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 β4 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). EVT’s 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 αβ1 and up-regulation of integrin αβ3 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 αβ3 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; Nachitagall et al., 1996; Ren et al., 1997; Sharkey et al., 1999; Kayisl 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 (Piccinini 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 placent 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 10⁶ 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-hybridxoma cell-line AC1M88 (Briese et al., 2005; Hanann 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 Ca²⁺ and Mg²⁺ (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 (IgG) (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 anti-CK7 antibody at 1:500 overnight. The slides were then washed in TBS and incubated with non-immune block for 30 min. Primary antibody was applied and incubated at 4°C for 1 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 (DakoCytomation) 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 RNaseasy Minikit (QIAGEN) according to the manufacturer’s protocol. Genomic DNA contamination was removed by RNA-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 μg of cDNA was added to a PCR mix made with the five times Fast-Start DNA 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 (ΔΔCt) 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.

**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 × 10⁴ cells/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 NaH₂PO₄ containing 50% ethanol. TIMP-1 and -2 protein secreted from primary EVT cells was assayed by enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN). All samples for comparison were run on the same gel.

**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-quantitated 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). The entire real-time RT–PCR run was performed twice.

**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     | AGCCCTGCGCTTTGCGGA/GCGCGGGATCATCATC     |
| ITGA1 | Integrin α1 subunit | GAAAAACGTGACCCTAGTTC/TACCTAGCGGAA/GAAACCATTTGGAACCAATC |
| ITGA5 | Integrin α5 subunit | GCGCTTGAGGATCAAGTCTT/AAATCGGGTGAAATTACGTTGG |
| ITGA6 | Integrin α6 subunit | GCTGTTTATAATCTTCATATCATCAATTG/TTGGGTCAGAACCTTGTTT |
| ITGB1 | Integrin β1 subunit | GTGTGTTCTGGAATTTCTTATT/TTTCCCCCATACCTGCAGATTGAC |
| ITGB4 | Integrin β4 subunit | CTGTGTCGACAGGGACATT/AAAGGCTACTGGTGGGAA |
| ITGA7 | Integrin αv subunit | GAAAAGATTGACAGCAGTTC/GAGTATGAGATGTCCCGC |
| ITB3 | Integrin β3 subunit | ACTGCCCTGACCTGACCT/CGCCGTGACGTCGACCTG |

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. 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).
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-glycerol phosphate (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](https://academic.oup.com/humrep/article-abstract/23/8/1724/2913928)

**Figure 1:** Immunocytochemical staining of cytokeratin (CK7) 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.

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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

| Symbol     | UniGene | RefSeq   | Mean C Ct |
|------------|---------|----------|-----------|
| COL1A1     | Hs.172928 | NM_000088 | 1.8       |
| FN1        | Hs.203717 | NM_001266 | 9.4       |
| THBS1      | Hs.164226 | NM_000326 | 5.6       |
| COL6A2     | Hs.420269 | NM_001649 | 9.0       |
| COL5A1     | Hs.210283 | NM_000093 | 8.2       |
| TIMP1      | Hs.522632 | NM_003254 | 8.1 x 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_004570 | 2.5       |
| CTNNB1     | Hs.476018 | NM_001904 | 2.3       |
| CSPG2      | Hs.445681 | 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 x 10^-3 |
| LAM1       | Hs.498946 | NM_002291 | 8.6       |
| MMP1       | Hs.83169  | NM_004210 | 8.0       |
| ITGA3      | Hs.265829 | NM_002204 | 7.1       |
| ITGB5      | Hs.13155  | NM_002213 | 4.5       |
| ITGA6      | Hs.436873 | NM_002210 | 4.5       |
| COL6A1     | Hs.134830 | NM_001850 | 3.7       |
| ADAMTS1    | Hs.534115 | NM_006988 | 2.7       |
| KAL1       | Hs.521869 | NM_002026 | 2.5       |
| COL16A1    | Hs.369212 | 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_002021 | 1.0       |
| VCAM1      | Hs.109225 | NM_001078 | 9.2 x 10^-4 |
| LAMC1      | Hs.497039 | NM_002293 | 8.7       |
| SGCE       | Hs.371199 | NM_003919 | 6.6       |
| ITGA4      | Hs.553495 | NM_000885 | 6.0       |
| SPPI       | Hs.313    | NM_000582 | 5.8       |
| THBS3      | Hs.169785 | NM_007112 | 5.0       |
| ITGB3      | Hs.218040 | NM_002122 | 4.7       |
| MMP13      | Hs.20266  | NM_004271 | 4.0       |
| ITGA6      | Hs.133397 | NM_000120 | 3.9       |
| COL6A1     | Hs.474053 | NM_001848 | 3.3       |
| COL11A1    | Hs.523446 | NM_080629 | 3.7       |
| MMP15      | Hs.80343  | NM_004230 | 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 x 10^-5 |
| ADAMTS13   | Hs.131433 | NM_139028 | 6.7       |
| ITGA5      | Hs.505654 | NM_000205 | 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 | 1.3       |
| 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_000245 | 6.2 x 10^-6 |
| MMP14      | Hs.2399   | NM_004995 | 4.1       |
| PECAM1      | Hs.514412 | NM_004350 | 4.0       |
| CDH1       | Hs.461086 | NM_001430 | 3.8       |
| LAMB3      | Hs.497636 | NM_000228 | 3.5       |

Continued

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.
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 \( \beta_1 \) showed the highest mRNA expression compared to all other integrins screened. Integrins \( \alpha_1, \alpha_6 \) mRNA showed moderate expression, while integrins \( \alpha_5 \) and \( \beta_4 \) showed low levels of gene expression.

Quantitative analysis of mRNA for integrin subunits in EVT

We investigate 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 \( \beta_4 \) whose expression level decreased approximately 50% on treatment with LIF (\( P < 0.05 \), Fig. 3).

![Figure 3](https://example.com/figure3.png)

**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) \( \alpha_1, \alpha_5, \alpha_6, \beta_1, \beta_3, \beta_4 \) are presented as ratios obtained by the comparative cycle threshold method (\( \Delta \Delta C_t \)) method, corrected to \( \beta\)-actin. Data show three independent experiments. Each sample was assayed in triplicate. *\( P < 0.001 \) compared to control.

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   | \( 7.6 \times 10^{-7} \) |
| 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_000994   | undetectable    |
| ITGB2    | Hs.375957 | NM_000211   | undetectable    |
| LAMA1    | Hs.270364 | NM_005559   | undetectable    |
| SELP     | Hs.82848 | NM_000655   | undetectable    |
| VTN      | Hs.2257  | NM_000638   | undetectable    |
| ADAMTS6  | Hs.271605 | NM_007037   | 7.6              |
| 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_000994   | undetectable    |
| ITGB2    | Hs.375957 | NM_000211   | undetectable    |
| LAMA1    | Hs.270364 | NM_005559   | undetectable    |
| SELP     | Hs.82848 | NM_000655   | undetectable    |
| VTN      | Hs.2257  | NM_000638   | undetectable    |
| ADAMTS6  | Hs.271605 | NM_007037   | 7.6              |
| 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_000994   | undetectable    |
| ITGB2    | Hs.375957 | NM_000211   | undetectable    |
| LAMA1    | Hs.270364 | NM_005559   | undetectable    |
| SELP     | Hs.82848 | NM_000655   | 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 \( \beta\)-actin (mean C \( C_t \)).

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 with LIF was examined by gelatin zymography. Unstimulated EVT cells secreted both 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.

![Figure 4](https://example.com/figure4.png)

**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 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...
The effect of LIF on TIMP-1 and -2 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 5: Effect of leukemia inhibitory factor (LIF) on tissue inhibitors of matrix metalloproteinase (TIMP)-1 and -2 protein secretion from primary EVT cells in vitro. The increase in TIMP-1 and TIMP-2 secretion was observed when LIF was added to EVT (Fig. 6, bottom).

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 β4 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 α6β4, 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, α5/β1 (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 β1 mRNA was maximally expressed, while integrin α5 mRNA was minimally expressed. Integrins α6 and β4 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 EVT.

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 cytrophoblast progression through the cell column is associated with a downregulation of the integrin α6β4 (a likely LN receptor), our demonstration of a reduction in expression of the β4 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 α1, α5 and β1 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...
integron 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 β4 subunit, that is regulated by LIF, dimerizes with the integrin α6 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 α6β4 cell surface protein production. It is also likely that factors other than LIF affect the affinity of integrin α6β4 on trophoblast cells to LN. In addition, integrin α6β4 also potentially binds other ECM components suggesting that EVT cells bind to LN via mechanisms other than via integrin α6β4.

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 retardation (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 β4 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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