Abstract—Preeclampsia is a pregnancy-specific syndrome manifested by the onset of hypertension and proteinuria after the 20th week of gestation. Abnormal placenta development has been generally accepted as the initial cause of the disorder. Recently, microRNA-210 (miR-210) has been found to be upregulated in preeclamptic placentas compared with normal placentas, indicating a possible association of this small molecule with the placental pathology of preeclampsia. However, the function of miR-210 in the development of the placenta remains elusive. The aim of this study was to characterize the molecular mechanism of preeclampsia development by examining the role of miR-210. In this study, miR-210 and potassium channel modulatory factor 1 (KCMF1) expressions were compared in placentas from healthy pregnant individuals and patients with preeclampsia, and the role of miR-210 in trophoblast cell invasion via the downregulation of KCMF1 was investigated in the immortal trophoblast cell line HTR8/SVneo. The levels of KCMF1 were significantly lower in preeclamptic placenta tissues than in gestational week–matched normal placentas, which was inversely correlated with the level of miR-210. KCMF1 was validated as the direct target of miR-210 using real-time polymerase chain reaction, Western blotting, and dual luciferase assay in HTR8/SVneo cells. miR-210 inhibited the invasion of trophoblast cells, and this inhibition was abrogated by the overexpression of KCMF1. The inflammatory factor tumor necrosis factor-α could upregulate miR-210 while suppressing KCMF1 expression in HTR8/SVneo cells. This is the first report on the function of KCMF1 in human placental trophoblast cells, and the data indicate that aberrant miR-210 expression may contribute to the occurrence of preeclampsia by interfering with KCMF1-mediated signaling in the human placenta. (Hypertension. 2014;64:00-00.) • Online Data Supplement

Key Words: KCMF1 ■ miR-210 ■ placenta ■ preeclampsia ■ TNF-a

Complicating 7% to 10% of pregnancies,1 preeclampsia, along with other hypertensive disorders of pregnancy, is a major contributor to maternal morbidity worldwide.2,3 Although abnormal placentation is thought to play a major role in the development of preeclampsia, the pathogenesis of this disorder is not clearly understood.1 Defects of trophoblast cell function, such as reduced proliferation,4 excessive apoptosis,5 aberrant differentiation,6 limited migration and invasion of the uterus, and poor remodeling of spiral arteries,7,8 have been considered to be associated with preeclampsia. Nevertheless, the molecular mechanisms underlying the onset and progression of preeclampsia remain largely unknown.

MicroRNAs are 22- to 24-nucleotide noncoding RNAs that regulate gene expression by seed sequence pairing with the 3′-untranslated region (UTR) of target mRNAs, leading to repressed translation or induced mRNA cleavage of the target genes.9,11 MicroRNAs have been identified as essential mediators of numerous cellular processes,12 potentially including the response to hypoxia. In particular, microRNA-210 (miR-210) is specifically induced by hypoxia-inducible factor-1α during hypoxia13 and regulates many hypoxia response pathways, including cell survival,14 angiogenesis, mitochondrial metabolism,15 and DNA repair.16 miR-210 can target and suppress a large number of genes, including the cell cycle regulator E2F transcription factor 3,17 the receptor tyrosine kinase ligand ephrinA3,18 the DNA repair protein RAD52,19 and the iron-sulfur cluster assembly proteins 1/2.15 Recent studies revealed that miR-210 was upregulated in patients with preeclampsia20–24 and demonstrated that hypoxia-inducible miR-210 might participate in the occurrence of preeclampsia via the downregulation of ephrin-A3 and homeobox-A9.24 The repressive effect of miR-210 on primary trophoblast cell invasion was also reported.25 In addition, the study by us and others revealed that miR-210 could be a potential serum biomarker...
for preeclampsia.21,25 Despite these findings, the details of the role played by miR-210 in preeclampsia development remain largely unknown.

We performed bioinformatic analyses to predict target genes for miR-210 using 4 different programs, miRanda, TargetScan, miRBase, and PicTar, and found that potassium channel modulatory factor 1 (KCMF1) is one of the commonly predicted targets of miR-210. KCMF1 is a 42-kDa zinc finger protein comprising 381 amino acids with a high serine and threonine content. In 2001, KCMF1 was first mentioned to be potential in the regulation of potassium channels in Aplysia californica.26 Shortly thereafter, KCMF1 was demonstrated to be downregulated in the Ewing sarcoma cell lines after the overexpression of CD99.27 As a result of its upregulation by fibroblast growth factor receptor 2 signaling pathways in gastric cancer cells, KCMF1 was also named FGIC (basic fibroblast growth factor–induced gene in gastric cancer).28 Interestingly, KCMF1 could enhance proliferation, migration, and invasion in epithelial cancers, especially in human and mouse pancreatic cancer.29 However, the function of KCMF1 in the placenta remains unclear.

Based on the evidence noted above, we propose that miR-210 might regulate trophoblast cell invasion by targeting KCMF1 and that the pathway may play a role in the development of preeclampsia. To test this hypothesis, we used human trophoblast cell line, HTR8/SVneo, to investigate the mechanisms by which miR-210 exerts its function via the downregulation of KCMF1. We also explored the association of aberrant miR-210 and KCMF1 expression during abnormal placentation in preeclampsia.

Materials and Methods

Detailed Materials and Methods are provided in the online-only Data Supplement.

Patients and Placental Collection

Placental samples, delivered at 36 to 40 weeks, were collected from 15 severe preeclamptic patients and 26 normal pregnant women in the Department of Obstetrics and Gynecology, Peking University Third Hospital, China. Severe preeclampsia was defined according to the definition in Williams Obstetrics (23rd edition). Briefly, these patients had no history of pre-existing or chronic hypertension but exhibited systolic blood pressure ≥160 mm Hg or diastolic blood pressure ≥110 mm Hg on ≥2 occasions, accompanying severe proteinuria (urinary protein excretion, ≥2 g/24 hours) after the 20th week of gestation. Patients with renal disease, transient hypertension in pregnancy, gestational diabetes mellitus, spontaneous abortion, intrauterine fetal death, fetal chromosomal or congenital abnormalities, or pregnancies conceived by fertility treatment were excluded from this study. The blood pressure of all patients returned to normal, and symptoms of proteinuria disappeared 6 weeks postpartum. The placentas were collected within 1 hour of cesarean birth, and specimens at the chorionic plate and basal plate were separately taken from the placenta disc near the position of umbilical cord insertion. The specimens were snap-frozen in liquid nitrogen and subjected to RNA extraction. The clinical characteristics of the patients enrolled in this study are summarized in the Table.

Statistical Analysis

All experiments were repeated ≥3 times. The results are expressed as the mean±SEM. Statistical analysis was performed using 1-way ANOVA or 2-sided Student t test, and differences with P<0.05 were considered statistically significant. All statistical analyses were performed using SPSS Statistics 22.0 for Windows (IBM, Armonk, NY).

| Table. Clinical Characteristics of the Pregnant Women Enrolled in This Study |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Characteristics | Normal Pregnancy | Preeclampsia | P Value |
| Maternal age, y | 31.6±0.9 | 29.3±1.3 | 0.153 |
| BMI, kg/m² | 22.0±0.2 | 21.7±0.8 | 0.787 |
| Systolic blood pressure, mm Hg | 115.7±1.1 | 156.2±3.5* | <0.001 |
| Diastolic blood pressure, mm Hg | 76.0±1.1 | 100.8±2.1* | <0.001 |
| 50 g GCT, mmol/L | 7.4±0.3 | 6.6±0.3 | 0.138 |
| 24-h urine protein, g | 0.03±0.02 | 4.2±0.41* | <0.001 |
| Primiparous percentage, % | 71.8 | 61 | NA |
| Gestational day at delivery, d | 267.5±1.5 | 255.7±1.6 | 0.87 |
| Infant birth weight, g | 3544±98 | 2588±161* | 0.004 |

Data are shown as the mean±SEM, and significant difference between groups was analyzed with 1-way ANOVA. BMI indicates body mass index, indicating the weight in kilograms divided by the square of the height in meters; and GCT, glucose challenge test.

*Compared with normal pregnancy, P<0.05.

Results

Expressions of KCMF1 and miR-210 Exhibited an Inverse Correlation in Placentas From Patients With Severe Preeclampsia

We examined KCMF1 and miR-210 expression levels in control and severe preeclamptic placentas using quantitative real-time polymerase chain reaction. Similar to our previous report, the level of miR-210 was significantly higher in the basal plate of severe preeclamptic placentas than in that of the normal pregnant controls (Figure 1A; P<0.05); however, its level in the chorionic plate seemed to be the same between the 2 groups (Figure 1B).

The mRNA level of KCMF1 was significantly lower in both the basal plate and the chorionic plate of severe preeclamptic placentas than in the corresponding control groups (Figure 1C and 1D; P<0.05). The KCMF1 protein level, as examined using Western blotting, also decreased in severe preeclamptic placentas (Figure 1E and 1F). Correlation analysis revealed an inverse correlation in miR-210 and KCMF1 expressions in the placental basal plate of the studied individuals (Figure 1G).

Localization of KCMF1 in Human Placenta

As shown in Figure 2, immunohistochemistry revealed that KCMF1 localized in both the nucleus and cytoplasm in various subtypes of trophoblast cells, including villous cytotrophoblasts, column trophoblast cells, interstitial extravillous trophoblast cells, and endovascular trophoblast cells.

Validation of KCMF1 as the Direct Target of miR-210 in Human Trophoblast Cells

As shown in Figure 3A and 3B, the levels of both KCMF1 mRNA and protein were ≈40% and 60% lower in HTR8/SVneo cells transfected with miR-210 mimics (miR-210) than the scramble control cells. Bioinformatics analysis reveals that KCMF1 is a potential target gene of miR-210. The seed sequence of miR-210 is complementary to the 554- to 561-nucleotide sequence of the 3′-UTR in KCMF1 mRNA. As shown in Figure 3C, we constructed a luciferase reporter vector by cloning a 240-bp DNA fragment, including binding site (BD) of the 3′-UTR in human KCMF1 mRNA.
downstream of the firefly luciferase reporter gene (the reporter vector was named BD-WT). A point mutation was incorporated into the binding sites of the 3′-UTR in the KCMF1 gene to generate BD-MUT reporter vector. As shown in Figure 3D, miR-210 mimics significantly reduced the relative luciferase activity of the BD-WT construct by ≈40% compared with the scramble control but did not influence the luciferase activity of the BD-MUT construct. These data strongly suggest that KCMF1 can be the target gene of miR-210 in human trophoblast cells and that the 554- to 561-nucleotide region of the 3′-UTR in the KCMF1 gene is the real binding site for miR-210.

Effects of miR-210 on Cell Invasion in HTR8/SV neo Cells

The effect of miR-210 on trophoblast cell invasion was further examined. Data of quantitative real-time polymerase chain reaction showed that the level of miR-210 increased to ≈400x that of the scramble control cells after miR-210 mimics transfection (Figure 4A) and decreased by ≈70% by miR-210 inhibitor compared with the inhibitor scramble control cells (Figure 4B). As shown in Figure 4C and 4D, transfection with miR-210 mimics or inhibitors could clearly repress or induce the cell invasiveness in HTR8/SVneo cells. To avoid any influence of cell growth on the results of the cell invasion assay, we treated the cells with mitomycin C when conducting the
miR-210 is one of the significantly overexpressed microRNAs participating in the pathogenesis of preeclampsia. We treated HTR8/SVneo cells with recombinant human TNF-α and examined miR-210 and KCMF1 levels after 24 hours. As shown in Figure 1, TNF-α significantly increased the expression of miR-210 by 1.8-fold over the control (Figure 1A), whereas it decreased KCMF1 mRNA and protein levels to 60% to 70% of the control (Figure 1B and 1C). The inverse change pattern of miR-210 in KCMF1 mRNA was specifically responsive to miR-210 overexpression, as shown by inhibition of the luciferase activity of the BD–wild type (WT), and mutant construct is shown as BD-MUT (*, mutation sites). The luciferase assay in HTR8/SVneo cells transfected with the BD-WT and BD-MUT reporter constructs together with miR-210 or NC. The results are presented as the means±SEM according to 3 independent experiments. **Compared with corresponding NC, P<0.01. CMV indicates cytomegalovirus.

Discussion

Several investigators have reported that some microRNAs are irregularly expressed in preeclamptic placentas compared with normal placentas. Thus far, it has remained unclear how the deregulated microRNAs participate in the pathogenesis of preeclampsia. miR-210 is one of the significantly overexpressed small RNA in preeclamptic placentas. Until now, functional studies on miR-210 have mainly focused on its role in cancer. The reasons that miR-210 attracted our attention in the study of placentation development were as follows: (1) In our previous study, we found that the levels of miR-210 increased in the plasma of preeclamptic individuals early in gestation (15–18 weeks), indicating its possible participation in the cause of preeclampsia at early gestation. (2) Target prediction results show that KCMF1 is one of the possible targets of miR-210, and the predicted binding site in the 3′-UTR of KCMF1 mRNA is evolutionarily conserved from amphibians to humans. The evidence suggests that the regulation of KCMF1 by miR-210 might be conserved during evolution. (3) KCMF1 and miR-210 were all localized in various subtypes of human trophoblast cells. The KCMF1 and miR-210 expressions in the basal plate of preeclamptic placentas exhibited inverse association. The placental basal plate mainly consists of invasive interstitial trophoblast cells, indicating that miR-210–KCMF1 pathway may be involved in regulating trophoblast invasion.

Target exploration is critical for clarifying the mechanism of microRNAs. Several lines of evidence in this study prove that KCMF1 is a functional target of miR-210 in human trophoblasts. First, KCMF1 expression could be suppressed by miR-210 in HTR8/SVneo cells. Second, the luciferase report construct containing the putative miR-210–binding sequence in KCMF1 mRNA was specifically responsive to miR-210 overexpression, as shown by inhibition of the luciferase activity. The inhibitory effect of miR-210 on the luciferase activity was abolished on mutation of the binding sites paired by the seed sequence of miR-210. Third, functional study in HTR8/SVneo cells transfected with NC and miR-210 mimics (miR-210). **Compared with NC, P<0.01. B, Western blot analysis to show KCMF1 protein levels in HTR8/SVneo cells transfected with NC and miR-210. Left, A typical Western blotting result; Right, Bar chart showing the statistical results based on 3 independent experiments. The density of KCMF1 was adjusted based on that of GAPDH in the same blot, and the values are presented as the means±SEM. **Compared with NC, P<0.01. C, Schematic map of the luciferase assay constructs. The construct containing the region complementary to the seed sequence for miR-210 in the 3′-untranslated region (UTR) segment of the human KCMF1 gene is shown as BD–wild type (WT), and mutant construct is shown as BD-MUT (*, mutation sites). D, Luciferase assay in HTR8/SVneo cells transfected with the BD-WT and BD-MUT reporter constructs together with miR-210 or NC. The results are presented as the means±SEM according to 3 independent experiments. **Compared with corresponding NC, P<0.01. CMV indicates cytomegalovirus.

Figure 3. Validation of potassium channel modulatory factor 1 (KCMF1) as a target gene of microRNA-210 (miR-210) in HTR8/SVneo cells. A, Quantitative real-time polymerase chain reaction to show KCMF1 mRNA levels in HTR8/SVneo cells transfected with scramble control (NC) and miR-210 mimics (miR-210). **Compared with NC, P<0.01. B, Western blot analysis to show KCMF1 protein levels in HTR8/SVneo cells transfected with NC and miR-210. Left, A typical Western blotting result; Right, Bar chart showing the statistical results based on 3 independent experiments. **Compared with NC, P<0.01. C, Schematic map of the luciferase assay constructs. The construct containing the region complementary to the seed sequence for miR-210 in the 3′-untranslated region (UTR) segment of the human KCMF1 gene is shown as BD–wild type (WT), and mutant construct is shown as BD-MUT (*, mutation sites). D, Luciferase assay in HTR8/SVneo cells transfected with the BD-WT and BD-MUT reporter constructs together with miR-210 or NC. The results are presented as the means±SEM according to 3 independent experiments. **Compared with corresponding NC, P<0.01. CMV indicates cytomegalovirus.
miR-210 in human trophoblasts. The invasion-repressing effect of miR-210 that was observed in HTR8/SVneo cells could, at least in part, result from the interference with the signaling mediated by KCMF1. Abnormal placental development, especially the shallow invasion of trophoblasts into the decidual stroma and spiral arteries during early gestation, has been generally accepted as the major causal factor for preeclampsia. The findings in the present study provided evidence to indicate the involvement of miR-210–KCMF1 pathway in the shallow trophoblast cell invasion in preeclamptic placenta.

Functional studies of miR-210 have been largely performed in cancer cells, and the data demonstrated its participation in regulating various cell behaviors, including proliferation, migration, apoptosis, differentiation, DNA repair, and cell metabolism. Interestingly, the function of miR-210 could be opposite in different cell types being tested. For instance, miR-210 could target proteins that are crucial for the cell cycle, such as E2F transcription factor 3, fibroblast growth factor receptor–like 1, or homeobox A1, resulting in the inhibition of cell growth in epithelial cells, human esophageal squamous cell carcinoma HE3 cells, or FaDu cells. However, it had growth-stimulating effect in HCT116 Dicerex5, RKO Dicerex5, and DLD-1 Dicer ex5 cells via targeting the v-myc avian myelocytomatosis viral oncogene homolog antagonist MAX network transcriptional repressor. Human placental trophoblast cells possess similar properties to tumor cells in terms of proliferation and invasion, although their behaviors are temporally and spatially restricted during pregnancy. We did not find any influence of miR-210 on cell proliferation in HTR8/SVneo cells. Our finding of the inhibition on human trophoblast cell invasion by miR-210 was consistent with the recent reports by Zhang et al and Anton et al but were contradictory to the observations in endothelial cells and renal carcinoma cells. It is most likely that miR-210 works in a unique way in human trophoblast cells, different from that in cancer cells. Considering the fact that 1 microRNA may regulate diverse targets in different cellular contexts, experimental validation of miR-210 targets in trophoblast cells remains to be...
advanced, although large amounts of the targets for this small RNA have been described in cancer cells and other cell types.

The mechanism of miR-210 upregulation in the preeclamptic placenta remains elusive. The vascular remodeling at the failed placental site has been the target of intense scrutiny in studies of preeclampsia. It seems that the remodeling of spiral arteries is largely a result of cytotrophoblast invasion. The cellular control of trophoblast invasion depends on interactions between the maternal decidua and the fetal trophoblast, and immune maladaptation is an important factor that contributes to the inadequate invasion of cytotrophoblasts into the uterine decidua. Local oxygen tension and immune- or inflammatory-mediated interactions are primary determinants of the process. Several cytokines, such as interleukin-2, interferon-γ, and TNF-α, are well-known mediators of immune maladaptation in preeclampsia. TNF-α has been shown to decrease the motility of HTR-8/SVneo cells in vitro and to negatively affect the migration and invasion of trophoblasts. It has been reported that TNF-α stimulates reactive oxygen species production by several mechanisms, including direct toxicity and its effects on mitochondrial function. In addition, reactive oxygen species generation by diverse sources induces miR-210 expression in adipose-derived stem cells via the platelet-derived growth factor receptor-β, Akt, and Elk-related tyrosine kinase pathways. Transcription of miR-210 is regulated by nuclear factor-xB and Elk1. Kopriva et al. observed that toll-like receptor 3 activation induces placental miR-210 through hypoxia-inducible factor-1α and the nuclear factor-xB–p50 pathway, leading to a decrease in signal transducer and activator of transcription 6, interleukin-4 induced, and interleukin-4 levels, which may contribute to the development of preeclampsia. In this study, we found that TNF-α significantly increased the expression of miR-210 in HTR-8/SVneo cells, which is similar to the observation of Kagiya et al. in murine macrophage cell line, RAW264.7 cells. Meanwhile, the KCMF1 expression was downregulated on TNF-α treatment. We assume that the increased miR-210–KCMF1 pathway may also be involved in the immune maladaptation at the fetal–maternal interface during the occurrence of preeclampsia.

In general, this study extends our knowledge on the functions of miR-210 and KCMF1 in the human placenta. Our findings provide new evidence that aberrantly expressed miR-210, via downregulating KCMF1, may play an important role in the development of preeclampsia.

Figure 6. Regulation of the expressions of microRNA-210 (miR-210) and potassium channel modulatory factor 1 (KCMF1) by tumor necrosis factor-α (TNF-α) in HTR8/SVneo cells. A and B, Quantitative real-time polymerase chain reaction to reveal the expressions of miR-210 (A) and KCMF1 (B) in HTR8/SVneo cells treated with TNF-α. **Compared with the control, P<0.01. C, Western blot analysis showing alterations in KCMF1 protein levels in HTR8/SVneo cells treated with TNF-α. Left, A typical Western blotting result; Right, Bar chart of the statistical results based on 3 independent experiments. The density of KCMF1 was adjusted by that of GAPDH in the same blot, and the values are presented as the mean±SEM. *Compared with scramble control, P<0.05.

Perspectives

Additional in vivo studies using miR-210 transgene animals are warranted to comprehensively understand the roles of the aberrantly expressed miR-210 in the onset and progression of preeclampsia.

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Disclosures

None.

References

Novelty and Significance

What Is New?
- MicroRNA-210 inhibits trophoblast cell invasion via targeting and suppressing potassium channel modulatory factor 1 (KCMF1) expression.
- KCMF1 is specifically localized in human placental cytotothrophoblast cells and invasive extravillous trophoblast cells. KCMF1 contributes to the regulation of trophoblast cell invasion.
- aberrant overexpression of microRNA-210 and the subsequent downregulation of KCMF1, which are partly caused by tumor necrosis factor-α, may participate in shallow trophoblast cell invasion in preeclamptic placenta.

What Is Relevant?
- Preeclampsia is a serious hypertensive disorder in pregnancy and is a major cause of maternal and fetal mortality and morbidity worldwide. A large number of differentially expressed microRNAs have been found in the preeclamptic placenta. Further demonstrating the relevance of the differential microRNAs and their functional targets in the preeclamptic placenta would be of great significance to elucidate the molecular mechanisms involved in the pathogenesis of preeclampsia.

Summary
This study extends our knowledge on the functions of microRNA-210 and KCMF1 in the human placenta. Our findings provide new evidence that aberrantly expressed microRNA-210, via down-regulating KCMF1, may play an important role in the development of preeclampsia.
MicroRNA-210 Contributes to Preeclampsia by Downregulating Potassium Channel Modulatory Factor 1
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MicroRNA-210 contributes to preeclampsia by down-regulating KCMF1

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Expanded Materials and Methods

Study subjects
In this study, all the collection of human placenta tissue specimens were performed with the permission of the Local Ethical Committee in the Institute of Zoology, Chinese Academy of Sciences, and the informed consents were obtained from all patients enrolled in this study.

Tissues of human chorionic villi at gestational weeks 7–8 were obtained at Beijing Haidian Hospital (Beijing, China) from patients who underwent therapeutic termination of pregnancy. All the patients accepted no special medical treatment before termination of pregnancy. The gestational week of specimens was determined according to morphological observation of the villi and pathological examination, with the record of menstrual cycles as a reference.

Placentas from normal pregnancies and severe preeclamptic women were obtained from a pregnant cohort that included 2500 pregnant women who underwent perinatal care in Peking University Third Hospital from August 2010 to October 2012. The placentas of the pregnant women were collected within 1h of caesarean birth, and specimens at the chorionic plate and basal plate were separately taken from the placenta disc near the position of umbilical cord insertion. The specimens were snap-frozen in liquid nitrogen and subjected to RNA extraction. Totally 15 severe preeclamptic patients delivered at the 35-39th weeks and 26 normal pregnant women delivered at the 37-39th weeks were randomly selected from the cohort and enrolled in this study. The clinical characteristics of the patients were summarized in Table 1.

Severe PE was diagnosed according to the definition in Williams Obstetrics (23rd edition)\(^1\). In brief, the patients had no history of preexisting or chronic hypertension, but they showed systolic blood pressure of >160 mm Hg or diastolic blood pressure of >110 mm Hg 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\(^2\). 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.

Cell line and culture condition
Immortalized human trophoblast cell line, HTR8/SVneo, was a kind gift from Dr. CH Graham at Queen’s University, Canada\(^3\) and cultured according to the supplier’s protocols using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).

RNA extraction and quantitative real-time PCR
Total RNA was extracted using the TRIzol reagent (Invitrogen), and reverse transcription was carried out using Superscript II (Invitrogen) following the manufacturer’s
Two micrograms of total RNA were reverse-transcribed into cDNA using oligo(dT) primer (Tiangen Biotech Co, Beijing, China) and Moloney murine leukemia virus reverse transcriptase (Promega Corporation, Madison, USA). Reverse transcription of miRNA was performed according to the instruction of MiRcute MiRNA First-strand cDNA Synthesis Kit (Tiangen Biotech). In short, poly (A) was added to 3’ end of microRNA, and then oligo(dT)-universal tag was used as specific primer to generate cDNA for miRNA.

Quantitative real-time PCR (qRT-PCR) analysis was performed using an Roche LightCycler®480 II detection system (Roche, Basel, Switzerland), and the reaction mixture contained SYBR Green for cDNA (Takara Biotechnology Co, Dalian, China) or MiRcute MiRNA Premix for miRNA cDNA (Tiangen Biotech). For the detection of cDNA, the experiment was carried out following the instructions of the SYBR® Premix Ex Taq™ kit (Takara, Dalian, China), the reaction for each sample was carried out in duplicate at 95°C for 30s, followed by 40 cycles of 95°C for 5s, 60°C for 31s. While for the quantitation of miRNA cDNA, the reaction was performed according to the instructions of the miRcute miRNA qRT-PCR detection kit (Tiangen, Beijing, China). The reaction for each sample was carried out in duplicate at 94°C for 2 min, followed by 40 cycles of 94°C for 20s, 60°C for 30s and 72°C for 30s. Specific primers were used in the PCR: (KCMF1), 5’-TGAGAGGGGCTATACGGAGA-3’ (forward) and 5’-AGGTAACGCACATATTGGG-3’ (reverse); human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5’-AAGGTCATCCCTGAGCTGAAC-3’ (forward) and 5’-ACGCCTGCTTCAACCTTCT-3’ (reverse); hsa-miR-210, 5’-CGTGAGCGCTGAGGACTGAGC-3’ (forward) and 5’-CGCAAGGATGACACGGGACACAT-3’ (reverse); hsa-U6, 5’-CGCAAGGATGACACGGGACACAT-3’ (forward).

The expression of the detected genes were normalized to endogenous GAPDH or U6 levels. The mRNA and miRNA expression were calculated using the 2^−ΔΔCT method, where ΔCT indicated the subtraction of the CT of GAPDH or U6 from the mRNA or miRNA of interest, and ΔΔCT was calculated by subtracting the ΔCT.

Because the SYBR Green-based technique used to measure mRNA and miRNA levels might generate false positive results false positive results due to problems in the specificity or efficiency of primers. Several preliminary experiments were performed to avoid the influence of these problems, and the information was shown in Figure S1.

Western blot analysis

Protein extracts were prepared from cells and placental tissues using RIPA lysis buffer. Cell or tissue lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blotting was carried out as described previously. The primary antibodies used were mouse anti-human KCMF1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human GAPDH antibody (Ambion, Austin, Texas, USA) and horseradish peroxidase-conjugated secondary antibody (Promega, Madison, WI). Signals were detected using an Enhanced Chemiluminescence Plus kit (Thermo Scientific, Rockford, USA) and visualized after exposure to a Kodak film. The membranes were then scanned and signal intensities were analyzed by the Gel-Pro Analyzer (software version 4.0; United Bio). The relative densities of the KCMF1 were determined by normalization with the density value of GAPDH in the same blot.

Immunohistochemistry
Early placenta villi and decidual tissues were fixed in 4% paraformaldehyde (Sigma) and embedded in paraffin wax. Paraffin sections at 5μm thickness were subjected to routine rehydration and antigen retrieval before being incubated with antibodies against human cytokeratin 8 (CK8) (Novus Biologicals, Novus, USA) or human KCMF1 (Santa Cruz Biotechnology, Santa Cruz, CA). The sections were further incubated with secondary antibody conjugated to horseradish peroxidase (Zhongshan Goldenbridge Biotechnology Co., Beijing, China) and visualized with diaminobenzidine (Zhongshan Goldenbridge) as substrate.

Sequences and constructs

The mature microRNA mimics for miR-210, miR-210 inhibitor and the scramble control (NC) were designed and purchased from GenePharma (Shanghai, China). The sequences of miRNA and miRNA inhibitor are: negative control, 5’-UUCUCCGAACGUCCGUACCGU-3’(sense) and 5’-ACGUGACACGUUCGGAGAATT-3’(anti-sense); miR-210 mimics, 5’-CUUGUGCGUGACACCGGCUA-3’(sense) and 5’-AGCCGCUGUCACACGCAAGU-3’(anti-sense); miRNA inhibitor NC, 5’-CAGUACUUAUGUAGUAGUACAA-3’(sense); miR-210 inhibitor, 5’-UCAGCCGCUGACACGCAAGU-3’(sense).

For the construction of the KCMF1 expressing plasmid (pKCMF1), the coding sequence of KCMF1 was amplified and inserted into pcDNA4.0 vector (Invitrogen, Carlsbad, CA) at the Not I and Xho I restriction sites. To generate pMIR-REPORT Luciferase plasmids for KCMF1, 3’UTR segments of human KCMF1 mRNA (554-561nt, Genbank accession no. NM_020122) containing the one putative miR-210 binding sequence was amplified and cloned into pMIR-REPORT Luciferase plasmid (Ambion, Austin, Texas, USA) at the Mlu I and Spe I sites. The construct was named BD-WT. The mutated pMIR-REPORT plasmid, which carry site mutations in the 3’UTR segments of human KCMF1 mRNA being complementary to the seed sequence of miR-210, was generated based on BD-WT plasmid by QuikChange Lightning Site-Directed Mutagenesis Kit following the manufacture’s instruction (Stratagene, La Jolla, California, USA). The construct was named BD-MUT. The primers for vectors construction are shown in below: KCMF1 BD construct, 5’-GACTAGTAAGGAGTAAAAACTTAAAAAAA-3’ (Forward) and 5’-CGACGCGGT ACAGGCTGAGCTCCACACAGG-3’(Reverse); KCMF1 BD MUT construct, 5’-CTTTTGTGGTGGCC TCCTACCCAGAAAGCCAGCTCTGAG-3’(Forward) and 5’-CTGCAGACTGGGTAGGAGGCACACAAAG-3’(Reverse); KCMF1 expressing plasmid, 5’-ATAAGGTGCGGCCGCATGTCCCGACATGAAGGTGTC-3’(Forward) and 5’-CCGCTCGAGTCAAGAGGAGGTGGTGGAGG-3’(Reverse). All of constructs were confirmed by DNA sequencing.

Dual luciferase assay

HTR8/SVneo cells were plated into 24-well plates at a density of 4.5×10^4 cells/well. Transfection was performed 24 hours after seeding using Lipofectamine 2000 (Invitrogen) with 100 ng of pMIR-REPORT plasmid construct, 10 ng of pRL-TK control vector (encoding Renilla luciferase) and 40nM of miR-210. Five hours after transfection, the cells were recovered in complete medium. Luciferase activities were measured by the Dual-Glo luciferase assay system according to the manufacturer’s instructions (Promega, Madison,
Wisconsin, USA) 48 hours after transfection.

**Transwell insert invasion assay**

In vitro cell invasion was assayed by determining the ability of cells to invade a synthetic basement membrane, growth factor-reduced Matrigel matrix (BD Biosciences, Franklin, NJ). The experiment was carried out as described previously. In short, 48 hours after transfection, cells were treated cells with 10 mg/ml mitomycin C for another 2 hours. Then the cells were trypsinized and seeded into transwell insert pre-coated with 200 mg/ml matrigel (BD Biosciences, USA) at 5×10^4 cells per insert. The inserts contained RPMI 1640 medium (Invitrogen, Carlsbad, CA) plus 1% fetal bovine serum, and the lower chambers were loaded with RPMI1640 medium plus 10% fetal bovine serum. 24 hours after incubating, the cells were fixed and stained with hematoxylin. Non-invaded cells on the upper surface of the membrane were completely removed. The number of stained cells at the lower surface of the membrane was counted under light microscope. Cell invasion index was presented as the percentage of invaded cell number compared with the corresponding control.
Supplementary References


Supplementary Figures

A. GAPDH

B. KCMF1

C. U6

D. miR-210

E. Standard Curve

F. Standard Curve

G. Standard Curve

H. Standard Curve
Figure S1.
Technical considerations for the measurement of microRNA and mRNA by quantitative PCR. A-D, the specificity of the primers were determined by melting curves which showed single peaks for the amplification of specific microRNA or mRNA. Negative control was set using RNA templates without reverse transcription. E-H, the efficiencies of the primers for target gene (miR-210 and KCMF1) and reference gene (U6 and GAPDH) were determined by using the method of “standard curve”. As shown, the efficiency of primers for GAPDH and KCMF1 are 99.05% (Slope=-3.369) and 97% (Slope=-3.474) respectively; and 96.05% (Slope=-3.526) and 95.55% (Slope=-3.555) for primers of U6 and miR-210, respectively.
Figure S2.
Dose-dependent decrease in miR-210 level and gradual increase in KCMF1 expression
We transfected HTR8/SVneo cells with different concentration of miR-210 inhibitor of NC-inhibitor (5, 10, 20 and 40 nM), and measured miR-210 and KCMF1 levels by qRT-PCR. We found a dose-dependent decrease in miR-210 level (A) and gradual increase in KCMF1 expression (B). While in NC-inhibitor treated cells, neither miR-210 nor KCMF1 level had any change.