Original Paper

CDX2 Enhances HTR-8/SVneo Trophoblast Cell Invasion by Altering the Expression of Matrix Metalloproteinases

Rui-Zhe Jia^a Gui-Chun Ding^b Chun-Mei Gu^b Tao Huang^b Can Rui^{a,b}
Yi-Xiong Wang^b Qin Lu^c

^aDepartment of Obstetrics, Nanjing Maternal and Child Health Hospital, Affiliated with Nanjing Medical University, Nanjing, ^bDepartment of Obstetrics and Gynecology, Yangzhou Maternal and Child Health Hospital, Affiliated with Yangzhou Medical University, Yangzhou, ^cDepartment of Obstetrics and Gynecology, Shanghai Jiao Tong University Affiliated Tongren Hospital, Shanghai, China

Key Words

CDX2 • HTR-8/SVneo cell line • Trophoblast invasion • PI3K/Akt

Abstract

Background/Aims: The invasion of trophoblast cells into the maternal uterine decidua is critical for normal placentation, establishment of pregnancy and maintenance of fetal growth in humans. Several growth factors and cytokines have been implicated in trophoblast invasion, but the underlying regulatory mechanisms of invasion are not fully understood. Our earlier studies have found that caudal-related homeobox transcription factor 2 (CDX2) is hypomethylated in human pre-eclampsia placental tissues. However, whether CDX2 is involved in trophoblast invasion was unclear. **Methods and Results:** In this study, we investigated CDX2 function using a human HTR-8/SVneo cell line that overexpressed CDX2. Cell invasion assays demonstrated that CDX2 enhanced trophoblast cell invasiveness. Meanwhile, MTT assays revealed that CDX2 did not affect cell proliferation. Western blot analysis and quantitative real-time PCR demonstrated that the expression level of matrix metalloproteinase-9 (MMP-9) was significantly increased, whereas the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) was markedly suppressed in the CDX2-overexpressing trophoblast cells. The phosphoinositide-3-kinase (PI3K)/Akt signaling pathway is involved in proliferation, migration, metastasis and invasion. Our study showed that inhibition of PI3K/Akt signaling led to decreased expression of CDX2. **Conclusion:** We concluded that CDX2 is likely regulated by the PI3K/Akt signaling pathway during trophoblast cell invasion. Our findings may reveal new insights into the regulatory mechanisms of trophoblast cell invasion and may be an important contributor to the pathogenesis of pregnancy-related diseases.

Copyright © 2014 S. Karger AG, Basel

R.-Z. Jia and G.-C. Ding contributed equally to this work.

Qin Lu
and Yi-Xiong Wang

Department of Obstetrics and Gynecology, Shanghai Jiao Tong University Affiliated Tongren Hospital, Shanghai 200336 (China)
and Department of Obstetrics and Gynecology, Yangzhou Maternal and Child Health Hospital Affiliated with Yangzhou Medical University, Yangzhou 225002 (China)
E-Mail cuixianwei198615@163.com and E-Mail sbyywyx@163.com

Introduction

Trophoblast cells are the main component of the placenta. Normal placental development is characterized by extravillous trophoblast cells penetrating into the maternal decidua and myometrium [1]. Invading trophoblast cells alter the vasculature inside the uterus and partially replace the endothelial cells of the uterine spiral arteries. These activities of trophoblast cells are key events during the establishment, maintenance and termination of physiological pregnancy [2, 3]. Invasiveness is a characteristic of trophoblast cells; however, trophoblast invasion is a strictly controlled physiological event. Any changes in the trophoblast invasion capacity can seriously affect placental function and can threaten maternal and child health. Defective trophoblast invasion may contribute to many pregnancy-related diseases [4]. For example, excessive invasion can lead to gestational trophoblastic diseases [5], whereas inadequate invasion, specifically restricted endovascular invasion, may cause miscarriage [6], pre-eclampsia [7], fetal growth restriction [8] and other pathological events during pregnancy [9].

Multiple factors including growth factors, cytokines and chemokines are involved in the regulation of trophoblast invasion, such as corin [10], E-cadherin [11], tumor necrosis factor- α (TNF α) [12], matrix metalloproteinase (MMP)-2/MMP-9 [13], interleukin-1- β (IL-1 β)/transforming growth factor- β 1 (TGF- β 1) [14] and tissue inhibitor of metalloproteinase-1 (TIMP-1) [15]. Through the expression and secretion of MMPs, trophoblast cells gain the capacity to degrade extracellular matrix. As a key enzyme during trophoblast invasion, MMP-9 is a member of the MMP family that can efficiently degrade type IV collagen, a main component of the basement membrane [13]. TIMPs are specific inhibitors of MMP-9 that act by forming complexes with MMP-9 [13, 15]. MMP-9 and TIMP-1 are both closely associated with trophoblast invasion. Down-regulation of MMP-9 and up-regulation of TIMP-1 may both lead to decreased trophoblast invasion [16-18].

Caudal-related homeobox transcription factor 2 (CDX2) is a member of the caudal-related homeobox gene family, with multiple functions in mammalian development, such as proliferation and differentiation [19-22]. Our previous study found that CDX2 is hypomethylated in human severe pre-eclampsia placenta [23]. Additionally, CDX2 is a transcriptional regulator essential for the trophoblast lineage [24, 25]. However, the specific role CDX2 plays in trophoblast invasion is still poorly understood. In this study, we hypothesized that CDX2 is a regulator of trophoblast invasion and that abnormal CDX2 expression may be associated with the pathogenesis of pregnancy-related diseases.

To test our hypothesis, we used the HTR-8/SVneo trophoblast cell line to investigate CDX2 function. By using MTT assay and cell invasion assay, the proliferation and invasive capacity of the HTR-8/SVneo cells were investigated. We detected the expressions of MMP-9 and TIMP-1 by Western blot analysis and quantitative real-time PCR. Meanwhile, we evaluated PI3K/AKT signaling in trophoblast cells. Our findings indicate that CDX2 regulates the invasive capacity of the HTR-8/SVneo trophoblasts by altering the expression of MMPs.

Materials and Methods

Reagents and antibodies

Primary polyclonal antibodies against CDX2 (Abcam, ab76541, USA), MMP-9 (Abcam, ab38898, USA), TIMP-1 (Cell Signaling, #8946, USA), PI3K (Abcam, ab22653, USA), and AKT1 (phospho S473, Abcam, ab66138) were used at 1:1000 dilution. Rabbit polyclonal anti- β -actin antibody (Beyotime, AA128) was used at 1:1,000 dilution (final concentration 1 μ g/ml) as a loading control for western blotting. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Abcam, ab6721, USA) was used at 1:5,000 dilution (final concentration 0.2 μ g/ml) as the secondary antibody.

Cell culture and transfection

The HTR-8/SVneo cells (purchased from the Cell Bank of the Chinese Academy of Sciences, China) were maintained in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 U/mL penicillin under standard culture conditions (37°C and 5% CO₂ incubator) [26]. The CDX2 expression plasmid was constructed by inserting the entire coding region of the human CDX2 cDNA (synthesized by Shanghai Generay Biotech Co., Ltd., China) into the pBMN-GFP vector (The Phoenix™ retroviral system, Allele Biotech, USA) using the BamH I and EcoR I endonucleases (TaKaRa, Dalian, China) and was verified by DNA sequencing (Sangon, China). For transfection, the cells were seeded in 6-well plates at a density of 1×10⁴ cells in 2 mL of culture medium per well and were allowed to attach for 24 h. The cells were then transfected with either the CDX2 expression plasmid or a lentiviral vector control plasmid according to the manufacturer's protocol. To evaluate the role of the PI3K pathway in CDX2 regulation and trophoblast invasion, the CDX2-overexpressing cells were treated with LY294002 (Sigma, USA), which is a highly selective inhibitor of the PI3K pathway. After treatment with 0.1 µM LY294002 for 24 h, then harvested for further testing.

In vitro cell invasion assay

The invasive capacity of the HTR-8/SVneo cells was tested after transfection. The cell invasion assay was performed using a QCM ECMatrix 24-well kit (Chemicon, ECM550, USA) according to the manufacturer's instructions. The cells were resuspended in RPMI-1640 medium without FBS. They were then seeded into the upper compartment of the invasion chamber. The lower compartment was then immediately loaded with 600 µL medium containing 10% FBS as a chemoattractant. After 24 h of incubation at 37°C in a humidified atmosphere containing 5% CO₂, non-migrating cells in the top compartment were completely removed by a sterile cotton swab, while those cells that had invaded into the lower chambers were stained with 0.04% Trypan Blue solution, washed with distilled water, and dried at room temperature. The invading cells were eluted with 100 µL of 10% acetic acid. The absorbances were then measured at 546 nm and 578 nm using a spectrophotometer (MK3, Thermo, USA). Each experiment was performed in triplicate.

MTT assay for cell proliferation

The proliferation capacity of the trophoblast cells was assessed using an MTT (Sigma, MO, USA) assay according to the manufacturer's instructions. The CDX2-overexpressing HTR-8/SVneo cells and the control cells were harvested with 0.05% trypsin-EDTA and were resuspended in medium. A total of 10⁴ cells in 100 µL medium per well were seeded into 96-well plates and incubated for 48 h at 37 °C and 5% CO₂ in a humidified incubator. Twenty microliters of MTT (5 mg/mL stock) was then added to each well, and the plates were incubated for another 4 h. The medium was then removed, and 150 µL of dimethyl sulfoxide (DMSO) was added. The plates were gently mixed on a shaker at room temperature for 10 min. Finally, the absorbances were measured at 490 nm using a spectrophotometer. There were five technical replicates per sample in each experiment, and each experiment was independently repeated three times.

RNA extraction and quantitative real-time PCR

TRIzol (Invitrogen, USA) was used to extract total RNA from approximately 5×10⁶ cells. Following extraction, 1 µg of RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, USA) and a random primer according to the manufacturer's instructions. Quantitative real-time PCR reactions were performed using SYBR Green (Applied Biosystems, USA) on an ABI 7500 system. Melting curve analyses were performed to evaluate the primer specificities. The PCR primers are listed in Table 1. The results were normalized to the level of 18S rRNA. There were three technical replicates and at least three biological replicates per sample.

Western blotting

Protein extracts were prepared from transfected HTR-8/SVneo cells using RIPA lysis buffer (Sigma, USA). Protein concentration was measured using the bicinchoninic acid (BCA) method. Samples containing equal amounts of proteins (100 µg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk in a Tris-buffer containing 0.05% Tween-20 (TBST) for 1 h and were then incubated with the primary antibodies at 1:1000 dilution. The membranes were then

Table 1. The primer sequences used for the quantitative real-time PCR (F: forward primer and R: reverse primer)

Gene	Primer sequence	Product size (bp)
Cdx2	F : 5'-GTCCTCGGCAGCCAAGTGAAC-3'	120
	R : 5'-TCCGGATGGTGATGTAGCGACTGTAG-3'	
MMP-9	F : 5'-TGCCCGGACCAAGGATACAGTTT-3'	184
	R : 5'-GTTTCAGGGCAGGACCATAGAGG-3'	
TIMP-1	F : 5'-ACCGCAGCGAGGAGTTTCT-3'	185
	R : 5'-CAGTTTGAGGGGATGGATAA-3'	
PI3K	F : 5'-ATGGGGATGATTACGGCAAGATA-3'	151
	R : 5'-TTTCGCACCACTCAATAAGTC-3'	
AKT	F : 5'-CCCCGAGGTGCTGGAGGACAAT-3'	97
	R : 5'-AAGGGCAGGCGACCGCATCAT-3'	
18S	F : 5'-AGTCGCCGTGCCTACCAT-3'	129
	R : 5'-CGGGTCGGGAGTGGGTAAT-3'	

incubated with HRP-conjugated goat anti-rabbit IgG. Chemiluminescence detection and quantification of protein bands were performed as described previously [27]. Densitometry analysis of relative protein levels was performed using the Image J software [28, 29]. All experiments were repeated at least three times.

Statistical analysis

We used the SPSS 20.0 statistical software for data analysis. The data are expressed as the means $\pm$ standard deviations (SD). Where appropriate, statistical analysis were performed with analysis of variance (ANOVA) test. Otherwise, comparisons between groups were conducted using Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Overexpression of CDX2 in HTR-8/SVneo cells

The HTR-8/SVneo cells were seeded into 6-well plates and transfected with either the CDX2 expression plasmid or the control plasmid. After 48 h, the cells were harvested for RNA and protein extraction. The protein expression of CDX2 was verified by western blotting (Fig. 1A). The mRNA expression of CDX2 was assessed by quantitative real-time PCR, and the level was 5.1-fold higher (Fig. 1B, Student's *t* test, $**p < 0.01$) in the CDX2-overexpressing cells than in the control.

Effects of CDX2 overexpression on trophoblast invasion and proliferation

We transiently overexpressed CDX2 in the HTR-8/SVneo cells to study the roles of CDX2 in trophoblast invasion and proliferation using the assays described above. We found that the invasion capacity of the CDX2-overexpressing cells was significantly higher than that of the control. The absorbances of the invaded cells were measured at 546 nm and 578 nm. The data are presented as means $\pm$ SD, and the difference between the two groups of samples was statistically significant at $p < 0.05$ (Student's *t* test, Fig. 2A, 2B). An MTT assay was performed to determine whether the increase in invasion by the CDX2-overexpressing

Fig. 1. CDX2 overexpression in HTR-8/SVneo cells. HTR-8/SVneo trophoblast cells were transfected with pBMN-CDX2-GFP (CDX2) or the empty vector pBMN-GFP (Control). (A) Western blot analysis of CDX2 expression in the CDX2-overexpressing HTR-8/SVneo cells and the control cells. β -Actin was used as an internal control. (B) The relative mRNA expression level of CDX2 was measured by quantitative real-time PCR. Data are represented as means $\pm$ SD. Student's *t* test, ** $p < 0.01$ compared with the control.

Fig. 2. Effects of CDX2 expression on HTR-8/SVneo cell invasion and proliferation. (A and B) Overexpression of CDX2 enhanced trophoblast invasion. The absorbances of invaded cells were measured at 546 nm and 578 nm. Data are represented as means $\pm$ SD; Student's *t* test, * $p < 0.05$ compared with the control. (C) Cell proliferation measurements revealed no significant difference between the two groups (Student's *t* test, $p > 0.05$). Three independent experiments produced similar results.

cells was caused by increased cell proliferation. The results indicated that the proliferation capacity of the cells was not significantly changed when CDX2 was overexpressed (Fig. 2C). Three independent experiments produced similar results.

The effects of CDX2 on MMP-9 and TIMP-1 expression

We measured the protein expression levels of MMP-9 and TIMP-1 by western blotting. Overexpressing CDX2 significantly increased MMP-9 expression and decreased TIMP-1 (the inhibitor of MMP-9) expression in the HTR-8/SVneo cells (Fig. 3A). Densitometry analysis revealed that MMP-9 was increased to 340% compared with the control, whereas TIMP-1 was decreased to 35% relative to the control (Fig. 3B). Additionally, quantitative real-time PCR analysis showed the same trend as the western blots, with MMP-9 mRNA up-regulated and TIMP-1 mRNA down-regulated in CDX2-overexpressing HTR-8/SVneo cells (Fig. 3C).

Regulation of CDX2 expression via the PI3K/AKT pathway

To elucidate the mechanisms through which CDX2 promotes trophoblast invasion, we evaluated PI3K/AKT signaling in trophoblast cells. We measured PI3K and p-AKT expression by western blotting and quantitative real-time PCR in the CDX2-overexpressing HTR-8/

Fig. 3. Effects of CDX2 on MMP-9 and TIMP-1 expression. (A) MMP-9 and TIMP-1 protein expression was assessed by western blots in HTR-8/SVneo trophoblast cells transfected with pBMN-GFP (control) or pBMN-CDX2-GFP (CDX2). (B) MMP-9 and TIMP-1 protein expression in control and CDX2-overexpressing HTR-8/SVneo trophoblasts. Three independent experiments produced similar results. Data are represented as means $\pm$ SD. ANOVA-test, ** $p < 0.01$ compared with the control. (C) Quantitative real-time PCR analysis of MMP-9 and TIMP-1 mRNA expression in CDX2-overexpressing HTR-8/SVneo cells and the control cells (ANOVA-test, ** $p < 0.01$). Data are represented as means $\pm$ SD from three independent experiments.

Fig. 4. Regulation of CDX2 expression via the PI3K/AKT pathway in HTR-8/SVneo cells. (A) PI3K and p-AKT protein expression in control and CDX2-overexpressing HTR-8/SVneo trophoblasts. No significant difference was detected between the two groups. (B) Inhibition of PI3K signaling by LY294002 (ly) decreased CDX2 mRNA expression in HTR-8/SVneo trophoblast cells. (C and D) Effect of LY294002 on the invasion capacity of CDX2-overexpressing HTR-8/SVneo trophoblast cells. Data are represented as means $\pm$ SD from three independent experiments (ANOVA-test, * $p < 0.05$).

SVneo cells and the control cells. Surprisingly, CDX2 did not affect PI3K/AKT signaling. No significant difference was observed for PI3K or p-AKT at either the mRNA or the protein level (Fig. 4A). However, when PI3K signaling was blocked by the specific inhibitor LY294002 in the HTR-8/SVneo cells, CDX2 expression was reduced (Fig. 4B) and the invasion capacity of the trophoblasts was decreased compared with the control cells (Fig. 4C, Fig. 4D: $0.173 \pm 0.041 / 0.281 \pm 0.042$ vs. $0.259 \pm 0.033 / 0.339 \pm 0.035$ $p < 0.05$). Invasion was also reduced when PI3K signaling was inhibited by LY294002 in the CDX2-overexpressing cells (Fig. 4C, Fig. 4D: $0.231 \pm 0.035 / 0.264 \pm 0.038$ vs. $0.322 \pm 0.045 / 0.435 \pm 0.045$, $p < 0.05$). The absorbances of the invaded cells were measured at 546 nm and 578 nm using a spectrophotometer (Fig. 4C, 4D). Taken together, these data suggested that PI3K/AKT signaling might regulate CDX2

expression in HTR-8/SVneo cells; however, CDX2 does not appear to reciprocally regulate the PI3K/AKT pathway.

Discussion

Like tumor cells, invasiveness is a feature of trophoblasts; however, unlike tumor invasion, trophoblast invasion is a strictly controlled physiological event. The placenta is a critical organ for the development of the fetus in utero. Trophoblast invasion into the uterine spiral arteries and the rebuilding of the arteries are crucial for normal placental development [2, 3, 30]. Imperfect uterine spiral artery rebuilding and inadequate trophoblast invasion have been associated with a range of pregnancy-related diseases [5-9].

CDX2 is a member of the caudal-related homeobox gene family and is involved in differentiation, proliferation, and maintenance of intestinal epithelial cells [19-22]. Using intestinal cell lines, numerous studies have indicated that CDX2 acts as a tumor suppressor by inhibiting cell proliferation [21, 22]. However, it has also been reported that CDX2 is associated with trophoblast differentiation [24, 25, 31]. Our previous study found that CDX2 is hypomethylated in human severe pre-eclampsia placental tissues. Therefore, we sought to further investigate the effect of CDX2 on trophoblast invasion and proliferation. In this study, we found that CDX2 overexpression increased the invasiveness of the HTR-8/SVneo cells (Fig. 2A, 2B) but did not affect cell proliferation (Fig. 2C). CDX2 may function in trophoblast invasion to prevent shallow placenta implantation and other pregnancy-related diseases.

At present, it is known that several cytokines and growth factors are associated with the regulation of trophoblast invasion [10-15, 32, 33]. However, very few of these regulatory factors have been investigated in depth. Recent reports have demonstrated that MMP-9 down-regulation and TIMP-1 up-regulation can both lead to reduced trophoblast invasiveness [15-17]. We have also investigated the roles of MMP-9 and TIMP-1 in trophoblast invasion. Previous reports have shown that CDX2 inhibits invasion of Lovo cells by reducing expression of MMP-2 [34] and CDX1 restricts the invasion of HTR-8/SVneo trophoblast cells by inhibiting MMP-9 expression [35]. So we investigate the effects of CDX2 on MMP-9 and TIMP-1 expression. Our present study shows that CDX2 overexpression significantly promotes MMP-9 expression and inhibits TIMP-1 expression in HTR-8/SVneo trophoblast cells (Fig. 3B, 3C). These data suggest that CDX2 enhances trophoblast invasion by stimulating MMP-9 secretion while inhibiting TIMP-1 expression.

The PI3K/AKT signaling pathway plays important roles in cell growth, proliferation, migration and invasion [27, 36]. This pathway is activated in pre-eclamptic placentas to regulate cell proliferation [37]. Previous reports have shown that PI3K and AKT play an important role in regulating trophoblast invasion through the upregulation of MMP-9/TIMP-1 expression by EGF [38]. Literature also suggests that HCG stimulates trophoblast invasion through PI3K and AKT signaling [39]. Recent studies have shown that CDX-2 was a target of PI3K signaling [40, 41]. We investigated the effect of PI3K/Akt signaling on CDX2 expression in HTR8/SVneo cells. Inhibition of PI3K signaling with the specific inhibitor LY294002 decreased CDX2 expression (Fig. 4B). In contrast, CDX2 overexpression did not affect PI3K expression (Fig. 4A). These data suggest that the PI3K/AKT pathway could regulate CDX2 expression.

In summary, our results demonstrated that CDX2 overexpression in HTR-8/SVneo cells enhanced cell invasion by promoting MMP-9 expression and inhibiting TIMP-1 expression. Therefore, the expression level of CDX2 may have a significant impact on trophoblast invasion and may be associated with pregnancy-related diseases. Our findings may reveal new insights into the regulatory mechanisms of trophoblast invasion and may be an important contributor to the pathogenesis of pregnancy-related diseases.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (No. 81200442), the Natural Science Foundation of Jiangsu Province, China (No. BK2011106), the Scientific and Technological Personnel Start Projects Foundation of Nanjing Health Bureau, Jiangsu Province, China (No. QYK11141), and the Selected Member of Nanjing Health Youth Talent Training Project (QRX11209).

References

- Chen JZ, Sheehan PM, Brennecke SP, Keogh RJ: Vessel remodeling, pregnancy hormones and extravillous trophoblast function. *Mol Cell Endocrinol* 2012;349:138-144.
- Hunkapiller NM, Fisher SJ: Placental remodeling of the uterine vasculature. *Method Enzymol* 2008;445:281-302.
- Knöfler M, Pollheimer J: IFPA Award in Placentology lecture: molecular regulation of human trophoblast invasion. *Placenta* 2012;33:S55-S62.
- Norwitz ER: Defective implantation and placentation: laying the blueprint for pregnancy complications. *Reprod Biomed Online* 2006;13:591-599.
- Hidden U, Bilban M, Knöfler M, Desoye G: Kisspeptins and the placenta: regulation of trophoblast invasion. *Rev Endocr Metab Dis* 2007;8:31-39.
- Ball E, Bulmer JN, Ayis S, Lyall F, Robson SC: Late sporadic miscarriage is associated with abnormalities in spiral artery transformation and trophoblast invasion. *AM J Pathol* 2006;208:535-542.
- Goldman-Wohl D, Yagel S: Regulation of trophoblast invasion: from normal implantation to pre-eclampsia. *Mol Cell Endocrinol* 2002;187:233-238.
- Plaks V, Rinkenberger J, Dai J, Flannery M, Sund M, Kanasaki K, Werb, Z: Matrix metalloproteinase-9 deficiency phenocopies features of preeclampsia and intrauterine growth restriction. *P Natl Acad Sci USA* 2013;110:11109-11114.
- Wells M: The pathology of gestational trophoblastic disease: recent advances. *Pathology* 2007;39:88-96.
- Cui Y, Wang W, Dong N, Lou J, Srinivasan DK, Cheng W, Wu, Q: Role of corin in trophoblast invasion and uterine spiral artery remodelling in pregnancy. *Nature* 2012;484:246-250.
- Shih IM, Hsu MY, Oldt III RJ, Herlyn M, Gearhart JD, Kurman RJ: The role of E-cadherin in the motility and invasion of implantation site intermediate trophoblast. *Placenta* 2002;23:706-715.
- Huber AV, Saleh L, Bauer S, Husslein P, Knöfler M: TNF α -mediated induction of PAI-1 restricts invasion of HTR-8/SVneo trophoblast cells. *Placenta* 2006;27:127-136.
- Staun-Ram E, Goldman S, Gabarin D, Shalev E: Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and-9) in human trophoblast invasion. *Reprod Biol Endocrin* 2004;2:59.
- [14] Karmakar S, Das C: Regulation of Trophoblast Invasion by IL-1 β and TGF- β 1. *Am J Reprod Immunol* 2002; 48: 210-219.
- Luo J, Qiao F, Yin X: Hypoxia induces FGF2 production by vascular endothelial cells and alters MMP9 and TIMP1 expression in extravillous trophoblasts and their invasiveness in a cocultured model. *J Reprod Develop* 2011;57:84-91.
- Singh M, Kindelberger D, Nagymanyoki Z, Ng SW, Quick CM, Elias KM, Berkowitz, RS: Matrix metalloproteinases and their inhibitors and inducer in gestational trophoblastic diseases and normal placenta. *Gynecol Oncol* 2011;122:178-182.
- Karthikeyan VJ, Lane DA, Beevers DG, Lip GYH, Blann AD: Matrix metalloproteinases and their tissue inhibitors in hypertension-related pregnancy complications. *J Hum Hypertens* 2013;27:72-78.
- Zhu JY, Pang ZJ, Yu Y: Regulation of trophoblast invasion: the role of matrix metalloproteinases. *Reviews in obstetrics and gynecology* 2012;5:e137.
- Beck F, Stringer EJ: The role of Cdx genes in the gut and in axial development. *Biochem Soc T* 2010;38:353-357.
- Silberg DG, Swain GP, Suh ER, Traber PG: Cdx1 and cdx2 expression during intestinal development. *Gastroenterology* 2000;119:961-971.
- Eda A, Osawa H, Yanaka I, Satoh K, Mutoh H, Kihira K: Expression of homeobox gene CDX2 precedes that of CDX1 during the progression of intestinal metaplasia. *J Gastroentero* 2002;37:94-100.

- 22 Park SY, Jeong MS, Yoo M, Jang SB: Caudal-related homeodomain proteins CDX1/2 bind to DNA replication-related element binding factor. *BBA-Proteins Proteom* 2011;1814:1891-1899.
- 23 Jia, RZ, Zhang X, Hu P, Liu XM, Hua XD, Wang X, Ding HJ: Screening for differential methylation status in human placenta in preeclampsia using a CpG island plus promoter microarray. *Int J Mol Med* 2012;30:133.
- 24 Tolkunova E, Cavaleri F, Eckardt S, Reinbold R, Christenson LK, Schöler HR, Tomilin, A: The Caudal-Related Protein Cdx2 Promotes Trophoblast Differentiation of Mouse Embryonic Stem Cells. *Stem Cells* 2006;24:139-144.
- 25 Murohashi M, Nakamura T, Tanaka S, Ichise T, Yoshida N, Yamamoto T, Gotoh, N: An FGF4-FRS2 α -Cdx2 Axis in Trophoblast Stem Cells Induces Bmp4 to Regulate Proper Growth of Early Mouse Embryos. *Stem Cells* 2010;28:113-121.
- 26 Suman P, Gupta SK: Comparative analysis of the invasion-associated genes expression pattern in first trimester trophoblastic (HTR-8/SVneo) and JEG-3 choriocarcinoma cells. *Placenta* 2012;3:874-877.
- 27 Wu WL, Gan WH, Tong ML, Li XL, Dai JZ, Zhang CM, Guo, XR: Over-expression of NYGGF4 (PID1) inhibits glucose transport in skeletal myotubes by blocking the IRS1/PI3K/AKT insulin pathway. *Mol Genet Metab* 2011;102:374-377.
- 28 Lee G, Papapetrou EP, Kim H, Chambers SM, Tomishima MJ, Fasano CA, Studer L: Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature* 2009;461:402-406.
- 29 Nakagawa I, Amano A, Mizushima N, Yamamoto A, Yamaguchi H, Kamimoto T, Yoshimori T: Autophagy defends cells against invading group A Streptococcus. *Science* 2004;306:1037-1040.
- 30 Ren L, Liu YQ, Zhou WH, Zhang YZ: Trophoblast-derived chemokine CXCL12 promotes CXCR4 expression and invasion of human first-trimester decidual stromal cells. *Hum Reprod* 2012;27:366-374.
- 31 Thompson, Megan Elizabeth: Epigenetic Control of Gene Expression in the Placental Trophoblast (Doctoral dissertation). 2012.
- 32 Lala PK, Chakraborty C: Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury. *Placenta* 2003;24:575-587.
- 33 Bischoff P, Meisser A, Campana A: Paracrine and autocrine regulators of trophoblast invasion-a review. *Placenta* 2000; 1:S55-S60.
- 34 Zheng JB, Sun XJ, Qij, Li SS, Wang W, Ren HL, Du JK: Effects of homeodomain protein CDX2 expression on the proliferation and migration of lovo colon cancer cells. *Pathology & Oncology Research* 2011;17:743-751.
- 35 Jia RZ, Rui C, Li JY, Cui XW, Wang X: CDX1 restricts the invasion of HTR-8/SVneo trophoblast cells by inhibiting MMP-9 expression. *Placenta* 2014;35:450-454.
- 36 Duan C, Bauchat JR, Hsieh T: Phosphatidylinositol 3-kinase is required for insulin-like growth factor-1-induced vascular smooth muscle cell proliferation and migration. *Circ Res* 2000;86:15-23.
- 37 Park JK, Jeong JW, Kang MY, Baek JC, Shin JK, Lee SA, Paik, WY: Inhibition of the PI3K-Akt Pathway Suppresses sFlt1 Expression in Human Placental Hypoxia Models *In Vitro*. *Placenta* 2010;31:621-629.
- 38 Qiu Q, Yang M, Tsang BK, Gruslin A: EGF-induced trophoblast secretion of MMP-9 and TIMP-1 involves activation of both PI3K and MAPK signalling pathways. *Reproduction* 2004;128:355-363.
- 39 Prast J, Saleh L, Husslein H, Sonderegger S, Helmer H, Knofler M: Human chorionic gonadotropin stimulates trophoblast invasion through extracellularly regulated kinase and AKT signaling. *Endocrinology* 2008;149:979-987.
- 40 Bai ZG, Ye YJ, Shen DH, Lu YY, Zhang ZT, Wang S: PTEN expression and suppression of proliferation are associated with Cdx2 overexpression in gastric cancer cells. *International journal of oncology* 2013;42:1682-1691.
- 41 Kim S1, Domon-Dell C, Wang Q, Chung DH, Di Cristofano A, Pandolfi PP, Freund JN, Evers BM: PTEN and TNF-alpha regulation of the intestinal-specific Cdx-2 homeobox gene through a PI3K, PKB/Akt, and NF-kappaB-dependent pathway. *Gastroenterology* 2002;123:1163-1178.