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Down-Regulated Long Non-Coding RNA MEG3 and its Effect on Promoting Apoptosis and Suppressing Migration of Trophoblast Cells

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ABSTRACT

Preeclampsia is characterized by hypertension and proteinuria twenty weeks into pregnancy. Failure of uterine spiral artery remodeling contributes to preeclampsia's development. The development might be associated with trophoblast cells functioning abnormally. Long non-coding RNAs (lncRNAs) are aberrantly expressed in many diseases. Maternally expressed gene 3 (MEG3), one of these lncRNAs, might function as a tumor suppressor. Aberrant expression of MEG3 induces prenatal death, and little is known of MEG3's role in preeclampsia. This study aims to identify the role of lncRNA MEG3 on apoptosis and the migration of human trophoblast cells, and to investigate the involvement of lncRNA MEG3 in pathogenic mechanisms underlying preeclampsia. In this study, we found MEG3 levels were down-regulated by approximately 80% in placental samples collected from preeclamptic patients (n = 30) compared to samples collected from normotensive patients (n = 30) by qRT-PCR analysis. By designing RNA interference species to suppress MEG3 and specific plasmids designed to over-express MEG3, we explored the role of MEG3 on the functions of two trophoblast cell-lines, HTR-8/SVneo and JEG3 cells. Over-expression of MEG3 reduced apoptosis and promoted migration of HTR-8/SVneo and JEG3 cells. Furthermore, inhibition of endogenous MEG3 increased apoptosis and decreased migration of HTR-8/SVneo and JEG3 cells. Additionally, lncRNA MEG3 influenced expression of NF-κB, Caspase-3, and Bax protein expressions in trophoblast cells. Our findings highlight that abnormal levels of lncRNA MEG3 might lead to aberrant conditions in HTR-8/SVneo and JEG3 trophoblast cells, which might be associated with uterine spiral artery remodeling failure and its contribution to preeclampsia. J. Cell. Biochem. 116: 542–550, 2015. © 2014 Wiley Periodicals, Inc.

KEY WORDS: PREECLAMPSIA; LONG NON-CODING RNA MEG3; TROPHOBLAST; APOPTOSIS; MIGRATION

Preeclampsia is a pregnancy-specific syndrome characterized by new-onset hypertension and proteinuria after 20 weeks of gestation. This is a major cause of maternal, fetal, and neonatal mortality worldwide [Sibai et al., 2005].

Despite advances made in this field, the underlying mechanism of preeclampsia remains poorly understood. It is widely accepted that the placenta is central to preeclampsia's pathogenesis. During normal early placental development, extravillous trophoblasts (EVTs) of fetal origin begin to migrate and invade the uterine spiral arteries of the decidua and the myometrium, which then replace the endothelial layers of the maternal spiral arteries. This process is referred to as uterine spiral artery remodeling, which is crucial for decreasing maternal blood flow resistance and increasing uteroplacental perfusion [Brosens and Renaer, 1972]. In preeclampsia, this process is incomplete [Meekins et al., 1994]. Increased apoptosis [Myatt, 2002], decreased proliferation of EVTs [Redline and Patterson, 1995], and altered migration and invasive abilities of EVTs [de Groot et al., 1996] will prevent them to effectively invade the myometrial spiral arteries. Thus, considerable efforts are required to achieve a deeper understanding of the molecular mechanisms of altered cellular functions of trophoblast cells.

Recent large-scale complementary DNA cloning studies identified that a large portion of the mammalian genome is transcribed, and

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only a small fraction of these transcripts represent protein-coding genes, with the remaining transcripts being non-protein coding genes [Kawaji et al., 2011]. LncRNAs represent a subgroup of non-coding RNAs that are longer than 200 nucleotides. So far, multiple lines of evidences link dysfunction of lncRNAs to the pathogenesis and progression of a broad range of diseases, including cancer [Wang et al., 2010], cardiovascular disease [Leung et al., 2013]and neurodegenerative diseases [Johnson, 2012]. Moreover, many studies have demonstrated that lncRNAs are involved in development [Eissmann et al., 2012] and are associated with diverse cellular processes like stem cell pluripotency, cell apoptosis, cell proliferation, and cell migration [Gibb et al., 2011; Kim et al., 2013]. However, few long non-coding RNAs (lncRNAs) have been identified that modulate the function of trophoblasts and participate in preeclampsia, with the exception of H19 and SPRY4-IT1 [Gao et al., 2012; Zou et al., 2013].

Maternally expressed gene 3 (MEG3), which encodes a non-coding RNA, is an imprinted gene belonging to the DLK1-MEG3 locus located on chromosome 14q32.3 in humans with maternal expression [Miyoshi et al., 2000]. LncRNA MEG3 is expressed in many normal tissues including the placenta, while it was found to be lost in various types of human tumors and tumor cells [Zhang et al., 2003]. In addition, aberrant MEG3 affects biological functions of tumor cells, including apoptosis [Braconi et al., 2011], and proliferation [Wang et al., 2012]. Furthermore, MEG3 is essential for normal development and growth [da Rocha et al., 2008]. Studies also show that maternal deletion of the MEG3 gene in mice results in skeletal muscle defects and prenatal death [Zhou et al., 2010]. Nevertheless, little is known regarding whether lncRNA MEG3 expression levels are abnormal in preeclampsia, its biological role in modulating functions of trophoblasts, or its clinical significance in preeclampsia.

In this study, we determined that severe preeclamptic placentas exhibited lower levels of MEG3 compared to controls. Moreover, we also determined the effects of MEG3 on trophoblast apoptosis, migration, and the possible mechanisms that might be involved. Our data suggests that low levels of lncRNA MEG3 might lead to aberrant conditions of the trophoblast cells. This might be associated with failure in uterine spiral artery remodeling observed in preeclampsia.

MATERIALS AND METHODS

ETHICS STATEMENT

The Ethics Board of the First Affiliated Hospital of Nanjing Medical University approved all of the experiments, and informed consent was obtained from each patient. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

COLLECTION OF PLACENTAL SAMPLES AND CLINICAL CHARACTERISTICS

All the placental samples were collected from the Department of Obstetrics and Gynecology of the First Affiliated Hospital of Nanjing Medical University, China between 2012 and 2013. Thirty placental tissues were obtained from nulliparous women with preeclampsia undergoing cesarean deliveries without labor (preeclampsia group). Another thirty placental tissues were obtained from nulliparous women with normal pregnancies who presented for delivery by cesarean sections without labor at term (control group). The clinical data was obtained from all of the patients sampled.

Preeclampsia was diagnosed based on the standard criteria: a systolic blood pressure $\geq$140 mm Hg and/or a diastolic blood pressure $\geq$90 mm Hg in two successive measurements 4–6 h apart, and a urinary protein level that was >0.3 g in a 24 h urine collection. Additionally, the two groups did not have any other complications, including maternal history of hypertension and/or renal disease, severe intrauterine growth retardation (IUGR), diabetics, smoking, alcoholism, chemical dependency, and fetal congenital abnormalities.

The placental specimens were taken from the center of the placenta near the umbilical cord, avoiding areas of infarcts and thromboses immediately after extraction of the placenta from the uterus. The specimens were then washed with sterile phosphate-buffered saline before storing them in liquid nitrogen for future RNA and protein analyses.

CELL CULTURE, TRANSFECTION, SMALL INTERFERING RNAs, AND PLASMIDS

We chose four cell-lines which were associated with pregnancy, including the human EVT-derived cell-line HTR-8/SVneo, the human choriocarcinoma cell-line JEG-3, human amniotic epithelial cells (WISH), and human umbilical vein endothelial cells (HUVEC) for investigation. HTR-8/SVneo (kindly provided by Dr. Charles Graham, Queen′s University, Canada) was derived from a short-lived primary EVT cell-line, maintained in RPMI-1640 medium, and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 ug/ml streptomycin. JEG-3 (obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) was maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 ug/ml streptomycin. WISH (obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) was maintained in RPMI-1640 medium and supplemented with 10% heat-inactivated FBS. HUVEC (obtained from the Department of Biochemistry and Molecular Biology, Nanjing Medical University, China) was cultured in endothelial cell growth medium (Sciencell). All cells were cultured at 37°C with 5% CO_2.

For the RNAi-mediated knockdown of MEG3, Invitrogen provided three different siRNAs against MEG3. Among these, si-MEG3–2 had the highest transfection efficiency. The target sequence of the si-MEG3 was 5′-UUAGGUAAGAGGGACAGCUGGCUGG -3′. To overexpress MEG3, a plasmid vector expressing the full-length MEG3 (1595 bp) was constructed by Invitrogen Inc. and named pCDNA-MEG3. An empty vector was used as the control. HTR-8/SVneo and JEG-3 cells were transiently transfected with 100 nM si-RNAs and a negative control respectively using the Lipofectamine 2000 transfection reagent (Invitrogen, Shanghai, China). A plasmid over-expressing MEG3 and an empty vector were cultured using the FuGENE® HD Transfection Reagent (Roche, Germany). Twenty-four hours after transfection with the si-RNAs and negative control, or 48 h after transfection with plasmids and the empty vector, the cells were harvested so that the transfection efficiency could be detected by quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

RNA EXTRACTION AND qRT-PCR

Total RNA was extracted from the placental tissues or cells using the Trizol reagent (Invitrogen Life Technologies). A Reverse Transcription Kit (Takara, RR047A) was used for the synthesis of cDNA. After reverse transcription (20 μl reaction volume), the concentration (cDNA) of vast majority of the samples was 1 μg/μl (sample test). Then 1 μl of template was used for subsequent qRT-PCR reactions. QRT-PCR was performed using a Power SYBR Green PCR kit (Takara). According to the manufacturer's instruction, the cycling was performed at 95°C for 30 s, 90°C for 15 s, 60°C for 34 s, and 68°C for 30 s. An ABI 7500 was used to carry out the qRT-PCR and data collections. For quantification, the relative CT method ($2^{-\Delta CT}$ method) was applied. All of the experiments were performed according to the protocols provided by the respective manufacturers. The primers of MEG3 were designed according to Prime 5.0, and validated by BLAST. The real-time PCR results were normalized with GAPDH following the general practice [Schoof et al., 2001; Zhang et al., 2010; Zhou et al., 2013]. To validate the efficiencies of the primes, a standard curve was made. The slope of curve was −3.4, and amplification efficiency was 97%.The sequences of the primers used were:

MEG3 (Forward: 5′-ATGAGAGCAACCTCCTAGGGTTGTTGT-GAG-3′, Reverse:5′- CCCGCCAGGAAGAAGNACTTGGGTCCGG-3′), GAPDH (Forward: 5′-GACTCATGACCACAGTCCATGC-3′, Reverse: 5′-AGAGGCAGGGATGATGTTCTG-3′). The assays were performed in triplicate and each experiment was repeated at least three times.

FLOW CYTOMETRY

The HTR-8/SVneo and JEG-3 cells were transfected with si-NC and si-MEG3 for 24 h or with the empty vector and pCDNA-MEG3 for 48 h, respectively. For supplement control, two cell-lines were first treated with doxorubicin (Doxo, Sigma) 20μM for 8 h, followed by transfection with empty vector and pCDNA-MEG3 for 48 h. Then, the cells were harvested using trypsin without EDTA; cells were double stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Shanghai, China) according to the manufacturer's instructions. Cell analysis was conducted by flow cytometry (FACScan; BD Biosciences) applying the CellQuest analysis software tool (BD Biosciences). The cells were then sorted into viable, necrotic, early apoptotic, and late apoptotic cells. The relative ratio of apoptotic cells was counted for further comparisons. This assay was repeated in three independent experiments.

TERMINAL DEOXYRIBONUCLEOTIDE TRANSFERASE-MEDIATED DUTP NICK-END LABELING (TUNEL) ASSAY

According to the manufacturer's instructions, the 3′-OH of the DNA fragments of apoptotic cells were labeled and stained by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling method using an apoptosis in situ detection kit (Roche, Germany). Fluorescent microscopy (Nikon Corporation, Tokyo, Japan) was used to capture the images of the FITC-labeled TUNEL-positive cells. The nuclei were counter-labeled blue by DAPI, and the nick-ends were labeled as green fluorescence. An emergence between the nucleus (blue) and nick-end (green) labeling was colored blue-green. All of the experiments were repeated at least three times.

IN VITRO CELL MIGRATION ASSAYS

Twenty-four or 48 h after being transfected with the two groups, as described previously, the HTR-8/SVneo cells were resuspended in RPMI-1640 culture medium containing 1% FBS. The cells were trypsinized, and 5×10^4 cells were seeded into the upper chamber (8μm pore size, Millipore, Billerica, MA) placed in the 24 well-plate with a medium containing 1% FBS. The lower chamber contained 10% FBS. After 24 h of incubation, the remaining cells from the upper surface of the membrane were removed, and the cells that had migrated to the lower surface were fixed in methanol and stained with crystal violet. A digital microscope was used to capture 12 random high-power fields (100 ×) from each well. The number of migrated cells on the entire membrane was counted using Image-Pro Plus (Media Cybernetics, American). Each experiment was conducted in triplicate and repeated for three times.

WESTERN IMMUNOBLOT ASSAY

We transfected HTR-8/SVneo cells with si-MEG3 and si-NC, while JEG-3 were transfected with pCDNA-MEG3 and an empty vector. Next, the two trophoblastic cells were lysed to enable protein extraction. SDS-PAGE and Western immunoblots were performed according to standard procedures [Xiang et al., 2014]. At last, the reactive bands were visualized using an ECL kit. And the density of the bands was computer-analyzed by a densitometer (Quantity One, Bio-Rad). Specific antibodies (Caspase-3 [#9662] and NF-κB [#4764 s], obtained from Cell Signaling Technology, Inc.; Bax [#ab5714], obtained from Abcam plc.) were used at a dilution of 1:1000. The secondary antibody used in these assays was horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (dilution of 1:1,000; Beijing ZhongShan Biotechnology CO., Beijing, China). A GAPDH antibody (#sc-47724, Santa Cruz Biotechnology, Inc., CA, USA, dilution 1:1000) was used as the control. All of the experiments were repeated at least three times.

STATISTICAL ANALYSIS

All data were expressed as mean ± SD (standard deviation, SD). Student's *t*-test or one-way ANOVA analysis was performed to analyze the data using the SPSS statistical software tool (SPSS Inc., Chicago, IL). Data was considered statistically different at alpha values of $P < 0.05$.

RESULTS

DEREGULATED EXPRESSION OF THE LONG NON-CODING RNA MEG3 IN PREECLAMPSIA

To investigate the level of lncRNA MEG3 in preeclampsia, we analyzed MEG3 levels in 30 placental tissues from women with preeclampsia and 30 placental tissues from healthy pregnant women as quantified by qRT-PCR. There were no significant differences between preeclampsia and the normal pregnancy group in maternal age, and gestation age ($P > 0.05$) Table 1 shows the details of patient clinical characteristics. Notably, the expression of MEG3 was remarkably reduced in pregnant women with preeclampsia compared to the controls (Fig. 1A).

TABLE 1. Clinical characteristics of normal and preeclamptic pregnancies

Variable	PE (n = 30)	Control (n = 30)	*P* value[a] Normal vs PE
Maternal age (y)	30.5 ± 5.6	30.3 ± 3.5	>0.05
Maternal weight (kg)	79.3 ± 8.1	74.7 ± 4.2	>0.05
Gestational age (week)	35.9 ± 3.4	38.1 ± 1.3	>0.05
Systolic blood pressure (mm Hg)	166 ± 19	114 ± 7	<0.01
Diastolic blood pressure (mm Hg)	111 ± 11	73 ± 7	<0.01
Proteinuria (g/day)	>0.3	<0.3	<0.01
Body weight of infant (g)	2550 ± 745	3395 ± 410	<0.05

All results are presented as mean ± SD. All the patients of 2 groups are nulliparous women.
PE, preeclampsia; SD, standard deviation.
Obtained by T-Test analysis of variance using SPSS 18.0 software (SPSS Inc, Chicago, IL).

THE EFFECT OF MEG3 ON TROPHOBLAST CELL APOPTOSIS

The significant decrease in MEG3 expression in preeclampsia samples prompted us to explore the possible biological significance of MEG3 in preeclampsia. To assess the potential functional role of MEG3 in vitro, we first measured MEG3 expression in two trophoblast cell-lines (e.g., HTR-8/SVneo and JEG-3) and another two cell-lines also associated with pregnancy for comparison (e.g., WISH and HUVEC). As shown in Figure 1B, we found that the expression of MEG3 in JEG-3 cells was lower compared to WISH and HUVEC. By contrast, the expression of MEG3 in HTR-8/SVneo was the highest in the four cell-lines.

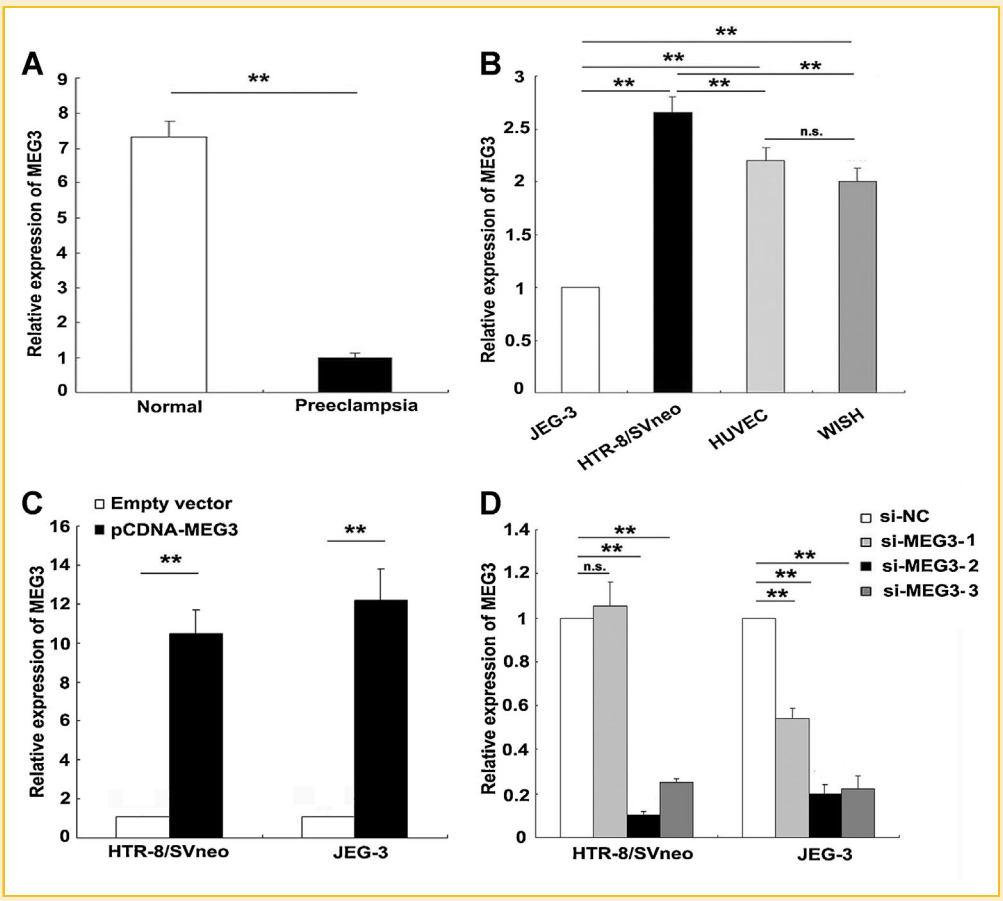

Figure 1. LncRNA MEG3 expression is reduced in preeclampsia. (A) The relative expression of lncRNA MEG3 was assessed by qRT-PCR using SYBR green and normalized to GAPDH. The levels of MEG3 were lower in preeclamptic placentas (n = 30) than in normotensive placentas (n = 30). (B) MEG3 expression levels were determined by qRT-PCR in four cell-lines. The relative expressions of MEG3 in HTR-8/SVneo, WISH, and HUVEC were normalized to that in JEG-3. Three times of biological replicates have been conducted and displayed. (C) HTR-8/SVneo cells and JEG-3 cells were transfected with an empty vector or pCDNA-MEG3, and the transfection efficiency was assessed by qRT-PCR. (D) HTR-8/SVneo and JEG-3 cells were transfected with si-NC or si-RNAs, and the transfection efficiency was assessed by qRT-PCR. (Values are mean ± SEM; **: P < 0.01; n.s.: not significant).

In order to manipulate the MEG3 levels in trophoblast cells, we designed plasmids specific for MEG3. The expression of MEG3 in JEG-3 and HTR-8/SVneo cells was increased by 12.2-fold and 10.6-fold respectively after 48 h of transfection with the pCDNA-MEG3 vector as compared to the empty vector (Fig. 1C; $P < 0.01$). In addition, we designed three siRNAs specific for MEG3 to investigate the biological effects of MEG3 on trophoblasts. These siRNA species were tested for their knockdown efficiency in HTR-8/SVneo and JEG-3 cells. As shown in Figure 1D, the MEG3 levels of the two trophoblast cell-lines were diminished by 90% and 80% respectively, which were then inhibited by si-MEG3-2 as compared to the negative control. Thus, we selected si-MEG3-2 for all subsequent studies.

Next, flow cytometry and TUNEL assays were respectively used to assess the influence of MEG3 on trophoblast apoptosis. In general, the two methods both indicated that upregulated MEG3 using transfection with pCDNA-MEG3 vector significantly inhibited HTR-8/SVneo and JEG-3 cell apoptosis compared to those transfected with an empty vector (Fig. 2A and C). On the other hand, the inhibition of MEG3 using siRNAs greatly promoted trophoblast cell apoptosis in both trophoblast cell-lines (Fig. 2B and D). The same results were also found in HUVEC cell-line (Fig. S1).

MEG3′s anti-apoptotic effect was further verified by the mitigated apoptosis observed in using MEG3 to rescue pro-apoptotic factor treated doxorubicin (Doxo) in both trophoblast cell-lines (Fig. 2E and F).

THE IMPACT OF MEG3 ON TROPHOBLAST CELL MIGRATION

Considering that HTR-8/SVneo cells are extravillous trophoblasts [Chau et al., 2013; Tapia-Pizarro et al., 2013], and the invasive ability of HTR-8/SVneo cells is higher than that of JEG-3 cells [Suman and Gupta, 2012], we selected the HTR-8/SVneo cell-line as the model of choice to perform in vitro migration assays. The HTR-8/SVneo cells were treated with plasmids and si-MEG3–2 separately. As shown in Figure 3, when HTR-8/SVneo cells were transfected with the pCDNA-MEG3 vector, a significant increase in cell migration was observed compared to those cells transfected with the empty vector (3.6 fold, $P < 0.01$). By contrast, down-regulation of MEG3 with siRNAs induced a 65% decrease in the migration capacity of HTR-8/SVneo cells in the transwell assays.

MEG3 INFLUENCES NF-κB, CASPASE-3, AND BAX IN VITRO

To further investigate the potential mechanism of lncRNA MEG3 on apoptosis and migration, we performed Western immunoblotting assay in trophoblast cells. Since HTR-8/SVneo and JEG-3 cells express the highest and lowest levels of MEG3 respectively, we treated HTR-8/SVneo cells with si-MEG3 or si-NC. We similarly treated JEG-3 cells with an empty vector or the pCDNA-MEG3 vector. Results showed that the protein levels of NF-κB, Caspase-3, and Bax were increased in the HTR-8/SVneo cells when treated with si-MEG3 as compared to si-NC, while these protein levels were significantly decreased following ectopic expression of MEG3 in JEG-3 cells (Fig. 4).

DISCUSSION

A growing body of evidence suggests that lncRNAs are molecules of previously unrecognized major biological significance. Lately, researchers are beginning to reveal the value of lncRNAs in different physiological processes, including embryonic stem cell [Dinger et al., 2008] and T-cell differentiation [Pang et al., 2009]. The abnormal expression of lncRNAs could be involved in tumorigenesis [Hall and Russell, 2005]. H19, the lncRNA that has been studied in preeclampsia, is an imprinted gene, and has been implicated in the regulation of human placental trophoblast proliferation [Gao et al., 2012]. H19 is also involved in tumor suppression and has oncogenic properties in cancer [Luo et al., 2013].

MEG3 is the first lncRNA proposed to function as a tumor suppressor, and is an imprinted gene. Others have demonstrated its potential role in the suppression of cell growth [Zhang et al., 2003]. Since tumor progression and pregnancy share many common features, such as immune tolerance and invasion [Louwen et al., 2012], we began to explore MEG3 in preeclampsia pathogenesis. Preeclampsia and IUGR are both pregnancy-specific syndromes, and there is evidence that the biologic pathways leading to preeclampsia and IUGR are similar [Papageorghiou et al., 2001 Dugoff, 2010; Schoofs et al., 2014]. Meanwhile, MEG3 is also associated with IUGR [McMinn et al., 2006]. We identified that the expression of MEG3 was markedly reduced in 30 human preeclamptic placentas as compared with normal pregnancies.

The normal capabilities of trophoblasts include apoptosis, proliferation, and migratory properties, which are essential for deep placental formation that facilitates normal growth and development of the fetus [Redline and Patterson, 1995; de Groot et al., 1996; Myatt, 2002]. In this study, we focused on the identification and characteristics of lncRNAs MEG3 in apoptosis and migration of trophoblast HTR-8/SVneo and JEG3 cell-lines. According to our data, ectopic expression of MEG3 inhibited apoptosis of the trophoblasts and promoted cellular migration. By contrast, down-regulation of MEG3 could promote trophoblast apoptosis and inhibit cell migration in vitro. These findings indicated that reduced MEG3 might play an important role in increasing apoptosis and decreasing migration of trophoblasts. This might lead to failure in remodeling uterus spiral arteries, thereby giving rise to defective deep placental formation. The defective formation might be associated with the pathogenesis of preeclampsia.

In this study, we also determined whether MEG3 has an effect on the proliferative capability by MTT assay. However, there was no significant difference between the treatment and control groups in the context of both trophoblast cell-lines (Fig. S2).

The signaling pathways regulating apoptosis and migration in humans are complex and involve an expanding list of molecules. Caspase-3 and Bax are placental apoptotic biomarkers, and Caspase-3 is a central executor of the mitochondrial-dependent apoptosis pathway [Tait and Green, 2010]. Bax is also involved in inactivation of the intrinsic apoptosis pathway. A significant increase in NF-κB expression was detected in the placentas of women with pre-eclampsia [Vaughan and Walsh, 2012], and this expression can affect apoptosis and the migratory capability of cells.

Although we demonstrated that MEG3 might have a major effect on the biological functions of trophoblasts, including apoptosis and migration, the underlying mechanism of how MEG3 is involved in NF-κB, Bax and Caspase-3 signaling pathways should be investigated in more detail going forward. Previous studies of lncRNAs

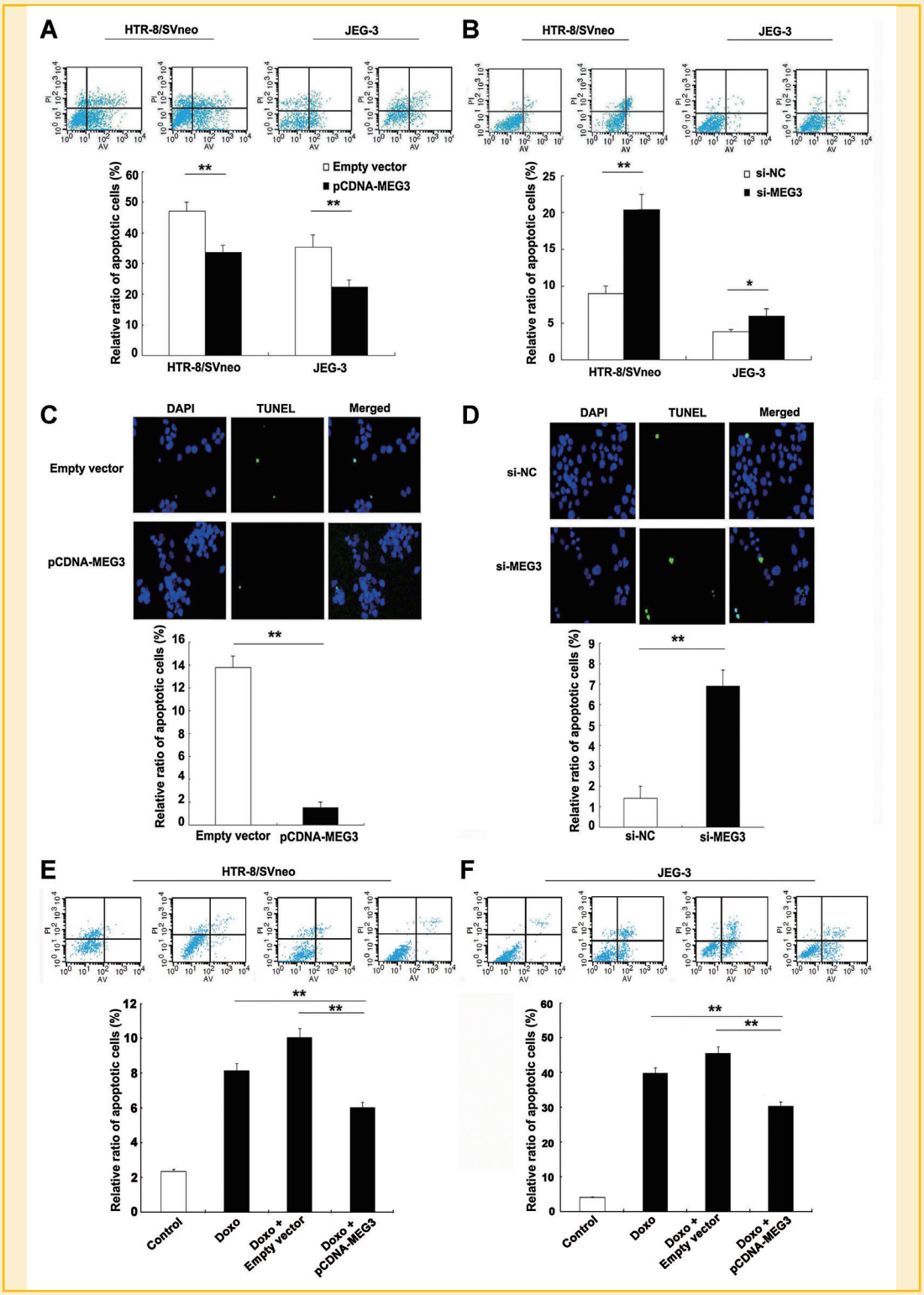

Figure 2. The effect of MEG3 on apoptosis of trophoblast. (A) Flow cytometry was performed to determine the apoptotic rates of pCDNA-MEG3 vector tranfected HTR-8/SVneo (Left) and JEG-3 cells (Right) as compared to those infected with an empty vector. (B) Results from flow cytometry demonstrate the apoptotic rates of si-MEG3 tranfected HTR-8/SVneo (Left) and JEG-3 cells (Right) as compared to those infected with si-NC. (C) TUNEL was performed to determine the apoptotic rates of the pCDNA-MEG3 vector tranfected JEG-3 cells as compared to those transfected with an empty vector (original magnification: $\times$200). (D) Results from TUNEL demonstrate the apoptotic rates of si-MEG3 transfected HTR-8/SVneo cells as compared to those transfected with si-NC (original magnification: $\times$200). (E, F): Flow cytometry was carried out to determine the anti-apoptotic effect of MEG3 on two trophobalst cell-lines using pCDNA-MEG3 vector tranfection after doxorubicin (Doxo) treated. (Values are mean $\pm$ SEM; *: $P < 0.05$; **: $P < 0.01$).

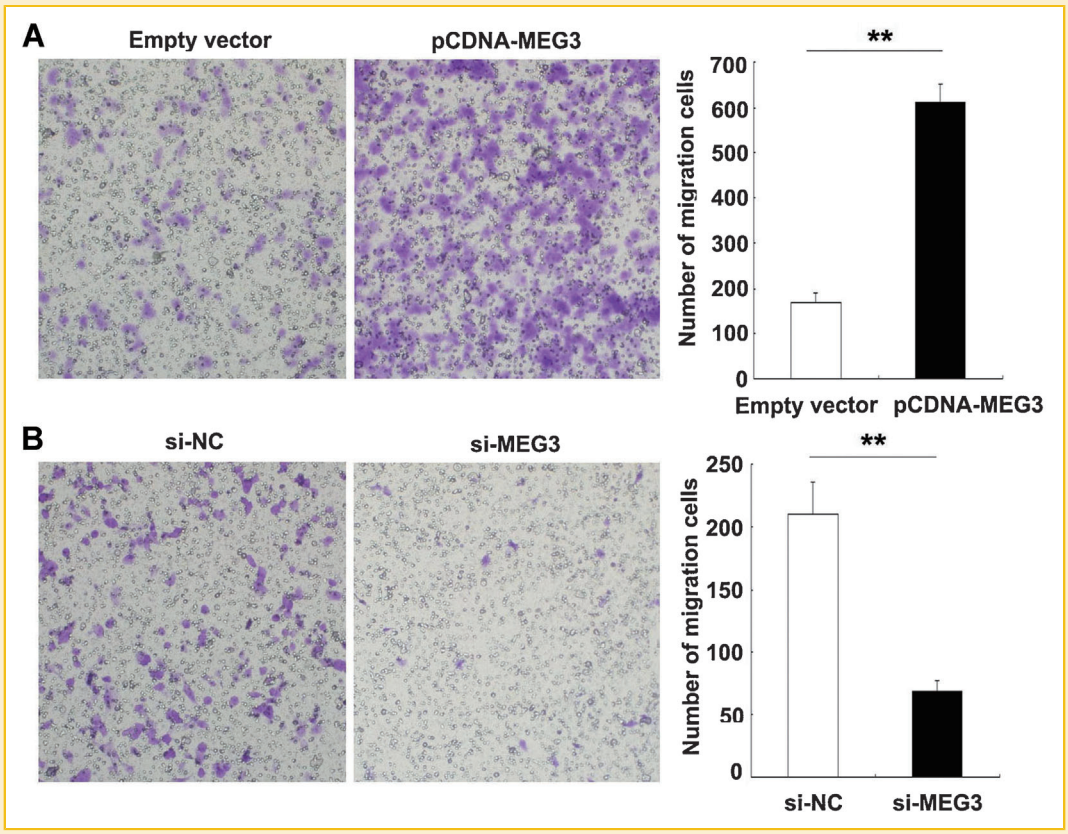

Figure 3. The impact of MEG3 on trophoblast migration. (A) HTR-8/SVneo cells transfected with an empty vector or pCDNA-MEG3 for 48 hrs were seeded on the membranes in the upper chamber of a transwell insert (original magnification $\times 100$). The columns indicate the number of migrated cells in pCDNA- MEG3 and in empty vector groups. (B) HTR-8/SVneo cells transfected with si-NC or si-MEG3 for 24 hrs were seeded on membranes in the upper chamber of a transwell insert (original magnification: $\times 100$). The columns indicated that the number of migrated cells in the si-NC and si-MEG3 groups. (Values are mean $\pm$ SEM; **: $P < 0.01$).

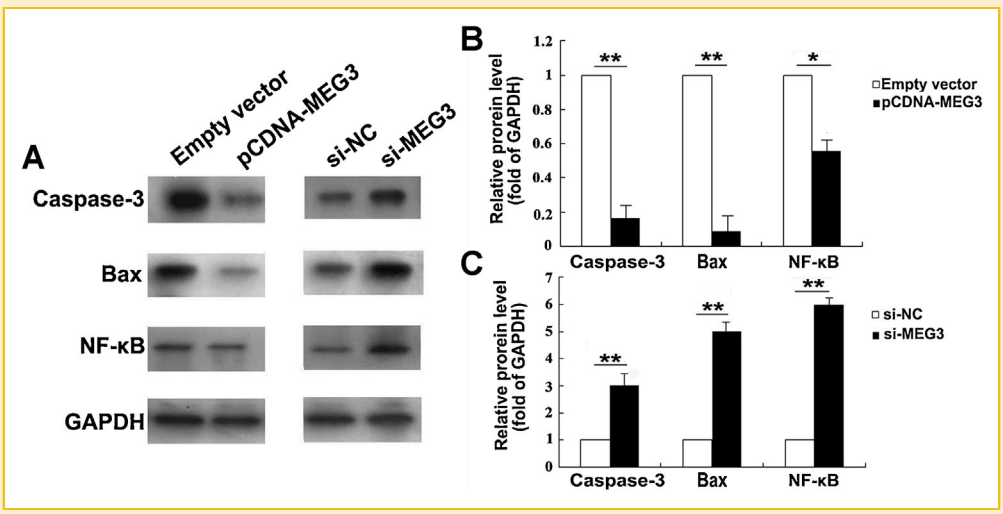

Figure 4. The proteins regulated by MEG3. (A) Western blot assay of Caspase-3, Bax, and NF-κB expression after si-MEG3 or si-NC was transfected into HTR-8/SVneo cells, and after pCDNA-MEG3 vector or empty vector tranfection of JEG-3 cells. GAPDH protein was used as an internal control. (B, C) The columns indicate the relative expression of Caspase-3, Bax, and NF-κB in trophoblasts in different groups. Results shown are from three independent experiments. (Values are mean $\pm$ SEM; *: $P < 0.05$; **: $P < 0.01$).

have shown that they are localized in specific subcellular localizations. The subcellular localization of lncRNAs can provide major insights into their potential function. When the lncRNAs are within the cytoplasm, they might function as protein scaffolds [Clemson et al., 2009], signaling molecules, and act as "miRNA sponge" which sequesters miRNAs and deactivates these small regulatory RNAs [Wang et al., 2010]. By contrast, when transcripts are located in the nucleus, they can interact with chromatin remodeling complexes and induce local or global changes in chromatin packaging [Rinn et al., 2007], and regulate other imprinted genes that will have an impact on placental growth [Zhou et al., 2010]. Experiments confirming the subcellular localization of MEG3 in trophoblasts are needed to confirm the molecular mechanisms of MEG3 in preeclampsia.

In conclusion, our observations demonstrate that MEG3 expression is substantially decreased in female subjects with preeclampsia compared to healthy pregnant women. By applying gain-of-function and loss-of-function approaches, we also showed that MEG3 expression levels could alter the biological functions of trophoblasts in vitro and the possible mechanisms involved. These data may provide a potential molecular mechanism of aberrant cell function of trophoblasts, which might confer a failure in remodeling uterine spiral arteries associated with the pathogenesis of preeclampsia.

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