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Synthetic progestins induce growth and metastasis of BT-474 human breast cancer xenografts in nude mice

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Abstract

Objective—Previous studies showed that sequential exposure to estrogen and progesterone or medroxyprogesterone acetate (MPA) stimulates vascularization and promotes the progression of BT-474 and T47-D human breast cancer cell xenografts in nude mice (Liang et al, *Cancer Res* 2007, 67:9929). In this follow-up study, the effects of progesterone, MPA, norgestrel (N-EL) and norethindrone (N-ONE) on BT-474 xenograft tumors were compared in the context of several different hormonal environments. N-EL and N-ONE were included in the study since synthetic progestins vary considerably in their biological effects and the effects of these two progestins on the growth of human tumor xenografts are not known.

Methods—Estradiol-supplemented intact and ovariectomized Immunodeficient mice were implanted with BT-474 cells. Progestin pellets were implanted either simultaneously with estradiol pellets 2-days prior to tumor cell injection (i.e. combined), or 5-days following tumor cell injections (i.e. sequentially).

Results—Progestins stimulated the growth of BT-474 xenograft tumors independent of exposure timing and protocol, MPA stimulated the growth of BT-474 xenograft tumors in ovariectomized mice and progestins stimulated VEGF elaboration and increased tumor vascularity. Progestins also increased lymph node metastasis of BT-474 cells. Therefore, progestins, including N-EL and N-ONE, induce the progression of breast cancer xenografts in nude mice and promote tumor metastasis.

Conclusions—These observations suggests that women who ingest progestins for HT or oral contraception could be more at risk for developing breast cancer as a result of proliferation of existing latent tumor cells. Such risks should be considered in the clinical setting.

Keywords

Hormone Therapy; Breast Cancer; Progestin; MPA; xenograft

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BACKGROUND

Recent studies reported development of a mouse model for studying the effect of progestins on growth of breast cancer xenograft tumors in the context of different hormonal environments (1). In this model, nude mice are sequentially implanted with estradiol prior to inoculation with tumor cells followed by implantation with progestin pellets. Estradiol supplementation supports a short burst of tumor cell growth, followed by regression and tumor cell senescence and/or apoptosis. However, supplementation with progesterone or MPA rescues tumor growth, thus providing a good model in which to examine how the hormonal environment modulates tumor growth and progression. Here, this model is further developed to compare effects of clinically-relevant progestins that are common in clinical use.

Progestins, which are widely administered to post-menopausal women in the context of hormone therapy (HT), prevent estrogen-induced proliferation of uterine cells, which may play a role in endometrial abnormalities including endometrial cancer (2,3). However, epidemiological studies suggest that progestin-containing HT may have the adverse effect of increasing breast cancer risk (4–6). Our experimental data, as described above, are consistent with this epidemiological finding; namely, that progesterone and medroxyprogesterone acetate (MPA), a synthetic progestin, induce progression of p53-deficient, but not p53-proficient human tumor xenografts (from MCF-7 cells) in nude mice (1).

The goal of this study was to compare the effects of commonly used progestins, which vary in their biological properties (7–10), on xenograft tumor growth in intact and ovarectomized nude mice. Two androgenic progestins, norgestrel (N-EL) and norethindrone (N-ONE), as well as MPA were used in this study. Prior to inoculation, hormone pellets were administered either sequentially or simultaneously. Animals were inoculated with estrogen and progesterone receptor positive, and p53 deficient, BT-474 breast cancer cells to establish xenografts and tumor size, expression of vascular endothelial growth factor (VEGF), and tumor vascularity were monitored over time. MPA was included in some experiments as a reference compound, because it is the most commonly used progestin in HT, and we have previously documented that MPA increases proliferation of BT-474 cells in vivo (1). Exposure of ovariectomized mice to MPA mimics the hormonal environment of HT-treated post-menopausal women.

MATERIALS AND METHODS

Cell lines and culture

BT-474 breast cancer cells, which contain both estrogen and progesterone receptors, were obtained from ATCC (Manassas, VA). Cells were maintained in phenol red-free DMEM: F12 medium (Invitrogen Corporation & Life Technologies, Grand Island, NY) supplemented with 10% FBS (JRH Biosciences Lenexa, KS) in 100 × 20 mm tissue culture dishes. Cells were harvested using 0.05% trypsin-EDTA (Invitrogen Corporation & Life Technologies, Grand Island, NY).

Hormone pellets

Sixty-day release pellets containing 17β-Estradiol (1.7mg), progesterone (10 mg), MPA (10mg), norgestrel (10 mg), norethindrone (10 mg) or placebo were from Innovative Research of America (Sarasota, FL). After implantation, steady-state blood levels of the injected compounds reaches 2–10 ng/ml (Innovative Research, USA).

Progestin-dependent growth of BT-474 human breast xenografts

Intact and ovariectomized female athymic nu/nu mice, 5–6 weeks old (18–22 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Mice were housed in a laminar air-flow

cabinet under specific pathogen-free conditions. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current federal regulations and standards and all procedures were approved by an institutional ACUC committee. Nude mice were inoculated with 17β-estradiol pellets 48 h before implantation of BT-474 cells. Cells were harvested by trypsinization and washed twice with DMEM/F12 medium. Cell pellets ($(1\times 10^7$ cells) were re-suspended in 0.15 ml DMEM/F12 medium, and injected subcutaneously into left and right flanks of each mouse (0.15 ml/injection). Between 3–6 mice were used for each group resulting in 6–12 tumors in each treatment group as indicated by n numbers in figures. Tumor size was measured every three days using a digital caliper and tumor volume was calculated using the formula $(L \times W \times H) \times \pi/6$ as previously described (1,11). Tumors began to regress after reaching 60–100 mm^3 in size (approximately 6–10 days). When tumor volume had decreased by approximately 50%, mice were inoculated with placebo or progestin pellets. This is referred to as the *sequential protocol*. In some experiments estrogen and progestin or estrogen and placebo pellets were implanted at the same time, *i.e.*, 48 h before tumor cell inoculation. This is referred to as the *combined protocol*. Throughout each study animal weight and behavior was monitored as an index of toxicity. At the end of the treatment period (between days 50–60 as indicated in figures), animals were sacrificed and tumors harvested and weighed. Fresh tumor tissue was immediately placed in 4% paraformaldehyde for immunohistochemical analysis (IHC).

Immunohistochemical Assays

Tumor tissue was fixed overnight in 4% paraformaldehyde, followed by paraffin infiltration and embedding. Five μm sections were mounted onto ProbeOn Plus microscope slides (Fisher Scientific Inc., Pittsburgh, PA), stained with hematoxylin-eosin and examined for cellularity by light microscopy. Sections were de-waxed in xylene, rehydrated through graded concentrations of ethanol, and rinsed in distilled water. Sections were subjected to heat-induced epitope retrieval in 10 mM citrate buffer (pH 6.0) for 30 min and then cooled to room temperature prior to treatment with 3% hydrogen peroxide in absolute methanol (to inactivate endogenousperoxidase activity). Sections were then washed 3X with PBS, incubated in blocking buffer with 5% bovine serum albumin for 20 min and probed for 60 minutes at room temperature with one of the following antibodies: VEGF (1:200 dilution of a rabbit anti-VEGF polyclonal antibody [sc-152], Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and CD34 (1:25 dilution of a rat monoclonal anti-CD34 [ab8158–100], Abcam, Inc., Cambridge, MA). Sections were then washed and sequentially incubated with a secondary antibody. VEGF-labeled sections were incubated for 30 minutes with EnVision+, a horseradish peroxidase–labeled polymer conjugated to anti-rabbit antibodies (DAKO). Sections probed with anti-CD34 were incubated for 30 minutes with a biotinylated rabbit anti-rat IgG [DAKO] and after a wash, with a streptavidin-linked horseradish peroxidase product (DAKO) for another 30 minutes at room temperature. Bound antibodies were visualized following incubation with 3, 3′-diaminobenzidine solution (0.05% with 0.015% H_2O_2 in PBS; DAKO) or NovaRED substrate (Vector Laboratories, Inc., Burlingame, CA) for 3–5 minutes. Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared, and cover-slipped for microscopic examination.

The distribution of immunolabeled cells in histologic sections of tumors was determined by use of a morphometric software (FoveaPro 3.0, ©2005 Reindeer Graphics) on images photographed at 20x magnification. Nine to 12 images from 3–5 tumors per treatment group were analyzed. VEGF distribution was determined on all cells in each tumor image. Results are expressed as area in square pixels.

For blood vessel enumeration, images of CD34-labeled sections from three to four tumors per treatment group were photographed at 20x magnification. Investigators performing this

determination were blinded to treatment group assignments. Total number of vessels was counted in 3 to 4 fields from each tumor section (3–4 tumors; 15 sections in total). Vessel density was calculated using vessel number per field and plotted as mean ± SEM. Data was analyzed using ANOVA and $p<0.05$ was considered significant.

Lymph node Metastasis

Inguinal lymph nodes were collected from the nude mice at the end of experiment and analyzed by one of us (CBW) for the presence of tumor cells following H&E staining. Investigator performing this determination was blinded to treatment group assignments. Tumor cells were identified as cohesive pleomorphic polygonal to spindle-shaped cells with large vesicular nuclei and moderate to abundant cytoplasm. Immunohistochemical labeling of MHC-1 (anti-MHC-1, sc-25619, Santa Cruz Biotechnology, Inc, Santa Cruz, CA) on tumor cells was performed on a subset of tissues to verify identity of the cells (data not shown).

Statistical Analysis

Statistical significance was tested using one-way analysis of variance (ANOVA) using SigmaStat Software version 3.5 (Sigstat Software Inc., Richmond, CA, USA). For ANOVA, the assumption of analysis of variance was examined and non-parametric measure based on ranks was used, as needed. Values were reported as mean ± SE. When ANOVA indicated significant effect (F-ratio, $p<0.05$), the Student-Newman-Keuls multirange test was employed to compare the means of individual groups. When normality using Student-Newman-Keuls test failed, significance was determined by Kruskal Wallis test (one-way ANOVA by ranks) with Dunn's test. Differences in the ability of progestins to increase lymph node metastasis was conducted using logistic analysis of variances performed using GENMOD procedure in the SAS program, Cary, NC.

RESULTS

Synthetic progestins rescue growth of BT-474 xenografts: sequential protocol

The goal of this study was to compare the effects of norgestrel (N-EL), norethindrone (N-ONE), and MPA on the growth of BT-474 tumor xenografts in nude mice. Figure 1A shows the change in tumor size over a 50 day treatment period in animals implanted sequentially with estrogen and N-EL or N-ONE hormone pellets. In animals implanted only with an estrogen pellet, tumors regressed from 6 to 10 days post-injection. In contrast, injection with N-EL or N-ONE pellets at day 8 rescued regressing tumors and promoted continued increase in the size of BT-474 xenograft tumors. Representative tumor size and morphology are shown in Figure 1B. After dosing with N-EL or N-ONE, tumor size and vascularization increased significantly. Average animal weights per treatment group over the course of the experiment are shown in Figure 1C. Progestin treatment did not reduce animal weight significantly due to toxicity. Thus treatment of BT-474 xenografts with N-EL and N-ONE led to progression of tumor growth as was observed with MPA in our previous study (1).

Synthetic progestins rescue growth of BT-474 xenografts: combined protocol

The effect of a different hormonal environment on tumor growth was tested by co-injecting mice with estradiol and progesterone prior to tumor cell inoculation. This combined treatment protocol was compared to the sequential treatment protocol, and the results are shown in Figure 2A. The results show that sequential or combined exposure to estrogen and progesterone promotes growth of BT-474 xenograft tumors to a similar extent. Similar results were obtained using the combined treatment protocol, when MPA or norgestrel was substituted for progesterone (Figure 2C). Animal weight did not vary significantly between any treatment groups (Figures 2B and 2D).

MPA enhances BT-474 tumor xenografts in ovariectomized nude mice

The effect of sequential or combined exposure to estrogen and MPA was also examined in ovariectomized nude mice. The results show that MPA promotes growth of BT-474 xenografts in ovariectomized nude mice, independent of the exposure protocol used in the experiment (Figure 3A). Animal weight was not adversely affected by hormone treatment during this experiment (Figure 3B).

Synthetic progestins increase VEGF and tumor blood vessel density

Previous *in vitro* and *in vivo* studies show that progestins induce VEGF and stimulate tumor vascularization (12–14). Therefore, the effect of N-EL and N-ONE on VEGF expression and vascularization was examined in the experimental system used here. The results (Fig 4A) showed that VEGF expression was 2- to 4-fold higher in tumors exposed to estrogen plus MPA, N-EL or N-ONE than in tumors exposed only to estrogen. Higher VEGF expression is expected to correlate with increased density of blood vessels and increased tumor vascularization. This expectation was verified by immunohistochemical quantification of CD-34, an indictor of blood vessel density (Fig 4B). The results show significantly higher expression of CD-34 in tumors exposed to estrogen plus MPA, N-EL or N-ONE than in tumors exposed only to estrogen.

Synthetic progestins increase metastasis of tumor cells to lymph nodes

Since there have been suggestions of increased progestin-dependent metastasis in various models (15,16), we isolated inguinal nodes from animals shown in Fig 2C and examined these for the presence of tumor cells. N-EL treatment significantly increased the frequency of tumor metastasis when compared to controls (Fig 5; $p<0.01$). While MPA increased lymph node infiltration of tumor cells this did not reach statistical significance compared with controls (Fig 5). However based on the differences of least squares means, the odds of MPA treatment increasing metastatic growth was 3.33 times greater than controls, and N-EL increased metastatic growth 8 times compared to MPA.

DISCUSSION

The role of progestins in the development of human breast cancer is controversial. Several recent clinical trials and clinical studies suggest that the risk of breast cancer is higher in post-menopausal women exposed to estrogen/progestin-containing HT than in those taking estrogen alone or placebo (4–6). These studies have led to a significant decrease in the use of HT, including those involving progestins, amongst postmenopausal women (17). While MPA is the most widely used progestin in HT and contraception, other progestins, including norgestrel and norethindrone are also used (18,19). These progestins are thought to promote uterine cell differentiation, which antagonizes the proliferative effects of estrogens in the uterus. Although the exact role of progestins in the human breast is not known, a number of reports suggest they play a pro-proliferative, anti-apoptotic role in both human and rodent mammary gland (1,20–25).

Tumors appear rapidly in post-menopausal women exposed to estrogen/progestin-containing HT (4), suggesting that exposure to progestins promotes the proliferation of latent tumor cells within breast tissue (1,6,15). In cell culture and *in vivo* models, progestins induce VEGF expression in PR-positive/p53-deficient tumor cells (12,13), which could further stimulate tumor cell growth via a paracrine mechanism (26). In a mouse model of human tumor cell-induced xenografts, the elaboration of progestin-induced VEGF was shown to be derived from the injected tumor cells and not stromal mouse cells, because the induced VEGF was targeted by an antibody to human (not murine) VEGF (1).

Due to the possibility of different synthetic progestins having varying effects on breast tumor growth (8–10), this study compared the effects of two commonly used progestins, norgestrel and norethindrone, on human breast cancer cell xenografts in nude mice. We used Her/2-neu p53-deficient BT-474 cells for this study since these cells grow aggressively and secrete VEGF in response to exposure to progestins (1). We have previously shown that RU-486, an anti-progestin, arrests progestin-dependent breast cancer cell progression, demonstrating the involvement of PR (1). Both norgestrel and norethindrone are as effective as MPA in promoting the growth of human breast cancer xenograft tumors in nude mice (Figures 1A and 1B). Not surprisingly, tumors exposed to treatment regimens containing a synthetic progestin and estrogen secrete higher levels of VEGF and express higher levels of CD34 than tumors exposed to estrogen alone, suggesting that all three progestins promote tumor vascularization (Figures 4 and 5).

In this study, the ability of progestins to rescue growth of regressing BT-474 xenograft tumors was determined, using two different hormone exposure protocols. In initial studies, progestin exposure was initiated after tumor regression began (*i.e.*, approximately 6 days after tumor cell inoculation). However, a different hormonal environment, generated by co-implantation of estrogen and progestin pellets prior to inoculation with tumor cells, was equally effective in supporting tumor growth. Thus, in both of these hormonal environments, tumors are capable of recruiting components of extracellular matrix and stromal cells, forming blood vessels, and proliferating at a sufficient rate to support tumor growth. We believe that progestins play an active role in this process, at least in part by stimulating stromal cell functions in the vicinity of the tumor, as has been suggested by others (27). In addition, it has also been suggested that progestins may influence the epithelial-mesenchymal transition without having a direct effect on tumor cell proliferation (28). Both these possibilities will be examined further in future studies.

This study also shows that MPA promotes the growth of BT-474 xenografts in ovariectomized nude mice, independent of the exposure protocol used in the experiment. Thus, it is unlikely that intact and/or functional ovaries play a role in tumor progression in this experimental model. However, progestin-induced expression and secretion of VEGF by tumor epithelial cells is likely to be required for tumor growth and progression in this model.

Interestingly, our study demonstrated that progestins can increase the metastasis of breast cancer cells to lymph node. Although we did not detect significant differences in elevated VEGF levels and increased numbers of blood vessels in response to the different progestins (Fig 4), norgestrel exposure did lead to much bigger tumors (250 mm^3) compared with MPA (150 mm^3) or controls (50 mm^3). Thus the increased infiltration of tumor cells observed with this particular progestin could arise as a consequence of a more aggressive growth of BT-474 tumors. It is known that progestins increase the invasiveness of tumor cells (7) and it may be that norgestrel is more potent than MPA in terms of its ability to induce such an effect, though this remains to be established. Further studies are required to determine the role played by PR in promoting metastasis. With this in mind we will use this model together with anti-progestins such as RU-486 to ascertain whether increased metastasis is PR dependent.

CONCLUSION

In conclusion, this study shows that several clinically-relevant progestins promote the growth of human breast cancer cells as xenograft tumors in nude mice. These synthetic progestins, which are administered widely as components of HT and oral contraception, could exert similar effects in human breast tissue that carry latent tumor cells or micro-tumors. Progestins also appear to increase the metastasis of breast cancer cells into lymph node. Women who are genetically-predisposed to developing breast cancer could be particularly at risk of adverse

effects from progestin-containing HT. It is not yet known whether progestin-containing HT stimulates the growth of breast cancer cells at a certain pathological stage or within a critical kinetic "window" of exposure. Nevertheless, there is a need for selective progesterone receptor modulators that do not induce breast tumor cell proliferation but do inhibit estrogen-dependent proliferation of uterine cells. Recent studies have identified natural non-steroidal compounds of interest for such purposes, but their mechanism(s) of action and their ability to block estrogen-dependent proliferation of uterine cells are not yet known (29–31). Future studies should also examine the effect of progestins in the context of orthotopic cancer models in the mouse or other animal model systems.

Acknowledgments

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Fig. 1A

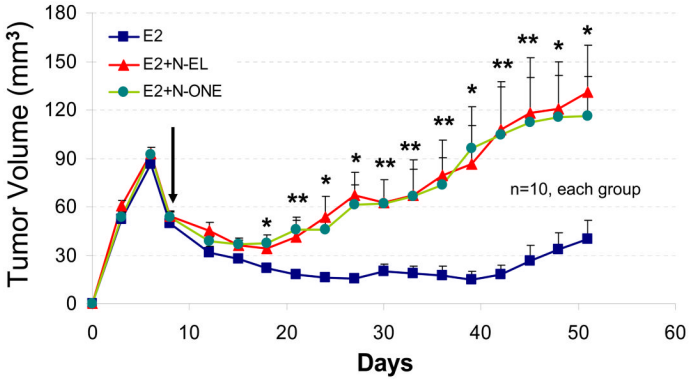

Fig 1B

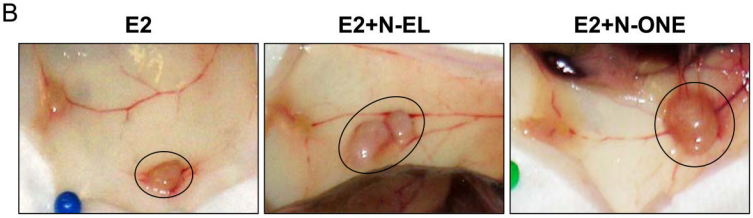

Fig 1C

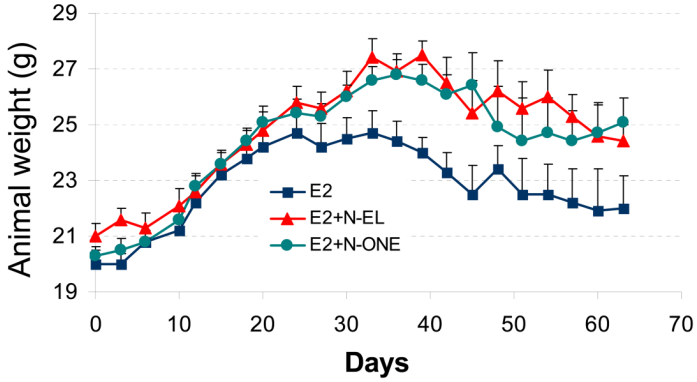

Figure 1. Synthetic progestins rescue growth of BT-474 xenograft tumors sequential protocol

(A) Cells were injected into estradiol (E2)-supplemented intact nude mice (as described in Methods) followed by implantation of a norgestrel (N-EL) or norethindrone (N-ONE) pellet (arrow). Tumor size was monitored for 50 days, as indicated. (B) Representative tumors from each treatment group are shown. (C) Average body weight was calculated for each treatment group at the indicated time points. Five animals were used and n represents number of tumors in each group. *$p < 0.05$, significantly different from E2 group (one-way ANOVA, Student-Newman-Keuls test), **$p < 0.05$, significantly different from E2 group (one-way ANOVA, Kruskal-Wallis test). Significance values apply to both N-EL and N-ONE groups.

Fig 2A

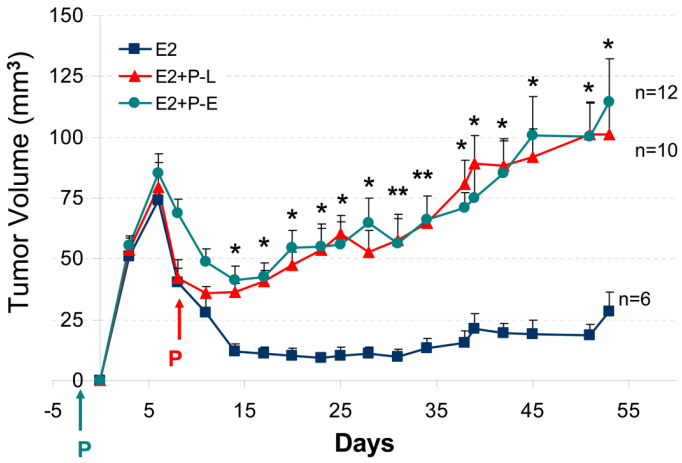

Fig 2B

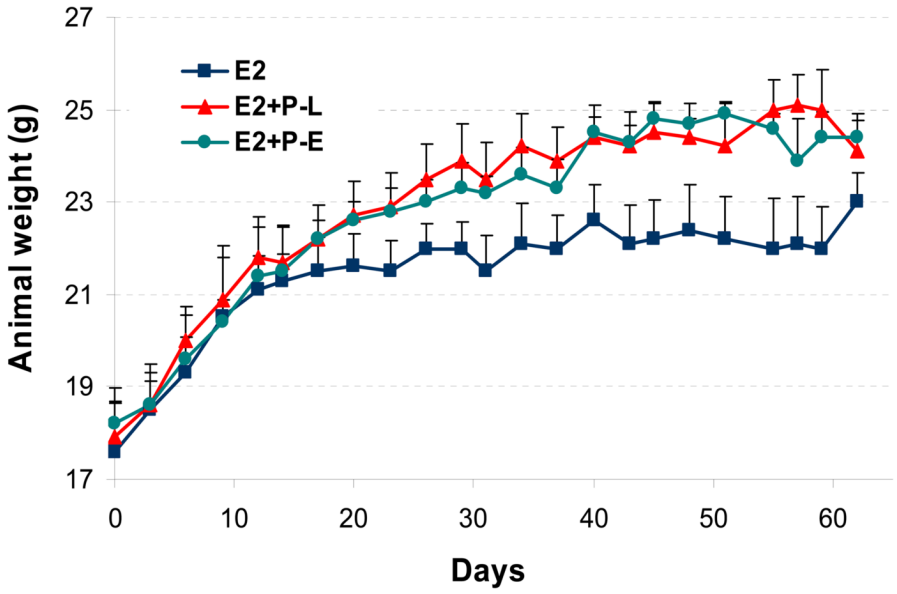

Fig 2C

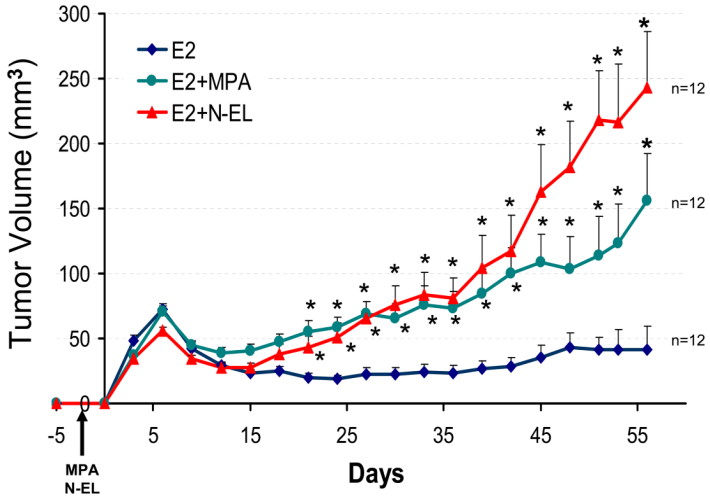

Fig 2D

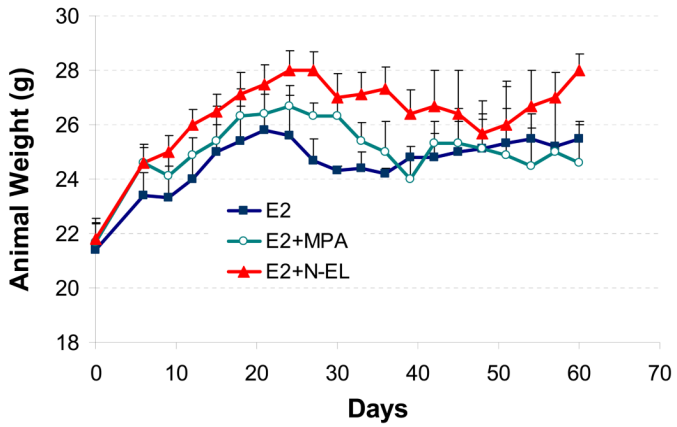

Figure 2. Synthetic progestins rescue growth of BT-474 xenograft tumors: combined protocol

(A) BT-474 cells were injected into estrogen-supplemented intact nude mice as described in Methods and tumor growth monitored. A progesterone (P) pellet (arrow) was implanted on day 8 (red arrow; E2 +P-L) or co-implanted with estradiol (E2 +P-E) 48 h prior to injection with tumor cells (green arrow). *$p < 0.05$, significantly different from E2 group (one-way ANOVA, Student-Newman-Keuls test), **$p < 0.05$, significantly different from E2 group (one-way ANOVA, Kruskal-Wallis test). Significance values apply to both P-L and P-E groups. (B) Average body weight was calculated for each treatment group shown in (A) at the indicated time points. (C) MPA or norgestrel (N-EL) pellets (arrow) were co-implanted with estradiol 48 h prior to injection with tumor cells (arrow). *$p < 0.05$, significantly different from E2 group (one-way ANOVA, Student-Newman-Keuls test) (D) Average body weight was calculated for each treatment group shown in (C) at the indicated time points.

Fig 3A

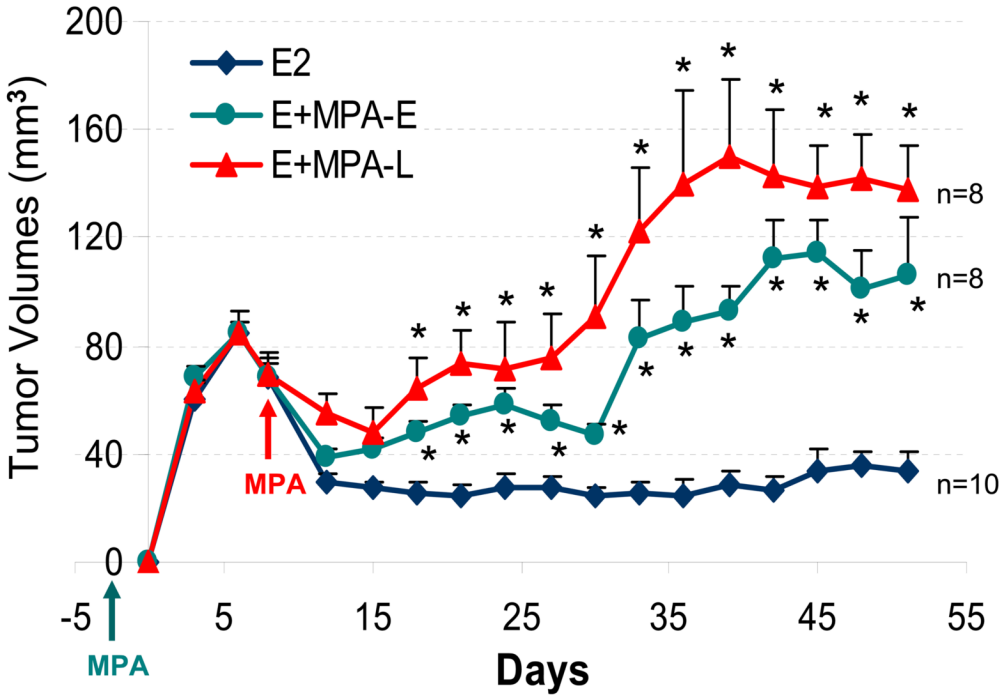

Fig 3B

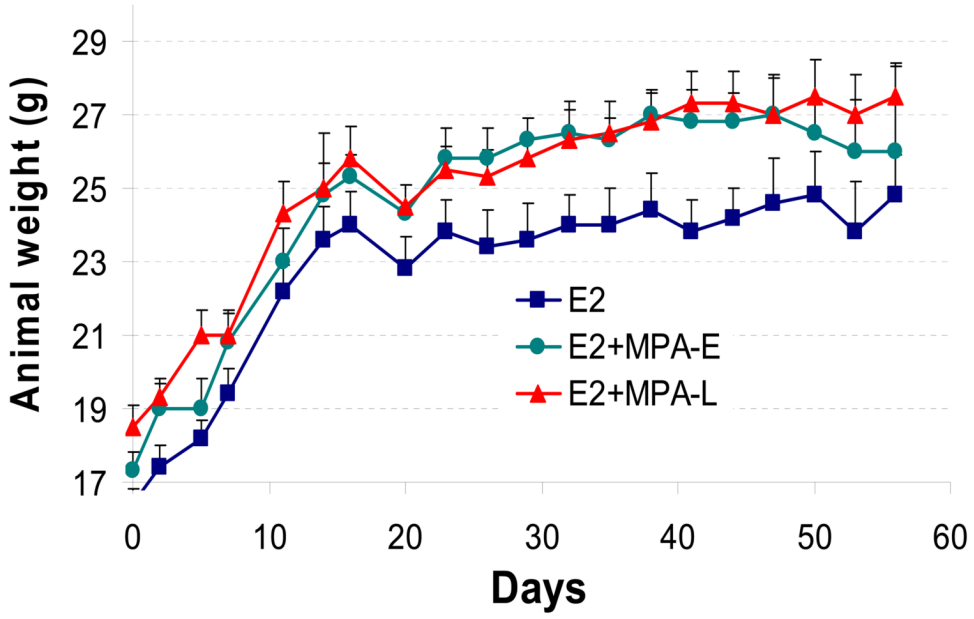

Figure 3. MPA rescues growth of BT-474 xenograft tumors in ovariectomized mice

(A) As in Figure 2A, except MPA pellets were implanted in ovariectomized mice 8 days after (MPA-L, red arrow) or 48 h before (MPA-E, green arrow) inoculation with tumor cells. Control animals (blue line) were co-injected with estrogen- and placebo-containing pellets. (B) Average body weight was calculated for each treatment group at the indicated time points. *p <0.05, significantly different from E2 only group ANOVA (Student-Newman-Keuls test).

Fig 4A

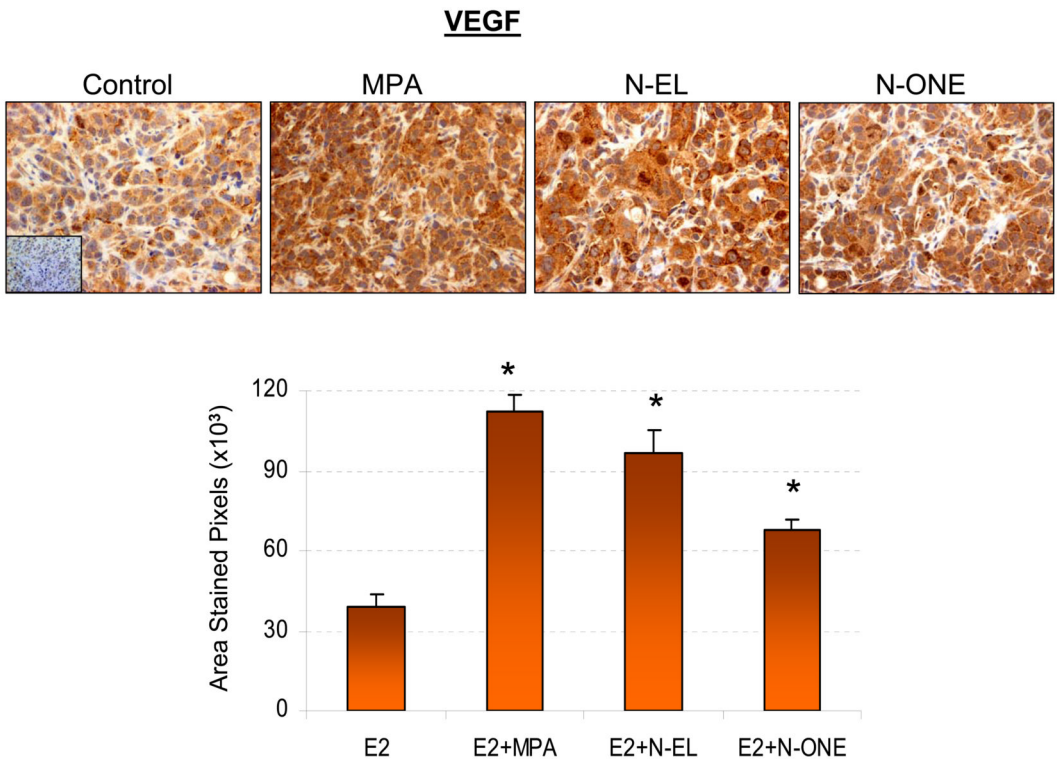

Fig 4B

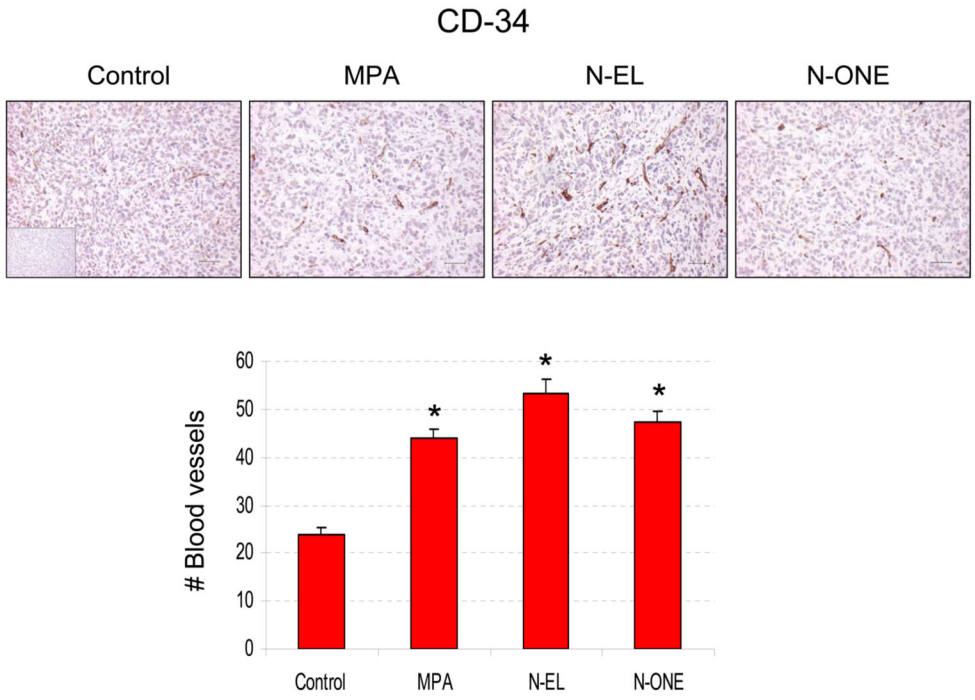

Figure 4. Synthetic progestins stimulate VEGF expression (A) and CD-34 expression in BT-474 xenograft tumors

(A) Animals were sacrificed and tumor samples collected at the end of the treatment period. Tissues were prepared and stained for VEGF (upper panel) as described in Methods. Quantification of VEGF immunoreactivity is shown in lower panel. *p<0.05, ANOVA; significantly different from E2 only group. (B) Animals were sacrificed and tumor samples collected at the end of the treatment period. Tissues were prepared and stained for CD-34 (upper panel) as described in Methods. Quantification of CD-34 immunoreactivity is shown in lower panel. **p* <0.05, ANOVA (Student-Newman-Keuls test); significantly different from E2 only group. *Insets*, negative controls without primary antibody.

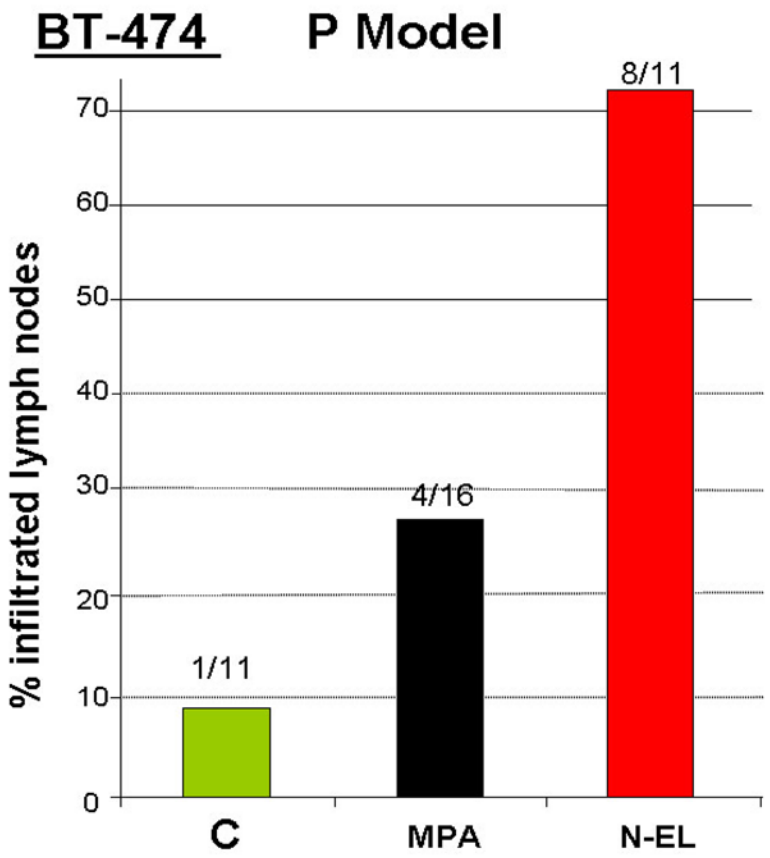

Figure 5. Incidence of lymph node metastasis in MPA- and norgestrel-stimulated BT-474 xenografts in nude mice

Nude mice were implanted with an E2 and progestin pellet and inoculated with BT-474 cells as described in the legend to Figure 2. Inguinal lymph glands were collected at the end of the experiment (Day 57) and the percentage of lymph nodes infiltrated with tumor cells quantified. Average tumor volume at day 57 was 50, 150, and 250 mm^3 in control (C), MPA-, and norgestrel (N-EL)-treated animals, respectively. The incidence of lymph node infiltration in the norgestrel group was significantly different from control ($p < 0.05$, logistic analysis of variances performed using GENMOD procedure in the SAS program).