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Original Article

Role of kisspeptin/GPR54 in the first trimester trophoblast of women with a history of recurrent spontaneous abortion

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Abstract: Trophoblast migration and invasion during pregnancy are under strict physiological control, both temporally and spatially. The trophoblast's ability to invade the endometrium is regulated by the dynamic interaction between invasion-related genes. Once this dynamic balance is broken, the trophoblast exhibits abnormal invasion ability, often resulting in recurrent spontaneous abortion (RSA). Kisspeptins, products of the KISS1 gene, were originally identified as metastasis suppressor peptides with the ability to bind G protein-coupled receptors (i.e., GPR54). The human placenta expresses both KISS1 and kisspeptin receptor (KISS1R) mRNA within the trophoblast compartment, where it is thought to inhibit physiological invasion. In order to explore the effects of KISS1 on the biological behavior of human trophoblasts and its association with RSA, we used immunohistochemistry to compare the expression of kisspeptin and GPR54 at the maternal-fetal interface in RSA cases and cases with normal pregnancy. Abortion-prone CBA/J×DBA/2 matings were established as a model for spontaneous abortion, while non-abortion-prone CBA/J×BALB/c matings were used as a model for normal pregnancy. The expression of kisspeptin and GPR54 in mice placental tissues was compared by immunohistochemistry. Gene recombination and transfection technology were used to evaluate the effects of the KISS1 gene and kisspeptin on JAR cells in terms of proliferation, colony formation ability, and migration and invasion abilities. Kisspeptin/GPR54 revealed lower levels of expression at the maternal-fetal surface in RSA patients compared to controls ($P<0.001$). Similarly, the expression of kisspeptin/GPR54 at the maternal-fetal interface of spontaneous-abortion mice (CBA/J×DBA/2) was remarkably lower than the group of mice that experienced normal pregnancy (CBA/J×BALB/c) ($P<0.05$). Data indicated that the KISS1 gene and kisspeptin play a significant role in the inhibition of trophoblast migration and invasion propensity *in vitro* without affecting cell growth or proliferation. Moreover, kisspeptin appeared to exert an effect in a dose-dependent manner. These data support the fact that the downregulation of kisspeptin/GPR54 may be related to RSA, and that the abnormal expression of KISS1 acts as an invasion-inhibitor gene. Consequently, KISS1 possesses the ability to interfere with normal homeostasis of trophoblast regulation, ultimately resulting in miscarriage.

Keywords: KISS1, kisspeptin, GPR54, recurrent spontaneous abortion

Introduction

Kisspeptins, which are peptide products of the KISS1 gene, and their receptor, G-protein-coupled receptor-54 (GPR54), are known to play a key role in fertility. Recurrent spontaneous abortion (RSA) occurs in approximately 1% of all pregnancies, and is defined as three or more pregnancy losses prior to 20 gestational weeks [1]. Although RSA has been attributed to various hematologic, anatomic, hormonal, immune and genetic defects, in most cases the etiology remains unknown. This is a condition referred to as unexplained RSA (URSA). Furthermore, there is a high incidence of depression and anxiety in women experiencing URSA [2].

A successful pregnancy requires a variety of mandatory conditions. A healthy embryo maintains the intrinsic ability to attach, migrate and invade. Initially, the fertilized egg divides rapidly, interacting first with the epithelial component of the endometrium, followed by the stromal and endothelial components [3]. During this process, the extravillous cytotrophoblast of

the embryo migrates and invades into the endometrial tissue, which anchors and differentiates into an endovascular cytotrophoblast that remodels spiral arteries into a primitive placental vasculature, thus, allowing for a dramatic increase in blood flow to support fetal growth [4-6]. Defective formation of the placental vasculature may lead to a variety of pregnancy complications including infertility, miscarriage, pre-eclampsia, fetal growth restriction, and death [5, 6]. Among these conditions, RSA in the first trimester of pregnancy is the most common. The invasion ability of the cytotrophoblast plays a critical role in the proce ss of embryo implantation. Krieg [7] used microarray analysis to identify changes in gene expression in the decidua of RSA patients. A total of 155 genes were identified as being significantly dysregulated in the decidua of RSA patients, and genes related to cell invasion accounted for 17.1% of this cohort of defective genes. This suggests that the successful invasion of trophoblasts in the first trimester represents a critical basis for a successful pregnancy. Furthermore, abnormalities in genes known to regulate the ability to invade could cause recurrent miscarriage.

KISS1, which maps to chromosome 1q32-q41, encodes a 145-amino acid peptide referred to as kisspeptin. The peptide products of the KISS1 gene can be hydrolyzed into a variety of truncated fragments: kisspeptin-54 (metastin, 54 amino acids), kisspeptin-14 (14 amino acids), kisspeptin-13 (13 amino acids) and kisspeptin-10 (10 amino acids) [8, 9]. It has been reported that kisspeptins conduct their biological activities by binding to a G protein-coupled receptor known as GPR54 [8, 10], and that all kisspeptins are equally potent in terms of their ability to activate GPR54. Since KISS1 was identified as a metastasis suppressor gene in human melanoma [9], a series of studies have investigated the function of KISS1 in the metastasis of a variety of human cancers such as thyroid [11], breast [12], hepatocellular [13] and bladder cancer [14]. However, the precise mechanism underlying remains unclear.

More recent studies have demonstrated that kisspeptin/GPR54 signaling may participate in human pregnancy including embryo implantation, placental function and the maintenance of pregnancy. Both KISS1 and its receptor gene are highly expressed in the placenta [8, 10]. Indeed, plasma metastin concentration has been shown to increase significantly during the first trimester compared to non-pregnant conditions, reaching a maximal level in the third trimester. However, it returns to non-pregnant levels 5 days after delivery [15]. Ramaesh et al. used complementary *ex vivo* and *in vitro* assays to further conclude that kisspeptin-10 (Kp-10) inhibited angiogenesis, and that Kp-10 may contribute to the regulation of angiogenesis in the placenta [16].

In addition, several studies have shown that kisspeptin/GPR54 may be related to miscarriage or adverse pregnancy outcomes. For example, low levels of metastin in maternal plasma during the first trimester were associated with small for gestational age neonates [17]. Another study revealed that serum kisspeptin levels between 16-20 weeks of gestation were significantly lower in those women who subsequently developed pre-eclampsia and intrauterine growth restriction than in controls [18]. Both mRNA and protein expression levels of KISS1 were also reduced in cases of pre-eclampsia, compared with normal term pregnancies, although GPR54 was able to reverse this effect [19]. Mouse models have also revealed that loss-of-function mutations in the gene encoding GPR54 results in miscarriage without forming a normal placenta [20]. Furthermore, our previous study has shown that kisspeptin/GPR54 may be closely related to RSA, and that the abnormal expression of kisspeptin during pregnancy was highly likely to be associated with adverse pregnancy [21].

In this study, we hypothesize that kisspeptin/GPR54 represent critical factors in the proliferation and invasion of trophoblast during the first trimester of pregnancy, and that the expression of KISS1 at the maternal-fetal interface may be involved in the pathogenesis of RSA. In order to investigate the physiological significance of kisspeptin/GPR54 in women with RSA, we used a combination of mouse models and *in vitro* studies to investigate the role of kisspeptin/GPR54 in the first trimester trophoblast.

Materials and methods

Human subjects

The present study involved chorionic villous and uterine decidua tissues were obtained

from 45 RSA patients, in which pregnancy loss occurred during the first trimester (RSA group), and 42 patients undergoing voluntary abortion during the first trimester without history of spontaneous pregnancy loss or receiving artificial reproductive technology (control group). All subjects were selected from archived material (2009-2011) at the Department of Pathology, the Second Affiliated Hospital of Soochow University. All RSA patients that recruited into the study met all of the following requirements. RSA subjects must have experienced two or more spontaneous abortions prior to 20 weeks of gestation. All these women had regular menstruation and underwent a series of examinations including ultrasound, hysteroscopy and conventional blood analyses for RSA screening; and these patients were ultimately diagnosed as RSA with unexplained etiology. Blood analysis included screening for TORCH infection (toxoplasma, rubella, cytomegalovirus, and herpes), antibodies such as lupus anticoagulant, anti-cardiolipin and anti-β2 glycoprotein I, and endocrine hormones such as LH, androgen, prolactin, and thyroid-stimulating hormone. Cases with abnormal uterus, endocrine or chromosomal abnormality, infectious diseases, or immune problems were rejected. Age of RSA patients ranged from 21 to 35 years, with a mean age of 28.41 years. Median age of control subjects was 28.43 years (range, 18-40 years).

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University, and all human specimens were used in accordance with committee guidelines regarding the use of human subjects in research and the secondary use of pathologic or surgical tissue.

Immunohistochemistry for kisspeptin and GPR54

Trophoblast and decidua tissues were obtained following dilatation and curettage, washed in normal saline, and formalin-fixed and paraffin-embedded for further experiments. Paraffin-embedded samples were cut into 4 μm tissue sections and incubated with antibodies against kisspeptin (Biorbyt, Cambridge, UK; rabbit polyclonal; 1:150) and GPR54 (MBL International Corporation, Woburn, USA; rabbit polyclonal; 1:100). All steps were carried out in accordance with manufacturer's protocols.

All stained cells were counted at 400× magnification using a double-blinded method. Each tissue section was given an integrated score (staining intensity × proportion of the number of positive cells). Staining intensity was scored based on four levels: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining [22]. The proportion (%) of positive cells was determined as follows: 0 point (≤10%), 1 point (11%-50%), 2 points (51%-75%), 3 points (>75%). It was considered positive when the score was greater than 2 points.

Animals

An abortion-prone CBA/J×DBA/2 matings were established as a model for spontaneous abortion, while a non-abortion-prone CBA/J×BALB/c matings were used as the model for normal pregnancy. Mice were provided by the Experimental Animals Center of Soochow University (certificate No. 2012-0045). All experimental procedures were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee of Soochow University. Placenta tissues were collected on day 9 (early pregnancy) and day 14 (late pregnancy) of gestation. Embryo resorption rate was counted separately on each of day 9 and 14. All placental tissues obtained from pregnant CBA/J mice were detected and prepared as 4 μhm tissue sections for immunohistochemistry of kisspeptin and GPR54.

Cell culture and kisspeptin

The human trophoblastic JAR cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Biochrom, Germany) along with 100 IU/mL of penicillin and 100 μg/mL of streptomycin (Beyotime, Jiangsu, China). Cells were routinely maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Human Kp-10 (Tocris, Bristol, UK) with the sequence YNWNSFGLRF was prepared as a 1 mM stock solution in phosphate buffer saline (PBS, PH 7.4). Cells were subsequently treated with different concentrations of Kp-10 (0 nM, 10 nM, 100 nM and 1 μM) in culture medium.

Construction of recombinant expression vectors and stable transfection JAR cells

Total KISS1 cDNA was amplified from human term placenta tissue by reverse transcription-polymerase chain reaction (RT-PCR) with a forward primer (5'-CCCAAGCTTGGGATGAACTCACTGG-3', carrying a Hind III site) and a reverse primer (5'-CGGGATCCGGTCAGCCCCGCCC-3', carrying a BamH I site). RT-PCR was performed as follows: denaturing at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds with 30 cycles. PCR products and plasmid pcDNA3.1 (+) were subjected to double digestion with Hind III and BamH I restriction enzymes (Invitrogen, Carlsbad, CA, USA), and were ligated together with the T4 ligase enzyme. Finally, DNA sequencing was used to verify that KISS1 cDNA had successfully been incorporated into the pcDNA3.1 (+) expression plasmid.

The pcDNA3.1 and pcDNA3.1-KISS1 vectors were separately transfected into JAR cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. Then, transfected JAR cells containing pcDNA3.1 or pcDNA3.1-KISS1 were selected by adding 600 μg/mL of G418 (Amresco, Solon, USA) to the culture medium. Stably transfected cells were harvested following culture for two weeks in a selection medium containing G418 (at concentrations that gradually increased from 600 μg/mL to 1000 μg/mL). Subclones were isolated by limited dilution and cultivated by routine methods.

The detection of KISS1 mRNA by RT-PCR and the detection of kisspeptin protein by Western blotting were used to determine whether the KISS1 gene had been transfected successfully.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). 1 μg of RNA was reverse transcribed into cDNA, and KISS1 cDNA (438 bp) was amplified using the following primers: forward primer (5'-GTAGATCCAACTCACTGGTTTCGTGGCAG-3'), reverse primer (5'-GCTAAGCTTTCACTGCCCCGCACCTG-3'). Duplex amplification was performed for 30 cycles according to the following program: 30 seconds at 95°C, 30 seconds at 54°C, and 30 seconds at 72°C. PCR fragments were separated by electrophoresis on 1.5% agarose gel, with β-actin (315 bp) as an internal standard.

Western blotting

Total cell lysates were harvested in RIPA lysis buffer. After protein concentrations were determined, total cell lysates (50 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12%) to detect kisspeptin expression. Proteins were electrophoretically transferred onto a polyvinylidenedifluoride (PVDF) membrane. Then, membranes were blocked at room temperature for 2 hours in blocking buffer containing Tris buffered saline (TBS, PH 7.4) and 0.1% Tween (TBS-T), with 5% non-fat milk. Proteins were then incubated at 4°C overnight with rabbit polyclonal KISS1 antibody (Biorbyt, Cambridge, UK; 1:300). The membrane was rinsed three times with TBS-T for 10 minutes and incubated with goat anti-rabbit antibody (Santa Cruz, CA, USA; 1:2000) for 1 hour at room temperature. Finally, protein bands were detected by chemiluminescence. β-actin was used as a loading control.

Cell proliferation assay

For cell proliferation assays, the effects of Kp-10 and KISS1 on JAR cell viability was measured by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan) according to manufacturer's instructions. JAR cells (3×10^3 cells/well) were seeded into 96-well plates. Cells were treated with Kp-10 (0 nM to 1 μM) for 24, 48 or 72 hours. Then, CCK-8 solution (10 μL/well) was added to the culture medium and incubated for 2 hours in the incubator. Absorbance (OD) was read at 450 nm by a microplate reader (Tecan, infinite 200 PRO Nano Quant, Switzerland). Each group was replicated for five times, and the experiment was performed in triplicate.

Non-transfected cells and cells transfected with pcDNA3.1-KISS1 or pcDNA3.1 (3×10^3 cells/well) were seeded into 96-well plates, incubated for 24, 48 or 72 hours, and evaluated by cell proliferation assay, as detailed above.

Colony formation assay

Clonogenic soft agar assays were performed as previously described [23] with some modifica-

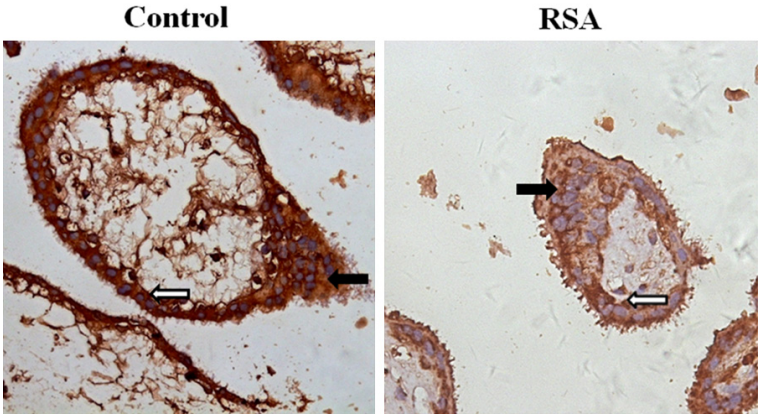

Figure 1. Immunohistochemical staining of kisspeptin in the first trimester trophoblasts. Strong kisspeptin staining was shown in trophoblasts in the control group. Trophoblasts in the RSA group reveal a low positive expression of kisspeptin. The black arrow indicates kisspeptin-positive syncytiotrophoblast. The white arrow indicates kisspeptin-positive cytotrophoblast. Magnification, ×400.

tions. Culture medium containing 10% FBS and 1.2% soft agar (Amresco, Solon, USA) was set into 6-well dishes. Then, a specific number of cells (5×10^2 cells/well) were embedded in 0.7% soft agar were overlaid onto the basal layer. Kp-10 (0 nM to 1 μM) was added to the cell suspension before cells were plated. Cultures were incubated at 37°C in a 5% CO_2 atmosphere, and colonies were counted after 14 days. Colony data per well are presented as mean ± standard error of mean (SEM) for three independent experiments.

Cell migration and matrigel invasion assays

Migration assays were performed with transwell polycarbonate filters with 8-μm pore size (Corning, New York, USA). A total of 5×10^4 cells in 100 μL of serum-free medium were seeded into the upper chamber. Medium containing 10% FBS was added into the lower chamber as a chemo-attractant. Kp-10 (0 nM to 1 μM) was added into the upper chamber, and the cultures were incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. Cells that migrated to the lower surface were fixed and stained with hexamethylpararosaniline, and counted under a microscope (×200, Observer A1, Zeiss, Germany). The experiment was performed in triplicate.

For invasion assays, the experiment was performed as described previously with minor modifications [24]. Before cells were seeded, 80 μL of matrigel (BD Biosciences, Franklin Lakes, USA) was added into serum-free medium at a dilution of 1:8 and air-dried for 12 hours. After incubation for 48 hours, cells were stained and counted in the same way described in the migration assays.

Statistical analysis

Data were analyzed using SPSS17.0 software and presented as mean ± SEM. Differences in basic characteristics of patients were analyzed by Analysis of Variance (ANOVA) and Student's *t*-test. Immunohistochemical differences in kisspeptin and GPR54 expression were analyzed by Chi-square test. Differences between the groups of assayed cells were analyzed by independent sample *t*-tests. $P<0.05$ was considered statistically significant.

Results

Expression of kisspeptin and GPR54 in trophoblasts and decidual tissues

Kisspeptin expression in trophoblasts and the decidua was detected by immunohistochemistry (**Figures 1** and **2**). The kisspeptin positive rate was 73.3% (33/45) in trophoblasts and 22.2% (10/45) in decidua in the RSA group, which were significantly lower compare with the control group (100% and 59.5%, $P<0.001$).

GPR54 was expressed in 17 out of 45 (37.8%) trophoblasts in RSA cases, which was significantly lower than cases in the control group (76.2%, $P<0.001$). However, there was no significant difference in terms of GPR54 expression in decidua between the RSA and control groups.

Embryo resorption rate and kisspeptin/GPR54 expression in CBA/J×DBA/2 mice and CBA/J×BALB/c mice

Embryo resorption rate in abortion-prone CBA/J×DBA/2 matings on day 9 and day 14 of gestation were significantly higher than in non-abortion-prone CBA/J×BALB/c matings ($P<$

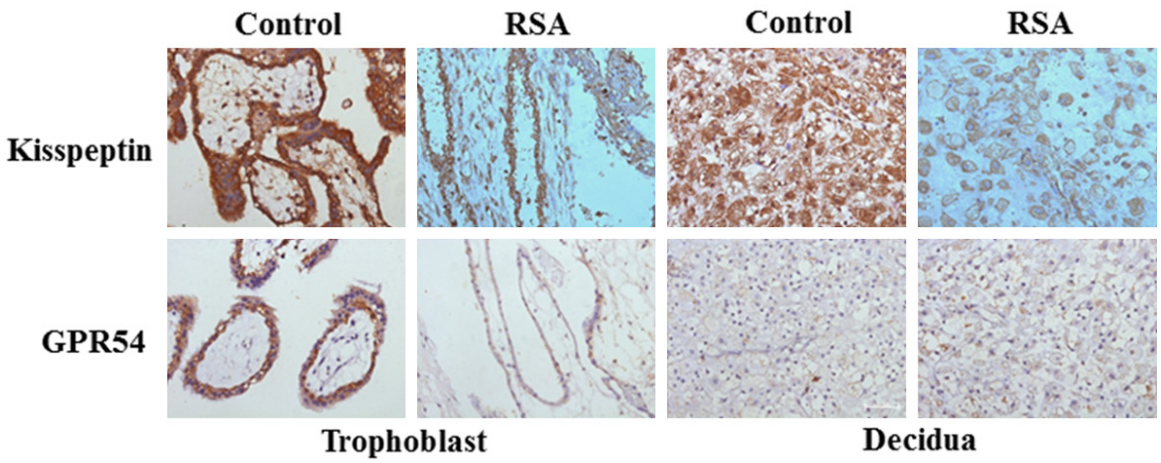

Figure 2. Immunohistochemistry staining of kisspeptin and GPR54 in trophoblasts and decidua. The expression of kisspeptin and GPR54 in trophoblasts in the RSA group was lower than in the control group. The expression of kisspeptinin decidua in the RSA group was lower than in the control group. There was no significant difference in terms of GPR54 expression in decidua when compared between the RSA group and the control group. All images were acquired at ×400 magnification.

Table 1. Embryo resorption rate of CBA/J female mice

	Day 9 embryo resorption rate			Day 14 embryo resorption rate		
	Embryo Resorption n (%)	Embryo Survival n (%)	*P*	Embryo Resorption n (%)	Embryo Survival n (%)	*P*
CBA/J×DBA/2	14 (29.2)	34 (70.8)	0.0153	12 (27.3)	32 (72.9)	0.0095
CBA/J×BALB/c	4 (9.1)	40 (90.9)		2 (5.4)	35 (94.6)	

P: CBA/J×DBA/2 group *vs.* CBA/J×BALB/c group.

0.05, **Table 1**). The expression of kisspeptin and GPR54 at the maternal-fetal interface of the spontaneous abortion group (CBA/J×DBA/2) was significantly lower than in the normal pregnancy group (CBA/J×BALB/c). Interestingly, there was a remarkable reduction in the expression of kisspeptin and GPR54 on day 14 when compared to day 9 of gestation (**Figure 3**).

Identification of stable transfection in JAR cells with KISS1

KISS1 cDNA was successfully cloned into eukaryotic expression vector pcDNA3.1 (+), and recombinant pcDNA3.1-KISS1 was verified by gene sequencing. The obtained DNA sequences were analyzed by BLAST and compared to the KISS1 sequence published on the Gene Bank. Results revealed that the nucleotide sequence was accurate, indicating that the recombinant plasmid pcDNA3.1-KISS1 was constructed successfully.

JAR cells were stably transfected with pcDNA-3.1-KISS1 or pcDNA3.1 and three cell lines were derived for pcDNA3.1-KISS1 (pK-JAR1, pK-JAR2 and pK-JAR3) by G4-18 selection. Two further stable cell lines, p-JAR1 and p-JAR2, were produced from pcDNA3.1 transfected cells. KISS1 overexpression was observed in pK-JAR1, pK-JAR2 and pK-JAR3 by both PCR and western blotting. JAR cells and cells transfected with pcDNA3.1 did not express KISS1 (**Figure 4**).

Effects of kisspeptin-10 and KISS1 transfection on cell growth

Cell inhibition in vitro was examined via CCK-8 and colony formation assays at set time points after treatment with various concentrations of Kp10.

Kp-10 (10 nM to 1 μM) exhibited no inhibitory effect upon JAR cells compared with the control group (0 nM, *P*>0.05, **Table 2**). Moreover, no significant differences were identified among the three groups (pcDNA3.1-KISS1, pcDNA3.1 transfected cells, and non-transfected JAR cells, *P*>0.05, **Table 3**).

Colony formation assays revealed that Kp-10 treatment had no effect on colony form-

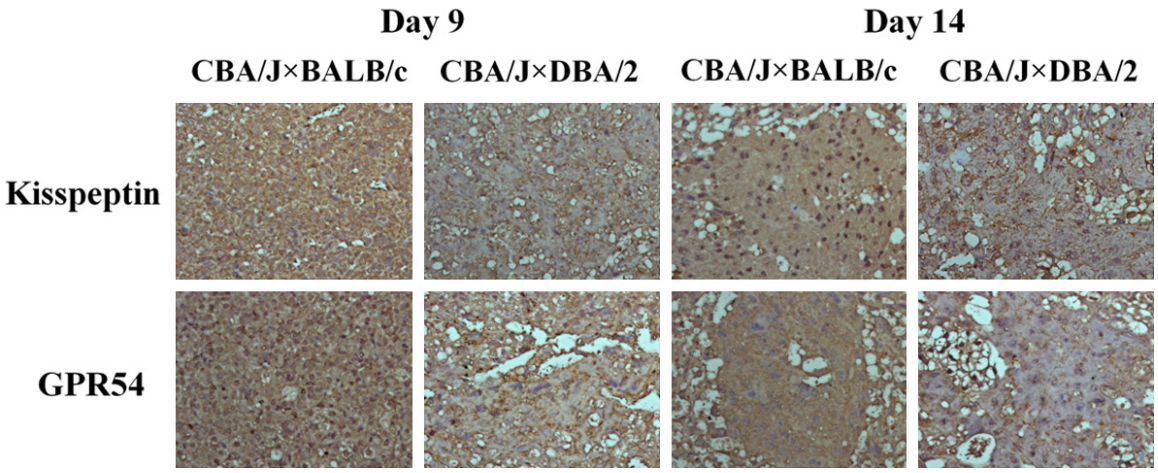

Figure 3. Immunohistochemical assay of kisspeptin and GPR54 at the maternal-fetal interface in mice. Among spontaneous abortion mice (CBA/J×DBA/2), the expression of kisspeptin on day 9 and day 14 of gestation, respectively, were significantly higher than in the control group (CBA/J×BALB/c). Moreover, there was a remarkable increase in GPR54 expression in the abortion group (CBA/J×DBA/2) compared with the control group (CBA/J×BALB/c) on day 9 and 14 of gestation. There was also a reduction in kisspeptin and GPR54 expression on day 14 compared with day 9 of gestation.

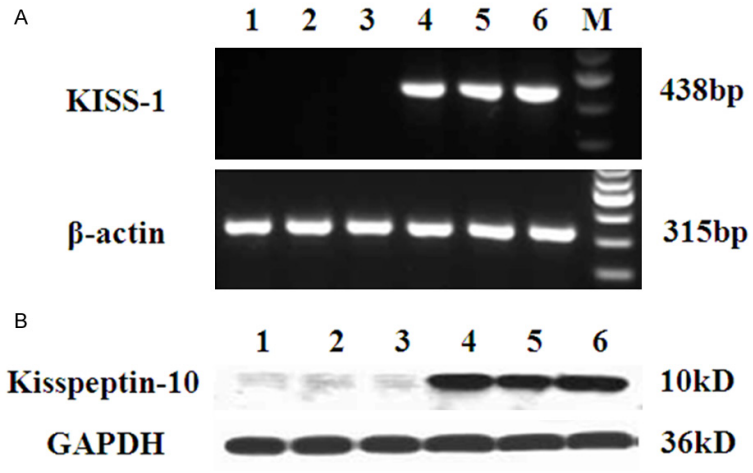

Figure 4. Identification of KISS1 and kisspeptin in stably transfected JAR cells. A: The mRNA level of KISS1 was distinctly evident in cells transfected with pcDNA3.1-KISS1. M, molecular marker; 1, non-transfected JAR cells; 2-3, JAR cells transfected with pcDNA3.1 (p-JAR1 and p-JAR2); 4-6, JAR cells transfected with pcDNA3.1-KISS1 (pK-JAR1, pK-JAR2 and pK-JAR3). B: The expression of KISS1 protein (kisspeptin) was also distinctly evident in cells transfected with pcDNA3.1-KISS1.

ation after incubation for 14 days (10 nM to 1 μM, $P>0.05$, **Table 4**). Compared with the control group, both pcDNA3.1-KISS1 and pcDNA3.1 transfected cells did not reveal a significant difference in their ability to form colonies ($P>0.05$, **Table 5**).

These results indicated that kisspeptin intervention and KISS1 expression did not significantly affect JAR cell growth.

Effect of kisspeptin-10 and KISS1 transfection on cell migration

Trans-well migration assays revealed that the number of chemo-attractive migration cells was significantly lower in groups with exogenous Kp-10 than in the control group. Taking the number of cells that migrated through the 8-μm pores of the filter in the control group (0 nM) as a standard, the migration rate of cells treated with 100 nM and 1 μM of Kp-10 was 79.6% and 73.8%, which show a significant reduction ($P<0.05$). In contrast, the 10 nM group (95.1%) did not show a remarkable difference ($P>0.05$, **Figure 5A**).

The cell migration rate of pcDNA3.1-KISS1 transfected cells was significantly lower than in the pcDNA3.1 transfected group and control group ($P<0.05$). However, there was no significant difference between the control group and pcDNA3.1 transfected group ($P>0.05$, **Figure 5B**). These data suggested that the intervention of kisspeptin and KISS1 overexpression resulted in a significant reduction in cell migration.

Table 2. Effects of Kisspeptin-10 on cell growth

	24 h		48 h		72 h	
	AV (mean ± SEM)	*P*	AV (mean ± SEM)	*P*	AV (mean ± SEM)	*P*
Control	0.431±0.017		1.321±0.044		2.701±0.199	
10 nM	0.458±0.013	0.228	1.341±0.020	0.684	2.900±0.122	0.427
100 nM	0.432±0.014	0.967	1.304±0.019	0.731	2.769±0.137	0.785
1 μM	0.455±0.015	0.319	1.319±0.018	0.960	2.781±0.161	0.762

P: Kisspeptin-10 groups *vs.* Control group.

Table 3. Effects of KISS1 transfection upon cell growth

	24 h		48 h		72 h	
	AV (mean ± SEM)	*P*	AV (mean ± SEM)	*P*	AV (mean ± SEM)	*P*
JAR	0.438±0.038		1.679±0.027		2.263±0.142	
pK-JAR1	0.409±0.011	0.497	1.650±0.034	0.526	2.176±0.074	0.603
pK-JAR2	0.433±0.010	0.893	1.679±0.028	0.999	2.353±0.106	0.625
pK-JAR3	0.446±0.023	0.873	1.699±0.018	0.548	2.368±0.039	0.497
p-JAR1	0.446±0.018	0.871	1.689±0.006	0.728	2.321±0.062	0.603
p-JAR2	0.422±0.039	0.768	1.706±0.038	0.725	2.343±0.016	0.607

P: KISS groups *vs.* Control group.

Table 4. Colony formation assays of JAR cells with Kisspeptin-10

	Colonies/well n (mean ± SEM)	Colony Formation (%)	*P*
Control	330.0±5.00	66.0	
10 nM	336.7±6.01	67.3	0.442
100 nM	327.7±4.33	65.5	0.742
1 μM	333.7±4.63	66.7	0.619

P: Kisspeptin-10 groups *vs.* Control group.

Table 5. Colony formation assay of JAR cells transfected with KISS1

	Colonies/well n (mean ± SEM)	Colony Formation Efficiency (%)	*P*
JAR	338.7±9.49	67.7	
pK-JAR1	328.0±6.50	65.6	0.406
pK-JAR2	321.7±3.33	64.3	0.208
pK-JAR3	336.3±8.69	67.3	0.865
p-JAR1	341.0±7.23	68.2	0.855
p-JAR2	334.0±4.93	66.8	0.685

P: KISS groups *vs.* Control group.

Effects of kisspeptin-10 and KISS1 transfection on cell invasion

Cell invasion ability was evaluated by the capacity of cells to digest matrigel. Cell invasion in the presence of Kp-10 (100 nM and 1 μM) was significantly less than the control group (0 nM, $P<0.05$). But 10 nM of Kp-10 did not exhibit a remarkable inhibitory effect on cell invasion ($P>0.05$, **Figure 6A**).

Cell invasion was significantly lower in the pcDNA3.1-KISS1 transfected group, compared with the pcDNA3.1 transfected group and control group ($P<0.05$, **Figure 6B**). These differences indicated that KISS1 and kisspeptin played an important role in cell invasion.

Discussion

It has been reported that kisspeptin and its receptor primarily act as metastasis suppressors in tumor cells [8, 9, 11-13]. Recently, the high expression of the kisspeptin/GPR54 system in the placenta [8, 10] and its effects in pregnancy complications [17-19] have attracted increasing attention. A number of studies have confirmed the strong expression of the kisspeptin/GPR54 system at the maternal-fetal interface, but its precise distribution remains controversial. In this study, we demonstrate that kisspeptin expression in RSA patients, both in trophoblasts and decidua was significantly lower than in patients with normal pregnancy. Compared with the control group, GPR54 expression in the RSA group was lower in trophoblasts, but not in decidua. These suggested that kisspeptin/GPR54 exhibited a low-

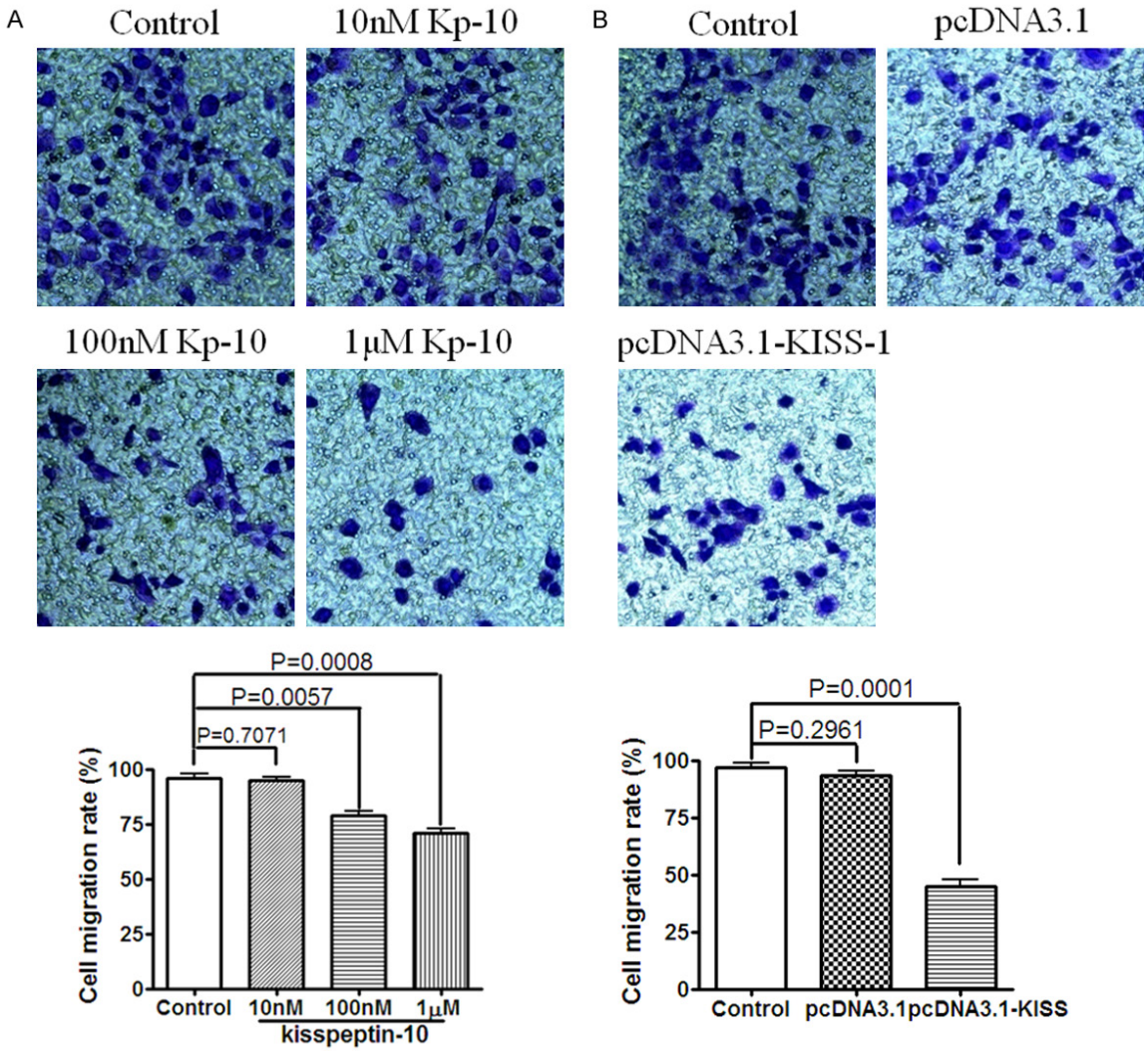

Figure 5. Suppression effects of Kp-10 and KISS-1 on migration capacity of JAR cells. A: Kp-10 (100 nM, 1 μM) suppressed the migration capacity of JAR cells. B: JAR cells transfected with pcDNA3.1-KISS-1 had lower migration capacity than control cells. pcDNA3.1 had no effect on migration capacity of JAR cells. Data are normalized to control group (%). Results are expressed as mean ± SEM.

er expression at the maternal-fetal surface in RSA patients.

Plasma kisspeptin concentration has been known to significantly increase during the first trimester and fall rapidly following delivery, and there is no difference in kisspeptin level when compared between non-pregnant women and men [15]. Bilban et al. [25] detected KISS1 mRNA and its encoded production in the syncytiotrophoblast during early pregnancy, while the cytotrophoblast was devoid of expression. However, GPR54-positive cells were readily detectable in the syncytiotrophoblasts and cytotrophoblasts. Park et al. [26] used immunohistochemical methods to find a different distribution of the kisspeptin/GPR54 system. It was possible to detect the expression of kisspeptin in the syncytiotrophoblasts, cytotrophoblasts and decidua, while GPR54 was only detectable in the syncytiotrophoblasts and decidua. However, it is very clear that, these data illustrate the positive state of kisspeptin/GPR54 at the maternal-fetal interface, with its predominant expression in invasive trophoblasts. Collectively, these data suggest that the abnormal expression of kisspeptin/GPR54 may have effect on placental function, and that normal placental function relies on the accurate expression of kisspeptin/GPR54.

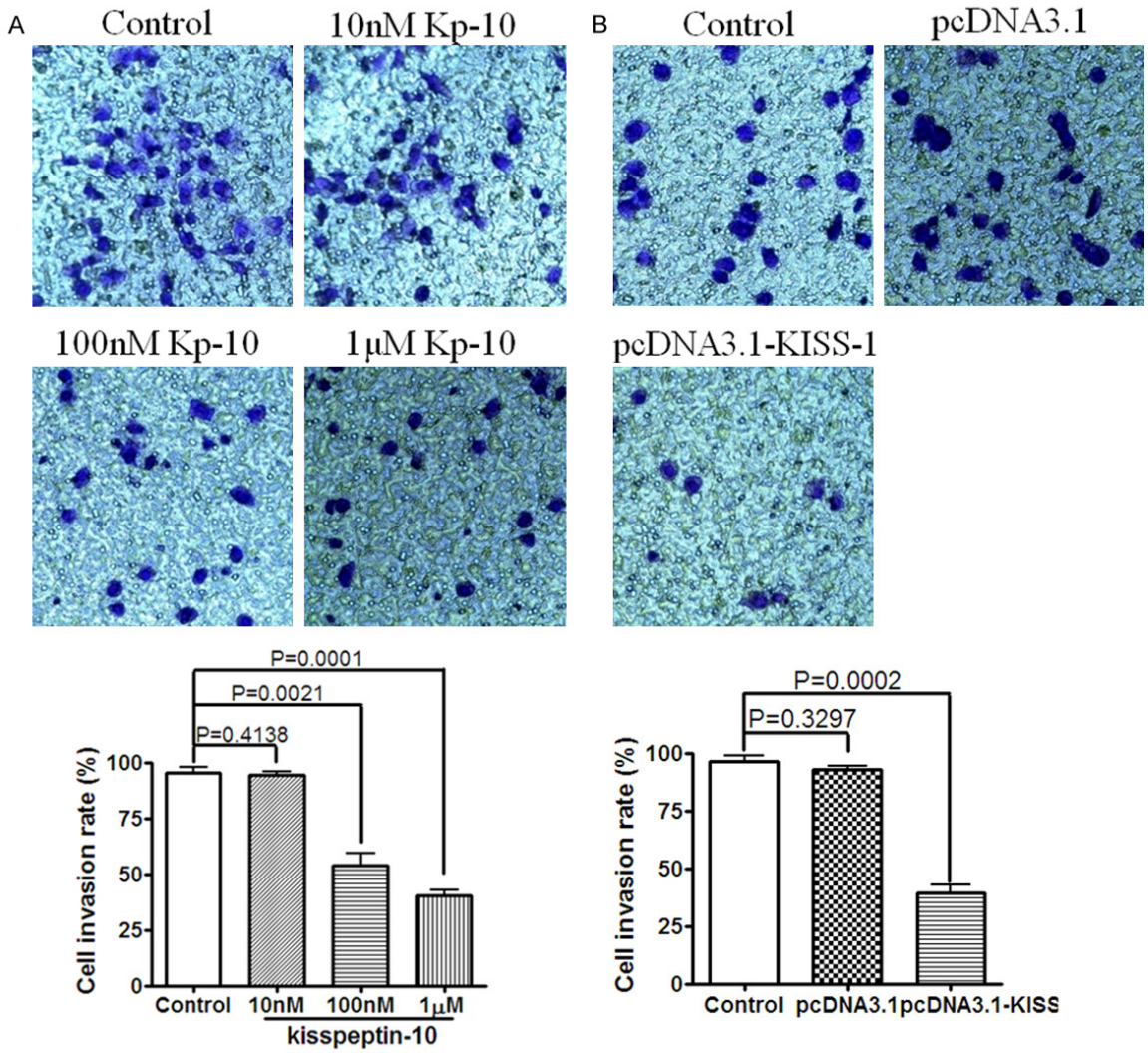

Figure 6. Suppression effects of Kp-10 and KiSS-1 on invasion capacity of JAR cells. A: Intervention of Kp-10 (100 nM, 1 μM) decreased the invasion capacity of JAR cells. B: JAR cells transfected with pcDNA3.1-KISS-1 had lower invasion capacity than control cells. pcDNA3.1 had no effect upon invasion capacity of JAR cells. Data are normalized to control group (%). Results are expressed as mean ± SEM.

A successful pregnancy requires the cooperation between the developing trophoblasts (exhibiting its special invasive ability) and receptive maternal tissue, accompanied by a range of regulatory factors that influence the biological behavior of trophoblasts. When equilibrium between these factors is disturbed during the first trimester, the probability of miscarriage significantly increases. It is supposed that the pathogenesis of RSA is associated with the regulation of kisspeptin/GPR54 at the maternal-fetal interface. CBA/J×BALB/c is a non-abortion-prone control mating combination, whereas CBA/J×DBA/2 crosses yield abortion-prone pregnancies. We observed a re-markable reduction in the expression of kisspeptin and GPR54 on day 14 (late pregnancy) compared to day 9 (early pregnancy) of gestation. Results arising from the animal model are consistent with immunohistochemical data from human samples. A research has indicated that mRNA levels of KISS1 and GPR54 in the placenta during first trimester is higher than in term of gestation [27]. Crucially, the expression of kisspeptin and GPR54 at the maternal-fetal interface in spontaneous abortion pregnancies (CBA/J×DBA/2) is markedly reduced compared to mice with normal pregnancies (CBA/J×BA-

LB/c). These data, arising from both human and animal tissues, indicated that lower kisspeptin and GPR54 expression may be related to spontaneous abortion. In particular, kisspeptin may play a relatively dominant role.

We then proceeded to explore how KISS1 and its product, kisspeptin, regulated the biological behavior of human trophoblasts in JAR cells. We transfected JAR cells with the recombinant plasmid pcDNA3.1 (+)-KISS1 and then used Kp-10 protein to interfere with these JAR cells. KISS1 and Kp-10 reduced the invasion propensity of JAR cells *in vitro* in a dose-dependent manner, without affecting cell growth and proliferation. These results are consistent with the study by Martin *et al.* [26, 28], in which a human first trimester trophoblast cell line was used in combination with a metastin-derived decapeptide. These data suggest that the KISS1 gene and its product, kisspeptin, play a significant role in the inhibition of trophoblast migration and invasion during the first trimester.

It is incompletely understood how kisspeptin regulates the trophoblast invasion. Importantly, the ability of trophoblasts to digest the surrounding matrix, migrate and invade into the uterine decidua, and angiogenesis is critical to the successful implantation and embryo development [29]. Embryo implantation and tumor invasion use the same biochemical mediators for invasion. However, unlike tumor infiltration, trophoblast invasion is strictly regulated both in time and space. Homeostasis between genes responsible for the promotion and inhibition of its invasion during pregnancy effectively limits trophoblast invasion to a specific range. Abnormalities in either promoter or inhibitor genes can disrupt such homeostasis, and result in irregular invasive ability that is either too strong or too weak, thus, compromising pregnancy outcome. If early trophoblast over-invades the endometrial tissue, it is possible for a placenta accreta or placenta implantation to occur.

The highly invasive capacity of trophoblast may rely on its ability to invade the basement membrane associated with the secretion of collagenases and gelatinases [29, 30]. Metalloprotease (MMPs) are known to degrade the extracellular matrix (ECM) to complete the process of invasion. Xu *et al.* reported that the secretion of MMP9 between week 6 and 11 of gestation, as well as incidences of pregnancy failure, dramatically increased when MMP9 was abnormally expressed [31]. Moreover, MMP9 plays a role in embryo development by promoting trophoblast differentiation and invasion [32]. It is suggested that invasion-inhibitor gene, MMP9, is expressed at low levels at the maternal-fetal interface in RSA patients. We speculate that abnormal KISS1 may influence the expression of MMP9, thus, breaking homeostasis between invasion-promoting and invasion-inhibiting genes, resulting in the defective regulation of trophoblast invasion, and ultimately leading to detrimental effects upon pregnancy. During normal pregnancy, it is likely that invasion-promoter gene, MMP9, is expressed in a dominant manner at the maternal-fetal interface to balance the strong expression of invasion-inhibitor gene, KISS1. And the trophoblast invades into the maternal tissue within a relatively specific range to maintain normal pregnancy. However, at the maternal-fetal interface in RSA patients, the low expression of the invasion-inhibitor gene, KISS1, results in low-level expression of the invasion-promoter gene, MMP9. Consistently, RSA occurs when trophoblasts fail to invade into the maternal tissue.

It is of great interest to investigate the expression of kisspeptin/GPR54 at the maternal-fetal interface and the regulation of KISS1 in relation to the biological behavior of trophoblasts in RSA. Our data suggest that KISS1 exerts a certain influence on the biological behavior of trophoblasts, and its abnormal expression is associated with RSA. The precise function of kisspeptin remains unclear and requires further investigation. We supposed that KISS1 acted as an invasion-inhibitor gene, which abnormal expression broke the homeostasis of trophoblast regulation, thus, resulting in miscarriage. We intend to carry out further investigations to continue development of kisspeptin as the potential therapeutic of RSA.

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Disclosure of conflict of interest

None.

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