Pigment Epithelium-Derived Factor Alleviates Tamoxifen-Induced Endometrial Hyperplasia

Keren Goldberg1, Hadas Bar-Joseph1, Hadas Grossman1, Noa Hasky1, Shiri Uri-Belapolsky1, Salomon M. Stemmer2, Dana Chuderland1, Ruth Shalgi1, and Irit Ben-Aharon2

Abstract

Tamoxifen is a cornerstone component of adjuvant endocrine therapy for patients with hormone-receptor–positive breast cancer. Its significant adverse effects include uterine hyperplasia, polyps, and increased risk of endometrial cancer. However, the underlying molecular mechanism remains unclear. Excessive angiogenesis, a hallmark of tumorigenesis, is a result of disrupted balance between pro- and anti-angiogenic factors. VEGF is a proangiogenic factor shown to be elevated by tamoxifen in the uterus. Pigment epithelium–derived factor (PEDF) is a potent anti-angiogenic factor that suppresses strong pro-angiogenic factors, such as VEGF. Our aim was to investigate whether angiogenic balance plays a role in tamoxifen-induced uterine pathologies, elucidate the molecular impairment in that network, and explore potential intervention to offset the proposed imbalance elicited by tamoxifen. Using in vivo mouse models, we demonstrated that tamoxifen induced a dose-dependent shift in endogenous uterine angiogenic balance favoring VEGF over PEDF. Treatment with recombinant PEDF (rPEDF) abrogated tamoxifen-induced uterine hyperplasia and VEGF elevation, resulting in reduction of blood vessels density. Exploring the molecular mechanism revealed that tamoxifen promoted survival and malignant transformation pathways, whereas rPEDF treatment prevents these changes. Activation of survival pathways was decreased, demonstrated by reduction in AKT phosphorylation concomitant with elevation in JNK phosphorylation. Estrogen receptor-α and c-Myc oncoprotein levels were reduced. Our findings provide novel insight into the molecular mechanisms tamoxifen induces in the uterus, which may become the precursor events of subsequent endometrial hyperplasia and cancer. We demonstrate that rPEDF may serve as a useful intervention to alleviate the risk of tamoxifen-induced endometrial pathologies. Mol Cancer Ther; 14(12); 2840–9. ©2015 AACR.

Introduction

Tamoxifen is considered a pivotal component of the hormonal therapy backbone commonly used for treatment of patients with hormone-receptor–positive breast cancer in all settings—adjuvant, metastatic, and as a “primary prevention” risk-reducing strategy for high-risk populations (1, 2). The current American Society of Clinical Oncology guidelines for adjuvant hormonal therapy by tamoxifen have been recently revised; for premenopausal patients for 10 years of adjuvant tamoxifen therapy rather than 5 years (3), whereas in postmenopausal patients aromatase inhibitors are considered an alternative, though tamoxifen represents a valid and effective treatment standard.

Tamoxifen belongs to a class of selective estrogen receptor modulators (SERM), it binds to estrogen receptor (ER) and elicits estrogen agonistic or antagonistic responses by recruiting diverse sets of corepressors and coactivators, depending on the target tissue (4). Tamoxifen serves as antagonist to ER in breast cancer cells, whereas in the uterus it exerts partial agonistic activity, resulting in a range of endometrial pathologies including hyperplasia, polyps, carcinomas, and sarcomas (5, 6). Several large-scale studies have reported a 2- to 7-fold increase in the incidence of endometrial cancer in patients treated with tamoxifen (7, 8) compared with control patients. Histopathologic changes in the endometrium were observed after tamoxifen administration mainly in postmenopausal patients (9), and because endometrial hyperplasia is viewed as a possible precursor for malignant transformation, patients with atypical hyperplasia are closely monitored.

Tamoxifen is known to exert its effect on the endometrium via activation of estrogen-regulated genes (10); however, recent observations indicate that tamoxifen can also activate different sets of genes that are not regulated by estrogen (11). As demonstrated by gene array profiling, these genes are related to cellular processes of DNA replication, cell-cycle progression, and cellular organization (12). Yet, the exact molecular mechanism by which tamoxifen exerts adverse effects on the uterus remains to be elucidated.

Angiogenesis, or formation of new blood vessels, is essential for tissue proliferation. Under physiologic conditions, endometrial angiogenesis is tightly regulated by pro- and anti-angiogenic factors (13); estrogen plays a pivotal role in establishing new vascular bed and promoting cellular growth and differentiation.
within the endometrium at each menstrual cycle (13). In ovariectomized mice, tamoxifen stimulates production of uterine VEGF (14), one of the major pro-angiogenic factors in the endometrium, known to be upregulated in many pathologies and malignancies (15).

Pigment epithelium–derived factor (PEDF) is a 50-kDa glycoprotein that belongs to the noninhibitory members of the serine protease inhibitors (serpin) superfamily. PEDF is one of the most potent physiologic inhibitors of angiogenesis (16). The anti-angiogenic effect of PEDF is associated with disruption of endothelial proliferation, promotion of endothelial cell apoptosis, and downregulation of pro-angiogenic factors. Increasing amount of evidence indicates that under physiologic conditions, an essential balance between PEDF and VEGF exists, and PEDF may counteract the angiogenic potential of VEGF (17). Several studies showed that PEDF level declines with age, and suggested that this decline may play a key role in the development of angiogenic-related pathologies (18). Moreover, it was shown that decrease in PEDF expression is one of the mechanisms that promote tumor growth (19), suggesting PEDF as an anticancer therapy (20, 21). We have recently characterized the expression and regulation of PEDF in human and rodent premenopausal endometrium, indicating that PEDF is dynamically expressed in mice endometrium throughout the estrous cycle, in reciprocity to VEGF, and that the balance between the two is regulated by estrogen and progesterone (22, 23). PEDF is a secreted glycoprotein that promotes a variety of activities upon binding to various receptors (20). In our previous studies, we showed that endometrial cells express Patatin-like phospholipase domain-containing protein 2 (PNPLA2; ref. 22), a PEDF receptor (PEDF-R) that mediates mainly prosurvival activity (24). Another key protein that regulates the anti-angiogenic activity of PEDF is laminin receptor (LR; ref. 25), which has not been characterized in the uterus. We have also demonstrated that stimulation of ECC-1 endometrial cell line with recombinant PEDF (rPEDF) induces a significant decrease in the level of VEGF expression (22). Finally, we established the role of PEDF as a potential therapeutic agent for endometriosis and ovarian hyperstimulation syndrome (23, 26).

In the current study, our aim was to explore the mechanism accountable for tamoxifen-induced endometrial pathologies in postmenopausal patients, among which these entities represent clinical concern. We hypothesized that tamoxifen alters uterine angiogenic balance and that the shift in regulatory angiogenic proteins contributes to tamoxifen-induced uterine pathologies. It is therefore suggested that upon this mechanism, a replacement therapy using rPEDF may reduce tamoxifen-induced adverse effects.

**Materials and Methods**

**Animals**

Institute for Cancer Research (ICR) female mice (8 weeks old; Harlan Laboratories) were housed in temperature- and humidity-controlled rooms at the animal facilities of the Sackler Faculty of Medicine, Tel-Aviv University, under artificial illumination for 12 hours daily. Food and water were available ad lib. Animal care was in accordance with institutional guidelines and approved by the local authorities.

**Tamoxifen treatment**

Tamoxifen tablets (Teva Pharmaceutical Industries) were pulverized to thin powder, dissolved in ethanol, and diluted in canola oil. Tamoxifen was administered per os by a blunt gavage needle. Control mice were administered with canola oil.

**Ovariectomy**

Mice were anesthetized by an intraperitoneal injection of a mixture of ketamine hydrochloride (Kepro) and xylazine hydrochloride (VMD). The dorsal mid-lumbar area was shaved to remove hair and prepared for aseptic surgery. A single skin incision at the dorsal end of the ribcage was made and muscle wall was incised, exposing the peri-ovarian fat pad region. The ovary was exteriorized by grasping the peri-ovarian fat with tissue forceps; the oviduct area was ligated and cauterized by extremely hot forceps to avoid bleeding, thus excising the ovary. The remaining tissue was returned into the peritoneal cavity. The procedure was repeated at the other side as well. The muscle incisions were closed with 4-0 absorbable suture (Ethicon) and skin incisions were closed with wound clips (Clay-Adams). Mice were allowed to rest for one week to ensure post-menopausal state, before experimental onset.

**RNA isolation, reverse transcription, and qRT-PCR**

RNA was extracted using Trizol reagent (Invitrogen), according to the manufacturer’s instructions, and quantified with the Nano-Drop spectrophotometer (ND-1000; Thermo Scientific). Total RNA was reverse transcribed using high capacity cDNA reverse transcription kit (Applied Biosystems; ABI). Changes in the level of mRNA expression were detected by SYBR green reagent (SYBR Green PCR Master Mix, Applied Biosystems) using 20-ng cDNA and specific primers, on an ABI Prism 7900 Sequence PCR machine (Applied Biosystems). Hypoxanthine phosphoribosyltransferase 1 (HPRT-1) served as an endogenous control.

**Western blot analysis**

Samples were subjected to SDS-PAGE and immunoblotted with the appropriate primary antibodies: anti-PEDF (1:200, sc-25594, Santa Cruz Biotechnology), anti-VEGF (1:200, sc-152, Santa Cruz Biotechnology), anti-phospho-AKT (1:1,000, P-4112, Sigma Chemical Company), anti-AKT (1:1,000, #2938, Cell Signaling Technology), anti-phospho-JNK (1:1,000, J-4750, Sigma), anti-JNK (1:1,000, J-4500, Sigma), anti-ER (1:1,000, ab 2746, Abcam), anti-c-Myc (1:1,000, sc-40, Santa Cruz Biotechnology), and anti-actin (1:10,000, MAB1501; Millipore). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch) and subjected to enhanced chemiluminescence assay (Thermo Scientific). The intensity of the bands was analyzed by the ImageJ software.

**Histologic evaluation**

Uteri were excised, fixed in 4% paraformaldehyde in PBS, dehydrated by passage through an ascending series of alcohols, embedded in paraffin, sectioned (6 μm), and placed on slides (Proper). Slides for histologic evaluation were stained with hematoxylin and eosin (Bio-Optica).

**IHC**

Sections were deparaffinized by running the slides through xylene (15 minutes) and a descending series of alcohols (5 minutes each) to a total re-hydration in distilled water. The
sections were placed into preheated antigen unmasking solution (H-3300; Vector Laboratories Inc.), cooled on ice to 50°C, re-heated for three more minutes and cooled on ice to room temperature. The slides were then rinsed in PBS and incubated for 1 hour with PBS/Tg (0.2% Tween-20, 0.2% gelatin in PBS), re-rinsed in PBS, blocked for 10 minutes in blocking solution (927B; Cell Marque), and incubated overnight at 4°C with anti-CD34 (1:400, Cell Signaling Technology) and a nuclear marker (Hoechst 3342; Sigma), sections were rinsed in PBS, and mounted with mowiol (Sigma) before being scanned by a laser confocal microscope (Leica TCS SP5).

rPEDF production

His-tagged human rPEDF (NM_002615.4) was expressed in Escherichia coli BL21. Bacteria were allowed to grow at 30°C to OD600 nm of 0.5 to 0.6, induced for 4 to 5 hours by isopropyl-L-thio-β-D-galactopyranoside (0.5 mmol/L; Sigma), centrifuged, and their pellets lysed. Recombinant protein was purified by ion metal affinity chromatography with Ni-NTA His-Bind resin (Merck KGaA) according to the manufacturer's protocol. Proteins of the eluted fractions were resolved by SDS-PAGE, dyed by GelCode (Blue Stain Reagent, Thermo scientific), or Western blotted with a specific anti-PEDF antibody (~57 kDa). Eluates with 90% purity were dialyzed against Tris buffer pH 10.

Statistical analysis

Data were expressed as mean ± SEM. A Student t test (equal variance, one-tailed) was used to statistically evaluate the differences between control and experimental results. Differences were considered statistically significant when P < 0.05.

Results

Short-term tamoxifen treatment modulates uterine VEGF/PEDF balance in vivo

We have established an in vivo mouse model for examining the effect of tamoxifen on postmenopausal uterine tissue (see Supplementary Fig. S1 for the experimental design). To study the subacute effect we used the short-term treatment model, which demonstrated a dose-dependent increase of uterine weight (Fig. 1A), as described in the literature (27). The histologic appearance (Supplementary Fig. S2) showed that tamoxifen caused an increase of uterine glands and epithelial height as well as enlargement of the stromal compartment (27). We found that the level of VEGF protein in the uterus was upregulated in a dose-dependent manner (Fig. 1B and E), correlated with uterine weight. Thereafter, we evaluated the effect of tamoxifen on the expression of PEDF protein, and found that PEDF levels were downregulated (Fig. 1C and E) as tamoxifen doses were increased, yielding a reciprocal manner of VEGF and PEDF expression (Fig. 1D), indicating impaired uterine angiogenic balance after tamoxifen administration.

Figure 1.

Short-term tamoxifen treatment modulates uterine VEGF/PEDF balance in vivo. Various concentrations of tamoxifen were administered for 7 days to ovariectomized mice. Uteri were excised and lysed. Proteins were resolved by SDS-PAGE and immunoblotted with anti-VEGF or anti-PEDF antibodies. mRNA was extracted using TRIZOL reagent and subjected to RT-PCR. A, uterine weight. B, VEGF protein expression. C, PEDF protein expression. D, VEGF/PEDF protein ratio. E, representative Western blot of VEGF, PEDF, and actin as a loading control. Protein expression was quantified by Image J and calibrated to actin as a loading control. F, PEDF-R mRNA calibrated with HPRT-1. n = 3–4 mice/group. The ratio between each treatment and control is plotted as mean ± SEM (*, P < 0.05; **, P < 0.01), statistically significantly different from control value, using t test.
We evaluated the expression of PEDF-Rs in the uterus and found that short-term tamoxifen treatment upregulated the expression level of both PNPLA2 and LR (Fig. 1F).

rPEDF downregulates both tamoxifen-induced hyperplasia and VEGF elevation, and modifies signaling pathways

We chose the dose of 2.5 μg/mouse tamoxifen as an optimal dose and added subcutaneous injections of rPEDF (10 mg/kg, every third day). We administered rPEDF to mice according to two regimens (see Supplementary Fig. S1 for experimental design): (1) starting on day 1 of tamoxifen administration (“rPEDF prevention”), (2) starting on day 4 of tamoxifen administration (“rPEDF treatment”). When rPEDF was added on days 4 to 7 of tamoxifen administration (“rPEDF treatment” regimen), significant decreases in uterine weight (Fig. 2A) and in uterine VEGF level (Fig. 2B and C) were observed, compared with the values obtained in mice treated with tamoxifen alone; suggesting that rPEDF had a significant favorable effect on tamoxifen-induced uterine hyperplasia. This effect may be attributed, at least in part, to PEDF’s ability to resist tamoxifen-induced VEGF elevation. No effects on uterine weight or uterine VEGF level were observed in mice treated according to the “rPEDF prevention” regimen (data shown in Supplementary Fig. S3).

Figure 2.
rPEDF downregulates both tamoxifen-induced hyperplasia and VEGF elevation, and modifies signaling pathways. Ovariectomized mice were administered for 7 days with tamoxifen (2.5 μg/mouse) or with a combination of tamoxifen and rPEDF (10 mg/kg; days 4–7). Uteri were excised, lysed, proteins resolved by SDS-PAGE, and immunoblotted with anti-VEGF antibody (B and C), anti-p-AKT (D and E), or with anti-p-JNK antibodies (F and G). A, uterine weight. B, VEGF protein expression. C, representative Western blot analyses of VEGF and actin as a loading control. Protein expression was quantified by ImageJ and calibrated to actin as a loading control. Each value is normalized to that of control (tamoxifen-only) level. Tamoxifen, n = 23; tamoxifen + rPEDF, n = 16. D, p-AKT protein expression. E, representative Western blot of p-AKT. Protein expression was quantified by ImageJ and calibrated to gen-AKT as a loading control. F, p-JNK protein expression. G, representative Western blot of p-JNK. Protein expression was quantified by ImageJ and calibrated to gen-JNK as a loading control. Each value is normalized to that of control (tamoxifen-only) level (n = 4 mice/treatment group). Data are plotted as mean ± SEM (*, P < 0.05; **, P < 0.01), statistically significantly different from control value, using t test.
Examination of signaling pathways known to mediate cellular growth and survival (PI3K/AKT/mTOR pathway) as well as apoptosis [c-Jun N-terminal kinases (JNK)] revealed a significant decrease in phosphorylation of AKT (Fig. 2D and E) and an increase in phosphorylation of JNK (Fig. 2F and G) in mice treated with the ‘rPEDF treatment’ regimen compared with mice treated with tamoxifen alone. Mice of the ‘rPEDF prevention’ cohort showed an increase in phosphorylation of JNK and no significant change in phosphorylation of AKT compared with mice treated with tamoxifen alone (data shown in Supplementary Fig. S3).

Prolonged tamoxifen treatment modulates uterine VEGF/PEDF balance in vivo

We established a prolonged-treatment model in which tamoxifen was administered to mice continuously for a period of 1 month. The effect of prolonged tamoxifen treatment was similar to that of the short-term treatment; namely, an increase in uterine weight (Fig. 3A), upregulation of VEGF protein (Fig. 3B and E), and downregulation of PEDF protein (Fig. 3C). VEGF/PEDF ratio was significantly increased (Fig. 3D), suggesting a long-lasting effect of tamoxifen.

Evaluation of the expression of PEDF-Rs after 1 month of tamoxifen treatment (Fig. 3F) revealed that PNPLA2 level was significantly increased, whereas that of LR did not change significantly. This suggests that following tamoxifen treatment endogenous PEDF will more likely bind to PNPLA2 and induce cellular survival than promote anti-angiogenic activity through LR.

A prolonged combined treatment of tamoxifen and rPEDF downregulates tamoxifen-induced hyperplasia, VEGF elevation, and blood vessels density

We examined the long-term effectiveness of rPEDF treatment administered in vivo according to the prolonged tamoxifen (2.5 μg/mouse) treatment model and added rPEDF injections (2 mg/kg, every third day). Based on our finding that ‘rPEDF treatment’ regimen was more effective than ‘rPEDF prevention’ regimen, we started rPEDF injections on day 8 of tamoxifen treatment (see Supplementary Fig. S1 for experimental design). Uterine weight did not differ in the group treated with the combination compared with tamoxifen-only (Fig. 4A; P = 0.2). The expression level of uterine VEGF protein was significantly decreased in mice of the combined “tamoxifen+rPEDF” mice compared with that of the “tamoxifen-only” group (Fig. 4B and C); suggesting a durable, long-lasting effect of rPEDF on the uterus after 1 month of treatment.

Changes in tissue vasculature were examined by IHC staining with CD34. Tamoxifen caused a remarkable increase in the density of uterine blood vessels (Fig. 4E and H) compared with control (Fig. 4D and G), whereas addition of rPEDF (tamoxifen+rPEDF treatment) caused a decrease of CD34 staining (Fig. 4F and I) compared with tamoxifen-only.

Figure 3.
Prolonged tamoxifen treatment modulates uterine VEGF/PEDF balance in vivo. Tamoxifen (2.5 μg/mouse) was administered for 1 month (5 days/week) to ovariectomized mice. Uteri were excised and lysed. Proteins were resolved by SDS-PAGE and immunoblotted with anti-VEGF or anti-PEDF antibodies.

mRNA was extracted using TRIzol reagent and subjected to RT-PCR. A, uterine weight. B, VEGF protein expression. C, PEDF protein expression. D, VEGF/PEDF protein ratio. E, representative Western blot of VEGF and actin as a loading control. F, PEDF-R mRNA calibrated with HPRT-1. n = 6–7 mice/treatment group. Protein expression was quantified by ImageJ and calibrated to actin as a loading control. The ratio between each treatment and control is plotted as mean ± SEM (*, P < 0.05; **, P < 0.01), statistically significantly different from control value, using t test.
Combined treatment of tamoxifen and rPEDF modulates ERα and c-Myc expression patterns

We used RT-PCR to examine the expression pattern of hormonal receptors in the uteri of mice treated according to the short-term and prolonged in vivo models; assessing the mRNA levels of ERα and ERβ and progesterone receptor (PR). Short-term tamoxifen treatment caused an upregulation of ERα mRNA level, but had no significant effect on that of ERβ and PR (Fig. 5A). rPEDF impaired the tamoxifen-induced upregulation of ERα mRNA. The levels of ERα protein along the various treatments resembled those of ERα mRNA, though with no statistical significance (Fig. 5B, P = 0.1).

Prolonged tamoxifen treatment upregulated mRNA levels of both ERα and ERβ, but had no significant effect on PR expression (Fig. 5D). The combined treatment of tamoxifen+rPEDF significantly reduced ERα mRNA level compared with tamoxifen. The level of ERα protein expression at the various treatments correlated with that of ERα mRNA (Fig. 5E).

Finally, we tested the effect of rPEDF on c-myc protein. Short-term tamoxifen treatment increased the level of c-Myc protein,
whereas rPEDF caused no significant change (Fig. 5C). The level of c-Myc was significantly increased after a prolonged tamoxifen treatment as well, and significantly decreased after a combined tamoxifen+tP tP PEDF treatment (Fig. 5F).

**Discussion**

Tamoxifen-induced uterine pathologies present a major clinical challenge. To date, the mechanism that accounts for the spectrum of tamoxifen-induced endometrial hyperplasia-metaplasia remains unclear. It is postulated that it involves alterations in uterine angiogenesis, though only paucity of supporting evidence exists. In the current study, we hypothesized that tamoxifen exerts its effects on the uterus by modulating uterine angiogenic balance; we studied the mechanisms using an in vivo mouse model. Because tamoxifen is prescribed in the clinical setting on a daily basis for an extended period, we established two models that represent the subacute and the long-term effects. Our results were in accordance with previous reports and showed that tamoxifen upregulates the level of uterine VEGF. We showed, for the first time, that the level of uterine PEDF is downregulated after tamoxifen treatment, resulting in an impaired balance of VEGF and PEDF, and demonstrated that tamoxifen-induced increase in uterine weight correlates with VEGF increase and PEDF decrease. PEDF was shown to be a potent effector of angiogenesis and was proposed as a potential therapeutic intervention for other angiogenesis-related pathologies. We suggested using rPEDF as a replacement therapy for reducing tamoxifen-induced adverse effects, and investigated its effect on key proteins involved in regulation of cells proliferation, survival, and apoptosis. Our research is the first to demonstrate the significant impact of tamoxifen on the expression of uterine blood vessels, revealed by enhanced staining of the endothelial marker CD34. We suggest that the increase in blood vessels density may be attributed to the impaired balance between pro- and anti-angiogenic factors, induced by tamoxifen. Moreover, treatment with rPEDF restored the angiogenic balance by resisting tamoxifen-induced uterine VEGF elevation, as seen by the decrease of both uterine weight and CD34-marked blood vessels. Former evidence characterizing the changes in uterine vasculature after tamoxifen treatment in post-menopausal patients was not persistent, possibly due to limited sample size and increased heterogeneity (28, 29).

Endometrial hyperplasia, especially atypical hyperplasia, is generally viewed as a possible precursor for malignant transformations (30). Expression of VEGF and vascular density, two important markers of angiogenesis, were found to be increased...
in many premalignant lesions (31) and are known to play a role in the spectrum of tissue malignant transformation. Our in vivo models verified that tamoxifen enhanced both hyperplasia and angiogenic activation in treated mice. Furthermore, administration of rPEDF reduced the levels of both parameters (vascular density and VEGF), implying a reduced risk of endometrial tumorigenesis following tamoxifen treatment.

Based on the ability of PEDF to elicit non-angiogenic cellular pathways as well, we examined its effect on signaling pathways mediating cell proliferation, survival, and apoptosis. Activation of the PI3K/AKT/mTOR pathway, a signaling pathway that plays an important role in cellular growth and survival, has recently been implicated in endometrial cancer (32). Activated AKT initiates a cascade of downstream signaling events in endometrial cancer cells that promote cellular growth, metabolism, proliferation, survival, migration, apoptosis, and angiogenesis. JNKs, key mediators of stress and inflammation, were implicated in apoptotic responses to DNA damage and to cytotoxic agents (33, 34). Activation of JNK correlates with induction of apoptosis in endometrial cancer cells (35). PEDF was previously shown to induce apoptosis by downregulation of phosphorylated-AKT (36) and upregulation of phosphorylated-JNK (37). Combined rPEDF–tamoxifen treatment causes a significant increase in the phosphorylation of the pro-apoptotic signaling molecule JNK, and a decrease in the phosphorylation of the pro-survival signaling molecule AKT, when compared with treatment with tamoxifen alone. Our data indicated that rPEDF treatment reduces activation of proliferation-associated signaling pathways.

Hormone receptors (ER and PR) expression is a fundamental principle in tamoxifen treatment for breast cancer. We hypothesized that due to its SERM traits, tamoxifen-induced effects on the endometrium may involve endometrial ER. The effect of tamoxifen on the ER may entail recruitment of processes such as tissue proliferation and VEGF regulation, two major pathways relevant to increased hyperplasia and possibly to malignant transformations. Moreover, a study conducted in Ishikawa endometrial cell line suggests that tamoxifen-induced upregulation of VEGF is mediated mainly through ERα, and not through ERβ (38). ERβ was shown to modulate, and possibly suppress, ERα-mediated uterine gene transcription (39); the activity of tamoxifen may therefore be determined by the relative expression of ERα and ERβ in the tissue (40). Our results showed that rPEDF treatment induces a specific decrease in the expression of uterine ERα, without modifying the expression of ERβ and PR, implying a shift toward higher activity of ERβ over ERα. The decrease in ERα expression confirms dual implication with regard to tamoxifen-treated uterus: First, the ER-related signaling, known to mediate hyperplastic response, is diminished, and, second, the uterine response to tamoxifen is reduced because there are less tamoxifen-binding receptors per cell. c-Myc protein, known to induce proliferation and to play a role in the development of endometrial carcinoma (41), is upregulated by tamoxifen (42, 43). In our mice
model tamoxifen treatment causes an increase in the level of c-Myc protein, compared with its level in control mice. Administration of rPEDF prevents c-Myc elevation, suggesting a decreased risk of malignant development. Our results also indicate that tamoxifen treatment changes the expression patterns of receptors known to mediate PEDF activity. By upregulating the level of PNPLA2, induction of prosurvival pathway is preferred instead of estrogen-related signal activation, and reducing susceptibility to tamoxifen-induced adverse effects. Moreover, the expression of c-Myc oncoprotein is downregulated after rPEDF treatment, possibly reducing the risk of endometrial malignant transformation (proposed mechanism of action is depicted in Fig. 6). By administering PEDF, we restored the altered angiogenic balance of uterine tissue to its natural state, and reduced the cascade of events leading to pathologic hyperplasia.

Disclosure of Potential Conflicts of Interest
K. Goldberg, H. Bar-Joseph, D. Chuderland, S.M. Stemmer, R. Shalgi and I. Ben-Aharon have ownership interest in U.S. Provisional Application no. 61/972,325. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: K. Goldberg, D. Chuderland, R. Shalgi, I. Ben-Aharon
Development of methodology: K. Goldberg, D. Chuderland, I. Ben-Aharon
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Goldberg, H. Bar-Joseph, H. Grossman, N. Haskey, S. Uri-Belashoky
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Goldberg, S.M. Stemmer, D. Chuderland, R. Shalgi, I. Ben-Aharon
Writing, review, and/or revision of the manuscript: K. Goldberg, S.M. Stemmer, D. Chuderland, R. Shalgi, I. Ben-Aharon
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Ben-Aharon

Study supervision: D. Chuderland, R. Shalgi, I. Ben-Aharon

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