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HLA-G expression is regulated by miR-365 in trophoblasts under hypoxic conditions

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ABSTRACT

Introduction: Hypoxia occurs in the first trimester of placental development and is implicated in the regulation of trophoblast differentiation. Prolonged hypoxic conditions in the placenta are related to the development of preeclampsia. MicroRNAs (miRNAs) are noncoding, single-stranded RNAs that modulate gene expression by targeting messenger RNA. We hypothesized that, under hypoxic conditions, trophoblasts may have a unique miRNA profile that may play a critical role in placental development.
Methods: Total RNA was extracted from human trophoblast, HChEpC1b, exposed to normoxia (20% O_2) or hypoxia (2% O_2) for 24 h, and the miRNA expression profiles were investigated using a microRNA array. Several differential miRNAs were selected and validated using real-time reverse transcription PCR. We identified potential targets of these miRNAs using in silico analysis. We confirmed a potential target protein by western blot analysis and luciferase assays.
Results: The expression of miR-365 was significantly upregulated under hypoxic conditions. In silico analysis showed that miR-365 targeted human leukocyte antigen (HLA)-G. Both hypoxic conditions and overexpression of miR-365 inhibited the expression of HLA-G proteins. The overexpression of miR-365 also decreased the activity of the luciferase reporter containing the 3′-untranslated region (UTR) of HLA-G with the predicted miR-365-binding site.
Discussion: HLA-G is a non-classical HLA class-Ib molecule that is expressed mainly in extravillous trophoblasts and which plays a key role in maintaining immune tolerance at the maternal–fetal interface. Our results indicate that miR-365 targets the HLA-G 3′ UTR to repress its expression. The expression of miR-365 may play an important role in human placental development and in immunoprotection of the semiallogenic embryo.

1. Introduction

Hypoxia occurs during the development of placenta in the first trimester and is implicated in trophoblast differentiation [1–4]. Intervillous blood flow increases after 10 weeks of gestation and results in exposure of trophoblast to oxygen [1]. Before this time, low oxygen appears to prevent trophoblast differentiation toward an invasive phenotype [1]. Prolonged hypoxic conditions in the placenta are known to be a major factor related to the development of preeclampsia, fetal growth restriction (FGR) and miscarriage [1–5]. Especially, preeclampsia is a syndrome characterized by the new onset of hypertension and proteinuria during pregnancy and is the leading cause of maternal and fetal morbidity and mortality [1–3]. In normal human pregnancies, trophoblasts infiltrate the decidua during early pregnancy, and the walls of the spiral arteries are replaced by trophoblasts [6]. The spiral arteries are consequently transformed into large-lumen vessels lined by extravillous trophoblasts (EVTs), thereby facilitating the circulation in the placenta. As a result, the maternal spiral arteries are converted from high- to low-resistance vessels that supply nutrients and oxygen to the fetus. Abnormally shallow EVT invasion impairs spiral artery remodeling, and the resistance of the spiral arteries remains high, and this may be part of the mechanism by which pre-eclampsia

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develops. This results in reduced blood flow in the placenta [7–11], which can lead to hypoxia. Hypoxia affects trophoblast gene expression, and consequently modulates cell survival, differentiation, metabolic function, and apoptosis [12]. Placental dysfunction may result from altered gene expression during hypoxia [13,14].

MicroRNAs (miRNAs) are noncoding ribonucleic acids of 20–24 nucleotides that usually negatively regulate gene expression by binding to sequences in the 3′ untranslated region (3′ UTR) of the target gene's messenger RNA (mRNA) [15–17]. Such binding results in either translational obstruction or degradation of the mRNA [18]. To date, more than 2500 different miRNAs have been identified in humans, each potentially controlling hundreds of target genes [15,19,20]. miRNAs play a key role in various pathological and physiological disorders, and there is increasing evidence that they are important regulators of placental development [18]. miRNAs are found in human placentas, and abnormal miRNA levels have been reported in placentas from pregnancies with complications, such as preeclampsia, FGR and recurrent miscarriage [21–26].

We hypothesized that trophoblasts under hypoxic conditions have a unique miRNA profile that may play a critical role in placental development. The aim of this study was to identify the miRNAs in trophoblasts that are affected by hypoxia and to determine their relationship with their target genes.

2. Material and methods

2.1. Cell lines and culture conditions

Experiments were carried out using HChEpC1b cells and JAR choriocarcinoma cells. HChEpC1b is a human extravillous trophoblast cell line immortalized by retroviral infection of E6, E7, and hTERT. The cell line was established using primary human trophoblast from the placenta of a pregnancy that had been electively aborted at the 7th week of gestation. HChEpC1b cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 50 IU/ml penicillin, and 50 μg/ml streptomycin (Invitrogen) and incubated in 5% CO_2 at 37 °C. JAR choriocarcinoma cells were cultured in RPMI1640 containing 10% FBS and incubated in 5% CO_2 at 37 °C. Both cell types were also cultured under hypoxic conditions using a hermetically enclosed incubator (ESPEC N_2-O_2-CO_2 BNP-110 incubator, TabaiESPEC, Osaka, Japan) that was continually flushed with a mixture of 5% CO_2, 2% O_2, and 93% N_2 for 24 h.

2.2. MicroRNA microarray profiling

Total RNA was extracted from HChEpC1b cells under normoxic and hypoxic conditions using ISOGEN reagent (Nippon Gene, Tokyo, Japan). Total RNA samples were labeled using a miRCURY LNA microRNA Power Hy3/Hy5 Labeling Kit (Exiqon, Vedbaek, Denmark), and miRNA expression profiles were assessed with a miRCURY LNA microRNA Array (Exiqon).

2.3. Real-time reverse transcription PCR analysis

The expression levels of these miRNAs were confirmed using real-time reverse transcription PCR (RT-PCR). Total RNA was extracted from HChEpC1b cells under normoxic and hypoxic conditions. RNA purity was then assessed using an Ultrospec 3300 pro (GE Healthcare UK, Little Chalfont, UK). Reverse transcription was performed with miRNA-specific stem-loop RT primers and a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), followed by RT-PCR using specific TaqMan Assays and a TaqMan Universal PCR Master Mix. All

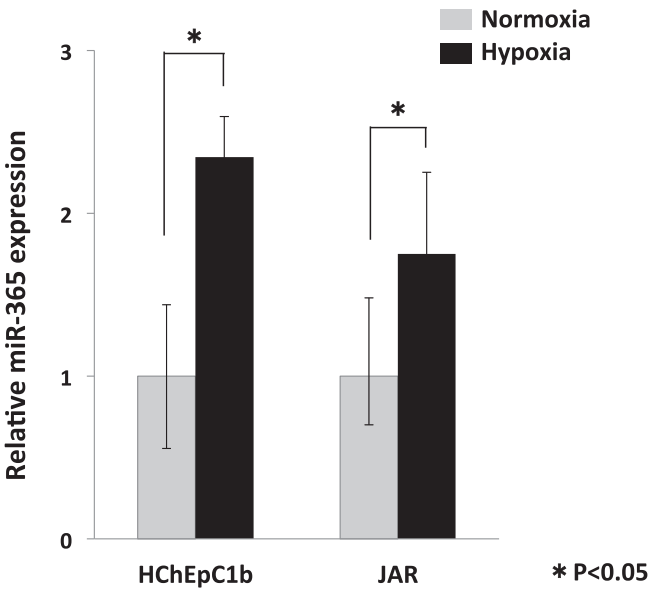

Fig. 1. Effect of hypoxia on miR-365 expression in HChEpC1b and JAR cells. Cells were cultured for 24 h under normoxic (20% O_2) or hypoxic (2% O_2) conditions. Total RNA was prepared and subjected to real-time RT-PCR analysis. Total RNA input was normalized using miR-16 as an endogenous control. Experiments were carried out in triplicate.

reactions were carried out in triplicate in 96-well plates using a real-time PCR System (Applied Biosystems). Fold changes were determined using the $2^{-\Delta\Delta CT}$ method [27]. Total RNA input was normalized using miR-16 as an endogenous control. Using miR-16 provided higher stability and expression in our model compared with RNU24 and RNU38B.

2.4. Prediction of miRNA targets

We searched for potential target genes of the identified miRNAs using in silico analysis (miRBLAST). We then selected the human leukocyte antigen (HLA)-G which was the potential target of miR-365 and performed western blot analysis.

2.5. Western blot analysis

Equal amounts of protein (20 μg/lane) were separated by SDS-PAGE using the NuPAGE System (Invitrogen). Each protein was transferred to polyvinylidene difluoride membranes (Invitrogen) at 30 V for 60 min. These membranes were blocked using 5% ECL Prime Blocking Reagent (GE Healthcare UK) in PBS-T overnight at 4 °C. The membranes were then washed in PBS-T, incubated with mouse monoclonal HLA-G antibody (clone MEM-G/1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:1000 dilution overnight, and reacted with Anti-Mouse IgG, HRP-Linked Whole Ab Sheep (GE Healthcare UK) at a 1:10000 dilution. To verify equal protein

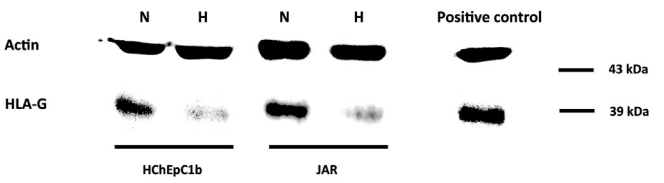

Fig. 2. HLA-G protein levels in hypoxic trophoblasts. Equal amounts of trophoblast proteins (20 μg/lane) were separated and subjected to western blot analysis under normoxic (N) or hypoxic (H) conditions. To verify equal protein loading, membranes were then stripped and reprobed with actin antibody.