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Regulation by Pink1 on the mitochondrial dysfunction in endothelial cells post the hypoxia mimetic agent $CoCl_2$ treatment

G.-H. LIU[1], Y. WEN[2], P. YANG[1], G.-F. LIU[3]

[1]Department of Cardiology, [2]Department of Endocrinology, [3]Department of Radiology; China-Japan Union Hospital of Jilin University, Changchun, China

Guohui Liu and Yan Wen equally contributed to this work

Abstract. – OBJECTIVE: To explore the role of miR-451a in the migration and invasion of non-small cell lung cancer (NSCLC) cells.

MATERIALS AND METHODS: Quantitative Real time-polymerase chain reaction (qRT-PCR) and Western blot were performed to detect the levels of miR-451a and activating transcription factor 2 (ATF2) in NSCLC. Transwell assay was employed to analyze the migratory and invasive abilities in NSCLC cells. Dual-luciferase reporter assay was applied to confirm the binding condition of miR-451 and its target gene in NSCLC cells.

RESULTS: MiR-451a was downregulated in NSCLC tissues and lung cancer cell lines A549 and NCI-H460, while ATF2 was upregulated. The mRNA level of miR-451a was negatively correlated to ATF2. Additionally, miR-451a regulated cell migration and invasion through targeting ATF2. Furthermore, ATF2 could reverse the inhibitory migration and invasion of A549 cells induced by miR-451a.

CONCLUSIONS: MiR-451a inhibits the migratory and invasive abilities of NSCLC cells through ATF2 regulation. The newly identified miR-451a/ATF2 axis provides a novel insight into the pathogenesis of NSCLC.

Key Words:
miR-451a, Migration, Invasion, ATF2, Non-small cell lung cancer.

Introduction

Lung cancer is the most common malignant tumor with the highest mortality rate[1,2]. Among all the diagnostic cases of the lung cancer, approximately 85% were non-small cell lung cancer (NSCLC), including adenocarcinoma and squamous cell carcinoma[3,4]. Most NSCLC patients are diagnosed at advanced stage and had a poor prognosis. Thus, it is necessary to find biological markers for the early diagnosis and survival prediction of NSCLC.

MicroRNAs (miRNAs), with a length of 22-28 oligonucleotides, could induce mRNA degradation or inhibit gene expression through binding to 3'-UTR of target mRNA[5,6]. Evidence has revealed that miRNAs may participate in almost 60% of all human genes at post-transcriptional level[7,8]. MiR-451a plays a crucial role in the occurrence and development in various tumors, including papillary thyroid carcinoma, renal cell carcinoma, gastric cancer and dilated cardiomyopathy[9-12]. MiR-451a could abrogate the treatment resistance in FLT3-ITD-positive acute myeloid leukemia[13]. Moreover, miR-451a is acted as a noninvasive biomarker for early prediction of recurrence and prognosis of pancreatic ductal adenocarcinoma[14]. Therefore, we strongly believed that miR-451a may play great roles in NSCLC. Activating transcription factor 2 (ATF2) belongs to the cAMP response element binding family, which is activated by c-Jun N-terminal kinases phosphorylation and responses to regulatory biological progresses[15,16]. ATF2 activates the transcription of genes by dimer formation with other members of the activator protein superfamily and stimulates cytokine productions in response to DNA damage and cell death[17,18]. ATF2, a paradigm of the multifaceted regulation of transcription factors, is involved in glucose metabolism and tumorigenesis[19,20]. In addition, ATF2 is associated with the malignant phenotypes and poor prognosis of ovarian adenocarcinoma patients and renal cell carcinoma[21,22]. Considering these functions, we proposed that miR-451a could regulate cell migration and invasion in NSCLC *via* regulating ATF2 expression.

Corresponding Authors: Ping Yang, MD; e-mail: yanglping87v@sohu.com
Guifeng Liu, MD; e-mail: jlfsliuguifeng@163.com

Materials and Methods

Tissue Specimens

In accordance to WHO classification, 55 paired lung carcinoma and paracancerous tissues were collected from the Affiliated Yantai Yuhuangding Hospital of Qingdao University, from 2015 to 2017. All the specimens were surgically resected and immediately stored at -80°C before RNA extraction. Patients did not receive preoperative therapies, including radiotherapy and chemotherapy. This study was approved by the Ethical Committee of Affiliated Yantai Yuhuangding Hospital of Qingdao University. All patients signed informed consent.

Cell Culture

We obtained two human lung cancer cell lines A549 and NCI-H460 and normal lung cells MRC-5 from American Type Culture Collection (ATCC, Rockville, MD, USA). All cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) cultured at 37°C with 5% CO_2.

Transfection

MiR-451a mimic, miR-451a inhibitor, pcDNA3.1-ATF2 and control vector were constructed.

A549 cells were seeded into 6-well plates and cultivated overnight before transfection. The plasmid vectors were transfected into A549 cells used Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA).

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) or MIRcute Extraction and Separation of miRNAs Kit (Tiangen, Beijing, China) were utilized to isolate and extract total RNAs or miRNAs, respectively. To detect the expression of miRNA or mRNA, PrimeScript™ II 1st Strand complementary Deoxyribose Nucleic Acid (cDNA) Synthesis Kit (TaKaRa, Otsu, Shiga, Japan) was conducted to reversely transcribed the first complementary Deoxyribose Nucleic Acid (cDNA) chain. Then, SYBR Premix kit or SYBR Prime Script miRNA RT-PCR kit (both purchased from TaKaRa, Otsu, Shiga, Japan) was applied to perform the RT-qPCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as the loading controls. The relative expression levels of miRNA and mRNA were calculated through $2^{-\Delta\Delta Ct}$ method. The primer sequences were as the follows: MiR-451a: F: 5'-ACACTCCAGCTGGGAAACCGTTACCATTACT-3', R: 5'-CTGGTGTCGTGGAGTCGGCAA-3'; ATF2: F: 5'-CCGGATCCATGAAATTCAAGTTACATGT-3', R: 5'-GGCTCGAGTCAACTTCCTGAGGGCTGTG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Protein Extraction and Western Blotting

Radioimmunoprecipitation assay (RIPA) Lysis Buffer supplemented with phenylmethylsulfonyl fluoride (PMSF) (both from Solarbio, Beijing, China) were employed to extract the total proteins from lung cancer cells. After centrifugation, protein concentration was measured by bicinchoninic acid (BCA) reagent kit (Solarbio, Beijing, China). After electrophoresis, the separated proteins were transferred to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was incubated with rabbit anti-ATF2 monoclonal antibody (1:1000; Abcam, Cambridge, MA, USA) at 4°C overnight. A rabbit secondary antibody (Santa Cruz, CA, USA) containing horseradish peroxidase (HRP)-conjugated was used to incubate the membrane for 1 h at room temperature. GAPDH mouse antibody (1:3000; ZSGB-BIO, Beijing, China) was used as internal control. The interest proteins were performed by electrochemiluminescence (ECL) Western Blotting Detection System (BestBio, Beijing, China).

Transwell Assay

Transwell chamber (8 μm in pore size, Costar, Beijing, China) was pre-coated with Matrigel (Clontech, Mountain View, CA, USA). A549 cells were prepared for cell suspension. 200 μL of cell suspension in serum-free medium were added in the upper chamber. Meanwhile, 500 μL of normal medium containing 15% FBS were added in the lower chamber. 24 h later, non-adherent cells were removed using cotton swab cautiously. Cells were fixed with methanol and stained with crystal violet. Cell counting was then carried out through the microscope.

Plasmid Construction and Luciferase Reporter Assay

TargetScan software (www.targetscan.org) was used to predict target genes of miR-451a; moreover, AFT2 was screened out. Plasmids with wild-type and mutant-type 3'UTR oligonucleotides fragment inserting in pmirGlvector of AFT2 were constructed (pmirGlo-ATF2-WT, pmirGlo-ATF2-MUT). Plasmid efficacy was verified

by sequencing. After co-transfection of miR-451a mimic or negative control (NC) and WT or MUT into A549 cells, the dual-luciferase activity was detected using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) with Renilla luciferase as normalization.

Statistical Analysis

Statistical analyses were performed using software Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA). Measurement data were compared by Student's *t*-test. The differences among groups were compared by one-way ANOVA, followed by LSD post-hoc test. $p<0.05$ indicated that the results were statistically significant.

Results

Correlation of miR-451a and ATF2 in NSCLC Tissues

To explore the function of miR-451a in NSCLC, the expression level of miR-451a was measured in 55 pairs of NSCLC tissues using qRT-PCR. The data showed that miR-451a was lowly expressed in NSCLC tissues compared with corresponding adjacent normal tissues ($p<0.0001$, Figure 1A). In addition, we also determined the mRNA level of ATF2 and it was higher in NSCLC tissues than that in corresponding adjacent normal tissues ($p<0.0001$, Figure 1B). Correlation analyses showed that miR-451a negatively correlated to ATF2 in NSCLC tissues ($p=0.0012$, r=-0.4252, Figure 1C). Besides, miR-451a was downregulated in NSCLC cells, A549 ($p=0.0035$) and NCI-H460 ($p=0.0004$) compared with that of normal lung epithelium cell MRC-5 (Figure 1D).

MiR-451a Inhibits Migration and Invasion of NSCLC Cells

To determine the function of miR-451a in NSCLC, the migratory and invasive abilities were evaluated. Transfection efficacies of miR-451a mimic and miR-451a inhibitor in A549 cells were verified by qRT-PCR. As expected, mRNA level of miR-451a was incre-

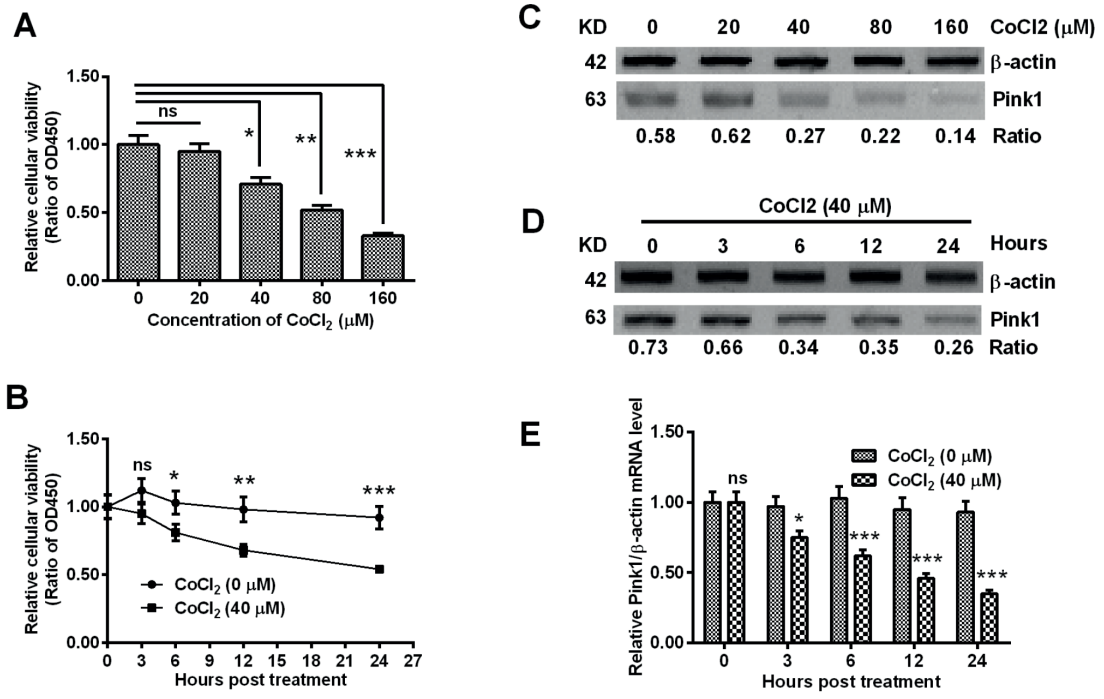

Figure 1. Regulation by cobalt chloride ($CoCl_2$) on cell viability and Pink1 expression in human endothelial EA.hy926 cells. Cell viability was tested with MTT assay for human endothelial EA.hy926 cells, which were treated with 20, 40, 80 or 160 μM $CoCl_2$ for 12 hours ***(A)***, with 40 μM $CoCl_2$ for 0, 3, 6, 12 or 24 hours ***(B)***. Western blotting assay was performed to quantified the Pink1 expression in protein level in the EA.hy926 cells, which were treated with 20, 40, 80 or 160 μM $CoCl_2$ for 12 hours ***(C)***, with 40 μM $CoCl_2$ for 0, 3, 6, 12 or 24 hours ***(D)***. Real-time quantitative PCR was performed to examine the relative mRNA level of Pink1 to β-actin in the EA.hy926 cells, which were treated with 40 μM $CoCl_2$ for 0, 3, 6, 12 or 24 hours ***(E)***. Experiments were performed independently for triplicate. $^{*}p<0.05$, $^{**}p<0.01$ or $^{***}p<0.001$, ns: no significance.

ased (p=0.0013) after transfection of miR-451a mimic, which was reduced (p=0.0080) after transfection of miR-451a inhibitor in A549 cells (Figure 2A). Subsequently, the migratory and invasive cell numbers were reduced (p=0.0015 and 0.0035) when A549 cells were transfected miR-451a mimic. On the contrary, the migratory and invasive cell numbers were increased after transfection of miR-451a inhibitor (p=0.00114 and 0.0043, Figure 2B). The above results indicated that miR-451a inhibits migration and invasion of NSCLC cells.

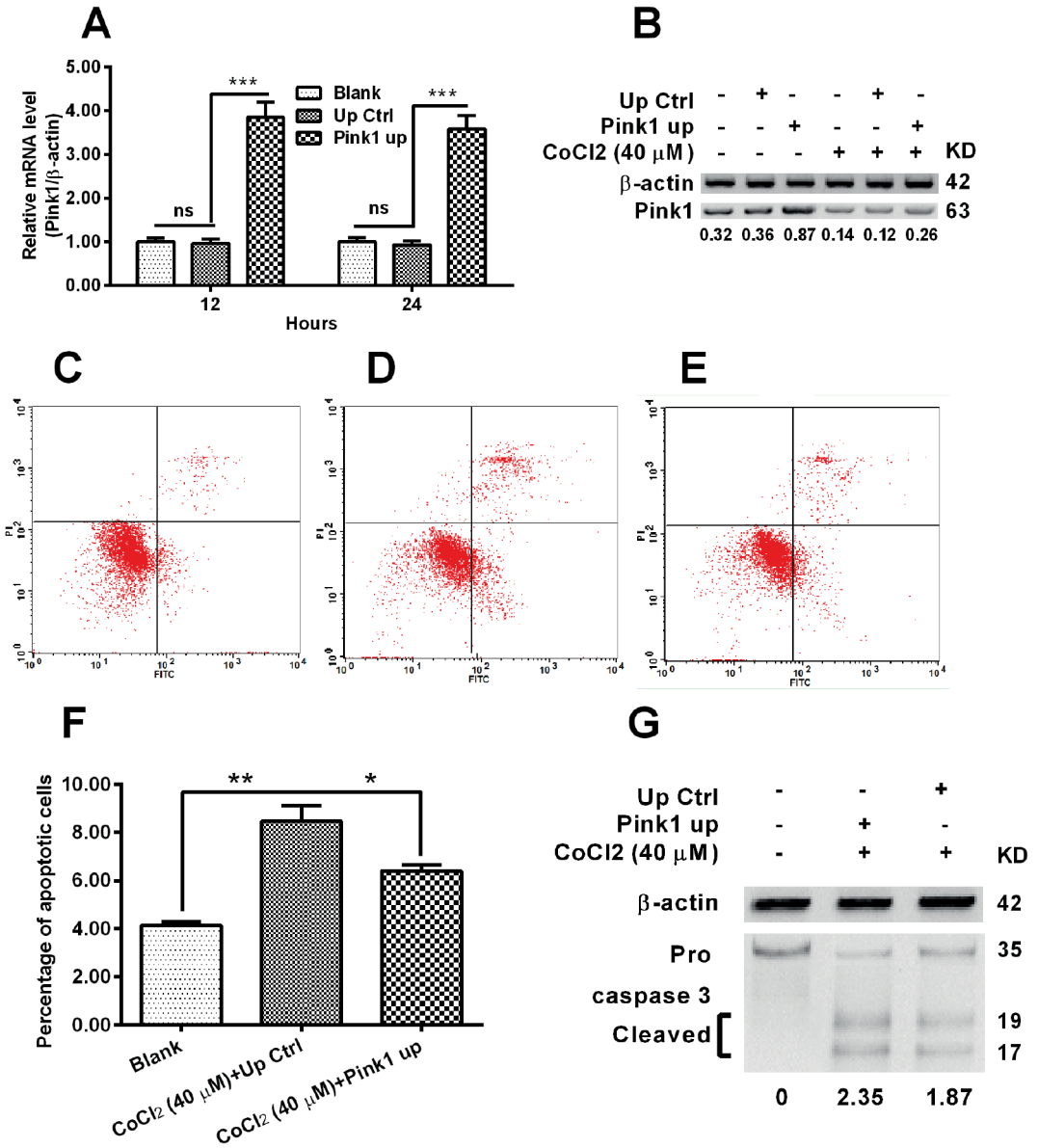

Figure 2. Apoptosis in the EA.hy926 cells post the $CoCl_2$ treatment and the Pink1 overexpression. A and B, Relative Pink1 to β-actin mRNA level ***(A)*** or Western blotting assay of Pink1 ***(B)*** in the blank or the $CoCl_2$-treated (40 μM) EA.hy926 cells, which were transfected with CAT-pcDNA3.1(+) (Up Ctrl), with Pink1-pcDNA3.1(+) (Pink1 up). ***C-E***, Apoptosis (Flow cytometry analysis) in the blank ***(C)*** or the $CoCl_2$-treated EA.hy926 cells, which were pre-transfected with CAT-pcDNA3.1(+) ***(D)***, with Pink1-pcDNA3.1(+) ***(E)*** for 12 hours. ***F,*** Apoptosis quantification of blank or the $CoCl_2$-treated EA.hy926 cells, which were pre-transfected with CAT-pcDNA3.1(+) ($CoCl_2$(40 μM)+Up Ctrl), with Pink1-pcDNA3.1(+) ($CoCl_2$(40 μM)+Pink1 up) for 12 hours. Early apoptotic cells were denoted in the upper right quadrant. ***G:*** Western blotting assay of cleaved caspase 3 in the three groups of EA.hy926 cells (blank, $CoCl_2$(40 μM)+Up Ctrl and $CoCl_2$(40 μM)+Pink1 up). Experiments were performed independently for triplicate. $^{*}p< 0.05$ or $^{**}p< 0.01$.

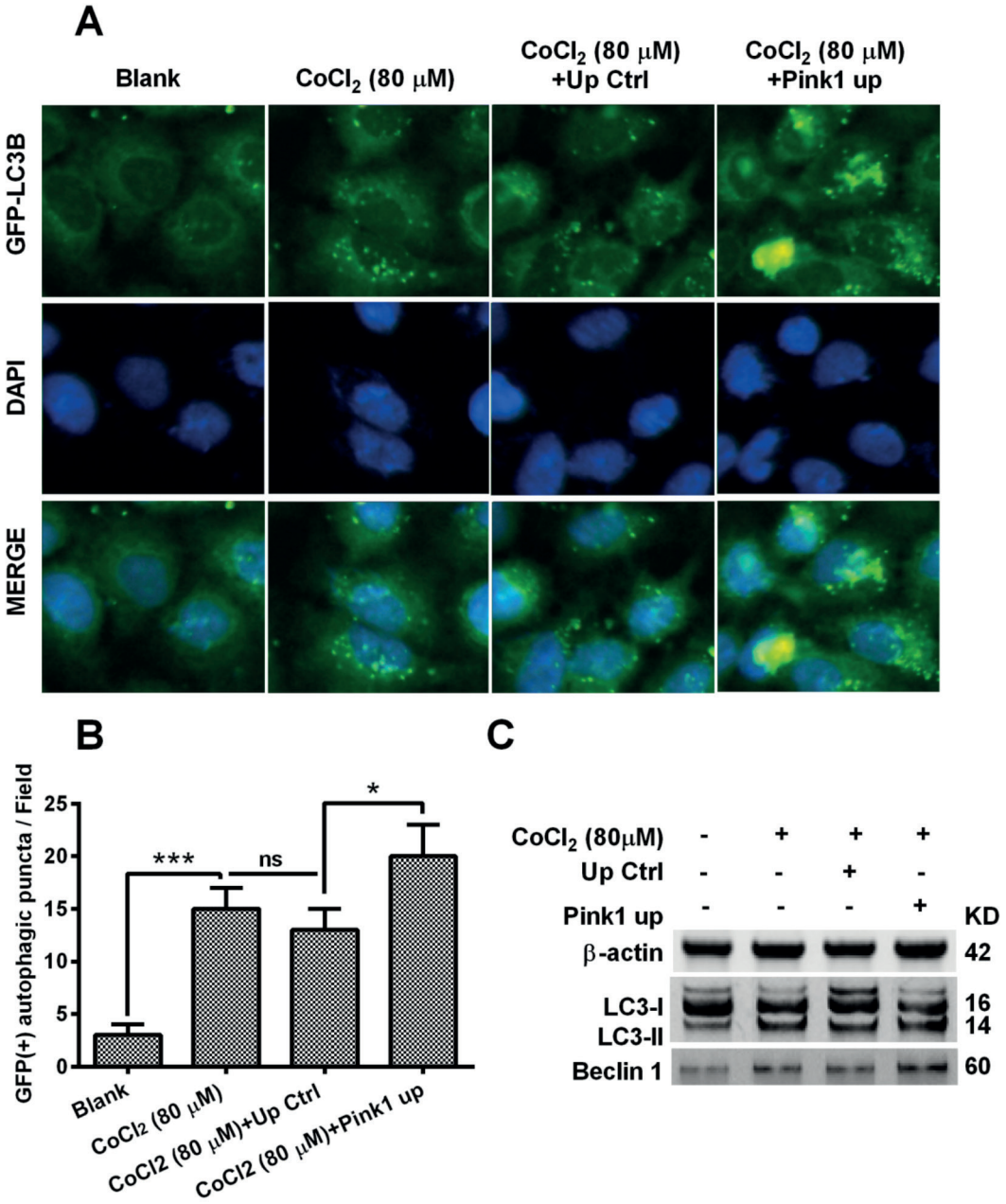

Figure 3. Autophagy in the EA.hy926 cells post the $CoCl_2$ treatment and the Pink1 overexpression. ***A,*** Autophagic vesicles were indicated as green fluorescence-positive puncta in the four groups of EA.hy926 cells (blank, $CoCl_2$ treatment with 80 μM (12 hours), $CoCl_2$ treatment and Up Ctrl, $CoCl_2$ treatment and Pink1 up), which were transfected with LC3-GFP reporter plasmid. ***B,*** Counting of autophagic vesicles in the four groups of EA.hy926 cells. ***C,*** Western blotting of autophagy-related proteins, LC3-I/II and Beclin 1 in the four groups of EA.hy926 cells; Experiments were performed independently for triplicate. $^{*}p< 0.05$ or $^{***}p< 0.001$, ns: no significance.

MiR-451a Targets ATF2 and Inhibits its Expression

ATF2 was predicted to be a target gene of miR-451a by TargetScan and the binding site was located at 2960-2967 in the mRNA of ATF2 3'-UTR. To verify whether miR-451a could direct target to ATF2, the binding pmirGlo-ATF2-WT (WT) and pmirGlo-ATF2-MUT (MUT) were constructed, respectively (Figure 3A). Subsequently, WT or MUT and miR-451a mimic were co-transfected into A549 cells and then luciferase abilities were detected. As expected, the luciferase activity was

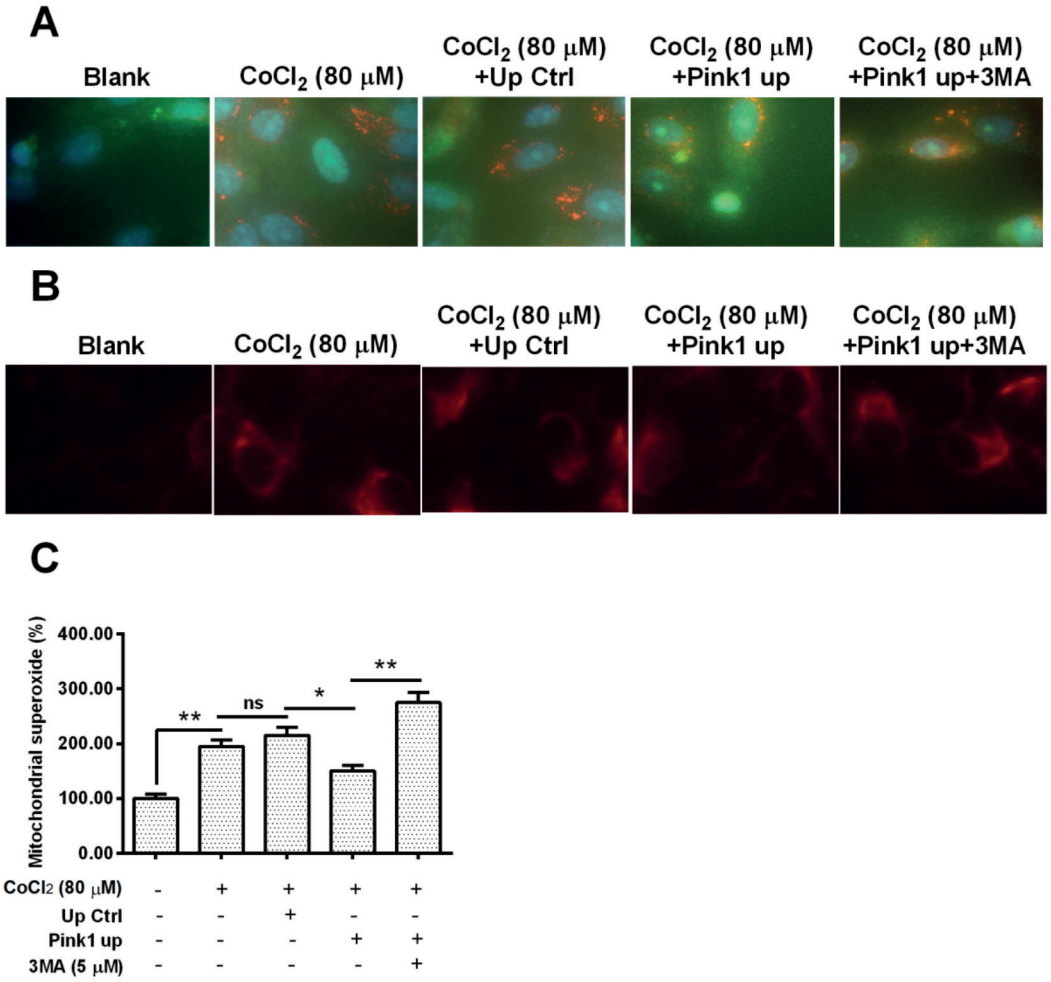

Figure 4. Regulation by Pink1 overexpression on the $CoCl_2$-induced mitochondrial dysfunction in EA.hy926 cells. EA.hy926 cells were transfected with CAT-pcDNA3.1(+) (Up Ctrl), with Pink1-pcDNA3.1(+) (Pink1 up) for 12 hours, were treated with CoCl2 (80 μM) and 3MA (0 or 5 μM) for another 12 hours (blank cells as blank control), then the JC-1 staining for mitochondrial membrane potential (MMP) ***(A)***, the fluorophore 5-(and-6)-chloromethyl-2,7-dichlorodi-hydrofluorescein diacetate (DCFH-DA) staining for mitochondrial reactive oxygen species (ROS) ***(B)*** and the MitoSOXTM Red staining for mitochondrial superoxide ***(C)*** was performed. All experiments were repeated independently in triplicate. $^{*}p< 0.05$ or $^{**}p< 0.01$, ns: no significance.

reduced (p=0.0011) in cells co-transfected with WT and miR-451a, while the luciferase activity of MUT showed no alteration (p=0.7717) in A549 cells (Figure 3B). To further determine whether miR-451a mediated ATF2, ATF2 expression was evaluated after exogenous alteration of miR-451a. As expected, mRNA level of ATF2 was repressed (p=0.0030) when cells were transfected with miR-451a mimic, which was increased (p=0.0017) after transfection with miR-451a inhibitor (Figure 3C).

ATF2 Could Reverse Partial Effects of miR-451a on Cell Migration and Invasion

To further explore the mechanism of miR-99a in regulating migration and invasion through ATF2, miR-99a mimic and pcDNA3.1-ATF2 were co-transfected into A549 cells. The mRNA level of ATF2 was decreased (p=0.0003) when transfected with miR-451a mimic and it was reversed after transfection of pcDNA3.1-ATF2 (p=0.089, Figure 4A). The protein level of ATF2 showed the same results with that of mRNA level (Figure 4B). Transwell assay revealed that the migratory number was increased (p=0.0224) when co-transfection with miR-451a mimic and pcDNA3.1-ATF2, which was reversed by miR-451a mimic transfection (Figure 4C). Similarly with migration results, the invasive ability was also enhanced (p=0.0199) when co-transfection with miR-451a mimic and ATF2 was compared with those only transfected with miR-451a mimic (Figure 4D).

Discussion

Non-small cell lung cancer accounts for 85% of lung cancer, including adenocarcinoma and squamous cell carcinoma[3,4]. Most NSCLC patients are diagnosed at advanced stage and had a poor prognosis. Thus, it is necessary to find biological markers for the early diagnosis and survival prediction of NSCLC.

MicroRNAs (miRNAs) could induce mRNA degradation or inhibit gene expression through binding to 3'-UTR of target mRNA. MicroRNAs participate in almost 60% of all human genes at post-transcriptional level[5,6]. MiR-451a is served as a noninvasive biomarker and it could alleviate drug resistance in miscellaneous cancers[13,4]. Liu et al[23,24] elucidated that miR-451a inhibited cell proliferation and enhanced tamoxifen sensitive in breast cancer. More importantly, miR-451a acted as a tumor suppressor by retarding cell migration and invasion in melanoma[25]. Our results were consistent with the previous findings that miR-451a was downregulated in NSCLC tissues and lung cancer cells. In lung cancer cells A549, miR-451a overexpression suppressed cell migration and invasion while knockdown of miR-451a obtained the opposite results. We first proposed that miR-451a regulated cell migration and invasion through targeting ATF2.

ATF2 is involved in multiple regulatory biological progresses, including gene transcription, DNA damage, metabolism and tumorigenesis[18,19]. Increasing evidences have revealed that ATF2 was a target of several miRNAs, including miR-26b, miR-204, miR-622 and miR-451[26-29]. Further study in our research found the similar results that ATF2 was a target of miR-451a and it was regulated by miR-451a in lung cancer cells A549. We first proposed the connection between miR-451a and ATF2 in NSCLC. In hepatocellular carcinoma, ATF2 knockdown promoted the anticancer activity of sorafenib[30]. Similar findings were discovered by Li et al[30], and they indicated that ATF2 knockdown suppressed cell growth and enhances sensitivity to chemotherapy in pancreatic cancer. Consistent with the previous results, we first verified that miR-451a mediated migration and invasion of A549 cells through regulating ATF2 expression.

Conclusions

We showed that miR-451a was downregulated in non-small cell lung cancer tissues and lung cancer cells A549 and NCI-H460, while ATF2 was upregulated. The mRNA level of miR-451a had negative correlation with ATF2. MiR-451a regulated the migration and invasion of lung cancer cells through targeting ATF2. In addition, ATF2 could reverse partial functions of miR-451a on migration and invasion of A549 cells.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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