Invasiveness of human endometrial stromal cells is promoted by decidualization and by trophoblast-derived signals

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BACKGROUND: Extensive invasion of the maternal decidua by extravillous trophoblast is considered of critical importance for implantation and placentation in humans, the decidua being viewed as a passively invaded tissue. In this study, we examined whether decidual cells might contribute to the highly dynamic processes at the fetal–maternal interface by active movement.

METHODS: Primary endometrial stromal cells (ESCs) or the telomerase-immortalized ESC line, St-T1b, was induced to decidualize or was left undifferentiated. The AC-1M88 cell line served as a model for extravillous trophoblast cells. Motility of ESCs and trophoblast cells was monitored in transwell invasion and migration assays under co-culture conditions. Secretion of matrix metalloproteinases (MMPs) was assessed by gelatin zymography.

RESULTS: AC-1M88 cell invasiveness was unaffected by the presence of ESCs, irrespective of their decidualization status. Surprisingly, decidualized ESCs were significantly more invasive than undifferentiated cells, and this invasive activity was strongly enhanced when cells were cultured in direct contact with AC-1M88 cells. Conditioned medium from AC-1M88 cells also stimulated migration and invasion of ESCs. Secretion of MMP-2 and -9 by ESCs was increased upon decidualization.

CONCLUSIONS: Enhanced motility and invasive capacity of decidualized ESCs in the presence of trophoblastic cells lead us to hypothesize a major contribution of the decidua in encapsulating the early conceptus and supporting subsequent trophoblast invasion. Our findings thus suggest a far more active role of the decidua in the implantation process than hitherto recognized.

Key words: human endometrial stroma / decidualization / invasion / trophoblast / motility

Introduction

Growth and survival of the developing fetus are critically dependent on the integrity of the fetal–maternal interface. 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. A subpopulation of EVT’s intravasates the decidual spiral arteries and initially plugs the vessels to restrict exposure of the early placenta and fetus to maternal arterial blood. Between 11 and 14 weeks of gestation, plugs are dislodged, the endovascular EVT’s replace the endothelial lining of the vessels and dramatically widen their diameter, ultimately establishing a high-flow, low-resistance utero-placental circulation to supply the fetus with nutrients and oxygen (Kliman, 2000; Kaufmann et al., 2003; Burton and Jauniaux, 2004; Chaddha et al., 2004; Red-Horse et al., 2004). Thus, the invasive nature of EVT’s is essential for the survival of the conceptus, yet the extent of invasion must be tightly controlled. Although the decidua is believed to provide a cytokine/chemokine environment that attracts invading EVT’s, it also poses a physical barrier on the way to gaining access to the maternal blood supply. One of the major constituents of decidual extracellular matrix (ECM) is collagen IV (Iwahashi et al., 1996), which is a substrate of matrix metalloproteinases (MMP’s) -2 and -9 (Sternlicht and Werb, 2001). These proteases are expressed by trophoblast cells (Cohen et al., 2006; Ferretti et al., 2007), whereas decidual cells produce tissue inhibitors of MMP’s (TIMP’s), antagonizing the proteolytic activity of EVT’s (Higuchi et al., 1995; Lockwood et al., 1998; Irwin et al., 2001; Dimitriadis et al., 2005).
Decidualization is a differentiation process of endometrial stromal cells (ESCs) that, in humans, is initiated in the secretory phase of the menstrual cycle in response to progesterone but independently of a blastocyst signal. The transformation of elongated stromal fibroblasts into secretory epitheloid-like decidual cells is first apparent in the vicinity of terminal spiral arteries and then spreads throughout the endometrial compartment via autocrine and paracrine signals. Typical secretory products of decidualized cells are insulin-like growth factor-binding protein-1 (IGFBP-1) and decidual prolactin (dPRL) (Gellersen and Brosens, 2003; Jones et al., 2006; Gellersen et al., 2007b). Decidual cells also express the tetraspinin CD82 (KAI-1), a cell surface protein that functions as a metastasis suppressor in tumour cells (Jackson et al., 2005; Gellersen et al., 2007a). Although migratory and invasive potential of ESCs has been recognized, its significance has only been discussed for processes outside pregnancy. After menstruation, there is a need for motile cells to regenerate the endometrial functional layer. The pathobiology of endometriosis involves the ability of endometriotic cells to migrate to distant sites (Matsumoto et al., 2005; Nasu et al., 2005; Banu et al., 2008; Ferreira et al., 2008). The role of decidualization for motile behaviour of ESCs has not been specifically addressed so far, and neither has the invasive potential of decidualized cells been considered in the context of implantation.

The initial contact of the implanting blastocyst during the steps of apposition and attachment is made with the luminal epithelium of the endometrial lining ~6–7 days after conception. But as soon as the epithelium is breached, the interface lies between trophoblast and decidual cells. It is therefore the decidua–trophoblast dialogue that orchestrates the remarkably dynamic process by which the blastocyst becomes completely embedded in the uterine wall within a few days, as early as 10 days after conception (Norwitz et al., 2001). The present study aimed at further exploring the role of the decidua in the remodelling of the fetal–maternal interface in early pregnancy.

Investigations into the intercellular communication between decidual and trophoblast cells in humans are largely restricted to cell culture experimentation. Differentiated invasive EVTs by nature are not proliferating, so the only way of procuring appreciable cell numbers is the outgrowth from villous explants (Genbacev et al., 1992, 1993; Kaufmann et al., 2003; Korgun et al., 2006; Mardon et al., 2007). To combine infinite proliferative capacity with a terminally differentiated invasive phenotype, the hybridoma cell line, AC-1M88, a fusion of primary EVTs from term placenta with a hypoxanthine-guanine phosphoribosyltransferase-deficient mutant of the JEG-3 choriocarcinoma cell line, was kindly provided by H.G. Frank and P. Kaufmann (University Hospital Aachen, Germany) (Funayama et al., 1997; Gaus et al., 1997) and was maintained in DMEM/Ham’s F12 with 10% steroid-depleted dialysed fetal bovine serum (FCS) (PromoCell, Heidelberg, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.2% Primocin (Invivogen, San Diego, CA, USA), and supplemented with insulin (1 μg/ml) and 17β-estradiol (E2; 1 nM) (Sigma-Aldrich, Deisenhofen, Germany). Decidualization was induced in minimal medium I (MM1; ESC medium without insulin and E2) by treatment with 0.5 mM 8-Br-cAMP for 4–5 days (Biolog, Bremen, Germany). The local ethics committee approved this study and patient consent was obtained before tissue collection.

The telomerase-immortalized ESC-derived cell line, St-T1b, was maintained and induced to decidualize as described for primary ESCs, except that Primocin was omitted (Samalecos et al., 2009).

In the present study, we assessed the response of ESCs (primary or immortalized) to trophoblastic cells, with a special emphasis on the role of decidualization for their migratory and invasive capacities.

Materials and Methods

Cell culture

Primary cultures of human ESCs were prepared from anonymized uterine biopsy samples obtained from premenopausal women at the time of hysterectomy for benign gynaecological disorders. Purified ESCs were prepared as described previously (Gellersen et al., 1994) and maintained in ESC medium: phenol red-free Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 with 10% steroid-depleted dialysed fetal bovine serum (FCS) (PromoCell, Heidelberg, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.2% Primocin (Invivogen, San Diego, CA, USA), and supplemented with insulin (1 μg/ml) and 17β-estradiol (E2; 1 nM) (Sigma-Aldrich, Deisenhofen, Germany). Decidualization was induced in minimal medium I (MM1; ESC medium without insulin and E2) by treatment with 0.5 mM 8-Br-cAMP for 4–5 days (Biolog, Bremen, Germany). The local ethics committee approved this study and patient consent was obtained before tissue collection.

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Matrigel invasion assay

Invasive capacity of cells was assessed in Biocoat Matrigel invasion chambers with 8 μm pore size (BD Biosciences, Heidelberg, Germany). After rehydration of the chambers according to the manufacturer’s instructions, 750 μl of medium (MM1) was placed into the lower well, the insert was put in place and 2.5 × 10^4 cells (AC-1M88, St-T1b or primary ESCs) were plated in 500 μl of MM1 into the insert. For each treatment condition, six inserts were plated in parallel. After 48 h of incubation at 37°C, the cells were fixed in methanol for 2.5 min, followed by washing in solutions II and III from the Diff-Quik Staining Kit (Siemens Healthcare Diagnostics, Eschborn, Germany) for 2.5 min each. After rinsing in water, six parallel inserts were split into two groups and processed as follows: from three inserts, the cells that had invaded to the lower side of the membrane were wiped off with a cotton swab, and from the remaining three inserts, the non-invasive cells on the upper side were removed (see Fig. 3). On each membrane, cells were counted in six randomly selected visual fields (×10 objective) and the mean number of cells per visual field (N) was determined. Numbers of cells on the upper side (N_u, from n = 3 membranes) added to numbers of cells on the lower side (N_l, from n = 3 membranes) yielded the total cell number (N_T) per visual field. The percent invasiveness (%) was
calculated as follows:

\[ I = \frac{N_I}{N_U} \times 100 \]

Because \( N_I \) is the sum of independent populations \( (N_{U} + N_{C}) \) with standard deviations \( S_{U} \) and \( S_{C} \), the Gaussian error propagation law was applied to calculate the standard deviation of \( N_I \), \( S_I \):

\[ S_I = \sqrt{\left(\frac{S_U}{N_U}\right)^2 + \left(\frac{S_C}{N_C}\right)^2} \times I \]

The Matrigel co-invasion assay

To assess invasiveness in a co-culture setting, a co-invasion assay was devised on the basis of the Matrigel invasion protocol with the following modifications: 750 \( \mu \)l of medium (MM1) was placed into the lower well, the insert was put in place, 300 \( \mu \)l of medium (MM1) was added to the insert and two cell types were plated simultaneously using 1.25 \( \times \) 10^4 cells each co-suspended in 200 \( \mu \)l of the same medium. After 48 h at 37°C, the inserts were transferred to wells of a 24-well plate containing formalin. Following fixation for at least 24 h, duplicate inserts (each duplex done in triplicate) were processed as follows: either the cells that had invaded to the lower side of the membrane or the non-invasive cells on the upper side were wiped off with a cotton swab. The membranes were excised from the inserts and placed into the wells of a 48-well plate such that the surface carrying the cells faced to the top. These membranes were then subjected to dual immunocytochemistry to identify the respective cell types, mounted on slides and cell numbers and invasive indices determined as described earlier (see Fig. 3).

Chemotactic Matrigel invasion assay

Invasiveness of primary ESCs or St-T1b cells in response to a gradient was monitored by plating 2.5 \( \times \) 10^4 cells per insert of Biocoat Matrigel invasion chambers in Opti-MEM serum-free medium. The lower well contained 37°C. The inserts were transferred to wells of a 24-well plate containing formalin. Following fixation for at least 24 h, duplicate inserts (each duplex done in triplicate) were processed as follows: either the cells that had invaded to the lower side of the membrane or the non-invasive cells on the upper side were wiped off with a cotton swab. The membranes were excised from the inserts and placed into the wells of a 48-well plate such that the surface carrying the cells faced to the top. These membranes were then subjected to dual immunocytochemistry to identify the respective cell types, mounted on slides and cell numbers and invasive indices determined as described earlier (see Fig. 3).

RNA extraction and RT–PCR

RNA was extracted from cultured cells with peqGold RNapure reagent (Peqlab, Erlangen, Germany) according to the manufacturer’s protocol, but the aqueous phase obtained after chloroform extraction was subjected to an additional purification step by phenol/chloroform/isoamylalcohol extraction. One microgram of RNA was used for oligo(dT)-primed cDNA synthesis with the ImProm-II Reverse Transcription System (Promega, Mannheim, Germany). Of the resulting 20 \( \mu \)l of cDNA, 0.5 \( \mu \)l was used per semi-quantitative PCR reaction. Primer sequences and PCR conditions were as described previously (Samalecos et al., 2009). PCR products were resolved in 2% agarose gels, stained with SYBR Gold (Molecular Probes, Invitrogen) and visualized in a Typhoon 8600 Imager (Amersham Biosciences, Freiburg, Germany).

Western blot

Whole-cell extracts were prepared in RIPA buffer and electrophoresed on 10% SDS–polyacrylamide gels (NuPage Bis–Tris; Invitrogen) as detailed previously (Samalecos et al., 2009). Immunodetection was performed with the enhanced chemiluminescence system (SuperSignal; Pierce, Bonn, Germany). KAI-1 was detected under non-reducing conditions (antibody TS82b; 1:500; Diacline, Besançon, France). CEACAM1 was detected under reducing conditions (clone 4D1C2; 0.5 \( \mu \)g/ml) (Bamberger et al., 2000); detection of GAPDH (clone 6C5, 1:10 000; HyTest, Turku, Finland) was used for normalization under both conditions.

Statistical analysis

Data were analysed by Student’s t-test or by one-way analysis of variance (ANOVA) followed by either Tukey’s multiple comparisons test, or Dunnnett’s test to compare with a control, using GraphPad Prism software (Version 5 for Mac OS X).
Results

Invasiveness of AC-1M88 cells and primary ESCs or St-T1b cells in co-culture

To study mutual influences of ESCs (primary or immortalized) and trophoblastic AC-1M88 cells on their invasive behaviour, we set up a Matrigel invasion assay with both cell types in co-culture. We have previously shown that the cell types can be distinguished by immunocytochemical staining for vimentin (St-T1b cells or primary ESCs) and CK7 (AC-1M88 cells) (Samalecos et al., 2009). ESCs are routinely maintained in media containing steroid-depleted FCS, and in the presence of 8-Br-cAMP if a decidual phenotype is desired. In the light of setting up a co-culture system, it was important to first assess the response of AC-1M88 cells to such culture conditions. To this end, the expression of CEACAM1 as a marker of EVTs, and of KAI-1 as a marker of decidualized ESCs, was investigated in both cell types under different culture conditions (Fig. 1). In serum-free media, CEACAM1 protein level was low in AC-1M88, whereas KAI-1 protein was highest in St-T1b cells. In the presence of FCS, be it untreated or steroid-depleted and added to 1, 5 or 10%, CEACAM1 protein was equally high in AC-1M88 cells. KAI-1 protein in St-T1b cells was lower when compared with serum-free conditions but not affected by the concentration or type of FCS. Under all culture conditions, CEACAM1 was not expressed in St-T1b, nor KAI-1 in AC-1M88 cells (Fig. 1). We then tested the effect of 8-Br-cAMP on AC-1M88 cells. Again, KAI-1 protein was not induced, but the concentration normally used to decidualize ESCs, 0.5 mM, was cytotoxic for AC-1M88 cells upon extended treatment (data not shown). It was therefore not possible to co-culture AC-1M88 with ESCs in the presence of the decidualization stimulus. We then tested whether decidualized ESCs would maintain their differentiated status after the withdrawal of the stimulus for at least 2 days. Treatment of primary ESCs with 8-Br-cAMP for 6 days induced KAI-1 protein and dPRL mRNA (Fig. 2A and B). KAI-1 protein began to disappear after 2 days of withdrawal; dPRL transcript levels faded more slowly and were still detectable after 6 days in the absence of cAMP analogue. St-T1b cells displayed a similarly strong induction of KAI-1 protein and dPRL mRNA with decidualization (Fig. 2C and D). KAI-1 protein expression faded more slowly than in primary cells and was maintained even after 3 days of cAMP withdrawal, whereas dPRL mRNA expression was not as long-lived as in primary ESCs. In both cell types, regulation of KAI-1 was exclusively at the protein but not the transcript level. Morphological decidualization of St-T1b cells was still retained after 3 days of withdrawal of 8-Br-cAMP (Fig. 2E). For primary ESCs, we have reported similar kinetics previously (Pohnke et al., 2004).

On the basis of these observations, we then devised a modified Matrigel invasion assay for co-culture of AC-1M88 and undifferentiated or decidualized St-T1b cells. An equal number of AC-1M88 cells and St-T1b cells, untreated or pretreated for 5 days with 0.5 mM 8-Br-cAMP to induce a decidual phenotype, were mixed and plated in Matrigel invasion chambers. Steroid-depleted FCS was used at 10% (MM1 medium) to maintain CEACAM1 in AC-1M88 cells and KAI-1 protein expression in decidualized St-T1b cells. Owing to its toxicity to AC-1M88 cells, 8-Br-cAMP was omitted for the duration of the invasion assay. To take into account potential effects of different culture conditions on proliferation rates, we prepared parallel inserts for each treatment to wipe off the cells on the upper or the lower surface and count both to obtain the total number of cells after the 48-h incubation (Fig. 3A). The invasive index was calculated as the ratio of cells counted on the bottom side relative to the total cell number. Representative pictures of stained cells on both surfaces of the membrane are shown in Fig. 3B. Invasiveness of AC-1M88 was not affected by the presence of St-T1b cells, irrespective of their decidualization status (Fig. 4A). Remarkably, when mixed with AC-1M88 cells, invasiveness of predecidualized St-T1b cells was much higher than that of undifferentiated cells. Furthermore, predecidualized St-T1b cells were dramatically more invasive in the presence of AC-1M88 cells than in their absence (76 versus 17%) (Fig. 4A). Proliferation of AC-1M88 was slightly enhanced by the presence of predecidualized but not undifferentiated St-T1b cells. The attenuated proliferation rate of St-T1b cells upon decidualization was not altered by the presence of AC-1M88 cells (Fig. 4B).

The same invasion/co-invasion protocol was then applied to primary ESCs (Fig. 4C and D). Proliferation of ESCs or of co-plated AC-1M88 after 48 h was not altered as a function of ESC decidualization. However, predecidualized ESCs, as described earlier for St-T1b cells, responded to the presence of AC-1M88 cells by a marked increase in invasive activity from 18 to 57%.

Taken together, observations made on St-T1b cells with respect to invasion were entirely recapitulated in primary ESCs. AC-1M88 invasiveness was not altered by the presence of ESCs. Decidualized ESCs were more invasive than non-decidualized cells, and the presence of AC-1M88 cells stimulated invasiveness of non-decidualized, and even more so, of decidualized ESCs.

Production of MMPs by AC-1M88, primary ESCs and St-T1b cells

Movement of cells through ECM requires the local production and activation of MMPs. We show by gelatin zymography that AC-1M88

![Figure 1](image-url) Cell type-specific expression of CEACAM1 and KAI-1 under different culture conditions in AC-1M88 and St-T1b cells. Whole-cell extracts were prepared from AC-1M88 and St-T1b cells maintained in different culture media for 2 days: serum-free Opti-MEM (lane 1), or DMEM/HF-12 supplemented with 10% FCS (lane 2), or with 1%, 5% or 10% steroid-depleted FCS (as in MM1) (lanes 3–5), respectively. CEACAM1, KAI-1 and GAPDH were detected by immunoblotting.
cells and undifferentiated ESCs secrete active MMP-2 and, to a lesser extent, MMP-9. In ESCs, MMP production and the activity of MMP-2 were up-regulated upon decidualization (Fig. 5A). St-T1b cells also secreted MMP-2 and more readily detectable levels of MMP-9. Again, MMP secretion was higher in decidualized cells (Fig. 5B). MMP isoforms were identified on the basis of their electrophoretic mobility and by incubation of zymograms with an inhibitor of MMP-2/MMP-9.

Migration and invasion of primary ESCs and St-T1b cells in response to AC-1M88 secretory products

In the initial Matrigel co-invasion assay, endometrial stromal and AC-1M88 trophoblastic cells had been in direct contact. We wished to assess whether cell–cell contact was required for the stimulation of ESC invasiveness by trophoblastic cells, or whether secreted factors derived from AC-1M88 cells elicited the response. To this end, primary ESCs or St-T1b cells were plated onto Matrigel invasion inserts in serum-free medium. The lower reservoirs contained MM1 only (controls), MM1 supplemented with conditioned medium from AC-1M88 cells or a monolayer of AC-1M88 cells, thus generating a chemotactic gradient. In parallel, migration assays were initiated using inserts without Matrigel coating. Invasion assays were terminated after 48 h, migrations assays after 24 h. Decidualized St-T1b cells and primary ESCs were dramatically more invasive than undifferentiated cells (14.1- and 22.9-fold increase upon decidualization, respectively) (Fig. 6A and B). Conditioned medium from AC-1M88 cells at 50 or 80%, or the presence of AC-1M88 cells in the lower reservoir, strongly enhanced invasion of undifferentiated St-T1b cells or primary ESCs. This response was significantly enhanced upon decidualization of the latter; in decidualized St-T1b, the tendency towards enhancement did not reach statistical significance.

In contrast, there was no significant difference in migration between undifferentiated and decidualized St-T1b cells or primary ESCs when exposed to control medium (Fig. 6C and D). Yet, the presence of AC-1M88-derived factors elicited a massive stimulation of migration in both cell types. Although the absolute numbers of migrated cells were distinctly higher in decidualized compared with undifferentiated St-T1b cells, such difference was not apparent in primary cells. However, when the migratory and invasive responses to AC-1M88 cells were expressed relative to those seen with control medium,
both cell types displayed a remarkably similar pattern (Table I). Migration towards AC-1M88 cells compared with control medium was 26-fold higher in undifferentiated St-T1b cells and primary ESCs, and 14- or 11-fold in decidualized cells. The presence of AC-1M88 cells increased invasive indices 17-fold in both cell types when left undifferentiated, and 1.5-fold when they had been decidualized (Table I). The relatively small response to AC-1M88 cells in decidualized cells is accounted for by the enormous increase in invasiveness caused by decidualization per se (Fig. 6A and B).

**Discussion**

Implantation and placenta formation in humans are signified by two distinctive features: decidual transformation of ESCs in preparation...
for implantation is initiated independent of a blastocyst-derived signal, and the invading trophoblast is particularly aggressive (Kliman, 2000; Fazleabas et al., 2004; Red-Horse et al., 2004; Ferretti et al., 2007; Gellersen et al., 2007b; Salamonsen et al., 2007). Processes occurring at the human maternal–fetal interface can therefore not adequately be studied using *in vivo* rodent models which lack these features. *In vitro* co-culture experiments using human cells are hampered by difficulties in procuring sufficient extravillous trophoblast material. Being well aware of the limitations inherent in the use of cell lines compared with primary cells, we decided to use the well-characterized AC-1M88 cell line as a model for trophoblast cells (Hannan et al., 2009). The cell adhesion molecule CEACAM1 present in AC-1M88 cells is implicated in invasive activity and, in the placenta, specifically localizes to EVTs at the invading front (Bamberger et al., 2000; Briese et al., 2005). Nothing is known to date about the regulation of CEACAM1 in trophoblast cells, although we show here that this adhesion molecule is induced by components present in FCS.

In order to assess mutual influences of ESCs and EVTs on invasive behaviour in a co-culture system allowing cell–cell contact, we devised a Matrigel co-invasion assay. For control purposes, cell types were also plated alone. This led to the surprising observation that predecidualization markedly promoted invasive behaviour of ESCs, be they primary or immortalized. Co-culture with AC-1M88 cells greatly

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**Figure 4** Effect of predecidualization on the invasiveness of St-T1b cells or primary ESCs and of co-plated AC-1M88 cells. Invasive indices (A and C) and total cell numbers (B and D) were determined from Matrigel invasion/co-invasion assays as described in Fig. 3 for St-T1b cells (A and B) and primary ESCs (C and D). (A) Invasiveness of AC-1M88 cells (M88) was not changed by the presence of undifferentiated (grey bars) or predecidualized (black bars) St-T1b cells, compared with AC-1M88 cells plated alone (white bar). Invasiveness of non-decidualized or predecidualized St-T1b cells was significantly increased by the presence of AC-1M88 cells; in the presence of AC-1M88 cells, predecidualized St-T1b cells were significantly more invasive than undifferentiated cells. Each data point represents the mean ± SD derived from six invasion membranes (three top, three bottom measurements). *P* < 0.05; **P** < 0.01 (ANOVA followed by Tukey’s test). (B) Total cell numbers (top plus bottom) are expressed as percent of controls (AC-1M88 cells plated with non-decidualized St-T1b cells; or non-decidualized St-T1b cells plated alone or in the presence of AC-1M88 cells, respectively). The number of AC-1M88 cells, when co-plated with St-T1b cells, was increased when the latter had been predecidualized. *P* < 0.05; **P** < 0.01 (Student’s t-test). (C) Invasiveness of AC-1M88 cells (M88) was not changed by the presence of primary ESCs, either non-decidualized (grey bars) or decidualized (black bars). Invasiveness of non-decidualized or predecidualized ESCs was significantly increased by the presence of AC-1M88 cells; in the presence of AC-1M88 cells, predecidualized ESCs were significantly more invasive than undifferentiated cells. Each data point represents the mean ± SD derived from six invasion membranes (three top, three bottom measurements). *P* < 0.05; **P** < 0.01; ***P** < 0.001 (ANOVA followed by Tukey’s test). (D) Total cell numbers (top plus bottom) are expressed as percent of controls (AC-1M88 cells plated with non-decidualized ESCs; or non-decidualized or predecidualized ESCs plated alone or in the presence of AC-1M88 cells.

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enhanced this invasive potential of decidualized ESCs, far more so than it did for undifferentiated ESCs.

A co-invasion assay similar to the one described in this study has been conducted on endometriotic stromal cells. These were seeded on a monolayer of peritoneal cells on the upper surface of a Matrigel invasion chamber. Under these circumstances, ESC invasiveness was enhanced by activin A, a member of the transforming growth factor-β family (Ferreira et al., 2008). Endometriosis arises by...
implantation of viable endometrial cells in retrograde menstrual effluent at ectopic pelvic sites. Because decidualization precedes menstrual shedding, it is tempting to speculate that an enhanced decidual response may be associated with increased invasive potential of cells in menstrual fluid (Brosens et al., 2009).

A recent study addressed the behaviour of villous trophoblast from early pregnancy when placed on the top of a layer of decidual fibroblasts isolated from term placenta (Fafet et al., 2008). The presence of trophoblast induced PRL immunoreactivity in these cells, indicating that placental signals support the maternal decidual response. Similarly, in a co-culture system of proliferative ESCs with first trimester trophoblast, gene expression profiling demonstrated induction of several decidual-specific products in the ESCs, including IGFBP-1 (Popovici et al., 2006). Confrontation assays have also been established, whereby decidual fragments are placed in contact with spheroids formed from an EVT cell line, allowing visualization of EVTs invading into the decidual explant (Helige et al., 2008). Generally, in co-culture systems of ESCs or decidual fragments with trophoblast, it is the movement of EVTs that is being monitored while the activity of the ESCs has not received attention (Mardon et al., 2007). The common concept derived from such in vitro co-culture models and from immunohistochemical analyses of human implantation sites has created a picture of motile and actively invading EVTs within a matrix of sedentary decidual cells that limit invasion by secreting protease inhibitors, and/or control directional invasion through chemotactant substances.

Our findings suggest a novel interpretation, namely that decidual cells themselves have not only migratory but also invasive potential, and are thus equipped to actively support the movement of EVTs into the decidual layer by encircling them. The decidualization process results in an enhancement of this ability which is even further augmented by trophoblastic signals. This concept is also supported by observations made in co-culture experiments using human hatched blastocysts placed on an ESC monolayer (Carver et al., 2003; Grewal et al., 2008). The interaction between ESCs and blastocyst leads to greatly elevated hCG production by the latter (Carver et al., 2003), and the gonadotrophin in turn may promote decidualization of ESCs, at least partly through the activation of the cAMP pathway (Tang and Gurpide, 1993; Han et al., 1999). Time-lapse imaging of ESCs in co-culture with blastocyst revealed increased motility of ESCs at the contact sites but was interpreted as moving away from the implanting embryo (Grewal et al., 2008). In our opinion, an alternative explanation is that decidual cells can move around the blastocyst in an engulfment process. Notably, we observed on the upper surface of the Matrigel invasion membranes that stromal and trophoblast cells, after 48 h in co-culture, were not evenly interspersed but that each cell population had migrated to organize into multi-cellular branches (Fig. 3). It has to be emphasized that there was no chemotactic gradient in the Matrigel co-invasion assay, and yet 60–80% of decidualized cells invaded through the porous membrane. This indicates that contact with trophoblastic cells elicits a highly motile behaviour. One might speculate that, in this setting, the trophoblast cells produced MMPs to degrade the ECM, thus paving the way for ESCs. We demonstrate, however, that decidualized ESCs possess intrinsic invasive capacity. In the chemotactic invasion assay, ~25% of cells invaded from the compartment containing serum-free medium towards the compartment with serum-containing medium. Moreover, zymography revealed elevated MMP-2/-9 production in decidualized ESCs. Motility alone was much less increased by decidualization than was invasiveness, yet both activities were strongly enforced by factors released from AC-1M88 cells.

Paracrine networks operating at the maternal–fetal interface in early placentaion involve a rich array of cytokines, chemokines and their receptors (Salamonsen et al., 2007; Hannan and Salamonsen, 2008), and AC-1M88 cells reflect the repertoire seen in primary EVTs to a considerable extent (Hannan et al., 2006). A candidate paracrine factor stimulating ESC migration and invasion is a platelet-derived growth factor which is also produced by first trimester

| Table I Effect of AC-1M88 secretory factors on migration and invasion of non-decidualized and decidualized St-T1b cells and primary ESCs |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Non-decidualized cells exposed to | Migration | Invasion |
| Control (MM1) | 1 | 1 | 1 | 1 |
| 50% CM | 5.4 | 23.2 | 9.8 | 30.0 |
| 80% CM | 14.1 | 32.2 | 16.2 | 35.7 |
| AC-1M88 | 26.4 | 26.5 | 17.4 | 17.1 |
| Decidualized cells exposed to | Migration | Invasion |
| Control (MM1) | 1 | 1 | 1 | 1 |
| 50% CM | 6.6 | 13.3 | 1.2 | 2.3 |
| 80% CM | 12.4 | 15.4 | 1.4 | 2.3 |
| AC-1M88 | 14.5 | 11.3 | 1.5 | 1.5 |

Migration and invasion of non-decidualized or decidualized St-T1b or primary ESCs in response to conditioned medium (CM) from AC-1M88 cells, or a monolayer of AC-1M88 cells plated in the lower reservoir. Data from the experiments shown in Fig. 6 are expressed as fold increase relative to control conditions (MM1 medium in the lower reservoir) of the migration or invasion assays, respectively.
Invasiveness of human endometrial stromal cells

References


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Submitted on November 24, 2009; resubmitted on December 12, 2009; accepted on December 15, 2009