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

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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 451 and its target gene in NSCLC cells.

RESULTS: MiR-451a was downregulated S-CLC tissues and lung cancer cell lines A549 NCI-H460, while ATF2 was upregulated. The NA level of miR-451a was negatively correlated ATF2. Additionally, miR-451a re ell migration and invasion through tar g A Furthermore, ATF2 could reverse th nhibitor gration and invasion of A549 cells uced by

CONCLUSIONS: MiR- inhi gratory and invasive hilit SCLC cells through ATF2 regu n. The identified miR-451a/ATF2 a rovides a n sight into the pathogen NSCLC.

Key Words:
miR-451 igration, Invasio , Non-small cell lung can

Intro tion

g can most common malignant tum ith the est mortality rate[1,2]. Among all t iagnostic cases of the lung cancer, ap- pr % were non-small cell lung can- (NSCLC), including adenocarcinoma and mous cell carcinoma[3,4]. Most NSCLC pa- t e diagnosed at advanced stage and had a poor prognosis. T is necessary to find bio markers for arly diagnosis and s al prediction of NSCLC.

licroRNAs (miRNAs), with a length of 22-28 onucleotides uld induce mRNA degradation or inhibit ge xpression through binding to 3 of targ RNA[5,6]. Evidence has revealed that y participate in almost 60% of all human genes at post-transcriptional level[7,8]. iR-451a plays a crucial role in the occurrence pment in various tumors, including pa- ary 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.

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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, [illegible]D-NA3.1-ATF2 and control vector were constru[illegible]

A549 cells were seeded into 6-well plates [illegible] cultivated overnight before transfection. The pl[illegible] smid vectors were transfecte[illegible]549 cells used Lipofectamine 2000 [illegible]gent ([illegible]itrogen, Carlsbad, CA, USA).

RNA Isolation and [illegible]eal Time-Polymerase [illegible]in Rea[illegible] (qRT-PCR)

TRIzol Reagent ([illegible]trogen, Carls[illegible]A, USA) or MIRcute Ext[illegible]d Separation [illegible]iRNAs Kit (Tiangen, [illegible]ng, [illegible] were utilized to isolate and extra[illegible]t total RNAs [illegible]NAs, respectively. To detect [illegible]expression of mi[illegible]r mRNA, PrimeScri[illegible]1 1st Strand comple[illegible]entary Deoxyribose [illegible]eic Ac[illegible]DNA) Synthesis Kit (TaKaRa, Ot[illegible]ga, J[illegible] was c[illegible]ucted to reversely transcribe [illegible] compl[illegible]tary Deoxyribose Nu-[illegible] Acid [illegible]A) cl[illegible] Then, SYBR Premix kit [illegible]R Pr[illegible] miRNA RT-PCR kit (both purc[illegible]d from [illegible]Ra, Otsu, Shiga, Japan) was appl[illegible] to perform the RT-qPCR. Glyceraldehyde 3-[illegible]drogenase (GAPDH) and U6 were [illegible]as the loading controls. The relative expression [illegible]s of miRNA and mRNA were calculated throug[illegible] method. The primer sequences were as the follo[illegible] MiR-451a: F: 5'-ACACTCCAGCTGGGAAACCGTTACCATTACT-3', R: 5'-CTGGTGTCGTGGAGTCGGCAA-3'; ATF2: F: [illegible] GATCCATGAAATTCAAGTTACAT[illegible], 5'-GGCTCGAGTCAACTTCCTGA[illegible]GCTGTG-3', U6: F: 5'-GCTTCGGCAGCA[illegible]TACTAAA-AT-3', R: 5'-CGCTTCAGAATTTC[illegible]TCAT-3'; GAPDH: F: 5'-CGCTCTCT[illegible]TCCTC[illegible]TC-3' R: 5'-ATCCGTTGACTCC[illegible]CTTCAC-[illegible]

Protein Extractio[illegible]d We[illegible]n Blotting

Radioimmunopre[illegible]ion[illegible]y (RIP[illegible]Lysis Buffer supplem[illegible]d w[illegible]hylmet[illegible]ulfonyl fluoride (PMSF[illegible]oth from[illegible]bio[illegible]ing, China) were em[illegible]d to extract th[illegible]oteins from lung canc[illegible]. After centrif[illegible]tion, protein concent[illegible]n was[illegible]ured by bicinchoninic acid (BCA) reagent kit (Sc[illegible] Beijing, China). After elec[illegible]esis, the sepa[illegible] proteins were trans[illegible]d to the polyvinylidene difluoride (PVDF) [illegible]mbrane (Milli[illegible]re, Billerica, MA, USA). The [illegible]mbrane was [illegible]bated with rabbit anti-ATF2 [illegible]oclonal antib[illegible] (1:1000; Abcam, Cambridge, M[illegible]SA) at 4[illegible]overnight. A rabbit secondary anti[illegible]Cruz, CA, USA) containing horseradish peroxidase (HRP)-conjugated was used to [illegible]ubate the membrane for 1 h at room temperature. [illegible]DH mouse antibody (1:3000; ZSGB-BIO, [illegible]ijing, 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.

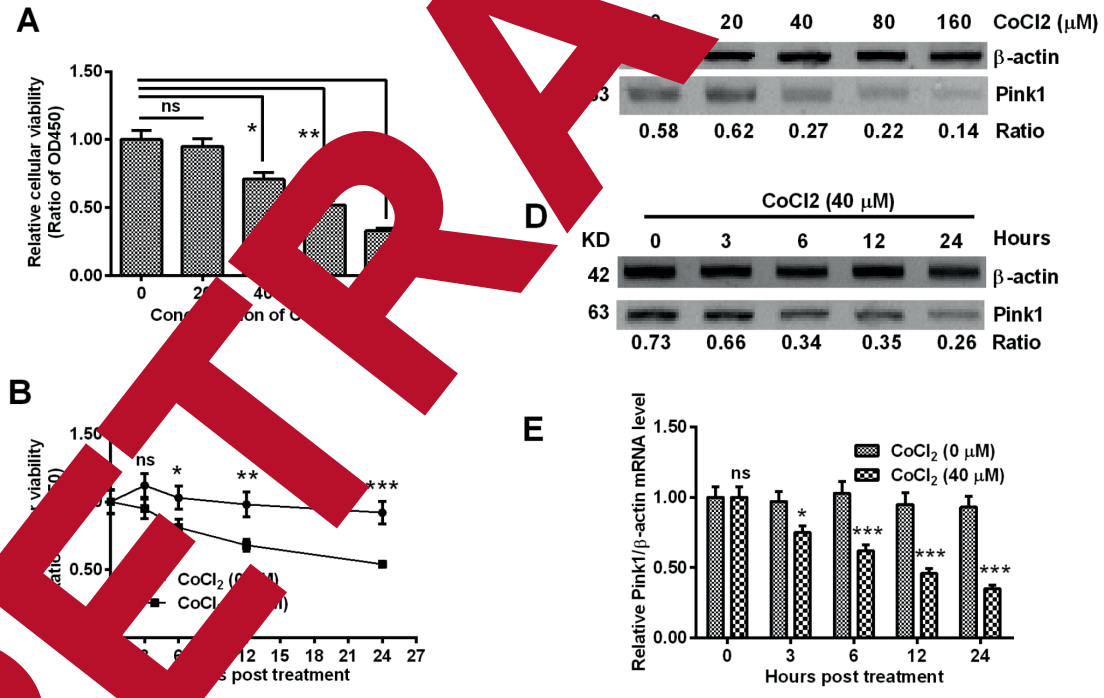
Results

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 lowl in NSCLC tissues compared with adjacent normal tissues ($p<0.00$ Figure 1A). In addition, we also determin mRNA level of ATF2 and it was higher in C tissues than that in correspondin djacent l tissues ($p<0.0001$, Figure 1 Correlation showed that miR-451 gativel correlate ATF2 in NSCLC tiss $p=0.0$ =-0.4252, Figure 1C). Besides, 51a down ated in NSCLC cells 549 (5) and -H460 ($p=0.0004$) c red wi of nal lung epithelium RC-5 (Figur

MiR-45 nhi igration and Invasion of Cells

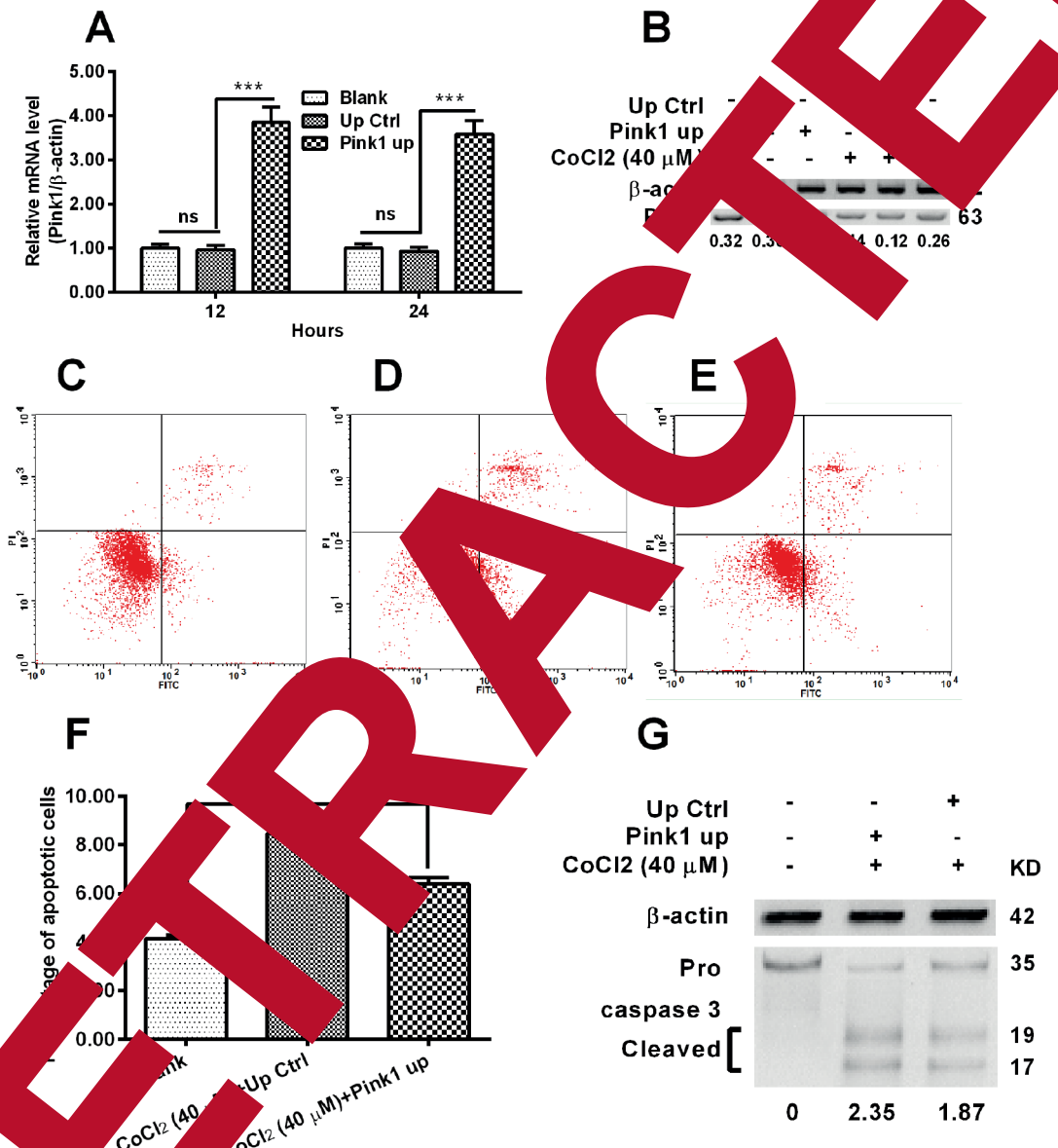
T mine the fu n of miR-451a in N C, the migratory and invasive abili- were eval ed. Transfection efficacies miR-451a m c and miR-451a inhibitor 549 cells v verified by qRT-PCR. As e d, mR evel of miR-451a was incre-



Figu Regulation by cobalt chloride ($CoCl_2$) on cell viability and Pink1 expression in human endothelial EA.hy926 cells. Ce sted with MTT assay for human endothelial EA.hy926 cells, which were treated with 20, 40, 80 or 160 μM C 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 ink1 expression in protein level in the EA.hy926 cells, which were treated with 20, 40, 80 or 160 μM $CoCl_2$ for 12 hours ith 40 μM $CoCl_2$ for 0, 3, 6, 12 or 24 hours ***(D)***. Real-time quantitative PCR was performed to examine the relative l el 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)***. Exper nts 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 w sed after transfection of miR-4 (p=0.00114 and 0.0043, Figure The above results indicated that miR-451 bits migration and invasion of NSCLC cel



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

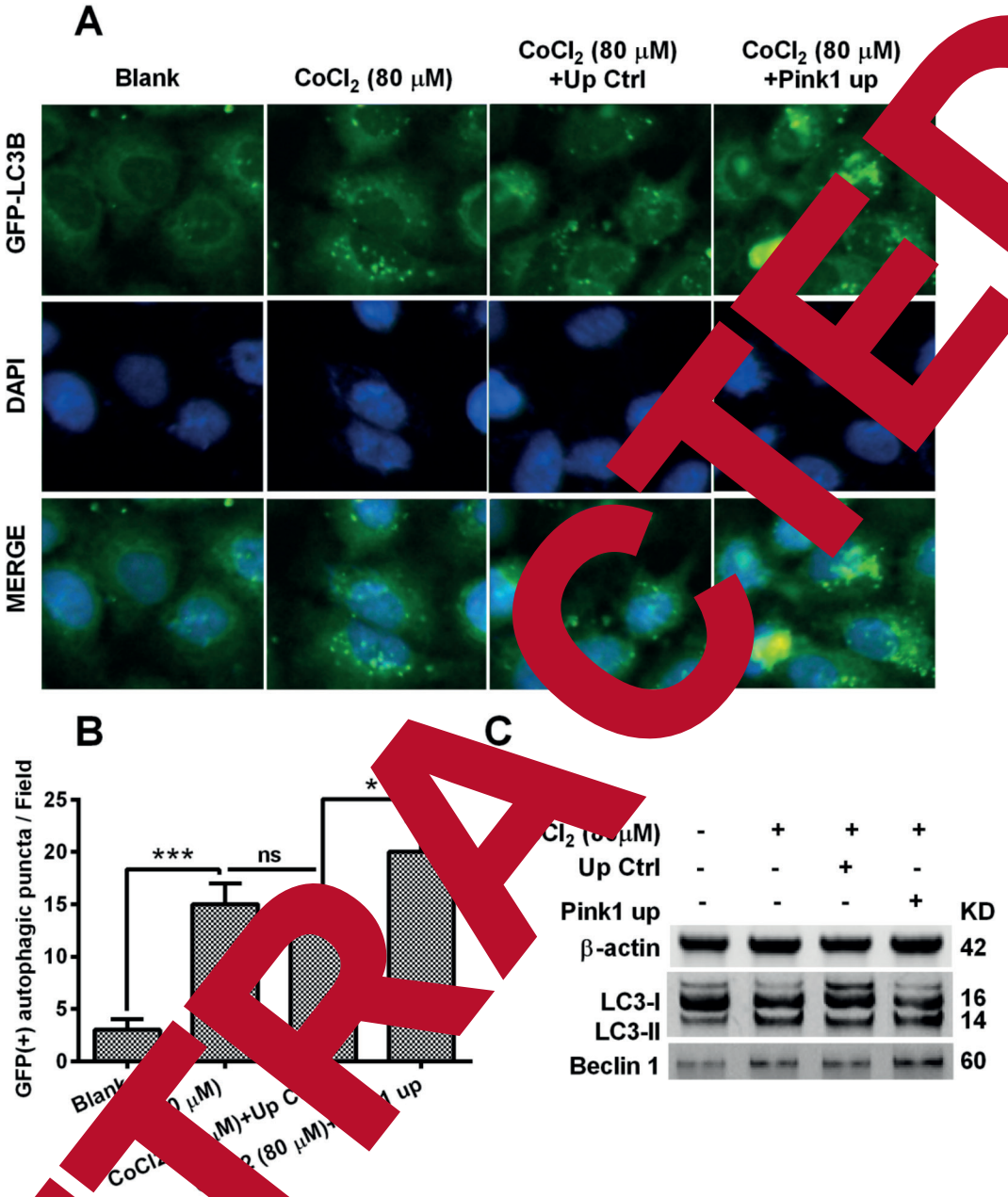


Figure tophagy in the EA.hy92 cells post the $CoCl_2$ treatment and the Pink1 overexpression. ***A,*** Autophagic vesicles were i ed as g fluorescence-positive puncta in the four groups of EA.hy926 cells (blank, $CoCl_2$ treatment with 80 μM urs), C reatment and Up Ctrl, $CoCl_2$ treatment and Pink1 up), which were transfected with LC3-GFP reporter plas Co g of aut ic vesicles in the four groups of EA.hy926 cells. ***C,*** Western blotting of autophagy-related protein and Bec n the four groups of EA.hy926 cells; Experiments were performed independently for triplica- $^*p < 0.05$ 0.001 no significance.

MiR 1a Targets ATF2 and Inhibits it

TF2 was predicted to be a target gene of miR- by TargetScan and the binding site was loca- 960-2967 in the mRNA of ATF2 3'-UTR. To v y 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

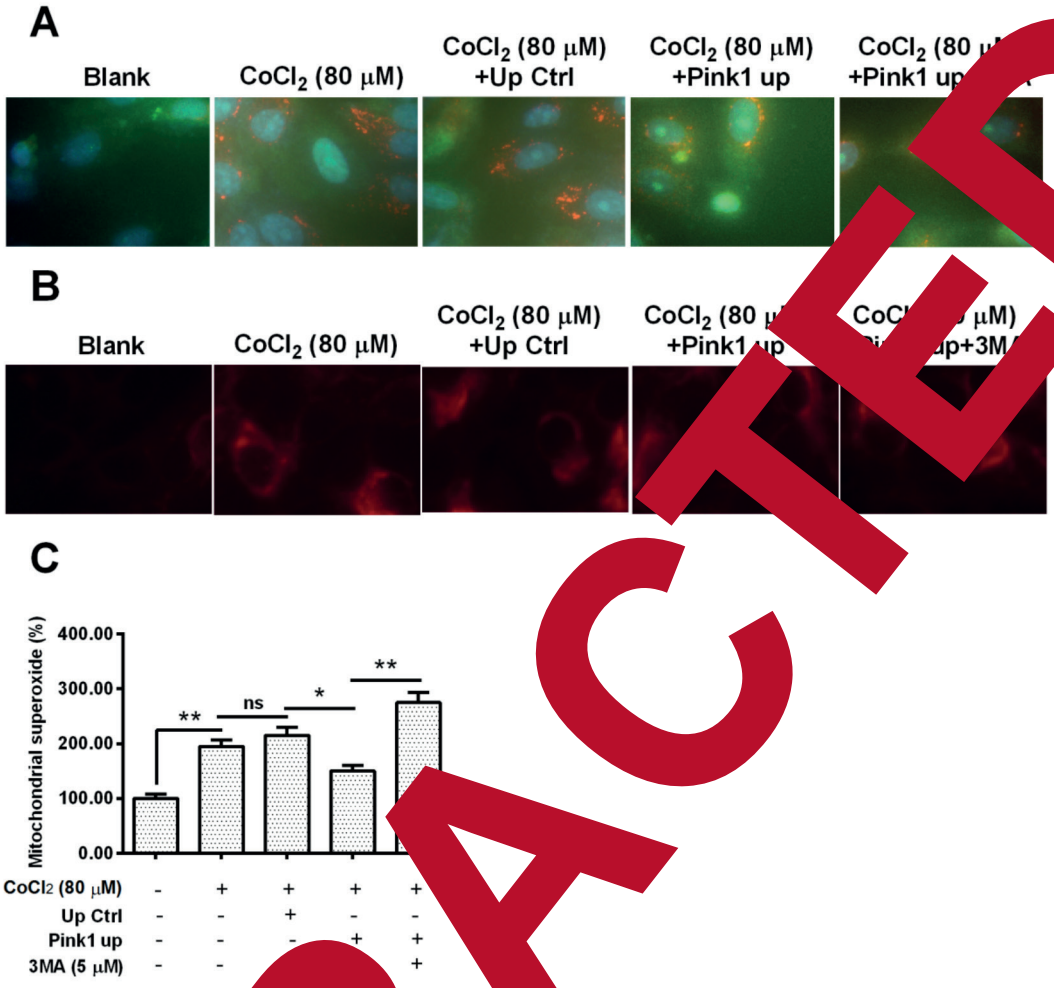


Figure 4. Regulation by Pink1 expressio the $CoCl_2$-in uced mitochondrial dysfunction in EA.hy926 cells. EA.hy926 cells were transfected with CA DNA3.1(- Pink1-pcDNA3.1(+) (Pink1 up) for 12 hours, were treated with CoCl2 (80 μM) and 3MA (0 or an ank cells as blank control), then the JC-1 staining for mitochondrial membrane potential (MM), th e 5-(and-6)-chloromethyl-2,7-dichlorodi-hydrofluorescein diacetate (DCFH-DA) staining for mitochondri ctive oxy ies (ROS) ***(B)*** and the MitoSOXTM Red staining for mitochondrial superoxide ***(C)*** was performed. A eriments were independently in triplicate. $^{*}p< 0.05$ or $^{**}p< 0.01$, ns: no significance.

reduced (p 0.0011) in c transfected with WT and -451a, while th ferase activity of MU owed no alteration (p 0.7717) in A549 cells gure 3D o further determine whether mi me d ATF ATF2 expression was evalu exogen lteration of miR-451a. A expec RNA l of ATF2 was repressed 030) w were transfected with miR-451a mic, whi as increased (p=0.0017) after tran ion with miR-451a inhibitor (Figure 3C).

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

rther explore the mechanism of miR-99a in regu g 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 ... tion and invasion while knockdown of m... obtained the opposite results. We first pr... ed that miR-451a regulated cell migration and ... sion through targeting ATF2.

ATF2 is involved in multiple regulatory bi... logical progresses, including ...cription, DNA damage, metabolism a... umor...esis[18,19]. Increasing evidences hav... vealed ... ATF2 was a target of several ... As, in... 26b, miR-204, miR-62... and ...[29]. Further study in our researc... nd the ... results that ATF2 was a targ... miR-451a a... as regulated by miR-45... g cancer cell... 549. We first propose... co... n between miR-451a and ATF2 i... NSCLC. In h... ellular carcinoma, ATF2 kn... own promoted ... icancer activity of so... nib[30]. Similar findings were discovered by Li... [30], an... y indicated that ATF2 knockdo... pres... ell gro... h and enhances sensitivity ... herapy ... ancreatic cancer. Consistent wi... previ... results, we first verified ...R-451... migration and invasion of A54... lls throu... egulating ATF2 expression.

Conclusions

...howed that miR-451a was downregulated in ... small cell lung cancer tissues and lung cancer cells A549 and NCI-H460, while ATF2 was upregulated. The mRNA level o... had negative correlation with AT... miR-4... regulated the migration and invas... of lung cancer cells through targeting A... In addition, ATF2 could reverse partial functi... miR-451a on migration and invasion ... A549 c...

Conflict of Interest

The Authors declare tha... have ... conflict o... est.

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