LIF upregulates poFUT1 expression and promotes trophoblast cell migration and invasion at the fetal–maternal interface

S Liu1,4, J Wang1,4, H-M Qin2, X-M Yan3, X-S Yang1, C Liu1 and Q Yan*1

Trophoblast cell migration and invasion are crucial for the establishment of a successful pregnancy. Protein O-fucosyltransferases, such as poFUT1 and poFUT2, catalyze the O-fucosylation of proteins and have important roles in embryonic development. Leukemia inhibitory factor (LIF) is a critical cytokine in the regulation of embryonic development and implantation. However, the exact roles of poFUTs in embryo migration and invasion and the effects of LIF on the expression of poFUTs have not been studied in detail. In the current study, we showed that poFUT1 and LIF were highly expressed in human trophoblast cells and in the serum of women during the first trimester of a normal pregnancy. However, in patients with threatened abortion, poFUT1 and LIF levels were found to be reduced. There were no significant differences in the expression levels of poFUT2 between the two groups. The migration and invasion potential of trophoblasts in an explant culture and in an in vitro implantation model was decreased or increased upon altering poFUT1 expression levels by siRNA or cDNA transfection. Our results also revealed that LIF upregulated the expression of poFUT1. The upregulation of poFUT1 by LIF promoted trophoblast cell migration and invasion at the fetal–maternal interface by activating the PI3K/Akt signaling pathway. Taken together, these study findings suggest that poFUT1 may be used as a marker of embryo implantation.

Cell Death and Disease (2014) 5, e1396; doi:10.1038/cddis.2014.335; published online 28 August 2014

Human pregnancy is a complex biological process that requires synchrony between the develop embryo and the receptive uterine endometrium. The fertilized egg divides into a 2-, 4-, and 8-cell embryo before developing into the morula and then into the mature blastocyst. In the mature blastocysts, the inner cell mass (ICM), which comprises embryonic stem cells, differentiates into various tissues in the fetus. The trophoblast is the outer layer of embryonic tissue (also called villi) that facilitates the adhesion and penetration of the blastocysts into the endometrium during implantation. During the implantation of a human embryo, the progenitor trophoblast cells differentiate into the syncytiotrophoblast (STB) and the invasive trophoblast (iCTB) by different developmental pathways. The STB covers the villi and develops into the placenta, which mediates the exchange of nutrients, oxygen, and waste between maternal and fetal blood. The iCTB, which is derived from the trophoblast by epithelial–mesenchymal transition (EMT), forms cell columns and has a highly invasive character, causing it to migrate away from the attached embryo, invade the uterine epithelium and uterine spiral arteries to establish the maternal–fetal interface. However, poor iCTB migration and invasion often result in a failure to establish the maternal–fetal connection and leads to abortion. Many factors at the maternal–fetal interface, such as hormones (e.g., progesterone), growth factors and their receptors (e.g., EGF and EGFR), and cytokines (e.g., leukemia inhibitory factor (LIF)), regulate trophoblast invasion. These molecules increase the invasion potential of the embryo by increasing the expression levels of matrix metalloproteinases (MMPs) and decreasing that of the tissue inhibitor of MMP (TIMP).

Protein glycosylation has an important role in many physiological and pathological processes, including inflammation, cancer metastasis, and embryo implantation. Protein fucosylation is one of the important types of glycosylation. It is classified into two kinds, N-fucosylation and O-fucosylation, which are catalyzed by fucosyltransferases (FUTs) and protein O-fucosyltransferases (poFUTs), respectively. It is known that FUTs are expressed at specific stages of the menstrual cycle and are associated with reproductive function. Ponnampalam et al. reported an increased expression level of FUT4 in the secretory phase as compared with that observed in the proliferative phase. We had previously shown that FUT4 and FUT7, which are expressed in uterine epithelial cells, influence the adhesion potential of the embryo. poFUTs include poFUT1 and poFUT2. poFUT1 transfers L-fucose directly to the glycoproteins with epidermal growth factor (EGF)-like repeats. A mutation in poFUT1 caused embryonic lethality in mice; silencing poFUT1 led to abnormal development in

1 Liaoning Provincial Core Lab of Glycobiology and Glycoengineering, Department of Biochemistry and Molecular Biology, Dalian Medical University, Dalian, People’s Republic of China; 2 Department of Pathology, The Secondary Affiliated Hospital of Dalian Medical University, Dalian, People’s Republic of China and 3 College of Medical Laboratory, Dalian Medical University, Dalian, People’s Republic of China

*Corresponding author: Q Yan, Liaoning Provincial Core Lab of Glycobiology and Glycoengineering, Department of Biochemistry and Molecular Biology, Dalian Medical University, Dalian, People’s Republic of China

1These authors contributed equally to this work.

Abbreviations: LIF, leukemia inhibitory factor; poFUT1, protein O-fucosyltransferase 1; ICM, inner cell mass; STB, syncytiotrophoblast; iCTB, invasive trophoblast; MMPs, matrix metalloproteinases; CK7, cytokeratin 7; TIMP, tissue inhibitor of MMP; EMT, epithelial–mesenchymal transition

Received 05.5.14; revised 02.7.14; accepted 03.7.14; Edited by A Finazzi-Agro
Drosophilas\textsuperscript{20,21} poFUT2 catalyzes the O-fucosylation of the glycoproteins with thrombospondin type 1 repeats (TSRs). The disruption of poFUT2 in mice resulted in teratomas.\textsuperscript{22,23} However, the exact roles of poFUT1 and poFUT2 in human trophoblast migration and invasion have not been studied in detail.

LIF, a pleiotropic cytokine of the interleukin-6 family, is essential for the successful completion of human pregnancy.\textsuperscript{24,25} LIF, secreted by both the trophoblast and the uterine endometrium, mediates embryonic development and implantation in an autocrine or paracrine manner at the maternal–fetal interface. In the mother, LIF level peaks during the secretory/postovulatory phase of the menstrual cycle and regulates uterine receptivity for blastocyst implantation. In the fetus, LIF promotes trophoblast proliferation, invasion, and differentiation. Reduced expression level of LIF led to incomplete embryonic development. A deficiency of LIF results in implantation failure and causes repeated abortions or unexplained infertility.\textsuperscript{26–29} However, the role of LIF-mediated regulation of poFUT1 and poFUT2 in embryo implantation has not been elucidated.

In this study, we report that the levels of poFUT1 and LIF were higher in trophoblasts obtained from women with a normal pregnancy than those from patients with threatened abortion. However, the expression level of poFUT2 was not significantly different between the two groups. The poFUT1 promoted the migration and invasion of trophoblasts in an explant culture and in an \textit{in vitro} implantation model. The upregulation of poFUT1 by LIF facilitated trophoblast cell migration and invasion through activating the PI3K/Akt signaling pathway.

Results

Expression of poFUT1 and LIF level was higher in women with normal pregnancy than in patients with threatened abortion. We analyzed the expression of poFUT1 and poFUT2 in villi of women in the first trimester of the normal pregnancy and threatened abortion by using immunohistochemistry (Figure 1A). Trophoblast cells were identified by cytokeratin 7 staining on a separate adjacent section (Figures 1Ac and d). The poFUT1 and poFUT2 could be detected in the villous trophoblast and the trophoblast column (TC) during the first trimester. In addition, the expression levels of poFUT1 were significantly lower in the villi of patients with threatened abortion than in the villi of women with normal pregnancy (Figures 1Ae and f). However, the expression levels of poFUT2 did not differ significantly between the two groups (Figures 1Ag and h). The serum level of LIF in the normal pregnancy and threatened abortion groups was analyzed by enzyme-linked immunosorbent assay (ELISA) (Figure 1B). The results showed that the LIF level was significantly lower in the threatened abortion group (5.08 $\pm$ 1.79 ng/l) than in the normal pregnancy group (10.60 $\pm$ 6.30 ng/l; $P<0.01$).

Expression level of poFUT1 in trophoblast tissues and cells. Immunofluorescent staining and western blot analysis were used to confirm the expression of poFUT1 in the frozen sections of human villi and cultured trophoblast cells (Figure 2). The fluorescent image showed that poFUT1 was expressed in human villi (Figure 2a). Immunofluorescent observation revealed that poFUT1 was expressed in JAR cells, where it was localized in the endoplasmic reticulum (Figure 2b). The results of the western blotting further
The successful transfection of siRNA into explant was determined by FITC-tagged CON siRNA, which exhibited green fluorescence (Figures 3Ca). The migration and invasion potentials of the trophoblast cells transfected with poFUT1 siRNA-1 and siRNA-2 were evaluated by the transwell migration and matrigel invasion assays, respectively. Compared with the CON siRNA, poFUT1 siRNAs significantly decreased the percentage of cells that showed migration (Figure 3D; **P<0.01) or invasion (Figure 3E; **P<0.01). The invasion potential of the trophoblast after poFUT1 siRNAs transfection was studied by assaying the activity of MMP-2 and the levels of TIMP-1 and -2 using gelatin zymography and western blotting, respectively. As illustrated in Figure 3F, the suspension medium from poFUT1 siRNA-treated cells exhibited much lower levels of MMP-2 activity than that from CON siRNA-treated cells. TIMP-1 and -2 levels were significantly higher in poFUT1 siRNA-transfected cells than in those transfected with control siRNA (Figure 3G).

However, transfection of trophoblasts with poFUT1 cDNA increased poFUT1 expression at both mRNA and protein levels, as detected by real-time PCR and western blot analysis (Figures 4a and b). Moreover, the upregulation of poFUT1 facilitated cell migration (Figure 4c; **P<0.01) and invasion (Figure 4d; **P<0.01) compared with the mock control, as analyzed by transwell cell migration assay and matrigel cell invasion models. Upregulation of poFUT1 also increased MMP-2 activity, as determined by gelatin zymography (Figure 4e), and TIMP-1 and -2 levels, as detected by western blotting (Figure 4f).

LIF upregulates poFUT1 expression in trophoblast cells and promotes trophoblast outgrowth and invasion. LIF is an essential factor for embryo implantation. To investigate whether LIF can regulate poFUT1 expression, trophoblast cells were treated with various concentrations of LIF (0, 10, and 50 ng/ml) for different time intervals (24, 48, and 72 h). Protein samples were collected and poFUT1 levels were analyzed by western blotting. The results revealed that LIF increased expression levels of poFUT1 (Figures 5a and b). The results also showed that LIF restored the reduced poFUT1 levels in poFUT1 siRNA-transfected trophoblast cells (Figure 5c), and further elevated the increased poFUT1 levels in poFUT1 cDNA-transfected trophoblast cells (Figure 5d).

To investigate whether the LIF-mediated expression of poFUT1 promoted trophoblast outgrowth and invasion, an extravillous explant culture and a matrigel invasion model were used. In Figure 6a, bright-field images (Figures 6Aa, f and k) showed that poFUT1 siRNA significantly decreased trophoblast outgrowth, while LIF restored the inhibition. Dark-field images showed the IF staining (Figures 6Ab–e, g–j, and i–o). CK7 (red) is a marker for trophoblast cells (Figures 6Ab, j, and I). Compared with the group treated with CON siRNA, poFUT1 siRNA was significantly decreased poFUT1 level (green) in trophoblast cells and LIF could restore the poFUT1 level (Figures 6Ac, h, and m). In the matrigel cell invasion model, LIF also restored the trophoblast cell invasion potential. LIF was found to regulate the activity of MMP-2 and the expression of TIMP-1 and -2 (Figures 6A, B, and D).
Figure 3  Silencing poFUT1 suppresses trophoblast outgrowth in extravillous explant cultures, migration and invasion of trophoblast cells. (A and B) Expression of poFUT1 in trophoblast cells after control (CON) or poFUT1 siRNA transfection by real-time PCR and western blotting. (C) (a) Villi transfected with FITC-tagged siRNA, showing the transfection efficiency. (b) Extravillous explants from 7 weeks of gestation maintained in culture on matrigel were incubated with CON siRNA or poFUT1 siRNA. Representative pictures of explants were taken under the microscope (× 10) after 24 and 48 h of culture in vitro. (D and E) Representative images of the cells treated with control (CON) or poFUT1 siRNA in transwell migration assay and matrigel invasion assay (× 100). (F) Gelatin zymography assay of MMP-2 in the culture medium collected from trophoblast cells which were treated with Con siRNA or poFUT1 siRNA-1, -2. (G) Western blot analysis of TIMP-1 and TIMP-2 in trophoblast cells transfected with poFUT1 siRNA-1, -2 or Con siRNA.
LIF further elevated the poFUT1 levels in poFUT1 cDNA-transfected trophoblast cells (Figure 5d), promoted trophoblast cell invasion, and regulated MMP-2 activity and TIMP-1 and -2 expression (Figures 6C and E).

Upregulation of poFUT1 by LIF promotes cell invasion through the activation of the PI3K/Akt signaling pathway. To explore the molecular mechanism underlying the LIF-mediated regulation of poFUT1 in trophoblast invasion, the activation of the PI3K/Akt signaling pathway was analyzed. Trophoblast cells were pretreated with poFUT1 siRNA, LIF, or LY294002 (an inhibitor of PI3K), and the presence PI3K/Akt signaling molecules, p-PDK, p-Akt (468), p-Akt (308), was detected by western blotting (Figure 7). The results showed that silencing poFUT1 resulted in the inhibition of p-PDK, p-Akt(468), and p-Akt(308), was detected by western blotting (Figure 7). The results showed that silencing poFUT1 resulted in the inhibition of p-PDK, p-Akt(468), and p-Akt(308), was detected by western blotting (Figure 7). The results showed that silencing poFUT1 resulted in the inhibition of p-PDK, p-Akt(468), and p-Akt(308), was detected by western blotting (Figure 7).

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Moreover, addition of PI3K/Akt inhibitor suppressed the invasion potential of the trophoblast cells (Figures 7c and e).

The pFUT1 cDNA transfection increased the expression levels of p-PDK, p-Akt(468), and p-Akt(308) and activated the PI3K/Akt signaling pathway, which in turn promoted trophoblast invasion (Figures 7b, d, and f).

Discussion

Embryo adhesion, migration, and invasion are regulated in a strict spatiotemporal manner. A variety of molecules, which are expressed at specific stages of the menstrual cycle, have important roles in the embryo implantation process, such as hormones, growth factors, growth factor receptors, and cytokines. Abnormal expression level of these factors may lead to the impaired function of the downstream molecules, which in turn may eventually result in infertility, abortion, or pregnancy-related complications. Glycosyltransferases catalyze protein glycosylation, which is involved in regulating the proliferation, migration, and invasion potential of the trophoblast. For example, polysialyltransferase IV (ST8SiaIV), which enhances trophoblast migration and invasion, is highly expressed in trophoblast cells during the first trimester and downregulated during the course of pregnancy. Yamamoto et al. reported that silencing N-acetylgalactosaminyltransferase V (GnT-V) inhibited the migration and invasion of human cytotrophoblasts. FUTs also have important roles during embryo implantation. FUT7 is highly expressed in both mature blastocysts in mice and chorionic tissues in humans, as well as in the embryonic trophoblast cells. The downregulation of FUT7 expression by antisense oligodeoxynucleic acid decreased the embryo adhesion rate in an in vitro implantation model. In our previous studies, we found that even after N-linked sugar chains were deleted by treatment with N-tunicamysin and PNGase, many fucosylated proteins could be detected in trophoblast cells in a UEA lectin blot (unpublished data). Moreover, embryo implantation was not completely inhibited in these experiments. O-fucosylation has an important role in embryo development and culmination. Therefore, we hypothesized that O-fucosylation is also closely related to embryo implantation. O-fucosylation is catalyzed by poFUT1 and poFUT2. In this study, we analyzed the expression of poFUT1 and poFUT2 in the villi of women with normal pregnancy and in patients with threatened abortion in the first trimester of pregnancy using immunohistochemistry. The results revealed that poFUT1 was highly expressed in the villi of women with normal pregnancy. However, villi from patients with threatened abortion showed decreased poFUT1 levels (Figure 1A). Moreover, silencing poFUT1 not only significantly inhibited trophoblast outgrowth in the extravillous explant culture model, but also suppressed the migration and invasion potentials of cultured trophoblast cells (Figure 3). To corroborate our hypothesis that decreased poFUT1 level may increase the risk of abortion, Serum LIF level was also simultaneously analyzed. It has been reported that LIF stimulates the proliferation, differentiation, and invasion of extravillous trophoblast explants and trophoblast cell lines. A mutation or deficiency in LIF is associated with embryo dormancy and pregnancy loss. Reduced levels of LIF increase the risk of miscarriage in patients undergoing in vitro fertilization. In this study, we found that as compared with the normal pregnancy group, the threatened abortion group had significantly diminished levels of serum LIF (Figure 1B). This result is consistent with the observation that poFUT1 level was reduced in patients with threatened abortion. Therefore, poFUT1, together with LIF, may be a novel marker of threatened abortion and may be useful for the evaluation of embryonic development.

LIF is an upstream regulatory factor. LIF activates embryo and promotes embryo implantation by regulating the expression levels and functions of specific molecules. For example, LIF decreases embryonic lethality by elevating SOCS3 expression, increases PAPPA level which is associated with embryonic development, and promotes embryo adhesion to the uterine epithelium by increasing the levels of integrin β3. LIF is also involved in immune tolerance through the regulation of HLA-G, a class I MHC molecule specifically...
Figure 6  LIF facilitates trophoblast outgrowth in extravillous explant cultures, migration and invasion of trophoblast cells through poFUT1. (A) Effect of LIF on outgrowth of trophoblast transfected with poFUT1 siRNA using extravillous explant culture. Whole mount immunofluorescent assay indicating the silencing of poFUT1 in the outgrowth. Bright-field images (a, f, and k) show the outgrowth of the explants. The boxed areas in (a, f, and k) are enlarged and shown in (b–d, gi, l–n), respectively (× 40). The antibodies used for immunofluorescent staining indicated on the images. (B and C) Effect of LIF on invasion in poFUT1 siRNA or poFUT1 cDNA-transfected trophoblast cells. (D and E) Effect of LIF on MMP-2 activity by gelatin zymography and TIMP-1 and -2 expression by western blotting in poFUT1 siRNA or poFUT1 cDNA-transfected trophoblast cells.
expressed by invasive cytotrophoblast cells. To the best of our knowledge, the role of LIF in poFUT1 regulation has not been reported to date. In our previous study, we found that LIF treatment upregulated the expression of FUT7, which has a role in embryo adhesion, in mouse embryos. In this study, trophoblast cells were used to analyze the regulatory effect of LIF on poFUT1. The results showed that LIF treatment upregulated the expression of poFUT1 in trophoblast cells (Figure 5). LIF also restored trophoblast outgrowth potential in a poFUT1 siRNA-transfected extravillous explant (Figure 6A). Consistent with this result, LIF could partially restore poFUT1 expression and invasion potential in poFUT1-silenced trophoblast cells (Figures 5 and 6). These results suggest that poFUT1 is one of the target molecules regulated by LIF.

Embryo invasion is a key step in the implantation process. The trophoblast secretes MMPs, which degrade the ECM.

Figure 7  Upregulation of poFUT1 by LIF promotes cell migration and invasion through PI3K/Akt signal pathway. (a) Trophoblast cells were pretreated by poFUT1-siRNA, LIF or inhibitor of PI3K (LY294002), and activation of PI3K/Akt signal molecules (p-PDK, p-Akt468, and p-Akt308) was measured by western blotting. (c and e) Invasion potential and MMP-2 activity and TIMP-1, -2 expression were detected. (b) Trophoblast cells pretreated by poFUT1 cDNA, LIF or inhibitor of PI3K, and activation of PI3K/Akt signal molecules was measured by western blotting. (d and f) Invasion potential and MMP-2 activity and TIMP-1, -2 expression were detected.
This facilitates blastocyst invasion through the decidua and into the maternal vasculature. MMP-2 and TIMPs are associated with cell invasiveness and metastasis potential. MMP-2 has been implicated in the remodeling of the ECM during the trophoblast invasion process. In this study, we found that silencing or overexpressing poFUT1 could downregulate or upregulate trophoblast invasion by altering the activity of MMP-2 (Figures 3F and 4e). In addition, in the poFUT1-silenced group, LIF increased the MMP-2 activity, further enhancing trophoblast invasion (Figure 6D). TIMPs, which are the major endogenous inhibitors of MMP activity in tissues, inhibit trophoblast invasion. In this study, poFUT1 siRNA increased TIMP-1 and TIMP-2 expression, further inhibited MMP-2 activity, and decreased trophoblast invasion (Figure 3G).

The present study also investigated the molecular mechanism by which LIF and poFUT1 regulates trophoblast invasiveness. LIF regulates embryo development and implantation through activating many signaling pathways. For example, LIF regulates trophoblast differentiation through activating the JAK/STAT and MAPK signaling pathways. LIF maintains pluripotency in mouse and primate embryonic stem cells by activating the PI3K/Akt signaling pathway. Activation of the PI3K/Akt signaling pathway was also reported to promote the metastasis ability and invasiveness of many tumors by enhancing the activity of MMPs. To a certain extent, trophoblast invasion in reproduction is similar to that of the tumor metastasis. Therefore, we hypothesized that trophoblast invasion promoted LIF correlated with activation of PI3K/Akt signaling pathway. However, the mediating molecules involving in this process remain not clear. The poFUT1 is a key enzyme in regulating the O-fucosylation of specific glycoproteins on the cell surface and the downstream signaling pathways, such as notch. Shi et al. reported that poFUT1 altered fucosylation of notch receptor could affect notch signaling and embryo development, but the exact signaling pathway remains not clear. In this study, silencing poFUT1 inhibited the activation of the signaling molecules (p-PDK, p-Akt468, and p-Akt308) in PI3K/Akt signaling pathway, which decreased the cell invasion potential (Figures 7a, c, and e). Whereas, the addition of LIF in the poFUT1 silencing group could upregulate the expression of poFUT1 (Figure 5c), and restore the activation of PI3K/Akt signaling pathway, as well as increase trophoblast cell invasion potential (Figure 7). The above results indicate that LIF upregulates poFUT1 expression, which further activates PI3K/Akt signaling pathway and promotes trophoblast invasion. Although our results have demonstrated that the upregulation of poFUT1 by LIF promotes embryo invasion through the activation of the PI3K/AKT signaling pathway, future investigation is required to explore the target O-fucosylated glycoproteins catalyzed by poFUT1 in PI3K/Akt signaling pathways.

In summary, to our knowledge, this study is the first to demonstrate that poFUT1 promotes trophoblast invasion and migration at the maternal–fetal interface. LIF also promotes trophoblast cell invasion and migration by upregulating the expression of poFUT1. Moreover, the upregulation of poFUT1 by LIF promotes cell invasion and migration through the PI3K/AKT signaling pathway. The correlation between the decreased expression of poFUT1 and abortion provides a pathological criterion that may be applied for the diagnosis and treatment of threatened abortion.

### Materials and Methods

**Serum and tissue sample.** The protocols for human study were in accordance with the Institutional Review Board of Dalain Medical University. Samples were obtained from Yingkou Central Hospital and the Secondary Affiliated Hospital of Dalian Medical University from 2012 to 2013. Women serum samples used in this study were obtained from the women at the ages of 25–35. The non-pregnant control group (n = 30) was excluded from other gynecological abnormalities. The pregnant women were confirmed by ultrasound detection at 6–10 gestational weeks. The serum samples collected from normal pregnant group (n = 30) and threatened abortion group (n = 30) were used to analyze LIF level. These paraffin-embedded human villi were used for keratin 7 (CK-7) and poFUT1/2 immunohistochemical and immunofluorescent staining. The fresh human villi tissues were collected from the non-drug abortion women.

**Cell culture.** The human embryonic JAR cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The JAR cells were maintained in EMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO2 in humidified air according to standard procedures. The growth medium was renewed every 2–3 days.

**ELISA.** Commercial ELISA kits (Rocho, Norcross, GA, USA) were used to detect the serological values of LIF according to the manufacturer’s instructions. The absorbance was measured at 450 nm using an ELISA microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Three samples were tested in each group for each time.

**Immunohistochemistry and immunofluorescence.** Tissue slides were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min. Tissues were blocked with 10% normal goat serum for 30 min, and then incubated with primary anti-human poFUT1 antibody (1 : 50; Protein Tech, Chicago, IL, USA) and anti-human keratin 7 antibody (1 : 50; Protein Tech) at 4 °C for 12 h. The slides were incubated with biotinylated secondary antibody at 37 °C for 15 min, and reacted with a streptavidin-peroxidase conjugate at 37 °C for 10 min, then used 3,3'-diaminobenzidine as a chromogen substrate. Mayer’s hematoxylin was used as a counterstained dye. A negative control was obtained by replacing the primary antibody with phosphate-buffered saline (PBS). Images were captured with the Olympus fluorescence microscope (Olympus, Tokyo, Japan).

**Frozen sections were fixed with 4% paraformaldehyde for 48 h at room temperature, and then treated with 20% sucrose for 48 h. Tissues were embedded with OCT and sliced to 18 μm at −20 °C.** After blocking with goat serum for 2 h at room temperature, the primary anti-human poFUT1 antibody (1 : 50) and anti-human keratin 7 (1 : 50) were incubated at 4 °C for 12 h. The next day, after 1 h incubation with phycoerythrine (PE)-conjugated anti-goat IgG (1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37 °C, slides were washed three times for 5 min with PBS, and then treated with DAPI (Santa Cruz Biotechnology) for 5 min at 37 °C. The poFUT1 and keratin 7 immunofluorescent staining was photographed with the inverted microscope (Olympus).

**Explant culture and whole mount immunofluorescence staining.** The explant culture was performed as described previously.

In brief, small pieces of tissues (2–3 mm) from tips of first-trimester human placental villi (7 weeks) were dissected and explanted in Millicell-CM culture dish inserts (0.4 mm pore size, Millipore, Carrigtwohill, Co., Cork, Ireland) pre-coated with phenol red-free matrigel substrate (Becton Dickinson, Bedford, MA, USA). Inserts were placed into 24-well culture dishes (Costar, Cambridge, MA, USA). The explants were cultured in serum-free DMEM/F12 media with 100 U/ml penicillin and 100 μg/ml streptomycin at 3% CO2/95% N2. Trophoblast cell sprouting and migration from the distal end of the villous tips were recorded for 24 and 48 h. To test the effect of poFUT1 on the trophoblast cell migration, siRNAs specifically targeting poFUT1 (500 nM), an equal concentration of the control siRNA and LIF was introduced into wells of culture media. All explant experiments with cultured villi were repeated three times.

Whole mount immunofluorescent staining was performed to confirm the role of poFUT1 siRNA and LIF on trophoblast outgrowth. The explants cultured for 48 h together with matrigel were fixed by 4% neutral PFA at room temperature for 30 min, and then washed 3 × 30 min in PBS with 1% Triton X-100. The explanted villi were blocked with goat serum for 1 h, and then incubated with primary anti-human poFUT1 antibody.
boFUT1 antibody (1 : 50) and anti-human keratin 7 (1 : 50) at 4 °C for 2 days. The explants were washed 3 × 1 h in PBS with 1% Triton X-100 and 10% FBS, and 3 × 10 min in PBS with 1% Triton X-100. Explants were then incubated with fluorescent secondary antibody for 1 day and DAPI for 1 hour sequentially. Finally, the fluorescent signals were photographed with the inverted microscope (Olympus).30

Real-time PCR. Cells were treated with RNAiso Plus reagent (Takara, Liaoning, China) for RNA extraction, and PrimeScript RT reagent Kit with gDNA Eraser kit (Takara) was used for synthesizing cDNA. SYBR Premix Ex Taq (Takara) was used for quantitative real-time PCR. Primers were as follows: poFUT1: 5’-CAGCGGAAGCGCGATTAGA-3’ (forward), and 5’-CTGTAGGAGAACCTGAAGAAAG-3’ (reverse). GAPDH: 5’-GGACCGTCAAGGCTGAGA-3’ (forward), and 5’-TGGTGAAGAGCGGCGAGTGA-3’ (reverse). The reactions were performed with Applied Biosystems 7500 Fast Real-time PCR System (Life Technologies, Carlsbad, CA, USA).

Transfection of poFUT1 siRNA and poFUT1 overexpression plasmid. poFUT1 siRNA and non-specific siRNA were dissolved in RNAse-free water provided by the manufacturer to a stock concentration of 20 nM. Cells were plated into 60-mm culture dishes and then transfected with 40 nM of siRNA using Lipofectamine 2000 following the manufacturer’s instructions, poFUT1 overexpression plasmid PEX-3-poFUT1 (GenePharma, Shanghai, China) was constructed and transfected into cells using Lipofectamine 2000 following the manufacturer’s instructions. All assays were performed 48 h after transfection. poFUT1 siRNA sequences were as follows: poFUT1 siRNA-1 (688) sense: 5’-GGGUCAGAACGAAUUGUGAATT-3’, antisense: 5’-UUCUCAACUGCUUGACGTCTT-3’; poFUT1 siRNA-2 (962) sense: 5’-GGGUCAGAACGAAUUGUGAATT-3’, antisense: 5’-UAUGCAACGUGCUAGGCTT-3’; poFUT1 siRNA-3 (1089) sense: 5’-GACCACUUUAUUGCGCAACUTT-3’, antisense: 5’-AGUUGCGCAAUAAGAUGGUGCTT-3’. Western blotting. To prepare whole-cell extracts, cells at 90% confluence were washed in PBS before incubation with lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM Na2VO4, 0.2 mM phenylmethylsulfonyl fluoride and 0.5% Nonidet P-40) on ice for 10 min. The cell lysates were clarified by centrifugation at 9000 × g for 10 min, and the supernatants were collected. Protein concentration was determined with the Bio-Rad assay. 20 μg of total protein was electrophoresed on 8% SDS-polyacrylamide gels

Matrigel cell invasion and transwell cell migration assay. For matrigel cell invasion assay, transwell inserts (8.0 μm pore size, 0.5 mm thick) containing polycarbonate filters with 8 mm pores were precoated with 50 μl of 1 mg/ml matrigel matrix (Becton Dickinson). For cell migration assay, the inserts were not precoated with matrigel. In all, 1.0 × 105 JAR cells in serum-free medium were plated in the upper chamber, whereas medium with 10% FBS was added to the lower chamber. After incubating for 24 h, the cells on the matrigel side of the inserts were removed by cotton swab. The inserts were fixed in methanol and stained with crystal violet. The number of invaded or migrated cells attached to the other side of the insert was counted under a light microscope (Olympus) in five random fields at a magnification of × 100. Three independent experiments were performed. Number of invasive or migrated cells under different treatments were normalized to the control and expressed as a means of invasion or migration percentage (%). ± S.D.

Gelatin zymography assay. MMP-2 activity was detected using the gelatin zymography assay. The supernatants were collected and used as the samples. Then, 10 μl total protein was electrophoresed on 8% SDS-polyacrylamide gels copolymerized with 1% gelatin. After electrophoresis, the gels were washed five times in 2.5% Triton X-100 (20 min each) and two times in buffer without Triton X-100 to remove Triton X-100, and then incubated in 50 mmol Tris-Cl pH 7.6, and 5 mmol CaCl2 (18 h, 37 °C). The gels were stained with 0.1% Coomassie blue R250 and destained in 10% isopropanol and 10% acetic acid in H2O. MMP-2 was detected as transparent bands on the blue background of a Coomassie blue-stained gel.

Statistical analysis. The quantitative data derived from three independent experiments are expressed as means (± S.D.). Unpaired Student’s t-tests were used to analyze between-group differences that are repeated and P-value < 0.05 was considered as statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by grants from National Natural Science Foundation of China (31070729, 31270866, and 31200608), Major State Basic Research Development Program of China (2012CB822103), Specialized Research Program for the Doctoral Program of Higher Education of China (2012110120003), and National Natural Science Foundation of Liaoning (2014023045).

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