Decidual HtrA3 negatively regulates trophoblast invasion during human placentation

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BACKGROUND: Controlled trophoblast invasion into the maternal decidua (interstitial invasion) is important for placental development. Factors secreted by the maternal decidual cells and the extravillous trophoblast can influence trophoblast invasion and abnormalities in the invasion process may lead to pregnancy complications. Serine protease HtrA3 is highly expressed in the decidual cells in the late secretory phase of the menstrual cycle and throughout pregnancy, and in most trophoblast cell types, apart from the invading interstitial trophoblast during the first trimester. HtrA3 and its family members are down-regulated in a number of cancers and are proposed as tumour suppressors. The current study aimed to investigate whether HtrA3 is secreted by decidual cells, and whether inhibiting such secretion alters trophoblast invasion.

METHODS AND RESULTS: Human endometrial stromal cells (HESCs) were decidualized with estradiol, medroxyprogesterone acetate and cyclic adenosine monophosphate. Real-time RT–PCR, western blotting and immunocytochemistry confirmed that HtrA3 mRNA and protein expression increased during decidualization. HtrA3 was also detected in the conditioned media (CM) of the decidualized HESCs, confirming its secretion. For functional studies, a protease-inactive mutant form of HtrA3 which was previously confirmed to be a dominant-negative inhibitor was produced using wheat germ cell-free technology. CM from decidualized HESCs significantly suppressed invasion of trophoblast HTR-8 cells (P, 0.01), whereas inhibition of HtrA3 in this CM by exogenous HtrA3 mutant resulted in increased trophoblast HTR-8 cell invasion (P, 0.001).

CONCLUSIONS: These results strongly support the hypothesis that decidual HtrA3 negatively regulates trophoblast invasion.

Key words: HtrA3 / trophoblast / invasion / protease / decidua

Introduction

Adequate invasion of the maternal decidua by extravillous trophoblast is of critical importance for implantation and placentation in humans. Interstitial extravillous trophoblast cells (EVTs) emanate from the cell columns of the anchoring chorionic villi and invade the maternal decidua as well as the inner third of the myometrium (Kaufmann and Castellucci, 1997). Paracrine interactions between the EVT and the maternal decidua are important for successful embryonic implantation, including establishing the placental vasculature and anchoring the placenta to the uterine wall (Pijnenborg, 1998; Lockwood et al., 2009).

During the invasive phase of implantation, both trophoblasts and maternal decidual stromal cells secrete products that regulate trophoblast differentiation and migration into the maternal endometrium (Graham and Lala, 1991; Bischof et al., 1995; Pijnenborg, 1998; Hannan et al., 2006; Paiva et al., 2009). Decidualization, which includes the transformation of stromal fibroblasts into decidual cells, remodelling of the vasculature, and extracellular matrix and infiltration and/or proliferation of macrophages and uterine natural killer cells, occurs during implantation and placentation (Gellersen and Brosens, 2003; Gellersen et al., 2007; Salamonsen et al., 2009). Although the decidua is believed to provide a cytokine/chemokine environment that attracts invading EVT (Red-Horse et al., 2004; Hannan et al., 2006), it also poses a physical barrier and restricts trophoblast invasion (Lala and Chakraborty, 2003; Fitzgerald et al., 2008; Orsi and Tribe, 2008; Knoeller, 2010). Impaired decidualization is associated with failure of placentation and pregnancy (Gendron et al., 1997; Lim et al., 1997; Francis et al., 2006; Salker et al., 2010).

HtrA3 is one of the four members of the HtrA family proteins, with a secreted multidomain and serine protease activity. Members of this family are conserved from bacteria to humans and serve dual functions: one as chaperone proteins at low/normal temperature and as...
proteases at high temperatures (Clausen et al., 2002). These members share two highly conserved domains, a trypsin-like catalytic domain followed by one or more C-terminal PDZ-binding domains (Zumbrunn and Trueb, 1996). Under conditions of cellular stress, the members of this protein family play a role in activating the cellular stress response and exhibit evidence of increasing proteolytic activity (Faccio et al., 2000; Gray et al., 2000; Chien et al., 2006). In humans, the HtrAs are involved in cell growth, apoptosis, invasion and inflammatory reactions (Clausen et al., 2002).

Expression of HtrA3 is developmentally regulated and localized to the endometrium and placenta during the first trimester of pregnancy (Nie et al., 2003b, 2006b). During the menstrual cycle, overall endometrial HtrA3 expression is highest in the late secretory phase, when the endometrium is prepared for maternal trophoblast interaction. It is strongly up-regulated in the decidual cells and selectively expressed in certain trophoblast subtypes at the maternal–fetal interface. Less invasive villous syncytiotrophoblast and cytotrophoblast express the highest levels of HtrA3, whereas the interstitial EVTs express the lowest levels (Nie et al., 2006b).

An interesting feature of the HtrA3 protein is that although it is a serine peptidase, it also contains an insulin-like growth factor (IGF)-binding domain at the N-terminal end immediately following the signal peptide (SP), suggesting that it can be secreted and may be involved in the IGF system (Nie et al., 2003a; Hou et al., 2005). The up-regulation of HtrA3 expression in association with placental development is accompanied by a significant elevation of this protein in the maternal serum during the first trimester (Nie et al., 2006b), supporting its secretion. HtrA3 is also secreted by the HTR-8 trophoblast cell line (Singh et al., 2010).

Mammalian HtrA1, the first discovered member of the HtrA family, shares identical domain organization with HtrA3, including an N-terminal SP, the insulin growth factor-binding domain (IGFBP) and a kazal-type S protease inhibitor domain, suggesting that they may have similar functions (Zumbrunn and Trueb, 1996; Nie et al., 2003a). However, they display different expression patterns among adult human tissues. The expression pattern of HtrA3 in the peri- and post-implantation uterus and also in non-villous trophoblast is much higher than that of HtrA1, suggesting that HtrA3 plays the more important role in the formation and function of the placenta (Nie et al., 2006a,b). In cancers, over-expression of either HtrA1 or HtrA3 inhibits cell growth and proliferation in vitro and in vivo, and also plays a protective role in various malignancies due to the tumour suppressive properties of these proteases (Baldi et al., 2002; Shridhar et al., 2002; Chien et al., 2004; Bowden et al., 2006; Beleford et al., 2010). We recently demonstrated a similar inhibitory effect of HtrA3 on trophoblast invasion in vitro (Singh et al., 2010).

The distinct distribution of HtrA3 at the maternal trophoblast interface suggests that decidual HtrA3 may restrict trophoblast invasion and play a role in placental development. The present study investigated the expression and regulation of HtrA3 mRNA and protein in the first trimester decidua and in in vitro decidualized primary human endometrial stromal cells (HESCs). Further, to investigate paracrine interactions at the decidua–trophoblast interface, we applied a specific inhibitor of HtrA3 (Singh et al., 2010) to determine whether HtrA3 secreted by primary decidualized HESCs would alter the invasive capacities of HTR-8 trophoblast cells.

### Materials and Methods

#### Tissues

Human endometrial tissues were obtained by curettage from women with normal menstrual cycles and no apparent endometrial dysfunction, undergoing minor gynaecological surgical procedures, such as laparoscopic sterilization or investigation of tubal patency. Decidual tissues were obtained from women undergoing elective termination of pregnancy (amenorrhea 8–12 weeks). Approval was obtained from the Ethics Committee at Monash Medical Centre, Melbourne and written informed consent was obtained from each participating patient.

For Northern analysis, tissues (n = 4) were immersed in RNA-Later (Ambion, Austin, TX, USA) immediately after collection and stored at −80°C for subsequent RNA extraction. For immunohistochemical analysis, tissues (n = 7) were fixed in buffered formalin (pH 7.4) at 4°C overnight, washed in Tris-buffered saline (TBS, pH 7.4) and processed to paraffin wax blocks. For cell culture experiments, endometrial biopsies (n = 4) were collected into Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, St. Louis, MO, USA) and processed within 24 h for stromal cell isolation.

#### Isolation and culture of HESCs

Endometrial stromal cells were isolated from tissue by enzymatic digestion and filtration as previously described (Heng et al., 2010) with the following modifications. Briefly, the tissues were washed with phosphate-buffered saline (PBS), finely minced and digested with PBS containing 0.03% collagenase type 3 (Worthington, Lakewood, NJ, USA), 0.02% deoxyribonuclease type I (Roche, Castle Hill, NSW, Australia) and 37°C shaking water bath for ~40 min. Digestion was stopped with serum-free DMEM/F12 and the digest was subjected to consecutive filtration through 44 and 11 micron nylon mesh to remove epithelial cells. Stromal cells were collected and plated in tissue culture flasks (775 cm²; Corning, Castle Hill, NSW, Australia) and cultured in DMEM/F12 (Sigma) medium containing 10% charcoal stripped-fetal calf serum (CS-FCS, Thermo Electron Corporation, Maple Plain, MN, USA), supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, Mulgrave, VIC, Australia). Stromal cell preparations obtained by this procedure were >97% pure as assessed by immunostaining for cytokeratin and vimentin as previously described (Dimitriadis et al., 2002).

Once 70–80% confluent, HESCs were trypsinized and then plated in T-25 cm² tissue culture flasks (Corning), cultured to 80% confluence and then treated with estradiol 17-β (E₂, 10⁻⁷ M), medroxy-progesterone acetate (MPA, 10⁻⁷ M) and cyclic adenosine monophosphate (cAMP, 500 μM) (E₂, MPA and cAMP, Sigma) for 0–96 h in DMEM/F12 containing 0.1% BSA at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Untreated cells served as non-decidualized controls. Following incubation, culture media was collected and centrifuged supernatant was assayed for the decidual cell marker prolactin (PRL) by ELISA (Biodclone Aust. Pty. Ltd., Marrickville, Australia) as per the manufacturer’s instructions. The cells were subjected to morphologic assessment, and then harvested for RNA isolation or protein extraction. Cell extracts and conditioned medium (CM) were centrifuged (900g) and supernatant protein was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. Four independent experiments were performed using different cell preparations. For immunostaining, cells were seeded on 14 mm sterile glass coverslips in 12-well plates (8 x 10⁴ cells/well) and a similar decidualization procedure was followed.

#### HTR-8/SVneo cell culture

The HTR-8/SVneo (HTR-8) trophoblast cell line was kindly provided by Dr. C.H. Graham (Queen’s University, Kingston, ON, Canada). Cells...
were cultured at 37°C in an atmosphere of 5% CO₂/95% air in RPMI 1640 medium (Sigma) containing 10% FCS (SAFC Biosciences, Kansas, USA), 20 mM HEPES, 7.5% (w/v) sodium bicarbonate (Sigma), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco). Cells were grown in 75 and 25-cm² culture flasks (Corning) for cell line maintenance and treatment.

**RNA extraction, northern blot analysis and RT–PCR**

Total RNA was extracted from human decidua (n = 4) and Northern analysis (15 µg/lane) was performed as previously published (Nie et al., 2003c). A cDNA fragment of 457 bp representing the common region of human HtrA3 mRNAs, capable of detecting both HtrA3 transcripts, was used as a probe (Nie et al., 2003a). To evaluate the quality of RNA on the membrane, each blot was hybridized with a cDNA probe for GAPDH.

HESCs were lysed and processed for total RNA isolation using an RNeasy MiniKit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. RNA was treated with ribonuclease-free deoxyribonuclease-I (DNA-free kit, Ambion) to remove any contaminating genomic DNA. RNA concentration was determined at 260 nm after deoxyribonuclease-1 (DNA-free kit, Ambion) to remove any contaminating genomic DNA. RNA concentration was determined at 260 nm using a ND1000 Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Total RNA (0.5 µg) was preheated at 65°C for 5 min, then reverse transcribed at 46°C for 2 h in a 20 µl reaction mixture using 100 ng random hexanucleotide primers, 5 IU avian myeloblastosis virus reverse transcriptase, 10 mM dNTPs, 100 mM dithiothreitol and 20 IU ribonuclease inhibitor in the presence of cDNA synthesis buffer (all from Roche, Mannheim, Germany). The resultant cDNA mixtures were heated at 95°C for 3 min before storage at −20°C. Negative controls were performed by omission of reverse transcriptase.

The cDNA product (1 µl) was amplified in a total volume of 26 µl using 13 µl GoTag green master mix (Promega, Hayward, VIC, Australia) and 10 pmol of forward and reverse primers for HtrA3 isoforms (long (L) and short (S)) and 18S (Table I; Nie et al., 2006b). A no template control in which deethyl pyrocarbonate water was substituted for RNA was included as a negative control. All PCRs were run in duplicate and products were analysed by electrophoresis on 1% agarose gel (Roche) and stained with Gel Red (Biotium, Stepney, SA, Australia). As a loading control, 18S was analysed by electrophoresis on 1% agarose gel (Roche) and stained with Gel Red (Biotium, Stepney, SA, Australia). As a loading control, 18S was included as a negative control. All PCRs were run in duplicate and products were analysed by electrophoresis on 1% agarose gel (Roche) and stained with Gel Red (Biotium, Stepney, SA, Australia). As a loading control, 18S was included as a negative control. All PCRs were run in duplicate and products were analysed by electrophoresis on 1% agarose gel (Roche) and stained with Gel Red (Biotium, Stepney, SA, Australia). As a loading control, 18S was included as a negative control. All PCRs were run in duplicate and products were analysed by electrophoresis on 1% agarose gel (Roche) and stained with Gel Red (Biotium, Stepney, SA, Australia). As a loading control, 18S was included as a negative control. All PCRs were run in duplicate and products were analysed by electrophoresis on 1% agarose gel (Roche) and stained with Gel Red (Biotium, Stepney, SA, Australia). As a loading control, 18S was included as a negative control. All PCRs were run in duplicate and products were analysed by electrophoresis on 1% agarose gel (Roche) and stained with Gel Red (Biotium, Stepney, SA, Australia). As a loading control, 18S was included as a negative control. All PCRs were run in duplicate and products were analysed by electrophoresis on 1% agarose gel (Roche) and stained with Gel Red (Biotium, Stepney, SA, Australia). As a loading control, 18S was included as a negative control. All PCRs were run in duplicate and products were analysed by electrophoresis on 1% agarose gel (Roche) and stained with Gel Red (Biotium, Stepney, SA, Australia). As a loading control, 18S was included as a negative control. All PCRs were run in duplicate and products were analysed by electrophoresis on 1% agarose gel (Roche) and stained with Gel Red (Biotium, Stepney, SA, Australia). As a loading control, 18S was included as a negative control. All PCRs were run in duplicate and products were analysed by electrophoresis on 1%

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences (5’–3’)</th>
<th>Annealing temperature (°C) and cycles (n)—Conventional</th>
<th>Annealing temperature (°C) and cycles (n)—Quantitative</th>
</tr>
</thead>
<tbody>
<tr>
<td>HtrA3 (Long)</td>
<td>F: ATG CGG ACG ATC ACA CCA AG 64 (26 cycles) 64 (26 cycles)</td>
<td>58 (30 cycles) 62 (44 cycles)</td>
<td>53 (35 cycles) 62 (44 cycles)</td>
</tr>
<tr>
<td>HtrA3 (short)</td>
<td>F: GAG GGC TGG TCA CAT GAA GA R: GCT CCG CTA ATT TCC AGT</td>
<td>64 (26 cycles)</td>
<td>64 (26 cycles)</td>
</tr>
<tr>
<td>18S</td>
<td>F: CGG GTA CAT CCA CAT ACC AGG AA R: GCT GGA ATT ACC GCG GCT</td>
<td>64 (26 cycles)</td>
<td>64 (26 cycles)</td>
</tr>
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F, forward; R, reverse.

Briefly, PCR of all standards and samples (n = 4) was performed using duplicate reactions; sample cDNA was diluted 1:5 fold (HtrA3 isoforms—L and S) and 1:500 fold (18S). The diluted cDNA template (2 µl) was added to PCR reaction tubes to a total volume of 20 µl containing PCR master mix (Roche), including SYBR Green I, dNTPs, Taq polymerase enzyme, and reaction buffer, supplemented with optimal concentrations of MgCl₂ (3 nmol) and specific primers (10 pmol) for forward and reverse (Table I).

The PCR reaction was initiated by a denaturing step of 10 min at 95°C followed by 26–44 cycles of 95°C for 10 s, 62–64°C for 5 s and 72°C for 6–16 s (specific parameters; Table I). The standard curve method was used to quantify the expression of HtrA3 (L and S) mRNA and 18S rRNA in each sample. Melt curve analysis was carried out to monitor PCR product purity. The ratio of HtrA3 mRNA level to that of 18S was calculated for each sample and the relative level of HtrA3 expression in decidualized HESCs was expressed as percentage of the non-decidualized controls.

**Immunohistochemistry**

Sections of 5 µm thickness were subjected to standard immunohistochemistry. Following antigen retrieval by microwaving the sections for 5 min in 0.01 M citric acid buffer (pH 6.0), non-specific binding was blocked by pre-incubation of tissue sections with a blocking buffer containing high-salt TBS (0.3 M NaCl in 50 mM Tris, pH 7.6). 0.1% Tween, 15% rabbit serum and 2% horse serum for 20 min at room temperature. The primary antibody (sheep anti-HtrA3 antibody (Nie et al., 2006b) or pre-immune sheep IgG as a negative control (both 0.5 µg/ml) was incubated in the blocking buffer at 37°C for 1 h and washed with high-salt TBS plus 0.6% Tween. The secondary antibody (biotinylated rabbit anti-sheep IgG, 1:200; Vector Laboratories, Burlingame, CA, USA) was applied in the blocking buffer for 30 min at room temperature. Positive immunostaining was revealed by incubating the sections with an avidin–biotin–complex conjugated to horseradish peroxidase (DakoCytomation, Botany, NSW, Australia) for 30 min at room temperature, following the application of the peroxidase substrate 3,3′-diaminobenzidine (DAB, DakoCytomation) leading to a brown precipitate for positive staining. The sections were counterstained with Harris haematoxylin. Microscopy was performed using an Olympus BH2 microscope fitted with a Fujix HC-2000 high-resolution digital camera (Fujix, Tokyo, Japan).

**Protein extraction and western blotting**

HESCs were lysed with RIPA buffer (65 mM Tris-base (pH 7.4), 1% Nonidet P40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) and HESCs CM was concentrated 10 times with Vivaspin-2 (Sartorius Stedim Biotech GmbH, Goettingen, Germany). Equal amount of total protein (36 µg/ml cell lysates and 50 µg/ml CM) were separated on
10% SDS–PAGE under reducing conditions and transferred to Hybond-P membranes (Amersham Life Science, Sydney, Australia). After overnight blocking at 4°C in a blocking buffer [5% (w/v) skimmed milk in TBS and 0.1% (v/v) Tween 20], the membrane was incubated with sheep anti-human HtrA3 antibody at 1:20 dilution in the blocking buffer for 2 h at room temperature, then with a HRP-conjugated donkey anti-sheep IgG (1:5000; Chemicon, Australia) for 1 h at room temperature, before being developed by chemiluminescence (ECL Plus system, Amersham).

**Immunofluorescence**

All procedures including incubations and washes were carried out at room temperature. HESCs were grown on 14 mm glass coverslips and decidualized as mentioned earlier. Following decidualization cells were washed twice in PBS, fixed with 4% paraformaldehyde (BDH, Poole, UK) for 30 min, washed twice with PBS and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 15 min. The cells were then blocked with 1:20 sheep serum for 30 min, incubated with HtrA3 antibody (1:20) for 2 h, washed in PBS for 3 × 5 min and finally incubated with 1:50 polyclonal goat anti-sheep Alexafluor 568 (Invitrogen) for 1 h in the dark. The nuclei were stained with 2 μM 4',6-diamidino-2-phenylindole dilactate (DAPI, Sigma) for 10 min and the coverslips were mounted with fluorescent reagent (Calbiochem, Kilsyth, Australia). Staining was examined with an Olympus BX60 fluorescent microscope and images were taken using an Olympus DP70 camera and DP controller imaging Leica software.

**Production of recombinant human HtrA3 mutant as an inhibitor**

A mutant form of human HtrA3 (long form), which was previously validated as a dominant-negative inhibitor of HtrA3 (Singh et al., 2010), was produced using wheat-germ cell-free technology (CellFree Sciences). In brief, the open reading frame of HtrA3-L cDNA was GST tagged and cloned into the pCFS-TRI-1240G kit (CellFree Sciences) per manufacture’s instructions. A mutant was generated by site-directed mutagenesis to substitute amino acid serine with alanine (S305A) within the trypsin catalytic triad to induce catalytic inactivation. The recombinant protein was synthesized in vitro, purified using glutathione sepharose 4B gel (GE Healthcare Bio-Sciences, Rydalmere, NSW, Australia), and analysed by SDS–PAGE to confirm its purity (Singh et al., 2010). Using an Olympus BX60 camera and DP controller imaging Leica software.

**Mitochondrial dehydrogenase assay**

The mitochondrial dehydrogenase (MMD) assay which is widely used as a measure of cell viability was performed as described previously (Harris et al., 2009). In brief, HTR-8 cells were seeded in 96-well plates and allowed to adhere. The culture medium was replaced with fresh medium containing 10% FBS, with the addition of 10% CM from HESCs (control and decidualized) or PBS. After 24 h, the culture medium was removed and replaced with medium containing 0.05% (w/v) MTT in PBS. HTR-8 cells were incubated for a further 3 h, then the culture medium was removed and DMSO (100 μL/well) was added to the cells to solubilize the formazan crystals generated. After 1 min on a shaker, the absorbance of each well at 550 nm was measured using a plate reader.

**In vitro matrigel invasion assay**

Cell invasion was determined using the QCM™ 24-well Cell Invasion Fluorimetric Assay with ECMMatrix™ matrigel coated inserts (Chemicon, Melbourne, Australia), according to the manufacturer’s instructions.

HTR-8 cells (70–80% confluent) were incubated for 24 h in culture medium (RPMI-1640) containing 1% FCS, then trypsinized and resuspended in culture medium (1.25 × 10^5–2.5 × 10^5 cells/250 μl). To eliminate any effect of exogenously added steroids, CM from non-decidualized HESCs was later supplemented with equivalent concentrations of E_2, MPA and CAMP prior to co-culturing with HTR-8 trophoblast cells. HTR-8 cells were preincubated with 10% CM from decidualized or non-decidualized HESCs for 15–30 min at room temperature prior to seeding them on the inserts pre-soaked with serum-free culture medium. About 5% FCS was added to the lower chamber as the chemotacticant. Controls were untreated HTR-8 cells supplemented with normal culture medium on culture inserts. After 48 h incubation at 37°C, the invaded cells on the lower surface of the inserts were detached by prewarmed detachment solution (supplied with the kit) and incubated at 37°C for 30 min, followed by incubation with a lysis buffer/CyQuant GR dye solution for 15 min at room temperature (as per the manufacturer’s instructions). Fluorescence measurements were reported as relative fluorescence units (Wallac plate reader, Victor 2 spectrofluorometer, Perkin Elmer, MA using a 485/525 nm filter set). To identify any effect of secreted HtrA3 on HTR-8 trophoblast cell invasion, a separate set of experiments were performed; CM from decidualized HESC was preincubated with or without mutant HtrA3-L (24 ng/ml) for 15–30 min at room temperature prior to incubation with cells. Each invasion assay was repeated five times and data expressed as percent change (±SEM) relative to vehicle controls.

**Statistics**

Data are expressed as mean ± SEM fold change for each treatment relative to vehicle control. Statistical analysis was performed on raw data using one-way ANOVA, followed by Tukey’s post hoc test (P < 0.05 taken as significant), after testing for normal distribution using PRISM version 5.00 (GraphPad Software, San Diego, CA, USA). To compare treatment and control, a t-test was performed and P < 0.05 was taken as significant.

**Results**

**HTRA3 isoforms are expressed by human first trimester decidual cells**

Two transcripts of ~2.8 and ~2.2 kb, representing the two alternatively spliced forms of HtrA3 mRNA [long (L) and short (S), respectively] were detected by northern blotting in the total RNA isolated from pregnant decidua (Fig. 1A). The long form transcript was at a slightly higher level than the short form in three of the four samples, although the difference was not significantly different. The subcellular localization of endogenous HtrA3 was confirmed by immunohistochemistry in the first trimester decidual tissue (n = 7) with a sheep anti-human HtrA3 antibody suitable for immunohistochemistry (Nie et al., 2006b). A homogenous pattern of HtrA3 staining in all samples was detected in the cytoplasm of decidualized stromal cells (Fig. 1B). Moreover, in accordance with HtrA3 secretory properties, immunostaining was also observed in the stromal intracellular compartment. The staining pattern was similar in all the tissues and also when repeated in the same tissue.

**HTRA3 mRNA is significantly up-regulated during decidualization of HESCs in culture**

Cultured primary HESCs undergo differentiation from typical elongated fibroblast to rounded enlarged decidual cells in response
to exogenous E2 (10^{-8} M), MPA (10^{-7} M) and cAMP (500 μM) and this changed morphology was clearly evident at 96 h (not shown). The success of decidualization was confirmed by an induction of the decidualization marker, PRL which was detected in the CM after 24 h with significant time dependent increases at 48 h \((P, 0.01)\) and a maximum at 96 h \((P, 0.001; \text{Fig. 2A})\). Non-decidualized control HESCs showed undetectable basal levels of PRL throughout this time.

The mRNA expression levels of both HtrA3 isoforms (L and S) corresponding to the expected sizes were detected by RT–PCR in the decidualizing HESCs with a time dependent increase (Fig. 2B). Elevated expression levels of both HTRA3 isoforms were observed after 24 h of decidualization reaching a maximum at later time points. In contrast, mRNA for both HtrA3 isoforms was undetectable in the non-decidualized cells. Quantitative real-time PCR at 96 h of decidualization demonstrated a significant increase \((P < 0.01)\) in mRNA for both isoforms relative to time-matched non-decidualized controls (Fig. 2C).

**HtrA3 protein is detected in in vitro decidualized HESC cell lysate and CM**

Western blot analysis using a polyclonal antibody capable of detecting both HtrA3 isoforms revealed two specific bands (~49 and ~39 kDa) in cell lysates from control and decidualized HESCs, confirming HtrA3 protein expression in these cells (Fig. 3A). In accordance with the
mRNA expression data, the band intensities were stronger in decidualized cell lysates.

The secretory nature of HtrA3 was confirmed by its detection in the conditioned media (CM) from HESCs. Two specific bands (~41 and ~30 kDa) were detected with the intensities stronger in CM from decidualized cells at 96 h compared with time-matched non-decidualized controls (Fig. 3A).

The cellular localization of HtrA3 in the cultured cells was demonstrated by immunofluorescence. A clearly different level and localization for HtrA3 was seen in non-decidualized and decidualized HESCs (Fig. 3B). An intense and homogeneous pattern of staining for HtrA3 was observed in all decidualized cells compared with the weak and mosaic pattern observed in non-decidualized controls.

CM from decidualized stromal cell inhibits invasiveness of HTR-8 trophoblast cells

Using an invasion assay, we assessed whether secreted factors (including HtrA3) derived from decidualized and non-decidualized HESCs would influence the invasiveness of HTR-8 trophoblast cells. A significant reduction (P < 0.001) in HTR-8 cell invasion was observed when incubated with CM from decidualized HESCs compared with the non-treated control, whereas CM from non-decidualized cells had a minimal effect (Fig. 4A). The reduction in HTR-8 cell invasion caused by decidual CM was highly significant compared with both non-treated and non-decidual CM-treated counterparts (Fig. 4A). When the HtrA3 specific inhibitor, mutant HtrA3-L [24 ng; HtrA3 dose selected based on our previous study (Singh et al., 2010)] was added to the CM from decidualized cells, the invasion was significantly increased (Fig. 4B). A MTT assay established that there was no difference in cell viability when HTR-8 cells were incubated with CM from decidualized HESCs compared with CM from non-decidualized HESCs (data not shown).

Discussion

This paper demonstrates that HtrA3 is up-regulated in decidual cells both in vivo and in vitro and that HtrA3 secreted from decidual cells contribute to the highly dynamic processes of trophoblast invasion at the fetal–maternal interface. We isolated primary HESCs from women and decidualized them in culture, then demonstrated that the invasive capacity of trophoblast cells (HTR-8) was inhibited by CM from in vitro decidualized HESCs. We further demonstrated that HtrA3 in the decidual cell CM contributed to this inhibition, as the inhibition was partially eliminated by inhibiting HtrA3 using a HtrA3 dominant-negative inhibitor. These results support the hypothesis that decidual HtrA3 negatively regulates trophoblast invasion.

Being aware of the limitations inherent in the use of cell lines compared with primary cells, we used the well-characterized human trophoblast-derived HTR-8 cell line as a model for trophoblast cells (Hannan et al., 2010). The HTR-8 cells exhibit markers of primary EVT cells in situ including cytokeratins, IGF2 and placental-type alkaline phosphatase and produce an appropriate repertoire of ECM, adhesion molecules and HLA-G (Irving et al., 1995; Kilburn et al., 2000; Horita et al., 2007; Lash et al., 2007; Jacob et al., 2008). These cells have a phenotype and physiological characteristics consistent with those of normal first trimester trophoblast (Irving et al., 1995) and have been widely used for migration and invasion studies (Gleeson et al., 2001; Chakraborty et al., 2003; Belkacemi et al., 2005; Huber et al., 2006; Horita et al., 2007; Lash et al., 2007; Zhou et al., 2009; Jovanovic et al., 2010).

First trimester human trophoblast cells share similar intrinsic invasive properties of tumour cells in vitro (Yagel et al., 1988). EVT invasion of the maternal decidua is a complex process and includes regulated proteolytic degradation of decidual ECM and altered adhesion to ECM, followed by cell migration and invasion through the degraded matrix (Mareel and Leroy, 2003). Decidua acts to alter the local microenvironment by secreting many factors which control trophoblast invasion.
Decidualization of stromal cells involves dramatic proliferation and differentiation of endometrial stromal fibroblasts (Gellersen and Brosens, 2003; Gellersen et al., 2007; Salamonsen et al., 2009). A strong link between HtrA3 expression and endometrial stromal cell decidualization was established here in an in vitro model using primary HESCs. HtrA3 mRNA increased in parallel with increasing levels of decidualization (detected by PRL secretion) and a similarly increased protein level was detected in both cell lysate and CM. HtrA3 is also detected with initiation of decidualization during the menstrual cycle, but immunoreactive intensity is much higher in the highly advanced decidual cells in early pregnancy (Nie et al., 2006b).

Since inhibitors of HtrA3 are not commercially available, our use of a previously validated recombinant HtrA3 mutant enabled us to determine the effect of secreted HtrA3 on trophoblast invasion. Our previous study confirmed that the mutant HtrA3 inhibited the activity of WT HtrA3 forms, providing a critical reagent for functional studies (Singh et al., 2010). The mutant HtrA3 generated by site-directed mutagenesis of the catalytic serine had no inherent protease activity, consistent with inactivation of other serine proteases by a similar strategy (Hu et al., 1998; Hara et al., 2009). Dominant-negative inhibition by co-expression of its non-functional mutant forms is a widely used strategy to inhibit the function of a protein (Tsaiavalis et al., 2002; Wu et al., 2004; Overall and Blobel, 2007), whereby the mutants bind to the enzyme substrate without cleaving it, thereby limiting substrate availability for the wild-type enzyme. Alternatively the mutant could form a non-functional complex with the enzyme with high affinity and specificity, thus blocking the interaction between the wild-type enzyme and the substrate (Kodadek, 2008). This mutant provided a powerful tool for the functional studies described here.

Trophoblast invasion during normal pregnancy is tightly regulated both spatially and temporally (Anin et al., 2004) and requires a fine balanced interplay between factors that promote and restrict invasion. Dysregulation of this process can lead to pathological conditions such as placental accreta and pre-eclampsia (McMaster et al., 2004; Cross, 2006; Lyall, 2006). Evidence suggests that control of trophoblast invasion is mainly regulated by products of the decidua (Graham and Lala, 1991) such as TIMPS (Zhu et al., 2009), IGFBP1 (Han et al., 1996), TGF-β1 (Zhu et al., 2009) and IL-11 (Dimitriadis et al., 2002), all of which inhibit trophoblast invasion in vitro (Graham and Lala, 1991; Paiva et al., 2009). On the other hand, consistent with the paradigm that factors that promote invasion are primarily thought to be trophoblast derived, EVTs secrete MMPs (Aplin, 1991) and IGF2 (Hamilton et al., 1998; McKinnon et al., 2001). In line with the previous findings, our study has also confirmed that factors secreted by HESC including HtrA3 have profound effects on trophoblast invasion. A significant increase in HTR-8 trophoblast invasion after HtrA3 activity in the CM was neutralized by the highly specific HtrA3 mutant provides direct experimental evidence that decidual HtrA3 can restrain trophoblast invasion. A recent study by Li et al. (2010) confirmed a strong correlation between abnormally high maternal serum levels of HtrA3 at 13–14 weeks of gestation and pre-eclampsia. We speculate that altered expression of HtrA3 by decidual cells due to inadequate levels of decidualization, uteroplacental ischaemia or abnormal cytokine/hormone expression may affect trophoblast invasion and migration and contribute to complications of pregnancy.

It is also evident that EVTs express negligible amounts of HtrA3 in comparison with that produced by the decidua. Previous studies have

(Knofer, 2010). CM from first trimester human decidual cells suppress invasion of trophoblast cells (Graham and Lala, 1991; Zhu et al., 2009). We have demonstrated that CM from decidualized HESCs decreases HTR-8 trophoblast cell invasion, extending the findings of Graham and Lala (1991) and Zhu et al. (2009). In contrast, Zhu et al. (2009) demonstrated that CM from decidualized HESCs stimulates trophoblast cell invasion. The difference is likely due to our use of cAMP in addition to E2 and P which considerably enhances decidualization of stromal cells in vitro beyond that with E2 and P alone, with the cells more closely representing the decidua of pregnancy. Importantly the cells used by Graham and Lala were a mixed population of epithelial cells, stromal cells and different subsets of leukocytes, whereas the present study used highly purified decidual stromal cells. The results indicated that the decidual cells may provide a key control to restrain invasion by trophoblast.

Figure 4 Effect of CM from decidual cells with and without inhibition of HtrA3 on HTR-8 cell invasion. (A) HTR-8 cells were incubated with CM from decidualized (Dec-CM) and non-decidualized (Non-Dec-CM) HESCs or control medium (Ctrl), and their invasive capacities were assessed by invasion assay. (B) Exogenous addition of HtrA3 inhibitor (24 ng mutant HtrA3-L) to Dec-CM significantly increased HTR8-cell invasion compared with Dec-CM alone control. Data is combined from five independent experiments and expressed as mean ± SEM (⁎⁎p < 0.01, ***p < 0.001).
reported that HtrA3 is involved in regulating both TGFβ1 and IGF/IGFBP systems at the maternal–trophoblast interface (Irving and Lala, 1995; Hamilton et al., 1998; Irwin et al., 1999; Chakraborty et al., 2002; Tocharus et al., 2004) and it is therefore likely that HtrA3 produced by decidua may modulate these signalling pathways to negatively regulate trophoblast invasion during pregnancy. However, the precise mechanism by which HtrA3 alters these signalling pathways remains elusive. In addition, HtrA3 may have roles in modulating ECM and ECM proteins (Lysiak et al., 1995; Tocharus et al., 2004; Iacob et al., 2008), implying that it may directly modulate the ECM microenvironment within the decidua, to regulate trophoblast invasion. Future studies are required to identify the mechanism of HtrA3 action in trophoblast invasion and also its interaction with both the TGF and IGF pathways.

In conclusion, this study demonstrated clearly that HtrA3, a serine protease produced by decidualized HESCs can influence trophoblast invasion in vitro and points to a similar role in vivo as the invading trophoblast journeys through the maternal decidua. The intrinsic loss of HtrA3 in invasive trophoblast and its up-regulation in decidualized HESCs suggest a contributing role for HtrA3 in regulating trophoblast invasion through both autocrine and paracrine mechanisms. Identification of HtrA3 substrates in the physiological context of placentation is now needed to establish the underlying molecular mechanism of HtrA3 action.

**Authors’ roles**

H.S. did all the cell experiments, analysed the data, and wrote the manuscript. Y.E. synthesized and purified the recombinant HtrA3. G.N. contributed towards study design, result interpretation and manuscript writing.

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