 interaction played an important role in the regulation of apoptosis as well as enhancement of drug sensitivity in renal cancer cells. Thus, miR-148a could be considered as a potential target for renal cancer therapy.

Acknowledgements

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