MiR-376a inhibits non-small cell lung cancer cell progression by regulating Rab1A

Zheng-Hao Cao

The First Affiliated Hospital of Zhengzhou University

Jing-Liang Cheng

The First Affiliated Hospital of Zhengzhou University

Xian-Zhao Zheng

The First Affiliated Hospital of Zhengzhou University

Qing-Qing Lv

The First Affiliated Hospital of Zhengzhou University

Jin-Xin Ma

The First Affiliated Hospital of Zhengzhou University

Yong Zhang (✉ moshanshan77+zhangy@gmail.com)

The First Affiliated Hospital of Zhengzhou University <https://orcid.org/0000-0003-3275-7203>

Research article

Keywords: miR-376a; Rab1A; non-small cell lung cancer; regulation

Posted Date: September 12th, 2019

DOI: <https://doi.org/10.21203/rs.2.14321/v1>

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Abstract

Post transcriptional gene regulation of microRNA-376a (miR-376a) plays a crucial role for tumorigenesis and cancer development. However, the potential role of miR-376a in non-small cell lung cancer (NSCLC) remains unclear. In this study, we investigated the crucial role of miR-376a in NSCLC by analyzing miR-376a expression as well as its target genes. Through overexpression strategies, we uncovered the molecular mechanisms underlying miR-376a-mediated tumorigenesis. Quantitative real-time PCR analysis demonstrated miR-376a levels to be significantly decreased in NSCLC cells compared with non-tumorigenic counterparts. Interestingly, miR-376a overexpression potentially repressed NSCLC cell proliferation, migration, and invasion, but increased apoptosis in A549 cells. Using bioinformatic approaches, we predicted that miR-376a targets Rab1A, and further luciferase fusion assay demonstrated Rab1A was a direct target of miR-376a and miR-376a inhibited cell proliferation by regulating the mRNA and protein levels of Rab1A in NSCLC cells. Overall, our findings uncover the miR-376a could suppress NSCLC cells progression via directly targeting Rab1A.

Introduction

Lung cancer is a common malignant tumor that accounts for nearly 10% of cancer cases and is the most deadly malignant tumor, posing a significant threat to human health worldwide, both in males and females. Lung cancer is generally classified based on histological type into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC has emerged as the most common type of lung cancer, and it accounts for nearly 85% of total cases. Despite the efficacy of surgical therapy for almost 50% of cases, many NSCLC patients still have a poor prognosis [1-2]. Thus, the findings of novel diagnosis and therapeutic target for NSCLC patients are extremely urgent.

MicroRNAs are single-stranded, non-coding RNAs of 22-25 nucleotides that are reported to post-transcriptionally modulate protein expression and are thereby involved in cancer development. Approximately 50% of miRNAs in the human genome are characterized tumor suppressors and play a crucial role in the regulation of gene expression involved in pivotal cellular mechanisms. Recent studies have demonstrated that miRNAs crucially govern the proliferation, invasion and immigration mechanisms of cancer cells and can thereby function as biomarkers in human cancers. For example, a previous study demonstrated that increased expression of miR-155 reduces expression of tumor suppressor proteins, thereby acting as a crucial diagnostic marker in B cell lymphoma [3]. Congruently, miR-15a and miR-16, which are expressed from the intron region of the prostate cancer gene, have been shown to act as tumor suppressor genes [4-5]. An increase in miR-216a and miR-217 levels in hepatocellular cancer cells leads to activation of the TGF- β and PI3K/Akt pathways, conferring drug resistance to sorafenib and causing neoplasm recurrence [6]. MiR-376a also regulates cell processes in several cancers via targeting of various genes, and low levels of miR-376a induce apoptosis in retinoblastoma cells by targeting caspase-3. Furthermore, Liu *et al.* reported that the transcript level of miR-376a was increased in murine malignant lung cancer compared to adjacent normal murine lung [7]. Overexpression of miR-376a has been reported to attenuate the proliferation and migration of melanoma

cells by targeting IGF1R, a tyrosine kinase receptor associated with tumorigenesis and metastasis [8]. MiR-376a also regulates proliferation, apoptosis, migration and invasion in metastatic prostate cancer and hepatocellular carcinoma [9-10], and functions as a tumor suppressor in nasopharyngeal carcinoma [11]. Nevertheless, few studies have focused on the function of miR-376a in lung cancer, especially NSCLC, despite its significance to human health. In this study, we aimed to uncover the relation between miR-376a and NSCLC tumorigenesis.

Materials And Methods

Cell culture

Human non-small cell lung cancer (NSCLC) cells line (A549) and normal line (NuLi-1) were purchased from the American Type Culture Collection (Manassas, VA, USA). The NuLi-1 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, USA), 100 units/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma). The A549 cells were grown in RPMI-1640 medium (Gibco, USA) and 10% FBS (HyClone, USA) with 100 units/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma). All the cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were digested by trypsinization and passaged *in vitro*. Cells in the logarithmic growth phase were collected and harvested for all experiments.

Cell transfection

MiR-376a mimics, inhibitor and miR negative control (miR-NC) were obtained from GenePharma Co., Ltd. (Shanghai, China). Small interfering RNA (siRNA) oligonucleotides of Rab1A (sense 5'-GGAAACCAGUGCUAAGAAUT-3', antisense 5'-AUUCUUAGCACUGGUUUCCTT-3'), which are used to inhibit Rab1A expression in the study was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). To gain an overexpressing vector of Rab1A, the cDNA of Rab1A was cloned into pFLAG-CMV vector (Sigma) and the resulting construct was named pCMV-Rab1A (Rab1A). When converged to 80%, cells were transfected with 50 nM using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. After transfection, the transfection efficiency was examined using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Quantitative RT-PCR analysis

The relative transcript abundance of miR-376a, Rab1A in cells was determined by quantitative real-time PCR (qRT-PCR) using an ABI PRISM® 7500 Sequence Detection System (ABI, USA). As for miRNA detection, cells were lysed and the total miRNA was extracted using RNAiso for Small RNA (Takara, Japan). RT-PCR was performed using Mir-X miRNA First-Strand Synthesis Kit (Takara, Japan) and TB Green Advantage qPCR Premix (Takara, Japan) according to the manufacturer's recommendation with GADPH as the internal reference. As for mRNA detection, total RNA from each group of cells was extracted using Trizol (Invitrogen, USA) according to the manufacturer's instructions, and reversed

transcribed to cDNA using PrimeScript[™] RT reagent Kit with gDNA Eraser (Takara, Japan). QRT-PCR was performed using SYBR Premix Ex Taq[™] Kit (Takara, Japan) with GAPDH as the internal reference. All qRT-PCR primers were purchased from Sangon (Shanghai, China) and were as follows: GAPDH forward, 5'-CAGGGCTGCTTTTAACTCTGGT-3' and reverse, 5'-GATTTTGGAGGGATCTCGCT-3'; miR-376a forward, 5'-GTGCAGGGTCCGAGGT-3' and reverse, 5'-ATCATAGAGGAAAATCCACG-3'; Rab1A forward, 5'-TTGCCTTCTTCTTAGGTTTGC-3' and reverse, 5'-GCTTGATTGTTTTCCCGTCT-3'.

Cell viability assay

The viability of the cell was performed by using Cell Counting Kit-8 (CCK-8; Dojindo, Japan) assay. Briefly, transfected cells were cultured for 72 h in RPMI 1640 medium containing 10% FBS (HyClone, USA) and then seeded into 96-well plates at a density of 5×10^3 cells per well, 10 μ L of fresh CCK-8 solution was added to each well and then co-incubated for 4 h at 37 °C. The optical density (OD) of each well was measured the absorbance at 450 nm using a microplate reader (Bio-Rad, USA).

Migration and invasion assays

Cell migration was determined using the scratch assay. After transfection, a 2-mL (1×10^5 cells/mL) sample of cells was seeded in 6-well plates and incubated for 24 h to allow attachment. The cell layer was scratched, down the middle of the plate well, and the medium was removed; the cells were then washed three times with PBS. Fresh medium containing 3% FBS was added, and the cells were visualized under microscopy after 24 h. To minimize deviation, at least triple views were visualized and captured, and the average number was used.

For invasion assay, dissolved ECM gel (Sigma, 100 mg/well) was added to a 24-Transwell Boyden chamber (Corning, USA) and incubated for 4-8 h at 37 °C. Post-transfection cells in logarithmic phase after trypsinization and suspension were seeded into the Transwell Boyden chamber using 100 μ L per well at 5×10^5 cells/mL, after which 500 μ L RPMI1640 medium containing 10% FBS was added to the lower chamber. The cells that did not invade the upper chamber were removed and fixed with methanol. Cells were stained with Giemsa solution and observed by optical microscopy (ECLIPSE Ti2, Nikon, Japan).

Dual-luciferase reporter assay

The luciferase reporter assay was performed according to the methods described previously [10]. In brief, the 3'-UTR of Rab1A with wild-type or mutant binding sites for miR-376a were cloned into pmiR-GLO-Dual-Luciferase miRNA target expression vector plasmid (E1330; Promega, USA) naming pmiR-GLO-Rab1A-WT and pmiR-GLO-Rab1A-Mut, respectively. The A549 cells were co-transfected with pmiR-GLO-Rab1A-WT or pmiR-GLO-Basic-Rab1A-MUT and miR-376a or miR-376a mimic using Lipofectamine[™] 2000 Transfection Reagent (Invitrogen, USA), according to the manufacturer's protocol. After incubation for 72 hours, the firefly and *Renilla* luciferase activities were measured using the Dual-Glo Luciferase System

(Promega) according to the manufacturer's protocol. The firefly luciferase activity was normalized with the *Renilla* luciferase activity.

Hoechst 33342 staining assay

Cell apoptosis was determined using the Hoechst 33342 staining assay. In brief, 2 mL (1×10^5 cells/mL) sample of cells was seeded in 12-well plates and incubated for 24 h to allow attachment. After transfection, 10 μ L of Hoechst 33342 live cell staining solution (100X) (Beyotime Biotechnology, Shanghai, China) was evenly added to each well. and then co-incubated for 10 minutes at 37 °C. The dye-containing medium was removed and the cells were washed three times with PBS and observed by fluorescence microscope. (ECLIPSE Ti2, Nikon, Japan)

Western blot assay

The total proteins were extracted from treated the cells using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease and phosphatase inhibitor (Biocolor Biosciences & Technology Company, Shanghai, China) according to the manufacturer's instructions. The protein concentration were detected by a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China), and then equal of total protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE; Biocolor Biosciences & Technology Company, Shanghai, China) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After The PVDF membranes were blocked with 5% skim milk for 3 h at room temperature and then incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: Rab1A (1:1000 dilution, Abcam, ab97956). GAPDH (1:1000 dilution, CST, 5174S) was used as an internal reference. The membrane was incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 dilution, Abcam, ab6721) for 2 h at 37 °C. Bands were visualized using NovexTM ECL Chemiluminescence Substrate Reagent kit (Invitrogen, USA) and scanned by InvitrogenTM E-GelTM Imager System. The bands were quantified and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Experiments in this study were repeated independently at least three times, and the results are expressed as the mean $\pm$ SD (standard deviation of mean). All data were analyzed using the SPSS statistical package. Univariate sample means were compared using one-way analysis of variance (ANOVA). Least significant difference (LSD) was used to compare multivariate sample means pairwise. $P < 0.05$ (*) or $P < 0.01$ (**) was considered statistically significant.

Results

MiR-376a is downregulated in NSCLC cells

To evaluate the expression of miR-376a in NSCLC cells and normal lung cells, quantitative real-time PCR (qRT-PCR) assay was performed. The results showed that miR-376a expression level was markedly lower in A549 cells, with the reduction of around 71.1%, compared with that in the NuLi-1 cells ($P<0.01$, **Fig. 1a**). The above data revealed that the miR-376a might play a protective role in NSCLC pathogenesis.

The overexpression of miR-376a inhibits cell proliferation, migration, invasion and promotes cell apoptosis

To further investigate whether the protective role of miR-376a on in NSCLC pathogenesis, we performed that A549 cells were transiently transfected with miR-376a mimics or inhibitor to study gain- and loss-of-function of miR-376a. The transfection efficiency of miR-376a mimics and inhibitor was confirmed by qRT-PCR (Online Resource 1). The viability of cells was detected using CCK-8 assay. As shown in **Fig. 1b**, miR-376a mimics inhibited cell proliferation ability compared with the miR-NC in A549 cells, while miR-376a inhibitor increased cell proliferation ability. To further assess the effect of miR-376a on cell migration by scratch wound healing assay, the results showed that miR-376a mimics in A549 cells could strongly suppress cell migration compared with the miR-NC group, while miR-376a inhibitor increased cell migration (**Fig. 1c**). Furthermore, transwell assay was performed to detect the cell invasive ability and the results showed that miR-376a mimics significantly inhibited cell invasion compared with control, while miR-376a inhibitor increased cell invasion (**Fig. 1d**). In addition, Hoechst 33342 staining was taken to detect the effect of miR-376a mimics on apoptosis in A549 cells. The results revealed that the percentage of apoptotic cells was markedly increased in A549 cells with miR-376a mimics (**Fig. 1e**). In brief, the above results revealed that miR-376a could inhibit cell proliferation, migration and invasion and promote cell apoptosis in A549 cells.

Rab1A is a target of miR-376a in NSCLC cells

To predict the potential target genes regulated by miR-376a in NSCLC cells, the predicted software targets can and miRanda were performed in the study. We found that 3'-UTR of Rab1A had complementary sites with miR-376a. We further determined the abundance of Rab1A in A549 cells. Both the level of mRNA and protein were measured. The level of Rab1A was higher in A549 cells than that in NuLi-1 cells (**Fig. 2a**), which was in good agreement with the Western blot analysis (**Fig. 2b**). To further verify the interaction between miR-376a and Rab1A, we determined the relative transcript abundance of Rab1A under different miR-376a levels. As shown in **Fig. 2c**, miR-376a mimics markedly downregulated the expression of Rab1A, whereas miR-376a inhibitor upregulated the expression of Rab1A. Western blot analysis was also performed to confirm the qPCR results, showing the same trends (**Fig. 2d**). To validate whether miR-376a regulates the expression of Rab1A, 3'-UTR of Rab1A with wild-type (Rab1A-WT) or mutant (Rab1A-Mut) binding sites for miR-376a were cloned into pmiR-GLO-Dual-Luciferase plasmid, respectively. Relative luciferase activity was determined after plasmids co-transfection with miR-376a mimics. As shown in **Fig. 2e and f**, miR-376a mimics significantly reduced the luciferase activity of the Rab1A-WT-transfected A549 cells, while no significant change was found in A549 cells transfected with Rab1A-Mut. Taken together, these findings suggested that Rab1A is a target of miR-376a.

Rab1A regulates tumorigenesis in NSCLC cells

To gain further insight into the mechanistic role of Rab1A in NSCLC tumorigenesis, we knocked down *Rab1A* or overexpressed Rab1A in A549 cells. The mRNA level and the protein expression of Rab1A were determined to verify the effects of different treatments. As shown in **Fig. 3a and b**, both mRNA and protein levels were reduced in A549 cells transfected with si-Rab1A, but increased in A549 cells transfected with Rab1A, indicating that Rab1A were successfully silenced and overexpressed in the transfected cells.

To determine the role of Rab1A in the life cycle of NSCLC cells, cell proliferation, migration, invasion and apoptosis were measured under different levels of Rab1A. Knockdown of Rab1A (si-Rab1A) significantly decreased the growth rate of cells, but overexpression (Rab1A) accelerated cell proliferation (**Fig. 3c**). si-Rab1A inhibited cell migration and invasion but enhanced apoptosis, whereas overexpressing Rab1A had the opposite effects (Fig. 3d, e and f). These results demonstrate that Rab1A participates in NSCLC pathogenesis.

MiR-376a inhibits the NSCLC cells proliferation by targeting Rab1A

To evaluate whether the effects of miR-376a on NSCLC proliferation via mediating Rab1A, we performed the CCK-8 cell proliferation assays (**Fig. 4**). The results showed that si-Rab1A inhibited the cell proliferation, but co-transfection of si-Rab1A and miR-376a inhibitor dismissed the effects. The Rab1A increased the cell proliferation, but co-transfection of Rab1A and miR-376a mimics dismissed the effects. The above results suggested that miR-376a inhibits cell proliferation by targeting Rab1A in NSCLC cells.

Discussion

Owing to the functional significance of miRNA on post-transcriptional regulation, governing crucial cellular mechanisms, investigations on miRNAs have garnered a great deal of research attention. Recent studies have explored the potential of miRNAs to regulate expression of crucial proteins involved in cancer and have demonstrated their efficacy as molecular markers for diagnosis of various cancers [12], including NSCLC, a kind of deadly lung cancer with few and unsatisfied therapeutic treatments. A diversity of miRNAs is involved in NSCLC cells, and their functions differ. For example, miR-33a targets the mRNA of Methyltransferase like 3 (METTL3), a key protein to upregulate several crucial oncoproteins in human lung cancer cells, leading to attenuate NSCLC cells proliferation [13]. MiR-142-3p overexpression in NSCLC cells is effective to inhibit anticancer drug-induced autophagy as well as increase chemo-sensitivity of NSCLC [14].

MiR-376a is considered a meaningful prognostic biomarker and a potential therapeutic target in various types of cancers. For example, miR-376a directly targets specificity protein 1, markedly decreasing the proliferation and invasion of glioblastoma multiforme cells in vitro [15]. In hepatocellular carcinoma tissues and cell lines, miR-376a was decreased by targeting p85 α [10]. Furthermore, it is reported that miR-376a regulates human erythropoiesis by targeting CDK2 and Ago2 [16]. Nonetheless, the molecular

mechanisms underlying the inhibition of tumorigenesis in NSCLC remain unknown, and the interaction and relationship between miR-376a and its target genes in NSCLC have not yet been explored.

In an effort to investigate the biological function of miR-376a in NSCLC, A549 cells were used as a model NSCLC cell, and we verified miR-376a to be downregulated in NSCLC cell lines using qRT-PCR, which is consistent with previous research in hepatocellular carcinoma, melanoma tissue and colorectal cancer [8, 10, 17]. It is reported that arsenic trioxide-mediated apoptosis of retinoblastoma cells was regulated by miR-376a, which targets caspase-3, a major effector of cell apoptosis pathway [18]. The effect of miR-376a expression on NSCLC cell proliferation and invasion may be associated with its target gene. Bioinformatic analysis predicted that miR-376a targets Rab1A. To the best of our knowledge, this study provides the first evidence that miR-376a regulates NSCLC by targeting Rab1A. The proteins of the Rab family are small GTPases regulating vesicle trafficking from the ER to Golgi apparatus, which are crucial for cell survival and growth [19]. Using an unbiased knockdown gene, Rab1a was identified as a novel candidate in cell migration [20]. Rab1A was also found to be overexpressed in HCC compared with normal livers, and Rab1A increased cell growth and migration, cell cycle progression and tumor formation *in vivo* and *in vitro* [21]. It is proved that Rab1A promotes mTORC1 signaling and oncogenic growth and enhances tumor progression and invasion in colorectal cancer [12]. In our study, experimental evidences corroborate that overexpression of miR-376a inhibited that of Rab1A, as indicated by qPCR and western blotting. These data provide valuable insight into the role of miR-376a through its ability to inhibit Rab1A expression, in turn leading to inhibiting NSCLC cell processes. Thus, targeting Rab1A by miR-376a are potential diagnostic and treatment approaches for NSCLC cells.

In summary, miR-376a regulates cell proliferation, migration, invasion and apoptosis in A549 cells by targeting Rab1A. These data were corroborated by overexpression and knockdown of these target genes in transfected cells. For the first time we also demonstrated the mechanistic role of miR-376a in regulating the target genes Rab1A, with a crucial role in NSCLC. Given the potential anti-tumorigenic properties of the tumorigenic properties of Rab1A, this study provides a meaningful foundation for diagnostic and therapeutic strategies for NSCLC.

Declarations

Author Contributions

Y.Z. conceived and designed the experiments. Z.H.C. and J.L.C. performed the experiments. X.Z.Z, Q.Q.L. and J.X.M. analyzed the data. Z.H.C., J.L.C. and Y.Z. wrote the manuscript. All authors read and approved the final manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

None

Compliance with ethical standards

Conflict of Interest

The authors declare that they have no competing interests.

Research involving human and animal rights

This work does not contain any studies with human participants or animals performed by any of the authors.

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Additional Materials

Online Resource 1 The expression of miR-376a in A549 cells transfected with scramble RNA, miR-376a mimics, miR-376a inhibitor, or miR-NC. Data were expressed as mean $\pm$ standard deviation of three independent experiments. **, $P < 0.01$; n.s., no significant difference.

Figures

Figure 1

MiR-376a inhibits proliferation, migration invasion and apoptosis of NSCLC cells. (a) MiR-376a expression levels in NuLi-1 cells and A549 cells. Equal amounts of cells were harvested and prepared for qRT-PCR. NuLi-1 was used as normal control line. (b) Effects of miR-376a on cell proliferation ability in A549 cells by CCK-8 kit assay. (c) Effects of miR-376a on cell migration in A549 cells by scratch assay. (d) Effects of miR-376a on cell invasion in A549 cells by transwell invasion assay. (e) Effects of miR-

376a on cell apoptosis in A549 cells by Hoechst33342 staining. Data were expressed as mean $\pm$ standard deviation of three independent experiments. **, $P < 0.01$; n.s., no significant difference. Mimics, miR-376a mimics; inhibitor, miR-376a inhibitor; miR-NC, miR negative control; control, no treatment. Bar, 200 μ m.

Figure 2

Direct interaction between miR-376a and the 3'-UTR of Rab1A in vitro. (a and b) The mRNA or protein expression level of Rab1A in NuLi-1 cells and A549 cells. (c and d) The mRNA or protein expression level

of Rab1A in A549 transfected with miR-376a mimics, miR-376a inhibitor or miR-NC. (e) Generation of Rab1A-WT- and Rab1A-Mut-containing luciferase reporter plasmids via sequential mutation of the predicted miR-376a binding site in the Rab1A 3' untranslated region. (f) Luciferase activity of Rab1A-WT and Rab1A-Mut co-transfected with miR-376a mimics or miR-NC. Data were expressed as mean $\pm$ standard deviation of three independent experiments. **, $P < 0.01$; n.s., no significant difference.

Figure 3

Rab1A regulate tumorigenesis. (a and b) mRNA and protein expression of Rab1A in A549 cells transfected with Rab1A or si-Rab1A. (c) Effects of Rab1A on cell proliferation ability in A549 cells by CCK-8 kit assay. (d) Effects of Rab1A a on cell migration in A549 cells by scratch assay. (e) Effects of Rab1A on cell invasion in A549 cells by transwell invasion assay. (f) Effects of Rab1A on cell apoptosis in A549 cells by Hoechst33342 staining. Data were expressed as mean $\pm$ standard deviation of three independent experiments. **, $P < 0.01$; n.s., no significant difference. Bar, 200 μm .

Figure 4

Rab1A MiR-376a inhibits the NSCLC cell proliferation by targeting Rab1A. Cell viability of A549 cells transfected with Rab1A, Rab1A and miR-376a mimics, si-Rab1A, si-Rab1A, miR-376a inhibitor, and miR-

NC. Data were expressed as mean $\pm$ standard deviation of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; n.s., no significant difference.

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