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Hedgehog signaling through GLI1 and GLI2 is required for epithelial–mesenchymal transition in human trophoblasts

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ABSTRACT

Background: Epithelial to mesenchymal transition (EMT) is critical for human placental development, trophoblastic differentiation, and pregnancy-associated diseases. Here, we investigated the effects of hedgehog (HH) signaling on EMT in human trophoblasts, and further explored the underlying mechanism.
Methods: Human primary cytotrophoblasts and trophoblast-like JEG-3 cells were used as in vitro models. Quantitative real-time RT-PCR and Western blot analysis were performed to examine mRNA and protein levels, respectively. Lentiviruses expressing short hairpin RNA were used to knock down the target genes. Reporter assays and chromatin immunoprecipitation were performed to determine the transactivity. Cell migration, invasion and colony formation were accessed by wound healing, Matrigel-coated transwell, and colony formation assays, respectively.
Results: Activation of HH signaling induced the transdifferentiation of cytotrophoblasts and trophoblast-like JEG-3 cells from epithelial to mesenchymal phenotypes, exhibiting the decreases in E-Cadherin expression as well as the increases in vimentin expression, invasion, migration and colony formation. Knockdown of GLI1 and GLI2 but not GLI3 attenuated HH-induced transdifferentiation, whereas GLI1 was responsible for the expression of HH-induced key EMT regulators including Snail1, Slug, and Twist, and both GLI1 and GLI2 acted directly as transcriptional repressor of *CDH1* gene encoding E-Cadherin.
Conclusion: HH through GLI1 and GLI2 acts as critical signals in supporting the physiological function of mature placenta.
General significance: HH signaling through GLI1 and GLI2 could be required for the maintenance of human pregnancy.

1. Introduction

Hedgehog (HH) signaling has conserved roles in the differentiation of various cells in metazoans ranging from *Drosophila* to humans [1,2]. The mammalian HH ligands consist of sonic hedgehog (SHH), indian hedgehog (IHH) and desert hedgehog (DHH). In vertebrates, in the absence of HH, patched-1 (PTC1) receptor represses smoothened (SMO) activity, and the GLI transcription factors (GLI2 and GLI3) are proteolytically cleaved into their repressors within the primary cilium. In the presence of HH, binding of HH to PTC1 relieves the suppression of SMO, resulting in activation of GLI transcription factors (GLI1, GLI2 and GLI3) and inducing the transcription of the target genes including cyclin D, cyclin E, myc as well as PTC1 and GLI1 [3,4]. Overall, the conserved effect of HH is to switch the GLI transcription factors from repressors into activators and allow for fine-tuned transcriptional events.

Epithelial–mesenchymal transition (EMT) is a process in which epithelial cells lose the polarity and adhesiveness, change into a mesenchymal phenotype and gain the capacity of increased mobility [5]. EMT plays crucial roles in development of multiple tissues and organs, and also contributes to tissue repair and carcinoma progression [6]. In human placental development, trophoblast cells differentiate into either the villous cytotrophoblast (CTB) lineage to form the syncytiotrophoblast (STB) that secretes the majority of placental hormones, or the invasive extravillous cytotrophoblast (EVT) lineage to anchor the chorionic villi in the uterus [7,8]. Invasive EVTs migrate through the endometrium, interact with decidual cells and immunocompetent cells, and differentiate into multinucleated placental bed giant cells. On the other hand, EVTs can invade the maternal spiral arteries, mediate the destruction of the arterial wall, and replace the endothelium, forming endovascular trophoblasts [9,10]. Upon acquisition of migratory ability, EVTs tend to lose their tight epithelial assembly and phenotype, becoming loosely

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attached, subsequently invading the maternal decidua as interstitial cytotrophoblasts. The process by which placental trophoblasts originate as epithelial cells and are subsequently triggered to change from an epithelial to a mesenchymal-like migratory phenotype, resembling EMT is identified in other developmental processes [7,8].

The link between EMT and HH signaling has been previously established in many pathological conditions. For instance, HH/GLI signals regulate the EMT in pancreatic carcinoma, hepatocellular carcinoma, gastrointestinal neuroendocrine tumors, and lung squamous cell carcinoma [11–13]; and HH signals regulate EMT in renal and biliary fibrosis [14,15]. To date, HH signaling regulating EMT in physiological conditions has not yet been studied. Hence, we investigated the roles of HH signaling in EMT in human primary cytotrophoblasts, and explored the underlying mechanisms in trophoblast-like cells. Our results demonstrate that HH signals stimulate EMT of placental cytotrophoblast possibly by inducing the GLI1-controlled expression of key transcriptional factors of EMT and both GLI1- and GLI2-controlled transcription of a downstream target gene, *CDH1*, encoding E-Cadherin. This work establishes HH signaling as an essential mechanism in maintaining the physiological functions of adult placentas.

2. Materials and methods

2.1. Cell lines

Trophoblast-like JEG-3 cells were obtained from ATCC (Manassas, VA), and cultured in DMEM/F12 = 1:1 medium (Hyclone, USA) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc., Grand Island, NY) at 37 °C with 5% CO_2 as described previously [16, 17]. 293 EcR SHH cells (SHH-expressing cells, ATCC) and control HEK 293 cells were used for the production of biologically active murine SHH conditional medium (SM) and control medium (CM), respectively, in the presence of ecdysone [18]. The specificity of SM was confirmed by 5E1 SHH blocking antibody (DSHB, Iowa City, Iowa). 293FT packaging cells (Life Technologies) and GP293 retroviral packaging cells (Clontech Laboratories, Inc., Mountain View, CA) for generating the lentiviruses and retroviruses were cultured as described previously [17,19].

2.2. Isolation and culture of cytotrophoblasts

Human placentas were obtained from uncomplicated normal term (38–40 W) pregnancy after elective cesarean section without labor, following a protocol approved by the Ethics Committee of School of Medicine of Zhejiang University. The primary cytotrophoblasts were isolated and purified as described previously [17]. Briefly, tissue aliquots were removed from the maternal side of the placenta and digested with 0.125% trypsin (Sigma, St. Louis, MO) in DMEM (Life Technologies). The placental cytotrophoblasts were purified using a 5–65% Percoll (Sigma) gradient at step increments of 5%. The cells were plated at a density of 1.5×10^6 cells per well in 6-well plates, and were cultured at 37 °C with 5% CO_2 and 95% air in DMEM containing 10% newborn calf serum (Life Technologies) to allow syncytialization in vitro.

2.3. RNA isolation, RT-PCR and quantitative real-time PCR

Total RNA was isolated from JEG-3 cells and cytotrophoblasts by using a TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. 5 μg total RNA in a volume of 20 μl was reversely transcribed by using SuperScript III reagent (Life Technologies) and the oligo-(deoxythymidine) primer with incubation at 42 °C for 1 h. After the termination of cDNA synthesis, each reaction mixture was diluted with 80 μl Tris–EDTA buffer. Messenger RNA levels of target genes were determined by RT-PCR and quantitative RT-PCR as described previously [17,20]. The relative amounts of the mRNA levels of the target genes were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels, respectively, and the relative difference in mRNA levels was calculated by $2^{-\Delta\Delta Ct}$ method [21]. The primers were listed in the Supplementary data (Table S1).

2.4. Western blotting

Total protein extracts were prepared in whole cell lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10 mM sodium pyrophosphate, 1.5 mM $MgCl_2$, 100 mM sodium fluoride, 10% glycerol, and 1% Triton X-100) containing an inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate). Protein concentrations were determined using a standard Bradford assay, and 50 μg of total protein was subjected to SDS-PAGE followed by a transfer onto PVDF membranes (Millipore, Bedford, MA). Membranes were incubated overnight at 4 °C with primary antibodies against E-Cadherin (SC-7870, Santa Cruz Biotechnology Inc., Santa Cruz, CA), vimentin (SC-6260, Santa Cruz), GLI1 (SC-20687, Santa Cruz), GLI2 (ab26056, Abcam Ltd., Cambridge, UK), GLI3 (ab69838, Abcam), and β-actin (SC-69879, Santa Cruz) followed by incubation in secondary antibodies. Signals were developed using the Enhanced Chemiluminescence System. National Institutes of Health Image software (ImageJ, http://rsb.info.nih.gov/ij/) was used to quantify the immunoreactive bands, and the normalized antigen signals were calculated from target protein-derived and β-actin-derived signals. The mean density of bands from the control cells was set to 1.

2.5. Immunohistochemistry and immunocytochemistry staining

Immunohistochemistry staining was performed by using the Histostain-Plus Kit (Kangwei Reagents, Beijing, China) according to the manufacturer's instructions. Briefly, paraffin-embedded placental sections (4 μm) were deparaffinized and rehydrated in xylene and a graded series of ethanol. After antigen retrieval in 10 mM sodium citrate and 10 mM citric acid, tissue sections were then incubated with 3% H_2O_2 in methanol to quench endogenous peroxidase followed by sequential incubation included with normal serum for 30 min, with control IgG and primary antibodies against SHH (06-1106, Millipore, Billerica, MA), IHH (ab39634, Abcam Ltd., Cambridge, UK), DHH (SC-271168, Santa Cruz), PTC1 (06-1102, Millipore), SMO (ab72130, Abcam), GLI1 (SC-20687, Santa Cruz), GLI2 (ab26056, Abcam Ltd., Cambridge, UK) and GLI3 (ab69838, Abcam) at 4 °C overnight, and with HRP-labeled secondary antibody (Life Technologies) for 30 min. The diaminobenzidine (DAB) solution was used for development of color, and the sections were counterstained with hematoxylin. Immunocytochemistry was performed on chamber slides (Nalge Nunc International, Naperville, IL). Either JEG-3 cells or cytotrophoblasts were fixed in ice-cold methanol and permeabilized with 0.1% Triton X-100 in PBS (PBST). After incubation with blocking buffer (1% bovine serum albumin), the cells were incubated with primary antibodies against E-Cadherin or vimentin. After washing with PBST, the cells were incubated with Alexa 488- or 555-conjugated secondary antibodies (Life Technologies). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Slides were analyzed by a laser scanning microscope.

2.6. Wound healing, Matrigel invasion and colony formation assays

For wound healing assays, the cells were seeded in 6-well plates (2×10^5 cells/well). After various treatments, the confluent monolayer of cells was scrapped with a sterile tip to create an artificial wound, and then incubated with either CM or SM and allowed to heal. Cell migration to the wounded surface was then monitored by microscopy after 48 h, and the distance between the edges of the wound was measured by Photoshop CS3. Invasion of cells was measured in Matrigel-coated Transwell inserts (6.5 μm, Costar, Cambridge) containing polycarbonate filters with 8-μm pores as detailed previously [22]. After various treatments, JEG-3 cells (2×10^5) in 200 μl of serum-free medium were plated in the upper chamber, whereas 600 μl of medium