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Original Article

Knockdown of miR-572 suppresses cell proliferation and promotes apoptosis in renal cell carcinoma cells by targeting the NF2/Hippo signaling pathway

Yongjun Guan[1*], Zhimin Gong[2*], Tianlin Xiao[2], Zhaoyuan Li[2]

Departments of [1]Urology Surgery, [2]Oncology, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, 441021, China. [*]Equal contributors.

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Abstract: *Background:* Renal cell carcinoma (RCC) is one of the most common types of cancer. miR-572 has been proposed to be implicated in a number of human cancers, including RCC. Nevertheless, the detailed functions and molecular mechanisms of miR-572 in RCC have not been well illustrated. *Methods:* qRT-PCR assay was used to assess the expression of miR-572 in RCC specimens and cell lines. Loss-of-function experiments were carried out to explore the effect of miR-572 on proliferation and apoptosis in 786-0 cells. Predicted by TargetScan, the interaction between miR-572 and neurofibromin 2 (NF2) was explored by dual-luciferase reporter assay and western blot analysis. To investigate whether the regulatory effect of miR-572 was mediated by NF2, 786-0 cells were transfected with anti-miR-572 alone, or together with si-NF2. After that, western blot assay was used to validate whethermiR-572 regulated proliferation and apoptosis of the RCC cell line through NF2/Hippo signaling. *Results:* miR-572 expression was upregulated in RCC specimens and cell lines, and miR-572 knockdown suppressed proliferation and enhanced apoptosis in 786-0 cells. miR-572 repressed NF2 expression by binding to NF2 mRNA 3'-UTR. Moreover, the anti-miR-572-mediated regulatory effect on proliferation and apoptosis was abated by the restoration of NF2 expression in RCC cells. Furthermore, miR-572 knockdown activated NF2/Hippo signaling pathway in RCC cells. *Conclusions:* The regulatory effect of miR-572 on proliferation and apoptosis is mediated through modulating NF2/Hippo signaling in RCC cell lines, providing a novel potential strategy for RCC.

Keywords: Renal cell carcinoma (RCC), miR-572, neurofibromin 2 (NF2), Hippo signaling pathway

Introduction

Renal cell carcinoma (RCC) originates from oncogenic transformation in the kidney tubules, and is among the top 10 most common malignancies [1]. Clear cell RCC is the most common and aggressive subtype (approximately 80%-90%) of RCC and is associated with a poor prognosis [2]. The 5-year survival rate for patients with RCC is estimated to be approximately 55% [3]. Due to lacking early-stage clinical symptoms, nearly 40% of RCC patients are diagnosed at an advanced stage when the tumor has progressed and metastasized extensively [4]. Although anti-angiogenic multi-tyrosine kinase inhibitors have been developed, their contribution to survival is still limited [5]. Therefore, a better understanding of the molecular mechanisms underlying RCC development and progression is necessary for improving diagnosis, therapy, and prevention of RCC.

MicroRNAs (miRNAs), a type of non-coding small RNAs of approximately 19-23 nucleotides (nt) in length, function in the posttranscriptional regulation of genes by binding to the 3'-untranslated regions (3'-UTR) of their target mRNAs, thereby participating in various physiological processes [6]. Growing evidence suggests that miRNAs play significant roles in many human cancers, including RCC, highlighting their potential use as biomarkers and therapeutic tools for RCC [7]. For instance, Li *et al* [8] discovered that miR-21 was upregulated in RCC tissues and cell lines, which promoted cell proliferation and transformation capacity by targeting PDCD4 expression, indicating that miR-21 contributed to tumor transformation and metastasis. Su *et al* [9] found that miR-451a level was increased in RCC tissues and the downregulation of miR-451a repressed cell proliferation and migration, and promoted apoptosis in renal cancer cells. Moreover, Chow *et*

al [10] demonstrated that miR-20a, which is a member of the miR-17-92 cluster, had increased expression in RCC specimens and miR-20a depletion suppressed the proliferation of ACHN cell line, suggesting miR-20a has an oncogenic effect on RCC. In addition, many other miRNAs also are described to be upregulated in RCC tissues by miRNA microarray chip assay, such as miR-193a-3p, miR-339-5p, and miR-652, whose expression might provide a useful clue for RCC research [11].

miR-572, an important determinant of cell development, has been implicated in a number of human cancers; for example, Zhang *et al* [12] elucidated that miR-572 expression was increased in human ovarian cancer (OC) cell lines and tissues, and upregulation of miR-572 contributed to the development and progression of OC by targeting SOCS1 and p21. Hui *et al* [13] revealed that the miR-572 level is upregulated in breast cancer tissues by TLDAs analysis, representing a potential therapeutic target for breast cancer therapy. A prior report demonstrated that miR-572 expression was significantly increased in serum of RCC patients, which has the potential to be used clinically as a novel diagnostic marker for RCC [14]. Nevertheless, further study is expected to address the role of miR-572 in RCC.

In this study, we demonstrated that miR-572 expression was elevated in RCC specimens and cell lines. Moreover, miR-572 depletion suppressed proliferation and triggered apoptosis in 786-0 cells. Furthermore, the effect of miR-572 on proliferation and apoptosis is realized via neurofibromin 2 (NF2)/Hippo signaling in RCC cell lines.

Materials and methods

Clinical specimens

A total of 24 pairs of RCC clinical specimens and adjacent non-cancerous specimens were collected from patients who underwent radical nephrectomy at Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science. No local or systemic treatment was conducted in these patients before surgery. All specimens were processed and stored in RNAlater™ Stabilization Solution (AM7021, Thermo Fisher Scientific, Waltham, MA, USA) at -80°C until RNA extraction. Written informed consents were provided by all participants prior to the study. The study was approved by the Institutional Review Board and Ethical Committee of Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science.

Cell culture

Human normal renal tubule epithelial cell line HK-2 (CRL-2190), and human RCC cell lines A498 (HTB-44), 786-O (CRL-1932), and ACHN (CRL-1611) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, 11885076, Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS, 10099141, Gibco), 1% Penicillin-Streptomycin (15070063, Gibco) in a humidified atmosphere of 5% CO_2 at 37°C.

Cell transfection

The miR-572 mimics, miR-572 inhibitor (anti-miR-572), NF2-special inhibitor (si-NF2) and all control oligonucleotides (miR-NC, anti-miR-NC, si-NC) were synthesized by GeneCoposia™ (Guangzhou, China). All oligonucleotides were transfected into 786-0 cells using Lipofectamine™ 2000 Transfection Reagent (11668019, Thermo Fisher Scientific) according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from RCC specimens and cell lines using mirVana™ Total RNA Isolation Kit (A27828, Applied Biosystem, Waltham, MA, USA) referring to the instructions of manufacturer and quantified using a NanoDrop 8000 Microvolume UV-Vis spectrophotometer (ND-8000-GL, Thermo Fisher Scientific). A total of 1 μg RNA was reverse transcribed into cDNA with the miScript Reverse Transcription kit (218061, Qiagen, Hilden, Germany). The relative expression of miR-572 was measured using qRT-PCR analysis with LightCycler®480 Fluorescent Quantitative PCR system (Roche Diagnostics GmbH, Mannheim, Germany) and mirVana®qRT-PCR miRNA Detection Kit (AM-1558, Thermo Fisher Scientific). Primers for the U6 non-coding small RNA were used as an internal control and the fold change of miR-572 was calculated using the $2^{-\Delta\Delta CT}$ method. Primers used for quantitative PCR were synthesized by

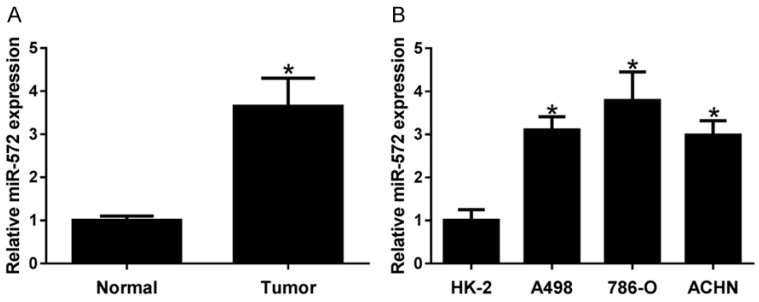

Figure 1. miR-572 expression was upregulated in RCC specimens and cell lines.Relative miR-572 expression was assessed by qRT-PCR assay in RCC clinical specimens and adjacent non-cancerous specimens (A), and RCC cell lines (A498, 786-0, ACHN) and human renal tubule epithelial cell line HK-2 (B). **P*<0.05 vs. negative control (normal specimens, HK-2 cell line).

The Beijing Genomics Institute (Beijing, China) and listed as follows: miR-572: 5'-TTAAAATAGAGAGCGGCGTC-3' (forward) and 5'-TAAACCGACTTCACGATACG-3' (reverse); U6: 5'-GCTTCGGCAGCACATATACTAAAAT-3' (forward) and 5'-CGCTTCACGAATTTGCGTGTCAT-3' (reverse). The PCR reaction was performed as follows: 95°C 10 min, followed by 35 cycles of 94°C 15 s, 55°C 30 s and 72°C 30 s.

Cell proliferation assay

Cell proliferation capacity of treated 786-0 cells was detected using Cell Counting Kit-8 (CCK-8, CK04, Dojindo Molecular Technologies, Shanghai, China) referring to the manufacturer's instructions. Briefly, 786-0 cells after transfection were seeded in 96-well plates in growth medium at approximately 5×10^3 cells/well. At incubation for 0, 24, 48, and 72 h, 20 μl of CCK-8 solution was added to the each well for 2 h at 37°C. Then, the absorbance at 450 nm was measured by a microplate reader (9200, Bio-Rad Laboratories, Hercules, CA, USA).

Flow cytometry

Cell apoptosis ability of treated 786-0 cells was assessed by flow cytometry with Annexin V-FITC Apoptosis Detection Kit (BMS500FI-100, Thermo Fisher Scientific) according to the manufacturer's instructions. In brief, at transfection 48 h, cells were harvested and washed with PBS, following by resuspension in binding buffer (1×). Then, 5 μl of Annexin V-FITC and 10 μl of Propidium Iodide were added to 185 μl of the cell suspension for 10 min. The fluorescence was measured using a flow cytometer (343098, FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA) with CellQuest software.

Dual-luciferase reporter assay

The online software TargetScan was used to search for the potential target genes of miR-572. Then, the 3'-UTR sequences of NF2 containing the putative miR-572 binding sites were commercially synthesized and inserted into downstream of the luciferase open reading frame in the psiCHECK-2 vector (C8021, Promega Corporation, Madison, WI, USA) to construct the NF2 3'-UTR wild-type reporter plasmid (NF2 3'-UTR-WT). Mutant form of the luciferase construct (NF2 3'-UTR mutant-type reporter plasmid, NF2 3'-UTR-MUT) was generated using a Q5 Site-Directed Mutagenesis Kit (E0554, New England Biolabs, Ipswich, MA, USA). 786-0 cells were cotransfected with NF2 3'-UTR-WT plasmid or NF2 3'-UTR-MUT plasmid and miR-572 mimics or anti-miR-572, following the measurement of relative luciferase activity using Dual-Glo Luciferase Assay System (E2920, Promega Corporation).

Western blot

Cells were lysed using RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate) supplemented with a protease inhibitor cocktail (4693116001, Roche, CA, USA). Protein concentration was measured with a BCA Protein Assay Kit (23235, Thermo Fisher Scientific). Equal amounts of extracted protein (50 μg) were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (SDS-PAGE) and then transferred onto PVDF membranes (IBFP0812C, Millipore, Billerica, MA, USA). Following immersed in blocking buffer (phosphate-buffered saline containing 0.1% Tween-20, and 5% nonfat milk) for 1 h, the membranes were incubated with rabbit anti-NF2 (ab109244, 1:50000, Abcam, Cambridge, UK), anti-Mst1 (ab124787, 1:1000, Abcam), anti-p-Mst1 (ab76323, 1:1000, Abcam) and

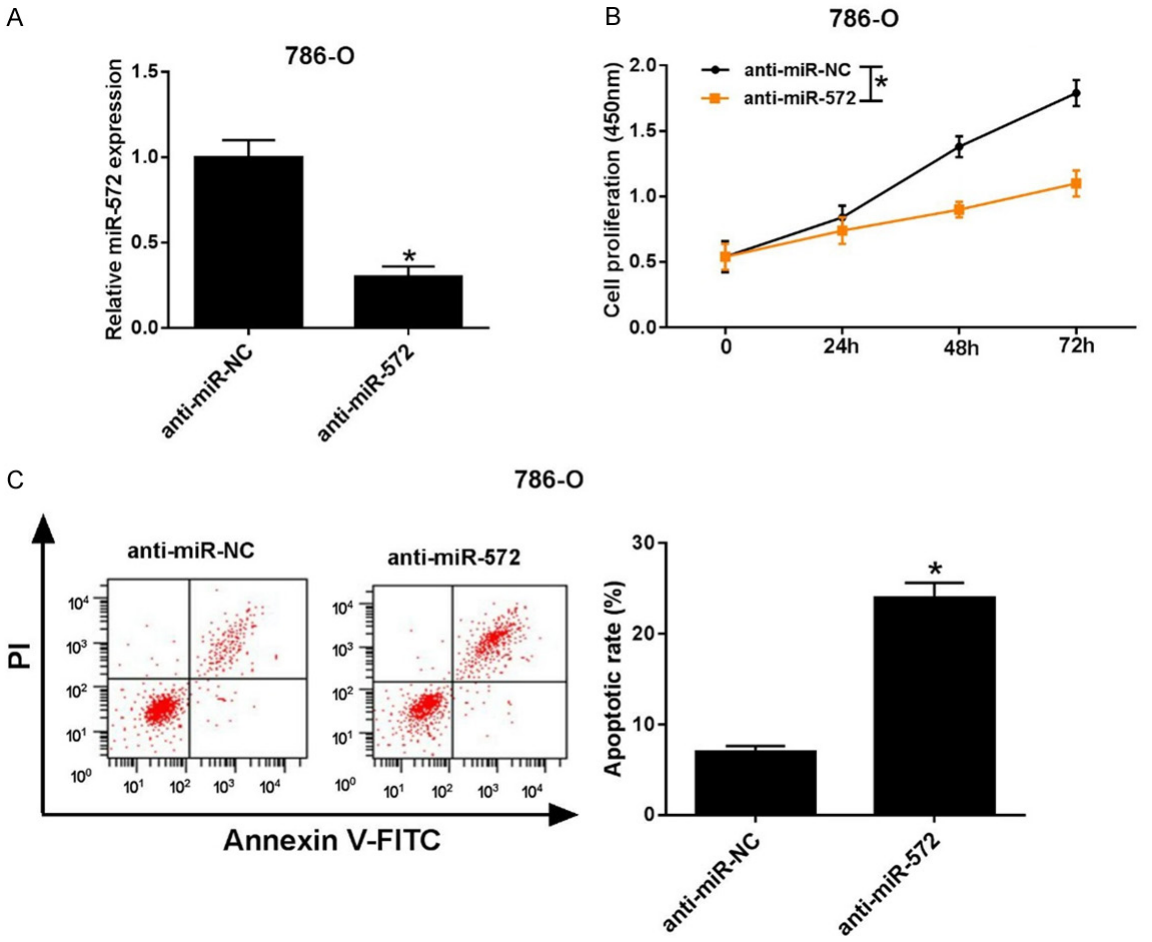

Figure 2. Knockdown of miR-572 suppressed proliferation and enhanced apoptosis in RCC cells. 786-O cells were transfected with anti-miR-572 or anti-miR-NC, following the detection of miR-572 expression by qRT-PCR analysis (A), cell proliferation capability by CCK-8 assay (B), and cell apoptosis ability by flow cytometry (C). *P<0.05 vs. anti-miR-NC.

anti-β-actin (ab179467, 1:5000, Abcam) overnight at 4°C. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (ab6721, 1:2000, Abcam) for 30 min, and the protein bands were visualized using a Pierce ECL Substrate Western Blot Detection system (Bio-Rad Laboratories).

Statistical analysis

All data were analyzed with SPSS version 13.0 (SPSS Inc., IL, USA) and all experiment graphs were performed by GraphPad Prism version 7.0 software (GraphPad Software Inc., CA, USA). For miRNA expression assay, the fold change of miR-572 was calculated using the $2^{-\Delta\Delta CT}$ method, and data was analyzed using two-tailed Student's t-test comparing between tumor specimens or cell lines and respective controls. A one way ANOVA was conducted for cell proliferation study. For cell apoptosis, protein expression, as well as luciferase activity, differences were measured via two-tailed Student's t-test between two groups, and P<0.05 was considered to indicate statistical significance. All data were presented as mean ± standard deviation (mean ± SD) from at least three independent assays.

Results

Upregulation of miR-572 in RCC specimens and cell lines

To investigate the function of miR-572 on the progression of RCC, qRT-PCR assay was first conducted to assess the expression of miR-572 in RCC clinical specimens and adjacent non-cancerous specimens. As shown in **Figure**

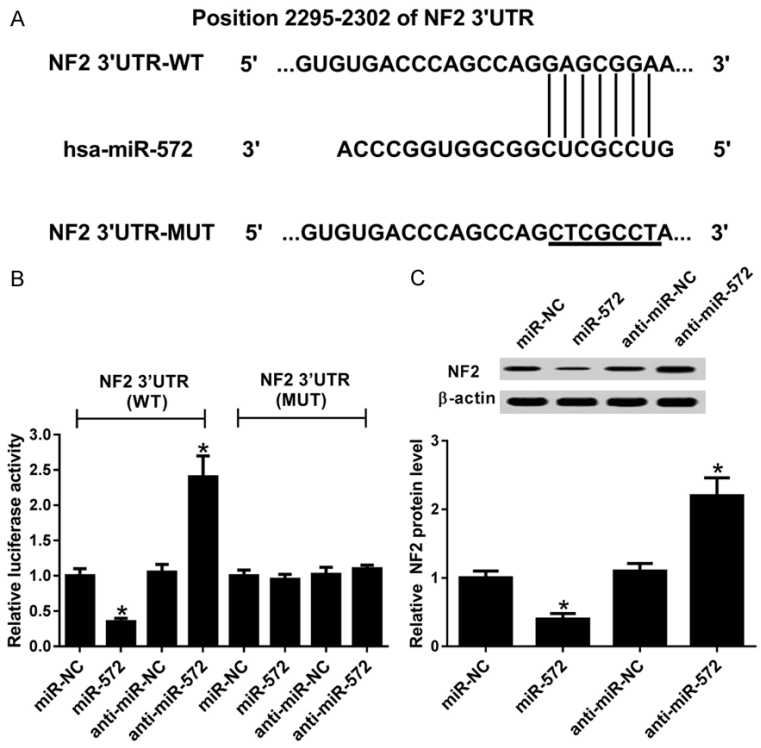

Figure 3. NF2 is a direct target of miR-572. A. Putative binding site of miR-572 on the NF2 3'-UTR along with the mutation in the predicted seed region. B. Dual-luciferase reporter assay in 786-O cells cotransfected with NF2 3'-UTR wild-type reporter plasmid (NF2 3'-UTR-WT) or NF2 3'-UTR mutant-type reporter plasmid (NF2 3'-UTR-MUT) and miR-572 mimics or anti-miR-572. C. Western blot analysis of NF2 expression in 786-O cells transfected with miR-572 mimics, anti-miR-572 and corresponding controls. **P*<0.05 vs. respective negative control.

1A, the data show that miR-572 expression was markedly increased in RCC specimens compared with the adjacent normal specimens. Then, miR-572 levels were detected by qRT-PCR in RCC cell lines compared with homologous control. Consistently, results revealed that miR-572 expression was about threefold in RCC cell lines than that in control group (**Figure 1B**). All these data suggested that miR-572 might play an important role in RCC progression.

Knockdown of miR-572 suppresses proliferation and enhances apoptosis in an RCC cell line

Loss-of-function experiments were carried out to explore the effect of miR-572 on proliferation and apoptosis in 786-O cells. As shown in **Figure 2A**, qRT-PCR assay revealed that miR-572 expression was strikingly suppressed by transfection with anti-miR-572 compared with negative control. Subsequently, CCK-8 assay and flow cytometry results demonstrated that miR-572 depletion significantly inhibited cell proliferation (**Figure 2B**) and promoted apoptosis (**Figure 2C**) in 786-O cells. Taken together, these results hinted that the knockdown of miR-572 represses proliferation and contributes to apoptosis in RCC cells.

NF2 was a direct target of miR-572

To further explore the molecular mechanism by which of miR-572 modulates proliferation and apoptosis of RCC cell line, the computational algorithm of TargetScan (Version 5.2) was used to research for the potential target genes of miR-572. The predicted data revealed that the 3'-UTR of NF2 mRNA had several complementary sites with miR-572, indicating that NF2 might be a target of miR-572 (**Figure 3A**). In order to verify the interaction between NF2 and miR-572, dual-luciferase reporter assay was performed by cotransfection with NF2 3'-UTR wild-type reporter plasmid (NF2 3'-UTR-WT) or NF2 3'-UTR mutant-type reporter plasmid (NF2 3'-UTR-MUT) to 786-O cells and miR-572 mimics or anti-miR-572. These data showed that the luciferase activity of NF2 3'-UTR-WT plasmid was markedly reduced by introduction of miR-572 mimics (**Figure 3B**), while it was significantly enhanced following miR-572 knockdown compared with corresponding counterparts (**Figure 3B**). However, little change was observed in the luciferase activity of NF2 3'-UTR-MUT plasmid in response to the alteration of miR-572 expression (**Figure 3B**). Then, miR-572 mimics or anti-miR-572 were transfected into 786-O cells to determine the regulatory effect of miR-572 on NF2 expression. Western blot analysis manifested that the NF2 level was remarkably reduced by miR-572 overexpression (**Figure 3C**), while it was significantly increased following miR-572 depletion (**Figure 3C**). All these results

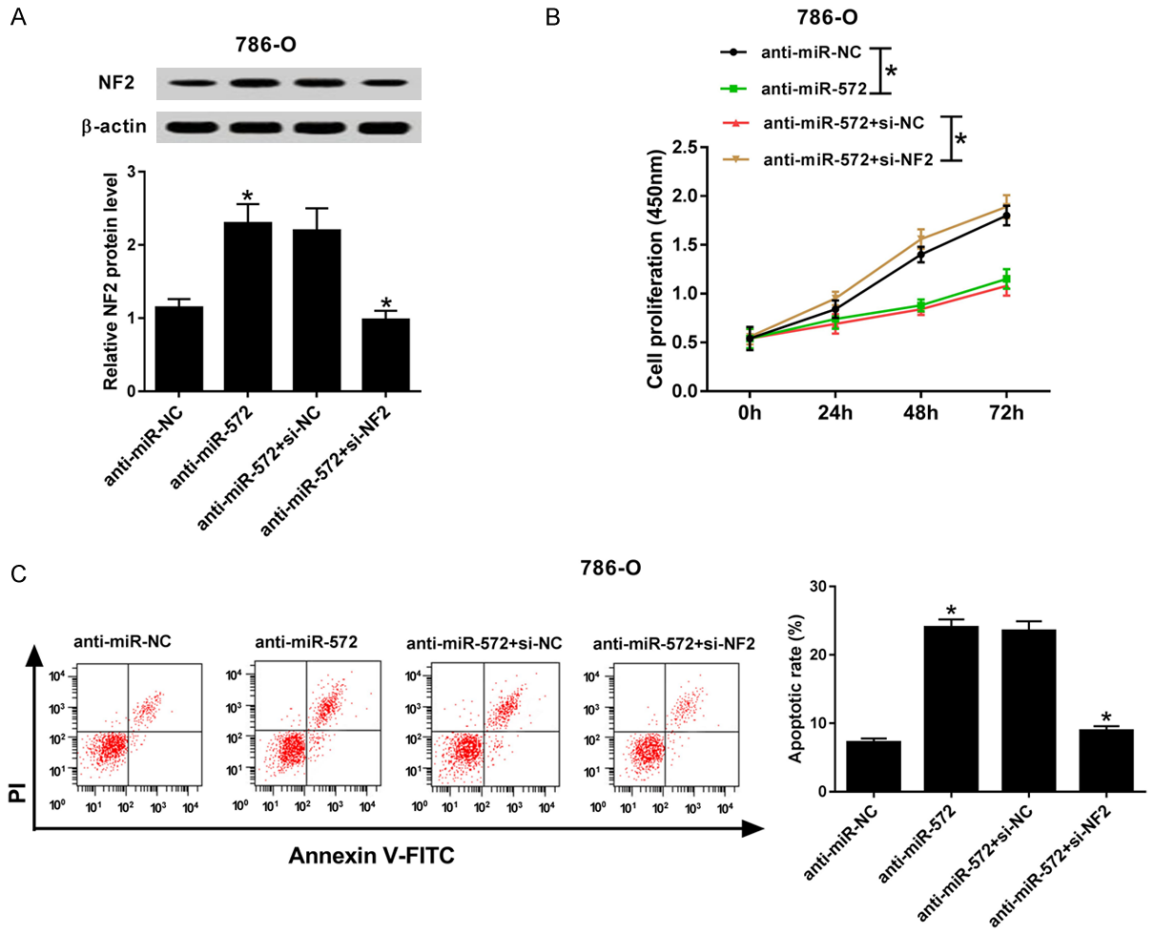

Figure 4. Anti-miR-572-mediates the regulatory effect on proliferation and apoptosis of RCC cells. This was abated by the restoration of NF2 expression. 786-O cells were transfected with anti-miR-572 alone or together with si-NF2, following the measurement of relative NF2 expression by qRT-PCR (A), cell proliferation capacity by CCK-8 assay (B), and cell apoptosis ability by flow cytometry (C). *P<0.05 vs. negative control (anti-miR-NC, anti-miR-572+si-NF2).

implied that NF2 was a direct target of miR-572 and miR-572 repressed NF2 expression by binding to NF2 mRNA 3'-UTR.

Anti-miR-572-mediated regulatory effect on proliferation and apoptosis was abated by the restoration of NF2 expression in an RCC cell line

To further evaluated whether the regulatory effect of miR-572 on proliferation and apoptosis of RCC cells was mediated by regulating NF2, 786-O cells were transfected with anti-miR-572 alone, or together with si-NF2. As shown in **Figure 4A**, qRT-PCR assay revealed that anti-miR-572-triggered the promotion of NF2 expression was significantly reversed by cotransfection with si-NF2, compared with that of control (**Figure 4A**). The results of CCK-8 assay and flow cytometry manifested that anti-miR-572-mediated effect on suppression of proliferation and enhancement of apoptosis in 786-O cells was significantly abated following the restoration of NF2 expression (**Figure 4B** and **4C**). All these data suggested that miR-572 exerted its regulatory effect on proliferation and apoptosis by modulating NF2 expression in RCC cells.

miR-572 knockdown activates NF2/Hippo signaling pathway in RCC cell line

The Hippo signaling pathway is a central mechanism that regulates organ size and tissue homeostasis in species spanning from *Drosophila* to mammals. It has been firmly established that its dysregulation underlies a series of human diseases including cancer [15].

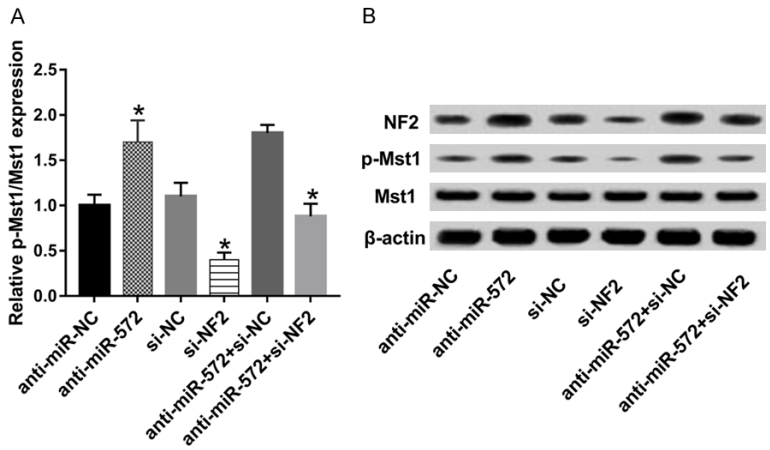

Figure 5. Anti-miR-572 activates the NF2/Hippo signaling pathway in RCC cell line. 786-O cells were transfected with anti-miR-572, si-NF2, anti-miR-572+si-NF2, or corresponding negative controls, following the determination of NF2 expression, Mst1 level and phosphorylation of Mst1 by western blot assay (A) and (B). β-actin was used as the internal normalizer. **P*<0.05 vs. corresponding control.

Moreover, NF2 was previously reported as an upstream regulator of Hippo signaling in multiple biological processes [16]. Therefore, we further validated whether miR-572 regulated proliferation and apoptosis of RCC cell line via NF2/Hippo signaling pathway, by analyzing the phosphorylation of mammalian Ste20-like Kinase 1 (Mst1). Western blot results demonstrated that the phosphorylation of Mst1 was markedly promoted following miR-572 downregulation, while it was significantly weakened by transfection with si-NF2 compared with respective controls (**Figure 5A** and **5B**). Conversely, anti-miR-572-triggered Hippo signaling was dramatically reversed by cotransfection with si-NF2 (**Figure 5A** and **5B**). Taken together, these results hinted that miR-572 depletion blocks proliferation and facilitates apoptosis through the Hippo signaling pathway activation via NF2 in RCC cells.

Discussion

Recently, growing evidence has suggested that miR-572 acts as an important regulation factor, participating in the regulation of cell progression in human carcinogenesis. For example, Liu *et al* [17] revealed that miR-572 expression was increased in the serum of patients with nasopharyngeal carcinoma. Wu *et al* [18] demonstrated that miR-572 enhanced cell proliferation by inhibiting PPP2R2C expression in OC cell lines. In the present study, we found that miR-572 expression was significantly upregulated in RCC specimens and cell lines. Moreover, the knockdown of miR-572 suppressed cell proliferation and promoted apoptosis in RCC cell line. Conversely, miR-572 level was reported to be downregulated in men with metastatic compared with non-metastatic prostate cancer, highlighting its role as potentially useful biomarkers for the staging in prostate cancer [19]. Additionally, miR-572, a member of downregulated miRNAs, was proposed to participate in the miRNAs regulatory network in bladder cancer [19].

Compared with classical transcription factors, miRNAs exert their functions by regulating the expression of endogenous target genes [20]. Thus, the online software TargetScan was used to search for the potential target genes of miR-572. Further, we validated that miR-572 directly interacts with the 3'-UTR of NF2 to suppress NF2 expression in RCC cells. NF2, a gene thaty encodes the protein Merlin, is able to transduce intra- and extracellular signals to modulate a series of cellular processes [21]. It was reported that mutant or inactivation of NF2 led to uncontrolled mitosis and tumorigenesis [22]. Moreover, NF2 has been proposed to exert its predominant tumor suppressive functions by regulating oncogenic gene expression [23]. Increasing evidence shows that NF2 functions as a tumor-suppressor gene and is linked to multiple human cancers [24]. For example, Ladanyi *et al* [25] demonstrated that about 35% to 40% of patients with malignant pleural mesothelioma (MPM) carried inactivating mutations of NF2 gene, indicating NF2 acted as a novel target for therapeutic development. The inactivation of NF2 also was found in familial multiple meningiomas, whose development might be caused by the presence of a predisposing germline mutation in NF2 gene [26]. Moreover, the mutation of NF2 directly contributed to tumor angiogenesis, and NF2/Merlin regulated angiogenesis via modulating Rho GTPase Rac1/semaphorin 3F axis in schwannoma [27]. A prior report said that NF2 was

recurrently mutated in papillary RCC by MutSigCV analysis, and NF2 was an upstream gene of Hippo signaling pathway [28]. In this study, our data revealed that anti-miR-572-mediated regulatory function on proliferation and apoptosis in RCC cell line was mitigated by the restoration of NF2 expression.

The Hippo signaling pathway was initially discovered in *Drosophila* as a valid mechanism to control organ size via governing cell proliferation and apoptosis [29]. The central axis of Hippo signaling is a kinase cascade that includes Mst1, Mob1a/b (Mob kinase activator 1a/b), Lats1/2 (serine/threonine-protein kinase 1/2) and downstream transcription coactivators (YAP and TAZ) [30]. Recently, Hippo signaling dysregulation has been shown to underly a series of human diseases including cancer [15]. In liver cancer, the deregulation of the Hippo signaling was reported to promote tumor development and malignancy by regulating downstream target YAP [31]. In glioblastoma, activation the of Hippo pathway was attenuated by CD44, thereby leading to the enhancement of tumor cell resistance to reactive oxygen species, the secretion of cytotoxic agent, and the generation of cell stress [32]. Moreover, Hippo signaling was reported to play a vital role in the development, progression, and metastasis of human gastric cancer [33]. The Hippo/YAP/TZA pathway also was involved in the breast tumorigenesis by acting as a key downstream signaling branch of the G protein coupled estrogen receptor (GPER) [34].

In addition, NF2 was previously reported as an upstream regulator of Hippo signaling in multiple biological processes [16]. A previous report described that the tumor-suppressor gene NF2 regulated cell proliferation and apoptosis via Hippo signaling in the development of *Drosophila* [35]. NF2 induced ischemia/reperfusion injury via enhancing the activation of Mst1 and inhibition of YAP, thereby activating Hippo signaling in the heart [36]. In our study, the data showed that the Hippo signaling was blocked following NF2 depletion, and it was activated by miR-572 downregulation. Subsequently, we found that the knockdown of miR-572 activated Hippo signaling pathway via NF2 in RCC cells. Similarly, miR-135b accelerated lung cancer metastasis through attenuating Hippo signaling, suggesting miR-135b might be an oncogenic miRNA and a potential therapeutic target [37]. MiR-130b enhanced glioblastoma stem cell self-renewal by directly inhibiting the phosphorylation of Mst1 and promoting the activation of YAP/TZP, providing a novel regulatory mechanism of Hippo signaling [38].

In conclusion, our study revealed that miR-572 level was upregulated in RCC specimens and cell lines, and miR-572 abrogation limited proliferation and facilitated apoptosis through driving NF2/Hippo signaling in RCC cells. Our study contributes to a better understanding of the molecular mechanisms involved in RCC progression and provides a novel potential treatment strategy for RCC.

Disclosure of conflict of interest

None.

Address correspondence to: Tianlin Xiao and Zhaoyuan Li, Department of Oncology, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, 441021, China. Tel: +86-136-07271396; E-mail: gsd1365@126.com (TLX); Tel: +86-13886270929; E-mail: 13797751612@163.com (ZYL)

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