miR-18a Contributes to Preeclampsia by Downregulating Smad2 (Full Length) and Reducing TGF-β Signaling

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INTRODUCTION

Preeclampsia (PE) is a multisystem disorder that affects about 2%–7% pregnancies worldwide and is considered one of the leading causes of maternal and neonatal mortality as well as morbidity.1,2 Although the etiology and the pathogenesis of PE are not well understood, it is generally accepted that placental defects, especially the dysregulation of the trophoblast behaviors, are the underlying mechanisms that lead to the disease. For instance, reduced trophoblast proliferation, excessive trophoblast cell apoptosis, as well as the insufficient trophoblast invasion have all been linked to the occurrence and the development of PE.3–5 MicroRNAs (miRNAs) are a subset of small, noncoding RNAs that are involved in numerous important biological events.6 A major mechanism by which miRNAs regulate gene expression is the partial base pairing to the 3′ untranslated region (3′ UTR) of the target mRNAs to repress their translation and/or to induce their degradation.7 Our and others8–10 previous work have demonstrated that a great number of miRNAs are differentially expressed in PE placentas, and some of these differentially expressed miRNAs may participate in the regulation of various trophoblast cell functions.7,10,11 miR-18a is predominantly downregulated in placental tissues and maternal plasma of patients with PE and is mainly localized in the various subtypes of trophoblast cells in the placenta.12 A series of in vitro and in vivo studies have demonstrated that miR-18a plays an important role in different cell functions via downregulating FGFI, Smad4, HIF-1α, and other target genes.12–14 Therefore, elucidation of the underlying mechanisms by which miR-18a regulates the functions of trophoblast cells may provide a better understanding of the pathophysiology of this disorder and uncover new targets for therapeutic intervention.

With the use of bioinformatics tool Targetscan,15,16 we found that Smad2 and Smad3 were among the most predicted targets of miR-18a. Smad2 and Smad3 are both central cytoplasmic mediators of the TGF-β signaling, leading to the enhancement of trophoblast cell invasion. A lack of miR-18a, which results in the upregulation of Smad2(FL), contributes to the development of PE.
pathway is responsible for the dysfunction of trophoblast cells and the pathophysiology of PE.21–27 We therefore speculated that miR-18a interferes with the TGF-β signaling in PE placentas via targeting Smad2 and/or Smad3.

Smad2 is composed of 12 exons, and exon3 is spliced out in about 10% of Smad2 in several human tissues, including heart and placenta.28 There exists two mature Smad2 isoforms in human placenta: full-length Smad2, Smad2(FL), and Smad2 lacking exon 3, Smad2(-Δ exon3). Perhaps due to the specificity of the antibody and/or the protein extraction method,29 our previous work just detected the expression patterns of the Smad2(FL) in human placenta.30 Despite that more than 90% sequences are similar between Smad2(FL) and Smad2(-Δ exon3), these two proteins have different expression patterns and distinguishing function features in several pathologies.29,30 However, the expression pattern and function of these two Smad2 isoforms in severe PE (sPE) placenta remain unknown.

In the present study, based on the evidence noted above, we have proposed that miR-18a regulated trophoblast cell function by targeting at one or more of the Smad proteins, which impaire the TGF-β signaling and lead to the disease. To test our hypothesis, we used human trophoblast cell line HTR8/SVneo to investigate whether miR-18a attenuated the TGF-β signaling and inhibited the expression of Smad protein(s), the disturbance of which favors the disease development.

RESULTS

miR-18a Was Significantly Downregulated in the Chorionic Plates, but Not the Basal Plates of the sPE Placentas

With the use of quantitative real-time PCR technology, we examined pri-miR-18a and miR-18a expression levels in the control and sPE placentas. The levels of pri-miR-18a and miR-18a were significantly downregulated in the chorionic plates (Figures 1A and 1C) but not in the basal plates (Figures 1B and 1D) of the sPE placentas.

The Expression Pattern of Placental miR-18a at Different Gestational Stages during Normal Gestation

The expression pattern of miR-18a at different gestational stages of normal pregnancy was examined by quantitative real-time PCR. At least 3 samples were collected and analyzed in each gestational week. As shown in Figure 1E, the level of miR-18a during gestational weeks 8–10 was significantly higher than that at gestational weeks 6–7, and the level of miR-18a began to decrease after gestational week 10. The level of miR-18a at mid-term and term was significantly decreased compared to that at gestational weeks 6–7.

Expressions of Smad2(FL) and Phosphorylated (p)-Smad2(FL) Proteins Are in Inverse Correlation with miR-18a in sPE Placentas

We compared the expression levels of Smad2(FL), Smad2(-Δ exon3), and Smad3 in placentas from 13 sPE patients and 32 normal pregnant women, both at mRNA and protein levels. The mRNA expression of Smad3, Smad2(FL), and Smad2(-Δ exon3) had no obvious difference between sPE and control placentas in neither chorionic nor basal plates (Figures 2A, 2B, 3A, and 3B). The protein levels of Smad3 and Smad2(-Δ exon3) also exhibited no significant difference in sPE and normal placentas in either chorionic or basal plates (Figures 2C, 2D, 3C, and 3D). However, the Smad2(FL) protein level was increased in the chorionic plate, but not basal plates, of sPE placentas (Figures 3C and 3D). Interestingly, the p-Smad2(FL) protein level in the sPE placentas was also significantly increased in the chorionic plate but not the basal plates. There were no significant differences of the p-Smad2(-Δ exon3) between normal and sPE placentas either in the chorionic plate or in the basal plate (Figures 3E and 3F). p-Smad2(-Δ exon3) and Smad2(-Δ exon3) could not be detected in the Htr8/SVneo cells (Figure 3C–3F).

A correlation analysis of miR-18a concentration and Smad2(FL) (or p-Smad2(FL)) level was also performed accordingly. The data revealed an inverse correlation between miR-18a and Smad2(FL) (or p-Smad2(FL)) expression in the placental chorionic but not in the basal plates of the studied individuals (Figures 3G–3I).

The antibody against Smad2 detected two bands on the western blots (Figure 3), which raises a question of whether it is due to an antibody-specificity issue or whether there are two variants of the protein. In addition, apart from Smad2(-Δ exon3) (composed of 437 amino acids), three other Smad2 variants (composed of 425 amino acids, 418 amino acids, and 414 amino acids, respectively) could also be retrieved in the NCBI database (https://www.ncbi.nlm.nih.gov). To verify that the lower band is Smad2(-Δ exon3), the corresponding protein spot was cut from the middle of the lanes and used for mass-spectrum analysis. As shown in Figure S1, five conserved peptides and one Smad2(-Δ exon3)-specific peptide were found, respectively. The five conserved peptide motifs were SLDGR, NATVEMTRR, RHIGR, MSFVK, and MSFVKGWGAERYR, respectively. The Smad2(-Δ exon3)-specific peptide motif was LDELEK. These results confirmed that there are two variants of Smad2, wild-type (WT) and mutant (MUT), and the MUT Smad2 isoform is Smad2(-Δ exon3).

Colocalization of miR-18a and Smad2 in Human Placenta

The localization of miR-18a and Smad2 in normal human placenta was examined by in situ hybridization and immunohistochemistry. Immunostaining for cytokeratin (CK)-8 was used as a marker for trophoblast cells (Figures 4E and 4F). miR-18a and Smad2 were both localized in the in placental tissues, derived from gestational weeks 7–8, in similar patterns. They were predominantly localized in various subtypes of trophoblasts, including villous cytotrophoblast cells, syncytiotrophoblasts (Figures 4A and 4C), and interstitial trophoblasts cells invading the decidual stroma (Figures 4B and 4D). They were also weekly expressed in some villous mesenchymal cells (Figures 4A and 4C). No hybridization signals were detected in decidual cells (Figures 4B and 4D). In the negative control (NC) hybridization and immunohistochemistry without probe or primary
antibody, no positive signals were observed (Figures 4G and 4H), showing the specificity of the experiments.

**Verification of Primary Syncytiotrophoblasts and Cytotrophoblasts**

We performed western blot analysis to detect syncytin-2 and human chorionic gonadotropin (hCG)-β of the primary syncytiotrophoblasts and cytotrophoblasts, which are markers for the cell type, which confirmed that syncytin-2 and hCG-β were present only in the primary cytotrophoblasts and syncytiotrophoblasts, respectively (Figure S2).

**HTR8/SVneo Cell Line Was Selected as the Suitable Trophoblast Cell Line in This Study**

We compared the expression level of miR-18a in three trophoblast cell lines (HTR8/SVneo, JEG-3, and B6-tert), as well as primary cultured cytotrophoblasts and syncytiotrophoblasts. We found that the HTR8/SVneo cells and the primary trophoblasts had comparable expression levels of miR-18a, whereas other two cell lines (JEG-3 and B6-tert) had a higher expression level than the primary cells (Figure S3), suggesting that HTR8/SVneo, rather than other two cell lines, had a miR-18a homeostasis similar to that in trophoblasts. Thus, the HTR8/SVneo cell line was selected as the experimental model to study the function of miR-18a in trophoblasts *in vitro*. 

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**Figure 1. Differential Expression Patterns of pri-miR-18a and Mature miR-18a in sPE Placentas**

(A–D) Quantitative real-time PCR experiments were performed to measure the expression levels of pri-miR-18a (A and B) and mature miR-18a (C and D) in the chrorionic plates (A and C) and basal plates (B and D) of the placentas derived from sPE patients (n = 13) and normal pregnant women (n = 32). Data are presented as mean ± SD. *p < 0.05. (E) miR-18a expression levels change across gestation. At least 3 samples were collected and analyzed in each gestational week. Data are presented as mean ± SD. *Compared to the miR-18a level at gestational weeks 6–7 (6–7w), p < 0.05.
Smad2(FL) Is the Direct Target of miR-18a in Human Trophoblast Cells

As shown in Figures 5A and 5B, the transfection of miR-18a downregulated the expression of Smad2(FL) at both mRNA and protein levels, up to 76% and 54%, respectively. According to bioinformatics analysis, the seed sequence of miR-18a was complementary to 136 to 142 nt of 3' UTR in Smad2(FL) mRNA. To further confirm that Smad2(FL) was the target of miR18a, the luciferase reporter construct carrying a 300-bp DNA fragment, including binding site (BD) of the 3' UTR in human Smad2 mRNA (the vector was named BD-WT) or the point-mutated report construct (the vector was named BD-MUT) was transfected into HTR8/SVneo cells together with miR-18a mimics and thymidine kinase promoter-Renilla luciferase reporter plasmid (pRL-TK). 48 h after transfection, miR-18a mimics could evidently reduce the relative luciferase activity of the BD-WT construct by ~35%, compared with scramble control, but could not affect the relative luciferase activity of the BD-MUT construct (Figures 5C and 5D).

miR-18a Promoted Trophoblast Cell Invasion but Had No Effect on Cell Growth or Cell Cycle

The effects of miR-18a on trophoblast cell invasion and proliferation were examined. Data from quantitative real-time PCR experiments showed that the level of miR-18a increased to ~690-fold of that of the scramble control cells after miR-18a mimics transfection (Figure 6A). miR-18a inhibitor decreased the level of miR18a by ~98% (Figure 6B). As shown in Figures 6C and 6D, overexpression of miR-18a in Htr8/SVneo cells significantly promoted cell invasiveness (Figure 6C). Conversely, inhibition of miR-18a could inhibit the cell-invasive potential (Figure 6D). In addition, miR-18a did not have an effect on cell growth (Figure 6E) or cell cycle (Figure 6F).

Overexpression of Smad2(FL) Rescued the Invasion-Promoting Effect of miR-18a in HTR8/SVneo Cells

We performed a rescue experiment by transfecting HTR8/SVneo cells with miR-18a together with pcDNA4-Smad2(FL). Interestingly, the invasion-promoting effect of miR-18a was inhibited by the overexpressed Smad2(FL) (Figures 6G, S4A, and S4B).

miR-18a Inhibits the Invasion Suppression by TGF-β Induced in Htr8/SVneo Cells

With the consideration of the antagonistic potential of TGF-β signaling on trophoblast cell invasion, we investigated whether miR-18a could counteract the invasion suppression activity of
(legend on next page)
TGF-β. The Htr8/SVneo cells were transfected with miR-18a mimics and then treated with recombinant TGF-β protein. We observed that TGF-β stimulated Smad2(FL) activation and inhibited the invasion in HTR8/SVneo cells (Figures 7A–7D). More importantly, transfection of miR-18a mimics inhibited the upregulation of p-Smad2(FL) and suppression of cell invasion by TGF-β (Figures 7A–7D).

DISCUSSION
Since the first major study conducted by Pineles et al., more and more differentially expressed miRNAs have been identified in the placenta from PE patients. However, despite the wealth of studies that have suggested an association of certain miRNAs with PE, consensus has not yet been reached on which appears to be the key contributor to the pathogenesis of the condition and what the underlying mechanisms are. miR-18a is among the top 20 members of a list of differentially expressed miRNAs in PE placentas. With the consideration of the primary expression of miR-18a in various subtypes of placental trophoblasts, it is likely that miR-18a may have functional roles in the regulation of trophoblast cell behaviors.

Despite previous studies have demonstrated that this small RNA has biological functions in different cells, the existing results are conflicting. We proposed that such discrepancies might be due to the differences in microenvironments and regulatory networks in different kinds of cells. For instance, human placenta trophoblast cells share properties, such as active proliferation and invasion with tumor cells. However, these tumor-like behaviors in trophoblasts are tightly organized in unique temporal and spatial manners during pregnancy. In this study, we examined the expression profile of miR-18a in placentas at different gestational weeks and found that miR-18a was significantly upregulated during gestational weeks 8–10, followed by a decrease until term (Figure 1C), which is consistent with the previous results. Since trophoblast cells are highly invasive at gestational weeks 8–10 during the whole gestation, miR-18a may regulate trophoblast cell invasion. However, unlike the report in tumor cells, miR-18a substantially improved cell invasion (Figures 6C and 6D), but had no influence on cell growth and cell cycle in trophoblast cells (Figures 6E and 6F). These observations...
indicate that this small molecule has unique functions in placental trophoblast cells.

Our results have shown that miR-18a promoted trophoblast invasion via targeting Smad2(FL): (1) The expression of Smad2(FL) was markedly upregulated in PE placentas, whereas miR-18a was decreased (Figures 1A, 1B, 3C, and 3D)\(^7\). (2) miR-18a inhibited the expression of Smad2(FL) (Figures 5A and 5B)\(^7\). (3) The luciferase reporter assay demonstrated the direct binding of miR-18a to the 3’ untranslated region (UTR) segment of the human Smad2(FL) gene (Figures 5C and 5D)\(^7\). (4) miR-18a was colocalized with Smad2 in human trophoblast cells (Figures 4A–4D). (5) The invasion-promoting effect of miR-18a could be partly attenuated by the overexpression of Smad2(FL) (Figure 6G)\(^7\).

Notably, there exists a great difference in the miR-18a expression levels of control placentas (Figure 1C), and this difference may be due to the different genetic backgrounds of these control placentas. The mRNA levels of Smad2(Δexon3) in sPE and control placentas were much lower than those of Smad2(FL) (Figures 3A and 3B). In contrast, the protein levels of these two molecules were comparable in both sPE and control placentas (Figures 3C and 3D). The inconsistency may be due to the differences of translation efficiency, mRNA, and protein stability of these two molecules in the sPE and control placenta tissues. In addition, the protein level of p-Smad2(Δexon3) in control and sPE placentas was much higher than those of p-Smad2(FL) (Figures 3E and 3F). This discrepancy may be caused by the activities and specificities of phosphokinases and/or phosphatases, as well as the stabilities of these two phosphor proteins in the sPE and control placenta tissues.

The next step was to clarify which signal pathway was regulated by miR-18a in PE placentas. Smad2(FL) is an important downstream effector of the TGF-β superfamily\(^18\). Aberrant expressions of several members in this superfamily have been implicated in PE pregnancies. For instance, productions of TGF-β and Nodal, as well as their receptors, were significantly enhanced in PE placentas, which may lead to over-activated signaling, excessive cell apoptosis, and less invasion\(^20,21,40\). More importantly, in human placenta, TGF-β primarily regulates trophoblast cell invasion\(^22–27\), and Nodal promotes cell apoptosis\(^41\). We found that miR-18a increased cell invasiveness (Figures 6C and 6D) but did not influence the cell growth or cell cycle (Figures 6E and 6F). Furthermore, members in the same miRNA
cluster have similar functions. \textsuperscript{42} miR-18a belongs to miR-17-92 cluster, \textsuperscript{42} and our previous bioinformatic analysis demonstrated that the miR-17-92 cluster may regulate placenta development via fine tuning TGF-\(\beta\) signaling. \textsuperscript{7} These provide clues that miR-18a has a vital role in TGF-\(\beta\) signaling and PE, which is indeed supported by the experimental findings (Figures 3E, 3F, \textsuperscript{7A}, \textsuperscript{7B}, \textsuperscript{7C}, and \textsuperscript{7D}).

Our previous results have demonstrated the varying expression pattern of miRNAs in differential sampling sites of sPE placentas. \textsuperscript{7} Consistent with this, in this study, miR-18a, Smad2(FL), and p-Smad2(FL) exhibited differential expression patterns in sPE placentas, and they are differentially expressed in the chorionic plates of sPE placentas (Figures 1A, 1B, \textsuperscript{3C}, \textsuperscript{3D}, \textsuperscript{3E}, and \textsuperscript{3F}). \textsuperscript{7} miR-18a could promote trophoblast cell invasion via downregulating Smad2(FL) (Figure 6G). The improper synthesis of hCG and the dysregulated expression of HLA-C gene and PPI3 genes in the chorionic plates of human placenta are linked to the insufficient trophoblast cell invasion and the occurrence of PE. \textsuperscript{43--45} Therefore, the differential expressions of miR-18a, Smad2(FL), and p-Smad2(FL) in the chorionic plates of the sPE placentas may interfere with the synthesis of hCG, as well as the expression of the HLA-C gene and PPI3 gene, which is implicated in the control of the trophoblast cell invasion and the occurrence of sPE.

One of the most complex properties of miRNAs is the large number of targets that may exist for one miRNA. Our previous results have revealed that several target genes of miR-195 and miR-210 are involved in PE. \textsuperscript{32,46--51} Furthermore, miR-195 can promote trophoblast cell invasion via downregulating activin receptor IIA (ActRIIA) and ActRIIB, \textsuperscript{46,48} whereas, miR-210 can inhibit trophoblast cell invasion via modulating potassium channel modulatory factor 1 and thrombospondin type I domain-containing 7A (PPI3). \textsuperscript{50,51} Our results have shown that Smad2 was not activated without TGF-\(\beta\) treatment (Figure 7C), whereas miR-18a promoted cell invasion independent of TGF-\(\beta\) treatment (Figure 7D). Previous studies have also shown that miR-18a may promote the trophoblast cell invasion via targeting estrogen receptor (ER)\(-\alpha\). \textsuperscript{32} Therefore, we propose that miR-18a regulates the trophoblast cell invasion via modulating other target genes, and further experimental studies are warranted to elucidate the way that miR-18a and its targets are involved in causing the trophoblast cell dysfunction associated with PE.

The mechanism of miR-18a downregulation in PE placentas remains unknown. Our finding of lowered expression of pri-miR-18a in sPE...
In conclusion, our results suggest that miR-18a inhibits Smad2(FL) expression and activation, which promote trophoblast cell invasion, which is likely via inhibition of TGF-β signaling. This, thus, plays an important role in the pathogenesis of PE (Figure 8).

In addition, it is well known that primary cells retain most of their physical characteristics in vivo, thus mirroring the status and functionality of these cells under normal physiological conditions, making them well suited for use in studies of cell biology. However, for the limited number of each separation and the finite lifespan, the selection of a suitable cell line is urgently needed. Recently, research into the function of human trophoblasts has been largely restrained by a lack of proper cell models, despite considerable efforts. In this study, by comparing the expression level restrained by a lack of proper cell models, despite considerable research into the function of human trophoblasts has been largely invasion of trophoblast cells.

In preeclamptic placenta, an increase in TGF-β expression and a decrease in miR-18a expression will cause overactivation of Smad2(FL), leading to insufficient invasion of trophoblast cells.

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**Table 1. Clinical Characteristics of the Pregnant Women Who Donated the Placentas for This Study**

| Characteristics | Normal Pregnancy (n = 32) | Preeclampsia (n = 13) | p Value |
|-----------------|--------------------------|-----------------------|---------|
| Maternal age (years) | 31 ± 4 | 29 ± 5 | 0.211 |
| BMI (kg m⁻²) | 22 ± 2 | 22 ± 3 | 0.661 |
| SBP (mmHg) | 116 ± 6 | 157 ± 13¹ | 1.549E−07 |
| DBP (mmHg) | 77 ± 5 | 101 ± 8² | 2.094E−14 |
| 24 h urine protein (g) | N/D | 3 ± 2³ | – |
| 50 g GCT (mM) | 7 ± 1 | 6 ± 2 | 0.062 |
| Gestational age at delivery (day) | 268 ± 7 | 256 ± 6⁴ | 2.020E−06 |

Data are expressed as mean ± SD and compared by one-way ANOVA. SBP, systolic blood pressure; DBP, diastolic blood pressure; N/D, no data; GCT, glucose challenge test.

¹p < 0.05 versus normal pregnancy.

**MATERIALS AND METHODS**

**Subjects**

In this study, all of the collection of human placenta tissues was performed with the permission of the Local Ethical Committee in the Institute of Zoology, Chinese Academy of Sciences, and in Peking University Third Hospital. The informed consents were obtained from all patients enrolled in this study.

Placenta samples from normal pregnancies and those from sPE women were obtained from Peking University Third Hospital. A total of 13 sPE patients and 32 normal pregnant women were enrolled in this study. sPE was diagnosed according to the definition in *Williams Obstetrics* (23rd edition). In brief, the patients had no history of pre-existing or chronic hypertension, but they showed systolic blood pressure of >160 mmHg or diastolic blood pressure of >110 mmHg on at least 2 occasions, accompanied by significant proteinuria (>2 g per 24 h or ≥ 3+ by dipstick in 2 random samples collected at >4-h intervals) after 20 weeks of gestation. Normal or uncomplicated pregnancy was defined as gestation in a previously normotensive woman who did not suffer from any complications during pregnancy and who delivered a healthy neonate with a weight adequate for a gestational age of more than 37 weeks of pregnancy. Women who developed renal disease, transient hypertension during pregnancy, multiple pregnancy, gestational diabetes, spontaneous abortion, intrauterine fetal death, fetal chromosomal or congenital abnormalities, or pregnancies conceived by fertility treatment were all excluded from this study. For each of the pregnant women in this cohort, placenta of the pregnant woman was collected within 1 h of caesarean birth, and specimens at the chorionic plate and the basal plate were separately taken from the placenta disc near the position of umbilical cord insertion. The specimens were snap frozen in liquid nitrogen until use. The clinical characteristics of the patients were summarized in Table 1.

Tissues of human chorionic villi at gestational weeks 6–12 and placenta tissues at gestational weeks 24–27 were obtained at Beijing
Table 2. Sequences of the Primers Used for Plasmid Construction or Real-Time PCR

| Product                  | Primer Sequence (5′ to 3′)  |
|-------------------------|----------------------------|
| GAPDH real-time PCR     | F: AAGTCTCATGCTGAGGTGAAC  |
|                         | R: AGCCCTGCTTACACCACTCTC  |
| pri-miR-18a real-time PCR| F: CGTAAAGTGAGAGGAGCTCA   |
|                         | R: ATCGT                   |
| miR-18a real-time PCR    | F: TAAGGTGATCATTAGTGAGATAG |
| U6 real-time PCR         | F: CGCAAGGATGACACGCAAATTC  |
| Smad2(FL)-expressing plasmid | F: GGAATTCAGAAGCTATTAT   |
|                         | R: AGGCAAGGAGAAGATGGGT     |
| Smad2(Δexon3)-real-time PCR | F: GGAGCTCATATGGAGTGGG   |
|                         | R: GGCCCTGTGTATCCCCACTGA   |
| Smad3 were listed in Table 2. |                        |

Western Blotting

The proteins were extracted by lysing the cells or the homogenized tissues with Radio-Immunoprecipitation Assay (RIPA) buffer containing 150 mM NaCl, 10 mM Tris (pH 7.6), 1% Nonidet P-40 (NP-40), 0.5% Na deoxycholate, 0.1% SDS, 1 mM NaF, Na3VO4 with 100× protease inhibitor cocktail (Sigma-Aldrich, MO, USA) and harvesting the supernatants after centrifuging. Protein concentration was measured by a bichinchoninic acid (BCA) protein assay (Boster Biological Technology, Wuhan, China), and 40 µg of protein from each cell lysate was subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare, CT, USA). The membrane was blocked for 1 h with 5% nonfat milk in PBS (PBS and 0.1% Tween 20) and incubated at 4°C overnight with Smad2 (R&D Systems, MN, USA), p-Smad2 (Cell Signaling Technology, MA, USA), Smad3 (Cell Signaling Technology, MA, USA), syncytin-2 (Abcam, Cambridge, UK), and GAPDH (Sigma-Aldrich, MO, USA) primary antibodies, respectively. The membrane was washed with PBS 3 times, incubated further with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, PA, USA) and GAPDH (Ambion, TX, USA) primary antibodies, respectively. The membrane was washed with PBS 3 times, incubated further with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, PA, USA) and analyzed by ImageJ software (NIH, MD, USA). The relative densities of Smad2, p-Smad2, and Smad3 protein were determined by normalization with a density value of GAPDH.

SDS-PAGE and Mass Spectrum Assays

40 µg of proteins for each sample was separated on a 10% SDS-PAGE gel. Protein bands were visualized by Coomassie blue R-250 staining. To identify the proteins, the corresponding protein spots were cut from the middle of the lanes, washed with water, and stored at −20°C. The samples were sent to Shanghai Applied Protein Technology (Shanghai, China) for mass spectrum analysis. Experiments were carried out on a Q Exactive mass spectrometer (Thermo Scientific, MA, USA), and the signals were captured on X-ray films (Kodak, NY, USA) and analyzed by Imagent software (NIH, MD, USA). The data of mass spectrum were analyzed by Mascot software.

In Situ Hybridization for miRNAs

Paraffin sections of 5 µm in thickness were subjected to routine deparaffinization and rehydration and treated with protease K, followed
by hybridization with the miRCURY LNA (locked nucleic acid) miRNA detection probes (Exiqon, Copenhagen, Denmark), specifically against miR-18a and NC, at 55°C overnight. The sections were serially washed in 5x, 2x, and 0.2x saline sodium citrate (SSC) buffer and then were incubated with alkaline phosphatase (AP)-conjugated anti-digoxigenin (DIG) antibody (Roche, IN, USA) at 37°C for 2 h. The stained signals were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium chloride (BCIP/NBT; Promega, WI, USA) as a substrate. The scramble miRNA (NC) probe was used as a NC. The probe sequences (5' to 3') were the following: miR-18a, CTAATCTGCACTAGATG, and NC, GTGTAACACGTCTATACGCCCA.

**Immunohistochemistry**
Paraffin sections of 5 μm in thickness were subjected to routine deparaffinization, rehydration, and antigen retrieval before being incubated with antibodies against human Smad2 (Proteintech, IL, USA) and CK-7 (Cell Signaling Technologies, MA, USA). The sections were further incubated with secondary antibody conjugated with horseradish peroxidase (Zhongshan Goldenbridge, Beijing, China) and were visualized with diaminobenzidine (Zhongshan Goldenbridge, Beijing, China) as the substrate.

**Cell Culture and Transient Transfection**
The human choriocarcinoma cell line, JEG-3, was purchased from American Type Culture Collection and cultured as previously described.11 The human trophoblast cell line HTR8/SVneo was kindly provided by Dr. C.H. Graham at Queen’s University, Canada, and cultured as previously described.7 Immortalized human cytotrophoblast cell line B6Tert-1 was cultured as previously described.54 After HTR8/SVneo cells were grown to 60%–70% confluence, transient transfection of small interfering (si)RNA or miRNA duplexes was carried out using Lipofectamine 2000 (Life Technologies, CA, USA) following the manufacturer’s protocol.

**Sequences and Constructs**
The siRNA duplex against human Smad2, mature miRNA mimics for miR-18a, mir-18a inhibitor, and the scramble control was designed and purchased from Shanghai GenePharma, China. The sequences of siRNA and miRNA are shown in Table 3.

| Table 3. The Sequences of siRNA and miRNA |
|------------------------------------------|
| Mimics | Sense (5’ to 3’) | Antisense (5’ to 3’) |
| Scramble control | UUCUCGGAACGUGUCAGGUTT | ACGUGACACGCUUGGAAGAATT |
| si-Smad2 | GGUUUCACUCCAAUGUATT | UAACAUUGGAGAGUAACCTA |
| hsa-miR-18a | UAAGGGUCAUCAUGGCGAGAUAG | AUCUGCAGUAAGCACCUCUAU |
| Inhibitor NC | CAGUAUUCUGUGUAGUACAA | |
| Inhibitor-18a | CUAUCUGCACUGAGCACCUCUA | |

To generate pMIR-REPORT Luciferase plasmids for Smad2(FL), 3’ UTR segments of human Smad2 mRNA (50–349 nt) containing putative miR-18a-binding sequences (136–142 nt) were amplified and cloned into pMIR-REPORT luciferase plasmid (Ambion, TX, USA) at the SpeI and HindIII sites. A point mutation was incorporated into the 139 nt of the 3’ UTR in the BD-WT plasmid to generate a BD-MUT construct. For the construction of the Smad2(FL)-expressing plasmid (pcDNA4-Smad2(FL)), the coding sequence of Smad2 was amplified and inserted into the pcDNA4.0 vector at the EcoRI and XhoI restriction sites. The sequences of primers for the construction of vectors are also listed in Table 2. All of the constructs were confirmed by DNA sequencing.

In addition, there exists a poorly conserved site in the 7,608–7,614 nt of the 3’ UTR of Smad2(FL) mRNA. The site type of this poorly conserved site is 7-mer-A1 but not 7-mer-m8, and several score values (such as context++ score, context++ score percentile) are too low. Therefore, we did not perform a luciferase assay to confirm this BD.

**Luciferase Assay**
For the luciferase assay, Htr8/SVneo cells (40,000 per well) were seeded into 24-well plates, 12 h before transfection. The cells were co-transfected with 80 ng of Smad2(FL) 3’ UTR luciferase reporter plasmid, 8 ng of Renilla luciferase reporter vectors (Promega, WI, USA), and 40 nM chemically synthesized, mature hsa-miR-18a or NC miRNAs with Lipofectamine 2000, according to the manufacturer’s protocol. Luciferase activity was assayed 48 h after transfection with a dual-luciferase reporter assay system (Promega, WI, USA) and was measured with a luminometer (Promega, WI, USA). All transfections were carried out with triplicate replicates in at least three independent experiments.
Transwell Invasion Assay
A Transwell insert invasion assay was conducted in 24-well fitted inserts (8 μm pore size; Millipore, MA, USA). Briefly, 48 h after transfection, HTR8/SVneo cells were treated with 10 μg/mL mitomycin C for another 2 h and were trypsinized and seeded into Transwell inserts, precoated with 20 μg Matrigel (BD Biosciences, NJ, USA) with 200 μL serum-free culture medium. Lower chambers were loaded with the same medium with 10% fetal bovine medium. After incubating for 28 h, cells on the upper surface of membranes were completely removed, and the migrated cells were fixed with 0.25% glutaraldehyde and stained with hematoxylin. The number of invaded cells was counted in the whole field of the membranes under light microscope. A cell-invasion index was presented as the percentage of cells that was counted in the whole.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium (MTT) Assay
HTR8/SVneo cells transfected with miR-18a or NC miRNAs for 48 h were trypsinized and plated on 24-well culture plates at a density of 3 × 10⁴ cells/well. Then after 24, 48, and 72 h, cells were incubated in 5 mg/mL MTT solution (Sigma Aldrich, CA, USA) for 4 h. After removal of MTT solution, dimethyl sulfoxide (DMSO) was added, and the absorbance at a wavelength of 490 nm (reference wavelength 690 nm) was recorded with a microplate reader (BioTek, VT, USA).

Cell-Cycle Analysis
As described by Xia et al.,⁵⁸ HTR8/SVneo cells transfected with miR-18a or NC miRNAs for 72 h were harvested with trypan blue (0.25% trypsin) and fixed with 70% ethanol overnight at 4°C. The cells were further incubated with 120 μg/mL RNase A (Sigma-Aldrich, CA, USA) and 120 μg/mL propidium iodide (Sigma-Aldrich, CA, USA) at room temperature for 30 min before cell-cycle analysis. Then the distribution of cells in the different phases of the cell cycle was determined with flow cytometry (BD Biosciences, NJ, USA) and analyzed by CellQuest software (BD Biosciences, NJ, USA).

TGF-β1 Treatment
To investigate whether miR-18a could interfere with the TGF-β1 signaling in human trophoblast cells, we measured p-Smad2 in the cells to monitor the activation of TGF-β1 signaling. After the transfection with miRNA duplexes for 24 h and then cell starvation for 24 h, cells were treated with 5 ng/mL TGF-β1 (R&D Systems, MN, USA) for 15 min before protein extraction.

We also investigated whether there existed an additive effect of miR-18a and TGF-β1 on cell invasion. Htr8/SVneo cells transfected with miRNA duplexes for 48 h were used for Transwell invasion assay, supplemented with or without 2.5 ng/mL TGF-β1.

Statistical Analysis
All statistical analyses were performed with Statistical Package for the Social Sciences software (version 17.0; SPSS, IL, USA). The comparison among groups was estimated by one-way ANOVA, and p <0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.09.019.

AUTHOR CONTRIBUTIONS
P.X., Y.W., C.P., Y.Z., and Y.-L.W. conceived and designed the experiments. P.X., Z.L., X.Y., X.S., and Y.-x.L. performed the experiments. Y.W. and Y.Z. contributed reagents/materials. P.X. and Y.-L.W. wrote the paper.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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