ORIGINAL RESEARCH

Elevated expression of KiSS-1 in placenta of preeclampsia and its effect on trophoblast

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SUMMARY
The expression of KiSS-1, MMP-9 and MMP-2 mRNAs and proteins was studied in placentas of women with preeclampsia (PE, n=47) and women of normal pregnancy (NP; n=30). In addition, KiSS-1 mRNA expression as well as cell growth, proliferation and invasion were examined in JAR cells (human trophoblast cell line) transfected with pcDNA3-KiSS-1vector. Expression of KiSS-1 mRNA and protein was higher ($p<0.05$) in women with PE compared with that of NP women. In contrast, expression of MMP-9 and MMP-2 was lower ($p<0.05$) in PE than in NP women. KiSS-1 mRNA was detected in JAR cells successfully transfected with pcDNA3-KiSS-1 gene (JAR-K1, JAR-K2, JAR-K3). KiSS-1 mRNA was not detected in JAR cells transfected with pcDNA3 gene (JAR-P1, JAR-P2) and non-transfected JAR cells. No difference ($p>0.05$) was observed in cell growth among these three cell types. Invasion ability was significantly lower ($p<0.01$) in JAR-K1, JAR-K2 and JAR-K3 cells compared to JAR-P cells and non-transfected JAR cells. Overexpression of KiSS-1 and insufficient expression of MMP-9 and MMP-2 in placenta were demonstrated in women with PE. The data

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suggests that KiSS-1 gene plays an important role in inhibiting trophoblast invasion during placental development. *Reproductive Biology **11** 2*: 99 -115.
Key words: preeclampsia, placenta, trophoblast invasion, KiSS-1, MMP-2, MMP-9

INTRODUCTION

Preeclampsia (PE), a pregnancy-specific syndrome characterized by hypertension and proteinuria is a leading cause of maternal and perinatal morbidity and mortality worldwide. The etiology of PE is poorly understood. Histological examination of placental bed biopsies from PE women demonstrated that limited migration of extravillous cytotrophoblasts into superficial deciduas (i.e. shallow implantation of placenta) was responsible for placental hypoxemia, endothelial cell dysfunction and consequent preeclampsia [4].

There are many similarities between trophoblast invasion and tumor metastasis. Overexpression of some oncogenes were detected in trophoblast during normal early pregnancy. However, the trophoblast invasion ability should be tightly controlled and a balance between the invasion promoting and suppressing genes should be kept [14]. A myriad of genes involved in modulating metastasis has been reported. KiSS-1 was first discovered in metastasis-suppressed melanoma [10] and further in other malignant tumors such as ovarian cancer, renal cell cancer, breast cancer, pancreatic cancer, gastric cancer, and hepatocellular carcinoma [5, 6, 9, 19]. Predominant expression of KiSS-1 mRNA in human placenta was first reported by Ohtaki et al. [15].

Kisspeptins comprise a family of peptides derived from the primary translation product of the KiSS-1 gene. The product is a 145 amino acid precursor polypeptide, and proteolytic processing results in formation of shorter kisspeptins with 54 (Kp-54; metastin), 14 (Kp-14), 13 (Kp-13) or 10 (Kp-10) amino acids [15]. Kisspeptin is a ligand for G-protein coupled receptor (GPR54, hOT7T175, AXOR12, KiSS-1R; [23]). Terao et al. [22] indicated that metastin and hOT7T175 signaling may participate in implantation of the mammalian embryo, placenta formation, and maintenance of pregnancy. The mean plasma concentration of kisspeptin in both men and non pregnant women is low (1.3 pmol/l). The mean concentration of kisspeptin in maternal plasma is 1 230 pmol/l during the first

trimester, 4 590 pmol/l during the second trimester and 9 590 pmol/l during the third trimester. After delivery, the plasma kisspeptin lowers to non pregnant level (7.6 pmol/l; [8]). Previously, we showed that expression of KiSS-1 mRNA increased gradually during pregnancy [17].

Matrix metalloproteinases (MMPs), zinc-dependent proteolytic enzymes that cleave constituents of the extracellular matrix and basement membranes, were known as key factors involved in promoting cancer spread. Two members of the family, gelatinase B (MMP-9) and gelatinase A (MMP-2), have been found to enhance cell invasion in tumor metastasis and normal pregnancy. The activity of MMPs may be specifically inhibited by tissue inhibitor of metalloproteinases (TIMP; [16, 26]). We demonstrated that the expression of MMP-9 and MMP-2 was elevated during the first trimester of normal pregnancy and gradually declined thereafter [17]. Hiden et al. [7] reported that the expression of kisspeptin and its receptor in humans and rats was the highest when invasiveness of trophoblast reached its peak. We hypothesize that KiSS-1 as well as MMP-9 and MMP-2 play an important role in the development of PE. Therefore, the aim of the present study was to compare expressions of KiSS-1, MMP-9 and MMP-2 mRNAs and proteins between PE patients and women with normal pregnancy. In addition, we transfected KiSS-1 gene in JAR cells (human trophoblast cell line) to explore the potential pathogenesis of KiSS-1 protein in preeclampsia.

MATERIALS AND METHODS

Placental samples collection

Placental samples were collected between March 2005 and September 2008, immediately after cesarean section from 47 women diagnosed with PE and 30 age-matched women with normal pregnancy (NP). Preeclampsia was defined as pregnancy-induced hypertension (blood pressure ≥140/90 mm Hg) and proteinuria (≥300 mg/24 hours) in women who were normotensive before pregnancy and had no other underlying clinical problems. All women were more than 35 weeks' gestation at delivery. The experiment was performed according to the guidelines of the Committee for the Rights of Human Subjects in the 2nd affiliated Hospital of Soochow University.

KiSS-1, MMP-9 and MMP-2 mRNA analysis (RT-PCR)

Immediately after delivery, the samples of placenta were dissected from the decidual basalis and rinsed. Total RNA was extracted from the placenta using the TRIZOL reagent (Invitrogen Co., Camarillo, USA). The RNA was reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega Co., Madison, USA). cDNAs were amplified under conditions described in Table 1. The PCR products were resolved on 1.5% agarose gel contained 0.5 μg/ml ethidium bromide (EB). The PCR products were normalized to β-actin.

Table 1. Characteristics of PCR reactions performed on the placental tissue of PE and NP women

Genes	Expected size	Forward primers	Reverse primers	Temperature and PCR cycles			
KiSS-1	438 bp	ATGAACTCACTGGTTTCTTGGCAG	TCACTGCCCCGCACCTG	94°C, 1 min	68°C, 50 s	72°C, 30 s	30 cycles
MMP-9	300 bp	GGTGGACCGGATGTTCCC	GCCCACCTCCACTCCTCC	94°C, 30 s	60°C, 30 s	72°C, 40 s	30 cycles
MMP-2	285 bp	AACCCTCAGAGCCACCCCTA	GTGCATACAAAGCAAACTGC	94°C, 40 s	55°C, 30 s	72°C, 40 s	30 cycles
β-actin	621 bp	ACACTGTGCCCATCTACGAGG	AGGGGCCGGACTCGTCATACT	94°C, 30 s	60°C, 30 s	72°C, 40 s	30 cycles

Total RNA was extracted from samples of PE and normal pregnancy and reverse transcribed into cDNA, then PCR was performed as described in materials and methods; PE: preeclampsia; NP: normal pregnancy

KiSS-1, MMP-9 and MMP-2 protein analysis (WB)

Placenta tissue was homogenized on ice for 1 min in cold lysis buffer [pH 7.5; 50 mM Tris-HCL, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 100 μM sodium orthovanadate (Sigma, St. Louis, USA), 50 μl/g tissue protease inhibitor cocktail]. Homogenates were centrifuged (4˚C; 12000×g; 10 min) and supernatants were collected. Protein concentrations were determined by the GeneQuant ultraviolet spectrophotometer. Samples were mixed 1:1 with loading buffer [pH 6.8; 250 mMTris-HCl, 20% glycerol, 10% sodium-dodecyl-sulfate (SDS), 0.2 mol/l dithiothreitol, 0.1% bromophenol blue] and boiled for 5 min before loading. Samples were separated on 10% SDS-

polyacrylamide resolving gels using Protean II apparatus (BioRad, Hemelhempstead, UK) at a constant current of 20 mA. Each well was loaded with 50 μg of protein. Low molecular weight range markers (19 to 118 kDa, MBI, Burlington, Canada) were loaded beside the samples. Protein was transferred in buffer containing 25 mmol/l Tris, 190 mmol/l glycine, 20% methanol at a constant 60 V to nitrocellulose membranes (Amersham Co, Uppsala, Sweden). Filters were blocked for 2h at room temperature (RT) in phosphate-buffered saline (PBS) containing 5% bovine serum albumin. The antibody [anti-MMP-9 and anti-MMP-2 (Oncogene Research Products, La Jolla, USA), anti-KiSS-1 (Phoenix Pharmaceuticals, Belmont, USA)] diluted 1:500 in TBST were added overnight at 4˚C. The filters were rinsed and washed five times for 5 min in TBST and then incubated with horseradish peroxidase-conjugated goat-anti-rabbit IgG diluted 1:1000 in TBST for 1 hour at RT. Blots were washed 5 times for 5 min. Proteins were detected using the Hyperfilm ECL (Amersham Co, Uppsala, Sweden) detection system and filters were exposed to Hyperfilm ECL. Protein expression was normalized to β-actin.

Stable transfection JAR Cells with KiSS-1

Total RNA was extracted from human term placenta tissue. The open reading frame (ORF) of KiSS-1 cDNA was isolated with RT-PCR, analyzed by Basic Local Alignment Search Tool (BLAST) to the NCBI ORF and demonstrated 100% identity with the sequence of KiSS-1 cDNA in human cDNA library. KiSS-1 cDNA was subcloned into the plasmid pcDNA3 with Hind III and BamH I (Invitrogen Co., Camarillo, USA) restriction enzymes. KiSS-1 cDNA was included in genome of eukaryotic expression vector pcDNA3 and confirmed by restriction site analysis and sequencing. The recombinant eukaryotic expression vector (pcDNA3-KiSS-1) with full-length cDNA encoding human KiSS-1 gene was constructed and confirmed by enzyme digest identification and DNA sequencing. Either blank pcDNA3 vector (as control) or pcDNA3-KiSS-1 vector were transfected into human trophoblast cell line JAR by lipofectamine. Transfected cells were selected in Iscove's Modified Dulbecco's Media with 0.35 mg/ml G418 (Life Technologies, Carlsbad, USA). Detection of neo gene was used to validate the success of transfection. Cell subclones were isolated by limited dilution and cultivated with routine methods.

Total RNA was extracted from untransfected cells and cells containing pcDNA3-KiSS-1 and pcDNA3 using the TRIZOL reagent (Invitrogen Co., Camarillo, USA). The RNA was reverse transcribed and amplified by PCR. Primers and PCR conditions of neo and β-actin analysis are presented in Table 2.

Table 2. Characteristics of PCR reactions performed in the cell transfection experiment

Genes	Expected size	Forward primers	Reverse primers	Temperature and PCR cycles			
neo	690 bp	GAGAGGCTATTCGGCTATGA	AGGAGCACGAAATGCCATAG	94°C, 1 min	55°C,1 min	72°C, 2 min	30 cycles
β-actin	267 bp	TCGACAACGGCTCCGGCAT	AAGGTGTGGTGCCAGATTTTC	94°C, 30 s	58°C, 30 s	72°C, 40 s	30 cycles

Total RNA was extracted from samples of JAR cells and reverse transcribed into cDNA, then PCR was performed as described in materials and methods.

Detection of cell morphology and viability

Morphology of pcDNA3-KiSS-1 and pcDNA3-treated cells as well as untreated JAR cells as controls were examined under light microscope. Cell viability was performed by MTT assay, which is a sensitive and specific method for examining cell survival rate and growth rate. Cells were plated at 1×10^3/well on 96-well plates. MTT dissolved in PBS was added to culture medium at 5 g/l at the end of each incubation time. Four hours later, incubation (37˚C, 5% CO_2 and saturation humidity) medium was removed and 200 µl DMSO was added to each well and mixed. Absorbance was read using spectrophotometer at wavelength 490 nm. The viability experiment was performed daily for eight days and repeated for each cell line six times.

In vitro cell migration assay

To investigate whether KISS-1 protein is involved in cell migration**,** cell invasion was measured by Matrigel Boyden Chamber Kit (Becton Dickinson Biosciences, Franklin Lakes, USA). A porous (8 µm in diameter) polyethylene terephthalate (PET) membrane was coated with Matrigel. A 0.5 ml of treated and untreated cell suspensions was added to the upper compartment respectively. After

incubation at 37˚C, 5% CO_2 and saturated humidity for 20 h, cells were fixed in formaldehyde and stained. The number of cells migrating through the membrane was counted under high power (×200) of light microscope. The experiment was repeated for every cell line 12 times (12 chambers).

Statistical analysis

Data are presented as means±SEM. Differences between groups were examined using Student t-test. Statistical significance was set at $p<0.05$. Calculations were performed using SPSS 11.0 Software (SPSS Software Development Co., Chicago, USA).

RESULTS

Placental expression of KiSS-1, MMP-9 and MMP-2

KiSS-1, MMP-9 and MMP-2 mRNA were detected in placentas of NP and PE women (fig. 1). Expression of KiSS-1 mRNA was significantly higher in the placentas of women with PE compared with NP women. Placental expression of MMP-9 and MMP-2 mRNA was significantly lower in PE than that in NP women (fig. 2).

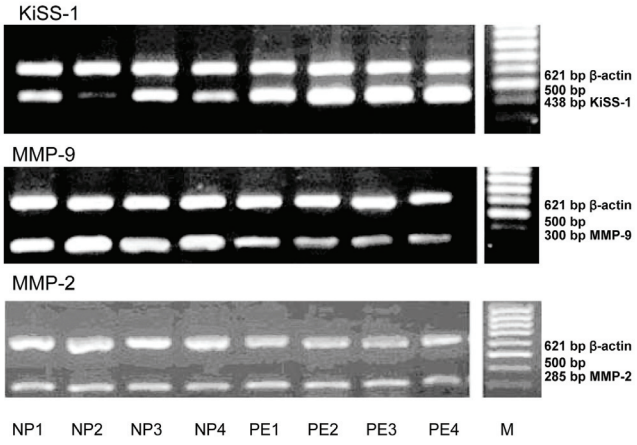

Figure 1. Expression of KiSS-1, MMP-9 and MMP-2 mRNA in placenta of normal pregnancy (NP) and in preeclampsia (PE). NP1-NP4: four examples of NP placentas; PE1-PE4: four examples of PE placentas; bp: base pairs; M: molecular marker (100-1000 bp)

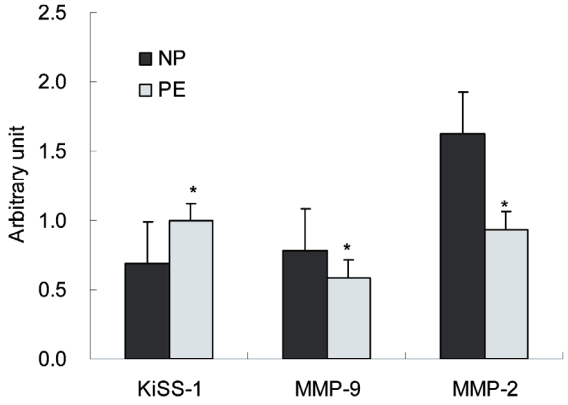

Figure 2. Semiquantitative analysis of KiSS-1, MMP-9 and MMP-2 mRNA expression (means±SEM) in the placenta of normal pregnancy (NP) and in preeclampsia (PE). The relative density of each gene product was compared between the two examined groups. *p<0.05

Expression of KiSS-1 protein was higher in placenta of PE women in comparison to NP women. Placental expression of MMP-9 and MMP-2 proteins was significantly lower in PE women than that in NP women (figs. 3 and 4).

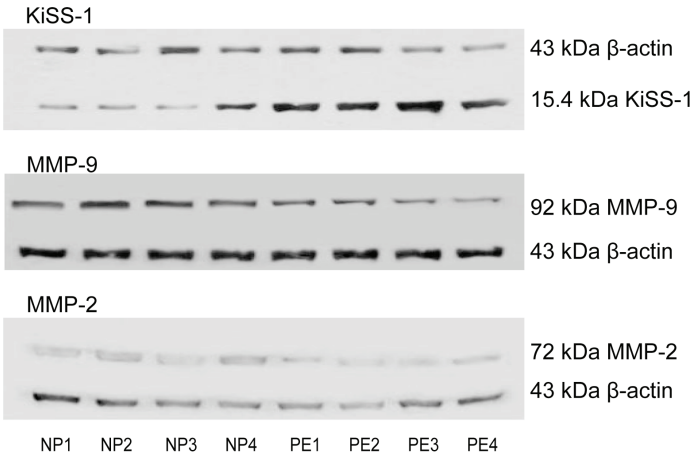

Figure 3. Expression of KiSS-1, MMP-9 and MMP-2 protein in placenta of normal pregnancy (NP) and in preeclampsia (PE). NP1-NP4: four examples of NP placentas; PE1-PE4: four examples of PE placentas.

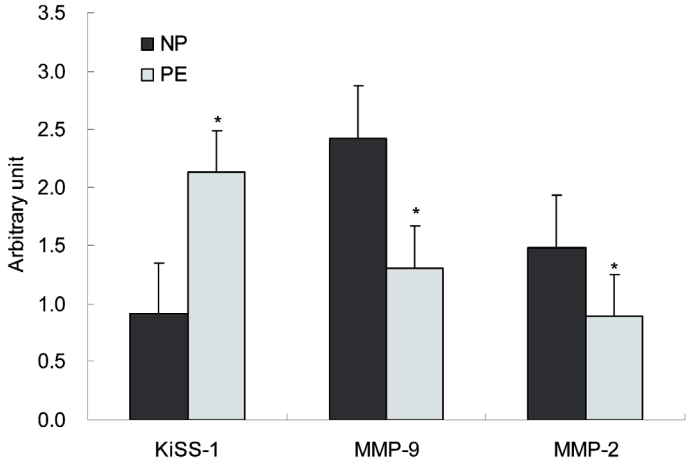

Figure 4. Semiquantitative analysis of KiSS-1, MMP-9 and MMP-2 protein expression (means±SEM) in placenta of normal pregnancy (NP) and in preeclampsia (PE). The relative density of each protein was compared between the two examined groups. *$p<0.05$

Identification of stable transfection in JAR cell with KiSS-1

JAR cells (human trophoblast cell line) were transfected with pcDNA3-KiSS-1 (pcDNA3 vector as control). After transfection, five clones with neo gene were selected: three from pcDNA3-KiSS-1 transfected group (JAR-K1, JAR-K2, JAR-K3) and two from pcDNA3 transfected group (controled group; JAR-P1, JAR-P2). JAR cells all died within a week as a result of non-resistance to G418.

The neo gene (690 bp) was included in pcDNA3vector. It has been successfully detected in the five clones selected (JAR-K1, JAR-K2, JAR-K3, JAR-P1, and JAR-P2) and was not detected in JAR cells, indicating a stable transfection of pcDNA3-KiSS-1 and pcDNA3 vector into JAR cells. Expression of KiSS-1 mRNA (438 bp) was positive in JAR-K1, JAR-K2, JAR-K3 while negative in JAR-P1, JAR-P2 and JAR cells (fig. 5).

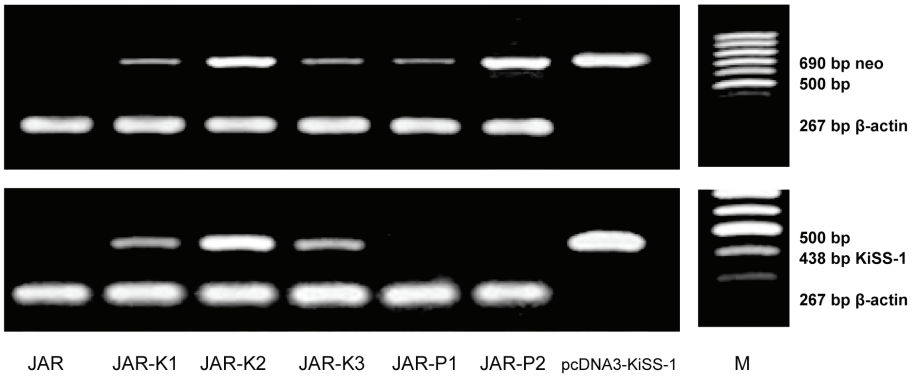

Figure 5. Expression of neo mRNA detected in five screened cell clones (JAR-K1, JAR-K2, JAR-K3, JAR-P1 and JAR-P2). M: molecular marker (100-1000 bp); JAR-K1, JAR-K2 and JAR-K3: JAR cells transfected with pcDNA3-KiSS-1 and successfully recombined KiSS-1 gene; JAR-P1, JAR-P2: JAR cells transfected with pcDNA3 and recombined pcDNA3 gene.

Cell morphology and viability

Cell morphology did not differ between transfected and non-transfected JAR cells. Growth curves measured by MTT did not reveal any significant differences among groups ($p>0.05$; fig. 6), indicating that KiSS-1 does not affect cell proliferation.

Invasion ability of the JAR cells

Boyden Chamber invasion assay showed that the number of invading cells was significantly lower in the groups with recombinant KiSS-1 gene (JAR-K1, JAR-K2, JAR-K3) compared with that in JAR-P1, JAR-P2 and JAR groups ($p<0.01$). The number of invading cells did not significantly differ among the JAR-P1, JAR-P2 and JAR cells ($p>0.05$; fig. 6).

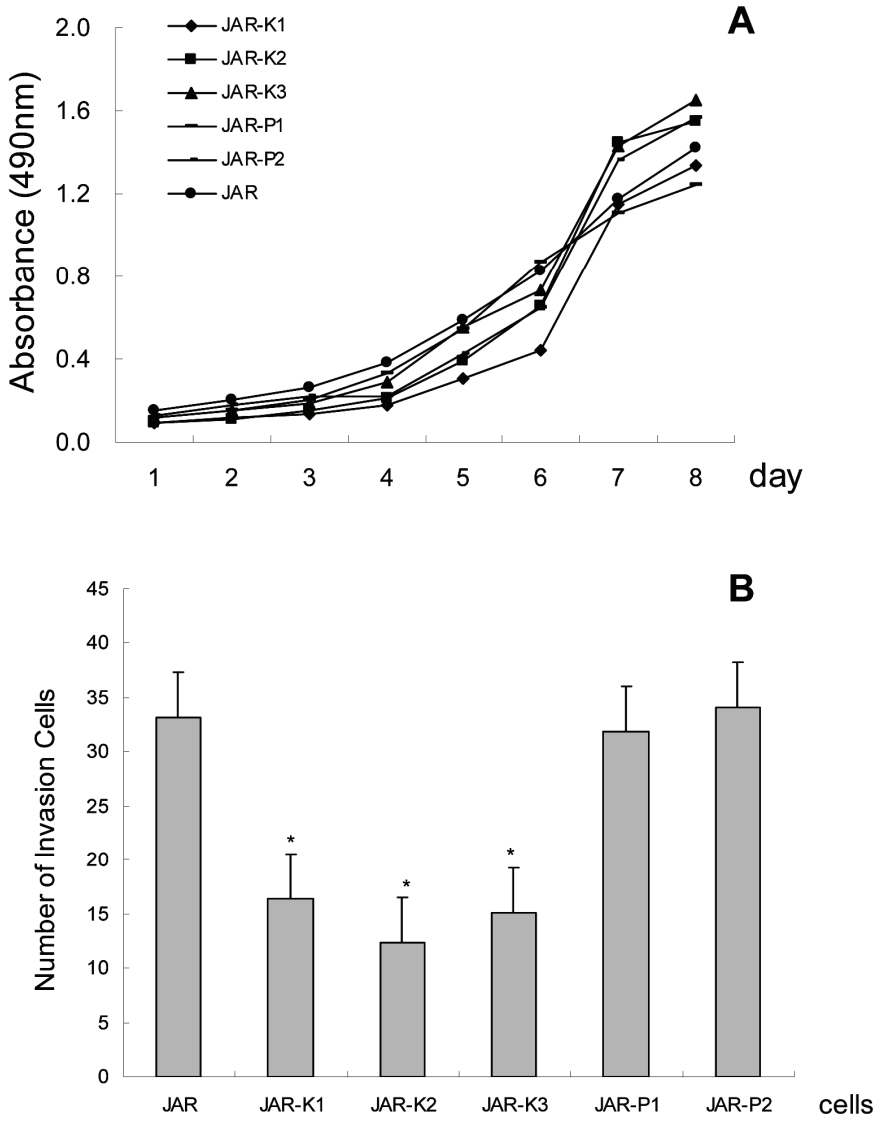

Figure 6. Cell growth and cell invasion ability of JAR cells transfected with or without pcDNA3-KiSS-1. A: cell growth ($p>0.05$); B: cell invasion ability. JAR-K1, JAR-K2 and JAR-K3: JAR cells transfected with pcDNA3-KiSS-1 and successfully recombined KiSS-1 gene; JAR-P1, JAR-P2: JAR cells transfected with pcDNA3 and recombined gene of pcDNA3; *indicates the significant difference compared with JAR cells ($p<0.01$)

DISCUSSION

Invasion of extravillous trophoblast into the uterus is a key process of successful placental and embryonic development in humans. Degradation of maternal smooth muscle around maternal spiral arteries and replacement of endothelial cells by endovascular trophoblast ultimately lead to the dilation of the vessel lumen, which is necessary to enhance intervillous blood flow. Shallow invasion of trophoblast and incomplete transformation of spiral arteries are hallmarks of maternal preeclampsia, a life threatening hypertensive disorder of the mother [2].

Recent research has suggested that kisspeptins may play a role in pregnancy and placentation. Our previous study demonstrated detectable expression of KiSS-1, MMP-9 and MMP-2 during the first trimester of normal pregnancy. We showed that expression of KiSS-1 mRNA increased gradually as gestational weeks (GW) increased, with the lowest level during fifth GW and the highest level at term [17]. At term, placental expression of KiSS-1 mRNA and protein was higher in PE patients compared with NP patients [26]. In order to explore the function of KiSS-1 in the trophoblast, the recombinant eukaryotic expression vector containing full-length cDNA encoding KiSS-1 gene (pcDNA3-KiSS-1) was transfected into human trophoblast cell line JAR. Constitutive stable of JAR cells were generated by transduction with the eukaryotic expression vector pcDNA3-KiSS-1. RT-PCR confirmed the expression of KiSS-1 mRNA in JAR-K cells. No differences were observed in cell proliferation between transfected and non-transfected cells. However, the invasion ability was significantly lower in the transfected cells compared with controls. This suggests that KiSS-1 gene suppressed trophoblast cell invasion *in vitro* without affecting cell growth and proliferation.

To mimic explanted human placental arteries Ramaesh et al. [18] designed an experiment with tube-like structure formation covered by human umbilical vein endothelial cells (HUVEC). They found that kisspeptin-10 inhibited the sprouting of new vessel from the explanted arteries in a dose-dependent manner. It suggests that kisspeptin-10 may contribute to the regulation of angiogenesis in placenta [18].

Behavior of trophoblast during early pregnancy strongly resemble that of cancer cells. The same genes are activated in these

two processes i.e. MMPs, insulin-like growth factor (IGF-I), vascular endothelial growth factor (VEGF), leukemia inhibitor (LIF), colony-stimulating factor-I (CSF-I), E-cadherin, and tumor necrosis factor (TNF). Trophoblast may undergo a molecular process similar to an epithelial-mesenchymal transition that is thought to play a critical role in cancer cell invasion and metastasis [1]. MMPs are produced by cytotrophoblastic cells (CTB) and are instrumental to their invasive behavior. However, in contrast to tumor invasion, trophoblast invasion is limited both in time and in space. It takes place during the first trimester of pregnancy and the invasion does not go beyond the proximal third of the myometrium. Factors regulating MMP expression are of maternal and fetal origin [3]. Dysregulation of these genes during early pregnancy has been implicated in a wide spectrum of abnormal pregnancies.

We found that placental expressions of MMP-9 and MMP-2 mRNAs and proteins were lower in PE women than in women with normal pregnancy, suggesting that MMP-9 and MMP-2 may contribute to the limited trophoblast invasion in PE. Since the invasive capacity of the trophoblast depends on the secretion of proteolytic enzymes such as MMPs to degrade components of the extracellular matrix (ECM), it is reasonable to infer that MMP-9 and MMP-2 genes are involved in promoting trophoblast invasion during normal pregnancy [12]. MMP-9 was reported to play a major role in human trophoblast invasiveness *in vitro*. There was further evidence for a role of MMP-2 in regulation of trophoblast invasion and spiral artery remodeling in early placentation [13]. Verlohren et al. [24] treated preeclamptic and control pregnant Sprague-Dawley rats with doxycycline, an inhibitor of matrix metalloproteinase activity. Inhibition of MMPs resulted in intrauterine growth retardation and lighter placentas in both groups as well as reduced trophoblast invasion, trophoblast-induced vascular remodeling and associated reduced placental perfusion in preeclamptic rats [24]. It suggests that down-regulations of MMP-2 and MMP-9 in trophoblast during early pregnancy in PE women may cause poor development of placental vessels, playing a role in the pathogenesis of preeclampsia.

Maintenance of normal pregnancy depends on the dynamic balance between expression of genes promoting and inhibiting invasion in placenta. We do not know whether the activation and inhibition of the invasion genes are controlled by independent factors.

Recent studies reported a negative regulation of MMP-2 and MMP-9 expression by KiSS-1. The product of KiSS-1 gene was found to form a stable complex with pro-MMP-2 and pro-MMP-9 *via* the propeptide domain of MMP. The affinity of KiSS-1 protein for pro-MMP was high and the complex was extremely stable. KiSS-1 diminished MMP-9 expression by reducing NF-κB binding to the promoter of MMP-9. KiSS-1 may be involved in one of many mechanisms responsible for diminished metastases [21]. Yoshioka et al. used metastin to down-regulate MMP-2 expression to suppress the motility and invasive ability of renal carcinoma cells, which suggested that metastin may have a potential therapeutic use [25]. Lee and Kim [11] demonstrated that KiSS-1 suppressed MMP-9 expression by activating p38 MAP kinase in human stomach cancer. Therefore, metastin in combination with a MMP inhibitor may be used as an antimetastatic agent, thus providing a potential therapeutic target to treat MMP overexpression-induced diseases [20].

We suggest that a tightly controlled balance between promoting and suppressing invasion genes is critical for temporal and spatial development of normal placenta. Disturbance of this balance is largely responsible for pathological pregnancies. The over-expression of KiSS-1 together with the down-regulation of MMP-2 and MMP-9 genes may account for the limited invasion ability of trophoblast and consequent spiral arties deficiency present in the pathogenesis of PE. It should be emphasized that we have examined late gestation tissue where trophoblast invasion is long established. Experiments performed in different stages of pregnancy should provide more data enabling better understanding of the molecular mechanisms underlying PE.

REFERENCES

1. Bilban M, Haslinger P, Prast J, Klinglmüller F, Woelfel T, Haider S, Sachs A, Otterbein LE, Desoye G, Hiden U, Wagner O, Knöfler M **2009** Identification of novel trophoblast invasion-related genes: heme oxygenase-1 controls motility via peroxisome proliferator-activated receptor gamma. *Endocrinology* **150** 1000-1013.
2. Brosens JJ, Pijnenborg R, Brosens IA **2002** The myometrial junctional zone spiral arteries in normal and abnormal pregnancies: a review of the literature. *American Journal of*

Obstetrics and Gynecology **187** 1416-1423.
3. Cohen M, Bischof P **2007** Factors regulating trophoblast invasion. *Gynecology and Obstetrics Investigation* **64** 126-130.
4. Dekker GA, Sibai BM **1998** Etiology and pathogenesis of preeclampsia: current concepts. *American Journal of Obstetrics and Gynecology* **179** 1359-1375.
5. Dhar DK, Naora H, Kubota H, Maruyama R, Yoshimura H, Tonomoto Y, Tachibana M, Ono T, Otani H, Nagasue N **2004** Downregulation of KiSS-1 expression is responsible for tumor invasion and worse prognosis in gastric carcinoma. *International Journal of Cancer* **111** 868-872.
6. Hata K, Dhar DK, Watanabe Y, Nakai H, Hoshiai H **2007** Expression of metastin and a G-protein-coupled receptor (AXOR12) in epithelial ovarian cancer. *European Journal of Cancer* **43** 1452-1459.
7. Hiden U, Bilban M, Knöfler M, Desoye G **2007** Kisspeptins and the placenta: regulation of trophoblast invasion. *Reviews in Endocrine and Metabolic Disorders* **8** 31-39.
8. Horikoshi Y, Matsumoto H, Takatsu Y **2003** Dramatic elevation of plasma metastin concentrations in human pregnancy: metastin as a novel palcenta-derived hormone in humans. *Journal of Clinical Endocrinology and Metabolism* **88** 914-919.
9. Katagiri F, Nagai K, Kida A, Tomita K **2009** Clinical significance of plasma metastin level in pancreatic cancer patients. *Oncology Reports* **21** 815-819.
10. Lee JH, Welch DR **1997** Identification of highly expressed genes in metastasis -suppressed chromosome 6/human malignant melanoma hybrid cells using subtractive hybridization and differential display. *International Journal of Cancer* **71** 1035-1044.
11. Lee KH, Kim JR **2009** KiSS-1 suppresses MMP-9 expression by activating p38 MAP kinase in human stomach cancer. *Oncology Research* **18** 107-116.
12. Nardo LG, Nikas G, Makrigiannakis A **2003** Molecules in blastocyst implantation. Role of matrix metalloproteinases, cytokines and growth factors. *Journal of Reproductive Medicine* **48** 137-147.
13. Naruse K, Lash GE, Innes BA, Otun HA, Searle RF, Robson SC, Bulmer JN **2009** Localization of matrix metalloproteinase

(MMP)-2, MMP-9 and tissue inhibitors for MMPs (TIMPs) in uterine natural killer cells in early human pregnancy. *Human Reproduction* **24** 553-561.
14. Novakovic B, Rakyan V, Ng HK, Manuelpillai U, Dewi C, Wong NC, Morley R, Down T, Beck S, Craig JM, Saffery R **2008** Specific tumour-associated methylation in normal human term placenta and first-trimester cytotrophoblasts. *Molecular Human Reproduction* **14** 547-554.
15. Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Kitada C, Usuki S, Kurokawa T, Onda H, Nishimura O, Fujino M **2001** Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* **411** 613-617.
16. Palei AC, Sandrim VC, Cavalli RC **2008** Comparative assessment of matrix metalloproteinase (MMP)-2 and MMP-9, and their inhibitors, tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in preeclampsia and gestational hypertension. *Clinical Biochemistry* **41** 875-880.
17. Qiao C, Lin QD **2003** The preliminary study on the mRNA expression of KiSS-1 gene in normal villi tissue of the first trimester. *Chinese Journal of Practical Gynecology and Obstetrics* **19** 289-290.
18. Ramaesh T, Logie JJ, Roseweir AK, Millar RP, Walker BR, Hadoke PW, Reynolds RM **2010** Kisspeptin-10 inhibits angiogenesis in human placental vessels ex vivo and endothelial cells in vitro. *Endocrinology* **151** 5927-5934.
19. Schmid K, Wang X, Haitel A, Sieghart W, Peck-Radosavljevic M, Bodingbauer M, Rasoul-Rockenschaub S, Wrba F **2007** KiSS-1 overexpression as an independent prognostic marker in hepatocellular carcinoma: an immunohistochemical study. *Virchows Archiv* **450** 143-149.
20. Shoji S, Tang XY, Umemura S, Itoh J, Takekoshi S, Shima M, Usui Y, Nagata Y, Uchida T, Osamura RY, Terachi T **2009** Metastin inhibits migration and invasion of renal cell carcinoma with overexpression of metastin receptor. *European Urology* **55** 441-449.
21. Takino T, Koshikawa N, Miyamori H, Tanaka M, Sasaki T, Okada Y, Seiki M, Sato H **2003** Cleavage of metastasis

suppressor gene product KiSS-1 protein/metastin by matrix metalloproteinases. *Oncogene* **22** 4617-4626.
22. Terao Y, Kumano S, Takatsu Y, Hattori M, Nishimura A, Ohtaki T, Shintani Y **2004** Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta. *Biochimica et Biophysica Acta- Gene Structure and Expression* **1678** 102-110.
23. Tomita K, Niida A, Oishi S, Ohno H, Cluzeau J, Navenot JM, Wang ZX, Peiper SC, Fujii N **2006** Structure-activity relationship study on small peptidic GPR54 agonists. *Bioorganic and Medicinal Chemistry* **14** 7595-7603.
24. Verlohren S, Geusens N, Morton J, Verhaegen I, Hering L, Herse F, Dudenhausen JW, Muller DN, Luft FC, Cartwright JE, Davidge ST, Pijnenborg R, Dechend R **2010** Inhibition of trophoblast-induced spiral artery remodeling reduces placental perfusion in rat pregnancy. *Hypertension* **56** 304-310.
25. Yoshioka K, Ohno Y, Horiguchi Y, Ozu C, Namiki K, Tachibana M **2008** Effects of a KiSS-1 peptide, a metastasis suppressor gene, on the invasive ability of renal cell carcinoma cells through a modulation of a matrix metalloproteinase 2 expression. *Life Science* **83** 332-338.
26. Zhang H, Lin QD, Qiao C **2006** Expression of trophoblast invasion related genes mRNA and protein in human placenta in preeclampsia. *Chinese Journal of Obstetrics and Gynecology* **41** 509-513.