Hormone-dependent placental manipulation of breast cancer cell migration

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BACKGROUND: Breast cancer during pregnancy is often more advanced than in non-pregnant women. Nevertheless, no case of metastasis inside the placenta has been reported. Previously, we showed that placental-explants eliminated breast cancer cells from their surroundings, due to cell-death and elevated migration. Our objective was to find the underlying mechanisms of these phenomena.

METHODS AND RESULTS: Our model contained Michigan Cancer Foundation 7 (MCF7) or T47D cells co-cultured with and without human placental explants. Microarray analysis, validated by quantitative PCR, of MCF7 following their placental co-culture suggested activation of estrogen (E2) signaling. As extensive cross-talk exists between E2 and progesterone, their involvement in mediating placental effects on breast cancer cells was tested. Indeed, addition of E2 and progesterone receptor (ER and PR) inhibitors to the co-culture system reduced cancer cell motility, yet did not alter cell-cycle or death. E2 and progesterone concentrations in placental media were found to be similar to those of early pregnancy blood levels. Interestingly, placental-breast cancer co-culture media contained lower progesterone (P<0.05) and higher E2 (200%, P<0.05) levels than placenta cultured separately. Placental supernatant and E2 and progesterone at placental levels were sufficient to increase MCF7 and T47D migration and invasion (P<0.05), yet did not alter MCF7 cell-cycle or death. Furthermore, placental supernatant elevated p38 and Jun N-terminal kinase (JNK) phosphorylation in both cell lines (P<0.05). Inhibitors of JNK, ER and PR reversed MCF7 and T47D motility induced by the placenta, suggesting their involvement.

CONCLUSIONS: We suggest that E2 and progesterone contribute to cell migration away from placental areas. We hypothesize that they may increase metastatic spread to other organs in pregnancy.

Key words: placenta / pregnancy / breast cancer cells / motility

Introduction

Breast cancer is one of the most common malignancies in pregnant women (Antonelli et al., 1996; Pavlidis, 2002; Smith et al., 2003; Jacobs et al., 2004; Pereg et al., 2008; Lyons et al., 2009). Numerous publications have shown that pregnant women diagnosed with breast cancer develop larger tumors and are more likely to develop metastases (Shousha, 2000; Woo et al., 2003; Pereg et al., 2008). These observations suggest that pregnancy may alter the prognosis of breast cancer. A possible cause may be the extensive physiological changes of the breast during pregnancy, which impairs diagnosis. In addition, the unique hormonal and gestation-related growth factor panel may also contribute to disease aggressiveness (Hulka and Stark, 1995; Schindler, 2005). Neoplastic cells of pregnant women have lower estrogen receptor alpha (ERα) levels compared with those of non-pregnant controls (Shousha, 2000; Woo et al., 2003; Pereg et al., 2008). Indeed, ERα negative breast cancers are often related to a poorer disease prognosis (Putti et al., 2005; Ulery et al., 2009). ER is a master transcriptional regulator of breast cancer phenotype (Carroll et al.,...
2006). Its levels can be regulated by estrogen (E2) (Smyth et al., 1988; Duong et al., 2007). Furthermore, extensive cross talk exists between E2 and progesterone. Progesterone reduces ERα levels and has some anti-estrogenic effects, whereas the opposite is evident regarding the effect of E2 on the progesterone receptor (PR) (Velarde et al., 2007; Sukhoeva et al., 2009).

A major concern is the possible effect of the malignancy on the fetus. Although the placental intervillous space is characterized by a slow flow of generous blood volumes in a large surface area, which creates an ideal biologic environment for metastasis initiation (Jackisch et al., 2003), metastasis to the placenta or embryo is rare (Jackisch et al., 2003). The limited number of metastases found on the placenta suggests that there are unknown placental factors that hinder cancer cell growth in its close proximity. In fact, less than 100 cases of maternal tumors metastasizing to the placenta or fetus have been reported since 1866 (Alexander et al., 2003; Al-Adnani et al., 2007; Shuhaila et al., 2008; Pages et al., 2010; Thelmo et al., 2010). If breast cancer is a rather common malignancy during pregnancy and usually diagnosed at advanced stages, it is surprising that no cases of fetal or villous invasion in these patients have ever been reported. Thus, this suggests that the placenta serves as a non-supportive microenvironment for malignant cells in general, and especially for breast cancer cells.

During placental implantation, extravillous trophoblast (EVT) cells invade the uterus, replace vessel endothelium and are eventually stationed at the placenta front line, facing the metastatic invasion, which suggests their involvement in metastatic inhibition.

In our previous study, we investigated the unique effect of the placenta on breast cancer cells. We have established a biological model in which human first trimester placental explants were co-cultured with Michigan Cancer Foundation (MCF7) or T47D cells on a ‘Matrigel’ substrate (Tartakover-Matalon et al., 2010). MCF7 and T47D cells are commonly used as a breast cancer cell model to study E2 and progesterone signaling, as they express ERα (Bourdeau et al., 2008) and PR (Sukhoeva et al., 2009). Our study has shown that the placenta enhanced MCF7 cell death. Moreover, it led to the formation of uniquely shaped breast cancer cell aggregates, and eliminated the MCF7 and T47D cancer cells from its proximity. Interestingly, breast cancer cells were most significantly affected in the areas of EVT cell differentiation (Tartakover-Matalon et al., 2010), suggesting that EVT cells or their invasion process could be a possible cause for these phenomena. In the current study, we aimed to establish mechanisms that would explain the earlier-mentioned observations.

First, we performed a microarray analysis of MCF7 cells pre-exposed to placental explants.

Manipulations of the breast cancer E2 pathway (demonstrated below) were shown using pathway analysis. On the basis of these results and taking into account the significance of hormones during pregnancy, we focused on E2 and progesterone involvement in mediating placental effects on MCF7 and T47D cells. In addition, we tested the involvement of these two hormones in the placental culture system by using specific ER and PR inhibitors.

This study provides an insight to the effect of pregnancy on cancer cell behavior, and suggests mechanisms underlying the placental defense against breast cancer metastasis.

**Materials and Methods**

**Cell culture**

T47D (kindly provided by Dr Ilan Tzarfati, Tel Aviv University) and MCF7 breast cancer cell lines (BCCL) (with and without enhanced green fluorescent protein (eGFP) tags) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM) and Pen-Strep-Nystatin antibiotics (Biological Industries, Israel). Cells were maintained in 5% CO2 at 37°C. Cells tagged with eGFP were supplemented with 300 μg/ml neomycin (G418 sulfate, Invivogen, San Diego, CA, USA).

**Transfection**

Commercial vectors eGFP-C1 (Clontech, Mountain View, CA, USA) were characterized with eGFP and neomycin (G418)-resistant genes. Three microliter of FuGENE six transfection reagent (Roche Diagnostics, Mannheim, Germany) was added to 97 μl of OPTI-MEM I (5 min, room temperature, GIBCO, USA) and 1 μl of plasmid DNA was added (45 min, room temperature). The entire volume (100 μl) was added to 105 MCF7 or T47D cells previously seeded in a 24-well plate. For selecting eGFP positive cells, cells were exposed to 300 μg/ml neomycin. Fluorescence was analyzed by fluorescence-activated cell sorter (FACS; Navios, Beckman Coulter, Miami, FL, USA).

**Placental tissue preparation and co-culture**

Culture techniques were based on previously described methods (Genba-cev et al., 1992, 1993; Genbacev and Miller, 1993). Placentae at 6–9 weeks of gestation were retrieved from normal pregnancies terminated for psychosocial reasons. The study was approved by the ethics commit-tee of Meir Medical Center. Placental villi of 10 mg wet weight were transferred into culture inserts (Millipore, Carrigtwohill Co., Cork, Ireland) previously layered with ‘Matrigel’ (BD Biosciences, Bedford, MA, USA) and placed into 24-well plates. After 1.5 h, 700 μl of DMEM/F-12, HAM, L-glutamine (2 mM), sodium pyruvate (1 mM), HEPES (25 mM), antibiotics and 10% FCS (Biological Industries, Beit Haemek, Israel) were added to the bottom of the culture dish and left overnight. After 12–16 h, 4 × 104 of T47D-eGFP or MCF7-eGFP cells with or without inhibitors were added. Breast cancer cells plated alone on ‘Matrigel’ served as controls. After 24 h, a bare area assay (See below) was performed, culture media were collected, replaced and stored at −80°C. The bottom well medium was also replaced. Following 60 h co-culture, the bare assay was performed again, media were collected and cells were retrieved by trypsin-EDTA (Biological Industries) from the ‘Matrigel’ and processed in one of the following ways:

1. MCF7-eGFP/T47D-eGFP were sorted out by FACS (BD FACS Aria, Tel Aviv University). Following sorting, protein and RNA extractions were performed.
2. MCF7-eGFP were analyzed by FACS (Navios, Beckman Coulter, USA), while ‘gating in’ only eGFP positive cells.

Placenta samples from different patients were used in each experiment.

**Hormone level analysis**

Progesterone and E2 levels were measured by Immulite 2000 analyzer (Endocrinology Laboratory, Meir Medical Center). The analysis was done using solid phase (bead) competitive chemiluminescent enzyme immunoassay kits L2KPV2 and L2KE22, respectively, according to the manufacturer’s instructions. Medium placed on ‘Matrigel’ for the same period provided the background value.
Exposing breast cancer cells (MCF7 or T47D) to placental or breast cancer cells upper media and to E2 and progesterone

MCF7 or T47D cells (2.5 x 10^5) were placed for 24 h in 96-well plates covered with ‘Matrigel’ in the presence of either: (i) media collected from placental or breast cancer cells (MCF7 or T47D), cultured on ‘Matrigel’ for 60 h and centrifuged to eliminate cells and debris (supernatant), or (ii) E2 and progesterone (Sigma Aldrich, USA) diluted in ethanol (EtOH) to ‘placental level’ concentrations (i.e. 100 nM progesterone, 500 nM E2) or ‘breast cancer cells level’ (10 nM E2).

In the experiments, three sources of placental supernatant were used: the first was collected from a single placenta, while the second and the third were pooled media from 5 or 6 placentae.

Inhibitors

Hormone receptor inhibitors Fulvestrant (ICI 182 780) and Mifepristone (RU486) (both purchased from Sigma Aldrich, USA) were diluted in EtOH to 100 and 10 nM, respectively. The same volume of EtOH served as control.

Mitogen-activated protein kinase (MAPK) inhibitors SB 202190 (p38 MAPK inhibitor) and SP600125 inhibitor (JNK) were dissolved in dimethylsulfoxide (DMSO) at 20 μM (SIGMA and Biomol Int., USA, respectively). MCF7 and T47D cells were exposed to inhibitors for 1 h prior to assays.

Visual assessment of MCF7 and T47D distribution (bare area assay)

Placental explant co-cultures were examined daily under a microscope (Olympus IX71). To assess bare areas around the placenta and EVT cell differentiation and invasion, each placental explant was divided into 12 sectors (like a clock). The number of sites from which MCF7-eGFP or T47D cells disappeared was calculated. (For example, nine sectors were considered as 75% effect (9/12 x 100 = 75%, Fig. 1). The effect of ER and PR inhibitors on bare area size was analyzed by comparing the size of the bare areas formed with and without the inhibitors. EVT cell migration from the placenta to the ‘Matrigel’ can be identified by microscopic observation as finger-like outgrowth projections that surround the placental explants and are located very close to the villous area/surface (Genbacev and Miller, 1993). As breast cancer cells migrate further than EVT, areas almost empty of cells were created that could be easily distinguished from areas that contained breast cancer cells. In addition, the eGFP tag in breast cancer cells was used to differentiate between them and the EVT cells that lack the eGFP tag.

Cell-cycle analysis

This was done as described by Chu et al. (1999). Briefly, retrieved cells were washed with phosphate-buffered saline (PBS), and fixed with 5% formaldehyde (Gadot, Israel) and 0.1% sodium azide (Sigma Aldrich, St Louis, MO, USA; 1 h, 4°C). Fixation was followed by cell perforation (70% EtOH in PBS, overnight, 20°C). The next day, cells were exposed to 40 μg/ml propidium iodide (PI) (Sigma Aldrich) and 100 μg/ml ribonuclease A (Sigma Aldrich) in PBS (30 min, room temperature, in the dark). Cell DNA content was analyzed using FACS (Navios, Beckman Coulter, USA). In co-culture experiments, only MCF7-eGFP-gated cells were tested. Results were analyzed using ModFit LT 3.0 (Verity Software House, ME, USA).

Assessment of apoptosis and necrosis

Annexin V-Alexa Fluor 680 (AF680; Invitrogen, Carlsbad, CA, USA) supplemented with 10 μg/ml PI (Sigma Aldrich) was used to detect cell death according to the manufacturer’s instructions and assayed for fluorescence by FACS (Navios, Beckman Coulter, Fullerton, CA, USA). In co-culture experiments, only MCF7-eGFP-gated cells were analyzed. For other systems that did not require the eGFP tag, we used Annexin V-PE (BioVision, Mountain View, CA, USA) supplemented with 0.1 μg 7-aminoactinomycin D (7AAD) (eBioscience, San Diego, CA, USA). Annexin V+/PI− (or Annexin V+/7AAD−) cells were considered apoptotic and Annexin V+/PI+ (or Annexin V+/7AAD+) cells were designated late apoptotic/necrotic. All results are expressed as the percent of total cell number.

Gelatin zymography

Media (20 μl) were electrophoresed at non-reducing conditions in 10% polyacrylamide gel containing 1 mg/ml gelatin type A (Sigma Aldrich). Gels were washed in 2.5% Triton X-100 and incubated overnight in 50 mM Tris–HCl (pH 7.5) and 5 mM CaCl2. Coomassie blue staining followed by destaining [20% methanol, 7% acetic acid in double distilled water (DDW)] allowed visualization of clear lysis zones against the blue background. Matrix metalloproteinases 2 and 9 (MMP2 and 9) were identified by a marker and by the migration of gelatinase zymogram standard (Chemicon, Temecula, CA, USA) for human MMP2 and MMP9 that were electrophoresed adjacent to the tested aliquot. Optical densities of clear zones were measured as arbitrary units by using LAS3000 Image reader (Fugifilm, Greenwood, SC, USA). Results were normalized to background values using the Multi-gauge V3.0 program (Fugifilm, Greenwood, SC, USA). Proactive bands were strong, whereas active forms were very weak and could not be separately evaluated.

Scratch test assay

Confluent MCF7 or T47D cell monolayers were wounded by pipette tip. Following wounding, placental or breast cancer cell (MCF7 or T47D) supernatant or E2 + progesterone with/without inhibitors was added to the cells. Wound closure was monitored by microscopy at 24 h
(MCF7) or 48 h (T47D) following plating. Results are presented as relative percentage of closure compared with time 0. The wound measurements were performed with the help of ImageJ software: http://rsweb.nih.gov/ij/.

### Transwell invasion assay
Invasion assays were done using Transwell plates (Costar, Corning, NY, USA) based on methods previously described (Shaw, 2005). The upper chamber of each well was coated with 40 μl ‘Matrigel’ (0.125 μg/μl) and left to dry overnight at 37°C. Dilution of ‘Matrigel’ was done using placental/BCCL supernatant. Then, wells were rehydrated with 40 μl of DMEM for 1 h, and 10^5 MCF7 or T47D cells were added. The lower chamber was filled with 600 μl media (10% FCS) supplemented with 5 μg/ml fibronectin (Sigma Aldrich). Plates were incubated at 37°C for 48 h. Non-invading cells from the upper chamber were removed using cotton swabs. Inserts were stained with Accustain (Sigma Aldrich, Steinheim, Germany) for 30 min. Invading cells were counted using an inverted microscope.

### RNA extraction and RT cDNA synthesis
MCF7 or MCF7-eGFP cells were washed with PBS, and RNA was extracted using RNeasy kit (QIAgen, Valencia, CA, USA) according to the manufacturer’s instructions. RNA concentrations were measured at 260/280 nm using Nanodrop-1000 (Thermo Scientific, Wilmington, DE, USA). Extracted RNA was converted to cDNA using Applied Biosystems kit according to the manufacturer’s instructions.

### Real-time quantitative PCR
Specific primers were designed according to ‘Primer Express 3.0’ software specifications. Quantitative PCR (qPCR) reactions were done using Power SYBR Green (Applied Biosystems, Warrington, UK) according to the manufacturer’s instructions. The following genes were tested: Id1, KLF9, ESR1, GREB1, MUC1 and PGR (Primers are listed in Table I). RPL18A housekeeping gene served as the control. Primers were normalized by specific cDNA formula: 

\[ q = \frac{P(A + B)}{P(A) + P(B) - P(A) \times P(B)} \]

where \(P(A)\) denotes placental hormones; \(P(B)\) BCCL hormones; \(P(A + B)\) co-culture hormones; \(q < 0.85\) was considered antagonistic interaction; \(q > 1.15\) was considered a synergistic interaction; \(1.15 > q > 0.85\) was interpreted as an additive interaction.

All the experiments were repeated a minimum of three times.

### Western blotting
MCF7, T47D, MCF7-eGFP or T47D-eGFP cells were washed with PBS and lysed in buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 10 mM NaPPi, 2 mM NaVO3, 10 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulphonyl fluoride and 10 μg/ml leupeptin) on ice for 10 min. Protein lysate from 2–3 × 10^6 cells was mixed (1:5) with loading buffer, denatured for 10 min at 65°C and separated on SDS–PAGE and transferred to a polyvinylidene difluoride membrane. After blocking (5% dry milk in tris-buffered saline-T), membranes were incubated at 4°C overnight with primary antibodies (Supplementary data, Table SII). Bound antibodies were visualized using peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) followed by enhanced chemiluminescence detection (Pierce, Rockford, IL, USA). Optical densities were visualized and measured as arbitrary units by LAS3000 Imager (Fugifilm, Greenwood, SC, USA). Results were normalized to Tubulin using Multi-gauge V3.0 program (Fugifilm).

### Microarray performance and bioinformatics analysis
Affymetrix GeneChip® Human Affymetrix GeneChip® Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) were used for gene expression analysis according to the instruction manual http://media.affymetrix.com/support/technical/datasheets/gene_1.0_st_datasheet.pdf. Microarray expression profiles were extracted from raw CEL files using Partek Genomic Suite (Partek® software, version 6.4 Copyright © 2009; Partek Inc., St. Louis, MO, USA; www.partek.com). Data were normalized and summarized with the robust multi-average algorithm to allow for data comparison across the different arrays (Irizarry et al., 2003) followed by one-way analysis of variance. Genes were identified as differentially expressed with a cut-off of \(P < 0.05\) and 1.25-fold expression difference. Gene ontology (GO) functional classification and pathway enrichment analysis of differentially expressed genes was assessed by the following tools: GENE CODIS: http://genecodis.dacya.ucm.es (Nogales-Cadenas et al., 2009), GSEA: http://www.broadinstitute.org/gsea/index.jsp and ToppGene: http://toppgene.cchmc.org (Chen et al., 2009).

### Statistical analysis
Paired Student’s t tests were used to test differences between treatments. An effect was considered significant when the \(P\)-value was ≤ 0.05. Interaction between treatments was assessed according to the following formula:

\[ q = \frac{P(A + B)}{P(A) + P(B) - P(A) \times P(B)} \]

\(q\) is the observed parameter, \(P(A)\) denotes placental hormones; \(P(B)\) BCCL hormones; \(P(A + B)\) co-culture hormones; \(q < 0.85\) was considered antagonistic interaction; \(q > 1.15\) was considered a synergistic interaction; \(1.15 > q > 0.85\) was interpreted as an additive interaction.

All the experiments were repeated a minimum of three times.

### Table I Primer table.

<table>
<thead>
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<th>Target</th>
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<th>Reverse</th>
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<td>GATGCTCCATGCTTTTGTATTACTC</td>
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<tr>
<td>PGR</td>
<td>GATTCAGAAGGCAAGGAG</td>
<td>CAGCTCCCAAGTGAAGGAC</td>
</tr>
<tr>
<td>GREB1</td>
<td>AAGCCCAACAGGTTGTTG</td>
<td>GACATGCCTGCCCTCTCAT</td>
</tr>
<tr>
<td>MUC1</td>
<td>CATTTCACCACCACCATGACA</td>
<td>GGTGTAGGGCTGTTGAGCT</td>
</tr>
<tr>
<td>RPL18A</td>
<td>CGAGCCCGATGCATTCGAG</td>
<td>GGGACCTGGATCTGGAGTGCT</td>
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<td>Id1</td>
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<td>TGGGTCACTTCCGGATCG</td>
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<tr>
<td>KLF9</td>
<td>CCATTACAGTGACATACAGGTA</td>
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</table>
Results

Microarray analysis demonstrated modulation of the ER signaling pathway in MCF7 cells co-cultured with placental explants

Placental explants secrete various cytokines and growth factors that may alter diverse MCF7 pathways. In order to study gene expression in MCF7 cells following co-culture, a whole human genome cDNA microarray analysis was done. MCF7-eGFP cells were cultured with and without placental explants (n = 3), purified by FACS and their total RNA was extracted. RNA was hybridized to GeneChip Human Gene 1.0 ST array kit (Affymetrix), and analyzed as described in Materials and Methods. Seven-hundred and eight genes were differentially expressed (fold change cut off 1.25 and $P \leq 0.05$) between MCF7-eGFP cells co-cultured with placental explants and control MCF7-eGFP cells cultured alone. GO comparative analysis between this gene set (708 genes) and previously published gene sets using Toppgene and GESA softwares revealed two major categories of significant similarities. The first category includes genes that deal with the effect of anti-estrogens and estrogen on breast cancer cells, as well as with the acquired endocrine therapy resistance (to anti-estrogen) (Table II, 2, 5–9, 12). The second category was enriched with genes that are changed in breast cancer cells as a result of ER down-regulation (Table II, 1, 3–4, 10–11). Functional analysis of these genes with Toppgene software (using FDR correction, $P < 0.05$) highlighted ‘Breast_cancer_estrogen_signaling’ as one of the most significantly changed pathways (Table II, 13, gene list at Supplementary data, Table SI). Most E2 responsive genes were up-regulated following placental co-culture (MUC1, C3, SLC7A5, GREB1, PGR, TGFA and CD44).

Whereas expression of ESR1 and CLU that are known to be down-regulated by E2 was reduced (Heikaus et al., 2002; Stoica et al., 2003), as were the remaining three E2 responsive genes (STC2, GSN and THBS1; Supplementary data, Table SI). Significant changes were also observed in the ‘androgen/estrogen/progesterone biosynthesis pathway’ and the ‘steroid biosynthesis pathway’ in the MCF7 cells co-cultured with the placenta (Toppgene and Genecodes 2, respectively, Table II, 14–15 and Supplementary data, Table SI ). Participation of the indicated pathways is attributed to a significant decrease in the LIPA and SOAT1 enzymes and an elevation in the CYP27B1 enzyme. LIPA and SOAT1 are known to be repressed by E2 (Tomita et al., 1996; Snyder et al., 2009), whereas CYP27B1 enzyme is known to be induced by E2 in MCF7 cells (Lechner et al., 2006). Furthermore, the Toppgene analysis of drug–gene interactions revealed genistein, diethylstilbestrol and progesterone among the 10

### Table II Microarray analysis.

<table>
<thead>
<tr>
<th>No</th>
<th>Computer overlapping (ref)</th>
<th>Software</th>
<th>P-value</th>
<th>Genes in query/genes in genome</th>
<th>Annotations in software</th>
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</tbody>
</table>

**Altered pathway**

| 13 | BREAST_CANCER_ESTROGEN_SIGNALING | Toppgene | 2.04E-04| 13/101                        | 1878                   |
| 14 | P02727 Androgen/estrogen/progesterone biosynthesis | Toppgene | 9.33E-03| 2/4                           | 1878                   |
| 15 | 00100 Steroid biosynthesis | GeneCodis2 | 2.02E-02| 3/19                          | KEGG Pathways          |

**Drug**

| 16 | Genistein | Toppgene | 5.75E-14| 83/912                        | 16297                  |
| 17 | Progestrone | Toppgene | 1.45E-10| 91/1209                       | 16297                  |
| 18 | Diethylstilbestrol | Toppgene | 3.24E-10| 60/664                        | 16297                  |

**Transcription factors binding sites**

| 19 | V5ER_Q6_01 | GeneCodis2 | 5.95E-04| 9/337                         | 615                    |
| 20 | V5ER_Q6_02 | GeneCodis2 | 6.85E-04| 9/337                         |                       |
| 21 | V5ER_Q6_01 | Toppgene   | 2.86E-02| 9/212                         |                       |
drugs with the highest probability of interacting with our gene set (708-gene set, Table II, 16–18). These three drugs modulate E2 signaling: genistein is an isoflavonoid that can bind to ER and may function as selective estrogen receptor modulators, diethylstilbestrol is a synthetic non-steroidal estrogen and progesterone is known to oppose ER-mediated gene-regulatory events (Graham and Clarke, 1997). Nevertheless, the promoter region analysis of the 708 genes did provide evidence of enrichment in the estrogen response elements motif (ERE motif). Bourdeau et al. (2008), who previously characterized the gene regulation in MCF7 cells by E2, found that the EREs sequences were enriched only in up-regulated genes. Indeed, we found an enrichment in the ERE motif in the up-regulated genes (V$ER_Q6_01 (GeneCodis2, Toppgene) and V$ER_Q6_02 (GeneCodis2), Table II, 19–21).

Validation of microarray results done by qPCR analysis demonstrated that MCF7 co-cultured with placental explants expressed reduced RNA levels of ESR1 (22% reduction, \( P < 0.05 \)) and elevated levels of its targets: PGR (284% elevation), GREB1 (251% elevation, \( P < 0.05 \)) and MUC1 (262% elevation, \( P < 0.05 \); see Fig. 2).

Taken together, the bioinformatics analyses and their validation support an active and significant effect of estrogen signaling in our research model. The published literature indicated that exposure to E2 provides evidence of enrichment in the estrogen response elements non-steroidal estrogen and progesterone is known to oppose selective estrogen receptor modulators, diethylstilbestrol is a synthetic isoflavonoid that can bind to ER and may function as selective estrogen receptor modulators, diethylstilbestrol is a synthetic non-steroidal estrogen and progesterone is known to oppose ER-mediated gene-regulatory events (Graham and Clarke, 1997). Hormone receptor expression on MCF7 and T47D cells following their culture on ‘Matrigel’ and placenta

Hormone receptor expression on MCF7 and T47D cells following their culture on ‘Matrigel’ with or without placental explants

Our biological model is unique because cells are layered on ‘Matrigel’. It is well established that MCF7 and T47D cells express ER\(_\alpha\), PR A and PR B. However, most studies examined cells cultured on plastic. Here, we analyzed ER\(_\alpha\), PR A and PR B expression on MCF7 and T47D cells layered on ‘Matrigel’ in the presence and absence of placental explants. Our previous study showed that MCF7 and T47D grown on ‘Matrigel’ express ER\(_\alpha\) and that placental explants reduced its expression (Tartakover-Matalon et al., 2010). Here, a microarray validated by qPCR showed that ER mRNA levels were reduced in MCF7 cells as well. Moreover, we found that MCF7 and T47D cells grown on ‘Matrigel’ were positive for PR A and PR B. PR A was more prominent than PR B in MCF7 cells and the opposite was observed with T47D cells (PR A/PR B = 2.9, PR A/PR B = 0.76, respectively, \( P < 0.05 \), Fig. 3A). Microarray results demonstrated that co-culture with placenta elevated PR mRNA levels of MCF7 (see above). Nevertheless, immunoblotting showed no change in PR levels or in PR A/PR B ratio in MCF7 cells following their placental co-culture (Fig. 3B and C). The PR levels in T47D are higher than in MCF7 cells (Lostumbo et al., 2006). After culturing the T47D cells with placental explants, the PR A and PR B levels were significantly reduced (55 and 88% reduction, respectively, \( P < 0.05 \)), and PR A became more prominent than PR B (PR A/PR B = 2.5, \( P < 0.05 \)). Since the ER and PR levels can be reduced as a result of exposure to high E2 and progesterone levels (Turgeon and Waring, 2000; Duong et al., 2007), we further analyzed the E2 and progesterone levels in breast cancer cells cultured with and without placental explants.

E2 and progesterone levels in media collected from breast cancer cells and placentae cultured together or separately

At 72 h, the supernatant of MCF7, T47D or placenta cultured on ‘Matrigel’ for 72 h (white bar). As a control, MCF7-eGFP were cultured separately under the same conditions (black bar). MCF7-eGFP were removed from the ‘Matrigel’, separated by FACS and their RNA was extracted. Messenger RNA levels of (A) the E2 targets PGR, GREB1 and MUC1 and (B) the progesterone targets ESR1, KLFA and Id1 were analyzed by RT-qPCR. Results were expressed as a percentage of the control value. Mean ± SEM. *Significantly different from control \( P < 0.05 \), paired ‘t’-test (ESR1, PGR, GREB1, MUC1; \( n = 4 \); Id1, KLFA; \( n = 3 \)).
(33 pg/ml) grown alone. T47D contained small amounts of progesterone (0.65 ng/ml), while MCF7 had none. Media from placentae grown separately contained high levels of E2 and progesterone (705 pg/ml and 67 ng/ml, respectively). Medium collected from placental-breast cancer cell co-culture contained significantly and synergistically higher E2 levels than those found in placentae or in cells grown separately (Fig. 3E, 1506 pg/ml and 1499 pg/ml for MCF7 and T47D, respectively, *P* < 0.05, *q* > 1). By contrast, media from placental-breast
cancer cell co-culture contained significantly less progesterone than media from placenta cultured alone (Fig. 3D, 44 ng/ml and 9 ng/ml for MCF7 and T47D, respectively, \( P < 0.05 \), q < 0.85).

This demonstrates that the breast cancer cells co-cultured with placental explants are exposed to \( E_2 \) and progesterone level similar to those found in blood of normal 6–9 week pregnancies (Schindler, 2005).

To investigate the reason for the earlier-mentioned effects of co-culture, we cultured MCF7 and T47D cells for 24 h on ‘Matrigel’ with placental levels of \( E_2 \) and progesterone and then measured hormone concentrations. As a control, \( E_2 \) and progesterone were added to a well coated with ‘Matrigel’ without any cells. We found that compared with the controls, progesterone and \( E_2 \) concentrations were decreased by the cells (MCF7: progesterone – 52%, \( E_2 \) – 15%; T47D: progesterone – 50%, \( E_2 \) – 23%, \( P < 0.05 \)). Thus, the reason for progesterone reduction in placental-breast cancer cell co-culture could be progesterone consumption by the cancer cells. However, the exact cause for \( E_2 \) elevation in the co-culture remains to be resolved but is probably a consequence of breast cancer cell–placenta interaction.

**FCS is needed for \( E_2 \) production by the placenta**

Progesterone can be produced by first trimester placenta. However, placental estrogens are usually derived from fetal androgens, placental proestogens or other steroid precursors (Boyd and Hamilton, 1970). As placental explants were cultured without the fetus, we wished to find the \( E_2 \) precursors. We speculated that the precursors originated from either FCS or phenol red. Thus, we cultured placenta in media with and without FCS and phenol red (a weak estrogen; Welshons et al., 1988). Placental explants cultured without FCS, and with and without phenol red produced significantly less \( E_2 \) than in full media (Fig. 3F, 81 and 86% reduction, respectively). Therefore, FCS is essential for placental \( E_2 \) production.

The elimination of breast cancer cells from the area around the EVT is mediated by \( E_2 \) and progesterone

Previously, we showed that the placenta eliminated MCF7 and T47D cells from EVT cell differentiation areas due to breast cancer cell migration away from the placenta (Tartakover-Matalon et al., 2010). Our aim was to analyze the involvement of \( E_2 \) and progesterone in mediating this effect.

The ER inhibitor (ICI 182 780) and the PR inhibitor (RU486) were added to the placental-breast cancer cell co-cultures. Areas of MCF7 and T47D elimination (‘bare area’) and MMP2 and MMP9 activity were assessed. After 24 h, both inhibitors reduced the ‘bare area’ around placental explants. Hundred nanometers RU486 produced a larger decrease than 10 nM ICI 182 780 (Fig. 4A and C, MCF7: – 95 versus –35%; T47D: –71 versus –45%, \( P < 0.05 \)). Both 10 and 100 nM ICI 182 780 had similar effects on the MCF7 cells but RU486 affected MCF7 in a dose-dependent manner so that 10 nM reduced the bare area by only 64%. After 72 h, both 10 nM ICI 182 780 and 100 nM RU486 still significantly decreased the ‘bare area’ with T47D cells (Fig. 4B, –62 and –47%, respectively, \( P < 0.05 \)), whereas only RU486 was still effective for MCF7 cells (Fig. 4B, –62%, \( P < 0.05 \)).

Moreover, both inhibitors decreased MMP2 and MMP9 activity in placental-MCF7 cell co-culture supernatants and again RU486 was more potent than ICI 182 780, decreases in MMP2 being 67 versus 43% and in MMP9 57 versus 40%, respectively. Effects on placental-T47D co-culture supernatants followed a similar pattern (Fig. 4D–F).

These results support the involvement of both progesterone and \( E_2 \), in mediating the MCF7 and T47D cell migration away from the placenta.

**Placental supernatants enhance breast cancer cell motility in an ER- and PR-dependent pathway**

As a next step, the effect of placental supernatants containing high \( E_2 \) and progesterone concentrations on breast cancer cell migration (scratch test) and invasion (transwell assay) was tested. We found that the placental supernatants increased the MCF7 and T47D migration (Fig. 5A, B and D, +33 and +51%, respectively, \( P < 0.05 \)) and invasion (Fig. 5E, F and H, +24 and +242%, respectively, \( P < 0.05 \)) compared with control MCF7 and T47D supernatants. Addition of ICI 182 780 to MCF7 and T47D supernatants did not affect migration, but it decreased the MCF7 cell migration induced by placental supernatant by 33% (\( P < 0.05 \), Fig. 5A).

RU486 did not affect MCF7 migration in the presence of MCF7 culture supernatant but inhibited T47D cell migration in the presence of T47D supernatant by 43% (Fig. 5B). RU486 completely blocked the stimulation of migration of MCF7 cells and markedly decreased stimulation of T47D cell migration by placental supernatant (Fig. 5A and B). Furthermore, adding RU486 to control MCF7 and T47D supernatants did not affect cell invasion; however, the addition of ICI 182 780 elevated MCF7 cell invasion (Fig. 5E), similar to results previously obtained by Thompson et al. (1988). The addition of either receptor inhibitor decreased the stimulation of breast cancer cell invasion caused by placental supernatant (Fig. 5E and F).

Therefore, placental induction of motility was reversed by ER and PR inhibitors. These results indicate that soluble factors alone can induce breast cancer cell motility without placenta–breast cancer cell contact or ‘Matrigel’ involvement.

**\( E_2 \) and progesterone in concentrations equivalent to placental culture increase breast cancer cell motility**

As a final validation of the influence of \( E_2 \) and progesterone on MCF7 and T47D cell motility, breast cancer cells were cultured in the presence of either ‘MCF7/T47D level’ or ‘placental’ level of \( E_2 \) and progesterone based on the findings reported earlier. The addition of ‘placental’ level \( E_2 \) and progesterone to MCF7 or T47D increased their migration (Fig. 5C) and invasion (Fig. 5G).

Taken together, we concluded that the induced motility of breast cancer cells around the placenta is a result of their exposure to elevated levels of \( E_2 \) and progesterone.
Placental supernatant activate MAPK that affects breast cancer cell migration

E2 and progesterone may activate MAP kinase (Faivre et al., 2005; Fu et al., 2008a,b; Li et al., 2010), and this stimulation is involved in breast cancer cell migration (Li et al., 2010). Therefore, protein levels and phosphorylation of ERK1/2, p38 and JNK were tested in MCF7 and T47D cells cultured with placental or breast cancer cell supernatant for 24 h. Placental supernatant elevated the phosphorylated forms of p38 and JNK in MCF7 and T47D cells (Fig. 6A and B) but had no effect on ERK1/2 phosphorylation. Thus, we added p38 and JNK inhibitors (SB 202190 and SP600125, respectively) to placental and breast cancer cell supernatants and analyzed their effect on...
Figure 5 Placental supernatants (UF) and placental levels of E2 + progesterone enhance breast cancer cell motility in an ER- and PR-dependent pathway. Breast cancer cell migration (A and B) and invasion (E and F) in the presence of supernatants from MCF7 or T47D (controls) or placental cultures were assayed by a scratch test and a transwell assay, respectively. Assays were done in the presence of ICI 182 780 (white bar), RU486 (gray bar) or ethanol solvent as a control (Eth, black bar). Results were expressed as a percentage of the values observed in supernatants from MCF7 or T47D cultured with Eth. Mean ± SEM, *Denotes significantly different from control P ≤ 0.05; **Indicates significant effect of ICI 182 780 or RU486 P < 0.05. Paired 't'-test (Scratch test: n = 5 and n = 4 for MCF7 and T47D, respectively; Transwell assay: n = 4 and n = 3 for MCF7 and T47D, respectively). The effect of placental concentrations of E2 and progesterone on breast cancer cell migration was tested by a scratch test (C) and invasion by a transwell assay (G). Breast cancer cells were cultured with E2 and progesterone concentrations equivalent to those measured in breast cancer cell (BCCL level) or placental (placental level) supernatants. Results were expressed as a percentage of the value observed with BCCL supernatant. Mean ± SEM, *Denotes significantly different from control P < 0.05, paired 't'-test." (Scratch test: MCF7 n = 5, T47D n = 4; Transwell: n = 3). (D) A representative photomicrograph of the scratch test taken at 0 and 24 h following assay. (H) A representative photomicrograph of the transwell assay taken at the end of the assay (48 h). BCCL, breast cancer cells, UF-Supematant.
MCF7 and T47D cell migration. Only the JNK inhibitor blocked the stimulation of migration by placental supernatant (Fig. 6C and D).

Thus, JNK activation is involved in the elevated migration caused by placental supernatants.

Hormones are not involved in mediating the placental effect on MCF7 cell death and elevated sub G1

Recently, we demonstrated that placental explants had no effect on T47D cell proliferation and almost no effect on their cell death (1.5% elevation; Tartakover-Matalon et al., 2010). However, placental explants increased the cell death and sub-G1 phase of the MCF7 cell cycle, yet had no effect on other cell-cycle phases (Tartakover-Matalon et al., 2010). In order to test the involvement of E2 and progesterone in mediating the sub-G1 phase and death elevation in the co-culture system, ICI 182.780 and RU486 were added to the co-culture and the cell cycle and death were reevaluated. ICI 182.780 caused elevated cell death (+7.5%, $P < 0.05$, Fig. 7A), increased sub-G1 phase (+3%, $P < 0.05$, Fig. 7B) and G1 cell-cycle phase (+19%, $P < 0.05$, Fig. 7C) in control cells, but had no effect on these parameters in placental-MCF7 co-cultures (Fig. 7A and C).
This is probably a result of the high E2 levels found in the co-culture competing with the inhibitor.

The only significant effect of RU486 was the G1 phase elevation, found in MCF7 co-cultured with placenta (Fig. 7C), suggesting that progesterone saved the cells from G1 cell-cycle arrest caused by the placenta. Once progesterone is blocked, this arrest was evident.

Further, we analyzed the effect of placental and MCF7 supernatant and of placental or MCF7 levels of E2 and progesterone on the MCF7 cell cycle and death. Placental supernatant did not elevate the MCF7 death rates or lead to any change in the cell-cycle phases in comparison with MCF7 cultured with MCF7 supernatant (data not shown). Furthermore, no differences were observed in the cell cycle or death following culture with E2 and progesterone in placental or MCF7 levels (24 and 72 h, Fig. 7D and E).

We can conclude that soluble factors alone (including the combination of E2 and progesterone) are not responsible for the increased cell death and elevated sub-G1 phases observed previously in the placental-MCF7 co-culture (Tartakover-Matalon et al., 2010).
ER and PR inhibitors do not reduce the EVT cell invasion

Our latest publication showed that MCF7 and T47D cell lines were most significantly affected near areas of EVT cell differentiation (Tartakover-Matalon et al., 2010). Thus, we had to confirm that ICI 182 780 and RU486 had no adverse effects on EVT cell differentiation and thereby affect the breast cancer cells. For this purpose, placental explants layered on ‘Matrigel’ were exposed to ICI 182 780 and RU486. A visual assessment of EVT cell differentiation was done at 24 h. Both inhibitors had no significant effect on the EVT cell invasion compared with control placental cultured without inhibitors. Surprisingly, increased MMP9 activity was found in the media collected from placenta cultured with ICI 182 780 versus control (50% elevation, \( P < 0.05 \)). These results confirmed that the decreased breast cancer cell elimination in the presence of inhibitors was not a result of inhibited EVT cell differentiation.

Discussion

Breast cancer during pregnancy is a rare event; yet with the current trend of delaying pregnancy to a later age, its incidence is expected to increase. Some studies suggest that cancer diagnosed during pregnancy is often more aggressive than that occurring in non-pregnant women (Shousha, 2000; Woo et al., 2003; Pereg et al., 2008). Nevertheless, there are very few reports of metastases to the products of conception. This fact suggests that the placenta is not a supportive environment for cancer cells.

This hypothesis was the basis of our previous research (Tartakover-Matalon et al., 2010). In this study, we demonstrated that placental explants induced breast cancer cell (MCF7 and T47D) elimination from sites of EVT cell invasion. This disappearance was shown to be mainly the result of migration and modest elevation in the cell death. After establishing and characterizing these phenomena, we conducted the current study to find the underlying mechanisms.

Our placental explant-breast cancer cell system is complex, involving various hormones, cytokines, growth factors and extracellular matrix (ECM) alterations. It has the advantage of being only one step away from in vivo conditions, but the complexity of this model has to be addressed. In order to deal with the numerous possibilities that exist in our system, we decided to use a non-biased microarray analysis. Microarray results suggested changes in the E2 signaling pathway. Since most E2 responsive genes were up-regulated while three were down-regulated, we could assume that additional factors were involved in the gene regulation. The importance of estrogen signaling to both breast cancer and pregnancy is well established (Folkerd et al., 2003; Pereg et al., 2008). Nevertheless, placental trophoblast cells lack the 17α-hydroxylase enzyme and, therefore cannot convert the C21-steroids to C19-steroids (Strauss et al., 1996). During pregnancy, the placenta produces E2 from the embryonic precursor. However, since our biological system did not contain the embryo, we attempted to find a source of immediate precursors of E2 in our placental explant culture. We found that FCS was responsible for 80% of the E2 production. We assume that the remaining 20% was derived from precursors that were in the original placental tissue; however, this needs to be confirmed. In contrast to the elevated E2 levels, the progesterone levels decreased in breast cancer-placenta co-culture compared with those found in placenta cultured alone. Our data suggest that this reduction was a result of progesterone usage by the breast cancer cells.

We then aimed to find out whether the higher levels of E2 and progesterone determined in our culture were responsible for the altered breast cancer cell phenotype caused by the placental explants. For this purpose, we cultured MCF7 and T47D cells with placental explants, placental supernatant and with E2 and progesterone at the same levels found in our placental cultures (‘placental level’), and compared their phenotype with that of the controls. The breast cancer cells cultured separately with or without breast cancer cell supernatant (collected previously) or with E2 and progesterone in levels found in breast cancer cell cultures (‘MCF7 and T47D level’) served as controls. All experiments were done on non-starved cells, and cultured on a ‘Matrigel’ substrate as in the co-culture model (except for the scratch assay, which was done on a plastic). In order to further confirm the hormone-specific effect, we used the steroid-hormone receptor inhibitors ICI 182 780 and RU486, for ER and PR, respectively.

Our results consistently showed that the placenta increased MCF7 and T47D cell motility, and that progesterone and E2 were significantly involved in mediating this effect. Furthermore, we demonstrated that JNK is specifically involved in mediating this effect.

Indeed, previous studies demonstrated that JNK facilitates E2 induced cell migration (Li et al., 2010). Nevertheless, as far as we know, its contribution to progesterone-derived migration had never been shown.

In our previous research, we also observed that the placenta increased MCF7 cell death. In the current study, we demonstrated that placental supernatant and the combination of placental hormones (E2 and progesterone) did not affect MCF7 cell death, suggesting that direct contact between the MCF7 cells and the placental explants, or to placental ECM embedded factors is needed to induce MCF7 cell death.

Although E2 and progesterone are known as mitogenic factors, no significant changes in the MCF7 cell-cycle phases were observed following their culture on ‘Matrigel’ with the placental explants or their supernatants.

Numerous studies have analyzed the effects of E2 or progesterone alone on breast cancer cells. It is mostly agreed that E2 induces breast cancer cell proliferation and cell-cycle progression. The effect of
Moreover, the existence of E2 and progesterone in our biological system that is layered on ‘Matrigel’ completely meets these requirements. Hence, it is necessary to take into account the involvement of the ECM is needed to analyze systems (Lange, 2008). This is why Lange (2008) suggested that a different experimental method including two- and three-dimensional systems (2D and 3D) with diverse substrates (Fu et al., 2008a,b; Lange, 2008). Indeed, the mitogenic actions of progesterone depend on the cell polarity, a property that is not supported in 2D culture systems (Lange, 2008). This is why Lange (2008) suggested that a system that will preserve the breast epithelial cell architecture and take into account the involvement of the ECM is needed to analyze the progesterone affect. Our established placental co-culture system that is layered on ‘Matrigel’ completely meets these requirements. Moreover, the existence of E2 and progesterone in our biological model did not modify MCF7 cell-cycle phases.

Several previous observations may explain our findings. Cross talk between the two hormones and their receptors is extensive and can eventually lead to variable outcomes (Chlebowski et al., 2003; McGowan et al., 2004). Generally, progesterone and E2 have opposing roles in the MCF7 cell death and proliferation; thus their combined effect may cancel each other. Secondly, hormone concentrations are critical for the final result. For example, E2 in small amounts can inhibit cell death; yet in large amounts, it can do the opposite (Lippman et al., 1976). Finally, the fact that our experiments were done on non-starved cells layered on ‘Matrigel’ in the presence of variable factors may mask part of the effects that could have been observed in a 2D plastic, starved culture.

The activity of progesterone and E2 also depends on the type of hormone receptor present (i.e. PR A, PR B, ERα, ERβ forms) and the relative expression of both forms in the cell (Fu et al., 2008a,b). MCF7 cells are known to contain only the α form of ER (Paruthiyil et al., 2004) and both forms of PR (Mullick and Katzenellenbogen, 1986). We found that MCF7 and T47D cells cultured on ‘Matrigel’ contain both forms of PR. The PR A form was found to be more prominent in MCF7 cells, while the opposite was observed with T47D. After co-culturing with the placenta, both MCF7 and T47D cells showed PR A prominence. PR isoform predominance, especially PR A or an increased PR A/PR B ratio, is observed in a high proportion of breast cancers. It correlates with invasive cell behavior (Fu et al., 2008a,b) and was shown to result in aberrant progesterone response, leading to loss of cell adhesion (McGowan et al., 2004). These previous studies (McGowan et al., 2004; Fu et al., 2008a,b) support our current findings, which showed that progesterone inhibitor affected MCF7 and T47D cell motility. In the current study, progesterone inhibitor was most effective in inhibiting the placent al effect on breast cancer cells. Nevertheless, although mRNA levels of PR were elevated, as expected from cells exposed to high E2 levels (Velarde et al., 2007), no elevated PR protein expression was found. However, as our previous studies demonstrated that like ER, which is down-regulated by E2 (Duong et al., 2007), PR levels are also decreased in the presence of high progesterone levels, and this effect can be independent of changes in PR mRNA (Turgeon and Waring, 2000). Furthermore, as E2 and progesterone levels are significantly higher in placental culture media in comparison with breast cancer cell media, the ligand-to-receptor ratio for cells co-cultured with placental explants is definitely elevated and may enhance progesterone signaling.

In conclusion, the involvement of both E2 and progesterone in mediating breast cancer cell motility is demonstrated throughout this study, and is supported by the initial microarray experiment results. Indeed, the bioinformatics provided the evidence of E2 pathway activation and involvement of additional factors that modulate E2 signaling.

This increased migration contributes to the elimination of breast cancer cells from the placental area (Tartakover-Matalon et al., 2010). In our previous study, we showed that MCF7 and T47D BCCLs were the most affected near areas of EVT cell differentiation. In the present study, we confirmed that ER and PR inhibitors did not inhibit EVT cell differentiation, suggesting a direct effect of the hormone on breast cancer cells. Since steroid hormones are soluble factors equally dispersed in the culture after 60–70 h, elimination of the breast cancer cells specifically from EVT implantation sites suggested involvement of additional factors. The combined effect of elevated migration caused by E2 + progesterone and cell elimination especially from EVT sites was responsible for creation of the bare areas. E2 and progesterone hormonal concentrations in our biological model are similar to those found in pregnant women in the early weeks of pregnancy. Thus, this may imply that whereas they contribute to breast cancer cell elimination from the placenta, they may also enhance breast cancer cell invasiveness and thus disease progression during pregnancy.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

**Authors’ roles**

G.E.S. performed the experiments, analyzed the data and wrote the manuscript. S.T.M. designed the experiments, analyzed the data and wrote the manuscript. M.L. and L.D. made substantial contributions to design, analysis, interpretation of data and revising the draft. M.P. and A.F. supplied the placenta and reviewed the manuscript. V.O.-K. performed the whole human genome cDNA microarray test. M.P.-C. participated in the bioinformatical analysis of the microarray results. G.R. participated in FACS analysis.

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