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miR-15b-AGO2 play a critical role in HTR8/SVneo invasion and in a model of angiogenesis defects related to inflammation

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ABSTRACT

Introduction: microRNAs (miRs) have been shown to play critical roles in the regulation of trophoblast and endothelial cell functions, and one significant finding concerning the miR-15/16 family is that most members of this family are highly expressed in endothelial cells and contribute to functions, such as tube formation. The interaction between trophoblast and endothelial cell play an important role in normal placentation process. Therefore, the aims of this study were to investigate the expression of miR-15b in human placenta and to uncover the potential role of miR-15b as well as its target functional loop in trophoblast and endothelial cells. Whether inflammation could modulate the expression of miR-15b and its down-stream target was further investigated. Additionally, the potential link between miR-15b deregulation and preeclampsia was also explored in the placenta of patients diagnosed with preeclampsia.

Methods: The expression of miR-15b was studied in the placental tissue of a normal pregnancy using *in situ* hybridization, and the effects of miR-15b on proliferation, invasion, and angiogenesis were further explored in vitro using HTR-8/SVneo and HUVEC cell line models. A Lipopolysaccharides (LPS) treatment model in HTR-8/SVneo cell was utilized to explore the mechanism of how LPS treatment could lead to the activation of miR-15b expression. Western blot was used to detect the expression of proteins related to miR-15b mediated pathway in preeclamptic placentas.

Results: miR-15b inhibits trophoblast cell invasion and endothelial cell tube formation by suppressing the expression of Argonaute 2 (AGO2), a major miRNA effecter protein. AGO2 is specifically localized to human placenta cytotrophoblast and endothelial cells, and it plays important roles in trophoblast cell invasion and endothelial cell tube formation. LPS treatment may lead to the overexpression of miR-15b and down-regulation of AGO2, which may be involved in shallow trophoblast cell invasion associated with the pathogenesis of preeclampsia. Chromatin immunoprecipitation assay indicates that increased occupancy of AGO2 to miR-15b promoter is responsible for the increased expression of miR-15b under the condition of LPS treatment. Furthermore, preeclamptic placentas have decreased expression of AGO2, but increased expression of miR-15b and TLR-4 compared to normal controls.

Discussion: This is the first report about the function of AGO2 in human trophoblast and endothelial cells in the placenta. The data indicates that the aberrant expression of miR-15b contributes to abnormal placentation by targeting *AGO2* mRNA. This study provides insight into the potential role of the miR-15b and AGO2 functional loop in the placentation process.

1. Introduction

Preeclampsia is the most common pregnancy related disorder among women, but its pathogenesis remains largely unknown; however, trophoblast cell dysfunction was reported to play a critical role in the etiology of this disorder [1]. The major pathologic

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characteristics include shallow trophoblast invasion and defective vascular remodeling [2]. Recently, microRNA (miR) has been shown to play critical roles in trophoblast cell migration and invasion as well as endothelial cell development. Specifically, miR array technology has been used to detect biomarkers that show promise for the early detection of preeclampsia [3]. Mechanistic understanding of the involvement of miRNAs and their target genes in the process of abnormal placentation during onset and progress of preeclampsia could contribute to the translation of mechanistic and biomarker validation studies.

MiR-15/16 is a conserved family of miRs, which is involved in a diverse array of cell behaviors, including cell proliferation, apoptosis, and cell invasion, by binding to the conserved seed region of targeted mRNAs(4). For example, miR-15b may inhibit cell proliferation by targeting cyclin E [5–7] and regulating apoptosis by targeting Bcl-2 [8–10]. One significant finding concerning the miR-15/16 family is that most members are highly expressed in endothelial cells and play important roles in the regulation of endothelial cell functions, such as tube formation [11].

AGO2 belongs to the Argonaute family and binds to a single RNA molecule and other proteins to form the RISC complex which mediates targeted RNA degradation. As a highly specialized member of the AGO family, only AGO2 possesses an essential Slicer-independent function within the mammalian miRNA biogenesis pathway, and it is important to regulate endothelial cell function [12]. Although previous studies have shown that AGO2 is expressed in trophoblasts, the exact role of AGO2 and other miRNA biogenesis proteins in the cell function of trophoblasts remain elusive [3]. Interestingly, miRNAs, such as miR-103/107, could target miRNA biogenesis proteins, including Dicer and AGO2, to control cancer metastasis, as shown in a mouse model [13]. Therefore, a conserved mechanism consisting of a negative regulatory loop could be involved in various pathophysiological processes.

In the present study, we investigated the effects of miR-15b on cell proliferation, invasion and angiogenesis in trophoblast and endothelial cell lines, both of which are major cell types involved in placental development. We further explored the potential connection between miR-15b deregulation and preeclampsia. Interestingly, mechanistic studies show that miR-15b can target AGO2 mRNA to regulate trophoblast cell invasion and endothelial cell tube formation. Our study identified a novel pathway that is important for miRNA function in placenta biology.

2. Materials and methods

2.1. Objective of study and diagnostic criteria

The study was approved by the ethics committee of West China Second University Hospital of Sichuan University and all consent forms were signed. All selected subjects were pregnant women with a scheduled cesarean section whose terminal weeks of pregnancy were between 35weeks and 39weeks in our hospital from March 2012 through May 2014. Preeclampsia was defined as systolic blood pressure>140 mmHg and/or diastolic blood pressure >90 mmHg on two occasions >6 h apart after 20 weeks of gestation before the onset of labor, plus proteinuria of >2+ (dipstick method) or >0.3 g/24 h (American College of Obstetricians and Gynecologists, 1996). Severe preeclampsia was defined as a higher blood pressure >160 mmHg systolic or>110 mmHg diastolic on two occasions >6 h apart and a proteinuria level >5 g/24 h or >3 + by dipstick testing on at least two separate occasions (American College of Obstetricians and Gynecologists, 1996). The gestation-matched placentas of normal pregnancy were chosen as normal controls. The two groups of pregnant women were singleton pregnancies and excluded patients with a previous history of heart, liver, kidney, thyroid disease or diabetes. A total of 11 controls and 13 patients were recruited for the study.

2.2. Specimen collection

Two groups of subjects were chosen, and the placentas were collected after cesarean section with tissue randomly selected from the material side around the central villous area and avoid of calcified foci; the samples were rinsed with saline twice, quickly placed in liquid nitrogen and then placed in a −80 °C refrigerator for storage. One part of fresh placentae was fixed in 4% paraformaldehyde and processed for immunohistochemical staining. For the RNA and protein analyses, term villous tissues from the middle of placenta were cut from the maternal side and a piece of tissue approximately 1 × 1 × 1 cm in size was collected, avoiding blood clots, infarctions, or calcified parts, and preserved at −80°C both RNA and protein lysates were prepared from it.

2.3. Cell line and proliferation assays

HTR-8/SVneo, an immortalized human trophoblast cell line, was cultured in RPMI-1640 medium (C11875500BT, Gibco, USA) supplemented with 10% fetal bovine serum (10099-141, Gibco, Australia) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. HUVEC cells (ATCC CRL-1730, USA) were cultured in F-12K medium and incubated at 37 °C in a humidified atmosphere with 5% CO_2. The proliferation activity was measured by the use of Cell Counting Kit-8 (CCK-8) (Dojindo, China). For the cell proliferation assay, 4000/well HTR-8/SVneo cells and 2400/well HUVEC cells were cultured in 100 μL of media in 96-well plates. The cells were cultured for four days, and the absorbance (OD values) was measured at 450 nm after 10 μl of CCK-8 solution was added to each well and incubated with the cells for 1 h every day.

2.4. Western blot analysis

Whole cell lysates were prepared with RIPA cell lysis buffer with the addition of PMSF and 1% proteinase inhibitors. Equal amounts of protein were resolved by 10%SDS-PAGE and then transferred to a PVDF membrane. The membranes were incubated with a primary AGO2 antibody at a concentration of 1:1000 (03-110, Millipore, USA) or TLR4 (617066, Zen BioScience, China) or VEGFR1 (Sangon Biotech, Shanghai, China) or VEGFR2 ((Sangon Biotech, Shanghai, China) and normalized to a β-actin control at a concentration of 1:5000 (20130822, Zen Bioscience, China). The primary antibody binding was visualized with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:10000, Zen Bioscience, China). The signal was labeled with HRP (1305702, Millipore Corporation, Billerica, USA), and band intensities were measured using image analysis software (ImageJ, NIH).

2.5. Real time PCR

Total placental RNA was prepared from frozen placental tissues with Trizol reagent (Life Technologies, USA). Five-hundred nanograms of RNA was used for reverse transcription, which was carried out using PrimeScript™ RT reagent Kit (Takara, Dalian, China) following the manufacturer's instructions, and reverse transcription of miRNA was performed according to manufacturer's instructions for M-MLV reverse transcriptase (Life Technologies, USA). Quantitative real-time PCR (qRT–PCR) analysis was performed using an Applied Biosystems 7500 detection system (Life Technologies, USA). For the detection of cDNA, the experiment was carried out following the manufacturer's instructions of the SYBR® select master mix (Life Technologies, USA). The specificity of