Pregnancy Hypertension 13 (2018) 37–45

Contents lists available at ScienceDirect

Pregnancy Hypertension

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

Dysfunction of pseudogene *PGK1P2* is involved in preeclampsia by acting as a competing endogenous RNA of *PGK1*

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ARTICLE INFO

Keywords:
Preeclampsia
Competing endogenous RNA
PGK1
PGK1P2
LncRNA
miRNA-330-5p

ABSTRACT

Objectives: Normal decidualization is essential for normal pregnancy and abnormal decidualization is thought to cause preeclampsia (PE). Phosphoglycerate kinase 1 (*PGK1*) is an enzyme involved in the glycolytic pathway which is the main metabolism process decidual cells exhibit. Phosphoglycerate kinase 1, pseudogene 2 (*PGK1P2*), which is also a long non-coding RNA (lncRNA), has a high sequence similarity to *PGK1* and therefore acquires an ability for sequence-specific regulation.

Methodology: The expression of *PGK1* and *PGK1P2* in human decidua, as well as their relationship and functions during decidualization was investigated using *in vitro* cultured human endometrial stromal cell lines (hESCs) and primary ESCs by real-time PCR, immunohistochemistry, western blotting, siRNA techniques and miRNA inhibitor or mimic transfection.

Results: The levels of *PGK1* and *PGK1P2* mRNA and PGK1 protein in severe preeclamptic decidua were lower than those in normal pregnant controls. *PGK1* and *PGK1P2* mRNAs were both induced after *in vitro* decidualization and their deficiency caused impaired decidualization in turn. We also found *PGK1P2* acted as a competing endogenous RNA (ceRNA) to regulate *PGK1* expression through miR-330-5p.

Conclusions: We proved that *PGK1* and *PGK1P2* are a pair of ceRNAs against miR-330-5p and they play a vital role in human decidualization by regulating angiogenesis and glycolysis metabolism. The deficiency of *PGK1* and *PGK1P2* in the decidua jeopardizes the decidualization process and subsequently might lead to the occurrence of PE. These findings may help in promoting novel predictive, diagnostic and prognostic biomarkers of PE in future.

1. Introduction

Preeclampsia (PE) is a disease unique to human pregnancy characterized by hypertension and/or proteinuria [1]. It is the major contributor to maternal death and perinatal mortality and morbidity worldwide and causes socioeconomic burden to the affected families and society consequently. To date, delivery of the placenta is the only effective treatment for PE. Most of the symptoms will disappear within a week after delivery. However, the pathogenesis of PE remains an enigma. It is widely believed that the genesis of preeclampsia resides in the placental bed during early pregnancy. Impaired implantation and deficient placentation in early pregnancy leads to disturbed placental function which is thought to be the major cause of PE. Deficient placentation is characteristic of PE, but the underlying causes are unknown [2].

In normal human placentation, uterine invasion by trophoblast cells and subsequent spiral artery remodeling depend on establishing a talk between fetal trophoblasts and maternal decidua. The major component of placental bed is the decidua into which trophoblasts invade [3]. Both successful embryo implantation and placentation relies on maternal adequate decidualization of endometrial stromal cells (ESCs) [4]. The extent of decidualization appears to correlate with the degree of trophoblast invasion and placenta formation. Accumulating attention has been given to decidua in PE researches. Impaired decidualization causes failed intravascular trophoblast invasion and deficient placentation which increases the risk of PE [5,6]. Furthermore, several

Abbreviations: PE, preeclampsia; ESCs, endometrial stromal cells; hESCs, human endometrial stromal cell lines; PGK1, phosphoglycerate kinase 1; PGK1P2, phosphoglycerate kinase 1, pseudogene 2; LncRNA, long non-coding RNA; ceRNA, competing endogenous RNA; SPE, severe preeclampsia; NP, normal pregnancies; VEGF, vascular endothelial growth factor; PRL, prolactin; IGFBP1, insulin-like growth factor binding protein 1

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https://doi.org/10.1016/j.preghy.2018.05.003
Received 26 October 2017; Received in revised form 21 April 2018; Accepted 2 May 2018
Available online 03 May 2018

knock-out mouse models have revealed that endometrial decidualization is vital for maintaining pregnancy [7,8]. Naturally normal decidualization is essential for normal pregnancy and abnormal decidualization caused by disorders of decidual tissues is thought to cause PE [6,9].

PGK1 (phosphoglycerate kinase 1) has traditionally been studied as an important adenosine triphosphate (ATP)-generating enzyme of the glycolytic pathway in which it catalyzes the conversion of 1, 3-diphosphoglycerate to 3-phosphoglycerate [10]. Furthermore, PGK1 can influence DNA replication and repair in the nucleus [11,12], it can be secreted extracellularly by tumors such as prostate cancer [13]. In cancer researches, PGK1 is found to be correlated with peritoneal dissemination in gastric cancer [10] and participates in the angiogenic process as a disulphide reductase [14]. *PGK1P2* (phosphoglycerate kinase 1, pseudogene 2), one of *PGK* pseudogenes, is a long non-coding RNA (LncRNA), it has a high sequence similarity to its parental protein-coding gene *PGK1*. Our previous research has demonstrated both *PGK1* and *PGK1P2* significantly decreased in the decidua of PE patients [15], however, their transcription and function in maternal decidua remains largely unknown.

Pseudogenes as well as LncRNAs are traditionally claimed to not yield functional mRNAs and not translated into proteins consequently, thus regarded as garbage fragments or dark matter in the genome, however, recent studies have demonstrated that they can regulate gene function in many ways [16]. Specifically, pseudogene and LncRNA transcripts can act as natural microRNA (miRNA) sponges to suppress intracellular miRNA function by using shared miRNA response elements, like competing endogenous RNA (ceRNA) does [17]. Its interaction with miR-330-5p has already been identified from a HITS-CLIP data [18]. Taken together, these previous observations encouraged us to investigate the role of *PGK1* and *PGK1P2* in decidualization process and their relationship with PE. We also focused on whether PGK1P2 can act as a ceRNA for PGK1 against miR-330-5p.

2. Methodology

2.1. Study population and decidual sample collection

Sixteen women with pregnancies complicated by severe preeclampsia (SPE) and sixteen women with normal pregnancies (NP) were recruited from the Department of Obstetrics and Gynecology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University between April 2015 and March 2016. SPE was defined as systolic blood pressure ≥160 mm Hg or diastolic blood pressure ≥110 mm Hg and/or proteinuria > 3+ protein on dip stick [19]. Only pregnancies delivered by caesarean section were included and none of them were in labor prior to caesarean section. Women with chronic hypertension, diabetes mellitus, renal disease, reduced thyroid, fetal and placental structural abnormalities, and other pregnancy related complications were excluded.

Decidual tissues were scrubbed from the placental bed at the time of caesarean section. The tissues were washed with sterilized water to remove blood, snap frozen in liquid nitrogen immediately and then were stored at −80 °C until use. The study was approved by the Ren Ji Hospital Research and Ethics Committees. Informed consent was obtained from all participants before the collection of decidual tissues.

2.2. Immunohistochemistry

PGK1 expression was studied by immunohistochemistry (IHC) on paraffin-embedded sections as described previously [20]. Tissue sections were deparaffinized and microwaved for 10 min in 10 mM sodium citrate buffer (PH 6.0) to retrieve antigen. Sections were blocked for endogenous peroxidase activity and incubated with anti-PGK1 (17811-1-AP, Proteintech) overnight at 4 °C. Subsequently, the sections were incubated with appropriate HRP-conjugated secondary antibody and developed by the diaminobenzidine (DAB)-HRP reaction system.

2.3. Cell culture and reagents

The immortalized human endometrial stromal cell lines (hESCs) was a kind gift from Dr. Haibin Wang (Xiamen University Medical College, China). The hESCs were cultured in Phenol Red-free DMEM/F12 containing glutamine (Life Technologies, Inc., Grand Island, NY), 10% dextran-coated charcoal-stripped FBS (Biological Industries, Beit Haemek, Israel), 5×10^{-2} g/L antibiotics (Gibco, Grand Island, NY, USA), 5×10^{-4} g/L puromycin (Gibco, Grand Island, NY, USA) and 1% Insulin-Transferrin-Selenium (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.4. Isolation and culture of primary human endometrial stromal cells (ESCs)

Endometrial tissues were obtained by dilatation and curettage between day 7–12 of the menstrual cycle from women with regular menstrual cycles and no apparent endometrial dysfunction at the time of diagnostic hysteroscopy. Primary ESCs were isolated from those proliferative endometrial tissues by enzymatic digestion and filtration which produces > 95% pure stromal cell cultures as described previously [21,22]. Briefly, tissues were washed twice with DMEM/F12 medium (Life Technologies, Inc., Grand Island, NY) and finely minced with scissors. Then they were digested by collagenase type I (c0130; Sigma Chemical Co., St. Louis, MO, USA) for 70 min at 37 °C and digested with deoxyribonuclease (DN25; Sigma Chemical Co., St. Louis, MO, USA) for 20 min at 37 °C. Then the digested tissues were filtered through 180 and 40 μm griddles sequentially to remove mucous and undigested tissues and to permit stromal cells to pass. Primary ESCs were seeded in 75 cm^2 tissue culture flasks and cultured in the same condition as hESCs did but free of 1% Insulin- Transferrin- Selenium.

2.5. In vitro decidualization and siRNA transfection

Cells were inoculated into 6-well plates (10^6 cells per well) for future manipulation. For *in vitro* decidualization inducing, the FBS's concentration were reduced to 2% (v/v) and the cells were treated with 10^{-6} mol/L medroxyprogesterone-17-acetate (MPA) (Sigma Chemical Co., St. Louis, MO, USA) dissolved in ethanol, and 5×10^{-4} mol/L N6, 2′-O-dibutyryladenosine cAMP sodium salt (cAMP) (Sigma Chemical Co., St. Louis, MO, USA) for 6 days. While control samples were only treated with 0.1% (v/v) ethanol.

As for siRNA transfection, cells were incubated in the antibiotics free medium, at 60% confluence, Lipofectamine 3000 Transfection Reagent (7.5 μl/well; Invitrogen) and 50 nM PGK1-siRNA (RiboBio, Guangzhou, China), PGK1P2-siRNA (GenePharma, Shanghai, China) or non-targeting control siRNA diluted in Opti-MEM (Invitrogen) were transfected to hESCs or primary ESCs. The medium was changed 24 h later, then the cells were induced to decidualization for 4 days.

2.6. Transient transfection

For the transfection of miR-330-5p mimic and inhibitor, hESCs and primary ESCs were seeded in 6-well plates (5×10^5 cells per well) with antibiotics free medium. Lipofectamine3000 Transfection was mixed with 50 nM miR-330-5p mimic, 100 nM miR-330-5p inhibitor or scrambled control (RiboBio, Guangzhou, China). The mixture was then added to hESCs or primary ESCs at 50% confluence. 6 h later, the medium was changed and the cells were induced to decidualization. As for hESCs, the *in vitro* decidualization lasted 4 days. While for primary ESCs, the *in vitro* decidualization continued 4 days for miR-330-5p mimic and 3 days for transfection of miR-330-5p inhibitor.