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Original Article

Long non-coding RNA MALAT-1 is downregulated in preeclampsia and regulates proliferation, apoptosis, migration and invasion of JEG-3 trophoblast cells

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Abstract: Long non-coding RNA (lncRNA), as a newly identified subset of the transcriptome, has been implicated in a variety of physiological and pathological processes. Metastasis associated lung adenocarcinoma transcript-1 (MALAT-1), a lncRNA that was initially detected in the metastatic lung cancer, was reported to be overexpressed in placenta previa increta/percreta (I/P), which is caused by excessive trophoblast invasion. However, the role of MALAT-1 in the regulation of trophoblast behavior is not fully understood. In this study, we first examined the expression of MALAT-1 in the placentas from the patients with preeclampsia, the pathology of which is associated with inadequate trophoblast invasion, and found that the expression of MALAT-1 was downregulated in the preeclamptic placentas as compared to the normal placentas. We further investigated the function of MALAT-1 in JEG-3 trophoblast cell line using short interfering RNA (siRNA) against MALAT-1 transcripts. Silencing of MALAT-1 in JEG-3 cells suppressed proliferation and induced cell cycle arrest at G_0/G_1 phase. Reduced expression of MALAT-1 by RNA interference resulted in enhanced apoptosis in JEG-3 cells, accompanied with elevated levels of the pro-apoptotic proteins including cleaved caspase-3, cleaved caspase-9 and cleaved poly (ADP-ribose) polymerase-1 (PARP-1). Moreover, the migration rate and the invasiveness of JEG-3 cells were suppressed when MALAT-1 was downregulated. In summary, our results suggest that MALAT-1 may play an important role in the regulation of proliferation, cell cycle, apoptosis, migration and invasion of trophoblast cells, and under-expression of MALAT-1 during early placentation may be involved in the pathogenesis of preeclampsia.

Keywords: Long non-coding RNA, MALAT-1, preeclampsia, trophoblast invasion, trophoblast proliferation

Introduction

Preeclampsia (PE) is a frequently encountered complication of pregnancy that occurs in 3-5% pregnant women [1]. PE classically manifests as new-onset hypertension and proteinuria after 20 weeks of gestation, and it is a leading cause of maternal and neonatal morbidity and mortality [2]. Inadequate trophoblast invasion, followed by abnormalities in the development of placental vasculature and resultant placental underperfusion has been implicated in the pathology of PE [3, 4]. However, the molecular mechanism for the regulation of trophoblast behavior and the pathogenesis of PE remains largely elusive.

Long non-coding RNA (lncRNA) refers to a class of non-coding RNA longer than 200 nucleotides and devoid of an open reading frame that can be translated into a protein [5, 6]. Although it was previously thought to be spurious transcriptional noise due to low specificity of RNA polymerase II [7], lncRNA was recently demonstrated to play functional roles in the regulation of gene expression by means of dosage compensation, imprinting, transcriptional regulation and nuclear organization [8, 9]. In addition, lncRNA is proposed to be involved in normal development and in the pathogenesis of multiple diseases [10, 11]. By screening the expression profile of lncRNAs in the placentas of PE patients and healthy subjects, He et al. identi-

fied 738 out of 28,443 lncRNAs that were differentially expressed in the PE placentas [12], suggesting that aberrant expression of lncRNAs may contribute to the pathogenesis of PE.

Metastasis associated lung adenocarcinoma transcript-1 (MALAT-1), named after its initially discovered function, is a lncRNA of over 8,000 bp [13]. MALAT-1 was later identified to be a nuclear enriched abundant transcript [14] and expressed in the lungs, pancreas, nerve system and other healthy organs [13, 15]. High expression of MALAT-1 has also been detected in various types of cancers, including lung cancer, endometrial stromal sarcoma, hepatocellular carcinoma, breast cancer and pancreatic cancer [13, 16, 17]. In addition, overexpression of MALAT-1 was found in placenta previa increta/percreta (I/P), a severe form of invasive placentation [18], implying a potential role of MALAT-1 in trophoblast invasion during placental development.

This study aimed to investigate the possible role of MALAT-1 in the pathophysiology of PE and its function in trophoblast biology. We first examined the expression of MALAT-1 in the placentas from PE patients and healthy subjects. We further studied the function of MALAT-1 in the proliferation, apoptosis, migration and invasion of trophoblast cells *in vitro* by targeting the expression MALAT-1 with short interfering RNA (siRNA) in JEG-3 trophoblast cell line.

Materials and methods

Placental tissue samples

Collection and experimentation of human tissues was conducted in strict accordance with the protocol approved by the Clinical Research Ethics Committee of China Medical University and written informed consent was obtained from all enrolled subjects. The placental tissues from PE patients and healthy pregnancy women (n=18 each) were collected immediately after delivery, and frozen in liquid nitrogen for later experiments. PE was diagnosed as systolic pressure ≥140 mmHg and/or diastolic pressure ≥90 mmHg, accompanied by proteinuria (urine protein ≥0.3 g/24 h, or ≥0.2 g/L in a random urine test).

Real-time polymerase chain reaction

Total RNA was extracted from primary tissues or cultured cells using the RNApure Total RNA Fast Extraction Kit (BioTeke, Beijing, China), and reverse transcribed into cDNA using Super M-MLV reverse transcriptase (BioTeke). The expression of MALAT-1 was measured in the Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Daejeon, Korea) using SYBR GREEN PCR Master Mix (Solarbio, Beijing, China) and the following primers: MALAT-1 forward, 5'-GACTTCAGGTCTGTCTGTTCT-3'; MALAT-1 reverse, 5'-CAACAATCACTACTCCAAGC-3'. -actin was used as the internal control, and the primers were as follows: -actin forward, 5'-CTTAGTTGCGTTACACCCTTTCTTG-3'; -actin reverse, 5'-CTGTCACCTTCACCGTTCCAGTTT-3'.

Cell culture and transfection

JEG-3 human trophoblast cell line was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). JEG-3 cells were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere consisting of 5% CO_2.

Two sets of short hairpin RNA (shRNA) targeting 5'-GGCTCTTCCTTCTGTTCTA-3' (6427-6445) and 5'-GAAGGAGCTTCCAGTTGAA-3' (7211-7229) on MALAT-1 transcript were cloned into the pRNA-H1.1/Neo siRNA expression vector (GenScript, Piscataway, NJ, USA) to make MALAT-1 shRNA-1 and MALAT-1 shRNA-2 respectively. The scramble shRNA with the sequence of 5'-TTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAA-3' was cloned into pRNA-H1.1/Neo as the negative control (NC). JEG-3 cells were transfected with the indicated vector using lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Prior to transfection, the cells were starved in serum-free medium for 1 h. The medium was replaced with fresh culture medium 6 h after transfection.

Proliferation assay

Cell proliferation was assessed by MTT assay. JEG-3 cells were seeded in a 96-well plate at a density of 3×10^3 cells/well, and subjected to transfection 24 h later. At post-transfection 24 h, 48 h, 72 h and 96 h, MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium to a final concentration of 0.2 mg/ml, followed by 4 h incubation at 37°C. Thereafter, the supernatant was removed, and 200 μL DMSO (Sigma-Aldrich) was added into each well to dissolve

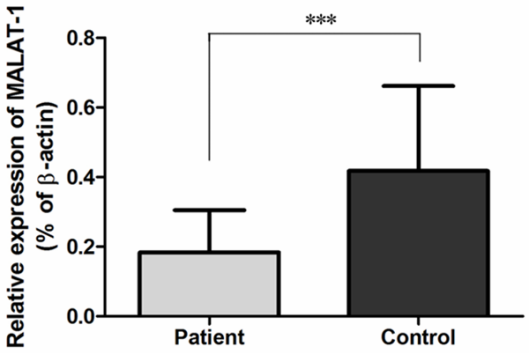

Figure 1. Dowregulation of MALAT-1 in PE placentas. Placentas of PE patients and healthy subjects were collected immediately after delivery (n=18 each). The expression levels of MALAT-1 in the placental tissues were determined by real-time PCR using -actin as the internal control. Data are expressed as the mean ± SD. ***P<0.001.

the formazan crystals. Optical density (OD) at 490 nm was measured with the ELX-800 microplate reader (BioTek, Winooski, VT, USA). Each time point was done in 5 replicates.

Cell cycle analysis by flow cytometry

At post-transfection 24 h, the cells were trypsinized, washed with PBS and fixed in 70% ethanol for 2 h at 4°C. Propidium iodide (PI) solution and RNase A from a Cell Cycle Analysis Kit (Beyotime) were added to the cells and incubated for 30 min at 37°C in the dark. The cells were then analyzed for the cell cycle state in FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Hoechst assay

JEG-3 cells were cultured on coated glass slides and subjected to transfection when reaching approximately 80% confluence. At post-transfection 24 h, the cells were fixed and stained with Hoechst stain using a Hoechst staining kit (Beyotime, Haimen, China) according to the manufacturer's protocol. The slides were mounted and observed under an Olympus fluorescence microscope.

Apoptosis analysis by flow cytometry

At 24 h after transfection, the cells were examined for apoptotic status by double-staining with Annexin V and PI using an Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen, Nanjing, China), followed by flow cytometric analysis.

Western blotting

At post-transfection 24 h, the cells were lysed with NP-40 lysis buffer (Beyotime) for total protein extraction. The concentration of the protein samples was determined with the BCA Assay Kit (Beyotime). A total of 40 g proteins from each sample were separated by SDS-PAGE, and transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat milk and incubated with anti-cleaved caspase-3 antibody (1:500; Abcam, Cambridge, MA, USA), anti-cleaved caspase-9 antibody (1:500; Santa Cruz, Dallas, TX, USA) or anti-PARP antibody (1:1000; Abcam) at 4°C overnight. Subsequently, the membrane was incubated with the respective horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Beyotime) for 45 min at 37°C, followed by visualization of the target protein using the ECL system (7Sea PharmTech, Shanghai, China). To verify equal loading and transfer, the membrane was incubated with the stripping buffer (Beyotime) for 15 min and re-probed with anti--actin antibody (Santa Cruz).

Migration assay

JEG-3 cells were transfected with MALAT-1 shRNA or NC when reaching 80-90% confluence. 24 h later, the cells were incubated with 5 M mitomycin-C (Sigma-Aldrich) for 2 h to inhibit cell proliferation. Thereafter, a scratch was created by dragging a 200 μl pipette tip across the surface of the monolayer. The cell monolayer were washed twice with serum-free medium and photographed under an optical microscope. The cells were then cultured in serum-free medium for 24 h at 37°C and photographed again. The migration rate is indicated by the percentage of wound closure which is calculated as (original gap distance-gap distance at 24 h)/original gap distance ×100%.

Matrigel-based invasion assay

Matrigel-based Transwell assay was performed to assess the invasion of JEG-3 cells 24 h after transfection. JEG-3 cells were pre-treated with 5 M mitomycin-C for 2 h. 3×10^4 JEG-3 cells resuspended in 200 μl serum-free medium were plated in the Matrigel (BD Biosciences)-coated Transwell chamber (Corning, New York, USA), which was placed in a well of the 24-well plate containing 800 μl culture medium supple-

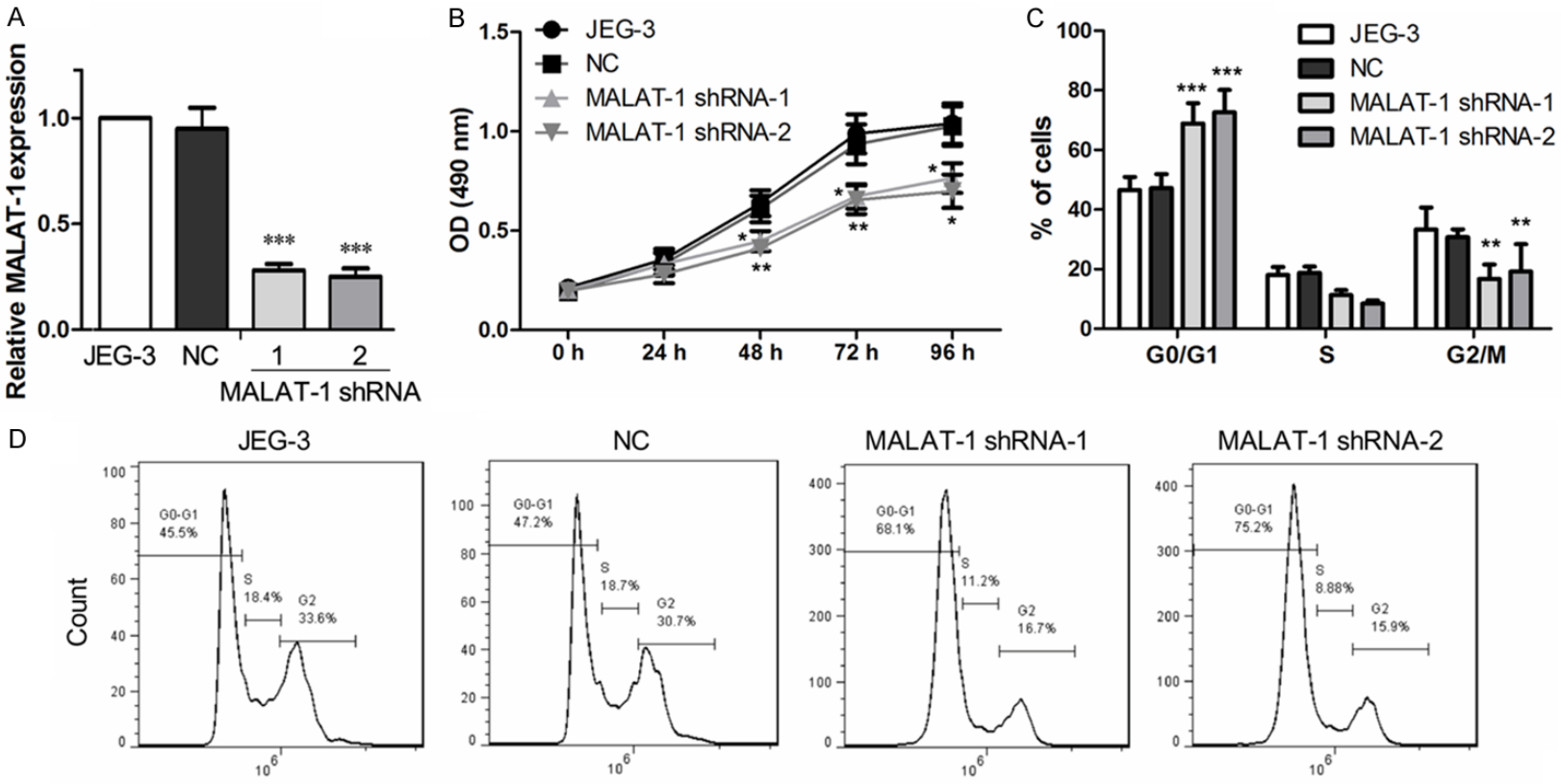

Figure 2. Silencing of MALAT-1 inhibited proliferation and cell cycle progression in JEG-3 cells. JEG-3 cells were transfected with siRNA expression vectors encoding MALAT-1 shRNA-1, MALAT-1 shRNA-2 or negative control (NC) sequence. (A) The expression level of MALAT-1 was determined by real-time PCR 24 h after transfection. -actin was used as the internal reference. (B) Cell proliferation was assessed by MTT assay. (C, D) Cell cycle was analyzed at post-transfection 24 h by flow cytometry. (C) P resents the statistical analysis of three independent experiments, and (D) shows the representative set of FACS results. Data are expressed as the mean ± SD. Compared with the non-transfected JEG-3 cells, **P*<0.05, ***P*<0.01, ****P*<0.001.

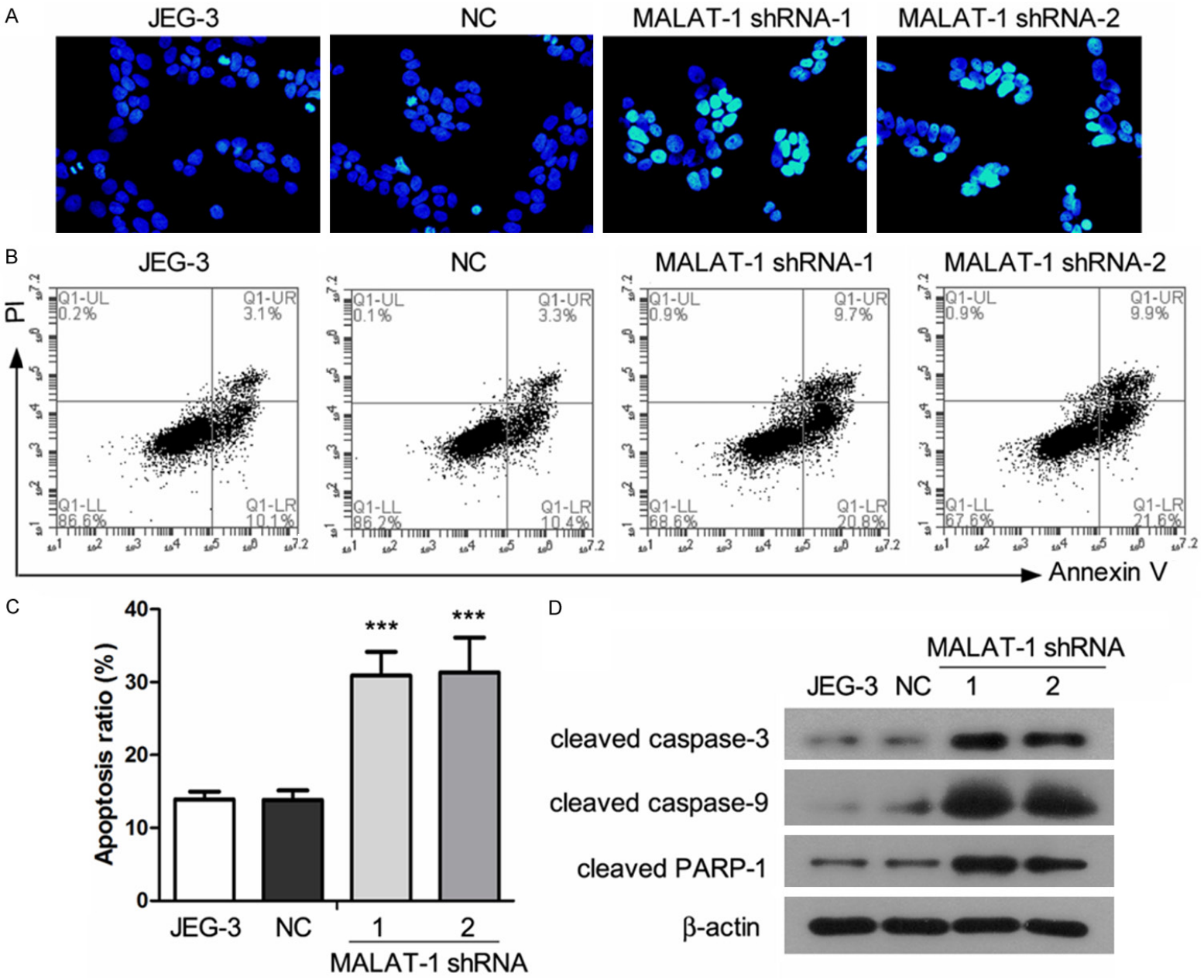
A
JEG-3
NC
MALAT-1 shRNA-1
MALAT-1 shRNA-2
B
JEG-3
NC
MALAT-1 shRNA-1
MALAT-1 shRNA-2
PI
Annexin V
Q1-UL 0.2%
Q1-UR 3.1%
Q1-LL 86.6%
Q1-LR 10.1%
Q1-UL 0.1%
Q1-UR 3.3%
Q1-LL 86.2%
Q1-LR 10.4%
Q1-UL 0.9%
Q1-UR 9.7%
Q1-LL 68.6%
Q1-LR 20.8%
Q1-UL 0.9%
Q1-UR 9.9%
Q1-LL 67.6%
Q1-LR 21.6%
C
Apoptosis ratio (%)
40
30
20
10
0
***
***
JEG-3
NC
1
2
MALAT-1 shRNA
D
MALAT-1 shRNA
JEG-3
NC
1
2
cleaved caspase-3
cleaved caspase-9
cleaved PARP-1
β-actin

Figure 3. Reduced expression of MALAT-1 promoted apoptosis in JEG-3 cells. A. Hoechst staining of the apoptotic cells in JEG-3 cells with and without reduction in MALAT-1 expression by RNAi. The cells were photographed at 400× magnification. B. At post-transfection 24 h, the cells were stained with anti-Annexin V antibody and PI, followed by FACS analysis. C. Statistical analysis of the FACS data from three independent experiments. Data are presented as the mean ± SD. Compared with the non-transfected JEG-3 cells, ***P<0.001. D. The levels of several key apoptotic proteins were examined by Western blot analysis. This figure shows the representative blots from three independent experiments.

mented with 20% FBS. The cells were cultured for 24 h at 37°C in an atmosphere of 5% CO_2. Following incubation, the cells and the Matrigel on the top surface of the Transwell membrane were wiped off, and the cells on the bottom surface of the membrane were fixed with paraformaldehyde and stained with crystal violet (Amresco, Solon, OH, USA). The number of invaded cells was counted from 5 fields on each membrane under 200× magnification.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). The difference in the expression levels of MALAT-1 between PE patients and healthy controls was analyzed by the unpaired student t test. For the in vitro assays, each assay was replicated for at least three times, and one way analysis of variance (ANOVA) was conducted to compare multiple groups, followed by Bonferroni post-hoc test for the comparison between two groups. GraphPad PRISM software (version 5.0; San Diego, CA, USA) was used for data analysis and plotting. $P \leq 0.05$ was considered statistically significant.

Results

Reduced expression of MALAT-1 in PE placentas

To investigate the potential role of MALAT-1 in the pathophysiology of PE, we first examined the expression of MALAT-1 in the placentas from PE patients and normal pregnancy. Real-time PCR results indicated that the level of MALAT-1 in the PE placentas was significantly lower compared with the healthy placentas (**Figure 1**), implying that the reduced level of MALAT-1 in the placenta may be involved in the pathogenesis of PE.

Downregulation of MALAT-1 suppressed proliferation and cell cycle of JEG-3 cells

To further study the role of MALAT-1 in the regulation of trophoblast behavior, the expression of MALAT-1 was reduced in JEG-3 trophoblast cell line by RNA interference (RNAi). MALAT-1 shRNA-1 and MALAT-1 shRNA-2, targeting two distinct sites on MALAT-1 transcript, were transfected into JEG-3 cells, and both shRNAs reduced the expression of MALAT-1 by over 70% (P<0.001; **Figure 2A**). Moreover, JEG-3 cells that were transfected with MALAT-1 shRNA-1 or MALAT-1 shRNA-2 displayed suppressed proliferation as demonstrated by MTT assay (P<0.05; **Figure 2B**). In addition, compared with the non-transfected cells, silencing of MALAT-1 by RNAi led to increased numbers of cells at G_0/G_1 phase (P<0.001) and reduced numbers of cells at G_2/M phase (P<0.01), indicating that the cell cycle was arrested due to the loss of MALAT-1 (**Figure 2C**, **2D**).

Reduction in MALAT-1 expression induced apoptosis in JEG-3 cells

Following transfection with MALAT-1 shRNA, the apoptosis in JEG-3 cells was determined by Hoechst staining and flow cytometric analysis. The fluorescent Hoechst stain that binds to DNA is preferentially taken by the apoptotic cells, the membrane integrity of which was disrupted. As shown in **Figure 3A**, the cells transfected with MALAT-1 shRNA-1 or MALAT-1 shRNA-2 showed more Hoechst-positive cells as compared to the control cells. Quantitative analysis of apoptosis by flow cytometry revealed that silencing of MALAT-1 markedly augmented apoptosis in JEG-3 cells (30.9%±3.2%, 31.3%±4.8%) compared with the non-transfected cells (13.9%±1.1%) (P<0.001; **Figure 3B**, **3C**). We further examined several key apoptotic players in JEG-3 cells with and without RNAi. Compared with the control cells, the levels of cleaved caspase-3, cleaved caspase-9 and cleaved poly(ADP-ribose) polymerase-1 (PARP-1) were significantly elevated in the cells transfected with MALAT-1 shRNA (**Figure 3D**), indicating that reduced expression of MALAT-1 led to activation of the apoptotic pathway in JEG-3 cells.

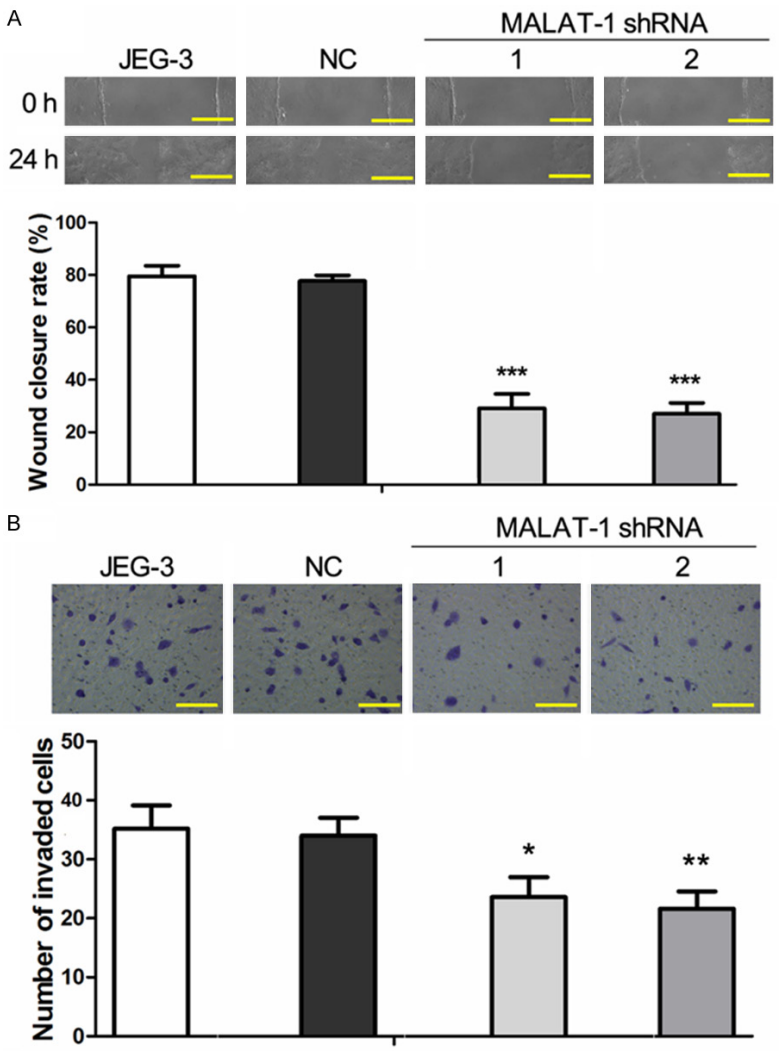

Figure 4. Downregulation of MALAT-1 by RNAi inhibited migration and invasion of JEG-3 cells. A. Cell migration was measured by the wound closure rate in the scratch assay. B. Matrigel-based Transwell invasion assay was performed to assess the invasiveness of JEG-3 cells with and without RNAi. The figure shows the representative images from three independent experiments, and the data are expressed as the mean ± SD. Compared with the non-transfected JEG-3 cells, **P*<0.05, ***P*<0.01, ****P*<0.001.

Reduced expression of MALAT-1 inhibited migration and invasion of JEG-3 cells

The migration and invasion of JEG-3 cells with and without RNAi were assessed by scratch assay and Matrigel-based Transwell assay. After 24 h migration, the scratch wound was closed by approximately by 80% for JEG-3 and NC cells, whereas the cells transfected with MALAT-1 shRNA only narrowed the scratch by 29.1%±5.5% and 2701%±4.1% respectively (*P*<0.001; **Figure 4A**). In addition, in the 24 h Matrigel invasion assay, the number of invaded cells was significantly reduced in JEG-3 cells with RNAi (23.6±3.4, 21.6±3.0) than that in non-transfected JEG-3 cells (35.2±4.0) (**Figure 4B**). These results demonstrated that downregulated expression of MALAT-1 impaired migration and invasion of JEG-3 cells.

Discussion

Since lncRNA was identified in the recent genome-wide studies, its importance in various cellular activities has been gradually recognized [8, 9]. In addition, aberrant expression of lncRNAs has been implicated in the pathogenesis of cancer and other diseases [10, 11, 19]. MALAT-1, which was first characterized for its role in lung cancer metastasis [13], was found to be associated with a placental disorder that is caused by excessive trophoblast invasion [18]. Inadequate trophoblast invasion was well documented in PE, and is recognized to be the main cause for the development of PE [3, 20]. To investigate the involvement of MALAT-1 in the regulation of trophoblast function and in the pathophysiology of PE, we first examined the expression of MALAT-1 in the PE placentas in comparison with normal placentas. The results indicated that MALAT-1 was significantly downregulated in the PE placentas. Moreover, by introducing MALAT-1 siRNA into JEG-3 cells, we demonstrated that reduction in the expression of MALAT-1 inhibited proliferation, suppressed cell cycle progression, promoted apoptosis, and impaired migration and invasion of JEG-3 cells. Our study suggests that MALAT-1 may play an important role in regulating the growth and invasion of trophoblasts, and that deregulated expression of MALAT-1 might contribute to the pathogenesis of PE.

The functional studies of trophoblasts have revealed the striking similarities in molecular circuits between trophoblasts and cancer cells, which may be largely related to the invasive nature inherited in both cell types [21, 22]. MALAT-1 was firstly identified in the highly metastatic lung cancer cells [13], and was later demonstrated to play a critical role in cancer cell invasion and metastasis in other cancers such as pancreatic cancer and melanoma [23, 24]. In our study, we showed that downregulation of MALAT-1 expression by RNAi significantly reduced the motility and the invasiveness of JEG-3 cells, which is consistent with a previous study by Tseng et al. [18]. Tseng et al. also reported that the activities of matrix metalloproteinase (MMP)-2 and MMP-9, the classic enzymes that degrade extracellular matrix and facilitate cell invasion, were not affected when MALAT-1 was downregulated, leaving the molecular targets of MALAT-1 a mystery. In another RNAi experiment in lung cancer cells, Tano et al. showed that silencing of MALAT-1 influenced the expression of CTHRC1, CCT4, HMMR and ROD1, knocking down any of which inhibited cell migration [25]. Thus, they proposed that MALAT-1 regulates cell motility via these novel targets. Nevertheless, the molecular mechanism underlying the regulatory function of MALAT-1 in cell migration and invasion is poorly understood at present and remains to be further explored.

MALAT-1 has been implicated in the regulation of cell proliferation, cell cycle and apoptosis. Yamada et al. reported that MALAT-1 was highly expressed in endometrial stromal sarcoma and normal endometrial stromal cells in the proliferative phase but weekly expressed in the endometrial stromal cells in the secretory phase [16], suggesting that MALAT-1 may be involved in cell proliferation. In our study, silencing of MALAT-1 in JEG-3 cells reduced the proliferation rate, supporting the notion that MALAT-3 may play an important role in promoting the proliferation of trophoblast cells. We further demonstrated that reduced expression of MALAT-1 in JEG-3 cells induced cell cycle arrest at G_0/G_1 stage, which may contribute to the deceleration of JEG-3 proliferation. Recently, Yang et al. reported that downregulation of MALAT-1 induced G_2/M arrest in HepG2 and HeLa cells via interaction with hnRNP C [26]. The discrepancy in the stage of action of MALAT-1 during cell cycle is probably attributed to the different cell types and differential interacting partners in the cells, which need to be characterized in future studies. In addition, we showed that loss of MALAT-1 promoted apoptosis in JEG-3 cells, and this observation is consistent with previous studies in various cancer cell lines [23, 27, 28].

In conclusion, we identifed that MALAT-1 was downregulated in the placentas of PE patients, and demonstrated that silencing the expression of MALAT-1 by RNAi suppressed proliferation, induced G_0/G_1 arrest, promoted apoptosis, and inhibited migration and invasion of JEG-3 cells. These results suggest that MALAT-1 may play an important role in the growth and motility of trophoblast cells and that the downregulated expression of MALAT-1 may be involved in the pathogenesis of PE.

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Disclosure of conflict of interest

None.

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