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Endogenous and exogenous *miR-520c-3p* modulates CD44-mediated extravillous trophoblast invasion

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ABSTRACT

Introduction: Adequate extravillous trophoblast (EVT) invasion is essential for successful placentation. Although *miR-520c-3p* plays an important role in CD44-mediated invasion in cancer cells, there is little information on whether *miR-520c-3p* is involved in the regulatory mechanisms of CD44-mediated EVT invasion.

Methods: We screened first trimester trophoblast cells and trophoblast cell lines for expression of *miR-520c-3p* using real-time polymerase chain reaction. The cell invasion assay was performed using EVT cell lines, HTR8/SVneo and HChEpC1b, to investigate the capability of suppressing EVT invasion by *miR-520c-3p*. Laser microdissection analysis was then used to determine whether *miR-520c-3p* was present in the first trimester decidua. Finally, the possibility of chorionic villous trophoblast (CVT)-EVT communication via exosomal *miR-520c-3p* was determined using an *in vitro* model based on BeWo exosomes and the EVT cell lines as recipient cells.

Results: The *miR-520c-3p* level was significantly downregulated in EVT cell lines and EVTs. Cell invasion was significantly inhibited in *miR-520c-3p*-overexpressing cell lines, involving a significant reduction of CD44. Laser microdissection analysis showed that *miR-520c-3p* in the periarterial area of the decidua was significantly higher than that in the non-periarterial area. Using an *in vitro* model system, BeWo exosomal *miR-520c-3p* was internalized into the EVT cells with subsequently reduced cell invasion via CD44 repression.

Conclusions: EVT invasion is synergistically enhanced by the reciprocal expression of endogenous *miR-520c-3p* and CD44. The present study supports a novel model involving a placenta-associated miRNA function in cell-cell communication in which CVT exosomal *miR-520c-3p* regulates cell invasion by targeting CD44 in EVTs.

1. Introduction

In the human placental villi that attach the placenta to the uterine decidua, cytotrophoblasts undergo an epithelial-mesenchymal transition (EMT) and differentiate into extravillous trophoblasts (EVTs). EVT invasion into the uterine spiral arteries of the decidua results in the remodeling of uterine vasculature and plays a crucial role in the establishment of successful pregnancy. EVT invasion is a well-orchestrated process that is tightly regulated via complex interactions involving temporal and spatial events [1–4]. Inadequate EVT invasion into the decidua and subsequent incomplete remodeling of the spiral arteries often results in poor placentation leading to pregnancy-related diseases such as pre-eclampsia [5]. We recently reported that as a new member of EVT-decidual extracellular matrix interactions, a CD44-hyaluronic acid (HA) interaction is involved in the molecular mechanism of EVT invasion [6]. In our previous study, an *in vitro* cell invasion assay with small interfering RNA knockdown of CD44 in EVTs showed that CD44 expression significantly promoted EVT invasion in an

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HA-dependent manner. However, there is little information on the regulatory mechanisms of this CD44/HA-mediated EVT event.

The aim of this study was therefore to identify key parameters involved in the regulation of CD44/HA-mediated EVT invasion. During our investigation of the functional roles of the human imprinted chromosomal 19 miRNA cluster (C19MC) miRNAs that are expressed predominantly in the placenta [7–9], (i.e., placenta-associated miRNAs), we found differential expression of a placenta-associated miRNA (*miR-520c-3p*) between chorionic villous trophoblasts (CVTs) and EVTs. Subsequent analyses revealed that downregulation of endogenous *miR-520c-3p* accelerated CD44/HA-mediated EVT invasion. In addition, we showed the possible participation of exosomal *miR-520c-3p* in CVT-EVT cell communication and EVT invasion.

2. Methods

2.1. Sample collection and isolation of EVTs

The study was performed according to the guidelines of the Declaration of Helsinki. Human placentas from patients who gave informed consent were obtained using protocols approved by the Nippon Medical School Ethics Committee and the Jichi Medical University Ethics Committee. First trimester placental tissues (at 7–13 weeks of gestation, n = 18) were aseptically obtained after legal abortions. EVTs grown from explanted human chorionic villi were isolated as previously described [6]; approximately 95% of the isolated cells were immunohistochemically positive for HLA-G. Minced chorionic villous tips were defined as first trimester CVTs.

2.2. Cell culture

BeWo (RIKEN Bioresource Center, Tsukuba-shi, Ibaraki, Japan) was used as a human CVT model cell line. HTR8/SVneo [10] and HChEpC1b [6,11] were used as human EVT model cell lines. These cell lines were cultured in appropriate culture media (37 °C and 5% CO_2) as previously described [6].

For miRNA overexpression studies, Pre-miR (mature miRNA mimics; Thermo Fisher Scientific, Waltham, MA, USA) was used. The EVT cell lines were transfected with Pre-miR-520c (30 nM; mature *miR-520c-3p* mimic) for 4 h using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Pre-miR negative control #1 (Pre-miR NC) was used as a negative control. Pre-miR-transfected cells were harvested 48 h after transfection and the total RNA was extracted as described below.

2.3. Real-time polymerase chain reaction (PCR)

Total RNAs were isolated from tissues using ISOGEN (Nippon Gene, Chiyoda-ku, Tokyo, Japan) or RNAiso Plus (Takara Bio, Kusatsu-shi, Shiga, Japan). Real-time PCR for miRNA and mRNA was performed using the TaqMan MicroRNA Assay (Thermo Fisher Scientific) and SYBR Premix Ex Taq (Takara Bio), respectively, as previously described [12]. To normalize expression levels of miRNAs and mRNAs, *SNORD44* and *GAPDH* were used as endogenous internal controls, respectively [12]. Primers for *miR-517a-3p*, *miR-520c-3p*, and *SNORD44* were from Thermo Fisher Scientific. Primers for *CD44* mRNA were as follows: forward 5′-CATTGCAGTCAACAGTCGAAGAAG-3′ and reverse 5′-GCTGTCATAAACTGGTCTGGAGTT-3′.

2.4. Western blotting and immunohistochemistry

Western blotting was performed as described previously [6]. The antibodies used for Western blotting were as follows: mouse *anti*-CD44 (Cell Signaling Technology, Danvers, MA, USA), mouse anti-GAPDH (Thermo Fisher Scientific), rabbit *anti*-CD81 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit *anti*-TSG101 (Abcam, Cambridge, MA, USA), and horseradish peroxidase-conjugated secondary antibodies (Thermo Fisher Scientific).

Immunohistochemistry was used to provide confirmation of CD44 expression in trophoblast cell lines as described previously [13]. Briefly, cells grown on coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Samples were incubated with rabbit *anti*-CD44 (Trans Genic, Kobe-shi, Hyogo, Japan) and subsequently with Alexa-Fluor 594-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA). Control sections were treated in the same manner except that the primary antibody was replaced with irrelevant IgG. The signals were detected with a fluorescence microscope (DM6 B; Leica Microsystems, Wetzlar, Hesse, Germany) or a confocal microscope (TCS SP5; Leica Microsystems).

2.5. Cell invasion assay

An invasion assay was performed as previously described [6]. Briefly, cell invasion was evaluated using a growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA)-coated Transwell assay. Cells (5×10^4) in RPMI1640 [with no fetal bovine serum (FBS)] were placed in the upper chamber, and RPMI 1640 medium supplemented with 5% FBS and 250 μg/mL hyaluronic acid (HA [~289 kDa]; R&D Systems, Minneapolis, MN, USA) was added to the lower chamber. After incubation for 20 h, migratory cells were counted using a light microscope (KX4; Olympus, Shinjuku-ku, Tokyo, Japan) under 200 × magnification.

2.6. Luciferase reporter assay

To construct a reporter plasmid, we first cloned the 3'-untranslated region (3′-UTR) of the human *CD44* gene (GenBank accession number NM_000610) into the pMIR-REPORT vector, containing the firefly luciferase reporter (Applied Biosystems, Foster City, CA, USA). The total RNA, isolated from HeLa cells, was reverse-transcribed to cDNA using PrimeScript Reverse Transcriptase (Takara Bio). The 3′-UTR of the *CD44* mRNA was amplified from the cDNA. After sequence verification by cloning into a pCR4-Blunt-TOPO vector (Thermo Fisher Scientific), the *CD44* 3′-UTR was cloned into pMIR-REPORT. This final construct was designated pMIR-CD44.

For the reporter assay, HTR8/SVneo cells were transfected with pMIR-CD44 or pMIR-cont (empty vector, pMIR-REPORT), and the control vector (pCMV SPORT-βgal; Thermo Fisher Scientific), together with Pre-miR-520c or Pre-miR-NC (50 nM), using Lipofectamine 2000 in 24-well plates. After transfection for 24 h, firefly luciferase and β-galactosidase (β-gal) activities in cell lysates were measured. Firefly luciferase activity was normalized to the β-gal activity.

2.7. Laser microdissection (LMD) analysis

To examine the *in situ* distribution of *miR-520c-3p* in the human first trimester decidua, LMD was performed using an LMD6000 system (Leica Microsystems). Briefly, 15-μm frozen sections of the first trimester decidua (7–9 weeks of gestation; n = 5) were mounted on polyethylene naphthalate foil membrane-coated glass slides (Leica Microsystems) and stained with hematoxylin and eosin. Then, the periarterial area (PA), defined as decidua stroma within an area of approximately 100 μm from the spiral artery wall in sections of the first trimester decidua, and non-PA (area more