Placenta 35 (2014) 450–454

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

CDX1 restricts the invasion of HTR-8/SVneo trophoblast cells by inhibiting MMP-9 expression

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ARTICLE INFO

Article history:
Accepted 21 April 2014

Keywords:
CDX1
HTR-8/SVneo
Trophoblast invasion
MMP-9
Preeclampsia

ABSTRACT

Introduction: Pathogenesis of early-onset preeclampsia (PE) is generally recognized by impaired trophoblast invasion of the myometrial arteries, which results in placental insufficiency. Recently, we reported that CDX1 is hypermethylated in the human preeclampsia placenta. However, whether CDX1 participates in trophoblast invasion has not been clearly elucidated.
Methods: We investigated the function of CDX1 in the extravillous trophoblast cell line HTR-8/SVneo using stable transfection of CDX1. Using a CDX1 stable transfected cell line, we determined the cell invasion using a QCM ECMatrix 24-well kit. The cell viability was detected using an MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. Quantitative RT-PCR and western blotting analyses were performed to examine the changes in the expression of downstream target genes and proteins. To disrupt PI3K/AKT signaling, we used the PI3K inhibitor perifosine.
Results: Cell invasion assays demonstrated that CDX1 restricts trophoblast cell invasiveness. In contrast, quantification of cumulative cell numbers revealed that CDX1 did not affect cell proliferation. Western blotting analysis and quantitative real time PCR demonstrated that MMP-9 expression was reduced, whereas TIMP-1 expression was increased in CDX1-overexpressed cells. However, overexpression of CDX1 did not affect PI3K/AKT signaling in HTR-8/SVneo trophoblast cells. In contrast, CDX1 was regulated by the PI3K/AKT signaling pathway.
Conclusions: Altogether, we found that in trophoblast cells, CDX1 reduced invasion independently of the PI3K/AKT signaling pathway. Furthermore, CDX1 functions in concert with PI3K/AKT signaling to regulate trophoblast invasion. We concluded that CDX1 restricts the invasion of HTR-8/SVneo trophoblast cells by inhibiting MMP-9 expression independently of the PI3K/AKT pathway.

1. Introduction

Preeclampsia (PE) affects approximately 6–8% of all pregnancies worldwide [1]. PE is characterized by maternal hypertension and proteinuria. It is a major contributor to maternal and perinatal mortality and morbidity. Although the precise origin of PE remains elusive, it is generally recognized that impaired trophoblast invasion of myometrial arteries and the resulting placental insufficiency are specifically associated with early-onset PE [2]. Invasion of placenta extravillous trophoblast cells to the maternal decidualized endometrial and myometrium plays an important role in embryo implantation [3]. The invasion process helps to connect the placenta to the uterine wall via blood flow to ensure adequate fetal nutrition and oxygen supply. However, either excessive or deficient invasion of extravillous trophoblast cells may result in several pathological conditions, such as placental bed tumors or preeclampsia [4,5]. Thus, the trophoblast invasion process is precisely controlled to avoid even the smallest disturbances.

Previous studies have reported that a number of factors are related to trophoblast invasion, such as KiSS-1 [6], CCN3 [7], fetuin-A [8], matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9) [9,10]. MMP-9 is a member of the gelatinase family, which consists of enzymes that can efficiently degrade native collagen type IV, causing degradation of basement membranes and the destruction of the extracellular matrix (ECM), which helps the trophoblast to

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http://dx.doi.org/10.1016/j.placenta.2014.04.011
0143-4004/

escape the primary site and initiate trophoblast invasion [11]. Tissue inhibitor of metalloproteinases (TIMP-1) is an inhibitor of MMP-9, which mediates trophoblast invasion. Thus, these two enzymes are considered to be crucial for human embryo implantation and trophoblast cell invasion.

Caudal-related homeobox transcription factor 1 (CDX1), one of the CDX group of ParaHox genes (CDX1, CDX2 and CDX4), plays a key role in multiple processes that contribute to mammalian development [12], in particular, processes involved in intestinal cell proliferation, differentiation, and neoplasia [13,14]. CDX1 can activate key signaling molecules, such as Ras, Rho and phosphoinositide 3-kinase (PI3K) in intestinal epithelial cells [15], as well as the PI3K/AKT pathway, which is a central feature of the signaling pathway used by trophoblast cells in proliferative, migratory and invasive processes [16]. Recently, our study found that the promoter CpG dinucleotides of CDX1 showed a significant higher DNA methylation level in PE patients compared to control healthy cases [17]. Typically, DNA hypermethylation recruits corepressors and histone deacetylation complexes to the target gene, which silences gene expression. We hypothesized that CDX1 can regulate trophoblast cell invasion, and abnormal expression of CDX1 is associated with PE pathogenesis.

In this study, we examined the function of CDX1 in the immortalized human first-trimester extravillous trophoblast cell line HTR-8/SVneo. These results revealed that overexpression of CDX1 in HTR-8/SVneo trophoblast cells resulted in reduced cell invasion *in vitro*. Consistent with these findings, the expression of MMP-9 was decreased, whereas the expression of TIMP-1 was increased in CDX1 overexpressed cells. In contrast, we found no change in the phosphoinositide 3-kinase (PI3K)/AKT pathway in HTR-8/SVneo trophoblast cells when CDX1 was overexpressed, but CDX1 could be regulated by the PI3K/AKT pathway. Altogether, these data suggested that CDX1 restricts the invasion of HTR-8/SVneo trophoblast cells by altering matrix metalloproteinase expression independent of the PI3K/AKT pathway.

2. Materials and methods

2.1. Antibodies

Primary polyclonal antibody against CDX1 (Abcam, ab126748, USA), MMP-9 (Abcam, ab38898, USA), TIMP-1 (Cell Signaling, #8946), PI3K (Abcam, ab22653, USA), and AKT1 (phospho-S473, Abcam, ab66138) were used at a 1:1000 dilution. As the loading control for western blotting analyses, rabbit polyclonal anti-β-ACTIN antibody (Abcam, ab1801, USA) was used at a 1:1000 dilution.

2.2. Cell culture and stable transfection

HTR-8/SVneo cells (purchased from Cell Bank of Chinese Academy of Sciences, China) were cultured in RPMI-1640 media (Invitrogen, USA) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin at 37 °C in 5% CO_2 [10].

For stable transfections, CDX1 expression plasmid was constructed by inserting the entire coding region of the human *CDX1* cDNA (synthesized by Shanghai Generay Biotech Co., Ltd., China) into a vector of pBMN-GFP (The Phoenix™ retroviral system, Allele Biotech, USA) using the restriction enzymes, BamH I and EcoR I (TaKaRa, Dalian, China). When the cells reached 70–80% confluence, transfection with CDX1 or empty vector was performed according to the manufacturer's protocol and the final constructs were confirmed using DNA sequencing (Sangon, China). The integrated cell line was screened with 2 μg/ml puromycin. The colonized cells were isolated, amplified and used for subsequent experiments.

2.3. Cell invasion assay

The cell invasion assay was performed using the QCM ECMatrix 24-well kit (Chemicon, ECM550, USA) according to the manufacturer's instructions. Each lower chamber contained an additional 600 μl of 0.5% FBS as the chemoattractant. CDX1 overexpressed or control HRT-8/SVneo cells were placed into the upper chamber and then incubated for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation, non-migrating cells in the top chambers were completely removed using a cotton swab. Cells that invaded into the lower chambers were fixed in 95% methanol for 5 min and then quantified using a colorimetric crystal violet assay. The cell number was quantified using light microscopy. For each experiment, the number of cells in seven randomly chosen fields of each filter was quantified. Three independent experimental results were presented as the percentage of cell invasion relative to the control (set as 100%).

2.4. Cell viability assay

The cell viability was determined using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay according to the manufacturer's protocol. Briefly, HRT-8/SVneo cells with overexpression of empty vector or CDX1 (packaging viral particle) were plated at a density of 2×10^5 cells/well and incubated 36 h at 37 °C in a 5% CO_2 atmosphere with 24-well plate. The cells were lysed with dimethyl sulphoxide (DMSO) after a 4 h incubation with MTT (Ameresco, USA), and the plates were subsequently incubated in a shaker at low speed for 10 min and read using a spectrophotometer (MK3, Thermo, USA). These results were expressed as the relative cell viability compared to the control (set as 100%). Each sample was performed in triplicate and the experiment was repeated three times.

2.5. Pharmacological treatment

To test the effect of PI3K inhibition on cell invasion, 5 μM perifosine (Selleck, USA) was applied in the upper chambers. To evaluate the effect of perifosine on trophoblast cells invasion when CDX1 was overexpressed, cells with or without CDX1 expression was treated with perifosine (5 μM) for 36 h in the upper chambers.

2.6. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from approximately 5×10^6 cells using Trizol Reagent (Invitrogen, USA). Superscript II reverse transcriptase (Invitrogen, USA) was used to generate cDNA using 1 μg of RNA and oligo dT primer, according to the manufacturer's instructions. PCR assays were performed using SYBR Green buffer (Applied Biosystems, USA) on the ABI 7500 system. To verify the primer specificities, melting curve analyses were performed. A standard curve was generated for each gene to evaluate the primer efficiency and for data analyses.

Sequences of PCR primer sets used for qRT-PCR included: for *CDX1*, 5′-CCAAAA CCGGCGGGCAAAGGAG-3′ (forward) and 5′-GCTGGGGTGGCCGTGATGTCGT-3′ (reverse); for *MMP-9*, 5′-TGCCCGGACCAAGGATACAGTTT-3′ (forward) and 5′-GTTCA GGGCGAGGACCATAGAGG-3′ (reverse); for *TIMP-1*, 5′-ACCGCAGCGAGGAGTTTCT-3′ (forward) and 5′-CAGTTTGCAGGGGATGGATAA-3′ (reverse); for *PI3K*, 5′-ATGGGGA TGATTTACGGCAAGATA-3′ (forward) and 5′-TTTCGCACCACCTCAATAAGTC-3′ (reverse); for *AKT*, 5′-CCCCCGAGGTGCTGGAGGACAAT-3′ (forward) and 5′-AAGGGC AGGCGACCGCACATCAT-3′ (reverse); for *18S*, 5′-AGTCGCCGTGCCTACCAT-3′ (forward) and 5′-CGGGTCGGGAGTGGGTAAT-3′ (reverse); The expected fragment length of *CDX1*, *MMP-9*, *TIMP-1*, *PI3K*, *AKT*, and *18S* was 102, 184, 185, 151, 97 and 129 bp, respectively. To rule out DNA contamination in the RNA preparations, quantitative RT-PCR controls were performed with RNA templates, which did not show any amplification. In all of the samples, the calculated concentration was normalized using 18S RNA as an internal control. In addition, qRT-PCR was performed in triplicate and was repeated at least three times.

2.7. Western blotting analyses

Transfected HTR-8/SVneo cell samples were homogenized in RIPA lysis buffer (Sigma, USA). The membranes were blocked for 1 h with 5% nonfat dry milk in Tris-buffer containing 0.05% Tween-20 (TBST), and then incubated with primary antibodies at the dilution indicated above followed by incubation with HRP conjugated goat anti-rabbit IgG (1:1000 dilution; Abcam, ab6721, USA). The chemiluminescence process and quantification of objective bands on the exposed films were performed as previously described [18].

2.8. Statistical analysis

Data were shown as the mean ± standard deviation (SD). Significant differences for the control and test conditions were identified using unpaired Student's *t*-test. All statistical analyses were performed using the SPSS software, version 17.0. A value of $p < 0.05$ was defined as statistically significant.

3. Results

3.1. Overexpression of CDX1 in stable HTR-8/SVneo cell lines

Stable HTR-8/SVneo cell transfection of CDX1 was established and maintained in RPMI-1640 media containing 2 μg/ml puromycin. Expression of CDX1 in the HTR-8/SVneo cells was verified using western blotting using an antibody against CDX1 (Fig. 1A). Analysis of the densitometry revealed that CDX1 was increased to be approximately 5-fold compared to control when CDX1 was overexpressed in HTR-8/SVneo cells (Fig. 1B).