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Human chorionic gonadotropin (hCG) modulation of TIMP1 secretion by human endometrial stromal cells facilitates extravillous trophoblast invasion *in vitro*

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STUDY QUESTION: Are secreted extracellular matrix (ECM) remodelling elements, relevant to embryo implantation and placentation, modified by hCG in endometrial stromal cells (ESCs)?

SUMMARY ANSWER: hCG decreases tissue inhibitor of metalloproteinase 1 (TIMP-1) secretion in ESCs, thereby facilitating extravillous trophoblast invasion *in vitro*.

WHAT IS KNOWN ALREADY: Successful embryo implantation and placentation depend on the appropriate invasion of the trophoblast into the maternal endometrial stroma. hCG is one of the earliest embryo-derived secreted signals in the endometrium which abundantly expresses hCG receptors. However, there is little data concerning the effects of hCG on endometrial ECM remodelling with respect to embryo implantation.

PARTICIPANTS/MATERIALS, SETTING, METHODS: This study was conducted in an academic research laboratory within a tertiary-care hospital. Samples were collected from 36 women undergoing benign gynaecological surgery during the mid-secretory phase. ESCs were isolated and stimulated with hCG (10 UI/ml) or vehicle. Conditioned media (CM) were analysed to determine changes in the secreted profile of nine matrix metalloproteinases (MMPs) and three tissue-specific inhibitors of MMPs (TIMPs) using an ELISA array. Data were confirmed by gelatine zymography, western blot and ELISA. The HTR8/SVneo cell line served as a model for trophoblast cells. The invasive potential of trophoblast cells was assessed using Transwell invasion assays under CM or co-culture conditions with ECS and the role of regulated molecules was examined by using immunoprecipitation in CM prior to the assessment of invasive potential.

MAIN RESULTS AND THE ROLE OF CHANCE: MMP-2 levels increased 30%, whereas TIMP-1 levels decreased 20% in CM from ESCs stimulated with hCG ($P < 0.05$). Gelatine zymography confirmed an increase in MMP-2 activity ($P < 0.05$). ELISA and western blotting also confirmed the reduction in TIMP-1 upon hCG treatment ($P < 0.05$). Invasion assays revealed a ~50% increase in invading HTR8/SVneo cells in chambers with hCG-stimulated ESCs compared with the control ($P < 0.05$). Immunodepletion of TIMP-1 from control ESC-CM partially resembled the effect of CM from hCG-stimulated ESCs in the trophoblast invasion assays.

LIMITATIONS, REASONS FOR CAUTION: The assays were performed *in vitro* and ESCs were not decidualized, therefore they reflected the very early stages of embryo implantation or the advanced stages when decidualization fails.

WIDER IMPLICATIONS OF THE FINDINGS: Our data suggest that hCG induces endometrial stromal extracellular remodelling by modulating secreted MMP-2 and TIMP-1. This regulation may be physiologically relevant because it increases the invasive potential of trophoblast-derived cells. At present, few data exist concerning the implications of hCG and endometrial ECM remodelling in embryo implantation. Hence, our results should be confirmed by further *in vivo* studies.

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Key words: chorionic gonadotropin / endometrium / trophoblast invasion / hCG / endometrial stromal cells

Introduction

Successful embryo implantation and placentation depends on the appropriate invasion of the trophoblast into the maternal endometrial stroma (Pijnenborg *et al.*, 1980; Aplin, 1991; Bischof *et al.*, 2001), which is a process that is initiated during the mid-secretory phase of the menstrual cycle. After the blastocyst attaches to the endometrial luminal epithelium, its trophectodermal cells acquire invasive properties that enable them to pass through the epithelium and its basal lamina to infiltrate the endometrial stromal tissue (Boyd and Hamilton, 1970). In humans, the process of decidual transformation begins independently of the presence of an embryo and is first apparent in stromal cells surrounding the spiral arteries ~10 days after the postovulatory rise in ovarian progesterone levels (de Ziegler *et al.*, 1998). However, this decidualization is not evident until some 5 days after the embryo makes contact with the stroma (Hertig *et al.*, 1956; Boyd and Hamilton, 1970). These observations reveal a condition occurring during early pregnancy in which the embryonic trophoblast faces both decidualized and non-decidualized stroma.

Matrix metalloproteinases (MMPs) are zinc endopeptidases that are required for the degradation of extracellular matrix (ECM) components during normal embryo development, morphogenesis and tissue remodelling. MMPs with collagenase activity, particularly MMP-2 and MMP-9, have been described in very early embryos, amnion and placenta (Puistola *et al.*, 1989; Autio-Harmainen *et al.*, 1992). Their activity underscores the role of proteolytic enzymes and MMPs in the breaching of the ECM barrier by trophoblasts during embryo implantation and early placenta development. The proteolytic activities of MMPs are precisely regulated at least in part by endogenous tissue inhibitors of metalloproteinases (TIMPs), which bind the MMPs with a 1:1 stoichiometry (Gomis-Ruth *et al.*, 1997). In fact, the expression of TIMP family members in the human endometrium during implantation represents a substantial regulatory barrier to trophoblast invasion (Zhang and Salamonsen, 1997). Hence, the balance between MMPs and TIMPs locally at the invasive site is a highly regulated process mediated by the spatiotemporal expression of MMPs involved in ECM remodelling, requiring the appropriate interaction between the trophoblast and the endometrium (Hulboy *et al.*, 1997).

hCG is a glycoprotein hormone, which is critical at the onset of pregnancy as it promotes the maintenance of progesterone production by the corpus luteum (Devoto *et al.*, 2002; Zeleznik and Pohl, 2006) and the establishment of the placental syncytium (Yang *et al.*, 2003). hCG production by human embryos begins at an early stage of development (Bonduelle *et al.*, 1988; Lopata and Oliva, 1993), although the intact protein first becomes detectable in the mother's blood and urine between 6 and 14 days after fertilization (Hay and Lopata, 1988; Lohstroh *et al.*, 2006), suggesting that the trophoblast secretes the hormone long before its detection in maternal serum (Lopata and Oliva, 1993). This early appearance of hCG suggests that it may exert paracrine effects regulating embryo implantation and early placental development. hCG/LH receptors are found in virtually all maternal tissues in close proximity to the embryo including the endometrium and myometrium (Reshef *et al.*, 1990). Both the glandular and stromal elements of the endometrium express hCG/LH-binding sites and receptor-like immunoreactivity (Reshef *et al.*, 1990; Lin *et al.*, 1994). Receptor expression appears to be cycle dependent with secretory endometria expressing higher levels compared with proliferative endometria or the endometria of post-menopausal women. Various lines of evidence support paracrine roles for hCG in the production of chemokines by endometrial epithelium relevant to embryo implantation (Fazleabas *et al.*, 1999; Licht *et al.*, 2001a,b; Zygmunt *et al.*, 2005; Fluhr *et al.*, 2006; Paiva *et al.*, 2011). Functionally, there is evidence that hCG promotes the decidualization of endometrial stromal cells (ESCs) *in vitro* (Han *et al.*, 1999) and up-regulates COX-2 gene expression (Han *et al.*, 1996) and endometrial prolactin production (Han *et al.*, 1997). Similar observations have been made in the baboon (Fazleabas *et al.*, 1999).

Intrauterine administration of hCG at low concentrations during the secretory phase also regulates the secretion of molecules related to endometrial differentiation and embryo implantation (Licht *et al.*, 1998). In addition, a regulatory role for hCG on the MMP/TIMP system in decidualized ESCs and trophoblast cells has been described (Fluhr *et al.*, 2008a). hCG increased the secretion of MMP-2 and -9 in cytotrophoblasts in a dose-dependent manner, while TIMP-1, -2 and -3 were significantly reduced by hCG in pre-decidualized ESCs in a time- and dose-dependent manner (Fluhr *et al.*, 2008a), suggesting a role for hCG in regulating the invasion of the trophoblast into the maternal endometrium. However, no evidence for such regulation has been shown so far. The objective of the present work was to determine whether hCG regulates ESC function and ECM remodelling to modulate trophoblast invasion.

Materials and Methods

Tissue collection and ESC culture

Endometrial tissue samples were obtained from ovulatory women (35–45 years old) and with an indication for benign gynaecological surgery (e.g. tubal ligation and leiomyoma). Signed informed consent was obtained from participating women under the approval of the Human Bioethics Committee of the University of Chile, Santiago, Chile and in accordance with the Declaration of Helsinki. All patients had not received any form of hormonal treatment in the last 3 months before participating in the study and were scheduled for surgery at the mid-secretory phase. Subjects, who had regular menstrual cycles (26–35 days) and normal body mass index, were documented not to be pregnant, and had no history of endometriosis. Endometrial biopsies were obtained from the uterine fundus at the time of surgery with Pipelle catheters under sterile conditions. Histological dating was performed for each biopsy according to the method of Noyes *et al.* (1951) to confirm their mid-secretory timing. ESCs were obtained as follows: the endometrial tissue was washed twice with phosphate-buffered saline (PBS) solution, minced into small pieces and digested for 1 h at 37° C in 0.5% collagenase type 3 (45 U/ml; Worthington Biochemical Corp., Lakewood, NJ, USA) and DNase I (Sigma, St. Louis, MO, USA). Subsequently, the suspension was filtered through a 100-μm nylon sieve (Falcon, BD, Franklin Lakes, NJ, USA) followed by a 40-μm nylon sieve (Falcon). Stromal cells that passed through the 40-μm sieve were thoroughly washed with PBS, resuspended in DMEM/F-12 (Gibco, Grand Island, NY, USA) 10% fetal bovine serum (FBS; Gibco) and 1% Antibiotic–Antimycotic (Gibco) and seeded in 75-cm^2 culture flasks (Orange Scientific, Braine-l'Alleud, Belgium). The purity of the isolated ESCs was assessed by immunocytochemistry using antibodies against vimentin (mouse monoclonal V-9; Calbiochem, Biosciences, Inc., La Jolla, CA, USA), cytokeratin (mouse monoclonal B311.1; Calbiochem), and PTPRC (mouse monoclonal CD45; Dako Cytomation, Carpinteria, CA, USA) resulting in >96% of purity for ESCs, <4% for endometrial epithelial cells and 0% for leucocytes in the obtained cell cultures.

For the experiments described, cell preparations were individually grown (not pooled) and then seeded in six-well tissue culture plates until 60–80% confluent, FBS starved for 12–14 h and then stimulated with urinary-purified hCG (Sigma) or vehicle for 48 h. The doses of hCG used were selected from previous publications (Fluhr *et al.*, 2008a; Paiva *et al.*, 2011). CM from ESC cultures stimulated with hCG (hCG-CM) or vehicle (V-CM) were harvested, centrifuged, aliquoted and stored at −80°C. All samples of CM were analysed individually (not pooled).

ELISA array

The multiplex sandwich fluorescent immunoassay ExcelArray Human MMP/TIMP Array (Thermo Scientific, Rockford, IL, USA) was used for the simultaneous quantitative measurement of MMP-1, -2, -3, -7, -8, -9, -10, -12, -13, TIMP-1, -2 and -4 according to the manufacturer's instructions. The ExcelArray slide contains 16 wells (subarrays) of 12 target-specific antibodies and a negative and positive control, all spotted in triplicate on a protein microarray slide (Garcia *et al.*, 2007; Giricz *et al.*, 2011). hCG-CM ($n = 5$) and V-CM ($n = 5$) were individually analysed with the ExcelArray using a subarray for each sample. The signal associated with each spot was measured by a fluorescence slide reader (ScanArray LITE; PerkinElmer Life Sciences, Inc., Boston, MA, USA). The intensity of the fluorescent signals is proportional to the amount of each MMP or TIMP in the standard or sample. The lower detection limit of all of the assayed molecules is 123 pg/ml except for MMP-2, TIMP-1 and TIMP-2 when it was 309 pg/ml.

Immortalized extravillous trophoblast cell line HTR-8/SVneo

The extravillous trophoblast (EVT) cell line HTR-8/SVneo was generously provided by Dr Peeyush Lala (Department of Anatomy and Cell Biology, University of Western Ontario, Canada). The cell line was produced from primary cultures of cytotrophoblast cells obtained from explant cultures of human first-trimester placenta as described previously (Graham *et al.*, 1993). The cell line expresses cytokeratin 7 (CK7, Maldonado-Estrada *et al.*, 2004), as well as other EVT markers (Irving and Lala, 1995; Irving *et al.*, 1995). HTR-8/SVneo cells, which have previously been reported to secrete hCG with culture medium concentrations of 42 ± 17 IU/l (Graham *et al.*, 1993), were grown in RPMI 1640 (Biological Industries, Israel) supplemented with 10% FBS (Gibco) and 1% Antibiotic–Antimycotic (Gibco).

Matrigel invasion assay of HTR8/SVneo cells co-cultured with ESCs

To assess invasiveness in a co-culture setting, an invasion assay was performed using a commercially available *in vitro* invasion assay (ECM 550; Chemicon, Millipore, CA, USA) consisting of membranes with 8 μm pores coated with Matrigel on the upper side. ESCs (1×10^4 per well) suspended in 250 μl of serum-free DMEM/F12 were seeded on to the Matrigel coated insert, while the lower well contained 500 μl of the same medium. After overnight incubation, ESCs were stimulated with urinary-purified hCG (Sigma) or vehicle for 48 h in a tissue culture incubator and then washed free of hCG or vehicle in the upper and lower wells. On top of the ESC monolayer, 2×10^4 HTR8/SVneo cells suspended in 250 μl of serum-free DMEM/F12 were seeded and 500 μl of DMEM/F12 culture medium supplemented with 10% FBS were added to the lower well as a chemoattractant. After 48 h of incubation at 37°C, the cells in the upper side of the insert were wiped off with a cotton swab and the cells in the lower side were washed with cold PBS and the fixed with cold acetone for 10 min. ESCs have shown invasive behaviour *in vitro* (Gellersen *et al.*, 2010) and so HTR8/SVneo cells were specifically detected in the lower side of inserts by immunocytochemistry using an antibody against CK7 (mouse anti-human monoclonal, clone OV-TL 12/30, 8.3 μg/ml; Dako). On each insert, only immunostained cells were counted in six randomly selected visual fields (×10 objective) and the mean number of cells per visual field was determined. The mean of the controls was set to 1, and the data are expressed as means of the fold change ± SD of the control values. No pools of ESCs from different endometrial preparations were prepared and experiments were performed at least three times, using different individual preparations of primary ESCs in triplicate.

Matrigel invasion assay of HTR8/SVneo cells in ESC-CM

Invasiveness of HTR8/SVneo cells in response to ESC-secreted molecules was assessed by plating 2×10^4 HTR8/SVneo cells suspended in 250 μl of one of the following culture media: hCG-CM, V-CM and non-conditioned culture medium with 10 IU/ml hCG (hCG-NC). In addition, HTR8/SVneo cells were suspended in V-CM depleted of TIMP-1 [by TIMP-1 immunoprecipitation (IP)] or a V-CM control for immunodepletion [non-specific IP (NS IP)]. CM were used individually for each assay (not pooled). Each cell suspension was seeded on a Matrigel coated insert from the ECM550 Invasion Assay (Millipore). The lower well contained DMEM/F12 culture medium supplemented with 10% FBS and 1% Antibiotic–Antimycotic (Gibco) as chemoattractant. The invasion assay was terminated after 48 h and the cells in the upper side of the insert were wiped off with a cotton swab and the cells on the lower side were stained with the solution included in the invasion assay. The number of invasive cells was determined as described earlier for the Matrigel invasion assays in co-culture.

TIMP-1 ELISA assay

Immunoreactive TIMP-1 in hCG-CM, V-CM, TIMP-1 IP and NS IP was measured quantitatively using a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA). The CM were thawed and concentrated 10-fold using a vacuum dryer prior to performing the assay for each individual sample in duplicate. The lower detection limit of the assay is 0.08 ng/ml. The mean of the controls was set to 1, and the data are expressed as means of the fold change ± SD of the control values.

Zymography

Proteinase activities in equal volumes of hCG-CM ($n = 9$) and V-CM ($n = 9$) were analysed in each individual sample in duplicate by zymography on 10% SDS-polyacrylamide gels containing 1 mg/ml gelatine (Sigma) or 2 mg/ml casein (Sigma) under non-reducing conditions. MMPs, both latent (pro-) and active forms, were identified by molecular weight using molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA). The MMP identity of the bands was confirmed by incubation of parallel gels in the presence of EDTA. Enzymatic activity was visualized by negative staining and semi-quantified by densitometric analysis of zymograms using the Image-Pro Plus Software (Media Cybernetics, Inc., Silver Spring, MA, USA). The mean of the controls was set to one, and the data are expressed as means of the fold change ± SD of the control values.

MMP activity assay

The fluorogenic substrate for MMP-2/MMP-9 (MOCAc-Pro-Leu-Gly-Leu-A2pr(Dnp)-Ala-Arg-NH2) and for MMP-3 (MOCAc-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH2) (Peptide Institute, Inc., Ibaraki, Japan) were used to analyse MMP activity in each individual sample of hCG-CM ($n = 3$) and V-CM ($n = 3$) as previously described (Nagase *et al.*, 1994). Briefly, 100 μl of ESC-CM was added to 100 μl of assay buffer containing the substrate and incubated for 15 min. The reaction was stopped by the addition of 800 μl of 10% acetic acid and the fluorescence excitation and emission were measured at wavelengths of 325 and 393 nm,

respectively. The mean of the controls was set to 1, and the data are expressed as the means of the fold change ± SD of the control values.

Plasminogen activator urokinase-type activity assay

Plasminogen activator urokinase-type (PLAU) activity was determined in hCG-CM ($n = 3$) and V-CM ($n = 3$) by measuring plasminogen activation for each individual sample using a commercially available kit (ECM 600; Chemicon) according to manufacturer's instructions. Briefly, 100 µl of standard or sample (i.e. CM, in duplicate) was added to sample buffer (100 mM Tris and 0.5% Triton X-100, pH 8.8) containing a chromogenic tripeptide PLAU substrate (supplied by the manufacturer) in a final volume of 200 µl. After overnight incubation at 37°C, samples were analysed spectrophotometrically at 405 nm. Data are expressed as the fold change from the control condition.

MTS assay for cell viability

The effect of hCG-CM ($n = 3$) and V-CM ($n = 3$) on HTR8/SVneo proliferation was measured using a conventional tetrazolium-based MTS assay. The cells were seeded in 96-well plates and incubated overnight. The next day, culture media in wells were replaced with V-CM or hCG-CM and the cells were incubated for 48 h. After culture, MTS solution (Promega, Madison, WI, USA) was added to each well and incubated for 4 h at 37°C. The formazan formed in each well was read at a wavelength of 490 nm using the Synergy 2 microplate reader (BioTek instruments, Winooski, VT, USA). All CM were analysed individually (not pooled).

ECM-degrading activity of ESC-CM in stroma of endometrial slices

Metalloproteinase activity from hCG-CM ($n = 3$) and V-CM ($n = 3$) was individually assayed in endometrial tissue in triplicate as described by Marbaix *et al.* (1996) with modifications. Sections (10 µm thick) were obtained from frozen mid-secretory endometrial tissue. Tissue slices were fixed in acetone for 5 min and air dried, and each section was incubated for 24 h at 37°C with 40 µl of hCG-CM, V-CM or non-conditioned culture media as control. All media were supplemented with 50 mM Tris–HCl (pH 7.5), 0.05% Triton X-100, 5 mM $CaCl_2$, 3 mM NaN_3, proteinase inhibitor (Complete, EDTA-Free protease inhibitor cocktail tablets, Roche, IN, USA) and, when indicated, 2 mM aminophenylmercuric acetate (APMA; Sigma) and/or 25 µM Ilomastat (GM6001; Millipore, Billerica, MA, USA). APMA converts latent (pro) forms of the MMPs to their active forms and Ilomastat is a synthetic broad-spectrum MMP inhibitor. Sections were later fixed overnight in 4% formaldehyde at room temperature and processed for the Van Gieson staining (Bancroft and Cook, 1984) for the identification of collagen fibres. Images from stained sections were obtained with an Olympus BX51 microscope (Olympus, Tokyo, Japan) and collagen fibre staining intensity in endometrial stroma was analysed using the Image-Pro Plus Software (Media Cybernetics, Inc., Silver Spring, MA, USA) by obtaining an expression Level Score (ELS) for each endometrial sample. The mean of ELS scores from the controls was set to 1, and the data are expressed as mean of fold change ± SD of the control values.

TIMP-1 immunoblot

Total protein was precipitated from individual samples of hCG-CM ($n = 3$) and V-CM ($n = 3$) for 10 min after the addition of four volumes of cold acetone and then centrifuged at 15 000*g* for 15 min at 4°C. The obtained pellet was dried in a speed-vac (Eppendorf 5301, Hamburg, Germany) and resuspended in deionized water with phenylmethanesulfonyl fluoride, and the total protein concentration was determined by the bicinchoninic acid method (Thermo Scientific). Protein samples (10 µg) were subjected to SDS–PAGE in triplicate, transferred to a nitrocellulose membrane (Whatman Protran, Sigma) and blocked with 5% BSA (Sigma) before being incubated overnight with primary antibody against TIMP-1 (mouse anti-human monoclonal IgG1k, MAB3301, 1 µg/ml; Millipore). After incubation with a secondary antibody conjugated with peroxidase (goat anti-mouse IgG, 115–35–3, 0.11 µg/ml; Jackson Immunoresearch, West Grove, PA, USA), detection was performed using the ECL Western Blotting Substrate Pierce Kit (Thermo Scientific) in the Discovery Team photodocumentation system (Ultralum, Paramount, CA, USA). The net intensity and area of the bands were analysed with KODAK 1D Image Analysis Software (Eastman Kodak, Rochester, NY, USA). ELS scores from the controls was set to 1, and the data are expressed as mean of fold change ± SD of the control values. Ponceau-stained bands served as loading controls.

IP of TIMP-1

TIMP-1 in V-CM was immunoprecipitated as previously described (Stilley and Sharpe-Timms, 2012), with modifications, in each individual V-CM (not pooled). Samples of V-CM (200 µl) and 40 µg of anti-TIMP1 antibody (MAB3301; Millipore) were incubated overnight at 4°C and then 20 µl of Protein G Plus-Agarose Suspension (IP04; Calbiochem, Darmstadt, Germany) were added and incubated for 3 h at room temperature. The mixture was centrifuged at 3000*g* for 3 min at 4°C and the supernatant tested by was ELISA (R&D) for TIMP-1 before being frozen at −80°C until use. As a non-specific IP control, 40 µg of Mouse azide-free IgG (PP54Z-K; Millipore) was used instead of anti-TIMP1 antibody. The TIMP-1 concentration in V-CM was set to 1, and the data are expressed as mean of fold change ± SD of V-CM values.

Statistical analysis

All of the experiments were performed in triplicate using cell preparations from at least three different endometrial biopsies ($n \geq 3$). Experimental data are presented as the mean ± SD, and the number of experiments is indicated in the figure legends as '*n*'. The significance of the assays was determined using a Kruskal–Wallis test followed by the Dunn's *post hoc* test for comparisons between control and treatment conditions; $P < 0.05$ was considered to be statistically significant.

Results

Secreted MMPs and TIMPs in ESC-CM

To determine the effect of hCG on the secreted profile of selected MMPs and TIMPs in cultured ESCs, the secreted profiles of MMP-1, -2, -3, -7, -8, -9, -10, -12 and -13, and TIMP-1, -2 and -4 in hCG-CM and V-CM were first assayed simultaneously by ELISA array (Fig. 1A). All MMPs and TIMPs analysed were detected with the exception of MMP-9 and -13 and TIMP-4. The most abundant ECM elements analysed were MMP-2 and -3 and TIMP-1 and -2 (Fig. 1A–C). Following the stimulation of ESCs with hCG (10 IU/ml for 48 h), MMP-2 increased by 18.3% ($P < 0.05$, Fig. 1D), MMP-8 decreased by 14.3% ($P < 0.05$, Fig. 1E) and TIMP-1 decreased by 32.2% ($P < 0.05$, Fig. 1F). Amongst the multiple MMPs produced by the human placenta, MMP-2 and -9 have been described as key for the invasive function of the cytotrophoblasts (Campbell *et al.*, 2003). We further analysed the hCG-CM and V-CM for latent and active MMP-2 by gelatine zymography to confirm the ELISA-array results. Both secreted pro-MMP-2 (Fig. 2A and E) and active MMP-2 (Fig. 2B and F) increased with hCG stimulation consistent with the results obtained. The decrease in TIMP-1 in hCG-CM was confirmed by conventional ELISA (Fig. 2C) and western blot (Fig. 2D and H),

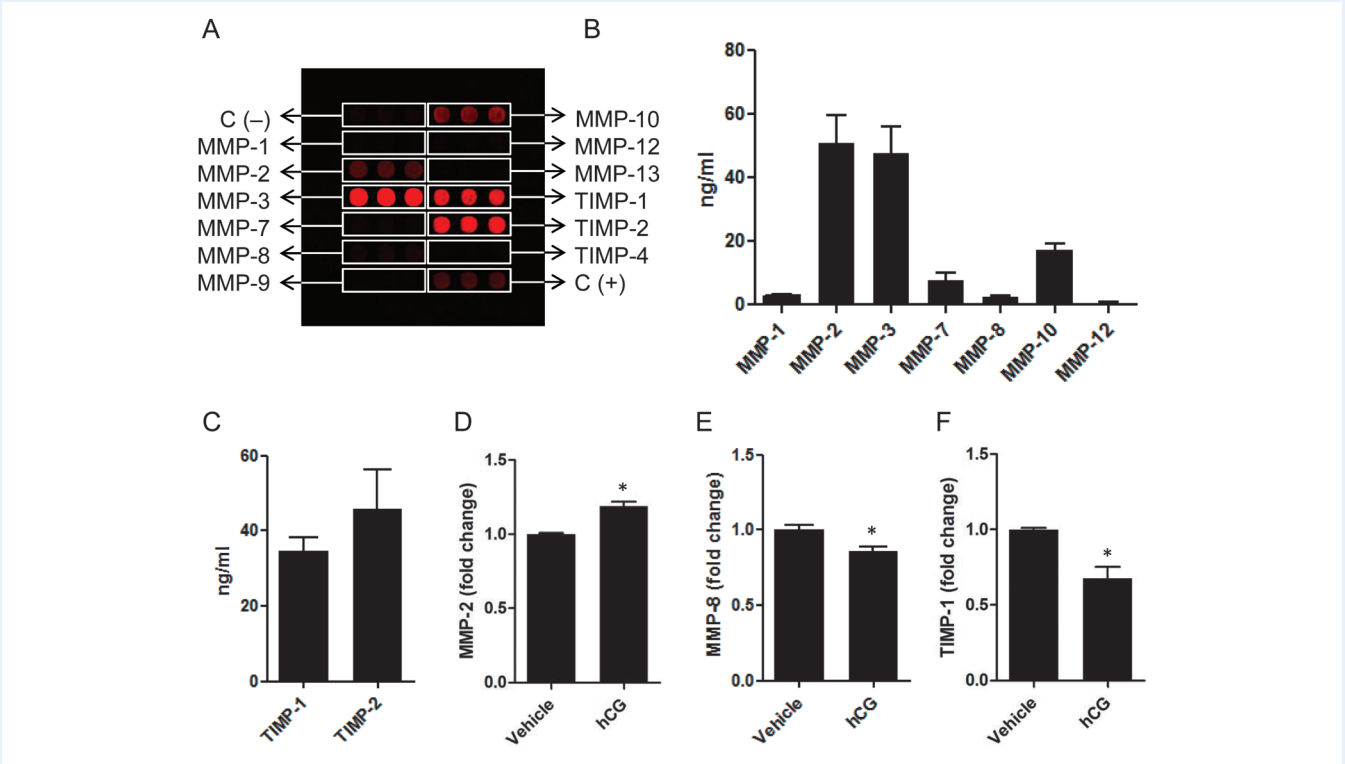

Figure 1 Secreted molecules from confluent ESCs cultured *in vitro* relevant to ECM remodelling. ESCs were isolated from normal secretory endometrium, serum starved for 12 h and incubated with fresh serum-free culture medium for 48 h. Culture medium was analysed using a fluorescent ELISA-Array from ESC cultures of five different preparations (not pooled) in triplicate spots. (**A**) Representative section of the ELISA array is shown for the detection of secreted MMPs from ESC-CM. The red fluorescent intensity correlates with the abundance of a specific analyte in the CM. (**B**) Profile of the secreted MMPs of ESCs in culture obtained with the ELISA-array assay. The corresponding measured concentrations are indicated for each analysed MMP except for MMP-9 and MMP-13, which could not be detected. (**C**) Profile of secreted inhibitors of MMPs (TIMPs)-1, -2 and -4 from ESCs. TIMP-4 levels were undetectable. (**D**), (**E**) and (**F**) Data for regulation of secreted MMP-2, MMP-8 and TIMP-1, respectively, induced by hCG. Data are expressed as the mean ± SD as fold change from the control condition, $^*P < 0.05$.

revealing a reduction of 36.3 and 52.1%, respectively, at the dose of 1 and 10 IU/ml hCG. MMP-3 secretion appeared to be one of the most abundantly secreted MMPs in the ELISA-array assay; however, its levels in CM did not changed upon hCG stimulation. A casein zymography for detection of pro- and active MMP-3 activities in hCG-CM and V-CM was also performed, confirming no significant variations (data not shown).

MMP-2/-9 enzymatic activity is increased in hCG-stimulated ESC-CM

Although the secreted profile of MMPs and TIMPs is apparently modified in favour of ECM-degrading capacity following hCG treatment of ESCs, the actual enzymatic activity of MMPs in the context of all the other secreted molecules by ESCs (particularly TIMPs) is not known. Again, because gelatinolytic (MMP-2/-9) activity is the most relevant degradative action of the extravillous trophoblast during its invasion into the endometrial stromal compartment, we applied an assay using a specific substrate for MMP-2/-9 that fluoresces once cleaved to analyse the gelatinolytic activity of hCG-CM (1 and 10 IU/ml hCG) or vehicle (V-CM). hCG stimulation increased the gelatinase activity by 16% (Fig. 2G) at 10 IU/ml.

PLAU, the presence of which has been reported in the human endometrium (Nordengren *et al.*, 2004), converts plasminogen into the active serine protease plasmin, which directly degrades ECM and catalyses the proteolytic activation of MMPs. We evaluated PLAU activity regulation in hCG-stimulated ESC-CM but found no significant changes in PLAU activity in ESC-CM upon hCG stimulation (data not shown). MMP-3 activity in ESC-CM was also assessed as a control using a specific substrate that also fluoresces after cleavage by this enzyme, but no effect of hCG was observed (data not shown).

Invasive potential of trophoblast-derived cells through a stromal cell layer and ECM

To elucidate the physiological meaning of our findings further, we evaluated the possible function of hCG modulation of ECM-degrading activity in ESCs in the context of trophoblast invasion. ESCs were plated on the surface of a Transwell insert coated with GFR-Matrigel and stimulated with 10 IU/ml hCG for 48 h. The ESCs subsequently were carefully washed free of hCG and HTR8/SVneo cells were plated on the ESC monolayer and incubated for an additional 48 h (Fig. 3A). HTR8/SVneo cells invaded inserts containing hCG-stimulated ESCs 75% more than with vehicle-stimulated ESCs (Fig. 3B–D). To address the

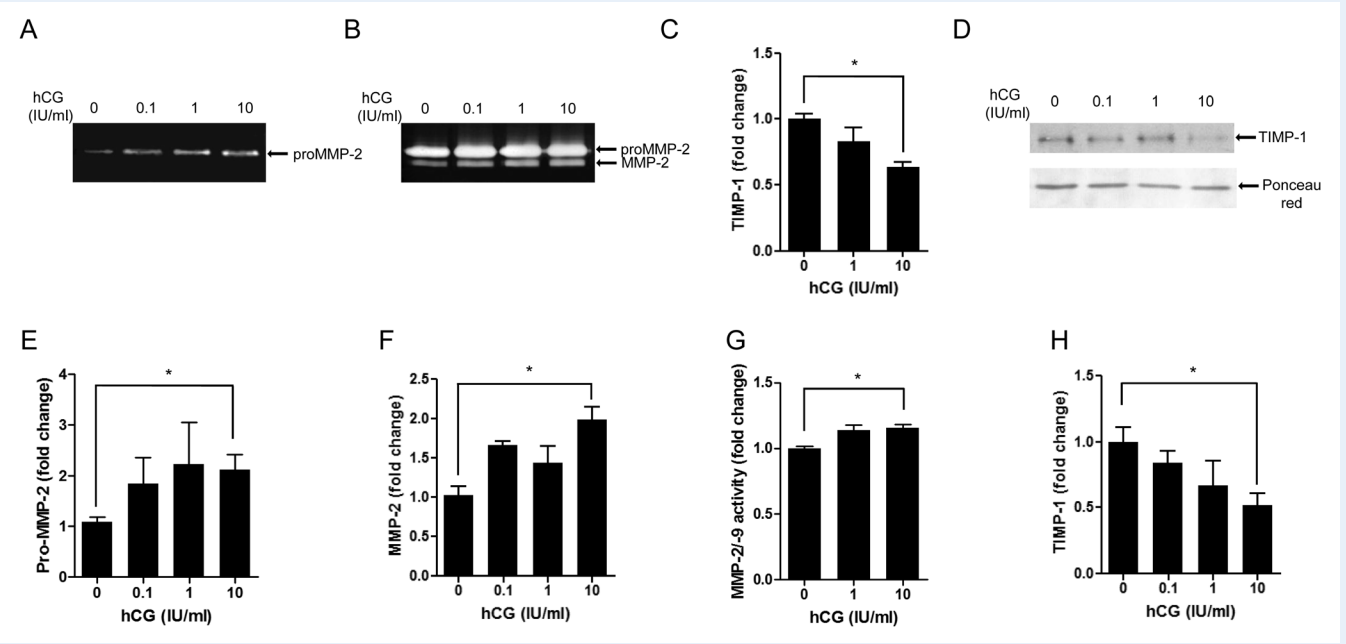

Figure 2 Effect of hCG on ESC-secreted pro-MMP-2, active MMP-2, TIMP-1 and MMP-2/-9 enzymatic activity. Confluent ESC cultures from the mid-secretory phase endometrial tissue were stimulated with vehicle or hCG (1–10 IU/ml). CM was harvested at 48 h and analysed. (**A**) and (**B**) Representative gelatine zymographies reporting the up-regulation of secreted pro-MMP-2 and active MMP-2, respectively. (**C**) Representative western blot for TIMP-1 in CM showing a decrease in CM from ESCs stimulated with 10 IU/ml hCG. (**D**) Decreased TIMP-1 in CM levels was confirmed by ELISA. (**E**) Semi-quantification of pro-MMP-2 bands in gelatine zymographies from CM. (**F**) Semi-quantification of gelatinase activity in CM corresponding to active MMP-2 bands. (**G**) Quantification of gelatinase activity in CM using a fluorogenic substrate displaying that 10 IU/ml hCG increases overall gelatinase enzymatic activity. (**H**) Semi-quantification of TIMP-1 levels in CM by western blot, displaying down-regulation at 10 IU/ml hCG. Gelatine zymographies were performed with CM from ESCs of nine different preparations (not pooled) and individually assayed in triplicate. Western blot, ELISA and fluorogenic gelatinase assays were performed with CM from ESCs of three different preparations (not pooled) and individually assayed in triplicate. Data for zymographies, ELISA and fluorogenic gelatinase assay are expressed as the mean ± SD as fold change from the control condition. Data from western blots are expressed as the mean ± SD of arbitrary units obtained from bands. $^{*}P < 0.05$.

possibility that HTR8/SVneo cells increase their invasive potential because they modify their own gelatinolytic capacity when co-cultured with hCG-stimulated ESCs in the invasion assay, ESCs were stimulated with hCG or vehicle for 48 h, then were carefully washed free of hCG and finally co-cultured with HTR8/SVneo cells in fresh medium for an additional 48 h. Neither the gelatinase activity nor TIMP-1 levels in the CM from the co-cultures exhibited significant changes (Fig. 4A and B) suggesting that ESCs in the presence of hCG modify the surrounding ECM to facilitate trophoblast invasion. This phenomenon was further examined using an ESC-free invasion assay in which HTR8/SVneo cells were plated on GFR-Matrigel coated inserts in hCG-CM or V-CM (Fig. 5A). The invasive capacity of HTR8/SVneo was increased in hCG-CM (Fig. 5B). hCG did not exert its pro-invasive effect directly on HTR8/SVneo cells evidenced by the use of non-CM with hCG (hCG-NC, Fig. 5B) or nor did it affect HTR8/SVneo cell proliferation, as assessed by MTS assay (Fig. 5C).

Histochemical study of endometrial ECM degradation

Human endometrial stromal ECM contains an extensive network of so-called 'reticular fibres' during the secretory phase which contain both type III and type I collagen (Marbaix *et al.*, 1996). The collagen fibrils are of about 20–40 nm in diameter, which run singly or in small bundles that constitute the reticular fibres. To characterize the endometrial ECM-degrading potential of CM from ESCs, we incubated mid-secretory endometrial sections with hCG-CM or V-CM for 48 h and semi-quantified total collagen fibre staining in the stromal compartment. Collagen fibres present in the endometrial stromal ECM are visualized as a dark grey thread-like network (Fig. 6A). Our analysis revealed decreased collagen fibre staining in endometrial stroma following incubation with hCG-CM (Fig. 6A–C). To address the specific contribution of MMPs to collagen fibre degradation in endometrial slices, ESC-CM were pre-treated with APMA (which converts the pro-MMP forms to the active MMP forms) and/or Ilomastat (a synthetic broad-spectrum MMP inhibitor) before they were incubated with the endometrial sections. hCG-CM reduced the collagen fibre staining by 34.6% compared with the basal condition (Fig. 6B), in keeping with our previous observation that hCG increases gelatinolytic activity in hCG-CM. Pretreatment of hCG-CM with Ilomastat revealed only a 9.7% decrease in fibre staining compared with the basal condition, suggesting that MMPs significantly contribute to the increase in gelatinolytic activity with hCG-CM. Conversely, pretreatment of V-CM with the organomercurial APMA reduced the fibre staining by 14.6% compared with the basal condition. This modest reduction suggests that although MMPs are active, their enzymatic activity in CM is inhibited in the absence of hCG.

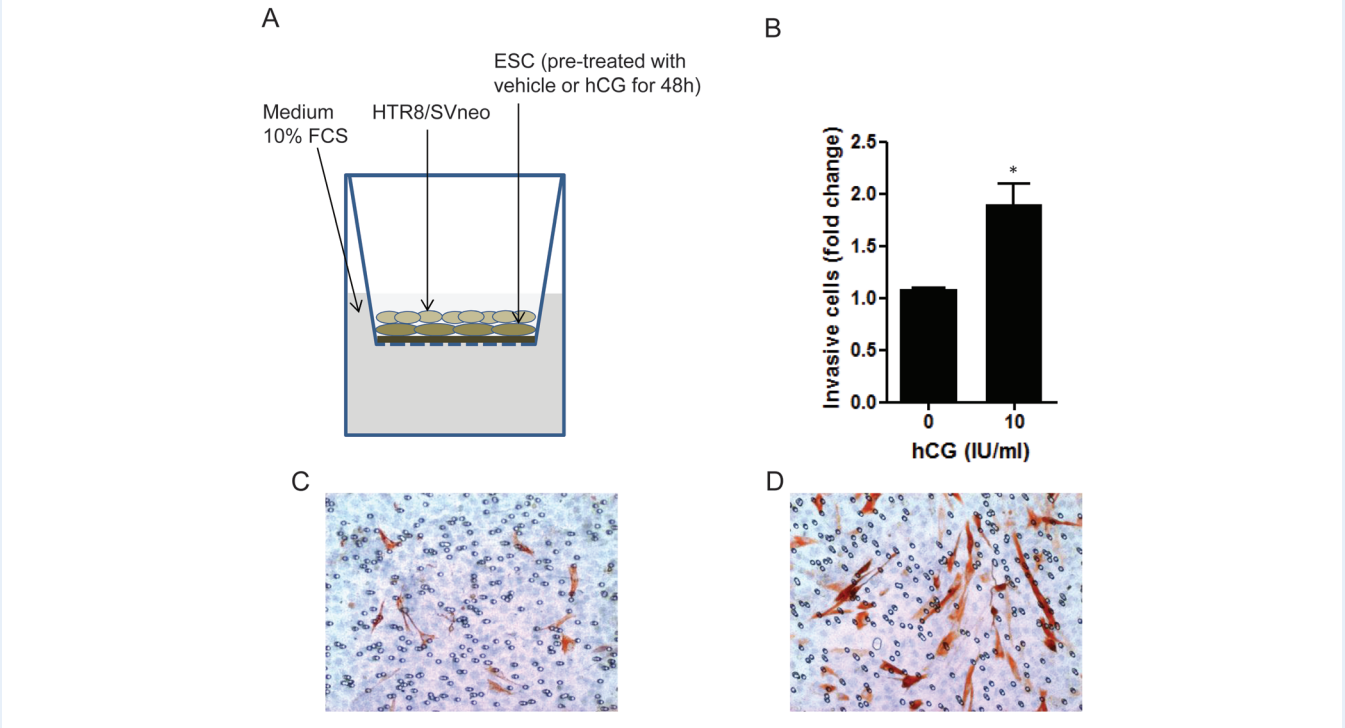

Figure 3 Invasion assays of trophoblast-derived cells co-cultured with ESCs. (**A**) Invasion assays were performed with HTR8/SVneo (derived from extravillous trophoblasts) co-cultured with ESCs from the mid-secretory phase endometrial tissue. ESCs plated in Boyden chamber inserts coated with Matrigel were stimulated with 10 IU/ml hCG or vehicle for 48 h. Subsequently, hCG was removed, and HTR8/SVneo cells were plated on top of ESCs and incubated for further 48 h. The total number of invading trophoblast cells on the bottom side of the insert was determined by immunostaining for cytokeratin 7 (CK7). (**B**) Quantification of invasive trophoblast cells co-cultured with ESCs depicted in (A). (**C**) and (**D**) Representative microphotographs showing fixed and stained HTR8/SVneo cells on the bottom side of an invasion membrane from the co-culture invasion assay depicted in (A) with ESCs pre-incubated with vehicle (C) or 10 IU/ml hCG (D). Immunostaining of HTR8/SVneo cells for CK7 resulted in a brown precipitate. Pores in the invasion membranes appear as small blue dots (original magnification $\times 100$). The data in (B) are expressed as the mean fold change $\pm$ SD of the number of invasive cells in each condition relative to the respective control. Invasion assays were performed with ESCs from three different preparations (not pooled) and assayed in triplicate. $^*P < 0.05$.

TIMP-1 immunodepletion in ESC-CM modifies the invasive potential of trophoblast-derived cells

The histochemical results of endometrial ECM degradation suggest that TIMP-1 reduction could contribute to endometrial ECM remodelling. Therefore, we analysed the effect of TIMP-1 reduction in V-CM on trophoblast invasive potential. TIMP-1 IP from V-CM resulted in TIMP-1 concentrations comparable to those in hCG-CM (Fig. 7A). Replacement of the primary anti-TIMP-1 antibody with normal mouse IgG did not result in modified TIMP-1 levels (Fig. 7A). Invasion assays were then performed using HTR8/SVneo cells resuspended in V-CM that was either TIMP1-depleted (TIMP-1 IP) or immunoprecipitated with normal mouse serum (NS IP). The invasive potential of HTR8/SVneo increased following TIMP1 removal, resulting in invasion levels similar to that observed with hCG-CM (Fig. 7B).

Discussion

The major findings of this study are that hCG can modulate MMP-2 activity and TIMP-1 secretion by ESCs, and hence alter trophoblast invasion, most likely by altering the surrounding ECM. Specifically, we demonstrated that hCG increased the secretion of MMP-2 and decreased TIMP-1 secretion by ESCs. This finding suggests that hCG might be physiologically relevant during embryo implantation and early placentation because it could facilitate trophoblast invasion by loosening the endometrial stromal ECM at the implantation site. The invasive process is dependent on the ability of the embryo to initiate the degradation of ECM proteins (Graham and Lala, 1992) through the secretion of MMPs at the blastocyst stage (Puistola *et al.*, 1989; Turpeenniemi-Hujanen *et al.*, 1992; Wang *et al.*, 2003) until the placenta has fully developed. Our *in vitro* trophoblast invasion assays corroborated this hypothesis; hCG treatment of ESCs increased the invasive potential of trophoblast cells through the ESCs by ~50%.

hCG is one of the most important signalling molecules produced by peri-implantation human embryos (Lopata and Oliva, 1993) and trophoblastic cells (Cole, 2009). In addition to the well-known endocrine effects of hCG on the corpus luteum maintenance, hCG may act as a growth and differentiation factor during pregnancy (Licht *et al.*, 2001b). Maternal serum hCG concentrations positively correlate with the extension of EVT infiltration in spiral arterioles and thus have been proposed to act as a promoter for EVT invasiveness (Hay, 1988). The hCG receptor

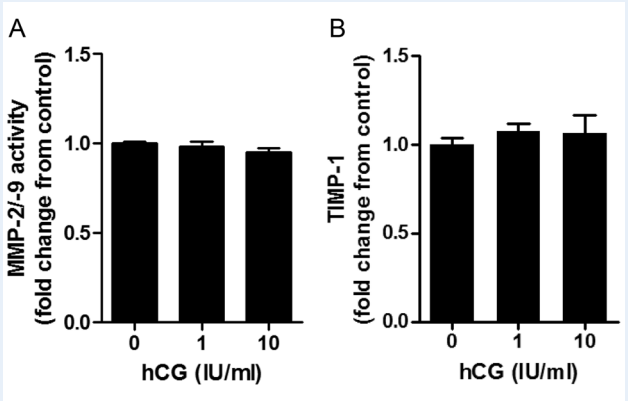

Figure 4 Combined MMP-2/-9 activity and TIMP-1 secretion in co-cultures. Gelatinase activity (**A**) and TIMP-1 levels (**B**) of CM obtained from co-cultures of ESCs and HTR-8/SVneo cells. ESC monolayers in culture were treated with hCG or vehicle for 48 h. Subsequently, monolayers were carefully washed free of hCG and HTR-8/SVneo cells were added to wells containing ESCs. The CM from the co-culture were collected after 48 h and analysed by a fluorescent assay for MMP-2/-9 activity and by ELISA for TIMP-1 quantification. Data are expressed as the fold change from the control condition and are reported as the mean ± SD. Gelatinase activity and TIMP-1 levels were determined in CM from ESCs of three different preparations (not pooled) co-cultured with HTR8/SVneo cells and assayed in triplicate. *$P < 0.05$.

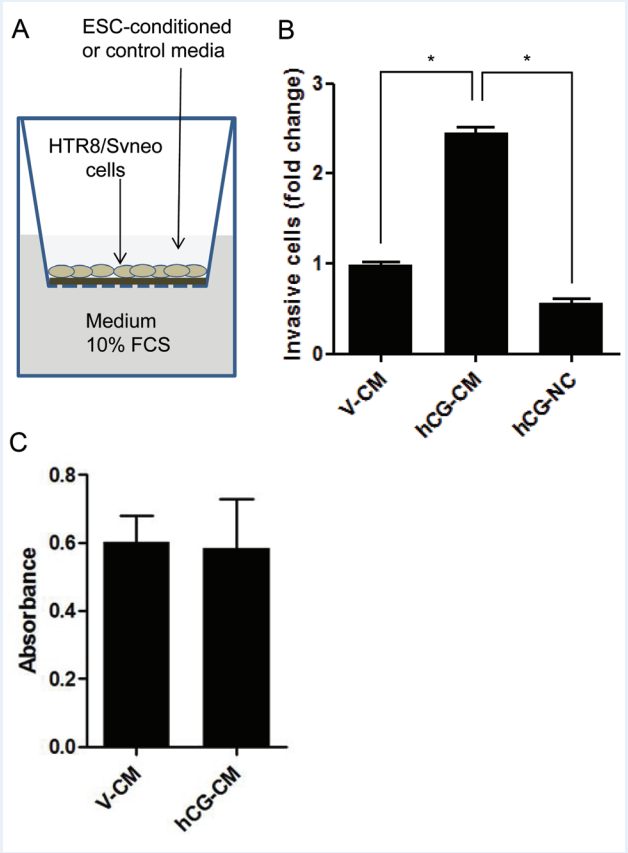

Figure 5 CM from ESCs stimulated with hCG increases the invasive potential of trophoblast cells without modifying their viability. (**A**) Invasion assays were performed with HTR8/SVneo cells resuspended in CM from ESCs stimulated with vehicle or 10 IU/ml hCG (V-CM and hCG-CM respectively). Non-CM with 10 IU/ml hCG (hCG-NC) was also used. HTR8/SVneo cells resuspended in the CM were plated in Boyden chamber inserts coated with Matrigel and DMEM/F-12 medium with 10% fetal calf serum was used as a chemoatractant in the lower vessel of the invasion chamber. Cells were incubated for 48 h, and the number of invading cell on the bottom of the inserts was counted after staining using a commercial cell staining dye. (**B**) Quantification of invasive trophoblast cells resuspended in the indicated culture media depicted in (A). (**C**) Effect of hCG-CM and V-CM on HTR8/SVneo cell viability. Cells were resuspended in V-CM or hCG-CM and seeded at a density of 2.5×10^3 cells/well, then incubated for 48 h. The MTS assay was performed and formazan absorbance was used as a measure of cell viability. Data in (B) are expressed as the mean fold change ± SD of invasive cells in each condition relative to their respective control. Data in (C) are expressed as the mean absorbance at 490 nm ± SD. Invasion and MTS assays were performed with CM from ESCs of three different preparations (not pooled) and assayed in triplicate. *$P < 0.05$.

(LH/CG-R) has been detected in trophoblast cells, allowing autocrine regulation of various functions in the trophoblast by hCG (Licht *et al.*, 2001b). However during the first 9 weeks of gestation, trophoblast cells express a truncated and inactive variant of the LH/CG-R before the full-length functional receptor is available (Licht *et al.*, 2001b), suggesting that during the implantation process, the earliest effects of hCG are exerted on the endometrial tissue. Functional hCG/LH receptors have been detected in human endometrial cells during the menstrual cycle with maximal expression being observed during the mid-secretory phase (Reshef *et al.*, 1990; Licht *et al.*, 2003; Evans *et al.*, 2009) and hCG has been shown to regulate key aspects of embryo implantation such as endometrial differentiation and receptivity (Fazleabas *et al.*, 1999; Licht *et al.*, 2001a,b; Perrier d'Hauterive *et al.*, 2004; Fluhr *et al.*, 2006; Evans *et al.*, 2009; Paiva *et al.*, 2011). Our results are in agreement with these observations and further underscore the role of paracrine hCG signalling in the endometrium during the implantation process. The observed effects of hCG on the invasive potential of the trophoblast appear to be largely exerted through the ESCs (Fig 5A and B).

The MMP/TIMP system has been documented to be related to physiological and pathological ECM remodelling in the endometrium (Curry and Osteen, 2003). The adequate regulation of MMP synthesis, activation and regulatory interactions with their specific inhibitors in the extracellular environment is necessary for decidualization of the endometrial stromal compartment during the secretory phase as well as for the process of tissue breakdown during the menstrual phase in a non-conceptive cycle (Hulboy *et al.*, 1997; Curry and Osteen, 2003; Strakova *et al.*, 2003). In addition, for successful embryo implantation leading to a viable pregnancy, a well-structured ECM surrounding the ESCs is essential (Aplin, 1991; Bischof and Campana, 1996), and *in vitro* experiments have demonstrated that ECM thickness exerts a major impact on the invasive phenotype of trophoblast cells (Kliman and Feinberg, 1990) indicating that some elements of the MMP/TIMP system must be precisely regulated in the endometrium during the embryo implantation process.

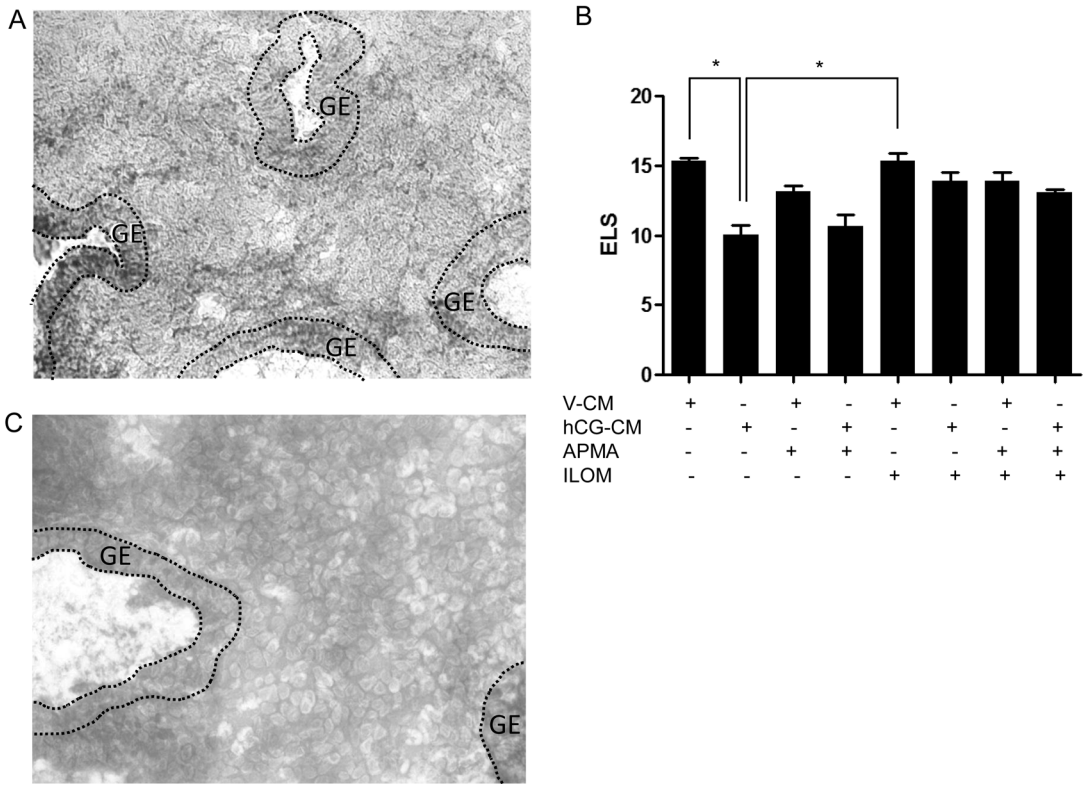

Figure 6 CM from ESCs stimulated with hCG increased the degradative activity of endometrial reticular fibres in stroma. Acetone-fixed histological sections from a mid-secretory endometrium were incubated at 37°C for 48 h with CM from ESCs stimulated with or without 10 IU/ml hCG for 48 h. MMPs in CM were activated prior to incubation with APMA when indicated. The MMPs inhibitor ILOMASTAT was added to CM when indicated. At the end of the culture time, endometrial sections were stained for collagen fibres. (**A**) and (**C**) Representative microphographs demonstrating fixed and stained endometrial sections for collagen fibres that were previously incubated with CM from ESCs stimulated with vehicle (A) or hCG (C). Original magnification ×40. **(B)** Semi-quantification of collagen fibre staining in endometrial sections incubated with CM. Data are expressed as the mean ± SD of the expression level score (ELS) for fibre staining obtained for each condition. Black dashed lines indicate endometrial glandular epithelium (GE), collagen fibres present in the endometrial stromal ECM are visualized as a dark grey thread-like network. Collagen-degradative activity in the endometrial stromal compartment was performed for CM from ESCs of three different preparations (not pooled) and assayed in triplicate. *$P < 0.05$.

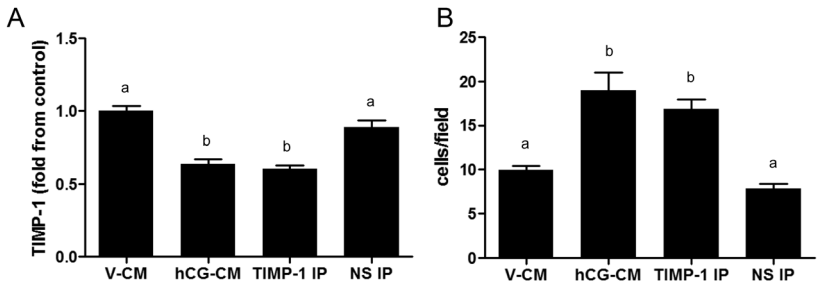

Figure 7 Reduced levels of TIMP-1 secreted by ESCs are involved in trophoblast invasion *in vitro*. (**A**) TIMP-1 levels in CM from ESCs stimulated with or without 10 IU/ml hCG for 48 h (hCG-CM and V-CM, respectively) were compared with those obtained after immunoprecipitating TIMP-1 from V-CM (TIMP-1 IP). TIMP-1 levels in CM were measured by ELISA. As a control for the IP, normal mouse IgG was used instead of the anti-TIMP1 primary antibody (NS IP). Data are expressed as the fold change from V-CM and are expressed as the mean ± SD. (**B**) Invasion assays using HTR8/SVneo cells resuspended in the indicated CM. HTR8/SVneo cells were incubated for 48 h, and the number of invading cells on the bottom of the inserts was counted after staining with a commercial cell staining dye. Immunoprecipation and invasion assays were performed with CM from ESCs of three different preparations (not pooled) and assayed in triplicate, a ≠ b, $P < 0.05$.

Licht *et al.* (2001a), using an intrauterine microdialysis system that analysed uterine flushing after the application of exogenous hCG in women during the secretory phase, reported an increase in secreted MMP-9. We did not observe such regulation in our ELISA-array experiments, which could be explained by the fact that uterine flushing contains mostly endometrial epithelial secretions, whereas our study used stromal fibroblasts, which secrete very low-to-non-detectable levels of MMP-9 *in vitro*. The MMPs we observed to be significantly regulated in ESCs by hCG included MMP-2 and MMP-8 which increased and decreased in CM, respectively. MMP-2 has been described in the human endometrium and exhibits a constitutive expression pattern during the menstrual cycle (Curry and Osteen, 2003), whereas the endometrial transcript levels of MMP-8 are largely absent during the menstrual cycle but become dramatically up-regulated toward the menstrual phase (Goffin *et al.*, 2003). This inverse regulation of MMPs with collagenase activity seems contradictory; however, because the secreted levels of MMP-8 are much lower than those of MMP-2 (Fig. 1B), the influence of MMP-8 on the overall ECM remodelling might be overridden by TIMP-1 and MMP-2 regulation, as reflected in the collagen fibre degradation of endometrial slices (Fig. 6A–C). In addition, TIMP-1 and -2 are inhibitors of many MMPs including MMP-8. Because TIMPs form 1:1 stoichiometric complexes with MMPs, the relatively high basal levels of TIMP-1 and -2 secreted by ESCs may result in a low MMP-8 activity in the endometrium.

Several studies have reported the expression of TIMPs in the human endometrium *in vivo* with a few discrepancies between the observed expression patterns of endometrial TIMPs (Henriet *et al.*, 2002; Jokimaa *et al.*, 2002; Goffin *et al.*, 2003; Boulday *et al.*, 2004; Vassilev *et al.*, 2005). However, TIMP-1 has been consistently reported to not be significantly regulated during the menstrual cycle, except during menses, where it becomes up-regulated. Data from *in vitro* studies strongly suggest that endometrial TIMPs play an important role as modulators of the implantation process (Pijnenborg *et al.*, 1981; Bischof and Campana, 1996; Zhang and Salamonsen, 1997) and TIMP-1 in particular has been demonstrated to limit the invasive behaviour of first trimester trophoblast cells in *in vitro* assays (Graham and Lala, 1991; Chakraborty *et al.*, 2002), which is consistent with our findings. Transforming growth factor β (TGF-β), which controls trophoblast invasiveness *in situ* (Graham and Lala, 1992), exerts its inhibitory effects by increasing TIMP-1 in both trophoblast and decidual cells (Graham and Lala, 1991), underscoring the role of TIMP-1 in trophoblast invasive behaviour. In addition to its role as an endogenous potent MMP inhibitor, TIMP-1 exhibits physiological roles that are independent of its ability to block MMP activities including direct anti-apoptotic and proliferation effects (Hayakawa *et al.*, 1992; Boulday *et al.*, 2004; Chromek *et al.*, 2004; Liu *et al.*, 2005). Our experiments demonstrate that hCG-CM does not affect HTR-8/SVneo cell viability (Fig. 5C), suggesting that the hCG-mediated regulation of TIMP-1 in ESCs increases the invasive potential of trophoblast cells through mechanisms not related to their cell viability. However, we cannot rule out the possibility that TIMP-1 modulation by hCG is mediating other or additional physiological effects that are unrelated to its regulatory ability on ECM remodelling (Martin *et al.*, 1999). However, the fact that hCG seems to antagonize the endometrial restraining mechanisms for trophoblast invasion by modulating the MMP/TIMP system toward ECM breakdown at the implantation site suggests that hCG may enhance ESC migration and invasion (Carver *et al.*, 2003; Grewal *et al.*, 2008; Gellersen *et al.*, 2010) and thus facilitate trophoblast invasion. The trophoblast invasion assays we performed using hCG-CM with no ESCs during the assay, directly increased trophoblast invasive potential (Fig. 4B and 7B), suggesting that the regulation of ECM remodelling elements in ESCs may play an important role during embryo implantation in addition to ESC motility (Carver *et al.*, 2003; Gellersen *et al.*, 2010). As demonstrated by our IP assays with V-CM, TIMP-1 modulation seems to be particularly relevant in trophoblast invasion regulation.

hCG has been reported to decrease the secretion of TIMP-1 and -2 in *in vitro* decidualized ESCs (Fluhr *et al.*, 2008a), which is consistent with our findings using non-decidualized ESCs with the exception that we did not detect the regulation of TIMP-2 secreted levels. This finding suggests differential effects of hCG in the regulation of the MMP/TIMP system depending on the differentiation status of the ESCs. In the present study, we not only detected an increase and a decrease in secreted levels of MMP-2 and TIMP-1, respectively, but we also have demonstrated that TIMP-1 regulation in ESCs is physiologically relevant to trophoblast invasive function *in vitro*. We did not observe direct effects of hCG on the invasive potential of the trophoblast cell line HTR8/SVneo, which contradict other studies that reported an increase in the migration potential of the same cell line stimulated with hCG (Zygmunt *et al.*, 2005; Chen *et al.*, 2011). These apparently discordant effects of hCG on trophoblast function may be explained by the fact that the previous studies evaluated only the migratory effects, whereas we have assessed the invasive potential of the cells, including ECM remodelling and cell motility. In addition, other *in vitro* studies using primary trophoblast cultures have demonstrated that hCG decreases trophoblast cell invasiveness (Milwidsky *et al.*, 1993; Yagel *et al.*, 1993).

During pregnancy, hCG is largely secreted by the syncytium and peaks in production at concentrations of 100–200 IU/ml in maternal serum at 8–10 weeks of gestation (Cole, 2009). To the best of our knowledge, there is no report of hCG levels at implantation sites during early pregnancy in humans. However, it has been estimated that the hCG concentration in the close proximity to the implanting embryos is ~8–62 IU/ml (Paiva *et al.*, 2011); hence, the doses used in this work are within this physiological range and have been shown to modulate endometrial cell function in previous reports (Perrier d'Hauterive *et al.*, 2004; Fluhr *et al.*, 2008b).

In this study we have used ESCs from the mid-secretory phase but they not decidualized *in vitro*. However, most of the trophoblast invasion process occurs after stromal fibroblasts has undergone the phenotypic switch to decidual cells as observed in early pregnancy. Because decidualization is initiated around the spiral arterioles in the mid-secretory phase, but is not widespread until a few days later (Noyes *et al.*, 1951; Hertig *et al.*, 1956; Enders, 1991), our research model reflects the physiological response of ESCs to hCG at the very early implantation stages or in situations when ESCs fail to decidualize during the implantation and early placentation processes.

In summary our data demonstrate that paracrine actions of hCG on ESCs induces changes in ECM remodelling and facilitates trophoblast invasion *in vitro*. These findings provide significant insights into the biology of embryo/maternal interactions underscoring the concept that high local concentrations of hCG produced at the implantation site by the invading human trophoblast are important for the successful establishment and maintenance of pregnancy and that TIMP-1 regulation in endometrial stromal ECM plays a major role in trophoblast function. The *in vivo* effects of hCG on the endometrial stroma might be more complex as many more signalling molecules mediate paracrine effects at the feto-maternal interphase.

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Authors' roles

A.T.-P. conceptualized the project, designed the study, performed the experiments, analysed the data and wrote the manuscript. F.A. participated in the acquisition of data. W.A.P. participated in the analysis of data and in critical discussions of the manuscript. L.D. conceptualized the project and participated in the design of the study, in critical discussions and in drafting the manuscript. All of the authors approved the final manuscript.

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Conflict of interest

The authors have no conflicts to declare.

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