The Attenuation of Trophoblast Invasion Caused by the Downregulation of EZH2 Is Involved in the Pathogenesis of Human Recurrent Miscarriage

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Recurrent miscarriage (RM) is currently defined as two or more losses of a clinically established intrauterine pregnancy. Despite years of research, RM continues to be a clinically frustrating challenge for patients and physicians, and its etiology remains poorly understood. Accumulating evidence has suggested that epigenetic modifications are involved in early embryogenesis, and defects in epigenetic patterning contribute to the development of RM. Here, we studied the role of enhancer of zeste homolog 2 (EZH2) in the pathogenesis of RM and found that the EZH2 expression was significantly decreased in the villi from women with RM compared with that in control villi. EZH2 promoted the invasion of trophoblast cells. Moreover, EZH2 could promote epithelial-mesenchymal transition by epigenetically silencing CDX1. Both chromatin immunoprecipitation (ChIP)-PCR and dual-luciferase report assays demonstrated that EZH2 repressed CDX1 transcription via direct binding to its promoter region and then trimethylating Histone3-Lysine27. Furthermore, we discovered that progesterone, which is used extensively in the treatment of miscarriage and RM, increased the expression of EZH2 via the extracellular signaling-regulated kinase (ERK1/2) pathway. These findings revealed that EZH2 may regulate trophoblast invasion as an epigenetic factor, suggesting that EZH2 might be a potential therapeutic target for RM.

INTRODUCTION

Recurrent miscarriage is defined as two or more failed clinical pregnancies, which are verified by ultrasonography or histopathologic examination.1 Recurrent miscarriage (RM) is one of the most common clinical problems in reproduction, and it affects 2%–5% of childbearing couples.2 Common established etiology includes advanced maternal ages, uterine anomalies, immune dysregulations, hormonal and metabolic disorders, environmental factors, and cytogenetic abnormalities. However, less than 50% of couples with RM are diagnosed with an exact etiology.1 Previous studies have revealed that shallow or insufficient extravillous trophoblast (EVT) invasion is implicated in the pathogenesis of RM.4,5 In recent years, it has been increasingly appreciated that epigenetic factors, such as histone modification and DNA methylation, are involved in pregnancy outcomes through regulating trophoblast functions, which are responsible for the healthy development of the fetus.6–9

Histone modification is one of the major epigenetic mechanisms.5,10 Specific histone modifications confer active or repressive transcriptional states.11 For instance, the trimethylation of Histone3-Lysine27 (H3K27me3) is a marker of tightly packaged heterochromatin and is associated with gene repression.12 Together with DNA hypermethylation, transcriptional factors, and other regulatory subunits, H3K27me3 typically forms complexes to control target gene expressions.5 In human placental villi, most of the EVTs are H3K27me3-positive when stained by immunohistochemistry.12

Enhancer of zeste homolog 2 (EZH2), the catalytic subunit of polycomb repressive complex 2, mediates the transcriptional silencing of target genes through H3K27me3. EZH2 overexpression is associated with aggressiveness and advanced diseases in some cancers.13–16 Additionally, EZH2 has extensive roles in reproduction. It is a driver of epithelial-mesenchymal transition (EMT) in endometriosis, which impairs fertility.17 EZH2 is also essential for the early embryogenesis of mice.18 EZH2-deficient embryos display impaired...
growth potential and embryonic lethality in mice. However, the functions and mechanisms of EZH2 in human trophoblasts remain unknown.

Caudal-related homeobox transcription factor 1 (CDX1) plays essential roles in anteroposterior vertebral patterning and intestinal homeostasis. Increasing evidence demonstrates that CDX1 and CDX2 function as tumor suppressors. In addition, the CpG islands of the CDX1 promoter showed abnormal DNA methylation levels in pre-eclampsia patients compared with those from healthy cases. Moreover, CDX1 restricts the invasion of HTR-8/SVneo trophoblast cells by inhibiting MMP-9 expression. However, the relationship between CDX1 and RM, as well as the upstream epigenetic regulation of CDX1, has not been sufficiently addressed. In this study, we demonstrate that the expression of EZH2 was reduced in the villi of patients with RM, and its downregulation affected trophoblast invasion by inhibiting EMT via epigenetically repressing CDX1. We also found that progesterone (P4) leads to the upregulation of EZH2 through the extracellular signaling-regulated kinase (ERK1/2) pathway in trophoblasts.

**RESULTS**

**The Expression of EZH2 Is Decreased in the Chorionic Villous Tissues of RM Patients**

To determine whether EZH2 contributes to the pathogenesis of RM, we first conducted qRT-PCR and western blotting of the villous tissues in first trimester. At both mRNA and protein levels, EZH2 was significantly decreased in the villous tissues of RM patients compared with those of healthy controls (HCs) (Figures 1A and 1B). The immunohistochemical staining of paraffin-embedded villous tissues was performed to further investigate the abundance of EZH2. After staining, the strong expression of EZH2 was observed in the HC group, with the staining mainly locating in the cytotrophoblast (Figure 1Ca1), whereas a much weaker staining was detected in the RM group (Figure 1Cc1).

**EZH2 Promotes the Invasion of Trophoblast Cells**

To evaluate the role of EZH2 in the invasion of trophoblast cells, transwell assays were performed using the cell line JAR as a model of EVT, owing to the difficulty of acquiring primary EVT cells. First, the knockdown efficiency of EZH2 was tested by western blotting, and the results showed that both EZH2 and its effecter target H3K27me3 were significantly reduced (Figures 2A and 2B). Then, transwell assays revealed that EZH2 knockdown significantly decreased the invasion of trophoblast cells, and treatment with EPZ005687 (an EZH2 inhibitor) also led to a significant reduction in invasiveness (Figures 2C and 2D). Moreover, the overexpression of EZH2 promoted trophoblast invasion, which was in line with the knockdown and inhibition results (Figure 2E). Considering the link between EZH2 overexpression and tumor invasion and proliferation in cancers, we also conducted CCK-8 assays and found that the knockdown of EZH2 did not influence the proliferation of trophoblast cells (Figure 2F).
EZH2 Regulates the Transcription and Expression of E-Cadherin and CDX1

Because EZH2 promoted cell invasion of trophoblast cells, to determine the mechanisms, we screened 15 anti-invasion genes based on the literature regarding trophoblast cells by qRT-PCR, and we found that EZH2 knockdown significantly increased the mRNA levels of TIAM-1, KLF17, CDH1 (the gene encoding E-cadherin), and CDX1 (Figure 3A). Among these genes, the last two changed most significantly; consequently, we focused on the roles of E-cadherin and CDX1. Earlier studies from other groups suggest an inverse relationship between EZH2 and E-cadherin expression, and they demonstrate that E-cadherin is downregulated in EZH2-overexpressing cells through H3K27 trimethylation of the E-box regions in the CDH1 promoter. Therefore, we analyzed whether the decrease in EZH2 protein level could restore E-cadherin protein using western blotting. The results showed that, following the knockdown of EZH2 by siRNA transfection, E-cadherin protein in the trophoblast cells was significantly upregulated (Figures 3B and 3C). Additionally, the expression of N-cadherin protein was reduced simultaneously (Figures 3B and 3C).

Because CDX1 plays a pivotal role as a tumor suppressor, we focused on whether CDX1 is another candidate target gene of EZH2. In accordance with the above result (Figure 3A), EZH2 inhibitors, including GSK343 and EPZ005687, both increased the mRNA level of CDX1 in trophoblast cells (Figure 3D). To further confirm the results, western blotting was performed, and the result showed that silencing EZH2 upregulated the expression of CDX1 at the protein level (Figure 3E). In light of the inverse relationship between EZH2 and CDX1, it is interesting to find that the levels of CDX1 increased significantly in the villous tissues of RM patients compared with those of HC (Figure 3F). Linear correlation analysis also showed that the CDX1 mRNA level was negatively correlated with that of EZH2 in villous tissue (Figure 3G). Taken together, these data suggested that the downregulation of EZH2 promoted CDX1 expression.

EZH2 Inhibits the Transcription of CDX1

To examine whether EZH2 repressed CDX1 expression by physical DNA binding to the CDX1 promoter, we performed chromatin immunoprecipitation (ChIP) assays. The isolated DNA was subjected to qPCR using primer sets (Table 2) designed to amplify the region of the CDX1 promoter harboring the EZH2 binding sites (Primer1 and Primer2 in the diagrammatic sketch of Figure 4A). Among the primer sets, MYOD1 was used as a positive control (Figure 4B), whereas
GAPDH was utilized as a negative control (Figure 4E). We found that EZH2 was directly recruited to the putative EZH2-binding regions of the CDX1 promoter, and this recruitment was accompanied by the detection of high levels of the repressive marker H3K27me3 (Figures 4C and 4D). To further determine the repression of CDX1 by EZH2, we next conducted dual-luciferase report assays in JAR cells using the luciferase reporter plasmid containing a 2-kb CDX1 promoter and EZH2-overexpressing plasmid. The transient enhanced expression of wild-type EZH2 resulted in significant inhibition of CDX1 promoter activity (Figure 4F). Thus, we speculated that EZH2 might regulate CDX1 expression through direct binding at the CDX1 promoter. These results suggested that EZH2 may promote invasion in trophoblast cells through downregulation of CDX1.

The Overexpression of CDX1 Upregulates E-Cadherin Expression

A previous study demonstrated that CDX1 is one of the EMT-related genes; it could induce E-cadherin adhesion activity by reducing beta-catenin and p120 tyrosine phosphorylation. In a mouse tumor model, CDX1 knockout tumors lose the expression of the epithelial marker E-cadherin and gain the expression of vimentin, Twist1, Zeb1, and Zeb2, as is typical of EMT. Consistent with the above findings, the enhanced expression of CDX1 by the transient transfection of CDX1 plasmids upregulated E-cadherin protein in the trophoblast cells and reduced the expression of N-cadherin (Figures 5A and 5B). The transwell assays further showed that CDX1 overexpression significantly decreased the invasion of trophoblast cells. Additionally, CDX1 overexpression also enhanced EZH2 siRNA-mediated deficient cell invasion (Figures 5C and 5D).
Progesterone Upregulates EZH2 Expression through ERK1/2 Activation

Progesterone has been proposed and used extensively in the treatment of different reproduction-associated pathologies, such as miscarriages, RMs in early pregnancy, and the maintenance of pregnancy. A close association has been recently described in mammary physiology during pregnancy between progesterone and EZH2 expression. Therefore, we explored whether progesterone augments EZH2 expression in trophoblast cells. A western blot analysis revealed significant increases in the level of EZH2 after treatment with progesterone for 24 h. Meanwhile, a striking decrease in CDX1 expression was detected (Figures 6A and 6B).

Figure 4. EZH2 Regulated CDX1 Expression via Direct Binding to Its Promoter

(A) Schematic diagram of the CDX1 promoter region showing the location of CpG islands, the putative EZH2-binding region, and the spanning region of primers used for ChIP assay and PCR analysis. (B–E) Knockdown of EZH2 decreased the binding of both EZH2 and H3K27me3 on the promoter of CDX1. Immuno-precipitated DNA fragments were analyzed by qPCR with specific primer sets. OMoratin obtained from trophoblast cells was precipitated using antibodies against EZH2, histone H3, and IgG. Each ChIP experiment was repeated three times. MYOD1 (B) was positive control; Primer1 (C) and Primer2 (D) were for EZH2; and GAPDH (E) was negative control. (F) Overexpression of EZH2 decreased the transcriptional activity of CDX1 promoter, as detected by dual-luciferase report assays. Approximately 2 kb upstream of the transcriptional start site (TSS) on the promoter of CDX1 was cloned into pGL3 vector. Three plasmids were co-transfected into trophoblast cells for 48 h. Fluorescence measurement was performed using dual-luciferase report assays. RLuc was used for the internal control. All data are reported as the mean ± SEM. *p < 0.05; **p < 0.01. FLuc, firefly luciferase; GAPDH, negative control; MYOD1, positive control; RLuc, Renilla luciferase.

It is well-known that both the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway and the mitogen-activated protein kinase (MAPK)/ERK signal pathway are critical for the proliferation and invasion of trophoblast cells. Some researchers have previously highlighted evidence in some cancer cells suggesting that EZH2 may be regulated by the above pathways. Promoter analysis shows that three Elk-1 binding motifs are located within the EZH2 promoter, and the MEK-ERK1/2-Elk-1 pathway leads to EZH2 upregulation, as detected by dual-luciferase assays. In our study, after treatment with progesterone for 24 h, we found that ERK1/2 signaling was intensely activated, whereas AKT signaling was not (Figures 6C and 6D). To further determine the upstream signaling pathway mediating the ERK1/2-dependent expression of EZH2, we blocked the ERK1/2 signaling pathway using U0126 (20 μM) and found a significant reduction in EZH2 protein, followed by increased expression of CDX1 (Figures 6E and 6F). These data suggest that progesterone upregulated the expression of EZH2 through the ERK1/2 signaling pathway. The schematic diagram of EZH2 mediating the invasion of trophoblast is shown in Figure 6G.

DISCUSSION

The resemblance in the invasive behavior between placental trophoblasts and cancer cells and the role of aberrant epigenetic regulation in cancer development are well-known. Delicate control of trophoblast...

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invasion is important for implantation, uterine spiral artery remodeling, and maternal-fetal communication. Moreover, numerous studies have established a strong link between epigenetic regulation and placental trophoblast function by demonstrating the critical role of epigenetic regulators in maintaining a healthy pregnancy.

In this study, we investigated the role of EZH2 in the invasiveness of trophoblast. Our data showed that the expression of EZH2 both at mRNA and protein levels was significantly decreased in the villi of women who suffered from RM compared with those from normal pregnant women. The ChIP and dual-luciferase report assays showed that EZH2 could inhibit CDX1 transcription directly through trimethylation of H3K27 at the promoter of CDX1, which inhibited EMT in trophoblast. Thus, EZH2 could indirectly promote trophoblast EMT via CDX1. Moreover, we found that progesterone, which is used to treat or prevent early miscarriages and RM upregulated EZH2 expression via the ERK1/2 signaling pathway, which means the epigenetic mechanisms in the pathogenesis of RM have long been targeted for its therapy to reverse this disadvantage.

It is now well established that impaired trophoblast invasion is implicated in the etiology of pregnancy complications, including RM. In our present study, trophoblast invasion was attenuated by silencing of EZH2 or inhibiting of EZH2 enzyme activity with inhibitors. EZH2 overexpression also promoted invasion of trophoblasts. EZH2 can promote the invasion of malignant cancer cells by silencing target genes that are tumor suppressors. Among these genes, CDH1 (the gene that encodes E-cadherin) is putatively one of the targets. EZH2 facilitates tumor metastasis by directly repressing CDH1 transcription via methylating Histone3-Lysine 27 at the promoter region mainly through E-box regions. E-cadherin is a marker of epithelial cells and is of crucial importance in cancer invasion and metastasis. EMT, featured by reduced E-cadherin and increased N-cadherin expression, has been identified as a characteristic of aggressive tumors; it entitles cells to migratory and invasive capability in carcinogenesis. Previous studies have shown that EZH2 physically associates with CDH1 and
inhibits E-cadherin transcription via H3K27me3. In this study, EZH2 also repressed E-cadherin transcription and protein expression, which was accompanied by the upregulation of N-cadherin and proved that EZH2 directly promoted EMT.

CDX1 is also a tumor suppressor gene; the ablation of CDX1 and CDX2 results in a significant increase in tumor formation in the colon. CDX1 is regulated by epigenetic modification; the hypermethylation of the CpG island at the promoter is positively associated with severe clinical phenotypes. In addition, it is one of the EMT-related genes. CDX1 or CDX2 expression enhances E-cadherin-mediated cell-cell adhesion in human COLO 205 cells. In our study, we found there exists a physical association between EZH2 and H3K27me3 at the CDX1 promoter in trophoblast cells by ChIP-qPCR. Furthermore, CDX1 promoter activity significantly diminished after the overexpression of EZH2, indicating the methylation of H3-K27 by EZH2 at the CDX1 promoter and consequent suppression of CDX1. Moreover, the expression of CDX1 increased in the villous tissues of RM patients, which was opposite to the EZH2 expression. In addition, our study also showed that enhanced CDX1 expression inhibited invasion of trophoblast, upregulated E-cadherin, and downregulated N-cadherin, which means that the CDX1 expression inhibited EMT. Overall, we speculate that EZH2 could promote trophoblast invasion via the indirect promotion of EMT by inhibiting the CDX1 expression.

The PI3K/AKT and ERK1/2 signaling pathways are both verified to be involved in the progress of cellular growth, proliferation, and invasion. Whereas EZH2 expression is regulated by the AKT and ERK1/2 pathways, phos (p)-AKT and p-ERK1/2 proteins are less expressed in the RM group compared with the control group. In addition, previous research has reported that trophoblast invasion and migration could be promoted by progesterone, which is widely used in clinical practice. Furthermore, Pal et al. found that the expression and phosphorylation of EZH2 (Thr487) coincide with H3K27me3 modification and peak during pregnancy, which is driven by progesterone. Therefore, we examined p-AKT and p-ERK1/2 expression after progesterone treatment. Our work indicated that progesterone could activate the ERK1/2 pathway and, thus, significantly increase the expression of EZH2 accompanied with the decreased level of
CDX1, but not the PI3K/AKT signaling pathway. This is in accordance with the results that report that progesterone is able to induce phosphorylation of ERK1/2 in two choriocarcinoma cell lines (BeWo and JEG-3)\(^{80}\) and is consistent with the reports demonstrating that the phosphorylation of EZH2 at serine 21 suppresses its methyltransferase activity responsible for H3-K27 trimethylation.\(^{50,51}\) The decreased expression of EZH2 in women with RM provides new insights into the epigenetic mechanisms underlying the pathogenesis of RM and paves new avenues for the development of novel therapeutics.

**MATERIALS AND METHODS**

**Villous Samples Collection**

Twenty-one patients with RM between the ages of 23 and 35 years (mean age 29.0 ± 3.4 years) who had been treated at the Department of Obstetrics and Gynecology of Renji Hospital between July 2016 and September 2016 were recruited for this study. Among these RM patients, 13 patients had two miscarriages, and 8 patients had three miscarriages. Twenty-two cases of RM at the ages of 23 and 35 years (mean age 28.1 ± 4.5 years) with healthy early pregnancies that were artificially terminated for nonmedical reasons were included as HCs. The gestational ages when the pregnancy was terminated for nonmedical reasons were artifically terminated for nonmedical reasons were included was 18.8 ± 4.5 weeks. Twenty-one patients with RM between the ages of 23 and 35 years (mean age 29.0 ± 3.4 years) who had been treated at the Department of Obstetrics and Gynecology of Renji Hospital between July 2016 and September 2016 were recruited for this study. Among these RM patients, 13 patients had two miscarriages, and 8 patients had three miscarriages. Twenty-two cases of RM at the ages of 23 and 35 years (mean age 28.1 ± 4.5 years) with healthy early pregnancies that were artificially terminated for nonmedical reasons were included as HCs. The gestational ages when the pregnancy was terminated for nonmedical reasons were artifically terminated for nonmedical reasons were included was 18.8 ± 4.5 weeks. Among these RM patients, 13 patients had two miscarriages, and 8 patients had three miscarriages. Twenty-two cases of RM at the ages of 23 and 35 years (mean age 28.1 ± 4.5 years) with healthy early pregnancies that were artificially terminated for nonmedical reasons were included as HCs. The gestational ages when the pregnancy was terminated for nonmedical reasons were artifically terminated for nonmedical reasons were included was 18.8 ± 4.5 weeks.

**Western Blot Analysis**

Total protein was isolated from villous tissues and cultured JAR cells using ice-cold radio immunoprecipitation assay (RIPA) (Beyotime Biotechnology, Haimen, China) containing protease inhibitor cocktail tablets and phosphatase inhibitor cocktail tablets (Roche, Basel, Switzerland). Twenty-five micrograms of protein was electrophoresed in SDS-polyacrylamide gels and transferred onto nitrocellulose filter membranes (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). Then the membranes were incubated with primary antibodies at 4°C overnight. After extensive washing, the bound primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (1:5,000; Proteintech) with a chemiluminescent detection system. The primary antibodies used were as previously described.\(^{56}\) In brief, the slides were deparaffinized, rehydrated in water, and washed in PBS thrice. After inactivation of endogenous peroxidase activity and antigen retrieval, the sections were subjected to incubation with 0.3% Triton X-100 for 20 min. After blocking with immunoglobulin G and incubation with the primary antibody overnight at 4°C, the samples were handled using a two-step plus Poly-HRP Anti-Mouse/Rabbit IgG Detection System (PV-9000; ZSGB-BIO, Beijing, China). Finally, the slides were counterstained with hematoxylin, dehydrated with 95% alcohol, and mounted in neutral balsam. Negative controls were treated with nonimmune serum instead of the primary antibody. The antibody against EZH2 used for immunohistochemistry was the same as that used for western blotting.

**qPCR Analysis**

The total RNA was prepared using an animal total RNA isolation kit (Foregene, Chengdu, China) according to the manufacturer’s instructions. After reverse transcription, the cDNAs were amplified on an ABI 7300 Real-Time PCR System (Applied Biosystems) under the following conditions: initial denaturation for 3 min at 95°C followed by 40 cycles of denaturation at 95°C for 40 s, annealing at 59°C for 40 s and extension at 72°C for 20 s, and a final extension for 5 min at 72°C. Beta-actin was used for normalization. The results were analyzed using the ΔΔCt method. The sequences of the primers are given in Table 1.

**Transwell Assay**

Cell invasion was measured using Matrigel precoated transwell inserts (24-well plates; Corning Life Sciences, Tewksbury, MA, USA), as previously described.\(^{56}\) In brief, the inserts (8-mm pore size; Corning) were precoated with 25 μL of 1:8 diluted Matrigel. After transfection with NC or siEZH2, EZH2 or vector plasmids (GV230-EZH2 or GV230, kindly provided by Rujuan Zuo, Shanghai Jiaotong University, China),\(^{57}\) CDX1 or vector plasmids (pIRES2-CDX1 or pIRES2) for 48 h, or treatment with EPZ005687 (20 μM; Selleckchem, Houston, TX, USA) for 24 h, the cells (1 × 10^5 in 200 μL of DMEM/F12 containing 1% FBS) were cultured in 1% FBS media and seeded in the inserts. Then the inserts were placed into 24-well plates containing 600 μL of 20% FBS media in each well, and the cells were incubated for 24 h. The inserts were then removed and washed in PBS, and the noninvasive cells, together with the Matrigel, were removed from the upper surface of the filter by wiping with a cotton bud. The inserts were then fixed in methanol for 10 min at room temperature and stained with crystalline iodine. The cells were observed under a fluorescence microscope (Zeiss, Germany). The cells that migrated to the lower surfaces were counted in five predetermined fields at a magnification of 100×. The ratio of the number of cells in the treated group to the number of cells in the control group was calculated as the “relative invasion.” Each experiment was carried out in triplicate and repeated at least three times. The sequences of EZH2 siRNA and CDX1 overexpression plasmids are in Tables S1 and S2.
Table 1. Primer Sequences for qRT-PCR

| Gene     | Primers                                                                 | Size (bp) |
|----------|-------------------------------------------------------------------------|-----------|
| EZH2     | F: 5'-TTTCTGGCAGACCCACACT-3'                                             | 96        |
|          | R: 5'-CTCGTCTCAACTGATCGGT-3'                                             |           |
| CDX1     | F: 5'-CCAAAGCGGGGCAAAAGGA-3'                                             | 102       |
|          | R: 5'-GCTTTGAGGCGGCTTAGGG-3'                                             |           |
| ACTB     | F: 5'-GGGAAATCTGCTGGACATTAG-3'                                           | 275       |
|          | R: 5'-GTTGTTGGCTAAGGCTTCTTG-3'                                           |           |
| TIMP2    | F: 5'-AGCATTTGAACCGAGTTGGA-3'                                            | 64        |
|          | R: 5'-CCAAAGGAAGAGCCTGAGG-3'                                             |           |
| TIMP3    | F: 5'-CTGGCAACTCCTGACATGG-3'                                             | 121       |
|          | R: 5'-TTTGGAGGCTCTGGATACAT-3'                                            |           |
| TIMP4    | F: 5'-GCTGGTGGAGGATCGACTG-3'                                             | 119       |
|          | R: 5'-CAGGTTCTCGAAGCTGCCGGA-3'                                           |           |
| TIAM1    | F: 5'-TGCATCTGCACTATCCTGAGG-3'                                           | 206       |
|          | R: 5'-GGCTGTCTCCATCTGACC-3'                                              |           |
| RAC1     | F: 5'-CTGCCAGCGCCTGAGG-3'                                                | 197       |
|          | R: 5'-ATGATTGCTGTGGTAATGTGATC-3'                                         |           |
| KKIP     | F: 5'-CTCACCCCTTGCTGAGCAGA-3'                                            | 138       |
|          | R: 5'-GAGCCGCAAGCATAAGCGGAA-3'                                           |           |
| SERPINB1 | F: 5'-TCAGCTGTCGCCAGGTTCAAACTG-3'                                         | 300       |
|          | R: 5'-GGATGCTACCTGAGGAAATTAGG-3'                                         |           |
| SERPINB2 | F: 5'-GCTGGGAAGATGATGTCAGTGTC-3'                                         | 300       |
|          | R: 5'-GGCTGTTGGAAACCTCTGCAAG-3'                                          |           |
| SERPINB5 | F: 5'-CATGGAGGGCAGCTGTGTTATG-3'                                          | 417       |
|          | R: 5'-CTGCCACCTGCTAGGATCAC-3'                                            |           |
| KLF2     | F: 5'-AGAGGGTTCTCTCGAGATTGAC-3'                                          | 193       |
|          | R: 5'-TCTCCCAACAGCTCAGCAGAG-3'                                           |           |
| KLF17    | F: 5'-GGCTCAGGAGGTCACACTCCTT-3'                                          | 74        |
|          | R: 5'-CAGACATCTCCTGCGCTGGA-3'                                            |           |
| BRMS1    | F: 5'-AAGGACACCTCTGTGGCTGGA-3'                                           | 146       |
|          | R: 5'-TGTTGACAGGCGGTCAGGAG-3'                                            |           |
| CDH1     | F: 5'-GGGTGTGAGGAGGAAAATAGG-3'                                           | 119       |
|          | R: 5'-GCGAGATGCCTCAGGACCGG-3'                                            |           |

ChIP Assay

In brief, the cells were harvested at 80% confluence in a 10-cm dish after 0.25% trypsin-EDTA digestion, washed twice with ice-cold PBS containing protease inhibitors, and centrifuged at 2,500 rpm for 3 min. The cell pellet was resuspended in buffer I (15 mM Tris-HCl [pH 7.5], 60 mM KCl, 5 mM MgCl2, 0.1 mM EGTA, and 0.3 M sucrose) and 250 μL of lysis buffer (15 mM Tris-HCl [pH 7.5], 60 mM KCl, 5 mM MgCl2, 0.1 mM EGTA, 0.3 M sucrose, 0.5% Nonidet P-40 [NP-40], and 1% sodium deoxycholate). Then, the mixture was lysed on ice for 10 min. After addition of 500 μL of MNase buffer (85 mM Tris-HCl [pH 7.5], 60 mM KCl, 5 mM MgCl2, 0.1 mM EGTA, and 0.3 M sucrose) containing 2 μg/mL BSA and 4000 U/mL Micrococcal Nuclease (M0247; NEB) per immunoprecipitation (IP) prep, the nuclei were then subjected to digestion for 10 min at 37°C, with frequent inversion every 3–5 min. The digestion was stopped by the addition of EDTA to a final concentration of 20 mM. The fragmented chromatin DNA was isolated by centrifugation at 12,000 rpm for 10 min at 4°C, then was immunoprecipitated with the antibodies of interest at 4°C overnight. The immune complexes were collected with protein A/G agarose beads (Santa Cruz) by incubating for 2 h at 4°C. The beads were first washed with washing buffer and were then eluted with elution buffer at 65°C for 10 min twice. The DNA was then subjected to RNase A treatment at 37°C for 30 min and was purified by Proteinase K digestion at 65°C for 2 h, followed by phenol-chloroform (Sigma) extraction and ethanol precipitation. The purified DNA was dissolved in sterile double-distilled water for further qPCR analysis. Similar amounts of fragmented DNA without precipitation were used as inputs. The primers designed for ChIP-qPCR are listed in Table 2. The primers for the GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) promoter were used as a negative control, and MYPD1 (Myoblast determination protein 1) was used as a positive control. qPCR results were analyzed using the following equation to express the abundance of the proteins enriched at the respective promoters: Percent input = 100% × 2^(-ΔΔCt) [Input Sample - C] [IP Sample].

Transient Transfection Assay and Dual-Luciferase Assay

The human placent choriocarcinoma cell line (JAR) was obtained from Shanghai Cellular Library of Chinese Academy of Sciences; the verification of the JAR cell line is supplied in the Supplemental Information. JAR cells were seeded in a 24-well culture plate in triplicate and cotransfected with EZH2 or vector plasmids (GV230-EZH2 or GV230) and CDX1-promoter plasmids (pGL3-CDX1-promoter; Transheep, Shanghai, China), along with phRL-TK plasmids (Transheep), which were used as internal controls. The ratio of the plasmid DNA (EZH2 [or Vector]:CDX1-promoter:phRL-TK) was 4:5:1. After 48 h of transfection by Lipofectamine 3000 Reagent (1.8 μL/well; Invitrogen), the cells were lysed for the measurement of luciferase activity using a Dual-Luciferase Reporter Assay System (E1910; Promega). CDX1-promoter-driven firefly luciferase activity was normalized for transfection efficiency by comparing with Renilla luciferase activity (indicated as “FLuc ratio to RLuc”). The sequences of CDX1-Luc plasmids are in Table S3.

Cell Culture and Treatment

JAR cells were maintained in six-well plates and cultured in phenol red-free DMEM/F-12 containing glucose (GIBCO, Grand Island, NY, USA), 1% Insulin-Transferrin-Selenium (Invitrogen, Carlsbad, CA, USA), 5 × 10^{-2} g/L antibiotics (GIBCO), and 10% charcoal-stripped FBS (Biological Industries, Beit Haemek, Israel) at 37°C in 5% CO2. After treatment with 10^{-3} M progesterone (Sigma Chemical, St. Louis, MO, USA) for 24 h, the cells were lysed with ice-cold RIPA for further analysis. For transient transfection, when cells reached 60%–70% confluence, synthetic siRNAs (GenePharma, Shanghai, China) or constructed plasmids were transfected using the Lipofectamine 3000 transfection kit (Invitrogen, Carlsbad, CA, USA) for 48 h according to the manufacturer’s instructions.
**Table 2. ChIP-qPCR Primer Sets**

| Gene      | Primers for ChIP | Size |
|-----------|------------------|------|
| MYOD1     | F: 5’-CGGCGTGAAGCTAAATGAG-3’<br>R: 5’-CAGGTGGAGCCCGCTG-3’ | 82   |
| CDX1-Primer1 | F: 5’-AGTTTCCTGTAAAGTCGCCGAAAG-3’<br>R: 5’-AAATGCTCAGGAGCCTTG-3’ | 80   |
| CDX1-Primer2 | F: 5’-AGCACAGGATACCTTCTCTCTG-3’<br>R: 5’-GGAGGCCCTGAGGATCCTG-3’ | 110  |
| GAPDH     | F: 5’-TACAGCGGTCTTTACGGCCG-3’<br>R: 5’-TCGAACAGGAGGAGCAGAGCGA-3’ | 166  |

**Statistical Analysis**
The statistical analysis was conducted using SPSS statistical software (version 22.0; IBM, Chicago, IL, USA). The differences were analyzed using an independent sample t test between two groups. Comparison among multiple groups was carried out by a one-way ANOVA followed by Tukey’s post hoc test. The data are reported as the mean ± SEM. A value of p < 0.05 was considered statistically significant.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes three tables and one STR profile report and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.12.011.

**AUTHOR CONTRIBUTIONS**
S.L. and C.Z. designed the experiments, which were conducted by S.L., N.W., H.L., J.Y., J.L., and C.Z. S.L., N.W., H.L., J.Y., J.L., and C.Z. wrote the manuscript. S.L., W.-P.L., and C.Z. edited the manuscript.

**CONFLICTS OF INTEREST**
The authors declare no competing interests.

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