# Keap1-targeting microRNA-941 protects endometrial cells from oxygen and glucose deprivation-re-oxygenation by activating Nrf2 signaling

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**Research**

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# Abstract

Background: Mimicking ischemia-reperfusion injury, oxygen and glucose deprivation (OGD)-re-oxygenation (OGDR) stimulation to endometrial cells induces significant oxidative stress and programmed necrosis, which can be inhibited by nuclear-factor-E2-related factor 2 (Nrf2) signaling activation. MicroRNA (miRNA)-induced silencing of the Nrf2 suppressor protein Keap1 is novel strategy to activate Nrf2 cascade.

Methods: microRNA-941 (miR-941) expression was exogenously altered in HESC cells and primary human endometrial cells, and cells treated with OGDR. Nrf2 pathway genes were examined by Western blotting assay and real-time quantitative PCR analysis. Endometrial cell programmed necrosis and apoptosis were tested.

Results: miR-941 is a novel Keap1-targeting miRNA, regulates Nrf2 signaling activation. In T-HESC cells and primary human endometrial cells, ectopic overexpression of miR-941 suppressed Keap1 3'-UTR (untranslated region) activity and downregulated its mRNA/protein expression, leading to Nrf2 cascade activation. Conversely, Keap1's 3'-UTR activity and expression were elevated in endometrial cells with miR-941 inhibition, whereas Nrf2 activation was inhibited. miR-941 overexpression in endometrial cells largely attenuated OGDR-induced oxidative stress and programmed necrosis, both were intensified with miR-941 inhibition. Further studies show that Keap1-Nrf2 cascade activation is absolutely required for miR-941-induced endometrial cell protection. MiR-941 overexpression and inhibition were completely ineffective in Keap1-/Nrf2-KO T-HESC cells (using CRISPR/Cas9 strategy). Restoring Keap1 expression, by an UTR-depleted Keap1 construct, abolished miR-941-induced anti-OGDR activity in T-HESC cells.

Conclusions: Targeting Keap1 by miR-941 activates Nrf2 cascade to protect human endometrial cells from OGDR-induced oxidative stress and programmed necrosis.


# 1. Background

Postpartum hemorrhage is one of the most common complications in the clinical obstetrics practices [1–3], causing ischemic injuries to human endometrium and necrosis to endometrial cells [1–3]. When followed with reperfusion, endometrium ischemia shall induce profound oxidative injury to the endometrial cells [1–3], leading to reactive oxygen species (ROS) accumulation, but depletion of certain antioxidants [4–6]. Unsolved oxidative injury will cause excessive DNA breaks, protein damages and mitochondrial dysfunction [4–6], eventually leading to endometrial cell necrosis [1–3]. Our lab [7, 8] and others have been using the oxygen and glucose deprivation (OGD)-re-oxygenation (OGDR) procedure to cultured endometrial cells, mimicking ischemia-reperfusion injury [9–12].

It has been recently shown that cell necrosis could be a programmed and active cell death form [13–16], also called "programmed necrosis". In the endometrial cells, OGDR stimulation will promote p53 translocation to cell mitochondria to form a complex with mitochondria permeability transition pore (mPTP) component proteins, cyclophilin-D (CypD) and adenine nucleotide translocator type 1 (ANT1) [17,

18]. It will then initiate mitochondrial depolarization, mPTP pore opening and cytochrome C release to cytosol, eventually leading to cell necrosis, but not apoptosis [13–15, 19–22]. Our previous studies have shown that CypD inhibition or silencing (by targeted shRNA) suppressed OGDR-induced cytotoxicity and programmed necrosis in endometrial cells [8]. Ginseng Rh2 (GRh2) and keratinocyte growth factor (KGF) also protected endometrial cells from OGDR by shutting down the programmed necrosis pathway [7, 8].

The transcription factor nuclear-factor-E2-related factor 2 (Nrf2) promotes the transcription and expression of multiple key anti-oxidant genes and detoxifying enzymes, protecting cells against various oxidative injuries [23–25]. In-activated Nrf2 accumulates in the cytoplasm, binds directly to its suppressor protein Kelch-like ECH-associated protein 1 (Keap1) [23–25] and Cul3 ubiquitin ligase to promote Nrf2 ubiquitination and proteasomal degradation [23–25]. Activated Nrf2 (i.e. via post-translational modifications) will be departed from Keap1-Cul3 complex, leading to its protein stabilization and accumulation [23–25], which then travels to cell nuclei, and binds to antioxidant responsive element (ARE) [23–25]. This will lead to transcription of Nrf2-depenedent anti-oxidant genes [26–28]. The majority of the Nrf2-dependent genes, including heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), γ-glutamyl cysteine ligase catalytic subunit (GCLC) [23], can exert potent anti-oxidant activity [29, 30].

Forced Nrf2 activation in endometrial cells shall provoke profound anti-oxidant activity and alleviate OGDR-induced oxidative injury. We have previously shown that KGF-induced Nrf2 activation efficiently protected endometrial cells from OGDR [7]. MicroRNAs (miRNAs) are essential and novel players in the regulating activation of the Keap1-Nrf2 pathway [31–36]. The 22-nt long single-strand non-coding RNAs (ncRNAs) physically bind to the 3'-untranslated region (3'-UTR) of the targeted mRNAs, causing degradation and/or translation suppression of target genes [37, 38]. One strategy to activate Nrf2 signaling cascade is to silence its suppressor protein Keap1, possibly by expressing Keap1-targeting miRNAs [31, 35, 36]. Here we will show that targeting Keap1 by microRNA-941 (“miR-941”) activates Nrf2 signaling to protect endometrial cells from OGDR-induced cytotoxicity.

# 2. Materials And Methods

**2.1. Chemical and reagents.** Puromycin and ploybrene were purchased from Sigma-Aldrich Chemicals Co. (St Louis, Mo). The fluorescent dye JC-1 was provided by Invitrogen Thermo-Fisher (Shanghai, China). All cell culture reagents and qPCR reagents were obtained from Gibco BRL Co. (Grand Island, NY). The antibodies of the present study were provided by Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Tech (Suzhou, China). The primers, sequences, constructs and virus were designed and provided by Shanghai Genechem Co. (Shanghai, China), unless otherwise motioned.

**2.2. T-HESC cell culture.** As described previously [7, 8], the immortalized human endometrial cell line, T-HESC [39], was cultured in regular DMEM/Hams F-12 nutrient medium plus 10% FBS.

**2.3. Culture of primary human endometrial cells.** The fresh human endometrial tissues, acquired from a written-informed consent uterine-bleeding patient (31-year old, administrated at Changzhou Second People's Hospital, undergoing the partial hysterectomy surgery) were digested with 0.15% trypsin-EDTA (Sigma) and Collagenase I (Sigma) for 1h, and then transferred to DMEM/Hams F-12 nutrient medium plus 15% FBS. Tissues were dissolved. Blood vessel cells, immune cells and other non-endometrial cells were abandoned through gravity sedimentation. The remaining human endometrial cells were resuspended and cultured in complete DMEM medium [8]. The primary human endometrial cells at passage 3-10 were utilized for biomedical assays. The protocols of using human tissues and cells were approved by the Ethics Committee at Changzhou Second People's Hospital.

**2.4. miR-941 overexpression or inhibition.** The pre-miR-941 nucleotide sequence and its anti-sense sequence were synthesized and sequence-verified by Shanghai Genechem Co. Each of the two was ligated to the GV248 construct (Shanghai Genechem Co.). The construct, along with the lentivirus-packing helper plasmids (psPAX2 and pMD2.G [33]), were co-transfected to HEK-293T cells, forming the pre-miR-941-expressing lentivirus ("lv-pre-miR-941") and the pre-miR-941 anti-sense lentivirus ("antagomiR-941"). Virus were enriched, filtered, and added directly to human endometrial cells (in the polybrene-containing medium). When necessary puromycin (5.0 μg/mL) was included in the medium to select stable cells, with miR-941 levels examined by qPCR.

**2.5. qPCR.** The human endometrial cells, with the applied treatments, were harvest and the total cellular RNA extracted using TRIzol protocol [8]. We utilized an ABI Prism 7600 Fast Real-Time PCR system to carry out the quantitative real time-PCR (qPCR) assay. To calculate product melting temperature we applied the melt curve analyses. mRNA primers of human Nrf2 pathway genes were provided by Dr. Jiang [40-42]. *Glyceraldehyde-3-phosphatedehydrogenase* (*GAPDH)* was always examined as the reference gene and the internal control, and the $2^{-\Delta\Delta Ct}$ method utilized for the quantification of targeted mRNAs. miR-941 was normalized to U6. miR-941 and U6 primers were obtained from OriGene (Beijing, China).

**2.6. Keap1 3'-UTR activity.** Keap1 3'-UTR reporter plasmid (containing the miR-941-binding sites, at position of 276-283) was generated using the same protocol described previously [31], which was transfected to human endometrial cells using the Lipofectamine 2000 protocol. Afterwards, cells were

subjected to the applied genetic modifications, with the Keap1 3'-UTR luciferase activity tested through the Promega kit [43].

**2.7. Transfection of miR-941 mimic.** Human endometrial cells were seeded into the six-well tissue culture plates (at $1 \times 10^5$ cells in each well). Lipofectamine 2000 was utilized for the transfection of 500 nM of the wild-type ("WT") or the mutant ("Mut") miR-941 mimics (synthesized by Shanghai Genechem Co.). After 48h, miR-941 levels were determined by qPCR.

**2.8. RNA-Pull down assay.** The RNA-Pull down assay was carried out through the previously-described protocol [44, 45], testing miR-941-bound mRNA using the Pierce Magnetic RNA Pull-Down Kit, Shanghai, China). In brief, T-HESC cells were transfected with biotinylated miR-941 mimic or control mimic (100 nmol/L) for 48h, and cells were harvested using the lysis buffer described early [45]. The biotin-captured RNA complex was pulled down by incubating the cell lysates (600 μg of each treatment) with the streptavidin-coated magnetic beads [44]. The bound mRNA was purified using the RNeasy Mini Kit (QIAGEN, Shanghai, China), with expression of *Keap1 mRNA*, in the bound fractions examined by qPCR. Its levels were normalized to the input controls.

**2.9. Cell viability.** Human endometrial cells were seeded into the 96-well tissue culture plates (3,000 cells in each well). Following the applied treatments, a cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) was utilized to examine cell viability [8], with CCK-8 optic density (OD) examined at test-wavelength of 450 nm.

**2.10. Lactate dehydrogenase (LDH) assay.** The human endometrial cells were seeded into the 12-well tissue culture plates (at $0.5 \times 10^5$ cells in each well). LDH release to the cell medium, the quantitative marker of cell necrosis [46], was tested through a two-step LDH detection kit (Promega, Shanghai, China) [8]. LDH contents in the medium were always normalized to total LDH levels.

**2.11. OGD/re-oxygenation (OGDR).** As described previously [7, 8], human endometrial cells with applied genetic treatments were initially placed into an airtight chamber (95% $N_2$/5% $CO_2$) for 4h, mimicking OGD. Thereafter, human endometrial cells were returned back to the complete medium and re-oxygenated. Cells were further cultured for applied time periods.

**2.12. Western blotting.** Human endometrial cells were seeded into the six-well tissue culture plates (at 1 × $10^5$ cells in each well). Following the applied treatments, cellular lysates were achieved and quantified [7, 8]. The lysates proteins (40 μg per treatment into each lane) were separated by SDS-PAGE gels, and transferring to polyvinylidene difluoride (PVDF) blots [47]. The detailed protocols for Western blotting and data quantification (through the Image J software) were previously described [40, 48]. Assaying of nuclear fraction proteins was described early [40].

**2.13. Mitochondrial immunoprecipitation (Mito-IP)**. T-HESC cells were harvested and resuspended [8], with the supernatants collected as the cytosolic fraction lysates. The pellets were resuspended to achieve mitochondrial fraction lysates and quantified [49]. Mitochondrial fraction lysates (300 μg per treatment) were pre-cleared (using anti-IgG Sepharose beads), and incubated with anti-CypD antibody (Santa Cruz Biotech). The mitochondrial complex was captured by anti-IgG. CypD-p53-ANT1 association was examined by Western blotting assaying of CypD-bound proteins.

**2.14. JC-1 mitochondrial depolarization assay.** The human endometrial cells were seeded into the 12-well tissue culture plates (at 0.5 × $10^5$ cells in each well). In OGDR-stimulated cells with mitochondrial depolarization ("ΔΨ") the JC-1 fluorescent dye shall aggregate, forming green monomers [50]. The detailed protocol of JC-1 protocol was discussed previously [8]. The JC-1 fluorescence intensity was examined at 530 nm (Titertek Fluoroscan, Germany). The representative JC-1 images, integrating both green and red fluorescence images, were also presented.

**2.15. Superoxide detection.** Endometrial cells with the applied genetic treatments were initially seeded onto 96-well tissue-culturing plates (at 3 × $10^3$ cells of each well). Following the applied OGDR stimulation, the superoxide colorimetric assay kit (BioVision, Shanghai, China) was applied to examine the cellular superoxide contents. In brief, the superoxide detection reagent (50 μL/well) was added for 15 min under the dark, with the superoxide's absorbance tested at the test-wavelength of 450 nm [31].

**2.16. Lipid peroxidation assay.** As reported [31] endometrial cells with the applied genetic treatments were initially seeded onto the six-well tissue-culturing plates (at 1 × $10^5$ cells per well). Following the applied OGDR stimulation, the lipid peroxidation assay kit (Abcam, Shanghai, China) was utilized to examine cellular lipid peroxidation levels, via the malondialdehyde method. The lipid peroxidation levels, determined by the thiobarbituric acid reactive concentration, were tested and quantified using the previously-described protocol [31, 51].

**2.17. NQO1 activity.** The detailed protocol for testing the relative NQO1 activity in human endometrial cells has been described elsewhere [52]. In brief, the inducer potency was quantified by using the NQO1 bioassay. T-HESC cells or the primary human endometrial cells ($10^4$ per well of a 96-well plate), with applied genetic treatments, were cultured for 24h. The NQO1 enzyme activity was tested and quantified in cell lysates using menadione as the substrate.

**2.18. Nrf2 or Keap1 knockout (KO).** The lentiCRISPR-Nrf2-KO-puro construct and the lentiCRISPR-Keap1-KO-puro construct were described early [31], each was transduced to T-HESC cells (cultured in the polybrene-containing complete medium). The GFP-positive T-HESC cells were sorted via FACS. The selected single cells were further incubated in complete medium with puromycin for 10 days, with Nrf2/Keap1 KO verified by Western blotting and/or qPCR assays.

**2.19. Keap1 re-expression.** The Keap1 (with no 3'-UTR region) expression GV248 construct was designed and provided by Shanghai Genechem, transduced to T-HESC cells with miR-941 overexpression. Cells were then selected by puromycin for 10 days, with Keap1 re-expression verified by qPCR and Western blotting assays.

**2.20. Statistical analysis.** Data were presented as mean ± standard deviation (SD). The repeated-measures analysis of variance (RMANOVA) with Dunnett's post hoc test for multiple comparisons (SPSS 16.0, SPSS Co. Chicago, CA) were utilized to evaluate statistical significance. The two-tailed unpaired T test (Excel 2013) was carried out to examine significance between two specific treatment groups. $P < 0.05$ was considered statistically significant.

# 3. Results

## 3.1. miR-941 targets Keap1 and activates Nrf2 signaling in human endometrial cells

The current study is to identify possible Keap1-targeting miRNAs. The miRNA database, TargetScan (V7.2, http://targetscan.org/) [53] was first consulted. Several miRNAs binding to 3'-UTR of Keap1 were indentified and further verified by other miRNA databases, including miRbase (http://www.mirbase.org/) and miRDB (http://www.mirdb.org/miRDB/policy.html). The genetic analyses discovered that miR-941 putatively targets the 3'-UTR of Keap1 (at position of 276–283), with context$^{++}$ score: -0.59 and the score percentage of 99% (from TargetScan V7.2 [53], Fig. 1A). Further experiments were performed to examine whether miR-941 could affect Keap1. T-HESC human endometrial cells [7, 8] were transduced with the lentivirus encoding the pre-microRNA-941 ("lv-pre-miR-941"). Followed by puromycin selection two stable

cell lines, “s-L1” and “s-L2”, were established. qPCR testing mature miR-941 expression, using the described primers [54], demonstrated that miR-941 levels increased over 20 folds in the lv-pre-miR-941-expresing stable cells (Fig. 1B). Significantly, the 3’-UTR activity of Keap1 decreased over 90% in miR-941-overexpressed cells (Fig. 1C), where Keap1 mRNA (Fig. 1D) and protein (Fig. 1E) levels were significantly downregulated. These results suggest that miR-941 selectively targets and downregulates Keap1 in endometrial T-HESC cells.

Testing the potential effect of miR-941 on Nrf2 signaling, we show that although Nrf2 mRNA levels (Fig. 1F) were unchanged in lv-pre-miR-941-expresing cells, its protein levels (Fig. 1E) were significant elevated (vs. control cells). Importantly, the stabilized Nrf2 protein translocated to the nuclei in T-HESC cells (testing nuclei lysates, Fig. 1G). Furthermore, mRNA and protein expression of two key Nrf2-dependent genes, including HO1 and NQO1 [33, 55–57], was significantly increased in miR-941-overexpressed cells (Fig. 1E and H), with the NQO1 activity significantly boosted (Fig. 1I) as well. These results suggest that miR-941 overexpression induced Nrf2 signaling cascade activation in T-HESC cells.

To further support that miR-941 targets Keap1, three miR-941 mutants in their binding sites to the Keap1 3’-UTR, “Mut1/2/3” (sequences listed in Fig. 1J), were synthesized. The wild-type (“WT”) and the mutant miR-941 s were individually transfected to T-HESC endometrial cells. As shown only the WT miR-941 decreased Keap1’s 3-UTR activity (Fig. 1K, the left panel) and Keap1 mRNA/protein expression (Fig. 1K, the right panel), while the mutant miR-941 s were ineffective (Fig. 1K). The results further support that miR-941 targets Keap1 mRNA in T-HESC cells. The nonsense control miRNA (“miRC”) exerted no significant effect on Keap1 expression and Nrf2 signaling cascade in T-HESC cells (Fig. 1B-K). To further support our hypothesis, the RNA-Pull down assay was performed. As shown, in T-HESC cells cells, the biotinylated-miR-941 directly associated with Keap1 mRNA (Fig. 1L). As the negatively control, the streptavidin-coated magnetic beads failed to bind to Keap1 mRNA (Fig. 1L).

In the primary human endometrial cells (“Endometrial cells”), infection of lv-pre-miR-941 led to over 10 folds increase in the expression of mature miR-941 (Fig. 1M), causing downregulation of Keap1 mRNA (Fig. 1N). miR-941 overexpression similarly induced Nrf2 signaling activation, leading to Nrf2 protein (but not mRNA) accumulation (Fig. 1O and P), HO1 and NQO1 upregulation (both mRNA and protein, Fig. 1P and Q) as well as an increase in NQO1 activity (Fig. 1R). Collectively, these results show that miR-941 targets Keap1 and activates Nrf2 signaling in human endometrial cells.

## 3.2. miR-941 overexpression protects human endometrial cells from OGDR

Our previous studies [7, 8] have shown that OGDR mainly induced programmed necrosis, but not apoptosis, in endometrial cells. Here in the control T-HESC cells (with lv-miRC), OGDR similarly induced programmed necrosis, leading to superoxide accumulation and lipid peroxidation (Fig. 2A) as well as mitochondrial CypD-ANT1-p53 association (Fig. 2B), accompanied by mitochondrial depolarization (tested by JC-1 green fluorescence accumulation, Fig. 2C) and cytosol cytochrome C release (Fig. 2D). Significantly, ectopic overexpression of miR-941, by lv-pre-miR-941, potently inhibited OGDR-induced

oxidative stress and programmed necrosis in T-HESC cells (Fig. 2A-D). The lv-pre-miR-941-expresing T-HESC cells were protected from OGDR, presented with reduced cell viability reduction (Fig. 2E) and necrosis (medium LDH release, Fig. 2F), when compared to control cells with same OGDR treatment. Next, T-HESC human endometrial cells, transfected with the wild-type ("WT") or the mutants miR-941 s (see Fig. 1), were subjected to OGDR stimulation. Results show that transfection of the WT-miR-941 potently inhibited OGDR-induced cytotoxicity in T-HESC cells (Fig. 2G and H), while the mutants were completely ineffective (Fig. 2G and H).

In the primary human endometrial cells OGDR-induced ROS production (tested by superoxide accumulation, Fig. 2I), mitochondrial depolarization (Fig. 2J) and cytosol cytochrome C release (Fig. 2K) were significantly attenuated by ectopic miR-941 overexpression. The latter also inhibited alleviated OGDR-induced cytotoxicity in the human endometrial cells (Fig. 2L). These results clearly show that miR-941 overexpression inhibited OGDR-induced oxidative stress, programmed necrosis and cytotoxicity in human endometrial cells.

## 3.3. miR-941 inhibition upregulates Keap1, suppressing Nrf2 signaling in human endometrial cells

To suppress miR-941 expression the pre-microRNA-941 anti-sense lentivirus ("antagomiR-941") was transduced to the T-HESC endometrial cells. With selection by puromycin two stable cell lines, "L1" and "L2", were established. As compared to control cells with miRNA anti-sense control lentivirus ("antaC"), mature miR-941 levels decreased over 90% in the antagomiR-941-expressing T-HESC cells (Fig. 3A). Significantly, miR-941 inhibition led to 3–4 folds increase of 3'-UTR activity of Keap1 in T-HESC cells (Fig. 3B), resulting in upregulation of Keap1 mRNA (Fig. 3C) and protein (Fig. 3D) levels. The antagomiR-941 did not affect Nrf2 mRNA levels (Fig. 3E), but did cause Nrf2 protein reduction (Fig. 3D). Furthermore, mRNA (Fig. 3F) and protein (Fig. 3D) expression of HO1-NQO1 as well as the NQO1 activity (Fig. 3G) were significantly decreased in T-HESC cells with antagomiR-941. These results suggested that miR-941 inhibition by antagomiR-941 led to Nrf2 signaling inhibition in T-HESC endometrial cells.

In the primary human endometrial cells antagomiR-941 infection similarly downregulated mature miR-941 expression (Fig. 3H). Contrarily, Keap1 mRNA (Fig. 3I) and protein (Fig. 3J) levels were increased. AntagomiR-941 inhibited Nrf2 signaling in the primary human endometrial cells, evidenced by decreased expression of Nrf2 protein (but not Nrf2 mRNA) (Fig. 3J and K), downregulation of HO1-NQO1 (Fig. 3J and L), and reduced NQO1 activity (Fig. 3M). Therefore, miR-941 inhibition upregulated Keap1 but inhibited Nrf2 signaling in primary human endometrial cells.

## 3.4. miR-941 inhibition intensifies OGDR-induced programmed necrosis in human endometrial cells

Since miR-941 inhibition suppresses Nrf2 signaling activation in human endometrial cells, it could then intensify OGDR-induced cytotoxicity. To verify this hypothesis, antagomiR-941-expressing T-HESC cells (see Fig. 3) were subjected to OGDR stimulation. As compared to the antaC control cells (see Fig. 3),

OGDR-induced ROS production (tested by superoxide accumulation) was significantly intensified in stable T-HESC cells with antagomiR-941 (Fig. 4A). Furthermore, OGDR-induced mitochondrial CypD-ANT1-p53 association (Fig. 4B), mitochondrial depolarization (JC-1 green fluorescence accumulation, Fig. 4C) and cytochrome c release to cytosol (Fig. 4D) were augmented with miR-941 inhibition. Consequently, antagomiR-941 potentiated OGDR-induced necrosis (LDH release to the medium, Fig. 4E) in T-HESC cells. In the primary human endometrial cells, miR-941 inhibition by antagomiR-941 similarly enhanced OGDR-induced ROS production (Fig. 4F), mitochondrial depolarization (JC-1 green fluorescence accumulation, Fig. 4G) and cytochrome C release to cytosol (Fig. 4H). Further studies show that OGDR-induced viability reduction (Fig. 4I) and necrosis (Fig. 4J) were exacerbated with miR-941 inhibition in primary endometrial cells. These results clearly show that miR-941 inhibition intensified OGDR-induced cytotoxicity in human endometrial cells.

## 3.5. miR-941 fails to affect OGDR-induced cytotoxicity in Keap1/Nrf2-KO human endometrial cells

Next we tested whether Nrf2 activation is essential for miR-941-induced endometrial cell protection against OGDR. The CRISPR/Cas9-Nrf2-KO construct [31] was transduced to T-HESC cells, and stable cells established. Compared to the Cas9 control cells (“Cas9-C”), Nrf2-KO cells were more sensitive to OGDR stimulation, presented with enhanced viability reduction (Fig. 5A) and cell necrosis (Fig. 5B). Significantly, exogenously altering miR-941 expression, via lv-pre-miR-941 or antagomiR-941 (see qPCR results in Fig. 5C), failed to affect OGDR-induced cytotoxicity in Nrf2-KO T-HESC cells (Fig. 5A and B). These results suggest that miR-941-induced endometrial cell protection against OGDR requires Nrf2 signaling activation. Western blotting assay results, Fig. 5D, confirmed Nrf2 depletion in the Nrf2-KO T-HESC cells. Nrf2 KO did not affect Keap1 regulation by lv-pre-miR-941 or antagomiR-941, as shown in Fig. 5D.

If targeting and silencing Keap1 is the primary mechanism of miR-941-induced endometrial cell protection against OGDR, Keap1 depletion should mimic miR-941’s actions. To test this hypothesis, the CRISPR-Cas9 method was again applied to knockout Keap1 in T-HESC cells (using a previously described protocol [31]). OGDR-induced cytotoxicity (Fig. 5E) and necrosis (Fig. 5F) were largely attenuated in Keap1-KO T-HESC cells (vs. Cas9-C cells), mimicking actions by miR-941 overexpression. Importantly, in the Keap1-KO cells, lv-pre-miR-941 and antagomiR-941 were ineffective on OGDR-induced actions (Fig. 5E and F), although both altered miR-941 expression (Fig. 5G). Results in Fig. 5H confirmed Keap1 depletion in the Keap1-KO T-HESC cells, resulting in significant Nrf2 protein accumulation.

To further support our hypothesis, the UTR-depleted Keap1 construct was transfected to lv-pre-miR-941-expressing T-HESC cells, resulting in complete Keap1 mRNA (Fig. 5I) and protein (Fig. 5J) re-expression, but Nrf2 protein reduction (Fig. 5J). Functional studies show that re-expression of Keap1 reversed lv-pre-miR-941-induced T-HESC cell protection against OGDR, leading to profound cell necrosis (Fig. 5K). Notably, Keap1 re-expression did not affect miR-941 overexpression in lv-pre-miR-941-T-HESC cells (Fig. 5L). These results further support that Keap1-Nrf2 cascade activation is essential for miR-941-induced endometrial cell protection against OGDR.

# 4. Discussion

The function of miR-941 is largely unknown in human cells. The study by Hu et al., has shown that miR-941 is a human-specific miRNA, highly expressed in the human brain and in other human cells [58]. It has regulatory effects on gene expression, participates in hedgehog- and insulin-signaling pathways, and thus regulating evolution of human longevity [58]. Zhang et al., have shown that miR-941 levels are significantly downregulated in hepatocellular carcinoma (HCC) tissues and cells. Functionally, miR-941 negatively regulated KDM6B (lysine (K)-specific demethylase 6B) to inhibit HCC cell epithelial-mesenchymal transition (EMT) and cell migration/invasion [59]. Bai et al., reported that miR-941 levels are significantly higher in acute coronary syndrome patients than those in healthy controls [60]. The expression and potential function of miR-941 in human endometrial cells have not been studied thus far.

Recent studies have implied that miRNA-induced silencing of Keap1, the Nrf2 suppressor protein, is a novel strategy to provoke Nrf2 cascade activation in human cells. In breast cancer cells miR-200a targeted Keap1 to activate Nrf2 signaling cascade [36]. In SH-SY5Y neuroblastoma cells and differentiated human neural progenitor cells, miR-7 activated Nrf2 signaling by targeting and silencing Keap1 [35]. In the retinal pigment epithelium cells (RPEs) and retinal ganglion cells (RGCs), miR-141 targeted Keap1 and activated Nrf2 signaling, protecting cells against ultra-violet (UV)-induced oxidative stress [41]. A very recent study by Xu et al., has shown that Keap1-targeting miR-626 activated Nrf2 signaling and protected RPEs from oxidative injury [31]. Therefore, Keap1-targeting miRNAs can induce significant Nrf2 cascade activation, protecting human cells from oxidative stress.

The results of this study confirm that miR-941 is a direct and specific Keap1-targeting miRNA, regulates Keap1-Nrf2 cascade in human endometrial cells. In T-HESC cells and primary human endometrial cells ectopic overexpression of miR-941 suppressed Keap1's 3'-UTR activity and downregulated its mRNA/protein expression, subsequently leading to Nrf2 activation. Conversely, Keap1's 3'-UTR activity and expression were elevated in endometrial cells with miR-941 inhibition, where Nrf2 activation was inhibited. RNA-Pull down experiments confirmed that miR-941 directly binds to Keap1 mRNA in T-HESC cells. Functional studies confirmed that miR-941 exerted significant endometrial cytoprotection against OGDR. Ectopic miR-941 overexpression in endometrial cells largely inhibited OGDR-induced ROS production and programmed necrosis, both were intensified with miR-941 inhibition. Thus, miR-941 provokes Nrf2 cascade activation by targeting and silencing Keap1. miR-941 should be a novel strategy to protect human endometrial cells from OGDR and possible ischemia-reperfusion injuries.

Our results support that activation of Keap1-Nrf2 cascade is absolutely required for miR-941-induced endometrial cell protection. Nrf2 KO, using the CRISPR/Cas9 method, abolished miR-941-induced endometrial cytoprotection against OGDR. Furthermore, CRISPR/Cas9-induced Keap1 KO mimicked miR-941's actions and largely attenuated OGDR-induced cytotoxicity in T-HESC cells. More importantly, ectopic miR-941 overexpression and miR-941 inhibition were both ineffective against OGDR in the Nrf2-KO and Keap1-KO T-HESC cells. To further support our conclusion, we show that restoring Keap1 expression, using an UTR-depleted Keap1 construct, abolished miR-941-induced endometrial cytoprotection against

OGDR. Furthermore, transfection of the miR-941 mimics, with mutations at the binding sites to Keap1's 3'-UTR, failed to protect endometrial cells from OGDR. Therefore, activation of the Keap1-Nrf2 cascade is responsible for miR-941-induced endometrial cell protection against OGDR.

# 5. Conclusion

MiR-941 negatively regulates Keap1 to activate Nrf2 signaling cascade, thus protecting human endometrial cells from OGDR-induced oxidative stress and programmed necrosis.

# Abbreviations

γ-glutamyl cysteine ligase catalytic subunit (GCLC); antioxidant responsive element (ARE); adenine nucleotide translocator type 1 (ANT1); cell counting kit-8 (CCK-8); cyclophilin-D (CypD); mitochondrial immunoprecipitation (Mito-IP); microRNA (miRNA); ginseng Rh2 (GRh2); heme oxygenase-1 (HO-1); Kelch-like ECH-associated protein 1 (Keap1); keratinocyte growth factor (KGF); knockout (KO); lactate dehydrogenase (LDH); microRNA-941 (miR-941); mitochondria permeability transition pore (mPTP); quantitative real time-PCR (qPCR); non-coding RNAs (ncRNAs); NAD(P)H
quinone oxidoreductase 1 (NQO1), nuclear-factor-E2-related factor 2 (Nrf2); oxygen and glucose deprivation (OGD); OGD-re-oxygenation (OGDR); untranslated region (UTR); optic density (OD); standard deviation (SD).

# Declarations

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Authors' contributions.
All listed authors designed the study, performed the experiments and the statistical analysis, and wrote the manuscript. All authors have read the manuscript and approved the final version.
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Authors' information. Not applicable.

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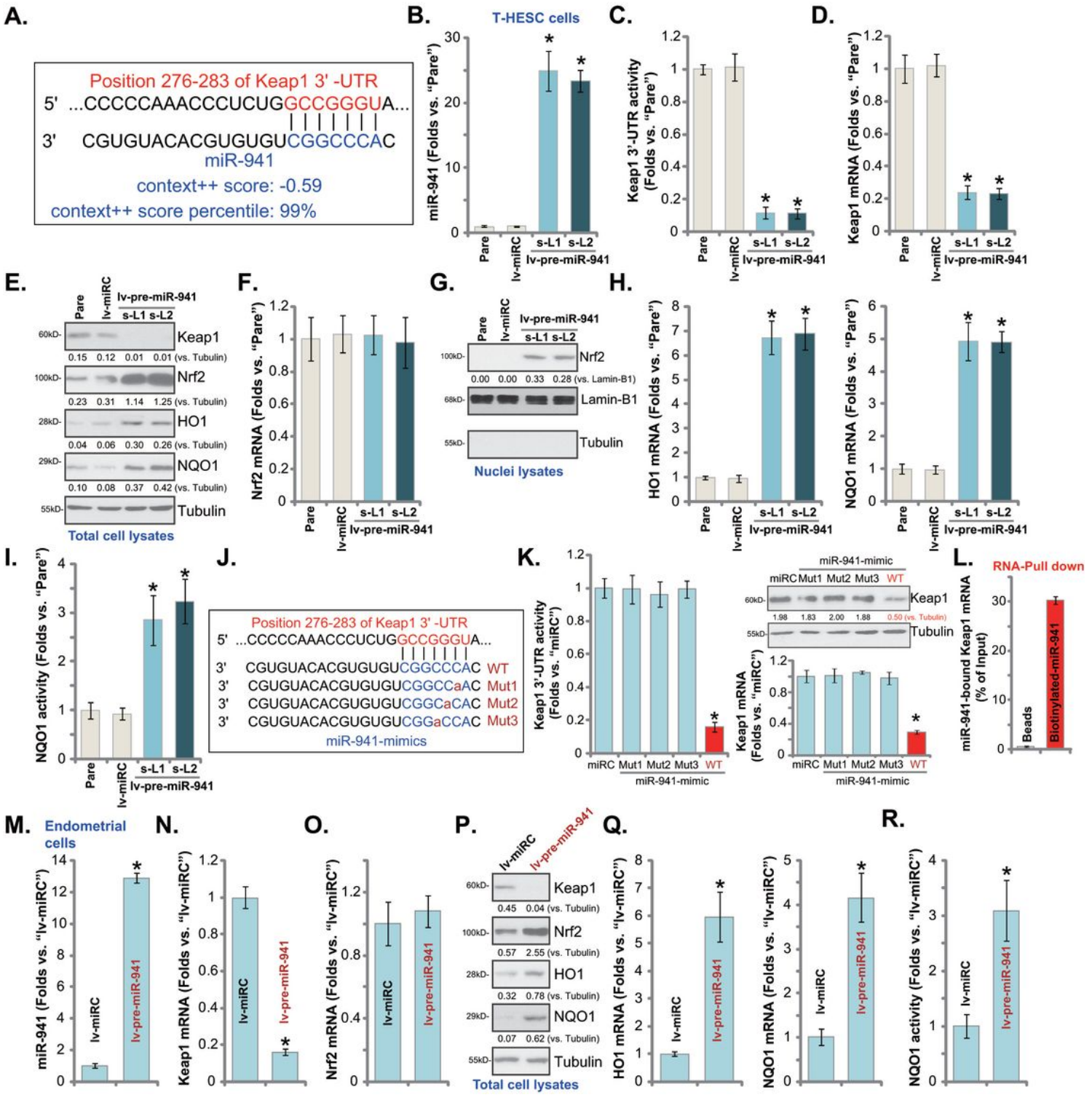
# Figures

## Figure 1




## Figure 1

miR-941 targets Keap1 and activates Nrf2 signaling in human endometrial cells. miRNA-941 putatively targets the 3'-UTR (untranslated region) of human Keap1 mRNA (at position of 276-283) (A). T-HESC human endometrial cells were transduced with lentiviral pre-microRNA-941 ("lv-pre-miR-941"), with selection by puromycin the stable cells were established, with control cells transduced with lentiviral non-sense microRNA ("lv-miRC"); Expression of mature miR-941 and listed mRNAs was tested by qPCR

assays (B, D, F and H); Keap1 3'-UTR activity was shown (C), with expression of listed proteins in total cell lysates (E) and nuclei lysates (G) tested by Western blotting; The relative NQO1 activity was tested as well (I). T-HESC cells were transfected with 500 nM of non-sense microRNA control ("miRC"), the wild-type ("WT") or the mutant miR-941 mimics (sequences listed in J), with Keap1 3'-UTR activity (K) and Keap1 mRNA/protein expression (K) tested after 48h. RNA-Pull down assay confirmed the direct association between biotinylated-miR-941 and Keap1 mRNA in T-HESC cells (L). The primary human endometrial cells ("Endometrial cells", same for all Figures) were infected with lv-pre-miR-941 or lv-miRC, with expression of listed genes tested by qPCR (M-O, and Q) and Western blotting (P) assays after 48h. The relative NQO1 activity was tested as well (R). Expression of listed protein was quantified and normalized (E, G, K and P). "Pare" stands for the parental control cells (same for all Figures). Data were presented as mean ± SD (n=5), and results were normalized. * P <0.05 vs. "lv-miRC"/"miRC" cells. Experiments in this figure were repeated five times with similar results obtained.

**Figure 2**

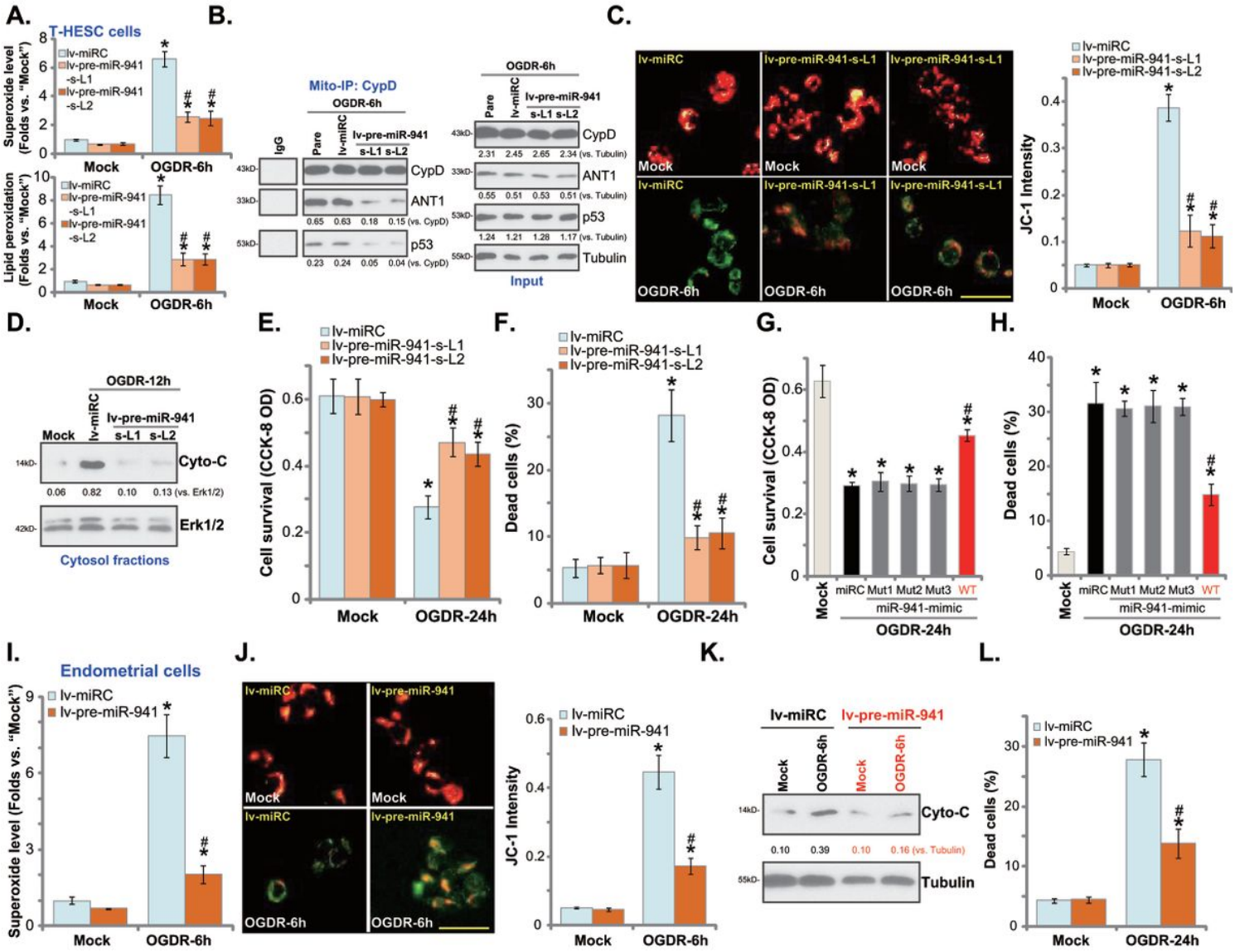
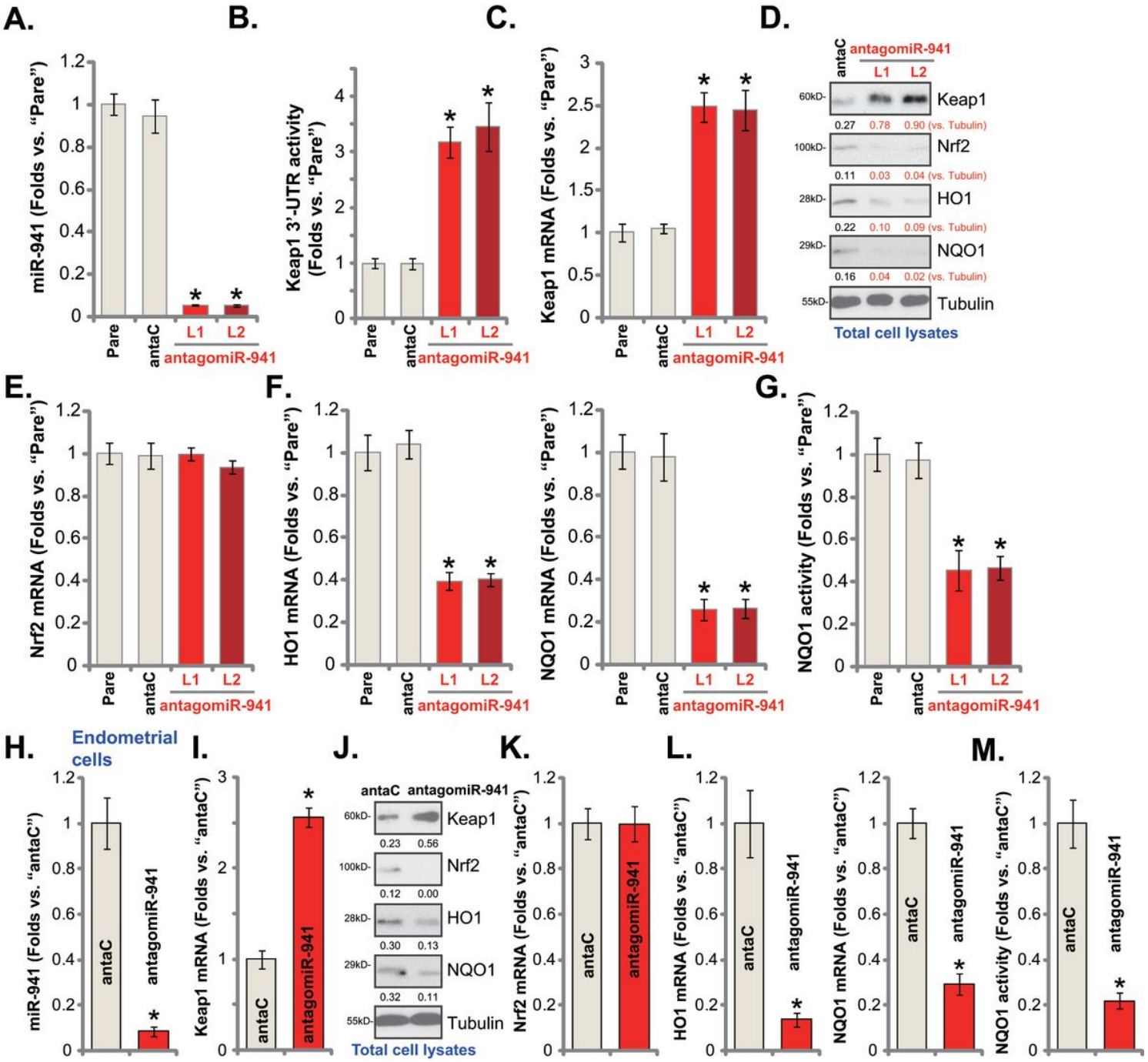



**Figure 2**

miR-941 overexpression protects human endometrial cells from OGDR. Stable T-HESC cells with lentiviral pre-microRNA-941 (“lv-pre-miR-941”, two lines) or the lentiviral non-sense microRNA (“lv-miRC”) were subjected to OGD exposure for 4h, followed by re-oxygenation (“OGDR”) for applied time periods, ROS production (superoxide and lipid peroxidation contents, A), mitochondrial CypD-ANT1-p53 association (tested by mito-IP assay, B) as well as mitochondrial depolarization (JC-1 green fluorescence accumulation, C) and cytochrome C release (D, testing cytosol proteins) were tested; Cell survival and necrosis were tested by CCK-8 assay (E) and LDH release assay (F), respectively. T-HESC cells were transfected with 500 nM of non-sense microRNA control (“miRC”), the wild-type (“WT”) or the mutant miR-941 mimics for 48h, followed by the same OGDR stimulation for another 24h, cell survival (G) and necrosis (H) were tested. The primary human endometrial cells, with lv-pre-miR-941 or lv-miRC, were subjected to the same OGDR procedure, ROS production (superoxide contents, I), mitochondrial depolarization (J) and cytosol cytochrome C release (K) were tested similarly, with cell necrosis tested by LDH release assay (L). For mito-IP assay, CypD-bound ANT1 and p53 were quantified (B), with total levels of CypD, ANT1 and p53 tested as the “Input” control (B). For the cytochrome C release assay, relative cytosol cytochrome C level was quantified (D and K). Data were presented as mean ± SD (n=5), and results were normalized. “Mock” stands for non-OGDR treatment (same for all Figures). * $P < 0.05$ vs. “Mock” treatment in “lv-miRC”/“miRC” cells. # $P < 0.05$ vs. OGDR treatment in “lv-miRC”/“miRC” cells. Experiments in this figure were repeated five times with similar results obtained. Bar= 50 μm (C and J).

# Figure 3




## Figure 3

miR-941 inhibition upregulates Keap1, suppressing Nrf2 signaling in human endometrial cells. T-HESC cells were transduced with the pre-microRNA-941 anti-sense lentivirus ("antagomiR-941"), with selection by puromycin the stable cells were established, with control cells transduced with microRNA anti-sense control lentivirus ("antaC"); Expression of mature miR-941 and listed mRNAs were tested by qPCR assays (A, C, E and F); Keap1 3'-UTR activity was shown (B), with expression of listed proteins in total cell lysates (D) tested by Western blotting; The relative NQO1 activity was tested as well (G). The primary human endometrial cells were infected with antagomiR-941 or antaC, with expression of listed genes tested by

qPCR (H, I, K, and L) and Western blotting (J) assays. The relative NQO1 activity was tested as well (M). Expression of listed protein was quantified and normalized (D and J). Data were presented as mean ± SD (n=5), and results were normalized. * P <0.05 vs. “antaC” cells. Experiments in this figure were repeated five times with similar results obtained.

**Figure 4**

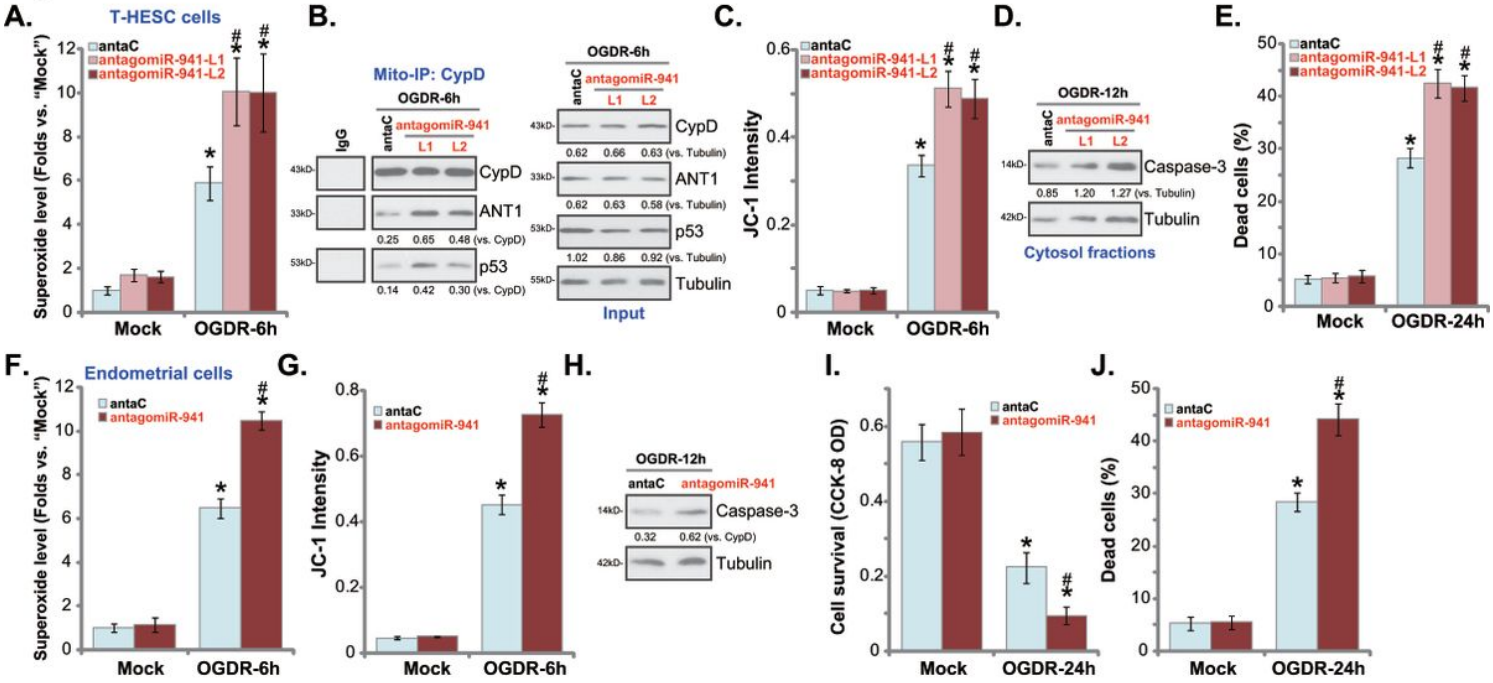
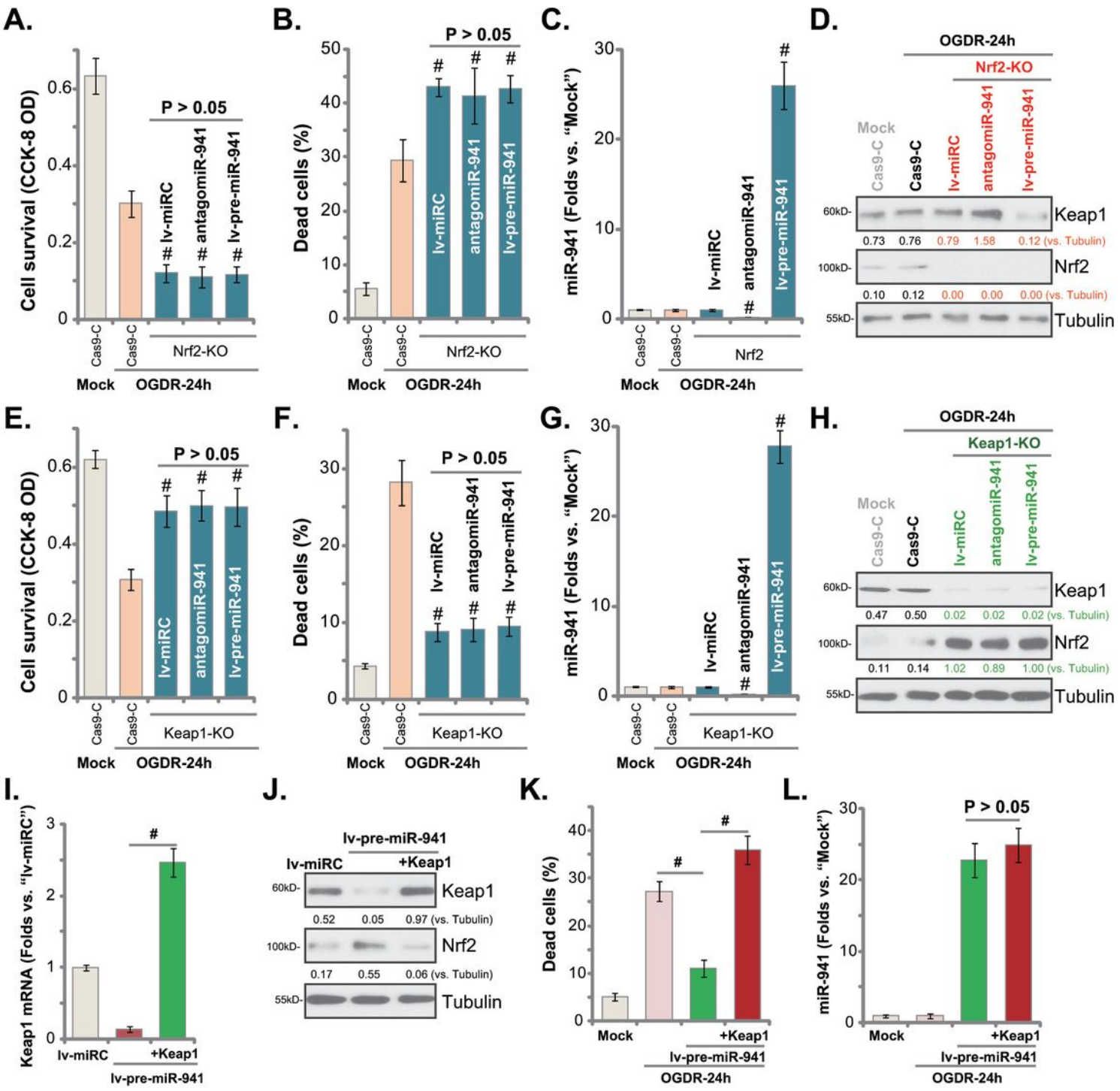



**Figure 4**

miR-941 inhibition intensifies OGDR-induced programmed necrosis in human endometrial cells. The stable T-HESC cells with the pre-microRNA-941 anti-sense lentivirus (“antagomiR-941”, two lines, “L1/L2”) or microRNA anti-sense control lentivirus (“antaC”) were subjected to OGD exposure for 4h, followed by re-oxygenation (“OGDR”) for applied time periods, ROS production (superoxide contents, A), mitochondrial CypD-ANT1-p53 association (tested by mito-IP assay, B) as well as mitochondrial depolarization (JC-1 green fluorescence accumulation, C) and cytochrome C release (D, testing cytosol proteins) were tested; Cell necrosis was tested by medium LDH release assay (E). The primary human endometrial cells were infected with antagomiR-941 or antaC for 48h, afterwards cells were subjected to the same OGDR stimulation and cultured for applied time periods, ROS production (F), mitochondrial depolarization (G) and cytosol cytochrome C release (H) were tested similarly, with cell survival and necrosis tested by CCK-8 (I) and LDH release (J) assays, respectively. For the mito-IP assay, CypD-bound ANT1 and p53 were quantified (B), with expression of CypD, ANT1 and p53 tested in the “Input” controls (B). For the cytochrome C release assay, relative cytosol cytochrome C level was quantified (D and H). Data were presented as mean ± SD (n=5), and results were normalized. * P <0.05 vs. “Mock” treatment in “antaC” cells. # P <0.05 vs. OGDR treatment in “antaC” cells. Experiments in this figure were repeated five times with similar results obtained.

## Figure 5




## Figure 5

miR-941 fails to affect OGDR-induced cytotoxicity in Keap1/Nrf2-KO human endometrial cells. Stable T-HESC cells with the CRISPR/Cas9-Nrf2-KO construct ("Nrf2-KO") (A-D) or the CRISPR/Cas9-Keap1-KO construct ("Keap1-KO") (E-H) were further infected with the pre-microRNA-941 anti-sense lentivirus ("antagomiR-941"), the pre-microRNA-941 lentivirus ("lv-pre-miR-941") or non-sense microRNA lentivirus ("lv-miRC") for 48h. These cells or the control cells with empty CRISPR/Cas9-KO construct ("Cas9-C")

were treated with OGD for 4h, followed by re-oxygenation (“OGDR”) for 24h, cell survival (A and E) and necrosis (B and F) were tested by CCK-8 and LDH release assays, respectively. Expression of mature miR-941 (C and G) and listed proteins (in total cell lysates, D and H) were shown. The lv-pre-miR-941-expressing T-HESC cells were further transfected with the UTR-depleted Keap1 construct (“+Keap1”) for 48h, expression of listed genes was shown (I and J); Cells were subjected to the same OGDR stimulation for 24h, cell necrosis (testing medium LDH release, K) and mature miR-941 expression (L) were tested similarly. Keap1 and Nrf2 protein expression was quantified and normalized to Tubulin (D, H and J). Data were presented as mean ± SD (n=5), and results were normalized.. # $P < 0.05$ vs. OGDR treatment in “Cas9-C” cells (A-C, E-G). # $P < 0.05$ (I and K). Experiments in this figure were repeated three times with similar results obtained.