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Glucocorticoid receptor-mediated insulin-like growth factor-I transcriptional regulation in BeWo trophoblast cells before and after syncytialisation

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ABSTRACT

Prenatal exposure to excessive glucocorticoids (GCs) leads to intrauterine growth retardation and fetal programming of adult health and disease through deregulation of placental functions. Placental secretion of insulin-like growth factor-I (IGF-I) plays a critical role in the regulation of placental development and function. However, it remains elusive whether GCs affect placental functions through glucocorticoid receptor (GR)-mediated transcriptional regulation of *IGF-I* gene. In this study, human placental choriocarcinoma (BeWo) cells before and after syncytialization were used as cytotrophoblast and syncytiotrophoblast models, respectively, to explore the effects of dexamethasone (Dex) on transcriptional regulation of *IGF-I* gene at both stages. Dex significantly inhibited ($P < 0.05$) cell proliferation in cytotrophoblasts and down-regulated amino acid transporter *SLC7A5* in syncytiotrophoblasts. Concurrently, the abundance of *IGF-I* mRNA and its transcript variants, together with IGF-I level in culture media, were significantly reduced, in association with significantly enhanced ($P < 0.05$) GR phosphorylation. GR antagonist RU486 was able to abolish all these effects. Two glucocorticoid response elements (GREs) were predicted in the promoter regions of *IGF-I* gene. GR binding to GRE1 was significantly enriched ($P < 0.05$) in both cytotrophoblasts and syncytiotrophoblasts, but that to GRE2 was significantly diminished ($P < 0.05$) in cytotrophoblasts but not in syncytiotrophoblasts, in response to Dex treatment. IGF-I supplementation completely rescued Dex-induced cell cycle arrest but not *SLC7A5* down-regulation, indicating different regulatory mechanisms. Taken together, our results suggest that GR-mediated transcriptional regulation of *IGF-I* is involved in Dex-induced inhibition of placental cell proliferation and function.

1. Introduction

Glucocorticoids (GCs) act as both environmental and maturational signals that affect the growth of placenta and fetus during intrauterine development [1–3]. Despite the clear physiological importance of endogenous GCs during development, there are a plethora of data showing that exposure to excess exogenous GCs during pregnancy correlates with reduced birth weight and adverse outcomes in the offspring [4–6]. Placenta is critical for the supplies of nutrients to the fetus and production of a range of hormones that may affect fetal development. Gestational exposure to low-dose of dexamethasone (Dex, a synthetic GCs that is little metabolized by 11β-hydroxysteroid dehydrogenase 2) led to reduction of placental weight and retardation of fetal growth in several species including sheep [7], rodents [8,9] and humans [10,11]. Excessive exposure to GCs during pregnancy led to decline of placental amino acid transport in women [12]. GCs can influence the placental functions either directly via glucocorticoid receptor (GR) on various cell types forming the placenta or indirectly by inducing physiological changes in the fetus and mother [13–15].

The syncytiotrophoblast, which derived from cytotrophoblast, is the major component of the human placenta, as it is central to placental nutrients transport and hormone production. Insulin-like growth factors (IGFs) are critically involved in the development of placenta and the function of syncytiotrophoblast cells [16–18]. During gestation, the placenta is one of the major sources of insulin-like growth factor-I (IGF-I) [19]. IGF-I stimulates a variety of cellular responses including proliferation, migration, and

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differentiation in trophoblast cell lineage [16,20]. Not only that, IGF-I is known to alter placental production of the fetal metabolic substrate, lactate, and its distribution between the uterine and umbilical circulations [21]. IGF-I is reported to regulate amino acid and glucose transporters in placental cells both in vivo and in vitro [17,18]. Previous studies demonstrated that the expression of *IGF-I* was regulated by Dex in several cell models [22,23] and animal models [24–26]. However, the relationship between GCs and IGF-I in placental cells and the mechanism by which GCs inhibits *IGF-I* expression remain elusive.

The BeWo cell line, derived from human choriocarcinoma, has been widely used as an in vitro model of human villous cytotrophoblast. Moreover, it can be induced to differentiate into syncytiotrophoblast by forskolin (FSK) and has therefore been used as in vitro model for placenta [27,28]. To further understand the effects of synthetic GCs on IGF-I secretion in placental cells, BeWo cells before and after syncytialisation were used as cytotrophoblast and syncytiotrophoblast models in the present study to test the hypothesis that GCs regulate *IGF-I* expression via bindings of GR to glucocorticoid response elements (GREs) in *IGF-I* gene promoter, thus suppressing the proliferation of cytotrophoblasts and the nutrient transport in syncytiotrophoblasts. The results will help to delineate the molecular mechanisms involved in the effects of Dex on placental/fetal growth.

2. Materials and methods

2.1. Cell culture, cell fusion and treatment

The trophoblast cell model, BeWo choriocarcinoma cell line was obtained from the Chinese Academy of Medical Sciences and Peking Union Medical College. In this experiment, cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA), supplemented with 10% (v/v) fetal calf serum (Invitrogen, Mt Waverley, VIC, Australia) under a humidified atmosphere of 5% CO_2 in air. Cells were sub-cultured (3:1) every 3 days. BeWo cells were treated with 100 μM of FSK (Sigma-Aldrich, F3917, USA) for 48 h to induce syncytialization and then used as a model of syncytiotrophoblast [29]. At the end of experiments, immunofluorescent staining and total RNA extraction were performed to determine the effect of FSK on cell fusion. After 24 h in culture, the cells were treated with the following reagents: Dex (100 nM, Sigma-Aldrich, D4902, USA), IGF-I (100 ng/mL, Prospect Biosystems, CYT-216, Israel) and RU486 (1 μM, Sigma-Aldrich, M8046, USA) for 24 h.

2.2. Immunofluorescence staining

BeWo cells (cytotrophoblasts) and differentiated BeWo cells (syncytiotrophoblasts) were fixed in 4% neutral paraformaldehyde for 30 min, blocked with 5% FBS and then incubated with rabbit anti- E-cadherin antibody (1:1000, Cell Signaling Technology, 3195, USA) in TBS-T containing 5% skim milk overnight at 4 °C. The TRITC-labeled goat anti-rabbit IgG (1:1000, KPL Inc., USA) was used as the second antibody. DAPI was used as a marker for cell nuclei.

2.3. RNA isolation, cDNA synthesis and real- time PCR

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, 15596-026, USA) and then treated with DNase I (RNase Free, D2215, Takara, Japan) to eliminate possible contamination of genomic DNA according to the manufacturer's instructions. Concentration of the extracted RNA was measured using NanoDrop 1000 Spectrophotometer (Thermo, ND-1000, USA). Ratios of absorption (260/280 nm) were between 1.9 and 2.1. RNA integrity was confirmed by denaturing agarose electrophoresis. Two micrograms of total RNA were reverse-transcribed in a final volume of 25 μL with M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random hexamer primers (SunShine, China) following the manufacturer's instructions. Reverse transcription was performed in a Thermal Cycler PTC0200 (Bio-Rad, USA). Two microliters of diluted cDNA (1:20) were used for real-time polymerase chain reaction (PCR). All primers (Table 1) were synthesized by Generay Biotech Co., Ltd. (Shanghai, China). Real-time quantitative PCR was performed with a Mx3000P real-time PCR detection system (Stratagene, USA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was chosen as a reference gene in quantitative mRNA profiling assays. The amplification specificity of each gene was checked by melting curve analysis.

2.4. Protein extraction and Western blot analysis

Cells were cultured in 6-well plates and treated with Dex (100 nM) with or without RU486 (1 μM) for 24 h. Media were removed and cells were covered with 200 μL RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM PMSF, 1 mM sodium orthovanadate with Roche EDTA-free complete mini protease inhibitor cocktail, No. 11836170001). Lysates were cleared by centrifugation, and protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). Protein (50 μg/lane) was used for electrophoresis on 10% SDS-PAGE gels. Primary antibodies, rabbit anti-GR antibody (1:500, Santa Cruz, sc-1004, USA), Phospho-GR (Ser211) (1:500, Cell Signaling Technology, 4161, USA) and β-actin (1:10000, Bioworld, AP0060, USA), were used in the Western blot analysis.

2.5. Radioimmunoassay analysis

IGF-I concentration was tested using an IGF-I radioimmunoassay (RIA) kit (JD-RF6, Beijing North Institute of Biological Technology Co. Ltd, China). The detection limit for IGF-I was 0.45 ng/mL and the intra- and inter-assay coefficients of variation were 5 and 10%, respectively.

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was performed according to a previous publication [13] with some modifications. Briefly, 1×10^8 BeWo cells were washed by PBS. After cross linking in 1% formaldehyde for 8 min, the reaction was stopped by 2.5 M glycine. The pellets were washed with pre-cooled PBS and lysed with SDS lysis buffer containing a protease inhibitor cocktail (Roche, 05892791001, Germany). Chromatin was sonicated to the length of 300–500 bp (5 min, 50% power, 5s on, 5s off) and the protein–DNA complex was diluted in ChIP dilution buffer pre-cleared with salmon sperm DNA/protein G agarose beads (40 μL, 50% slurry). The pre-cleared chromatin preparations were incubated with 3 μg rabbit anti-GR antibody (Santa cruz, sc1004x, USA) overnight at 4 °C. A negative control was included with normal rabbit IgG. Protein G agarose beads (40 μL, 50% slurry) were added to capture the immunoprecipitated chromatin complexes. Finally, reverse cross-linking was performed to release DNA fragments from the immunoprecipitated complex at 65 °C for 1 h and the DNA was purified. Immunoprecipitated DNA was used as a template for Real-time PCR using specific primers to amplify genomic sequences at the promoter region of *IGF-I* gene. All primers (Table 1) were synthesized by Generay Biotech Co., Ltd. The occupancy of each transcription factor at target gene promoter was presented as the fold enrichment that was derived by the method of cycle threshold (CT) in the formula of