

miR-148b Functions as a Tumor Suppressor by Targeting Endoplasmic Reticulum Metallo Protease 1 in Human Endometrial Cancer Cells

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This study investigated the tumor-suppressive role of miR-148b in regulating endoplasmic reticulum metalloprotease 1 (ERMP1) expression and the oxidative stress response in endometrial cancer cells. Human endometrial cancer RL95-2 cells were used and transfected with miR-148b mimic, miR-148b inhibitor, or their scrambled negative control. Thereafter, the transfection efficiency was determined by RT-qPCR, and cell proliferation was assessed by MTT assay. The dual-luciferase reporter assay, Western blot, and RT-qPCR were conducted to determine the target gene of miR-148b. ERMP1 is a putative target of miR-148b, and thereby the overexpression and downregulation of ERMP1 on the proliferation of RL95-2 cells were assessed. Next, the expressions of hypoxia-inducible factor 1 (HIF-1) and nuclear factor erythroid 2-related factor 2 (Nrf2) were analyzed by Western blot. Intracellular reactive oxygen species (ROS) was determined using dichlorofluorescein diacetate (DCFDA). Results showed that differential expression of miR-148b or ERMP1 was observed in normal endometrial tissues and endometrial cancerous tissues. Enhanced expression of miR-148b effectively inhibited proliferation of RL95-2 cells. ERMP1 was the target of miR-148b. ERMP1 silencing obviously suppressed proliferation of RL95-2 cells. Thus, miR-148b repressed cell proliferation, likely through downregulating ERMP1. Furthermore, it was observed that miR-148b significantly decreased expression of HIF-1 and Nrf2 by downregulating ERMP1. The intracellular ROS level was enhanced by miR-148b via downregulating ERMP1. To conclude, our results suggested that miR-148b suppressed cell proliferation and regulated the oxidative stress response in human endometrial cancer RL95-2 cells by inhibiting ERMP1.

Key words: Endometrial cancer cells; miR-148b; ERMP1; HIF; Nrf2; Oxidative stress

INTRODUCTION

Endoplasmic reticulum metalloprotease 1 (ERMP1; also known as Felix-ina or FXNA) is a zinc-binding protease that belongs to the peptidase M28 family¹. ERMP1 expression is needed in the ovaries for the organization of somatic cells and oocytes into discrete follicular structures². The ERMP1 gene maps at chromosome 9p24, a locus described as a novel amplicon in human esophageal and breast cancers³. A study by Grandi et al. demonstrated that ERMP1 protein is involved in cell proliferation, migration, and invasiveness⁴. The study dealt with characterization of ERMP1 and its role in cancers with high morbidity and mortality rate. Moreover, it was also observed that ERMP1 was highly expressed in a large fraction of breast, colon, lung, and ovary cancers⁴. In the present study, we have made an attempt to evaluate the role of ERMP1 in endometrial cancer, which is the most frequent form of malignant tumor of the female

reproductive tract, and overall, the endometrium is the fifth most common cancer in women, accounting for 4.8% of all women's cancers⁵.

Endometrial cancer is classified into types I and II⁶. Type I endometrial cancers are low-grade estrogen-related endometrioid carcinomas (EECs), which is reported to occur mostly in perimenopausal women. On the contrary, type II endometrial cancers are known to be aggressive nonendometrioid carcinomas (NEECs; mainly serous and clear cell carcinomas) that occur mostly in older women irrespective of estrogen stimulation. The most frequent type of endometrial cancer is endometrioid carcinoma, which accounts for more than 80% of all cases⁷. MicroRNAs (miRNAs) are potent targets as a therapeutic agent for different types of cancer. To date, there are a number of studies most commonly in mouse xenografts and in primates that deal with inhibition of oncogenic miRNAs (oncomiRs) that are overexpressed^{8,9}.

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miRNAs are known to play an important role in the posttranscriptional gene regulation in plants and animals. miRNAs are a family of small (i.e., with typical length of 19–25 nucleotides) non-protein-coding RNA molecules that play pivotal regulatory roles^{10,11}. miRNAs are complementary with specific protein-coding messenger RNAs (mRNAs) so as to induce mRNA degradation or translational repression¹². Mature miRNAs reportedly target the majority of all mRNAs¹³. There are studies demonstrating that miRNAs regulate a wide range of biological or cellular processes such as proliferation^{14,15}, metabolism¹⁶, differentiation¹⁷, development¹⁸, apoptosis¹⁹, cellular signaling²⁰, and cancer development and progression^{21–24}. Hence, in this study, we investigated the tumor-suppressive role of miR-148b in regulating ERMP1 expression and the oxidative stress response in endometrial cancer cells.

MATERIALS AND METHODS

Sample Collection

A total of 60 patients (all female, median age: 57.3 years) with endometrial cancer were enrolled in this study from November, 2015, to December, 2016, in Jinan Central Hospital. All patients received hysterectomy before other treatment strategy, including radiotherapy and chemotherapy. The cancerous and neighboring (<3 cm away from cancerous tissues) tissues were collected during hysterectomy. This study was approved by the ethics committee of Jinan Central Hospital and was performed under the ethical standards. Written informed consent was obtained from each patient for the use of their tissue samples. The collected tissues were washed twice with ice-cold PBS and were then stored at -70°C until use.

Cell Culture

Human endometrial cancer RL95-2 cells and other cell lines, including Ishikawa, HEC-1A, HEC-1B, KLE, and one normal endometrial cell line (EMC) were obtained from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences and cultured in DMEM/Hams F12 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C and 5% CO_2 ²⁵.

MicroRNA Transfection

Synthetic miR-148b mimic, miR-148b inhibitor, and scrambled negative control RNA (miR-NC) were bought from GenePharma (Shanghai, P.R. China). Cells were seeded in six-well plates and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) the next day when the cells were approximately 70% confluent. In each well, equal amounts (100 pmol) of miR-148b mimics, miR-148b inhibitor, and miR-NC were added.

The efficiency of downregulation and overexpression of miR-148b was evaluated by quantitative real-time PCR (RT-qPCR)²⁶.

ERMP1 Transfection

Full-length ERMP1 was cloned into the retroviral pBABE vector backbone. Retroviruses were generated from the 293T after transfection with pBABE. T24 cells were infected with virus containing ERMP1. The siRNA of ERMP1 and the corresponding NC were designed and synthesized by GenePharma. They were referred to as si-ERMP1 and si-NC, respectively. The cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after infection, the cells were selected using $2\mu\text{g}/\text{ml}$ puromycin for 10 days and then used for the experiments as described²⁷.

Western Blot Analysis

The cells were washed two times with PBS and then lysed with 1% SDS loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromophenol blue) as the whole-cell sample. The protein samples were then subjected to SDS-PAGE. Immunoblottings were carried out with primary antibodies [anti-ERMP1, anti-tubulin, anti-hypoxia-inducible factor 1 (HIF-1), and anti-nuclear factor erythroid 2-related factor 2 (Nrf2); Abcam, Cambridge, MA, USA]. The next day, the membrane was incubated with HRP-conjugated secondary antibodies (Abcam). The proteins were detected by enhanced chemiluminescence (ECL Plus; Amersham Pharmacia Biotech, Cardiff, UK)²⁸.

Dual-Luciferase Reporter Activity Assay

The 3'-UTR segment of the ERMP1 gene that contains the miR-148b binding site was amplified through PCR and inserted into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). Cells were then cotransfected with ERMP1 3'-UTR and pre-miR-148b or miR-NC using Lipofectamine 2000 (Invitrogen). Luciferase activity was analyzed at 48 h post-transfection using the Dual-Luciferase Reporter Assay System (Promega). For each transfection, the luciferase activity was averaged from three replicates²⁹.

Bromodeoxyuridine (BrdU) Staining to Determine Cell Proliferation

Cells were pulsed with $10\mu\text{M}$ BrdU (Sigma-Aldrich) for 1.5 h at 37°C . Cells were then fixed in cold 70% ethanol for 5 min. Denaturation of DNA was done by adding 1.5 M HCl to the cells for 30 min at room temperature. Immunostaining using anti-BrdU antibody at 1:1,000 dilution (A21300; Invitrogen) was performed. Primary antibody was followed by 1 h of incubation with

secondary green fluorescence dye-conjugated antibody (Alexa Fluor 488; Invitrogen). Mounting medium with DAPI was used to counterstain the nuclei. Images were taken using a Leica inverted fully automated microscope (DMI6000B) with digital camera DFC 420 RGB (Leica Microsystems, Wetzlar, Germany)³⁰.

MTT Assay to Determine Cell Proliferation

Cells were plated in 96-well culture plates (10,000 cells per well). MTT (5 mg/ml; Sigma-Aldrich) was dissolved in PBS and filter sterilized. Then 20 μ l of the prepared solution was added to each well. The plate was incubated until purple precipitate was visible. Subsequently, 100 μ l of Triton X-100 was added to each well and incubated in darkness for 2 h at room temperature. The absorbance was measured on an ELISA reader (MultiskanEX; Lab systems, Helsinki, Finland) at a test wavelength of 570 nm and a reference wavelength of 650 nm.

RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Grand Island, NY, USA). RNA (500 ng) was polyadenylated and reverse transcribed to cDNA using an NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen). cDNA was used as the template for real-time PCR FastStart Universal SYBR Green Master (Roche Applied Science, Mannheim, Germany) with the universal reverse primer provided in the kit. Real-time PCR was performed on an Applied Biosystems real-time detection system (Applied Biosystems, Foster City, CA, USA), and the thermocycling parameters were 95°C for 3 min and 40 cycles of 95°C for 15 s followed by 60°C for 30 s. Each sample was run in triplicate and was normalized to U6 small nuclear RNA (snRNA) levels [U6 primers 5'-CTTCGGCAGCACATATACT-3' (forward) and 5'-AAAATATGGAACGCTTCACG-3' (reverse)]. Melting curve analysis was performed to confirm the specificity of the PCR products. The replicates were then averaged, and fold induction was determined by a CT-based fold change calculation³¹.

Measurement of Intracellular ROS Using DCFDA

Cells were cultured for 24 h in the incubator at 20% O₂ or at 1% O₂, after which the cells were treated with 5 μ g/ml of 2',7'-dichlorofluorescein diacetate (DCFDA; Sigma-Aldrich) for 15 min, washed with PBS, and lysed in RIPA buffer. The fluorescence was detected by spectrophotometric analysis at 510 nm³².

Statistical Analysis

All data were expressed as means \pm SEM. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post hoc. Results with a value of $p < 0.05$ were considered statistically significant.

GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) was used for these analyses.

RESULTS

miR-148b Is a Tumor Suppressor in Human Endometrial Cancer RL95-2 Cells

miRNA was purified from the cancerous and neighboring tissues of 60 patients with endometrial cancer. RT-qPCR analysis was done to determine the expression levels of miR-148b, wherein a differential expression of miR-148b was observed in normal endometrial tissues and endometrial cancerous tissues, with a median value of 5.5 and 3.1, respectively (Fig. 1A). Significantly

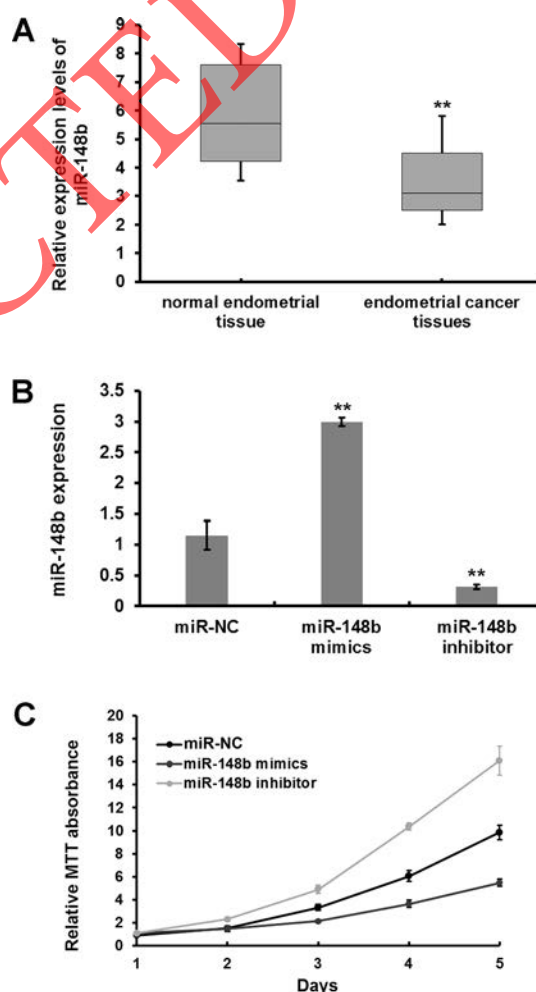


Figure 1. MicroRNA-148b (miR-148b) is a tumor suppressor in human endometrial cancer RL95-2 cells. (A) Quantitative real-time PCR (RT-qPCR) was performed to determine the expression level of miR-148b in cancer and neighboring tissues of 60 patients with endometrial cancer. (B) miR-NC, miR-148b mimic, or inhibitors were transfected into RL95-2 cells, and RT-PCR was conducted to measure miR-148b expression levels. (C) Effects of miR-148b on cell proliferation was determined by MTT assay. $**p < 0.01$.

enhanced expression of miR-148b was observed in endometrial cancer cells transfected with miR-148b mimic compared to miR-NC-transfected cells ($p < 0.01$) (Fig. 1B). In contrast, miR-148b expression was significantly reduced in miR-148b inhibitor-transfected cells compared to miR-NC-transfected cells ($p < 0.01$) (Fig. 1B). Cell proliferation was determined by MTT assay, and Figure 1C shows an enhanced proliferation in cells transfected with miR-148b inhibitor compared to miR-NC-transfected cells (Fig. 1C). Also, repressed proliferation was observed in cells transfected with miR-148b mimic compared to miR-NC-transfected cells (Fig. 1C).

ERMP1 Is a Direct Target of miR-148b in Human Endometrial Cancer RL95-2 Cells

Figure 2A shows miR-148b base pairs with the 3'-UTR of ERMP1. Moreover, the predicted binding relationship was confirmed by luciferase assay. Figure 2B shows that the luciferase activity was remarkably reduced in cells cotransfected with miR-148b and wild-type ERMP1 compared to the cells cotransfected with miR-148b and mutant 3'-UTR of ERMP1 (Fig. 2B). Additionally, ERMP1 expression was remarkably reduced in cells transfected with miR-148b mimic compared to miR-NC-transfected cells, and ERMP1 expression was increased in miR-148b inhibitor-transfected cells compared to miR-NC-transfected cells (Fig. 2C and D).

The Expression of miR-148b and ERMP1 in Several Endometrial Cancer Cell Lines Is Also Opposite

According to the RT-qPCR analysis, miR-148b was downregulated in some endometrial cancer cell lines, including Ishikawa, HEC-1A, HEC-1B, and KLE, compared to EMC (Fig. 3A). On the contrary, ERMP1 was upregulated in the tested cell lines (Fig. 3B). These data indicated that the interrelation of miR-148b and ERMP1 might occur in various endometrial cancer cell lines, from which we inferred that ERMP1 was a direct target of miR-148b in endometrial cancer, and endometrial cancer might be suppressed by targeting ERMP1 through regulating miR-148b.

ERMP1 Knockdown Inhibits Cell Proliferation of Human Endometrial Cancer RL95-2 Cells

Expression of ERMP1 was significantly enhanced in transfected cells relative to empty vectors. ERMP1 was dramatically downregulated after transfection with si-ERMP1 compared to si-NC. Tubulin was used as the internal standard (Fig. 4A). Stable transfection of RL95-2 cells with ERMP1, when evaluated for proliferation using MTT assay and BrdU staining, showed a significantly enhanced cell proliferation compared to empty vectors. However, downregulating ERMP1 exhibited completely contrary effects on RL95-2 cell proliferation (Fig. 4B and C). The data suggested that ERMP1 knockdown effectively inhibited cell proliferation of human endometrial cancer RL95-2 cells.

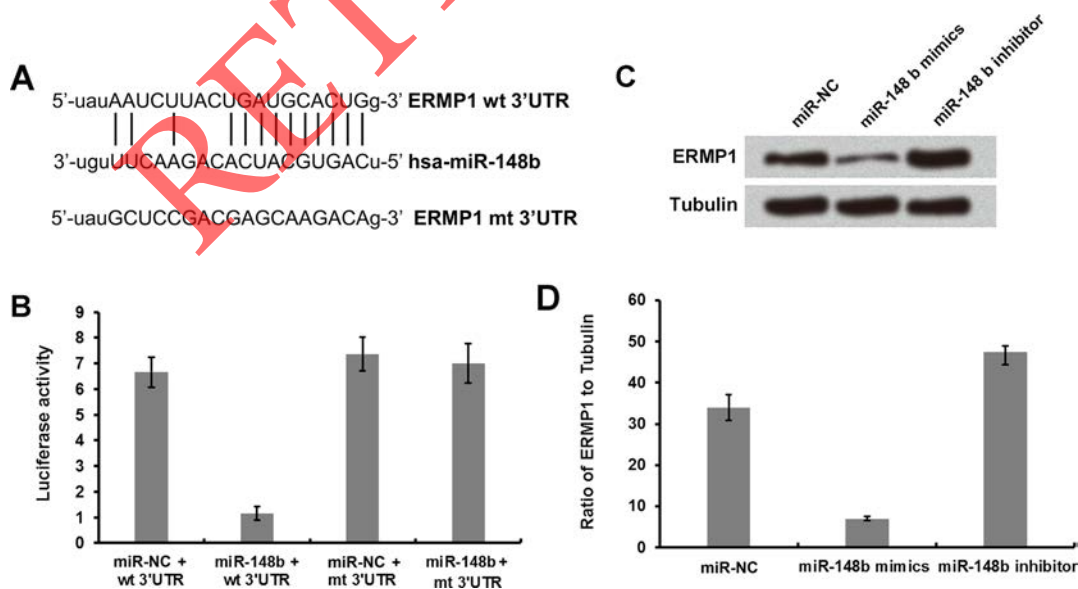


Figure 2. ERMP1 is a direct target of miR-148b in human endometrial cancer RL95-2 cells. (A) A diagram showing miR-148b that forms a base pair with the 3'-UTR of ERMP1. (B) miR-148b and wild-type or mutated 3'-UTR of ERMP1 were cotransfected into RL95-2 cells, and the luciferase activities were measured. (C, D) miR-148b mimic or inhibitor was transfected into RL95-2 cells, and Western blot analysis was performed to determine the expression of ERMP1 and tubulin.

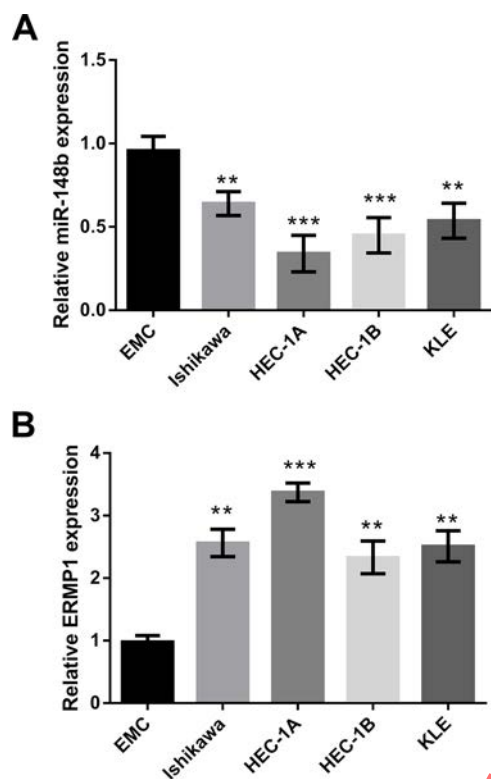


Figure 3. The expression of miR-148b was decreased, and ERMP1 was enhanced in some endometrial cancer cell lines. The expression of (A) miR-148b and (B) ERMP1 in the normal endometrial cell line (EMC), Ishikawa, HEC-1A, HEC-1B, and KLE was analyzed by RT-qPCR. ** $p < 0.01$ and *** $p < 0.001$.

miR-148b Regulates Oxidative Stress in Human Endometrial Cancer RL95-2 Cells

Cells transfected with miR-148b were cultured for 24 h at 20% O_2 and at 1% O_2 to induce oxidative stress. The cells were harvested and subjected to immunoblot analysis with HIF-1 and Nrf2, which were closely related to the oxidative stress of cancer cells. It was observed that HIF-1 and Nrf2 expression in miR-148b-overexpressing cells were decreased at O_2 concentrations of 1% and 20% compared to the miR-NC group (Fig. 5A). In addition, the ROS production was obviously enhanced when RL95-2 cells were transfected with miR-148b mimic (Fig. 5B).

miR-148b Enhances Oxidative Stress Response in Human Endometrial Cancer RL95-2 Cells by Downregulating ERMP1

Cells cotransfected with miR-148b and ERMP1 were cultured for 24 h at 20% O_2 or at 1% O_2 to analyze the effect on the oxidative stress of RL95-2 cells. It was observed that expression of HIF-1 and Nrf2 was significantly enhanced in cells cotransfected with miR-148b and ERMP1 at 20% and even more enhanced at 1% O_2

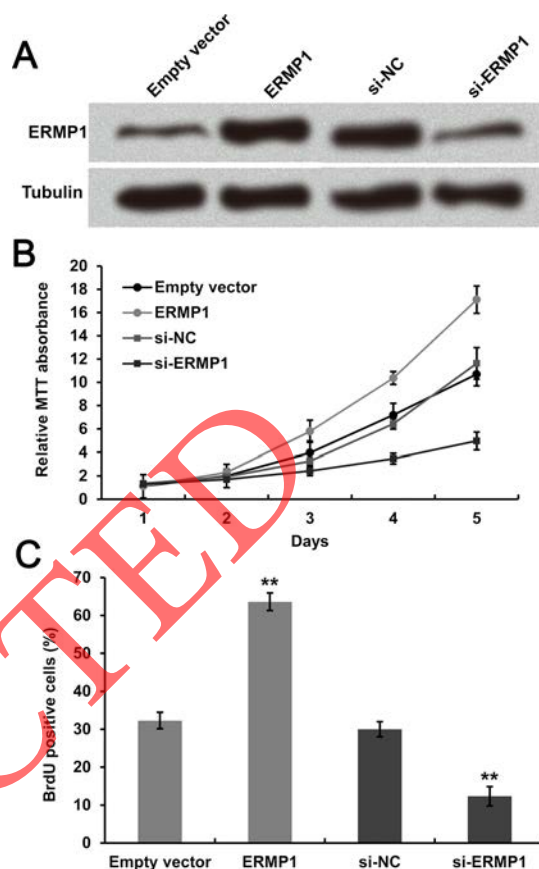


Figure 4. ERMP1 overexpression promotes cell proliferation of human endometrial cancer RL95-2 cells, whereas knock-down of ERMP1 inhibited cell proliferation. (A) The complete sequence of ERMP1 and si-ERMP1 was transfected into RL95-2 cells. Cell proliferation was determined using (B) MTT and (C) BrdU staining after transfection. ** $p < 0.01$.

concentration compared to cells transfected only with miR-148b (Fig. 6A and B).

DISCUSSION

Endometrial cancer is the most commonly reported cancer of the female genital tract⁵. In spite of a vast number of studies related to the understanding of endometrial cancer biology, therapeutic modality has remained the same over the past 40 years³³. Activation of unfolded protein response (UPR) and increased GRP78 due to endoplasmic reticulum stress have been identified recently as mechanisms that lead to growth, invasion, and resistance to therapy of different types of cancer³⁴⁻³⁷. However, a possible role of endoplasmic reticulum stress in endometrial cancer is yet to be established. Grandi et al. demonstrated that ERMP1 is well expressed in a high percentage of breast, colorectal, lung, and ovary cancers, irrespective of their stage and grade and also regulated

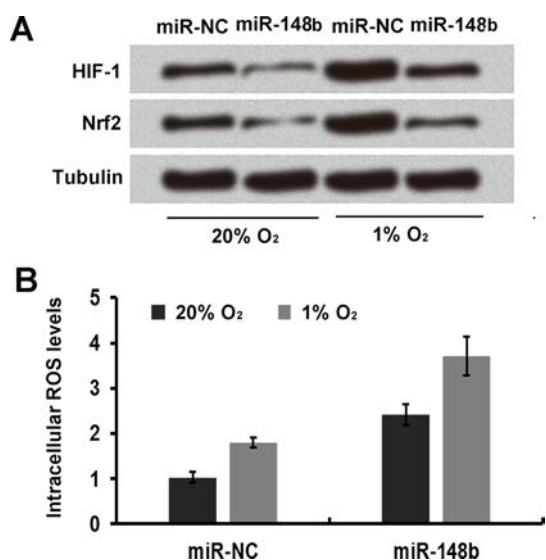


Figure 5. miR-148b regulates oxidative stress in human endometrial cancer RL95-2 cells. (A) Immunoblot analysis was used to determine the expression levels of HIF-1 and Nrf2 in miR-148b-transfected cells. (B) The ROS production in miR-148b-transfected cells was detected by the dichlorofluorescein diacetate (DCFDA) staining method.

cell proliferation, migration, and invasiveness of cancer cells⁴. Keeping this factor in mind, we investigated the role of ERMP1 in endometrial cancer.

The expression of ERMP1 was found to be enhanced in the human endometrial cancer cell line RL95-2 used in the study (data not shown). We have reported that ERMP1 overexpression significantly increased cell proliferation as observed by MTT assay and BrdU staining. However, the cell proliferation was obviously inhibited after silencing ERMP1. This finding was consistent with the other studies where ERMP1 silence repressed proliferation in endometrial cancer RK95-2 cells³⁸. Since it is an established fact that miRNAs play an important role in the posttranscriptional gene regulation, by targeting mRNA, we further evaluated the tumor-suppressive role of miR-148b in regulating ERMP expression and oxidative stress response in endometrial cancer cells. In our study, we have reported that miR-148b targeted ERMP1 and thereby downregulated the expression in cells that were transfected with both miR-148b and ERMP1. Moreover, this was confirmed by dual-luciferase reporter assay where we reported that miR-148b base paired with 3'-UTR of ERMP1 and a significantly reduced luminescence, whereas the luminescence significantly increased when miR-148b did not bind with the mutant 3'-UTR of ERMP1. Our findings displayed that the regulatory

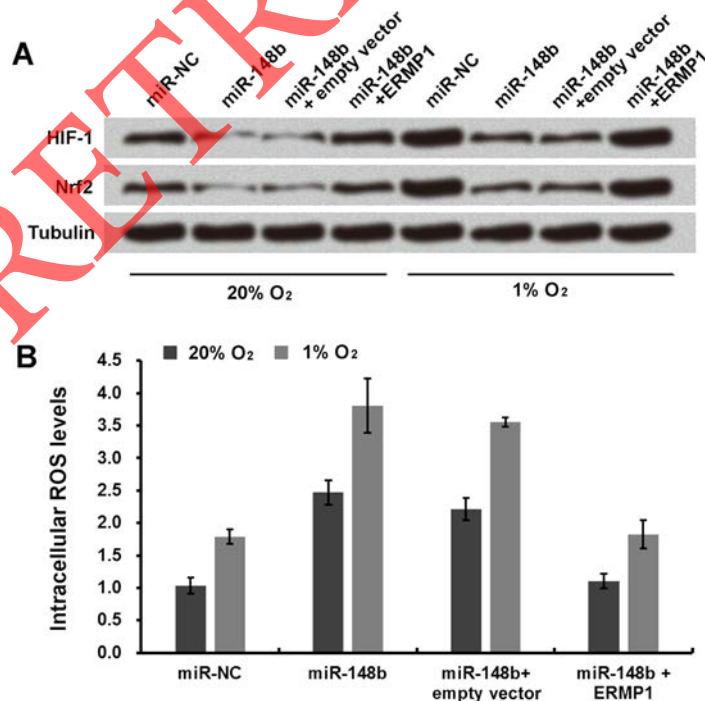


Figure 6. miR-148b enhances oxidative stress response in human endometrial cancer RL95-2 cells by downregulating ERMP1. (A) Immunoblot analysis was used to determine the expression levels of HIF-1 and Nrf2 in transfected miR-148b and ERMP1 cells. (B) The ROS level in miR-148b- and ERMP1-transfected cells was detected by the DCFDA staining method.

relationship between miR-148b and ERMP1 might occur in various endometrial cancer cell lines, not only the RL95-2 cell line.

ERMP1 expression is strongly affected by endoplasmic reticular stress induced by thapsigargin and other oxidative stresses⁴. Free radicals and oxidant species act as deleterious and toxic products, concerned with cellular and tissue dysfunction³⁹. Overproduction of the aforementioned species leads to DNA, lipid, and protein damage. Nevertheless, ROS or reactive nitrogen species (RNS) at low or moderate concentrations are also involved in physiological responses as part of the signaling processes and defense mechanisms against infectious agents⁴⁰. Irregularities of cellular oxygenation that cause intermittent hypoxia and oxidative stress thereby affect the regulation of HIF-1 and Nrf2. HIF-1 is primarily induced in hypoxia, and Nrf2 is induced in response to oxidative stress^{41,42}. Thus, in our study, we observed that expression of HIF-1 and Nrf2 was significantly decreased when miR-148b was upregulated, which was possibly realized by downregulation of ERMP1.

Going by our observations, we can conclude that miR-148a functions as a tumor suppressor by downregulating ERMP1 in human endometrial cancer. Additionally, miR-148b regulated the oxidative stress response by inhibiting ERMP1 in human endometrial cancer RL95-2 cells.

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