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Original Article

Upregulation of microRNA-155 inhibits trophoblast differentiation through the Wnt/β-catenin pathway

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Abstract: *Background and aim*: Trophoblasts play an important role in embryo implantation and make a connection with deciduas. Dysregulation of human trophoblast invasion and differentiation can result in pre-eclampsia (PE), a hypertensive disorder of pregnancy with significant morbidity and mortality for mothers and their offspring. In our study, we explored the role of the expression of microRNA-155 (miR-155) in trophoblast invasion and differentiation which is associated with PE pathology. *Methods:* Trophoblasts were isolated from placentas of patients with PE and the expression of miR-155 and its potential target calcium voltage-gated channel auxiliary subunit alpha 2 delta 1 (CACNA2D1) was assayed by real-time PCR. Computational analysis and luciferase reporter assays were then performed to identify the putative target of miR-155. Next, the proliferation and invasion of the primary trophoblast after transfection of miR-155 mimic were assayed by MTT and Transwell chambers, respectively. Beta-catenin was assayed by Western blot and real-time PCR. *Results*: The results show that miR-155 inhibits proliferation and cell invasion. Moreover, miR-155 inhibited mRNA and protein expression of β-catenin and inhibited the proliferation and migration of trophoblasts through Wnt/β-catenin transcriptional activity. *Conclusion*: The findings of our study suggest that miR-155 may play a key role in human trophoblast proliferation and invasion, which may contribute to the pathogenesis of PE.

Keywords: Pre-eclampsia, miR-155, calcium voltage-gated channel auxiliary subunit alpha 2 delta 1, trophoblast, Wnt/β-catenin

Introduction

Fetal growth and development depend on the normal proliferation and differentiation of placental trophoblast cells, maintaining the gas exchange between mother and fetus, the supply of nutrients, and elimination of waste. Proliferation, differentiation, and invasion of trophoblast cells in maternal uterus are the basic biological events in the process of placenta formation. Trophoblasts, the outer layer of a blastocyst, are involved in the initial adhesion to the uterine wall and subsequent implantation within the wall. During this period, the trophoblast layer proliferates and differentiates into two distinct layers such as syncitiotrophoblast and cytotrophoblast. The invasion of trophoblast cells in deciduas is a very essential step for establishment of pregnancy and failure of this step is crucial for development of PE. Recently, microRNAs (miRNAs) have been identified as pivotal regulators of proliferation, differentiation, and invasion of trophoblast cells which are involved in preeclampsia (PE) [1, 2]. miRNAs are small non-coding RNA that can suppress the expression of gene either suppressing mRNA translation or increasing mRNA degradation.

Wnt/β-catenin pathway is a key contributor in fetal growth and development and adult tissue homeostasis. Wnts comprise a family of palmitoylated cysteine-rich glycoproteins which are secreted in a lipoprotein-bound form due to their low solubility or through exosomes [3]. The first described member of this family of secreted ligands was the Wnt1 proto-oncogene, which is homologous to the Drosophila gene Wingless. Originally, the gene was named int-1 since it had been identified as an integration site for the murine mammary tumor virus which can provoke breast cancer [4]. In humans, 19 different Wnt ligands and 10 seven-transmembrane domains frizzled (Fzd) receptors have been

identified [5]. Following interaction of a Wnt-protein ligand to a Frizzled family receptor, the Wnt/β-catenin pathway becomes activated and passes the signal to the dishevelled protein which is located into cytoplasm. Wnt ligands trigger not only canonical Wnt signaling but also non-canonical β-catenin-independent signaling including the Wnt/Ca^{2+} and the Wnt/planar cell polarity (PCP) pathways [8]. The difference between these two pathways is the involvement of β-catenin protein. In canonical Wnt signaling, in the presence of Wnt ligand, the co-receptor LRP-5 or -6 (receptor related proteins) is brought in complex with Wnt-bound Frizzled. This leads to activation of Dishevelled (Dvl) by sequential phosphorylation, poly-ubiquitination, and polymerization which allows β-catenin to accumulate and localize to the nucleus [6]. It is likely that the complex interplay of different Wnts with Fzds provokes specific Wnt responses depending on the receptor context and the particular cell type. Stabilization and nuclear recruitment of β-catenin is a hallmark of the canonical pathway [7] and that nuclear β-catenin form complexes with TCF/LEF (T-cell factor/lymphoid enhancing factor) and activates Wnt target gene expression. Activation of the Wnt/β-catenin pathway induces trophoblast differentiation and invasion [9].

miR-155 has diverse effects on trophoblasts including inhibition of differentiation and proliferation and modulation of immune response [10]. Many targets of miR-155 have been verified to play a pivotal role in proliferation and invasion of trophoblasts [11, 12]. In the present paper, we tested a new target which was involved in miR-155-mediated trophoblast differentiation through inhibition of the Wnt/β-catenin pathway.

Materials and methods

Subjects

The study was approved by the Ethics Committee of Department of Obstetrics, the First Affiliated Hospital of Chongqing Medical University with obtained patient consent. Placental tissues were obtained from 25 patients with PE and 15 healthy individuals. Patients were free of endometriosis, malignant tumors, pelvic inflammatory diseases, gynecological endocrine diseases, rheumatic immune diseases, and other connective tissue diseases.

Trophoblast isolation

Primary trophoblasts were isolated from placental villous tissue using serial trypsin digestion methods. Briefly, villous tissue was cut into small pieces using scissors, washed using PBS, and incubated with 0.1% trypsin for 10 minutes, 0.2% trypsin for 10 minutes, 0.2% trypsin for 30 minutes, then sequentially four more times with 0.2% trypsin for 30 minutes with agitation. The cells that were incubated with trypsin the last four times were filtered through 4 layers of sterile gauze and 2 layers of sterile nylon mesh and then centrifuged at 1000 rpm for 5 minutes at 4°C. The cell pellets were layered on top of a 65%/30% Percoll step gradient and centrifuged at 1600 rpm for 20 minutes. The band of cells at the interface were collected, washed, and re-suspended in DMEM/ F12 medium supplemented with 10% FBS, 1 × Minimal Essential Amino Acids, L-glutamine, and 0.1% gentamycin (trophoblast medium). The cells were cryopreserved in 10% DMSO. Characterization of the isolated trophoblasts was carried out by immune staining for the trophoblast marker cytokeratin 7 (CK7) and the mesenchyme marker vimentin (Vim). Nuclei were stained using DAPI.

Cell culture and transfection

When confluence reached 80%, Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect trophoblasts with miR-155 mimics (5'UUAAUGCUAAUCGUGAUAGGGG3'), negative control (NC, 5'CAGUACUUUUGUGUAGUACAA3'), and miR-155 inhibitors (5'CCCCUAUCACGAUUAGCAUUAA3'), in accordance with the manufacturer's protocol (GenePharma, Shanghai, China). The final concentration of miR-155, NC, and inhibitor was 100 nM. Three independent experiments were performed.

Real time PCR

Total RNA was extracted from trophoblast cells using TRIzol (Qiagen, China) and cDNAs were obtained by reverse transcription (RT) using SuperscriptII reverse transcriptase (Life technologies, Grand Island NY, USA), according to manufacturer's guidelines. An Applied Biosystems 7500 Sequence Detection System (Thermo Fisher Scientific, Inc., USA) was used to perform qPCR (Thermo Fisher Scientific, Inc.,

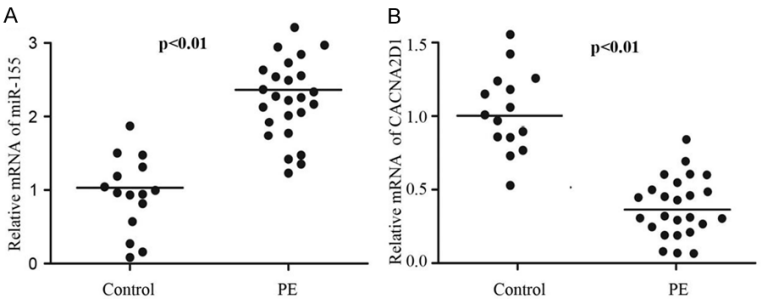

Figure 1. miR-155 (A) and CACNA2D1 (B) expression were assayed by real-time PCR (n=4). miR-155 exhibits markedly increase and CACNA2D1 expression exhibits markedly decrease in patients with PE.

USA), based on the guidelines indicated by the supplier. The thermocycler reaction involved 95°C for 10 minutes (initial denaturation), followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Small nuclear RNA U6 was used as an internal control to normalize the expression of miR-155 and GAPDH was used as an internal control for CACNA2D1 and β-catenin mRNA. Calculated ΔCt values were normalized against a GAPDH control. Fold change was calculated using ΔΔCt method [13].

Proliferation assay

In order to test the effect of miR-155 on cell proliferation, MTT assay was performed. 10 μl of MTT solution (5 mg/mL; Sigma, USA) was added to 100 μl culture media and cells were incubated for a further 4 hours at 37°C. After removing the culture medium, 100 μL DMSO was added to each well and the plates were shaken for 10 minutes. Absorbance (OD) value was then measured at A490 nm. The experiment was repeated 6 times.

Invasion assay

An invasion assay was performed using Transwell chambers with 24-well inserts (pore size, 8 μm; Corning Costar, Corning, NY, USA). Briefly, trophoblast cells were plated in the top chamber with Matrigel (BD Biosciences, San Jose, CA, USA) and incubated for 24 hours. Cells in the top chamber were plated in medium without serum or growth factors and medium supplemented with serum was used as a chemoattractant in the lower chamber. After 24 hours of incubation, invaded cells through the pores were counted under a light microscope after crystal violet staining.

Western blotting

Total lysates were obtained by incubating cells in RIPA lysis buffer (Dingguo, Beijing, China) supplemented with 1% Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis MO) for 1 hour at 0°C. The lysate was centrifuged at 10,000 × g for 30 minutes at 0°C and a protein assay was performed on the supernatant using the BCA assay kit (Thermo Scientific, China). Samples (50 μg) were loaded on 10% Bis-Tris SDS-PAGE gels. After electrophoresis, the proteins were transferred to PVDF. The membrane was blocked for 1 hour in 5% BSA. The blocked membrane was incubated overnight with primary antibody in PBS, then washed and incubated with secondary antibody labeled with horseradish peroxidase (ThermoScientific, USA). After further washing, the membrane was incubated with chemiluminescent substrate (WesternBright Quantum, E&K Scientific, Santa Clara, CA) and exposed to a Kodak imager (Kodak Imaging Systems, New Haven CT). Densitometry was performed using Kodak Carestream Molecular Imaging Software and normalized to GAPDH control.

Statistical analysis

All experiments were repeated at least 3 times. Statistical analysis was performed by two-way ANOVA with repeated measures for the time-course experiments and by Student's t-test for all other data using the Prism software program (GraphPad Inc., San Diego, CA). Data were expressed as mean ± SEMs and differences were considered significant if $p < 0.05$.

Results

Examination of miR-155 and CACNA2D1 expression

qRT-PCR was performed to analyze the expression of miR-155 and CACNA2D1 in subjects of control and patients with PE. miR-155 was significantly increased in patients with PE (**Figure 1A**). Pearson's correlation analysis among all participates revealed a positive association between miR-155 expression and PE occurrence (r=0.466, P=0.024). Furthermore, a mul-

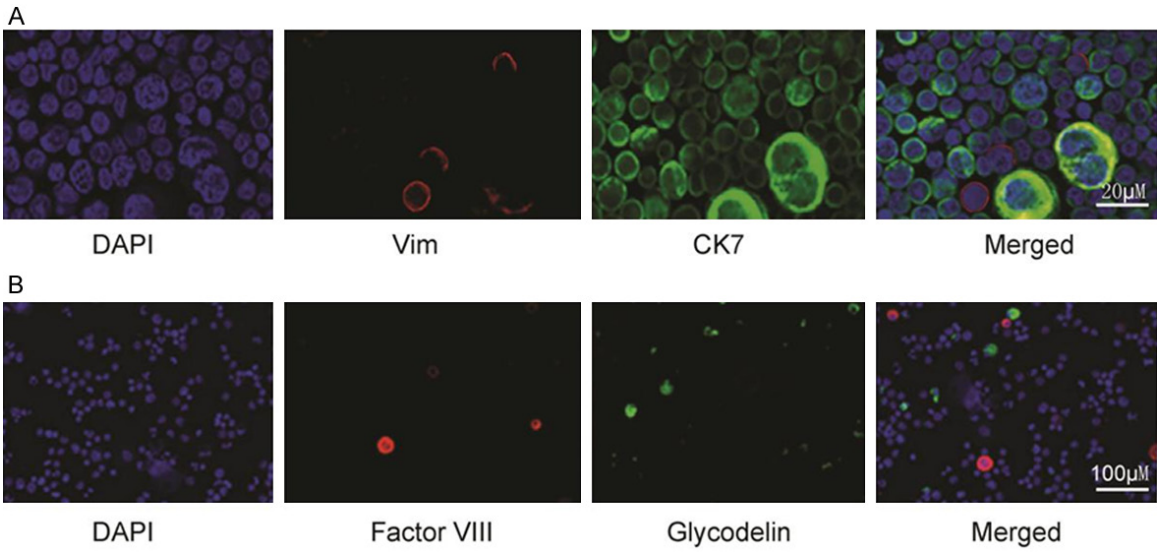

Figure 2. Identification of isolated trophoblasts by immunofluorescence analysis. A: CK7 (green; trophoblast marker) and Vim (red; mesenchyme marker) expression after culture of cells. B: Glycodelin (green; uterine epithelial marker) and Factor VIII (red; endothelial marker) expression after culture of cells.

tivariate linear model indicated the relationship between miR-155 and PE was persistent in the control (r=0.386, P=0.042) and PE groups (r=0.573, P=0.005), indicating that increased miR-155 expression may be associated with PE occurrence. CACNA2D1 mRNA expression level was also determined in the two groups using qRT-PCR (**Figure 1B**). Expression of CACNA2D1 mRNA in the control group was higher compared with the expression level in the PE group which suggested that higher SCN1A mRNA may be associated with inhibition of PE.

Characterization of isolated trophoblasts

Figure 2A shows immunofluorescence analysis of cytokeratin 7 (CK7; trophoblast marker) and vimentin (Vim; mesenchyme marker) expression after culture of cells on collagen-coated glass slides for 24 hours. The cells adhered well and consistently survived cryopreservation with little loss of viability. The cultures consisted of some single cells but large irregular-shaped cell colonies were predominate. Immunofluorescence analysis revealed no or few vimentin+ cells and the majority of cells expressed CK7 only consistent with trophoblasts (**Figure 2A**). Image analysis of the stained cells showed that 92.66% of the cells were $CK7^{+}Vim^{-}$. Only a few staining was found for the endothelial marker Factor VIII and the uterine epithelial marker, glycodelin (**Figure 2B**).

CACNA2D1 may be a target of miR-155

Online miRNA target prediction tools were used to search for target genes of miR-155 and CACNA2D1 was identified as a candidate target gene of miR-155, as it contained the appropriate seed sequence in the 3'UTR. In addition, it was confirmed that miR-155 mimics or miR-NC were successfully transfected into primary trophoblasts (**Figure 3A**). A luciferase reporter assay was used to investigate the regulatory relationship between miR-155 and CACNA2D1. The luciferase activity of cells transfected with wild-type CACNA2D1 3'UTR and miR-155 mimics was lower compared with cells transfected with mutated-type CACNA2D1 3'UTR and miR-NC (**Figure 3B**). These data indicate that CACNA2D1 may be a target of miR-155 with the binding sites located on the CACNA2D1 3'UTR.

Effect of miR-155 on proliferation and cell invasion

The results of MTT assay showed that cell proliferation in miR-155 group after 24 hours was decreased compared to that in the control group while proliferation in miR-155 inhibitor (100 nM) group showed increased proliferation, indicating that miR-155 inhibited proliferation of trophoblasts (**Figure 4A**). Cell invasion was detected after 48 hours. Each sample repeated three times. Five different areas were randomly selected under the microscope with a

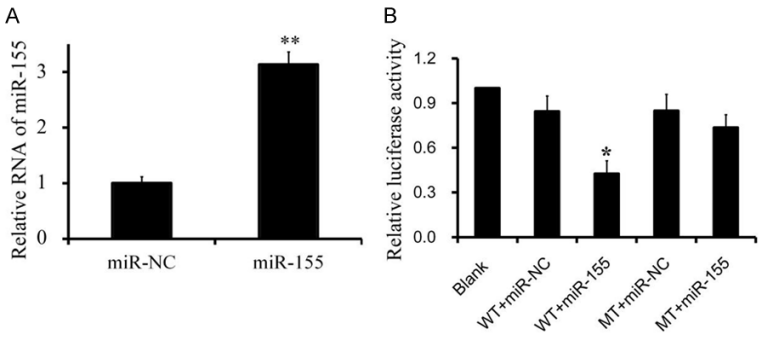

Figure 3. A: miR-155 expression after miR-155 mimics transfection was assayed by real-time PCR (n=4). **P < 0.01 *vs.* miR-NC. B: Luciferase reporter assay. Results indicated that the luciferase activity of trophoblasts transfected with miR-155 mimics was lower in WT CACNA2D1 3'UTR co-transfected cells compared with cells co-transfected with the MT CACNA2D13'UTR. *P < 0.05 *vs*. WT+miR-NC.

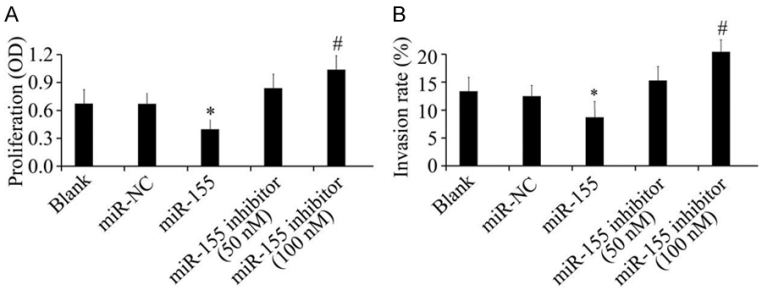

Figure 4. Proliferation and cell invasion were assayed by MTT (A) and Transwell chamber (B). The results showed that the cell proliferation and invasion in miR-155 group was decreased than those in miR-NC group. The proliferation and invasion rates in miR-155 inhibitor group were significantly higher than those in miR-NC group. *P < 0.05 *vs.* miR-NC and #P < 0.05 *vs.* miR-NC.

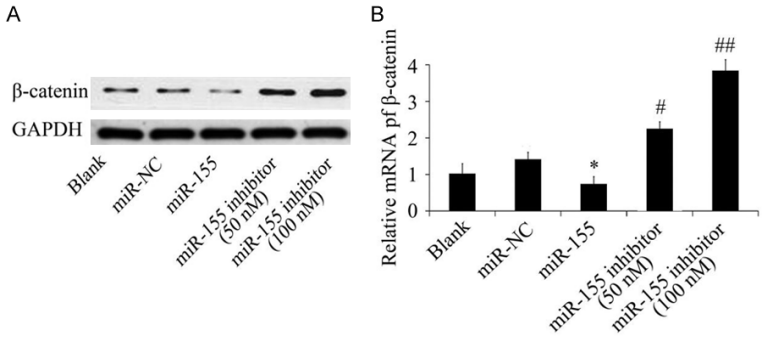

Figure 5. Expression of β-catenin were assayed by Western blot (A, n=4) and real-time PCR (B, n=4). The results showed that miR-155 inhibited the expression of β-catenin while miR-155 inhibitor promoted the expression of β-catenin. *P < 0.05 *vs.* miR-NC and #P < 0.05, ##P < 0.01 *vs.* miR-NC.

magnification of 200 times and invaded cells were calculated. Statistical analysis results (**Figure 4B**) showed that the invasion rate in miR-155 group is significantly lower than that in the control group (P < 0.05) while the invasion rate in miR-155 inhibitor (100 nM) group showed significant increase (P < 0.05).

miR-155 inhibits expression of β-catenin

Wnt signaling plays a major role in placental development and morphogenesis. To determine if miR-155 alters β-catenin protein levels, trophoblasts were analyzed for total β-catenin by Western blotting at 48 hours after transfection. There was a significant (up to 80%) reduction in the total levels of β-catenin protein in miR-155-trasfected cells and a significant increase in miR-155 inhibitor-transfected cells (**Figure 5A**). To determine if miR-155 affects β-catenin gene transcription, qRT-PCR was performed. As indicated in **Figure 5B**, β-catenin mRNA levels were slightly lower in miR-155-trasfected cells compared to controls.

Discussion

Trophoblast differentiation is a complex and partially understood process that is essential for normal placental development and successful pregnancy outcome. Embryo implantation and tumor invasion process have shown similar mechanisms as both trophoblast cells and tumor cells are very aggressive. Various proteolytic enzymes secreted by trophoblasts or tumor cells lead to degradation of extracellular matrix (ECM) that allows the migration of cells smoothly through that damaged ECM. However, there remains an essential difference between these two main processes. In the embryo implantation process, invasion of trophoblast cells is precisely con-

trolled so that it can only occur in a specific time and specific location. Trophoblast cells are short-lived, not like as with tumorigenicity. Therefore, finding out the mechanism of proliferation and migration of trophoblast cells will be helpful in understanding the diseases related with trophoblast differentiation.

In our experiment, to investigate the effects of miR-155 on trophoblast differentiation, trophoblasts of patients with PE were used and results showed that miR-155 was significantly increased while its potential target CACNA2D1 was significantly decreased. Moreover, the luciferase reporter assay also revealed a negative regulatory relationship between miR-155 and CACNA2D1. In the present study, we found that miR-155 decreased the expression of β-catenin consistent with its known ability to activate Wnt signaling [14]. miR-155 regulates Wnt signaling either directly by targeting β-catenin or indirectly by inhibiting expression of β-catenin associated proteins. There is good evidence that Wnt signaling plays a vital role in placental development and in the formation of trophoblastic giant cells [15, 16]. The Wnt pathway has been suggested to play a role in human trophoblast invasion and cell fusion [17]. The results show that Wnt activation is involved in both trophoblast differentiation and migration pathways.

In summary, the results presented here show that miR-155 has inhibitory effects on the differentiation of human trophoblasts through inducing nuclear accumulation of β-catenin. The ability of miR-155 to inhibit proliferation and invasion provides useful tools for manipulating trophoblast differentiation *in vitro* and may also assist in uncovering how trophoblast cell fate decisions are regulated during early pregnancy. It is suggested that future studies address the pathway effects of miR-155.

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