ORP8 induces apoptosis by releasing cytochrome *c* from mitochondria in non-small cell lung cancer

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Abstract. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, and numerous oncogenes are associated with this disease. Oxysterol-binding protein-related protein 8 (ORP8) is essential for cell growth, migration and the modulation of mitochondrial respiration and morphology. However, the underlying role of ORP8 in NSCLC remains unclear. In the present study, it was reported that the expression of ORP8 was low in NSCLC cells and tissues. The ORP8 expression levels were analyzed by immunohistochemistry (IHC), quantitative real-time PCR (qPCR) and western blot analysis. ORP8 overexpression inhibited cell growth and induced apoptosis in NSCLC cells with MTS, anchorage-independent growth and Hoechst 33342 staining assay. Further experiments demonstrated that ORP8 overexpression induced the apoptosis of NSCLC cells via the release of cytochrome *c* from mitochondria into the cytoplasm with western blot analysis and confocal microscopy results. In addition, qPCR analysis showed that miR-421 was upregulated in NSCLC cell lines, with the bioinformatics analysis, western blot analysis and Dual-Luciferase reporter assay, it was determined that miR-421 could target ORP8. The inhibition of cell proliferation via ORP8 overexpression was rescued by a miR-421 mimic, which aided in maintaining the proliferative potential of the cells. Overall, the present study revealed that ORP8 may be a candidate target in the prevention and treatment of NSCLC.

Introduction

Although surgical resection, radio chemotherapy and immunotherapy have considerably advanced, NSCLC still has a high mortality rate. The 5-year survival rate for NSCLC is only approximately 15% (1-3). The major challenge in lung cancer therapy is identifying new targets that can supplement the current treatment strategies (4), and exploring the factors affecting NSCLC progression is important for clinical therapy.

Apoptosis is known as programmed cell death, resulting in the efficient and orderly removal of damaged cells (5). Two primary molecular signaling pathways trigger cell apoptosis: The extrinsic (death receptor) and the intrinsic (mitochondrial) pathway (6). The intrinsic pathway is initiated by different stress responses and converges at mitochondria (7). Cytochrome *c* release from the mitochondria into the cytoplasm is crucial to initiate the apoptotic cascade (8). Cytochrome *c* binds to IP_3 receptors at the endoplasmic reticulum (ER) after release from the mitochondria, resulting in a localized increase of calcium (Ca^{2+}) concentration, which promotes more cytochrome *c* release (9,10). When cytochrome *c* reaches cytotoxic levels in the cytosol, it will activate cysteine proteases (caspase-3 and caspase-9) and eventually kill the cells (11,12).

In mammals, oxysterol-binding protein-related proteins (ORPs) are a 12-member gene family (13). ORP8 is one of the ORPs that locates to ER-mitochondria contact sites (14). A previous study indicated that ORP8 suppresses the expression of ATP binding cassette transporter A1 (ABCA1) (15). In hepatic cells, ORP8 modulated lipid homeostasis and sterol regulatory element-binding protein (SREBP) activity (16). Other functions of ORP8 have been reported, including inhibition of cell migration (17), regulation of the cell cycle in HepG2 cells (18) and regulation of phosphatidylinositol-4,5-biphosphate [PtdIns(4,5)P_2] levels at the plasma membrane (19). Notably, ORP8 induced hepatocellular carcinoma (HCC) cells to ER stress (20) and mediated 25-hydroxycholesterol (25-OHC) cytotoxicity (21). In gastric cancer, ORP8 inhibited cell growth and activated apoptosis (22).

In the present study, it was revealed that ORP8 plays a crucial role in NSCLC tumorigenesis. The overexpression of ORP8 markedly attenuated the malignant features of NSCLC cells by affecting cell proliferation, anchorage-independent growth and apoptosis induction. Furthermore, it was revealed that ORP8 induced NSCLC apoptosis through the

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mitochondrial signaling pathway. In addition, the relationship between ORP8 expression and miR-421 inhibition was assessed. The present findings indicated that ORP8 could be a novel target in NSCLC tumorigenesis.

Materials and methods

Cell lines and reagents. NSCLC cell lines (A549, H1975, HCC827) and normal lung cell line (NL20) were purchased from the American Type Culture Collection (ATCC). The cells were maintained in 5% CO_2 at 37˚C, in pH 7.4 DMEM with 10% FBS, penicillin (100 U/ml)/streptomycin (100 μg/ml) antibiotic mixture. Mito Tracker Red was obtained from Invitrogen; Thermo Fisher Scientific, Inc. Hoechst 33342 was purchased from Sigma-Aldrich; Merck KGaA.

Clinical specimens. Tissue specimens from adjacent tissue and matched primary lung adenocarcinoma tissues (median age was 56 years old) were collected from 15 patients between September 2017 and September 2018 at the First Affiliated Hospital of Zhengzhou University (Henan, China). Samples were immediately stored in liquid nitrogen. All samples were obtained after written informed consent was provided from each patient and approval for the study was obtained from the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Immunohistochemistry. Tissue samples were fixed in 10% (v/v) formaldehyde at room temperature for 24 h, embedded in paraffin and cut into 5-μm sections. IHC staining was performed according to a previous protocol (23) using ORP8 (dilution 1:200; product code ab99069; Abcam) antibody. The relative expression was assessed by the Image-Pro Premier software (v9.0) program (Media Cybernetics, Inc.).

Gene transfer. Lentiviruses carrying ORP8-cDNA (accession no. NM_001003712), miR-421 mimic and miR-421 inhibitor were obtained from Shanghai GenePharma Co., Ltd. The miR-421 mimic sequence was 5'-CGCGGGUUAAUUACAGACAACUA-3'. The miR-421 mimic negative control (NC) sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. The miR-421 inhibitor sequence was 5'-GCGCCCAAUUAAUGUCUGUUGAU-3'. The miR-421 inhibitor negative control (NC) sequence was 5'-CAGUACUUUUGUGUAGUACAA-3'. For lentiviral infection, $1x10^6$ cells were resuspended in 100 μl DMEM containing Polybrene (5 μg/ml) and lentivirus (MOI=10) in a 24-well plate. After 6 h of infection at 5% CO_2 and 37˚C, 400 μl DMEM was added. The infection efficiency was detected using western blotting or qPCR after 3 days of infection.

Quantitative real-time PCR (qPCR). Total RNA was extracted from cell lines using TRIzol reagent with a well-established method. The RNA samples were reverse-transcribed with the PrimeScript™ RT Reagent kit (DRR047A; Takara Bio, Inc.). qPCR was performed with the SYBR Advantage qPCR Premix (Takara Bio, Inc.) kit, following the manufacturer's protocol. qPCR was detected using the ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, inc.). The qPCR conditions were 95˚C for 2 min followed by 40 cycles at 95˚C for 15 sec and 60˚C for 40 sec and a final dissociation stage. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (24), and U6 or actin was used as the endogenous controls. The primer sequences are listed in Table I.

Western blot analysis. The cells were harvested and lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein concentrations were determined using a BCA kit (Beyotime Institute of Biotechnology). The amount of protein loaded per lane was 20 μg. The protein samples were separated by 10% SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc.). Membranes were blocked with bovine serum albumin (5%) for 1 h at room temperature with gentle shaking, incubated with the primary antibodies (1:1,000) at 4˚C for 12-16 h, and then incubated with the HRP-labeled secondary antibodies (goat anti-mouse 1:1,000 and goat anti-rabbit 1:3,000) for 1 h at 25˚C. Finally, the membranes were detected by enhanced chemiluminescence (ECL) plus western blot detection reagents (Thermo Fisher Scientific, Inc.). Proteins were quantified by a gel imager (Tanon 5200; Tanon Science and Technology). The corresponding primary antibodies were as follows: ORP8 (product code ab99069; Abcam), actin (product no. 3700), caspase-3 (product no. 14220), caspase-9 (product no. 9502), cytochrome *c* (product no. 12963), COX 4 (product no. 4844; all from Cell Signaling Technology, Inc.). The HRP-conjugated secondary antibodies (product nos. 7076 and 7074) were obtained from Cell Signaling Technology, Inc.

MTS assay. To detect cell proliferation, H1975, A549 and HCC827 cells ($1x10^3$ cells/well) stably expressing a control or ORP8 were seeded in 96-well plates. For each well, 20 μl of the MTS solution (Promega Corp.) was added. After 1 h of incubation, 25 μl of 10% SDS solution was added, and the absorbance was detected at 492 and 690 nm.

Anchorage-independent growth. Cells ($8x10^3$/ml/well) were suspended and added to the upper layer of solidified 1 ml DMEM/10% FBS/0.3% agar with 2 ml of DMEM/10% FBS/0.6% in the bottom layer of the agar in each well of a 6-well plate. After maintenance for 10-14 days in 5% CO_2 at 37˚C, the colonies were counted using the Image-Pro Plus software (version 6.2; Media Cybernetics, Inc.). The diameter of each colony was >25 μm.

Hoechst 33342 staining. Cell apoptosis analysis was performed with Hoechst 33342 staining in H1975, A549, and HCC827 cells. Cells ($1x10^6$) were collected, washed with cold PBS, stained with Hoechst 33342 (20 μg/ml) at 37˚C for 10 min, resuspended in 20 μl PBS, and immediately imaged using an Olympus BX53 fluorescence microscope (Olympus Corp.) at an x400 magnification. The number of apoptotic cells in each group were counted in 10 random fields with >500 cells.

Mitochondrial isolation. H1975 and A549 cells ($1x10^7$) were collected and washed with PBS, and cytochrome *c* release was determined using a mitochondrial isolation kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol.

Table I. The oligonucleotide primers used.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
Chop	GCCTTTCTCCTTTGGGACACTGTCCAGC	CTCGGCGAGTCGCCTCTACTTCCC
Bip	CCTGGGTGGCGGAACCTTCGATGTG	CTGGACGGGCTTCATAGTAGACCGG
ORP8	GAACAGGGAGATTTTGAATCA	TCCTGTGAGTGGATCAAGTTC
Actin	GGCATCCTCACCCTGAAGTA	AGGTGTGGTGCCAGATTTTC
miR-421	CTCACTCACATCAACAGACATTAATT	TATGGTTGTTCTGCTCTCTGTGTC
U6	CTCGCTTCGGCAGCACATATACT	ACGCTTCACGAATTTGCGTGTC

ORP8, oxysterol-binding protein-related protein 8.

Assessment of the intracellular cytochrome c distribution. To detect the intracellular cytochrome *c* distribution, confocal microscopy was used as previously described (25). Cells were incubated with Mito Tracker dye (Mito Tracker Red; Molecular Probes; Thermo Fisher Scientific, Inc.) for 10-15 min in an incubator in the dark. Then the slides were fixed with 4% formaldehyde at room tempetature for 30 min. The fixed slides were stained with an anti-cytochrome *c* antibody at 4˚C for 12-16 h, then with a FITC-labeled antibody at 37˚C for 30 min. The cytochrome *c* antibody (product no. 12963) was purchased from Cell Signaling Technology, the FITC-labeled antibody (product no. A-21202) was purchased from Thermo Fisher Scientific, Inc. Images were obtained using different excitation filters and merged.

Dual-luciferase reporter assay. The potential targets of miR-421 were predicted by miRanda (http://www.microrna.org/microrna/home.do), miRDB (http://mirdb.org/), miRWalk (http://mirwalk.umm.uni-heidelberg.de/) and TargetScan (http://www.targetscan.org/vert_72/). The 3'-UTR segments of miR-421 binding sites of ORP8 were inserted into the Dual-Glo luciferase assay system plasmid, pGLO (Promega Corporation). pGLO vectors and mimic-miR-421 were co-transfected into cells. Then, the cells were subjected to luciferase activity detection with a luciferase assay kit (Promega Corporation) according to the manufacturer's protocol. *Renilla* fluorescence was used as the internal standard.

Statistical analysis. The results are presented as the mean ± standard deviation (SD) of 3 independent experiments, and each dose or treatment was tested in triplicate. For the statistical analyses, all data were analyzed by Student's t-test for pairwise comparisons or one-way analysis of variance (ANOVA) followed by Bonferroni test for multivariate analysis. Differences were considered to be statistically significant when the P-value was <0.05.

Results

ORP8 is expressed at low levels in human NSCLC. NSCLC is the most common type of lung cancer, and numerous oncogenes are associated with this disease. ORP8 is a member of the oxysterol-binding protein-related protein (ORP) family of proteins that are expressed in the brain, liver, lung, spleen, and kidney in mice (15). However, ORP8 expression in human NSCLC has not been well defined. Initially, the expression of ORP8 was investigated in 15-paired NSCLC specimens and lung cancer cell lines. The results indicated that ORP8 was expressed at lower levels in the NSCLC tissues than in the adjacent non-tumor tissues (Fig. 1A). Similarly, ORP8 expression was downregulated in several NSCLC cell lines (HCC827, A549 and H1975) compared with that of the normal NL20 lung cell line both at the mRNA and protein levels (Fig. 1B and C). These results indicated that ORP8 downregulation may promote NSCLC progression.

ORP8 overexpression in human NSCLC cells reduces their tumorigenic properties. Aberrant cell proliferation is a hallmark of cancer, and the elimination of uncontrolled cell differentiation contributes to cancer treatment (26). In the present study, it was hypothesized that ORP8 may play a crucial role in cell growth. To assess the function of ORP8 in NSCLC, stable ORP8-overexpressing cells were generated. The overexpression efficiency was verified in H1975, A549 and HCC827 cells using western blotting (Figs. 2A and S1A and B). MTS and anchorage-independent cell growth assays were investigated to evaluate the effect of ORP8 expression. The results revealed that ORP8 overexpression inhibited proliferation in H1975, A549 and HCC827 cells (Fig. 2B, D and F). In addition, colony formation was reduced in ORP8-overexpressing cells (Fig. 2C, E and G), which indicated that increased ORP8 expression reduced cell transformation.

During the progression of transformation, cell growth is regulated not only by proliferation but also by cell death or apoptosis. Studies have revealed that ORP8 plays a key role in coordinating apoptosis in liver and gastric cancer (20,22). In the present study, the apoptosis assay results indicated that overexpression of ORP8 resulted in increased apoptosis in H1975, A549 and HCC827 lung cancer cell lines stained with Hoechst 33342 (Fig. 3A). The protein levels of cleaved caspase-3 and caspase-9 were analyzed using western blot analysis. The cleavage of caspases was significantly enhanced, indicating that apoptosis was induced in ORP8-overexpressing cells (Figs. 3B and S1C). Overall, the present data revealed that ORP8 was involved in modulating cell survival and apoptosis.

ORP8 overexpression releases cytochrome c from mitochondria. In liver cancer cells and gastric cancer cells, studies have shown that ORP8 overexpression could induce

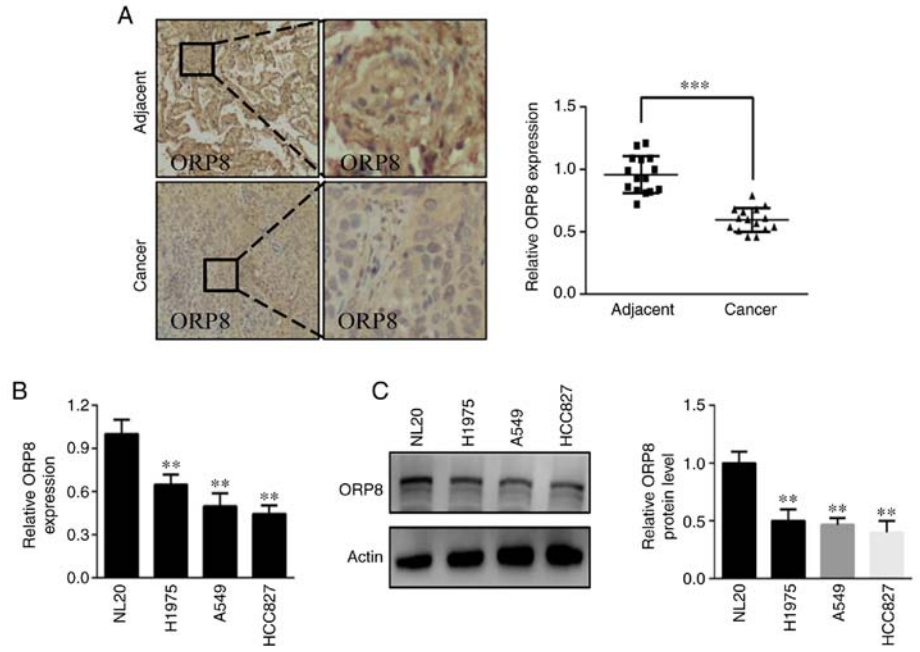

Figure 1. ORP8 is expressed at low levels in human NSCLC. (A) ORP8 expression in 15 NSCLC and matched adjacent patient samples was detected by IHC. The IOD was assessed by the Image Pro Premier software (v9.0). (B) The ORP8 mRNA level was assessed in NSCLC cell lines (H1975, A549, HCC827) and a normal lung cell line (NL20) by qPCR. (C) ORP8 protein levels were assessed in NSCLC cell lines and a normal lung cell line using western blotting. The data represent the mean ± SD of three individual experiments (**P<0.01 and ***P<0.001, n=3). ORP8, oxysterol-binding protein-related protein 8; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry; IOD, integrated optical density.

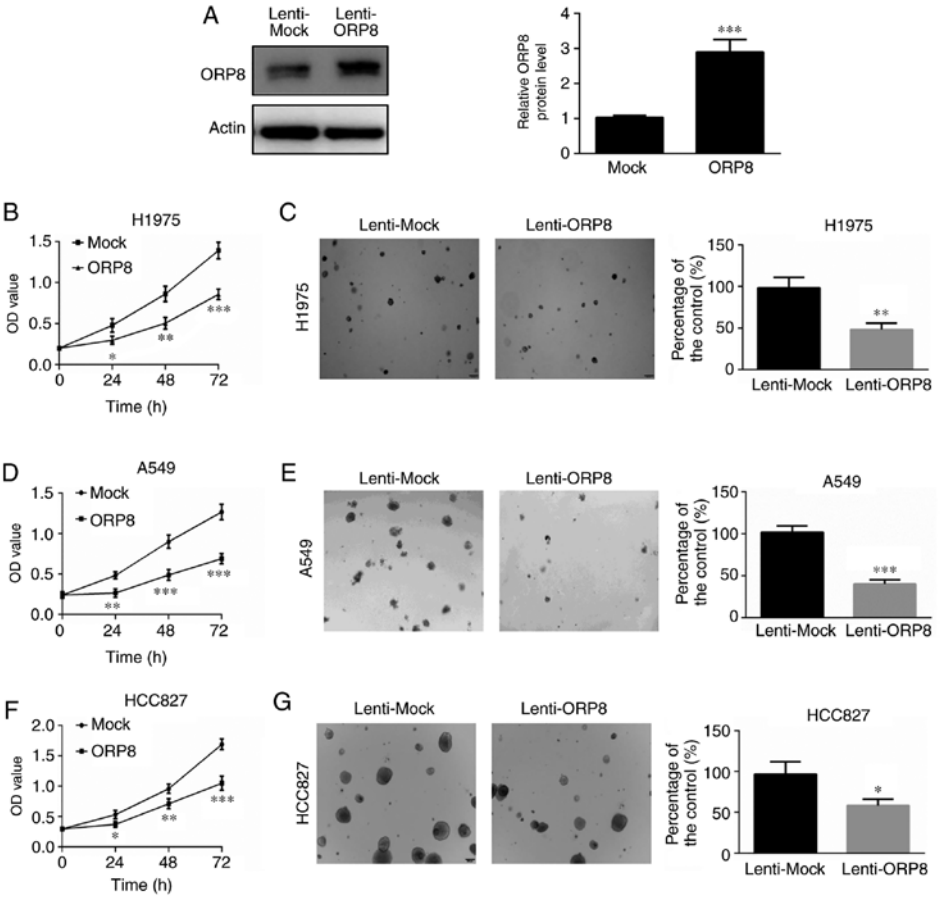

Figure 2. ORP8 overexpression inhibits H1975, A549 and HCC827 cell growth. (A) H1975 cells were infected with a lentivirus carrying ORP8 cDNA, and ORP8 overexpression efficiency was detected using western blotting. (B, D and F) Overexpression of ORP8 decreased the proliferation of H1975, A549 and HCC827 lung cancer cells. Cell growth was determined at 24, 48, and 72 h using an MTS assay. (C, E and G) Overexpression of ORP8 reduced the anchorage independent growth of H1975, A549 and HCC827 cells. Colonies were detected using a microscope and the Image Pro Plus (v.6) software. The data represent the mean ± SD of three individual experiments (*P<0.05, **P<0.01, and ***P<0.001, n=3). ORP8, oxysterol-binding protein-related protein 8.

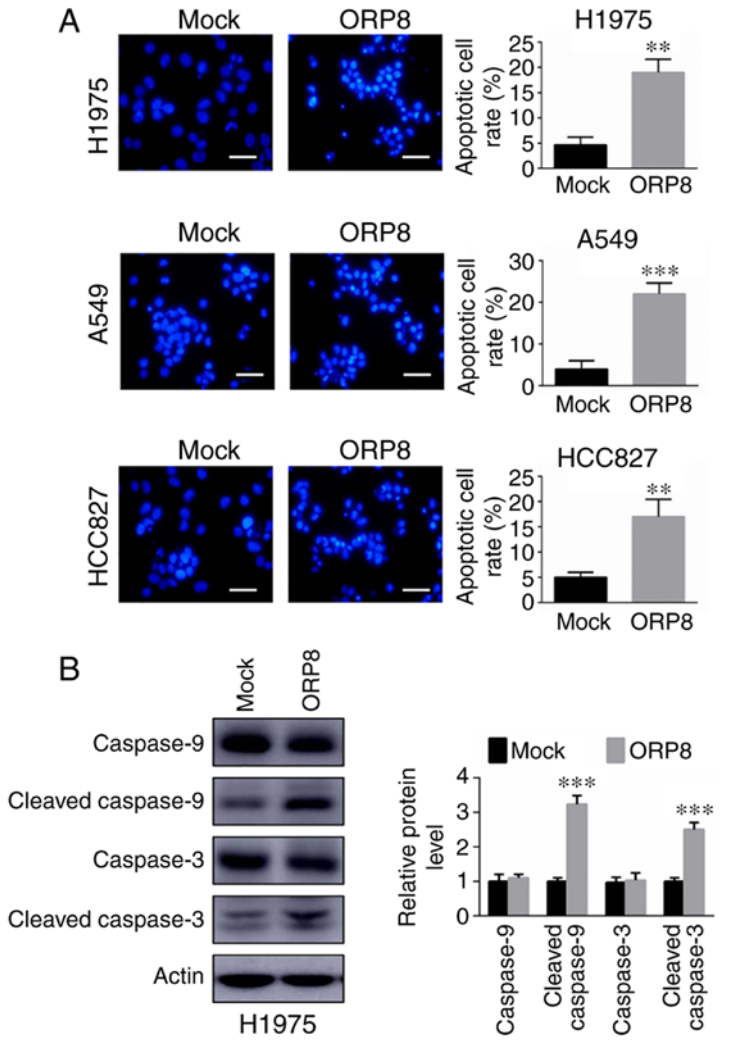

Figure 3. ORP8 overexpression induces H1975, A549 and HCC827 cell apoptosis. (A) H1975, A549 and HCC827 cells were infected with a lentivirus carrying ORP8 cDNA, and the nuclear morphology was detected after Hoechst 33342 staining analysis. Scale bars, 20 μm. (B) H1975 cells were infected with a lentivirus carrying ORP8 cDNA, and the cleaved caspase-3 and caspase-9 levels were assessed using western blotting. The data represent the mean ± SD of three individual experiments (**P<0.01, ***P<0.001, n=3). ORP8, oxysterol-binding protein-related protein 8.

ER stress responses (20-22) and that ORP8 localizes to ER-mitochondria contact sites (14). To determine whether ORP8 overexpression induced ER stress in NSCLC cells, the mRNA levels of Chop and Bip, which are the central components of ER stress responses, were detected (27). Results revealed that the expression of Chop and Bip was barely altered at both the mRNA and protein levels in H1975 and A549 cells (Figs. 4A and B and S2A and B). Mitochondria are the intracellular organelles that have critical roles in the apoptotic pathway (28), and the release of cytochrome *c* from the mitochondria into the cytoplasm is crucial to initiate the apoptotic cascade (8). Thus, to better understand the role of ORP8 in inducing cell apoptosis, cytochrome *c* release from mitochondria was assessed. Immunofluorescence staining was employed to observe the localization of cytochrome *c* relative to mitochondria. The present results revealed that cytochrome *c* co-localized with the mitochondrial marker (Mito Tracker) in the control H1975 and A549 cells (Fig. 4C and D, upper images), while cytosolic cytochrome *c* was increased in the ORP8-overexpressing cells (Fig. 4C and D, lower images). Western blot analysis similarly revealed that cytosolic cytochrome *c* levels were significantly enhanced after ORP8 overexpression in H1975, A549 and HCC827 cells (Figs. 4E and S2C and D). These data indicated that ORP8 upregulation promoted the release of cytochrome *c* from mitochondria, which induced cancer cell apoptosis.

ORP8 is a target of miR-421. Studies have revealed that upregulated miR-421 expression is associated with poor prognosis in NSCLC (29). Therefore, it was assessed whether the downregulation of ORP8 in NSCLC was due to miR-421 dysregulation. Notably, miR-421 was revealed to target ORP8 by using four different analytical programs (Fig. 5A). In addition, miR-421 was upregulated in NSCLC cells, compared with NL20 cells, in contrast to ORP8 downregulation in NSCLC (Fig. 5B). When cells were infected with miR-421 mimic or inhibitor, miR-421 expression was significantly increased or decreased, respectively (Fig. S3A and B). Moreover, ORP8 protein expression was significantly increased in H1975 and A549 cells treated with the miR-421 inhibitor (Figs. 5C and S3C), whereas ORP8 expression was significantly inhibited in H1975 and A549 cells treated with a miR-421 mimic (Figs. 5D and S3D). Moreover,

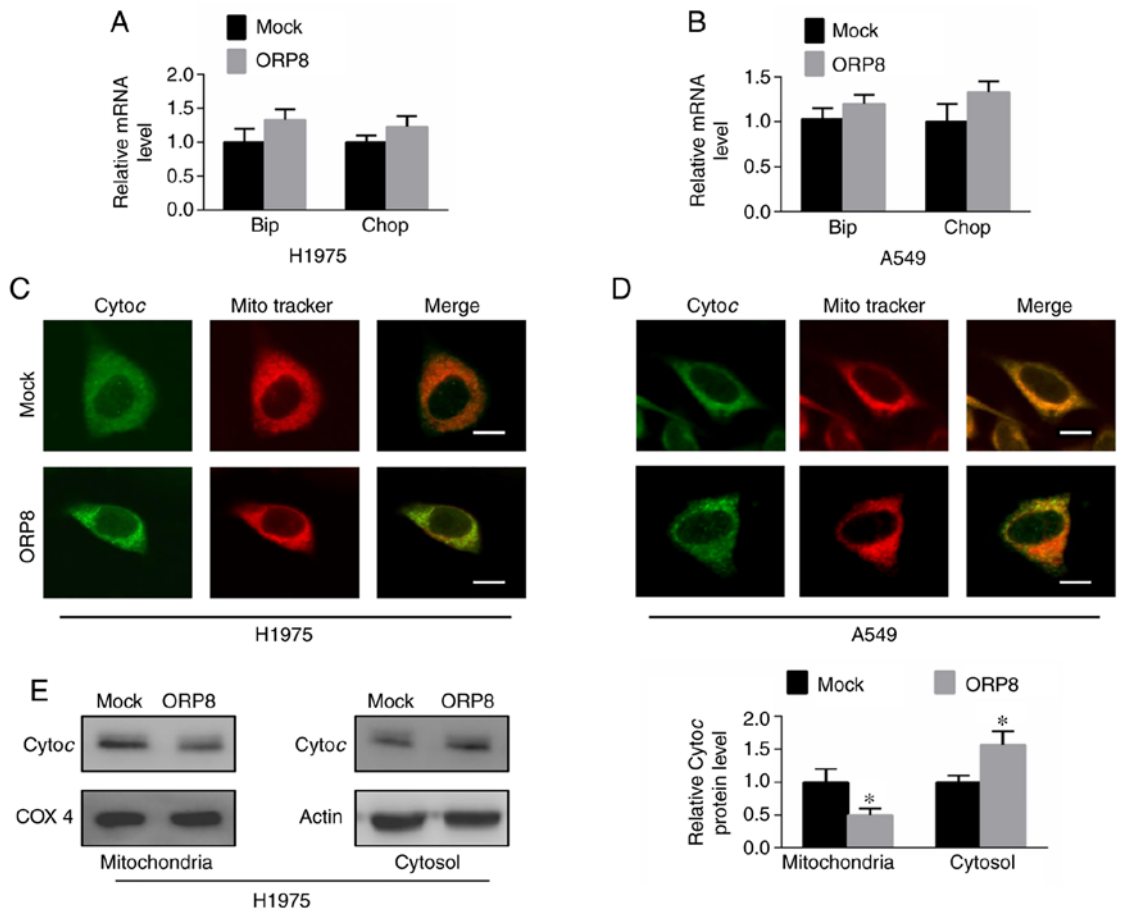

Figure 4. ORP8 overexpression increases the release of cytochrome *c* from mitochondria to the cytosol. (A and B) H1975 and A549 cells were infected with a lentivirus carrying ORP8 cDNA, and the relative Chop and Bip levels were detected by qPCR. (C and D) H1975 and A549 cells were infected with a lentivirus carrying ORP8 cDNA. The colocalization of cytochrome *c* with mitochondria is presented in cells labelled using Mito tracker dye and observed using a confocal microscope (mock, upper images), no colocalization of cytochrome *c* with mitochondria (ORP8, lower images), Scale bars, 50 μm. (E) Proteins from different organelles were extracted and detected by western blotting and normalized to COX4 in mitochondria and actin in the cytosol in H1975 cells. The data represent the mean ± SD of three individual experiments (*P<0.05, n=3). ORP8, oxysterol-binding protein-related protein 8.

H1975 cells were transfected with the mutant (MUT) and wild-type (WT) 3'-UTRs of ORP8. The luciferase activity was inhibited by miR-421 when transfected with the WT 3'-UTR, however, the activity was restored by the MUT 3'-UTR transfection (Fig. 5E). In addition, the inhibition of cell proliferation with ORP8 overexpression was rescued by the miR-421 mimic (Fig. 5F). These data indicated thatmiR-421 could be a potential target for ORP8 downregulation in NSCLC patients.

Discussion

NSCLC still has a high mortality rate. Targeted therapy, which depends on activated oncogenes and downstream signaling cascades, has emerged as an impressive approach for NSCLC. The present study demonstrated that ORP8 has a key role in NSCLC and maybe a potential therapeutic target for NSCLC.

ORP8 is an ORP family member that plays an important role in several signaling pathways. For instance, ORP8 regulates calcium signaling in specific cell compartments (30). ORP8 localizes to ER-mitochondria contact sites and is involved in mitochondrial function (14). ORP8 induces HCC cell ER stress (20), and the overexpression of ORP8 significantly increases the ER stress response induced by 25-OHC (21). Although ORP8 plays important roles in multiple signaling pathways and cancer development (31), its biological functions in NSCLC remain unclear. The present study was designed to study the function of ORP8 in NSCLC tumorigenesis.

The present data indicated that ORP8 was downregulated in NSCLC, both in cell lines and tissues. For functional study, stable ORP8-overexpressing H1975, A549 and HCC827 lung cancer cell lines were used. It was demonstrated that ORP8 overexpression inhibited cell growth and induced cell apoptosis in NSCLC. These results indicated that ORP8 overexpression markedly attenuated malignant features of NSCLC cells, and thus, an understanding of the mechanisms by which ORP8 regulates lung cancer development is required.

During tumor viability or progression, apoptosis plays a crucial role. A lack of apoptosis could lead to tumor development. Thus, inducing cell apoptosis could be a strategy for an oncotherapy approach (32). Disruption of the ER normal function induces a stress response, also known as an unfolded protein response (UPR), which initially compensates the damage of cells (33,34). When limiting the protein

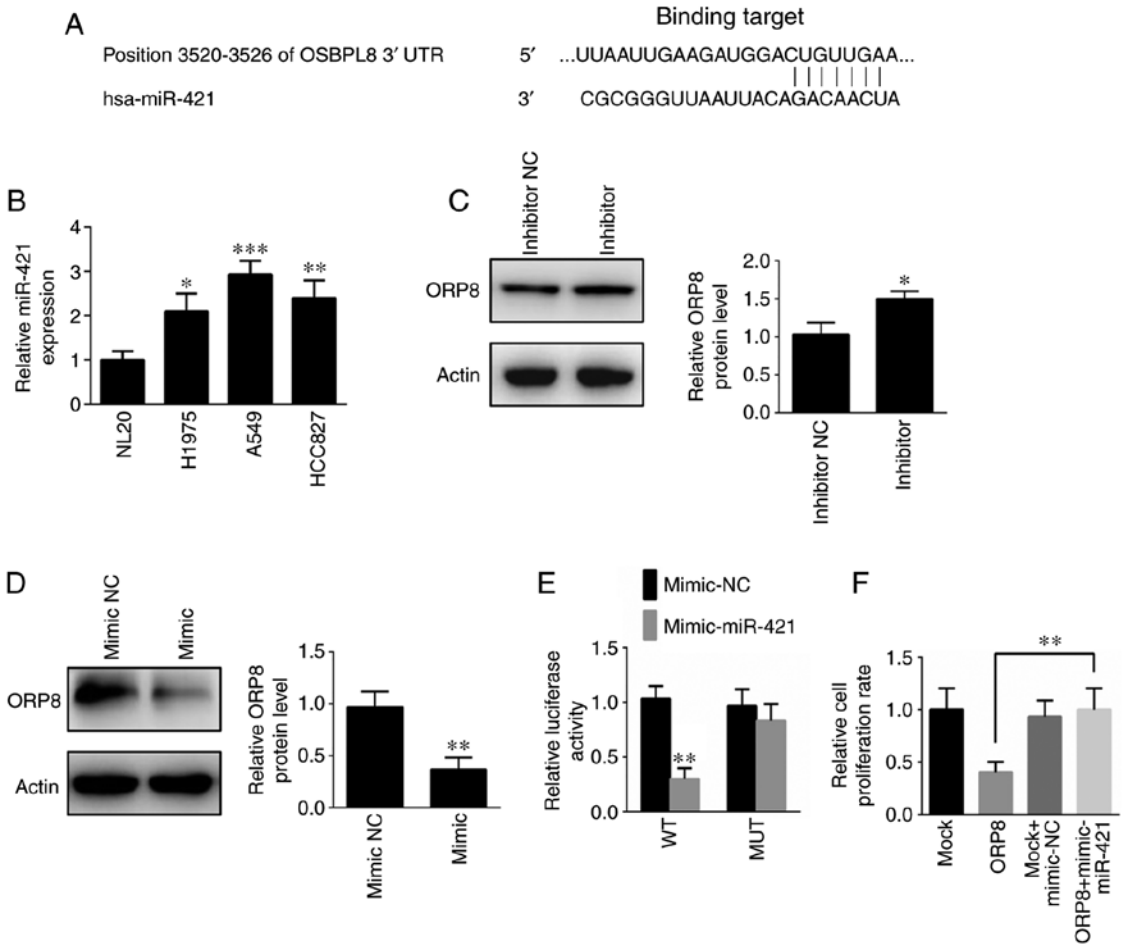

Figure 5. ORP8 is a target of miR-421. (A) Four software packages (miRanda, miRDB, miRWalk, Targetscan) were used to predict miR-421 targeting of ORP8. (B) The mRNA level of miR-421 was detected by qPCR in NSCLC cell lines (H1975, A549, and HCC827) and a normal lung cell line (NL20). (C) H1975 cells infected with an inhibitor NC or an inhibitor of miR-421, and the protein level of ORP8 was detected by western blotting. (D) H1975 cells infected with a mimic NC or miR-421 mimic, and the protein level of ORP8 was detected by western blotting. (E) The luciferase activity between miR-421 and the ORP8-3'-UTR was evaluated using a luciferase reporter assay. (F) H1975 cells were infected with a lentivirus carrying ORP8 cDNA or an inhibitor of miR-421, and cell growth was determined at 72 h using the MTS assay. The data represent the mean ± SD of three individual experiments (*P<0.05, **P<0.01 and ***P<0.001, n=3). ORP8, oxysterol-binding protein-related protein 8; NSCLC, non-small cell lung cancer.

folding capacity of the ER, ER stress causes the aberrant accumulation of misfolded and unfolded proteins (35,36). If the defensive UPR fails to deal with the misfolded proteins, ER stress will induce cell apoptosis (37). Evidence has indicated that ORP8 overexpression induces the ER stress response (20-22). Therefore, it was hypothesized that ER stress is induced by ORP8 overexpression in NSCLC cells. However, ER stress was not evident in H1975 and A549 cells. These data indicated that ER stress may not be the main mechanism for inducing apoptosis in NSCLC cells overexpressing ORP8.

The mitochondrial pathway induces cell apoptosis by decreasing mitochondrial membrane potential, increasing membrane permeability and decreasing ATP synthesis (38-40). Then, cytochrome *c* release from mitochondria into the cytoplasm could activate caspase-9. This cascade further activates caspase-3 and causes cell apoptosis (41-43). A previous study revealed that ORP8 localized to ER-mitochondria contact sites and was involved in mitochondrial function (14). Therefore, to better understand the role by which ORP8 regulates lung cancer apoptosis, cytochrome *c* release from mitochondria was evaluated. The present data revealed that cytosolic cytochrome *c* levels were significantly enhanced after ORP8 overexpression in H1975, A549 and HCC827 cells. Therefore, it was concluded that ORP8 overexpression induced mitochondrial-associated apoptosis by affecting the subcellular localization of cytochrome *c* in NSCLC cells.

Dysregulated miRNA expression has been implicated in cancer development (44). Four different analytical programs were used to reveal that miR-421 could target ORP8. Previous research has revealed that miR-421 expression is upregulated and associated with poor prognosis in non-small-cell lung cancer (29). The present experiments revealed the role of miR-421 by determining that it targets ORP8, indicating that the upregulated miR-421 expression led to the reduction of ORP8 expression in NSCLC cells.

Overall, the present data demonstrated that ORP8 regulated cytochrome *c* release from mitochondria and plays a critical role in human NSCLC carcinogenesis. ORP8 was revealed to be expressed at low levels in human NSCLC and inhibit cell growth and induce cell apoptosis. ORP8 downregulated expression in NSCLC coincided with the increased expression

of miR-421, and increased expression of miR-421 decreased ORP8 expression aiding in the maintenance of the proliferative potential. The present results indicated that ORP8 may be a candidate molecular target for NSCLC treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MZ, JiwL and JC conceived and designed the experiments. JiwL, ZL, XF, JinL and HL performed the experiments. JiwL, JC, SW and MZ analyzed the data. MZ, JiwL and SW contributed the reagents. JiwL and JC wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Written informed consent for the research was obtained from each patient. This retrospective study was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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