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Placental supernatants' enhancement of the metastatic potential of breast cancer cells: is estrogen receptor (ERα) essential for this phenomenon?

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Abstract

Purpose Pregnancy-associated breast cancer (PABC) is usually diagnosed at an advanced stage in comparison to non-pregnant women. The placenta secretes hormones and cytokines, which affect breast cancer progression. Previously, we demonstrated that human placental secretome facilitates the survival and migration of ERα+ breast cancer cells (BCCL), but pregnant women have a relatively high frequency of ERα-negative tumors. In the current study, we analyzed the effect of placental secretome on ERα-negative BCCL.

Methods BCCL [MCF-7(estrogen/progesterone receptor positive (ERα+/PR+), ERα reduced MCF-7 (siRNA, MCF-7 ERα−), HS-578 and BT-549 cells (both ER−/PR−)] were exposed to supernatants (collected from first trimester human placental explants and from control BCCL) or to E2 + P4 (estrogen + progesterone) in placental supernatant concentrations and then tested for cell proliferation (number, cell cycle, PCNA), cell-death, cell migration, STAT3 pathway activation and functionality.

Results Silencing ERα in the MCF-7 cells negated the placental supernatant and E2 + P4 enhancement of cell migration (> 130%, $p < 0.05$), number (> 120%) and survival (~ 130%). However, it had no such effect on MCF-7-ER− migration, which was still elevated in the presence of placental secretome. ER−/PR− BCCL were unaffected by the hormones, but placental secretome significantly elevated their migration (115%), number (140–170%), STAT3 phosphorylation (~ 180%) and BT-549 STAT3 level. These effects were negated by the STAT3 inhibitor.

Conclusions Placental supernatant facilitates BCCL malignant characteristics by activating ERα in estrogen responsive cells and STAT3 in ERα- BCCL. This indicates a possible mechanism that may underlie PABC's advanced state and suggests STAT3 pathway as a therapeutic target for PABC.

Keywords Breast cancer · Pregnancy · Placenta · ERα · STAT3

Introduction

Pregnancy-associated breast cancer (PABC) is often more advanced than breast cancer diagnosed in non-pregnant women. Pregnant women with breast cancer are usually diagnosed at an advanced stage, are more likely to develop metastases, and have inferior survival in comparison to age-matched non-pregnant women [1–3]. This may be due to the extensive physiological changes of the breast during pregnancy, which impairs diagnosis. Young age is an independent factor associated with higher risk of relapse and death from breast cancer [4]. Aggressive breast cancer subtypes are more common in younger women [4], who have a relatively high frequency of endocrine receptor-negative

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and basal-like tumors [5]. These tumor characteristics are also prevalent in pregnant women with breast cancer. Yet the aggression of the disease at a young age, which gets worse during pregnancy, suggests that the unique hormonal and gestation-related growth-factor panel during pregnancy might also contribute to the disease's aggressiveness. Moreover, though pregnancy reduces breast cancer patients' survival rate, no association has been observed between induction of abortion and prognosis, suggesting that BCCL biology is altered by events early in the pregnancy [1].

The placenta is a major source of hormones (such as estrogen (E2) and progesterone (P4)), growth-factors and cytokines during pregnancy [6, 7]. These factors play a role in breast cancer progression, since they are involved in cell proliferation, survival and motility. In a previous study, we demonstrated that first trimester human placental soluble factors facilitate the survival and migration of estrogen receptor positive (ERα+) BCCL [8, 9]. We found that estrogen (E2) and progesterone (P4) were involved in mediating these effects [8] and that the estrogen receptor inhibitor ICI 182 780 partially prevented the hormones' effect. ICI 182 780 is an ERα and GPR30 inhibitor. Young/pregnant women have a relatively high frequency of ERα negative and basal-like tumors [5]. Therefore, it remains to be determined whether ERα itself is crucial for mediating the placental soluble factors' effect, or whether there are other placental factors, bound to different receptor, that also contribute to the aggressive phenotype of the cancer cells.

In the study presented here, we analyzed the effect of first trimester human placental soluble factors on ER negative BCCL. We began using our previous model of ERα-positive BCCL cultured with full placental secretome, only this time we specifically reduced the ERα level of the cells using siRNA and reanalyzed the effect of the placental soluble factors on these cells. This experiment provided direct evidence for the specific involvement of this receptor in the cells response. However, since we found that some of the effects of the placental soluble factors still occurred in the silenced cells, we then also analyzed the placental secretome's effect on triple-negative (TN) cell lines. We chose the BT-549 and the HS-578 cell lines, which belong to the "mesenchymal-like/claudin-low" subtype, since claudin-low tumors are associated with a young-onset age, and are, therefore, more relevant to pregnancy [10].

Materials and methods

Cell culture

The study used MCF-7 (ERα+/PR+), BT-549 and HS-578 (both ERα−/PR−) cells, which underwent authentication (kindly provided by Dr. Ilan Tzarfati, Tel Aviv University). The cells 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). The cells were maintained in 5% CO_2 at 37 °C.

Placental explant cultures

Placental tissues were recovered surgically from normal pregnant women undergoing termination of pregnancy for psychosocial reasons at Meir Medical Center (in compliance with Helsinki regulations). Chorionic villi of 10 mg wet weight were isolated from first trimester placenta (6–9 weeks) and layered on growth-factor reduced (GFR) Matrigel (BD Biosciences) in a 24-well plate. Sixteen hours later, 700 µl of medium (DMEM/F-12 (HAM), L-glutamine (2 mM), sodium pyruvate (1 mM), HEPES (25 mM), antibiotics, and 10% FCS) (Biological Industries) was added to the placental wells and left overnight. Twenty-four hours later, the media were collected and stored at −20 °C.

ERα silencing

The ERα silencing was conducted with 4 sequences of ERα siRNAs (Qiagen, MA, USA), and commercially validated Alexa-labeled AllStars for the negative control. MCF-7 cells (4×10^4/well) were grown for 24 h in 0.5 ml medium without antibiotics. Then, 500 µl Optimem (GIBCO), 1.25 µl lipofectamine 2000 (Invitrogene, CA, USA) and 25 nM siRNA (combination of 2 sequences) were mixed for 20 min in RT and added to the cells. Transfection efficiency was analyzed by flow-cytometry (Navios, Beckman Coulter). Cells were cultured for 48 h (experiment and control). Then, they were suspended in equal volumes and used for phenotype analyses, or were lysed and their proteins extracted and measured by Western blot.

Placental/BCCL supernatant preparation

Placental explants or BCCL (control) were cultured on GFR Matrigel. After 24 h, the media were collected, centrifuged to eliminate cells and debris, and stored at − 80 °C.

Supernatant hormone level analysis

P4 and E2 levels were measured by the Immulite 2000 analyzer (Endocrinology Laboratory, Meir Medical Center). The analysis was done using solid phase (bead) L2KE22 and L2KPW2 competitive chemiluminescent enzyme immunoassay kits, respectively, according to manufacturer's instructions.

Exposing BCCLs to supernatants and to E2 + P4

Breast cancer cells (4×10^4) were placed for 24 h in 24-well plates in the presence of either (1) supernatants: BCCL/placenta with/without Stattic (20 µm), STAT inhibitor (Santa Cruz Biotech), or (2) E2 and P4 (Sigma Aldrich, USA) diluted in ethanol (Eth) to 'placental level' (i.e., 100 nM-P4, 5 nM-E2) concentrations. Ethanol in the same concentrations served as a control for the hormones. The hormone concentrations were detected as described in previous studies [11] and above.

Cell cycle analysis

Cells were perforated with cold 70% ethanol and exposed to 40 µg/ml Propidium iodide (PI (Sigma-Aldrich) and 100 µg/ml Ribonuclease (Sigma Aldrich) in PBS for 30 min (RT, in the dark), centrifuged, and the cell DNA content was analyzed using flow-cytometry (Navios, Beckman).

Cell count and death analysis

Trypan blue was mixed 1:1 with the BCCL and counted microscopically using automatic countess (Invitrogen). Live cells were unstained while dead cells assimilated the dye. Each treatment was counted twice.

Scratch test assay

To determine the extent of cell migration, confluent BCCL monolayers were wounded by a pipette tip. A black dot was marked under the scratch to accurately recognize the wound location. Following wounding, placental/BCCL supernatants with/without inhibitor or E2 + P4 were added to the cells. The wound closure was monitored by microscopy for 24 h following culture. Photographs of the marked areas were taken immediately after making the wound and 24 h later. The wound measurements were conducted using ImageJ software: https://rsbweb.nih.gov/ij/. Results were presented as the percentage of closure compared to time 0.

Western blotting

To assess the levels of particular proteins, BCCLs were washed with PBS and lysed in a 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 PMSF, 10 µg/ml Leupeptin) for 10 min on ice, and centrifuged (15 min, 12,000 RPM, 4 °C). Protein levels were determined by BCA assay with a Pierce BCA protein assay kit (Pierce, Rockford, IL, USA) according to manufacturer's instructions. Protein lysates were mixed (1:5) with sample buffer (250 mM Tris–HCl, pH 6.8, 400 mM DTT, 140 mM SDS, 60% glycerin, 0.02% bromophenol blue) and denatured for 10 min at 65 °C. Proteins (20 µg) from each sample were separated by electrophoresis on SDS-PAGE and transferred (wet-transfer) to a PVDF membrane. Validation of transfer efficiency was done by Ponceau staining (Sigma). After blocking (5% milk powder in Tris Buffer Saline containing 0.1% Tween (TBS-T)), the membranes were incubated with primary antibodies at 4 °C overnight (Table 1). Primary antibody was rinsed with TBS-T and TBS (Tris buffer saline). Bound antibodies were visualized using peroxidase-conjugated secondary antibody (Jackson Immune Research Laboratories) followed by enhanced chemiluminescence detection (Pierce). Optical densities were visualized and measured as arbitrary units by LAS3000 Imager. Results were normalized to Tubulin using a Multi-gauge V3.0 program (Fujifilm).

Ethical standards

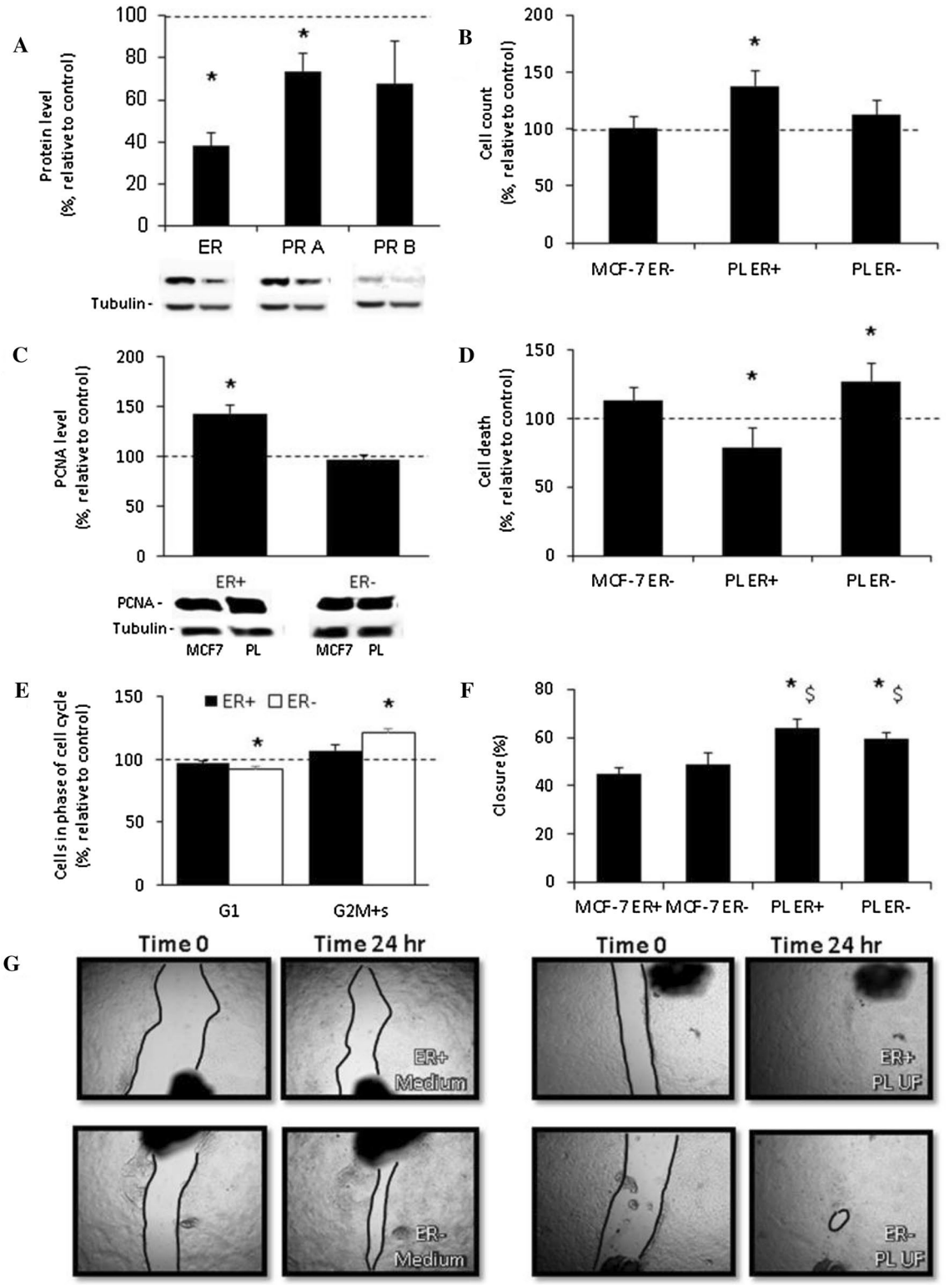
First trimester placenta (6–9 weeks of gestation) was donated from normal pregnancies terminated for psychosocial reasons, with a signed consent form from the donor. This study (0053-13-MMC) was approved by the ethics Helsinki committee of Meir Hospital.

Statistical analysis

Differences between the cohorts were analyzed using paired Student's *t* tests. An effect was considered significant when

Table 1 Antibodies used in this study

Target	Source	Isotype	Company	Dilution
ERα	Rabbit	mAb IgG	Millipore	1:1000
PR	Rabbit	mAb IgG	Cell Signaling Technology	1:1000
PCNA	Mouse	mAb IgG	Cell Signaling Technology	1:2000
Tubulin	Mouse	mAb IgG1	Sigma	1:8000
STAT3	Rabbit	Polyclonal IgG	Cell Signaling Technology	1:1000
Phospho-STAT3 (705)	Rabbit	Polyclonal IgG	Cell Signaling Technology	1:1000
Peroxidase-conjugated anti-mouse	Goat	Polyclonal IgG	Jackson Immuno-Research	1:40,000
Peroxidase-conjugated anti-rabbit	Goat	Polyclonal IgG	Jackson Immuno-Research	1:10,000


A
Protein level (%, relative to control)
100
80
60
40
20
0
*
*
ER
PR A
PR B
Tubulin -
B
Cell count (%, relative to control)
200
150
100
50
0
*
MCF-7 ER-
PL ER+
PL ER-
C
PCNA level (%, relative to control)
200
150
100
50
0
*
ER+
ER-
PCNA -
Tubulin -
MCF7
PL
MCF7
PL
D
Cell death (%, relative to control)
150
100
50
0
*
*
MCF-7 ER-
PL ER+
PL ER-
E
Cells in phase of cell cycle (%, relative to control)
150
100
50
0
ER+ ER-
*
*
G1
G2M+s
F
Closure (%)
80
60
40
20
0
*$
*$
MCF-7 ER+
MCF-7 ER-
PL ER+
PL ER-
G
Time 0
Time 24 hr
Time 0
Time 24 hr
ER+ Medium
ER+ PL UF
ER- Medium
ER- PL UF

◂**Fig. 1** The effect of ERα silencing on MCF-7 cells that were exposed to placental supernatant. MCF-7 cells (4×10^4) were cultured in a 24-well plate for 24 h, then transfected with ERα siRNA (ER−). Alexa-labeled siRNA served as a negative control (ER+). Forty-eight hours following transfection, upper fluids were replaced with placental/MCF-7 supernatants. Twenty-four hours later, cells were harvested, proteins were extracted and analyzed for ERα and PCNA levels by Western blot, or for cell number (countess), death (countess with trypan blue), cell cycle (FACS) and migration (scratch assay). Presented are Western blot analysis of ERα (**a**) and PCNA (**c**) levels, data on cell number (**b**), cell death (**d**) cell cycle (**e**), migration (**f**) and representative photomicrographs of the scratch assay at the beginning and end of the experiment (**g**). In **a**–**e**, data are relative to those obtained in ER+ cells cultured with MCF-7 supernatant (represented by the black line). In **f**, results are expressed as % closure of the scratch. MCF-7 ER−: MCF-7 cells transfected with ERα siRNA that were exposed to MCF-7 supernatant, PL ER+/PL ER−: MCF-7 cells transfected with control siRNA/ERα siRNA, respectively, that were exposed to placental supernatant. *Significantly different from MCF-7 ER+ ($p<0.05$), $^{\$}$significantly different from MCF-7 ER− ($p<0.05$)

the P value is <0.05. All experiments were conducted at least three times.

Results

The effect of placental supernatant on ERα-silenced MCF-7 cells

In a previous study, we demonstrated that exposing MCF-7 ERα+ cells to soluble factors that are produced by first-trimester human placental explants facilitated their survival and motility [8]. In the current study, we tested whether the ERα is indispensable to the placenta's ability to affect the MCF-7 phenotype. To do so, we used siRNA to reduce the ERα level in the MCF-7 cells (> 60% inhibition, $p<0.05$, Fig. 1a). Non-genomic siRNA tagged with Alexa monitored the introduction of the siRNA into the cells and served as a control. The transfection efficiency as measured by FACS was 97%. The level of the housekeeping gene, Tubulin (relative to protein concentration) did not change after transfection, suggesting that the effect could be attributed to the ERα siRNA specifically.

Progesterone receptor is an ERα target [12]. Since the placenta also produces progesterone (P4), we evaluated the effect of ERα siRNA transfection on MCF-7 PR level. We found that PRα and PRβ levels were slightly reduced (PRα: 22%↓, $p<0.05$; PRβ: 37%↓, Fig. 1a). ERα siRNA had no significant effect on MCF-7 number and survival in comparison to control (MCF-7 ER−, Fig. 1b, d).

Next, we exposed the ERα-silenced MCF-7 (MCF-7 ERα-) and the control cells (transfected with non-genomic siRNA, MCF-7) to supernatants collected from placental explant cultures or from MCF-7 cell cultures as a control. As it did in the wild-type MCF-7 cells [8], the placental supernatant increased the number of the control cells that were transfected with non-genomic siRNA (PL ER+, 38%↑, $p<0.05$, Fig. 1b). The increased cell number was a consequence of reduced death ($p<0.05$, Fig. 1d) and elevated proliferation, which was confirmed by increased expression of PCNA (30%↑, $p<0.05$, Fig. 1c). Silencing ERα in the MCF-7 cells prevented the increased cell number and PCNA level that were induced by the placental supernatant (ER−, Fig. 1b, c). Moreover, it elevated cell-death (PL ER−, 28%↑, $p<0.05$, Fig. 1d) and increased the fraction of cells in the S + G2M phases (20%↑, $p<0.05$, Fig. 1e), suggesting that the ERα- MCF-7 cells underwent G2M arrest. These results suggest that the effect of the placental supernatant on increasing MCF-7 cell survival and number is ERα dependent.

Our results also indicated the presence of additional placental factors that affect the cells' malignant behaviors through non-hormonal paths. First, we found that the absence of ERα did not just negate the increased cell survival, but produced an even higher rate of cell death than the control (35%, $p<0.05$, Fig. 1d). This suggests the presence of non-hormonal factors in the placenta that, in the *absence* of the ERα, *impair* cell survival. Second, we found that the placental supernatant increased MCF-7 cell migration (PL ER+, 42%, $p<0.05$, Fig. 1f, g). ERα siRNA transfection to MCF-7 cells had no significant impact on their migration compared to cells that were transfected with non-genomic siRNA (MCF-7 ER−, Fig. 1f). However, adding placental supernatant to the ER− MCF-7 cells increased their migration compared to the MCF-7 ERα− cells (22%↑, $p<0.05$, Fig. 1f, g) and compared to the control cells that contained normal ERα levels (MCF-7 ER+, 33%↑, $p<0.01$). These results suggest that placental factors facilitate MCF-7 cell migration through a mechanism that is independent of the ERα receptor.

The effect of estrogen + progesterone on ERα-silenced MCF-7 cells

In a previous study, we demonstrated that E2 + P4 in concentrations equal to those found in the placental secretome affect MCF-7 and T47D cell phenotype. In our study, we wished to confirm that ERα is the only receptor that mediated this effect. We added to the culture the combination of E2 and P4 because placental secretome contains both E2 and P4 [8], and because extensive cross-talk exists between E2 and P4. In the experiment, we exposed MCF-7 ERα− cells and control cells (transfected with non-genomic siRNA, MCF-7 ER+) to E2 + P4 combinations in concentrations equivalent to those found in the placental supernatant, or in MCF-7 cell supernatant as a control. Like the effects of the placental supernatant, E2 + P4 also

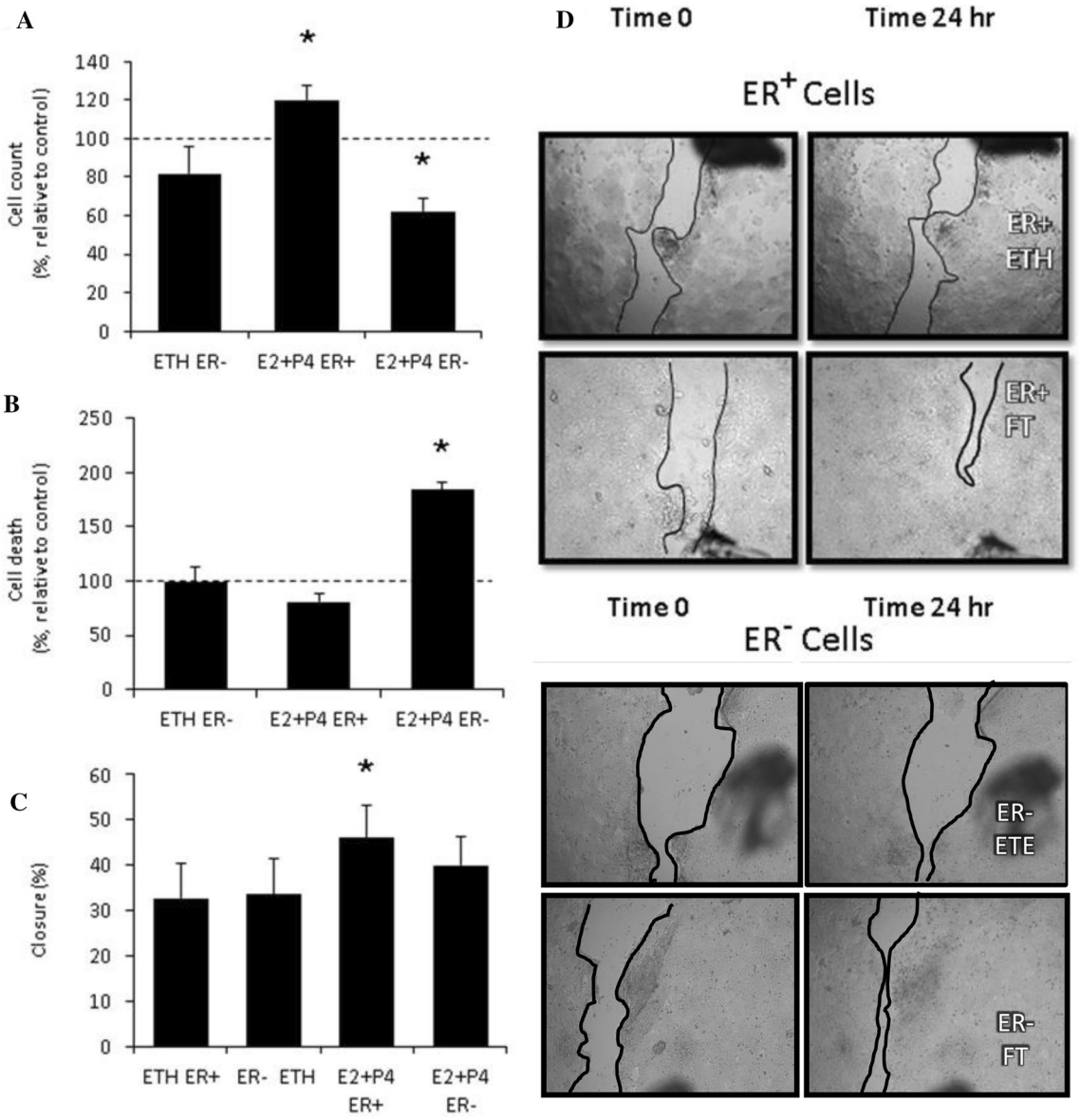


Fig. 2 The effect of ERα silencing on MCF-7 cells that were exposed to E2 + P4. MCF-7 cells (4×10^4) were cultured in a 24-well plate for 24 h, then transfected with ERα siRNA (ER−). Alexa-labeled siRNA served as a negative control (ER+). Forty-eight hours following transfection, upper fluids were replaced with medium that contain E2 + P4. As a control the cells were cultured with the solvent ethanol (ETH). Twenty-four hours later, cells were harvested and analyzed for cell number (countess), death (countess with trypan blue) and migration (scratch assay). Presented are data on cell number (**a**), death (**b**) migration (**c**) and representative photomicrographs of the scratch assay at the beginning and end of the experiment (**d**). In **a**, **b**, data are relative to those obtained in ETH ER+ cells (MCF-7 ER+ cells cultured with ethanol (represented by the line). In **c**, results are expressed as % closure of the scratch. ETH ER−: MCF-7 cells transfected with ERα siRNA and exposed to ethanol, E2 + P4 ER+/E2 + P4. ER−: MCF-7 cells transfected with control siRNA/ERα siRNA, respectively, which were exposed to E2 + P4 in levels equivalent to the first trimester of pregnancy. *Significantly different from control ($p < 0.05$)

increased the control MCF-7 number (E2 + P4 ER+, 20%↑, $p < 0.05$, Fig. 2a). ERα siRNA transfection in MCF-7 prevented the E2 + P4 dependent increase in cell number and induced cell-death (E2 + P4 ER−, 83%↑, $p < 0.05$, Fig. 2a, b). Interestingly, the E2 + P4 combination increased the MCF-7 ER− cell-death significantly more than the placental supernatant (83% vs 28%, $p < 0.05$). Moreover, like the placental supernatant, E2 + P4 increased the control MCF-7 cells' migration (E2 + P4 ER+, 40%, $p < 0.05$, Fig. 2c, d). ER siRNA transfection had no significant effect on MCF-7 cell migration compared to cells transfected with non-genomic siRNA (Fig. 2c, d). The addition of E2 + P4 to the MCF-7 ER− cells had no supplementary

effect on the cell motility compared to MCF-7 ER− cells (E2 + P4 ER−, Fig. 2c). These results suggest that E2 + P4 contributed to the increased cell number that was induced by the placental supernatant through ERα, but that there are additional placental factors, which do not depend on ERα, that facilitate the increased BCCL migration and reduced death.

Non-hormonal placental factors alter triple-negative (TN) BCCL phenotype

Our results demonstrated that, aside from E2 and P4, there are additional soluble placental factors that affect breast cancer cell survival and migration. This, and the fact that ER-negative tumors are more frequent in young and pregnant women, encouraged us to analyze the effect of placental supernatants on TN (ER−/PR−/HER2−) BCCL. We, therefore, exposed BT-549 and HS-578 cells to placental supernatants, or to their own supernatants as a control. We found that placental supernatants increased BT-549 and HS-578 cell number (35% and 69%, respectively, $p < 0.05$, Fig. 3a), as a result of the increased proliferation indicated by the elevated PCNA level (111%↑ and 30%↑, respectively, $p < 0.05$, Fig. 3b) and by increased cell number in the S + G2M phase of the cell cycle (21% and 13%, respectively, $p < 0.05$, Fig. 3c). The placental supernatant had no effect on HS-578 cell survival, but it did facilitate BT-549 cell-death ($p < 0.05$, Fig. 3d). Moreover, the placental supernatant also facilitated BT-549 and HS-578 cell migration (15%↑ for both cells, $p < 0.05$, Fig. 3e, f). As expected, E2 + P4 had no direct effects on the BT-549 and HS-578 cells' number and survival (data not shown).

Placental supernatants facilitate TN BCCL's malignant characteristics through activation of the STAT3 pathway

In previous studies, we performed a microarray assay to analyze the effect of placental explant co-culture on the MCF-7 cell's gene signature [8, 9]. Functional enrichment of the significantly altered genes demonstrated that the estrogen [8], interferon, STAT3 and JAK-STAT were among the most significantly changed pathways [9]. The microarray results showed an elevation in the STAT3 mRNA in MCF-7 cells that were cultured with placental explants (40%↑, $p < 0.05$). Since STAT3 is a key regulator of basal TN breast cancer phenotype [13], we next analyzed the effect of the placental supernatants on the levels of STAT3 and its phosphorylated forms in the TN cells.

We found increased STAT3 levels in BT-549 (47%, $p < 0.05$, Fig. 4a, b) and increased phospho-STAT3 levels in BT-549 and HS-578 cells that were exposed to the placental supernatants (69↑ and 44%↑, respectively, $p < 0.05$, Fig. 4a, b). These results demonstrated that the placental soluble factors activated the STAT3 pathway in the TN BCCL. To connect between the activation of this pathway and the elevated proliferation and migration that were caused in the TN BCCL by the placental supernatant, we added STAT3 inhibitor (Stattic) to the TN BCCL during their culture with the placental supernatant and reanalyzed BCCL number, and found that this addition inhibited the increased TN cell number (Fig. 4c, $p < 0.05$). Moreover, Stattic completely prevented the migration of the TN BCCL that were cultured alone or with the placental explant supernatant, demonstrating that no other placental factors were able to facilitate the cell migration (Fig. 4d).

Discussion

Placental factors facilitate the malignant characteristics of hormone receptor positive BCCL, as we have established in previously published research [8, 9, 14, 15]. Estrogen and progesterone are involved in mediating these effects [11]. In this study, we aimed to explore whether the ERα is indispensable to the placenta's ability to alter BCCL characteristics. We found that transfection of ERα siRNA into MCF-7 prevented the placental supernatant and E2 + P4's ability to increase MCF-7 cell survival and proliferation (Figs. 1, 2). Nevertheless, the ERα silencing did not change the placental factors' ability to facilitate cell migration, suggesting the involvement of additional factors in mediating this effect. Indeed, we demonstrated that the placental supernatant facilitated TN BCCL proliferation and migration in a non-hormone-dependent manner through the STAT3 pathway.

Interestingly, exposing MCF-7 ER− cells to placental supernatants that contained similar levels of E2 + P4 increased the cell-death significantly less than exposing them to E2 + P4. These results suggest that, in addition to the E2, the placental supernatants contain other pro-survival factors. Furthermore, E2 + P4 had no effect on MCF-7 ER− cell migration, while the placental supernatant significantly increased it. This suggests that the placental supernatant also contains non-hormonal pro-migratory factors. Altogether, our results suggest that ERα and E2 + P4 are indispensable to the placenta's ability to facilitate ERα-positive BCCL proliferation, but additional placental factors, which do not require ERα for their function, facilitate the BCCL motility and survival.

The fact that placental supernatant contains tumor-promoting factors that are not hormones is crucial, since in breast carcinomas in young women, and even more in pregnant ones, the ERα status is more frequently negative compared to older women [4, 16, 17]. Moreover, young patients with breast cancer tend to develop basal-like tumors at higher frequencies [4], which very often present

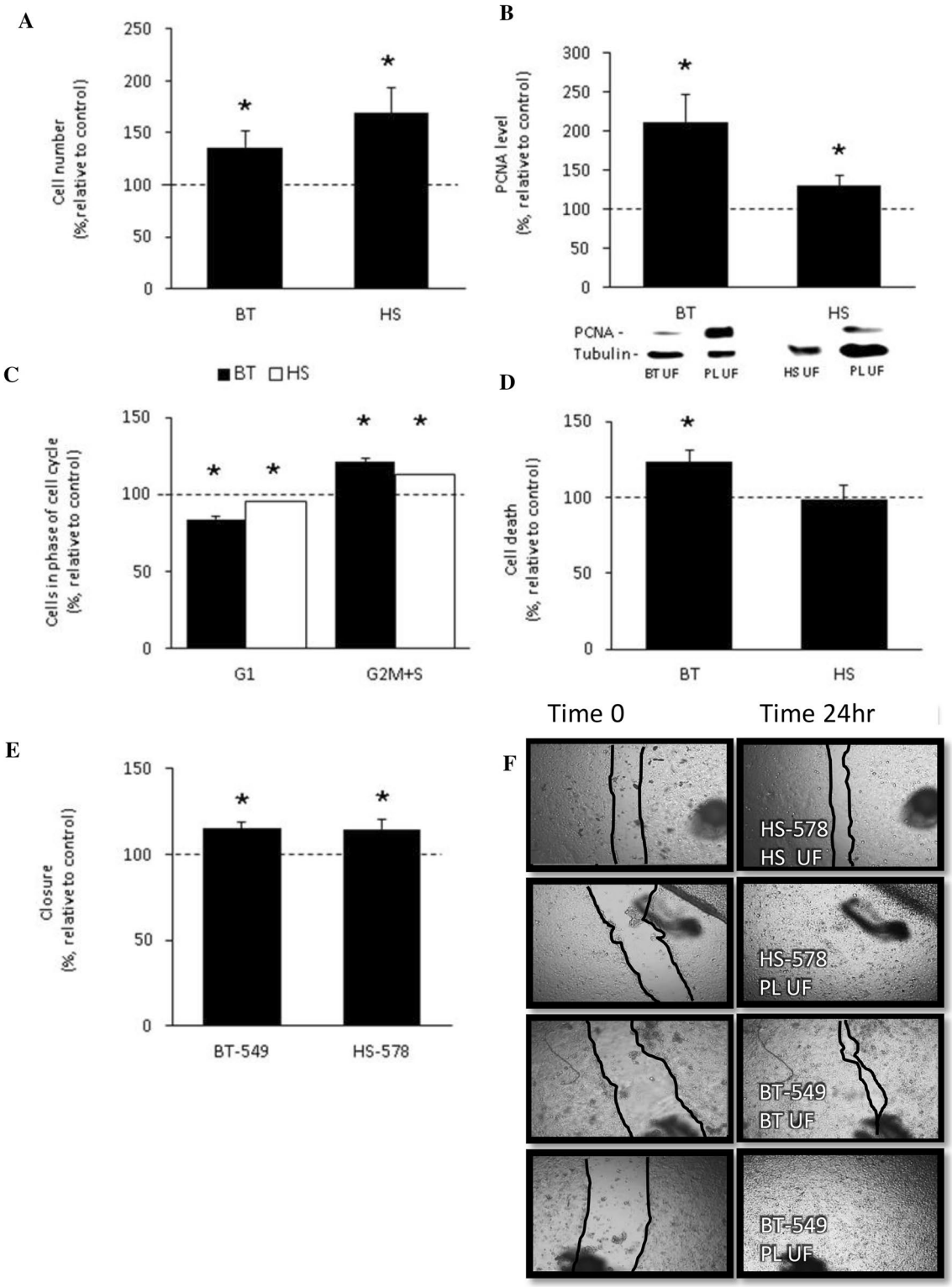


Fig. 3 The effect of placental supernatant on triple-negative breast cancer cells. BT-549 and HS-578 cells (4×10^4) were cultured for 24 h with placental/BCCL supernatants. Twenty-four hours later, cells were harvested and their proteins were extracted and analyzed for PCNA levels by Western blot, or for cell number (countess), death (countess with trypan blue), cell cycle (FACS) and migration (scratch assay). Presented are Western blot analysis of PCNA level (**b**), data on cell number (**a**), cell cycle (**c**), cell death (**d**) migration (**e**) and representative photomicrographs of the scratch assay at the beginning and end of the experiment (**f**). Data are relative to those obtained in cells cultured with BCCL supernatant (represented by the line). BT: BT-549, HS: HS-578 *significantly different from control ($p<0.05$)

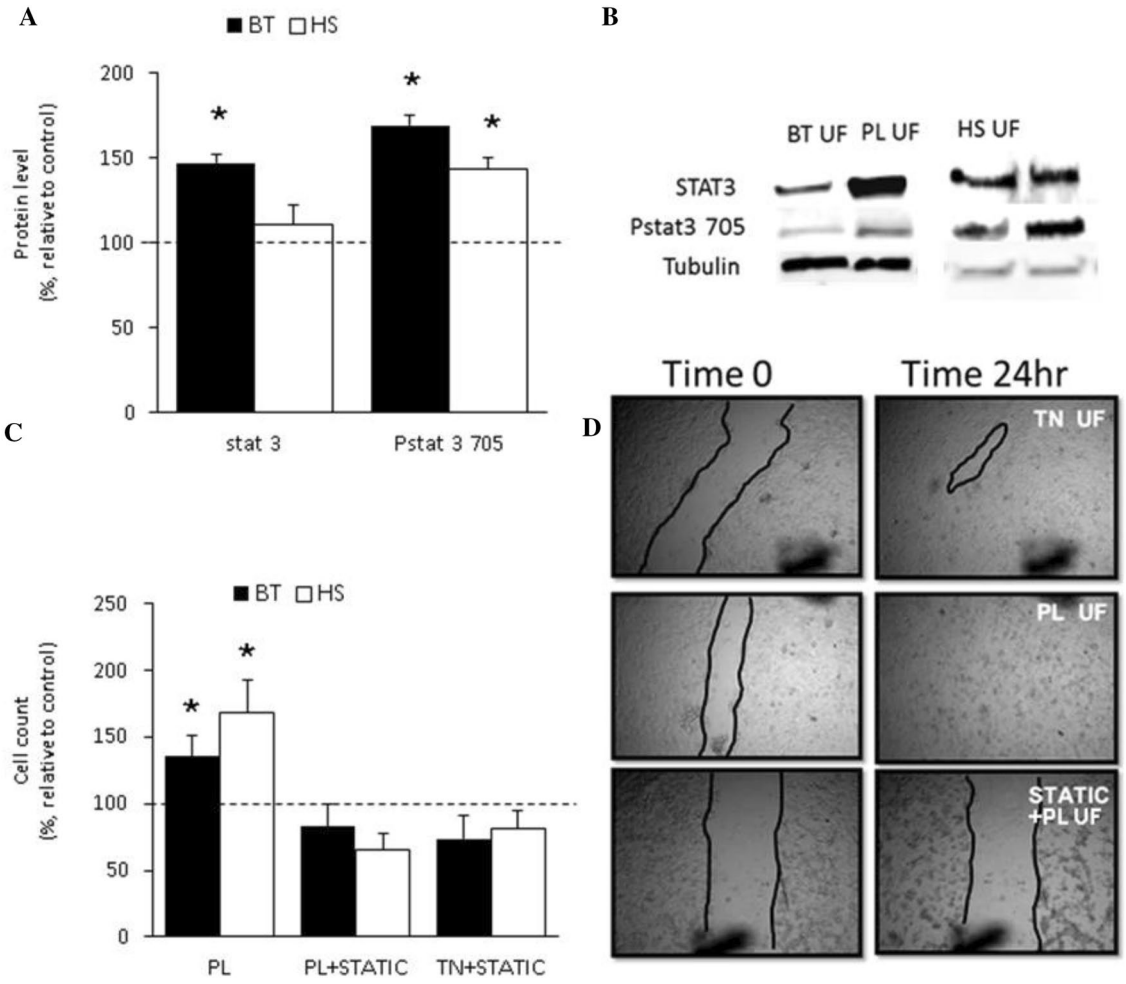


Fig. 4 The effect of placental supernatant on triple-negative breast cancer cells is mediated through STAT3 pathway. BT-549 and HS-578 cells (4×10^4) were cultured for 24 h with placental/BCCL supernatants with/without Stattic (20 µm, STAT inhibitor). Twenty-four hours later, cells were harvested and their proteins were extracted and analyzed for total STAT3 and PSTAT3 tyr705 levels by Western blot, or for cell number (countess). Presented are Western blot analysis of total STAT3 level (A-B), PSTAT3 tyr705 level (**a**, **b**) data on cell number (**c**) and representative photomicrographs of the scratch assay at the beginning and end of the experiment (**d**). Data are relative to those obtained in cells cultured with BCCL supernatant (represented by the line). BT: BT-549, HS: HS-578 *significantly different from control ($p<0.05$)

a triple-negative phenotype. Therefore, analyzing the effect of placental factors on BCCL that do not express ERα is an essential task.

Our results show that placental supernatants facilitate TN BCCL proliferation and migration by activating the STAT3 pathway. STAT3 has been widely recognized as an oncogene in various cancers and is activated in all breast cancer subtypes, especially in TN tumors [18, 19]. In breast cancer cells, STAT3 regulates proliferation, apoptosis, angiogenesis, immune response, metastasis, and drug resistance [18]. Although STAT3 is constitutively active in numerous types of cancers, including breast cancer and leukemia [20], only one report showed STAT3 mutation. Moreover, there is no difference in the expression levels of STAT3 in ER+, HER2, and TN breast cancer subtypes, though active phosphorylated STAT3 is restricted to basal TN breast cancer [19]. These data suggest that STAT3 is maintained in a constitutively active status through microenvironmental stimulation. Indeed, various factors, including cytokines (like IL6/interferon), growth-factors (like VEGF), hormones (progestins) and ECM proteins (fibronectin) are established regulators of STAT3 activity and/or transcription [21]. Our results suggest that human placenta is capable of activating the STAT3 pathway in neighboring cells. This is not surprising, since the STAT3 pathway is required for successful placental implantation [22, 23] and the human placenta secretes factors that activate the STAT3 pathway such as progesterone, VEGF, fibronectin and cytokines (like IL-6 and LIF), which may explain our results [6, 7, 24, 25].

In conclusion, our results suggest that placental soluble factors induce malignant characteristics in ER-positive and -negative breast cancer cells. While the improved ERα-positive MCF-7 growth and survival necessitate ERα expression, the effect of the placenta on TN BCCL is mediated through STAT3 signaling. In a previous publication, our microarray results demonstrated that first trimester human placenta also activates the STAT3 pathway in MCF-7 cells [9]. Altogether, our results provide an explanation for the advanced breast cancer found during pregnancy and suggest that STAT3 pathway plays an important role in mediating advanced breast cancer during pregnancy.

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Author contributions MB: data collection and management, data analysis, manuscript review and editing. OK: placentae collection, manuscript review and editing. MP: placentae collection, manuscript review and editing. AF: placentae collection, manuscript review and editing. LD: project development, analysis and manuscript review and editing. STM: protocol and project development, data analysis, and manuscript writing. ML: project development, analysis and manuscript review and editing.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval All the procedures performed in this study were in accordance with the ethical standards of the ethics Helsinki committee of Meir Hospital and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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