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Downregulated lncRNA *HOXA11-AS* Affects Trophoblast Cell Proliferation and Migration by Regulating *RND3* and *HOXA7* Expression in PE

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The long noncoding RNA *HOXA11-AS* displays abnormal expression in numerous human diseases. However, its function and biological mechanisms remain unclear in preeclampsia (PE). In this study, we report that *HOXA11-AS* is significantly downregulated in preeclamptic placental tissues and could contribute to the occurrence and development of PE. Silencing of *HOXA11-AS* expression could significantly suppress trophoblast cell growth and migration, whereas *HOXA11-AS* overexpression facilitated cell growth in the HTR-8/SVneo, JEG3, and JAR cell lines. RNA-seq analysis also indicated that *HOXA11-AS* silencing preferentially regulated numerous genes associated with cell proliferation and cell migration. Mechanistic analyses showed that *HOXA11-AS* could recruit Ezh2 and Lsd1 protein and regulate *RND3* mRNA expression in the nucleus. In the cytoplasm, *HOXA11-AS* modulates *HOXA7* expression by sponged miR-15b-5p, affecting trophoblast cell proliferation. Together, these data confirm that aberrant expression of *HOXA11-AS* is involved in the occurrence and development of PE and may act as a prospective diagnosis and therapeutic target in PE.

INTRODUCTION

Preeclampsia (PE), characterized by blood pressure higher than 140/90 mmHg after 20 weeks of pregnancy, is a major contributor of pregnancy-related death and fetal morbidity. PE afflicts nearly 3%–5% of pregnancies, especially in developing countries.[1,2] Despite the considerable morbidity and mortality, the cause of PE has been a mystery. Delivery of the placenta is the only known remedy for PE;[3] other effective prevention strategies have not yet been developed. The preferential clinical treatment for PE is to use a combination of labetalol with magnesium sulfate to slow down the progression of this disorder and extend the pregnancy period. After in-depth study, PE, which results from the aberrant expression of numerous PE-associated genes,[4–7] could be considered a heterogeneous disease with diverse clinical and molecular characteristics. Therefore, a deeper understanding of the biological mechanisms in PE might furnish more options for diagnosis and treatment.

Long noncoding RNAs (lncRNAs), which are longer than 200 bp with little or no protein-coding capacity, have intrinsic function as RNA.[8,9] Recently, technological advances have allowed the analysis of lncRNAs in diverse human diseases. Emerging studies have demonstrated that lncRNAs have been implicated in a variety of biological and pathological processes, including cell differentiation,[10] cell metabolism,[11] immune response,[12] and disease-associated development.[13–15] Additionally, aberrant levels of lncRNAs have been reported that positively or negatively affect gene expression in diverse human diseases, including PE.[16–19] Furthermore, a lot of studies have demonstrated that lncRNAs could act as significant regulatory molecules to regulate related gene expression at different levels, such as chromatin modification and transcriptional and post-transcriptional modification.[9,20] For instance, the lncRNA *CCAT1* modulates *SPRY4* and *HOXB13* expression by binding to *SUV39H1*(suppressor of variegation 3–9 homolog 1) and *EZH2* (enhancer of zeste 2 polycomb repressive complex 2 subunit) to affect cell growth and migration in esophageal squamous cell carcinoma.[21] Apart from their role in gene expression regulation, lncRNAs can also crosstalk with associated gene expression by competing for shared microRNAs (miRNAs) at post-transcriptional levels to affect the occurrence and development of various diseases.[22,23]

HOXA11-AS, a 1,628-bp lncRNA gene located on chromosome 7p15.2, plays a significant role in various disorders.[24–27] For instance,

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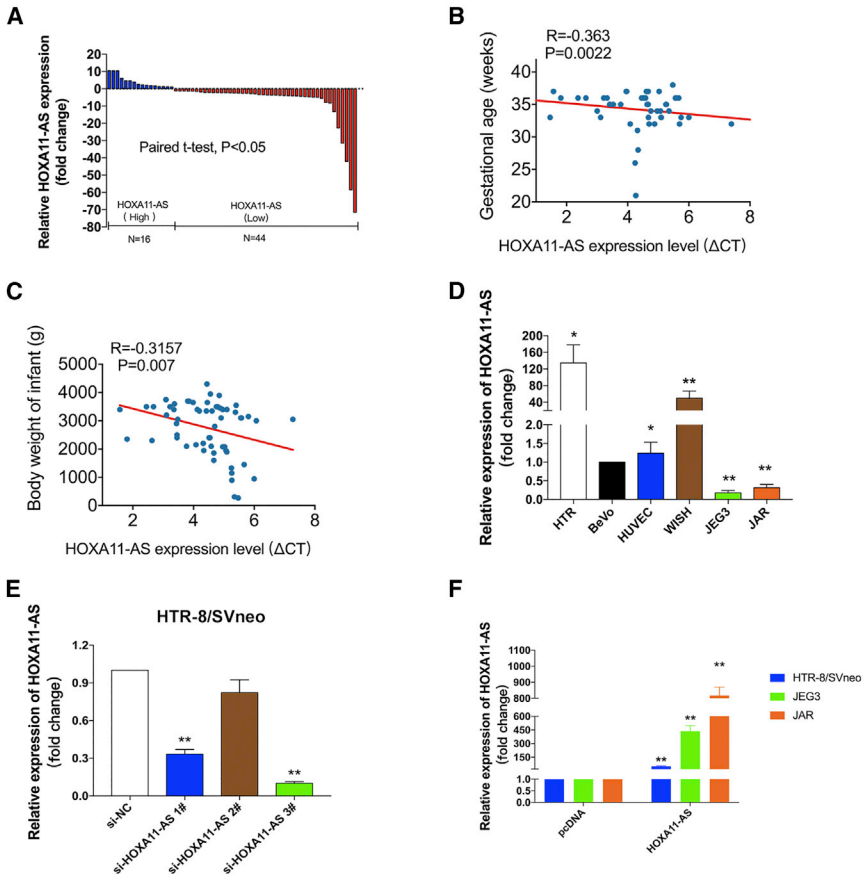

Figure 1. Relative *HOXA11-AS* Expression in PE

(A) The relative expression of *HOXA11-AS* was measured by qRT-PCR. The levels of *HOXA11-AS* were lower in preeclamptic placenta samples (n = 60) than in normal placentas (n = 60). (B and C) Correlations between HOXA11-AS and two clinical characteristics (B, gestational age; C, the body weight of the infant) were measured with one-tailed correlation analysis. (D) *HOXA11-AS* expression was detected by qRT-PCR in several cell lines and normalized to that in HTR-8/SVneo cells. (E)The expression of *HOXA11-AS* following treatment of HTR/Svneo cells with siRNAs. (F) The expression of *HOXA11-AS* following transfection of HTR/SVneo, JEG3, and JAR cells with pcDNA3.1+HOXA11-AS.. $^{**}p < 0.01$, $^{*}p < 0.05$.

HOXA11-AS can promote cell growth and invasion of gastric cancer by interacting with *EZH2* and *LSD1* (histone demethylase lysine-specific demethylase 1).[28] In addition, *HOXA11-AS* can compete for shared miR-140-5p to promote glioma tumorigenesis.[29] However, the biological functions of *HOXA11-AS* in PE remain unclear, which impels us to further explore the role and molecular mechanism of *HOXA11-AS* in PE.

In this study, we demonstrated that the expression level of *HOXA11-AS* was significantly downregulated in preeclamptic placental tissues compared with normal tissues. Furthermore, knockdown of *HOXA11-AS* could impair cell growth and migration in various trophoblast cell lines. Associated mechanistic exploration demonstrated that *HOXA11-AS* could exhibit different regulatory mechanisms in regulation of *RND3* and *HOXA7* expression in the nucleus and cytoplasm, thus being involved in the occurrence and development of PE. Unraveling the role of HOXA11-AS will provide novel insights for future PE treatments.

RESULTS

HOXA11-AS Is Downregulated in Human Preeclamptic Tissues

The expression level of *HOXA11-AS* was analyzed in 60 preeclamptic tissues and normal tissue samples by qRT-PCR. We found that the *HOXA11-AS* expression was significantly downregulated in preeclamptic tissues (Figure 1A). Furthermore, as shown in Figures 1B and 1C, HOXA11-AS expression levels also indicated a positive correlation with gestational age and the body weight of infants in the PE group. The detailed clinical characteristics of the patients who meet the criteria are listed in Table 1. In addition, we discovered that there were no significant differences between PE and the normal in gestational age and maternal age ($p > 0.05$). On the contrary, there were significant differences in systolic blood pressure, diastolic blood pressure, and body weight of infants between PE and the normal ($p < 0.05$).

HOXA11-AS Regulates Trophoblast Cell Proliferation and Migration *In Vitro*

Because human lncRNAs play essential roles in various cellular processes, we detected the expression of *HOXA11-AS* in four trophoblast cell lines and another two cell lines related to pregnancy, including HTR-8/SVneo, BeWo, JEG-3 and JAR, WISH, and HUVEC-C. As shown in Figure 1D, we found that the relative *HOXA11-AS* level in HTR-8/SVneo cells was higher than that in other cell lines, whereas the expression levels of *HOXA11-AS* in the BeWo, JEG3, and JAR cell lines were relatively lower compared with those in the WISH and HUVEC-C cell lines.

To explore the potential role of *HOXA11-AS* in trophoblast cells, we used an overexpression and knockdown model of HOXA11-AS *in vitro*. It was found that the expression levels of *HOXA11-AS* were exogenously influenced by specific small interfering RNAs (siRNAs) and overexpression plasmids in the HTR-8/SVneo, JEG3, and JAR cell lines (Figures 1E and 1F). Then we performed 3-(4,5)-dimethylthiahiazo (-z-y)-3,5-di-phenytetrazoliumromide (MTT) and colony formation assays to illustrate the effect of *HOXA11-AS*

Table 1. Clinical Characteristics of Preeclamptic and Normal Pregnancies

Variable	PE (n = 60)	Normal (n = 60)	p Value (Normal versus Preeclamptic)
Maternal age (year)	32.23 ± 4.526	34.62 ± 3.3763	$p > 0.05$
Maternal weight (kg)	75.99 ± 10.995	74.467 ± 7.979	$p > 0.05$
Smoking	0	0	$p > 0.05$
Systolic blood pressure (mmHg)	162.83 ± 15.103	116.6 ± 7.983	$p < 0.01$
Diastolic blood pressure (mmHg)	106.417 ± 10.807	71.54 ± 8.767	$p < 0.01$
Proteinuria (g/day)	>0.3	<0.3	$p < 0.05$
Body weight of infant (g)	2,287.33 ± 908.078	3,385.42 ± 372.269	$p < 0.05$
Gestational age (weeks)	33.93 ± 3.231	38.38 ± 0.958	$p < 0.05$

on the proliferation of HTR-8/SVneo, JEG3, and JAR trophoblast cells. The resulting data revealed that silencing of *HOXA11-AS* significantly retarded cell growth compared with controls, whereas upregulation of *HOXA11-AS* could enhance cell proliferation (Figures 2A and 2B). In addition, ethynyl deoxyuridine (EdU) staining assays and bromodeoxyuridine (BrdU) assays also demonstrated that *HOXA11-AS* knockdown inhibited trophoblast cell proliferation; however, *HOXA11-AS* overexpression boosted the rate of proliferating trophoblast cells (Figures 2C and 2D). These data indicate that downregulated *HOXA11-AS* might play a role as a suppressor in the inhibition of trophoblast cell proliferation.

Furthermore, transwell assays confirmed that silencing of *HOXA11-AS* significantly inhibited trophoblast cell migration and invasion compared with si-normal control (NC) treatment (Figure 3A). In contrast, upregulated *HOXA11-AS* could stimulate cell migration and invasion (Figure 3A).

The Effect of HOXA11-AS on the Cell Cycle and Apoptosis *In Vitro*

Because cell proliferation assays cannot thoroughly reflect cell cycle changes, we next performed flow cytometry analysis to detect whether cell cycle progression was affected after *HOXA11-AS* knockdown. The results revealed that cells transfected with specific siRNAs promoted cell accumulation in G0–G1 phase compared with those treated with si-normal control (NC). In contrast, *HOXA11-AS* overexpression could reduce cell cycle accumulation in G0–G1 phase (Figure 3B).

Also, flow cytometry assays were performed to investigate whether knockdown of *HOXA11-AS* affected cell apoptosis. The results showed that the ratio of total apoptotic cells was dramatically increased in HTR-8/SVneo cells transfected with siRNAs (Figure 3C). Similarly, we conducted flow cytometry assays to detect the protein expression level of cleaved caspase, which further confirmed that the apoptosis level was increased after HOXA11-AS knockdown (Figure 3D). These findings suggest that *HOXA11-AS* promotes proliferation and inhibits apoptosis in trophoblast cells.

Gene Expression Profiling

To investigate the *HOXA11-AS*-associated pathway on an unbiased basis in PE, we conducted RNA sequencing (RNA-seq) and evaluated the gene expression profiles of HTR-8/SVneo cells transfected with siRNAs against *HOXA11-AS*. After knockdown of HOXA11-AS, 131 mRNAs showed at least a 2-fold increased abundance, whereas a total ($\leq$2-fold) of 99 genes showed decreased abundance (Figure 4A). Gene ontology (GO) analysis showed that many significant biological processes were involved in cell proliferation, migration, as well as apoptosis (Figure 4B). Among all enriched genes, there are many well-known proliferation-related and migration-associated genes, such as *TNFSF9*, *TFPI2*, *CA9*, *IFITM1*, *TMEM158*, *RND3*, *ESM1*, *NAMPT1*, *HOXA7*, *PSAT1*, *CPA4*, *MEST*, *OLR1*, etc. We suspected that some altered genes might induce the occurrence and development of PE. The expression changes of these genes, therefore, were selectively demonstrated by qRT-PCR in *HOXA11-AS*-depleted and/or *HOXA11-AS*-overexpressing HTR-8/SVneo cells and *HOXA11-AS*-overexpressing JAR cells (Figures 4C and 4D). *RND3* and *HOXA7* have been identified as candidate factors involved in cell proliferation, apoptosis, and migration; therefore, we selected *RND3* and *HOXA7* for further study.

HOXA11-AS Could Recruit Lsd1 and Ezh2 in the Nucleus, Epigenetically Silencing *RND3*

To further explore the potential biological mechanisms of *HOXA11-AS*-mediated regulation in trophoblast cells, we first performed subcellular fractionation assays to assess the distribution of *HOXA11-AS* in nuclear and cytoplasmic fractions in HTR/SVneo, JEG-3, and JAR cells. As shown in Figure 5A, approximately 70% of *HOXA11-AS* is located in the trophoblast nucleus, and 30% of *HOXA11-AS* is in the cytoplasm. Therefore, these findings indicated that *HOXA11-AS* might play an essential role in transcriptional regulation. We employed bioinformatics analysis to predict possible RNA binding proteins, including Ezh2 (trimethylation of histone 3 lysine 27 [H3K27me3]), Suz12 (H3K27me3), Lsd1 (dimethylation of histone 3 lysine 4 [H3K4me2]), Dnmt1, Hur, Stau1, and Ago2 (http://pridb.gdcb.iastate.edu/RPISeq/references.php).[30] As shown in Figure 5B, *HOXA11-AS* may interact with Ago2, Ezh2 (H3K27me3), and Lsd1 (H3K4me2) in trophoblast cells. Previous studies have reported that *HOXA11-AS* can recruit Ezh2 and Lsd1 to epigenetically silenced targets in many tumor cell lines.

To examine the interaction probabilities of *HOXA11-AS* with target proteins, we performed RNA immunoprecipitation (RIP) assays with these antibodies. There was a substantial enrichment in RIPs of Ezh2, Lsd1, and Ago2 in HTR-8/SVneo cells (Figure 5C). We also conducted RIP assays in JEG3 and JAR cells that were transfected with an overexpression plasmid of *HOXA11-AS*. Our results demonstrated that *HOXA11-AS* directly interacted with Ezh2, Lsd1, and Ago2 (Figure 5C). Furthermore, RNA pulldown assays further confirmed that *HOXA11-AS* could interact with Ezh2, Lsd1, and Ago2 in

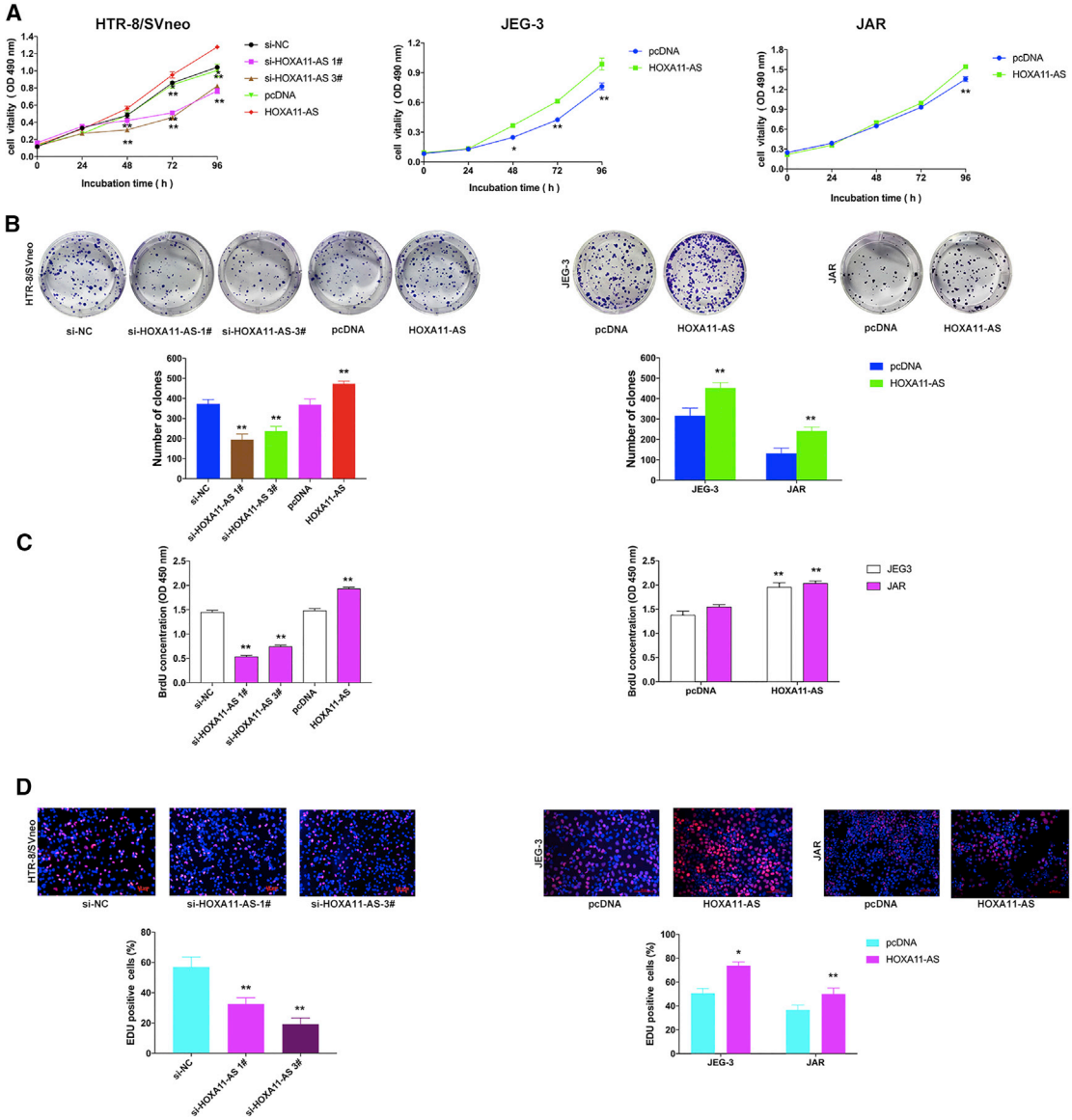

Figure 2. The Effect of *HOXA11-AS* on Proliferation in Trophoblast Cells

(A) MTT assays were used to determine the viability of si-*HOXA11-AS*-transfected trophoblast cells. (B) Colony formation assays were performed to determine the proliferation of si-*HOXA11-AS*-transfected HTR-8/SVneo, JEG-3, and JAR cells. Colonies were counted and captured. (C) BrdU assays were used to detect cell proliferation after transfection. (D) Proliferating trophoblast cells were labeled with EdU. The Click-it reaction revealed Edu staining (red). Cell nuclei were stained with DAPI (blue). $^{**}p < 0.01$, $^{*}p < 0.05$.

HTR-8/SVneo cells (Figure 5D). These data demonstrated that *HOXA11-AS* could directly bind Lsd1, Ezh2, and Ago2 in trophoblast cells. Previous studies have reported that Lsd1 and Ezh2 are negative regulators of transcription via H3K4me2 and H3K27me3, respectively.[31–33] Therefore, we further explored the mechanism of correlation among Ezh2, Lsd1, and *HOXA11-AS* using experimental methods.

We first suppressed the expression of Lsd1 and Ezh2 with effective siRNAs. The protein level of *RND3* was significantly upregulated after transfection with *EZH2* siRNAs and/or *LSD1* siRNAs in HTR-8/SVneo cells (Figure 5E). Then we hypothesized that *HOXA11-AS* may recruit Ezh2 and Lsd1 to the *RND3* promoter region, resulting in H3K27me3 and/or H3K4me2 in this region. Therefore, we performed chromatin immunoprecipitation (ChIP) assays to detect the enrichment of Ezh2 and H3K27me3 and Lsd1 and H3K4me2 in the promoter region of *RND3*. As shown in Figure 5F, the results showed that Lsd1 and Ezh2 protein could be directly recruited to the promoter region of the RND3 gene,

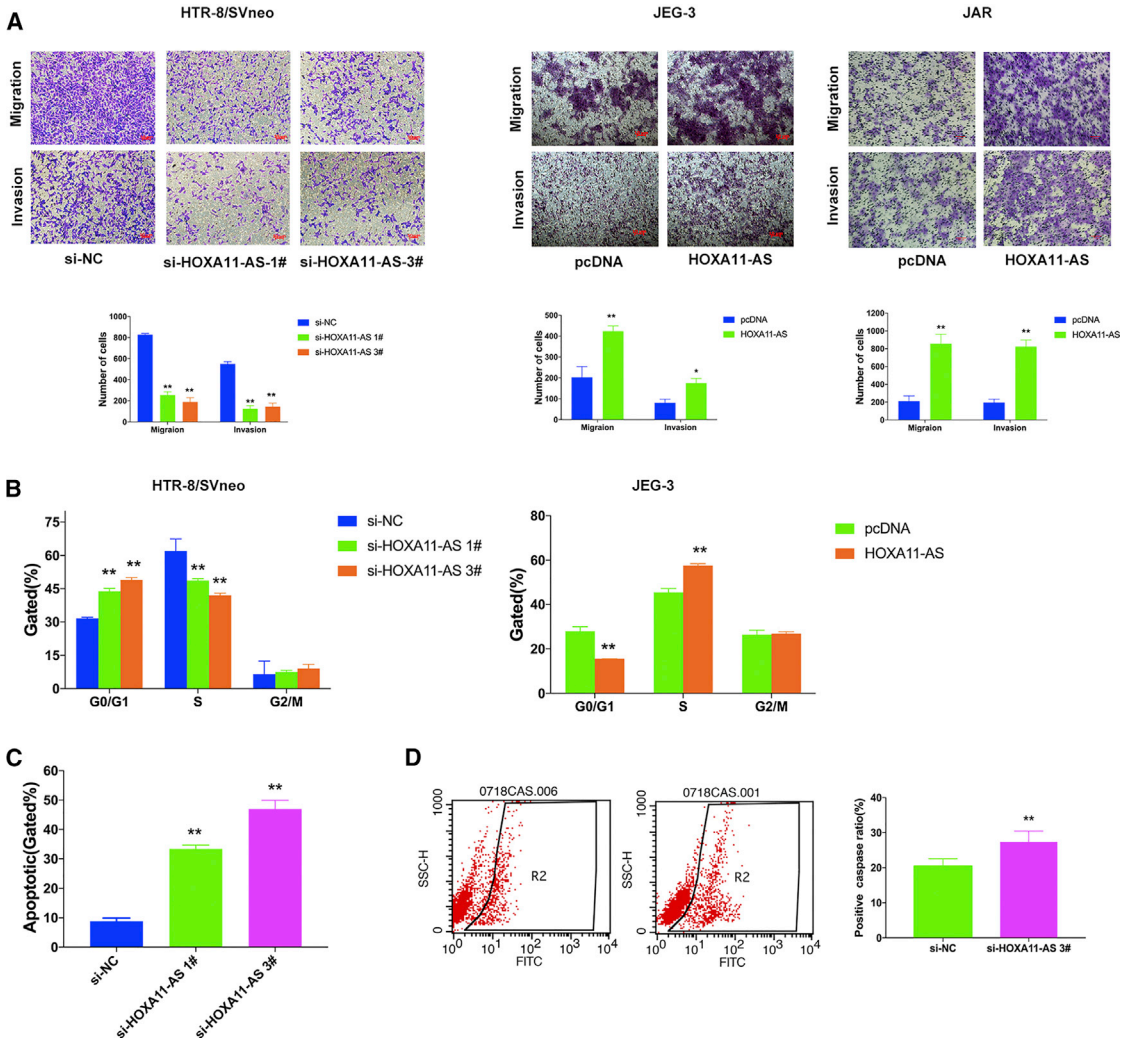

Figure 3. *HOXA11-AS* Regulates Trophoblast Cell Migration, the Cell Cycle, and Apoptosis *In Vitro*

(A) Trophoblast cells were treated with specific *HOXA11-AS* siRNAs and/or an overexpression plasmid. Transwell assays were used to investigate the changes in migratory and invasion abilities of trophoblast cells after transfection. (B) Cell cycle analysis by flow cytometry in HTR-8/SVneo and JEG-3 cells. (C) Flow cytometry was used to detect the apoptotic rates of cells. LR, early apoptotic cells; UR, terminal apoptotic cells. (D) Flow cytometry assays to detect the protein expression level of cleaved caspase after transfection with siRNAs against *HOXA11-AS* in HTR-8/SVneo cells. All experiments were performed in biological triplicates with three technical replicates. $^{**}p < 0.01$; $^{*}p < 0.05$; n.s., not significant).

silencing HOXA11-AS. Silencing of *HOXA11-AS* further suppressed Ezh2-mediated H3K27me3 demethylation and Lsd1-mediated H3K4me2 demethylation.

Our previous studies[34] have shown that the expression level of *RND3* is significantly increased in preeclamptic placental tissues compared with the controls. In this study, we also found that overexpression of RND3 could inhibit cell proliferation in the HTR-8/SVneo and JEG3 cell lines (Figures 6A–6C). Overexpression of *RND3* could also partly reverse *HOXA11-AS*-mediated growth promotion (Figures 6D and 6E). Together, these data suggest that *HOXA11-AS*-mediated cell growth could be reversed partly through epigenetic suppression of *RND3* by binding to Ezh2 and Lsd1 in the nucleus of trophoblast cells.

HOXA11-AS Promotes *HOXA7* Expression by Sponged miR-15b-5p, Affecting Trophoblast Cell Proliferation

Based on the RNA-seq analysis, numerous genes affecting cell phenotype were downregulated after silencing of HOXA11-AS. *HOXA7*, part of the cluster on chromosome 7, could promote cell proliferation and migration in various cell lines.[35–38] We next performed western blotting assays to further confirm the RNA-seq results. As shown in Figure 7A, we found that the protein level of *HOXA7* was significantly upregulated after *HOXA11-AS* overexpression, whereas the opposite

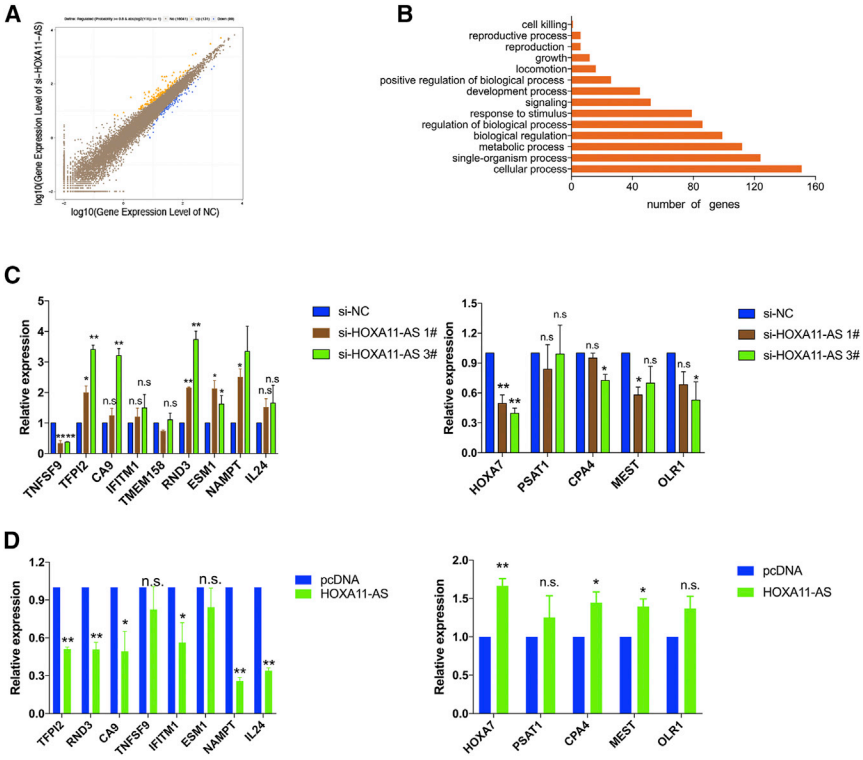

Figure 4. *HOXA11-AS* Knockdown Increases the Expression of Genes Involved in Cell Proliferation and Migration

(A) RNA transcriptome sequencing analysis was performed to analyze gene expression profiling in HTR-8/SVneo cells following *HOXA11-AS* knockdown. Shown are all of the differently expressed genes. (B) GO analysis for all genes with altered expression between the scrambled siRNA-treated and si-*HOXA11-AS*-treated cells *in vitro*. Cell growth was among the significant biological processes for genes whose transcript levels were changed in *HOXA11-AS*-depleted trophoblast cells. (C) qRT-PCR analysis of si-*HOXA11-AS*-treated trophoblast cells reveals altered mRNA levels of genes involved in cell proliferation and migration upon *HOXA11-AS* depletion. (D) qRT-PCR analysis of pcDNA3.1+HOXA11-AS-transfected trophoblast cells reveals altered mRNA levels of genes. $^{**}p < 0.01$; $^{*}p < 0.05$.

result was found after *HOXA11-AS* knockdown in HTR-8/SVneo cells.

An increasing number of studies have reported that lncRNAs could compete for specific miRNAs in the cytoplasm to mediate mRNA expression, further affecting cell phenotype.[22] There is evidence that miRNAs are found predominantly in the cytoplasm by binding to Ago2, which is the fundamental element of the RNA-induced silencing complex. Based on subcellular fractionation assays and RIP assays (Figure 5C), we found that 30% of *HOXA11-AS* is distributed in the cytoplasm and that *HOXA11-AS* could interact with Ago2 protein. Then we hypothesized that *HOXA11-AS* modulates *HOXA7* expression levels by sponged specific miRNA in HTR-8/SVneo cells.

To validate this concept, we first conducted bioinformatics analysis and found that miR-15b-5p was predicted to bind to the 3′ UTR of the downstream target gene of both *HOXA11-AS* and *HOXA7* (Figure 7B). Then we performed qRT-PCR, and the results showed that silencing of *HOXA11-AS* significantly upregulated the expression of miR-15b-5p; in contrast, the miR-15b-5p level was decreased after *HOXA11-AS* overexpression. Further experiments indicated that the RNA and protein levels of *HOXA11-AS* and *HOXA7* were significantly reduced after transfection with miR-15b-5p mimics (Figure 7C). Next we further explored the functions of miR-15b-5p in trophoblast cells. Diverse luciferase genes, including *HOXA11-AS*, mutant *HOXA11-AS*, the 3′ UTR of *HOXA7*, and the mutant 3′ UTR of *HOXA7*, were cloned and then co-transfected with miR-15b-5p in HTR-8/SVneo cells. Interestingly, we found that the relative luciferase activity of reporters of *HOXA11-AS* and the 3′ UTR of *HOXA7* were significantly abolished after treatment with miR-15b-5p (Figure 7D). In contrast, the relative luciferase activity on mutant reporters of both *HOXA11-AS* and the 3′ UTR of *HOXA7* showed no effect after treatment with miR-15b-5p (Figure 7D). Therefore, these data demonstrate that miR-15b-5p can bind to both *HOXA11-AS* and the *HOXA7* gene. Functional research further indicated that overexpression of miR-15b-5p could stimulate trophoblast cell growth and migration (Figure S1). In parallel, MTT and BrdU assays also indicated that knockdown of *HOXA7* could inhibit proliferation in HTR-8/SVneo cells and that silencing of *HOXA7* could reverse *HOXA11-AS*-induced cell proliferation (Figure 7E).

DISCUSSION

Recent studies have indicated that many lncRNAs have critical roles in PE. For example, the lncRNA *MALAT1* was recently determined to affect trophoblast cell proliferation and apoptosis, promoting PE development.[16] Our previous study also demonstrated that the lncRNAs *SPRY4-IT1* and *MEG3* modulate trophoblast cell proliferation and apoptosis as well as tube formation, invasion, and migration in this disorder.[14,39,40] Moreover, other lncRNAs, including *ATB*, Uc.187, and *RPAIN*, have been reported to regulate trophoblast cell growth and/or invasion and/or apoptosis in PE.[17,18,41] Therefore, more potential PE-associated lncRNAs need to be identified whose functions require further exploration of their underlying biological mechanisms.

In our study, we discovered that the level of *HOXA11-AS* was significantly downregulated in PE tissues compared with normal tissue samples. Also, lower mRNA of HOXA11-AS was related to the gestational age and lower birth weight of infants, indicating that

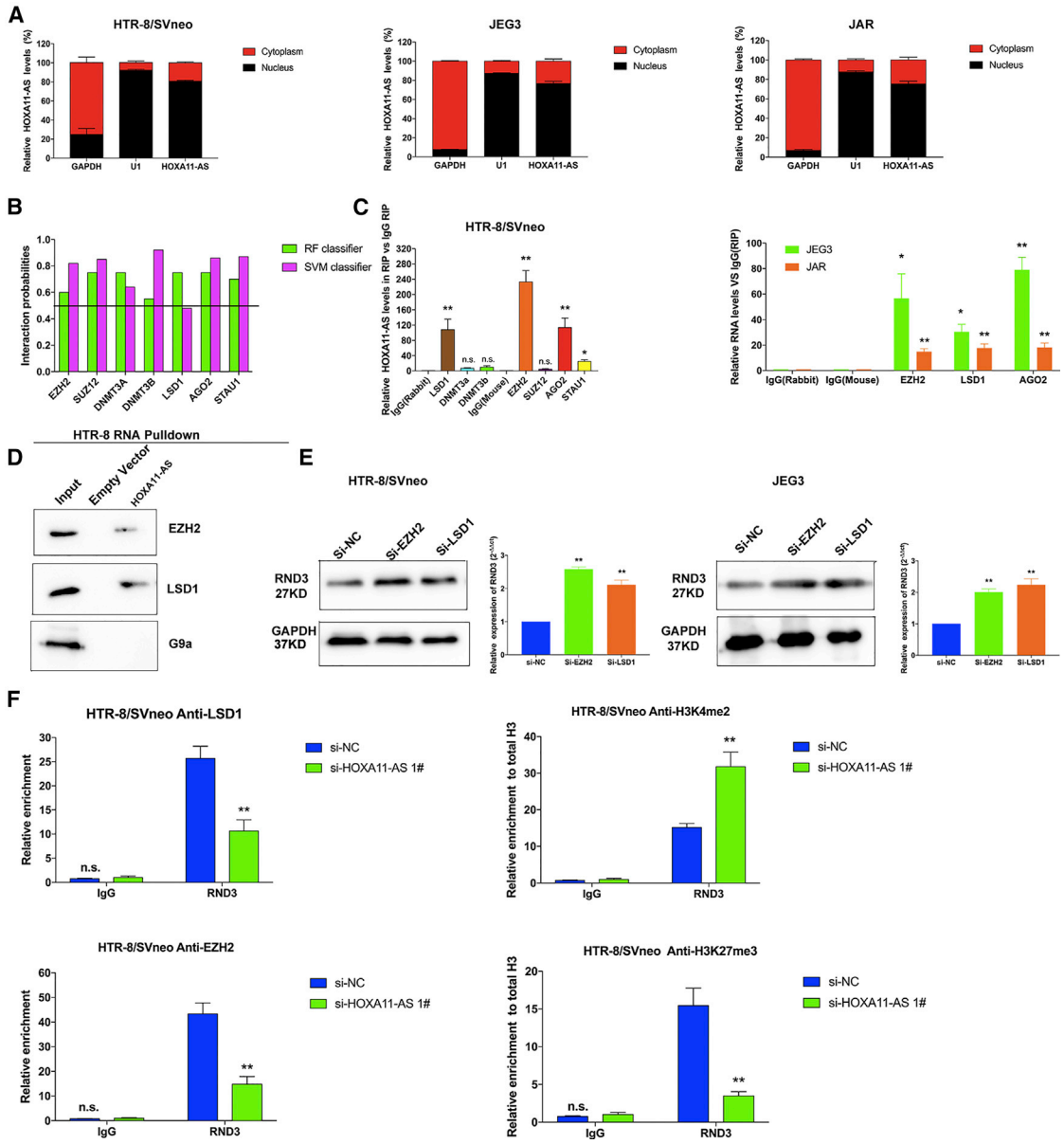

Figure 5. *HOXA11-AS* Can Recruit *EZH2* and *LSD1* to Silence *RND3* Expression

(A) Cell fractionation assays indicated that *HOXA11-AS* is mostly located in the nucleus. GAPDH and U1 acted as markers of the cytoplasm and nucleus, respectively. (B) Bioinformatics were used to predict the possibility of interaction of *HOXA11-AS*. Predictions with probabilities >0.5 were considered positive. RPISeq predictions are based on random forest (RF) or support vector machine (SVM). (C) RIP experiments were performed, and the coprecipitated RNA was detected by qRT-PCR. (D) *In vitro*-transcribed pulldown assays showed that *HOXA11-AS* could retrieve *EZH2* and *LSD1* in HTR-8/SVneo cells but not G9a. G9a was a negative control. (E) Western blot assays detected the expression of *RND3* after silenced *EZH2* after si-RNA transfection in HTR-8/SVneo cells. (F) The enrichment of *EZH2*/H3K27me3 and *LSD1*/H3K4me2 in the promoter regions of *RND3* was identified via ChIP assays, and this enrichment was decreased after *HOXA11-AS* knockdown in the HTR-8/SVneo cell line. Antibody directed against immunoglobulin G (IgG) was used as a negative control. Values represent the mean ± SEM from three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$.

downregulation of HOXA11-AS is positively related to early termination of pregnancy, which further suggests that downregulation of HOXA11-AS might lead to severe PE. Our data also revealed that knockdown of *HOXA11-AS* could impair trophoblast cell proliferation and migration *in vitro*, whereas *HOXA11-AS* overexpression could promote cell proliferation and migration. To investigate the *HOXA11-AS* -related pathway and downstream genes in PE, we conducted RNA transcriptome sequencing after transfecting a target cell line with specific siRNAs against *HOXA11-AS*; GO analysis suggested that gene expression profiles were primarily proliferation- and

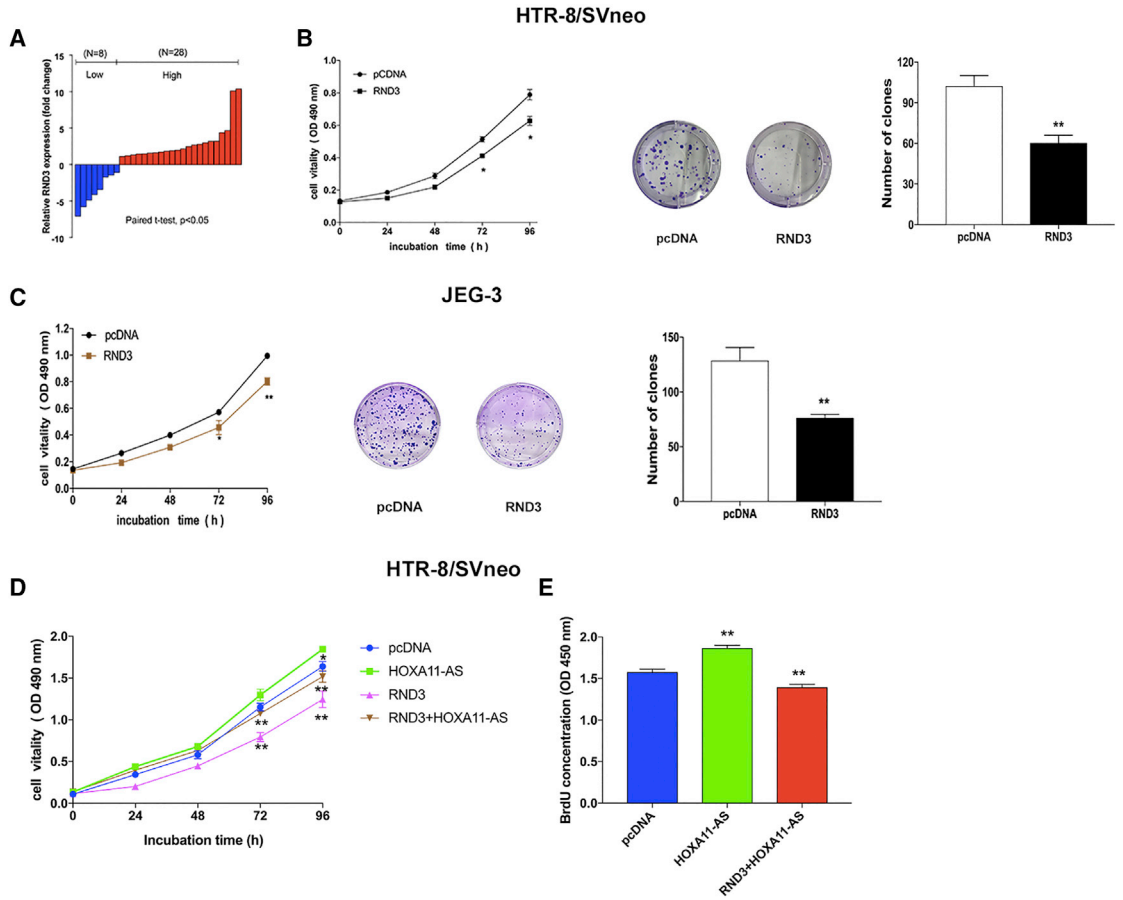

Figure 6. Upregulation of *RND3* Inhibits Trophoblast Cell Proliferation and Is Involved in the Function of *HOXA11-AS*

(A) *RND3* expression levels were presented as the fold-change in preeclampsia placental tissue samples compared to the control. (B) MTT assays and colony formation assays were performed to assess the cell proliferation after transfection with pcDNA3.1+RND3 in HTR-8/SVneo cells. (C) MTT assays and colony formation assays were conducted to detect the cell proliferation after transfection with pcDNA3.1+RND3 in JEG3 cells. MTT assays (D) and BrdU assays (E) were implemented to assess the cell viability for pcDNA-3.1+*HOXA11-AS* and pcDNA3.1+*RND3* co-transfected HTR-8/SVneo cells. $^{**}p < 0.01$, $^{*}p < 0.05$.

migration-associated. Previous studies have determined that many lncRNAs can bind with various chromatin-modifying enzymes to regulate related gene expression at the epigenetic level.[42] For instance, the pseudogene *DUXAP10* promotes an aggressive phenotype through binding with *LSD1* to repress *LATS2* and *RRAD* in non-small-cell lung cancer,[43] and the lncRNA *TUG1* is involved in cell proliferation of small-cell lung cancer by regulating *LIMK2b* via EZH2.[44] Our resulting data also revealed that *HOXA11-AS* could recruit and bind to two histone methylation modification complexes, including *EZH2* and *LSD1* in the nucleus, silencing genes expression. Further experiments were performed, indicating that these targets were affected by promoter H3K4me2 demethylation and H3K27me3 demethylation in trophoblast cells.

RND3/RhoE is a small GTPase that can exhibit biological functions as a suppressor gene in numerous diseases,[45–48] inhibiting multiple cellular processes such as actin cytoskeleton dynamics, the cell cycle,[45] migration, invasion,[49] and apoptosis.[45,50] However, the functions of RND3 in the pathological process of PE is still unclear. Our results revealed that *HOXA11-AS* can contribute to the downregulated expression of *RND3* by histone methylation in trophoblast cells. These results suggest that *HOXA11-AS* can bind to Ezh2 and Lsd1, epigenetically silencing *RND3* in the nucleus of trophoblast cells.

HOX genes, a highly conserved subgroup of the homeobox superfamily, are spatially and temporally regulated during embryonic development.[51] Abnormal expression of *HOXB7*[52] and *HOXB13*[53] has been reported to regulate many processes, including apoptosis, receptor signaling, and differentiation, in a myriad of disorders.[54] In our study, we found that low levels of *HOXA7* could affect proliferation in trophoblast cell lines, which implies that the post-transcriptional regulation of *HOXA7* is partly mediated by *HOXA11-AS* in development of PE through sponging miR-15b-5p in the cytoplasm, accelerating trophoblast cell growth. Furthermore, Sun et al.[28] have

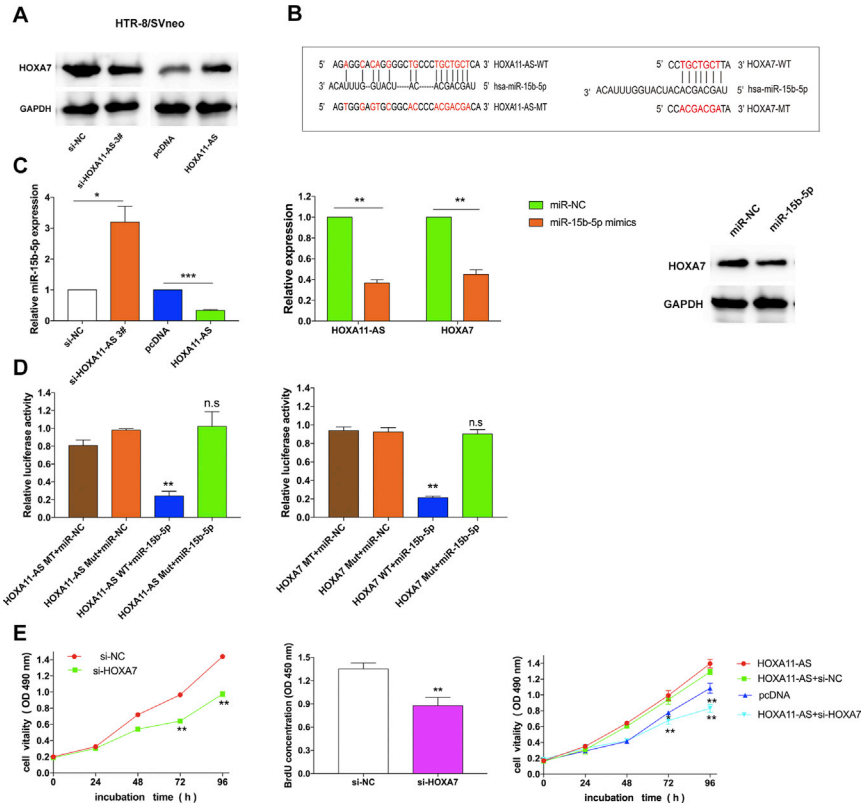

Figure 7. *HOXA11-AS* Promotes *HOXA7* Expression by Competing for miR-15b-5p in the Cytoplasm, Facilitating Trophoblast Cell Growth

(A) Western blot assays detected the expression of *HOXA7* after knockdown of *HOXA11-AS* and overexpression of *HOXA11-AS* in HTR-8/SVneo cells. (B) The RNAup algorithm predicted potential binding of miR-15b-5p to *HOXA11-AS* and *HOXA7*, with considerable sequence complementarity in the indicated regions. (C) qRT-PCR assays detected the expression of miR-15b-5p after knockdown of *HOXA11-AS* and overexpression of *HOXA11-AS* in HTR-8/SVneo cells. The expression of *HOXA11-AS/HOXA7* was detected after overexpression of miR-15b-5p in HTR-8/SVneo cells. Western blot assays detected the expression of *HOXA7* after knockdown of overexpression of miR-15b-5p in HTR-8/SVneo cells. (D) Luciferase reporter assays were used to determine the interacting activity between miR-15b-5p and *HOXA11-AS/HOXA7*. Luciferase activity is shown as relative luciferase activity normalized to *Renilla* activity. (E) MTT and BrdU assays were used to determine the viability of si-HOXA7-transfected HTR-8/SVneo cells and showed that knockdown *HOXA7* could reverse *HOXA11-AS*-mediated growth. *$p < 0.05$, **$p < 0.01$.

demonstrated that *HOXA11-AS* can promote gastric cancer tumorigenesis through sponging miR-1297. These findings demonstrated that *HOXA11-AS* simultaneously competes for miR-1297 and miR-15b-5p.

In brief, *HOXA11-AS* facilitates cell proliferation and migration by epigenetically regulating RND3 in the nucleus, and it can promote trophoblast cell growth through sponging miR-15b-5p in the cytoplasm. Our results indicate that *HOXA11-AS* can exhibit diverse biological regulatory mechanisms in PE (Figure 8). However, there are still many gaps in our current understanding of HOXA11-AS function and its biological mechanism in PE. Further studies, including clinical trials and animal research, are needed to elucidate whether HOXA11-AS might act as a prospective diagnosis and therapeutic target for PE.

MATERIALS AND METHODS

Tissue Samples and Ethics Statement

60 PE patients were selected for this study at the obstetrical department of the First Affiliated Hospital of Nanjing Medical University. Briefly, placenta tissue samples (about 1 cm × 1 cm × 1 cm in size) were taken from the central area of the placenta's maternal surface to avoid necrosis and calcification and placed in embedding molds that contained optimal cutting temperature (OCT) medium, frozen over a dry ice or ethanol slurry, stored at −80°C, and subsequently used for RNA and protein extraction. All patients provided written informed consent. The clinical characteristics of the PE patients are shown in Table 1. This research was authorized by the Ethics Board of the First Affiliated Hospital of Nanjing Medical University, China, and it was performed in compliance with the Declaration of Helsinki principles.

Cell Culture

The HTR-8, JEG3, and JAR cell lines were purchased from the Institute of the Chinese Academy of Sciences (Shanghai, China). HTR-8 and JAR cells were cultured in RPMI 1640 medium (Gibco, Nanjing, China) supplemented with 10% fetal bovine serum (FBS) (Gibco, BRL, Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin. JEG3 cells were cultured in MEM with 10% FBS. All cell lines were cultured in humidified air at 37°C/5% CO_2.

Plasmid Construction

Full-length cDNA of *HOXA11-AS* (1,628 bp, NR_002795.2), RND3 sequence (2807bp, NR_001254738) were synthesized and cloned into the pcDNA3.1(+) plasmid vector (Invitrogen). The resulting plasmids as well as the empty pcDNA3.1(+) vector were transfected into HTR-8/SVneo, JEG3, and JAR cells on 6-well plates and/or 24-well plates.

Cell Transfection

Lipofectamine 2000 and/or Lipofectamine 3000 transfection reagents (Life Technologies, Invitrogen, USA) were used to transfect the trophoblast cell lines with siRNAs targeting *HOXA11-AS* mRNA according to the manufacturer's protocol. All siRNA sequences are

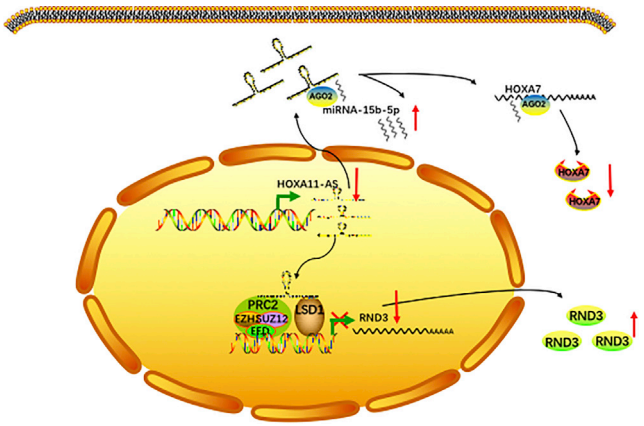

Figure 8. Proposed Model of Mediation by HOXA11-AS in Proliferation and Migration Progression of PE

listed in Table S1. Interference target sequences of *HOXA7* and *RND3* were purchased from Invitrogen. The transfected cells, on 6-well plates, were harvested for further experiments 48 hr post-transfection.

RNA Extraction and qRT-PCR Analyses

Total RNA from each treatment was extracted using TRIzol reagent (Thermo Fisher Scientific), and qRT-PCR analyses were conducted using SYBR Green Master Mix (TaKaRa Bio, Otsu, Japan) according to the protocol. The sequences of specific primers used are shown in Table S1.

Subcellular Fractionation Location

The nuclear and cytosolic fractions were separated and purified using the PARIS kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's manual.

Cell Viability Assays

Cell viability was detected with an MTT kit (Sigma) following the manual. For the colony formation assay, 600, 800, or 1,000 cells treated with siRNAs and/or plasmid were plated on 6-well plates and maintained in proper medium containing 10% FBS for 10–14 days, during which the medium was replaced every 4 days. Colonies were then fixed with methanol and stained with 0.1% crystal violet (Sigma) in PBS for 30 min. Colony formation was determined by counting the number of stained colonies.

BrdU experiments were performed using a BrdU cell proliferation assay kit (Millipore, catalog no. 2750) following the protocol. The higher optical density (OD) reading represents the higher BrdU concentration in each sample.

The EdU assay was implemented as a complementary method to authenticate the proliferation level. We exploited the 5-ethynyl-2-deoxyuridine labeling and detection kit (Ribobio, Guangzhou, China) to evaluate cell proliferation, following the manufacturer's manual.

Flow Cytometric Analysis of Cell Cycle and Apoptosis

After transfecting cells with siRNAs or plasmid, we performed fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) staining using the FITC-Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's instructions. Cell cycle level was determined by propidium oxide staining using the Cycle Test Plus DNA Reagent Kit (BD Biosciences, Franklin Lakes, NJ, USA), following the protocol, and analysis by FACScan. The ratio of the cells in G0/G1, S and G2/M phase were calculated and compared.

Transwell Assays

Cell Migration and invasion were detected and analyzed by transwell assays, as reported previously by Xu et al.[34] 24-well chambers were placed into the upper chamber of an insert with an 8-μm pore size polycarbonate membrane (Millipore, Billerica, MA, USA). Medium containing 15% FBS was added to the lower chamber. After incubation for 24–48 hr, the cells on the upper membrane were removed with a cotton swab. Cells that migrated or invaded through the polycarbonate membrane were stained with methanol and 0.1% crystal violet. The experiments were conducted three times.

Western Blot Assays

WB assays were conducted as reported previously by Xu et al.,[34] and the following primary antibodies were used: anti-EZH2, anti-LSD1, and anti-AGO2 (Millipore, USA) and anti-RND3 and anti-HOXA7 from Proteintech (WuHan, China). GAPDH (Cell Signaling Technology, San Jose, CA, USA) antibody was used as the control.

RNA-Seq Bioinformatic Analysis

The RNA-seq experiments were conducted by the Wuhan Genomics Institute (Wuhan, China). Culture cells that were treated were extracted. To establish the mRNA-seq library, the cDNAs were fragmented by nebulization following the protocol.

ChIP

ChIP assays were conducted as described previously by Xu et al.[34] According to the manual, experiments were performed using the EZ-CHIP kit (Millipore, USA). Relevant antibodies against H3K27me3, H3K4me2, and other target proteins were purchased from Millipore. The primer sequences of gene promoters are summarized in Table S1. Immunoprecipitated DNA were detected and analyzed by qRT-PCR. Experiments were repeated three times.

RIP Assays

RIP experiments were performed following the protocol of the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA). Antibodies, including EZH2, SUZ12, DNMT3a, DNMT3b, LSD1, AGO2, and STAU1, were purchased from Millipore.

Luciferase Reporter Assays

Luciferase reporter assays were performed as described previously by Zhang et al.[21] HOXA11-AS and HOXA7 3′ UTR cDNA fragments

were amplified by PCR assays and then subcloned downstream of the luciferase genes in the pGL3 plasmid. Mutant of plasmids, such as pGL3-HOXA7-3′ UTR MUT and pGL3-HOXA11-AS-MUT, were obtained by platinum pfx DNA polymerase according to the protocol. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Briefly, 1 × 105 HTR-8/SVneo cells were plated in 24-well plates for 36 hr. 48 hr post-transfection, the cells were retrieved and lysed for further experiments. The relative luciferase activity was normalized with *Renilla* luciferase activity.

Statistical Analysis

All statistical analyses in our experiment were performed using SPSS 20.0 software (IBM, SPSS, USA). The resulting data are represented as the mean ± SD. Statistical significance was ascribed at $^{*}p < 0.05$ or $^{**}p < 0.01$. Each experiment was repeated at least three times independently.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure, one table, and two data files and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.05.007.

AUTHOR CONTRIBUTIONS

Y.X., D.W., and J.L. performed most experiments. S.H., Q.Z., and Y.J. collected clinic tissues and analyzed data. X.X., Y.C., and T.W. conducted some experiments. Y.X. and L.S. designed the project and edited the manuscript.

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