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Twist1 is involved in trophoblast syncytialization by regulating GCM1

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ABSTRACT

Introduction: The multinucleated syncytiotrophoblast (STB) is maintained and regenerated by the fusion of underlying cytotrophoblast cells (CTBs) and is responsible for a number of functions in the human placenta. Deficiencies in this structure may result in pregnancy-associated diseases. However, the detailed mechanisms underlying trophoblast syncytialization await further investigation.
Methods: The location of the transcription factor Twist1 in human placental tissues was identified by immunohistochemistry. The expression of Twist1 and glial cells missing-1 (GCM1) was evaluated by qPCR or western blotting in two cell-fusion models including forskolin-induced fusion of BeWo cells and spontaneous syncytialization of CTBs. The key role of Twist1 in trophoblast differentiation was identified using BeWo cells transfected with Twist1-specific siRNA. We investigated the effect of hypoxia on the expression of Twist1 and GCM1 in primary CTBs cultured with 2% oxygen. The Twist1 binding region in the *GCM1* gene was detected by chromatin-immunoprecipitation.
Results: Twist1 was expressed in human placental tissues, and the expression of Twist1 and GCM1 increased in a time-dependent manner during spontaneous syncytialization of primary CTBs and forskolin-induced fusion of BeWo cells. A reduction in Twist1 and GCM1 expression was observed under hypoxic conditions and was accompanied by inhibition of trophoblast syncytialization. Moreover, siRNA-mediated silencing of Twist1 resulted in inhibition of BeWo cells fusion and down-regulation of GCM1 expression. Furthermore, Twist1 was found to bind to the E-box-enriched region in intron 2 of the *GCM1* gene during forskolin-induced fusion of BeWo cells.
Discussion: The above results suggest that Twist1 is required during trophoblast syncytialization. Twist1 may promote trophoblast syncytialization by regulating the expression of GCM1.

1. Introduction

The maintenance of healthy fetal development in the maternal uterus is highly dependent on proper placental growth throughout pregnancy. During the process of placenta formation, mononucleated cytotrophoblast cells (CTBs) either proliferate and differentiate into highly invasive extravillous trophoblast cells (EVTs), which can invade the maternal decidual stroma and remodel the spiral arteries of the deciduas, or fuse and form the continuous, uninterrupted, multinucleated syncytiotrophoblast

Abbreviations: ANOVA, one-way analysis of variance; β-hCG, β-human chorionic gonadotropin; ChIP, chromatin immunoprecipitation; CK7, cytokeratin7; CTB, cytotrophoblast; DAB, diaminobenzidine; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulfoxide; EMT, epithelial-to-mesenchymal transition; EVT, extravillous trophoblast; FBS, fetal bovine serum; FSK, forskolin; GCM1, glial cells missing-1; GSK-3β, Glycogen synthase kinase-3β; HERV, human endogenous retrovirus; HIF-2α, hypoxia inducible factor-2α; IgGs, immunoglobulins; NSFC, natural science foundation of china; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PFA, paraformaldehyde; qPCR, quantitative real-time PCR; SDS, sodium dodecyl sulfate; SP, streptavidin-peroxidase; SPSS, statistical package for social science; STB, syncytiotrophoblast; TBEs, T-cell factor-binding elements; TBP, TATA box binding protein.

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(STB) [1–3]. The syncytiotrophoblast, which forms the outermost surface of the placental chorionic villi, is located at the interface between maternal and fetal circulation. This multinucleated layer performs a number of specialized functions throughout pregnancy, such as the production and secretion of growth factors and hormones [4], the exchange of gases, nutrients and waste products [5], and the generation of an innate immunological barrier to protect the fetus against the maternal immune system and potential infections [6]. A better understanding of the process of differentiation and fusion of CTBs to form the STB is essential because disturbance of this regulation is thought to be associated with pregnancy disorders such as pre-eclampsia and intrauterine growth retardation [7,8].

A number of studies have examined the critical roles of the transcription factor glial cells missing-1 (GCM1) and its target syncytins in human placental villus syncytialization for many years following the examination of their localization in placental tissues [9,10]. GCM1 is a key transcription factor for placental development and is primarily expressed in placental tissue [10,11]. The cause of embryonic lethality in GCM1 null mice was the failure of placental development. Mutant placentas failed to transport nutrients and gases between maternal and fetal blood owing to a failure formation of the labyrinth layer [12,13]. In human placentas, the fusogenic syncytin proteins, including syncytin1 and syncytin2 encoded by the ENVELOPE genes of the human endogenous retrovirus family (HERV), have been demonstrated to be crucial initiators of human trophoblast syncytialization [9,14]; GCM1 was confirmed to promote trophoblast fusion by up-regulating the expression of syncytin proteins [11,15,16]. Reduced expression of GCM1 and syncytin proteins has been observed under hypoxic conditions [17,18] and is associated with pregnancy disorders such as pre-eclampsia and intrauterine growth restriction [8,19–21].

The epithelial-to-mesenchymal transition (EMT) plays an important role in promoting the invasion and metastasis of tumor cells [22]. During human placenta formation, cytotrophoblast cells also undergo a regulated and controlled EMT process. CTBs differentiate into EVTs, acquire migratory and invasive capabilities, and then invade into the maternal deciduas or spiral arteries to anchor the chorionic villi into the uterus [23]. Down-regulation of E-cadherin is considered a hallmark of EMT [24]. Transcription factors such as Snail, Slug, Zeb1 and Zeb2, and Twist1 have been reported to facilitate EMT by inhibiting the expression of E-cadherin proteins [25]. Interestingly, these putative EMT inducers may have alternative roles in the fetal–maternal interface. For example, Twist1 has been shown to promote the invasion of trophoblast cells into the maternal decidua [26]. Twist1 could also increase the fusion of human cytotrophoblast cells [27]. However, the detailed mechanisms involved in EMT inducer-promoted trophoblast syncytialization have not been clearly elucidated. The relationship between EMT inducers such as Twist1 and fusion-promoting proteins such as GCM1 during syncytialization remains unclear.

In this study, we show that the levels of Twist1 and GCM1 mRNAs, but not Snail, Slug, Zeb1 and Zeb2 mRNAs, were up-regulated during the spontaneous syncytialization of human primary cytotrophoblast cells. Increased levels of Twist1 and GCM1 were also observed during forskolin-induced BeWo cells fusion. The siRNA-mediated silencing of Twist1 resulted in the inhibition of BeWo cells fusion, accompanied by the down-regulation of GCM1 mRNA transcription. In addition, we have demonstrated that syncytialization of human cytotrophoblast cells was inhibited under hypoxic conditions and corresponded with decreased expression of Twist1 protein and GCM1 mRNA. Immunohistochemistry showed that the localization of Twist1 and GCM1 in the human first trimester placenta was similar and that both were detected in the nuclei of CTBs and the STB. Furthermore, we found through chromatin immunoprecipitation that GCM1 expression was enhanced possibly through the binding of Twist1 with a potential enhancer region in intron 2 of the *GCM1* gene. Our study demonstrated that Twist1 was involved in human placental trophoblast syncytialization, likely by up-regulating GCM1 expression.

2. Materials and methods

2.1. Placenta collection

The human placental tissues used in this study were collected in accordance with the policy of the Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University. Placentas were obtained from anonymous healthy patients aged 25–35 years with informed consent. The research protocols in this study were approved by the Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences. Nine normal placentas at different gestational stages (three at the first trimester (6–8 weeks), three at the second trimester (18–20 weeks) and three at full-term (38–40 weeks)) from patients who underwent legal abortion or cesarean section were used for the immunohistochemistry study. All of the placental tissues were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. For the spontaneous syncytialization studies, five full-term placentas were collected by cesarean section.

2.2. Isolation of human primary term cytotrophoblast cells and BeWo cell culture

Primary cytotrophoblast cells were obtained from human term placental tissues as previously described [28]. Briefly, after removal of the chorionic plate and deciduas, the placenta was cut into small pieces and washed with sterile saline. Placental villous tissue was digested 4 times in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, Thermo Fisher Scientific, Inc. Waltham, MA, USA) with 0.125% trypsin (Sigma–Aldrich Inc., St. Louis, MO, USA) and 0.02% DNase I (Sigma). The supernatant was centrifuged for 15 min at 1200 g, and the cell pellets were re-suspended in 5 ml DMEM and separated by centrifugation on a discontinuous Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient (5–65%) at 1200 g for 20 min. The bands between 30 and 50% Percoll were harvested, and then the primary cytotrophoblast cells were purified by centrifugation. Isolated trophoblast cells were plated onto 35 mm dishes (2×10^6 cells per dish) in DMEM (HyClone) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin and then cultured with different oxygen concentrations (2% or 20%) in a 5% CO_2 air incubator at 37 °C. More than 95% of the cells harvested were cytotrophoblast cells as determined by immunofluorescent staining of cytokeratin7 (CK7, ZM-0071, Zhongshan Golden Bridge Crop., Beijing, China), a marker for CTBs.

The human choriocarcinoma cell line BeWo was obtained from the American Type Culture Collection and was cultured in Ham's F-12K (Kaighn's) (Gibco BRL)/DMEM (1:1) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a 37 °C humidified incubator. BeWo cells were treated with 50 μM forskolin (FSK, Sigma–Aldrich) to induce cell fusion.

2.3. Quantitative real-time PCR

Total RNA of BeWo cells or primary cytotrophoblast cells under different treatments was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The concentration and purity of RNA samples was determined by a NanoDrop2000 spectrophotometer (Thermo