Breast cancer characteristics are modified by first trimester human placenta: in vitro co-culture study

S. Tartakover-Matalon1,2,*, A. Mizrahi1,2, G. Epstein1,2, A. Shneifi1,3, L. Drucker1,2, M. Pomeranz1,2,4, A. Fishman1,2,4, J. Radnay5, and M. Lishner1,2,3

1Oncogenetic Laboratory, Meir Medical Center, 45 Tschernovski St., Kfar Saba 44281, Israel 2Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel 3Department of Medicine A, Meir Medical Center, Kfar Saba 44281, Israel 4Department of Obstetrics & Gynecology, Meir Medical Center, Kfar Saba 44281, Israel 5Hematological Laboratory, Meir Medical Center, Kfar Saba 44281, Israel

*Correspondence address. Tel: +972-9-7472841; Fax: +972-9-7471145; E-mail: matalon.shelly@clalit.org.il

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BACKGROUND: Pregnant women with breast cancer present with a more advanced disease compared with non-pregnant women. Nevertheless, breast cancer metastasis to the placenta is rare. Trophoblast/tumor implantations share the same biochemical mediators, while only the first is stringently controlled. We hypothesized that the same mechanisms that affect/restrain placental implantation may inhibit metastatic growth in the placenta. We aimed to analyze the effects of human placenta on breast cancer cells.

METHODS: First trimester human placental explants were co-cultured with MCF-7/T47D-eGFP tagged cells. Following culture, placenta/cancer cells/both were fixed, paraffin embedded and sliced for immunohistochemical analysis or sorted by their eGFP expression for future analysis. The tested parameters were: proliferation (immunohistochemistry)/cell cycle (FACS), apoptosis (immunohistochemistry/FACS), cell count/adhesion/distribution around the placenta (cell sorter, visual observation and counting), matrix metalloproteinase activity (zymogram) and estrogen receptor (ER) expression (western blotting, immunohistochemistry).

RESULTS: Reduced breast cancer cell numbers (45%↓, 48%↓ for MCF-7/T47D, respectively, P<0.05) were observed near the placenta. The placenta elevated MCF-7 sub-G1 phase and modestly elevated apoptosis (3–17%↑ for T47D/MCF-7, respectively, P<0.05). Our findings demonstrate breast cancer cell migration from the placenta as: (i) T47D/MCF-7 cells changed their morphology to that of motile cells; (ii) elevated MMPs activity was found in the co-culture; (iii) placental soluble factors detached breast cancer cells; and (4) the placenta reduced MCF-7/T47D cells’ ER expression (a characteristic of motile cells).

CONCLUSIONS: MCF-7/T47D cells are eliminated from the placental surroundings. Analyzing the causes of these phenomena may suggest biological pathways for this event and raise new therapeutic targets.

Key words: pregnancy / breast cancer / apoptosis / migration

Introduction

Maternal malignancy during pregnancy is estimated to occur in 1 out of 1000 pregnancies (Al-Adnani et al., 2007). Since maternal age at conception continues to increase, the incidence of such cases is expected to rise (Pereg et al., 2008). Controversy exists regarding the effect of pregnancy on cancer prognosis, especially breast cancer and melanoma (Al-Adnani et al., 2007; Driscoll et al., 2009). Breast cancer is the most common malignancy affecting women worldwide (Keinan-Boker et al., 2008). Approximately 10% of breast cancer patients under the age of 40 are diagnosed during pregnancy (Beadle et al., 2009). Numerous studies have found that pregnant women with breast cancer have larger tumors and are more likely to have metastases and positive nodes compared with non-pregnant women (Woo et al., 2003; Pereg et al., 2008; Rodriguez et al., 2008). The reason for this is not yet clear, although, a delay in diagnosis due to pregnancy has been suggested as a possible cause. A theory that gestational hormones and pregnancy-related growth factors may induce a more aggressive behavior in malignant cells (Schedin et al., 2006; Rodriguez et al., 2008) is yet to be proven. Biologically, the level of estrogen receptor alpha (ERα) in the breast cancer cells of pregnant patients is lower than that in non-pregnant women (Shousha et al., 2000). Generally, ERα negative tumors are often less differentiated, and patients tend to have a decreased overall survival rate (Putti et al., 2005).
Despite the advanced breast cancer found in pregnancy, metastasis to the products of conception is rare (Al-Adnani et al., 2007). Approximately 80 cases of placental and/or fetal metastatic involvement originating from maternal cancer have been reported since 1866 (Al-Adnani et al., 2007).

Malignant melanoma is the most common malignancy involving the placenta, accounting for 30% of placental metastasis, followed by lung (16%), hematological malignancies (15%), breast (13%) and other cancers (26%) (Al-Adnani et al., 2007). The uteroplacental blood flow represents 10% of maternal cardiac output and thus, hemogenous spread of maternal tumor-emboli to the placenta is not unexpected (Tolar et al., 2003). Therefore, the limited number of placental metastasis from maternal tumors suggests that the placenta may be a non-supporting microenvironment for cancer cells. Investigation of the causes of impaired cancer cell growth near the placenta may suggest biological pathways for this phenomenon and future therapeutic targets.

Metastases cause 90% of all cancer deaths (Yilmaz et al., 2007). Secondary tumors are formed by cancer cells that have left the primary tumor mass and traveled to new sites, mainly through blood and lymphatic vessels. Metastasis occurs when cancer cells detach from neighboring tumor cells and extracellular matrix (ECM), and gain migratory and invasive capabilities; processes that demand changes in adhesion molecules and tight junctions (Kopfstein et al., 2006). Cell migration can be broadly divided into collective or single cell. Collective migration of cells, observed in several carcinomas, including mammary, consists of multiple cells that are mobile, yet simultaneously connected by cell—cell junctions (Friedl et al., 2009). Migration is regulated by signaling cascades such as Rho small GTPases and by activating proteases, especially matrix metalloproteinase 2 (MMP2) (Wolf et al., 2007). Following detachment and migration, cancer cells extravasate into a new environment where they seed and grow as secondary tumors. Neighboring cells in the metastatic niche influence cancer cell behavior by secreting various ECM proteins, chemokines, cytokines, growth factors, proteases and protease inhibitors (Schedin et al., 2004), which may restrain or encourage cancer cell growth in the new metastatic sites (Paget et al., 1989; Hu et al., 2008). Thus, the ability of cancer cells to form new colonies in the placental area may be related to the placenta’s ability to support cancer growth.

Placental cells (trophoblasts) are capable of invasive processes and are able to protect themselves from the maternal immune system. Malignant tumor cells have similar characteristics (Soundararajan et al., 2004; Cohen et al., 2007) and tumoric and trophoblastic invasions share the same biochemical mediators (Bilban et al., 2009). However, trophoblastic invasion during implantation is stringently controlled (Cohen et al., 2007). We hypothesized that the mechanisms that restrain trophoblast cell invasion into maternal tissue may also be responsible for the inhibition of metastatic growth in the placental area.

Human trophoblast stem cells differentiate along two pathways: syncytiotrophoblast and extravillous trophoblast (EVT) cells. Cytotrophoblast cells within the placental villi differentiate into syncytiotrophoblasts and mediate nutrient and gas exchange between mother and fetus. A second pathway involves the differentiation of cytrophoblast cells in the anchoring placental villi into EVT cells, which invade through the maternal tissue and associate with the decidual blood vessels (Yamamoto et al., 2009). First trimester human placenta secretes several factors, including hormones (such as progesterone and estrogen), growth factors (such as GM-CSF) and cytokines (such as IL6, LIF) (Bowen et al., 2002; Cohen et al., 2007; Fitzgerald et al., 2008). Placental cells may also synthesize a number of apoptosis triggering molecules, such as TNF-α (Bowen et al., 2002) and induce death of the neighboring cells (Bowen et al., 2002). These factors may also affect the migration, invasion, proliferation and death of breast cancer cells.

In this study, we wished to evaluate the effects of first trimester human placental explants on breast cancer cells. We used the placental explant model that was developed by Miller (Genbacev et al., 1992), where trophoblast cells differentiate into EVT cells and are released from the anchoring villi to the matrigel substrate. We co-cultured breast cancer cells (MCF-7/T47D cell lines) with the placental explants during EVT cell differentiation and invasion. Our study demonstrates that MCF-7/T47D cells are eliminated from the area surrounding the EVT cells, especially by migrating/detaching from this area.

Materials and Methods

Cell lines
MCF-7/T47D breast cancer cells (kindly provided by Prof. Z. Malik, Bar Ilan University and by Dr Ilan Tzarfati, Tel Aviv University) were maintained in DMEM medium supplemented with L-glutamine (2 mM), fetal calf serum (FCS:10%) and antibiotics. MCF-7 eGFP/T47D-eGFP tagged cells were cultured in the presence of G418 antibiotics (Biological Industries, Beit Haemek, Israel).

Antibodies
A list of the antibodies used is represented in Supplementary, Table S1.

Transient and stable transfection of plasmid DNAs
The eGFP expression vector eGFP-C1 (C1 vector) was purchased from Clontech (Mountain View, CA, USA). The plasmid was introduced into T47D/MCF-7 cells by FuGENE 6 transfection reagent (Roche), according to the manufacturer’s instructions. Briefly, 3 x 10^5 T47D/MCF-7 cells were grown in 2 ml of growth medium overnight. Three microliters of plasmid DNA was added to the mix and incubated for 30 min at room temperature. Then, the entire volume of the tube was added to the well that contained breast cancer cells. Forty-eight hours following transfection, cells were sorted by FL1 (FITC) fluorescence with Coulter Flow Cytometer (FACS; EPICS-XL, Beckman Coulter, Fullerton, CA, USA). Following transfection, T47D/MCF-7 cells were exposed to 300 μg/ml G418 (Clontech) for 2 weeks. The cells were sorted (cell sorter) and only eGFP expressing cells continued to grow in medium containing G418.

Placental culture and co-culture experiments
The local ethics committee approved the use of placental tissues. Placentae, 6–9 weeks gestational age, were retrieved from terminated normal pregnancies. Placental villi were dissected from the fetal membranes. Explants of 10 mg wet weight were transferred into culture dish inserts.
(Millipore Corporation) that were previously layered with matrigel (BD Biosciences). Medium [DMEM/F-12 (HAM)-1:1, l-glutamine (2 mM), sodium pyruvate (1 mM), antibiotics and FCS (10%), (Biological Industries, Beit Haemek, Israel)] was added to the lower well of the culture dish (bottom media) and no medium was added above the explants (top medium). Cultures were pre-incubated overnight in a 5% CO2 incubator. After 12–16 h, top media containing MCF7/MCF-7 eGFP/T47D/T47D-eGFP cells or CAG myeloma cells (all 4 × 104) were added to the placenta. As a control, breast cancer cells, CAG cells and placenta were cultured alone on matrigel. Media from the well (bottom) and the inserts (top) were replaced 24 and 72 h following placental culture and top media were collected and stored at −80°C for further MMP analysis. After 96 h of placental culture, inserts containing placental explants, breast cancer cells, or both were fixed, paraffin embedded and sectioned for further immunohistochemical analysis. Breast cancer cells tagged with eGFP were either treated the same as the non-tagged cells or retrieved from the matrigel by trypsin and sorted by their eGFP expression for future cell cycle and protein analyses.

**Cell sorting**

Breast cancer and placental derived cells were harvested 96 h post-seeding and passed several times through a syringe for clump dispersion. Next, cells (5 × 10^6 per ml in PBS supplemented with 10% FCS) were isolated and collected using a BD FACSARia cell sorter (BD biosciences). Matching cells treated with respective transfection reagent only, were considered negative for eGFP and used for calibration of eGFP cell threshold.

**Breast cancer cell adhesion to plastic platform**

Breast cancer cells (4 × 10^4 cells per well) were incubated for 24 h in plastic plates (24 wells) in the presence of 20% placental medium [DMEM/F-12 (HAM) + supplements] as described above, and mixed with 80% of one of the following media:

- (i) Medium alone [DMEM/F-12 (HAM) + supplements] (Medium control).
- (ii) Medium that was previously collected from inserts layered with matrigel for 24 h (Matrigel conditioned medium).
- (iii) Medium containing placental derived soluble factors that was previously collected from placental explants that were grown on matrigel for 24 h (Placental condition medium).

Following incubation, cultures were inspected under contrast phase microscope and photographed (Nikon ‘Labphot’ light microscope). Quantitative analysis was achieved by counting adhered versus non-adhered (round) T47D/MCF-7 cells in 4 mm² squares that were marked on the middle of the photograph. The 4 mm² squares were always positioned in the same photographic location.

**Immunohistochemistry**

Immunohistochemistry procedures were employed for: MCF-7/T47D cells and placental proliferation (using anti-Ki67), MCF-7/T47D cells and placental apoptosis (using anti-fragmented Parp/anti-fragmented caspase 3), MCF-7/T47D cells ERα analysis and for CD138 a marker for Myeloma cells (in CAG Myeloma co-culture).

Since MCF-7 cells do not contain caspase 3 and Parp is only partially cleaved in T47D cells, we had to evaluate fragmented Parp to identify MCF7 apoptotic cells and fragmented caspase 3 for analysis of T47D and apoptotic trophoblast cells.

In order to differentiate between EVT cells and breast cancer cells, double-staining procedures, which included eGFP staining of the breast cancer cells (positive to eGFP and negative to HLA-G) or HLA-G staining of the EVT cells (positive to HLA-G and negative to eGFP) were used in the co-culture experiments. Both systems provided a control for each other: whereas eGFP identifies only MCF-7/T47D-eGFP cells, the other staining combination avoids any suspected cross-reaction that could occur because of the addition of the eGFP tag to the breast cancer cells.

A total of six co-culture experiments were done, three of each method. Both sets of experiments gave the same results and thus they were combined (proliferation/apoptosis analysis of MCF-7 cells, apoptosis analysis of T47D cells, proliferation analysis of T47D cells was done only with non-tagged T47D cells). For both systems, paraffin-embedded sections were deparaffinized in xylene and alcohol, rinsed in phosphate buffered saline (PBS), immersed in EDTA buffer (pH 8) and heated in a 700 W microwave oven for 15 min. Endogenous peroxide activity was quenched in 3% H2O2 (in PBS). Samples were covered with normal blocker serum and incubated with the appropriate primary antibody (Ki67, caspase 3, fragmented Parp, or ERα) overnight. Following washing, the slides were incubated with horseradish peroxidase labeled polymer conjugated to the second antibody, washed and developed with AEC (ECL kit, Santa Cruz, CA, USA). After rinsing, denaturing solution for double stain procedures was added and washed (Biocare Medical, Concord, CA, USA).

Then, the slides were incubated with the second primary antibody (anti-eGFP in the co-culture system of eGFP tagged cells or anti-HLA-G in the co-culture system of non-tagged cells). Following rinsing with PBS, the slides were incubated with biotinylated antibody covered with streptavidin alkaline phosphatase and developed with BCIP/NBT-chromogen (Chemicon International, Temecula, CA, USA). The same procedures were used to evaluate trophoblast cell proliferation (Ki67 staining) and apoptosis (caspase 3 and fragmented Parp staining) following their co-culture with the breast cancer cells and in controls. Isotype-matched control antibodies were used and excluded non-specific staining of the tested antibodies.

**Cell counting**

Cell counting procedures were employed for evaluation of immunohistochemistry procedures and breast cancer cell distribution around the placental explants. Immunohistochemistry: microscopic evaluation (>400) allowed enumeration of stained from non-stained cells. We evaluated breast cancer staining (Ki67/caspase 3/Parp/ERα) in co-cultures (placental explants + breast cancer cells) versus breast cancer cells that were cultured alone. We also evaluated the number of Ki67 and caspase 3 stained trophoblast cells versus non-stained placental cells in placental explants that were co-cultured with breast cancer cells and on placenta that were cultured alone as a control. Three to six different placenta were used for each analysis. Every test for each treatment and experiment was performed on three to four explants. Thus, at least 18 slices were counted for every treatment (3 placenta × 3 explants × duplicate for each explant).

In order to evaluate the number of MCF-7 cells located around different areas of placenta (with/without EVT cells) we combined cell number data from all double-staining procedures (Ki67/HLA-G, Parp/HLA-G, ER/HLA-G, altogether: 18 slides × 3) and counted all the cells that were located around the explants; separating between areas that contained EVT cells (positive for HLA-G) and areas that did not contain EVT cells (negative for HLA-G).

**Breast cancer cell distribution around the placental explants**

T47D/MCF-7 cell distribution was evaluated by visual observation and cell counting under a contrast phase microscope (Nikon ‘Labphot’ light microscope). After 96 h in culture, the wells were photographed. Quantitative analysis was achieved by dividing the area surrounding the placental explant into 1 mm squares and counting the number of cancer cells in...
each and in squares positioned in photographs of control wells (breast cancer cells cultured alone). EVT differentiation and migration can be morphologically identified by microscopic observation as fingerlike outgrowth projections that surround the placental explants, which are later accompanied by individual EVT cells that bud from the villous tips into the matrigel (Methods in Toxology, 38: 246, 1993). Thus, we could discriminate between placentral areas from which EVT cells differentiated and migrated to the matrigel and other placental areas that did not contain EVT cells. Furthermore, fluorescence microscopy demonstrated that these extravillous cells were indeed negative for eGFP and were not of breast origin.

**Cell cycle analysis**

Cells were retrieved from the matrigel by trypsinization and suspended in PBS (Biological Industries, Beit Haemek, Israel) containing 0.5% formaldehyde (Gadot, Biochemical Industries, Haifa Bay, Israel) and 0.1% sodium azide (Sigma Aldrich, St Louis, MO, USA) for 1 h at 4°C. Fixation was followed by washing in ice-cold PBS and then the cells were re-suspended in 70% ethanol/PBS overnight at −20°C. The following day, cells were exposed to 40 µg/ml propidium iodide (PI) (Sigma Aldrich) and 100 µg/ml Ribonuclease A in PBS for 30 min at room temperature in the dark, and later read by flow cytometry (EPICS-XL, Beckman Coulter). Results were analyzed using either WinMDI 2.8 or ModFit LT 3.0 programs (Venty Software House, Inc., Topsham, ME, USA). Cell cycle analysis was done only to the eGFP tagged cells (breast cancer cells).

**Cell death analysis**

Annexin V-PE (BioVision, Mountain View) supplemented with 0.1 µg/ml 7AAD (eBioscience, San Diego, CA, USA) was used to detect exposed phosphatidylserine according to the manufacturer’s instructions and assayed for fluorescence by FACS (EPICS-XL, Beckman Coulter, UK). Cells that were positive for Annexin V and negative for 7AAD were considered apoptotic, whereas cells positive for both were defined as late apoptotic or necrotic cells.

**Western blotting**

MCF7 eGFP/T47D-eGFP cells were separated from the placent al explants by cell sorter and lysed in buffer that contained 50 mM Hepes, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 10 mM NaPPi, 2 mM NaVO3, 20 mM EDTA, 1 mM PMSF, 10 µg/ml Leupeptin for 10 min on ice. Then 20–30 µg protein lysate mixed 1:5 with loading buffer was denatured for 10 min at 65°C and separated on SDS–PAGE followed by semi-dry transfer to PVDF membrane. After blocking the non-specific binding sites with 5% milk powder, the membranes were incubated overnight at 4°C, with the anti-ERα antibody. Equal protein loading was confirmed with the anti-tubulin antibody. Primary antibody was rinsed with Tris-buffered saline (TBS) with and without 0.01% Tween. Bound antibodies were visualized using horseradish peroxidase conjugated secondary antibody (Vector Laboratories, Marion, IA, USA) and enhanced chemiluminescence (ECL) detection (ECL kit, Amersham Pharmacia, Piscataway, NJ, USA). Optical densities of immunoreactive protein bands were measured using Gel Analyzer software (Media Cybernetics, Bethesda, MD, USA) (arbitrary units) and normalized to tubulin values.

**Gelatin zymography**

Collected supernatants were assayed for MMP2/9 gelatinase activity. Aliquots (20 µl) of the media were electrophoresed at non-reducing conditions in 10% polyacrylamide gel containing 1 mg/ml gelatin type A (Sigma). Gels were washed twice in 2.5% Triton X-100 for gelatinase renaturation and incubated overnight in 50 mM Tris–HCl (pH 7.5) and 5 mM CaCl2. Coomassie blue staining followed by destaining allowed visualization of clear lysis zones against a blue background. Band intensity was calculated for each example. Standardized activated and proactivated MMP2/9 (Chemicon, Temecula, CA, USA) was electrophoresed near the experimental samples to validate the identity of the MMP. Gels were analyzed employing Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA, USA).

**Statistical analysis**

Paired Student’s t-tests were used to analyze differences between cohorts. A P-value of <0.05 was considered significant. All experiments were repeated separately three to six times.

**Results**

The area around the placent al explant had fewer breast cancer cells

Following 96 h of MCF-7/T47D cell culture on matrigel, most of the cells were arranged in aggregates, the remainder as single cells. Counting colonies similar in photographs of placenta-breast cancer cell co-culture and sorter data demonstrated reduced breast cancer cell numbers near the area of the placental explant (>40% reduction, P < 0.05, Fig. 1a–g). Additional studies with MCF-7 cells demonstrated that breast cancer cells did not contain placental explants in slides stained for HLA-G (EVT marker) of placental-MCF-7 cell co-culture and controls. Results showed significantly reduced MCF-7 cells number near placental areas with EVT cells compared with placental area that did not contain EVT cells and compared with control slides (42 ± 5.7, 107 ± 8.6, 219 ± 37.6 cells/slide, respectively, P < 0.05).

Placental explant reduced the number of breast cancer proliferating cells

The decrease in the number of breast cancer cells in the area surrounding the placental explant could be a result of reduced proliferation, increased apoptosis and/or cell detachment/migration from the placental area. To evaluate the effect of placental explants on the breast cancer cell proliferation rate, inserts with the co-cultured cells were fixed and stained. Proliferation was determined based on Ki67 staining (Fig. 2a–c). Decreased Ki67 staining was detected in MCF-7 cells located near the placenta compared with cells cultured alone (11% decrease, P < 0.05, Fig. 2d). A similar, yet not statistically significant decrease, was observed in T47D cells (10% decrease, P = 0.14, Fig. 2d). Additional studies with MCF-7 cells demonstrated that the reduced Ki67 staining was observed in aggregates located near EVT releasing villi (Fig. 2e), whereas MCF-7 single cells that were located near the placental area in which EVT cells were not observed had increased proliferation (Fig. 2e, “MCF-7 + Placenta No EVT”). Furthermore, cell cycle analysis (FACS) of MCF-7 eGFP cells cultured with/without placental explants showed the placenta to elevate the
sub-G1 fraction in comparison with control (Table I, Fig. 2f), indicating an elevated cell death rate. However, analysis of the cell cycle data without the sub-G1 phase (i.e. 100% cells = all phases except sub-G1) demonstrated no differences in the percentage of G1 and S phases in MCF-7 cells cultured with placental explants compared with controls, indicating that MCF-7 cell proliferation was not arrested by the placenta. Thus, the decreased Ki67 expression could be a result of the elevated sub-G1 fraction.
The placental explant increased breast cancer cell apoptosis

Since we observed decreased numbers of MCF-7/T47D cells and an elevated sub-G1 phase in MCF-7 cells that were located near placental explants, we analyzed their apoptotic rates. Apoptosis was determined with Parp p85 fragment staining in MCF-7 cells and with caspase 3 staining in T47D cells (Fig. 3a–c). A significant increase in apoptosis was found in MCF-7 cells located near placental explants compared with MCF-7 cells cultured alone (Fig. 3d, 17 versus 3%, respectively, *P* < 0.05). These results match those observed in the cell cycle analysis (17 versus 4.7%, sub-G1 phase in MCF-7 cells cultured with and without placental explants, respectively). A small but significant increase in apoptosis was also observed in T47D cells located near placental explants compared with T47D cells cultured
alone (3.2 versus 1.5%, respectively, \( P < 0.05 \); Fig. 3d). Additional studies with MCF-7 cells demonstrated that increased fragmented Parp staining was observed especially in single cells located near placental EVT releasing villi (Fig. 3e).

**The effect of coculture on breast cancer cell distribution, morphology and MMP secretion**

The reduced proliferation and elevated cell death of MCF-7/T47D cells co-cultured with placental explants afforded partial and limited explanation for the cancer cells’ absence from the EVT area. Therefore, we assumed that other mechanisms such as cell migration/detachment were responsible for these phenomena. In an attempt to understand the chronology of events in the elimination of breast cancer cells, we examined MCF-7/T47D cells cultured with and without placental explants at earlier time points (0, 2, 4, 6, 24 and

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**Table 1** Cell cycle analysis of MCF-7 cells with and without placental explants (72 h).

<table>
<thead>
<tr>
<th>Cell phase</th>
<th>MCF-7</th>
<th>MCF-7 + Placenta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-G1</td>
<td>4.7</td>
<td>17*</td>
</tr>
<tr>
<td>G1 (All cells)</td>
<td>62.6</td>
<td>54.9</td>
</tr>
<tr>
<td>S + G2/M (All cells)</td>
<td>32.6</td>
<td>27.8</td>
</tr>
<tr>
<td>G1 + S + G2M (All cells)</td>
<td>93.8</td>
<td>81.5*</td>
</tr>
<tr>
<td>G1 (without sub-G1)</td>
<td>64.7</td>
<td>65.2</td>
</tr>
<tr>
<td>S + G2/M (without sub-G1)</td>
<td>33.6</td>
<td>32.8</td>
</tr>
</tbody>
</table>

*In order to analyze cell cycle data without pre G1 cells, we considered 100% of the cells as the eGFP tagged cells in all phases, except for sub-G1 phase. Bold values are indicated statistically significant differences \(* P < 0.05\).
Placental explant decreased the ERα level of breast cancer cells

Since our results suggest increased breast cancer cell motility near the placental explants, we analyzed the effect of first trimester human placental explants on MCF-7/T47D ERα levels. Decreased ERα expression was found in MCF-7/T47D cells co-cultured with placental explants compared with MCF-7/T47D cells cultured alone (Fig. 5a–e). Immunohistochemistry, 48 and 93% decreased ER expression in MCF-7 and T47D cells, respectively, <0.05, Fig. 5d and e, western blotting, 65% decrease in MCF-7 cells, P < 0.05. Additional studies with MCF-7 cells demonstrated that reduced ER staining was observed all around the placental explants and not only in cells that were located near EVT releasing villi (data not shown).

The effect of first trimester placental soluble factors on MCF-7/T47D cells cultured on plastic substrate

The placenta could have affected the phenotype of breast cancer cells through direct cell to cell contact, by secreting soluble factors, or both. To evaluate the effect of placental soluble factors on MCF-7/T47D cells, they were exposed to media collected during placental explant proliferation and apoptosis rates. Co-culturing placenta with both T47D and MCF-7 cell lines did not change the proliferation and apoptosis rates of the trophoblast cells. Data are presented in Table II.

The area around the placental explant had fewer myeloma cells

Our data support the assumption that the placenta is a cancer-non-supportive microenvironment. Thus, we wished to test its effect on non-epithelial cells. We analyzed the effect of placental explants on CAG cell (myeloma cell line) distribution. We found that myeloma cells were absent from regions located near EVT releasing villi (Fig. 7a–c). Further immunohistochemistry studies demonstrated that the area proximate to the EVT cells (positive for HLA-G but negative for CD138) was indeed empty of CAG cells (negative for HLA-G but positive for CD138); however, CAG cells were found near other placental areas that did not contain EVT cells.

Discussion

Breast cancer is often diagnosed at a more advanced stage in pregnant compared with matched non-pregnant women (Pereg et al., 2008). However, the placenta and embryo are only rarely involved with metastatic disease and are described as a non-supportive microenvironment for cancer cells (Al-Adnani et al., 2007). To study the unique effect of the placenta on breast cancer cells, we co-cultured human first trimester placental explants with MCF-7/T47D cells (breast cancer cell lines that express ERα). In this model, the placental explants are located above the matrigel, and EVT cells differentiate and migrate from the placental villi to the surrounding matrigel (Genbacev et al., 1992). We showed that most of the breast cancer cells disappeared from areas proximate to the EVT cells, starting 6–24 h post-seeding. In contrast, more than 90% of the MCF-7/T47D cell that were cultured without the placenta (controls) formed aggregates and distributed equally in the wells, whereas the rest remained as single cells. Furthermore, apoptosis and reduced proliferation of breast cancer cells only partially contributed to their reduced numbers around the placental explants. Our findings suggest that breast cancer cells migrated/detached from the area of the EVT cells because (i) T47D cells changed their morphology to spindle-like cells that characterize motile cells (Tiezzi et al., 2007). To study the unique effect of the placenta on breast cancer cells, we co-cultured human first trimester placental explants with MCF-7/T47D cells (breast cancer cell lines that express ERα). In this model, the placental explants are located above the matrigel, and EVT cells differentiate and migrate from the placental villi to the surrounding matrigel (Genbacev et al., 1992). We showed that most of the breast cancer cells disappeared from areas proximate to the EVT cells, starting 6–24 h post-seeding. In contrast, more than 90% of the MCF-7/T47D cell that were cultured without the placenta (controls) formed aggregates and distributed equally in the wells, whereas the rest remained as single cells. Furthermore, apoptosis and reduced proliferation of breast cancer cells only partially contributed to their reduced numbers around the placental explants. Our findings suggest that breast cancer cells migrated/detached from the area of the EVT cells because (i) T47D cells changed their morphology to spindle-like cells that characterize motile cells (Tiezzi et al., 2007).
Figure 4  MCF-7/T47D cells changed morphology, distribution and MMPs activity around the placental explants. (a–d) Representative photomicrographs of MCF-7 (a and b) and T47D (c and d) cells cultured with (a and b) and without placental explants (c and d) taken at several time points. (e) Representative photomicrographs of MMP2 activity (experiments with MCF-7) and MMP9 activity (experiments with T47D) in media collected from BCCLs cultured with/without placental explants for 72 h and of placental explants cultured alone. Furthermore, quantification of MMP2/9 activity in media collected from breast cancer cells or from placental explants cultured alone and together; experiments with MCF-7 (n = 5) (4 h), experiments with T47D (n = 4–6) (4 h). Results were normalized to the total activity of breast cancer cells + placental explants. Statistically significant differences are indicated (*, P < 0.05).
et al., 2007) and MCF-7 cells rapidly formed elongated aggregates; (ii) This process was accompanied by elevated MMPs activity that is known to facilitate cell invasion through the matrigel; (iii) Placental soluble factors caused breast cancer cells to detach from plastic substrate; and (iv) breast cancer cells co-cultured with the placenta had reduced ERα expression.

Generally, ERα negative tumors are less differentiated, more aggressive, and have higher metastatic potential than ERα positive tumors (Putti et al., 2005; Bentzon et al., 2008). In vitro studies showed that breast cancer cell motility is inhibited by ERα (Platet et al., 2004). Thus, the reduced ERα levels found in breast cancer cells following their co-culture with the placenta may have supported their migration. However, since breast cancer cells were eliminated, especially from areas located near EVT cells, whereas the reduced ERα expression of the breast cancer cells was found around all the placental explants, additional placental factors must have been involved in their elimination. Interestingly, decreased ERα was also found during normal pregnancies in the receptive uterus (Bazer et al., 2008). Furthermore, decreased expression of ERα levels in breast cancer cells of pregnant women has been suggested previously (Shousha, 2000). Our study is
The effect of human placenta on breast cancer cells

Results are mean + matrigel compared with cells that were exposed to media (suggested as the ratio of cells exposed to placental factors/treatments and average of 44 middle of each well photomicrograph. Duplicates were made of all. Placental cells may synthesize a number of apoptotic triggering molecules, such as TNF-α, FasL, TRAIL (TNF-related apoptosis-inducing ligand) and INF and induce the death of neighboring cells (Aboagye-Mathiesen et al., 1996; Bowen et al., 2002; Fluhr et al., 2007; Bazer et al., 2009). EVT cells produce elevated levels of INFs compared with other trophoblast cells in the presence of growth factors (GM-CSF + PDGF), (Aboagye-Mathiesen et al., 1994; Aboagye-Mathiesen et al., 1996; Sodek et al., 2008; Bazer et al., 2009) and INFγ/β has tumor suppressor activities (Nicolini et al., 2006).

We found that placental explants changed the morphology of T47D and MCF-7 cells. Instead of round MCF-7 colonies that were formed on matrigel, elongated aggregates were seen near the placental explants. Similar changes in MCF-7 aggregate elongation were demonstrated previously, as a result of the combined exposure of breast cancer cells to estrogen and progesterone (Sukococheva et al., 2009). Noel et al. (1988) demonstrated that rapid MCF-7 cluster formation on the matrigel is a result of cell aggregation and not cell proliferation. Indeed, in order to rapidly form aggregates (6 h), the cells should have migrated on the matrigel. The MCF-7 cell cluster formed strand-like structures which soon moved away. MMPs are critical to the collective migration of tumor cells (Rorth et al., 2009). Furthermore, past studies found that MT1-MMP (MMP2 activator) is a key enzyme in the proteolysis of collagen-rich ECM to generate space for migration and invasion of cell masses (Nabeshima et al., 2000; Wolf et al., 2007). Indeed, we found elevated MMP2 activity, which supports migration of MCF-7 aggregates through the matrigel. T47D cells created very small aggregates and demonstrated a non-significant elevation of MMP2 activity levels in their medium. Instead, elevated MMP9 activity was found in T47D-placenta co-culture. MMP9, like MMP2, supports breast cancer cell invasion (Kunigal et al., 2007) and thus, their elevated levels together with the T47D morphological change to spindle-like cells and their rapid disappearance from the EVT area suggest that they migrated from this area. Both T47D and MCF-7 cells were eliminated from the placental area. However, few differences were observed between the placental effects on these two cell lines. The placenta had less of an effect on T47D cell death compared with MCF-7 cells. Furthermore, the placenta induced elevated MMP9 and had only a non-significant effect on MMP2 levels in T47D cells, whereas significantly elevated levels of MMP2, but not of MMP9, were observed in MCF-7 cells that were co-cultured with placenta. The reason for these differences may be attributed to the mutant p53 that is expressed by T47D cells (Lim et al., 2009), while MCF-7 cells express wild-type p53. Mutant p53 have pro-survival effect which may protect cells from apoptosis (Lim et al., 2009). Moreover, previous studies with sarcoma demonstrated that cells with mutant p53 overexpress MMP9, whereas wild-type 53 activate MMP2

![Figure 6](image)

**Figure 6** The effect of placental CM on breast cancer cells adhesion and apoptosis. (a) Quantification of breast cancer cell adhesion achieved by microscopic observation and cell counting in breast cancer cells cultured with and without placental CM. Results are presented as the ratio of cells exposed to placental factors/matrigel compared with cells that were exposed to media (y-axis). Results are mean ± SE [n = 7–11 (MCF-7); n = 3 (T47D)]. Cells were counted in 4 x 1 mm² squares that were positioned in the middle of each well photomicrograph. Duplicates were made of all treatments and average of 44 ± 7, 49 ± 9, 28 ± 8 cells (MCF-7) and 25 ± 8, 42 ± 3, 35 ± 4 (T47D) were counted in each square (medium, matrigel CM, placental CM, respectively). Thus, 352 ± 56, 392 ± 72, 222 ± 64 MCF-7 cells and 200 ± 64, 336 ± 24, 280 ± 32 T47D cells were counted in each experiment, respectively [cells/square x 4 (squares) x 2 (duplicate)] (b) Representative photomicrographs of MCF-7/T47D cells cultured with medium collected from placental explants cultured on matrigel (Placenta CM) or with medium that was cultured with matrigel only (Control). (c) Analysis of MCF-7 cell apoptosis in cultures exposed to placental soluble factors and control media. Analyzed by 7AAD/Annexin V staining and FACS (n = 7). Statistically significant differences are indicated *(P < 0.05)*. CM, conditioned media.
Table II  The effect of breast cancer cells on trophoblast cell apoptosis and proliferation.

<table>
<thead>
<tr>
<th>Cells</th>
<th>With/without Placenta</th>
<th>Apoptosis Assay</th>
<th>Cells (%)</th>
<th>Proliferation Assay</th>
<th>Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>+Placenta</td>
<td>Parp</td>
<td>8.4 ± 4.8</td>
<td>Ki67</td>
<td>57.4 ± 3.5</td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
<td>Parp</td>
<td>3.5 ± 1.2</td>
<td>Ki67</td>
<td>53.2 ± 2.6</td>
</tr>
<tr>
<td>T47D</td>
<td>+Placenta</td>
<td>Caspase 3</td>
<td>0.25 ± 0.12</td>
<td>Ki67</td>
<td>72.3 ± 5.6</td>
</tr>
<tr>
<td>T47D</td>
<td></td>
<td>Caspase 3</td>
<td>0.22 ± 0.1</td>
<td>Ki67</td>
<td>74.8 ± 3.5</td>
</tr>
</tbody>
</table>

Figure 7  Distribution of myeloma cells around the placental explant. (a) Representative photomicrographs of CAG cells cultured with/without placental explants. CAG cells are eliminated from EVT cell area. (b) Representative photomicrographs of CD138 and HLAG immunohistochemistry staining of CAG cells and placental explants. CAG cells express CD138 but do not express HLAG, whereas EVT cells express only HLAG. (c) Representative photomicrographs of CD138 (brown staining) and HLAG (black staining) double-staining procedure of placental explants co-cultured with CAG cells. NO CAG cells are located near the EVT cells.
promoter but inhibit MMP9 expression, both being observed in our system (Liu et al., 2006).

Malignant cells were eliminated mainly from the areas of EVT cell differentiation and implantation in the matrigel. Moreover, increased breast cancer cell apoptosis and decreased proliferation were also notable in the same areas, highlighting the unique effect of the EVT cells compared with the rest of the placental epithelial cells.

First trimester human placentas secrete a variety of factors. Many of these, including progesterone, IL-6, (epidermal growth factor) EGF, LiF and IGFII have been shown to modulate MMPs secretion and/or trophoblast invasion and implantation (Goldman et al., 2006; Cohen et al., 2007). During implantation, placental factors activate signaling pathways that modulate the expression of genes that are important for attachment of the trophoderm to the uterine wall, silence progesterone and ERα genes, induce apoptosis and promote other pregnancy-supporting functions (Galan et al., 2000; Bazer et al., 2008, 2009). In our biological system, placental explants modulated phenotypes of breast cancer cell lines in a similar way to its effect on the uterus during embryonic implantation (i.e. cell adhesion, ERα expression and apoptosis).

Breast cancer cells had no effect on proliferation and apoptosis of trophoblast cells, suggesting that the placental effect on neighboring cells is indeed specific for this organ and not a result of deficiency in the biological system, such as nutrient limitation.

In conclusion, our study demonstrates for the first time that embryonic organ/pregnancy conditions affect the phenotype of breast cancer cells. Furthermore, we propose that breast cancer cells migrated from the implantation site of the placenta (that contains EVT cells), suggesting it is a non-supporting microenvironment for breast cancer cells. Elimination of myeloma cells from the placental area supports previously published data, which suggested that the placental non-supporting effect on cancer cells is a general phenomenon.

We hypothesize that factors and pathways involved in normal placental implantation also modified biological characteristics of the malignant cells in the placental area. The molecular pathways responsible for the altered breast cancer phenotype are now being explored. Identification of these pathways may enable the development of new therapeutic approaches.

Authors’ roles

S.T.-M. designed and performed part of the experiments, analyzed the data and wrote the manuscript. A.M., G.E. and A.S. performed the experiments and analyzed the data. M.P. and A.F. supplied the placenta and reviewed the manuscript. M.L. and L.D. made substantial contributions to the design, analysis and data interpretation and revised the draft. J.R. participated in the FACS analysis.

Supplementary data

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

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