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Leukemia inhibitory factor promotes human first trimester extravillous trophoblast adhesion to extracellular matrix and secretion of tissue inhibitor of metalloproteinases-1 and -2

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BACKGROUND: Leukemia inhibitory factor (LIF) is a pleiotropic cytokine that is essential for blastocyst implantation in mice. It has been suggested that LIF may play a role in human first trimester extravillous trophoblast (EVT) invasion. The aim of the present study was to establish whether LIF induces changes in EVT function related to invasiveness. METHODS: Primary first trimester human EVT cell cultures were treated with/without LIF and the effects on cell adhesion to fibronectin (FN), vitronectin (VN) and laminin (LN) were assessed. Transcript levels of integrin subunits that mediate cell adhesion to these extracellular matrix (ECM) elements were determined by real-time RT–PCR. Matrix metalloproteinase (MMP)2 and MMP9 secretion was assessed by gelatine zymography and tissue inhibitors matrix metalloproteinase (TIMP) -1 and TIMP-2 secretion by enzyme-linked immunosorbent assay. RESULTS: EVT cells showed increased adhesion to FN, VN and LN ECM elements in response to LIF (20, 20 and 29%, respectively, $P < 0.05$ FN and VN compared to control; and $P < 0.001$ LN compared to control). Integrin β_4 mRNA levels decreased by 50% following LIF treatment ($P < 0.001$ versus control). MMP2 and MMP9 secretion was not affected by LIF but LIF did increase secretion of TIMP-1 and -2 ($P < 0.001$ versus control). LIF stimulated the phosphorylation of signal transducer and activator of transcription (STAT) 3 protein while it did not affect STAT3 protein abundance. The addition of a LIF inhibitor attenuated the LIF-induced STAT3 phosphorylation in EVT. CONCLUSION: The results suggest that LIF can regulate EVT invasion, suggesting an important role in early placental development.

Keywords: leukemia inhibitory factor; extravillous trophoblasts; trophoblast invasion; cell adhesion; matrix metalloproteinases

Introduction

In human placenta, cytotrophoblasts show two distinct patterns of differentiation. In floating villi, they differentiate into syncytiotrophoblast and form the syncytial layer. In contrast, at villus-anchoring sites, cytotrophoblasts form the stratified structure known as the cell column and differentiate into extravillous trophoblasts (EVTs) (Kaufmann and Castellucci, 1997). EVTs acquire invasive activity in the distal part of the cell column (Damsky *et al*., 1992) and begin to migrate towards maternal tissue. Those that migrate into the decidual tissue are called interstitial trophoblasts and those that migrate into the trophoblast shell and invade along the maternal vessels are known as endovascular trophoblasts.

Normal placental development depends on the ability of EVTs to attach to and then invade into the maternal decidua and the first third of the myometrium. This occurs in order to anchor the fetus to the maternal endometrium and then invade the maternal spiral arteries creating large-diameter, low resistance vessels that provide a means of steady perfusion of the floating villi with maternal blood (Genbacev *et al*., 1992; Irving *et al*., 1995; Aboagye-Mathiesen *et al*., 1996). Insufficient trophoblast invasion of the spiral arteries has been associated with pre-eclampsia and fetal growth restriction (Hustin *et al*., 1990; Kadyrov *et al*., 2006). A recent study shows that late sporadic miscarriage is associated with abnormal trophoblast invasion and insufficient myometrial spiral artery transformation (Ball *et al*., 2006). Identification of the factors that control trophoblast

function with regard to differentiation and invasion will thus enable better understanding of the etiology of these disorders.

Trophoblast invasion depends on a tightly controlled program of intercellular signaling mediated by growth factors, cytokines and hormones of both fetal and maternal origin (Meisser *et al*., 1999; Bischof and Campana, 2000). Cell adhesion molecules on the surface of EVTs play an important role in the process of entry of EVTs into the maternal decidua (Damsky *et al*., 1993; Zhou *et al*., 1993; Irving *et al*., 1995). For the invasion of EVTs, cell–cell and/or cell-extracellular matrix (ECM) interactions mediated by adhesion molecules, such as cadherins and/or integrins, has been considered to be important (Burrows *et al*., 1996). These molecules provide the appropriate adhesion to enable traction and movement of the cells and/or impart signal between the ECM and the cell interior. When trophoblast cells acquire the invasive phenotype in the cell column, down-regulation of the integrin $\alpha_6\beta_4$ and up-regulation of integrin $\alpha_5\beta_1$ occurs (Damsky *et al*., 1992). In addition, these cell surface integrins modulate trophoblast motility (Damsky *et al*., 1994) by interactions with certain ECM components (Irving and Lala, 1995). For example, fibronectin (FN) is abundant where anchoring villi are formed *in vivo* and both trophoblast and maternal derived FN contribute to the ECM encountered by EVT as they infiltrate the decidua. Thus, FN acts as a bridging ligand mediating anchorage and/or migratory activity following the interaction with the cognate $\alpha_5\beta_1$ integrin receptor of EVTs (Ilic *et al*., 2001).

Invasion also involves degradation of ECM elements in the direction of migration and this requires the action of proteases, particularly the matrix metalloproteinases (MMP)2 and 9 (Shimonovitz *et al*., 1994; Staun-Ram *et al*., 2004), whose activity is physiologically regulated, at least in part, by tissue inhibitors of metalloproteinases (TIMP) which bind the MMPs with a 1:1 stoichiometry: thus the balance between MMPs and TIMPs locally at the invasive site is important.

Leukemia inhibitory factor (LIF) is a pleiotrophic cytokine and has known roles in implantation in mice (Bhatt *et al*., 1991; Shen and Leder, 1992; Stewart *et al*., 1992; Vogiagis and Salamonsen, 1999; Kimber, 2005) and has parallel functions in humans (Charnock-Jones *et al*., 1994; Kojima *et al*., 1994; Arici *et al*., 1995; Delage *et al*., 1995; Laird *et al*., 1997; Hambartsoumian, 1998). LIF signal transduction occurs via the JAK/STAT signal transduction pathway (Heinrich *et al*., 1998; White *et al*., 2007). LIF is highly expressed in first trimester decidua and the localization of its receptor on trophoblast cells suggests paracrine actions during placentation (Kojima *et al*., 1994; Sawai *et al*., 1995; Nachtigall *et al*., 1996; Ren *et al*., 1997; Sharkey *et al*., 1999; Kayisli *et al*., 2002; Chen *et al*., 2004). Strong expression of LIF mRNA has also been detected in decidual leukocytes, which are abundant at the implantation site, suggesting that LIF may mediate interactions between maternal decidual leukocytes and invading trophoblast cells (Sharkey *et al*., 1999). In this context, decreased production of LIF mRNA by decidual T cells is associated with unexplained recurrent abortions (Piccinni *et al*., 2000).

To determine whether LIF acts in a paracrine manner within the developing placenta to modulate the invasive capacity of trophoblast, we examined the effects of LIF on primary trophoblast cells derived from first trimester decidua, with a focus on their adhesive capacity, expression of adhesion molecules and production of MMPs and TIMPs.

Materials and Methods

Tissue collection

Normal first trimester placental tissue was obtained from healthy women of reproductive age who underwent elective termination of pregnancy (amenorrhea: 8–12 week, $n = 14$). The study was approved by the Southern Health Human Research and Ethics committee. Written informed consent was obtained from each patient before surgical intervention. Chorionic villi were washed four times in sterile saline and transferred to ice-cold medium 199 (M199) containing antibiotic–antimycotic (Invitrogen, Carlsbad, CA, USA) before transporting to the laboratory for further processing within 2 h.

EVT cell preparation

Primary EVT cells were prepared from chorionic villi as previously described (Irving *et al*., 1995). Briefly, terminal villi trees were excised, washed twice and resuspended in diluted 1:3 growth factor reduced (GFR) matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The villous tips were then transferred to flasks and maintained at 37°C coated with GFR matrigel for 20 min to allow the matrigel to set, followed by addition of Dulbecco's modified Eagle's medium (DMEM)/F12 and 10% fetal calf serum (FCS) (Invitrogen). Medium was changed every 3 days for 10–14 days to allow extensive outgrowth. Outgrowing cells were detached by digestion with 0.03% trypsin–EDTA (Invitrogen) in 0.9% phosphate-buffered saline (PBS) for 5 min at 37°C followed by neutralization of trypsin activity with 10% FCS/M199. The dispersed cells were separated from large tissue remnants using a 40 μm filter (BD Biosciences) and then placed in culture to allow expansion for another 2–3 weeks. Cell cultures were detached as above, incubated with anti-CD9 (Biosource International, Camarillo, CA, USA) and subjected to negative immunomagnetic separation using MiniMacs columns (Miltenyi Biotech, Bergisch-Gladbach, Germany) to eliminate CD9-positive placental fibroblast mesenchymal cells (Morrish *et al*., 1991; Yui *et al*., 1994).

The trophoblast cell fraction was washed three times and cells seeded at a density of 10^6 cells/ml in DMEM/F12 supplemented as above. To determine the purity of the immunopurified population of EVT cells, cells were seeded on GFR Matrigel-precoated eight-chamber culture slides, (Becton–Dickinson Labware). The human EVT-hybridoma cell-line AC1M88 (Briese *et al*., 2005; Hannan *et al*., 2006), to be used as a positive control, was seeded on uncoated culture slides. AC1M88 and EVT cells were incubated in DMEM/F12 medium (Sigma), supplemented with 10% FCS under standard conditions for 24–48 h. Cells were then washed three times with PBS, fixed for 5 min with ice-cold ethanol, dried and stored at −20°C until immunohistochemistry for cytokeratin (CK7) and HLA-G was performed. Only cell preparations in which >95% of the cells were positive for CK7 were used for subsequent experiments.

EVT cells were cultured in T25 flasks (Nunc) and grown to 60–70% confluency in DMEM/F12 containing 1% antibiotics (penicillin, streptomycin and fungizone, Commonwealth Serum Laboratories, Melbourne, VIC, Australia) and 10% FCS (complete medium). The cells were washed twice with PBS free of Ca^{2+} and Mg^{2+} (PBS-) and stepped down to DMEM/F12 supplemented with 0.2% FCS and 1% of antibiotics for 24 h. This medium was changed and LIF (50 ng/ml; AMRAD, Melbourne, Australia) or vehicle (DMEM/F12)

added. After a further 24 h cells were harvested, and conditioned medium stored as aliquots −20°C until assayed.

Immunocytochemistry

Immunocytochemistry for the trophoblast-specific CK7 and the EVT-specific marker HLA-G was performed on the cells using anti-human CK7 (1:100 dilution, clone OV-TL 12/30, DakoCytomation, Glostrup, Denmark) and anti-human HLA-G (1:200 dilution, clone 4H84, BD Pharmingen, San Diego, CA, USA), respectively. The human EVT-hybridoma cell-line ACI.88 described elsewhere (Hannan *et al*., 2006; Paiva *et al*., 2007) as used as a positive control for the detection of both antigens. For the negative control, non-immune mouse immunoglobulin (Ig)G (DakoCytomation) was substituted for primary antibody at the same protein concentration.

Fixed cells on culture slides were rehydrated and treated with 3% hydrogen peroxide in distilled water for 10 min to block endogenous peroxidase activity. Cells were then washed with tris buffered saline (TBS) and incubated with non-immune block for 30 min. Primary antibody was applied and incubated at 4°C for 16 h followed by biotinylated horse anti-mouse IgG (1:200) for 30 min, then streptavidin–biotin–peroxidase complex ABC (DakoCytomation) according to the manufacturer's instructions. Peroxidase activity was visualized by application of diaminobenzidine substrate (DakoCtyomation) for 3–5 min. Cells were counterstained with Harris haematoxylin (Sigma), air dried and mounted. Only cell preparations in which >95% of the cells were positive for CK7 were used for subsequent experiments.

RNA preparation and quantitative real-time RT–PCR

EVT cells were treated with or without LIF (50 ng/ml) for 24 h. EVT cell cultures were lysed with RLT buffer (QIAGEN, Germantown, MD, USA) and stored at −80°C until used. Total RNA was extracted from samples with the RNeasy Minikit (QIAGEN) according to the manufacturer's protocol. Genomic DNA contamination was removed by RNase-free DNase set (QIAGEN). RNA yield, purity and concentration were determined by spectrophotometry and 1 μg of each RNA was run on a 1% agarose gel (Roche, Castle Hill, NSW, Australia) to ensure RNA integrity.

Two micrograms of RNA was converted to complementary DNA (cDNA) by using Superscript III RNA polymerase, random primer, RNase inhibitor (RNaseOUT), deoxynucleoside triphosphates and First-Strand buffer (Invitrogen) according to the manufacturer's protocol. Real-time PCR analyses were performed on the Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) in triplicate (final reaction volume, 25 μl) in 96-well Micro Optical plates (Applied Biosystems). For each sample, 25 ng of cDNA was added to a PCR mix made with the five times Fast-Start SYBR green master mix containing Rox (Roche Diagnostics, Indianapolis, IN, USA) and 400 nM of primers. The sequences for the primers are shown in Table I. The PCR protocol was: 50°C for 2 min; 95°C for 10 min; and 40 cycles of 95°C for 15 s followed by 60°C for 1 min. Relative expression levels were calculated by the comparative cycle threshold method ($\Delta\Delta C_t$) as outlined in the manufacturer's user manual, with β-actin serving as the endogenous control for normalization. The entire real-time RT–PCR run was performed twice.

ECM and adhesion molecule quantitative PCR arrays

Screening for the expression of 83 genes associated with cell adhesion and ECM molecules in EVT cell cultures under control conditions ($n = 2$, triplicate arrays per EVT culture) was conducted using the Human ECM and Adhesion Molecules RT2 Profiler PCR array (SuperArray Bioscience Corp., Frederick, MD, USA) according to the manufacturer's instructions. In brief, cDNA was prepared from 1 μg total RNA by using a RT2 PCR array first strand kit. PCR amplification was conducted with an initial 10-min step at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescent SYBR Green signal was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. Data were imported into an Excel database and analysed using the comparative cycle threshold method with normalization of the raw data to β-actin.

Table I. Sequences for primers used in the quantitative analysis of mRNA by real-time RT–PCR.

Gene	Description	Primer sequences 5′-3′ (forward/reverse)
ACTB	β-Actin	AGCCTCGCCTTTGCCGA/ GCGCGGCGATATCATCATC
ITGA1	Integrin α_1 subunit	GAAAACGTGACCCATGAGTTCA/ GAAAACGTGACCCATGAGTTCA
ITGA5	Integrin α_5 subunit	GCCTGTGGAGTACAAGTCCTT/ AATTCGGGTGAAGTTATCTGTGG
ITGA6	Integrin α_6 subunit	GCTGGTTATAATCCTTCAATATCAATTGT/ TTGGGCTCAGAACCTTGGTTT
ITGB1	Integrin β_1 subunit	GTGGTTGCTGGAATTGTTCTTATT/ TTTTCCCTCATACTTCGGATTGAC
ITGB4	Integrin β_4 subunit	CTGTGTGCACGAGGGACATT/ AAGGCTGACTCGGTGGAGAA
ITGAV	Integrin α_V subunit	GAAAAGAATGACACGGTTGC/ AGTGATGAGATGGTCCCGCT
ITB3	Integrin β_3 subunit	ACTGCCTGTGACTCCGACT/ CGCGTGGTACAGTTGCAGTAG

Cell adhesion assays

EVT cells were treated with or without LIF (50 ng/ml) for 24 h. Cells were then washed with PBS-, dissociated in trypsin–EDTA, rinsed twice in PBS- and resuspended in DMEM. EVT cells were plated in triplicate at 2×10^4cells/well on 96-well FN, vitronectin (VN), laminin (LN) or bovine serum albumin-coated CytoMatrix cell adhesion strips (Chemicon International, Inc.) according to the manufacturer's instructions and incubated at 37°C for 1 h. Cells were rinsed three times with PBS containing calcium and magnesium and stained for 5 min with 0.2% crystal violet dissolved in 10% ethanol. After five washes with PBS, crystal violet was eluted with 0.1 M NaH_2PO_4 containing 50% ethanol for 15 min and the optical densities (OD) were measured at 570 nm.

Gelatin zymography for latent and active forms of MMP2 and MMP9

Both latent and active forms of MMP2 and MMP9 were analysed in culture medium by zymography on 10% sodium dodecyl sulphate (SDS)–polyacrylamide gels containing 1 mg/ml gelatine (all reagents from Bio-Rad, North Ryde, Australia) under non-reducing conditions. Culture medium from baby hamster kidney cells that had been stably transfected with human MMP9 (Edwards, University of East Anglia, Norwich, UK) was used for MMP2 and MMP9 standards as described previously (Salamonsen *et al*., 1997). MMP identity of all bands was confirmed by incubation of parallel gels in the presence of EDTA (5 mmol/l) and by activation of latent enzymes by incubation of the culture medium with 4-aminophenyl mercuric acid (APMA) for 3 h at 37°C prior to running on a gel. Gelatinase activity was visualized by negative staining and semi-quantiated by densitometric analysis of zymograms as previously described (Salamonsen *et al*., 1997). All samples for comparison were run on the same gel.

TIMP-1 and -2 enzyme-linked immunosorbent assay

TIMP-1 and -2 protein secreted from primary EVT cells was assayed by enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc.,

Minneapolis, MN, USA). The conditioned media from primary EVT cells were used in a 1:10 dilution to ensure that TIMP levels fell within the range of the standards in the assays. The lower detection limit of the assay was 0.08 ng/ml, and inter- and intra-assay variability were <7 and <5%, respectively.

Western blot analysis for signal transducer and activator of transcription 3 (STAT3) protein and phosphorylated STAT3 in primary EVT cells

Confluent EVT cells were either untreated or treated with LIF (50 ng/ml) with or without a newly described superpotent LIF antagonist (LA) (20 nM, White *et al*., 2007) for 15 min. The medium was aspirated, and cells washed with ice-cold sterile PBS, twice on ice. Cells were lysed and scraped in ice-cold lysis buffer containing 50 mM Tris base, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerolphosphate (pH 7.5) and 2 μl/well protease inhibitors cocktail set III [4-(2-aminoethyl)benzenesulfonyl fluoride, 100 mM HCl, 80 μM aprotinin, 5 μM bestatin, 1.5 mm E-64, 2 mM leupeptin hemisulfate and 1 mm pepstatin A (Calbiochem, San Diego, CA, USA)]. Cell extracts were then centrifuged and supernatant protein was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of total protein were then resolved by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. All membranes were incubated with Ponceau S (Sigma) to ensure equal protein loading in all lanes (results not shown). The membranes were blocked with 5% non-fat dry milk in TBS, washed and probed separately with antibodies specific for phosphorylated STAT3 (Tyr705; Cell Signaling Technology Inc., Beverly, MA, USA) (1:1000) or total STAT3 (Cell Signaling Technology) (1:1000). The membranes were washed and then incubated with horse-radish peroxidase (HRP)-conjugated rabbit secondary antibody (DakoCytomation, Glostrup, Denmark) (1:1500). Finally, the HRP activity was detected using enhanced chemiluminescence reagent (Pierce). The membranes were exposed to autoradiographic film (Amersham Biosciences, Little Chalfont, UK) with the exposure time adjusted to keep the integrated OD within a linear and non-saturated range.

Statistical analyses

Data are expressed as mean ± SEM fold change for each treatment compared to control. For each independent experiment treatment, values were expressed as a percentage of controls and statistical analysis was then carried out on the data from different experiments. Statistical analysis was performed using the Mann–Whitney test when comparing more than two groups or Student's *t*-test when comparing two groups ($P < 0.05$ was taken as significant) using PRISM version 3.00 for Windows (GraphPad).

Results

Characterization of EVT cells

Immunocytochemical analyses of the cells cultured on GFR-matrigel coated slides showed that CK7 and HLA-G were present in >80% of the attached cells (Fig. 1A and B, respectively). The choriocarcinoma-fusion cell line AC1M88 was used as a positive control for both antigens (Fig. 1D and E). Replacement of primary antibodies with non-immune IgG resulted in a lack of immunostaining (Fig. 1C and F).

Effect of LIF on EVT adhesion

To investigate whether LIF had an effect on the adhesion of trophoblast to different ECM elements, trophoblast cells were incubated on FN, LN or VN coated wells. In the unstimulated state, trophoblast cells adhered more to FN compared to VN and LN (Fig. 2A). When trophoblast cells were treated with 50 ng/ml of LIF for 24 h, there was a significant increase of

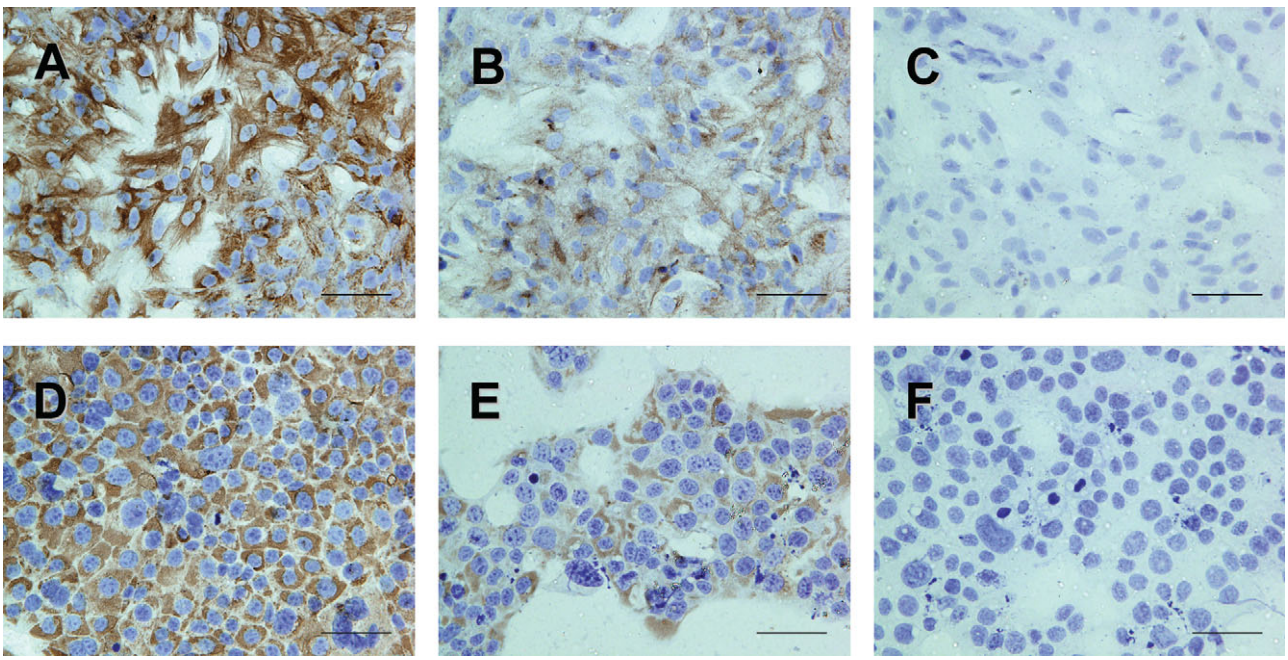

Figure 1: Immunocytochemical staining of cytokeratin (CK)7 and HLA-G in primary extravillous trophoblast (EVT).
(**A**–**C**) Primary EVT cells. (A) CK7 staining. (**B**) HLA-G staining. (C) Negative control for **B**. (D-F) AC1M88 cells (human EVT-hybridoma) (**D**) CK7 staining. (**E**) HLA-G staining. (**F**) Negative control for D. Scale bar = 50 μm. Positive staining is brown.

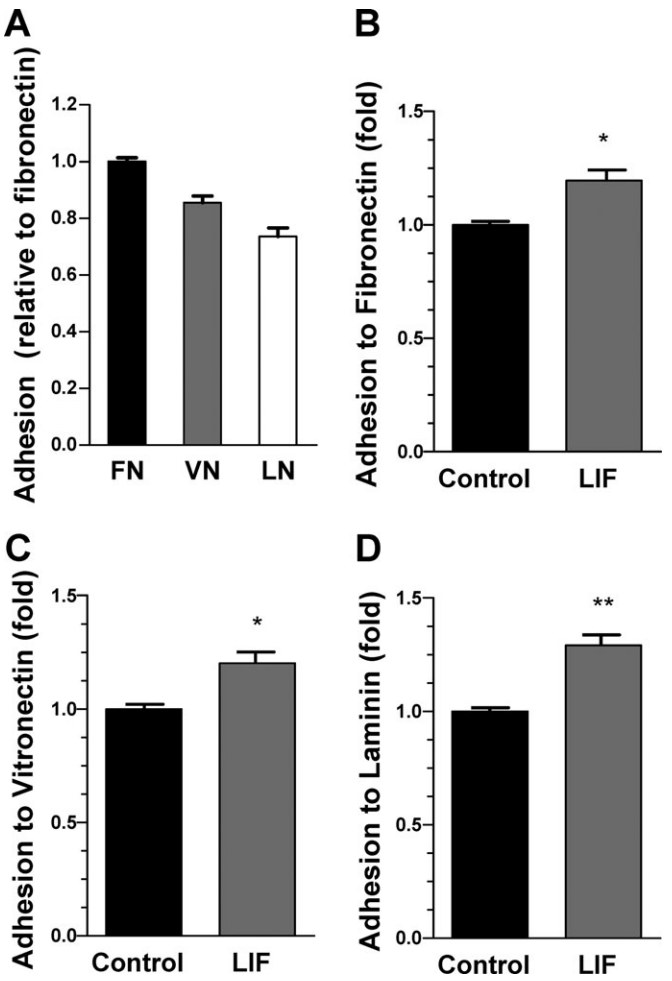

Figure 2: Effect of leukemia inhibitory factor (LIF) on adhesion of primary EVT cells to fibronectin (FN), vitronectin (VN) and laminin (LN).
(**A**) Adhesion profile of EVT cells to FN, VN and LN under unstimulated conditions. Data show two separate experiments assayed in triplicate. (**B**–**D**) EVT cells were cultured with/without LIF (50 ng/ml) for 24 h and the adhesion to FN (B), VN (**C**) and LN (D) screened. Data represented as mean ± SEM. Data show three independent experiments. Each sample was assayed in triplicate. $^{*}P < 0.005$, $^{**}P < 0.001$ compared to control.

binding to FN, VN and LN of 20, 20 and 29%, respectively ($P < 0.05$ for FN and VN and $P < 0.001$ for LN, Mann–Whitney test; Fig. 2B–D).

Expression profile of adhesion molecules and ECM elements in EVT cell cultures using PCR array

Since EVT cell cultures increased their adhesion to FN, LN and VN upon treatment with LIF we reasoned that such changes could be exerted via transcriptional regulation of those integrin subunits that mediate the cell adhesion to these ECM elements. As a first step, we investigated whether the ECM and adhesion molecule transcriptional expression profile of cultured EVT cells was similar to that of EVTs *in vivo* by a PCR array. The expression of each screened gene is shown corrected for β-actin in Table II. Signals were detected for 98.2% of the genes screened in EVT cells under unstimulated culture conditions. Genes were ranked from highest to lowest expression

Table II. Extracellular matrix and adhesion molecule mRNA expression in primary human EVT cells studied using PCR array.

Symbol	UniGene	RefSeq	Mean C C_t
COL1A1	Hs.172928	NM_000088	1.8
FN1	Hs.203717	NM_002026	2.9×10^{-1}
THBS1	Hs.164226	NM_003246	1.4
COL6A2	Hs.420269	NM_001849	9.0
COL5A1	Hs.210283	NM_000093	8.2
TIMP1	Hs.522632	NM_003254	8.1×10^{-2}
TGFBI	Hs.369397	NM_000358	7.3
MMP2	Hs.513617	NM_004530	6.8
ITGB1	Hs.429052	NM_002211	6.8
CTGF	Hs.410037	NM_001901	6.6
TIMP2	Hs.104839	NM_003255	5.1
COL4A2	Hs.508716	NM_001846	5.1
SPARC	Hs.111779	NM_003118	4.2
COL12A1	Hs.101302	NM_004370	2.5
CTNNB1	Hs.476018	NM_001904	2.3
CSPG2	Hs.443681	NM_004385	1.6
CTNNA1	Hs.445981	NM_001903	1.6
TNC	Hs.143250	NM_002160	1.5
THBS2	Hs.371147	NM_003247	9.1×10^{-3}
LAMB1	Hs.489646	NM_002291	8.6
MMP1	Hs.83169	NM_002421	8.0
ITGA3	Hs.265829	NM_002204	7.1
ITGB5	Hs.13155	NM_002213	4.3
ITGAV	Hs.436873	NM_002210	4.0
COL8A1	Hs.134830	NM_001850	3.7
ADAMTS1	Hs.534115	NM_006988	2.7
KAL1	Hs.521869	NM_000216	2.5
COL16A1	Hs.368921	NM_001856	2.4
ECM1	Hs.81071	NM_004425	2.2
ITGA2	Hs.482077	NM_002203	2.2
MMP16	Hs.546267	NM_005941	1.8
SPG7	Hs.185597	NM_003119	1.8
ITGA1	Hs.519304	NM_181501	1.2
ICAM1	Hs.515126	NM_000201	1.0
VCAM1	Hs.109225	NM_001078	9.2×10^{-4}
LAMC1	Hs.497039	NM_002293	8.7
SGCE	Hs.371199	NM_003919	6.6
ITGA4	Hs.553495	NM_000885	6.0
SPP1	Hs.313	NM_000582	5.8
THBS3	Hs.169875	NM_007112	5.0
ITGB3	Hs.218040	NM_000212	4.7
MMP13	Hs.2936	NM_002427	4.0
ITGA6	Hs.133397	NM_000210	3.9
COL6A1	Hs.474053	NM_001848	3.3
COL11A1	Hs.523446	NM_080629	2.7
MMP15	Hs.80343	NM_002428	2.5
MMP9	Hs.297413	NM_004994	2.0
NCAM1	Hs.503878	NM_000615	1.2
MMP11	Hs.143751	NM_005940	1.0
CTNND1	Hs.166011	NM_001331	7.7×10^{-5}
ADAMTS13	Hs.131433	NM_139028	6.7
ITGA5	Hs.505654	NM_002205	6.6
COL14A1	Hs.409662	NM_021110	6.6
COL15A1	Hs.409034	NM_001855	5.7
LAMA2	Hs.200841	NM_000426	3.8
CNTN1	Hs.567249	NM_001843	2.9
ITGA7	Hs.524484	NM_002206	2.9
ITGB4	Hs.370255	NM_000213	2.8
ITGA8	Hs.171311	NM_003638	2.4
HAS1	Hs.57697	NM_001523	1.6
CLEC3B	Hs.476092	NM_003278	1.4
LAMA3	Hs.436367	NM_000227	1.2
MMP3	Hs.375129	NM_002422	1.1
MMP8	Hs.534479	NM_002424	1.0
MMP10	Hs.2258	NM_002425	6.2×10^{-6}
MMP14	Hs.2399	NM_004995	4.1
PECAM1	Hs.514412	NM_000442	4.0
CDH1	Hs.461086	NM_004360	3.8
LAMB3	Hs.497636	NM_000228	3.5

Continued

Table II. *Continued*

Symbol	UniGene	RefSeq	Mean C C_t
CTNND2	Hs.314543	NM_001332	2.4
MMP7	Hs.2256	NM_002423	2.2
ADAMTS8	Hs.271605	NM_007037	7.6×10^{-7}
TIMP3	Hs.297324	NM_000362	6.6
SELE	Hs.89546	NM_000450	4.5
MMP12	Hs.1695	NM_002426	3.8
ITGAL	Hs.174103	NM_002209	1.9
ITGAM	Hs.172631	NM_000632	1.2
COL7A1	Hs.476218	NM_000094	undetectable
ITGB2	Hs.375957	NM_000211	undetectable
LAMA1	Hs.270364	NM_005559	undetectable
SELL	Hs.82848	NM_000655	undetectable
SELP	Hs.73800	NM_003005	undetectable
VTN	Hs.2257	NM_000638	undetectable

Ranked gene expression in human extravillous trophoblast cells. Data is presented as the mean of the threshold cycle values for each transcript corrected to β-actin (mean C C_t).

levels. The protease inhibitors were some of the most highly expressed genes in the EVT cells. The FN gene was also highly expressed. Interestingly, VN was not detectable. Integrin β_1 showed the highest mRNA expression compared to all other integrins screened. Integrins α_1, α_6 mRNA showed moderate expression, while integrins α_5 and β_4 showed low levels of gene expression.

Quantitative analysis of mRNA for integrin subunits in EVT

We investigates whether LIF regulated EVT integrin mRNA expression in EVTs. Real-time RT–PCR was performed on EVT cell cultures untreated and treated with LIF for those integrin subunits listed in Table I. A mean $\Delta\Delta C_t$ value of >1 reflects increased and a value of <1 points to a decreased gene expression compared to control. The calculated ratios of corrected transcript levels between treated and control cells showed a change only for integrin β_4 whose expression level decreased approximately 50% on treatment with LIF ($P < 0.05$, Fig. 3).

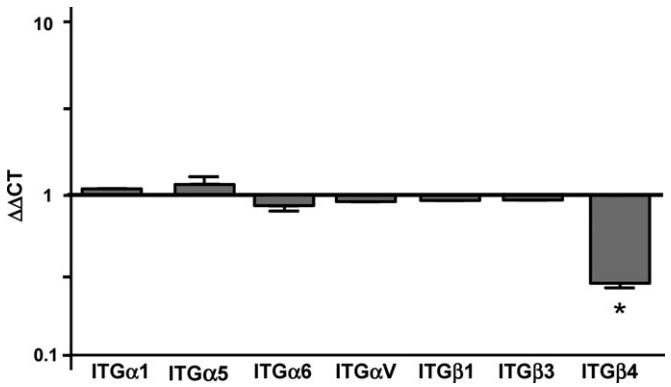

Figure 3: Effect of leukemia inhibitory factor (LIF) on integrin mRNA expression in primary EVT cells.
EVT cells were treated with LIF (50 ng/ml for 24 h). Real-time RT–PCR data for integrin (ITG) α_1, α_5, α_6, α_V, β_1, β_3, β_4 are presented as ratios obtained by the comparative cycle threshold method ($\Delta\Delta C_t$) method, corrected to β-actin. Data represented as mean ± SEM. Data show three independent experiments. Each sample was assayed in triplicate. *$P < 0.001$ compared to control.

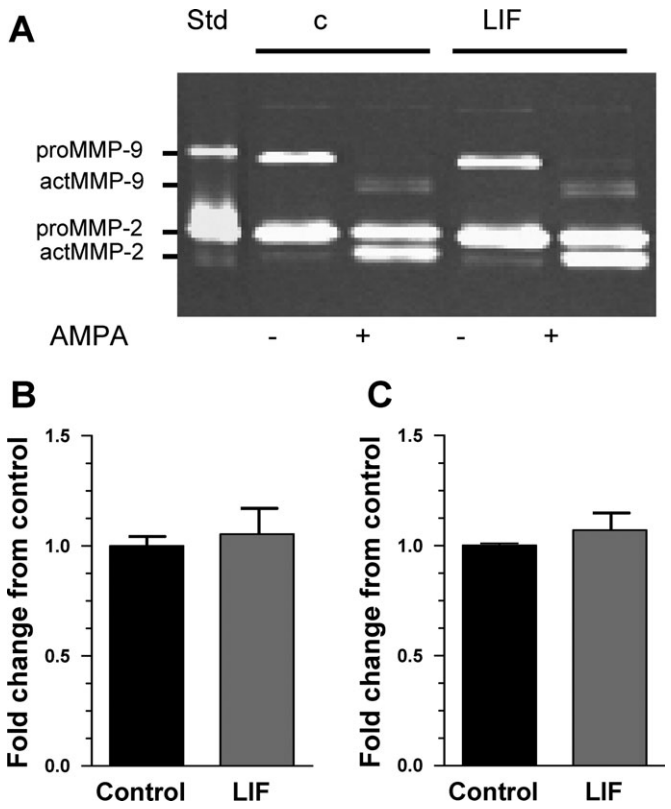

Figure 4: Effect of leukemia inhibitory factor (LIF) on matrix metalloproteinase (MMP)2 and MMP9 secretion from primary EVT cells. Cells were treated with/without LIF (LIF, 50 ng/ml) for 24 h. Cells were treated with aminomercuric acid (APMA) for 3 h at 37°C to activate MMP2 and MMP9. Standard (Std) from conditioned culture medium of BHK9 breast carcinoma cells. (**A**) Representative gelatin zymogram of three separate experiments illustrating pro (or latent) and active (act)MMP2 and MMP9. (**B**) Densitometric analysis of proMMP2. (**C**) Densitometric analysis of proMMP9. (B and C) Data represented as mean ± SEM. Data show three independent experiments. Each sample was assayed in duplicate.

Effect of LIF on MMP2 and MMP9 mRNA expression and enzyme production in EVT

The profile of MMP2 and MMP9 secreted from EVT cells treated and untreated with LIF was examined by gelatine zymography. Unstimulated EVT cells secreted both MMP2 and MMP9 in their latent forms and in some cultures a weak band corresponding to active MMP2 was found. No active MMP9 was detected. However, the active forms of both MMP2 and MMP9 could be induced by APMA treatment *in vitro* (Fig. 4A). There were no significant differences in MMP secretion following LIF treatment of EVT cells as shown by densitometric analyses of zymograms (Fig. 4B and C).

Effect of LIF on TIMP-1 and -2 secretion from EVT

Since the activities of MMP2 and MMP9 after activation are regulated by TIMPs, we analysed the levels of TIMP-1 and TIMP-2 protein in culture medium from EVT cells, without and following LIF treatment, by ELISA. Both TIMPs were significantly increased following treatment of EVT cells with LIF (Fig. 5, $P < 0.05$). Real-time RT–PCR measurement of TIMP-1 and -2 mRNAs was also carried out in the treated

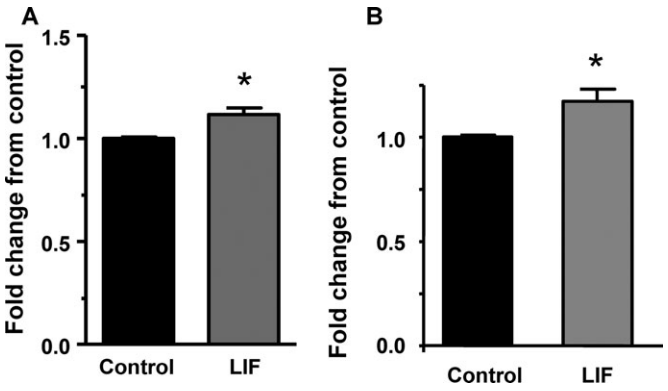

Figure 5: Effect of leukemia inhibitory factor (LIF) on tissue inhibitors of matrix metalloproteinase (TIMP)-1 and -2 protein secretion from primary EVT cells into culture medium.
Cells were treated with/without LIF (LIF, 50 ng/ml) for 24 h. (**A**) TIMP-1 secretion. (**B**) TIMP-2 secretion. Data represented as mean ± SEM. Data show three independent experiments. Each sample was assayed in duplicate. $*P < 0.001$ compared to control.

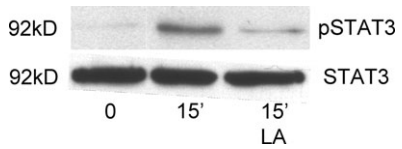

Figure 6: Leukemia inhibitory factor (LIF) induced phosphorylation in EVT cells.
EVT cells were cultured with LIF (50 ng/ml) for 15 min, and LIF plus superpotent LIF antagonist (LA, 20 mM) for 15 min. Cell lysates were electrophoresed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotted with anti-p(Tyr705)STAT3 (phosphorylated signal transducer and activator of transcription, top) or anti-STAT3 (bottom) followed by horse-radish peroxidase-conjugated rabbit antiserum and chemiluminescence visualization. The data shown is from a single experiment but is representative of two independent experiments.

and untreated EVT but these were not significantly altered by LIF treatment (data not shown).

Effect of LIF on STAT3 phosphorylation in EVT

To determine the specificity and response of LIF action in EVT cells we examined the effect of blocking LIF action with a newly described LA, on LIF induced STAT3 phosphorylation. The effect of LIF on phosphorylation of STAT3 and STAT3 in EVT was examined over a 15-min time period by Western blot (Fig. 6). Addition of LIF to EVT cells stimulated phosphorylation of STAT3 at 15 min while co-culture of cells with LIF and a superpotent LA diminished the effect, compared with cells treated with LIF alone for 15 min (Fig. 6, top). STAT3 protein abundance was not affected at any time point when LIF was added to EVT (Fig. 6, bottom).

Discussion

This study examined effects of LIF on human EVT cells and demonstrated that LIF stimulated their adhesion to ECM elements, decreased integrin β_4 mRNA expression and stimulated production of TIMP-1 and -2, suggesting a role for LIF during human placental development. The effect of LIF on the invasive potential of human EVT cells has been controversial. LIF has been reported to increase fetal FN secretion and decrease gelatinase activity by first-trimester trophoblast cells, suggesting an overall inhibitory effect on trophoblast invasion (Bischof *et al*., 1995; Nachtigall *et al*., 1996). However, another report showed that LIF increased the invasion of first trimester EVT cells *in vitro* (Poehlmann *et al*., 2005).

EVT cell invasion through the maternal decidua involves the adhesion, migration and invasion of EVT. EVT cell invasion is regulated by locally derived factors as well as by interactions of EVT with certain ECM components through integrins expressed in the cell surface (Irving and Lala, 1995). In the first trimester of pregnancy trophoblast invasion is tightly controlled. Taking a functional approach, we investigated the effect of LIF stimulation on EVT cell adhesion to some components of the ECM. We found that LIF increased the adhesion of EVT cells, a result that is relevant in the context of cell migration. Pregnancies complicated by pre-eclampsia or intrauterine growth retardation are characterized by shallow trophoblast invasion, and may result from failure to express the correct spectrum of integrins for trophoblast invasion (Nachtigall *et al*., 1996). Integrins change in expression as trophoblast differentiates from the villous to extravillous phenotype. Cytotrophoblast progression through the cell column and differentiation into the invasive phenotype is normally characterized by down-regulation of the integrin $\alpha_6\beta_4$, which may be a receptor of LN (Damsky *et al*., 1992; Aplin, 1997). Conversely, cells in the column up-regulate their production of the major FN receptor, α_5/β_1 (Damsky *et al*., 1992). The profile of integrin mRNA expressed by the EVT cells in our study suggest that they are of the invasive phenotype. Integrin β_1 mRNA was maximally expressed, while integrin α_5 mRNA was minimally expressed. Integrins α_6 and β_4 mRNA were moderately to minimally expressed relative to the other integrins screened.

The adhesion profile on untreated resting EVT cell cultures in the present study was FN > VN > LN, similar to the profile previously demonstrated for HTR8/SVneo trophoblast cells that closely resemble (Spessotto *et al*., 2006) the phenotype of EVT cells. To identify relative levels of ECM and adhesion molecule mRNA expression in EVT cells we used real-time RT–PCR ECM and adhesion molecule specific arrays. We found that all the major adhesion molecules were expressed by EVTs.

Importantly, when EVT cells were treated with LIF we found an increase in adhesion of the cells to all three matrices with the greatest increase in adhesion to LN. Since cytotrophoblast progression through the cell column is associated with a downregulation of the integrin $\alpha_6\beta_4$ (a likely LN receptor), our demonstration of a reduction in expression of the β_4 subunit, is consistent with a role for LIF in the differentiation of trophoblast cells to the invasive phenotype. The lack of effect of LIF on other integrin subunits in our study supports previous findings using fluorescence-activated cell sorter analysis, which showed no effect of LIF on the expression of the integrin subunits α_1, α_5 and β_1 by EVT cells (Sharkey *et al*., 1999).

The increase in cell adhesion to ECM elements without changes in the expression levels of the respective integrins subunits is interesting. In this regard, it has been shown that EVT

integrin expression can be regulated by a variety factors including chemokines (Luo *et al*., 2007). Thus, it is likely that *in vivo*, a variety of factors, including LIF, act in concert to regulate EVT integrins.

Paradoxically, the integrin β_4 subunit, that is regulated by LIF, dimerizes with the integrin α_6 subunit to form the possible LN receptor. At the same time, the highest increase in EVT cell adhesion upon LIF treatment was to LN. Cells control adhesion via integrins by regulating the amount of integrins expressed on the cell surface and also by modulating the affinity of integrins to their substrates. It is possible that LIF does not regulate integrin $\alpha_6\beta_4$ cell surface protein production. It is also likely that factors other than LIF affect the affinity of integrin $\alpha_6\beta_4$ on trophoblast cells to LN. In addition, integrin $\alpha_6\beta_4$ also potentially binds other ECM components suggesting that EVT cells bind to LN via mechanisms other than via integrin $\alpha_6\beta_4$.

MMP2 and MMP9 have been implicated in trophoblast invasion and migration (Campbell *et al*., 2003; Nardo *et al*., 2003) and in one study LIF decreased the overall gelatinolytic activity of trophoblast cells (Bischof *et al*., 1995). We also showed that secretion of latent and active forms of MMP2 and MMP9 by first trimester human EVT cells were not affected by LIF stimulation although LIF increased both TIMP-1 and -2 secretion. Importantly, secreted MMP2 and MMP9 were predominantly in latent forms, suggesting that an important level of regulation for their activity is by extracellular activation, probably by factors of maternal origin. The stimulation of TIMP secretion by LIF would provide further modulation of MMP action within the microenvironment of the EVT. This supports the previous report that gelatinase activity is inhibited in EVT cell cultures treated with LIF (Bischof *et al*., 1995). We also verified that LIF was active in the EVT cells since co-culture of the EVT cells with LIF and LA reduced LIF-induced STAT3 activation and suggests that LIF regulated EVT function via pSTAT3.

Our findings support a role for LIF on adhesion and differentiation of trophoblast cells to the invasive phenotype, both of which are involved in trophoblast invasion: a process that if not finely regulated can result in abnormal placentation. Indeed, an abnormally persistent expression of LIF receptor on EVT cells in placental bed biopsies has been demonstrated in patients with early onset pre-eclampsia combined with intra-uterine growth reduction (Reister *et al*., 2006).

In conclusion, our data showed that LIF stimulated primary first trimester EVT cell adhesion to LN, VN and FN along with reduced expression of the integrin β_4 subunit. Furthermore, LIF altered the balance between MMPs and TIMPs by increasing the secretion of TIMP-1 and -2 without alteration of MMP2 or MMP9 secretion. Overall, these changes suggest that LIF stimulates the differentiation of EVT cells toward the invasive phenotype. The study suggests that LIF has an important role in early placentation in women.

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References

Aboagye-Mathiesen G, Laugesen J, Zdravkovic M, Ebbesen P. Isolation and characterization of human placental trophoblast subpopulations from first-trimester chorionic villi. *Clin Diagn Lab Immunol* 1996;**3**:14–22.

Aplin JD. Adhesion molecules in implantation. *Rev Reprod* 1997;**2**:84–93.

Arici A, Engin O, Attar E, Olive DL. Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in human endometrium. *J Clin Endocrinol Metab* 1995;**80**:1908–1915.

Ball E, Bulmer JN, Ayis S, Lyall F, Robson SC. Late sporadic miscarriage is associated with abnormalities in spiral artery transformation and trophoblast invasion. *J Pathol* 2006;**208**:535–542.

Bhatt H, Brunet LJ, Stewart CL. Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proc Natl Acad Sci USA* 1991;**88**:11408–11412.

Bischof P, Campana A. A putative role for oncogenes in trophoblast invasion? *Hum Reprod* 2000;**15**(Suppl 6):51–58.

Bischof P, Haenggeli L, Campana A. Effect of leukemia inhibitory factor on human cytotrophoblast differentiation along the invasive pathway. *Am J Reprod Immunol* 1995;**34**:225–230.

Briese J, Oberndorfer M, Patschenik C, Schulte HM, Makrigiannakis A, Loning T, Bamberger AM. Osteopontin is colocalized with the adhesion molecule CEACAM1 in the extravillous trophoblast of the human placenta and enhances invasion of CEACAM1-expressing placental cells. *J Clin Endocrinol Metab* 2005;**90**:5407–5413.

Burrows TD, King A, Loke YW. Trophoblast migration during human placental implantation. *Hum Reprod Update* 1996;**2**:307–321.

Campbell S, Rowe J, Jackson CJ, Gallery ED. In vitro migration of cytotrophoblasts through a decidual endothelial cell monolayer: the role of matrix metalloproteinases. *Placenta* 2003;**24**:306–315.

Charnock-Jones DS, Sharkey AM, Fenwick P, Smith SK. Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *J Reprod Fertil* 1994;**101**:421–426.

Chen HF, Chao KH, Shew JY, Yang YS, Ho HN. Expression of leukemia inhibitory factor and its receptor is not altered in the decidua and chorionic villi of human anembryonic pregnancy. *Hum Reprod* 2004;**19**:1647–1654.

Damsky CH, Fitzgerald ML, Fisher SJ. Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. *J Clin Invest* 1992;**89**:210–222.

Damsky C, Sutherland A, Fisher S. Extracellular matrix 5: adhesive interactions in early mammalian embryogenesis, implantation, and placentation. *FASEB J* 1993;**7**:1320–1329.

Damsky CH, Librach C, Lim KH, Fitzgerald ML, McMaster MT, Janatpour M, Zhou Y, Logan SK, Fisher SJ. Integrin switching regulates normal trophoblast invasion. *Development* 1994;**120**:3657–3666.

Delage G, Moreau JF, Taupin JL, Freitas S, Hambartsoumian E, Olivennes F, Fanchin R, Letur-Konirsch H, Frydman R, Chaouat G. In-vitro endometrial secretion of human interleukin for DA cells/leukaemia inhibitory factor by explant cultures from fertile and infertile women. *Hum Reprod* 1995;**10**:2483–2488.

Genbacev O, Schubach SA, Miller RK. Villous culture of first trimester human placenta–model to study extravillous trophoblast (EVT) differentiation. *Placenta* 1992;**13**:439–461.

Hambartsoumian E. Endometrial leukemia inhibitory factor (LIF) as a possible cause of unexplained infertility and multiple failures of implantation. *Am J Reprod Immunol* 1998;**39**:137–143.

Hannan NJ, Jones RL, White CA, Salamonsen LA. The chemokines, CX3CL1, CCL14, and CCL4, promote human trophoblast migration at the feto-maternal interface. *Biol Reprod* 2006;**74**:896–904.

Heinrich PC, Behrmann I, Muller-Newman G, Schaper F, Graeve L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J* 1998;**334**:297–314.

Hustin J, Jauniaux E, Schaaps JP. Histological study of the materno-embryonic interface in spontaneous abortion. *Placenta* 1990;**11**:477–486.

Ilic D, Genbacev O, Jin F, Caceres E, Almeida EA, Bellingard-Dubouchaud V, Schaefer EM, Damsky CH, Fisher SJ. Plasma membrane-associated pY397FAK is a marker of cytotrophoblast invasion in vivo and in vitro. *Am J Pathol* 2001;**159**:93–108.

Irving JA, Lala PK. Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-beta, IGF-II, and IGFBP-1. *Exp Cell Res* 1995;**217**:419–427.

Irving JA, Lysiak JJ, Graham CH, Hearn S, Han VK, Lala PK. Characteristics of trophoblast cells migrating from first trimester chorionic villus explants and propagated in culture. *Placenta* 1995;**16**:413–433.

Kadyrov M, Kingdom JC, Huppertz B. Divergent trophoblast invasion and apoptosis in placental bed spiral arteries from pregnancies complicated by maternal anemia and early-onset preeclampsia/intrauterine growth restriction. *Am J Obstet Gynecol* 2006;**194**:557–563.

Kaufmann P, Castellucci M. Extravillous trophoblast in the placenta. *Trophoblast Res* 1997;**10**:21–65.

Kayisli UA, Selam B, Demir R, Arici A. Expression of vasodilator-stimulated phosphoprotein in human placenta: possible implications in trophoblast invasion. *Mol Hum Reprod* 2002;**8**:88–94.

Kimber SJ. Leukaemia inhibitory factor in implantation and uterine biology. *Reproduction* 2005;**130**:131–145.

Kojima K, Kanzaki H, Iwai M, Hatayama H, Fujimoto M, Inoue T, Horie K, Nakayama H, Fujita J, Mori T. Expression of leukemia inhibitory factor in human endometrium and placenta. *Biol Reprod* 1994;**50**:882–887.

Laird SM, Tuckerman EM, Dalton CF, Dunphy BC, Li TC, Zhang X. The production of leukaemia inhibitory factor by human endometrium: presence in uterine flushings and production by cells in culture. *Hum Reprod* 1997;**12**:569–574.

Luo BH, Carman CV, Springer TA. Structural basis of integrin regulation and signaling. *Annu Rev Immunol* 2007;**25**:619–647.

Meisser A, Cameo P, Islami D, Campana A, Bischof P. Effects of interleukin-6 (IL-6) on cytotrophoblastic cells. *Mol Hum Reprod* 1999;**5**:1055–1058.

Morrish DW, Shaw AR, Seehafer J, Bhardwaj D, Paras MT. Preparation of fibroblast-free cytotrophoblast cultures utilizing differential expression of the CD9 antigen. *In Vitro Cell Dev Biol* 1991;**27A**:303–306.

Nachtigall MJ, Kliman HJ, Feinberg RF, Olive DL, Engin O, Arici A. The effect of leukemia inhibitory factor (LIF) on trophoblast differentiation: a potential role in human implantation. *J Clin Endocrinol Metab* 1996;**81**:801–806.

Nardo LG, Nikas G, Makrigiannakis A. Molecules in blastocyst implantation. Role of matrix metalloproteinases, cytokines and growth factors. *J Reprod Med* 2003;**48**:137–147.

Paiva P, Salamonsen LA, Manuelpillai U, Walker C, Tapia A, Wallace EM, Dimitriadis E. Interleukin-11 promotes migration, but not proliferation, of human trophoblast cells, implying a role in placentation. *Endocrinol* 2007;**148**:5566–5572.

Piccinni MP, Maggi E, Romagnani S. Role of hormone-controlled T-cell cytokines in the maintenance of pregnancy. *Biochem Soc Trans* 2000;**28**:212–215.

Poehlmann TG, Fitzgerald JS, Meissner A, Wengenmayer T, Schleussner E, Friedrich K, Markert UR. Trophoblast invasion: tuning through LIF, signalling via Stat3. *Placenta* 2005;**26**(Suppl A):S37–S41.

Reister F, Kingdom JC, Ruck P, Marzusch K, Heyl W, Pauer U, Kaufmann P, Rath W, Huppertz B. Altered protease expression by periarterial trophoblast cells in severe early-onset preeclampsia with IUGR. *J Perinat Med* 2006;**34**:272–279.

Ren SG, Melmed S, Braunstein GD. Decidual leukemia inhibitory factor production and action on human chorionic gonadotropin secretion at different stages of gestation in vitro. *Early Pregnancy* 1997;**3**:102–108.

Salamonsen LA, Butt AR, Hammond FR, Garcia S, Zhang J. Production of endometrial matrix metalloproteinases, but not their tissue inhibitors, is modulated by progesterone withdrawal in an in vitro model for menstruation. *J Clin Endocrinol Metab* 1997;**82**:1409–1415.

Sawai K, Matsuzaki N, Kameda T, Hashimoto K, Okada T, Shimoya K, Nobunaga T, Taga T, Kishimoto T, Saji F. Leukemia inhibitory factor produced at the fetomaternal interface stimulates chorionic gonadotropin production: its possible implication during pregnancy, including implantation period. *J Clin Endocrinol Metab* 1995;**80**:1449–1456.

Sharkey AM, King A, Clark DE, Burrows TD, Jokhi PP, Charnock-Jones DS, Loke YW, Smith SK. Localization of leukemia inhibitory factor and its receptor in human placenta throughout pregnancy. *Biol Reprod* 1999;**60**:355–364.

Shen MM, Leder P. Leukemia inhibitory factor is expressed by the preimplantation uterus and selectively blocks primitive ectoderm formation in vitro. *Proc Natl Acad Sci USA* 1992;**89**:8240–8244.

Shimonovitz S, Hurwitz A, Dushnik M, Anteby E, Geva-Eldar T, Yagel S. Developmental regulation of the expression of 72 and 92 kd type IV collagenases in human trophoblasts: a possible mechanism for control of trophoblast invasion. *Am J Obstet Gynecol* 1994;**171**:832–838.

Spessotto P, Bulla R, Danussi C, Radillo O, Cervi M, Monami G, Bossi F, Tedesco F, Doliana R, Colombatti A. EMILIN1 represents a major stromal element determining human trophoblast invasion of the uterine wall. *J Cell Sci* 2006;**119**:4574–4584.

Staun-Ram E, Goldman S, Gabarin D, Shalev E. Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. *Reprod Biol Endocrinol* 2004;**2**:59.

Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, Abbondanzo SJ. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* 1992;**359**:76–79.

Vogiagis D, Salamonsen LA. Review: the role of leukaemia inhibitory factor in the establishment of pregnancy. *J Endocrinol* 1999;**160**:181–190.

White CA, Zhang JG, Salamonsen LA, Baca M, Fairlie WD, Metcalf D, Nicola NA, Robb L, Dimitriadis E. Blocking LIF action in the uterus by using a PEGylated antagonist prevents implantation: a nonhormonal contraceptive strategy. *Proc Natl Acad Sci USA* 2007;**104**:19357–19362.

Yui J, Garcia-Lloret M, Brown AJ, Berdan RC, Morrish DW, Wegmann TG, Guilbert LJ. Functional, long-term cultures of human term trophoblasts purified by column-elimination of CD9 expressing cells. *Placenta* 1994;**15**:231–246.

Zhou Y, Damsky CH, Chiu K, Roberts JM, Fisher SJ. Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts. *J Clin Invest* 1993;**91**:950–960.

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